
Structural modification of proteins and peptides for the creation of nanomaterials

Megan Garvey

Under the joint supervision of:

Professor John Carver
Executive Deputy Dean
School of Chemistry & Physics
The University of Adelaide
Adelaide, South Australia 5005, AUSTRALIA

and

Professor Juliet A. Gerrard
School of Biological Sciences
The University of Canterbury
Christchurch, NEW ZEALAND

*A dissertation submitted in January 2009 to The University of
Adelaide, in collaboration with The University of Canterbury,
for the degree of Doctor of Philosophy.*



Table of Contents

Table of Contents	i
Table of Figures	v
Table of Tables	vii
Abbreviations	viii
Synopsis	ix
Declaration	xi
Acknowledgements	xii

Chapter 1 Introduction and literature review

<i>1.1 Protein folding</i>	2
<i>1.2 Protein aggregation</i>	3
1.2.1 Amorphous protein aggregation.....	4
1.2.1.1 Amorphous aggregation related disease processes	5
1.2.2 Ordered protein aggregation (amyloid fibril formation).....	6
1.2.2.1 Disease relationship	7
1.2.2.1.1 <i>Dialysis-related amyloidosis</i>	10
1.2.2.2 Amyloid and amyloid-like fibril structure	10
1.2.2.3 Amyloid and amyloid-like fibril formation	13
1.2.2.4 Nanobiotechnological potential	13
<i>1.3 Crystallin proteins</i>	16
1.3.1 The eye lens.....	17
1.3.2 Nomenclature of lens crystallin proteins.....	18
1.3.3 Characteristics of lens crystallin proteins	18
1.3.3.1 α -Crystallin	19
1.3.3.1.1 <i>Structure of α-crystallin</i>	19
1.3.3.1.2 <i>Function of α-crystallin</i>	22
1.3.3.2 β/γ -Crystallin family	23
<i>1.4 Conclusion</i>	25

Chapter 2 Experimental procedures

<i>2.1 Materials</i>	27
<i>2.2 Basic protein purification techniques</i>	27
2.2.1 <i>Ex vivo</i> mammalian protein sources.....	27
2.2.1.1 Mammalian crystallin protein extraction.....	27
2.2.1.2 Mammalian crystallin separation.....	28
2.2.2 Recombinant protein sources.....	29
2.2.2.1 Human wild type α B-crystallin expression	29
2.2.2.2 Human wild type α B-crystallin purification	30
2.2.3 Reduction and carboxymethylation of κ -casein.....	31
2.2.4 SDS-PAGE Protocol.....	31
<i>2.3 Protein aggregation monitoring</i>	32
2.3.1 Amorphous protein aggregation.....	32
2.3.1.1 Induction of amorphous aggregation.....	32
2.3.1.1.1 <i>Light scatter assessment of thermally induced amorphous aggregation</i>	32

2.3.1.1.2	<i>Light scatter assessment of disulfide-bond reduction induced amorphous aggregation</i>	33
2.3.1.1.3	<i>Refolded aggregate formation for use as chaperones</i>	33
2.3.1.1.4	<i>Amorphous aggregate formation for use as chaperones</i>	33
2.3.2	Fibrillar protein aggregation	34
2.3.2.1	Aggregation induction	34
2.3.2.1.1	<i>Nanofibre formation by RsAFP-19</i>	34
2.3.2.1.2	<i>Nanofibre formation by R3A β2-microglobulin</i>	34
2.3.2.1.3	<i>Nanofibre formation by seeded β2-microglobulin</i>	34
2.3.2.1.4	<i>Nanofibre formation by pure crystallin solutions</i>	34
2.3.2.1.5	<i>Nanofibre formation by semi-pure crystallin mixtures</i>	35
2.3.2.1.6	<i>Nanofibre formation by crude crystallin mixtures</i>	35
2.3.2.1.7	<i>Nanofibre formation by α-crystallin using GdnHCl</i>	35
2.3.2.1.8	<i>Nanofibre formation by RCM κ-casein</i>	35
2.3.2.2	Assessment of amyloid characteristics of nanofibre formation	35
2.3.2.2.1	<i>Thioflavin t</i>	35
2.3.2.2.2	<i>Transmission electron microscopy</i>	37
2.3.2.2.3	<i>Atomic force microscopy</i>	37
2.3.2.2.4	<i>Protease treatment</i>	37
2.3.2.2.5	<i>X-ray fibre diffraction</i>	38
2.3.3	Chaperone activity quantification	38
2.3.4	<i>Removal of GdnHCl from α-crystallin samples for use as molecular chaperones</i>	39
2.4	Protein immobilisation techniques	39
2.4.1	Protein immobilisation	39
2.4.1.1	<i>Plasma generated aldehyde polymerisation onto solid supports</i>	39
2.4.1.2	<i>Covalent immobilisation of proteins to aldehyde polymer coated surfaces</i>	40
2.4.2	Plasma polymer and protein immobilisation assessment	40
2.4.2.1	<i>X-ray photoelectron spectroscopy</i>	40
2.4.2.2	<i>Liquid phase atomic force microscopy</i>	40
2.4.2.3	<i>Quartz Crystal Microgravimetry</i>	41

Chapter 3 Synthetic peptides for nanofibre formation

3.1	<i>Nanofibre forming peptide systems</i>	44
3.2	<i>Plant defensin proteins and peptides</i>	45
3.3	<i>Assessment of peptide nanofibre formation</i>	46
3.4	<i>Nanofibre formation by Raphanus sativus Antifungal Peptide (RsAFP-19)</i>	48
3.4.1	<i>RsAFP-19 Structure</i>	48
3.4.2	<i>RsAFP-19 Amyloidogenicity</i>	48
3.4.3	Characterisation of nanofibre formation by RsAFP-19	51
3.4.3.1	<i>Thermal destabilisation induced nanofibre formation of RsAFP-19</i> ..	51
3.4.3.2	<i>Freeze-thaw induced nanofibre formation of RsAFP-19</i>	55
3.4.3.3	<i>X-ray fibre diffraction analysis of RsAFP-19 nanofibres</i>	56
3.4.3.4	<i>Examination of RsAFP-19 nanofibres</i>	58
3.5	<i>Nicotinia glauca Defensin 1 modified peptide (NaD1-19)</i>	59
3.5.1	<i>NaD1-19 Structure</i>	59
3.5.2	<i>NaD1-19 Amyloidogenicity</i>	61
3.6	<i>Discussion of nanofibre formation by RsAFP-19 and NaD1-19</i>	62

Chapter 4 Nanofibre formation from disease-associated mutant protein variants

4.1	<i>Structure and function of β2-microglobulin</i>	66
4.1.1	Structure of mutant R3A β 2-microglobulin.....	68
4.2	<i>Amyloidogenicity of β2-microglobulin and R3A β2-microglobulin</i>	68
4.3	<i>R3A β2-microglobulin nanofibre formation</i>	69
4.3.1	β 2-microglobulin nanofibre formation.....	74
4.4	<i>Discussion of nanofibre formation by R3A β2-microglobulin and seeded wild-type β2-microglobulin</i>	76

Chapter 5 Nanofibres of defined morphology prepared by structural modification of crude crystallin mixtures

5.1	<i>Nanofibres as bionanomaterials</i>	81
5.1.1	Sourcing protein nanofibres	83
5.2	<i>Nanofibre formation from crude crystallin mixtures</i>	85
5.2.1	Extraction of crystallin proteins	85
5.2.2	Separation and purification of crystallin proteins	85
5.2.3	Formation of protein nanofibres from pure crystallin proteins	90
5.2.4	Formation of protein nanofibres from semi-pure crystallin mixtures	91
5.2.5	Formation of protein nanofibres from crude crystallin mixtures	95
5.2.5.1	Protein nanofibres with clumping morphology formed from crude crystallin mixtures.....	95
5.2.5.2	Protein nanofibres with individual fibril morphology formed from crude crystallin mixtures.....	96
5.2.5.3	Protein nanofibres with curly and loop morphology from crude mixtures of bovine crystallins	101
5.2.5.4	Thioflavin t assessment of protein nanofibres formed from crude bovine mixtures	102
5.2.5.5	Protease resistance of protein nanofibres formed from crude mixtures of bovine crystallins	103
5.2.5.6	X-ray fibre diffraction of protein nanofibres from crude mixtures of bovine crystallin	104
5.3	<i>Discussion of protein nanofibre formation by crystallin proteins</i>	105

Chapter 6 Retention of biological chaperone activity of α -crystallin after modification into an amyloid fibrillar structure

6.1	<i>Structure and function of α-crystallin</i>	110
6.2	<i>αT-Crystallin amyloid fibril chaperone assessment</i>	113
6.2.1	α T-Crystallin amyloid fibril formation.....	113
6.2.2	Chaperone activity assessment of native and fibrillar α T-crystallin against amorphous aggregation	113
6.2.3	Amorphous aggregate and β H-crystallin amyloid fibril controlled assays of α T-crystallin fibril chaperones	118
6.2.4	Chaperone activity of α B-crystallin fibrils and other species	127
6.3	<i>Discussion of chaperone activity by α-crystallin aggregate species</i>	130

Chapter 7	Structural modification of αB-crystallin by immobilisation onto a solid carrier leads to enhanced chaperone activity	
7.1	<i>Structure of αB-crystallin</i>	138
7.2	<i>Protein immobilisation</i>	140
7.2.1	Aldehyde polymerisation onto solid carriers.....	140
7.2.2	Covalent immobilisation of proteins to aldehyde polymer layers on solid carriers	141
7.2.2.1	Assessment of protein immobilisation by X-ray Photoelectron Spectroscopy	142
7.2.2.2	Atomic Force Microscopic analysis of protein immobilisation	144
7.2.2.3	Quartz crystal microbalance assessment of protein immobilisation..	145
7.3	<i>Chaperone activity of immobilised αB-crystallin</i>	146
7.3.1	Reduction-induced amorphous aggregation.....	147
7.3.2	Thermally induced amorphous aggregation.....	149
7.3.3	Amyloid fibrillar aggregation of κ -casein.....	151
7.3.4	Mutation induced fibrillar aggregation of β 2-microglobulin.....	151
7.4	<i>Discussion of immobilisation of αB-crystallin and its effect on chaperone activity of this protein</i>	156
Chapter 8	Conclusions and future work	161
Chapter 9	References	166

Table of Figures

1.1	Energy landscape of protein folding.....	3
1.2	Diagram of the protein folding and off-pathway folding.....	4
1.3	Cataract grades for selenium induced murine crystallin cataract.....	6
1.4	Diagram of the packing of an SH3 domain amyloid fibril.....	12
1.5	Stages of amyloid fibril formation.....	14
1.6	Morphological variation of amyloid and amyloid-like fibril protein nanomaterials.....	15
1.7	Structure of the eye lens.....	17
1.8	Primary sequence and putative features of bovine α -crystallins.....	20
1.9	Structural similarity of β - and γ -crystallin monomeric units.....	23
3.1	Structure of RsAFP proteins and peptide.....	48
3.2	TANGO β -aggregation scores for RsAFP proteins and peptides.....	50
3.3	ThT fluorescence of RsAFP-19 incubated at 60 °C.....	51
3.4	TEM of RsAFP-19 nanofibres formed at 60 °C (0 – 8 hours).....	53
3.5	TEM of RsAFP-19 nanofibres formed at 60 °C (12 – 20 hours).....	54
3.6	TEM and AFM of RsAFP-19 nanofibres formed by freeze-thaw.....	55
3.7	TEM and AFM of RsAFP-19 nanofibres formed at high temperature and concentration.....	56
3.8	XRD of RsAFP-19 nanofibres formed at high temperature and concentration.....	57
3.9	TEM and AFM of RsAFP-19 nanofibres showing protofilament twisting.....	58
3.10	Sequence similarity of RsAFP and NaD1 proteins and peptides.....	59
3.11	ThT fluorescence of NaD1-19 incubated at 60 °C.....	61
4.1	Crystal structures of human β 2-microglobulin.....	66
4.2	ThT fluorescence of R3A β 2-microglobulin nanofibre formation.....	69
4.3	Light scattering of R3A β 2-microglobulin nanofibre formation.....	70
4.4	TEM of R3A β 2-microglobulin nanofibre fibril formation.....	71
4.5	TEM of R3A β 2-microglobulin nanofibre mesh and seed fibrils.....	73
4.6	ThT fluorescence of seeded β 2-microglobulin nanofibre formation.....	74
4.7	Light scattering of seeded β 2-microglobulin nanofibre formation.....	75
4.8	TEM of seeded β 2-microglobulin nanofibre formation.....	76
5.1	Concentration of crystallin protein homogenate.....	85
5.2	Separation of bovine lens crystallin proteins.....	87
5.3	Separation of ovine lens crystallin proteins.....	88
5.4	Separation of cervine lens crystallin proteins.....	89
5.5	TEM of nanofibres formed from pure crystallin protein stocks.....	91
5.6	SDS-PAGE gel of semi-pure crystallin mixtures.....	92
5.7	TEM of nanofibres formed from semi-pure crystallin mixtures.....	93
5.8	TEM of nanofibres with clumping morphology formed from crude crystallin mixtures.....	96
5.9	TEM of nanofibres with individual straight or curled morphology formed from crude bovine crystallin mixtures.....	98
5.10	TEM of nanofibres with individual straight or curled morphology formed from ovine, cervine and combined crude crystallin mixtures.....	99

5.11	SDS-PAGE of heat treated crystallin proteins.....	100
5.12	TEM of nanofibres with loop morphology formed from treated bovine crude crystallin mixtures.....	101
5.13	ThT fluorescence of native and nanofibre solutions of crude bovine crystallin mixtures.....	102
5.14	TEM of protease treated nanofibre formed from crude bovine crystallin mixtures.....	103
5.15	XRD of native and nanofibre solutions of crude bovine crystallin mixtures.....	104
6.1	TEM of α T-crystallin amyloid fibrils.....	113
6.2	Native and fibrillar α T-crystallin chaperone protection of catalase amorphous aggregation.....	115
6.3	Native and fibrillar α T-crystallin chaperone protection of insulin amorphous aggregation.....	116
6.4	TEM of α T-crystallin species formed to assess the effects of structural variation on chaperone activity.....	119
6.5	TEM of α T-crystallin and non-chaperone protein species formed to assess the effects of structural variation on chaperone activity.....	120
6.6	Chaperone protection provided by various native, fibrillar and amorphous species against amorphous aggregation of catalase and insulin.....	121
6.7	Native and fibrillar α T-crystallin chaperone protection of RCM κ -casein amyloid fibril formation.....	124
6.8	Chaperone protection provided by various native, fibrillar and amorphous species against amyloid fibril formation of RCM κ -casein.....	125
6.9	TEM of α B-crystallin species formed to assess the effects of structural variation on chaperone activity.....	128
6.10	Chaperone protection provided by various native, fibrillar and amorphous species against amorphous aggregation of insulin and amyloid fibril formation of RCM κ -casein.....	130
6.11	Amyloid aggregation profiles for α A- and α B-crystallin proteins.....	134
7.1	Primary sequence and putative structural features of α B-crystallin.....	138
7.2	AFM of aldehyde polymer layer on silicon wafer.....	141
7.3	XPS chromatograph of atomic composition of aldehyde polymer layer with and without immobilised α B-crystallin.....	142
7.4	AFM of aldehyde polymer layer with immobilised α B-crystallin on silicon wafer.....	144
7.5	QCM of immobilisation of α B-crystallin onto aldehyde polymer coated quartz crystals.....	146
7.6	Chaperone protection provided by α B-crystallin, immobilised and in solution, against insulin amorphous aggregation.....	148
7.7	Chaperone protection provided by α B-crystallin, immobilised and in solution, against catalase amorphous aggregation.....	150
7.8	Chaperone protection provided by α B-crystallin, immobilised and in solution, against RCM κ -casein amyloid fibril formation.....	152
7.9	Chaperone protection provided by α B-crystallin, immobilised and in solution, against R3A β 2-microglobulin amyloid-like fibril formation..	154
7.10	TEM of R3A β 2-microglobulin amyloid-like fibrils formed in the presence of immobilised proteins.....	155

Table of Tables

1.1	Recognised forms of amyloidin.....	7
1.2	Amyloid diseases and associated proteins and peptides.....	8
1.3	Human crystallin proteins and their characteristics.....	18
5.1	Nanofibre formation conditions trialled with crude bovine lens mixtures.....	97
5.2	Summary of the components of each crystallin protein solution used to form nanofibres	106
6.1	α T-crystallin species formed for chaperone assessment of the effects of structural variation.....	118
6.2	α B-crystallin species formed for chaperone assessment of the effects of structural variation.....	127
6.3	Chaperone activity of α T- and α B-crystallin species.....	132
7.1	XPS derived atomic concentrations for aldehyde and α B-crystallin immobilised FEP.....	142
7.2	Summary of chaperone activity of α B-crystallin, immobilised and in solution.....	156

Abbreviations

AFM	Atomic force microscopy
Cryo-EM	Cryo-electron microscopy
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FTIR	Fourier transform infrared spectroscopy
GdnHCl	Guanidine hydrochloride
HMW	High molecular weight
HOPG	Highly ordered pyrolytic graphite
NaD	<i>Nicotinia alata</i> defensin
NMR	Nuclear magnetic resonance
QCM-D	Quartz crystal microgravimetry with dissipation
RsAFP	<i>Raphanus sativus</i> antifungal protein/peptide
RCM	Reduced and carboxymethylated
sHSP	Small heat shock protein
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ThT	Thioflavin T
TEM	Transmission electron microscopy
TFE	Trifluoroethanol
XPS	X-ray photoelectron spectroscopy

Synopsis

Amyloid fibrils are highly ordered β -sheet structures that are formed from a variety of proteins *in vivo*, where they may have biological roles or, more commonly, are found associated with a broad range of diseases (known as amyloidoses). Due to the role of amyloid fibrils in disease and their potential as bionanomaterials, formation of amyloid and amyloid-like fibrils *in vitro* are exciting areas of research activity. This thesis explores the formation and characterisation of nanofibre structures, including amyloid and amyloid-like fibrils, from diverse peptides and proteins.

The growing interest in protein nanofibres in the bionanotechnology industry has led to research into new nanofibre forming target peptides and proteins. In this thesis, *Raphanus sativus* antifungal peptide 19mer (RsAFP-19) and a mutant of β 2-microglobulin where the 3rd arginine was replaced with an alanine (R3A β 2-microglobulin), were examined for their amyloigenicity and nanofibre-forming propensity. Generation of nanofibre structures was successful for both, and the characteristics of formation, fibril structure and morphology were explored *via* thioflavin t binding (ThT), transmission electron microscopy (TEM), atomic force microscopy (AFM) and X-ray fibre diffraction. Nanofibre formation was also further characterised for mammalian lens crystallin proteins. Their ability to form protein nanofibres from semi-pure and crude protein mixtures was established, and the resulting fibrils characterised, including for their amyloid-like characteristics. This work demonstrates the ability of crystallin proteins to form inexpensive base materials for use in the bionanotechnology industry.

Inhibition of amyloid fibril formation is an area of current research activity for therapeutic purposes (against amyloidoses). α -Crystallin is a well-known molecular chaperone which traps intermediately structured target proteins, preventing them from both amorphous and ordered (amyloid fibril) aggregation. Like other crystallin proteins, α -crystallin can itself form amyloid fibrils under mildly denaturing conditions. The chaperone activity of α T- and α B-crystallin, in native, amorphously aggregated and fibrillar forms were assessed. Amyloid fibrils

and amorphous aggregates derived from α T and α B-crystallin acted as chaperones, although with modified activity to their native (non-fibrillar) structures. α B-Crystallin fibrils displayed enhanced chaperone activity, compared to native α B-crystallin. The chaperone activity of α B-crystallin was also assessed after its immobilisation onto solid surfaces. Protein immobilisation of the chaperone α B-crystallin was achieved using plasma generated aldehyde polymerisation and Schiff-based covalent bonding of the proteins. Immobilisation was characterised using X-ray photoelectron spectroscopy, AFM and quartz crystal micrography. Immobilised α B-crystallin was shown to act 100-fold to 5000-fold more effectively as a chaperone than native solution α B-crystallin (dependent upon target protein and the type of stress it was exposed to). This research has established that α T- and α B-crystallin can retain chaperone activity, or may even show enhanced activity, under conditions of extreme structural perturbation.

This thesis explores both the induction and inhibition of amyloid fibril, amyloid-like fibril and nanofibre formation, using a range of peptides and proteins, both pure and in crude mixtures. These protein nanofibre structures were characterised, to establish potential future use in bionanotechnology, kinetics and activity of amyloid and amyloid-like fibril formation and assessment of amyloid fibril inhibitors. This work further delineated aspects of α T- and α B-crystallin chaperone ability, highlighting these proteins' ability to act as effective chaperones under a broad range of conditions.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to current copyright laws (Copyright Act, 1968).

Megan Garvey

Adelaide
2009

Acknowledgements

Over the course of this PhD I have had help, advice, assistance and encouragement from many wonderful people. I would primarily like to thank my two supervisors, John Carver and Juliet Gerrard, who have been fantastic in their support, guidance and leadership. John and Juliet provided interesting and successful projects, insight when I was lost, encouragement to follow my own ideas, understanding when things went wrong and good wine with loud laughter at my parties. They have my heartfelt gratitude.

I would also like to thank numerous people who I worked under or with, including: Hans Griesser, Stefani Griesser and Benjamin Thierry, who's assistance and guidance made the immobilised chaperone project very successful and equally fun; Sally Gras who introduced me to XRD and was a joy to work with; Heath Ecroyd and Sarah Meehan, who both taught me so much, especially the glories of α -crystallin and the beauty of amyloid; Grant Pearce and Suzie Meade who provided knowledge and insight into amyloid fibrils and great company for lunch; Matt Nussio, Joe Shapter, Paula Brooksby, Leanne Britcher, Lyn Waterhouse, Daniel Bilusich, Phil Clements and Manfred Ingerfeld, who all provided much needed expertise in a range of highly skilled fields.

I have had the privilege of working as part of two great research groups over the past few years. I'd like to thank everyone who was part of Lab 7 or The Purple Bubble in that time. In particular (other than those already mentioned): Chris Brockwell who kept things real; David Thorn who is my angel; Scott Buckley for all the walks home; Tom Kouldelka for mass spec and tea breaks; Danielle Williams for making me smile so often; Yanqin Liu for so many lovely meals; Francis Dehle and Sean Hudson for being such good fun in very different ways; Shiva Rao for our fibril work; Sean Devenish and Ren Dobson for providing entertainment and advice; and Jackie Healy for looking after me in the lab.

Much of the last few years have been spent thinking and talking about work even when I shouldn't. For their understanding in such matters I'd like to thank all my friends, particularly: Christabel Hough who inspired me; Keina Tiaon for those Melbourne visits; Owen Powell who is always there for me; Thivanka Dedigama, Cat Hyndman, Gajan Tyagarajah and Kim Hynes for drinks and tasty brunches; Mike Maclean and the CMC for scotch and understanding; Kate Langdon for Grace-ful moments; Bruce May for everything except the stalking; Jess 'Sugar' Young for many fanciful tales; Simone Haigh and her ambulances; Steven Poon for all those small joys; Jens Kroeske for all the music and sunny days; Joel Rathus for ukeles and debates; Amy O'Loughlin for the laughter and the puppies; Justin Williams for tea-chats and understanding; Yallana Burgess who makes life shine; Simon Grealy for some champagne moments; and Markus Pietsch with whom each day is brighter and happier than the last.

Finally, I could never have done anything like this if not for my family: my brothers and their wives (Adam, Amor, Nathan and Tara) who are all wonderful and inspirational people; but especially my parents, Ian and Janet Garvey – their love, friendship, advice and support has been strong and constant, through this PhD and for all of my life. I am grateful in so many ways to them all and proud to be part of their family and able to share their lives.