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School of Pharmacy

Polymer-Mediated
Crystallisation of Proteins

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Contents

Abstract ......................................................................................................................... 7
Acknowledgments ........................................................................................................ 9
List of Abbreviations .................................................................................................. 10
List of Figures .............................................................................................................. 14
List of Tables ............................................................................................................... 33
Chapter 1. Introduction .............................................................................................. 37
  1.1 Introduction to chapter ....................................................................................... 37
  1.2 General protein introduction ........................................................................... 37
    1.2.1 Analysis of protein structure ................................................................. 37
    1.2.2 Protein therapeutics .............................................................................. 42
  1.3 Crystals .................................................................................................................. 46
  1.4 Protein crystals .................................................................................................... 52
    1.4.1 Some history of protein crystallisation .................................................. 53
    1.4.2 Principle of protein crystallisation ......................................................... 55
    1.4.3 Methods of protein crystallisation ......................................................... 56
  1.5 Use of additives in protein crystallisation ....................................................... 62
    1.5.1 Non polymer methods ............................................................................ 62
      1.5.1.1 Salts .................................................................................................. 62
      1.5.1.2 Organic solvents ............................................................................. 64
      1.5.1.3 Insoluble surfaces .......................................................................... 64
    1.5.2 Polymer additives ...................................................................................... 68
      1.5.2.1 Insoluble polymer surfaces ............................................................. 69
      1.5.2.2 Soluble polymer additives ............................................................... 72
  1.6 Polymers and polymer synthesis methods ..................................................... 76
    1.6.1 Atom transfer radical polymerisation (ATRP) ....................................... 77
    1.6.2 Reversible addition-fragmentation chain transfer polymerisation (RAFT) ...................................................................................................................... 80
  1.7 Protein targets .................................................................................................... 83
1.7.1 Hen egg-white lysozyme (HEWL) ................................................................. 84
1.7.2 Concanavalin a (Con A) .......................................................... 86
1.7.3 Bovine liver catalase (BLC) ......................................................... 87

1.8 Aims and objectives of the project ......................................................... 89

Chapter 2. Materials, Instrumentation and General Methods 93
2.1 Materials .......................................................................................... 93
2.2 Instrumentation .................................................................................. 96
2.3 General methods ............................................................................... 101

Chapter 3. Preparation and Characterisation of Monomers and Polymers 103
3.1 Introduction ...................................................................................... 103
3.2 Monomer synthesis ......................................................................... 105

3.2.1 2-Acrylamidoacetic acid (2-AmAA) ................................................. 105
3.2.2 4-Acrylamidobutanoic acid (4-AmBA) ............................................. 107

3.3 Polymer synthesis ............................................................................. 110

3.3.1 Atom transfer radical polymerisation (ATRP) method ................. 110

3.3.1.1 Polymer conversion calculations ............................................... 110

3.3.1.2 Poly[poly(ethylene glycol) methyl ether methacrylate$_{475}$]
[p(PEGMA$_{475}$)] (P1) ........................................................................ 111

3.3.1.3 Poly[2-(dimethylamino)ethyl methacrylate] [p(DMAEMA)]
(P2) .................................................................................................. 116

3.3.1.4 Quaternisation of p(DMAEMA) (P2) ...................................... 120

3.3.1.5 Poly[2-(dimethylamino)ethyl methacrylate-stat-poly(ethylene glycol) methyl ether methacrylate$_{475}$]
[p(DMAEMA-stat-PEGMA$_{475}$)] (P3) ................................................ 122

3.3.2 Reversible addition-fragmentation chain transfer Polymerisation
(RAFT) method .................................................................................. 125

3.3.2.1 Polymer conversion calculations ............................................. 126

3.3.2.2 Poly[N-(3-(dimethylamido)propyl)methacrylamide]
[p(DMAPMAm)] (P4) ......................................................................... 126

3.3.2.3 Poly(2-acrylamido-2-methyl-1-propanesulfonic acid)
[p(AMPS)] (P5) ................................................................................ 130

3.3.2.4 Poly(2-acrylamidoacetic acid) [p(2-AmAA)] (P6) .................... 133

3.3.2.5 Poly(4-acrylamidobutanoic acid) [p(4-AmBA)] (P7) ............... 136
3.3.3 Poly[[2-(methacryloyloxy)ethyl]trimethylammonium chloride-co-N-[2-(methacryloyloxy)ethyl]-dimethyl-(2-sulfoethan)aminium hydroxide] [p(METMAC-co-MEDSAH)] (P8) .......................................................... 138

3.4 General discussion and conclusions ............................................................ 141

Chapter 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation ............................................. 145

4.1 Introduction ........................................................................................................ 145

4.2 Methods .............................................................................................................. 148

4.2.1 Crystallisation technique and plate ............................................................... 148

4.2.2 Preparation of polymer solutions and stock buffer solutions .......... 150

4.2.3 Crystallisation protocol for tetragonal HEWL crystals .................. 151

4.2.4 Crystal imaging .............................................................................................. 152

4.2.5 X-ray crystallography .................................................................................... 153

4.2.6 Dynamic light scattering (DLS) studies ..................................................... 153

4.2.7 Scanning electron microscope (SEM) analysis ....................................... 155

4.3 Results and discussion ........................................................................................ 156

4.3.1 HEWL crystallisation in the presence of neutral polymer in solution: p(PEGMA475) (P1) .......................................................... 156

4.3.2 HEWL crystallisation in the presence of cationic polymers in solution ......................................................................................................................... 167

4.3.2.1 HEWL crystallisation in the presence of quaternised p(DMAEMA) (P2) .................................................................................................................. 167

4.3.2.2 HEWL crystallisation in the presence of p(DMAEMA-stat-PEGMA475) (P3) .................................................................................................................. 195

4.3.2.3 HEWL crystallisation in the presence of a mixture of two polymers: p(PEGMA475) and p(DMAEMA) (P1 & P2) (molar ratio 9:1) .......................................................... 200

4.3.2.4 HEWL crystallisation in the presence of p(DMAPMAm) (P4) .......................................................................................................................... 206

4.3.3 HEWL crystallisation in the presence of anionic polymers in solution .......................................................................................................................... 216

4.3.3.1 HEWL crystallisation in the presence of p(AMPS) (P5) .. 216

4.3.3.2 HEWL crystallisation in the presence of p(2-AmAA) (P6) ......................................................................................................................... 225
4.3.3.3 HEWL crystallisation in the presence of p(4-AmBA) (P7) ................................................................. 237

4.3.4 HEWL crystallisation in the presence of cationic/zwitterionic polymer in solution: p(METMAC-co-MEDSAH) (P8) ............. 250

4.4 Conclusions ............................................................................................................................................. 255

Chapter 5. Application of Polymers to Concanavalin A (Con A)

Crystallisation .......................................................................................................................................... 258

5.1 Introduction ........................................................................................................................................... 258

5.2 Methods .............................................................................................................................................. 261
  5.2.1 Crystallisation technique and plate ............................................................................................... 261
  5.2.2 Preparation of polymer solutions and stock buffer solutions ...................................................... 261
  5.2.3 Crystallisation protocol for Con A crystals .................................................................................. 262
  5.2.4 Crystal imaging ............................................................................................................................. 262
  5.2.5 X-ray crystallography ................................................................................................................... 263
  5.2.6 DLS studies .................................................................................................................................... 263
  5.2.7 SEM analysis ............................................................................................................................... 263

5.3 Results and discussion ........................................................................................................................... 264
  5.3.1 Con A crystallisation in the presence of neutral polymer in solution: p(PEGMA_{475}) (P1) .............. 264
  5.3.2 Con A crystallisation in the presence of cationic polymer in solution: quaternised p(DMAEMA) (P2) (quaternised ratio: 75%)... 271
  5.3.3 Con A crystallisation in the presence of anionic polymer in solution: p(AMPS) (P5) ...................... 278

5.4 Conclusions ......................................................................................................................................... 284

Chapter 6. Application of Polymers to Bovine Liver Catalase (BLC) Crystallisation ......................................................... 286

6.1 Introduction .......................................................................................................................................... 286

6.2 Methods ............................................................................................................................................. 289
  6.2.1 Crystallisation technique and plate ............................................................................................... 289
  6.2.2 Preparation of polymer solutions and stock buffer solutions ...................................................... 289
  6.2.3 Crystallisation protocol for BLC crystals .................................................................................... 290
  6.2.4 Crystal imaging ............................................................................................................................. 290
  6.2.5 X-ray crystallography ................................................................................................................... 290
6.2.6 DLS studies .............................................................. 291
6.3 Results and discussion......................................................... 291
  6.3.1 BLC crystallisation in the presence of neutral polymer in solution:
p(PEGMA475) (P1) ................................................................ 291
  6.3.2 BLC crystallisation in the presence of cationic polymers in
solution: quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %)........ 298
  6.3.3 BLC crystallisation in the presence of anionic polymers in solution:
p(AMPS) (P5) ........................................................................ 303
6.4 Conclusions ........................................................................... 309

Chapter 7. Discussion, Conclusions and Future Work .......... 311
  7.1 Discussion and conclusions.................................................. 311
  7.2 Future work ....................................................................... 327

Appendix .................................................................................... 330
  Supporting spectroscopy graphs............................................... 330

References ............................................................................... 340
Abstract

Proteins are the functional machines of nature and play an essential role in life. Understanding the structure and function of proteins is central to numerous areas of science and technology, including biotechnological, pharmaceutical and chemical industries. Protein crystals are an important objective for many researchers, but even though there are many examples of protein crystals reported, there are still many uncertainties in controlling the crystallisation process. In addition, while protein crystals have mostly been obtained for characterisation purposes, there are other applications where defined assemblies of proteins, either crystals or self-assembled protein nanoparticles, are desirable. These include industrial biocatalysis, therapeutic protein formulations, and even energy-harvesting systems. Accordingly, there remains a high demand for well-defined protein crystals or nanoscale aggregates.

A variety of techniques have been used to produce protein crystals, the typical strategy involves the addition of nucleants surface, or surface-active materials into the crystallisation solution. Solution ‘additives’ of diverse types have proven crucial in the control of protein crystallisation. These additives can act via multiple roles in a crystallisation process, such as by enhancing intermolecular contacts between protein macromolecules, or disrupting unfavourable intermolecular association, or diminishing interactions between protein and solvent. Synthetic polymers are important additives for protein
crystallisation control as there are many possible chemistries which can be introduced into the backbone and side-chains, giving a very wide range of functional behavior. However, because there are so many factors which can affect crystallisation, many of which are still poorly understood, to date only a very few classes of polymers have been studied as additives for protein crystallisation. Moreover, the mechanisms governing their interactions with protein crystals or surrounding solvent are elusive.

In the study presented here, we show that polymers designed with varying degrees of charge, molecular weight and backbone structure influenced the crystallisation of three target proteins: hen-egg white lysozyme (HEWL); concanavalin A (Con A); and bovine liver catalase (BLC). Polymers were prepared as ‘additives’ into protein solution to influence the proteins crystallisation process, leading to changes in size, habit, morphology and even polymorph of the final crystals. A simple model linking polymer structure and charge to protein isoelectric point (pI) and crystallisation rate is proposed.
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List of Abbreviations

2-AmAA – 2-Acrylamidoacetic acid
4-ABA – 4-Aminobutanoic acid
4-AmBA – 4-Acrylamidobutanoic acid
Å – Angstrom
AMPS – 2-Acrylamido-2-methyl-1-propanesulfonic acid
ATRP – Atom transfer radical polymerisation
Bipy – 2,2-Bipyridine
BLC – Bovine liver catalase
CaF₂ – Calcium fluoride
CAT – 2-(Ethylthiocarbonothioylthio)-2-methylpropanoic acid
CDCl₃ – Chloroform-d
CHCl₃ – Chloroform
Con A – Concanavalin A
Cryo – Cryogenic
CTA – Chain transfer agent
DLS – Dynamic light scattering
DMAEMA – 2-(Dimethylamino)ethyl methacrylate
DMAPMAm – N-[3-(dimethylamino)propyl]methacrylamide
DMSO – Dimethyl sulfoxide
DP – Degree of polymerisation
DPBS – Dulbecco’s phosphate buffered saline

E.C. – Enzyme commission (number)

EtOAc – Ethyl acetate

FT-IR – Fourier transform infrared spectroscopy

G-CSF – Granulocyte-colony stimulating factor

GPC – Gel permeation chromatography

HEWL – Hen egg-white lysozyme

[I] – [Initiator]

IR – Infrared spectroscopy

Kcal – Kilocalorie

KDa – Kilodalton

[M] – [Monomer]

MCT – Mercury cadmium telluride

MEDSAH – N-[2-(methacryloyloxy)ethyl]-dimethyl-(2-sulfoethan)aminium hydroxide

METMAC – [2-(Methacryloyloxy)ethyl]trimethylammonium chloride

MIPs – Molecularly imprinted polymers

M\text{\textsubscript{n}} – Number average molecular weight

M\text{\textsubscript{w}} – Molecular weight

MWCO – Molecular weight cut off

NaCl – Sodium chloride

NMR – Nuclear magnetic resonance
P(2-AmAA) – Poly(2-acrylamidoacetic acid)

P(4-AmBA) – Poly(4-acrylamidobutanoic acid)

P(AMPS) – Poly(2-acrylamido-2-methyl-1-propanesulfonic acid)

PBS – Phosphate-buffered saline

PDB – Protein data bank

PDI – Polydispersity index

P(DMAEMA) – Poly[2-(dimethylamino)ethyl methacrylate]

P(DMAPMAm) – Poly[N-(3-(dimethylamido)propyl)methacrylamide]

PEG – Polyethylene glycol

PEGMA,475 – Poly(ethylene glycol) methyl ether methacrylate $M_w \sim 475$

PEO – Poly(ethylene oxide)

PI – Isoelectric point

PLGA – Poly(lactic-co-glycolic acid)

P(PEGMA,475) – Poly[poly(ethylene glycol) methyl ether methacrylate $M_w \sim 475$]

PVP – Polyvinylpyrrolidone

RAFT – Reversible addition fragmentation chain transfer

RI – Refractive index

Rpm – Revolutions per minute

SEM – Scanning electron microscope

TFA – Trifluoroacetic acid

THF – Tetrahydrofuran
TMS – Tetramethysilane

Tris – Tris(hydroxymethyl)aminomethane

UVP – UV compatible

V-501 – 4,4′-Azobis(4-cyanvaleric acid)

W/v – Weight/volume
List of Figures

Figure 1-1. Main protein structure levels ................................................................. 39

Figure 1-2. Use of protein crystallography .............................................................. 41

Figure 1-3. Encapsulation of therapeutic protein into polymeric particles ...... 44

Figure 1-4. The crystal structure of Fluorite (CaF$_2$) ............................................ 47

Figure 1-5. Diagram showing the transfer of molecules from solution to crystal according to Gibbs energy change ....................................................... 49

Figure 1-6. Thermodynamics of nucleation, according to Gibbs free energy .. 50

Figure 1-7. A view of crystal structure of chymotrypsinogen ............................ 53

Figure 1-8. Crystallisation phase diagram of solubility curve showing how the solubility varies with the concentration of crystallising agent ..... 55

Figure 1-9. Micro-batch crystallisation technique ................................................... 59

Figure 1-10. Button dialysis crystallisation technique ........................................ 60

Figure 1-11. Vapour diffusion techniques: hanging drop, sandwich drop and sitting drop ............................................................................................................. 61

Figure 1-12. The solubility curves of a typical protein, enolase, as a function of ionic strength produced by two types of salts ................................. 63

Figure 1-13. Schematic representation of heterogeneous nucleation facilitate by the insoluble solid surface ......................................................... 65

Figure 1-14. Some example images of protein crystals the nucleated and grew on the mineral substrate surfaces ................................................. 66
Figure 1-15. Schematic representation of crystal nucleation in a pore, which explains the mechanism of porous substrates induce protein crystallisation.............................................................. 67

Figure 1-16. Schematic representation of the molecular imprinting process and selective crystallisation of calcium carbonate .................. 71

Figure 1-17. General ATRP reaction scheme .................................................. 78

Figure 1-18. Structures of PEGMA$_{475}$ and DMAEMA monomers .............. 80

Figure 1-19. Mechanism of RAFT polymerisation ........................................ 81

Figure 1-20. Structures of DMAPMAm, AMPS, 2-AmAA and 4-AmBA monomers ............................................................................. 83

Figure 1-21. The three-dimensional structure of HEWL, obtained by crystal X-ray diffraction .............................................................................. 85

Figure 1-22. Crystallographic structure of a Con A tetramer (from jack bean), obtained by crystal X-ray diffraction .............................................. 87

Figure 1-23. Crystallographic structure of a BLC tetramer ......................... 88

Figure 1-24. Schematic representation of protein crystallisation utilising polymer additives ............................................................................ 90

Figure 2-1. Bragg’s diffraction ....................................................................... 101

Figure 3-1. Structures of polymers, named P1 to P8 ................................. 104

Figure 3-2. Reaction scheme for the synthesis of 2-AmAA .......................... 105

Figure 3-3. $^1$H-NMR spectra of 2-AmAA .................................................... 106

Figure 3-4. $^{13}$C-NMR spectra of 2-AmAA .................................................. 106
Figure 3-5. Scheme of the mechanism of synthesis of 2-AmAA .............. 107
Figure 3-6. Reaction scheme for the synthesis of 4-AmBA ..................... 107
Figure 3-7. $^1$H-NMR spectra of 4-AmBA ........................................ 108
Figure 3-8. $^{13}$C-NMR spectra of 4-AmBA ........................................ 109
Figure 3-9. Reaction scheme for the synthesis of p(PEGMA$_{475}$) homopolymer (P1) via ATRP ................................................................. 111
Figure 3-10. $^1$H-NMR spectra for the polymerisation of PEGMA$_{475}$ to yield p(PEGMA$_{475}$) (P1) ......................................................... 113
Figure 3-11. Representative FTIR for p(PEGMA$_{475}$) (P1) ...................... 113
Figure 3-12. Kinetic plots for the synthesis of p(PEGMA$_{475}$) (P1) in this study ...................................................................................... 115
Figure 3-13. Reaction scheme for the synthesis of p(DMAEMA) (P2) via ATRP ................................................................................. 116
Figure 3-14. $^1$H-NMR spectra for the polymerisation of DMAEMA to yield p(DMAEMA) (P2) ............................................................... 117
Figure 3-15. Representative FTIR for p(DMAEMA) (P2) ....................... 117
Figure 3-16. Kinetic plots for the synthesis of p(DMAEMA) (P2) via ATRP method .............................................................. 119
Figure 3-17. Reaction scheme for quaternisation of p(DMAEMA) (P2) ...... 120
Figure 3-18. $^1$H-NMR spectra for quaternised p(DMAEMA) (P2) ........... 121
Figure 3-19. Representative FTIR for quaternised p(DMAEMA) (P2) ....... 122
Figure 3-20. Reaction scheme for the synthesis of a random copolymer:
p(DMAEMA-stat-PEGMA_{475}) (P3) via ATRP .......................... 122

Figure 3-21. $^1$H-NMR spectra for the polymerisation of DMAMEA and PEGMA_{475} to yield p(DMAEMA-stat-PEGMA_{475}) (P3) .......... 124

Figure 3-22. Reaction schemes for the synthesis of p(DMAPMAm) (P4) via RAFT method and the removal of RAFT end-group .......... 126

Figure 3-23. $^1$H-NMR spectra for the polymerisation of DMAPMAm to yield p(DMAPMAm) (P4) ................................................................. 128

Figure 3-24. Representative FTIR for p(DMAPMAm) (P4) ............... 128

Figure 3-25. Reaction schemes for the synthesis of p(AMPS) (P5) via RAFT method and removal of RAFT end-group ......................... 130

Figure 3-26. $^1$H-NMR spectra for the polymerisation of AMPS to yield p(AMPS) (P5) ................................................................. 132

Figure 3-27. Representative FTIR for p(AMPS) (P5) ....................... 132

Figure 3-28. Reaction schemes for the synthesis of p(2-AmAA) (P6) homopolymer via RAFT method and removal of RAFT end-group .................................................................................. 133

Figure 3-29. $^1$H-NMR spectra for p(2-AmAA) (P6) ...................... 134

Figure 3-30. Representative FTIR for p(2-AmAA) (P6) ................. 135

Figure 3-31. Reaction schemes for the synthesis of p(4-AmBA) (P7) homopolymer via RAFT method and removal of RAFT end-group .................................................................................. 136

Figure 3-32. $^1$H-NMR spectra for p(4-AmBA) (P7) ...................... 137
Figure 3-33. Representative FTIR for p(4-AmBA) (P7).............................. 137

Figure 3-34. Reaction scheme for the synthesis of p(METMAC-co-MEDSAH) (P8) by Dr. E. Peter Magennis .................................................. 139

Figure 3-35. Representative $^1$H-NMR spectra of p(METMAC-co-MEDSAH) (P8) by Dr. E. Peter Magennis .................................................. 140

Figure 3-36. Representative FTIR for p(METMAC-co-MEDSAH) (P8)...... 140

Figure 4-1. Schematic representation of the interaction of various polymers with cationic HEWL in the protein crystallisation process. ....... 147

Figure 4-2. MRC 2 Well Crystallisation Plate (Swissci), commercially available from Hampton Research................................................. 148

Figure 4-3. Diagram depicting the sitting drop vapour diffusion technique .. 149

Figure 4-4. The schematic representation of real-time protein concentration changes in the sitting drop................................................... 150

Figure 4-5. Schematic representation of protein crystallisation in the DLS quartz cell................................................................. 155

Figure 4-6. Photomicrographs of HEWL crystals, grown in the presence of p(PEGMA$_{475}$) (P1) at various concentrations......................... 159

Figure 4-7. Summary of photomicrograph analysis showing changes in the size of HEWL crystals, grown in the presence of neutral p(PEGMA$_{475}$) (P1) at various concentrations ............................................ 160

Figure 4-8. HEWL crystal photomicrographs and X-ray diffraction pattern yielded by the single HEWL crystal in the presence of neutral
Figure 4-9. Summary of DLS data of HEWL crystallisation in the presence of neutral p(PEGMA\textsubscript{475}) (P1).......................... 163

Figure 4-10. Variations in the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by neutral p(PEGMA\textsubscript{475}) (P1).......................... 164

Figure 4-11. SEM micrographs of HEWL crystals produced under tetragonal conditions, by sitting drop method ......................... 165

Figure 4-12. Schematic representation of hypothesis of HEWL crystallisation process without polymer (A); and the interaction of p(PEGMA\textsubscript{475}) (P1) with HEWL in the protein crystallisation process (B)....... 166

Figure 4-13. Photomicrographs showing changes in the size and shape of HEWL crystals, grown under the tetragonal protocol, in the presence of quaternised p(DMAEMA) (P2) (M\textsubscript{n}~46.3 kDa) (quaternised ratio: 50\%) at various concentrations ................. 170

Figure 4-14a. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M\textsubscript{n}~17.1 kDa) (quaternised ratio: 25\%) at various concentrations, with lysozyme concentration: 25 mg/mL............................................................ 173

Figure 4-14b. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M\textsubscript{n}~17.1 kDa) (quaternised ratio: 25\%) at various concentrations, with lysozyme
concentration: 50 mg/mL................................. 174

Figure 4-14c. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 25 %) at various concentrations, with lysozyme concentration: 75 mg/mL................................. 175

Figure 4-14d. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 25 %) at various concentrations, with lysozyme concentration: 100 mg/mL................................. 176

Figure 4-15a. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with lysozyme concentration: 25 mg/mL................................. 178

Figure 4-15b. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with lysozyme concentration: 50 mg/mL................................. 179

Figure 4-15c. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with lysozyme concentration: 75 mg/mL................................. 180

Figure 4-15d. Photomicrographs of HEWL crystals, grown under the tetragonal
protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with lysozyme concentration: 100 mg/mL.............................................. 181

Figure 4-16a. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 75 %) at various concentrations, with lysozyme concentration: 25 mg/mL......................................................... 183

Figure 4-16b. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 75 %) at various concentrations, with lysozyme concentration: 50 mg/mL......................................................... 184

Figure 4-16c & d. Photomicrographs showing changes in the size and shape of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 75 %) at various concentrations, with lysozyme concentration: 75 and 100 mg/mL................................................................. 185

Figure 4-17a. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 100 %) at various concentrations, with lysozyme concentration: 25 mg/mL ................................................. 187

Figure 4-17b. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa)
(quaternised ratio: 100%) at various concentrations, with lysozyme concentration: 50 mg/mL

Figure 4-17c & d. Photomicrographs showing changes in the size and shape of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 100%) at various concentrations, with HEWL concentration: 75 and 100 mg/mL

Figure 4-18. Schematic triangle graphs showing the trends of HEWL crystals obtained in the presence of different quaternised ratios of p(DMAEMA) (P2) (25%, 50%, 75% and 100%) by using different concentrations of HEWL

Figure 4-19. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of quaternised p(DMAEMA) (quaternised ratio: 75%) at various concentrations, with HEWL concentration 50 mg/mL

Figure 4-20. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75%)....

Figure 4-21. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiment induced by cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75%)
Figure 4-22. SEM micrographs of HEWL needles produced under tetragonal conditions in the presence of quaternised p(DMAEMA)......... 195

Figure 4-23. Photomicrographs of HEWL crystals, grown from the tetragonal protocol in the presence of p(DMAEMA-stat-PEGMA₄₇₅) (P3) (Mₙ~52.9 kDa) with various concentrations................................. 197

Figure 4-24. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(DMAEMA-stat-PEGMA₄₇₅) (P3) at various concentrations. . 198

Figure 4-25. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal formed in the presence of cationic p(DMAEMA-stat-PEGMA₄₇₅) (P3) ................................. 199

Figure 4-26. Photomicrographs of HEWL crystals, grown in the presence of a mixture of two polymers: p(PEGMA₄₇₅) and p(DMAEMA) (P1 & P2) (molar ratio 9:1) at various concentrations........................................ 203

Figure 4-27. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of a mixture of two polymers: p(PEGMA₄₇₅) and p(DMAEMA) (P1 & P2) at various concentrations..................................................... 204

Figure 4-28. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of a mixture of two polymers: p(PEGMA₄₇₅) and p(DMAEMA) (P1 & P2)...... 205

Figure 4-29. Variations of the size of HEWL aggregates or crystals in the
solution as a function of time during crystallisation experiments induced by a mixture of two polymers: p(PEGMA$_{475}$) and p(DMAEMA) (P1 & P2) .............................................................. 206

Figure 4-30a. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(DMAPMAm) (P4) (DP 38) at various concentrations ......................................................... 211

Figure 4-30b. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(DMAPMAm) (P4) (DP 83) at various concentrations ......................................................... 212

Figure 4-30c. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(DMAPMAm) (P4) (DP 150) at various concentrations ......................................................... 213

Figure 4-31. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(DMAPMAm) (P4) (DP 83) at various concentrations ........... 214

Figure 4-32. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by p(DMAPMAm) (P4) .............................................................. 215

Figure 4-33. Hypothesis of mechanism of cationic polymer mediated HEWL crystallisation ...................................................................................................................... 216

Figure 4-34a. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(AMPS) (P5) (DP 53) at
various concentrations ................................................................. 220

Figure 4-34b. Photomicrographs of HEWL crystals, grown under tetragonal
crystal condition, in the presence of p(AMPS) (P5) (DP 99) at
various concentrations ................................................................. 221

Figure 4-34c. Photomicrographs of HEWL crystals, grown under tetragonal
crystal condition, in the presence of p(AMPS) (P5) (DP 200) at
various concentrations ................................................................. 222

Figure 4-35. Summary of photomicrograph analysis showing changes in the
size and number of HEWL crystals, grown in the presence of
p(AMPS) (P5) (DP 99) at various concentrations ......................... 223

Figure 4-36. Variations of the size of HEWL aggregates or crystals in the
solution as a function of time during crystallisation experiments
induced by p(AMPS) (P5) (DP 99). ................................................. 224

Figure 4-37. SEM micrograph of HEWL crystal produced under tetragonal
conditions in the presence of p(AMPS) (P5), -- x 500
magnification; the scale bar was 50 μm ................................. 225

Figure 4-38a. Photomicrographs of HEWL crystals, grown under tetragonal
crystal condition, in the presence of p(2-AmAA) (P6) (DP 18) at
various concentrations ................................................................. 231

Figure 4-38b. Photomicrographs of HEWL crystals, grown under tetragonal
crystal condition, in the presence of p(2-AmAA) (P6) (DP 46) at
various concentrations ................................................................. 232
Figure 4-38c. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(2-AmAA) (P6) (DP 72) at various concentrations .......................................................... 233

Figure 4-38d. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(2-AmAA) (P6) DP (150) at various concentrations .......................................................... 234

Figure 4-39. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(2-AmAA) (P6) (DP 72) at various concentrations ............... 235

Figure 4-40. HEWL crystal photomicrographs and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of p(2-AmAA) (P6) with DPs 18 and 46................................................................. 236

Figure 4-41. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by p(2-AmAA) (P6)................................................................. 237

Figure 4-42a. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(4-AmBA) (P7) (DP 21) at various concentrations .......................................................... 243

Figure 4-42b. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(4-AmBA) (P7) (DP 50) at various concentrations .......................................................... 244

Figure 4-42c. Photomicrographs of HEWL crystals, grown under tetragonal
crystal condition, in the presence of p(4-AmBA) (P7) (DP 100) at various concentrations ........................................... 245

Figure 4-42d. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(4-AmBA) (P7) (DP 200) at various concentrations ........................................... 246

Figure 4-43. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(4-AmBA) (P7) (DP 100) at various concentrations ............... 247

Figure 4-44. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of p(4-AmBA) (P7) ........................................................................ 248

Figure 4-45. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by p(4-AmBA) (P7) ......................................................... 249

Figure 4-46. Hypothesis of mechanism of anionic polymer mediated HEWL crystallisation .................................................. 250

Figure 4-47. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(METMAC-co-MEDSAH) (P8) at various concentrations ................................................. 252

Figure 4-48. Summary of photomicrograph analysis of HEWL crystals, grown in the presence of p(METMAC-co-MEDSAH) (P8) at various concentrations ................................................................. 253
Figure 4-49. Hypothesis of mechanisms of cationic/zwitterionic polymer mediated HEWL crystallisation .................................................. 254

Figure 5-1. Schematic representation of the interaction of various polymers with anionic concanavalin A (Con A) in the protein crystallisation process ........................................................................................................ 260

Figure 5-2. Selected photomicrographs of Con A crystals, grown in the presence of p(PEGMA475) (P1) at various concentrations ........ 265

Figure 5-3. Summary of photomicrograph analysis of Con A crystals, grown in the presence of p(PEGMA475) (P1) at various concentrations.... 266

Figure 5-4. Con A crystals photomicrographs and X-ray diffraction patterns yielded by the single Con A crystal in the presence of neutral p(PEGMA475) (P1) ................................................................................ 267

Figure 5-5. Summary of DLS data of Con A crystallisation under neutral p(PEGMA475) (P1) at time points T_0 and T_{120min} .................. 268

Figure 5-6. Variations of the size of Con A aggregates or crystals in the solution as a function of time during crystallisation experiments induced by neutral p(PEGMA475) (P1) ................................................................................ 269

Figure 5-7. SEM micrographs of Con A crystals formed by sitting drop method .................................................................................. 270

Figure 5-8. Schematic representation of hypothesis of anionic Con A crystallisation process without polymer (A); and the interaction of neutral p(PEGMA475) (P1) with anionic Con A in the protein
crystallisation process (B).............................................................. 271

Figure 5-9. Photomicrographs of Con A crystals, grown in the presence of quaternised p(DMAEMA) (P2) at various concentrations ....... 273

Figure 5-10. Summary of photomicrograph analysis of Con A crystals, grown in the presence of quaternised p(DMAEMA) (P2) at various concentrations................................................................. 274

Figure 5-11. Summary of DLS data of Con A crystallisation under quaternised p(DMAEMA) (P2) at time points T₀ and T₁₂₀min................. 275

Figure 5-12. Variations of the size of Con A aggregates or crystals in the solution as a function of time during crystallisation experiments induced by cationic quaternised p(DMAEMA) (P2).............. 276

Figure 5-13. SEM micrographs of Con A crystals formed in the presence of quaternised p(DMAEMA) (P2).............................................. 276

Figure 5-14. Hypothesis of mechanism of cationic polymer mediated Con A crystallisation................................................................. 277

Figure 5-15. Photomicrographs of Con A crystals, grown in the presence of p(AMPS) (P5) (DP 99) at various concentrations ...................... 279

Figure 5-16. Summary of photomicrograph analysis of Con A crystals, grown in the presence of p(AMPS) (P5) at various concentrations ...... 280

Figure 5-17. Summary of DLS data of Con A crystallisation under anionic p(AMPS) (P5) (DP 99) at time points T₀ and T₁₀₀min ................. 281

Figure 5-18. Variations of the size of Con A aggregates or crystals in the
solution as a function of time during crystallisation experiments induced by anionic p(AMPS) (P5) ........................................... 282

Figure 5-19. SEM micrographs of Con A crystals formed in the presence of p(AMPS) (P5)................................................................. 283

Figure 5-20. Hypothesis of mechanism of anionic polymer mediated Con A crystallisation................................................................. 284

Figure 6-1. Schematic representation of the interaction of various polymers with anionic bovine liver catalase (BLC) in the protein crystallisation process................................................................. 288

Figure 6-2. Photomicrographs of BLC crystals, grown in the presence of p(PEGMA$_{475}$) (P1) at various concentrations ......................... 293

Figure 6-3. Summary of photomicrograph analysis of BLC crystals, grown in the presence of p(PEGMA$_{475}$) (P1) at various concentrations.... 295

Figure 6-4. Summary of DLS data of BLC crystallisation under neutral p(PEGMA$_{475}$) (P1) at time points T$_0$ and T$_{120\text{min}}$ ...................... 296

Figure 6-5. Variations of the size of BLC aggregates or crystals in the solution as a function of time during crystallisation experiments induced by neutral p(PEGMA$_{475}$) (P1)......................................................... 297

Figure 6-6. Schematic representation of hypothesis of anionic BLC crystallisation process in the absence of polymer (A); and the interaction of neutral p(PEGMA$_{475}$) (P1) with anionic BLC in the protein crystallisation process (B) ......................................................... 298

30
Figure 6-7. Hypothesis of mechanism of anionic polymer mediated Con A crystallisation................................................................. 300

Figure 6-8. Summary of DLS data of BLC crystallisation under cationic quaternised p(DMAEMA) (P2) at time points T₀ and T₁₂₀min .... 302

Figure 6-9. Variations of the size of BLC aggregates or crystals in the solution as a function of time during crystallisation experiments induced by cationic quaternised p(DMAEMA) (P2) ........................................... 302

Figure 6-10. Photomicrographs of BLC crystals formed in the presence of p(AMPS) (P5) at various concentrations.............................. 304

Figure 6-11. Summary of photomicrograph analysis of BLC crystals, grown in the presence of p(AMPS) (P5) at various concentrations........ 305

Figure 6-12. BLC crystal photomicrograph and X-ray diffraction patterns yielded by the single BLC crystal in the presence of anionic p(AMPS) (P5)................................................................. 306

Figure 6-13. Summary of DLS data of BLC crystallisation under anionic p(AMPS) (P5) at time points T₀ and T₁₂₀min............................ 307

Figure 6-14. Variations of the size of BLC aggregates or crystals in the solution as a function of time during crystallisation experiments induced by anionic p(AMPS) (P5)..................................................... 308

Figure 6-15. Hypothesis of mechanism of anionic polymer mediated BLC crystallisation............................................................... 309

Figure 7-1. Selected optical photomicrographs of HEWL crystals formed under
conditions of tetragonal crystal-inducing protocol, in the presence of neutral, cationic, anionic and cationic/zwitterionic polymers as solutes in the crystallisation solutions .............................................. 315

Figure 7-2. Schematic representation of the interaction of various polymers with cationic HEWL in the protein crystallisation process ....... 319

Figure 7-3. Summary of photomicrograph analysis showing changes in the size of HEWL crystals, grown in the presence of different classes of polymers at various concentrations .............................................. 321

Figure 7-4. Optical images showing changes of Con A crystals, in the presence of different polymers: neutral p(PEGMA$_{475}$) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5) ...... 322

Figure 7-5. Summary of photomicrograph analysis showing changes in the size of Con A crystals, grown in the presence of different classes of polymers at various concentrations .............................................. 323

Figure 7-6. Optical images showing changes of BLC crystals, in the presence of different polymers: neutral p(PEGMA$_{475}$) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5) ...... 324

Figure 7-7. Predicted growth of HEWL ((i)A) and Con A ((ii)A) particles during crystallisation; and actual variations in the size of HEWL ((i)B-D) and Con A ((ii)B-D) aggregates or crystals in the solution as a function of time during crystallisation experiments induced by different polymers by DLS ......................................................... 325
List of Tables

Table 1-1. Seven crystal lattice systems in three dimensions .......................... 48
Table 1-2. Types of chain growth polymerisation ........................................... 77
Table 3-1. Summary of synthesis of p(PEGMA_{475}) (P1) via ATRP ............... 114
Table 3-2. Summary of synthesis of p(DMAEMA) (P2) via ATRP .................. 118
Table 3-3. Summary of synthesis of p(DMAEMA-stat-PEGMA_{475}) (P3) ..... 125
Table 3-4. Summary of synthesis of p(DMAPMAm) (P4) via RAFT ............... 130
Table 3-5. Summary of synthesis of p(AMPS) (P5) via RAFT ................... 133
Table 3-6. Summary of synthesis of p(2-AmAA) (P6) via RAFT ................. 135
Table 3-7. Summary of synthesis of p(4-AmBA) (P7) via RAFT ................. 138
Table 4-1. Summary of hen egg-white lysozyme (HEWL) ......................... 146
Table 4-2. HEWL crystallisation plate set up: p(PEGMA_{475}) .................... 157
Table 4-3. Unit cell dimensions of HEWL crystals formed in the absence
   (Control) and presence of neutral p(PEGMA_{475}) (P1) ..................... 161
Table 4-4. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2)
   (M_n\sim46.3 \text{kDa}) (quaternised ratio: 50 \%) .............................. 168
Table 4-5. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2)
   (M_n\sim17.1 \text{kDa}) (quaternised ratio: 25 \%) ............................ 172
Table 4-6. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2)
   (M_n\sim17.1 \text{kDa}) (quaternised ratio: 50 \%) ............................ 177
Table 4-7. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2)
(Mₙ~17.1 kDa) (quaternised ratio: 75 %) ........................................ 182

Table 4-8. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2)

(Mₙ~17.1 kDa) (quaternised ratio: 100 %) ........................................ 186

Table 4-9. Unit cell dimension of HEWL crystal formed in the presence of cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %)

.............................................................. 193

Table 4-10. HEWL crystallisation plate: p(DMAEMA-stat-PEGMA₄₇₅) (P3)

.............................................................. 196

Table 4-11. Unit cell dimension of HEWL crystal in the presence of cationic p(DMAEMA-stat-PEGMA₄₇₅) (P3) ........................................ 199

Table 4-12. HEWL crystallisation plate set up: a mixture of two polymers:
p(PEGMA₄₇₅) and p(DMAEMA) (P1 & P2) ........................................ 201

Table 4-13. Unit cell dimension of HEWL crystal in the presence of a mixture of two polymers: p(PEGMA₄₇₅) and p(DMAEMA) (P1 & P2). 205

Table 4-14a. HEWL crystallisation plate set up: p( DMAPMAm) (P4) (DP 38)

.............................................................. 207

Table 4-14b. HEWL crystallisation plate set up: p( DMAPMAm) (P4) (DP 83)

.............................................................. 208

Table 4-14c. HEWL crystallisation plate set up: p( DMAPMAm) (P4) (DP 150)

.............................................................. 209

Table 4-15a. HEWL crystallisation plate set up: p(AMPS) (P5) (DP 53) ..... 217

Table 4-15b. HEWL crystallisation plate set up: p(AMPS) (P5) (DP 99) ..... 218
Table 4-15c. HEWL crystallisation plate set up: p(AMPS) (P5) (DP 200) .... 219
Table 4-16a. HEWL crystallisation plate set up: p(2-AmAA) (P6) (DP 18). 226
Table 4-16b. HEWL crystallisation plate set up: p(2-AmAA) (P6) (DP 46). 227
Table 4-16c. HEWL crystallisation plate set up: p(2-AmAA) (P6) (DP 72). 228
Table 4-16d. HEWL crystallisation plate set up: p(2-AmAA) (P6) (DP 150) 229
Table 4-17. Unit cell dimensions of HEWL crystal in the presence of anionic
p(2-AmAA) (P6) .............................................................. 236
Table 4-18a. HEWL crystallisation plate set up: p(4-AmBA) (P7) (DP 21). 238
Table 4-18b. HEWL crystallisation plate set up: p(4-AmBA) (P7) (DP 50). 239
Table 4-18c. HEWL crystallisation plate set up: p(4-AmBA) (P7) (DP 100) 240
Table 4-18d. HEWL crystallisation plate set up: p(4-AmBA) (P7) (DP 200) 241
Table 4-19. Unit cell dimension of HEWL crystal in the presence of anionic
p(4-AmBA) (P7) .............................................................. 248
Table 4-20. HEWL crystallisation plate set up: p(METMAC-co-MEDSAH) (P8) .............................................................. 251
Table 4-21. Summary of polymers applied to HEWL crystallisation .......... 255
Table 5-1. Summary of concanavalin A (Con A) ........................................ 259
Table 5-2. Con A crystallisation plate set up: p(PEGMA475) ...................... 264
Table 5-3. Diffraction data collection for Con A crystals formed in the absence
(Control) and presence of neutral p(PEGMA475) (P1) ...................... 267
Table 5-4. Con A crystallisation plate set up: quaternised p(DMAEMA) (P2)
(quaternised ratio: 75 %) .............................................................. 272
Table 5-5. Con A crystallisation plate set up: p(AMPS) (P5) (DP 99) .......... 278

Table 6-1. Summary of bovine liver catalase (BLC) ............................................. 287

Table 6-2. BLC crystallisation plate set up: p(PEGMA₄₇₅) ........................................ 292

Table 6-3. BLC crystallisation plate set up: quaternised p(DMAEMA) (P2) 299

Table 6-4. BLC crystallisation plate set up: p(AMPS) (P5) (DP 99) .......... 303

Table 6-5. Diffraction data collection for BLC crystal ............................................. 306
1. Introduction

1.1 Introduction to chapter

The overall aim of this project was to generate various polymers with varying degrees of charge, molecular weight and backbone structure, and then to study their roles as solution ‘additives’ in model protein crystallisation process. This chapter will explain why we need protein crystals, the importance of protein crystallisation and will focus on polymer additives in protein crystallisation. It will cover general principles of crystallisation, various crystallisation techniques, research and challenge in the crystallisation of protein so far, and an introduction of synthetic methods of polymers and three different model protein targets: hen egg white lysozyme (HEWL), concanavalin A (Con A) and bovine liver catalase (BLC).

1.2 General protein introduction

1.2.1 Analysis of protein structure

Proteins are a heterogeneous class of biological macromolecules with diameters of 1-100 nm. They are polypeptides made of many amino acids, which linked together via peptide bonds in a linear chain-like arrangement. Natural proteins are formed by up to 20 standard L-alpha amino acids, which also referred to as ‘residues’. Protein differs from one another due to the
difference of their order of the amino acid residues, which is defined by the sequence of their gene. A specific sequence of the amino acid residues in a protein is called ‘primary structure’. The ‘secondary structure’ refers to local sub-structures. Some areas within a protein are folded or coiled to form secondary structure, which are maintained by hydrogen bonding between amino acids in different regions. There are two kinds of secondary structure: alpha-helices or beta-pleated sheets.

The ‘tertiary structure’ is the final three-dimensional structure of a protein. The alpha-helices or beta-pleated sheets are folded further to form a compact globular structure, which driven by various interactions between atoms and molecules, including van der Waals forces, hydrogen bonding, electronic interactions between charged groups and hydrophobic interactions; and, stabilised by covalent disulphide bonds or salt bridges. Moreover, some proteins, which consist of several protein molecules (so called multi-subunit) as single large proteins have another level of structure: ‘quaternary structure’.

In this protein, each subunit has its own three-dimensional tertiary structure. All subunits associated with each other to form the final functional protein by the same non-covalent interactions or disulphide bonds as that stabilise the tertiary structure. The level of protein structures is shown in Figure 1-1.
Figure 1-1. Main protein structure levels.\(^1\) The amino acid sequence of a protein’s polypeptide chain is called primary structure and F, T, P, A, V etc. represent different amino acids: F represents phenylalanine, T represents threonine, P represents proline, A represents alanine, V represents valine, H represents histidine, L represents leucine, D represents aspartic acid, K represents lysine, S represents serine, Y represents tyrosine. C represents carboxyl group, N represents amino group.

The tertiary or quaternary structure of a protein can play a vital role to understanding the function of protein macromolecules. Currently, two main methods have been employed for determining the three-dimensional structure of a protein: nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. Although the NMR method can resolve the atomic structure of many small proteins, there is an upper molecular weight limit of about 25 kDa, or approximately 220 amino acids long for the NMR method. However, the primary tool for determining the three dimensional structure of proteins at the moment is X-ray crystallography, which is much more suitable for determining the structure of larger proteins (> 20 kDa),\(^2\) but is dependent on the availability
of good quality protein crystals. For the X-ray crystallography method, the target proteins must undergo crystallisation first, in order to provide a high quality single crystal. X-rays can be considered waves of electromagnetic radiation. The regular arrays of atoms in the crystal cause the beams of X-ray to diffract into many directions. By measuring the intensities and angles of these diffracted beams, a three-dimensional picture of electron density within the crystal can be obtained by a crystallographer. Then this three-dimensional image can be used to identify the protein structure. Thus, protein crystallisation serves as a basis for X-ray crystallography. By using X-ray method, the studies of the structures of myoglobin in 1950 and hemoglobin in 1955 were awarded the Nobel Prize in Chemistry in 1962. Now, almost 80% of all protein structures are determined by X-ray crystallography.

The information of tertiary or quaternary structure of the protein is useful in numerous ways. Furthermore, protein crystallographic study is becoming an essential interest in many science and technology areas today, including biotechnological, pharmaceutical and chemical industries, such as drug design and protein engineering (Figure 1-2).
Figure 1-2. Use of protein crystallography.\(^5\) Diagram showing the application of the crystalline protein in various science areas, by the pathway of combination of X-ray crystallography, computer graphic analysis and synthetic chemistry.

One application of protein crystallography is the rational drug design. Once the tertiary or quaternary structure of the protein is elucidated, it can be used to identify their active sites, possible areas of interactions with other macromolecules, or similarities in structures with other known proteins. For example, in some diseases, such as cancer, some proteins are always considered as drug targets, because they can activate certain biological pathways of the disease. Once the structure of these proteins is known, the
information can be used to guide the design of molecules to fit the active sites of the protein, thereby modifying its function. Typically these designed molecules will be inhibitors of protein function, thus the molecules act as drugs. This kind of method for drug development is known as structure-based design. For example, imatinib, marketed as Glivec or Gleevec, is a tyrosine kinase inhibitor used in the treatment of multiple cancers.\footnote{6}

Besides structure and function characterisation purposes, protein crystallography is also important in industrial biocatalysis. The stability of biocatalysts in a reaction environment is a key issue in industrial biocatalysis. One approach to increasing their operational stability is crystallisation.\footnote{7}

Cross-linked protein crystals (CLPCs) with high activity and stability have been studied in biocatalytic applications. For instance, xylose isomerase crystals cross-linked with glutaraldehyde and L-lysine have been used as a catalyst for the isomerization of D-glucose to D-fructose.\footnote{8, 9}

Aside from the above aspects, the isolation of proteins in crystalline form is essential in many key pharmaceutical areas.\footnote{5} There are other applications where defined assemblies of proteins, either crystals or self-assembled protein nanoparticles, are desirable. These include protein formulations.

\textbf{1.2.2 Protein therapeutics}

Proteins are involved in key cellular functions in the body, which provide the foundations to maintain the homeostasis of the organism. An increased
understanding of the roles of proteins in health and disease is crucial for the identification of new treatment options. Currently, the use of proteins as novel drugs is now well accepted in the treatment of certain diseases. Moreover, recent recombinant DNA techniques allow the production of novel proteins in large quantities.\textsuperscript{10, 11} Protein drugs have several advantages over small drug molecules, such as proteins often have highly specific action, do not interfere with normal biological processes, and therefore, adverse effects are decreased. Moreover, protein therapeutics is more tolerated, because the body produces a lot of proteins naturally. Despite these advantages, the use of protein drugs \textit{in vivo} still has some obstacles, including high molecular weight, structural fragility, short life time and highly sensitive to enzymes.\textsuperscript{12, 13, 14} The oral route of administration for proteins is problematic; generally they have very poor pharmacokinetics. Protein drugs macromolecules are easily degraded and denatured by the enzymes; the high acidity of the stomach could destroy them before they reach the intestine for absorption; and they must cross several biological barriers before reach the site of action.\textsuperscript{15} Therefore, improving the stability of protein drugs is very essential for their applications.

One method is encapsulating proteins inside biodegradable polymer shells, which serves to protect the protein from clearing by the body and allow for the sustained and targeted delivery of protein drugs. Poly(lactic-co-glycolic acid) (PLGA) polymers have been studied and used for encapsulating of proteins.\textsuperscript{16} The encapsulation of the protein is typically via emulsification\textsuperscript{17, 18} or direct
incorporation of solid protein\textsuperscript{19, 20} as shown in Figure 1-3\textsuperscript{21}

![Diagram showing encapsulation methods](image)

Figure 1-3. Encapsulation of therapeutic protein into polymeric particles by a) emulsification and b) solid incorporation method, taken from Reference\textsuperscript{21}

For the emulsification method, the protein is dissolved in an aqueous solution first and then dispersed in an organic polymer solution to get a protein in polymer solution. After evaporated the organic solvent, PLGA was precipitated around protein macromolecules to form microspheres.\textsuperscript{17, 18} For the direct incorporation of solid protein method, micronised solid protein is directly
suspended in an organic polymer solution via sonication. Then microspheres are obtained after the solution is frozen and organic solvent is extracted.\textsuperscript{19, 20} This encapsulating protein inside biodegradable polymer shells method does not alter the native properties of the protein. Moreover, it can provide a sustained release of the protein over a few hours or days.\textsuperscript{21} Another method is making the protein more stable by changing some of its amino acids, which can optimize the hydrogen bonding and electrostatic interactions of protein.\textsuperscript{22, 23} For instance, granulocyte-colony stimulating factor (G-CSF) is limited by its low stability, by engineering of its structure, its thermal stability was enhanced of up to 13 °C; the shelf time was improved, and the activity was retained \textit{in vitro}.\textsuperscript{23} Moreover, attaching and conjugating the protein to a polymer chain is a successful approach. Polyethylene glycol (PEG) is the most commonly employed polymer. The polymer can alter the solubility of protein and creates a protection barrier on the surface of the protein.\textsuperscript{24} In addition of all above methods, making protein nanocrystals or nanoaggregates (i.e. particles bigger than an individual protein molecule, but small enough to be colloidally stable) is another essential approach in protein therapeutics. Crystallised protein drug macromolecules remain in active form within the crystal lattice and hence are protected during storage and drug release process.\textsuperscript{25} Moreover, protein crystals can act as carrier-free delivery systems; have the ability of sustained release for the protein drugs, which
reduces in frequency of intakes, adverse effects and patient compliance. Furthermore, subtle changes in protein crystallisation conditions may result in multiple crystal structures from the same crystallising molecules, so called ‘polymorphs’. Different polymorphs have different physical properties, for instance crystal habit, melting point and dissolution rate. If produced different polymorphs have different dissolution rate and solubility, they can alter important pharmacokinetic factors, such as absorption rate, drug availability. For some unstable protein crystals, if different polymorphs of proteins were produced, which can enhance their stability; this might change the storage shelf life of the proteins similar as small drug molecules. Therefore, proteins as drugs, in the form of crystals, can show promise in controlled and sustained delivery and aid in their formulation.

1.3 Crystals

Crystals are physically homogeneous solids, whose atoms, ions, or molecules are in accordance with an ordered pattern extending in all three spatial dimensions. Many of them have a particular well-defined geometrical shape, with regular faces and sharp edges. Crystals exist widely in nature; the vast majority of solids are crystals, such as ice, salts and rocks. Figure 1-4 is a model of crystal structure of calcium fluoride.
Figure 1-4. The crystal structure of Fluorite (CaF$_2$). The lattice is a cubic crystal system with unit cell dimensions of $a = b = c = 5.46$ Å, $\alpha = \beta = \gamma = 90^\circ$. Calcium ions (red) reside at the vertices and face centers of a cube, with fluorine ions (green) in the tetrahedral interstices. Calcium ion centers are 8-coordinate, being centered in a ‘box’ for 8 fluorine ions. Fluorine ion center is coordinate to 4 calcium ions.

Crystallisation is the process by which crystals are formed. The crystallisation process of molecules from a solution is a reversible equilibrium phenomenon, driven by the minimization of the free energy of the system.$^{30}$ For a stable solution, the solute is fully dissolved, and the system is at equilibrium. However, if more solute molecules are added to the solution, and they could not dissolve completely, thus, a new state (the so-called ‘supersaturated state’) will appear. A supersaturated solution represents a chemical system which is not at equilibrium.$^{31, 32, 33}$ The system is thermodynamically driven to a new
equilibrium state with a new minimized free energy. Particular interactions occur between individual solute molecules and they are forced out from the solution and start to form amorphous aggregates. If these interactions are geometrically favorable, these amorphous aggregates will form crystal nuclei. As a result, individual solute molecules align themselves in a repeating series of ‘unit cells’ by adopting a consistent orientation. The crystalline ‘lattice’ then forms together by non-covalent interactions.34

There are seven crystal lattice systems including triclinic, monoclinic, orthorhombic, rhombohedral, tetragonal, hexagonal and cubic as shown in Table 1-1. One type of symmetry used to categorize crystal structures: axis; and the parameters used to give unit cell, which are the lengths of the cell edges (a, b and c) and the angles between them (α, β and γ).

Table 1-1. Seven crystal lattice systems in three dimensions

<table>
<thead>
<tr>
<th>Lattice System</th>
<th>Axis of Symmetry</th>
<th>Axial Length of Unit Cell</th>
<th>Inter Axial Angles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triclinic</td>
<td>0 axes</td>
<td>a ≠ b ≠ c</td>
<td>α ≠ β ≠ γ ≠ 90°</td>
</tr>
<tr>
<td>Monoclinic</td>
<td>1 two-fold axis</td>
<td>a ≠ b ≠ c</td>
<td>α = γ = 90°, β ≠ 90°</td>
</tr>
<tr>
<td>Orthorhombic</td>
<td>3 two-fold axes</td>
<td>a ≠ b ≠ c</td>
<td>α = β = γ = 90°</td>
</tr>
<tr>
<td>Rhombohedral</td>
<td>1 three-fold axis</td>
<td>a = b = c</td>
<td>α = β = γ ≠ 90°</td>
</tr>
<tr>
<td>Tetragonal</td>
<td>1 four-fold axis</td>
<td>a = b ≠ c</td>
<td>α = β = γ = 90°</td>
</tr>
<tr>
<td>Hexagonal</td>
<td>1 six-fold axis</td>
<td>a = b ≠ c</td>
<td>α = β = γ = 90°</td>
</tr>
<tr>
<td>Cubic</td>
<td>Minimum of 4 three-fold axes</td>
<td>a= b = c</td>
<td>α = β = γ = 90°</td>
</tr>
</tbody>
</table>

There are three stages of crystallisation: nucleation, growth, and cessation of growth. Nucleation is the initial step of the formation of a crystal from a solution. It normally occurs at nucleation sites on surfaces contacting the liquid.
Figure 1-5 shows the process of molecules transfer from solution to crystal. First, individual solute molecules come together at a supersaturated state and produce a stable aggregate. The aggregate must get large enough and first reach a ‘critical size’, which is defined by the competition of the ratio of the surface area of the aggregate to its volume.\textsuperscript{35,36} Once the critical size is reached and exceeded, the aggregate becomes a critical nucleus and can grow further. If the size of the aggregate is smaller than the critical nucleus size, it would spontaneously dissolve in the solution.\textsuperscript{37} This phenomenon is known as Gibbs-Thomson effect; and small free Gibbs energy change is the driven force of molecules transfer from solution to crystal.

Figure 1-5. Diagram showing the transfer of molecules from solution to crystal according to Gibbs energy change.
For a spherical nucleus with radius $r$ in the classic case:

$$\Delta G_r = (4/3) \pi r^3 \Delta G_v + 4 \pi r^2 \gamma$$

Where $G_v$ is the Gibbs volume energy and $\gamma$ is the surface energy. The graph (Figure 1-6) below shows the variation in the Gibbs free energy of nucleation with the increasing size of the radius.

Figure 1-6. Thermodynamics of nucleation, according to Gibbs free energy.

For a spherical nucleus with a radius $r > r^*$, the Gibbs free energy will decrease if the nucleus grows. The $r^*$ is the critical nucleus size; $\Delta G^*$ is the nucleation barrier.

Since the degree of occurrence of nucleation and crystal size are determined by the degree of supersaturation of the solution, which is in turn related to the solubility of solute molecules, to some extent by the degree of supersaturation this means that the nucleation can be controlled. The solution with a higher
solubility has many more molecules when at a supersaturated state than the solution with lower solubility, can increase the chance of collisions of diffusing molecules. This leads to an increase in a probability of the formation of stable aggregate. If the number of solute molecules was finite, in this case, it would result in the formation of many small crystals. While, at lower solute concentrations, it is not easy to form individual stable aggregate; thus this condition is much more favoring the formation of single crystals.

Once the critical nuclei are formed, then by adding more atoms, ions and molecules into the characteristic arrangement of the crystalline lattice, crystal growth stage happens. When the solution is supersaturated, nucleation and crystal growth occur simultaneously. They all are driven by the existence of supersaturation. Thus, either of them can be predominant. This means crystal size can in principle be controlled. If the nucleation is predominant over crystal growth, the system tends to produce a large number of small crystals. Otherwise, if the crystal growth is predominant, a small number of large crystals tends to result.

The growth of crystals is strongly affected by diffusion and convection effects. The concentration of solute molecules in the regions near the growing crystal is lower than the rest of the solution, leading to the formation of density gradients in the solution, under the effects of gravity convection currents form in these zones. These convection currents in turn dominate the diffusion rate.

Finally, crystal growth will be terminated for several reasons. The main reason
is that, as the crystallising solute concentration decreases, the solution system reaches equilibrium, or the crystal has reached a particular size and cannot grow further. This may be a result of the ‘poisoning’ of the growth surface. For example, some damaged crystallising solute molecules, which are on the growth surface of crystal, could result in the interruption of the crystal lattice growth.

1.4 Protein crystals

Proteins have become particular targets for crystallisation in recent years owing to their growing importance in pharmaceutical applications. Protein crystals contain protein molecules, similar as other materials, but much larger. However, unlike other compound crystals, protein crystals are composed of about 60%-70% solvent content. The entire crystal consists of large solvent channels that solvent and other small molecules can diffuse through freely. Thus, their packing in the crystals composes of many holes that are filled with water molecules (ordered or disordered), thus, protein crystals are extremely fragile and instable. An example of protein crystal lattice structure is shown in Figure 1-7, the structure of chymotrypsinogen crystal, showing the large solvent channels penetrating the unit cell.
Figure 1-7. A view of the crystal structure of chymotrypsinogen. The molecular packing in the unit cell of chymotrypsinogen crystal consists of several large holes of solvent channels.43

In the protein crystal, the contacts between protein molecules are quite complex, involving a delicate balance of specific and nonspecific flexible interactions,44,45 such as hydrogen bonds, salt bridges, electrostatic interactions, van der Waals interactions and hydrophobic interactions. All these interactions together provide for the maintenance of the crystal. In the entire crystal system, the number of these protein-protein interactions is far exceeding the number of crystalline protein molecules, this can explain the differences between protein molecules and small molecules crystals.46

1.4.1 Some history of protein crystallisation

Protein crystallisation started at 19th century. The first reported protein crystallisation was by Hünefeld in 1840, even before X-ray was known. The
protein was hemoglobin, which was from the earthworm. Hünefeld pressed the worm’s blood between two slides of glass and caused the dehydration of the protein, resulting in flat plate-like hemoglobin crystals. After that, many researchers focused on studying the crystallisation of hemoglobin from various sources and tried to find a standard procedure in the next several years. However, there was no general procedure for hemoglobin crystal growth, until in 1851, Fünke found and reported the successful and reproducible methods for the crystallisation of hemoglobin.

Following hemoglobin, other proteins such as plant seed reserve proteins were widely investigated in the last quarter of the 19th century by many researchers such as Osborne. The meaning of these researches for us today is that researchers developed several crystallisation approaches and now still used in common, included slow cooling, dialysis against low concentration of an ionic solution and use of organic solvents such as alcohol as precipitants.

Between 1900 and 1940, researchers started to turn their attention on enzymes, such as urease and insulin. Crystallisation was then considered as an important tool in purifying complex proteins. After the use of X-rays for medical purposes, Laue reported the diffraction of X-rays by crystals for the first time in 1912. Then Ewald and Bragg gave birth to the field of X-ray crystallography, which considered as an important tool for structure analysis of proteins. Today, about 83% of protein structures deposited in the Protein Data Bank (PDB) was obtained by X-ray crystallography.
1.4.2 Principle of protein crystallisation

In general, protein crystallisation from solution is an extremely complex process. As mentioned in section 1.3, nucleation is the vital step in the crystallisation process and has been well studied in detail. The crystallisation solution must reach a supersaturation state and overcome the free energy barrier for aggregation first, and then nucleation could occur. There is a classical phase diagram of the solubility curve showing the explanation of crystal nuclei formation and growth, which illustrate the change of protein molecules concentration against crystallising agent concentration (Figure 1-8).

![Crystallisation phase diagram of solubility curve showing how the solubility varies with the concentration of crystallising agent. The concentration area is divided by the solubility curve into two spaces: undersaturated and supersaturated zone. The supersaturated zone comprises of metastable, nucleation and precipitation zones.](image-url)
The whole concentration space is divided by the solubility curve into two areas: the undersaturated and supersaturated zones. The area under the solubility curve is undersaturated zone, where the protein concentrations below its solubility limit. In such solution, no crystallisation will take place. Above the solubility curve is the supersaturation zone, which comprises of three zones: metastable zone, nucleation zone and precipitation zone depending on the level of supersaturation. In the metastable zone, the supersaturation is too small; nucleation does not spontaneously occur. However, if the solution is seeded first, crystals may grow. It is therefore possible to induce nucleation in a selective and deliberate way to control the number of nuclei and the supersaturation point at which they will grow.\textsuperscript{58} In the nucleation zone, the supersaturation is large enough, critical nuclei can form, spontaneous nucleation occurs, and crystals can grow rapidly. In the precipitation zone, the supersaturation is too large, protein aggregates rapidly precipitate from the solution.\textsuperscript{32}

1.4.3 Methods of protein crystallisation

Protein crystallisation from a solution can be affected by many factors, including biochemical factors, such as protein sample purity, storage and pI; chemical factors, such as sample concentration, precipitant type, precipitant concentration, ionic strength, environment buffer and pH; physical factors, such as temperature, time, viscosity, sample handling and methodology.\textsuperscript{59, 60}
Besides these most well-known factors, there are still many other factors which can in principle influence protein crystallisation, including exactly known ones or not known ones. There are no systematic studies of all these factors on protein crystallisation due to the amount of parameter space to cover.

In order to form high quality crystals, the protein must ideally 100 % pure without any contaminations. Any contamination may lead to poorer crystals, or an amorphous precipitate, or no precipitation at all. Based on the above parameters, many different methods and approaches for creating supersaturation and thus inducing the nucleation process are possible.

One method for protein crystallisation is controlling the solubility of the protein (that is, alter the supersaturation), which is always very sensitive to temperature and pH as known. Protein solubility is temperature dependent, due to protonation and deprotonation reaction constants of the amino acid side chains in the protein structure being temperature dependent. In many cases, temperature maintains constant during crystallisation to obtain fine crystals. Most proteins are crystallised at 4 °C or 18-22 °C. Temperature also affects the kinetic energy of crystallisation, at lower temperatures the protein molecules have lower kinetic energy and can aggregate in an ordered manner more easily. Thus, many earlier proteins crystallisation were induced by slow cooling. For example, glucagon crystallised by dissolving in solution and cooling from 60 °C to room temperature.

Another method for inducing protein crystallisation is to control the
environment pH. Most proteins tend to share the ability to form crystals and crystallise at biological pH of 6.5-7.5. Different pH can produce different packing orientations of unit cell,\textsuperscript{67} because of that different pH can change the electrostatic character of protein macromolecules. The choice of pH is according to the optimum pH required (does not denature the protein) and also considering the isoelectric point of the protein (pI), because of solubility minimum at its pI.

Besides the control of temperature and pH, chemical precipitating agents are common used methods for achieving supersaturation of proteins and inducing crystallisation. Precipitating agents for protein crystallisation are generally divided into three main categories: salts, such as ammonium sulfate; organic solvents, such as ethanol; and large polymer precipitating agents.\textsuperscript{68}

Moreover, various physical techniques have been developed for reaching supersaturation thus inducing protein crystallisation. Among them, three main methods are commonly used: batch and micro-batch; dialysis; and vapour diffusion.

Batch method is a simple and old crystallisation technique. In this method, by mixing the concentrated protein with concentrated precipitating agent, the solution immediately reaches a high supersaturated state and therefore, crystallisation is induced. This method usually needs large volumes of solutions (upward of 1 mL), and typically results in larger crystals. Chayen designed micro-batch method (1992)\textsuperscript{69} as shown in Figure 1-9 and stated an
approach of rapid protein crystallisation by using very small sample droplets as little as 1 µl into an inert oil. The oil can prevent the evaporation of solvent.

![Diagram](Image)

**Figure 1-9.** Micro-batch crystallisation technique.

There are some disadvantages by using micro-batch method. First, if the precipitant concentration which chosen isn’t high enough to obtain supersaturation, crystallisation will never occurs. Furthermore, it is very difficult to manipulate of the crystals from the droplet covered by the oil.

Contrary to batch method, in dialysis method, the solution composition is changed by diffusion of small molecules. In a dialysis crystallisation experiment, the protein solution is contained in a dialysis semipermeable membrane, surrounded by a large volume of precipitant solution. For example, in button dialysis crystallisation (Figure 1-10), a dialysis membrane covers the dialysis button, which allowing the diffusion of solvent into the protein solution through the dialysis membrane. The solute molecules in the protein solution slowly equilibrate against the large volume of precipitant solution, leading to protein crystallisation within the dialysis button.
However, dialysis method does not work with some precipitant solutions, such as concentrated PEG solutions. Because PEG solutions prefer to draw all the water out of the button faster than dialysis occurs, thus, protein will precipitate rapidly rather than crystallise.

The most popular technique is vapour diffusion method. By using this method, we can screen a large number of crystallisation conditions by varying the composition of each well solution. In the vapour diffusion method, protein solutions achieve supersaturation by the evaporation and diffusion of water between solutions with different concentrations slowly. The technique used during this research was the vapour diffusion method. There are three kinds of vapour diffusion methods: hanging drop, sandwich drop and sitting drop (Figure 1-11).
Figure 1-11. Vapour diffusion techniques: hanging drop, sandwich drop and sitting drop.

A drop of 1:1 protein: precipitant solution is normally used in the sample well in the crystallisation experiment. In the hanging drop method, the protein and precipitant solution droplet is suspended from a coverslip. The volume of the droplet is limited; otherwise, it will fall down. In the sandwich drop method, protein and precipitant solution droplet is in contact with both an upper and a lower surface. This method is rarely used due to the two coverslips will reduce the exposure area of the droplet and slow down the rate of water evaporation. In the sitting drop method, protein and precipitant solution droplet is supported by a surface. It is easy to conduct and require very small amount of sample. Using this method, crystallisation takes place when the concentrations of
protein and precipitant increase in the droplet by water vapor from the droplet to the reservoir solution. This process will last until the concentration of precipitant in the droplet equals that of the reservoir solution.

1.5 Use of additives in protein crystallisation

A variety of approaches have been used to aid the production of protein crystals; the most common strategy involves the addition of nucleants, typically surfaces or surface-active materials into the crystallisation solution. Solution ‘additives’ of many types have proven useful in the control of protein crystallisation. These additives can act via multiple mechanisms, i.e. they can serve to bridge protein segments, electrostatically, or via hydrogen bonding or hydrophobic interactions. It is also possible for additives to diminish interactions between protein macromolecules and solvent to act as solubility-modifying detergents; or to stabilise intermediate structures/conformations of proteins during the early stages of crystallisation. There are two principal types of additives, including soluble materials, which added into the crystallisation solution directly, such as precipitants; and non-dissolved solid surfaces, which act as heterogeneous nucleants.

1.5.1 Non polymer methods

1.5.1.1 Salts

Salts act as precipitating agents, according to the ‘salting out’ principle.
ionic strength of the solution increases by adding salts, which in turn leads to a decrease in protein solubility. A simple explanation of the salting out effect is that salt ions, principally anions, compete with protein for solvent molecules, i.e. water, thus, salts can dehydrate the protein macromolecules. When the concentration of salts is sufficiently high and the competition is intense enough, protein molecules are forced to neutralise their surface charges by interacting with one another and then precipitate from the solution, which may result in an ordered arrangement of proteins in the crystalline form. Figure 1-12 shows the typical protein solubility behavior over the entire range of salt concentrations, including ‘salting in’ and ‘salting out’ regions.

Figure 1-12. The solubility curves of a typical protein, enolase, as a function of ionic strength produced by two types of salts. The regions of the curves with lower ionic strength where the solubility increases are called ‘salting in’ region; the regions of the curves with higher ionic strength where the solubility decreases are called the ‘salting out’ region.

The ‘Hofmeister series’ shows the order of salting out effect of the ions.
SO$_4^{2-}$ > HPO$_4^{2-}$ > CH$_3$COO$^-$ > citrate$^{3-}$ > tartrate$^{2-}$ > HCO$_3^-$ > CrCO$^{3-}$ > Cl$^-$ > NO$_3^-$ > ClO$_3^-$ for anionic proteins, while cationic ones are reverse.$^{63, 82}$ The salts commonly used in protein crystallisation involve ammonium or sodium sulfate, ammonium or sodium acetate, sodium or potassium phosphate, sodium or ammonium citrate and sodium chloride.$^{59, 60}$

1.5.1.2 Organic solvents

Organic solvents can also promote protein crystallisation by a similar competition with protein for water molecules. They can bind to water molecules and alter the interaction between water and protein molecules by H-bonding, thus leading to solvent competition and forcing protein molecules out of the solution. Besides, they may also have ‘excluded volume effects’ by hydrophobic exclusion of protein solutes. Furthermore, they can reduce the dielectric constant of the medium. Coulomb interactions between the individual protein molecules become stronger. Thus, they decrease the capacity of protein molecules and force them out of the solution.$^{83, 84}$ Organic solvents should be added very slowly to mix thoroughly and used at very low temperature or below 0 °C to avoid volatilization.$^5$ The common organic solvents are ethanol, acetone and dioxane.$^{85}$

1.5.1.3 Insoluble surfaces

Crystallisation controlled by insoluble solid surfaces is an approach to template
protein crystal growth via heterogeneous nucleation. A number of insoluble solid additives has been studied in protein crystallisation, such as minerals,\textsuperscript{86,87,88} human hair,\textsuperscript{89} hydrophobic self-assembled monolayers,\textsuperscript{90} porous glass and silicon,\textsuperscript{91,92} silicon-based devices,\textsuperscript{93} and chemically treated substrate surfaces, i.e. glass\textsuperscript{94} and mica.\textsuperscript{95}

Figure 1-13 is an example shows heterogeneous nucleation facilitated by the insoluble solid surface. First, the heterogeneous nucleant attracts and immobilises protein molecules on to its surface through some chemical, electrostatical or structural favor interactions to form protein aggregates. Then these protein aggregates start to form ordered nuclei by adopting a consistent orientation, and are capable of further growth.\textsuperscript{5}

![Figure 1-13. Schematic representation of heterogeneous nucleation facilitate by the insoluble solid surface.](image)

The mechanisms by which these insoluble solid surfaces induce heterogeneous nucleation are various. They potentially include: some materials with similar lattice characteristics with protein crystalline lattice that may affect protein crystallisation process,\textsuperscript{87} utilisation of the surface microstructure,\textsuperscript{92,96} i.e. roughness to promote protein nucleation, or use of surface chemistry properties to afford some specific interactions with protein molecules.\textsuperscript{95}
One example of insoluble solid additives is mineral. For instance, McPherson et al. tested fifty different mineral samples as heterogeneous nucleants to mediate crystallisation of four common proteins: canavalin, concanavalin B, beef liver catalase and hen egg lysozyme. Some example images of protein crystals are shown in Figure 1-14. They stated that, by the use of mineral surfaces, nucleation of canavalin and beef liver catalase was promoted at lower critical supersaturation levels, and the crystal habit of each of four proteins was modified. Moreover, the mineral apophyllite with a close lattice match to lysozyme, had a clear influence on the nucleation and crystal growth of protein crystal, which could illustrate the possible mechanism involved a direct lattice match.

Figure 1-14. Some example images of protein crystals the nucleated and grew on the mineral substrate surfaces. A and D: Lysozyme crystals on lepidolite and magnetite. B and C: Concanavalin B crystals on gypsum and aragonite.

Another example of insoluble solid additives for enhancing the nucleation of protein crystals is porous substrate, i.e. glass and silicon. For example,
Chayen et al. used porous silicon, which composed of nanoscale crystalline silicon wires and dots surrounded by voids, to enhance nucleation and crystallisation of proteins. The mechanism they proposed involved local supersaturation of protein molecules associated with the fractality of the porous substrate. The local concentration of protein molecules, which were inside and in the close vicinity of the pores was sufficient for aggregation and possibly induced nucleation (Figure 1-15).

![Schematic representation of crystal nucleation in a pore](image)

**Figure 1-15.** Schematic representation of crystal nucleation in a pore, which explains the mechanism of porous substrates induce protein crystallisation. (a) Protein molecules reside in the corner of the pore and start to aggregate until a critical nucleus forms. (b) The crystal keeps on growing until the entire pore is full of protein molecules. (c) In the close vicinity of the pore, another critical nucleus is then formed, thus protein crystal can breakout from the pore.

Chemically treated substrate surfaces are another type of commonly used insoluble solid additives for facilitating protein crystallisation. For instance, Ripamonti et al. prepared chemically modified mica sheets by mixed mica with n-propyltriethoxysilane and 3-aminopropyltriethoxysilane in different ratios to
vary the density of ionisable groups on the mica surfaces; and then tested them as heterogeneous nucleant surfaces for proteins crystallisation. They suggested a possible mechanism may involve that the chemically treated mica sheet surfaces could afford some interactions between the ionisable groups on the mica surfaces and charged residues of the protein, which may facilitate the aggregation and nucleation of protein molecules.

1.5.2 Polymer additives

Although the above additives have effectiveness on enhancing protein crystallisation, however, they still have some disadvantages. For example, some of them require high concentration, meaning that their affinity is low, such as salts and organic solvents. They must interact with a large number of water molecules to alter the free energy of protein molecules. For non-polymeric insoluble solid surfaces, they are random substances, have no designed specificity for proteins. Moreover, it is not easy to apply a systematic range of non-polymeric insoluble solid surfaces for screening experiments to find an optimized crystallisation condition that would have otherwise been missed. None of them has been proven as ‘universal’ heteronucleants to enhance the crystallisation for the majority of proteins. Synthetic polymers are important additives for protein crystallisation control as there are many possible chemistries which can be introduced into the backbone and side-chains, giving a very wide range of functional behaviour.
Polymers have also been successfully applied as additives in mediating crystallisation of inorganic materials,\textsuperscript{102} organic molecules,\textsuperscript{101, 103, 104, 105} as well as some key drugs such as paracetamol.\textsuperscript{106, 107} Proteins\textsuperscript{35, 108} and nucleic acids\textsuperscript{109} have become particular targets for polymer-mediated crystallisation in recent years owing to their growing importance in pharmaceutical applications. Polymers provide an ideal approach to control and investigate the majority of protein crystallisations in a reproducible and universal manner; including finding the optimal crystallisation conditions by a systematic screening experiment of altering the chemical characteristics and functionalities. Fundamentally, there are two types of polymers to be distinguished, insoluble polymer surfaces and soluble polymer additives.

1.5.2.1 Insoluble polymer surfaces

Protein crystallisation control by insoluble polymer additives is an approach by which the insoluble polymer surfaces serve as templates, scaffolds or heterogeneous nucleants to promote protein nucleation or template selective polymorphs of protein crystals.\textsuperscript{71, 100} One application of insoluble polymer surfaces is the use of molecularly imprinted polymers (MIPs) as surfaces for the selective nucleation of crystals via heterogeneous nucleation. Molecular imprinting is a technique by which selective recognition sites can be constructed in polymer matrices, and then used for molecular recognition of memorable template molecules.\textsuperscript{110, 111}
Molecularly imprinted polymers are prepared via molecular imprinting technique. They are formed in the presence of the template molecule, which will be extracted afterwards, resulting in complementary cavities in the polymer with high affinity for the templating species.\textsuperscript{112,113,114} Vulfson \textit{et al.} reported that polymers, when imprinted with inorganic material, calcite, were able to induce the nucleation of calcite under conditions favouring the growth of aragonite (Figure 1-16).\textsuperscript{115} Followed the same principle of MIPs employed in the crystallisation of inorganic small molecules, MIPs was also studied in protein crystallisation. Takeuchi \textit{et al.} demonstrated the effectiveness of different protein-imprinted polymer arrays with acidic or basic functional monomers to classify five tested proteins. They concluded that protein-imprinted polymers could offer a new approach to characterise and profile various proteins in nature.\textsuperscript{116} Furthermore, Chayen \textit{et al.} found that some MIPs induced crystallisation of proteins and yielded crystals in the conditions that didn’t afford crystals otherwise. Moreover, MIPs acted as heterogeneous nucleants and could facilitate the formation of protein crystals in the metastable zone of the crystallisation phase diagram, which would otherwise been missed.\textsuperscript{114}
Figure 1-16. Schematic representation of the molecular imprinting process and selective crystallisation of calcium carbonate. Functional monomers adsorbed to a calcite crystal surface, followed by cross-linking to convert monomer self-assemblies into a polymer matrix. Template calcite crystal was removed by washing with acidified aqueous methanol solution to produce imprinted polymer surfaces. Thus, the imprinted polymer surfaces can be used to template selective polymorphs of calcium carbonate crystal.\textsuperscript{115}

In a separate but related approach, a range of insoluble polymer surface libraries has been used to facilitate proteins crystallisation via heterogeneous nucleation. For example, Matzger \textit{et al.} reported a heteronucleant approach by applying polymers as heteronucleant surfaces to achieve functional diversity.
and flexibility of crystallisation formats. They applied functionally diverse polymer libraries, such as commercially available polymers and cross-linked copolymers for hen egg-white lysozyme, concanavalin A and bovine liver catalase crystallisation and concluded that the insoluble polymer surface libraries can be successfully applied, such as allowing nucleation rate control of lysozyme, improved lysozyme crystal size, new form access of concanavalin A and formation of high quality bovine liver catalase crystal.99

All the above mentioned previous work shows that the use of polymers as solid surfaces is an important and successful approach to mediate protein crystallisation via heterogeneous nucleation. Currently, there is a lack of surface characterisation on polymer surfaces. Moreover, by using this method, there is a great need for the clear understanding of the mechanisms of how these polymers work.

1.5.2.2 Soluble polymer additives

Soluble polymer additives have attracted interest from researchers in polymer-mediated protein crystallisation. Compared to insoluble polymer surfaces that act as templates or heterogeneous nucleants, soluble polymeric additives can influence the biomolecule crystallisation process leading to changes in size, habit and even polymorph of the final crystals.71 The mechanisms by which polymers act include complexation of electrolytes, thus changing the local ionic strength;71 the masking or neutralizing of charges on
proteins, which in turn enhances the role of hydrophobic association between protein chains,\textsuperscript{117, 118} or via hydrogen bonding or hydrophobic interactions to diminish the interactions between protein macromolecules and the solvent,\textsuperscript{5, 72, 73} or by simple viscosity modification which changes the kinetics of crystal growth. Polymeric additives can also stabilise transient protein-protein aggregates by adsorption on their surfaces, leading to faster nucleation and altered crystalline polymorph,\textsuperscript{78, 119, 120} or through attachment to specific crystal faces which then influences the direction of further crystal growth.\textsuperscript{100}

Cölfen \textit{et al.} used a variety of polymers, such as glycopolymers, low-molar-mass polymers and block copolymers to investigate additives controlled mineral crystallisation, such as barium and calcium carbonate. Compared to insoluble polymer surfaces, different types of soluble polymer additives with diverse functional groups can have big influences on crystallisation of minerals, even can alter the final crystal morphology.\textsuperscript{71, 100, 121, 122}

Crystallisation of proteins would follow the same general principle as that of small molecules.\textsuperscript{109, 123} Some polymers have been studied as additives i.e. nucleants/precipitants for protein crystallisation. They have been pioneered by Polson and co-workers. They used many polymers, such as polyethylene glycol (PEG), dextran, polyvinyl alcohol and polyvinyl pyrrolidone, as precipitants for protein fractionation. They proposed that, among the polymers they had used, PEG 6,000 was appeared to be the most suitable protein precipitant.
because its low viscosity, stability and because it did not denature the proteins at room temperature.\textsuperscript{124}

Inspired by Polson’s results, McPherson \textit{et al.} applied different sizes of PEG: 400, 1,000, 4,000, 6,000 and 20,000 to mediate 22 proteins, such as gene, \( \alpha \)-amylase, a protein of unknown function from turkey liver, catalase, abrin, ovalbumin, canavalin and horse albumin. They reported that six proteins were crystallised for the first time by screening of five different molecular weight sizes of PEG; one protein: \( \alpha \)-amylase easily afforded crystals when PEG was employed; one other protein that of unknown function from turkey liver yielded excellent quality crystals whereas only poor-quality crystals have been obtained previously. Moreover, they found that the PEGs were active in helping to crystallise a wide range of proteins and exhibited greater applicability than other previously used reagents.\textsuperscript{125} PEG remains in widespread use to aid protein crystallisation because it is stable, neutral, hydrophilic and its solutions are of low viscosity compared to other polymers.\textsuperscript{124, 125}

Based on the previous studies, McPherson \textit{et al.} also screened and assessed various chemicals mixed with PEG 3,350 acting as mixed additives on 81 different proteins. They stated that mixed additives, which composed of polyvalent, charged groups, i.e. di and tri carboxylic acids, diamino compounds, or bearing one or more sulfonyl or phosphate groups were the most promising types of additives to affect protein crystallisation.\textsuperscript{72} They proposed a novel
approach to crystallise proteins by combination of small molecules with simple polymer additives.

More recently, Yao et al. used some functional polymers, such as poly(PEGMA), PDMAEMA and some block copolymers to control of lysozyme crystallisation. They showed that only poly(PEGMA) mixed with lysozyme solution could not afford any lysozyme crystals, but PDMAEMA and some block copolymers, which contained high content of the PDMAEMA, such as PDMAEMA-b-poly(PEGMA-OH) and PDMAEMA-b-PBMA-b-poly(PEGMA), had a clear influence on the crystallisation of lysozyme and were able to modulate the shape of crystals but depending on their concentration in protein solution.\textsuperscript{108}

However, because there are so many factors which can affect crystallisation processes from solution, many of which are still poorly understand, to date only a limited number of polymer additives have been successfully applied in proteins crystallisation, most are PEGs and PEG derivatives. In addition, some of the previous work did not show clear crystal packing information.\textsuperscript{108}

Moreover, most of the studies are descriptive; the interactions between some of the existing polymer additives and protein macromolecules or surrounding solvent are still unclear, and the mechanisms by which protein crystals form in the presence of these polymers needs to be further investigated.\textsuperscript{100, 108}
1.6 Polymers and polymer synthesis

Polymers are macromolecules, which are composed of a large number of small molecules combined with each other in a repeating order. These small molecules are so called monomers, and the reactions by which they link together are so called polymerisations. There may be hundreds, thousands, tens of thousands, or more monomer molecules in a polymer molecule. There are two common laboratory synthetic methods used to made polymers: stepwise and chain polymerisation.\textsuperscript{126}

The first polymerisation method is stepwise polymerisation, which sometimes classified as ‘condensation polymerisation’. It is defined that monomers all combine together, thus the molecular weight increases slowly by losing small molecules, such as water. With this method, the molecular weight of polymer increases at a very slow rate at lower conversions, and only at very high conversion (i.e., > 95 \%) can reach relatively high molecular weight. Step growth polymerisation is always used to yield branched and networked polymers.\textsuperscript{127}

The second one is chain growth polymerisation. It is generally following three steps: chain initiation, chain propagation and termination of the active chain ends. There are several types of synthesis depending on the active site (Table 1-2).
Table 1-2. Types of chain growth polymerisation

<table>
<thead>
<tr>
<th>Active sites</th>
<th>Synthesis type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbanion</td>
<td>Anionic polymerisation</td>
</tr>
<tr>
<td>Carbenium ion</td>
<td>Cationic polymerisation</td>
</tr>
<tr>
<td>Free radical</td>
<td>Free radical polymerisation</td>
</tr>
</tbody>
</table>

The initiation of anionic polymerisation is usually by strong anions. Theoretically, there is no formal termination for anionic polymerisation method, because of that proton transfer from the solvent or other positive materials rarely take place. The initiation of cationic polymerisation is by strong protic acid. However, this method is extreme sensitive to the reaction conditions, such as solvent types, solvent purity and temperature. Among the above chain growth polymerisations, the most significant method is free radical polymerisation. Atom transfer radical polymerisation (ATRP) and reversible addition-fragmentation chain transfer polymerisation (RAFT) are the most extensively studied synthetic methods.

1.6.1 Atom transfer radical polymerisation (ATRP)

ATRP was first reported by Wang and Matyjaszewski in 1994,\textsuperscript{128} which is one of the most commonly used methods of controlled radical polymerisation. It provides a simple route to many well-defined functional polymers with controlled molecular weight and narrow molecular weight distribution.\textsuperscript{129,130} A general ATRP reaction usually comprises of a transition metal and ligand based catalyst and alkyl halides act as the initiator.\textsuperscript{129}
The ATRP polymerisation as shown in Figure 1-17 starts with the homolytic cleavage of the carbon-halogen bond of the initiator R-X, resulting in a single electron i.e. free radical at the carbon center (R•) and a halogen radical (X•). The halogen radical can bind to metal to change its oxidation state\textsuperscript{131} (Figure 1-17A). This process is reversible; the halogen can reunite with the initiator to make it inactive again (Figure 1-17B). Thus, the initiator is only active for a short time before becoming dormant.\textsuperscript{132} When the initiator is active, it can consume monomers and propagate (Figure 1-17C).

\[ R-X + \text{Cu( I)-X/Ligand} \rightleftharpoons R\cdot + \text{Cu ( II)-X}_2/Ligand \]

\[ P-X + \text{Cu( I)-X/Ligand} \rightleftharpoons P\cdot + \text{Cu ( II)-X}_2/Ligand \]

Figure 1-17. General scheme of an ATRP reaction. A: Initiation; B: Dormant species in ATRP; C: Propagation. M represents monomer; P represents a polymer with a number of monomer units.

Several parameters can affect an ATRP reaction. The initiator can affect the initiating efficiency. The faster the initiation, the slower termination and the faster propagation, more monomers are combined together to form a polymer with a narrow molecular weight distribution.\textsuperscript{133} The catalyst is a very important parameter in an ATRP reaction, because it determines the equilibrium constant.
between active and deactivated species. The catalyst system usually contains a transition metal and a ligand. The most commonly studied transition metal in an ATRP reaction is copper, due to its low cost and readily accessible redox character. The ligand must have strong complexion with copper\textsuperscript{134} and can stabilise Cu (II) compared to Cu (I). Besides, the choice of monomers is also very crucial. Monomers that are typically used in ATRP involve various vinyl polymers, which can stabilise the propagating radicals,\textsuperscript{135} such as styrene,\textsuperscript{136,137} methacrylate,\textsuperscript{137} 2-(dimethylamino)ethyl methacrylate\textsuperscript{139} and acrylonitrile.\textsuperscript{140} In addition to initiator, catalyst, ligand and monomer, solvent and temperature can influence the kinetics of ATRP as well.\textsuperscript{135} A wide range of polymers has been created with controlled functionalities and compositions via functionalised initiators.\textsuperscript{141,142} This approach has been used for the synthesis of methacrylate monomers showing fast ATRP kinetics with low polydispersities by using Cu (I) Br/2,2'-bipyridine catalytic system.\textsuperscript{143} Polymers synthesised with ATRP in this project include poly(ethyleneglycol)methyl ether-methacrylate [p(PEGMA\textsubscript{475})], poly[2-(dimethylamino)ethyl methacrylate] [p(DMAEMA)] and a random copolymer of PEGMA\textsubscript{475} & DMAEMA (Figure 1-18). From previous researchers’ studies, PEG has been found to be an effective additive in promoting the crystallisation of a range of proteins. However, PEG itself has limited chemical functionality, and cannot be derivatised easily to introduce groups which can form specific bonds with protein peptide residues. We thus
chose PEGMA\textsubscript{475}, as a neutral methacrylate derivative of PEG to obtain a polymer, p(PEGMA\textsubscript{475}), analogous to PEG but with hydrophilic side-chain functionality and a hydrophobic main-chain. Poly(PEGMA)-based materials have been shown to mediate protein crystallisation in solution\textsuperscript{108}. To test the effects of varying degrees of charge on protein crystallisation, another methacrylate monomer: DMAEMA was either homopolymerised or copolymerised with PEGMA\textsubscript{475} to form cationic polymers. The amine functionalities were designed to enhance water solubility of the polymers\textsuperscript{144} and also to provide lone-pair donor or charged residues dependent on pH and degree of protonation or quaternisation.

![Figure 1-18. Structures of PEGMA\textsubscript{475} and DMAEMA monomers. X denotes the number of repeating units.](image)

1.6.2 **Reversible addition-fragmentation chain transfer polymerisation (RAFT)**

Reversible addition-fragmentation chain transfer polymerisation (RAFT) is another most important controlled radical polymerisation. It was discovered at CSIRO in 1998\textsuperscript{145}. It commonly uses thiocarbonylthio compounds\textsuperscript{146} such as
dithioesters as the RAFT agents to mediate the polymerisation via a reversible chain-transfer process (Figure 1-19).

Figure 1-19. Mechanism of an ideal RAFT polymerisation.\textsuperscript{147} $P_n\cdot$ represents a polymeric radical with $n$ monomer units; $P_m\cdot$ represents a polymeric radical with $m$ monomer units; $X$ normally represents sulfur; the $Z$ group primarily affects the stability and activity of the $\text{S}=\text{C}$ bond.\textsuperscript{145}

In an ideal RAFT reaction, initiation is started by an initiator, which decomposes to form two free radicals ($I\cdot$), these then react with monomer ($M$) to obtain a chain growth polymeric free radical ($P_n\cdot$) (Figure 1-19a). Then the chain growth polymeric free radical ($P_n\cdot$) starts to propagate and reacts with the RAFT agent, to form an intermediate RAFT adduct radical. There is a RAFT pre-equilibrium here between the intermediate RAFT adduct radical and
a polymeric RAFT agent & a leaving group radical (R•) (Figure 1-19b). This leaving group radical (R•) then reacts with the monomer to form another chain growth polymeric free radical (Pm•) (Figure 1-19c). Group R must be a good leaving group and able to react with monomer to aid the fragmentation process. Then the main RAFT equilibrium process takes place, in which all chain growth polymeric free radicals share the radical among them, leading to chain growth (Figure 1-19d). It is very essential that the radicals are shared equally, thus, all the chains have the equal opportunities to propagate, and maintain well defined polymer characteristics. The termination of RAFT polymerisation involves a process called bi-radical termination to form dead polymers (Figure 1-19e).

In comparison to ATRP, a larger variety of monomers can be synthesised via RAFT polymerisation, especially water-soluble polymers, such as vinyl aromatics, acrylates, acrylamides, acrylonitrile, vinyl acetate and vinyl amides. Polymers synthesised via RAFT in this project include poly[N-(3-(dimethylamido)propyl) methacrylamide] [p(DMAPMAm)], poly(2-acrylamido-2-methyl-1-propanesulfonic acid) [p(AMPS)], poly(2-acrylamidoacetic acid) [p(2-AmAA)] and poly(4-acrylamidobutanoic acid) [p(4-AmBA)]. Similar as DMAEMA, a methacrylamide monomer: DMAPMAm, which has tertiary amines groups, was selected to afford positive charges and form another type of cationic polymer. For the choice of anionic polymers, they were chosen according to the pKa of the polymers, and also
considering the protein crystallisation pH, at which the polymers could provide charged residues. Three acrylamide monomers, including AMPS, 2-AmAA and 4-AmBA, which have ionisable sulfonic acid or carboxyl groups were homopolymerised to generate anionic water soluble homopolymers in this project (Figure 1-20).

![Figure 1-20. Structures of DMAPMAm, AMPS, 2-AmAA and 4-AmBA monomers.](image)

1.7 Protein targets

Hen egg-white lysozyme (HEWL), concanavalin A (Con A) and bovine liver catalase (BLC) were the protein targets used for crystallisation during this research, because of their range of size and charge. The isoelectric point (pI) of HEWL is 11.35, when at its crystallisation pH 4.8, HEWL macromolecules are positively charged, i.e. cationic.\textsuperscript{152} The isoelectric point (pI) of Con A is 4.5,\textsuperscript{153} when at crystallisation pH 8.5, Con A macromolecules are anionic. The isoelectric point (pI) of BLC is 5.4,\textsuperscript{154} and when at crystallisation pH of 6.8, BLC macromolecules may carry some negative charges, hence being a weekly
anionic protein at this pH.

1.7.1 *Hen egg-white lysozyme (HEWL)*

Lysozyme is a single chain enzyme and 129 amino acid in length\(^{155}\) (E.C. 3.2.1.17). It has a molecular weight (Mw) of 14.3 kDa, pI of 11.35, four disulphide bridges, formed by α-helices, and was discovered in 1922 by Fleming.\(^{156, 157}\) It is present in tears, saliva, mucus and the egg-white of chickens. Lysozyme protects the body from bacterial infection as a natural antibiotic. It can attack the cell wall of the bacteria by catalysing the hydrolytic cleavage of polysaccharides in the cell walls, leading to bacterial lysis.\(^{158}\)

HEWL has five structurally characterised crystal forms: monoclinic, triclinic, hexagonal, orthorhombic and tetragonal.\(^{159}\) It has been the most studied crystallisation system for the last 60 years. Alderton and Fevold first reported tetragonal HEWL crystals in 1946.\(^{160}\) Phillips and Johnson first applied X-ray crystallography to elucidate the three-dimensional structure of HEWL in 1965.\(^{161, 162}\) So far there are about 750 lysozyme crystallographic structures which have been obtained and are available in the PDB (Berman *et al.*, 2000). A model of the three-dimensional structure of HEWL is presented in Figure 1-21.
Figure 1-21. The three-dimensional structure of HEWL obtained by crystal X-ray diffraction, at a resolution of 1.8 Å and a crystallographic R factor of 17.3 %. This polymorph is the orthorhombic $P2_12_12_1$, with unit cell dimensions of $a = 30.6$ Å, $b = 56.3$ Å, $c = 73.2$ Å, and one molecule per asymmetric unit. Structure includes Mg$^{2+}$ and SO$_4^{2-}$ ions.$^{163}$

As mentioned in section 1.5.2, Matzger et al. applied a series of insoluble polymer surfaces to influence lysozyme crystallisation. They reported that HEWL crystallisation under conditions favouring the tetragonal form resulted in exclusively tetragonal crystals, but the insoluble polymer surfaces could successfully control lysozyme nucleation rate and improve lysozyme crystal size.$^{159,99}$ Moreover, Yao et al. used some soluble polymer additives to control lysozyme crystallisation via tetragonal crystal growth as well and stated that some polymers were able to modulate the shape of crystals.$^{108}$ With this in mind, we chose HEWL as the first protein target. The main reason for the use of HEWL in this research is that it has become the most preferred crystallisation system for the majority of protein crystallisation studies. This is
because the fact that it is easily purified for commercially use, low cost and widespread. Moreover, from the literature HEWL is relatively straightforward to crystallise in a specific polymorph (the tetragonal form) within one day with little effort.

1.7.2 Concanavalin A (Con A)

Concanavalin A (Con A) is a lectin (carbohydrate-binding protein), which is extracted from the jack-bean. It binds to certain structures that are found in various sugars, glycoproteins, and glycolipids specifically.\textsuperscript{164} Like most lectins, Con A is a homotetramer, each sub-unit has a molecular weight of 25.5 kDa, two chains and 235 amino acids. Each monomer of Con A binds two metallic ions: usually Mn\textsuperscript{2+} at site S1 and Ca\textsuperscript{2+} at site S2.\textsuperscript{165} Con A was the first lectin which used as commercial basis, and now it is widely applied in biology and biochemistry. For example, Con A can be used to characterise surface structure of glycoproteins and other sugar-containing entities exposed on the membrane of various cells;\textsuperscript{166} and some research showed that the cancer cells can be aggregated readily by Con A while other cells cannot.\textsuperscript{167}

Con A was first crystallised by Sumner and Howell in 1936.\textsuperscript{168, 169} A later investigation of the structure of Con A was conducted by Greer \textit{et al.} in 1970. They studied the X-ray diffraction of Con A crystal and found Con A crystallised in two space groups \textit{I}2\textit{2}2 or \textit{I}2\textit{1}2\textit{1}.\textsuperscript{170} For the last 60 years, Con A has been studied widely and yielded more than 50 X-ray crystal structures,
including the native state of Con A bound to manganese and calcium, Con A bound with carbohydrates, metal free Con A, and Con A bound to other transition metals. Currently, there are several common structurally characterised crystal forms of Con A, bound to manganese and calcium ions, including triclinic $P1$, orthorhombic $I222$, orthorhombic $C222_1$ and orthorhombic $P2_12_12_1$. A model of crystallographic structure of Con A bound to manganese and calcium ions is presented in Figure 1-22.

Figure 1-22. Crystallographic structure of a Con A tetramer (from jack bean), obtained by crystal X-ray diffraction, at a resolution of 2.4 Å. The space group $I222$ with unit cell dimensions of $a = 63.15$ Å, $b = 86.91$ Å, $c = 89.25$ Å. Monomers with color cyan, green, red and magenta; Calcium (gold) and manganese cations (grey) are depicted as spheres.

1.7.3 Bovine liver catalase (BLC)

Catalase is a common enzyme in all living organisms. It protects cells against toxic effects of hydrogen peroxide to proteins, lipids, and nucleic acids, by catalysing the reaction of decomposition of hydrogen peroxide to water and oxygen. Catalase from bovine liver is a tetramer of four equal polypeptide
chains: each subunit is over 500 amino acids long\textsuperscript{184} and has a molecular weight of 60 kDa.

Catalase from bovine liver was firstly crystallised by Sumner and Dounce in 1937.\textsuperscript{185} After that, McPherson \textit{et al.} introduced PEG 6,000 and 20,000 as additives to mediate catalase from sheep or deer liver crystallisation, and produced large size of sheep liver catalase crystal and deer liver catalase crystal with complex shape.\textsuperscript{125} Like Con A, the crystallisation and structural characterisation of catalase has been studied for almost 60 years. There were two common structurally characterised crystal forms of BLC crystal: trigonal \textit{P}\textsubscript{3}\textsubscript{2}1\textsubscript{2}1\textsubscript{2}1 (form I)\textsuperscript{186} and orthorhombic \textit{P}2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} (form II)\textsuperscript{187}. Recently, Matzger \textit{et al.} applied polymer-induced heteronucleant for BLC crystallisation and obtained another orthorhombic form (form III).\textsuperscript{99, 188} A model of crystallographic structure of BLC (form II) is presented in Figure 1-23.

![Figure 1-23. Crystallographic structure of a BLC tetramer, at a resolution of 2.69 Å and a crystallographic R factor of 20.5 %. It adopts the space group \textit{P}2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} with unit cell dimensions of a = 68.65 Å, b = 173.74 Å, c = 186.32 Å. Monomers with different colors are equal.\textsuperscript{99}](image-url)
1.8 Aims and objectives of the project

In this project, in order to probe some of the factors involved in polymer-mediated protein crystallisation, we set out to prepare different classes of polymers with varying degrees of charge, molecular weight and backbone structure, and then to study their roles as additives in model protein solutions. The guiding hypothesis was that specific functional polymers in solution could alter the rate of protein crystallisation from solutions, and thereby influence the shape, size, and/or polymorph of the resultant crystals.

The aim of this project was threefold. The first two objectives are shown in Figure 1-24. The first aim of this project was to design some key polymers with varying degrees of charge, molecular weight and backbone structure via proper ATRP or RAFT methods (Figure 1-24A). The polymers should be hydrophilic and stable when dissolved in protein crystallisation buffer solutions with different pH. They also need to be compatible with protein structures.
Figure 1-24. Schematic representation of protein crystallisation utilising polymer additives. A) Polymerisation of functional monomers to yield the polymer. B) 96-well crystallisation plate, each sample well (red) composes of protein and polymer additives. C) Polymer acts as ‘additive’ to mediate protein crystallisation.
The second aim was to use these polymers as solution additives to mediate various model proteins crystallisation via sitting drop vapour diffusion techniques (Figure 1-24C). In this research, hen egg white lysozyme (HEWL), concanavalin A (Con A) and bovine liver catalase (BLC) were selected as target proteins for crystallisation experiments. The first model protein was HEWL. HEWL macromolecules are positively charged at tetragonal crystallisation pH of 4.8, i.e. cationic. It is readily available and relatively straightforward to crystallise in the tetragonal form within one day. These facts allow us to develop experimental protocols to investigate the effects of these polymers successfully, such as alter protein crystal size, morphology, even crystal packing motifs, and obtain multiple forms from the same crystallisation condition. Besides the exploration of HEWL, we also aim to evaluate whether these polymers were suitable for other proteins, especially anionic proteins. Con A and BLC were selected as anionic model proteins for further crystallisation.

The final objective was to examine the mechanisms of how these polymers affect protein crystallisation and to develop an understanding of the interactions between polymer and protein macromolecules or the surrounding solvent. Moreover, we hoped to establish structure-function relationships linking the types of polymers used in these experiments and the protein crystal habits produced would generated. These relationships might be used as models to guide a wide range of protein crystallisation experiments in the future.
Accordingly, with the polymer additives, proteins can be nucleated into particles (amorphous or crystalline) and obtain polymer & protein nanoaggregates or nanocrystals, which will be useful in pharmaceutical, biotechnological or chemical applications.
2. Materials, Instrumentation and General Methods

2.1 Materials

4,4’-Azobis(4-cyanvaleric acid) (V-501) was purchased from Fluka®, UK and recrystallised from MeOH by Dr. Francisco Fernandez-Trillo. 2-(Ethylthiocarbonothioylthio)-2-methylpropanoic acid (CTA) was also kindly supplied by Dr. Francisco Fernandez-Trillo. A cationic/zwitterionic copolymer of \([2-(\text{methacryloyloxy})\text{ethyl}][\text{trimethylammonium chloride and N-[2-(\text{methacryloyloxy})\text{ethyl}]dimethyl-(2-sulfoethan)aminium hydroxide [p(METMAC-co-MEDSAH)}]\) was kindly supplied by Dr. E. Peter Magennis.

HEWL, Con A and BLC were purchased from Sigma-Aldrich®, UK (L6876, C7275 and C40, respectively). They were all used without further purification. MRC 2 Well Crystallisation Plates (Swissci) (UVP plate, 96 reservoir wells, reservoir volume: 50 to 100 µl, with 2 corresponding sample wells: 5 µl max fill volume) were purchased from Hampton Research, UK. Dulbecco’s Phosphate Buffered Saline (DPBS) was purchased from Lonza Group Ltd. All solvents, reagents and proteins, were of analytical or HPLC grade and purchased from Sigma-Aldrich®, Acros® or Fisher Scientific®, UK without further purification unless otherwise.

- 2-(Dimethylamino)ethyl methacrylate (DMAEMA) (98 %) – Fluka
- 2-(Ethylthiocarbonothioylthio)-2-methylpropanoic acid (CTA) –
synthesised by Dr. Francisco Fernandez-Trillo, School of Pharmacy, the University of Nottingham

- 2,2-Bipyridine (≥ 99 %) – Sigma Aldrich
- 2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPS) – Sigma Aldrich
- 4,4′-Azobis(4-cyanovaleric acid) (V-501) (recrystallised from MeOH by Dr. Francisco Fernandez-Trillo) – Fluka
- 4-Aminobutanoic acid (≥ 99 %) – Acros Organics
- Acrylic chloride (96 %) – Alfa Aesar
- Aluminium stubs with carbon tabs – Agar Scientific
- Aluminum oxide - Neutral grade – Sigma Aldrich
- Ammonium sulfate (≥ 99 %) – Sigma
- Bovine liver catalase (BLC) (C40, ≥10,000 units/mg protein) – Sigma Aldrich
- Cellulose acetate membrane 0.2 μm – Aldrich
- Chloroform (≥ 99.5 %) – Sigma Aldrich
- Chloroform-d – Sigma Aldrich
- Concanavalin A (Con A) from Canavalia ensiformis (Jack bean) (C7275) – Sigma Aldrich
- Copper (II) Bromide – (CuBr₂, 99 %) - Alfa Aesar
- Deuterium oxide (D₂O) – Sigma Aldrich
- Dialysis membrane (MWCO 1000, regenerated cellulose) – Spectrapor
- Diethyl ether (anhydrous, ≥ 99 %) – Sigma Aldrich
CHAPTER 2. Materials, Instrumentation and General Methods

- Dimethyl sulfoxide-d6 – Sigma Aldrich
- Dulbecco’s phosphate buffered saline (DPBS) – Lonza Group Ltd
- Ethanol (anhydrous, ≥ 99.5 %) – Sigma Aldrich
- Ethyl acetate (anhydrous, 99.8 %) – Sigma Aldrich
- Ethylene glycol (anhydrous, 99.8 %) – Sigma Aldrich
- Glycerol anhydrous puriss – (≥99.5 %) - Fluka
- Glycine (≥ 98.5 %) – Sigma Aldrich
- Hen egg white lysozyme (HEWL) (L6876, ≥100,000 units/mg protein) – Sigma Aldrich
- Hydrochloric acid (HCl) (ACS reagent, 37 %) – Sigma Aldrich
- Methyl 2-bromopropionate (98 %) – Sigma Aldrich
- Methyl iodide (99 %) – Sigma Aldrich
- MRC 2 Well Crystallisation 96 Plate – Hampton Research
- N-(3-(dimethylamido)propyl)methacrylamide (DMAPMAm) – Sigma Aldrich
- Petroleum ether – Sigma Aldrich
- Poly(ethylene glycol) (PEG 4000) – Sigma Aldrich
- Poly(ethylene glycol) methyl ether methacrylate (PEGMA₄₇₅) – Sigma Aldrich
- Poly(ethylene oxide) (PEO) – Sigma Aldrich
- Polystyrene – Sigma Aldrich
- Polyvinylpyrrolidone – Sigma Aldrich
CHAPTER 2. Materials, Instrumentation and General Methods

- Sodium acetate (anhydrous) – Sigma Aldrich
- Sodium chloride (NaCl) – Fisher Scientific
- Sodium phosphate (≥ 99 %) – Sigma Aldrich
- Tetrahydrofuran (THF) (anhydrous, ≥ 99.9 %) – Sigma Aldrich
- Toluene (anhydrous, 99.8 %) – Sigma Aldrich
- Trifluoroacetic acid (≥ 99 %) – Sigma Aldrich
- Trizma base (≥ 99.9 %) – Sigma

2.2 Instrumentation

**Gel permeation chromatography (GPC)**

The polydispersity index (PDI) and molecular mass of the polymers were determined using Polymer Laboratories GPC-50 (Varian, Inc.) plus fitted with differential refractometer (RI), capillary viscometer (DP) and dual angle laser light-scattering (15° and 90°) detectors. The flow rate was 1.00 mL/min or 0.5 mL/min and experiments were conducted at room temperature. Organic soluble polymer analysis was carried out on the GPC-50 Plus with Resipore Mixed-D columns (Agilent Tech. Inc.) (eluent-chloroform/5 % Et₃N) calibrated with linear polystyrene standards. Cationic polymer analysis was carried out on the same GPC-50 Plus with CATSEC-300 (250 mm × 4.5 mm ID) column (eluent-200 mM NaCl with 0.1 % trifluoroacetic acid (v/v)) calibrated with polyvinylpyrrolidone (PVP) standards. Anionic polymer analysis was carried out on the same GPC-50 Plus with Polymer Labs aquagel-OH guard columns.
(50 × 7.5 mm, 8 μm, Polymer Labs UK) (eluent-Dulbecco’s PBS without Ca$^{2+}$ and Mg$^{2+}$ ions) calibrated with a single, narrow poly(ethylene oxide) (PEO) standard (Polymer Labs, Mp 128 kDa, [η] 1.2968 dL/g) using a dn/dc value of 0.133 g/mL. Samples were prepared at 1-5 mg/mL in the mobile phase and injected 100 µL onto the column. Molecular weight and polydispersity index were calculated using Polymer Labs Cirrus 3.0 Software.

**NMR spectroscopy**

$^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer. The instrument is fitted with a 5mm switchable quadruple probe for the detection of $^1$H, $^{13}$C, $^{19}$F and $^{31}$P, and also with z-axis pulsed field gradients for gradient shimming. Chemical shifts were reported at 400 MHz ($^1$H) and 101 MHz ($^{13}$C) in ppm (δ units) relative to TMS in chloroform-d (CDCl$_3$), tetramethylsilane (DMSO-d$_6$), Methanol-d$_4$, or D$_2$O solutions.

**Infrared spectroscopy (IR)**

IR spectra of polymer samples (approximate 5 mg) were recorded on KBR discs on a Bio-rad FTS6000 Fourier Transform Spectrometer instrument. It is a high performance research grade multi-range FT-IR spectrometer, covering the spectral range 11,000-400 cm$^{-1}$ capable of both rapid scan and step scan operation. Its high quality 60° Michelson air bearing
piezoscan interferometer features piezoelectric-based continuous dynamic alignment (which simultaneously maximizes the spectral throughput and minimizes the noise in the measurements) and a KBr beam splitter for the mid IR. This FTIR instrument employs external reflection, micro-ATR equipped using a silicon crystal. It is interfaced with a Nicolet Avatar 360 spectrometer and uses a liquid nitrogen cooled MCT (HgCdTe) detector. It focuses more than 150 mW of infrared power at the sample, by using a high wattage water-cooled ceramic source rather than air-cooled sources for the mid IR and a two-inch clear aperture through the interferometer. The system was cooled by liquid nitrogen for 10 min before the detection of the polymer sample.

**Dynamic light scattering (DLS)**

Dynamic Light Scattering (DLS) was measured by using a Viscotec Model 802 instrument (Viscotek Europe Ltd.), equipped with an internal laser (825-832 nm) and a maximum radiation power of 60 mW. From standard autocorrelation functions, measured diffusion coefficients were related to particle hydrodynamic radius via the Stokes-Einstein Equation.

$$RH = \frac{kT}{6\pi\eta D}$$

Where RH is the hydrodynamic radius, k is the Boltzmann constant, T is the temperature, and η is the viscosity of the solvent. In addition, it was assumed that particles were spherical and non-interacting.

Measurements quoted are the averages of samples with at least ten readings of
particle size recorded at 19 °C. Data processing was performed with the software program OmniSize 3.0 (Viscotek Europe Ltd.).

**Freeze drier**

Water was removed by immersing reagent tubes containing the samples in liquid nitrogen, once frozen the samples were placed on an Edwards Modulyo freeze drier equipped with an Edwards high vacuum pump for 3 days at -25 °C and the water was then removed.

**Rotary evaporator**

Solvents evaporated under reduced pressure on a Buchi Rotavapor R-200 equipped with a B490 heating bath with 4 °C.

**Centrifuge**

Centrifugation was carried out on an Eppendorf Refrigerated Microcentrifuge. All protein solutions were centrifuged at 13,000 rpm for 5 minutes at 4 °C. Polypropylene micro-centrifuge tubes in size 1.5 mL (Sigma-Aldrich, T3406) with an integral flip cap were used here.

**Optical microscopy**

Protein crystals in the crystallisation plates were quantitatively observed using a Leica Stereomicroscope (63x magnification) with a Leica CLS1 50X
fan-cooled light source. A Nikon Coolpix 4500 digital camera was connected to image the crystals.

**Scanning electron microscope (SEM)**

The samples were loaded onto aluminium stubs with carbon tabs pre-fixed. These were then gold coated using an SCD 030 Blazers sputter coater for 5 minutes at 30mA. The coated samples were transferred into a JEOL JSM 6060 LV Scanning electron microscope and imaged at 20-30 kV. Prior to gold coating, protein crystals were taken out from the crystallisation plates, washed with crystallisation buffer solutions.

**X-ray crystallography**

Protein crystallographic analysis was carried out on a Rigaku micromax 007 microfocus X-ray generator with high-flux Osmic confocal multi-layer optics. X-rays were collimated to a 0.3 mm beam with X-rays from the copper K alpha emission (1.5418 Angstroms). Diffraction patterns were collected on Rigaku R-Axis 4++ image plates. Crystals were maintained at temperatures of -180 °C by an X-stream 2000 nitrogen vapour system.

In X-ray crystallography, X-ray beam is employed to hit the crystal samples. Atoms in the crystal scatter X-ray waves and will produce secondary spherical waves emanating from electrons. Those regular arrays of spherical waves normally in some specific directions, determined by Bragg’s law (Figure 2-1):
\[ 2d \sin \theta = n \lambda \]

Here, \( d \) is the distance between diffracting planes; \( \theta \) is the incident angle; \( n \) is any integer; and \( \lambda \) is the wavelength of the beam.

Figure 2-1. Bragg’s diffraction. Two incident beams (wavelength \( \lambda \)) scattered off two atoms in the crystalline.

Therefore, X-rays are diffracted resulting in a pattern on the detector. Then the crystallographer can get a three-dimensional image of the density of electrons within the crystal by measuring the angles and intensities of these diffracted patterns. Thus, the mean atom positions, their chemical bonds and other information within the crystals can be determined.

2.3 General methods

*General synthetic methods*

Poly[poly(ethylene glycol) methyl ether methacrylate\(_{475}\)] [p(PEGMA\(_{475}\))], poly[2-(dimethylamino)ethyl methacrylate] [p(DMAEMA)] and a random copolymer of PEGMA\(_{475}\) and DMAEMA [p(DMAEMA-stat-PEGMA\(_{475}\))] were prepared by a variant of published ATRP route by using
Cu(I)Br/2,2-bipyridine (Bipy) catalytic system and methyl 2-bromopropionate (MBrP) as the initiator. Poly[N-(3-(dimethylamido)propyl)methacrylamide] [p(DMAPMAm)], poly(2-acrylamido-2-methyl-1-propanesulfonic acid) [p(AMPS)], poly(2-acrylamidoacetic acid) [p(2-AmAA)] and poly(4-acrylamidobutanoic acid) [p(4-AmBA)] were synthesised using RAFT methods. The polymerisation employed 2-(ethylthiocarbonothioylthio)-2-methylpropanoic acid (CAT) as the RAFT agent and 4,4′-azobis(4-cyanovaleric acid) (V-501) as the initiator. A hybrid copolymer of [2-(methacryloyloxy)ethyl]trimethylammonium chloride and N-[2-(methacryloyloxy)ethyl]-dimethyl-(2-sulfoethan)aminium hydroxide [p(METMAC-co-MEDSAHH)] was kindly supplied by Dr. Eugene Peter Magennis. For more details see Chapter 3.

**General crystallisation method**

Crystallisation was performed in 96-well crystallisation plates, using the sitting drop vapour diffusion technique. Specifically, a solution (50 µL) of tested polymer buffer was pipetted in triplicate in the reservoir wells; protein solution, (1 µL) and buffered polymer solution (as described above, 1 µL) were pipetted into sample wells of the crystallisation plate. The plate was then sealed and incubated at 19 °C. More details are shown in Chapter 4.
CHAPTER 3

3. Preparation and Characterisation of Monomers and Polymers

3.1 Introduction

As mentioned in ‘Chapter 1’, although there are many examples of protein crystals reported, successful crystallisation of proteins from solution is still an extremely difficult task. Only a small number of techniques are suitable for helping proteins crystallise; besides, there are many uncertain and poorly understanding factors can affect and govern the crystallisation process from solution.\(^5\)

One essential strategy for protein crystallisation is using polymers as ‘additives’ into the protein solution.\(^{189}\) Synthetic polymers are important additives for protein crystallisation control as there are many possible functionalities can be introduced into the backbone and side-chains, giving a very wide range of functional behaviour.\(^5,100,101\) They can influence protein crystallisation process to a large extent in terms of size, habit, morphology and even polymorph of the crystal.\(^71\) Although a number of polymeric additives have been studied to influence protein crystallisation, most of the studies are descriptive; the interactions between some of the existing polymeric additives and protein macromolecules or surrounding solvent are still unclear, and the mechanisms by which protein crystals form in the presence of these polymers needs to be
In order to investigate some of the factors involved in polymer-mediated protein crystallisation, we designed various classes of polymers with varying degrees of charge, molecular weight and backbone structure, and then to study their role as additives in model protein solutions. In this paper, four classes of materials, including neutral, cationic, anionic and cationic/zwitterionic polymers based on methacrylate or (meth)acrylamide monomers were successfully prepared and shown in Figure 3-1.

![Figure 3-1. Structures of polymers, named P1 to P8. Neutral polymer: P1; cationic polymers: P2-4; anionic polymers: P5-7 and cationic/zwitterionic copolymer: P8.](image-url)
3.2 Monomer synthesis

3.2.1 2-Acrylamidoacetic acid (2-AmAA)\textsuperscript{190,191}

Figure 3-2. Reaction scheme for the synthesis of 2-AmAA.

2-AmAA was prepared in a 500 mL round bottomed flask with a magnetic stirrer, by adding glycine (22 mL, 133 mmol) to NaOH (2M, 264 mL) and cooling to 0 °C by using an ice bath. After glycine dissolved completely, acrylic chloride (24.1 g, 266 mmol) was added dropwise over a period of 30 minutes via an addition funnel. The reaction mixture was stirred for 1 hour. The resulting mixture was acidified to pH 1.0 with HCl (1 M). The crude product was saturated with NaCl and extracted with ethyl acetate (EtOAc) five times from the acidified aqueous solution and then dried over magnesium sulfate. The solution volume was reduced to 25 mL with a rotary evaporator. The obtained solution was diluted with ethyl acetate and cool to 0 °C with vigorous stirring to precipitate a solid. After filtering, then the formed suspension was washed with ethyl acetate/diethyl ether (1:1) (200mL). The solvent was removed with a rotary evaporator. Precipitation was carried out another time, and the final white solid (6.62 g, 39 \%) was dried under vacuum.
overnight. Characterisation was carried out by $^1$H-NMR (Figure 3-3) and $^{13}$C-NMR (Figure 3-4). $^1$H-NMR (400 MHz, DMSO) $\delta$ (ppm) 12.58 (s-board, 1H, COOH), 8.43 (s, 1H, NH), 6.29 (dd, $J = 16.9$, $10.2$ Hz, 1H, CH=CH$_2$), 6.11 (d, $J = 17.0$ Hz, 1H, CH$_2$=CH), 5.62 (d, $J = 9.9$ Hz, 1H, CH$_2$=CH), 3.84 (d, $J = 5.3$ Hz, 2H, CH$_2$). $^{13}$C-NMR (101 MHz, DMSO) $\delta$ (ppm) 171.66 (COOH), 165.42 (CONH), 131.68 (-C=), 126.19 (=CH$_2$), 40.00 (CH$_2$).

![Figure 3-3. $^1$H-NMR spectra of 2-AmAA.](image)

![Figure 3-4. $^{13}$C-NMR spectra of 2-AmAA.](image)
CHAPTER 3. Preparation and Characterisation of Monomers and Polymers

2-AmAA monomer was synthesised by using similar conditions according to the reference with modification. The reaction was carried out in sodium hydroxide (2 M) solution using 1:2:4 molar ratio of glycine to acrylic chloride to sodium hydroxide at 0 °C. The mechanism of this acylation reaction is shown in Figure 3-5.

![Figure 3-5. Scheme of the mechanism of synthesis of 2-AmAA.](image)

3.2.2 4-Acrylamidobutanoic acid (4-AmBA)

![Figure 3-6. Reaction scheme for the synthesis of 4-AmBA.](image)

Similar to 2-AmAA, 4-AmBA synthesis was carried out in a 500 mL round bottomed flask with a magnetic stirrer, by adding 4-aminobutanoic acid (4-ABA) (10 g, 97.0 mmol) to NaOH (2M, 194 mL) and cooling to 0 °C by using an ice bath. After glyline dissolved completely, acrylic chloride (19 mL,
194 mmol) was added dropwise over a period of 30 min via an addition funnel. The reaction mixture was stirred for 1 hour. The pH of the resulting mixture was adjusted to 1.0 using HCl (1 M). The aqueous phase was saturated with NaCl and extracted with EtOAc (5 x), then dried over magnesium sulfate. The solution volume reduced in the rotary evaporator to 25mL. The obtained solution was allowed to precipitate in the freezer overnight, filtered, washed with cold EtOAc and dried under vacuum. The final white solid (7.2 g, 47 %) was yielded. Characterisation was carried out by $^1$H-NMR (Figure 3-7) and $^{13}$C-NMR (Figure 3-8). $^1$H-NMR (400 MHz, DMSO) δ (ppm) 12.08 (s-board, 1H, COOH), 8.10 (s, 1H, NH), 6.20 (dd, $J = 16.8, 10.0$ Hz, 1H, $\text{CH}_2\text{=CH}_2$), 6.07 (d, $J = 16.8$ Hz, 1H, $\text{CH}_2\text{=CH}_2$), 5.56 (d, $J = 9.7$ Hz, 1H, $\text{CH}_2\text{=CH}_2$), 3.14 (d, $J = 5.8$ Hz, 2H, $\text{CH}_2\text{-NH}$), 2.23 (t, $J = 6.9$ Hz, 2H, $\text{CH}_2\text{-COOH}$), 1.70 (dd, $J = 38.8, 32.1$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$). $^{13}$C-NMR (101 MHz, DMSO) δ (ppm) 174.60 (COOH), 165.11 (CONH), 132.15 ($\text{-C}$=), 125.39 ($\text{=C}$H), 31.26 ($\text{CH}_2\text{COOH}$), 24.93 ($\text{CH}_2\text{-CH}_2\text{-CH}_2$).

Figure 3-7. $^1$H-NMR spectra of 4-AmBA.
As mentioned in the introduction chapter, the effect of polymer on protein crystallisation is likely to depend on the exact polymer functionality. Acrylamide monomers 2-AmAA and 4-AmBA were selected as anionic monomers, due to the presence of ionisable sulfonic groups, which could introduce some negative charges during the protein crystallisation process. Then those two monomers were used for RAFT polymerisation to form hydrophilic homopolymers. However, the product yield of those monomers was not as high as literature reported (70 %), this may be because of the inevitable loss during purification process.
3.3 Polymer synthesis

3.3.1 Atom transfer radical polymerisation (ATRP) method

General protocol for ATRP: 143

In a typical procedure, Cu(I)Br/2,2'-bipyridine (Bipy) was the catalytic system and methyl 2-bromopropionate (MBrP) was the initiator. Polymerisation was conducted in a tube fitted with a two way stopcock and sealed with a septum. The polymerisation solution was degassed by three vacuum-nitrogen cycles and placed into an oil bath at 70 °C. An NMR spectrum was recorded at the beginning of the experiment. The reaction was stopped by removing the septum thus exposing the reaction to oxygen. After reaction, an NMR spectrum was recorded to calculate the degree of conversion. The reaction mixture was passed through aluminum oxide and washed through with chloroform to remove the catalyst. The polymer was precipitated in a 100:1 excess of petroleum ether to remove the un-reacted monomer.

3.3.1.1 Polymer conversion calculations

The overall monomer conversion was calculated by 1H-NMR spectra by comparing the vinyl proton signals from the monomers (5.6 and 6.1 ppm) to the overall integration from the OCH$_2$ adjacent to the methacrylate group (4.01-4.48 ppm) for methacrylate-based monomers: PEGMA$_{475}$ and DMAEMA.
3.3.1.2 Poly[poly(ethylene glycol) methyl ether methacrylate]$_{475}$

[p(PEGMA)$_{475}$] (P1)$^{193,194}$

![Reaction scheme for the synthesis of p(PEGMA)$_{475}$ homopolymer (P1) via ATRP.](image)

In a typical ATRP procedure, 2,2-Bipyridine (0.13 g, 0.84 mmol), PEGMA$_{475}$ (3 g, 6.32 mmol), toluene (3.75 mL) and methyl 2-bromopropionate (MBrP) (0.07 g, 0.42 mmol) were added to a 10 mL tube fitted with a two way stopcock and sealed with a septum. The tube was connected to both a vacuum line and a nitrogen pump. The mixture was degassed by three freeze thaw cycles in liquid nitrogen. The frozen mixture was charged with nitrogen before the addition of Cu(I)Br (0.06 g, 0.42 mmol). The mixture was then degassed by three vacuum-nitrogen cycles and placed into an oil bath at 70 °C for 6 h. An NMR spectrum was recorded at the beginning of the experiment. Samples were taken at specific time points by piercing the septum with a syringe; the septum was resealed using parafilm. Air was removed from the syringe by purging it with nitrogen three times. The reaction was stopped by removing the septum thus exposing the reaction to oxygen. After reaction, NMR spectrum was
recorded to enable calculation of degree of conversion. The reaction mixture was passed through aluminum oxide and washed through with chloroform to remove the catalyst. The mixture was precipitated in a 100:1 excess of petroleum ether to remove the un-reacted monomer, and then yielded the polymer (1.9 g, 63 %). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 4.28-3.92 (m, 2H, COO-CH$_2$-CH$_2$O), 3.72-3.52 (m, 2xH, OCH$_2$-(CH$_2$)$_2$O-CH$_3$), 3.41-3.36 (m, 3H, OCH$_3$), 2.39-1.56 (m, 2H, CH$_2$-MA backbone), 1.18-0.55 (m, 3H, CH$_3$-MA). IR $\nu$ (cm$^{-1}$) 2849 (C-H), 1721 (C=O), 1464, 1346 (C-H), 1240, 1089 (C-O), 1026, 934, 846 (C-C). The $^1$H-NMR and $^{13}$C-NMR spectra of monomer PEGMA$_{475}$ are shown in Appendix (Page 330).

Overall monomer conversion was calculated from $^1$H-NMR spectra by comparing the vinyl proton signals from the monomers (5.6 and 6.1 ppm, Figure 3-10, signals a and b) to the overall integration from the OCH$_2$ across the range 3.9 to 4.4 ppm (Figure 3-10, signal d). In the region of 3.9-4.4 ppm two protons of the remaining monomers resonate as well as two protons from the polymer.
CHAPTER 3. Preparation and Characterisation of Monomers and Polymers

Figure 3-10. $^1$H-NMR spectra for the polymerisation of PEGMA$_{475}$ to yield p(PEGMA$_{475}$) (P1). Top: Reaction mixture at the beginning of the polymerisation experiment. Bottom: Purified polymer P1.

Figure 3-11. Representative FTIR for p(PEGMA$_{475}$) (P1).

P(PEGMA$_{475}$) was synthesised by using the general ATRP method as above shown. The size of the polymer was controlled by the ratio of
[Monomer]:[Initiator]. Several experiments with different [Monomer]:[Initiator] ratios for the synthesis of p(PEGMA<sub>475</sub>) were carried out (Table 3-1).

Table 3-1. Summary of synthesis of p(PEGMA<sub>475</sub>) (P1) via ATRP

<table>
<thead>
<tr>
<th>No.</th>
<th>[M]:[I]&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Conversion&lt;sup&gt;1H-NMR&lt;/sup&gt;</th>
<th>Time in minutes</th>
<th>&lt;sup&gt;a&lt;/sup&gt;M&lt;sub&gt;n&lt;/sub&gt; (GPC)</th>
<th>&lt;sup&gt;a&lt;/sup&gt;M&lt;sub&gt;n&lt;/sub&gt; (Theoretical)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50:1</td>
<td>30 %</td>
<td>360</td>
<td>27109</td>
<td>7196</td>
<td>1.15</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15:1</td>
<td>60 %</td>
<td>120</td>
<td>30815</td>
<td>4275</td>
<td>1.20</td>
</tr>
<tr>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20:1</td>
<td>83 %</td>
<td>180</td>
<td>36388</td>
<td>7885</td>
<td>1.26</td>
</tr>
<tr>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100:1</td>
<td>84 %</td>
<td>540</td>
<td>39405</td>
<td>39900</td>
<td>1.23</td>
</tr>
</tbody>
</table>

a) Experimental conditions: 70 °C; Monomer/Toluene=1:2 (w/v); [I]/[CuBr]/[Bipy]=1/1/2. b) Experimental conditions: 70 °C; monomer/toluene=1:1.25 (w/v); [I]/[CuBr]/[Bipy]=1/1/2. c) Molar ratio of [Monomer]:[Initiator]. Theoretical, from monomer/initiator ratio and monomer conversion. GPC analysis (eluent-chloroform, poly(styrene) standards). Polydispersity index (PDI) from GPC.

Table 3-1 shows a summary of p(PEGMA<sub>475</sub>) (P1) synthesised in this study and Figure 3-12 shows their kinetic plots. Generally, a conversion of 60 % was targeted to decrease the possibility of side reactions. Experiment 1 with [Monomer]:[Initiator] = 50:1, the conversion was about 30 % after 6 hours, and the PDI measured by GPC was found about 1.15 (Table 3-1, entry 1). The kinetic plot shows its slow initiation (Figure 3-12, 1). As the conversion of this experiment was relatively low, experiments 2-4 with different [Monomer]:[Initiator] ratios and a smaller amount of toluene were carried out. Generally the conversions were above 60 %, and the PDI measured by GPC were found about 1.20 (Table 3-1, entries 2-4). The polymerisation of p(PEGMA<sub>475</sub>) (P1) exhibited pseudo-first order kinetics and a linear increase of molecular weight with conversion (Figure 3-12) while the polydispersity of the
polymers was narrow throughout the polymerisation. In this way, several materials could be prepared, with different molecular weights.

![Figure 3-12. Some examples of kinetic plots for the synthesis of p(PEGMA\textsubscript{475}) (P1) in this study.](image)

The GPC used to determine molecular weight data (M\textsubscript{n} GPC) for the synthesis of p(PEGMA\textsubscript{475}) showed large discrepancies compared to the theoretical number molecular weight (M\textsubscript{n} Theoretical) in some experiments (Table 3-1, entries 1-3). Molar masses determined by GPC in chloroform will depend on how the polymer interacts with the column and how similar the polymer compared to the standards (polystyrene). From previous researchers’ study, polymers with macromolecular brushes may provide unusual GPC results;\textsuperscript{195} and the PDI may have been influenced by chain branching, thus, some side reactions may take place due to the presence of PEG-bismethacrylates as impurities in the PEGMA\textsubscript{475} monomer supplied.
3.3.1.3 Poly[2-(dimethylamino)ethyl methacrylate] [p(DMAEMA)]

(P2)\(^{196,197,198}\)

![Reaction scheme for the synthesis of p(DMAEMA) (P2) via ATRP.](image)

Typically, the same ATRP procedure as described above (Section 3.3.1.2) was carried out. 2,2-Bipyridine (0.12 g, 0.76 mmol), DMAEMA (6 g, 38.2 mmol), toluene (6 mL), methyl 2-bromopropionate (MBrP) (0.06 g, 0.38 mmol) and Cu(I)Br (0.05 g, 0.38 mmol) were added to a tube fitted with a two way stopcock and sealed with a septum. After being degassed, the reaction mixture was charged with nitrogen and placed into an oil bath at 70 °C overnight. The reaction mixture was passed through aluminum oxide and washed through with chloroform to remove the catalyst. The polymer was precipitated in a 100:1 excess of petroleum ether to remove the un-reacted monomer. Then a yellow compound was obtained (4.7 g, 78 %). \(^1\)H-NMR (400 MHz, CDCl\(_3\)) \(\delta\) (ppm) 4.28-3.90 (m, 2H, OCH\(_2\)), 2.73-2.42 (m, 2H, CH\(_2\)N), 2.40-2.13 (m, 6H, N(CH\(_3\))\(_2\)), 2.08-1.55 (m, 2H, CH\(_2\)-MA backbone), 1.17-0.62 (m, 3H, CH\(_3\)-MA).
IR ν (cm$^{-1}$) 2933, 2762 (C-H), 1720 (C=O), 1447, 1384 (C-H), 1262, 1140 (C-O), 1231 (C-N), 1013 (C-C). The $^{13}$C-NMR spectra of monomer DMAEMA is shown in Appendix (Page 331).

Figure 3-14. $^1$H-NMR spectra for the polymerisation of DMAEMA to yield p(DMAEMA) (P2). Top: Monomer DMAEMA. Bottom: Purified polymer p(DMAEMA) (P2).

Figure 3-15. Representative FTIR for p(DMAEMA) (P2).
Table 3-2 shows selected properties of p(DMAEMA) (P2) synthesised in this study. The size of the polymer was also controlled by the [Monomer]:[Initiator] ratio. Two repeat experiments with different [Monomer]:[Initiator] ratios were carried out. The PDI was measured by CHCl₃ GPC (polystyrene standard) and found in the range 1.20-1.60. Experiments 1 and 2 with a molar ratio of [Monomer]:[Initiator] = 100:1, generally the conversion was about 28 % based on monomer masses after 6 hours, and the PDI measured by GPC was found about 1.30 (Table 3-2, entries 1 and 2). The conversions of these experiments were relatively low, a common method for resolving this problem is either prolong the reaction time or lower the ratio of [Monomer]:[Initiator]. Then, experiments 3 and 4 with a molar ratio of [Monomer]:[Initiator] = 50:1 were carried out (Table 3-2, entries 3 and 4). The conversion was about 70 % after 6 hours, and the PDI measured by GPC was found about 1.52.

Table 3-2. Summary of synthesis of p(DMAEMA) (P2) via ATRP

<table>
<thead>
<tr>
<th>No.</th>
<th>[M]:[I]b</th>
<th>Conversion</th>
<th>Time (min)</th>
<th>Mn (GPC)</th>
<th>Mn (Theoretical)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>100:1</td>
<td>16 %</td>
<td>210</td>
<td>40816</td>
<td>2562</td>
<td>1.25</td>
</tr>
<tr>
<td>2a</td>
<td>100:1</td>
<td>28 %</td>
<td>360</td>
<td>42403</td>
<td>4402</td>
<td>1.30</td>
</tr>
<tr>
<td>3a</td>
<td>50:1</td>
<td>70 %</td>
<td>360</td>
<td>44171</td>
<td>5502</td>
<td>1.52</td>
</tr>
<tr>
<td>4a</td>
<td>50:1</td>
<td>68 %</td>
<td>360</td>
<td>46299</td>
<td>5345</td>
<td>1.52</td>
</tr>
</tbody>
</table>

a) Experimental conditions: 70 °C; Monomer/Toluene=1:1 (w/v); [I]/[CuBr]/[Bipy]=1/1/2. b) Molar ratio of [Monomer]:[Initiator]. Theoretical, from monomer/initiator ratio and monomer conversion. GPC analysis (chloroform, poly(styrene) standards). Polydispersity index (PDI) from GPC.

Figure 3-16 shows selected kinetic plots for the synthesis of p(DMAEMA) (P2) by ATRP. The polymerisation of p(DMAEMA) (P2) exhibited pseudo-first
order kinetics and a linear increase of molecular weight with conversion. In this way, several materials could be prepared, with different molecular weights.

Figure 3-16. Kinetic plots for the synthesis of p(DMAEMA) (P2) via ATRP method. Blue represents experiment 2 (Table 3-2, entry 2). Black represents experiment 3 (Table 3-2, entry 3)

However, the number average molecular weights determined from GPC ($M_n$ GPC) was much higher compared to the theoretical molecular weights ($M_n$ Theoretical) based on monomer conversion (Table 3-2). Also, polymers had slightly broad molecular weight distributions. These results suggested that GPC with polystyrene standard and CHCl$_3$ as the solvent may not a proper method for characterising this kind of polymer. Further GPC investigation may need by using more closely matched calibration standard rather than polystyrene.
CHAPTER 3. Preparation and Characterisation of Monomers and Polymers

3.3.1.4 Quaternisation of p(DMAEMA) (P2)*

In a round bottom flask with a magnetic stirring bar, p(DMAEMA) homopolymer (3 g) was dissolved in tetrahydrofuran (THF) (90 mL). The flask was sealed with a rubber septum and a molar equivalent of methyl iodide calculated was added via syringe. The mixture was left to react for 48 hours at room temperature. The color of the reaction mixture changed from dark yellow to light yellow. After this time the flask was opened and the solvent was removed under reduced pressure to yield a light yellow solid. Then around 10 mL of deionised water was added and freeze dried to yield the desired polymer.

\[ ^1H-NMR \ (400 \ MHz, D_2O) \delta \ (ppm) \ 4.61-4.31 \ (m, \ 2H, \ OCH_2-quadersied \ DMAEMA), \ 4.23-4.08 \ (m, \ 2H, \ OCH_2-DMAEMA), \ 4.00-3.64 \ (m, \ 2H, \ CH_2N(CH_3)_3), \ 3.41-3.03 \ (m, \ 9H, \ N(CH_3)_3), \ 2.94-2.80 \ (m, \ 2H, \ CH_2N(CH_3)_2), \ 2.54-2.33 \ (m, \ 6H, \ N(CH_3)_2), \ 2.19-1.77 \ (m, \ 4H, \ CH_2-MA backbone), \ 1.23-0.71 \ (m, \ 6H, \ CH_3-MA). IR \ \nu \ (cm^{-1}) \ 3433 \ (R_4N^+), \ 2952 \ (C-H), \ 1720 \ (C=O), \ 1468 \ (C-H), \ 1261, \ 1141 \ (C-O), \ 1226 \ (C-N), \ 946 \ (C-C). \]

In this study, a modification was carried out on p(DMAEMA) (P2) to introduce
permanent positive charges by the addition of a methyl group to the p(DMAEMA) tertiary amine repeating unit with CH₃I, i.e. quaternisation. The degrees of quaternisation were targeted at 25 %, 50 %, 75 % and 100 %. Characterisation was carried out by ¹H-NMR and IR. An example of ¹H-NMR spectra is shown in Figure 3-18. The experimental level of quaternisation was calculated through ¹H-NMR by comparing relevant peaks in the quaternised (Figure 3-18, signal g) and un-quaternised p(DMAEMA) repeating units (Figure 3-18, signal d).

Figure 3-18. ¹H-NMR spectra for quaternised p(DMAEMA) (P2).
3.3.1.5 Poly[2-(dimethylamino)ethyl methacrylate-stat-poly(ethylene glycol) methyl ether methacrylate]\textsuperscript{475} [p(DMAEMA-stat-PEGMA\textsubscript{475})] (P3)\textsuperscript{190,191,201}

\[
\begin{align*}
\text{DMAEMA} + \text{PEGMA}_{475} \xrightarrow{70 \, ^\circ \text{C/Toluene \, Cu(I)Br/2,2-Bipyridine}} \text{p(DMAEMA-stat-PEGMA}_{475})
\end{align*}
\]

Figure 3-20. Reaction scheme for the synthesis of a random copolymer: p(DMAEMA-stat-PEGMA\textsubscript{475}) (P3) via ATRP.

2,2-Bipyridine (0.02 g, 0.14 mmol), DMAEMA (0.11 g, 0.70 mmol), PEGMA\textsubscript{475} (3.01 g, 6.34 mmol), toluene (3.12 mL), methyl 2-bromopropionate (MBrP) (0.01 g, 0.07 mmol) and Cu(I)Br (0.01 g, 0.07 mmol) were added to a 10 mL tube fitted with a two way stopcock and sealed with a septum and placed into an oil bath at 70 °C for 5 h 50 min. The reaction mixture was
passed through aluminum oxide and washed through with chloroform to remove the catalyst. The polymer was precipitated in a 100:1 excess of petroleum ether to remove the un-reacted monomer. This produced 0.78 g of polymer (25 % yield). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 4.31-4.05 (m, 4H, CO-OCH$_2$) (both), 3.92-3.61 (m, 2xH, CO-OCH$_2$-(CH$_2$)$_2$O-CH$_3$), 3.60-3.51 (m, 3H, OCH$_3$), 2.52-2.32 (m, 2H, CH$_2$N), 2.15-1.90 (m, 6H, N(CH$_3$)$_2$), 1.31-0.85 (m, 4H, CH$_2$-MA backbone) (Appendix Page 331).

Figure 3-21 shows an example of $^1$H-NMR spectra for the polymerisation of DMAMEA and PEGMA$_{475}$ to yield p(DMAEMA-\textit{stat}-PEGMA$_{475}$) (P3). Overall monomer conversion was calculated from $^1$H-NMR spectra (Figure 3-21, signals a and b) by comparing the vinyl proton signals from the monomers (5.4-6.4 ppm) to the overall integration across the range 4.1 to 4.6 ppm (Figure 3-21, signals c and f).
A random copolymer of DMAEMA and PEGMA\textsubscript{475} was synthesised via ATRP method as above and selected properties are shown in Table 3-3. With the ratio of $[\text{DMAEMA}]:[\text{PEGMA}_{475}]:[\text{Initiator}] = 10:90:1$, the polymerisation of p(DMAEMA\textit{-stat}-PEGMA\textsubscript{475}) (P3) proceeded slowly, only 25\% of conversion was achieved after 6 h, and the PDI measured by GPC was found about 1.29.
Further experiments with a longer reaction time or different [DMAEMA]:[PEGMA\textsubscript{475}]:[Initiator] ratios may need to be carried out.

<table>
<thead>
<tr>
<th>DMAEMA</th>
<th>PEGMA\textsubscript{475}</th>
<th>Conversion</th>
<th>Time (min)</th>
<th>(M_n) (GPC)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 %</td>
<td>90 %</td>
<td>25 %</td>
<td>350</td>
<td>52915</td>
<td>1.29</td>
</tr>
</tbody>
</table>

Experimental conditions: 70 °C; Monomers/Toluene=1:1 (w/v); [I]/[CuBr]/[Bipy]=1/1/2. GPC analysis (chloroform, poly(styrene) standards). Polydispersity index (PDI) from GPC.

### 3.3.2 Reversible addition-fragmentation chain transfer polymerisation (RAFT) method

General protocol for RAFT polymerisation:\textsuperscript{202}

2-(ethylthiocarbonothioylthio)-2-methylpropanoic acid (CAT) was employed as the RAFT agent, 4,4′-azobis(4-cynovaleric acid) (V-501) as the initiator. Polymerisations were conducted in round bottom flasks sealed with a rubber septum and parafilm. An NMR spectrum was recorded at the beginning of the experiment. The polymerisation solutions were degassed using argon for at least 10 min and transferred to an oil bath preheated to 70 °C. After reaction, the solution was quenched by cooling in ice-water and opening to air. Another NMR spectrum was recorded to enable calculation of degree of conversion. The polymer was purified by dialysis in water with dialysis membrane (MWCO 1000). The molar mass of all the polymers made was estimated from the monomer conversion and GPC data. For the removal of a RAFT end-group, the reaction was carried out at 70 °C.
3.3.2.1 Polymer conversion calculations

The overall monomer conversion was calculated by $^1$H-NMR spectra by comparing the vinyl proton signals from the monomers (5.6 and 6.1 ppm) to the overall integration from CHCl$_3$ (around 7.26 ppm) (50 µL was added to each NMR sample as the standard for calculation) in the case of (meth)acrylamide-based monomers, including DMAPMAm, AMPS, 2-AmAA and 4-AmBA.

3.3.2.2 Poly[N-(3-(dimethylamido)propyl)methacrylamide]

[p(DMAPMAm)] (P4)

Figure 3-22. Reaction schemes for the synthesis of p(DMAPMAm) (P4) via RAFT method and the removal of RAFT end-group.
In a typical RAFT polymer synthesis experiment, DMAPMAm (1000 mg, 5.874 mmol, 0.75 M) in acetate buffer solution (10 mM, 6.768 mL), 2-(ethylthiocarbonothioylthio)-2-methylpropanoic acid (CAT) (0.059 mmol, 0.24 M) in ethanol (0.245 mL) and V-501 (1.65 mg, 0.006 mmol, 0.03 M) in ethanol (0.196 mL) were prepared separately and then mixed together (to make a final ratio 100:1 of monomer to RAFT agent). The polymerisation was carried out overnight. The p(DMAPMAm)-RAFT was purified by dialysis against water and recovered as a light yellow powder (716.8 mg, 72 %) after freeze-drying from water (3 days). The p(DMAPMAm)-RAFT polymer (428 mg) in water and V-501 (258 mg) in ethanol were prepared separately and then mixed together (to make a final ratio 1:30 of polymer to initiator). In order to remove the RAFT end-group, this mixture was degassed and reacted at 70 °C overnight. The title compound p(DMAPMAm) (P4) was purified by dialysis against water and recovered as a white powder (369 mg, 86 %) after freeze-drying from water (3 days). $^1$H-NMR (400 MHz, D$_2$O) $\delta$ (ppm) 3.33-2.95 (m, 4H, NH-CH$_2$-CH$_2$-CH$_2$), 2.96-2.71 (m, 6H, N(CH$_3$)$_2$), 2.04-1.48 (m, 2H, NH-CH$_2$-CH$_2$), 1.18-0.69 (m, 2H, CH$_2$-MAm backbone). IR $\nu$ (cm$^{-1}$) 3329 (N-H), 2963, 2687 (C-H), 1618 (C=O), 1522 (N-H), 1462 (C-H), 1202 (C-N). The spectra of $^{13}$C-NMR of monomer DMAPMAm and GPC of polymer p(DMAPMAm) are shown in Appendix Page 332.
Figure 3-23. $^1$H-NMR spectra for the polymerisation of DMAPMAm to yield p(DMAPMAm) (P4). Top: Monomer DMAPMAm. Bottom: Purified polymer p(DMAPMAm) (P4).

Figure 3-24. Representative FTIR for p(DMAPMAm) (P4).
P(DMAPMAm) (P4) homopolymers were synthesised using the general polymerisation procedure as described above. In the reaction conditions, CAT was used as chain transfer agent (CTA) and V-501 was used as a water-soluble initiator. DMAPMAm monomer was initially dissolved in 10 mM acetate buffer solution pH 5.5. CAT and V-501 were dissolved in ethanol to reach the concentration of 0.24 M and 0.03 M respectively as stock solutions. The above three portions were mixed to get a final molar ratio of 100:1:0.1 (monomer:CAT:V-501) and monomer concentration of 0.75 M. Temperature was controlled to be 70 °C all through the reaction. After 16 hours reaction, the mixture was cloudy and hard to dissolve in any solvent. This phenomenon suggested that polymerisation was occurred. In order to obtain polymers with different molecular weights, synthesis of p(DMAPMAm) (P4) with three targeted degrees of polymerisation (DPs) 50, 100 and 200 were carried out. The stock solutions of CAT and initiator were fresh made for each polymerisation. Also, the oxygen-excluding process was checked and carried out very carefully. Cationic aqueous GPC was used to characterise p(DMAPMAm) (P4). A summary of properties for the synthesis of p(DMAPMAm) (P4) is shown in Table 3-4. Generally the conversion was above 70 % after 16 h
Table 3-4. Summary of synthesis of p(DMAPMAm) (P4) via RAFT

<table>
<thead>
<tr>
<th>No.</th>
<th>[M]:[CAT]a</th>
<th>DPb</th>
<th>Conversion (1H-NMR) %</th>
<th>Time (h)</th>
<th>Mn (GPC)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>50</td>
<td>45</td>
<td>91%</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1b</td>
<td>38</td>
<td>38</td>
<td>77%</td>
<td>19</td>
<td>5820</td>
<td>1.43</td>
</tr>
<tr>
<td>2a</td>
<td>27</td>
<td>27</td>
<td>27%</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2b</td>
<td>100</td>
<td>88</td>
<td>88%</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2c</td>
<td>83</td>
<td>83</td>
<td>83%</td>
<td>19</td>
<td>9253</td>
<td>1.50</td>
</tr>
<tr>
<td>3a</td>
<td>167</td>
<td>84</td>
<td>84%</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3b</td>
<td>200</td>
<td>150</td>
<td>75%</td>
<td>19</td>
<td>15641</td>
<td>1.69</td>
</tr>
</tbody>
</table>

a Molar ratio of [Monomer]:[Initiator] (targeted DP). b The actual degree of polymerisation (DP) in the brackets is the final value calculated from 1H-NMR. GPC analysis (eluent-200 mM NaCl with 0.1 % trifluoroacetic acid, polyvinylpyrrolidone standards). Polydispersity index (PDI) from GPC.

3.3.2.3 Poly(2-acrylamido-2-methyl-1-propanesulfonic acid) [p(AMPS)] (P5)

Figure 3-25. Reaction schemes for the synthesis of p(AMPS) (P5) via RAFT method and the removal of RAFT end-group.
Similar RAFT experiment as DMAPMAm was carried out. AMPS (1000 mg, 4.83 mmol, 0.75 M) in acetate buffer solution (10 mM, 6.43 mL), CAT (0.048 mmol, 0.24 M) in ethanol (0.2 mL) and V-501 (1.4 mg, 0.005 mmol, 0.03 M) in ethanol (0.167 mL) were prepared separately and then mixed together (to make a final ratio 100:1 of monomer to RAFT agent). The polymerisation was carried out overnight. The p(AMPS)-RAFT was purified by dialysis against water and recovered as a light yellow powder (869 mg, 87 %) after freeze-drying from water (3 days). The p(AMPS)-RAFT (255 mg) in water and V-501 (200 mg) in ethanol were prepared separately and then mixed together (to make a final ratio 1:30 of polymer to initiator). In order to remove the RAFT end-group, this mixture was degassed and reacted at 70 ºC overnight. The title compound p(AMPS) (P5) was purified by dialysis against water and recovered as a white powder (230 mg, 90 %) after freeze-drying from water (3 days). $^1$H-NMR (400 MHz, D$_2$O) δ (ppm) 7.82-7.20 (m, 1H, NH), 3.65-3.02 (m, 2H, CH$_2$-SO$_3$H), 2.27-1.84 (m, 1H, CH-Am backbone), 1.61-1.30 (m, 6H, C(CH$_3$)$_2$). IR ν (cm$^{-1}$) 3298 (N-H), 2916 (C-H), 1638 (C=O), 1531 (N-H), 1439 (C-H), 1368 (C-N), 1179, 1040 (SO$_2$), 621 (S-O). The spectra of $^{13}$C-NMR of monomer AMPS and GPC of polymer p(AMPS) are shown in Appendix Page 333.
Figure 3-26. $^1$H-NMR spectra for the polymerisation of AMPS to yield p(AMPS) (P5). Top: Monomer AMPS. Bottom: Purified polymer p(AMPS) (P5).

P(AMPS) (P5) homopolymers were synthesised by using the general polymerisation procedure as described above. After 3 hours reaction, the mixture was cloudy and hard to dissolve in any solvent. This phenomenon suggested that polymerisation was occurred. In order to obtain polymers with
different molecular weights, synthesis of p(AMPS) homopolymer with four targeted degrees of polymerisation (DPs) 40, 70, 100 and 200 were carried out, generally the conversion was above 75%. Aqueous GPC was used to characterise p(AMPS) (P5). A summary of synthesis of pAMPS (P5) is shown in Table 3-5.

Table 3-5. Summary of synthesis of p(AMPS) (P5) via RAFT

<table>
<thead>
<tr>
<th>No.</th>
<th>Monomer</th>
<th>DP</th>
<th>Conversion (1H-NMR)</th>
<th>Time (h)</th>
<th>( M_n ) (Theoretical)</th>
<th>( M_n ) (GPC)</th>
<th>Polydispersity Index (PDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AMPS</td>
<td>38</td>
<td>96 %</td>
<td>2</td>
<td>8100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>AMPS</td>
<td>53</td>
<td>75 %</td>
<td>1</td>
<td>11208</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>AMPS</td>
<td>99</td>
<td>99 %</td>
<td>3</td>
<td>20120</td>
<td>21787</td>
<td>1.14</td>
</tr>
<tr>
<td>4</td>
<td>AMPS</td>
<td>200</td>
<td>100 %</td>
<td>16</td>
<td>41674</td>
<td>47561</td>
<td>1.19</td>
</tr>
</tbody>
</table>

The actual degree of polymerisation (DP) in the brackets is the final value calculated from \(^1\)H-NMR. Theoretical, from monomer/initiator ratio and monomer conversion. GPC analysis (eluent-DPBS, poly(ethylene oxide) standards). Polydispersity index (PDI) from GPC.

3.3.2.4 Poly(2-acrylamidoacetic acid) [p(2-AmAA)] (P6)

Figure 3-28. Reaction schemes for the synthesis of p(2-AmAA) (P6) homopolymer via RAFT method and the removal of RAFT end-group.
2-AmAA (1000 mg, 7.75 mmol, 0.75 M) in acetate buffer solution (10 mM, 10.3 mL), CAT (0.077 mmol, 0.24 M) in ethanol (0.322 mL) and V-501 (2.17 mg, 0.008 mmol, 0.03 M) in ethanol (0.226 mL) were prepared separately and then mixed together. The polymerisation was carried out for 3 h. The polymer was purified by dialysis against water and recovered as a light yellow powder (542 mg, 54 %). In order to remove the RAFT end-group, the p(2-AmAA)-RAFT polymer (542 mg) in water and V-501 (150 mg) in ethanol were prepared separately and then mixed together. The title compound p(2-AmAA) was purified by dialysis against water and recovered as a white powder (490.8 mg, 91 %) after freeze-drying from water (3 days). $^1$H-NMR (400 MHz, D$_2$O) $\delta$ (ppm) 4.09-3.27 (m, 2H, NH-CH$_2$), 2.38-1.88 (m, 1H, CH-Am backbone), 1.79-1.17 (m, 2H, CH$_2$-Am backbone). IR $\nu$ (cm$^{-1}$) 3250 (N-H), 2932 (C-H), 1549 (N-H), 1383 (C-O), 1233 (O-H), 1018 (C-N). The GPC spectra of polymer p(2-AmAA) are shown in Appendix Page 334.

Figure 3-29. $^1$H-NMR spectra for p(2-AmAA) (P6).
P(2-AmAA) (P6) homopolymers were also prepared by using the above polymerisation procedure similar as described in section 3.3.2.2. Table 3-6 shows some properties of p(2-AmAA) (P6) prepared in this study. Synthesis of p(2-AmAA) homopolymer with several targeted degrees of polymerisation (DPs) 20, 50, 100 and 200 were carried out. Aqueous GPC was used to characterise p(2-AmAA) (P6).

Table 3-6. Summary of synthesis of p(2-AmAA) (P6) via RAFT

<table>
<thead>
<tr>
<th>No.</th>
<th>Monomer</th>
<th>DP</th>
<th>Conversion (1H-NMR)</th>
<th>Time (hours)</th>
<th>(M_n) (Theoretical)</th>
<th>(M_n) (GPC)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-AmAA</td>
<td>18</td>
<td>90 %</td>
<td>3</td>
<td>2322</td>
<td>2501</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2-AmAA</td>
<td>46</td>
<td>80 %</td>
<td>2.5</td>
<td>6158</td>
<td>6153</td>
<td>1.07</td>
</tr>
<tr>
<td>3</td>
<td>2-AmAA</td>
<td>72</td>
<td>72 %</td>
<td>2.5</td>
<td>9296</td>
<td>14513</td>
<td>1.05</td>
</tr>
<tr>
<td>4</td>
<td>2-AmAA</td>
<td>150</td>
<td>75 %</td>
<td>16</td>
<td>19350</td>
<td>26205</td>
<td>1.09</td>
</tr>
</tbody>
</table>

*The actual degree of polymerisation (DP) in the brackets is the final value calculated from 1H-NMR. Theoretical, from monomer/initiator ratio and monomer conversion. GPC analysis (eluent-DPBS, poly(ethylene oxide) standards). Polydispersity Index (PDI) from GPC.*
3.3.2.5 Poly(4-acrylamidobutanoic acid) [p(4-AmBA)] (P7)

![Reaction scheme](image)

Figure 3-31. Reaction schemes for the synthesis of p(4-AmBA) (P7) homopolymer via RAFT method and the removal of RAFT end-group.

4-AmBA (2000 mg, 12.7 mmol, 0.75 M) in acetate buffer solution (10 mM, 17.0 mL), CAT (0.127 mmol, 0.24 M) in ethanol (0.529 mL) and V-501 (3.57 mg, 0.013 mmol, 0.03 M) in ethanol (0.372 mL) were prepared separately and then mixed together. The polymerisation was carried out overnight. The polymer was purified by dialysis against water and recovered as a light yellow powder (1804 mg, 90 %) after freeze-drying from water (3 days). The p(4-AmBA)-RAFT polymer (200 mg) in water and V-501 (150 mg) in ethanol were prepared separately and then mixed together to remove the RAFT end-group. The title compound p(4-AmBA) was purified by dialysis against water and recovered as a white powder (192.2 mg, 96 %) after freeze-drying.
from water (3 days). $^1$H-NMR (400 MHz, D$_2$O) $\delta$ (ppm) 3.21-2.92 (m, 2H, NH-CH$_2$), 2.29-2.14 (m, 2H, CH$_2$-COOH), 2.00-1.79 (m, 1H, CH-Am backbone), 1.79-1.57 (m, 2H, CH$_2$-CH$_2$-CH$_2$), 1.58-1.25 (m, 2H, CH$_2$-Am backbone). IR $\nu$ (cm$^{-1}$) 3240 (N-H), 2916 (C-H), 1636 (C=O), 1535 (N-H), 1395 (C-O), 1175 (O-H), 1042 (C-N). The GPC spectra of polymer p(4-AmBA) are shown in Appendix Page 334.

![Figure 3-32. $^1$H-NMR spectra for p(4-AmBA) (P7).](image)

Figure 3-32. $^1$H-NMR spectra for p(4-AmBA) (P7).

![Figure 3-33. Representative FTIR for p(4-AmBA) (P7).](image)

Figure 3-33. Representative FTIR for p(4-AmBA) (P7).
Similar as described in section 3.3.2.2, \( p(4\text{-AmBA}) \) (P7) homopolymers were synthesised with four targeted (theoretical) DPs 20, 50, 100 and 200. Aqueous GPC was used to characterise \( p(4\text{-AmBA}) \) (P7). Generally the conversion was around 90\% as shown in Table 3-7.

Table 3-7. Summary of synthesis of \( p4\text{-AmBA} \) (P7) via RAFT

<table>
<thead>
<tr>
<th>No.</th>
<th>Monomer</th>
<th>DP(^a)</th>
<th>Conversion ((^1)H-NMR)</th>
<th>Time (h)</th>
<th>( M_n ) (Theoretical)</th>
<th>( M_n ) (GPC)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-AmBA</td>
<td>21</td>
<td>92%</td>
<td>3</td>
<td>3521</td>
<td>3492</td>
<td>1.09</td>
</tr>
<tr>
<td>2</td>
<td>4-AmBA</td>
<td>50</td>
<td>100%</td>
<td>16</td>
<td>8074</td>
<td>10829</td>
<td>1.04</td>
</tr>
<tr>
<td>3</td>
<td>4-AmBA</td>
<td>100</td>
<td>100%</td>
<td>16</td>
<td>15924</td>
<td>16804</td>
<td>1.09</td>
</tr>
<tr>
<td>4</td>
<td>4-AmBA</td>
<td>200</td>
<td>100%</td>
<td>16</td>
<td>31624</td>
<td>31132</td>
<td>1.06</td>
</tr>
</tbody>
</table>

\(^a\) The actual degree of polymerisation (DP) in the brackets is the final value calculated from \(^1\)H-NMR. Theoretical, from monomer/initiator ratio and monomer conversion. GPC analysis (eluent-DPBS, poly(ethylene oxide) standards). Polydispersity index (PDI) from GPC.

3.3.3 Poly[[2-(methacryloyloxy)ethyl]trimethylammonium chloride-co-N-[2-(methacryloyloxy)ethyl]-dimethyl-(2-sulfoethan)aminium hydroxide] \( [p(METMAC-co-MEDSAH)] \) (P8)

A cationic/zwitterionic copolymer of [2-(methacryloyloxy)ethyl]trimethylammonium chloride and N-[2-(methacryloyloxy)ethyl]-dimethyl-(2-sulfoethan)aminium hydroxide \( [p(METMAC-co-MEDSAH)] \) (P8) was synthesised and kindly supplied by Dr. Eugene Peter Magennis. Molar ratio of METMAC: MEDSAH was 1:1.
Figure 3.34. Reaction scheme for the synthesis of p(METMAC-co-MEDSAH) (P8) by Dr. Eugene Peter Magennis.

To a reaction flask, 144 mg (0.695 mmol) of METMAC, 194 mg (0.695 mmol) of MEDSAH, 1.554 mg (5.6 μmol) of 2-(N-Morpholino)ethyl-2-bromobutyrate (1), 200 μL of a 0.069 M aqueous solution with CuBr₂ and Tris(2-pyridylmethyl)amine (TPA), and 50 μL of DMSO were added. This mixture was degassed for 30 minutes over ice after which 270 μL of a degassed 1 mg/mL solution of ascorbic acid were added to begin the polymerisation. The polymerisation was monitored by ¹H-NMR spectroscopy over time and terminated by exposing to air. The polymers were obtained by dialysis against water for 3 days followed by freeze drying to yield a white amorphous solid (P8). ¹H-NMR (D₂O, 400 MHz) δ (ppm) 4.49 (m, 4H, COCH₂) (METMAC and MEDSHA), 3.78 (m, 4H, NCH₂) (METMAC and MEDSAH), 3.60 (m, 2H, N(CH₃)₂CH₂) (MEDSAH), 3.2 (m, 15H, N(CH₃)) (both), 2.98 (m, 2H, CH₂SO₃) (MEDSAH), 2.28 (m, 2H, CH₂CH₂CH₂) (MEDSAH), 2.30-1.75 (m, 4H, CH₂-MA) (METMAC and MEDSAH), 0.99-2.00 (m, 6H, CH₃) (METMAC
and MEDSAH). IR ν (cm\(^{-1}\)) 3393 (R\(_4\)N\(^+\)), 2941 (C-H), 1715 (C=O), 1474 (C-H), 1153, 1032 (SO\(_2\)), 946 (C-C), 713 (S-O).

![Figure 3-35. Representative \(^1\)H-NMR spectra of p(METMAC-co-MEDSAH) (P8) by Dr. E. Peter Magennis.](image)

![Figure 3-36. Representative FTIR for p(METMAC-co-MEDSAH) (P8).](image)
3.4 General discussion and conclusions

As mentioned in Introduction Chapter, the effects of polymers on protein crystallisation are expected to depend strongly on the polymer functionalities. From previous researchers’ studies, PEG has been found to be an effective additive in promoting the crystallisation of a range of proteins. However, the PEG has a high molecular weight limitation of around 40 kDa, because PEG above that molecular weight will no longer cleared from the kidney. Moreover, PEG itself has limited chemical functionality, and cannot be derivatised easily to introduce groups which can form specific bonds with protein peptide residues. We thus chose poly(ethylene glycol) methyl ether methacrylate (PEGMA), as a neutral methacrylate derivative of PEG to obtain a polymer, p(PEGMA) (P1), analogous to PEG but with hydrophilic side-chain functionality and a hydrophobic main-chain. The PEGMA exhibits a similar biocompatible profile as PEG however since the PEG side chain of the PEGMA is linked by a cleavable ester bond the size limitations for poly(PEGMA) will not be the same as for PEG. Moreover, poly(PEGMA)-based materials have been shown to mediate protein crystallisation in solution.

To test the effects of varying degrees of charge on protein crystallisation, monomers containing tertiary amines groups were selected to afford positive charges, including one methacrylate monomer 2-(dimethylamino)ethyl methacrylate (DMAEMA) was either homopolymerised (P2) and quaternised
or copolymerised with PEGMA_{475} (P3) and one methacrylamide monomer N-(3-(dimethylamido)propyl)methacrylamide (DMAPMAm), which was also homopolymerised (P4) to form cationic polymers. The tertiary amines functionalities were designed to enhance water solubility of the polymers and also to provide lone-pair donor or charged residues dependent on pH and degree of protonation or quaternisation.

Anionic polymers were chosen according to the pKa of the polymers, and also by considering the protein crystallisation pH, such that the polymers could provide charged residues under relevant crystallising conditions. We chose three acrylamide-based main chain monomers, including 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), 2-acrylamidoacetic acid (2-AmAA) and 4-acrylamidobutanoic acid (4-AmBA), due to the presence of ionisable sulfonic acid or carboxyl groups, which could introduce some negative charges and generate anionic water soluble homopolymers (P5-7). Moreover, a cationic/zwitterionic copolymer of [2-(methacryloyloxy)ethyl]trimethylammonium chloride (METMAC) and N-[2-(methacryloyloxy)ethyl]-dimethyl-(2-sulfoethan)aminium hydroxide (MEDSAH) with both positive charges and zwitterionic components was prepared in order to span an even wider range of charge-charge interactions (P8).

P(PEGMA_{475}) (P1), p(DMAEMA) (P2), and a random copolymer of p(DMAEMA-stat-PEGMA_{475}) (molar ratio of 1:9) (P3) were prepared by a
variant of published ATRP route, which has been used for the synthesis of
methacrylate monomers showing fast ATRP kinetics with low polydispersities
by using Cu(I)Br/2,2'-bipyridine (Bipy) catalytic system and methyl
2-bromopropionate (MBrP) as the initiator. N-Methylation of p(DMAEMA)
with different degrees of quaternisation was carried out to vary the total
permanent cation content by the addition of a methyl group to the tertiary
amine repeating unit with CH$_3$I according to literature procedure. CHCl$_3$
was employed for the GPC analysis of those methacrylate polymers. However,
those polymers produced had slightly broad PDIs from GPC analysis. Also the
M$_n$ differed from that calculated by $^1$H-NMR, is explained by the conventional
calibration used on the GPC. This broadening suggests that more closely
matched calibration standard rather than polystyrene may need in the future
studies.

Monomers 2-AmAA and 4-AmBA were prepared in good yields and high
purity by modified literature protocols. P(DMAPMAm) (P4), p(AMPS)
(P5), p(2-AmAA) (P6) and p(4-AmBA) (P7) were synthesised using RAFT
methods according to protocols described in the literature, because RAFT
polymerisation is probably the most versatile and suitable for the synthesis of
acrylamide based polymers. The polymerisation employed
2-(Ethylthiocarbonothioylthio)-2-methylpropanoic acid (CAT) as RAFT agent
and 4,4’-azobis(4-cyanovaleric acid) (V-501) as the initiator. Aqueous
conditions (eluent-200 mM NaCl with 0.1 % TFA or DPBS) were applied for
the GPC analysis of those (meth)acrylamide polymers. It is known that RAFT agent reacts with a propagating polymer chain and forms a macro-RAFT agent. In order to preclude the influence of dithioester on proteins, we used the same initiator to substitute dithioester on the polymer. A cationic/zwitterionic copolymer of p(METMAC-co-MEDSAH) (P8) was kindly supplied by Dr. E. Peter Magennis; METMAC: MEDSAH molar ratio is 1:1.

In these ways, four classes of materials, including neutral, cationic, anionic and cationic/zwitterionic polymers based on methacrylate or (meth)acrylamide monomers with varying degrees of charge, molecular weight and backbone structure were successfully prepared and then investigated for crystallisation studies.
CHAPTER 4

4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

4.1 Introduction

As ‘Chapter 3’ mentioned, four classes of materials, including neutral, cationic, anionic and cationic/zwitterionic polymers based on methacrylate or (meth)acrylamide monomers were successfully prepared. In the present chapter their effect as additives in crystallisation of HEWL is investigated.

The first model protein was hen egg-white lysozyme (HEWL), as this protein has become the most preferred crystallisation system for the majority of protein crystallisation studies. It is readily available and relatively straightforward to crystallise in a specific polymorph (the tetragonal form) within one day. We then designed experimental protocols to investigate the effects of polymers on protein crystal size, crystal packing motifs, and polymorphic forms under crystallisation conditions normally favouring the tetragonal HEWL polymorph.

The isoelectric point (pI) of HEWL is 11.35, therefore when at its crystallisation pH 4.8, HEWL macromolecules are positively charged, i.e. cationic (Table 4-1).\textsuperscript{152}
In order to investigate the effects of polymers on protein crystallisation, such as the use of polymers to help proteins crystallise, or the use of polymers to guide proteins to associate into protein-protein dimer, trimer and higher order complexes, we prepared different classes of polymers with varying degrees of charge, molecular weight and backbone structure, and then studied their role as additives in model protein solutions. The guiding hypothesis was that select functional polymers in the solution could enhance the rate of protein crystallisation from solutions, as well as influence morphology shape, size, habit and polymorph of the crystal. A simple hypothesis linking polymer structure and charge to the effect on protein crystallisation is proposed and shown in Figure 4-1. Details will discuss later. Protein crystallisation experiments were performed on 96-well plates, by using the sitting drop vapour diffusion technique.

Table 4-1. Summary of hen egg-white lysozyme (HEWL)

<table>
<thead>
<tr>
<th>Protein</th>
<th>pI ( ^a )</th>
<th>Mw (kDa)</th>
<th>Crystallisation pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEWL</td>
<td>11.35</td>
<td>14.3</td>
<td>4.8</td>
</tr>
</tbody>
</table>

\(^a\) Isoelectric point (pI) is the pH at which a particular molecule or surface carries no net electrical charge.
Figure 4-1. Schematic representation of the interaction of various polymers with cationic HEWL in the protein crystallisation process. (A) Neutral polymer: H-bonding with water and thus promoting protein crystallisation via a dehydration mechanism. (B) Cationic polymer: charge-charge repulsions between cationic polymer and cationic HEWL. (C) Anionic polymer: attracting cationic HEWL for charge compensation. HEWL macromolecules are positively charged at tetragonal crystallisation pH of 4.8, i.e. cationic. Cl⁻ ions are derived from the crystallisation buffer solutions.
4.2 Methods

4.2.1 Crystallisation technique and plate

The technique used for protein crystallisation was the sitting drop vapour diffusion method, which is the most popular, as it can be used to screen a broad range of crystallisation conditions, using small amounts of materials, but can also be scaled up in order to obtain large crystals for X-ray diffraction analysis. MRC 2 Well Crystallisation Plates (Swissci) (UVP plate, 96 reservoir wells, reservoir volume: 50 to 100 µl, with 2 corresponding sample wells: 5 µl max fill volume) were purchased from Hampton Research (Figure 4-2). Specifically, a solution (50 µL) of tested polymer buffer was pipetted in triplicate in the reservoir wells. Protein solution (1 µL) and buffered polymer solution (as described above, 1 µL) were pipetted into sample wells of the crystallisation plate. Generally, a drop of 1:1 protein: polymer solution was usually used here.

![MRC 2 Well Crystallisation Plate](image)

Figure 4-2. MRC 2 Well Crystallisation Plate (Swissci), commercially available from Hampton Research.
The entire crystallisation plate system was sealed using transparent sealing tape (with a chemically inert adhesive). This prevents solvent being lost to the external environment. The differences in polymer and ions concentration between the drop and the reservoir solution causes water to evaporate from the drop, resulting in an increase of the supersaturation level within the sitting drop. This continues until the concentration of the polymer in the drop is the same as in the reservoir well solution. Thus, equilibration takes place through the vapour phase. Over time, protein nucleation will take place (Figure 4-3).

![Diagram](image)

Figure 4-3. Diagram depicting the sitting drop vapour diffusion technique. The reservoir has usually twice the polymer concentration than the sitting drop. Water vapour diffusion takes place in a single well environment.

At the beginning of the experiment, water vapour diffusion from the sitting drop to the reservoir solution causes a reduction in the volume of the sitting drop, which in turn increases the supersaturation level of all the solutes within it. Over time, the rate of supersaturation eventually decreases due to the precipitation of protein or other solutes (Figure 4-4).
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

Figure 4-4. The schematic representation of real-time protein concentration changes in the sitting drop.²⁰⁷

4.2.2 Preparation of polymer solutions and stock buffer solutions

Four classes of polymers including neutral, cationic, anionic and cationic/zwitterionic shown below were applied for HEWL crystallisation:

- P(PEGMA₄₇₅) (P1) – Neutral;
- Quaternised p(DMAEMA) (P2):
  - Degrees of quaternisation: 25 %, 50 %, 75 % and 100 %;
- A random copolymer [p(DMAEMA-stat-PEGMA₄₇₅)] (P3);
- P(DMAPMAm) (DPs: 38, 83 and 150) (P4);
- P(AMPS) (DPs: 53, 99 and 200) (P5);
- P(2-AmAA) (DPs: 18, 46, 72 and 150) (P6);
- P(4-AmBA) (DPs: 21, 50, 100 and 200) (P7);
- P(METMAC-co-MEDSAH) (P8) – Cationic/zwitterionic.
The stock polymer solutions in H₂O at a concentration of ~ 40 % (w/v) were prepared. In order to use polymers as part of the reservoir and sample solutions in HEWL crystallisation, it was necessary, to alter the pH to around 4.8, which is chosen from the optimum pH (4.2-4.8) for the tetragonal crystal-inducing protocol of HEWL; and also considering the solubility of polymers. The stock buffer solutions of 0.25 M sodium acetate and 20 % (w/v) NaCl at pH 4.8 and 0.1 M sodium acetate at pH 4.8 were also prepared and filtered using a 0.2 μm cellulose acetate membrane filter.

By using the above mentioned pH-altered polymer solutions and the stock buffer solutions, the following polymer solutions were prepared at a final volume of 2 mL, 1 mL, 500 μL or 250 μL: 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 %, 18 %, 20 %, 22 % and 24 % (w/v) in 0.25 M sodium acetate and 20 % (w/v) NaCl at pH 4.8. All polymers with various concentrations were completely dissolved in the buffer solutions.

4.2.3 Crystallisation protocol for tetragonal HEWL crystals

HEWL was purchased from Sigma (catalogue number: L6876, EC: 3.2.1.17, pI: 11.35, lysozyme activity: ~ 50,000 units/mg protein). 0.1 M sodium acetate solution at pH 4.8 was prepared and filtered through a 0.2 μm cellulose acetate membrane filter. This was used to prepare the 50 mg/mL HEWL solution, which was then centrifuged at 13,000 rpm for 5 min at 4 °C.

HEWL crystallisation was performed on a 96-well crystallisation plate, using
the sitting drop vapour diffusion technique, grown under conditions favouring
the tetragonal form. All manual pipetting was conducted on an open lab bench.
Specifically, 50 μL of polymer buffer solution (0.25 M sodium acetate and 20 %
w/v NaCl at pH 4.8) was pipetted in triplicate in the reservoir well; 1 μL of
protein (50 mg/mL HEWL in 0.1 M sodium acetate solution at pH 4.8) and 1
μL polymer buffer solution taken from each reservoir were pipetted into
corresponding sample well of the crystallisation plate. In each sample well, the
polymer concentration was half of that in the reservoir well, the final
concentration of HEWL in each sample well was 25 mg/mL. For control
purposes, some wells contained no polymer in their solutions. The plate was
then sealed and incubated at 19 °C. Crystals formed within 1-2 days.

4.2.4 Crystal imaging

For optical microscopy, HEWL crystals formed in the crystallisation plate were
directly observed by using a Leica Stereomicroscope, with a Leica fan-cooled
light source, connected to a Nikon Coolpix 4500 digital camera (4.0
megapixels). 63 x magnifications were used to obtain the image of the whole
sample well. Furthermore, in order to compare the effects of those polymers on
protein crystallisation, we selected polymers with similar DP across the range
of 80-100 for further image analysis by ImageJ.
4.2.5 X-ray crystallography

In this study, HEWL crystals were determined by single crystal X-ray diffraction recorded at room temperature on an X-ray generator with high-flux Osmic confocal multi-layer optics. Crystals first were mounted in a loop, which is made of plastic or nylon and attached to a solid rod. Thus, HEWL crystals for diffraction were cryoprotected with mother liquor containing 30 % glycerol, then protein crystals were maintained at temperatures of -180 °C by an X-stream 2000 nitrogen vapour system. By this freezing process, the radiation damage of the X-rays and the noise in the Bragg peaks due to thermal motion can be reduced.

4.2.6 Dynamic light scattering (DLS) studies

During crystallisation experiments, complex processes take place in the protein solution with an increase of supersaturation. The onset and rate of protein crystallisation in solution depend on the mechanism of how protein macromolecules incorporate with each other. This interaction among protein macromolecules can govern them aggregate together to form crystals or amorphous aggregates in a period time.

In the present study, dynamic light scattering (DLS) is a sensitive and widely used method to determine the size and mode of aggregation of proteins and other biomolecules in solution, by detecting variations in size of macromolecules. DLS has already been successfully used to distinguish
between protein solutions leading to crystallisation or to amorphous precipitation. Moreover, it is a very helpful method to screen for the probability for crystal formation in the protein solution, for example, dispersity analysis to optimize the crystallisation conditions.

Therefore, in this research, DLS was applied to monitor model protein crystallisation process by determining the particle size directly in the protein crystallisation solution. HEWL was crystallised using batch crystallisation method, where the protein solution, the protein fully dissolved in the buffer, and the precipitant solution (e.g., salts, soluble polymer, and small organic molecules) are directly added together, immediately leading to a supersaturated protein solution. As the time increased, protein aggregates grew in size until the critical nucleus was reached and then capable of further growth. The increase size of aggregates in the crystallisation solution could be easily monitored by DLS.

Selected polymers with similar DP across the range of 80-100 were employed for DLS studies, including p(PEGMA\textsubscript{475}) (P1), quaternised p(DMAEMA) (P2) (quaternised ratio 75 %), a mixture of two polymers: p(PEGMA\textsubscript{475}) and p(DMAEMA) (P1 & P2) (molar ratio 9:1), p(DMAPAM) (P4) (DP 83), p(AMPS) (P5) (DP 99), p(2-AmAA) (P6) (DP 72) and p(4-AmBA) (P7) (DP 100). For each experiment, 100 μL of 15 mg/mL HEWL in 0.1 M sodium acetate buffer at pH 4.8 was mixed with 100 μL of 1 mg/mL of polymer in 0.25 M sodium acetate and 20 % (w/v) NaCl at pH 4.8 directly in the VISCOTEK
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

DLS Quartz cell and then monitored by VISCOTEK DLS (Figure 4-5).

Temperature: 19 °C; Crystal Growth time: 7 hours.

Figure 4-5. Schematic representation of protein crystallisation in the DLS quartz cell.

4.2.7 Scanning electron microscope (SEM) analysis

Scanning electron microscopy (SEM) is a technique that can be used to tackle the limited resolution of optical microscopy. It plays an essential role in the study of biological or pharmaceutical crystals. The output signals, which make up the SEM images, are reflected from an opaque surface. SEM images are then formed by topographic contrast. Thus, SEM is a very useful method to observe morphological characters of the crystals.

Representative HEWL crystals grown in the presence of three selected polymers from each class, containing neutral p(PEGMA$_{475}$) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5) using sitting drop vapor diffusion method, were subjected to SEM analysis in this project. Prior
to gold coating, protein crystals were taken out from the crystallisation plates, washed with crystallisation buffer solutions (mother liquor). The samples (protein crystals) were loaded onto aluminium stubs with carbon tabs pre-fixed.\textsuperscript{220,221} These were then gold coated using an SCD 030 Blazers sputter coater for 5 min at 30 mA. The coated samples were transferred into a JEOL JSM 6060 LV scanning electron microscope and imaged at 15-30 kV.

4.3 Results and discussion

4.3.1 HEWL crystallisation in the presence of neutral polymer in solution: p(PEGMA\textsubscript{475}) (P1)

In order to investigate the influence of neutral p(PEGMA\textsubscript{475}) (P1) on HEWL crystallisation, we set out to prepare neutral p(PEGMA\textsubscript{475}) (P1) with a range of concentrations. By using the above mentioned pH-altered stock buffer solution, the following p(PEGMA\textsubscript{475}) (P1) (\(M_n\sim39.4\) kDa) solutions with various concentration were prepared at a final volume of 1 mL, 500 \(\mu\)L or 250 \(\mu\)L: 0.02 \%, 0.2 \%, 2 \%, 4 \%, 6 \%, 8 \%, 10 \%, 12 \%, 14 \%, 16 \%, 18 \%, 20 \%, 22 \% and 24 \% (w/v) in 0.25 M sodium acetate and 20 \% (w/v) NaCl at pH 4.8. 50 \(\mu\)L of polymer buffer solution was pipetted in triplicate in the reservoir well; 1 \(\mu\)L of protein (50 mg/mL HEWL in 0.1 M sodium acetate solution at pH 4.8) and 1 \(\mu\)L polymer buffer solution taken from each reservoir were pipetted into corresponding sample well of the crystallisation plate. Some sample wells contained no polymer as the control. Table 4-2 shows HEWL crystallisation
<table>
<thead>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M Na Acetate, 20% NaCl</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
</tr>
<tr>
<td>pPEGMA475 (P1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>1190 µL</td>
<td>5905 µL</td>
<td>275 µL</td>
<td>250 µL</td>
<td>225 µL</td>
<td>175 µL</td>
<td>150 µL</td>
<td>150 µL</td>
<td>125 µL</td>
<td>175 µL</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>pH 4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Table 4.2: HEWL crystallisation plate set up: p(PEGMA475)**
Figure 4-6 shows the photomicrographs of HEWL crystals obtained in the presence of p(PEGMA$_{475}$) (P1) ($M_n$~39.4 kDa) with various concentrations, as solute in the crystallisation solutions. HEWL crystallisation under conditions favouring the tetragonal form resulted in exclusively tetragonal crystals within 1-2 days without any polymer (Figure 4-6, Control). Neutral p(PEGMA$_{475}$) (P1) nucleated a few large crystals, most of the HEWL crystals were readily identified by their distinctive morphology as tetragonal. As the concentration of p(PEGMA$_{475}$) (P1) in solution was increased, a similar increase in size and decrease in number of HEWL crystals were found. Moreover, the shape of crystals was quite different from that obtained in control experiments. Lower concentration of p(PEGMA$_{475}$) (P1) in solution (< 2%, w/v) resulted in concomitant formation of needles and tetragonal HEWL crystals. However, as the concentration of p(PEGMA$_{475}$) (P1) was increased, only tetragonal crystals were formed. It is reported that HEWL needles have formed in solutions with high salt concentrations, and in the presence of acid-functionalized polymeric surfaces, such as cross-linked acrylic acid and methacrylic acid.
Figure 4-6. Selected photomicrographs of HEWL crystals, grown in the presence of p(PEGMA₄₇₅) (P1) (Mₙ~39.4 kDa) at various concentrations. Image labelled ‘Control’ represents HEWL crystals grown from sample wells without polymer. The scale bar was 500 µm.
Figure 4-7 presents a summary of HEWL crystal size distribution by measuring the average length (μm) of the largest diameter of each crystal at each polymer concentration and number distribution as well. Image analysis of photomicrograph data was done by using ImageJ software. There was a clear effect on HEWL crystallisation depending on the concentration of neutral p(PEGMA_{475}) (P1). For cationic HEWL, as the concentration of neutral p(PEGMA_{475}) (P1) in solution was increased, the size of crystals increased and the number decreased very clearly.

Figure 4-7. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of neutral p(PEGMA_{475}) (P1) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well.

Diffraction data were collected for HEWL crystals using single crystal X-ray diffraction recorded at room temperature. Figure 4-8 illustrates the diffraction
patterns of HEWL crystals obtained by X-ray crystallography and Table 4-3 shows unit cell dimensions of HEWL crystals. Therefore, HEWL crystallisation in the presence of neutral p(PEGMA\textsubscript{475}) (P1) under the tetragonal-inducing protocol, resulted in tetragonal crystals, which were confirmed by single crystal X-ray diffraction.

![HEWL Crystal Photomicrographs and X-ray Diffraction Patterns](image)

Figure 4-8. HEWL crystal photomicrographs and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of neutral p(PEGMA\textsubscript{475}) (P1). Image labelled ‘Control’ represents HEWL crystals grown from sample wells without p(PEGMA\textsubscript{475}) (P1). Two scattering angles: 0±1 degrees and 90±1 degrees.

<table>
<thead>
<tr>
<th>Control</th>
<th>p(PEGMA\textsubscript{475})</th>
</tr>
</thead>
<tbody>
<tr>
<td>a, b, c (Å)</td>
<td>78.64, 78.64, 37.07</td>
</tr>
<tr>
<td>α, β, γ</td>
<td>90°, 90°, 90°</td>
</tr>
<tr>
<td>Volume</td>
<td>229,289</td>
</tr>
<tr>
<td>Unit cell lattice</td>
<td>Tetragonal</td>
</tr>
<tr>
<td>p(PEGMA\textsubscript{475})</td>
<td>78.42, 78.42, 37.17</td>
</tr>
<tr>
<td>α, β, γ</td>
<td>90°, 90°, 90°</td>
</tr>
<tr>
<td>Volume</td>
<td>228,638</td>
</tr>
<tr>
<td>Unit cell lattice</td>
<td>Tetragonal</td>
</tr>
</tbody>
</table>
Figure 4-9 shows a summary of DLS data of HEWL crystallisation under neutral p(PEGMA_{475}) (P1). Figure 4-9a shows autocorrelation functions, which were analysed to obtain the distribution of particle radii, as shown in Figure 4-9b and Figure 4-9c. At the beginning of the experiment (T_0), HEWL macromolecules were non-interacting in the solution. The scattering from rapidly diffusing small individual macromolecules, only remained correlated for very short times and the decay times were short. The average size of the particles in the crystallisation solution was around 200 nm, measured by size distribution. With the addition of p(PEGMA_{475}) (P1), HEWL crystallisation initiated and aggregates formed. As the time increased, HEWL aggregates grew in size. Large particles diffused slower than small particles, and the correlation function decayed at a slower rate. After 420 minutes (T_{420min}), the average size of the particles in the crystallisation solution was around 900 nm. The size of pure neutral p(PEGMA_{475}) (P1) in the solution was also characterised by DLS and was found at around 7 nm.
Figure 4-9. Summary of DLS data of HEWL crystallisation in the presence of neutral p(PEGMA<sub>475</sub>) (P1). Pure neutral p(PEGMA<sub>475</sub>) (P1) in the solution was also characterised by DLS. a) representative autocorrelation functions; b) radii distribution by intensity; c) radii distribution by number.

All other DLS data details, including autocorrelation functions and radii distribution by intensity at each time points are shown in the Appendix Pages 335-336. Representative Tukey plots of DLS data show protein aggregates growth in size as a function of time during HEWL crystallisation experiments.
with p(PEGMA$_{475}$) (P1) (Figure 4-10). After the addition of neutral p(PEGMA$_{475}$) (P1), a characteristic increase in protein aggregate size can be observed. For control purpose, HEWL crystallisation experiment in the absence of polymer was carried out as well.

![Graph showing variations in the size of HEWL aggregates or crystals](image.png)

Figure 4-10. Variations in the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by neutral p(PEGMA$_{475}$) (P1). Control experiment contained no polymer.

HEWL crystals, which grown in the presence of neutral p(PEGMA$_{475}$) (P1) using sitting drop vapor diffusion method were then subjected to SEM analysis. The SEM results (Figure 4-11) show that HEWL crystals had not been purified completely, there were still some salts left in the samples. However, all images showed a striking feature of crystals: all HEWL crystals, formed without polymer (Control) or when in the presence of neutral p(PEGMA$_{475}$) (P1) had sharp edges, even though the edges of HEWL crystals with slight etch defects.
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

Figure 4-11. SEM micrographs of HEWL crystals produced under tetragonal conditions: a) control, without any polymer. b) in the presence of p(PEGMA$_{475}$) (P1). The scale bar was 100 μm.

Compared with optical microscopy images, which protein crystals have striking features, including the presence of sharp edges and observation of nearly monodisperse crystallites, in SEM the HEWL crystals do not look so well defined. This may because of poor mechanical properties and stability of protein crystals. When protein crystals were taken out from the crystallisation plates and washed with crystallisation buffer solution, they were not handled easily and dehydrated slightly. Thus, a treatment for improving the stability of protein crystals may need in future study. There was insufficient time in the thesis period to conclude these experiments, but future work should consider this.

All above results together illustrate that p(PEGMA$_{475}$) (P1) acted as a solution additive and had the ability to influence HEWL crystallisation. We might expect that neutral p(PEGMA$_{475}$), as a neutral methacrylate derivative of PEG, was non-ionic and thus would compete with protein solutes for water and
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

dehydrated protein macromolecules.\textsuperscript{125} Moreover, \textit{p}(PEGMA\textsubscript{475}) may have ‘volume-excluded effects’ by hydrophobic exclusion of protein solutes.\textsuperscript{224} Neutral polymers of this type could also reduce the dielectric constant of the crystallisation solution, leading to an increase of the effective distance over which protein electrostatic effects would take place,\textsuperscript{225} consequently protein aggregation and phase separation was promoted.\textsuperscript{108} In turn, HEWL macromolecules may have been more able to associate with each other (Figure 4-12).

Figure 4-12. Schematic representation of hypothesis of HEWL crystallisation process without any polymer (A); and the interaction of \textit{p}(PEGMA\textsubscript{475}) (P1) with HEWL in the protein crystallisation process (B). HEWL macromolecules are positively charged at tetragonal crystallisation pH of 4.8, i.e. cationic. Na\textsuperscript{+} and Cl\textsuperscript{−} ions are derived from the crystallisation buffer solutions.

166
4.3.2 HEWL crystallisation in the presence of cationic polymers in solution

4.3.2.1 HEWL crystallisation in the presence of quaternised p(DMAEMA) (P2)

Quaternisation was carried out on p(DMAEMA) (P2) to introduce permanent positive charges by an addition of a methyl group to the p(DMAEMA) tertiary amine repeating unit. We used methyl iodide to quaternise two sizes of p(DMAEMA) with different molecular weights. Typically, similar crystallisation procedures as described in Section 4.3.1 were carried out. Five quaternised p(DMAEMA) (P2) with different quaternised ratios were applied for HEWL crystallisation:

i) Quaternised p(DMAEMA) (M_n~46.3 kDa) (quaternised ratio: 50 %);

ii) Quaternised p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 25 %);

iii) Quaternised p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 50 %);

iv) Quaternised p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 75 %);

v) Quaternised p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 100 %).

i) Quaternised p(DMAEMA) (P2) (M_n~46.3 kDa) (quaternised ratio: 50 %)

The following polymer solutions were prepared by using the above pH-altered buffer solution: quaternised p(DMAEMA) (P2) (M_n~46.3 kDa) (quaternised ratio: 50 %) 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 %, 18 % and 20 % (w/v) (Table 4-4). HEWL concentration: 50 mg/mL.
<table>
<thead>
<tr>
<th>pH</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8</td>
<td>800 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>400 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
</tr>
<tr>
<td>199 µL</td>
<td>5.0 µL (0.02 %)</td>
<td>594 µL</td>
<td>29 µL</td>
<td>57 µL</td>
<td>243 µL</td>
<td>214 µL</td>
<td>156 µL</td>
<td>128 µL</td>
<td>99 µL</td>
<td>79 µL</td>
<td>41 µL</td>
<td>13 µL</td>
</tr>
<tr>
<td>4.8</td>
<td>1 µL</td>
<td>6 µL</td>
<td>29 µL</td>
<td>57 µL</td>
<td>243 µL</td>
<td>214 µL</td>
<td>156 µL</td>
<td>128 µL</td>
<td>99 µL</td>
<td>79 µL</td>
<td>41 µL</td>
<td>13 µL</td>
</tr>
</tbody>
</table>
| 4.8 | 86 µL (5 %) | 115 µL (6 %) | 144 µL (10 %) | 172 µL (12 %) | 291 µL (14 %) | 230 µL (15 %) | 259 µL (18 %) | 287 µL (20 %)
| 4.8 | 1199 µL | 6.0 µL (0.2 %) | 271 µL | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |

**Table 4.4:** HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2) (Mw~46 kDa) (quaternised ratio: 50 %)

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**CHAPTER 4. Application of Polymers to Hen Egg White Lysozyme (HEWL) Crystallisation**
Figure 4-13 shows HEWL crystals obtained in the presence of quaternised p(DMAEMA) (P2) (Mₙ~46.3 kDa) (quaternised ratio: 50 %) at various concentrations (0.02 % to 20 % (w/v)), as solute in the crystallisation solutions. Each concentration was repeated six times for reproducibility. Cationic quaternised p(DMAEMA) (P2) (Mₙ~46.3 kDa) (quaternised ratio: 50 %) had an increased propensity for nucleating many small crystals. Along with the concentration of quaternised p(DMAEMA) (P2) in solution was increased, the number of HEWL crystals appeared to increase; the size of crystals appeared to decrease. Instead of tetragonal, two other forms of HEWL crystals, including needles and monoclinic crystals were obtained, which was confirmed by single crystal X-ray diffraction results later. As mentioned in Section 4.3.1, HEWL needles can be grown in solutions with high salt concentrations,²²² or in the presence of acid-functionalised polymeric surfaces, such as cross-linked acrylic acid and methacrylic acid.¹⁵⁹ However, HEWL monoclinic crystals have rarely been observed previously under conditions which otherwise promote tetragonal crystal-polymorphs.
Quaternised p(DMAEMA) (P2) ($M_n \sim 46.3$ kDa) (quaternised ratio: 50 %)

Figure 4-13. Photomicrographs showing changes in the size and shape of HEWL crystals, grown under the tetragonal protocol, in the presence of quaternised p(DMAEMA) (P2) ($M_n \sim 46.3$ kDa) (quaternised ratio: 50 %) at various concentrations. HEWL concentration: 50 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer.
It was immediately clear that quaternised p(DMAEMA) had influenced the results of HEWL crystallisation experiments, especially the crystal morphology and form. For better understanding the mechanism of how quaternised p(DMAEMA) affected HEWL crystallisation, a series of crystallisation experiments in the presence of different quaternised p(DMAEMA) (Mn~17.1 kDa) systems (quaternised ratios: 25 %, 50 %, 75 % and 100 %), with various concentrations of HEWL (25, 50, 75 and 100 mg/mL) were carried out (Section ii-v).

ii) Quaternised p(DMAEMA) (P2) (Mn~17.1 kDa) (quaternised ratio: 25 %)

The following polymer solutions were prepared by using the above pH-altered buffer solution: quaternised p(DMAEMA) (Mn~17.1 kDa) (quaternised ratio: 25 %) 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 % and 18 % (w/v). Table 4-5 presents the crystallisation plating method and Figures 4-14a-d show images of HEWL crystals obtained in the presence of quaternised p(DMAEMA) (Mn~17.1 kDa) (quaternised ratio: 25 %) at various concentrations. HEWL concentrations: 25, 50, 75 and 100 mg/mL.
# Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

| Table 4-5: HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2) (Mw~17.1 kDa) (quaternised ratio: 25 %) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **0.25 M Na Acetate, 20 % NaCl**<br>Quaternised p(DMAEMA) (25 %) 31.84 % H$_2$O pH | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 800 µL (0.25 M Na Acetate, 20 % NaCl) | 1.3 µL (0.62 %) | 6.3 µL (0.2 %) | 31.4 µL (2 %) | 63 µL (4 %) | 94 µL (6 %) | 126 µL (8 %) | 157 µL (10 %) | 188 µL (12 %) | 220 µL (14 %) | 251 µL (16 %) | 283 µL (18 %) |

| **0.25 M Na Acetate, 20 % NaCl**<br>Quaternised p(DMAEMA) (25 %) 31.84 % H$_2$O pH | | | | | | | | | | | |
| 593.7 µL | 268.6 µL | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |

| **0.25 M Na Acetate, 20 % NaCl**<br>Quaternised p(DMAEMA) (25 %) 31.84 % H$_2$O pH | | | | | | | | | | | |
| 1198.7 µL | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |

| **0.25 M Na Acetate, 20 % NaCl**<br>Quaternised p(DMAEMA) (25 %) 31.84 % H$_2$O pH | | | | | | | | | | | |
| 3.1 µL (0.2 %) | 6.3 µL (0.2 %) | 31.4 µL (2 %) | 63 µL (4 %) | 94 µL (6 %) | 126 µL (8 %) | 157 µL (10 %) | 188 µL (12 %) | 220 µL (14 %) | 251 µL (16 %) | 283 µL (18 %) |

| **0.25 M Na Acetate, 20 % NaCl**<br>Quaternised p(DMAEMA) (25 %) 31.84 % H$_2$O pH | | | | | | | | | | | |
| 200 µL (0.25 M Na Acetate, 20 % NaCl) | 0 µL | 300 µL | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |

| **0.25 M Na Acetate, 20 % NaCl**<br>Quaternised p(DMAEMA) (25 %) 31.84 % H$_2$O pH | | | | | | | | | | | |
| 200 µL (0.25 M Na Acetate, 20 % NaCl) | 0 µL | 300 µL | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |
Quaternised p(DMAEMA) (P2) ($M_n \approx 17.1 \text{ kDa}$) (quaternised ratio: 25 %)

a) HEWL concentration: 25 mg/mL

Figure 4-14a. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) ($M_n \approx 17.1 \text{ kDa}$) (quaternised ratio: 25 %) at various concentrations, with HEWL concentration: 25 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Quaternised p(DMAEMA) (P2) (Mₐ~17.1 kDa) (quaternised ratio: 25%)

b) HEWL concentration: 50 mg/mL

Figure 4-14b. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (Mₐ~17.1 kDa) (quaternised ratio: 25%) at various concentrations, with HEWL concentration: 50 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Quaternised p(DMAEMA) (P2) ($M_n \sim 17.1$ kDa) (quaternised ratio: 25%)

c) HEWL concentration: 75 mg/mL

Figure 4-14c. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of (DMAEMA) ($M_n \sim 17.1$ kDa) (quaternised ratio: 25%) at various concentrations, with HEWL concentration: 75 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Quaternised p(DMAEMA) (P2) (\(M_n\sim17.1\) kDa) (quaternised ratio: 25 %)

d) HEWL concentration: 100 mg/mL

Figure 4-14d. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of (DMAEMA) (\(M_n\sim17.1\) kDa) (quaternised ratio: 25 %) at various concentrations, with HEWL concentration: 100 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
iii) Quaternised p(DMAEMA) (P2) (Mₙ≈17.1 kDa) (quaternised ratio: 50 %)

The following polymer solutions were prepared: quaternised p(DMAEMA) (Mₙ≈17.1 kDa) (quaternised ratio: 50 %) 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 % and 12 % (w/v) (Table 4-6).

Table 4-6. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2) (Mₙ≈17.1 kDa) (quaternised ratio: 50 %)
Figures 4-15a-d show the optical photomicrographs of HEWL crystals formed under quaternised p(DMAEMA) (Mₙ~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with HEWL concentrations: 25, 50, 75 and 100 mg/mL.

Quaternised p(DMAEMA) (P2) (Mₙ~17.1 kDa) (quaternised ratio: 50 %)

a) HEWL concentration: 25 mg/mL

Figure 4-15a. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (Mₙ~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with HEWL concentration: 25 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Quaternised p(DMAEMA) (P2) ($M_n \sim 17.1$ kDa) (quaternised ratio: 50 %)

b) HEWL concentration: 50 mg/mL

Figure 4-15b. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) ($M_n \sim 17.1$ kDa) (quaternised ratio: 50 %) at various concentrations, with HEWL concentration: 50 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Quaternised p(DMAEMA) (P2) (Mₙ~17.1 kDa) (quaternised ratio: 50 %)

c) HEWL concentration: 75 mg/mL

Figure 4-15c. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (Mₙ~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with HEWL concentration: 75 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 μm.
Quaternised p(DMAEMA) (P2) (M_n~17.1 kDa) (quaternised ratio: 50 %)

d) HEWL concentration: 100 mg/mL

Figure 4-15d. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 50 %) at various concentrations, with HEWL concentration: 100 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
iv) Quaternised p(DMAEMA) (P2) \((M_n \sim 17.1 \text{ kDa})\) (quaternised ratio: 75 %)

The following polymer solutions were prepared: quaternised p(DMAEMA) (P2) \((M_n \sim 17.1 \text{ kDa})\) (quaternised ratio: 75 %) 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 %, 18 % and 20 % (w/v) (Table 4-7).

Table 4-7. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2)
\((M_n \sim 17.1 \text{ kDa})\) (quaternised ratio: 75 %)
Figures 4-16a-d show images of HEWL crystals obtained under quaternised p(DMAEMA) (P2) ($M_n$~17.1 kDa) (quaternised ratio: 75 %), with HEWL concentrations of 25, 50, 75 and 100 mg/mL.

Quaternised p(DMAEMA) (P2) ($M_n$~17.1 kDa) (quaternised ratio: 75 %)

a) HEWL concentration: 25 mg/mL

![Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (P2) ($M_n$~17.1 kDa) (quaternised ratio: 75 %) at various concentrations, with HEWL concentration: 25 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.](image-url)
Quaternised p(DMAEMA) (P2) (M_n~17.1 kDa) (quaternised ratio: 75 %)

b) Lysozyme concentration: 50 mg/mL

Figure 4-16b. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 75 %) at various concentrations, with HEWL concentration: 50 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL)
Crystallisation

Quaternised p(DMAEMA) (P2) ($M_n$~17.1 kDa) (quaternised ratio: 75 %)

c) HEWL concentration: 75 mg/mL; d) HEWL concentration: 100 mg/mL

c) HEWL concentration: 75 mg/ml

Figure 4-16c & d. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) ($M_n$~17.1 kDa) (quaternised ratio: 75 %) at various concentrations, with HEWL concentrations: 75 and 100 mg/mL. Images labelled ‘Control’ represent crystals grown from sample wells without polymer. The scale bar was 500 µm.
v) Quaternised p(DMAEMA) (P2) (Mₙ~17.1 kDa) (quaternised ratio: 100 %)

The following polymer solutions were prepared: quaternised p(DMAEMA) (Mₙ~17.1 kDa) (quaternised ratio: 100 %) 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 %, 18 % and 20 % (w/v) (Table 4-8).

Table 4-8. HEWL crystallisation plate set up: quaternised p(DMAEMA) (P2) (Mₙ~17.1 kDa) (quaternised ratio: 100 %)
Figures 4-17a-d present optical micrographs of HEWL crystals obtained under quaternised p(DMAEMA) (P2) (Mₙ~17.1 kDa) (quaternised ratio: 100 %), with HEWL concentrations: 25, 50, 75 and 100 mg/mL.

Quaternised p(DMAEMA) (P2) (Mₙ~17.1 kDa) (quaternised ratio: 100 %)

a) HEWL concentration: 25 mg/mL

Figure 4-17a. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (Mₙ~17.1 kDa) (quaternised ratio: 100 %) at various concentrations, with HEWL concentration: 25 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

Quaternised p(DMAEMA) (P2) (\(M_n\sim 17.1\) kDa) (quaternised ratio: 100 %)

b) HEWL concentration: 50 mg/mL

![Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (\(M_n\sim 17.1\) kDa) (quaternised ratio: 100 %) at various concentrations, with HEWL concentration: 50 mg/mL. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.](image-url)
Quaternised p(DMAEMA) (P2) (M_n~17.1 kDa) (quaternised ratio: 100%)

c) HEWL concentration: 75 mg/mL; d) HEWL concentration: 100 mg/mL

c) HEWL concentration: 75 mg/ml

![Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 100%) at various concentrations, with HEWL concentrations: 75 and 100 mg/mL. Images labelled ‘Control’ represent crystals grown from sample wells without polymer. The scale bar was 500 µm.](image)

d) HEWL concentration: 100 mg/ml

![Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA) (M_n~17.1 kDa) (quaternised ratio: 100%) at various concentrations, with HEWL concentrations: 75 and 100 mg/mL. Images labelled ‘Control’ represent crystals grown from sample wells without polymer. The scale bar was 500 µm.](image)
All above HEWL crystal images, including Figures 4-14a-d, 4-15a-d, 4-16a-d and 4-17a-d, show that HEWL crystallisation was clearly affected by the presence of quaternised p(DMAEMA) in solution. The rate of HEWL crystals formed in sample wells containing quaternised p(DMAEMA) was faster than that without added polymer. Various quaternised ratios of p(DMAEMA) had similar effects on HEWL crystallisation, even with different HEWL concentrations. Tetragonal crystals only were formed at lower concentrations (0.02 % ~ 0.2 % (w/v)). Upon increasing the concentration to above 2 %, quaternised p(DMAEMA) showed a visible and pronounced effect on the HEWL crystal formation; needles and monoclinic crystals were obtained (Figures 4-14b & c, 4-15a-c, 4-16b and 4-17b). Therefore, quaternised p(DMAEMA) acted as solution additives significantly influenced the results of HEWL crystallisation. Moreover, quaternised p(DMAEMA) (P2) mediated-HEWL crystallisation followed a general morphology trend along with increasing polymer concentration: tetragonal → monoclinic & needle → needle, with tetragonal crystals forming at lower concentrations and monoclinic crystals & needle forming at higher polymer concentrations (Figure 4-18). In previous research, the absorption behavior of a high content of the cationic PDMAEMA on silicon wafers was controlled by a striving of the cationic segments for charge compensation by negative surface. Addition of quaternised p(DMAEMA), which exhibited a high cationic charge density, into HEWL solution, is therefore expected to alter the
electrostatic interactions between HEWL molecules and the solution.\textsuperscript{100, 118}

Figure 4-18. Schematic triangle graphs showing the changes in the morphology of HEWL crystals obtained in the presence of p(DMAEMA) with different degrees of quaternisation (25\%, 50\%, 75\% and 100\%). A) HEWL concentration: 25 mg/mL; B) HEWL concentration: 50 mg/mL; C) HEWL concentration: 75 mg/mL; D) HEWL concentration: 100 mg/mL. Three morphology phases: tetragonal, monoclinic and needle reside at the vertices of the triangle. The size of the colored squares quantitatively represents the possibility of crystal morphology shape.

Among all the above HEWL crystallisation experiments under quaternised p(DMAEMA) (P2), the experiment with quaternised ratio 75\% and HEWL concentration 50 mg/mL was the best condition for the formation of
monoclinic HEWL crystals. Therefore, photomicrograph data of this experiment (Figure 4-16b) was chosen for further image analysis. Figure 4-19 shows a full summary of HEWL crystals size and number distribution (tetragonal or monoclinic crystal). There was a significant effect on HEWL crystallisation depending on the concentration of cationic quaternised p(DMAEMA). For cationic HEWL, as the quaternised p(DMAEMA) (P2) concentration was increased, the size of monoclinic crystals decreased and the number of monoclinic crystals increased.

Figure 4-19. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of quaternised p(DMAEMA) (quaternised ratio: 75 %) at various concentrations, with HEWL concentration 50 mg/mL. a) Average length (μm) of the largest crystal (tetragonal or monoclinic) diameter; b) Experiment count for the number of crystals (tetragonal or monoclinic) per sample well. The scale bar was 500μm.
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

Figure 4-20 illustrates the diffraction patterns of HEWL crystal, grown in the presence of quaternised p(DMAEMA) (quaternised ratio: 75 %) by X-ray crystallography and Table 4-9 shows unit cell dimension of the HEWL crystal. Therefore, HEWL crystals obtained from quaternised p(DMAEMA) (P2)-mediated HEWL crystallisation, were confirmed as the monoclinic form by single crystal X-ray diffraction.

Figure 4-20. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %). Two scattering angles: $0 \pm 1$ degrees and $90 \pm 1$ degrees.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>a, b, c (Å)</th>
<th>$\alpha, \beta, \gamma$</th>
<th>Volume</th>
<th>Unit cell lattice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quaternised p(DMAEMA)</td>
<td>26.82, 63.09, 58.73</td>
<td>$90^\circ, 93.2^\circ, 90^\circ$</td>
<td>99,213</td>
<td>Monoclinic</td>
</tr>
</tbody>
</table>
Representative Tukey plots of DLS data show protein aggregate growth in size as a function of time during crystallisation experiment (Figure 4-21). At the beginning of the experiment ($T_0$), the average size of the particles in the crystallisation solution was around 100 nm. After the addition of cationic quaternised p(DMAEMA) (P2), as the time increased, the rate of HEWL aggregates size increase was very slow. This may because cationic quaternised p(DMAEMA) (P2) could lower the interfacial energy between cationic HEWL macromolecules and water by charge-charge repulsions, thus promoting nucleation and the formation of multiple crystal nuclei. Therefore, the size of each HEWL aggregate or crystal would increase very slowly. The size of pure quaternised p(DMAEMA) (P2) in solution was around 18 nm. DLS data details, including autocorrelation functions and radii distribution by intensity are shown in the Appendix Page 337.

![Figure 4-21](image-url)

Figure 4-21. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiment induced by cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %).
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

Figure 4-22 presents SEM micrograph of HEWL crystal formed in the presence of quaternised p(DMAEMA) (P2). The SEM result shows that with the addition of cationic quaternised p(DMAEMA), the HEWL produced poor needles. Because of the poor stability of monoclinic crystals, and because they were normally surrounded by lots of HEWL needles, monoclinic crystals were not handled easily and it proved impossible to obtain SEM images in this research.

Figure 4-22. SEM micrograph of HEWL needles produced under tetragonal conditions in the presence of quaternised p(DMAEMA), -- x 4000 magnifications; the scale bar was 5 μm.

4.3.2.2 HEWL crystallisation in the presence of p(DMAEMA-stat-PEGMA_{475}) (P3)

P(DMAEMA-stat-PEGMA_{475}) (P3) (M_n~52.9 kDa) solutions with concentrations of 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 %, 18 %, 20 % and 22 % (w/v) in 0.25 M sodium acetate, 20 % NaCl at pH 4.8 were prepared (Table 4-10). Each concentration was in triplicate for reproducibility.
Table 4-10. HEWL crystallisation plate: pDMAEMA-star-PEGMA475 (P3) at various concentrations as solute in the

<table>
<thead>
<tr>
<th>0.25 M Na Acetate, 20 % NaCl Copolymer 38.96 % H2O %</th>
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<tr>
<td>199 µL (0.25 M Na Acetate, 20 % NaCl) 1 µL (0.02 %)</td>
<td>408 µL (0.25 M Na Acetate, 20 % NaCl)</td>
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<td>199 µL (0.25 M Na Acetate, 20 % NaCl) 5.1 µL (0.2 %)</td>
<td>274 µL (0.25 M Na Acetate, 20 % NaCl)</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 51 µL (4 %)</td>
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<td>199 µL (0.25 M Na Acetate, 20 % NaCl) 77 µL (6 %)</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 233 µL (8 %)</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 197 µL (10 %)</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 172 µL (12 %)</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 146 µL (14 %)</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 120 µL (16 %)</td>
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<td>69 µL 4.3</td>
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<td>43 µL 4.8</td>
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Figure 4-23 shows images of HEWL crystals obtained in the presence of

[pDMAEMA-star-PEGMA475 (P3) at various concentrations] as solute in the
crystallisation solutions. Regular and small tetragonal HEWL crystals with the size of ~ 100 μm formed within one day.

\[ \text{p(DMAEMA-stat-PEGMA}_{475}\text{)} \] (P3) copolymer

Figure 4-23. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of p(DMAEMA-stat-PEGMA\textsubscript{475}) (P3) copolymer (M\textsubscript{n}~52.9 kDa) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 μm.
A full summary of HEWL crystals size distribution by measuring the average length (μm) of the largest diameter of each crystal and number distribution depending on p(DMAEMA-stat-PEGMA₄₇₅) (P3) copolymer concentration was obtained by further image analysis (Figure 4-24). As the concentration of p(DMAEMA-stat-PEGMA₄₇₅) (P3) copolymer in solution was increased, the number of HEWL crystals appeared to increase and the size of the crystals decreased very clearly. Upon increasing the copolymer concentration to 20 %, the size of HEWL crystal decreased to ~ 100 μm.

Figure 4-24. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(DMAEMA-stat-PEGMA₄₇₅) (P3) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well. The scale bar was 500μm.
Figure 4-25 illustrates the diffraction patterns of HEWL crystal, grown in the presence of p(DMAEMA-stat-PEGMA_{475}) (P3) by X-ray crystallography and Table 4-11 shows unit cell dimension of the HEWL crystal. Crystallisation of HEWL from the tetragonal-inducing protocol, when in the presence of cationic p(DMAEMA-stat-PEGMA_{475}) (P3), resulted in tetragonal crystals, which were confirmed by single crystal X-ray diffraction.

Figure 4-25. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal formed in the presence of cationic p(DMAEMA-stat-PEGMA_{475}) (P3). Two scattering angles: 0±1 degrees and 90±1 degrees.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>a, b, c (Å)</th>
<th>α , β, γ</th>
<th>Volume</th>
<th>Unit cell lattice</th>
</tr>
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<tbody>
<tr>
<td>P3</td>
<td>78.49, 78.49, 36.71</td>
<td>90°, 90°, 90°</td>
<td>226,172</td>
<td>Tetragonal</td>
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Table 4-11. Unit cell dimension of HEWL crystal in the presence of cationic p(DMAEMA-stat-PEGMA_{475}) (P3)

P(DMAEMA-stat-PEGMA_{475}) (P3) copolymer might have different functions during HEWL crystallisation, due to the two components: neutral PEGMA_{475}
and cationic DMAEMA. As mentioned earlier, the neutral PEGMA<sub>475</sub> section might bind to water by H-bonding, compete with protein solutes for water and thus force protein solutes come out from the water solution. It could also lower the dielectric constant of the solution, leading to protein aggregation being promoted. At the crystallisation pH of 4.8, the cationic DMAEMA section had quaternary nitrogen with some positive charges, which could alter the electrostatic interactions between ionic HEWL macromolecules and the solution. There were likely to have been charge-charge repulsions between cationic DMAEMA and cationic HEWL macromolecules, which would lower the interface energy between HEWL macromolecules and water, consequently accelerated protein aggregation and the formation of multiple crystal nuclei for HEWL nucleation.

**4.3.2.3 HEWL crystallisation in the presence of a mixture of two polymers:**

p(PEGMA<sub>475</sub>) and p(DMAEMA) (P1 & P2) (molar ratio 9:1)

As mentioned above, p(DMAEMA-stat-PEGMA<sub>475</sub>) (P3) affected the size of HEWL crystals very significantly. In order to compare the effects with p(DMAEMA-stat-PEGMA<sub>475</sub>) copolymer, we set out to prepare a mixture of two polymers: p(PEGMA<sub>475</sub>) and p(DMAEMA) (P1 & P2) for HEWL crystallisation. The following polymer solutions with a range of concentrations 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 %, 18 % and 20 % (w/v) were prepared. Table 4-12 shows HEWL crystallisation plate in the
### Table 4.12. HEWL crystallisation plate set up: a mixture of two polymers: p(PEGMA-475) and pDMAEMA (P1 & P2)

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<tr>
<th>0.25 M Na Acetate, 20 % NaCl</th>
<th>Mixed polymers 33.52 % H2O pH</th>
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<tr>
<td>800 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>1.2 µL (0.2 %) 594 µL 4.8</td>
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<tr>
<td>400 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>20 µL (60 %) 270 µL 4.8</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>60 µL (10 %) 211 µL 4.8</td>
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<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>89 µL (6 %) 181 µL 4.8</td>
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<tr>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>209 µL (14 %) 91 µL 4.8</td>
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<tr>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>339 µL (16 %) 61 µL 4.8</td>
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<tr>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>298 µL (20 %) 2 µL 4.8</td>
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Each concentration was in triplicate for reproducibility.
Figure 4-26 shows the photomicrographs of HEWL crystals formed under a mixture of two polymers: p(PEGMA\textsubscript{475}) and p(DMAEMA) (P1 & P2). A few large HEWL crystals formed within one day. From visual inspection, it is immediately clear that the results of crystallisation experiments are different from that obtained in the presents of p(DMAEMA-stat-PEGMA\textsubscript{475}) (P3) copolymer, especially the crystal size and morphology shape. When the concentration of a mixture of two polymers: pPEGMA\textsubscript{475} and p(DMAEMA) (P1 & P2) in solution was increased to 20 % (w/v), the size of HEWL crystal was around 300 \(\mu\text{m}\) and the shape of some crystals was rhombohedral. But when in the presents of p(DMAEMA-stat-PEGMA\textsubscript{475}) (P3) copolymer, the size was around 100 \(\mu\text{m}\) and the shape was exclusively tetragonal.

A mixture of two polymers: p(PEGMA\textsubscript{475}) and p(DMAEMA) (P1 & P2) had two separate sections: neutral p(PEGMA\textsubscript{475}) and cationic p(DMAEMA). However, unlike p(DMAEMA-stat-PEGMA\textsubscript{475}) copolymer, those two sections affected HEWL crystallisation separately. It seems the influence of the neutral p(PEGMA\textsubscript{475}) section was dominant in the crystallisation process.
A mixture of two polymers: p(PEGMA$_{475}$) and p(DMAEMA) (P1 & P2)

Figure 4-26. Photomicrographs of HEWL crystals, grown under the tetragonal protocol, in the presence of a mixture of two polymers: p(PEGMA$_{475}$) and p(DMAEMA) (P1 & P2) (molar ratio 9:1) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
A full summary of HEWL crystals size distribution by measuring the average length (μm) of the largest diameter of each crystal and number distribution depending on a mixture of two polymers: p(PEGMA\(_{475}\)) and p(DMAEMA) (P1 & P2) concentration was obtained by further image analysis (Figure 4-27). As the concentration of a mixture of two polymers: p(PEGMA\(_{475}\)) and p(DMAEMA) (P1 & P2) in solution was increased, the number of HEWL crystals appeared to increase and the size of the crystals appeared to decrease.

Figure 4-27. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of a mixture of two polymers: p(PEGMA\(_{475}\)) and p(DMAEMA) (P1 & P2) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well. The scale bar was 500μm.
Figure 4-28 illustrates the diffraction patterns of HEWL crystal, grown in the presence of a mixture of two polymers: p(PEGMA$\text{}_{475}$) and p(DMAEMA) (P1 & P2). Table 4-13 shows unit cell dimension of the HEWL crystal, identified as tetragonal by single crystal X-ray diffraction.

![HEWL crystal photomicrograph and X-ray diffraction patterns](image)

Figure 4-28. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of a mixture of two polymers: p(PEGMA$\text{}_{475}$) and p(DMAEMA) (P1 & P2). Two scattering angles: 0±1 degrees and 90±1 degrees.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>a, b, c (Å)</th>
<th>α, β, γ</th>
<th>Volume</th>
<th>Unit cell lattice</th>
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<tbody>
<tr>
<td>A mixture of two polymers:</td>
<td>78.85,</td>
<td>90°,</td>
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<tr>
<td>p(PEGMA$\text{}_{475}$) and</td>
<td>78.85,</td>
<td>90°,</td>
<td>230,779</td>
<td>Tetragonal</td>
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<tr>
<td>p(DMAEMA)</td>
<td>37.12</td>
<td>90°</td>
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Figure 4-29 shows Tukey plots of DLS data when in the presence of a mixture of two polymers: p(PEGMA$\text{}_{475}$) and p(DMAEMA) (P1 & P2). At the beginning of the experiment ($T_0$), the average size of the particles in the crystallisation
solution was around 180 nm. After the addition of a mixture of two polymers: p(PEGMA_{475}) and p(DMAEMA) (P1 & P2), as the time increased, a characteristic increase in HEWL aggregate size can be observed. After 395 minutes (T_{395min}), the average size of the particles in the crystallisation solution was around 900 nm. DLS data details, including autocorrelation functions and radii distribution by intensity are shown in the Appendix Page 337.

![Graph showing variations in particle size over time](image)

Figure 4-29. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by a mixture of two polymers: p(PEGMA_{475}) and p(DMAEMA) (P1 & P2).

### 4.3.2.4 HEWL crystallisation in the presence of p(DMAPMAm) (P4)

DMAPMAm, containing tertiary amines group was homopolymerised to form another cationic polymer. The following polymer solutions were prepared: p(DMAPMAm) (P4) (DPs 38, 83 and 150) 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v) in 0.25 M sodium acetate and 20 % NaCl at pH 4.8 buffer solution. Tables 4-14a-c show HEWL crystallisation plating methods under p(DMAPMAm) (P4) (DPs 38, 83 and 150).
### Table 4.14a. HEWL crystallisation plate set up: p(DMAPMAm) (P4) (DP 38)

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<td>0.25 M Na Acetate, 20 % NaCl</td>
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Table 4-14b. HEWL crystallisation plate set up: p(DMAPMAm) (P4) (DP 83)
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<tr>
<td>(0.25 \text{ M} \ \text{Na Acetate}, 20 % \ \text{NaCl})</td>
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<td>(p(\text{DMAPAm}) \ (\text{DP} \ 150))</td>
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<td>0.25 \text{ M} \ \text{Na Acetate}, 20 % \ \text{NaCl})</td>
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<td>(p(\text{DMAPAm}) \ (\text{DP} \ 150))</td>
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<td>(\text{H}_2\text{O})</td>
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Table 4.14c. HEWL crystallisation plate set up: \(p(\text{DMAPAm}) \ (\text{DP} \ 150)\)
Previous results showed that cationic quaternised p(DMAEMA) was able to control the morphology shape and alter the size of HEWL crystals. Similar as DMAEMA, monomer DMAPMAm has tertiary amine functionality, which can afford positive charges at crystallisation pH of 4.8. Figures 4-30a-c show the HEWL crystals obtained in the presence of p(DMAPMAm) (P4) (DPs 38, 83 and 150) with various concentrations (0.02 % ~ 20 % (w/v)), as solute in crystallisation solutions. From visual observation, cationic p(DMAPMAm) had an increased propensity for nucleating much more small crystals. It nucleated many uniform and small tetragonal HEWL crystals with the size of ~ 100 μm within one day. These HEWL crystals were identified as tetragonal by single crystal X-ray diffraction. Upon increasing the DP of p(DMAPMAm), the size of HEWL crystals slightly increased. The possible reason is that p(DMAPMAm) with higher DP had a larger molecular weight, which would increase the viscosity of the solution and slow the rate of protein crystal growth.227
a) p(DMAPMAm) (P4) (DP 38)

Figure 4-30a. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(DMAPMAm) (P4) (DP 38) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
b) p(DMAPAm) (P4) (DP 83)

Figure 4-30b. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(DMAPAm) (P4) (DP 83) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Figure 4-30c. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(DMAPMAm) (P4) (DP 150) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.

A full coverage of crystal size distribution by measuring the average length (µm) of the largest crystal diameter and number distribution was obtained from p(DMAPMAm) (P4) (DP 83) (Figure 4-31). As the p(DMAPMAm) concentration was increased, the size of crystals decrease and the number of
crystals increased.

Figure 4-31. Summary of photomicrograph analysis of HEWL crystals, grown in the presence of p(DMAPMAm) (P4) (DP 83) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well. The scale bar was 500μm.

Figure 4-32 shows Tukey plots of DLS data of HEWL crystallisation in the presence of p(DMAPMAm) (P4). P(DMAPMAm) (P4) did not alter the size of HEWL aggregates or crystals clearly after 420 min. The size of pure p(DMAPMAm) (P4) in the solution without any protein was around 3 nm. Similar as quaternised p(DMAEMA) (P2), cationic p(DMAPMAm) (P4) could lower the interfacial energy between cationic HEWL macromolecules and water by charge-charge repulsions, thus promoted nucleation and the formation of multiple crystal nuclei. In turn, HEWL aggregates or crystals would grow.
very slowly. DLS data details, including autocorrelation functions and radii distribution by intensity are shown in the Appendix Page 338.

Figure 4-32. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by p(DMAPMAm) (P4).

In summary, cationic polymers had an increased propensity for nucleating small crystals. Figure 4-33 shows the hypothesis of mechanism of how cationic polymers mediated HEWL crystallisation.\textsuperscript{108, 226, 228, 229} There were likely to have been charge-charge repulsions between cationic polymer molecules and cationic HEWL macromolecules, lowering the interfacial energy between HEWL macromolecules and water, thus promoting protein-protein interactions at hydrophobic sites or anionic-cationic pair sites and subsequent crystallisation. In addition, cationic polymers might have bound to anionic residues on the HEWL chain leading to charge compensation by electrostatic interaction,\textsuperscript{108} an increase of local concentration of HEWL macromolecules and the promotion of nucleation.
Figure 4-33. Hypothesis of mechanism of cationic polymer mediated HEWL crystallisation. Cationic polymer: charge-charge repulsions between cationic polymer and cationic HEWL. HEWL macromolecules are positively charged at tetragonal crystallisation pH of 4.8, i.e. cationic. Na\(^+\) and Cl\(^-\) ions are from the crystallisation buffer solutions.

4.3.3 HEWL crystallisation in the presence of anionic polymers in solution

4.3.3.1 HEWL crystallisation in the presence of p(AMPS) (P5)

Monomer AMPS, containing ionisable sulfonic acid group can introduce some negative charges and homopolymerise to generate an anionic polymer P5. The following polymer solutions were prepared: p(AMPS) (P5) (DPs 53, 99 and 200): 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v) in 0.25 M sodium acetate, 20 % NaCl, pH 4.8 buffer solution (Tables 4-15a-c).
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Table 4-15a. HEWL crystallisation plate set up p(AMPS) (P5) (DP 53)
### Table 4-15b: HEWL crystallisation plate set up p(AMPS) (P5) (DP 99)

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<tr>
<td>0.25 M Na Acetate, 20 % NaCl</td>
<td>400 µL (0.25 M Na Acetate, 20 % NaCl) 0.7 µL (0.02 %) 599.3 µL 4.8</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 3 µL (0.2 %) 797 µL 4.8</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 17 µL (2 %) 133 µL 4.8</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 34 µL (4 %) 116 µL 4.8</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 50 µL (6 %) 100 µL 4.8</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 67 µL (8 %) 83 µL 4.8</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 84 µL (10 %) 66 µL 4.8</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 101 µL (12 %) 49 µL 4.8</td>
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### Table 4-15c. HEWL crystallisation plate set up: p(AMPS) (P5) (DP 200)

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<td>0.25 M Na Acetate, 20 % NaCl</td>
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<td>0.25 M Na Acetate, 20 % NaCl</td>
<td>200 μL (0.25 M Na Acetate, 20 % NaCl)</td>
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*Note: * HEWL = Hen Egg-white Lysozyme, NaCl = Sodium Chloride, AMPS = Acrylamide, Na Acetate, pH = Hydrogen Ion Concentration, DP = Degree of Polymerisation.
Figures 4-34a-c show the images of HEWL crystals obtained in the presence of p(AMPS) (P5) (DPs 53, 99 and 200) with various concentrations (0.02 % ~ 20 % (w/v)). Anionic p(AMPS) nucleated a few large crystals, which were then identified as tetragonal by single crystal X-ray diffraction.

Figure 4-34a. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(AMPS) (P5) (DP 53) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Figure 4-34b. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(AMPS) (P5) (DP 99) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
c) p(AMPS) (P5) (DP 200)

Figure 4-34c. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(AMPS) (P5) (DP 200) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.

The HEWL images data obtained from the experiment in the presence of p(AMPS) (P5) (DP 99) was used for further image analysis. Figure 4-35 shows crystal size distribution by measuring the average length (µm) of the largest crystal diameter and number distribution was obtained from p(AMPS) (P5)
As the concentration of p(AMPS) (P5) in solution was increased, the number of HEWL crystals appeared to decrease and the size of the crystals appeared to increase.

Figure 4-35. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(AMPS) (P5) (DP 99) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well. The scale bar was 500μm.

Figure 4-36 presents Tukey plots of DLS data when crystallised in the presence of p(AMPS) (P5). DLS data details, including autocorrelation functions and radii distribution by intensity are shown in the Appendix Page 338. At the beginning of the experiment (T₀), HEWL macromolecules were non-interacting in the solution. The average size of the particles in the
crystallisation solution was around 100 nm, measured by size distribution. With the addition of p(AMPS) (P5), HEWL crystallisation initiated and aggregates formed. As the time increased, HEWL aggregates grew in size. After 360 minutes, the average size of the particles in the crystallisation solution was around 400 nm. The size of pure p(AMPS) (P5) (DP 99) without any protein in the solution was around 4 nm.

![Graph](image)

Figure 4-36. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by p(AMPS) (P5) (DP 99).

HEWL crystals were then taken out from the crystallisation plate, washed with crystallisation buffer solution. Then they were loaded onto aluminium stubs for SEM analysis. The SEM micrograph result (Figure 4-37) shows that with the addition of anionic p(AMPS) (P5), high quality HEWL crystal with sharp edges was formed. Moreover, HEWL crystals formed in the presence of anionic p(AMPS) (P5) were much more stable than that obtained without polymer.
4.3.3.2 HEWL crystallisation in the presence of p(2-AmAA) (P6)

Another acrylamide monomer 2-AmAA, which has carboxyl group, was chosen to generate anionic homopolymers. The following polymer solutions were prepared: p(2-AmAA) (P6) (DP 18): 0.02 %, 0.2 %, 2 %, 3 %, 4 %, 5 %, 6 %, 7 % and 8 % (w/v); p(2-AmAA) (P6) (DP 46): 0.02 %, 0.2 %, 2 %, 3 %, 4 %, 5 %, 6 %, 7 % and 7.5 % (w/v); p(2-AmAA) (P6) (DP 72): 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v); p(2-AmAA) (P6) (DP 150): 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v) in 0.25 M sodium acetate and 20 % NaCl at pH 4.8 buffer solution. Tables 4-16a-d show HEWL crystallisation plates contained p(2-AmAA) (P6) (DPs 18, 46, 72 and 150) as solution additives.
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<td>114 µL 4.8</td>
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Table 4-16a. HEWL crystallisation plate set up: p(2-AmAA) (DP 18)
### CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

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<td>H$_2$O pH</td>
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<td></td>
<td>400 μL (0.25 M Na Acetate, 20 % NaCl) 0.5 μL (0.02 %) 599.5 μL 4.8</td>
<td>200 μL (0.25 M Na Acetate, 20 % NaCl) 3 μL (0.2 %) 297 μL 4.8</td>
<td>100 μL (0.25 M Na Acetate, 20 % NaCl) 13 μL (2 %) 137 μL 4.8</td>
<td>100 μL (0.25 M Na Acetate, 20 % NaCl) 25 μL (4 %) 125 μL 4.8</td>
<td>100 μL (0.25 M Na Acetate, 20 % NaCl) 38 μL (6 %) 112 μL 4.8</td>
<td>100 μL (0.25 M Na Acetate, 20 % NaCl) 50 μL (8 %) 100 μL 4.8</td>
<td>100 μL (0.25 M Na Acetate, 20 % NaCl) 63 μL (10 %) 87 μL 4.8</td>
<td>100 μL (0.25 M Na Acetate, 20 % NaCl) 75 μL (12 %) 75 μL 4.8</td>
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<td><strong>0.25 M Na Acetate, 20 % NaCl</strong></td>
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<tr>
<td>p(2-AmAA) (DP 72)</td>
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<td><strong>0.25 M Na Acetate, 20 % NaCl</strong></td>
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<td>p(2-AmAA) (DP 72)</td>
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<td>H$_2$O pH</td>
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<tr>
<td></td>
<td>200 μL (0.25 M Na Acetate, 20 % NaCl) 0 μL 300 μL 4.8</td>
<td>→</td>
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</tr>
</tbody>
</table>

Table 4.16c. HEWL crystallisation plate set up: p(2-AmAA) (P6) (DP 72)
Table 4-16d. HEWL crystallisation plate set up: p(2-AmAA) (P6) (DP 150)
Figures 4-38a-d show the images of HEWL crystals formed in the presence of p(2-AmAA) (P6) (DPs 18, 46, 72 and 150) at various concentrations. From visual observation, HEWL needles formed under p(2-AmAA) (P6) with lower DPs of 18 and 46 at lower concentrations (Figures 4-38a, < 4 %; Figures 4-38b, < 7 % (w/v)). However, as the concentration of p(2-AmAA) (P6) with DPs of 18 and 46 was increased, there were no HEWL needles formed. It is reported that HEWL needles can be grown in the presence of acid-functionalised polymers.\textsuperscript{104} When HEWL crystallisation in the presence of p(2-AmAA) (P6) with higher DPs of 79 and 108, smaller uniform HEWL crystals were obtained (Figure 4-38c and d). The pKa of p(2-AmAA) (P6) was 5.92, measured by titration. At pH 4.8, p(2-AmAA) (P6) was a weakly anionic polymer. Because the crystallisation pH of HEWL being close (pH 4.8) to the pKa value of p(2-AmAA) (P6), and thus a smaller number of anionic carboxyls remaining on the p(2-AmAA) (P6) at pH of 4.8 compared to anionic p(AMPS) (P5). All these small HEWL crystals formed in the presence of anionic p(2-AmAA) (P6) were identified as tetragonal by single crystal X-ray diffraction later.
Figure 4-38a. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(2-AmAA) (P6) (DP 18) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
b) p(2-AmAA) (P6) (DP 46)

Figure 4-38b. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(2-AmAA) (P6) (DP 46) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
c) p(2-AmAA) (P6) (DP 72)

Figure 4-38c. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(2-AmAA) (P6) (DP 72) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Figure 4-38d. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(2-AmAA) (P6) (DP 150) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.

The HEWL images data obtained from p(2-AmAA) (P6) (DP 72) was used for further image analysis. Figure 4-39 shows crystal size distribution by measuring the average length (µm) of the largest crystal diameter and number distribution was obtained from p(2-AmAA) (P6) (DP 72). As the concentration
of p(2-AmAA) (P6) in solution was increased, the number of HEWL crystals decreased and the size of the crystals appeared to increase slightly.

Figure 4-39. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(2-AmAA) (P6) (DP 72) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well. The scale bar was 500 μm.

Figure 4-40 illustrates the diffraction patterns of HEWL crystal grown in the presence of p(2-AmAA) (P6) (DPs 18 and 46) using X-ray crystallography. Table 4-17 shows unit cell dimensions of those HEWL crystals. All the HEWL crystals formed in the presence of anionic p(2-AmAA) (P6) were identified as tetragonal by single crystal X-ray diffraction.
Figure 4-40. HEWL crystal photomicrographs and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of p(2-AmAA) (P6) with DP of 18 and 46. Two scattering angles: 0±1 degrees and 90±1 degrees.

Table 4-17. Unit cell dimensions of HEWL crystal in the presence of anionic p(2-AmAA) (P6) with DP of 18 and 46

<table>
<thead>
<tr>
<th>Polymer</th>
<th>a, b, c (Å)</th>
<th>α , β, γ</th>
<th>Volume</th>
<th>Unit cell lattice</th>
</tr>
</thead>
<tbody>
<tr>
<td>p(2-AmAA)</td>
<td>78.87, 78.87, 37.10</td>
<td>90°, 90°, 90°</td>
<td>230,794</td>
<td>Tetragonal</td>
</tr>
<tr>
<td>DP 18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p(2-AmAA)</td>
<td>79.13, 79.13, 37.16</td>
<td>90°, 90°, 90°</td>
<td>232,678</td>
<td>Tetragonal</td>
</tr>
<tr>
<td>DP 46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4-41 shows Tukey plots of DLS data when crystallised under p(2-AmAA) (P6). DLS data details, including autocorrelation functions and radii distribution by intensity are shown in the Appendix Page 339. At the beginning of the experiment (T₀), the average size of the particles in
crystallisation solution was around 30 nm, measured by size distribution. With the addition of p(2-AmAA) (P6), HEWL aggregates grew in size. After 360 minutes, the average size of the particles in crystallisation solution was around 180 nm. The size of pure p(2-AmAA) (P6) without any protein was around 3 nm.

Figure 4-41. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by p(2-AmAA) (P6).

4.3.3.3 HEWL crystallisation in the presence of p(4-AmBA) (P7)

4-AmBA, which has the carboxyl group and a higher molar mass than 2-AmAA, was also chosen to generate anionic homopolymers. The following polymer solutions were prepared: p(4-AmBA) (P7) (DP 21): 0.02 %, 0.2 %, 2 %, 3 %, 4 %, 5 %, 6 %, 7 % and 8 % (w/v); p(4-AmBA) (P7) (DPs 50, 100 and 200): 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v) in 0.25 M sodium acetate, 20 % NaCl, pH 4.8 buffer solution. Tables 4-18a-d show HEWL crystallisation plates contained p(4-AmBA) (P7) (DPs 21, 50,
Table 4. HEWL crystallisation plate setup: p(4-AmBA) (P7) (DP 21)

<table>
<thead>
<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M Na Acetate, 20 % NaCl</td>
<td>400 µL (0.25 M Na Acetate, 20 % NaCl) 1.2 µL (0.02 %)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl) 6 µL (0.2 %)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 30 µL (2 %)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 61 µL (4 %)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 91 µL (6 %)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl) 122 µL (8 %)</td>
</tr>
<tr>
<td>p(4-AmBA) (DP 21) 16.41 % H₂O pH</td>
<td>598.8 µL 4.8</td>
<td>294 µL 4.8</td>
<td>120 µL 4.8</td>
<td>89 µL 4.8</td>
<td>59 µL 4.8</td>
<td>28 µL 4.8</td>
</tr>
</tbody>
</table>

0.25 M Na Acetate, 20 % NaCl

p(4-AmBA) (DP 21) 16.41 % H₂O pH

0.25 M Na Acetate, 20 % NaCl

p(4-AmBA) (DP 21) 16.41 % H₂O pH

0.25 M Na Acetate, 20 % NaCl

p(4-AmBA) (DP 21) 16.41 % H₂O pH

0.25 M Na Acetate, 20 % NaCl

p(4-AmBA) (DP 21) 16.41 % H₂O pH

0.25 M Na Acetate, 20 % NaCl

p(4-AmBA) (DP 21) 16.41 % H₂O pH
### Table 4.18b. HEWL crystallisation plate setup: p(4-AmBA) (P7) (DP 50)

<table>
<thead>
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<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.25 M Na Acetate, 20 % NaCl</strong></td>
<td>400 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>200 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl)</td>
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<td>100 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>100 µL (0.25 M Na Acetate, 20 % NaCl)</td>
</tr>
<tr>
<td><strong>p(4-AmBA) (DP 50)</strong></td>
<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
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<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
</tr>
<tr>
<td><strong>H₂O</strong></td>
<td>599.5 µL (0.02 %)</td>
<td>13 µL (2 %)</td>
<td>125 µL (4 %)</td>
<td>112 µL (4.8)</td>
<td>51 µL (8 %)</td>
<td>63 µL (10 %)</td>
<td>74 µL (4.8)</td>
<td>87 µL (4.8)</td>
<td>62 µL (4.8)</td>
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<tr>
<td><strong>pH</strong></td>
<td>4.8</td>
<td>3.9</td>
<td>4.8</td>
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<tr>
<td><strong>0.25 M Na Acetate, 20 % NaCl</strong></td>
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<tr>
<td><strong>p(4-AmBA) (DP 50)</strong></td>
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<tr>
<td><strong>H₂O</strong></td>
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<tr>
<td><strong>pH</strong></td>
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<tr>
<td><strong>0.25 M Na Acetate, 20 % NaCl</strong></td>
<td>300 µL (0.25 M Na Acetate, 20 % NaCl)</td>
<td>63 µL (0.2 %)</td>
<td>137 µL (2 %)</td>
<td>105 µL (4 %)</td>
<td>100 µL (6 %)</td>
<td>100 µL (8 %)</td>
<td>100 µL (10 %)</td>
<td>100 µL (12 %)</td>
<td>100 µL (14 %)</td>
</tr>
<tr>
<td><strong>p(4-AmBA) (DP 50)</strong></td>
<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
<td>p(4-AmBA) (DP 50)</td>
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<td>p(4-AmBA) (DP 50)</td>
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<tr>
<td><strong>H₂O</strong></td>
<td>397 µL (0.02 %)</td>
<td>127 µL (2 %)</td>
<td>113 µL (4 %)</td>
<td>114 µL (4.8)</td>
<td>98 µL (6 %)</td>
<td>98 µL (8 %)</td>
<td>98 µL (10 %)</td>
<td>98 µL (12 %)</td>
<td>98 µL (14 %)</td>
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<tr>
<td><strong>pH</strong></td>
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Table 4-18c. HEWL crystallisation plate set up: p(4-AmBA) (P7) (DP 100)

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<th>0.25 M Na Acetate, 20% NaCl</th>
<th>P(4-AmBA) (DP 100)</th>
<th>pH 0.25 M Na Acetate, 20% NaCl</th>
<th>H2O</th>
<th>0.5 µL (0.02%)</th>
<th>3 µL (0.2%)</th>
<th>599.5 µL</th>
<th>4.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
<td>0.5 µL (0.02%)</td>
<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>200 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
<td>0.5 µL (0.02%)</td>
<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
</tr>
<tr>
<td>3</td>
<td>100 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
<td>0.5 µL (0.02%)</td>
<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>50 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
<td>0.5 µL (0.02%)</td>
<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>25 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
<td>0.5 µL (0.02%)</td>
<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
</tr>
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<td>6</td>
<td>10 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
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<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
</tr>
<tr>
<td>7</td>
<td>5 µL (0.25 M Na Acetate, 20% NaCl)</td>
<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
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<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
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<td>8</td>
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<td>P(4-AmBA) (DP 100)</td>
<td>pH 0.25 M Na Acetate, 20% NaCl</td>
<td>H2O</td>
<td>0.5 µL (0.02%)</td>
<td>3 µL (0.2%)</td>
<td>599.5 µL</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Legend: →: X, ↑: Y, †: Z
Table 4-18d. HEWL crystallisation plate set up: p(4-AmBA) (P7) (DP 200)

<table>
<thead>
<tr>
<th>Well</th>
<th>Mono</th>
<th>Poly</th>
<th>pH</th>
<th>Volume (μL)</th>
<th>% NaCl</th>
<th>% NaAcetate</th>
<th>Ammonium Formate (mM)</th>
<th>Phosphate Buffer (mM)</th>
</tr>
</thead>
<tbody>
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<td>400</td>
<td>H2O</td>
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<td>NaCl</td>
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<td>0.2</td>
<td>0.02</td>
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<td>3</td>
<td>0.25</td>
<td>0.25</td>
<td>NaAcetate</td>
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<td>4</td>
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<td>0.25</td>
<td>NaAcetate</td>
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<td>0.02</td>
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<td>NaAcetate</td>
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<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Figures 4-42a-d present images of HEWL crystals obtained in the presence of p(4-AmBA) (P7) (DPs 21, 50, 100 and 200) at various concentrations. From visual observation, large HEWL crystals were formed under p(4-AmBA) with lower DP of 21 (Figure 4-42a). However, smaller uniform HEWL crystals were obtained when in the presence of p(4-AmBA) (P7) with higher DPs of 50, 100 and 200 (Figure 4-42b-d). The pKa of p(4-AmBA) (P7) was 6.26, measured by titration. At pH 4.8, p(4-AmBA) (P7) was a weakly anionic polymer. Similar to p(2-AmAA) (P6), due to the crystallisation pH of HEWL being close (pH 4.8) to the pKa value of p(4-AmBA) (P7), a small number of anionic carboxyls remaining on the p(4-AmBA) (P7) at pH of 4.8. p(4-AmBA) with lower DP of 21 might have very few anionic carboxyls, and therefore might compete with protein solutes for water and result in dehydration of protein macromolecules rather than by electrostatic interaction. All these HEWL crystals formed in the presence of anionic p(4-AmBA) (P7) were identified as tetragonal by single crystal X-ray diffraction.
Figure 4-42a. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(4-AmBA) (P7) (DP 21) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
Figure 4-42b. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(4-AmBA) (P7) (DP 50) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
c) p(4-AmBA) (P7) (DP 100)

Figure 4-42c. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(4-AmBA) (P7) (DP 100) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
d) p(4-AmBA) (P7) (DP 200)

Figure 4-42d. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(4-AmBA) (P7) (DP 200) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
The HEWL images data obtained from p(4-AmBA) (P7) (DP 100) was used for further image analysis. Crystal size and number distribution data (Figure 4-43) shows that as the concentration of p(4-AmBA) (P7) (DP 100) in solution was increased, the number of HEWL crystals decreased and the size of the crystals appeared to increase slightly.

![Photomicrograph showing HEWL crystals grown in the presence of p(4-AmBA) (P7) at various concentrations.](image)

Figure 4-43. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(4-AmBA) (P7) (DP 100) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well. The scale bar was 500μm.

Figure 4-44 illustrates the diffraction patterns of HEWL crystal, grown in the presence of p(4-AmBA) (P7) using X-ray crystallography. Table 4-19 shows
unit cell dimension of the HEWL tetragonal crystal.

Figure 4-44. HEWL crystal photomicrograph and X-ray diffraction patterns yielded by the single HEWL crystal in the presence of p(4-AmBA) (P7). Two scattering angles: 0±1 degrees and 90±1 degrees.

Table 4-19. Unit cell dimension of HEWL crystal in the presence of anionic p(4-AmBA) (P7)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>a, b, c (Å)</th>
<th>α, β, γ</th>
<th>Volume</th>
<th>Unit cell lattice</th>
</tr>
</thead>
<tbody>
<tr>
<td>p(4-AmBA)</td>
<td>78.75, 78.75, 37.08</td>
<td>90°, 90°, 90°</td>
<td>229,956</td>
<td>Tetragonal</td>
</tr>
</tbody>
</table>

Figure 4-45 shows Tukey plots of DLS data when HEWL crystallised in the presence of p(4-AmBA) (P7). DLS data details, including autocorrelation functions and radii distribution by intensity are shown in the Appendix Page 339. At the beginning of the experiment (T₀), the average size of the particles in crystallisation solution was around 20 nm, measured by size distribution. With the addition of p(4-AmBA) (P7), HEWL aggregates grew in size. After 360 minutes, the average size of the particles in crystallisation solution was around 70 nm. The size of pure p(4-AmBA) (P7) without any protein was around 3 nm.
Figure 4-45. Variations of the size of HEWL aggregates or crystals in the solution as a function of time during crystallisation experiments induced by p(4-AmBA) (P7).

In summary, the influence of anionic polymers on HEWL crystallisation was less than that of the cationic polymers regarding crystal shape, but the anionic polymers did alter the size of HEWL crystals. Figure 4-46 shows the hypothesis of how anionic polymers mediated HEWL crystallisation. As mentioned earlier, some researchers reported polymers containing cationic segments had an absorption behavior on negative surface, which controlled by charge compensation.\textsuperscript{226, 108} We proposed that anionic polymers might favor binding cationic HEWL macromolecules for charge compensation via electrostatic interaction,\textsuperscript{108} thus, they would either lower the protein and water interfacial energy and act as nucleation centers by the absorption of HEWL macromolecules, or slow down the rate of HEWL crystal further growth. Therefore, they acted as not only nucleators in solution but also as inhibitors.\textsuperscript{100}
Figure 4-6. Hypothesis of mechanism of anionic polymer mediated HEWL crystallisation. Anionic polymer: binding cationic HEWL for charge compensation. HEWL macromolecules are positively charged at tetragonal crystallisation pH of 4.8, i.e. cationic. Na\(^+\) and Cl\(^-\) ions come from the crystallisation buffer solutions.

### 4.3.4 HEWL crystallisation in the presence of cationic/zwitterionic polymer in solution: p(METMAC-co-MEDSAH) (P8)

A cationic/zwitterionic copolymer with both positive charges and zwitterionic components was applied to HEWL crystallisation. The following polymer solutions were prepared: p(METMAC-co-MEDSAH) (P8): 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v) in 0.25 M sodium acetate, 20 % NaCl, pH 4.8 buffer solution (Table 4-20).
### Table 4.20. HEWL crystallisation plate: p(METMAC-co-MEDSAH) (P8)

<table>
<thead>
<tr>
<th>p(METMAC-co-MEDSAH)</th>
<th>pH</th>
<th>NaCl</th>
<th>NaAcetate</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>3.84</td>
<td>0.25%</td>
<td>20%</td>
<td>123.4 µL</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>4.8</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>5.1</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>5.4</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>5.7</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>6.0</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>6.3</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>6.6</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>6.9</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>7.2</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>7.5</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>7.8</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>8.1</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>8.4</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>8.7</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>9.0</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>9.3</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>9.6</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>p(METMAC-co-MEDSAH)</td>
<td>9.9</td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
</tbody>
</table>

**Note:** The table entries are placeholders for actual data. The pH, NaCl, NaAcetate, and volume are shown in µL.
Figure 4-47 shows the HEWL crystals obtained in the presence of p(METMAC-co-MEDSAH) (P8) at various concentrations. With the effects of both cation and zwitterionic sulfobetaine, p(METMAC-co-MEDSAH) (P8) nucleated a few crystals with complex shape. There were significant etch defects on the surface of crystals.

\[ \text{p(METMAC-co-MEDSAH) (P8)} \]

Figure 4-47. Photomicrographs of HEWL crystals, grown under tetragonal crystal condition, in the presence of p(METMAC-co-MEDSAH) (P8) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 500 µm.
CHAPTER 4. Application of Polymers to Hen Egg-white Lysozyme (HEWL) Crystallisation

Figure 4-48 presents image analysis of HEWL crystals grown in the presence of p(METMAC-co-MEDSAH) (P8). As the concentration of p(METMAC-co-MEDSAH) (P8) in solution was increased, the size of the crystals did not vary clearly, but, the shape of crystals appeared to be more complex.

Figure 4-48. Summary of photomicrograph analysis showing changes in the size and number of HEWL crystals, grown in the presence of p(METMAC-co-MEDSAH) (P8). a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well.

Cationic/zwitterionic copolymer might have different functions during HEWL crystallisation (Figure 4-49). It might complex with HEWL macromolecules for charge compensation and promoted nucleation; or may have charge-charge repulsions between cationic/zwitterionic polymer and cationic HEWL
macromolecules; after nucleation, it also might absorb on certain faces of crystals and influence the direction of crystal further growth. In these ways, the HEWL crystals might have complex shape; however, the crystal form was still tetragonal.

Figure 4-49. Hypothesis of mechanisms of cationic/zwitterionic polymer mediated HEWL crystallisation. It can act via multiple mechanisms, i.e. charge-charge repulsions between cationic/zwitterionic polymer and cationic HEWL macromolecules; or it can complex with HEWL macromolecules for charge compensation; or it can absorb on certain faces of crystals and influence the direction of crystal further growth. HEWL macromolecules are positively charged at tetragonal crystallisation pH of 4.8, i.e. cationic. Na\(^+\) and Cl\(^-\) ions are from the crystallisation buffer solutions.
4.4 Conclusions

In this chapter, four classes of materials with varying degrees of charge, molecular weight and backbone structure were investigated for their roles as additives in HEWL solution, including one neutral polymer: p(PEGMA$_{475}$) (P1); three cationic polymers: quaternised p(DMAEMA) (P2) with a series of quaternised ratios: 25 %, 50 %, 75 % and 100 %, a random copolymer p(DMAEMA-stat-PEGMA$_{475}$) (P3) and p(DMAPMAm) (P4) (DPs: 38, 83 and 150); three anionic polymers: p(AMPS) (P5) (DPs: 53, 99 and 200), p(2-AmAA) (P6) (DPs: 18, 46, 72 and 150) and p(4-AmBA) (P7) (DPs: 21, 50, 100 and 200); and one cationic/zwitterionic copolymer: p(METMAC-co-MEDSAH) (P8) (Table 4-21). Crystallisation experiments were performed on 96-well plates, by using the sitting drop vapour diffusion technique. The results show that these polymers have different effects on protein crystallisation, depending on the functionality and charge of polymers.

Table 4-21. Summary of polymers applied to HEWL crystallisation

<table>
<thead>
<tr>
<th>Type</th>
<th>Polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-neutral</td>
<td>p(PEGMA$_{475}$)</td>
</tr>
<tr>
<td>Poly-cationic</td>
<td>Quaternised p(DMAEMA) (quaternised ratios: 25 %, 50 %, 75 % and 100 %)</td>
</tr>
<tr>
<td></td>
<td>p(DMAEMA-stat-PEGMA$_{475}$)</td>
</tr>
<tr>
<td></td>
<td>p(DMAPMAm) (DPs: 38, 83 and 150)</td>
</tr>
<tr>
<td>Poly-anionic</td>
<td>p(AMPS) (DPs: 53, 99 and 200)</td>
</tr>
<tr>
<td></td>
<td>p(2-AmAA) (DPs: 18, 46, 72 and 150)</td>
</tr>
<tr>
<td></td>
<td>p(4-AmBA) (DPs: 21, 50, 100 and 200)</td>
</tr>
<tr>
<td>Poly-cationic/zwitterionic</td>
<td>p(METMAC-co-MEDSAH)</td>
</tr>
</tbody>
</table>
The first protein we selected was HEWL. The pI of HEWL is 11.35, therefore when at its crystallisation pH 4.8, HEWL macromolecules are positively charged, i.e. cationic. HEWL crystallisation under conditions favouring the tetragonal form resulted in exclusively tetragonal crystals within 1-2 days without any polymer. Neutral p(PEGMA$_{475}$) (P1) as a precipitant, competed with protein solutes for water and thus promoted HEWL crystallisation via a dehydration mechanism,\textsuperscript{125} resulting in a few large tetragonal crystals. Cationic polymers had an increased propensity for nucleating many small crystals by altering the electrostatic interactions between HEWL molecules and the solution. Moreover, instead of tetragonal crystals, two other forms of HEWL crystals were obtained when crystallised in the presence of quaternised p(DMAEMA) (P2): needles and monoclinic crystals. Anionic polymers could influence the size of HEWL crystals by attracting cationic HEWL macromolecules for charge compensation.\textsuperscript{108} Anionic p(AMPS) (P5) nucleated a few large crystals. However, smaller HEWL crystals were obtained when applied weakly anionic p(2-AmAA) (P6) and p(4-AmBA) (P7), due to the pKa values of p(2-AmAA) (P6) and p(4-AmBA) (P7) being close to the crystallisation pH 4.8. With the effects of both cation and zwitterionic sulfobetaine, p(METMAC-$co$-MEDSAH) (P8) nucleated a few crystals with complex shape.

In conclusion, all these results illustrate that polymers acting as solution additives can mediate protein crystallisation through favourable interactions.
between polymer and protein or the surrounding solvent system. They are found to influence strongly the resultant crystal size and shape, even crystal packing motifs; and have the ability to obtain multiple polymorphs from one crystallisation condition, which could save time for new constructs.
CHAPTER 5

5. Application of Polymers to Concanavalin A (Con A) Crystallisation

5.1 Introduction

Concanavalin A (Con A) was first crystallised by Sumner and Howell, \(^{168, 169}\) and Greer \textit{et al.} preliminarily reported its crystallographic studies. \(^{170}\) Currently, there are several common structurally characterised crystal forms of concanavalin A (Con A), which bound to manganese and calcium ions, including orthorhombic \textit{I}$\overline{2}22$ (form I), \(^{181}\) triclinic \textit{P}$\overline{1}$ (form II), \(^{171}\) orthorhombic \textit{C}$222_1$ (form III)\(^{172}\), orthorhombic \textit{C}$222_1$ (form IV)\(^{99}\) and orthorhombic \textit{P}$2_12_12_1$ (form V). \(^{99}\)

As mentioned in Chapter 4, various polymers we prepared have clear effects on HEWL crystal size, shape, crystal packing motifs, and polymorphic forms under crystallisation conditions favouring HEWL tetragonal polymorph. In addition to HEWL, which is cationic under conditions in which it is normally crystallised, we also aimed to evaluate whether those polymers were suitable for other proteins, especially anionic proteins. Therefore, concanavalin A (Con A) was chosen as first model anionic protein for further crystallisation studies. The isoelectric point (pI) of Con A is 4.5, \(^{227}\) but crystallisation of this protein is normally carried out at pH 8.5, at which point Con A molecules are negatively charged overall (Table 5-1).
### Table 5-1. Summary of concanavalin A (Con A)

<table>
<thead>
<tr>
<th>Protein</th>
<th>pI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mw (kDa)</th>
<th>Crystallisation pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>4.5</td>
<td>25.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isoelectric point (pI) is the pH at which a particular molecule or surface carries no net electrical charge.

Three classes of polymers were selected for further Con A crystallisation experiments, including neutral, cationic and anionic polymers. Based on the results of HEWL crystallisation, polymers with different degrees of charge and backbone structure have dramatically influenced the crystallisation results, especially the cationic quaternised p(DMAEMA) (P2). Among the anionic polymers we prepared, the expected pKa value of p(AMPS) (P5) (~ 0.86) was the smallest; thus, it would have more negative charges at the crystallisation pH of 8.5 than other two anionic polymers: p(2-AmAA) (P6) and p(4-AmBA) (P7). Therefore, in this chapter, we chose three key polymers from each class for their role as additives in Con A crystallisation: neutral p(PEGMA<sub>475</sub>) (P1), cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %) and anionic p(AMPS) (P5) (DP 99). Figure 5-1 shows a summary of hypothesis of the interaction of various polymers with anionic concanavalin A (Con A) in the protein crystallisation process.
Figure 5-1. Schematic representation of the interaction of various polymers with anionic concanavalin A (Con A) in the protein crystallisation process. (A) Neutral polymer: H-bonding with water and thus promoting protein crystallisation via a dehydration mechanism. (B) Cationic polymer: attracting anionic Con A for charge compensation. (C) Anionic polymer: charge-charge repulsions between anionic polymer and anionic Con A. Con A macromolecules are anionic when at crystallisation pH 8.5. NH$_4^+$ ions are derived from the crystallisation buffer solutions.
5.2 Methods

5.2.1 Crystallisation technique and plate

Con A was crystallised using the sitting drop vapour diffusion technique as described in Chapter 4 for HEWL. MRC 2 Well Crystallisation Plates (Swissci) (UVP plate, 96 reservoir wells) were purchased from Hampton Research. Specifically, a solution (50 µL) of tested polymer buffer was pipetted in triplicate in the reservoir wells. Protein solution (1 µL) and buffered polymer solution (as described above, 1 µL) were pipetted into sample wells of the crystallisation plate.

5.2.2 Preparation of polymer solutions and stock buffer solutions

The stock polymer solutions in H2O at a concentration of ~ 40 % (w/v) were prepared. In order to use polymers as part of the solution in Con A crystallisation, it was necessary, to alter the pH to around 8.5 by using HCl (1 M) or NaOH (2 M). The stock buffer solutions of 1 M ammonium sulfate and 10 mM Tris buffer at pH 8.5 and 20 mM Tris buffer at pH 8.0 were also prepared and filtered using a 0.2 µm cellulose acetate membrane filter.

The following polymer solutions were prepared at a final volume of 2 mL, 1 mL, 500 µL or 250 µL: 0.02 % (w/v), 0.2 % (w/v), 2 % (w/v), 4 % (w/v), 6 % (w/v), 8 % (w/v), 10 % (w/v), 12 % (w/v), 14 % (w/v), 16 % (w/v), 18 % (w/v), 20 % (w/v), 22 % (w/v) and 24 % (w/v) in 1 M ammonium sulfate and 10 mM Tris buffer at pH 8.5. Polymers had dissolved completely in all solutions.
5.2.3 Crystallisation protocol for Con A crystals\textsuperscript{181,209}

Con A from \textit{Canavalia ensiformis} (Jack bean) was purchased from Sigma (Product Number: C7275, CAS Number: 11028-71-0, pI: 4.5, 4.7, 5.1, 5.5). 20 mM Tris buffer at pH 8.0 was prepared and filtered through a 0.2 \( \mu \)m cellulose acetate membrane filter. This was used to prepare the 70 mg/mL Con A solution, which was then centrifuged at 13,000 rpm for 5 min at 4 °C.

Con A was crystallised using the sitting drop vapour diffusion technique as described in Chapter 4 for HEWL. A drop of 1:1 protein:polymer solution was usually used here. Con A solution (1 \( \mu \)L, 70 mg/mL Con A in 20 mM Tris buffer at pH 8.0) and buffered polymer solution (1 \( \mu \)L, 1 M ammonium sulfate and 10 mM Tris buffer at pH 8.5) were pipetted into each sample well, and buffered polymer solution (50 \( \mu \)L) was pipetted into each reservoir well of the crystallisation plate. Con A in each sample well was 35 mg/mL. For control, some wells contained no polymer in their reservoir and sample solutions. The entire crystallisation plate system was sealed using transparent sealing tape and monitored using optical microscopy. Crystallisation occurred over a couple of days at 19 °C.

5.2.4 Crystal imaging

Optical images of Con A crystals were directly collected by a Leica Stereomicroscope (63 x magnifications), with a Leica fan-cooled light source, connected to a Nikon Coolpix 4500 digital camera (4.0 megapixels).
5.2.5 X-ray crystallography

Con A crystals were determined by single crystal X-ray diffraction recorded at room temperature on an X-ray generator with high-flux Osmic confocal multi-layer optics. Con A crystals were cryoprotected with mother liquor containing 30% glycerol, then flash-frozen in a nitrogen-gas stream.99,210

5.2.6 DLS studies

For the DLS experiments on Con A crystal growth, Con A was crystallised using the batch crystallisation method. A protein solution of 30 mg/mL Con A in 20 mM Tris buffer at pH 8.0 was mixed with a polymer solution of 1 M ammonium sulfate and 10 mM Tris buffer at pH 8.5. Three classes of polymers, containing neutral polymer: p(PEGMA475) (P1), cationic polymer: quaternised p(DMAEMA) (P2) (quaternised ratio: 75%) and anionic polymer: p(AMPS) (P5) (DP 99) were used here. Crystal growth time: 2 hours. Control experiments contained no polymer in the crystallisation solution.

5.2.7 SEM analysis

Con A crystals grown in the presence of selected polymers neutral p(PEGMA475) (P1), cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75%) and anionic p(AMPS) (P5) by using sitting drop vapour diffusion method were subjected to SEM analysis in this project.
CHAPTER 5. Application of Polymers to Concanavalin A (Con A) Crystallisation

5.3 Results and discussion

5.3.1 Con A crystallisation in the presence of neutral polymer in solution: p(PEGMA<sub>475</sub>) (P1)

By using the above mentioned pH-altered stock buffer solution, the following p(PEGMA<sub>475</sub>) (P1) solutions with various concentrations were prepared: 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 % and 12 % (w/v) (Table 5-2).

Table 5-2. Con A crystallisation plate set up: p(PEGMA<sub>475</sub>)
CHAPTER 5. Application of Polymers to Concanavalin A (Con A) Crystallisation

Figure 5-2 shows images of Con A crystals formed in the presence of p(PEGMA_{475}) (P1). Con A solutions produced small crystals with sizes of around 60 \( \mu \text{m} \) within 1 week in the absence of polymer (Figure 5-2, Control). When p(PEGMA_{475}) (P1) was added to the solution, the size and number of Con A crystals were found to vary very clearly. Neutral polymer p(PEGMA_{475}) (P1) resulted in a few large rectangular crystals.

![Figure 5-2](image)

**Control ~ 60 \( \mu \text{m} \)**

Figure 5-2. Selected photomicrographs of Con A crystals, grown in the presence of p(PEGMA_{475}) (P1) at various concentrations. Image labelled ‘Control’ represents Con A crystals grown from sample wells without polymer. The scale bar was 200 \( \mu \text{m} \).
Con A crystal size and number distribution depending on neutral p(PEGMA\textsubscript{475}) (P1) concentration was obtained by further image analysis (Figure 5-3). For anionic Con A, as the concentration of p(PEGMA\textsubscript{475}) (P1) in solution was increased, the number of Con A crystals appeared to decrease, and the size of the crystals appeared to increase.

![Figure 5-3. Summary of photomicrograph analysis of Con A crystals, grown in the presence of p(PEGMA\textsubscript{475}) (P1) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well.](image)

Diffraction data were collected for Con A crystals by single crystal X-ray diffraction recorded at room temperature. Figure 5-4 illustrates the diffraction patterns of Con A crystals formed in the absence of polymer (Control) and in the presence of p(PEGMA\textsubscript{475}) (P1) by X-ray crystallography; and Table 5-3 shows diffraction data of Con A crystals, which confirmed as orthorhombic (space group: C\textit{C}2\textit{C}2\textit{C}_1, form IV).
CHAPTER 5. Application of Polymers to Concanavalin A (Con A) Crystallisation

Figure 5-4. Con A crystals photomicrographs and X-ray diffraction patterns yielded by the single Con A crystal in the presence of neutral p(PEGMA$_{475}$) (P1). Image labelled ‘Control’ represents Con A crystals grown from sample wells without p(PEGMA$_{475}$) (P1). Two scattering angles: 0±1 degrees and 90±1 degrees.

Table 5-3. Diffraction data collection for Con A crystals formed in the absence (Control) and presence of neutral p(PEGMA$_{475}$) (P1)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>p(PEGMA$_{475}$) (P1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>C222$_1$</td>
<td>C222$_1$</td>
</tr>
<tr>
<td>Unit cell lattice</td>
<td>Orthorhombic</td>
<td>Orthorhombic</td>
</tr>
<tr>
<td>Cell dimensions: a, b, c (Å)</td>
<td>101.4, 119.2, 250.2</td>
<td>102.0, 119.4, 251.0</td>
</tr>
<tr>
<td>$\alpha$, $\beta$, $\gamma$</td>
<td>90°, 90°, 90°</td>
<td>90°, 90°, 90°</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50-2.8</td>
<td>50-2.8</td>
</tr>
<tr>
<td>Crystal-to-detector distance</td>
<td>170 mm</td>
<td>170 mm</td>
</tr>
<tr>
<td>Con A form</td>
<td>Form IV</td>
<td>Form IV</td>
</tr>
</tbody>
</table>
100 μL of 2 mg/mL p(PEGMA475) (P1) in 1 M ammonium sulfate and 10 mM Tris buffer at pH 8.5 was mixed with 100 μL of 30 mg/mL Con A in 20 mM Tris buffer at pH 8.0; then dispensed to a VISCOTEK DLS Quartz cell. Figure 5-5 shows a summary of DLS data of Con A crystallisation under neutral p(PEGMA475) (P1) at time points T₀ and T₁₂₀min. Figure 5-5a shows autocorrelation functions, which were analysed by DLS to obtain the distribution of particle radii, as shown in Figure 5-5b.

Figure 5-5. Summary of DLS data of Con A crystallisation under neutral p(PEGMA475) (P1) at time points T₀ and T₁₂₀min: a) representative autocorrelation functions; b) radii distribution by intensity.

Representative Tukey plots of DLS data further illustrate protein aggregates growing in size as a function of time during Con A crystallisation experiments.
under neutral p(PEGMA<sub>475</sub>) (P1) (Figure 5-6). At the beginning of the experiment (T₀), the average size of the particles in the crystallisation solution was around 4 µm, measured by size distribution. With the addition of p(PEGMA<sub>475</sub>) (P1), Con A crystallisation initiated and aggregates formed. As the time increased, Con A aggregates grew in size. After 120 minutes, the average size of the particles in the crystallisation solution was around 9 µm. The size of pure neutral p(PEGMA<sub>475</sub>) (P1) without any protein in solution was around 7 nm.

Figure 5-6. Variations of the size of Con A aggregates or crystals in the solution as a function of time during crystallisation experiments induced by neutral p(PEGMA<sub>475</sub>) (P1). Tukey plots labelled ‘Control’ represent Con A aggregates or crystals grown from in the absence of polymer.

Con A crystals were taken out from the crystallisation plates then washed with crystallisation buffer solutions for SEM analysis. Figure 5-7 contains micrographs of Con A crystals grown in the absence (a) and presence of neutral p(PEGMA<sub>475</sub>) (P1) (b). Con A crystals were poorly defined and had significant
etch defects without applied polymer (Figure 5-7a). With the addition of neutral p(PEGMA<sub>475</sub>) (P1), Con A crystals had round edges with significant etch defects, however, p(PEGMA<sub>475</sub>) (P1) still improved the size and shape of Con A crystal (Figure 5-7b). Compared with optical microscopy images, SEM images of Con A crystals did not look so well. This may because of poor mechanical properties and stability of protein crystals.

Figure 5-7. SEM micrographs of Con A crystals formed by sitting drop method: a) control, without any polymer, -- x 600 magnifications; the scale bar was 20 μm; b) in the presence of p(PEGMA<sub>475</sub>) (P1), -- x 330 magnifications; the scale bar was 50 μm.

All above results suggest neutral p(PEGMA<sub>475</sub>) (P1) had a similar effect on crystal formation to that which occurred in HEWL crystallisation. Figure 5-8 presents a proposed mechanism of neutral polymer mediated Con A crystallisation. Neutral p(PEGMA<sub>475</sub>) (P1) competed with protein solutes for water and dehydrated protein macromolecules. Moreover, p(PEGMA<sub>475</sub>) might have ‘volume-excluded effects’ by hydrophobic exclusion of protein
solute.\textsuperscript{224} It could also lower the dielectric constant of the solution, consequently protein aggregation and phase separation was promoted. Therefore, Con A macromolecules were compelled to associate with each other.

Figure 5-8. Schematic representation of hypothesis of anionic Con A crystallisation process in the absence of polymer (A); and the interaction of neutral p(PEGMA\textsubscript{475}) (P1) with anionic Con A in the protein crystallisation process (B). Con A macromolecules are negatively charged at crystallisation pH of 8.5, i.e. anionic. NH\textsubscript{4}\textsuperscript{+} and SO\textsubscript{4}\textsuperscript{2-} ions come from the crystallisation buffer solutions.

5.3.2 Con A crystallisation in the presence of cationic polymer in solution: quaternised p(DMAEMA) (P2)

The following quaternised p(DMAEMA) (P2) (quaternised ratio: 75 \%) solutions with various concentrations were prepared: 0.02 \%, 0.2 \%, 2 \%, 4 \%, 6 \%, 8 \%, 10 \%, 12 \% and 14 \% (w/v) (Table 5-4).
Table 5-4. Con A crystallisation plate set up: quaternised p(DMAEMA) (P2)
Figure 5-9 shows Con A crystals formed in the presence of quaternised p(DMAEMA) (P2). Cationic polymer was likely to nucleate a few large block shape crystals.

![Photomicrographs showing changes in the size and shape of Con A crystals, grown in the presence of quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %) at various concentrations. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. The scale bar was 200 µm.](image-url)
CHAPTER 5. Application of Polymers to Concanavalin A (Con A) Crystallisation

Figure 5-10 shows image analysis of Con A crystal size distribution by measuring the average length (μm) of the largest diameter of each crystal and number distribution depending on quaternised p(DMAEMA) (P2) concentration. For anionic Con A, as the concentration of quaternised p(DMAEMA) (P2) in solution was increased, the number of Con A crystals appeared to decrease, and the size of the crystals appeared to increase.

Figure 5-10. Summary of photomicrograph analysis of Con A crystals, grown in the presence of quaternised p(DMAEMA) (P2) at various concentrations. a) Average length (μm) of the largest crystal diameter. b) Experiment count for the number of crystals per sample well.

100 μL of 2 mg/mL quaternised p(DMAEMA) (P2) in 1 M ammonium sulfate and 10 mM Tris buffer at pH 8.5 was mixed with 100 μL of 30 mg/mL Con A in 20 mM Tris buffer at pH 8.0 for DLS study. Figure 5-11 shows a summary of DLS data of Con A crystallisation under cationic quaternised p(DMAEMA) (P2) at time points T₀ and T₁₂₀min. Figure 5-11a shows autocorrelation functions, which were analysed by DLS to obtain the distribution of particle radii, as
shown in Figure 5-11b.

Figure 5-11. Summary of DLS data of Con A crystallisation under quaternised p(DMAEMA) (P2) at time points T₀ and T₁₂₀min: a) representative autocorrelation functions; b) radii distribution by intensity.

Representative Tukey plots of DLS data show Con A aggregates growing in size in the presence of quaternised p(DMAEMA) (P2) as a function of time during crystallisation experiments as shown in Figure 5-12. At the beginning of the experiment (T₀), the average size of the particles in the crystallisation solution was around 300 nm, measured by size distribution. With the addition of quaternised p(DMAEMA) (P2), an increase in size of the Con A aggregates was observed. After 120 minutes (T₁₂₀min), the average size of the aggregates in the crystallisation solution was around 1000 nm. The size of pure quaternised p(DMAEMA) (P2) in solution was around 18 nm.
Figure 5-12. Variations of the size of Con A aggregates or crystals in the solution as a function of time during crystallisation experiments induced by cationic quaternised p(DMAEMA) (P2).

The SEM micrograph result of Con A crystals is shown in Figure 5-13. Con A crystals formed under quaternised p(DMAEMA) (P2) also had round edges with significant etch defects, due to poor mechanical properties and stability of protein crystals.

Figure 5-13. SEM micrographs of Con A crystals formed in the presence of quaternised p(DMAEMA) (P2), -- x 200 magnifications; the scale bar was 100 μm.
In summary of all above results obtained in the presence of cationic quaternised p(DMAEMA) (P2), for anionic proteins, such as Con A, the cationic polymer was likely to have attracted anionic Con A macromolecules by electrostatic interaction for charge compensation in the crystallisation process (Figure 5-14). Therefore, the cationic polymer would either lower the protein and water interfacial energy and act as nucleation centers by the absorption of Con A macromolecules, or slow down the rate of further growth of Con A crystal.

Figure 5-14. Hypothesis of mechanism of cationic polymer mediated Con A crystallisation. Cationic polymer attracted anionic Con A for charge compensation. Con A macromolecules were anionic when at crystallisation pH 8.5. NH$_4^+$ and SO$_4^{2-}$ ions were from the crystallisation buffer solutions.
5.3.3 Con A crystallisation in the presence of anionic polymer in solution:

p(AMPS) (P5)

The following p(AMPS) (P5) (DP 99) solutions with various concentrations were prepared: 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v) (Table 5-5).

Table 5-5. Con A crystallisation plate set up: p(AMPS) (P5) (DP 99)

<table>
<thead>
<tr>
<th>p(AMPS) (P5)</th>
<th>0.02%</th>
<th>0.2%</th>
<th>2%</th>
<th>4%</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>14%</th>
</tr>
</thead>
<tbody>
<tr>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
<td>100μL</td>
</tr>
<tr>
<td>1M</td>
<td>1M</td>
<td>1M</td>
<td>1M</td>
<td>1M</td>
<td>1M</td>
<td>1M</td>
<td>1M</td>
<td>1M</td>
<td>1M</td>
</tr>
<tr>
<td>HEPES buffer (200mM)</td>
<td>HEPES buffer (200mM)</td>
<td>HEPES buffer (200mM)</td>
<td>HEPES buffer (200mM)</td>
<td>HEPES buffer (200mM)</td>
<td>HEPES buffer (200mM)</td>
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<tr>
<td>pH 8.5</td>
<td>pH 8.5</td>
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<td>pH 8.5</td>
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<td>pH 8.5</td>
<td>pH 8.5</td>
<td>pH 8.5</td>
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</tbody>
</table>

...
Figure 5-15 shows images of Con A crystals formed in the presence of p(AMPS) (P5) (DP 99). Anionic p(AMPS) (P5) accelerated Con A aggregation and the formation of multiple nuclei, produced many small crystals with sizes of ~ 20µm.

\[ \text{p(AMPS) (P5) (DP 99)} \]

Figure 5-15. Photomicrographs of Con A crystals, grown in the presence of p(AMPS) (P5) (DP 99) at various concentrations. Images labelled ‘Control’ represent crystals grown from sample wells without polymer. The scale bar was 200 µm.
CHAPTER 5. Application of Polymers to Concanavalin A (Con A) Crystallisation

Image analysis of photomicrograph data by measuring the average length (μm) of the largest diameter of each crystal depending on p(AMPS) (P5) concentration was obtained (Figure 5-16). However, it was not easy to obtain the number distribution, due to the amount of small crystals in the whole sample well was too large. For anionic Con A, as the concentration of p(AMPS) (P5) in solution was increased, the size of crystals appeared to decrease. At the concentration of 14 % (w/v), the size of Con crystal was around 20 μm.

![Graph showing average crystal size vs. p(AMPS) concentration]

Figure 5-16. Summary of photomicrograph analysis of Con A crystals, grown in the presence of p(AMPS) (P5) at various concentrations.

100 μL of 2 mg/mL p(AMPS) (P5) in 1 M ammonium sulfate and 10 mM Tris buffer at pH 8.5 was mixed with 100 μL of 30 mg/mL Con A in 20 mM Tris buffer at pH 8.0; then dispensed to a VISCOTEK DLS Quartz cell for DLS study. Figure 5-17 shows a summary of DLS data of Con A crystallisation under anionic p(AMPS) (P5) at time points $T_0$ and $T_{100\text{min}}$. Figure 5-17a shows autocorrelation functions, which were analysed by DLS to obtain the distribution of particle radii, as shown in Figure 5-17b and c.
Figure 5-17. Summary of DLS data of Con A crystallisation under anionic p(AMPS) (P5) DP (99) at time points $T_0$ and $T_{100\text{min}}$: a) representative autocorrelation functions; b) radii distribution by intensity; c) radii distribution by number.

Representative Tukey plots of above DLS data show an increase in the size of protein aggregates when in the presence of p(AMPS) (P5) as a function of time during crystallisation experiments (Figure 5-18). Con A aggregates growing in size can be observed during the crystallisation experiment. At the beginning of
the experiment ($T_0$), Con A macromolecules were non-interacting in the solution. The decay time was very short. The average size of the particles in crystallisation solution was around 5 nm, measured by size distribution. With the addition of anionic p(AMPS) (P5), Con A crystallisation initiated and aggregates formed. As the time increased, Con A aggregates grew in size. Large particles diffused slower than small particles, and the correlation function decayed at a slower rate. After 120 minutes ($T_{120\text{min}}$), the average size of the particles in crystallisation solution was around 700 nm. The size of pure p(AMPS) (P5) (DP 99) without any protein was around 4 nm.

![Figure 5-18. Variations of the size of Con A aggregates or crystals in the solution as a function of time during crystallisation experiments induced by anionic p(AMPS) (P5).](image)

Con A crystals were then taken out from the crystallisation plates, washed with 1 M ammonium sulfate and 10 mM Tris buffer solution at pH 8.5 for SEM study. The SEM micrograph result (Figure 5-19) shows that although Con A
crystals had slight rounded edges under p(AMPS) (P5), however, they were better than that obtained under neutral p(PEGMA<sub>475</sub>) (P1) and cationic quaternised p(DMAEMA) (P2). This may suggest that p(AMPS) (P5) could enhance the stability of Con A crystals.

![Figure 5-19. SEM micrograph of Con A crystals formed in the presence of p(AMPS) (P5), -- x 220 magnifications; the scale bar was 100 μm.](image)

In summary of the above results obtained in the presence of p(AMPS) (P5), we proposed that at Con A crystallisation pH of 8.5, anionic polymer molecules had some negative charges, resulting in ‘charge-charge’ repulsions between anionic polymer molecules and anionic Con A macromolecules, lowering the interfacial energy between Con A macromolecules and water, thus, leading to the promotion of the nucleation rate and formation of multiple nuclei (Figure 5-20).<sup>226, 108</sup>
CHAPTER 5. Application of Polymers to Concanavalin A (Con A) Crystallisation

Figure 5-20. Hypothesis of mechanism of anionic polymer mediated Con A crystallisation. Anionic polymer: charge-charge repulsions between anionic polymer and anionic Con A. Con A macromolecules were anionic when at crystallisation pH 8.5. NH$_4^+$ and SO$_4^{2-}$ ions were from the crystallisation buffer solutions.

5.4 Conclusions

In this chapter, three polymers with varying degrees of charge, molecular weight and backbone structure were selected from each class and investigated as solution additives for Con A crystallisation, including neutral p(PEGMA$_{475}$) (P1), cationic quaternised p(DMAEMA) (P2) with quaternised ratio: 75 % and anionic p(AMPS) (P5). Crystallisation experiments were performed on 96-well plates using the sitting drop vapour diffusion technique as well.

Con A crystallisation experiments produced small orthorhombic crystals (space group: $C222_1$, form IV) with sizes of ~ 60 μm within 1 week in the absence of
polymer. When polymers were utilised, the sizes and numbers of crystals were found to vary with the types of polymers added to solution, however, the form of crystals was still orthorhombic. Neutral polymers resulted in a few large block-shaped crystals with sizes of ~200 μm, suggesting a similar effect on crystal formation to that which occurred in HEWL crystallisation. Cationic polymers were likely to have attracted anionic Con A macromolecules by electrostatic interaction for charge compensation, and nucleated a few large crystals as well with sizes of ~150 μm. Anionic polymers accelerated Con A aggregation and produced many smaller crystals with sizes of ~20 μm within four days. In conclusion, all these results illustrate that polymers with varying net charges, acted as solution additives to mediate protein crystallisation and have the ability to alter the size of protein crystals.
6. Application of Polymers to Bovine Liver Catalase (BLC) Crystallisation

6.1 Introduction

Bovine liver catalase (BLC) was crystallised by Sumner and Dounce in 1937.\textsuperscript{185} BLC has three structurally characterised crystal forms: trigonal $P\overline{3}21$ (form I),\textsuperscript{186} orthorhombic $P2_12_12_1$ (form II)\textsuperscript{187} and another orthorhombic (form III).\textsuperscript{188} Forms I and II of BLC have been fully characterised using single X-ray diffraction previously. Form III only has previously been analysed by electron microscopy and powder X-ray diffraction.\textsuperscript{230, 231, 232, 233} It is very difficult to obtain high quality single BLC crystals, by using traditional growth methods. Moreover, BLC crystals are extremely thin (<1 μm),\textsuperscript{231} it is therefore hard to get X-ray diffraction pattern. Recently, Matzger \textit{et al.} utilised polymer-induced heteronucleation (PIHn) approach; i.e. cross-linked polymer films as heteronucleants, for BLC crystallisation, and got crystals suitable for single crystal X-ray diffraction (form III).\textsuperscript{188, 99} However, whether polymers were used as ‘additives’ in solution can mediate BLC crystallisation successfully or not, rarely has been studied.

Therefore, in addition to HEWL and Con A, a third model protein: bovine liver catalase (BLC), was chosen as model anionic protein for further crystallisation studies. The isoelectric point (pI) of BLC is 5.4,\textsuperscript{222} and when at crystallisation
CHAPTER 6. Application of Polymers to Bovine Liver Catalase (BLC) Crystallisation

pH of 6.8, BLC macromolecules will carry some negative charges, hence being a weekly anionic protein at this pH (Table 6-1).

![Image]

Table 6-1. Summary of bovine liver catalase (BLC)

<table>
<thead>
<tr>
<th>Protein</th>
<th>pI(^a)</th>
<th>Mw (kDa)</th>
<th>Crystallisation pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLC</td>
<td>5.4</td>
<td>60</td>
<td>6.8</td>
</tr>
</tbody>
</table>

\(^a\) Isoelectric point (pI) is the pH at which a particular molecule or surface carries no net electrical charge.

As described in Chapter 5, in order to evaluate the effect of those polymers with different degrees of charge and backbone structure on other proteins, especially anionic proteins, three key polymers from each class, neutral p(PEGMA\(_{475}\)) (P1), cationic quaternised p(DMAEMA) (P2) (quaternised ratio: 75 \%) and anionic p(AMPS) (P5) (DP 99) were also applied for BLC crystallisation by using sitting drop vapour diffusion crystallisation protocols. The crystallisation condition used here was first reported by McPherson and co-workers.\(^{210}\) Figure 6-1 shows a summary of hypothesis of the interaction of various polymers with weakly anionic bovine liver catalase (BLC) in the protein crystallisation process.
CHAPTER 6. Application of Polymers to Bovine Liver Catalase (BLC) Crystallisation

Figure 6-1. Schematic representation of the interaction of various polymers with anionic bovine liver catalase (BLC) in the protein crystallisation process. (A) Neutral polymer: H-bonding with water and thus promoting protein crystallisation via a dehydration mechanism. (B) Cationic polymer: attracting anionic BLC for charge compensation. (C) Anionic polymer: charge-charge repulsions between anionic polymer and anionic BLC. BLC macromolecules are weakly anionic when at crystallisation pH 6.8. Na⁺ ions are derived from the crystallisation buffer solutions.
CHAPTER 6. Application of Polymers to Bovine Liver Catalase (BLC) Crystallisation

6.2 Methods

6.2.1 Crystallisation technique and plate

BLC was also crystallised using the sitting drop vapour diffusion technique as described in Chapter 4 for HEWL. MRC 2 Well Crystallisation Plates (Swissci) (UVP plate, 96 reservoir wells) were purchased from Hampton Research. Specifically, a solution (50 µL) of tested polymer buffer was pipetted in triplicate in the reservoir wells. Protein solution (1 µL) and buffered polymer solution (as described above, 1 µL) were pipetted into sample wells of the crystallisation plate.

6.2.2 Preparation of polymer solutions and stock buffer solutions

Similar as procedures described in Chapter 4, the stock polymer solution was prepared and adjusted to the BLC crystallisation pH of 6.8. The stock buffer solutions of 12 % PEG 4000 and 0.05 M sodium phosphate buffer at pH 6.8 and 0.05 M sodium phosphate buffer at pH 6.8 were prepared and filtered using a 0.2 µm cellulose acetate membrane filter. By using the above mentioned pH-altered polymer solutions and the stock buffer solutions, the following polymer solutions were prepared: 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 %, 14 %, 16 %, 18 %, 20 %, 22 % and 24 % (w/v) in 12 % PEG 4000 and 0.05 M sodium phosphate buffer at pH 6.8. All polymers dissolved completely in the buffer solutions.
6.2.3 Crystallisation protocol for BLC crystals

Catalase from bovine liver (BLC) was purchased from Sigma (Product Number: C40, EC Number: 1.11.1.6, CAS Number: 9001-05-2, pI: 5.4). 0.05 M sodium phosphate buffer at pH 6.8 was prepared and filtered through a 0.2 μm cellulose acetate membrane filter. A protein solution of 40 mg/mL BLC was prepared and then centrifuged at 13,000 rpm for 5 min at 4 °C.

BLC was crystallised following the sitting drop vapour diffusion procedures, in a similar manner to HEWL crystallisation in Chapter 4, with a BLC solution of 40 mg/mL BLC in 0.05 M sodium phosphate buffer at pH 6.8 and a polymer solution of 12 % PEG 4000 and 0.05 M sodium phosphate buffer at pH 6.8 in the sample well, and 50 μL of tested polymer buffer in the reservoir well.

For control experiments, some reservoir and sample wells contained no polymer. The entire crystallisation plate system was sealed using transparent sealing tape and incubated at 19 °C.

6.2.4 Crystal imaging

Optical photomicrographs of BLC crystals were directly collected by a Leica Stereomicroscope (63 x magnifications), with a Leica fan-cooled light source, connected to a Nikon Coolpix 4500 digital camera.

6.2.5 X-ray crystallography

BLC crystallographic parameters were determined by single crystal X-ray
diffraction recorded at room temperature on an X-ray generator with high-flux Osmic confocal multi-layer optics. BLC crystals were cryoprotected with mother liquor containing 30 % ethylene glycol, then flash-frozen in a nitrogen-gas stream.99,210

6.2.6 DLS studies

Similar to HEWL, the batch crystallisation method was applied for a DLS time study of BLC crystallisation. Because the optimum intensity range for DLS measurement is 300-8000, a protein solution of 20 mg/mL BLC in 0.05 M sodium phosphate buffer at pH 6.8 was mixed with a polymer solution in 12 % PEG 4000 and 0.05 M sodium phosphate buffer at pH 6.8 to induce BLC crystallisation. Three classes of polymers, comprising neutral polymer: p(PEGMA₄₇₅) (P1), cationic polymer: quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %) and anionic polymer: p(AMPS) (P5) (DP 99) were utilised here. Temperature: 19 °C. Crystal growth time: 2 hours.

6.3 Results and discussion

6.3.1 BLC crystallisation in the presence of neutral polymer in solution:

p(PEGMA₄₇₅) (P1)

By using the above mentioned pH-altered stock buffer solution, the following p(PEGMA₄₇₅) (P1) solutions with various concentrations were prepared: 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 %, 12 % and 14 % (w/v) in 12 % PEG
### Table 6.2. BLC crystallisation plate set up: p(PEGMA<sub>475</sub>) (P).

<table>
<thead>
<tr>
<th>pH</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8</td>
<td>12 % PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>100 μL (12 % PEG 4000 and 0.05 M sodium phosphate buffer) 140.9 μL</td>
<td>140.9 μL</td>
<td>0.1 μL (0.02 %)</td>
<td>14.3 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
</tr>
<tr>
<td></td>
<td>p(PEGMA&lt;sub&gt;475&lt;/sub&gt;) 37 % H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.1 μL (0.2 %)</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
</tr>
<tr>
<td></td>
<td>pH 6.8</td>
<td>12 % PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>100 μL (12 % PEG 4000 and 0.05 M sodium phosphate buffer) 140.9 μL</td>
<td>140.9 μL</td>
<td>0.1 μL (0.02 %)</td>
<td>14.3 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
</tr>
<tr>
<td></td>
<td>p(PEGMA&lt;sub&gt;475&lt;/sub&gt;) 37 % H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.1 μL (0.2 %)</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
<td>0.1 μL</td>
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</tbody>
</table>

Table 6.2. BLC crystallisation plate set up and Figure 6.2 shows images of BLC crystals grown from solutions containing neutral p(PEGMA<sub>475</sub>) (P).
CHAPTER 6. Application of Polymers to Bovine Liver Catalase (BLC) Crystallisation

\( p(\text{PEGMA}_{475}) \) (P1)

Figure 6-2. Selected photomicrographs of BLC crystals, grown in the presence of \( p(\text{PEGMA}_{475}) \) (P1) at various concentrations. Image labelled ‘Control’ represents BLC crystals grown from sample wells without polymer. The scale bar was 200µm.

BLC crystal formation in the absence of added polymer occurred after two weeks and resulted in needle crystals outgrowth from a single point in a ‘wheatsheaf’ morphology (Figure 6-2, Control). However, it proved difficult to obtain good quality single crystals without any added polymer. The literature
reports that orthorhombic BLC crystals typically exhibit an extremely high defect density and incorporate great numbers of misoriented microcrystals.\textsuperscript{210} Neutral polymer p(PEGMA\textsubscript{475}) (P1) nucleated a few long crystals at lower concentrations (< 4 \%, w/v). However, the quality of these crystals was poor and it was not possible to determine their structure by single crystal X-ray diffraction.

The solutions in the sample wells were not clear enough for fully automated image analysis; therefore, we did both manual and automated analysis. A full summary of BLC crystals size distribution by measuring the average length (\(\mu m\)) of the largest diameter of each crystal and number distribution depending on neutral p(PEGMA\textsubscript{475}) (P1) concentration was obtained by further image analysis (Figure 6-3). For anionic BLC, as the concentration of p(PEGMA\textsubscript{475}) (P1) in solution was increased, the size of crystals increased first. However, as shown in Figure 6-2 above, when the concentration of neutral p(PEGMA\textsubscript{475}) (P1) was above 4 \%, ‘wheatsheaf’ shape BLC crystals formed rather than large and thin crystals. As the concentration of p(PEGMA\textsubscript{475}) (P1) was increased, the size of ‘wheatsheaf’ shape BLC crystals decreased slightly.
Figure 6-3. Summary of photomicrograph analysis of BLC crystals, grown in the presence of p(PEGMA₄₇₅) (P1) at various concentrations. a) Average length (μm) of the largest crystal diameter. b) Experiment count for the number of crystals per sample well.

For the DLS study, a protein solution of 20 mg/mL BLC in 0.05 M sodium phosphate buffer at pH 6.8 mixed with a polymer solution in 12 % PEG 4000 and 0.05 M sodium phosphate buffer at pH 6.8 was employed, which was then dispensed to a VISCOTEK DLS Quartz cell. The control experiment contained no polymer in the crystallisation solution. Figure 6-4 shows a summary of DLS data of BLC crystallisation under neutral p(PEGMA₄₇₅) (P1) at time points T₀ and T₁₂₀ₘᵣᵢₙ. Figure 6-4a shows autocorrelation functions, which were analysed by DLS to obtain the distribution of particle radii, as shown in Figure 6-4b.
Figure 6-4. Summary of DLS data of BLC crystallisation under neutral p(PEGMA<sub>475</sub>) (P1) at time points T<sub>0</sub> and T<sub>120 min</sub>: a) representative autocorrelation functions; b) radii distribution by intensity.

Figure 6-5 illustrate representative Tukey plots of the above DLS data. The characteristic increase in the size of BLC aggregates can be observed in all cases. At the beginning of the experiment (T<sub>0</sub>) under p(PEGMA<sub>475</sub>) (P1), the average size of the particles in the crystallisation solution was around 0.9 µm, measured by size distribution. With the addition of p(PEGMA<sub>475</sub>) (P1), BLC crystallisation initiated and aggregates formed. As the time increased, BLC aggregates grew in size. After 120 minutes (T<sub>120 min</sub>), the average size of the particles in the crystallisation solution was around 2 µm. As a control, the experiment was also carried out without polymer.
Figure 6-5. Variations of the size of BLC aggregates or crystals in the solution as a function of time during crystallisation experiments induced by neutral p(PEGMA_{475}) (P1). Tukey plots labelled ‘Control’ represent crystals grown from solutions without polymer.

The above results suggest that neutral p(PEGMA_{475}) (P1) competed with protein solutes for water, and thereby acted as a precipitant to mediate BLC crystallisation, leading to protein aggregation similar to HEWL and Con A (Figure 6-6B).\(^{125}\) Therefore, BLC macromolecules were compelled to associate with each other. However, as the concentration of p(PEGMA_{475}) (P1) in solution increased, the increased number of nucleation sites caused by the high concentration of precipitant in combination with the increased viscosity of these solutions, promoted the formation of needle crystal outgrowth from a single point in the ‘wheatsheaf’ morphology.\(^{210}\)
Figure 6-6. Schematic representation of hypothesis of anionic BLC crystallisation process in the absence of polymer (A); and the interaction of neutral p(PEGMA\textsubscript{475}) (P1) with anionic BLC in the protein crystallisation process (B). BLC macromolecules are negatively charged at crystallisation pH of 6.8, i.e. weakly anionic. Na\textsuperscript{+} and PO\textsubscript{4}\textsuperscript{3-} ions are from the crystallisation buffer solutions.

6.3.2 BLC crystallisation in the presence of cationic polymer in solution: quaternised p(DMAEMA) (P2)

The following quaternised p(DMAEMA) (P2) (quaternised ratio: 75 %) solutions with various concentration were prepared: 0.02 %, 0.2 %, 2 %, 4 %, 6 %, 8 %, 10 % and 12 % (w/v). Table 6-3 shows BLC crystallisation plate set up and Figure 6-7 shows images of BLC crystals formed in the presence of quaternised p(DMAEMA) (P2).
Table 6-3. BLC crystallisation plate set up: quaternised p(DMAEMA) (P2)

<table>
<thead>
<tr>
<th></th>
<th>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</th>
<th>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</th>
<th>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</th>
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<tbody>
<tr>
<td>1</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
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<tr>
<td>2</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
</tr>
<tr>
<td>3</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
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<tr>
<td>4</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
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<tr>
<td>5</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
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<tr>
<td>6</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
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<tr>
<td>7</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
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<tr>
<td>8</td>
<td>100 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>125 µL (10%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
<td>150 µL (12%) PEG 4000 and 0.05 M sodium phosphate buffer</td>
</tr>
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</table>

Quaternised p(DMAEMA) (75%) dissolved in H2O: 150 µL (6.8 µl)
Figure 6-7. Photomicrographs of BLC crystals, grown in the presence of quaternised p(DMAEMA) (P2) at various concentrations. Images labelled ‘Control’ represent crystals grown from sample wells without polymer. The scale bar was 200 μm.
From visual inspection of Figure 6-7, when cationic quaternised p(DMAEMA) (P2) was utilised, long and thin crystals were formed at concentration of 0.02 % (w/v). A few needles with a ‘sea-urchin’ morphology formed at lower concentrations (0.2 % ~ 2 %, w/v). However, as the concentration of cationic quaternised p(DMAEMA) (P2) in solution increased, it was hard to get BLC crystals, almost no BLC crystals were formed by using cationic quaternised p(DMAEMA) (P2) at higher concentrations. There may be several reasons. First, BLC was not easy to crystallise by using orthorhombic $P2_12_12_1$ crystallisation condition because orthorhombic BLC growth mechanism was complicated.210 Moreover, at pH 8.5, cationic quaternised p(DMAEMA) (P2) might favor attracting anionic BLC macromolecules by electrostatic interaction, resulting in the suppression of BLC crystallisation. Thus, insufficient BLC macromolecules could aggregate together and form nuclei.

Figure 6-8 shows a summary of DLS data of BLC crystallisation from solution containing cationic quaternised p(DMAEMA) (P2) at time points $T_0$ and $T_{120\text{min}}$. Moreover, representative Tukey plots of DLS data are shown in Figure 6-9. An increase in size of BLC aggregates was observed during the crystallisation experiments. At the beginning of the experiment ($T_0$), the average size of the particles in the crystallisation solution was around 400 nm, measured by size distribution. With the addition of quaternised p(DMAEMA) (P2), BLC aggregates grew in size. After 120 minutes ($T_{120\text{min}}$), the average size of the particles in crystallisation solution was around 8000 nm.
Figure 6-8. Summary of DLS data of BLC crystallisation under cationic quaternised p(DMAEMA) (P2) at time points T₀ and T₁₂₀min: a) representative autocorrelation functions; b) radii distribution by intensity.

Figure 6-9. Variations of the size of BLC aggregates or crystals in the solution as a function of time during crystallisation experiments induced by cationic quaternised p(DMAEMA) (P2).
6.3.3 BLC crystallisation in the presence of anionic polymer in solution:

p(AMPS) (P5)

The following p(AMPS) (P5) solutions with various concentrations were prepared: 0.02%, 0.2%, 2%, 4%, 6%, 8%, 10%, 12%, 14% and 16% (w/v) in 12% PEG 4000 and 0.05 M sodium phosphate buffer at pH 6.8 (Table 6-4).

Table 6-4. BLC crystallisation plate set up: p(AMPS) (P5) (DP 99)
Figure 6-10 shows photomicrographs of BLC crystals formed in the presence of p(AMPS) (P5). When p(AMPS) (P5) was used for BLC crystallisation, the size and shape of BLC crystals were found to vary very clearly. Anionic p(AMPS) (P5) promoted BLC aggregation, with single crystals being obtained in 10 days.

Figure 6-10. Photomicrographs of BLC crystals formed in the presence of p(AMPS) (P5) at various concentrations. Images labelled ‘Control’ represent crystals grown from sample wells without polymer. The scale bar was 200 µm.
CHAPTER 6. Application of Polymers to Bovine Liver Catalase (BLC) Crystallisation

Figure 6-11 shows image analysis of BLC crystals size distribution by measuring the average length (μm) of the largest diameter of each crystal and number distribution depending on anionic p(AMPS) (P5) concentration. For weakly anionic BLC, as the concentration of p(AMPS) (P5) in solution was increased, the number of BLC crystals appeared to increase and the size of the crystals appeared to decrease slightly.

Figure 6-11. Summary of photomicrograph analysis of BLC crystals, grown in the presence of p(AMPS) (P5) at various concentrations. a) Average length (μm) of the largest crystal diameter; b) Experiment count for the number of crystals per sample well.

Figure 6-12 illustrates the diffraction patterns of BLC crystal formed in the presence of anionic p(AMPS) (P5) by X-ray crystallography. X-ray diffraction identified the crystal form was orthorhombic (space group: $P2_12_12_1$, form III) (Table 6-5). BLC crystals of form III reported previously were extremely thin (< 1 μm) and hard to characterise fully using single crystal X-ray diffraction.\textsuperscript{231} Furthermore, BLC form III has rarely been studied; thus the use of polymers as...
solution additives might be useful in further BLC crystal characterisation.

Figure 6-12. BLC crystal photomicrograph and X-ray diffraction patterns yielded by the single BLC crystal in the presence of anionic p(AMPS) (P5). Two scattering angles: 0±1 degrees and 90±1 degrees.

<table>
<thead>
<tr>
<th>Table 6-5. Diffraction data collection for BLC crystal</th>
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<tbody>
<tr>
<td><strong>Space group</strong></td>
</tr>
<tr>
<td><strong>Unit cell lattice</strong></td>
</tr>
<tr>
<td><strong>Cell dimensions: a, b, c (Å)</strong></td>
</tr>
<tr>
<td><strong>α, β, γ</strong></td>
</tr>
<tr>
<td><strong>Resolution (Å)</strong></td>
</tr>
<tr>
<td><strong>Crystal-to-detector distance</strong></td>
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</table>

Figure 6-13 shows a summary of DLS data of BLC crystallisation under anionic p(AMPS) (P5) at time points T₀ and T₁₂₀min. Figure 6-13a shows autocorrelation functions, which were analysed by DLS to obtain the distribution of particle radii, as shown in Figure 6-13b.
Figure 6-13. Summary of DLS data of BLC crystallisation under anionic p(AMPS) (P5) at time points T₀ and T₁₂₀min: a) representative autocorrelation functions; b) radii distribution by intensity.

Furthermore, representative Tukey plots of above DLS data are shown in Figure 6-14. At the beginning of the experiment (T₀), the average size of the particles in the crystallisation solution was around 1.5 µm, measured by size distribution. With the addition of anionic p(AMPS) (P5), BLC aggregates grew in size. After 120 minutes (T₁₂₀min), the average size of the particles in the crystallisation solution was around 5 µm.
Figure 6-14. Variations of the size of BLC aggregates or crystals in the solution as a function of time during crystallisation experiments induced by anionic p(AMPS) (P5).

Figure 6-15 illustrates the proposed mechanism of anionic polymer mediated BLC crystallisation. Similar to Con A, at BLC crystallisation pH of 6.8, anionic p(AMPS) (P5) molecules had some negative charges. There were expected to be ‘charge-charge’ repulsions between anionic polymer molecules and anionic BLC macromolecules, thus, anionic polymer lowered the interfacial energy between BLC macromolecules and water, leading to the promotion of BLC nucleation rate.\textsuperscript{226, 108}
Figure 6-15. Hypothesis of mechanism of anionic polymer mediated BLC crystallisation. Anionic polymer: charge-charge repulsions between anionic polymer and anionic BLC. BLC macromolecules are weakly anionic when at crystallisation pH 6.8. Na$^+$ and PO$_4^{3-}$ ions come from the crystallisation buffer solutions.

6.4 Conclusions

In this chapter, similar to Con A, three key polymers with varying degrees of charge were selected from each class and utilised as solution additives for BLC crystallisation: neutral p(PEGMA$_{475}$) (P1), cationic quaternised p(DMAEMA) (P2) with quaternised ratio: 75 % and anionic p(AMPS) (P5). BLC crystallisation experiments were also performed on 96-well plates using the sitting drop vapour diffusion technique.
The isoelectric point (pI) of BLC is 5.4 therefore at its crystallisation pH of 6.8 BLC is a weakly anionic protein. BLC crystal formation in the absence of added polymers occurred after two weeks and resulted in needle crystals outgrowth in a ‘wheatsheaf’ morphology. Neutral p(PEGMA475) (P1) nucleated a few long crystals at lower concentrations (< 4 %, w/v). However, it was not possible to determine their structure by single crystal X-ray diffraction. When cationic quaternised p(DMAEMA) (P2) was utilised, a few needles with a ‘sea-urchin’ morphology formed at lower concentrations (< 2 %, w/v). However, as the concentration of quaternised p(DMAEMA) (P2) in solution increased, suppression of BLC crystallisation occurred. By contrast, anionic polymers promoted BLC aggregation, with single crystals being obtained in 10 days. X-ray diffraction identified these crystals as orthorhombic (space group: \(P2_12_12_1\), form III). These results illustrate that polymers can nucleate protein crystals produce diffraction-quality single crystals, which makes structural elucidation possible.
CHAPTER 7

7. Discussion, Conclusions and Future Work

7.1 Discussion and conclusions

Protein assemblies, either in the form of crystals or supramolecular nanoparticles, are important in areas ranging from basic biochemical research through to pharmaceutical application. In addition to characterisation purposes, there are other applications where defined assemblies of proteins, either crystals or self-assembled protein nanoparticles are desirable, including industrial biocatalysis and therapeutic protein formulations. The ability to prepare a given crystal or self-assembled structure from protein molecules, however, remains extremely challenging. A variety of approaches have been used to aid the production of protein crystals; the most common involves the addition of nucleants, or surface-active materials into the crystallisation solution. Synthetic polymers are important additives for protein crystallisation control as there are many possible chemistries which can be introduced into the backbone and side-chains, giving a very wide range of functional behaviour. Proteins have become particular targets for polymer-mediated crystallisation in recent years owing to their growing importance in pharmaceutical applications. Soluble polymeric additives can influence the biomolecule crystallisation process leading to changes in size, habit and even polymorph of the final crystals. Literature reviews in Chapter
1 introduced previous studies of synthetic polymers used as solution additives for the control of crystallisation. Although tremendous efforts have been made, only a very few functional polymers have been studied as additives in protein crystallisation. There are many factors which can affect crystallisation, the majority of which are still poorly understood. Moreover, the mechanisms governing the interactions between polymer additives and emerging protein assemblies are elusive and need to be further confirmed.

The work presented in this thesis has concentrated on the application of polymers as ‘additives’ into the protein solution, i.e. dissolved polymers in the solution to mediate proteins crystallisation. In order to probe some of the factors involved in polymer-mediated protein crystallisation, we set out to prepare different classes of polymers with varying degrees of charge, molecular weight and backbone structure, and then to study their roles as additives in model protein solutions. The guiding hypothesis was that specific functional polymers in solution could alter the rate of protein crystallisation from solutions, and thereby influence the shape, size, and/or polymorph of the resultant crystals.

Four classes of materials were investigated for their effects on protein crystallisation studies: neutral, cationic, anionic and cationic/zwitterionic polymers. We chose poly(ethylene glycol) methyl ether methacrylate,\textsubscript{475} (PEGMA\textsubscript{475}), as a neutral methacrylate derivative of PEG to obtain a polymer, \textit{p}(PEGMA\textsubscript{475}), analogous to PEG but with hydrophilic side-chain functionality
and a hydrophobic main-chain. Two monomers containing tertiary amines groups were selected to afford positive charges, including one methacrylate monomer 2-(dimethylamino)ethyl methacrylate (DMAEMA) which was either homopolymerised or copolymerised with PEGMA₄₇₅, and one methacrylamide monomer N-(3-(dimethylamido)propyl)methacrylamide (DMAPMAm), which was also homopolymerised to form cationic polymers. Three acrylamide-based main chain monomers, including 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), 2- acrylamidoacetic acid (2-AmAA) and 4- acrylamidobutanoic acid (4-AmBA), which have ionisable sulfonic acid or carboxyl groups, were selected to generate anionic water soluble homopolymers. Moreover, a cationic/zwitterionic copolymer of [2-(methacryloyloxy)ethyl]trimethylammonium chloride (METMAC) and N-[2-(methacryloyloxy)ethyl]-dimethyl-(2-sulfoethan)aminium hydroxide (MEDSAH) with both positive charges and zwitterionic components was prepared in order to span an even wider range of charge-charge interactions.

P(PEGMA₄₇₅) (P1), p(DMAEMA) (P2), and p(DMAEMA-stat-PEGMA₄₇₅) (P3) were prepared via ATRP routes.¹⁴³ N-Methylation of p(DMAEMA) with different degrees of quaternisation was carried out to vary the total permanent cation content.¹⁹⁹,²⁰⁰ P(DMAPMAm) (P4), p(AMPS) (P5), p(2-AmAA) (P6) and p(4-AmBA) (P7) were synthesised using RAFT methods.²⁰² A cationic/zwitterionic copolymer of p(METMAC-co-MEDSAH) (P8) was prepared at a METMAC:MEDSAH molar ratio of 1:1. Polymers with different...
charge, molecular weight and backbone structure were prepared in good yields.

Three model proteins crystallisation systems were studied here: hen egg white lysozyme (HEWL); concanavalin A (Con A); and bovine liver catalase (BLC). A simple model linking polymer charge to protein pI and crystallisation rate was proposed. Crystallisation experiments were performed on 96-well plates, by using the sitting drop vapour diffusion technique.

The first study was carried out on HEWL crystallisation. Figure 7-1 presents selected optical photomicrographs of HEWL crystals grown utilising different polymers as additives in the crystallisation process. The pI of HEWL is 11.35, therefore when at its crystallisation pH 4.8, HEWL macromolecules are positively charged, i.e. cationic. HEWL crystallisation under conditions favouring the tetragonal form resulted in exclusively tetragonal crystals within 1-2 days without any polymer (Figure 7-1, Control). When in the presence of polymers, most of the HEWL crystals were readily identified by their distinctive morphology as tetragonal. However, different polymers have clearly influenced the results of the crystallisation experiments, especially the crystal morphology.
Figure 7-1. Selected optical photomicrographs of HEWL crystals formed under conditions of tetragonal crystal-inducing protocol, in the presence of neutral, cationic, anionic and cationic/zwitterionic polymers as solutes in the crystallisation solutions. Image labelled ‘Control’ represents crystals grown from sample wells without polymer. Neutral polymer: P1. Cationic polymers: P2-4. P2a: 0.02 % w/v p(DMAEMA), P2b: 2 % w/v p(DMAEMA), P2c: 12 % w/v p(DMAEMA). Anionic polymers: P5-7, P6 and P7 were weakly anionic at pH 4.8. Cationic/zwitterionic polymer: P8.

Neutral p(PEGMA\textsubscript{475}) (P1) nucleated a few large HEWL tetragonal crystals (Figure 7-1, P1). It was expected that p(PEGMA\textsubscript{475}), as a neutral methacrylate derivative of PEG and an H-bond acceptor, might competed with protein solutes for water and thus promote protein crystallisation via a dehydration
mechanism (Figure 7-2, A). Moreover, p(PEGMA$_{475}$) may have ‘volume-excluded effects’ by hydrophobic exclusion of protein solutes.$^{224}$ Neutral polymers of this type could also reduce the dielectric constant of the crystallisation solution, leading to an increase of the effective distance over which protein electrostatic effects would take place.$^{225}$ Consequently protein aggregation and phase separation was promoted.$^{108}$ In turn, HEWL macromolecules may have been more able to associate with each other.

Cationic polymers had an increased propensity for nucleating many small crystals (Figure 7-1, P2c, P3 and P4). In Figure 7-1, P2a-c show the images of HEWL crystals obtained using quaternised p(DMAEMA) at three different concentrations. The HEWL crystal form was tetragonal when crystallised from 0.02% (w/v) quaternised p(DMAEMA) (Figure 7-1, P2a). Upon increasing the concentration to above 2% (w/v), the HEWL crystal form was clearly different from a typical tetragonal character (Figure 7-1, P2b). Instead of tetragonal, two other forms of HEWL crystals, including needles and monoclinic crystals were obtained, when crystallised from otherwise identical solutions in the presence of 12% (w/v) quaternised p(DMAEMA) (Figure 7-1, P2c). Moreover, quaternised p(DMAEMA) mediated-HEWL crystallisation followed a general trend, with tetragonal crystals forming at lower concentrations of polymer and monoclinic crystals & needle forming at higher polymer concentrations. Addition of quaternised p(DMAEMA) into HEWL solution, which exhibited a high cationic charge density, was expected to alter the electrostatic interactions
between HEWL molecules and the solution.\textsuperscript{118, 100} Cationic polymer ((DMAEMA)-stat-PEGMA\textsubscript{475}) (P3) and p(DMAPMAm) (P4) nucleated many uniform and small tetragonal HEWL crystals with the sizes around 100 \(\mu m\) within one day (Figure 7-1, P3 and P4). There were likely to have been charge-charge repulsions between cationic polymers molecules and cationic HEWL macromolecules, lowering the interfacial energy between HEWL macromolecules and water, thus promoting protein-protein interactions at hydrophobic sites or anionic-cationic pair sites and subsequent crystallisation (Figure 7-2, B). In addition, cationic polymers might have bound to anionic residues on the HEWL chain leading to charge compensation by electrostatic interaction,\textsuperscript{108} an increase of local concentration of HEWL macromolecules and the promotion of nucleation. Mixed polymers solutions of p(DMAEMA) and p(PEGMA\textsubscript{475}) (molar ratio 1:9) were also applied for HEWL crystallisation to compare the effects with P3 and formed a few large tetragonal crystals with sizes of \(\sim 300 \mu m\), implying the influence of the neutral p(PEGMA\textsubscript{475}) section was dominant in the crystallisation process (Figure 7-1, P1 & P2 mix).

The influence of anionic polymers on HEWL crystallisation was less than that of the cationic polymers regarding crystal shape, but the anionic polymers did alter the size of HEWL crystals (Figure 7-1, P5-7). Anionic p(AMPS) nucleated a few large crystals with mean size around 300 \(\mu m\) (Figure 7-1, P5). However, smaller HEWL crystals were obtained when p(2-AmAA) and p(4-AmBA) were added (Figure 7-1, P6 and P7). This was likely a
consequence of the crystallisation pH of HEWL being close (pH 4.8) to the
expected pKa values of p(2-AmAA) and p(4-AmBA), and thus a smaller
number of anionic carboxyl groups remaining on these polymers compared to
the cationic polymers at the same pH. The HEWL crystals formed in the
presence of anionic polymers were identified as tetragonal by single crystal
X-ray diffraction. Anionic polymers might favor attracting cationic HEWL
macromolecules for charge compensation by electrostatic interaction (Figure
7-2, C), thus, they would either lower the protein and water interfacial
energy and act as nucleation centers by the absorption of HEWL
macromolecules, or slow down the rate of further growth. Therefore, they acted
as not only nucleators in solution but also as inhibitors.

Cationic and zwitterionic sulfobetaine p(METMAC-co-MEDSAH) nucleated a
few crystals with complex shape (Figure 7-1, P8). The combined
cationic/zwitterionic copolymer was expected to have a variety of functions
during HEWL crystallisation (Figure 7-2, D). One expected action was
complexation with HEWL macromolecules, leading to charge compensation
and promotion of nucleation. After nucleation, this polymer also might absorb
on certain faces of crystals and influence the direction of crystal further
growth. From a first principles hypothesis, the HEWL crystals would thus be
expected to be of different shape when crystallised with zwitterionic polymers
compared to the purely cationic; however, the crystal form was still tetragonal.
Figure 7-2. Schematic representation of the interaction of various polymers with cationic HEWL in the protein crystallisation process. (A) Neutral polymer: H-bonding with water and thus promoting protein crystallisation via a dehydration mechanism. (B) Cationic polymer: charge-charge repulsions between cationic polymer and cationic HEWL. (C) Anionic polymer: binding cationic HEWL for charge compensation. (D) Cationic/zwitterionic polymer: have a variety of functions during HEWL crystallisation. HEWL macromolecules are positively charged at tetragonal crystallisation pH of 4.8.
To investigate further the influence of these polymers on protein crystallisation, we selected representative polymers from each class and carried out crystallisation assays with a range of polymer concentrations. Figure 7-3 outlines a summary evaluation of HEWL crystal size distribution, which was obtained by measuring the average length (μm) of the largest diameter of each crystal at each polymer concentration using ImageJ software. There was a clear effect on HEWL crystallisation depending on the concentration of polymer. For HEWL (cationic at crystallisation pH), as the concentration of neutral polymer in solution was increased, the size of crystals increased (Figure 7-3, P1). However, the size of HEWL crystals decreased when the concentration of cationic polymer was increased (Figure 7-3, P2-4). A slight increase in average size of HEWL crystals formed was observed when anionic polymer concentration increased (Figure 7-3, P5-7). Perhaps surprisingly, the hybrid cationic/zwitterionic copolymer P8 caused a slight increase in crystal size, suggesting perhaps a greater role for the pendant sulfonate anions at the side-chain termini than the trimethylammonium headgroups on the co-monomer (Figure 7-3, P8). At lower concentrations, polymers may have served as nucleation centres via bridging interactions to promote nucleation, whereas when the concentration was increased, it is possible that charged polymers could stabilise or coat emergent small protein aggregates and inhibit further growth of crystals.\textsuperscript{100,234} and that multiple mechanisms can be involved. Indeed, the influence of polymers in protein crystallisation has been suggested
to include the stages before nucleation, as well as initial nucleation and subsequent crystal growth. Therefore, in addition to the chemical functionality of polymers, it is likely that the concentration of polymers can play significant roles in modifying the protein crystallisation process.

Figure 7-3. Summary of photomicrograph analysis showing changes in the size of HEWL crystals, grown in the presence of different classes of polymers at various concentrations. Neutral polymer: P1; cationic polymers: P2-4; anionic polymers: P5-7, P6 and P7 were weakly anionic at pH 4.8. Cationic/zwitterionic polymer: P8

The second model protein investigated was Con A. The pI of Con A is 4.5, but crystallisation of this protein is normally carried out at pH 8.5, at which point Con A molecules are negatively charged overall. Three polymers were then selected from each class for further experiments: neutral p(PEGMA475) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5). Con A
solutions produced small crystals with sizes of ~ 60 μm within 1 week without any polymer, which were orthorhombic (Figure 7-4, Control). When polymers were utilised, the sizes and numbers of crystals were found to vary with the types of polymers added to solution, however, the form of the crystals was still orthorhombic. Neutral polymer P1 resulted in a few large block-shaped crystals (Figure 7-4, P1), suggesting a similar dehydration mechanism to that which occurred in HEWL crystallisation. Cationic polymer P2 was expected to bind anionic Con A and nucleated a few large crystals (Figure 7-4, P2). By contrast the anionic polymer P5 was postulated to decrease the interfacial energy between Con A macromolecules and water by charge-charge repulsions between anionic polymer and anionic Con A, resulting in the promotion of protein aggregation and formation of many smaller crystals within four days (Figure 7-4, P5).

Figure 7-4. Optical images showing changes of Con A crystals, in the presence of different polymers: neutral p(PEGMA_{475}) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5). Image labelled ‘Control’ represents crystals grown from sample wells without polymer.

Moreover, for anionic Con A, as the concentration of neutral polymer in
solution was increased, the size of Con A crystals increased (Figure 7-5, P1). The effects of cationic and anionic polymers on Con A crystallisation were predicted from this simple model to be opposite from the effects on HEWL. The size of Con A crystals increased when the concentration of cationic polymer was increased (Figure 7-5 P2). However, the size of Con A crystals decreased when the concentration of anionic polymer was increased (Figure 7-5, P5). All these results together illustrate that polymers with varying net charges have the ability to control the size of protein crystals.

Figure 7-5. Summary of photomicrograph analysis showing changes in the size of Con A crystals, grown in the presence of different classes of polymers at various concentrations. Neutral polymer: P1; cationic polymer: P2; anionic polymer: P5.

The third model protein was BLC. The pI of BLC is 5.4 but at its crystallisation pH of 6.8 BLC is a weakly anionic protein. Similar to Con A, three polymers were also selected from each class for further BLC crystallisation experiments: neutral p(PEGMA) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5). BLC crystal formation in the
absence of added polymers occurred after two weeks and resulted in needle crystal outgrowth with a ‘wheatsheaf’ morphology (Figure 7-6, Control). It was hard to get single crystals without any polymer. When in the presence of neutral p(PEGMA$_{475}$) (P1), poor quality crystals were obtained and it was not possible to determine their structure by single crystal X-ray diffraction. Cationic quaternised p(DMAEMA) (P2) produced a few needles with a ‘sea-urchin’ morphology formed at lower concentrations, but suppression of BLC crystallisation occurred at higher concentration. By contrast, anionic polymers promoted BLC aggregation and produced single crystals in 10 days, which were identified as orthorhombic (space group: $P2_12_12_1$, form III) by X-ray diffraction. BLC crystals of form III reported previously were extremely thin (< 1 μm) and hard to characterise fully using single crystal X-ray diffraction.$^{231}$ It should be noted that BLC form III has rarely been studied; thus the use of polymers as solution additives might be useful in further BLC crystal characterisation.

![Figure 7-6. Optical images showing changes of BLC crystals, in the presence of different polymers: neutral p(PEGMA$_{475}$) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5). Image labelled ‘Control’ represents crystals grown from sample wells without polymer.](image)
Light scattering experiments were carried out to evaluate the HEWL and Con A aggregation process directly in the presence of crystallisation solutions and polymers with varying net charges. Protein crystallisation was initiated by mixing protein with polymer solutions, leading to immediate supersaturation and formation of protein aggregates. Based on the effects of each polymer on crystal size and morphology in prior experiments, we predicted (Figure 7-7, A) and subsequently evaluated (Figure 7-7, B-D) the effects of each polymer on the growth of cationic HEWL and anionic Con A crystals.

![Diagram](image)

Figure 7-7. Predicted growth of HEWL ((i)A) and Con A ((ii)A) particles during crystallisation; and actual variations in the size of HEWL ((i)B-D) and Con A ((ii)B-D) aggregates or crystals in the solution as a function of time during crystallisation experiments induced by different polymers by DLS. Black represents the application of neutral polymers; blue represents the application of cationic polymers; red represents the application of anionic polymers.
At the beginning of the experiment, protein macromolecules were free and non-interacting in the solution. After the addition of various polymers, a characteristic increase in protein aggregate size can be observed in all cases. As time increased, the rate of protein aggregation growth followed two distinct sequences. With the addition of neutral p(PEGMA\textsubscript{475}) (P1), cationic quaternised p(DMAEMA) (P2) and anionic p(AMPS) (P5) in crystallisation solutions, the size of HEWL aggregates increased up to around 600 nm, 120 nm and 400 nm respectively, while Con A aggregates increased up to around 10000 nm, 1000 nm and 800 nm. Therefore, for HEWL, particle size increased most rapidly for neutral polymer > anionic polymer > cationic polymer (Figure 7-7(i), B-D), whereas for the rate of the growth of Con A aggregation the sequence was: neutral polymer > cationic polymer > anionic polymer (Figure 7-7(ii), B-D). These DLS data indicated that as time increased, protein aggregates increased in size, presumably until the critical nuclei were reached and crystals began to grow. As mentioned above, neutral polymers competed with proteins for water and thus promoted protein crystallisation. Cationic polymers were expected to lower the interfacial energy between cationic HEWL macromolecules and water by charge-charge repulsions, thus promoting nucleation; while the same polymers were likely to attract anionic Con A macromolecules for charge compensation, thus slowing down the rate of crystal growth. The effects of anionic polymer on HEWL and Con A crystallisation were predicted from this simple model to be opposite from
cationic polymers, and to a first approximation this pattern was observed. We thus propose that DLS measurements can be used as a simple qualitative screen to predict the outcome for protein crystallisation experiments.

In conclusion, a series of polymers with varying degrees of charge, molecular weight and backbone structure have been successfully prepared and applied to the crystallisation of HEWL, Con A and BLC via sitting drop vapour diffusion techniques. These polymers acted as solution additives to mediate protein crystallisation through favourable interactions between polymer and protein or the surrounding solvent system; and were found to influence strongly the resultant crystal sizes, shapes and polymorphs. Experiments in which polymers and proteins exhibited different charges suggested possible mechanisms of how these polymers might affect protein crystallisation. Moreover, ‘first principles’ structure-function relationships linking the types of polymers used in these experiments and the protein crystal habits produced were generated. These relationships might be used as models to guide a wide range of protein crystallisation experiments in the future. Therefore, polymers as solution additives may perhaps be tuned to control the size, quality, shape and even polymorph of protein crystals for use in pharmaceutical, biotechnological or chemical applications.

7.2 Future work

In this study, we used the whole range of polymers with varying degrees of
charge, molecular weight and backbone structure as solution ‘additives’ for HEWL crystallisation experiments, however, due to the time limit, we selected three key polymers from each class for Con A and BLC crystallisation experiments. Therefore, the whole range of polymers should be applied in the future Con A and BLC crystallisation experiments.

Based on the effects of each polymer on crystal size and morphology in the experiments, we suggested some mechanisms of how the polymers affect protein crystallisation, and proposed possible models of ‘first principles’ structure-function relationships linking the types of polymers used in these experiments and the protein crystal habits produced. However, the study of these models is still on its early stage. Because there are so many factors which can affect crystallisation, many of which are still poorly understood, these mechanisms are still somewhat tenuous and need to be further investigated. Obviously, this is the key issue to solve in the future.

A series of polymers were prepared and successfully applied as ‘additives’ to influence protein crystal size, outer crystal shape, even crystal packing motifs. However, future work should involve the investigation of more polymer additives with various possible functionalities, including soluble polymer additives and insoluble polymer surfaces, such as molecularly imprinted polymers (MIPs). The results will aid in better understanding of the mechanisms we proposed.

Moreover, the structure-function relationships linking the types of polymers
and the protein crystal habits produced might be used as models to guide a wide range of protein crystallisation experiments in the future. Therefore, polymers as solution additives may perhaps be tuned to control the size, quality, shape and even polymorph of protein crystals for use in pharmaceutical, biotechnological or chemical applications.
APPENDIX

Supporting spectroscopy graphs

Monomer PEGMA_{475}

Proton NMR

Carbon NMR
Monomer DMAEMA

Carbon NMR

Polymer p(DMAEMA-stat-PEGMA$_{475}$)

Proton NMR
Monomer DMAPMAm

Carbon NMR

Polymer p(DMAPMAm)

GPC
APPENDIX – Supporting spectroscopy graphs

Monomer AMPS

Carbon NMR

![Carbon NMR spectrum with peaks labeled a, b, c, d, e, f.]

Polymer p(AMPS)

GPC

![GPC chromatogram with molecular weight distribution.]

333
Polymer p(2-AmAA)

GPC

Polymer p(4-AmBA)

GPC
DLS analysis - HEWL crystal growth: control without polymer

**Combined correlation function**

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**Intensity distribution**

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DLS analysis - HEWL+ p(PEGMA$_{475}$)

Combined correlation function

Intensity distribution
DLS analysis - HEWL+ quaternised p(DMAEMA)

Combined correlation function

Intensity distribution

DLS analysis - HEWL+ mixed polymers of p(PEGMA$_{475}$) & p(DMAEMA)

Combined correlation function

Intensity distribution
DLS analysis - HEWL + p(DMAPMAm)

Combined correlation function

Intensity distribution

DLS analysis - HEWL + p(AMPS)

Combined correlation function

Intensity distribution
DLS analysis - HEWL + p(2-AmAA)

Combined correlation function

Intensity distribution

DLS analysis - HEWL + p(4-AmBA)

Combined correlation function

Intensity distribution
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