The functional studies of amyloid fibrils and their toxicity

By

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ABSTRACT

Amyloid fibrils are a form of highly ordered, β-sheet protein structure found in many sites in the body. Fibril formation occurs when intermediates along the protein-folding pathway irreversibly enter the off-folding pathway to form highly ordered amyloid fibrils. Amyloid fibril formation is of considerable research interest because of its intimate association with a wide range of debilitating diseases, including Alzheimer's, Huntington's and Parkinson's diseases and type II diabetes. Recently, it has been found that amyloid fibrils enhance human immunodeficiency virus (HIV-1) infection. Semen contains a fibril forming component that significantly increases the ability of HIV-1 to infect cells. This component is associated with peptide fragments of prostatic acid phosphatase and has been termed semen-derived enhancer of viral infection (SEVI). SEVI acts at the virus entry stage and only boots infectivity when the peptide has folded into highly structured arrays of amyloid fibrils. The work presented in this thesis describes the broader roles of SEVI in HIV-1 infection including its toxicity to neuronal and epithelial cells as well as the toxicity of α_{s2}-casein (milk fibril forming protein).

Firstly, the amyloidogenic regions of SEVI are identified by the use of computer algorithms. Accordingly, these regions were synthesised to examine their individual fibril-forming propensity. Fragments from the central regions formed fibrils of similar morphology to SEVI at physiological pH and temperature. Fibril formation was assessed via thioflavin T assay, circular dichroism spectrometry and transmission electron microscopy (TEM). In this study the toxicity of SEVI and its amyloidogenic fragments to neuronal and epithelial cells was investigated. SEVI and its fragments were toxic to neuronal cells but not to confluent epithelial cells.

Secondly, the coreceptors used by SEVI and its amyloidogenic fragments are identified. HIV enters the cell by the interaction of glycoprotein (gp) 120 envelope with the cellular

differentiation (CD) 4 protein and secondary coreceptors. Affinofile assays showed that SEVI and its fragments use CCR5 and CXCR4 secondary coreceptors to enhance HIV-1 entry to the host cells. Additionally the ability of clusterin to inhibit fibril formation by SEVI was investigated. Clusterin inhibited fibril formation by SEVI in a concentration dependent manner thereby inhibiting the cytotoxicity associated with the fibrils.

Lastly, work detailing the toxicity of fibrils formed by milk protein α_{s2}-casein is presented. α_{s2}-Casein forms fibrils spontaneously under physiological conditions. These fibrils have been found in *corpora amylacea*, an amyloid condition that infrequently develops within the mammary tissue of cows. The use of cell toxicity assays show that fibrils formed by α_{s2}-casein were toxic to pheochromocytoma (PC) 12 cells. Furthermore, the use of Thioflavin T assay and TEM showed that a polyphenol epigallocatechin-3-gallate (EGCG), a component found in green tea extracts, inhibits fibril formation by α_{s2}-casein. Previous research has found that, EGCG can reduce fibril formation and cellular toxicity of various fibril-forming proteins. EGCG has also been shown to inhibit SEVI enhancement of HIV infection in a manner dependent on the ability of EGCG to disrupt SEVI fibril formation, providing proof of principle for the potential of anti-fibril agents as inhibitors of HIV infection.

DECLARATION

I certify that this work contains no material that 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. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abigail Kegomoditswe Elias

31st January 2015

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To my late Papa and Mama, I am proud and forever thankful for what you have done for me. Thank you for your sacrifices so that I can have better education. I believe I have made you proud. I will always love you and may souls rest in peace. Thanks also to by two sisters Mosarwana and Kerapetse and my brother Outlwile for unconditional love and encouragement.

Finally, thanks be to God who gives victory through our Lord Jesus Christ. This work has been possible because of your grace, mercies and blessings.

Abbreviations

α_{s2}-CN	alpha$_{S2}$-casein
κ-CN	kappa-casein
AIDS	acquired immunodeficiency syndrome
CACO-2	epithelial colon carcinoma cells
CA	*corpora amylacea*
CA	caspid
CCR5	C chemokine receptor 5 (R5)
CXCR4	CXC chemokine receptor 4 (X4)
CD4	cluster of differentiation
CD	circular dichroism
CLI	complement-lysis inhibitor
CMA	chaperone-mediated autophagy
CMV	cytomegalovirus
CSF	cerebrospinal fluid
DNA	deoxy ribonucleic acid
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide

EGCG	(-)-epigallocatechin-3-gallate
Env	envelope
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated protein degradation
FPLC	fast protein liquid chromatography
gp	glycoprotein
HIV	human immune virus
HPLC	high performance liquid chromatography
HSPs	heat-shock proteins
LAMP2A	lysosome-associated membrane protein type-2A
MA	matrix
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	nucleocaspid
PAP	prostatic acid phosphatase
PC12	pheochromocytoma-12
PCD	protein conformational disorders
PCP	*Pneumocystis carinii pneumonia*
PR	protease
QTOF2	quadruple time of flight

RCM_K-CN reduced and carboxymethylated kappa-casein

RNA ribonucleic acid

RT reverse transcriptase

SAP serum amyloid P component

SD standard deviation

SEVI semen-derived enhancer of viral infection

TEM transmission electron microscopy

ThT thioflavin T

UV ultraviolet

1. INTRODUCTION

1.1 Background

The number of different proteins in the body is around 100 000 (Dobson, 1999). Proteins control or promote almost every event up on which our lives depend. Biological systems have procedures to ensure that proteins fold correctly or if they do not, that they are detected and degraded before any serious harm can ensue to the host organism (Plemper and Wolf, 1999). Protein networks consisting of cytoplasmic and endoplasmic reticulum (ER)-resident chaperones ensure proper folding of biologically active native proteins in a crowded cellular environment that can reach up to 300-400 grams per litre of proteins (Hartl et al., 2011).

Quality control mechanisms recognize misfolded proteins and mediate their degradation by the proteasome, lysosome and macroautophagy pathways (Hetz and Mollereau, 2014). Efficient protein folding and prevention of abnormal aggregation relies on the proteostasis network, which provides a dynamic interconnection between cytoplasmic and endoplasmic reticulum quality control and folding mechanisms (Hetz and Mollereau, 2014). When the protein folding capacity is saturated (such as with mutant proteins associated with neurodegenerative diseases), the proteasome and autophagy pathway act as a second barrier to degrade unfolded proteins and restore proteostasis (Hetz et al., 2013). Despite these controls, many human diseases are associated with incorrect protein folding (misfolding) that results in malfunctioning of cells. Misfolded proteins can accumulate within the ER thereby causing direct mutations in disease-related genes or perturbations in the function of the secretory pathway at different levels. The load of misfolded protein in the ER is reduced by ER-associated protein degradation (ERAD), a process in which these proteins are targeted to the cytoplasm for proteasome-mediated degradation (Hetz and Mollereau, 2014).

Misfolded proteins tend to form toxic oligomers and larger aggregates that can be eliminated by autophagy if there is an excessive reactive oxygen species in the cytoplasm, or if the proteasome is defective or saturated. An overload of misfolded proteins in the cytoplasm can also saturate the proteasome and induce compensatory autophagy, inhibit ERAD function and promote ER stress (Hetz and Mollereau, 2014, Hetz et al., 2013). Most attention has been on a group of diseases where proteasome activity and autophagy finally decline or are inhibited, which contributes to overload of insoluble species comprised of aggregates of misfolded proteins (Fig. 1.1), generating chronic ER stress and cell demise.

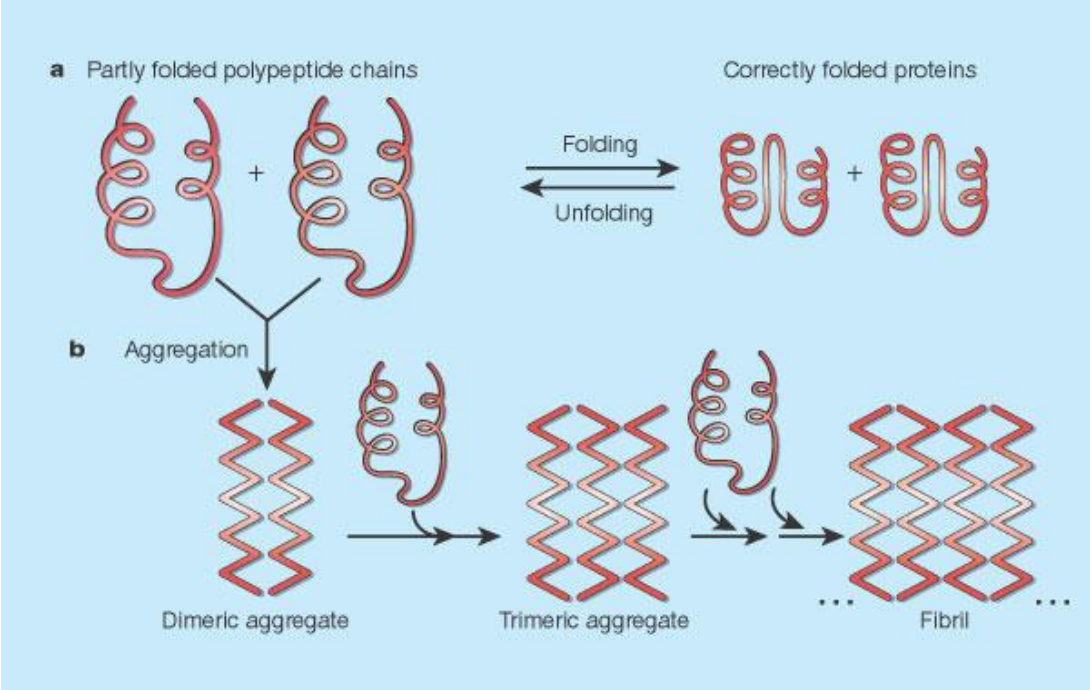

Figure 1.1: Schematic diagram of protein folding and misfolding. a, Partly folded polypeptide chains, upon release from ribosome (the protein-synthesizing machine), normally rapidly collapse into correctly folded, functional proteins. b, However, partly folded polypeptides may sometimes associate with similar chains to form aggregates. Partial unfolding of correctly folded proteins may also lead to aggregation. Aggregates vary in size from soluble dimers and trimers up to insoluble fibrillar and amorphous structures. Figure from Ellis and Pinheiro (2002).

1.2 Protein folding

The major functions of proteins in the cells include signalling, transport, catalysis, membrane fusion, cell regulation, and protection (Amm et al., 2014). After synthesis from the ribosome proteins must fold to their active native state (Bai et al., 1995). The folding process is complex and biological health depends on its success and disease on its failure. The complex and cooperative transition between the unfolded and the folded conformations of a protein have made it difficult to determine experimentally how it occurs. The complexity arises from different conformations a polypeptide chain can adopt while the cooperativity arises from the relative instability of all partially-folded conformations (Adesnik and Levinthal, 1969, Anfinsen, 1973), but it is only by elucidating the conformations and the kinetic roles of the intermediate conformations that the folding pathway will be elucidated. Amino acid sequence has all the information required for an unfolded protein to refold to its native conformation under appropriate conditions (Sali et al., 1994). Proteins are observed to refold on the second to minute time scale, depending on the protein and the conditions (Creighton, 1988).

In the early stages of folding, the formation of secondary structure is coordinated with the development of nonpolar surfaces. The stability of this species varies from sequence to sequence (Creighton, 1988). Those proteins that do have marginally stable early intermediates then fold into more stable species in the 5-1000 millisecond time range (Hartl and Hayer-Hartl, 2009). The final step in folding involves the attainment of native side-chain packing and hydrogen bond formation at many different sites throughout a protein. The packing of the nonpolar side chains results in the dehydration events which occur mainly as the transition state is reached.

1.3 Amyloid fibril formation

The folded, protein conformation (its tertiary structure) is always determined by its primary structure (amino acid sequence) (Anfinsen, 1973). The mechanism by which the final folded conformation of protein is encoded in its amino acid sequence and the folding process are still unclear. Energy landscapes are used to describe the transition of a high energy unfolded structure down a funnel-like energy profile to yield a native, biologically functional low-energy state (Dobson, 2004b, Dobson, 2001b, Hartl and Hayer-Hartl, 2009, Jahn and Radford, 2005) (Fig 1.2).

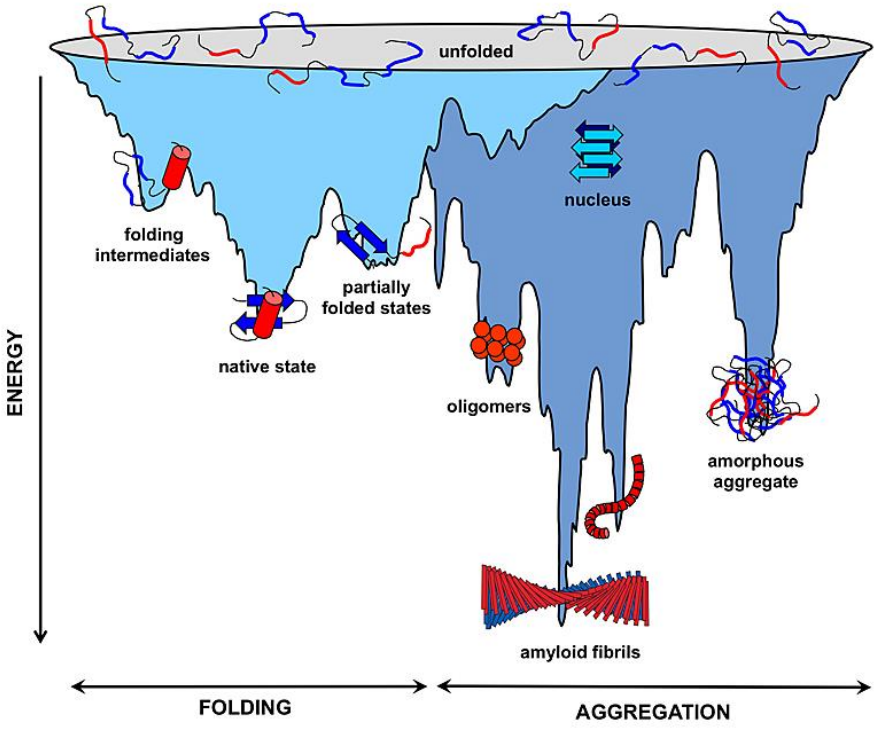

Figure 1.2: The difference between amorphous and amyloid fibrillar aggregation. The light blue surface shows that many intermediate conformations can lead to the native state (which is in a local energy minimum) via intramolecular interactions. The dark blue surface shows the conformations that can lead to amorphous aggregation or amyloid fibril formation via intermolecular interactions. From Hartl *et al* (2009).

Amyloid fibril formation is thought to arise when proteins adopt intermediately folded or molten globule states, either via partial unfolding of a natively folded protein, or directly via the adoption of structure of a natively unfolded protein. The proteins enter the off-folding pathway, driven by hydrophobic interactions between folding intermediates (Fig. 1.2 and 1.3), often these intermediate conformations arise due to cellular stress, such as elevated temperature, oxidative stress and deviation from physiological pH. The off-folding pathway can lead to amorphous aggregation, oligomeric pre-fibrillar species and thermodynamically stable amyloid fibrils (Chiti and Dobson, 2006, Dobson, 2004b, Dobson, 2001b, Jahn and Radford, 2005, Ecroyd and Carver, 2008, Hartl et al., 2011, Rambaran and Serpell, 2008). Amorphous aggregates are non-toxic and are able to be cleared by normal cellular degradation pathways e.g. via ubiquitination and the proteome (Ecroyd and Carver, 2008). However amyloid fibrils cannot be cleared by such cellular degradation pathways.

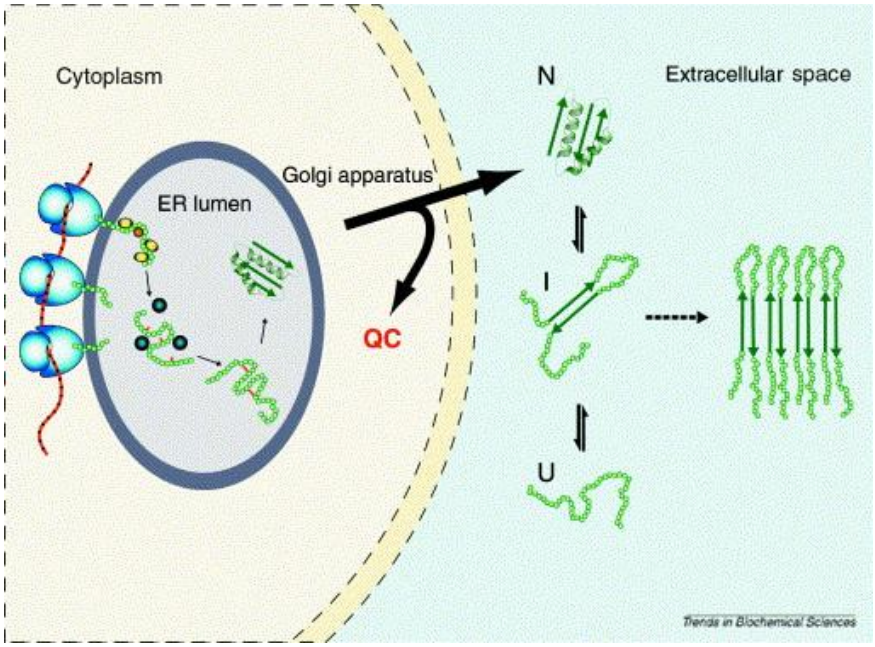

Figure 1.3: Schematic representation of the possible mechanism of formation of amyloid fibrils by a globular protein. Protein synthesis takes place on the ribosome, then protein folds in the endoplasmic reticulum (ER), aided by molecular chaperones that prevents

aggregation of incompletely folded species. The correctly folded protein is secreted from the cell and functions normally in its extracellular environment. Under certain conditions (e.g stress) the protein unfolds, at least partially, and becomes prone to aggregation. This can result in the formation of amyloid fibrils and other aggregates (Dobson, 1999). QC refers to quality control that prevents incompletely folded proteins being secreted to ER while, N, I, and U refer to native, intermediate (partially folded) and unfolded states respectively. Figure from Dobson (1999).

1.4 Structure of amyloid fibrils

Amyloid fibrils consist of many-chain aggregates and are composed of various numbers and arrangements of smaller structural units called protofilaments. Surprisingly, irrespective of the given amino acid sequence, fibrils formed by different polypeptides have very similar morphologies. Ultra-structurally, the fibrils have diameter of 7-10 nm, and structural studies of amyloids with high resolution methods has typically revealed a cross-β-pleated sheet conformation. The protofilaments assemble into the cross-β fibre structure, with β-strands extending perpendicular and β-sheets extending parallel to the axis. Two densely packed β-sheets (all yellow and all green arrows in Figure 1.4), whose strands run perpendicular to the fibre axis lead to the so called β-cross structure with a characteristic X-ray diffraction pattern. The intermolecular interactions between the polymer chains differ from the intramolecular ones within the same chain and dominate the formation of the protein aggregates. Thus, β-strands of different peptides within the fibril interact via hydrogen-bonds which are parallel to the fibril axis. In Figure 1.4, the corresponding β-strands (yellow and green arrows) of the example amyloid-β (1-40) peptide are parallel aligned to each other in each sheet.

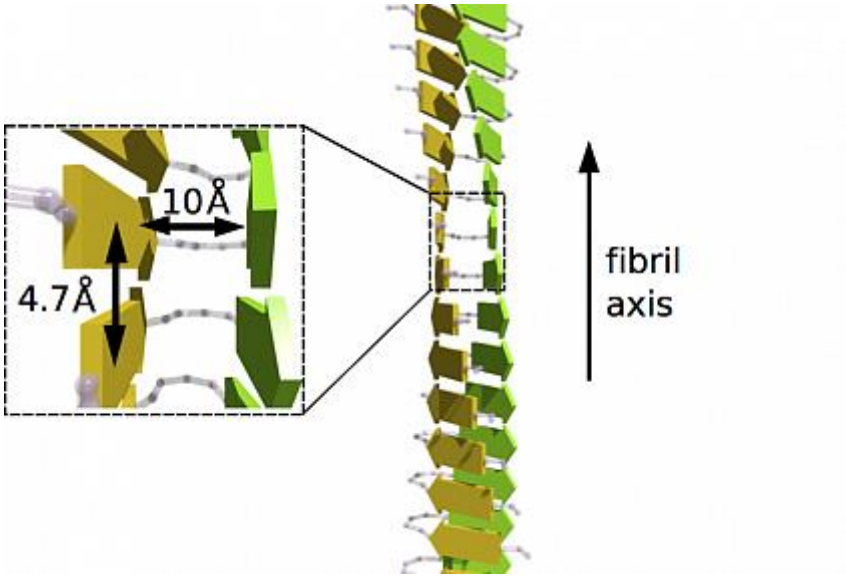

Figure 1.4: Scheme of cross-β structure of a mature amyloid fibril with a densely packed two-layer β-sheet structure. Each β-sheet is indicated by a yellow or green colour and shows a parallel alignment of the β-strands (yellow and green arrows). Distance between the sheets (~10Å) and between the strands (~4.7Å). Figure from Scheidt *et al* (2012).

Adjacent β-strands are separated by a regular repeat of approximately 4.7 Å, which is predetermined by the standard length of hydrogen bonds between the main chain carboxyl and amide groups. The protofibrils contain two or three sets of β-sheets that are stacked face-to-face at an inter-sheet spacing of 9-11 Å (Figure 1.4)(Sunde and Blake, 1997).

1.5 Diseases associated with amyloid fibrils

A range of debilitating human diseases, which result in the malfunctioning of the cell in the body, are associated with protein misfolding (Thomas et al., 1995). The mechanism by which protein aggregation results in disease is still not clear. On the other hand, in some of these diseases, the accumulation of aggregates is a merely consequence of these diseases, rather than the cause (Caughey and Lansbury, 2003, Clark and Muchowski, 2000). In some cases, the sheer volume of material involved, sometimes kilograms being deposited, can physically

disrupt the functions of organs (Tan and Pepys, 1994). The insoluble fibrils or plaques accumulate in a variety of organs including the liver, spleen and brain (Tan and Pepys, 1994, Kelly, 1998, Lansbury, 1999, Perutz, 1999). The term amyloidosis is used to describe many of the clinical conditions associated with amyloid. These diseases can be sporadic, inherited or even infectious, and often manifest late in life. Each disease is associated with a particular protein (table 1 and 2) and aggregates of these proteins are thought to be the direct or indirect origin of the pathological conditions associated with the disease in question (Dobson, 1999). Despite the range of proteins involved in these diseases, all of which have unique and characteristic native folds but the amyloid fibrils formed from each protein are similar in their overall morphology (Sunde et al., 1997). There are more than 20 diseases that are caused by amyloid fibrils which encompass both neurodegenerative and non-neurodegenerative disorders.

Neurodegenerative disorders caused by amyloid fibrils exhibit intra- and extracellular brain lesions consisting of abnormally folded and aggregated proteins and peptides (Muchowski, 2002). Table 1 lists neurodegenerative diseases, the protein involved and the location and/or name of the deposits.

Table 1-1: Human neurodegenerative diseases associated with the deposition in brain tissue of amyloid fibril aggregates (Chiti and Dobson, 2006, Taylor et al., 2002).

Disease	Protein Deposits	Protein or Peptide
Alzheimer's disease	Extracellular plaques Intracellular tangles	Amyloid β Tau
Parkinson's disease	Lewy bodies	α-synuclein
Creutzfeldt-Jakob disease	Prion plaque	Prion
Huntington's disease	Intranuclear and Cytoplasmic Inclusions	Huntingtin
Familial amyloid polyneuropathy I	Peripheral nerve plaques	Transthyretin
Familial British dementia	Extracellular plaques and intracellular tangles	British amyloid
Frontotemporal dementia	Cytoplasmic tangles	Tau
Amyotrophic lateral sclerosis	Bunina bodies	Superoxide dismutase I
Spinocerebellar ataxia	Intranuclear inclusions	Ataxins

While neurons are vulnerable to the toxic effects of misfolded proteins (Taylor et al., 2002), protein misfolding can affect other tissues including the liver, heart, kidneys, skin and

gastrointestinal tract. (Dobson, 1999). They include most of debilitating conditions in modern society, particularly those associated with an ageing population (Table 1.2).

Table 1-2: Human non- neuropathic diseases associated with amyloid fibril deposits (Chiti and Dobson, 2006).

Disease	**Affected Tissue**	**Protein or Peptide**
Type II diabetes	Pancreas	Islet amyloid polypeptide
Pulmonary alveolar proteinosis	Lungs	Lung surfactant protein C
Cataract	Eye/lens	γ-Crystallin
Aortic medial amyloidosis	Heart	Medin
Cutaneous lichen amyloidosis	Skin	Keratins
Hemodialysis-related amyloidosis	Blood	β2-microglobulin
Medullary thyroid Carcinoma	Thyroid	Calcitonin
Pituitary prolactinoma	Pituitary	Prolactin
AA amyloidosis	Systemic	Serum amyloid A

A large number of amyloidogenic proteins and peptides induce pathogenic cellular processes including, oxidative stress (Schubert et al., 1995), inappropriate signalling pathways (Saitoh et al., 1993, Mattson, 1995), intracellular calcium dyshomeostasis (homeostasis imbalance) (Mattson et al., 1992) and mitochondrial dysfunction (Shoffner, 1997), which lead to cell death. In most cases of neurodegenerative disorders, studies have shown that impairment of neural function is due to the interaction between cellular components and smaller aggregated specifically, smaller forms of the protein, which have somehow acquired a neurotoxic activity (Dobson, 2001a).

Studies (Chimon et al., 2007, Hoshi et al., 2003, Simoneau et al., 2007, Bucciantini et al., 2002, Hartley et al., 1999, Lambert et al., 1998, Lashuel et al., 2002, Olofsson et al., 2002, Walsh et al., 2002) have shown that intermediately structured species (oligomers and small pre-fibrillar protein aggregates), rather than mature fibrils, are the primary pathogenic species. On the other hand, others have reported that mature fibrils are the pathogenic species (Novitskaya et al., 2006, Dehle et al., 2010). In summary, therefore the role of amyloid fibrils in disease is complex and may vary from protein to protein.

Amyloid fibrils have been also implicated in the enhancement of viral infections. SEVI has been found to enhance HIV-1 (Münch et al., 2007) (chapter 2 and 3) and cytomegalovirus (CMV) (Tang et al., 2013) infections. β-amyloid peptide, a Alzheimer's disease marker, also enhances infections of HIV, amphotrophic Moloney leukemia virus, herpes simplex virus, and viruses with the envelope glycoprotein of vesicular stomatitis virus (Wojtowicz et al., 2002).

1.6 Protein quality system

Nature has the means to avoid proteins aggregating into amyloid fibrils under normal physiological conditions. Many factors are involved in these protective mechanisms, but the selection of sequences during evolution that can fold efficiently to a globular form in which the polypeptide chain and the hydrophobic residues are hidden in the interior is likely to be particularly important (Chiti et al., 1999). Thus, except when the protein is exposed to denaturing conditions, the peptide backbone is not accessible to form the interchain hydrogen bonds associated with amyloid fibrils Fig. 1.5) (Dobson, 1999).

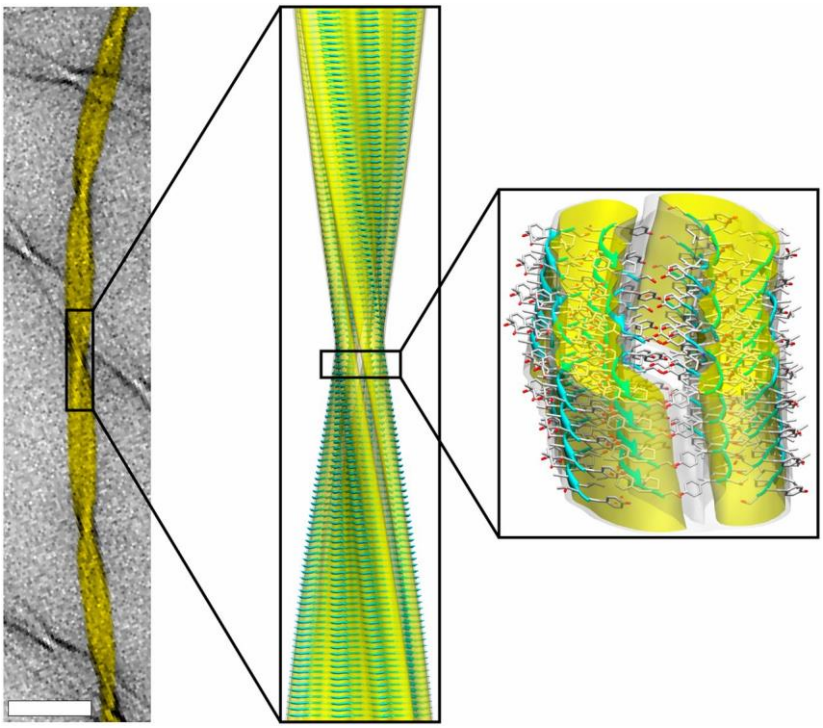

Figure 1.5: Molecular model of an amyloid fibril derived from cryo-EM analysis of SH3 domain fibrils (center). The background image of the fibril (left) was taken using TEM. The model shows a ribbon presentation (right) of the β-sheet structure assembled within the fibrils, with oxygen, carbon and nitrogen atoms are shown in red, gray and blue respectively. Figure from Fitzpatrick *et al*. (2013).

Another very important requisite is that, in a cellular environment denaturation of proteins does not normally occur under conditions where unfolded chains tend to aggregate. In particular, pH and temperature are generally controlled, and molecular chaperones and degradation mechanisms are present to cope with the majority of aggregation-prone species (Dobson, 1999).

Molecular chaperones are a diverse group of proteins that stabilise unfolded and partly folded proteins to prevent interactions that would otherwise result in protein misfolding and aggregation (Ehrnsperger et al., 1997). Molecular chaperones provide the first line of defence against protein misfolding by associating with nascent polypeptides as they come out from the ribosome (Taylor et al., 2002). Molecular chaperones ensure high fidelity in protein folding and assembly without becoming part of the final product (Carver et al., 2002). Protein folding is driven by the free energy change that accompanies the conversion of a linear, unstructured polypeptide chain into its native conformation (Anfinsen, 1973), but during its normal folding process, hydrophobic regions of the polypeptide are exposed to the solvent and must pass through partially folded intermediates to the fully native state. Molecular chaperones such as Hsp70 and Hsp60, act on the folding protein to encourage productive processes that result in the correctly folded form of protein (Carver et al., 2002). Many of molecular chaperones are called heat-shock proteins (HSPs), or stress proteins, because their expression is upregulated under stress conditions such as elevated temperatures (Jakob and Buchner, 1994). Molecular chaperones expression is elevated in the disease states that are associated with protein misfolding and precipitation, and are associated with the neural plaques found in Alzheimer's, Parkinson's, and Huntington's disease (Muchowski, 2002, Ganea, 2001).

1.7 Purpose of the study

All amyloid fibril folds, independent of their origin, bear structural similarities including the cross beta sheet secondary structure. They are also associated with degenerative diseases. Amyloid fibrils or at least their prefibrillar forms have been found to be toxic to neuronal cells (Chiti and Dobson, 2006, Dehle et al., 2010, Ecroyd and Carver, 2008). Clusterin and EGCG exhibit properties that play a role in the prevention of amyloid fibrils. The major objective of this project therefore is to (i) investigate the toxicity of Semen-derived Enhancer of Virus Infection (SEVI) to different cell lines (neuronal and epithelial cells), (ii) investigate whether clusterin, which has similar role to small heat shock protein prevents formation of SEVI fibrils and, (iii) determine the toxicity of milk protein α_{s2}-casein to neuronal cells, and, investigate the effect of EGCG on fibrils formed by α_{s2}-casein.

In Chapter 2, a fragment of Prostatic Acidic Phosphatase (PAP), PAP248-286, is characterised in greater detail using computer algorithms to determine its amyloidogenic regions. The regions are synthesised along with PAP248-286 and incubated to induce fibril formation. Finally the toxicity of SEVI and fibrils formed by its fragments to PC 12 and CACO-2 cells are examined.

In Chapter 3, the enhancement of HIV-1 infection by SEVI and its fragment PAP248-271 is examined further by examining infection with viruses that utilise the coreceptors CCR5 and CXCR4.

In Chapter 4, the ability of clusterin to prevent SEVI formation thereby inhibiting its cytotoxicity is examined.

In Chapter 5, cytotoxicity of α_{s2}-casein to PC12 cells is examined. Additionally, the ability of ECGC to prevent α_{s2}-casein fibril formation and cytotoxicity is examined.

It is concluded (in Chapter 6) that SEVI and its amyloidogenic fragments, PAP248-271 and PAP257-267, form fibrils that are toxic to neuronal cells but are not toxic to confluent epithelial cells. The fibril-forming propensity of SEVI is prevented by clusterin.

The milk protein α_{s2}-casein is toxic to both the neuronal and confluent epithelial cells. Investigation of the fibril-forming and fibril-inhibiting abilities of amyloid forming peptides and proteins is crucial in combating degenerative diseases

1.8 Linkage between Publications

Listed below are the three papers that comprise chapters 2, 3 and 4, which were completed during candidature. Chapter 2 has been published in Biochimica et biophysica acta. Chapter 3 and 4 are unpublished manuscripts that are to be submitted for publication.

Chapter 2. Elias, A. K., Scanlon, D., Musgrave, I. F. & Carver, J. A. 2014. SEVI, the semen enhancer of HIV infection along with fragments from its central region, form amyloid fibrils that are toxic to neuronal cells. *Biochimica et biophysica acta,* 1844**,** 1591-8.

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Chapter 3. Elias, A. K., Wilson, R. M., Carver, J.A., Musgrave, I. F., (2014) Clusterin inhibits fibril formation and suppresses cytotoxicity associated with SEVI (**to be submitted**).

Chapter 4. Elias, A. K., Carver, J. A., Musgrave, I. F., Amyloid fibrils formed by Bovine Milk α_s2-Casein are toxic to PC12 cells (**to be submitted**)

2 SEVI, the semen enhancer of HIV infection along with fragments from its central region, form amyloid fibrils that are toxic to neuronal cells

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2.1 Abstract

Semen-derived enhancer of viral infection (SEVI) is the term given to the amyloid fibrils formed by a 39-amino acid fragment (PAP248-286) of prostatic acidic phosphatase (PAP) found in human semen. SEVI enhances human immunodeficiency virus (HIV) infectivity by four to five orders of magnitude (Münch *et al.* (2007). Here, we show by various biophysical techniques including Thioflavin T fluorescence, circular dichroism spectroscopy and transmission electron microscopy that fragments encompassing the central region of SEVI, i.e. PAP248-271 and PAP257-267, form fibrils of similar morphology to SEVI. Our results show that the central region, residues PAP267-271, is crucially important in promoting SEVI fibril formation. Furthermore, SEVI and fibrillar forms of these peptide fragments are toxic to neuronal pheochromocytoma 12 cells but not to epithelial colon carcinoma cells. These findings imply that although SEVI assists in the attachment of HIV-1 to immune cells, it may

not facilitate HIV entry by damaging the epithelial cell layer that presents a barrier to the HIV.

Keywords: SEVI; amyloid fibril; cell toxicity; HIV; protein aggregation.

Abbreviations: Aβ, Amyloid β; AIDS, Acquired Immunodeficiency Syndrome; COCA-2, Epithelial colon carcinoma cells; HIV, Human Immunodeficiency Virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAP, Prostatic acid phosphatase; PC 12, Pheochromocytoma 12 ; SEVI, Semen-derived Enhancer of Viral Infection; TEM, Transmission electron microscopy; ThT, Thioflavin T.

2.2 Introduction

The human immunodeficiency virus (HIV) was first reported in the 1980s. It is the causative agent in acquired immunodeficiency syndrome (AIDS), which is amongst the top ten killer diseases worldwide, particularly in developing countries. Approximately 2.5 million people were reported to be newly infected with HIV in 2011 alone and adult deaths from AIDS have left approximately 16.6 million children without parents needs a reference 2011 . Sexual intercourse is the major route for the transmission of HIV. The risk of being infected from a single sexual act varies from one person to another and depends on various risk factors: stage of disease in the male, seminal viral load, cervico-vaginal infections in the female, mucosal epithelial integrity and susceptibility of the partner to HIV (Royce, 1997, Galvin and Cohen, 2004, Münch et al., 2007). Most infections are due to genital exposure to semen of HIV positive men and 60% of women who acquired HIV-1 in Africa do so via vaginal intercourse (Münch et al., 2007, Haase, 2005). Münch and colleagues (2007) showed that a 39-amino acid fragment (residues 248 to 286) of the enzyme Prostatic Acid Phosphatase (PAP) in semen, PAP 248-286, forms amyloid fibrils that enhance HIV infection by up to five orders of magnitude. The fibrils have therefore been termed semen-derived enhancer of virus infection (SEVI).

Amyloid fibrils are associated with more than 40 different diseases in humans including Parkinson's disease, Huntington's disease, Alzheimer's disease, prion diseases and diabetes mellitus type 2 (Caughey and Lansbury, 2003, Chiti and Dobson, 2006, Dehle et al., 2010, Dobson, 2004a, Scherzinger et al., 1997, Stefani and Dobson, 2003, Tan and Pepys, 1994, Thomas et al., 1995, Yankner and Lu, 2009) . An amyloid fibril is a highly-ordered, aggregated protein state that forms when partially folded intermediates mutually associate and enter an off-folding pathway (Chiti and Dobson, 2006, Dobson, 2004a, Ecroyd and

Carver, 2008). The fibrils are commonly about 10 nm in diameter, and they consist of between two and six protofibrils that are twisted around each other. X-ray fibre diffraction analysis indicates that the organised fibril core has a cross-β structure in which sheets are assembled from β-strands that run perpendicular to the fibril axis (Sunde et al., 1997). Even though different peptides and proteins have significant variation in amino acid sequences and structures in their biologically active states, they form amyloid fibrils that have remarkably similar essential features. Therefore, investigation of the mechanism of fibril formation and its role in human pathology is of great importance in understanding the fundamental processes that lead to the progression of diseases associated with amyloid fibril formation.

The rationale for this study is that detailed reasons for the cytotoxicity of SEVI or its amyloidogenic fragments are unknown. In an activity context, this is important as compromising the mucosal epithelial barrier may play a significant role in HIV transmission (Shattock and Moore, 2003). The central region of PAP248-286 encompassing residues G260-N265 has been proposed as an initiator of fibril formation (Sievers et al., 2011). Nanga *et al*. (Nanga et al., 2009) similarly identified V262 to H270 as an amyloidogenic region along with S279 to L283. We first sought to refine and extend these studies by identifying potential amyloidogenic regions in SEVI using three computer algorithms and determine whether these putative amyloidogenic regions are able to form amyloid fibrils.

The formation of amyloid deposits is linked to cell death, yet the mechanism by which fibrillar species cause cytotoxicity is unclear (Chiti and Dobson, 2006, Ecroyd and Carver, 2008, Stefani and Dobson, 2003, Hudson et al., 2009). Previous cell culture studies have shown that oligomers, protofibrils and mature fibrils are toxic to neuronal cells and the level of toxicity of these species is dependent on the specific fibril-forming protein. In this study, we investigated whether SEVI and its fragments were toxic to neuronal pheochromocytoma 12 (PC 12) cells, a standard model for the toxicity of amyloid β (Aβ, the putative causative

agent in Alzheimer's disease), and human epithelial CACO-2 cells as a model of epithelial cells. Fibrils formed by SEVI and its amyloidogenic fragments were toxic to neuronal pheochromocytoma 12 (PC 12) cells, with the fragments being more toxic than the entire SEVI peptide. However, both the native and fibrillar forms of SEVI and its fragments (PAP248-271 and 257-267) were not toxic to CACO-2 cells. Our results show that neuronal PC 12 cells are more susceptible to SEVI amyloid fibrils than non-neuronal epithelial CACO-2 cells, implying that SEVI is unlikely to facilitate HIV transmission by compromising the mucosal epithelial barrier.

2.3 Experimental Procedures

2.3.1 Predictions of amyloidogenic regions

The amino acid sequence of SEVI (Fig.1) was entered into three different computer algorithms, Zipper DB, Zyggregator, and Tango (Goldschmidt et al., 2010, Tartaglia and Vendruscolo, 2008, Rousseau et al., 2006) to predict the aggregation-prone regions of the SEVI peptide.

2.3.2 Peptide synthesis

Two different batches of peptides were used. One was synthesized in our laboratory and the other was purchased from Mimotopes Pty Ltd, Australia. Peptides were synthesized by standard Fmoc solid-phase peptide synthesis and purified using semi-preparative high-performance liquid chromatography (HPLC) and analyzed by analytical HPLC and hybrid quadruple time of flight (QTOF2) mass spectrometer for purity.

2.3.3 Amyloid fibril formation and Thioflavin T (ThT) fluorescence assay

PAP 248-286, fragments PAP248-271 and PAP257-267 were dissolved in 200 mM phosphate buffer, pH 7.2, 37 ºC at a concentration of 1 mM and agitated at 700 rpm for five days. The requirement for vigorous shaking to form SEVI fibrils precluded the use of *in situ* assays. As an alternative, 10 µL of aliquots were withdrawn from the solutions after every 20 min for 8 h and snap frozen at -20 ºC. After this time, each sample was thawed and then mixed with 10 µM ThT in 200 mM phosphate buffer, pH 7.2. The fluorescence was then measured in triplicate wells using FLUOstar and POLARstar optima microplate reader (BMG Labtechnologies, Melbourne Australia) with the excitation and emission wavelengths set at 440 and 490 nm, respectively.

Fresh samples of αs_2-casein (a gift from Dr David Thorn) were dissolved in 50 mM phosphate buffer, pH 7.2. α_{s2}-Casein was incubated at 50 ºC without shaking and aliquots

were taken after 24 hours and snap frozen at -20 ºC for 5 days. Samples were then thawed and mixed with 10 µM ThT in 50 mM phosphate buffer, pH 7.2 and fluorescence measured using a glass cuvette (10 mm light path) with excitation and emission wavelengths set at 442 and 490 nm using with Cary Eclipse spectrofluorimeter (Thorn et al., 2008).

For fibril formation of RCMκ-CN, the protein was dissolved in 50 mM phosphate buffer, pH 7.2. Samples were incubated for 24 hours with 10 µM ThT at 50 ºC and the fluorescence levels were measured with a FLUOstar and POLARstar optima microplate reader with the excitation and emission wavelengths set at 440 and 490 nm respectively.

2.3.4 Transmission electron microscopy

2 µL of each solution which potentially contained fibrillar species was added to formavar and carbon-coated nickel grids (SPI supplies, West Chester, USA) for 2 min. The grids were washed three times with 10 µL of water and negatively stained with 10 µL uranyl acetate (2% w/v). Excess stain was removed with filter paper and the grids were air dried and viewed under 10,000-64,000 times magnification at 80 kV excitation voltages using a CM100 transmission electron microscope (Philips, Eindhoven, Netherlands).

2.3.5 Circular Dichroism (CD) spectroscopy

For far-UV CD measurements, both the prefibrillar and fibrillar forms of each peptide were dissolved in 10 mM phosphate buffer at pH 7.2 at 0.25 mg/mL. An average of four wavelength scans were acquired for each spectrum from 190-260 nm at a rate of 10 nm/min in a 1 mm pathlength quartz cuvette at 25 ºC using a Jasco J-815 CD spectrophotometer.

2.3.6 Cell toxicity assays

PC 12 and CACO-2 cells were grown in RPMI (Roswell Park Memorial Institute) media containing 10% v/v fetal calf serum, 1% v/v glutamate, 1% v/v non-essential amino acids and 1% v/v penicillin and streptomycin in 75 cm^3 flasks at 37 ºC in an incubator with 95% air and

5% carbon dioxide. The medium was changed after 2 to 3 days. The cells were plated into a 96 well plate at a density of 2×10^4 cells per well and incubated for 24 h. The cells were then treated with 20, 25, 40, 50, 75 and 100 µM of native, prefibrillar and fibrillar SEVI and its fragments, PAP248-271 and PAP257-267, RCMκ- and α_{s2}-Casein (six replicates per treatment) and incubated for 48 h. Following incubation, the media was removed by gentle aspiration.

2.3.7 Cell viability

Cell viability was evaluated by using a 3-(4,5–dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay relies on the reduction of yellow tetrazolium MTT to a purple formazan dye by the mitochondrial dehydrogenase enzyme. The amount of formazan produced reflects the number of metabolically active viable cells (Fye Lau et al., 2013). 100 of µL MTT (0.25 mg/mL) containing media was added to each well and the plate was further incubated for 3 h at 37 ºC. The MTT containing media was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan. Formazan absorption was measured at 560 nm using POLARstar Optima microplate reader (BMG Labtechnologies, Offenburg, Germany). The mean of six replicate readings was taken and cell viability (%) was calculated by dividing the average absorption readings of treated wells by the average absorption readings of untreated wells (phosphate buffer only) and multiplying by 100.

2.4 Results

2.4.1 SEVI is predicted to have two distinct amyloidogenic regions

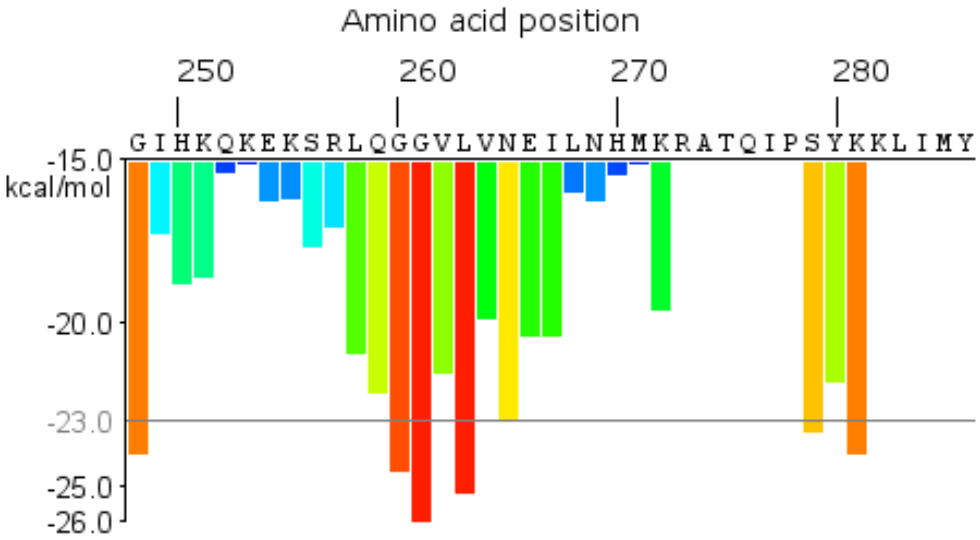
To gain insight into the region(s) of the SEVI peptide that have amyloidogenic propensity, we used three computer algorithms: Zipper DB, Zyggregator and Tango. Each algorithm uses different methods to identify peptide regions that are prone to aggregation. Zipper DB scans the peptide of interest by sliding a window of six residues (GIHKQK) over the sequence. Then, it maps each peptide onto the templates in the ensemble (Goldschmidt et al., 2010). The energy of each mapping of sequence to template is evaluated by the computer program ROSETTADESIGN. Segments with energies equal or below -23 kcal/mol are predicted to be amyloidogenic. For SEVI, the regions that are predicted to have high fibrillation propensity via Zipper DB are mostly in the middle of the peptide (PAP260-265) and its C-terminus (PAP279-281) (Fig. 2.1a).

The Zyggregator algorithm utilises physico-chemical properties of amino acids to determine changes in the aggregation rates of peptides as a result of individual amino acid substitutions. It predicts whether the rates correspond to the process of either folding or aggregation into oligomers or fibrils (Tartaglia and Vendruscolo, 2008). As shown in Fig. 2.1b, Zyggregator predicts that amyloidogenic regions of SEVI encompass PAP258-267 and PAP282-286, with a small region around residue 274.

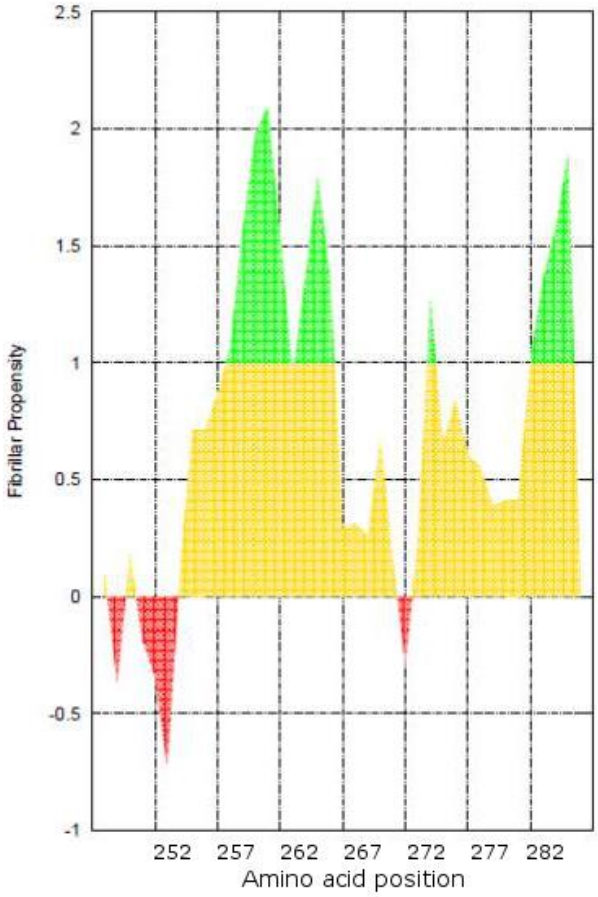
The Tango algorithm predicts the cross-beta sheet aggregation propensity of peptides by the assumption that the core regions of a fibrillar aggregate are fully buried (Rousseau et al., 2006). Tango predicts that amyloidogenic regions of SEVI are PAP261-266 and PAP274-286 (particularly towards its C-terminus), on the basis that these regions have an aggregation score of greater than zero (Fig. 2.1c). Thus, all three algorithms produced similar results, i.e., regions of high fibril-forming propensity in the middle and C-terminal regions of the SEVI

peptide. Accordingly, we synthesized the entire PAP248-286 (SEVI) peptide, and its fragments, PAP248-256, PAP257-267, PAP248-271 (Fig. 2.1d) that contained the N-terminus of the peptide with the middle (core) region and PAP272-286, encompassing the last 15 amino acids.

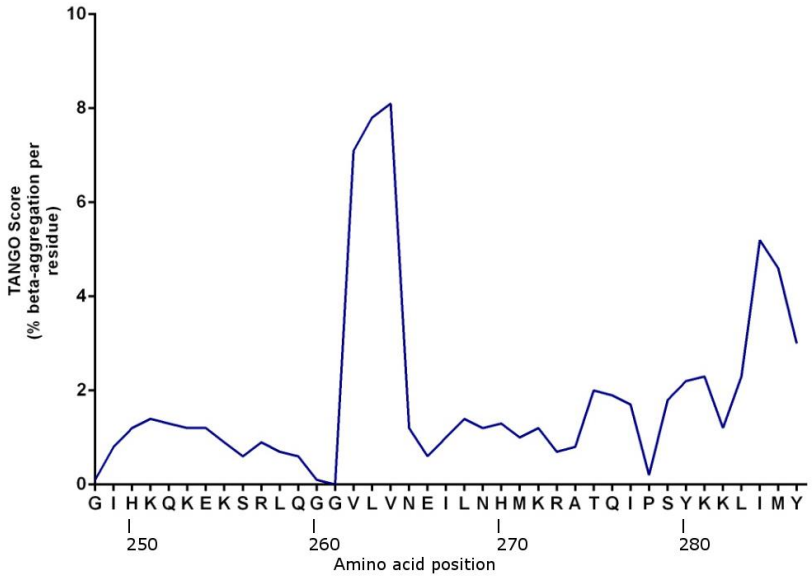
a.



b.


2.5
2
1.5
1
0.5
0
-0.5
-1
Fibrillar Propensity
252
257
262
267
272
277
282
Amino acid position

c.



d.

		250	260	270	280
PAP 248-286 (SEVI)	^{248}GIHKQKEKSRLQGGVLVNEILNHMKRATQIPSYKKLIMY^{286}				
PAP 248-256	^{248}GIHKQKEKS^{256}				
PAP 248-271	^{248}GIHKQKEKSRLQGGVLVNEILNHM^{271}				
PAP 257-267		^{257}RLQGGVLVNEI^{267}			
PAP 272-286				^{272}KRATQIPSYKKLIMY^{286}	

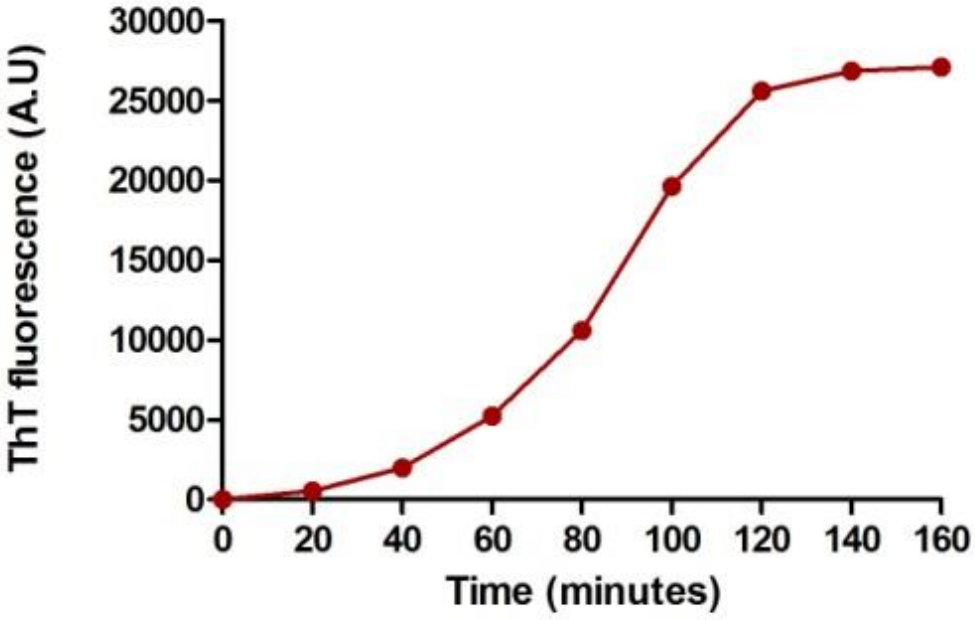
Figure 2.1: Amyloidogenic propensity of SEVI. a. Orange and red bars have energy levels equal to or less than -23 kcal/mol and indicate potential fibril-forming regions of SEVI as analyzed by ZipperDB. b. Aggregation profile of SEVI as analyzed by Zyggregator showing the intrinsic aggregation propensity profile, Z_i^{agg}. The green colour indicates regions of the sequence most likely to form amyloid fibrils, i.e. with a Z_i^{agg} score above 1.0. c. Tango

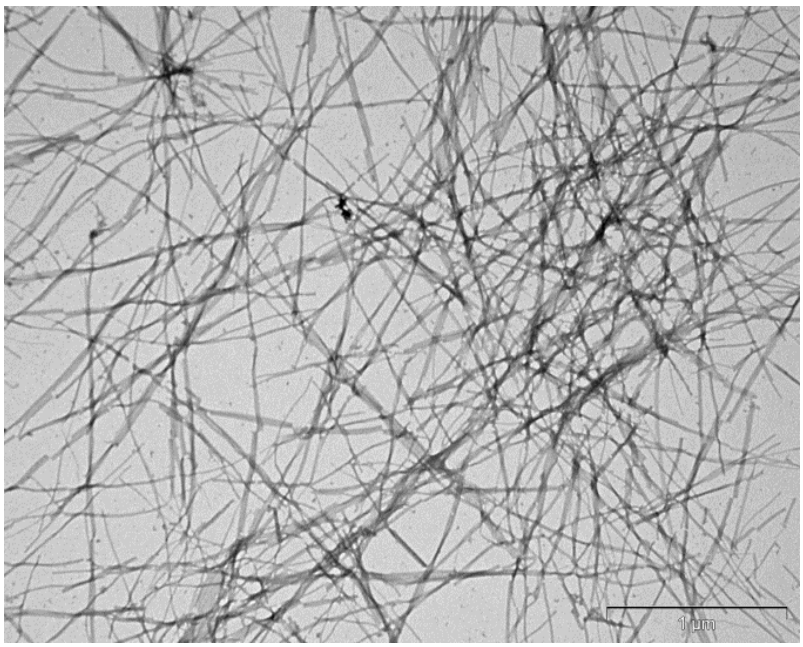
prediction of the regions of SEVI that are prone to fibril formation. d. Amino acid sequence of SEVI and its fragments that were synthesized for this study.

2.4.2 SEVI peptide fragments PAP248-271 and PAP257-267 form amyloid fibrils

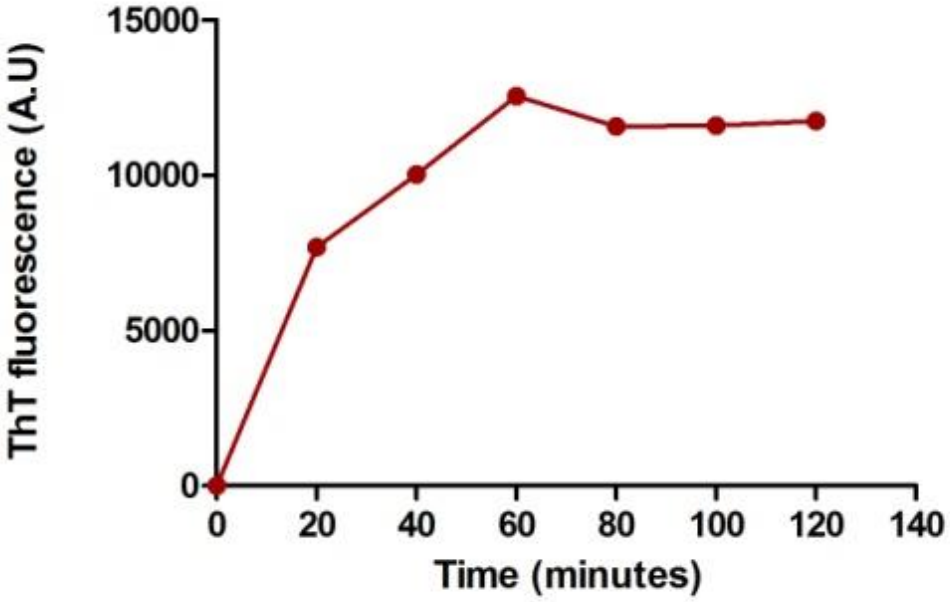
The propensity of SEVI (PAP 248-286), and its fragments PAP248-256, PAP248-271, PAP257-267 and PAP272-286 to form fibrils upon shaking at 700rpm for 5 h was determined. Aliquots were taken every 20 min and immediately frozen to stop fibril formation. At each time-point sample, Thioflavin T (ThT) was added and its fluorescence was measured as an indicator of the presence of amyloid fibrils. ThT is a fluorescent probe that binds to the cross-β sheet structure common to amyloid fibrils, with only the fibrillar forms of a peptide or protein giving rise to significant fluorescence (Naiki et al., 1990, Ye et al., 2009). Figure 2.2 shows the time course of ThT binding for SEVI and its fragments PAP248-271 and PAP257-267. In each case, ThT binding increased over time and reached a plateau. For SEVI, this occurred after approximately 100 min following incubation which included a lag phase of 40 min. PAP248-271 formed fibrils immediately and reached a plateau after 60 min while PAP257-267 had the longest lag phase of ~150 min and reached a plateau after 240 min. PAP248-271 aggregated at the fastest rate, compared to the SEVI peptide and PAP257-267 but exhibited lower final ThT fluorescence. PAP248-256 and PAP272-286 did not form fibrils as indicated by the absence of an increase in ThT fluorescence for either peptide (results not shown).

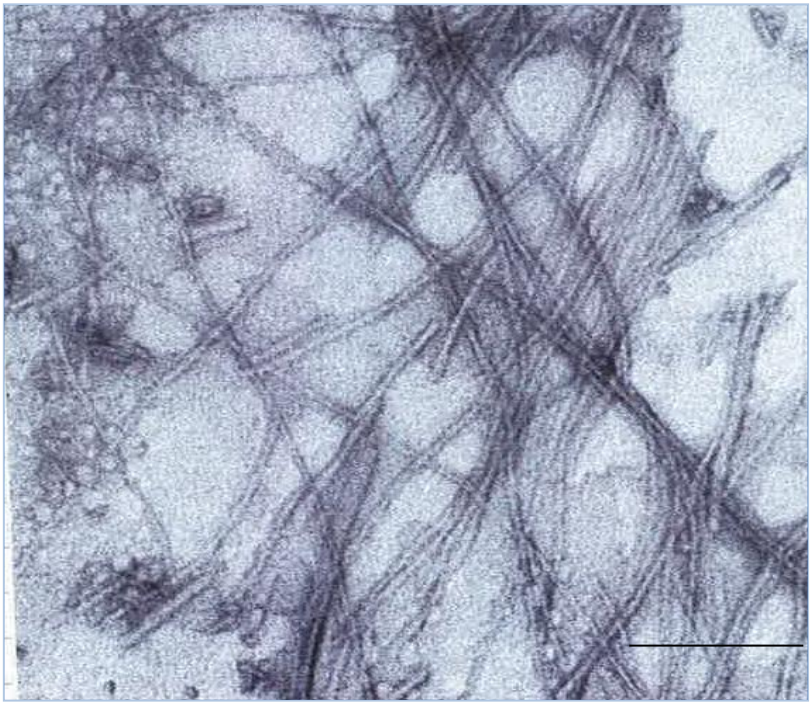
a. SEVI



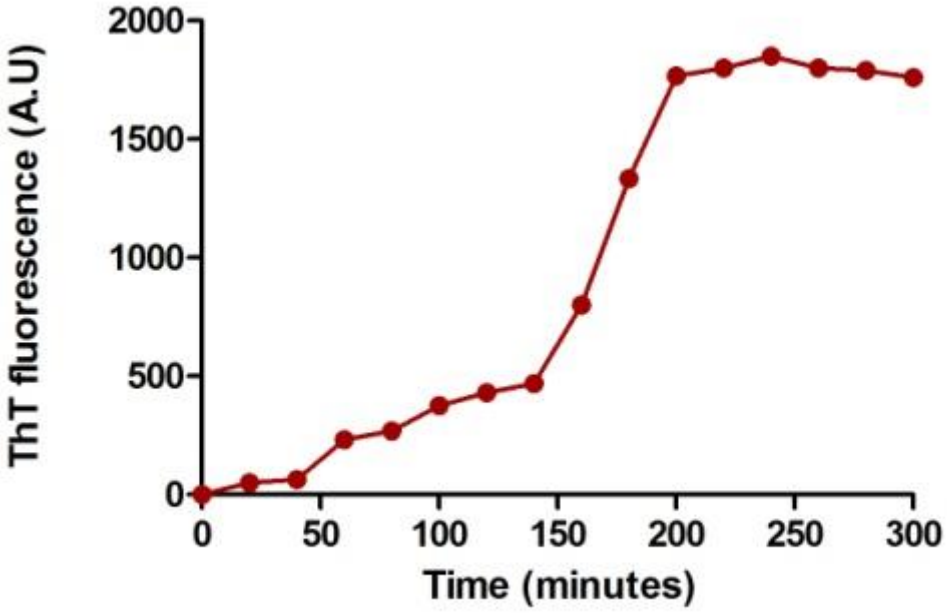


b. PAP248-271


ThT fluorescence (A.U)
15000
10000
5000
0
0
20
40
60
80
100
120
140
Time (minutes)



c. PAP257-267



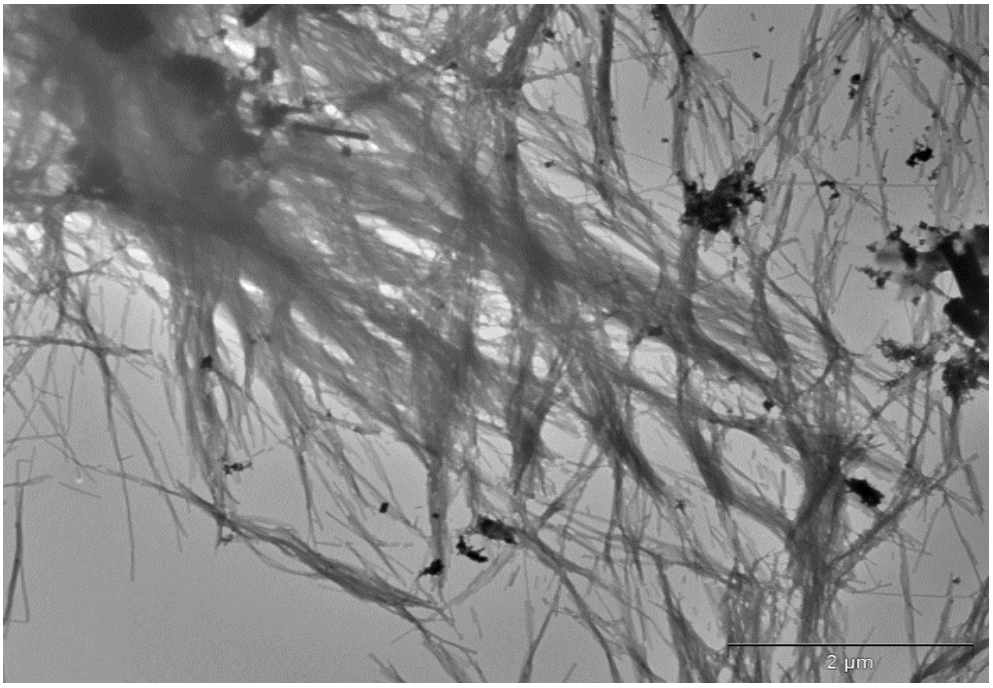


Figure 2.2: Characterization of SEVI and its fragments after incubation at 37 ºC with shaking at 700 rpm. (a) ThT fluorescence profile and TEM micrograph for SEVI. (b) ThT

fluorescence profile and TEM micrograph of PAP248-271. (c) ThT fluorescence profile and TEM micrograph for PAP257-267. The scale bar represents 2 μm (a, c) and 1 μm (b).

Transmission electron microscopy (TEM) was used to characterize the fibrils of SEVI and its fragments whose presence was implied by the ThT binding experiments. As shown in Fig.2.2, SEVI and PAP248-271 form long unbranched fibrils. The fibrils from PAP248-271 were much thicker than those formed from SEVI. In contrast, those formed by PAP257-267 were of very different morphology to both SEVI and PAP248-271 in being very clumped together.

Amyloid fibrils have a highly ordered, cross β-sheet secondary structure (Sunde et al., 1997, Sipe and Cohen, 2000, Nilsson, 2004). Previous studies have shown that PAP248-286 adopts an unfolded conformation in solution (Brender et al., 2011, Nanga et al., 2009). We used far-UV CD spectroscopy to examine the secondary structure of both the native and fibrillar forms of SEVI, PAP248-271 and PAP257-267. CD spectra of both the prefibrillar and fibrillar peptides (0.25 mg/mL) were acquired. A minimum in ellipticity at 200 nm for all peptides in the prefibrillar forms is indicative of a predominantly unstructured (random coil) conformation, although a slight minimum at ~220 nm in the two fragments implies some β-sheet structure is present in these species (Fig 2.3). The conversion to these species (Nanga et al., 2009)to a β-sheet rich conformation after incubation is readily apparent from the spectra of the fibrillar forms (Fig 2.3), all of which have large minima at 218 nm and the absence of a minimum at ~200 nm, particularly so for the two SEVI fragments.

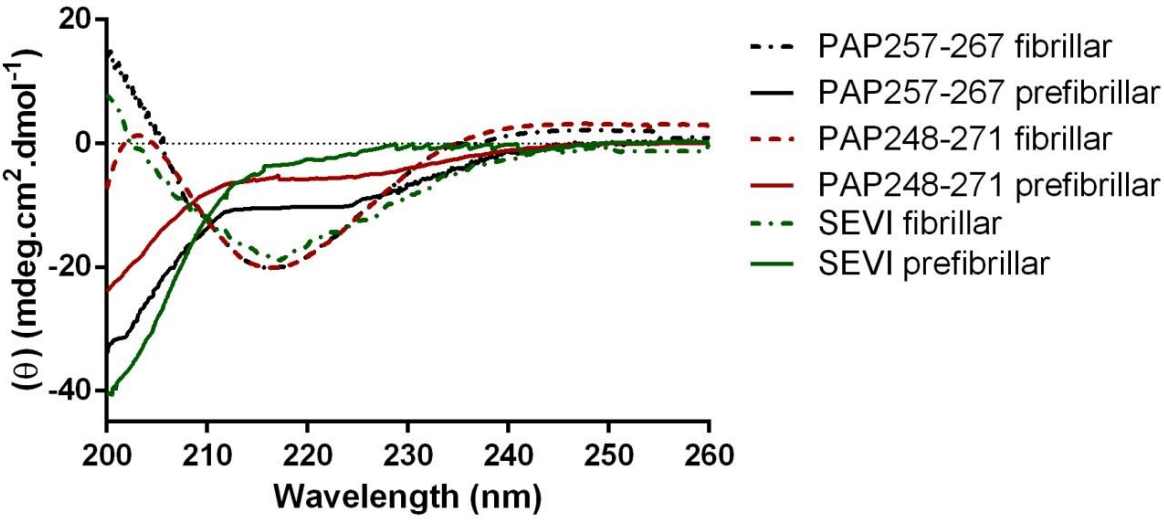


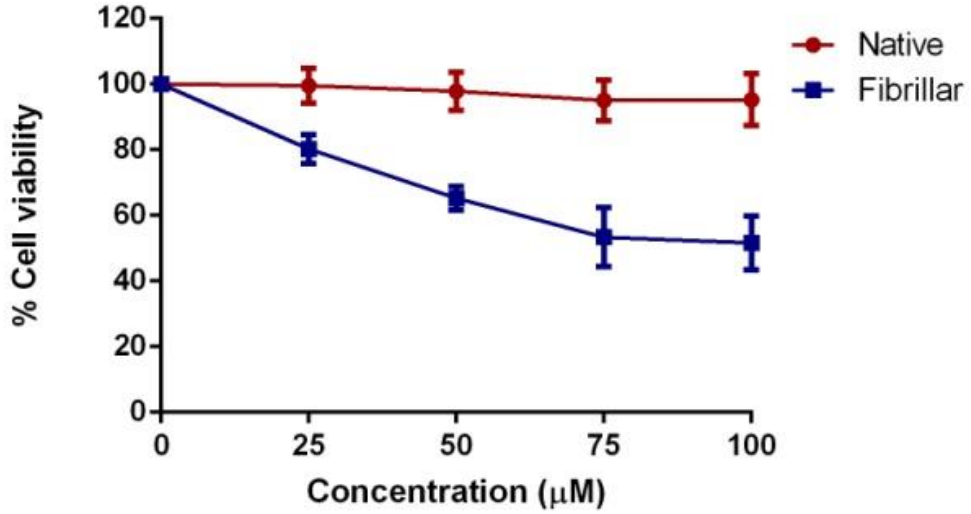
Figure 2.3: Far-UV CD spectra of 0.25 mg/mL of the peptides. SEVI (green), PAP248-271 (red) and PAP257-267 (black), before incubation (solid lines) and following incubation (dotted lines) at 37 ºC for five days in 10 mM phosphate buffer pH 7.2.

2.4.3 SEVI and its fibrillar fragments PAP248-271 and PAP257-267 are toxic to PC 12 cells

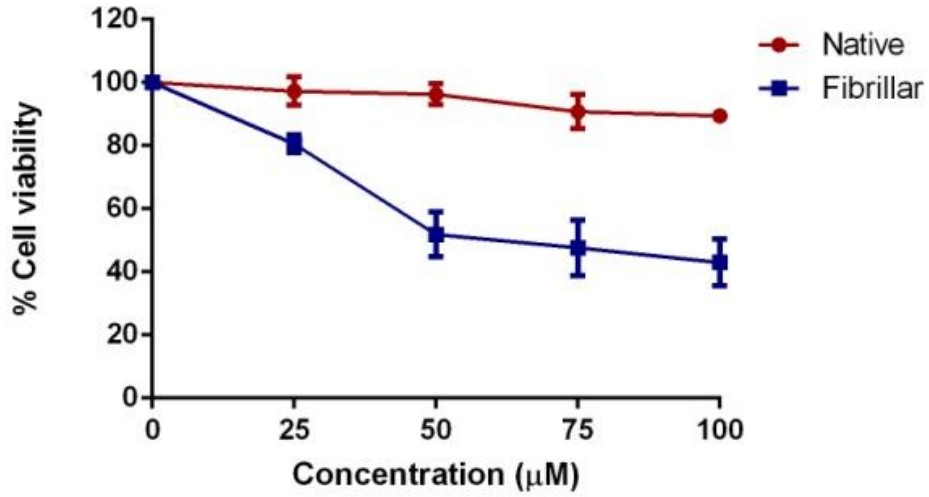
The fibrillar peptides caused cell death in neuronal model PC 12 cells in a concentration-dependent manner, as has been observed for other fibril-forming peptides and proteins (Dehle et al., 2010, Bucciantini et al., 2002). At the lowest concentration (25 µM), fibrils from SEVI produced 20% cell death compared to a control, which was similar to the effect produced by PAP248-271 while PAP257-267 was more toxic in causing around 30% cell death. As the fibril concentration was increased, the percentage cell death produced by the fibrils formed by PAP248-271 and PAP257-267 was similar, e.g. around 50% cell death was caused at a concentration of 50 µM. By contrast, SEVI caused 50% cell death at a higher concentration of 100 µM (Fig 2.4), implying that fibrils formed by the N-terminal fragments of SEVI were more toxic to PC 12 cells than SEVI. The native (prefibrillar) peptides were not cytotoxic

apart from the 10% cell death that was produced at higher concentrations of PAP248-271 (75-100 μM).

a. SEVI



b. PAP248-271



c. PAP257-267

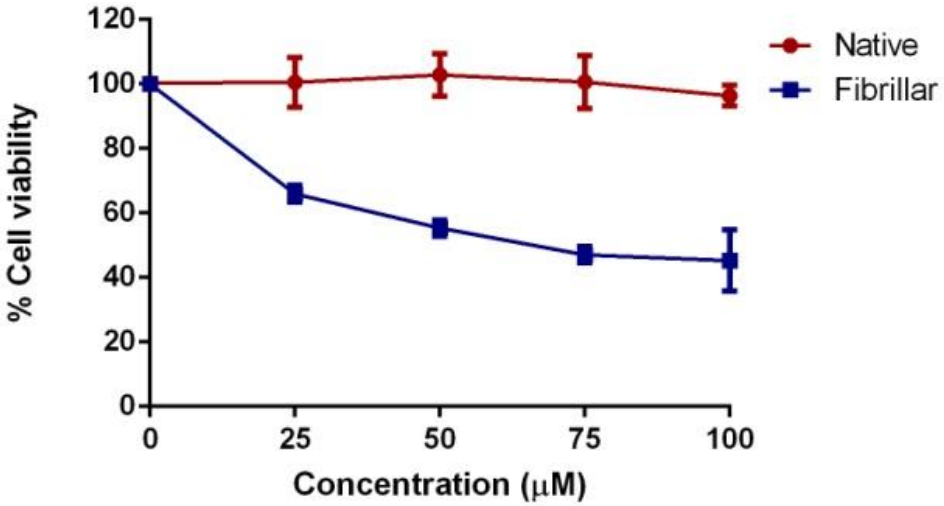
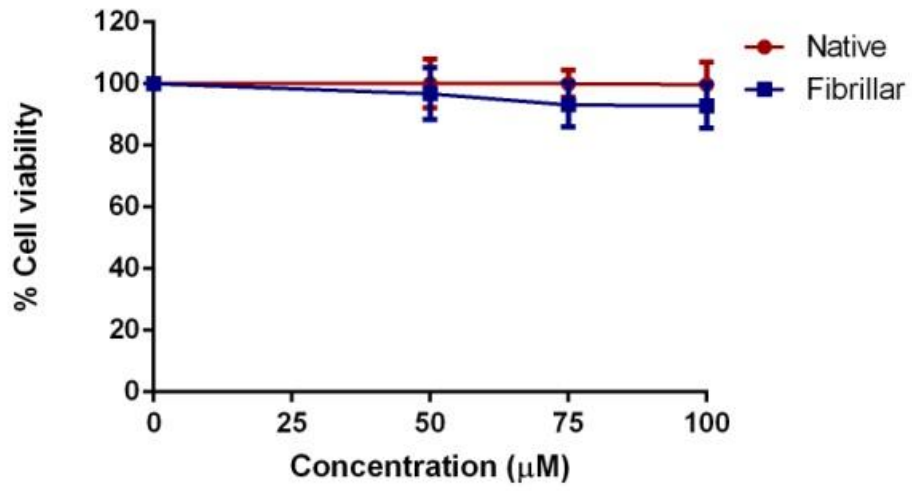


Figure 2.4: Concentration-dependent cytotoxicity of native (prefibrillar) and fibrillar SEVI. (a), PAP248-271 (b) and PAP257-267 (c) towards PC 12 cells. Peptides (1 mM) were dissolved in phosphate buffer (200 mM, pH 7.2) and either used in their native form or incubated for five days at 37 ºC to induce fibril formation. a. SEVI, b. PAP248-271 and c. PAP257-267. Native (red) and fibrillar (blue) forms were then added to the cell culture media of PC 12 cells and incubated for 48 h. Cell survival was assessed using the MTT assay. Values are presented as percentage of cell survival compared with control. Results are expressed as mean ± standard error of three independent experiments (One-way ANOVA, $p < 0.05$).

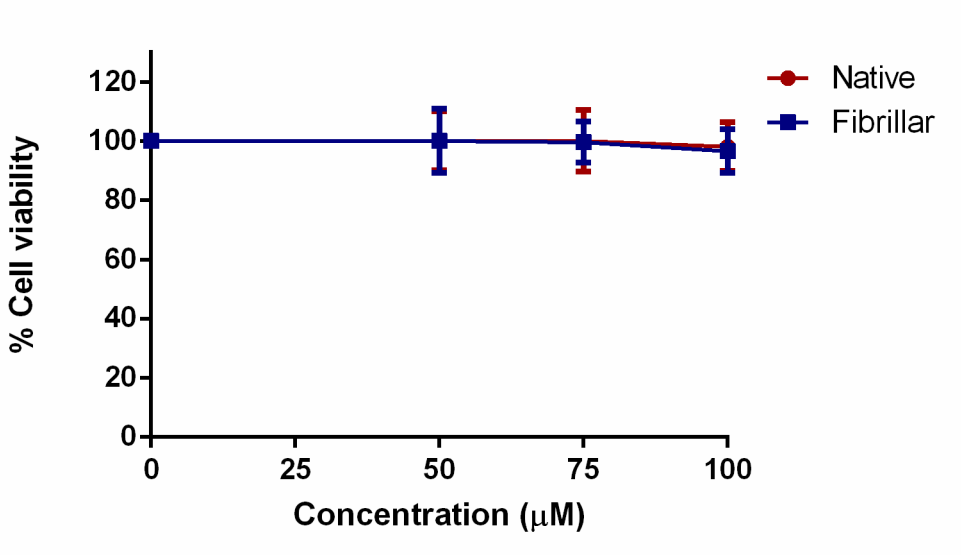
The toxicity of a variety of amyloid fibrillar species to neuronal cells is well established (Chiti and Dobson, 2006, Dehle et al., 2010, Yankner and Lu, 2009, Lorenzo and Yankner, 1994). However, their toxicity to other cell types is not so clear. Of relevance to this study, SEVI fibrils will encounter epithelial cells *in vivo*, rather than neuronal cells. While the primary mechanism of amyloid-induced increase in HIV infectivity appears to arise from increased viral binding to target cells (Münch et al., 2007), toxicity to epithelial cells could

plausibly also increase HIV entry by damaging the mucosal barrier. Indeed, anything that compromises mucosal epithelial integrity significantly increases susceptibility to HIV infection (Shattock and Moore, 2003). To investigate whether SEVI and its fragments were toxic to epithelial cells, we used immortalized intestine epithelial cells, CACO-2. When CACO-2 cells are cultured in a dish they differentiate to form a confluent monolayer consisting of columnar and polarized cells that express microvilli on the apical membrane and tight junctions between neighbouring cells (Hidalgo et al., 1989, Sun et al., 2008). The SEVI peptides were dissolved and incubated to form fibrils and added to the CACO-2 cells to final concentrations of 50, 75 and 100 µM. It was observed that native and fibrillar SEVI and its fragments were not toxic to confluent CACO-2 cells over this concentration range (Fig.2.5).

a. SEVI



b. PAP248-271



c. PAP257-267

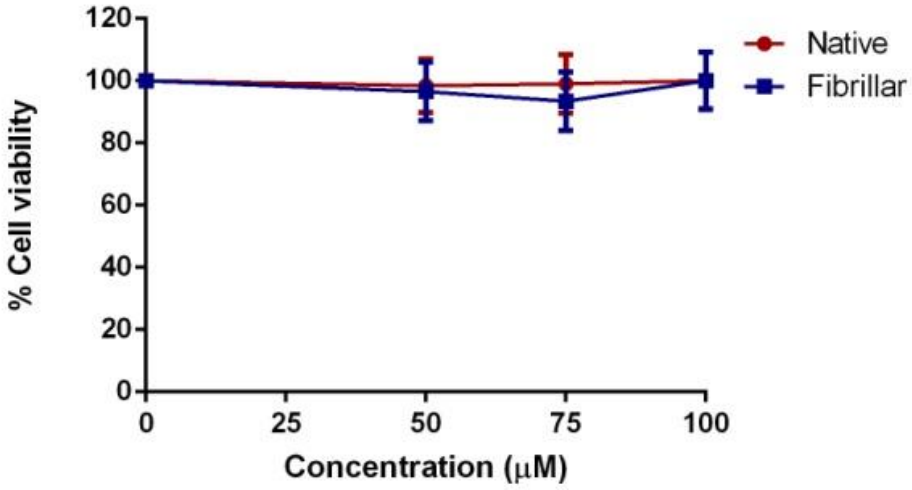


Figure 2.5: Concentration-dependent cytotoxicity of native and fibrillar SEVI, PAP248-271 and PAP257-267 to CACO-2 cells. Peptides (1 mM) were dissolved in phosphate buffer (200 mM, pH 7.2) and either used in its native form or incubated for 5 days at 37 ºC to induce fibril formation. a. SEVI, b. PAP248-271 and c. PAP257-267. Native (red) and fibrillar (blue) forms were then added to cell culture media of CACO-2 cells and incubated for 48 h and cell

survival was assessed using the MTT assay. Values are presented as percentage of cell survival compared with control. Results are expressed as a mean ± standard error of three independent experiments**.** SEVI, PAP248-271 and PAP257-267 did not decrease cell viability (One-way ANOVA, (p<0.05).

We also investigated whether non-toxicity to CACO-2 cells is specific to SEVI and its PAP248-271 and PAP257-267 fragments, that is, whether CACO-2 cells are resistant to fibrillar species in general. We performed toxicity assays with fibrils formed by the milk proteins α_{S2}-casein (Thorn et al., 2008) and reduced and carboxymethylated κ-casein (RCMκ-CN) (Ecroyd et al., 2008). Fibrils formed by α_{S2}-casein and RCMκ-CN are toxic to PC12 cells (Dehle et al., 2010)(Elias *et al*, unpublished results). As with SEVI and its fragments, the native and fibrillar forms of RCMκ-CN caused little death to CACO-2 cells over a wide concentration range (50-100 μM, results not shown) but fibrillar α_{S2}-casein was toxic to CACO-2 cells (Fig.2.6), with a fibril concentration of 5 μM leading to 28% cell death and 50 μM causing 33% cell death. At lower fibril concentrations, no cell death observed.

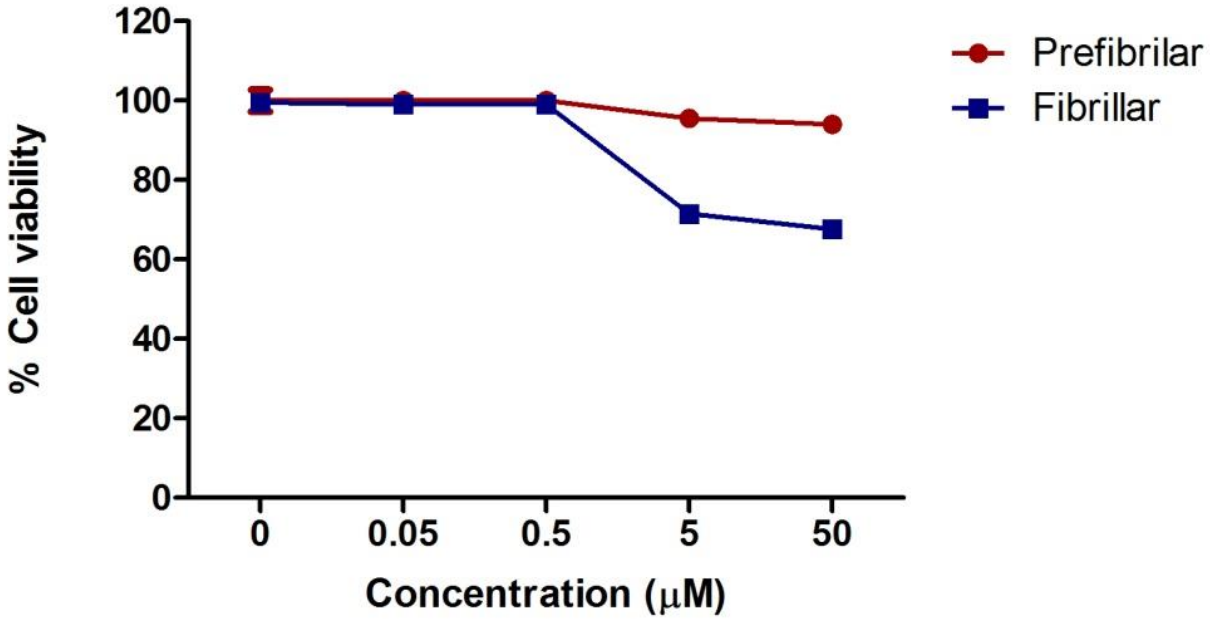


Figure 2.6: Concentration-dependent cytotoxicity of native and fibrillar α_{s2}-casein (0.5 mM) to CACO-2 cells. α_{s2}-Casein was dissolved in phosphate buffer (50 mM, pH 7.2) and either used in its native form or incubated for five days at 50 ºC to induce fibril formation. Native (red) and fibrillar (blue) forms were then added to cell culture media of CACO-2 cells and incubated for 48 h and cell survival was assessed using the MTT assay. Values are presented as percentage of cell survival compared with control. Results are expressed as a mean ± standard error of three independent experiments. α_{s2}-Casein decreased cell viability in concentration dependant manner (One-way ANOVA, $p < 0.05$) Errors are less in magnitude that the size of the individual data points.

2.5 DISCUSSION

A range of studies have shown that PAP248-286 forms fibrils termed SEVI (Münch et al., 2007, Popovych et al., 2012, Olsen et al., 2012, Hartjen et al., 2012, Kim et al., 2010, Capule et al., 2012, Easterhoff et al., 2011, Roan et al., 2010, Hauber et al., 2009). In this study, we confirmed and extended these findings to show that SEVI contains fragments (PAP 248-271 and PAP 257-267) that readily form amyloid fibrils. Furthermore, SEVI fibrils and the fibrils formed by these fragments are toxic to neuronal model PC 12 cells.

Structure prediction algorithms imply that PAP257-267 (R257-I267) is amyloidogenic, as was observed for this peptide. This region is similar in sequence to the fragments V261-H270 and G260-N265 which Nanga *et al*. (2009) and Sievers *et al*. (2011) respectively showed, to be amyloidogenic regions of SEVI precursor peptide PAP248-286. Our results imply that the preceding N-terminal residues promote fibril formation, due to the high fibril-forming propensity of PAP248-271, even though this region (PAP248-256), on its own, has no fibril forming propensity. Münch *et al.* (2007) investigated other fragments of SEVI (PAP250-286, PAP251-286, PAP252-286, PAP253-286, PAP248-282, PAP248-266 and PAP267-282) and found that length variation at the N-terminus of the PAP fragments did not impair fibril formation but deletion of the four C-terminal residues of SEVI (L283 to Y286) reduced the degree of fibril formation. Others have shown that a C-terminally truncated fragment of SEVI, PAP248-266, does not form fibrils (Roan et al., 2009, Martellini et al., 2011, Münch et al., 2007). In our study, the presence of I267-M271, when placed on the end of the first 19 amino acids of SEVI led to rapid fibril formation of PAP248-271. It is therefore concluded that these five amino acids are crucially important in reducing the lag phase thereby leading to rapid fibril formation by SEVI. The C-terminal region (PAP272-286) does not form fibrils and accordingly has a moderating effect on fibril formation of the entire SEVI peptide

(PAP248-286). The C-terminal region has a proline at position 278, a residue which has a significant role in the prevention of aggregation during fibril formation and protein folding *in vitro* (Samuel et al., 2000, Morimoto et al., 2004), as well as sequential lysine residues K281 and K282 which limit aggregation of PAP272-286 (Nanga et al., 2009). It is concluded that these three residues (P278, K281 and K282) moderate the predicted aggregation-prone propensity of other more C-terminal residues of PAP248-286 (Figure 2.1b,c), as speculated by Nanga *et al.* (Nanga et al., 2009) for the fragment K281-Y286.

The integrity of the mucosal epithelial barrier is a critical determinant of susceptibility to HIV infection (Shattock and Moore, 2003). Thus, determining if SEVI and its fragments are cytotoxic is important to fully understanding their role in HIV transmission. Fibrillar SEVI and its fragments were toxic to neuronal PC 12 cells. Toxicity to neuronal cells has been found for all fibrillar proteins examined to date, and SEVI is no exception. The amount of PAP produced in semen (1-2 mg/mL) (Ronnberg et al., 1981) considerably exceeds the concentration of SEVI (0.46 mg/mL) and its fragments (0.06-0.14mg/mL) required to cause 50% neuronal cell death in the assay undertaken in this study. The α-helical conformation has been implicated in the binding and membrane-perturbing effects of a variety of amyloid proteins (Knight et al., 2006, Jo et al., 2000, Terzi et al., 1997). Brender *et al*. (2009) showed that monomeric PAP248-286 binds to membranes in a partial α-helical conformation and weakens the integrity of the membrane in such a way that may promote fusion by the HIV gp41 protein. Thus, most likely SEVI (and its fragments) adopts a helical conformation when interacting with the membrane of PC 12 cells which, by mechanism(s) that may involve oligomerisation and pore formation, lead to cell disruption (lysis).

In contrast, there was little cytotoxicity of SEVI and its fragments to confluent CACO-2 cells, our model for mucosal epithelial cells. Another fibril-forming protein, κ-casein that is also toxic to PC 12 cells, had no toxic effect in confluent CACO-2 cells. These results are

consistent with the finding that Aβ, the neurotoxic peptide (Yankner and Lu, 2009, Dehle et al., 2010, Chiti and Dobson, 2006) that is the major component of extracellular plaques in Alzheimer's disease (Vivekanandan et al., 2011), is not toxic to endothelial cells and marginally toxic to confluent lung epithelial cells, although its more toxic to non-confluent cells (Balcells et al., 2008). Munch *et al*. (2007) also found that mixtures of 10% semen pellet containing the spermatocytes and large protein aggregates displayed only weak cytotoxic effects to TZM-bl cell cultures at high cell density. Balcell *et al.* (2008) speculated that fibrillar species may be more toxic to growing or damaged cells. Our findings suggest that even though SEVI may assist HIV-1 to attach to cells (Münch et al., 2007, Roan et al., 2009), it does not in itself harm healthy, confluent epithelial cells. However, in the presence of a damaged epithelial cell layer it might cause further damage, increasing the access of HIV-1 to its target cells. Studies are required to determine the toxicity, or otherwise, of SEVI and its fragments to non-confluent epithelial cells.

It is possible that PAP248-286 (which forms SEVI), is broken down into smaller fragments in semen via further proteolytic activity. The unstructured nature of native PAP248-286 would make it highly susceptible to proteolytic attack. Fragmentation is a secondary nucleation step that is essential for seeding and amyloid fibril growth in general (Knowles et al., 2009). Hence SEVI fragments, for example PAP248-271 which has little to no lag phase and is highly amyloidogenic, might act as a seed and thereby promote fibril formation of SEVI in semen. The results of this study indicate that there are fibril-forming fragments of SEVI that are toxic to neuronal cells but not to confluent epithelial cells. Potentially, the presence of these SEVI fragments in semen could provide additional avenues for promotion of HIV infection.

2.6 ACKNOWLEDGEMENTS

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3 SEVI and its fragment help HIV-1 attach to host cells via coreceptors CCR5 and CXCR4

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3.1 Abstract

Human Immunodeficiency virus type 1 (HIV-1) enters the cells by the interaction of the viral gp120 envelope (Env) glycoproteins with cellular cluster of differentiation (CD) 4 protein and a secondary coreceptor (Cashin et al., 2013). Secondary coreceptors are typically one of the chemokine receptors CCR5 (R5) and CXCR4 (X4). The other alternative coreceptors that can act as lentiviral coreceptors and mediate the entry of certain HIV-1, HIV-2 and simian immunodeficiency virus (SIV) includes CCR1, CCR2, CCR3, CCR8, CX3CR1, CXCR6, FPRL1, GPR15, APJ, STRL33 and D6 (Gorry et al., 2007, Farzan et al., 1997, McKnight et al., 1998, Reeves et al., 1999). HIV-1 Envs are characterized by their ability to use CCR5, CXCR4 or both coreceptors for entry. We show that amyloidogenic fragments known as

semen-derived enhancer of virus infection (SEVI) originating from prostatic acid phosphatase use both R5 and X4 to enhance HIV-1 entry to the host cells. These results show that even though SEVI amyloid fibrils enhance HIV-1 infection, it does not bypass the requirement for the appropriate coreceptor. Therefore, SEVI may promote HIV-1 attachment to genital surfaces and penetration of mucosal barrier by increasing HIV-1 virion binding to epithelial cells and migrating dendritic cells. Human immunodeficiency virus envelope proteins using R5, X4 or both coreceptors were used to determine the coreceptors used by SEVI and PAP248-271 for entry into the host cells. Our results provide additional insights into virus-cell interactions with the help of amyloidogenic peptides.

3.2 Introduction

3.2.1 The HIV epidemic

The first cases of acquired immunodeficiency syndrome (AIDS) were discovered in healthy American men suffering from *Pneumocystis carinii* pneumonia (PCP) in 1981 (Gottlieb et al., 1981). The causative agent, human immunodeficiency virus type-1 (HIV-1) was identified a few years later (Barre-Sinoussi et al., 1983). The HIV epidemic now affects more than 35 million people worldwide (UNAIDS, 2013). Since the discovery of HIV and AIDS more than 25 million people have died because of HIV-related causes. Sub-Saharan Africa is the region that is mostly affected with 67% of the worlds HIV epidemic. Teenage girls and young women account for one in four new HIV infections in sub-Saharan Africa. There were approximately 2.1 million (1.9-2.4 million) new HIV infections in 2013 (UNAIDS, 2013). Even though HIV-1 infections has killed millions and rapidly spreading in the human population, the efficiency of HIV-1 transmission via the sexual route is poorly understood. Despite the growth and prevalence of HIV-1 infections, HIV-1 is an unexpectedly pathogen with low infectivity (Castellano and Shorter, 2012, Dimitrov et al., 1993, Rusert et al., 2004). For instance, it is estimated that the risk of male-to-female intravaginal HIV-1 transmission is about 1 event per 200-2000 coital acts (Gray et al., 2001). However this rate is about 10-fold increased (Pilcher et al., 2004) during acute infection when the viral load is particularly high and further enhancement occur when sexual practices are associated with bleeding and lesions of the mucosal barrier as well as the presence of other sexual transmitted diseases (Galvin and Cohen, 2004). In vitro, semen can enhance the infection of different cell types including CD4+, T cells and macrophages.

3.2.2 Human Immunodeficiency Virus (HIV)

HIV is a member of the genus lentivirus of the retroviridae family. The primary targets of HIV are cells of the human immune system including monocytes (macrophages), lymphocytes (CD4+ T cells) and dendritic cells (Barre-Sinoussi et al., 1983, Grimwood et al., 1983). HIV is a descendent of simian immunodeficiency virus (SIV) which infects non human primates (Fig 3.1). There are two major types of HIV, HIV-1 and HIV-2. HIV-1 is classified into three groups including M, N and O (Fig 3.1) (Kuiken, 1999). The three groups represent the transmission of SIV into humans. 90% of all HIV-1 infections are from viruses belonging to group M (major) while groups N and O are restricted to certain geographic regions and occur rarely. Group M can be further subtyped based on the gene sequence. These subtypes are referred to as clades A, B, C, D, E, F, G, H, J, K (Fig 3.1) and hybrid forms known as *circulating recombinant forms* (CRFs) (Dalgleish et al., 1984). Clades A and D are predominant in Africa and clade C, which causes most of all infections worldwide is predominantly found in East and Southern Africa, Nepal and India (Dalgleish et al., 1984, Kuiken, 1999). Subtype B is most predominant in western countries such as Australia and all the strains used in this study are subtype B.

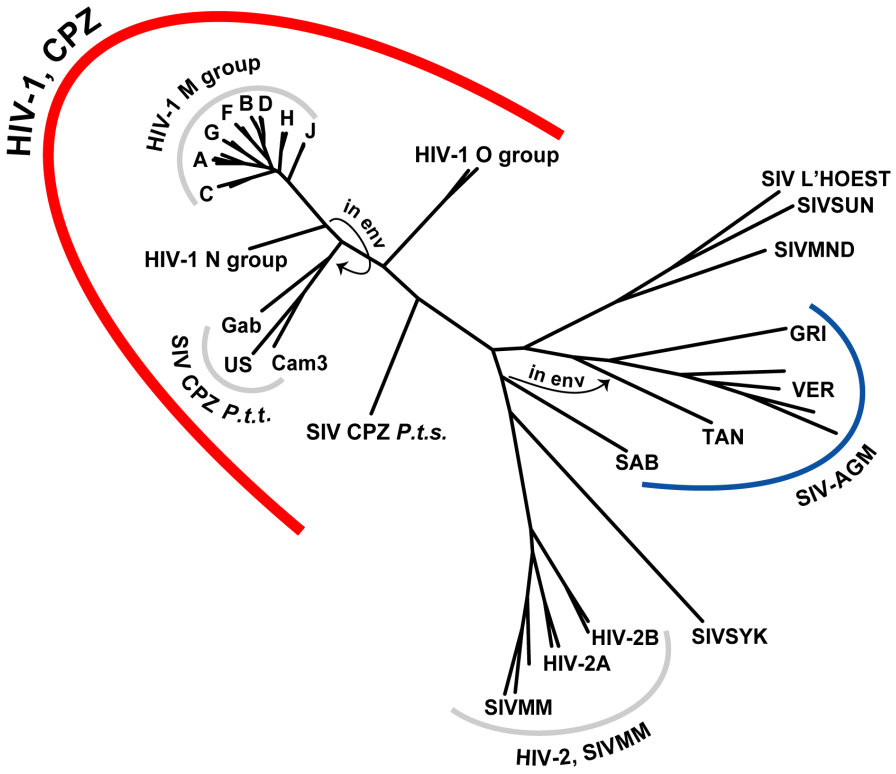

Figure 3.1: Phylogenetic tree of HIV and SIV. Figure from Kuiken (1999)

3.2.3 HIV structure

HIV has a roughly spherical structure of 100 nm in diameter. The genetic information of HIV is in a diploid single-stranded RNA that contains approximately 9,700 basepairs which encodes nine open reading frames (Frankel and Young, 1998). Three of the reading frames encode the Gag, Pol and Env polyproteins which are subsequently proteolyzed into individual proteins (Fig 3.2). As, shown in Fig 3.2, the virion is enveloped by a lipid membrane acquired from the host cell. The four Gag protein, MA (matrix), CA (capsid), NC (nucleocapsid), and p6, and the two Env proteins which includes SU (surface or gp 120) and TM (transmembrane or gp41), make up the core structure of the virion and the outer membrane envelope (Frankel and Young, 1998). Trimetric viral complexes of gp 120 and gp proteins are integrated into this membrane. The enzymatic functions are encapsulated by the

three Pol proteins, PR (protease), RT (reverse transcriptase) and IN (integrase) within the particle (Frankel and Young, 1998). HIV-1encodes six additional proteins found in the viral particle called accessory proteins which include Vif, Vpr and Nef. The two other accessory proteins, Tat and Rev, provide regulatory functions while the last protein, Vpu, indirectly assists in assembly of the virion (Gorry et al., 2007).

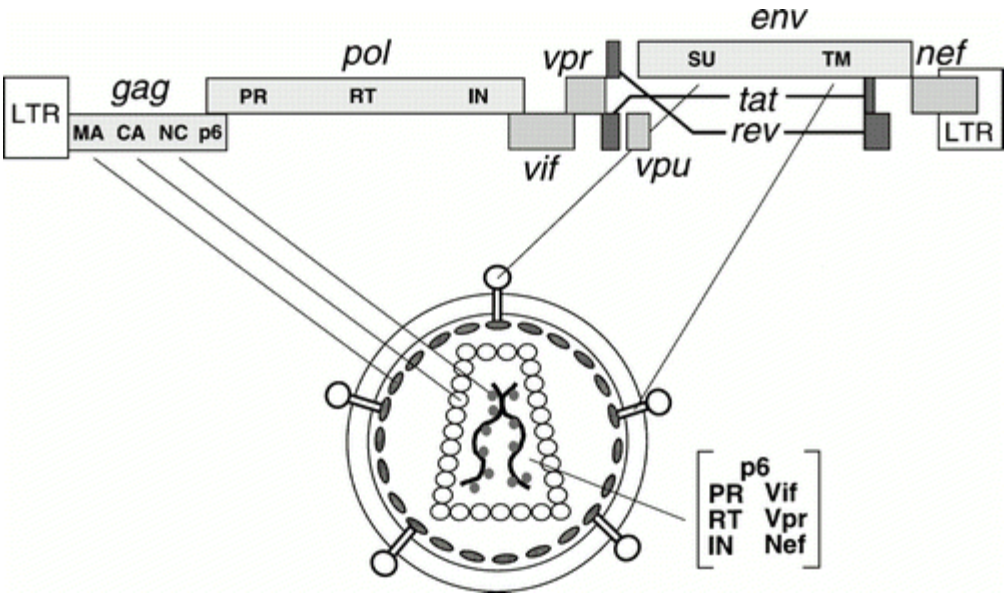

Figure 3.2: The HIV genome and virion. Adapted from (Kuiken, 1999).

3.2.4 Sexual transmission of HIV

Sexual transmission of HIV-1 is between 75-85% of the 35 million infections that have occurred so far (UNAIDS, 1996). The remaining percentage is shared between other routes including needle sharing, mother to infant and transfusion. HIV infectivity is the average probability of transmission to another person after that person is exposed to an infected host (Royce, 1997). There are two parameters together with infectivity that determines whether the epidemic slows or grows. The parameters are duration of infectiousness and the average

rate at which susceptible people change partners (Anderson et al., 1991). Host-related and environmental factors such as social, cultural and political milieu can amplify the epidemic.

Susceptibility of the host depends on the entry of the virus in to cells through CD4 and chemokine surface receptors (Weiss, 1996, Dragic et al., 1996). These cells include CD4 T lymphocytes, macrophages and Langerhan's cells. HIV receptive cells are found in cervicovaginal, foreskin, rectal epithelia and urethral.

3.2.5 Replication strategy for HIV-1

HIV is a retrovirus, i.e. an RNA virus that replicate in a host cell via the enzyme reverse transcriptase (RT) to produce DNA from its RNA genome (Engelman and Cherepanov, 2012). After the complementary DNA synthesis, the DNA is then integrated into the host's cell nucleus by the viral enzyme integrase (IN) (Fig 3.3). Once integrated the virus is called provirus. Then the DNA hijacks the host cell, and directs the cell to produce multiple copies of viral RNA. The process of reverse transcription is very error-prone due to lack of proof-reading capacity by RT. As a result, HIV is highly variable and mutation prone, therefore able to evade neutralizing antibodies and to develop resistance to antiretroviral agents (Preston et al., 1988, Roberts et al., 1988).

Like most viruses, HIV does not have the ability to reproduce independently. Its replication cycle involves the recognition and invasion, reverse transcription, integration to the host cells and the release of new virions (Fig 3.3). During infection, the viral surface protein gp120 binds to CD4 receptor on the host cell (Engelman and Cherepanov, 2012). The CD4 receptor is expressed on the surface of various cells in the body including monocytes, macrophages, T lymphocytes, microglia and dendritic cells (Berger et al., 1999). The binding of gp120 to CD4 induces a conformational change in the viral envelope glycoprotein complex that enables binding to a β-chemokine coreceptor, either CCR5 (R5) or CXCR4 (X4) (Berger et

al., 1999). Coreceptor binding results in additional conformational changes in the envelope spike and expose the trimeric gp41 structure leading to fusion of the viral envelope with the host ‘s cell membrane thereby allowing the viral genome and associated viral proteins to enter the cytoplasm (Moseri et al., 2014).

The HIV-1 Env protein determines the entry phenotype of the virus, typically using CD4 as the receptor and CCR5 as the coreceptor. (Joseph et al., 2014). The ability of HIV-1 to replicate in a particular cell type requires adaptation of the viral envelope protein to effectively utilize the receptor and the coreceptor on that cell type. CXCR4-using virus in late infection has long been thought to show adaptation to infect a host (Arrildt et al., 2012), most likely CD4+ naïve T cells, which are known to express high levels of CXCR4 and very little CCR5 (Zamarchi et al., 2002).

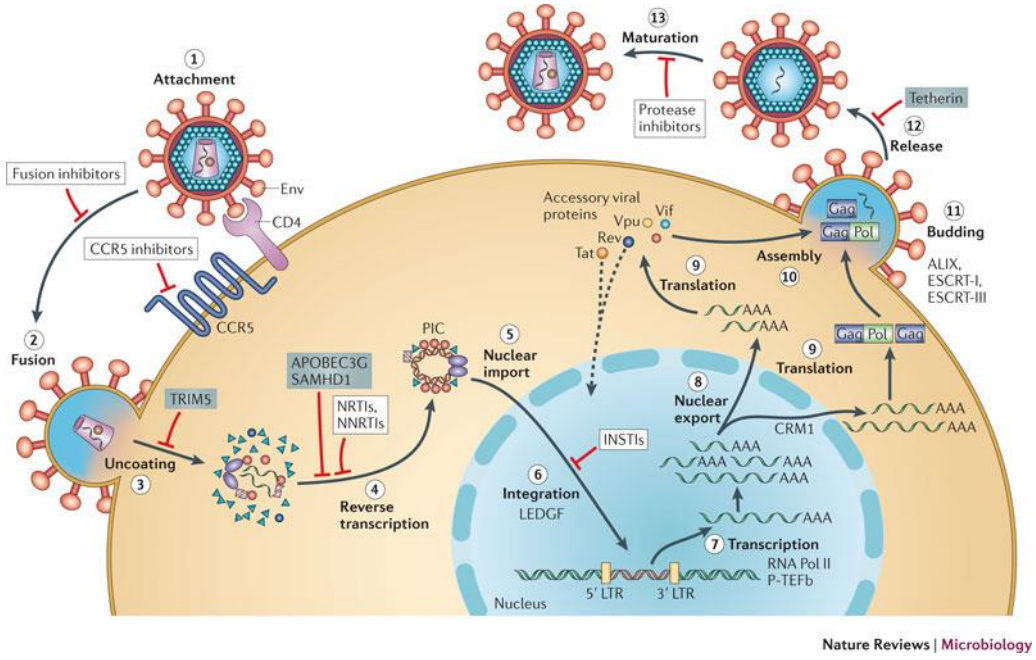

Figure 3.3: Schematic overview of the HIV-1 replication cycle. Figure from Engelman and Cherepanov (2012).

It has been shown that a fragment of prostatic acid phosphatase (PAP), forms fibrils (SEVI) that drastically enhances HIV-1 infectivity by several orders of magnitude by capturing HIV-

1 virions and promoting their attachment to target cells (Münch et al., 2007). SEVI promotes the interaction between virions and the cell surface and is independent of the viral glycoprotein and hence is not restricted to HIV-1, although fusion between the viral and cellular membranes still requires gp120, CD4, and an appropriate coreceptor (Münch et al., 2007, Hong et al., 2009).

In this study we determine the appropriate coreceptor that SEVI and its fragment PAP248-271 use to promote fusion and entry of the virions to the host cells thereby enhancing HIV infection. The use of Env offered an opportunity to elucidate whether Env are selected for during the enhancement of HIV infection by SEVI and PAP248-267 that is reflected in an increased ability to utilise alternative coreceptors *in vitro*, and whether particular patterns of alternative coreceptor engagement are linked to the preference of the virus for CCR5 or CXCR4. We demonstrate that SEVI and PAP248-271 greatly enhances the infectivity of HIV-1 by binding to both CCR5 and CXCR4 coreceptors.

3.3 Materials and Methods

3.3.1 Virus Stocks

Virus stocks were titrated by infecting cells expressing CD4 and/or CCR5. The volume of each virus stock (YU-2, ADA, HXB2, 89.6 and JRCSF) was calculated by the volume of each virus stock that generated 800,000 relative light units (RLU) of luciferase expression when infecting maximally induced Affinofile cells. Affinofile cells are a stable cell line that can be effectively induced to express combinatorial amounts of CD4 and CCR5 receptor cells. These experiments were performed in a PC3 laboratory at the Burnet Institute in Melbourne

3.3.2 Cell culture

JC53 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FBS) and 1% penicillin and streptomycin. Affinofile cells were maintained in DMEM with 4.5 g/L glucose supplemented with 10% dialyzed FBS and 50 mg/mL blasticidin.

3.3.3 Affinofile cell assays

Affinofile cells were plated at the concentration of $1.8x10^4$ cells per well in a 96 well plates and incubated for 24 hours. After incubation, ponasterone and doxycycline were added to the cells at various concentrations to induce CD4 and CCR5 expression and incubated further for 24 hours. The medium was removed and replaced with medium lacking ponasterone and doxycycline and viruses were added to the plates. Cells were spinoculated (i.e. the centrifugation of cells together with inoculated virus) at 2,000 rpm for 2 h at 37°C and then incubated at 37°C for 48h. After 48 h, the cells were washed twice with phosphate buffer and lysed with 50 µL of 1x reporter lysis buffer and the lysate was stored at -80°C. Virus entry

was then assessed by thawing the lysates and quantifying luciferase expression by using assay system.

3.3.4 Effect of SEVI on viral infectivity

Synthetic PAP fragments were purchased from Mimotopes Australia. Peptides were dissolved in phosphate buffer (200 mM, pH 7.2) and incubated at 37°C at 700 rpm for 48 hours to induce fibril formation. Virus infections were performed in triplicates in 12-well plates with 2.5×10^4 cells plated 24 hours before infection. To determine the coreceptors used by SEVI and PAP248-271, we mixed the peptides with virus preparations that are representative for R5 HIV-1 Env (YU-2, JRCSF and ADA isolates), X4 HIV-1 Env (HXB2 isolates) and R5X4 (dual) HIV-1 (89.6 isolates).

3.3.5 Cytotoxicity assays

JC 53 cells (1.5×10^5) were cultured in a 96 well plate to confluency. Serially diluted pre-incubated and incubated peptides (SEVI and PAP248-271) (six replicates per treatment) were added to the cells and incubated for 48 h. Following incubation media was removed gentle aspiration. Cell viability was evaluated using a 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

3.3.6 MTT assay

100 µL MTT (0.25 mg/mL) containing media was added to each well and the plate was for 3 hours at 37 ºC. After incubation, media was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan. Formazan absorption was measured at 560 nm using Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany). The mean of six replicate readings was taken and cell viability (%) was calculated by dividing the average absorption readings of treated wells by the average absorption readings of untreated wells (phosphate buffer only) and multiplying by 100.

3.4 Results

3.4.1 SEVI and its fragments are not toxic to JC 53 cells

Toxicity of SEVI and PAP248-271 to JC 53 cells, to be used for the virus infectivity assays was determined. Serial diluted SEVI and PAP248-271 (0.0001-1000 µg/mL) pre-incubated and incubated fibrils were added to JC 53 cells and incubated for 48h and cell viability determined as outlined in the material and methods section (Cytotoxicity). Fibrils formed by SEVI and PAP248-271 were not toxic to the cells (Fig 3.4). The higher concentration of both peptides, 100 µg/mL, showed a little toxicity with cell viability of around 80%. Pre-incubated peptides were not toxic to the cells (results not shown). Overall, the cells showed minimal toxicity to the fibrils formed by both peptides and therefore were suitable for use in affinofile assays.

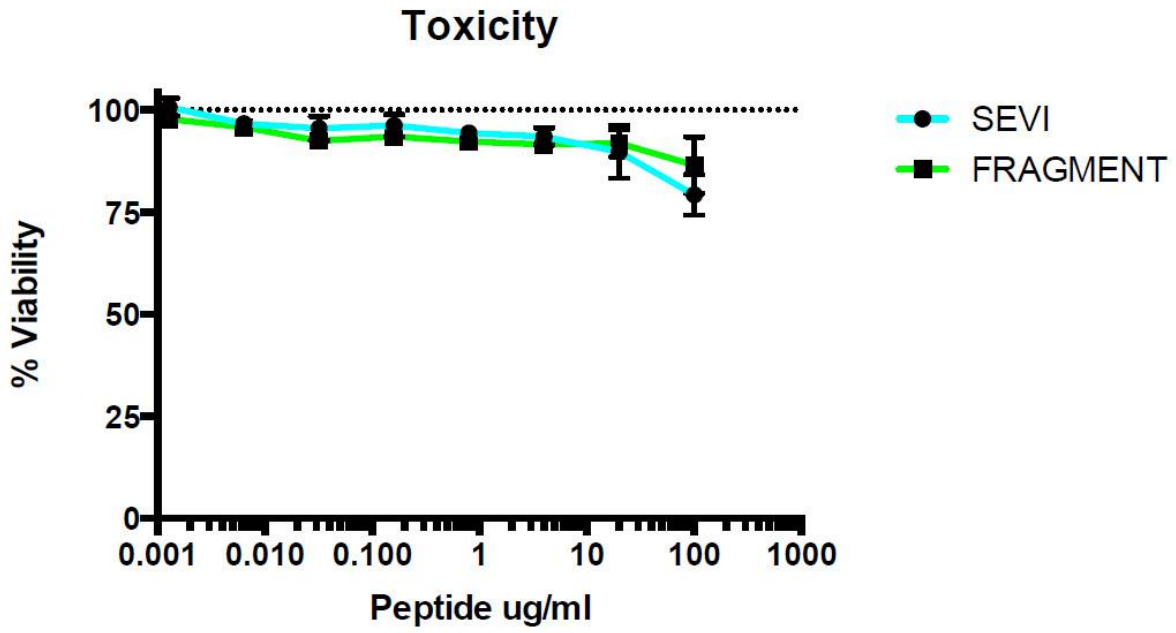

Figure 3.4: Concentration-dependent cytotoxicity of SEVI (●) and PAP248-271 (■) to JC 53 cells. Cell survival was assessed using MTT assay. Values are presented as percentage of cell survival compared with control. Results are expressed as mean ± standard error of three independent experiments ($p<0.05$).

To assess whether the virus use a particular receptor to infect the cells while in the presence of SEVI and PAP248-271, we analysed SEVI and PAP248-271's effect on a large panel of HIV with different viral envelope proteins that uses either R5 (YU-2, ADA, JRCSF), X4 (HXB2) or dual, R5/X4 (89.6) coreceptors. We found that SEVI (Fig 3.5) and PAP248-271 (Fig 3.6) enhanced infection by R5, X4 and dual-tropic HIV-I clones at different concentrations. SEVI enhanced infection was seen at concentrations ≥ 25 µg/mL with the most enhancement at the highest concentration of 100 µg/mL (Fig 3.5). With amyloidogenic PAP248-271, enhanced infection was observed at a very low concentration of ≥5 µg/mL. The most enhancement was shown by ADA virus. The most dramatic effects were observed after

infection with high concentrations (≥ 50µg/mL) of PAP24-271 in all the viruses (Fig 3.5). The magnitude of PAP248-271-mediated HIV-1 infectivity enhancement of this high concentration of the fragment decreased from 29-60% in between the different viruses. Thus, the enhancing activity of PAP248-271 is most pronounced when the concentration is low.

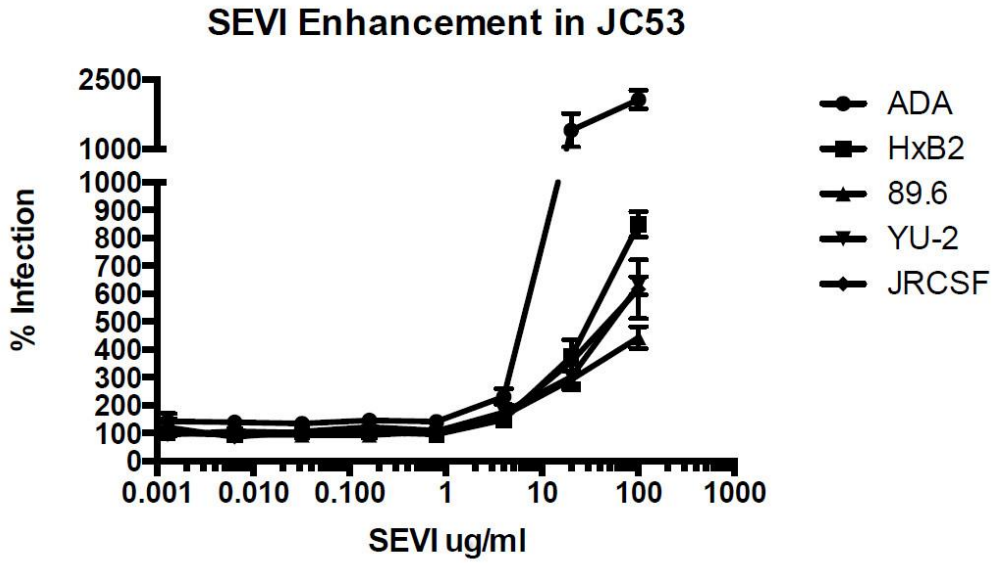

Figure 3.5: SEVI average enhancement (n=3) of HIV-1 infection of JC 53 cells by R5-(ADA (●), YU-2 (▼), JRCSF (♦)), X4 (HXB2 (■)), and dual (89.6 (▲)) viruses. JC 53 cells were infected with HIV-1 (5 ng) of R5 and X4 HIV-1 the presence of indicated concentration of SEVI. Values are represented as % infection compared with control. All data shown in this figure give average values ± SD obtained from triplicate infections.

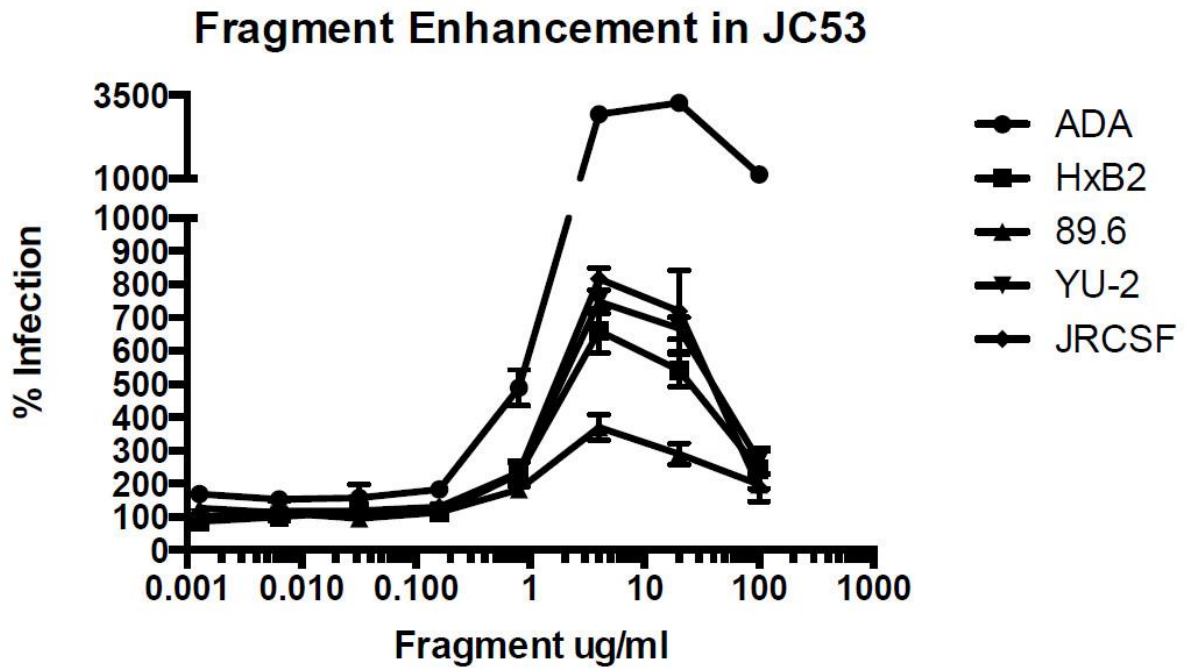

Figure 3.6: Average enhancement (n=3) of HIV-1 infection of JC 53 cells by R5- (ADA (●), YU-2 (▼), JRCSF (♦)), X4 (HXB2 (■)), and dual (89.6 (▲)) viruses in the presence of varying concentrations of amyloidogenic PAP248-271. JC 53 cells were infected with HIV-1 (5 ng) of R5 and X4 HIV-1 the presence of indicated concentrations of PAP 248-271. Values are represented as % infection compared with control. All data shown in this figure give average values ± SD obtained from triplicate infections.

3.5 Discussion

HIV-1 has a limited ability to cross the mucosal barrier in the genital tract to infect sufficient numbers of cells and establish a sustained infection (Haase, 2005). The first step in HIV-1 infection involves the binding of gp120 to the cell surface molecule CD4 of the target cell. CD4 serves as major receptor for both HIV-1 and HIV-2 and simian immunodeficiency viruses (SIVs) (Klatzmann et al., 1984, Dalgleish et al., 1984, Maddon et al., 1986). The binding of CD4 to gp120 and gp41 results in the exposure of Env domains that are thought to be involved directly with membrane fusion reaction (Sattentau and Moore, 1991, Sattentau et al., 1993, Kang et al., 1993, Thali et al., 1993). gp120 and gp41 are organised into trimeric complexes on the virion surface (Robey et al., 1985).

The gp120 is heavily glycosylated and contains protruding variable loops (Leonard et al., 1990). Gp120 interacts with CD4 via surface-exposed residues within three separate regions distributed over six segments of gp120 (Wyatt et al., 1993, Wyatt et al., 1998). The interaction between gp120 and CD4 promote conformational changes that expose the binding site for chemokine coreceptors CCR5 and CXCR4 (Wu et al., 1996, Trkola et al., 1996). The chemokine receptor CCR5 is the major coreceptor for primary HIV-1 isolates (Dalgleish et al., 1984, Klatzmann et al., 1984, Maddon et al., 1986) while CXCR4 is the predominant coreceptor used by the primary T-cell tropic and laboratory-adapted HIV-1 strains (Feng et al., 1996).

In chapter 2 we found that SEVI does not enhance HIV infection by compromising an intact layer of epithelium cells. An intact mucosal epithelium provides a strong physical barrier to HIV-1 infection (Miller and Shattock, 2003). However, there are number of things that compromise epithelium integrity including sexual intercourse and the presence of ulcerative

sexually transmitted diseases (Münch et al., 2007). Moreover, semen might cause local inflammation or epithelial breaks and induce DC projections to the luminal surface (Sharkey et al., 2007). Therefore, with compromised epithelium integrity, semen components such as SEVI might frequently be able to access $CD4^+$ T cells, macrophages and dendritic cells (which have R5 and X4 receptors) in the subepithelium to enhance HIV-1 attachment, infection and dissemination.

In this study, we show that SEVI and its fragment PAP248-271 enhanced HIV-1 infectivity of both R5 and X4 *in vitro*. Münch et al (2007), explored the ability of SEVI to enhance HIV-1 infection *in vivo* by infecting hCD4/hCCR5-transgenic rats with HIV-1 YU2 and found that SEVI significantly enhanced the infectivity of CCR5 HIV-1 *in vivo*. Our *in vitro* results support the findings by Münch and his collegues but do show the same magnitude of enhancement to CXCR4 HIV-1. On the other hand, it has been suggested that shorter fibrils are particularly effective in promoting the infectiousness of HIV-1 (Wojtowicz et al., 2002). Our data is in agreement with this suggestion as PAP248-271 (≥ 5μg/mL), which is smaller in size compared to SEVI (≥25 μg/mL), was more potent in assisting HIV-1 infection than the large SEVI fibrils obtained *in vitro* (Fig 3.5 and 3.6). SEVI and PAP are highly positively charged there the charge may be of assistance in reducing the repulsion of negatively charged cell membrane and the HIV therefore helping the virus to infect the cells. Elevated levels of PAP can usually be detected in the vagina for about 24 h after sexual intercourse (Collins and Bennett, 2001), therefore concentrations of SEVI and PAP248-271 may increase after sexual intercourse as PAP is degraded in the vaginal, rectal or oral environment. The dilution by vaginal fluids would not diminish the potency of amyloidogenic SEVI and PAP248-271 that form prior, during or after sexual intercourse in virion attachment and infectivity since ~2 mg/mL of PAP is produced in semen. Lower concentrations of both SEVI and PAP248-271,

≥25 and ≥5 μg/mL respectively are enough for infection enhancement, so it is probably that sufficient SEVI and fragments are produced to enhance HIV infectivity.

So much effort has been conducted to find a solution to stop HIV infection. Current researchers have put more effort to develop therapeutic agents that target coreceptors. The results of this study may aid in finding the agents that block enhancing activity of SEVI and its smaller fragments to prevent new HIV infections. One of these agents, clusterin has been investigated further in chapter 4 for its ability to stop fibril formation by SEVI thereby blocking the enhancement of HIV infection by these amyloid fibrils.

3.6 Acknowledgement

We thank Dr Michael Roche and Anne Witt for their technical assistance.

4 The extracellular molecular chaperone, Clusterin inhibits fibril formation and suppresses cytotoxicity associated with the SEVI peptide.

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4.1 Abstract

The mechanisms enabling correct translation, modification, assembly and transport of synthesized proteins inside the cell have been extensively studied. However, mechanisms to control protein folding and unfolding in the extracellular environment need further investigation. It has recently been proposed that extracellular molecular chaperones play an important part in extracellular quality control of protein folding (Carver et al., 2002). Clusterin is a glycoprotein found in most extracellular fluids and its increased expression accompanies most disease-associated extracellular amyloid deposits in particular Alzheimer's disease (McGeer et al., 1992). However, its roles in amyloid formation *in vivo* are poorly understood.

Clusterin has molecular chaperone activity whereby; similarly to small heat-shock proteins, clusterin potently prevents stress-induced amorphous and amyloid fibrillar aggregation of unfolding, non-native proteins by binding to their exposed hydrophobic regions. In this study, we show that clusterin potently inhibits the formation of amyloid fibrils termed semen-derived enhancer of virus infection (SEVI). The monomeric form of SEVI, PAP248-286 is a 39 amino acid fragment of prostatic acidic phosphatase found in semen. The fibrillar form of this fragment, SEVI, has been found to enhance HIV infection (Münch et al., 2007). The results presented here demonstrate that clusterin prevents the SEVI-induced cytotoxicity to pheochromocytoma 12 cells. At physiological concentrations, clusterin protected PC12 cells by potently inhibiting SEVI fibril formation. Thioflavin T assay and TEM data showed that clusterin halted SEVI fibril formation in a concentration dependent manner with the highest concentration of clusterin showing amorphous aggregates with no fibrils present. *In vivo*, these findings suggest that clusterin, which is abundant in semen, plays an important role inhibiting amyloid fibril formation by SEVI and, in doing so, provides protection to the cells.

Keywords: protein aggregation, SEVI, Clusterin, amyloid fibrils, cytotoxicity, cytoprotection, molecular chaperone

4.2 Introduction

Numerous debilitating and incurable human diseases are associated with extracellular depositions of highly structured protein aggregates in a variety of organs and tissues, including the brain (e.g. Alzheimer's disease), skeletal tissues and joints (e.g. haemodialysis-related amyloidosis) and peripheral organs such as liver, spleen and heart (e.g. systemic amyloidosis) (Chiti and Dobson, 2006, Yerbury et al., 2007, Carver et al., 2003). The protein aggregates are termed amyloid fibrils and arise when, for example, a specific protein or protein fragment loses its correct conformation, subsequently aggregates and forms insoluble deposits. The trigger for misfolding may be stresses such as elevated temperature, low pH, infection, reactive oxygen species and inherited mutations (Dobson, 1999). The intermediately folded proteins that result expose greater areas of hydrophobic regions to solution which encourages their mutual association (Fig 4.1) and hence aggregation (Carver et al., 2003).

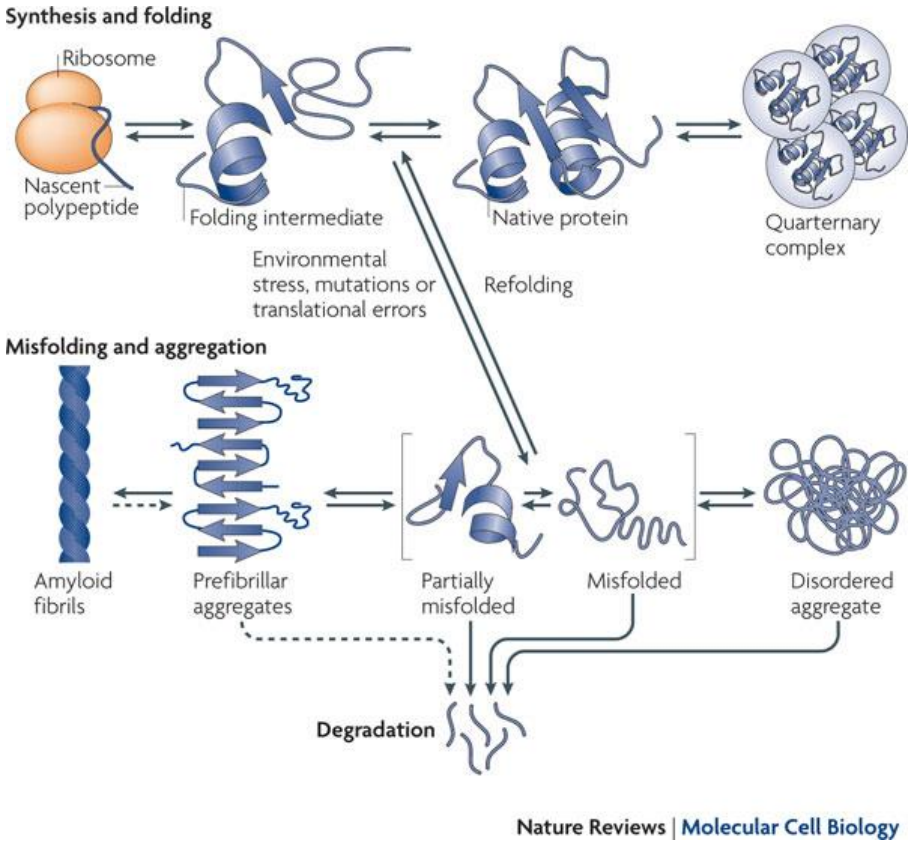


Figure 4-1: A schematic representation of the protein folding and off-folding pathway. A protein folds through different intermediates to its native, three-dimensional structure during and after its synthesis at the ribosome. Under conditions of stresses like decreased pH, mutations in the synthesized protein or translational errors can cause protein misfolding. Once present, misfolded intermediates can be refolded to the native state or be degraded by different cellular proteolysis systems that prevent the accumulation of misfolded proteins. One the quality-control network is overwhelmed, aggregates can form. Arrows indicate a process that can include several single steps; dashed arrows indicate a process of minor significance. Figure from Hertz *et al*. (2013)

In vivo, various mechanisms have evolved to prevent protein aggregation both intra- and extra-cellularly. These mechanisms include quality control and degradation processes and the production of molecular chaperones.

4.2.1 Intracellular quality control mechanisms

After protein synthesis, proteins undergo a variety of specific processes to mature and be functional. The maturation and correct folding of proteins often requires the assistance of molecular chaperone proteins. Molecular chaperones are a large group of proteins whose function is to interact with destabilized proteins to prevent their aggregation and in some cases, encourage the proteins towards their correct folding pathway (Yerbury et al., 2005b). There are different factors that cause misfolding of proteins. They include; mutation in the encoding genes, errors during transcription and/or translation, or structural alteration to the protein resulting from environmental stresses inside or outside the cell (Hampton, 2002). When proteins misfold, they are either degraded by energy-dependent proteases, rescued by molecular chaperones or they form aggregates (Fig.4.2).

An overall protein quality system has been identified in the cytoplasm, the secretory pathway and mitochondria (Hampton, 2002). Homeostasis within the cell is maintained by the continual synthesis and degradation of proteins by internal quality control mechanisms. The control mechanisms include the ubiquitin-proteasome system (Ciechanover, 1998), lysosomes (Brandes and Bertini, 1964), molecular chaperones (Carver et al., 2002) and aggresomes (Johnston et al., 1998). Endoplasmic reticulum (ER)- associated degradation is another system, in which misfolded proteins are retrotranslocated across the ER-membrane before degradation by the proteosome (Welch and Brown, 1996). If there is an imbalance between each of the control mechanisms, such as an overwhelming of the degradative capacity of cells, protein conformational disorders (PCDs) can result such as Parkinson's disease in which the intracellular aggregates contain ubiquitin and chaperones in addition to the main protein component, α-synuclein. Parkinson's disease has also been linked with genetic mutations related to the ubiquitin-proteasome pathway (Ross and Pickart, 2004).

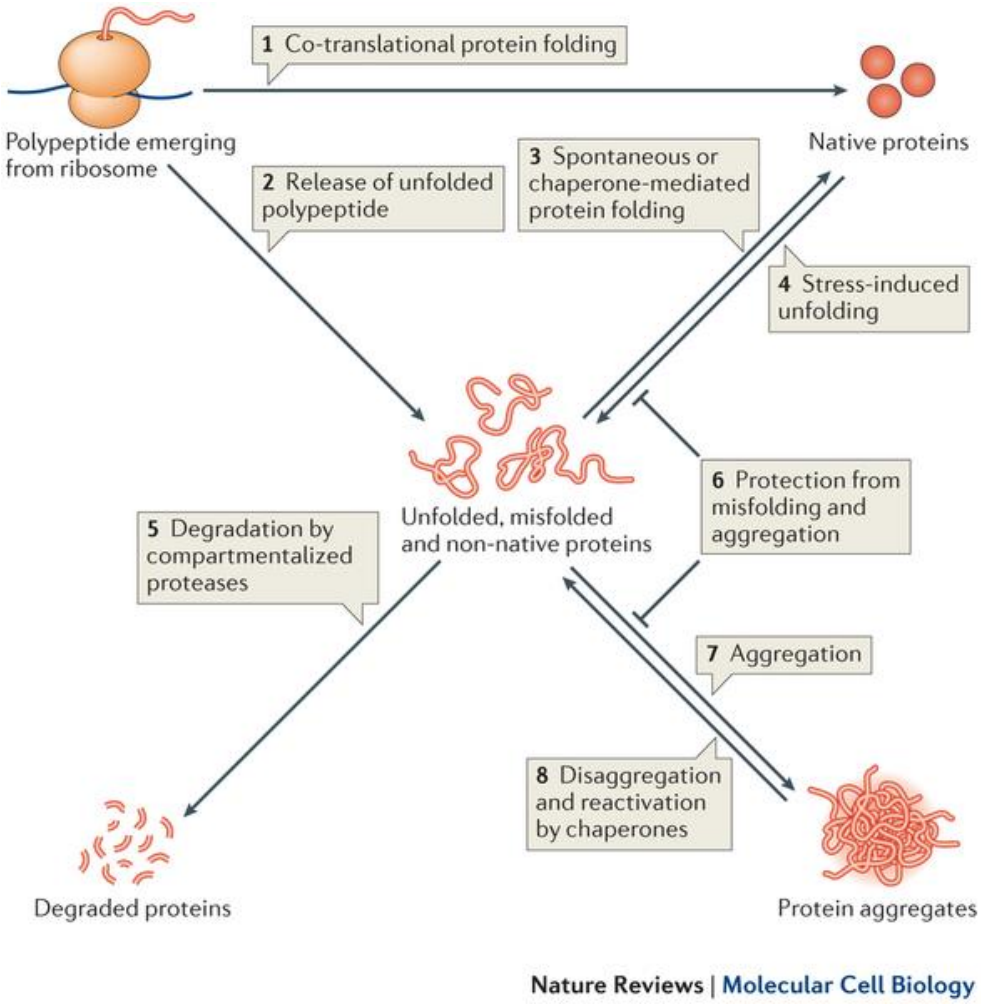


Figure 4-2: The protein quality control network involves the actions of many molecular chaperones to assist the folding of proteins into their native conformations to prevent misfolding of proteins. Chaperones aid in the folding of newly synthesized proteins, of unfolded proteins recently released from the ribosome and the remodelling of non-native proteins , which are often caused by heat stress and other stress conditions (Hartl et al., 2011, Lindquist and Kelly, 2011, Mayer, 2010). If the chaperone network becomes overwhelmed during stress, non-native proteins may form large, amorphous aggregates (Finley, 2009). Figure from Doyle *et al* (2013).

4.2.1.1 The Ubiquitin-Proteasome System

In the cytoplasm and endoplasmic reticulum, protein quality control is brought about by the ubiquitin- proteasome system that marks proteins for proteasomal degradation (Hampton, 2002, McDonough and Patterson, 2003, Trombetta and Parodi, 2003). Ubiquitin-mediated proteasomal degradation is one major proteolytic pathway in eukaryotes. This system recognises protein substrates, due to their hydrophobic residues (Herrmann et al., 2008). Ubiquitinylation is a highly regulated ATP-dependent process, involving the covalent tagging of proteins (Fig. 4.3). Labelling with less than four ubiquitin polypeptides targets proteins for other regulating processes within cells (Johnson, 2002, Aguilar and Wendland, 2003), whereas labelling with four or more ubiquitin molecules targets proteins for degradation. This occurs after subsequent recognition by the proteasome whilst the ubiquitin is recycled. Cytosolic proteins are targeted for ubiquitination under a number of different circumstances; recognition of exposed hydrophobic regions (Johnson et al., 1998) of misfolded proteins in the cytosol, or after association with chaperones (Cyr et al., 2002).

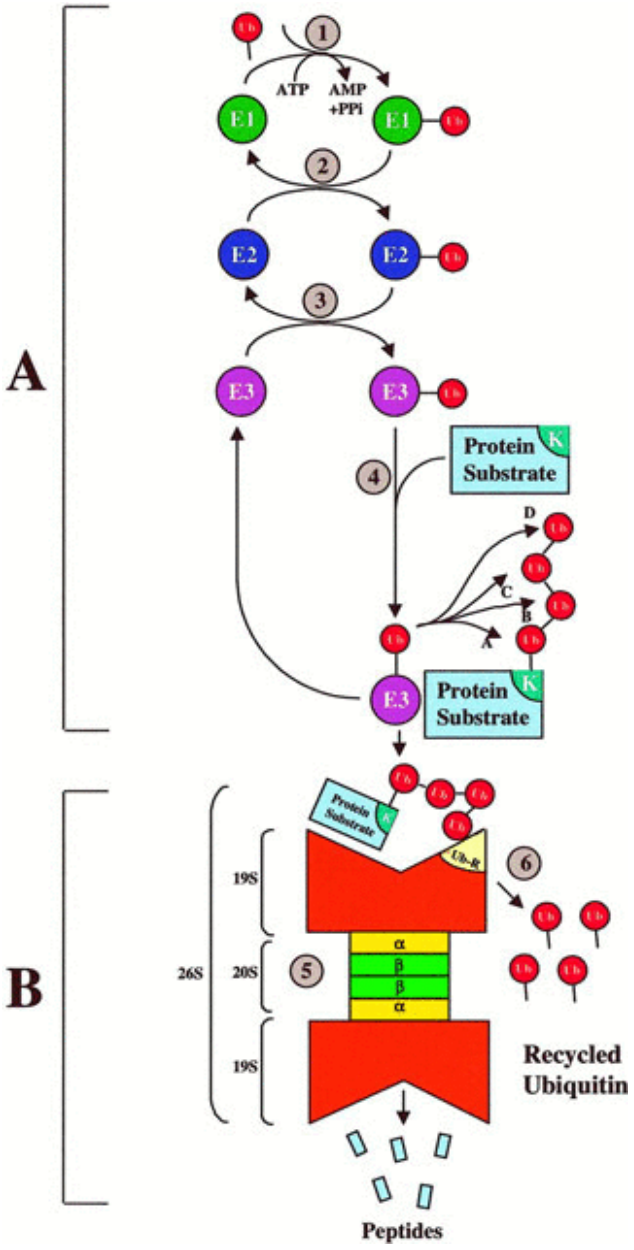


Figure 4-3: The ubiquitin–proteasome pathway. Degradation of a protein involves covalent attachment of ubiquitin molecules to the target protein (A) and degradation of the tagged protein by the 26S proteasome (B). Initially, ubiquitin is activated by the ubiquitin-activating enzyme (E1) and transferred by several Ubiquitin-carrier proteins (E2 enzymes) to a member of the ubiquitin-protein ligase family (E3) to which the substrate protein is specifically bound. E3 enzymes catalyse the last step in the conjugation process, covalent attachment of ubiquitin to the substrate. The binding of the polyubiquitinated substrate to the

ubiquitin receptor subunit in the 19S complex of the 26S leads to degradation of the substrate to short peptides by the 20S complex. Figure from Ciechanover (1998).

4.2.1.2 Molecular chaperones

One essential group of proteins involved in proteome quality control comprises molecular chaperones, which facilitate protein stabilisation, folding, unfolding and remodelling (Hartl et al., 2011, Lindquist and Kelly, 2011, Mayer, 2010, Priya et al., 2013). These molecular machines modulate the kinetic partitioning of polypeptides between pathways that lead to active proteins with native conformations and inactive proteins that are unfolded, misfolded or aggregated (Fig. 4.3). Chaperones are abundant cellular proteins, and for many their expression levels are further increased by stress conditions. Those that were initially identified as heat inducible are labelled heat-shock proteins (HSPs). There are four large and ubiquitous families of ATP-dependent molecular chaperones, namely HSP100 (which are often called Clps), HSP90, HSP70 and HSP60 proteins (Doyle et al., 2013). Within an organism there are likely to be several examples of each family. In addition to the ATP-dependent chaperones, there are ATP-independent chaperones, including the small HSPs (sHSPs) which are specifically involved in stabilisation of proteins rather than folding or refolding. There are also dedicated molecular chaperones that remodel a specific substrate protein or complex. Chaperones are known to have a range of different functions, including assisting protein folding (Hartl et al., 2011), stabilising proteins and thereby inhibiting protein aggregation (Broadley and Hartl, 2009), causing the disaggregation of aberrant protein oligomers (Weibezahn et al., 2005), and facilitating the degradation of misfolded proteins (Pickart and Cohen, 2004). Molecular chaperones are of great importance in maintaining protein function, however the majority of identified chaperones are found intracellularly while many amyloid fibrillar aggregates may accumulate in extracellular space (Kumita et al., 2007, Carver et al., 2003).

4.2.1.3 Aggresomes

The aggresome-macroautophagy pathway is another defence mechanism for sequestration and clearance of toxic protein aggregates inside the cells by formation of aggresomes (Garcia-Mata et al., 2002). Aggresome structure comprises of loosely packed particles of aggregated protein indicating a storage mechanism shielding the hydrophobic regions of individual aggregates which thereby prevents further aggregation (Garcia-Mata et al., 2002). Inhibition of the proteasome and/or over-expression of protein normally degraded by the proteasome induces aggresome formation (Wojcik and DeMartino, 2003). Aggresomes are capable of recruiting other cytosolic components to aid in the clearance of aggregated proteins as chaperones, including Hsc70, Hsp70 and sHSPs (Garcia-Mata et al., 2002).

4.2.1.4 Lysosomes

Lysosomes are membraneous organelles with many different hydrolytic enzymes including protein-degrading enzymes including acid phosphatase (De Duve et al., 1955). A very selective form of autophagy called chaperone-mediated Autophagy (CMA), target specific pool of intracellular proteins to the lysosome for degradation (Kaushik and Cuervo, 2012). Selectivity of the protein is determined by the presence of a penta-peptide amino acid sequence (biochemically similar to KFERQ) on the substrate proteins of a CMA targeting motif (Dice, 1990). This motif is recognized by the constitutively expressed intracellular heat shock-cognate chaperone hsc70 (Chiang et al., 1989). After binding of hsc70, substrates are targeted to the lysosomal membrane for docking at the cytosolic tail of the monomeric lysosome-associated membrane protein type-2A (LAMP2A) (Cuervo and Dice, 1996). Binding of the substrate initiates multimerization of the LAMP2A monomers and CMA translocation complex at the lysosomal membrane (Bandyopadhyay et al., 2008). Translocation into lysosomes of the substrate proteins is assisted by a lysosome resident

hsc70 (lys-hsc70), and leads to the complete degradation of the substrate into its constitutive amino acids by the luminal proteases known as cathepsins (Patel and Cuervo, 2015) .

4.2.2 Extracellular quality control mechanisms

There is an extensive knowledge about the intracellular mechanisms controlling protein folding but much less is known about the extracellular mechanisms. There has not yet been a confirmed mechanism that controls protein folding outside the cells. In extracellular fluids, the low levels (7.7-200 ng/mL) of proteasome/ubiquitin and the absence of ATP (Savas et al., 2003, Akarsu et al., 2001) suggest that protein refolding and degradation via the proteasome are unlikely to occur at a significant level in this environment. However extracellular chaperones are present in body fluids and interstitial spaces where the pathology of a number of protein-folding related diseases occurs. The ability of these extracellular chaperones to form complexes with stressed proteins and also bind to endocytic receptors may indicate that they are part of specific extracellular protein folding quality control mechanism. The discovery of extracellular molecular chaperones and understanding their mechanism of action has significant importance and implications for amyloid-based diseases.

These extracellular molecular chaperones include clusterin (Humphreys et al., 1999), haptoglobin (Yerbury et al., 2005a), serum amyloid P component (SAP) (Coker et al., 2000), α_2-macroglobulin (French et al., 2008) and the milk proteins β- and α_{s1}-casein (Matsudomi et al., 2004, Zhang et al., 2005, Morgan et al., 2005). Clusterin, haptoglobin and α_2-macroglobulin colocalise with amyloid plaques in Alzheimer's disease (Kida et al., 1995, Powers et al., 1981, Strauss et al., 1992), inhibit Aβ fibril formation *in vitro* (Du et al., 1998, Hughes et al., 1998, Kumita et al., 2007, Yerbury et al., 2007) and promote the clearance of protein aggregates via endocytosis (Hammad et al., 1997, Narita et al., 1997, Zlokovic, 1996). It is unclear, however, whether extracellular chaperones inhibit directly the toxicity of Aβ oligomers, as reports have been contradictory, with some claiming a protective action (Du

et al., 1998, Boggs et al., 1996, Mannini et al., 2012) and one report describing a dose-dependent effect (Yerbury et al., 2007).

4.2.2.1 Haptoglobin

Haptoglobin (Hp) is an acidic glycoprotein produced in the liver and secreted mainly in human serum at a concentration of 0.3-2.0 mg/mL, cerebrospinal and synovial fluids (Bowman and Kurosky, 1982, Dobryszycka, 1997). Hp is expressed as a single polypeptide which is internally cleaved to form α and β subunits. It possesses a tetrameric structure that is linked by disulfide bridges among two α chains and two β chains (Kristiansen et al., 2001, Wejman et al., 1984). Based on the length of the α chain, three phenotypes of Hp are known to occur in the human population: Hp1-1, Hp2-1, and Hp2-2. All of the phenotypes share the same 40-kDa β chain (243 amino acids), but there are two types of α chains: α_1 (83 amino acids), and α_2 (142 amino acids) (Sultan et al., 2013). The Hp1-1 phenotype has two β chains and two α_1 chains; the Hp2-1 phenotype has two β chains, one α_1 and one α_2 chain, whereas the Hp2-2 phenotype has two β chains and two α_2 chains. Hp binds tightly and specifically to haemoglobin (Hb) forming stable complexes which are cleared via receptor mediated mechanisms in the liver. Due to the formation of Hp and Hb complexes, researches have proposed that Hp is involved in the clearance of damaged Hb from the red cells (Bowman and Kurosky, 1982).

Hp has been termed an 'acute phase protein' because its level is elevated up to 8-fold in inflammation (Dobryszycka, 1997), infectious disease, tissue breakdown and leukemia and other cancers (Bowman and Kurosky, 1982) and is, thus, a useful marker for inflammation-related diseases (Arredouani et al., 2005, Engstrom et al., 2003, Sadrzadeh and Bozorgmehr, 2004). The three phenotypes of Hp exhibit chaperone activity in preventing the amorphous aggregation of target proteins (Yerbury et al., 2005a, Sultan et al., 2013, Ettrich et al., 2002). Yerbury *et al*. (2005b) have shown that extracellular chaperones recognize exposed

hydrophobic surfaces on non-native proteins in the extracellular space and mediate their clearance by targeting them for degradation in lysosomes via receptor-mediated endocytosis. A recent study has shown that the Hp2-1 phenotype exhibits molecular chaperone activity in preventing the amyloid aggregation of a range of proteins including amyloid β 1-42, calcitonin and lysozyme (Yerbury et al., 2009).

4.2.2.2 Serum Amyloid P Component (SAP)

SAP is a universal constituent of amyloid deposits *in vivo*, including the plaques, amorphous Aβ deposits, and neurofibrillary tangles of Alzheimer's disease (Coria et al., 1988, Iseki et al., 1988, Duong et al., 1989, Kalaria et al., 1991, Akiyama et al., 1991). SAP undergoes reversible calcium-dependent binding to all types of amyloid fibrils *in vivo* (Pepys, 1979) and comprises up to 15% of the mass of amyloid deposits *in vivo*. The physiological role of SAP is not known, and researchers have previously proposed that SAP might protect amyloid fibres from degradation *in vivo* by masking the abnormal fibrillar conformation that would otherwise be expected to trigger phagocytic clearance mechanisms (Hind et al., 1984, Pepys, 1988). SAP molecules deposited in amyloid are not catabolised and are broken down only when they return to circulation. SAP is also highly resistant to proteolysis due to its flattened β-jelly roll structure in which the β-strands are joined by compact loops tightly bonded to the body of the oligomeric assembly (Emsley et al., 1994).

4.2.2.3 Clusterin

The most well characterized of the extracellular chaperones is clusterin (Carver et al., 2003, Humphreys et al., 1999). It was the first extracellular chaperone to be identified (Humphreys et al., 1999). The efficiency on a molar basis of clusterin, serum amyloid P component (SAP) and haptoglobin at inhibiting precipitation of stressed proteins has been determined. Against the same target proteins, clusterin is up to 20 times more efficient than SAP (Coker et al., 2000) and up to 5 times more efficient than haptoglobin (Ettrich et al., 2002). Clusterin

significantly inhibits stress-induced protein precipitation in undiluted human serum (Poon et al., 2000).

Clusterin was first identified in ram rete testis fluid and was named for its ability to elicit clustering among Sertoli cells (Han et al., 2012, Blaschuk et al., 1983). Clusterin is implicated in a number of disease states based on its association with disease pathogenesis and has therefore been given a variety of alternative names (Table 1). Clusterin is thought to be in all mammalian species (Collard and Griswold, 1987). In human clusterin, synthesis and secretion is complex and exhibits different patterns in different tissues and cells. The human homolog of clusterin has been given other names, including complement-lysis inhibitor (CLI), secreted protein 40 (SP-40), and apolipoprotein J (ApoJ) (Han et al., 2012). Clusterin has been found in all body fluids including cerebrospinal fluid (CSF), human plasma and seminal fluid at concentrations of approximately 2, 100 and 1000 µg/mL respectively (Trougakos and Gonos, 2002, Fritz et al., 1983, Choi-Miura et al., 1992, Murphy et al., 1988). The expression of clusterin is increased in stress and disease conditions including exposure to noxious agents and withdrawal of growth factors.

4.2.2.3.1 Structure of Clusterin

Clusterin is highly conserved with 70-80% sequence conservation across different mammalian species. Understanding the functional relationship between the structure and function of clusterin is important in understanding the role of clusterin in protein folding control. The clusterin protein is a disulphide-linked heterodimer of α- and β- polypeptide chains derived from a single protein precursor (Michel et al., 1997). The heterodimer polypeptide chains are produced by posttranslational cleavage of the primary translation product at the Arg205-Ser206 bond (Burkey et al., 1991, Hochgrebe et al., 2000). Clusterin exists in a range of oligomeric forms in aqueous physiological pH; mildly aqueous pH favours partial dissociation into individual α-β heterodimers. Sequence analysis of clusterin

suggests that it has contiguous regions of disordered conformation that separate other regions of well defined secondary structure such as coiled-coil α-helices and amphipathic α-helical regions (Dunker et al., 2002, Wilson et al., 2008). The amphipathic possibly, forming a coiled-coil α-helix should explain the stability of clusterin when interacting with lipids and hydrophobic domains in other proteins (Michel et al., 1997). The structure of clusterin is conserved among vertebrates, and includes myosin-like coiled –coil domains, amphipathic α-helices with high hydrophobic moments, a nucleotide binding site and a motif also present in the cytolytic components of the complement membrane-attack complex (Jenne and Tschopp, 1992). There has been suggestion that clusterin acts as a 'biological detergent' based on the prolific binding of clusterin to many unrelated ligands. Hochgrebe *et al.* (2000) showed that at slightly acidic pH, clusterin binds more strongly to a number of macromolecules and at mildly acidic pH, clusterin exists in the form of α-β monomers and in this state exposes hydrophobic residues that are normally buried in the homopolymer state.

Table 4-1: Alternative names given to clusterin (Jenne and Tschopp, 1992).

NAME	**SPECIES**	**CHARACTERIZED AS**
Clusterin	Sheep	Protein from testis fluid with cell aggregating activity
SGPO-2 (sulphated glycoprotein 2)	Rat	Major sulphated Sertoli cell secretion product
CLI (cytolysis inhibitor)	Human	Consistent of the soluble , non lytic terminal complement complex (non-cell associated)
TRPM-2 (testosterone-	Rat	mRNA induced involution of the prostate after

repressed prostatic messenger 2)		androgen withdrawal
gp-IIII	Bovine	Constituent of chromatin granules
Apo-J	Human	Apolipoprotein
gp-80	Dog	Glycoprotein secreted at the apical surface of the kidney epithelial cells
oADHC-9	Human	mRNA from the hippocampus of a patient suffering from Alzheimer's disease
oTB16	Human	cDNA clone isolated from glioma library; mRNA highly expressed in epileptic foci
HISL-19 (secretograin IV)	Human	Immunocytochemical marker of neuroendocrine cells

4.2.3 Function of clusterin

The clusterin gene, in the genome, is expressed during tissue regression in many organs including the retina, brain (Duguid et al., 1989, Danik et al., 1993), uterus, mammary gland, temporary embryonic structures, kidney and prostate (Norman et al., 1995). A definitive biological function has not yet been established for clusterin, however its properties have suggested activity in a number of roles. Clusterin and other ECs may help control the folding of extracellular proteins by binding to the hydrophobic regions of the peptide to mediate endocytosis and intracellular lysosomal degradation of the misfolded peptide/protein (Stewart et al., 2007, Yerbury et al., 2005b). ECs stabilize proteins and prevent their stress-induced aggregation that can lead to formation of amorphous and amyloid structures (Wyatt et al., 2011).

Clusterin stabilizes a wide range of stressed, target proteins when they are aggregating amorphously sequestering them into soluble high-molecular weight complexes (Fig.4.1). Clusterin's chaperone action has many similarities to that of intracellular sHsps but the proteins share no sequence similarity although they are both found as polydisperse (heterogeneous) and oligomers and are highly dynamic. Clusterin is involved in many biological processes including phagocyte recruitment signal (Buttyan et al., 1989), lipid transportation (de Silva et al., 1990), complement inhibition (Jenne et al., 1991), cell adhesion and membrane recycling and apoptosis (Silkensen et al., 1995) and spermatogenesis (Hermo et al., 1994) and maturation (Sylvester et al., 1984). Among the multiple functions, clusterin may also have a role in cholesterol uptake and recycling by membranes during times of cellular stress and change (Rosenberg and Silkensen, 1995, Wilson and Easterbrook-Smith, 2000, Kapron et al., 1997). All these diverse properties are consistent with clusterin having molecular chaperone ability in which it stabilises target proteins to prevent their unfolding and possible aggregation.

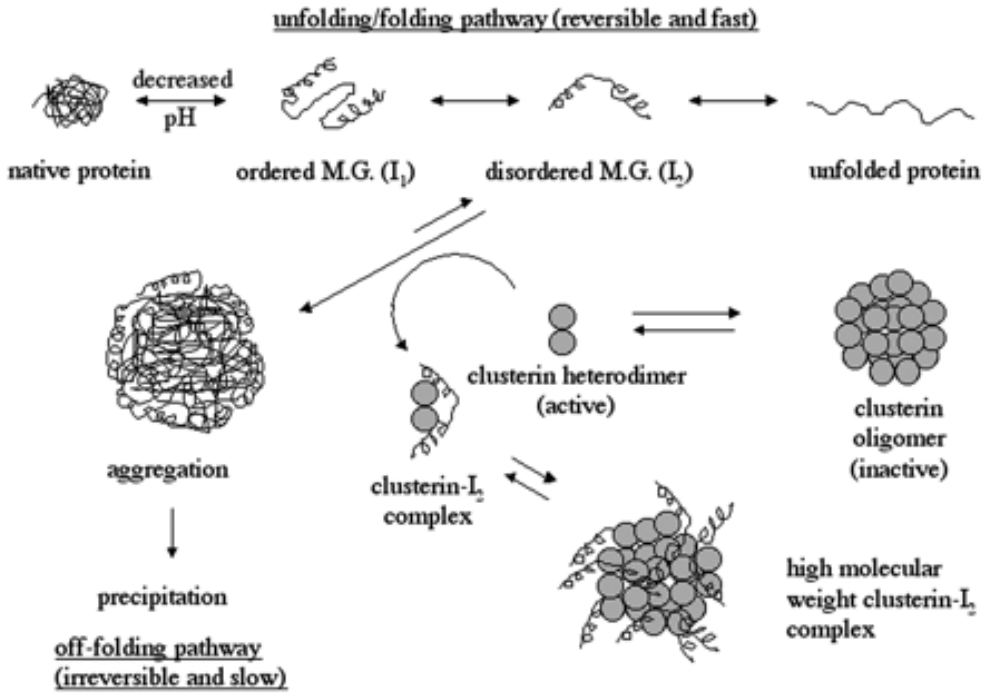


Figure 4-4: A schematic representation of mechanism for the chaperone action of clusterin to prevent the amorphous aggregation of target proteins. Under conditions of stress, a native protein unfolds and adopts a series of partly folded intermediate (molten globule) states which expose significant hydrophobicity to solution and enter the slow off-folding pathway that can potentially lead to irreversible aggregation. Along this pathway, the chaperone-active heterodimeric form of clusterin interacts and binds to I_2, which is then sequestered into a large clusterin-I_2 complex. The mechanism requires no input of energy. Figure from Poon *et al*. (2002)

Clusterin has attracted a lot of interest lately because it is a biomarker for Alzheimer's (its level is raised significantly in CSF in Alzheimer's sufferers) (Desikan et al., 2014). Studies show that clusterin inhibits aggregation of different proteins that are partially denatured by reducing agents or heat-shock (Humphreys et al., 1999). Clusterin has also been shown to inhibit amyloid fibril formation of prion protein (McHattie and Edington, 1999) and apolipoprotein (Hatters et al., 2002) at a substoichiometric concentrations. The aggregation of the Aβ amyloid peptide with different concentrations of clusterin revealed that clusterin also

inhibits amyloid formation by Aβ (Oda et al., 1995). In view of the ability of clusterin to prevent aggregation of various proteins, we investigated whether amyloid formation by SEVI is affected by the chaperone activity of clusterin. Thus, clusterin is highly prevalent in seminal fluid and is very good at preventing the fibrillar aggregation of various proteins; hence, there is direct physiological significance for the interaction between SEVI and clusterin.

4.3 Experimental Methods

4.3.1 Fibril formation

The PAP248-286 peptide was purchased from Mimotopes, The Peptide Company, Melbourne Australia. For aggregation mixtures, PAP248-286 (2 mg/mL, at the approximate concentration that is present in semen) was dissolved in 200mM phosphate buffer, pH 7.2. Clusterin was also dissolved in a same buffer and added to SEVI to a final concentration of 0, 3, 20, 40 and 200 µg/mL. Samples were incubated at 37^0C with shaking (700 rpm) for up to 2 days to allow for fibril formation. Aliquots (40 µL) were taken from the solution every 8 hours and snap frozen at -20^0C.

4.3.2 Thioflavin T binding assay

Amyloid fibril formation was monitored using a fluorometric assay that relies on the enhanced fluorescence properties of the dye Thioflavin T (ThT) when bound to amyloid fibrils. ThT binds selectively to β-pleated sheets, the major structural element of amyloid fibrils, and fibril formation is accompanied by an increase in the ThT fluorescence. Samples that were frozen as outlined above were unfrozen and prepared in duplicate by adding 10 µL of each sample to 25 µL of ThT (0.5 mM) and made to final volume of 200 µL with 200 mM phosphate buffer, pH 7.2 in Greiner black µClear 96-microwell plates (Interpath Services, Australia). Plates were sealed with ThinSeal to prevent evaporation and incubated at 37°C. The increase in fluorescence intensity of each sample was monitored (excitation 440 nm, emission 490 nm) in triplicate wells using FLUOstar and POLARstar optima microplate readers (BMG Labtechnologies, Melbourne, Australia).

4.3.3 Transmission electron microscopy

2 µl of aggregate samples from the ThT assay were added to formavar- and carbon-coated nickel grids (SPI supplies, West Chester, USA) for 2 minutes. The grids were washed three times with 10 µl of water and negatively stained with 10 µl uranyl acetate (2% w/v, Agar Scientific, UK). Excess stain was removed with filter paper and the grids were air dried and viewed using Tecnai G2 Spirit transmission electron microscope at 10500x magnification (Philips, Eindhoven, Netherlands).

4.3.4 Cellular Toxicity

PC12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% v/v fetal calf serum, 1% v/v glutamate, non essential amino acids and penicillin and streptomycin in a 75 cm^3 flasks at 37^0C in an incubator with 95% air and 5% carbon dioxide. The medium was changed after two to three days. The cells were plated into a 96 well plate at a density of 2×10^4 cells/well and incubated for 24 hours. The cells were then treated with samples of SEVI with varying clusterin concentration (six replicates per treatment) and incubated for 48 hours. Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl (MTT) assay.

4.3.5 MTT assay

3 mg of MTT powder was dissolved in 12 mL of serum-free Roswell Park Memorial Institute (RPMI) medium. Media was aspirated from the 96 well plate and 100 µL of 0.25mg/mL MTT containing media was added to each well (Sigma Aldrich, Sydney, Australia). The plate was incubated for three hours at 37^0C. The MTT-containing media was aspirated and 100 µL of DMSO was added to each well. Formazan absorption was measured at 560 nm using a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany). The mean of six replicates readings was taken and cell viability was calculated by dividing the average absorption readings of treated wells with average absorption readings of untreated wells (200 mM phosphate buffer only).

4.4 Results

4.4.1 Fibril formation of SEVI with varying concentration of clusterin

The effect of clusterin on the formation of SEVI was determined. SEVI, in its monomeric state, PAP248-286, is inactive in enhancing HIV infection and must first aggregate to form the active SEVI species to promote viral infection (Münch et al., 2007, Roan et al., 2010, Hauber et al., 2009). PAP248-286 is toxic to cells in the form of large aggregates with the characteristic β-sheet conformation of amyloid proteins (Münch et al., 2007, Kim et al., 2010), i.e. SEVI. Varying concentrations (3, 20, 40, 200 µg/mL) of clusterin were mixed with 2 mg/mL of PAP248-286 (Fig.4.5) dissolved in 200mM of phosphate buffer (pH 7.2) and incubated at 37°C with vigorous shaking at 700rpm.

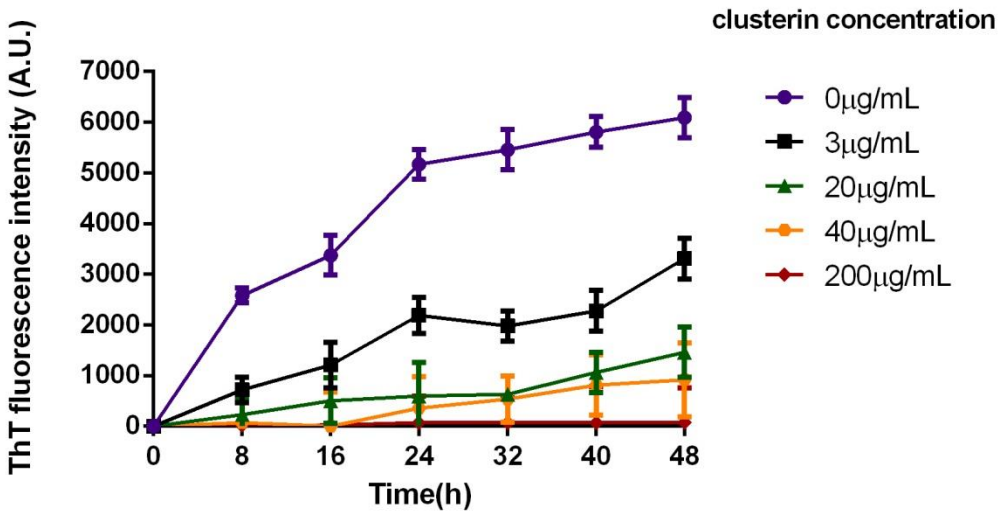
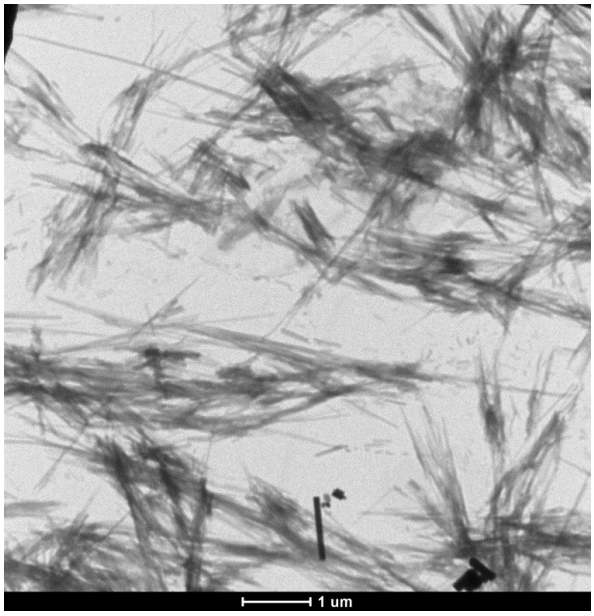


Figure 4-5: Effect of clusterin on fibril formation of 2 mg/mL SEVI at varying concentrations of clusterin incubated at 37º C for two days at pH 7.0. The data shown are means of triplicates ±SD.

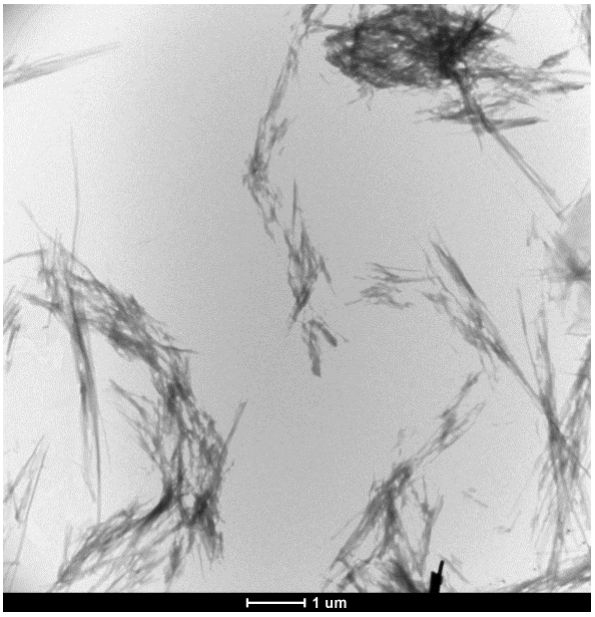
Incubation of 2mg/mL SEVI (blue) induced an increase in ThT fluorescence intensity which reached a plateau approximately after 24 hours on its own. This result is indicative of maximum fibril formation of SEVI after 24 hours which remained almost constant for another 24 hours. With the addition of 3 µg/mL of clusterin, there was still an increase in the intensity of ThT fluorescence but to less that 50% intensity induced by SEVI on its own (Fig 4.5). Thus, on this base, addition of 3µg/mL of clusterin inhibited half the ThT fluorescence intensity of SEVI. With the increase in the concentration of clusterin to 20 and 40 µg/mL there was a very little increase in ThT fluorescence. When 200 µg/mL of clusterin was added to SEVI, SEVI fibril formation was completely abrogated.

The effects of clusterin on SEVI fibril morphology (using samples from the ThT assays), were assessed by TEM (Fig.4.6). In accordance with conclusions from the ThT assay, the SEVI sample without clusterin showed long fibrillar aggregates of dimensions characteristic of amyloid fibrils as shown in Fig. 4.6a. The morphology of SEVI incubated with 3 µg/mL clusterin (Fig 4.6b) was similar to SEVI without clusterin but with fewer fibrils. The micrographs of SEVI in the presence of 20 µg/mL (Fig 4.6c) and 40 µg/mL clusterin (Fig 4.6d) showed scattered short fibrils in comparison to the dense long fibrils of SEVI on its own. The presence of 40µg/mL clusterin produced shorter fibrils than in the presence of 20µg/mL. At 200µg/mL clusterin (Fig. 4.6e), amorphous aggregates, instead were observed, with no fibrils present.

0 µg/mL clusterin

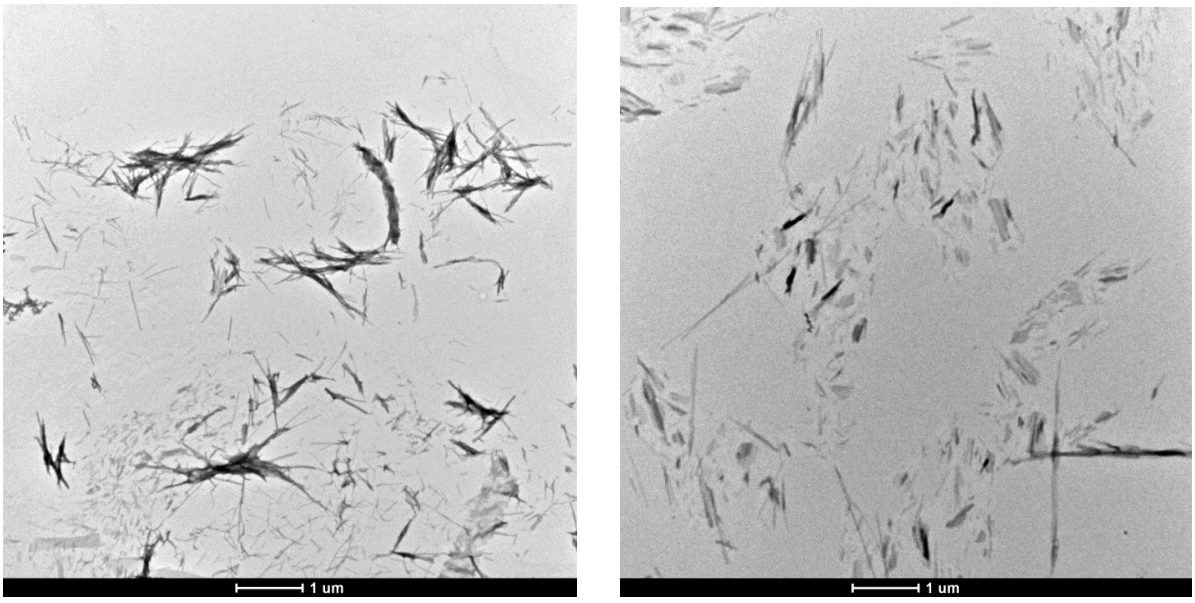


3 µg/mL clusterin



20 µg/mL clusterin

40 µg/mL clusterin



200 µg/mL clusterin

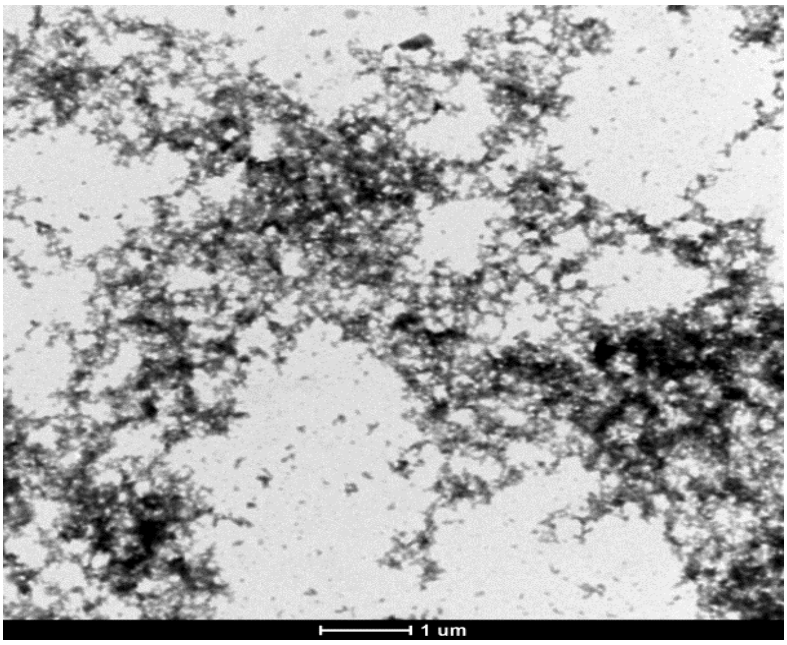


Figure 4-6: TEM of the effect of different concentrations of clusterin on SEVI fibril formation. Samples were taken at the end of aggregation reactions. the concentration of SEVI was kept constant at 2 mg/mL with varying concentration of clusterin; a, 0 b, 3 c, 20 d,

40 and e, 200 µg/mL. The results are representatives of three or more individual experiments. The scale bar shown represents 1µm.

We determined whether longer incubation of 3 days or higher concentration of clusterin (400 µg/mL) would dissolve all the fibrils formed. We found that with 3 days of incubation of SEVI with 200µg/mL clusterin, the results looked the same (not shown), while addition of higher concentration of SEVI did reduce the amount of fibrils formed but did not clear all the fibrils (results not shown). A sample of SEVI without clusterin was incubated along with the preformed fibrils for 3 days as a control to determine that the fibrils did not degrade on its own.

4.4.2 The effect of clusterin to preformed SEVI fibrils

To determine the effect of clusterin on preformed SEVI fibrils, we formed fibrils for 48 hours and added 200 µg/mL of clusterin to the sample. 200 µg/mL clusterin halted further fibril formation by SEVI as shown in Fig 4.7. The fibrils without clusterin present were further incubated for another 48 hours as a control to exclude deterioration of fibrils by themselves. After further 48 hour's incubation of the samples, the pre-formed fibrils with SEVI showed a decrease in ThT fluorescence as shown in Fig 4.7. Initially ThT fluorescence was high at approximately intensity of 8000 due to the presence of many fibrils. After addition of clusterin, fluorescence decreased with time by 77% implying a reduction in fibrils. Furthermore, clusterin incubated alone did not change its ThT fluorescence with time, indicating that the SEVI fibrils were still present after this time.

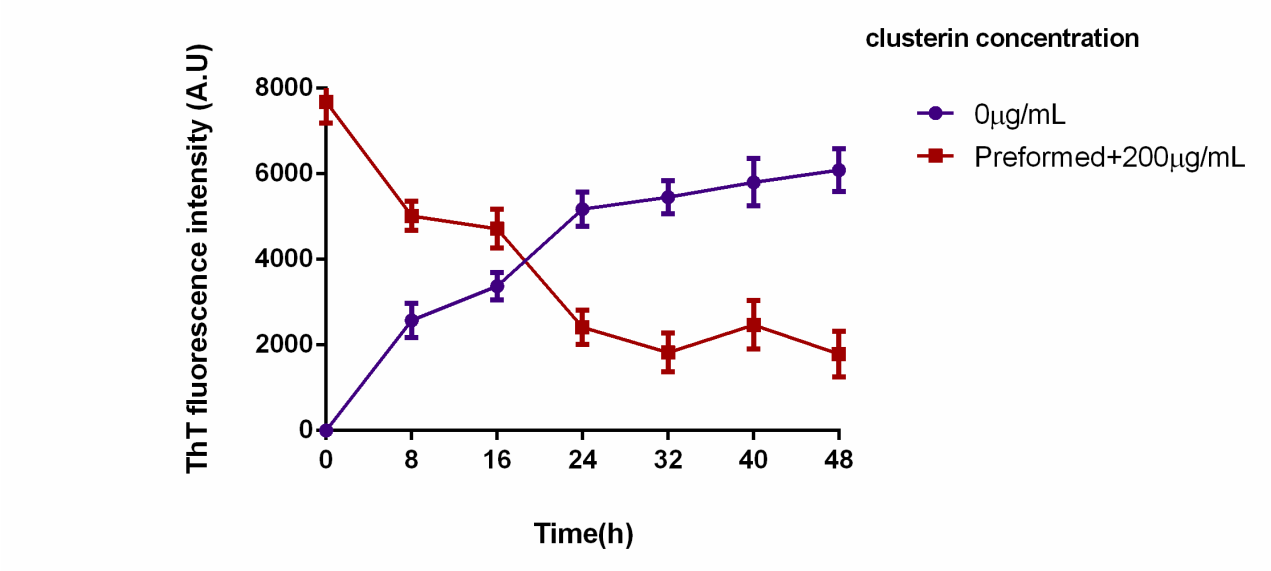


Figure 4-7: The effect of clusterin on pre-formed SEVI. SEVI (red squares) incubated at 37º C for two days at pH 7.2. 200 µg/mL of clusterin was mixed to 2 mg/mL of SEVI fibrils and incubated for 48 hours. The data shown are means of triplicates ±SD.

The samples from the ThT assay were viewed via TEM. The fibrils were present as very small fragments as shown in Fig 4.8.

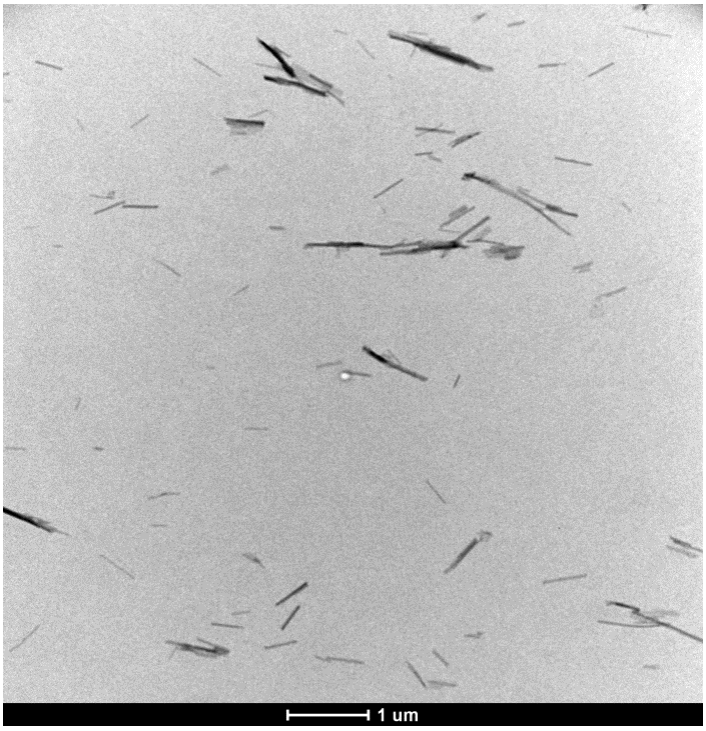


Figure 4-8: The TEM micrographs of clusterin on pre-formed SEVI incubated at 37º C for two days at pH 7.2.

4.4.3 Clusterin inhibits the toxicity of SEVI to PC 12 cells

As human semen has a very high clusterin concentration (0.5-3.5 mg/mL) and we have previously shown that SEVI is toxic to PC 12 cells (Elias et al., 2014), we investigated the effect of clusterin on the cytotoxicity of SEVI. Samples from the ThT assay were added to PC12 cells as outlined in Materials and Methods section. The toxicity of samples with clusterin was compared to that of SEVI without clusterin. SEVI (2 mg/mL) caused 34±3% cell death while 3 and 20 µg/mL of clusterin caused approximately 30±5% cell death (Fig 4.9). 40 µg/mL of clusterin provided some cyto protection i.e. around 25±2% cell death occurred, whereas total suppression of toxicity was observed at a 200µg/mL (Fig 4.9). Clusterin added to preformed SEVI fibrils (which are toxic to cells (Elias et al., 2014) had little, if any, impact on the cytotoxicity, despite being able to partially dissociate the fibrillar

species (as demonstrated in the TEM images in Fig 4.8). The addition of clusterin alone to the cell culture did not have a significant effect on the cells and results were not shown.

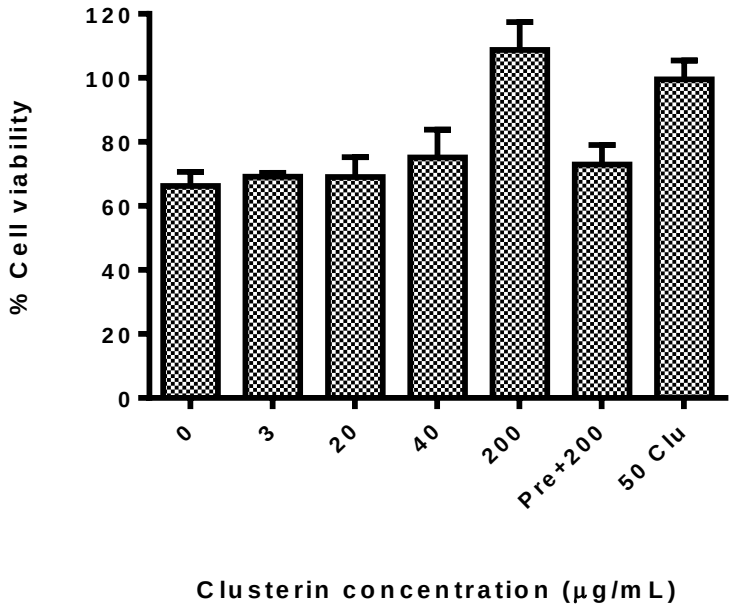


Figure 4-9: Effects of clusterin on cytotoxicity of SEVI (2mg/mL). PAP248-286 was dissolved in 200mM phosphate buffer, pH 7 and different concentration of clusterin added and incubated at 37ºC for two days to induce fibril formation. The sample labelled Pre+200 were SEVI fibrils mixed with 200 µg/mL clusterin added after fibril formation and incubated for 2 days. Samples were then added to cell culture media of PC 12 cells and incubated for 48 hours. The MTT assay was used to assess cell viability and values are presented as percentage of cell survival compared to buffer only (control). The data shown are means of triplicates ±SD (p<0.05)

4.5 Discussion

4.5.1 The effect of clusterin on the aggregation and fibril formation of SEVI

Clusterin is found associated with the extracellular Aβ plaques in Alzheimer's disease, suggesting a physiological interaction with Aβ (Kim et al., 2010, Powers et al., 1981, Strauss et al., 1992, Barral et al., 2004, Businaro et al., 1992, Trougakos and Gonos, 2006). Indeed clusterin is markedly induced in neuropathological conditions such as Alzheimer's disease and is a recognised biomarker for this disease due to its elevated levels in Alzheimer's disease sufferers. Clusterin also inhibits fibril formation *in vitro* of a variety of peptides and proteins, of disease and non-disease origin e.g. α-lactalbumin (Du et al., 1998, Hughes et al., 1998, Kumita et al., 2007, Yerbury et al., 2007, Yerbury et al., 2009, Humphreys et al., 1999) . In particular , clusterin has been shown to bind to all types of soluble oligomers present during the aggregation and disaggregation of $A\beta_{40}$, preventing proliferation and further growth of aggregated species (Cascella et al., 2013, Humphreys et al., 1999, Luheshi et al., 2010, Nielsen et al., 2009, Narayan et al., 2012, Wyatt et al., 2011).

A 39 amino acid fragment of Prostatic acidic phosphatase (PAP), forms fibrils termed SEVI. We sought to determine whether the anti-aggregation activity of clusterin applies to SEVI by the use ThT fluorescence assay and TEM with a mixture of SEVI (2mg/mL) and varying concentration of clusterin (Fig 4.7 and 4.8). In the presence of increasing clusterin concentration, SEVI exhibited a concentration-dependent decrease in ThT fluorescence while SEVI fibril morphology appeared chopped with shorter fragments in 40 µg/mL. With the highest clusterin concentration of 200µg/mL (Fig 4.6) large amorphous aggregates not fibrils were observed. DeMattos *et al*, (2002) showed that clusterin expression facilitates Aβ fibril formation *in vivo* by using the mice models that develops Alzheimer's disease-like

neuropathology. On the other hand *in vitro* studies have shown that clusterin suppress amyloid formation of apolipoprotein C-ll (Hatters et al., 2002), prion protein (McHattie and Edington, 1999), κ-Casein and Aβ (Matsubara et al., 1996, Yerbury et al., 2007). Hatters *et al.* (2002) proposed that clusterin inhibits apoC-ll amyloid fibril formation by interacting stoichiometrically with amyloidogenic precursors (nuclei), leading to dissociation of the nuclei back to monomer thereby inhibiting fibril growth but does not dissociate apoc-II amyloid fibrils.

This study shows that, at the highest concentration of clusterin (200 µg/mL), clusterin did inhibit fibril formation of SEVI maybe by the mechanism proposed, however at lower concentration of clusterin (20 and 40 µg/mL) the results showed that clusterin was able to disassemble fibrils to small fragments thus inhibiting fibril growth. The TEM results of the preformed fibrils of SEVI (2mg/mL) with 200 µg/mL of clusterin showed results similar to the 40 µg/mL of clusterin, with smaller fibril fragments (Fig 4.8).

Thus, the coupled results of ThT and TEM show that clusterin prevents fibril formation of SEVI. TEM results indicate that the stabilising action of clusterin does not significantly modify the structure of SEVI but causes SEVI to form short, stunted fibrils while high concentration of clusterin redirect SEVI aggregation down the amorphous pathway. This is in contrast to the action of (-)-Epigallocatechin-3-Gallate (EGCG) towards SEVI (Popovych et al., 2012) , in which EGCG binds to PAP248-286 in the monomeric form, disaggregating fibrils completely to form small EGCG/PAP248-286 complexes. The ability of clusterin to redirect SEVI aggregation down the amorphous pathway is similar to the interaction of αβ-crystallin with α-synuclein (Rekas et al., 2004).

4.5.2 Clusterin inhibits the toxicity of SEVI to cells

Clusterin and α2M mediate the clearance of extracellular target protein oligomers via endocytosis (Cascella et al., 2013, Hammad et al., 1997, Zlokovic, 1996). However, it is not clear if extracellular chaperones have an ability to inhibit intrinsically the toxicity of Aβ misfolded oligomers, as the evidence has been very sparse and often contradictory. Some research has supported a toxicity-enhancing role for extracellular chaperones (Lambert et al., 1998, Fabrizi et al., 2001, Oda et al., 1995), others claimed a protective action (Du et al., 1998, Boggs et al., 1996, Mannini et al., 2012) while one study demonstrated that the ratio of chaperone (including clusterin): Aβ could determine whether the effect was protective or enhanced toxicity (Yerbury et al., 2007).

This results show that clusterin has a significant protective effect on the cytotoxicity of SEVI towards PC 12 cells. The effect is concentration dependent, with higher concentrations of clusterin offering more protection. The results of these toxicity experiments supports the theory that cytotoxicity is correlated with exposed regions of hydophobicity (Chiti and Dobson, 2006). Narayan *et al.* (2014) have recently shown that clusterin prevents damage induced by amyloid β oligomers by preventing calcium influx, ROS production, and caspase 3 activation.

The clusterin concentration shown here to prevent fibril formation by SEVI and protect the cells is less than the range normally found in human seminal fluid (0.5-15.0 mg/mL) (O'Bryan et al., 1990). These results suggest that semen clusterin might be able to prevent SEVI fibril formation in semen. Because the concentration of PAP secreted in semen is 1-2 mg/mL, this raises the open question of why SEVI fibrils form and enhancement of HIV infection occurs in the presence of clusterin. Does SEVI aggregation overwhelm the concentration of clusterin *in vivo*? We speculate that SEVI formation in semen might be rapid and does not allow enough time for binding as clusterin binds preferably to intermediate

folding states of proteins that are slowly aggregating (Humphreys et al., 1999). Moreover environmental conditions *in vivo* e.g. crowding and other seminal proteins might interfere with the ability of clusterin to prevent fibril formation SEVI.

The results of this study suggest that clusterin prevents SEVI fibril formation thereby reducing and preventing cytotoxicity of SEVI. *In vivo*, at least in semen, the ability of clusterin to prevent the formation of SEVI fibrils would thereby effectively abrogate their HIV-1 enhancement activity. Potentially, clusterin therefore could be used as a supplement to antiretroviral microbicides, whereby increasing the levels of clusterin in the semen may provide a valuable natural inhibitor of sexual transmission of HIV-1.

4.6 Acknowledgements

The authors thank Dr Yanqin Liu and Lyn Waterhouse for their technical assistance.

5 Amyloid fibrils formed by bovine milk α_s2-casein are toxic to PC-12 cells

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5.1 ABSTRACT

α_s-Casein is a major milk protein that oligomerises and is comprised of the unrelated, unstructured proteins α_{s1} and α_{s2} casein. While their function is presumed to be primarily nutritional, caseins have a remarkable ability to stabilise proteins, i.e., to inhibit protein aggregation and precipitation, that is comparable in efficacy to molecular chaperones of the small heat-shock protein (sHsp) family. sHsps prevent the formation of amyloid fibrils by a range of proteins. When separated from α_{s1}-casein, α_{s2}- casein (α_{s2}-CN) forms amyloid fibrils spontaneously under physiological conditions (Thorn et al., 2008). Here, we show by cell toxicity assays and transmission electron microscopy that fibrils formed by α_{s2}-CN, a protein synthesized and secreted by mammary epithelial cells, are toxic to pheochromocytoma (PC)-12 cells. Furthermore, the time-dependent increase in Thioflavin T fluorescence and TEM results show that a polyphenol EGCG (epigallocatechin-3- gallate) inhibits amyloid fibril formation by α_{s2}-CN and its associated cytotoxicity to PC-12 cells. Our findings suggest that

EGCG is a potent inhibitor of α_{s2}-CN fibril formation and that it may have applications *in vivo* as a general inhibitor of fibril formation.

Keywords: α_{s2}- casein, amyloid fibrils, cytotoxicity, protein aggregation, EGCG

5.2 INTRODUCTION

Bovine milk is a mixture of proteins that provides the sole nutrient requirements to newborns. Milk contains proteins and encrypted biologically active peptides, which are released upon hydrolysis (Clare and Swaisgood, 2000). Bioactive peptides are functional foods with health-modulating benefits (Meisel, 2004, Wong and Ng, 2006). The active peptides have been identified and studied from in vitro enzymatic digests, *in vivo* gastro-intestinal digests, and or by chemical synthesis (Gobbetti et al., 2002, Sistla, 2013). The major bioactive proteins from bovine milk are the caseins (α_{s1}, α_{s2}, κ and β). The casein proteins lack a well-defined tertiary structure under physiological conditions (Uversky, 2002, Dunker et al., 2002) and therefore are classified as intrinsically or natively disordered proteins. Caseins associate together and form micelles (spherical particles) which give white appearance to milk and skim milk due to their ability to scatter light. The micelles are large and spherical with radii of 600 nm or less.

In the bovine casein micelle, approximately 80% of the particle volume is water because of the unfolded nature of the casein polypeptide chains and about 7% of the solute mass is calcium phosphate (Holt and Carver, 2012). Each casein micelle contains amorphous calcium phosphate nanoclusters bound tightly to short phosphorylated sequences called phosphate centre (PC) sequences which are present in the α_{s1}-, α_{s2}- and β-caseins. The remaining casein, κ, is partly present in the micelle surface region and limits the size of the micelles (Holt and Carver, 2012) probably via its hydrophilic C-terminal half which forms a diffuse outer region to the micelle (Fig 5.1) (Holt, 2004, Holt, 1993).

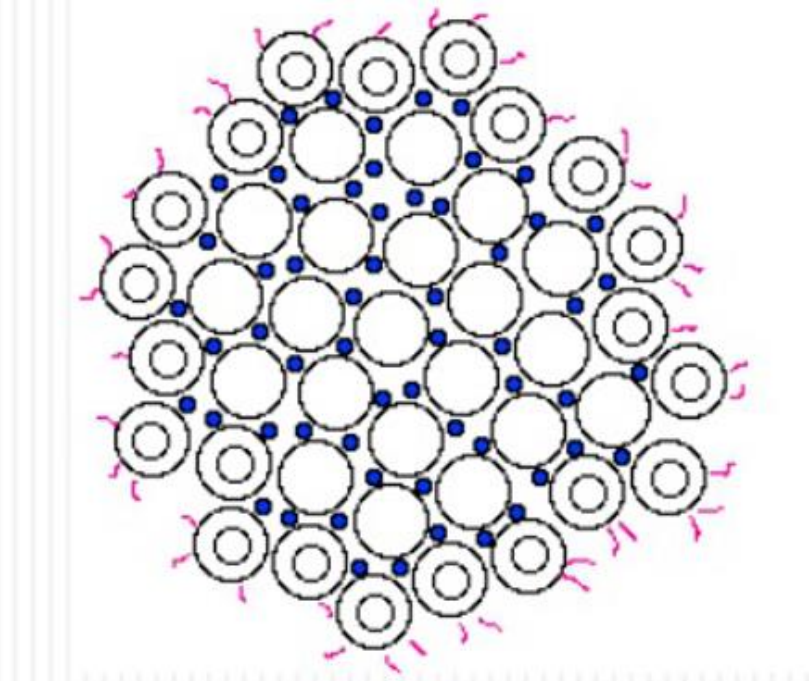


Figure 5.1: Molecular model showing structure of casein micelle. The hydrophobic regions of the protein are largely buried within the interior of the spherical particle and are shielded from solution by hydrophilic regions. The hydrophilic regions are represented by the 'hairs' protruding from the surface. Adapted from Holt *et al*. (2013)

5.2.1 Casein Amyloid formation

As stated in Chapter 1, 2 and 3, a broad range of human diseases arise from the failure of specific peptides or proteins to adopt, or remain in, their native functional conformation causing protein conformational/ misfolding diseases. *Corpora amylacea* (CA), mineralized amyloid stones containing casein peptides, are formed in the mammary glands during late lactation and during involution (Niewold et al., 1999), but they seldom affect the efficiency of lactation of other species, including cows, rats and canines. These CA deposits share many characteristics to amyloid fibrils associated with human disease (Goldsbury et al., 2000).

Potential amyloid folding proteins are numerous. The primary structure of most proteins examined contains at least one potential amyloid-forming subsequence (Goldschmidt et al.,

2010). However, different mechanisms have evolved to protect proteins against aggregation including burying the subsequence in a folded domain and the deployment of folding assistants such as molecular chaperones. Some molecular chaperone such as small heat-shock proteins (sHsps), have extensive regions that are disordered or unfolded in conformation (Bagneris et al., 2009, Jehle et al., 2010, Laganowsky et al., 2010), which facilitates their interaction with and stabilization of, a diversity of target proteins during chaperone action. With caseins being unfolded, they lack the option to bury amyloidogenic subsequences within their own structure. As a solution, they form an amorphous, nontoxic aggregate (i.e. the casein micelle) to isolate them (Holt et al., 2013).

Native α_{s2}-CN exists mostly as dimers formed by two intermolecular disulfide bonds while κ-CN has a series of disulfide-linked multimers i.e. dimers to decamers or higher (Rasmussen et al., 1994). These disulfide linkages dictate the oligomeric state of the precursor protein, which is a critical determinant for fibril formation. Fibril formation by the disulfide-linked form of κ-CN found in micelles is therefore relatively limited at 37°C but increases with incubation temperature and disulfide bond reduction (Thorn et al., 2005).

Milk also contains various globular proteins called whey proteins which include α-lactalbumin, β-lactoglobulin and serum albumin. These whey proteins are highly structured and heavily disulfide-bonded and are sensitive to temperature, pH and other conditions (Bhattacharyya and Das, 1999). Whey proteins adopt a number of partially unfolded intermediates when placed in various destabilising environments, and some of these species are prone to the formation of fibrils (Arnaudov et al., 2003, Bolder et al., 2006, Goers et al., 2002, Hamada and Dobson, 2002, Oboroceanu et al., 2010, Pearce et al., 2007). However under physiological conditions, whey proteins have little to no tendency to aggregate, and are therefore unlikely to give rise to the fibrils observed in CA mammary tissue.

5.2.2 Casein micelle structure and functions

The structure and biological functions of the casein micelle are closely related. About 95% of casein in milk is incorporated into micelle (spherical particles) which average around 10^5 kDa in mass (Fox and McSweeney, 2003). The three biological functions of the casein micelles are (1) the retention of micelles in the stomach of the neonate for digestion and absorption of nutrients, (2) the secretion of high concentrations of calcium and phosphate to prevent calcification of the mammary gland, and (3) the safe secretion of high concentrations of potentially fibrillogenic casein proteins through the mammary gland (Holt et al., 2013). Molecular models of the micelles generally include at least one of the following features; (1) the casein molecules are held together, to some degree by the colloidal calcium phosphate or the micelle exterior is rich in κ-casein while the interior contains mostly calcium-sensitive α_{s1}-, α_{s2}-, and β-caseins (Fox and McSweeney, 2003). Figure 5.2, outlines schematically the functional pathway leading to the formation of casein micelles and two dysfunctional pathways leading to either large amorphous precipitate or ordered fibrillar structures.

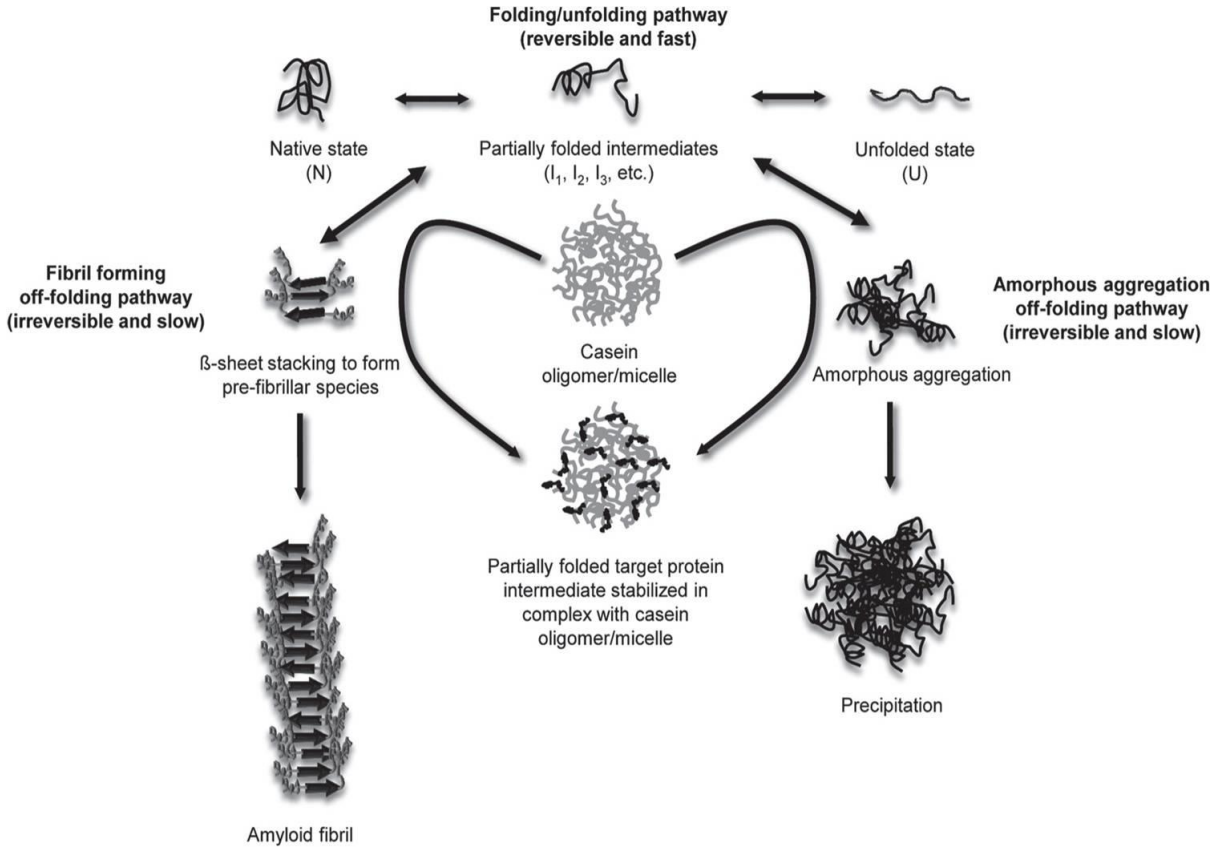


Figure 5.2: Schematic representation of the formation of a casein micelle. A native protein (N) unfolds via a variety of intermediate (I1, I, I3 etc) to the unfolded state (U). Under stressful conditions or mutation, the unfolded intermediate can self associate via exposed hydrophobic regions that are normally buried in the native state. When self assembly occurs, a partially folded intermediate enters the off-folding pathway to form either an amorphous insoluble protein precipitate or ordered amyloid fibril. Figure from Holt *et al.* (2013).

A partially folded intermediate form can also interact with native casein micelles or oligomers of α_S-CN (a mixture of α_{S1}- and α_{S2}-CN) to prevent it from self-associating. In doing so, the caseins form high-molecular-mass complexes with the target protein.

Caseins have extremely open, dynamic and flexible conformations which have led to suggestions that they exist in a type of molten globule with some elements of secondary structure but little to no tertiary structure (Farrell et al., 1996). The other molten globule

characteristic the caseins share is significant solvent-exposed regions of hydophobicity (Kuwajima, 1996). Caseins have distinct hydrophobic and hydrophilic regions because the polar and apolar residues are not uniformly distributed but occur in clusters (Fox and McSweeney, 2003). The open conformation of natively folded caseins is maintained by the high charge densities in the highly phosphorylated sequences and a high proportion of proline residues in the sequences of low charge density (Sawyer, 1993). The high proline content serves to disrupt α-helical and β-sheet secondary structure (Sawyer, 1993), while the presence of negatively charged phosphate groups causes intramolecular repulsion and opposes hydrophobic-driven collapse to a more compact structure (Uversky, 2002). The open conformation of caseins helps in its rapid degradation to smaller peptides by proteolytic enzymes thereby improved digestibility and is considered important for neonatal nutrition (Sawyer, 1993).

5.2.3 Caseins as molecular chaperones

The production and maintenance of proteins in a solution and biological state *in vivo* requires the coordinated action of a number of systems spanning transcription, RNA processing and transport, protein translation, folding, transport and degradation (Holt et al., 2013). A molecular chaperone (chapter 3) is a protein that can interact with a target protein to either help it acquire its native conformation or stabilize the target protein against aggregation (Hartl et al., 2011). Caseins have many of the properties exhibited by molecular chaperones. In particular, α-crystallin and other sHsps have significant hydophobicity exposed to solution which is vital for their recognition and association with partially unfolded target proteins (Treweek et al., 2011). Caseins and sHsps have extensive regions of disordered structure and tendency to self-associate into micelle-like aggregates (Bhattacharyya and Das, 1999).

Caseins function as molecular chaperones by stabilising partially denatured and aggregating proteins under a variety of destabilising conditions in a similar manner to sHsps (Carver et al., 2003, Morgan et al., 2005, Treweek et al., 2011), e.g. a mixture of α_s-casein (α_{s1}- and α_{s2}-casein, 4:1 w/w) protects a variety of proteins including the whey proteins against chemical-, thermal- and light-induced amorphous aggregation (Bhattacharyya and Das, 1999, Morgan et al., 2005, Matsudomi et al., 2004). On the other hand, β- and κ- casein show comparable effectiveness in preventing the amorphous aggregation of whey and unrelated proteins destabilised by heat and disulfide bond reduction (Morgan et al., 2005, Matsudomi et al., 2004, Guyomarc'h et al., 2009, Zhang et al., 2005, Thorn et al., 2008)

α_{s2}-CN is the least abundant of casein proteins and most difficult to isolate in a pure form, it therefore has been the least studied. Thorn *et al*.,(2008) showed that α_{s2}-CN forms fibrils under physiological conditions. Cell culture studies imply that prefibrillar and mature fibrils are toxic to cells (Bucciantini et al., 2002). On the other hand, amyloid formation pathways can be manipulated by using small molecules and molecular chaperones (Cohen and Kelly, 2003, Ehrnhoefer et al., 2008) thereby preventing cell toxicity.

5.2.4 Amyloid fibrils and EGCG

Polyphenolic compounds have been found to be effective anti-aggregative agents in inhibiting aggregation of a broad spectrum of amyloidogenic proteins (Porat et al., 2006). Natural polyphenols comprise over 8000 plant-derived compounds found in high concentrations in tea, wine, berries, spices and a other plants (Porat et al., 2006). These compounds also possess anti-oxidant activity , anti-carcinogenic, anti-inflammatory and anti-viral properties which are of potential health benefit (Porat et al., 2006, Chen et al., 2008).The polyphenol (-)-epigallocatechin-3-gallate (EGCG) (Fig. 5.3), the major catechin

found in green tea, has recently attracted much interest in protein misfolding research due to its potent anti-amyloid fibril activity. EGCG has inhibits fibril formation of various amyloid-forming proteins and peptides including amyloid-β, α-synuclein, κ-casein and huntingtin (Popovych et al., 2012, Hauber et al., 2009, Ehrnhoefer et al., 2008, Hudson et al., 2009). Herein, we analysed the potential of EGCG to interact with α_{s2}-CN derived fibrils and to interfere with cytotoxicity of the α_{s2}-CN to PC12 cells. Our results highlight the importance of EGCG in preventing amyloid fibril formation thereby inhibiting the general mechanism of toxicity associated with the process.

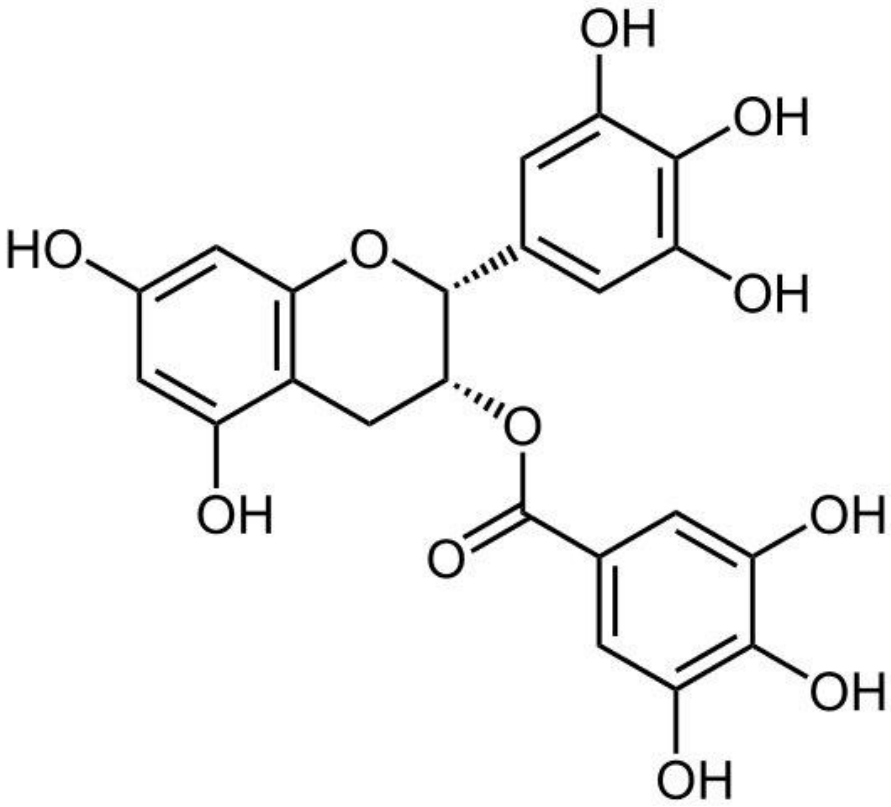


Figure 5.3: The structure of EGCG.

5.3 MATERIALS AND METHODS

5.3.1 Purification of α_{s1} and α_{s2} casein from α_s-casein

Bovine α_s-CN was separated by Dr David Thorn into its major components α_{s1} and α_{s2} by cation-exchange chromatography using the method described by Rasmussen *et al*. (1994) and Thorn *et al.*(2008).

5.3.2 Thioflavin T binding

To generate amyloid fibrils, purified α_{s2}-CN was dissolved in 200 mM phosphate buffer (pH 7.2) and incubated without shaking at 50°C. Samples were periodically withdrawn from the stock solution and snap frozen at -20°C. After the completion of the time course, samples were thawed and mixed with 1.6 mL of 10 µM of ThT in 200 mM phosphate buffer, pH 7.2. The fluorescence was measured using a glass cuvette (10 mm light path) with excitation and emission wavelengths set at 442 and 490 nm using with Cary Eclipse spectrofluorimeter.

To determine the effect of EGCG on fibril formation of α_{s2}-CN, 0.05, 0.5, 1 and 2 µM of EGCG was added to 500 µM of α_{s2}-CN in 200 mM phosphate buffer and incubated under the same conditions and ThT fluorescence determined as above.

5.3.3 Transmission Electron Microscopy

Fibril formation of the peptides was performed as outlined above. 2 µL of aggregates were diluted to 1 mg/mL and added to formavar and carbon-coated nickel grids (SPI supple, West Chester, USA) for 2 minutes. The grids were washed three times with 10 µL of water and

negatively stained with 10 µL of uranyl acetate (2% w/v). Excess stain was removed with filter paper and the grids were air dried and viewed with a CM 100 transmission electron microscope (Philips, Eindhoven, The Netherlands).

5.3.4 Cellular toxicity of α_{s2}-casein

Cells were grown in Roswell Park Memorial Institute (RPMI) media containing 5%v/v fetal calf serum, 1% v/v glutamate, penicillin and streptomycin in a 75 mL flask at 37 ^{0}C in an incubator with 95% air and 5% carbon dioxide. The cells were plated into a 96 well plate at a density of 2×10^4 cells per well and incubated for 24 hours. α_{s2}-CN (500 µM) was dissolved in 200 mM phosphate buffer (pH 7.2). To determine the concentration-dependent toxicity of α_{s2}-CN to PC12 cells, α_{s2}-CN was incubated at 50°C for 144 h to induce fibril formation. Following incubation, both the fibrillar and native α_{s2}-CN samples (dissolved in phosphate buffer) were diluted further in phosphate buffer and added to the cells to the final concentrations of 0, 0.05, 0.5, 5.0, 50 µM and incubated for 48 h. Cell viability was determined by the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.

To determine whether the toxicity of the species formed by incubation of α_{s2}-CN was time-dependent, the protein was incubated under the same conditions as described above, and samples were taken after 24, 48, 72, 96, 108 and 144 h of incubation and snap frozen. These aliquots from various time points were thawed and added to the cells to the final concentration of 5 µM. The cells were incubated at 37°C for 48 h followed by MTT assay.

To determine the effect of EGCG on the cell toxicity of α_{s2}-CN, samples of varying concentrations of EGCG (0.05, 0.5, 1.0 and 2.0 µM) and 5.0 µM α_{s2}-CN, taken from ThT

assay after incubation at 50°C for 144h, were added to the cells and incubated at 37°C for 48 h followed by MTT assay.

5.3.5 MTT assay

3 mg of MTT was dissolved in 12 mL of serum-free RPMI media. After 48 h incubation of treated cells, the media was aspirated and 100 µL of serum-free media containing MTT was added to each well. The plate was incubated for three hours and the media was removed and replaced with 100 µL of dimethyl sulfoxide (DMSO). Absorbance was then measured at 560 nm using BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany).

5.4 RESULTS

5.4.1 Toxicity of α_{s2}-CN to PC-12 cells

To determine whether amyloid fibrils by α_{s2}-CN were toxic to cells- PC 12 cells were treated with α_{s2}-CN in its native form and after incubation in 200 mM (pH 7.2) phosphate buffer at 50°C for 144 hours. Both native and fibrillar α_{s2}-CN caused a concentration-dependent decrease in cell survival (Fig. 5.4). However, fibrillar α_{s2}-CN was much more toxic compared with native state. At 0.05 µM, fibrillar α_{s2}-CN reduced cell viability to 82±6% compared with 88±5% for the native. A big difference was observed at the concentration of 5 µM where fibrillar α_{s2}-CN reduced cell viability to 47±8% compared with 81±6% for the native species (Fig 5.4). The native form at the highest concentration of 50 µM was more toxic to the cells with 62±8% cell viability compared to the fibrillar form, which led to 20±5% cell viability.

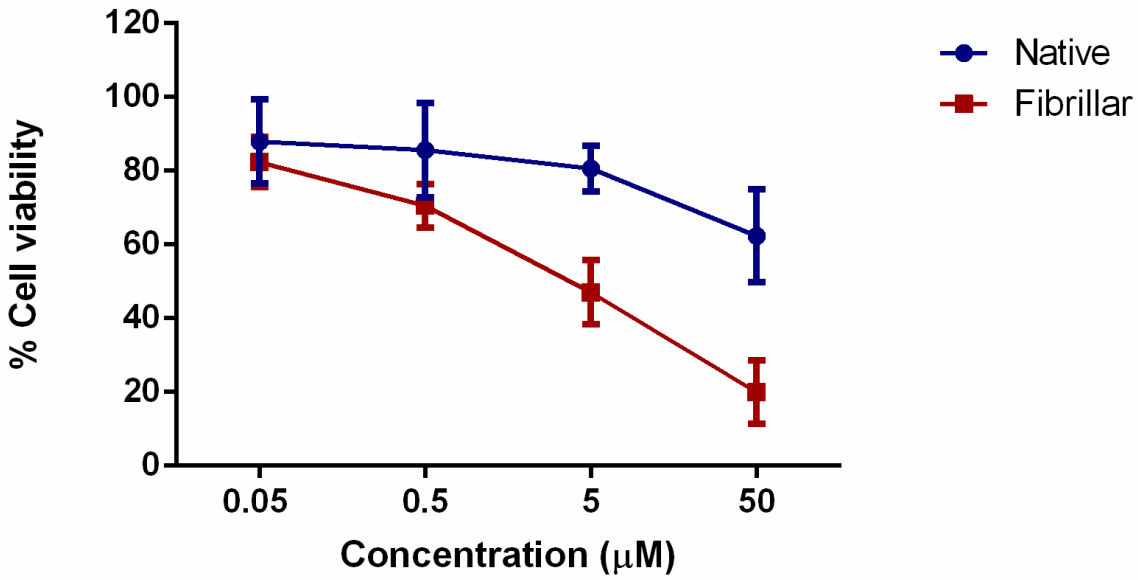


Figure 5.4: Concentration-dependent toxicity of α_{s2}-CN in both the native and fibrillar forms. α_{s2}-CN (500 µM) was dissolved in 200 mM phosphate (pH 7.2) buffer in its native form or incubated for 144 h at 50°C to induce fibril formation. Native (circle) and fibrillar

(square) forms of α_{s2}-CN (0-50 µM) were then added to PC-12 cells containing media and incubated for 48 hours. Cell viability was then assessed by MTT assay. Values are presented as percentage of cell viability compared with control. Results are expressed as mean ± standard error of three independent experiments ($p < 0.05$).

Prefibrillar species, including oligomers and protofibrils of some of amyloid fibril-forming proteins, have been shown to be as toxic as, or more toxic than, mature fibrils (Lansbury and Lashuel, 2006, Dehle et al., 2010, Bucciantini et al., 2002, Chimon et al., 2007, Simoneau et al., 2007). Therefore, we examined whether the toxicity of α_{s2}-casein to cells was due to prefibrillar species or mature fibrils. Samples taken from ThT assay that had been snap frozen at different stages of the fibril-forming process, were thawed and immediately added to PC-12 cells containing media to a final concentration of 5.0 µM α_{s2}-CN and incubated as described in the material and method section. The cell viability of the pre-incubated samples at different times (24, 48, 72, 96, 120 and 144 h) were toxic to the cells with cell viability between 45±5 - 49±5% (Fig 5.5). There was no significant change in cell viability due to time of incubation. The native form did not cause any significant reduction in viability of the cells (results not shown). This results show that both the prefibrillar and fibrillar species of α_{s2}-casein are toxic to PC-12 cells.

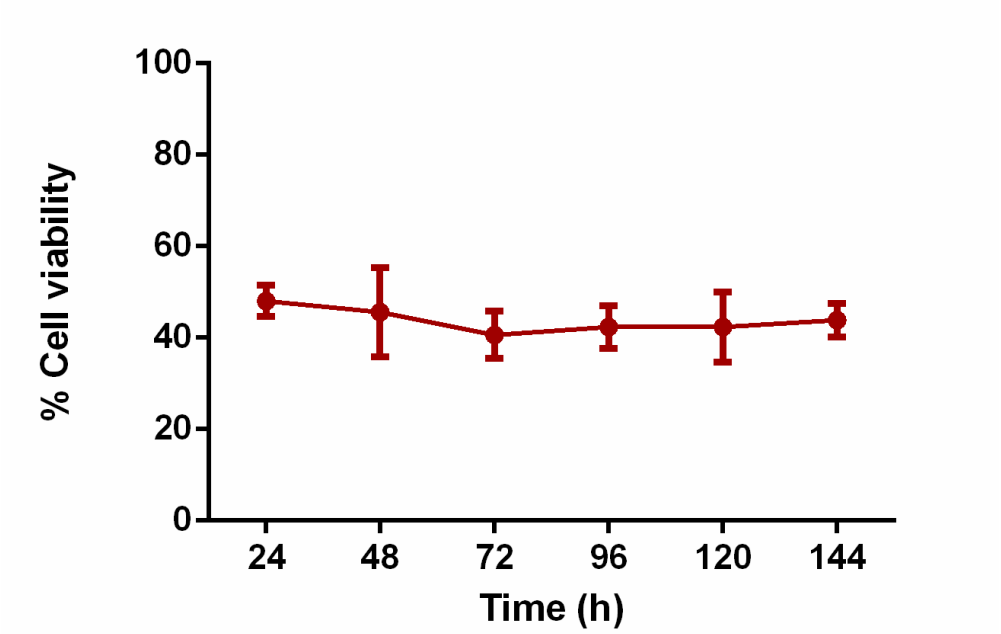
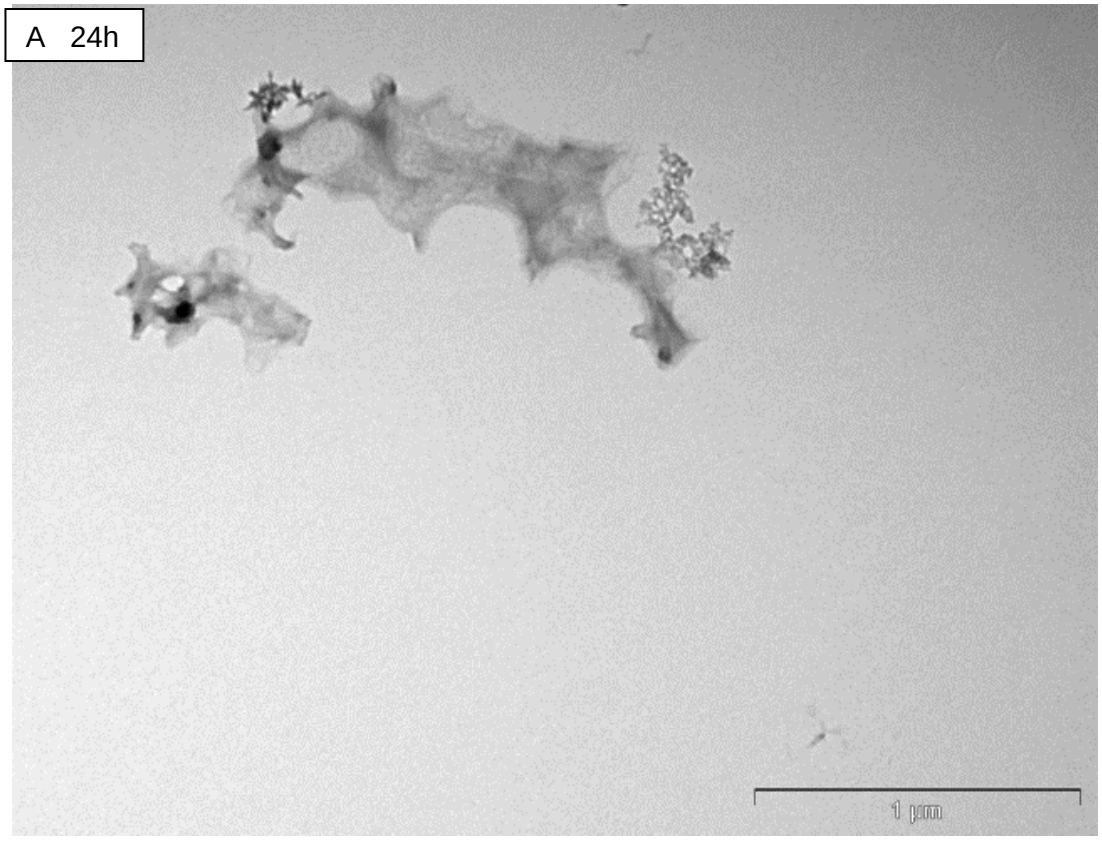
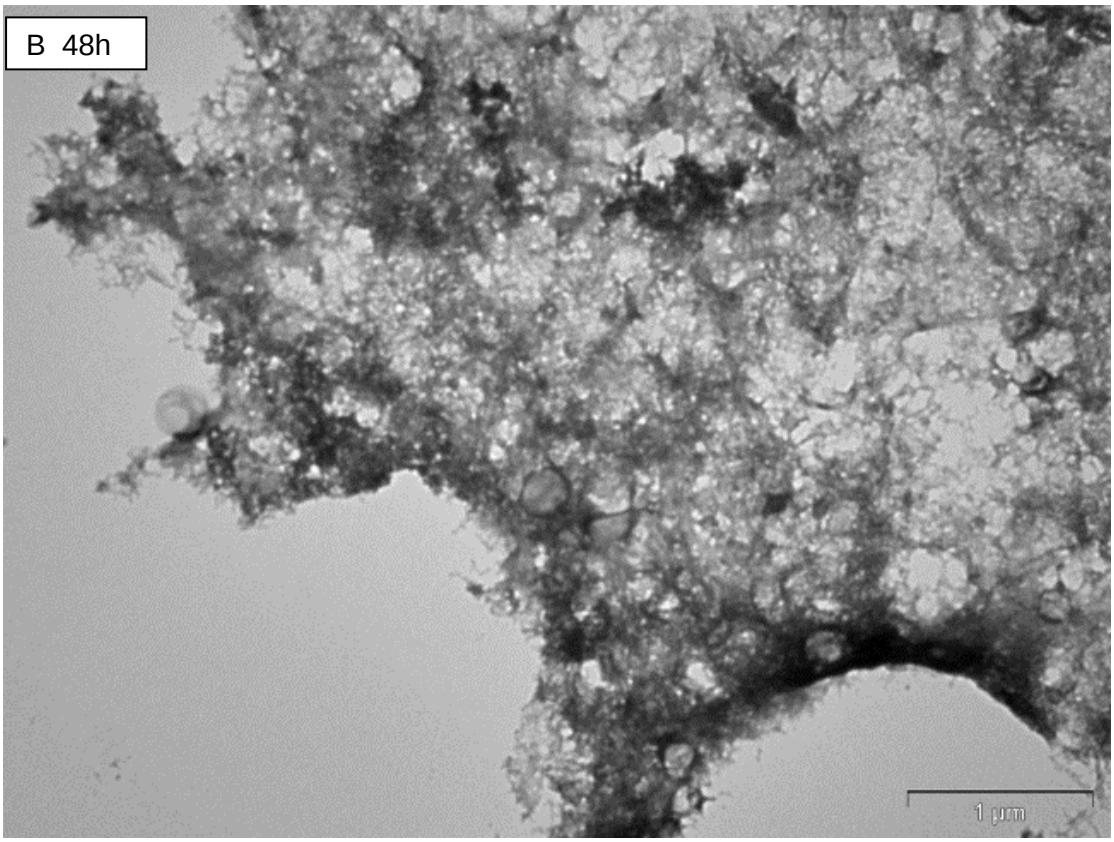


Figure 5.5: Time-dependent toxicity of pre-incubated α_{s2}-CN. α_{s2}-CN (500 µM) was dissolved in phosphate buffer (200 mM, pH 7.2) and incubated for 24, 48, 72, 96, 120 and 144 h at 50°C to induce fibril formation. The pre-incubated form of varying time course were then added to the culture media of PC-12 cells to final concentration of 5 µM and the cells were incubated for 48 h. Cell viability was assessed using the MTT assay. Values are presented as percentage survival compared with the control. Results are expressed as mean ± standard error of three independent experiments.

TEM analysis (Fig.5.6) confirmed fibril formation by α_{s2}-CN. The morphology of α_{s2}-CN was different depending on the length of incubation. After 24 hours of incubation, α_{s2}-CN exhibited a dense intact structure with no signs of amyloid fibrils (Fig 5.6A). With further incubation up to 48 hours, the structure that was observed in 24 h was still present but the structure was loose (Fig 5.6B). After 96 h of incubation, α_{s2}-CN showed the dense loose structure breaking away with some fibre-like structures (Fig 5.6C). After 144 h, there was a dramatic change in morphology with evidence of mature fibrils as shown in (Fig 5.6D).


A 24h
1 µm


B 48h
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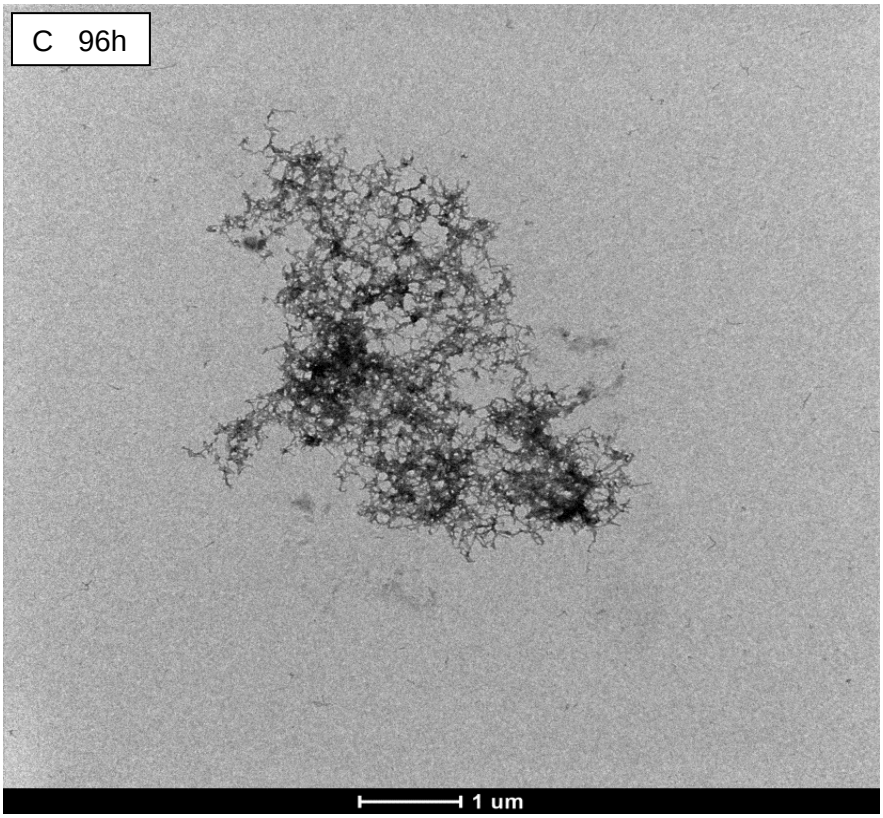

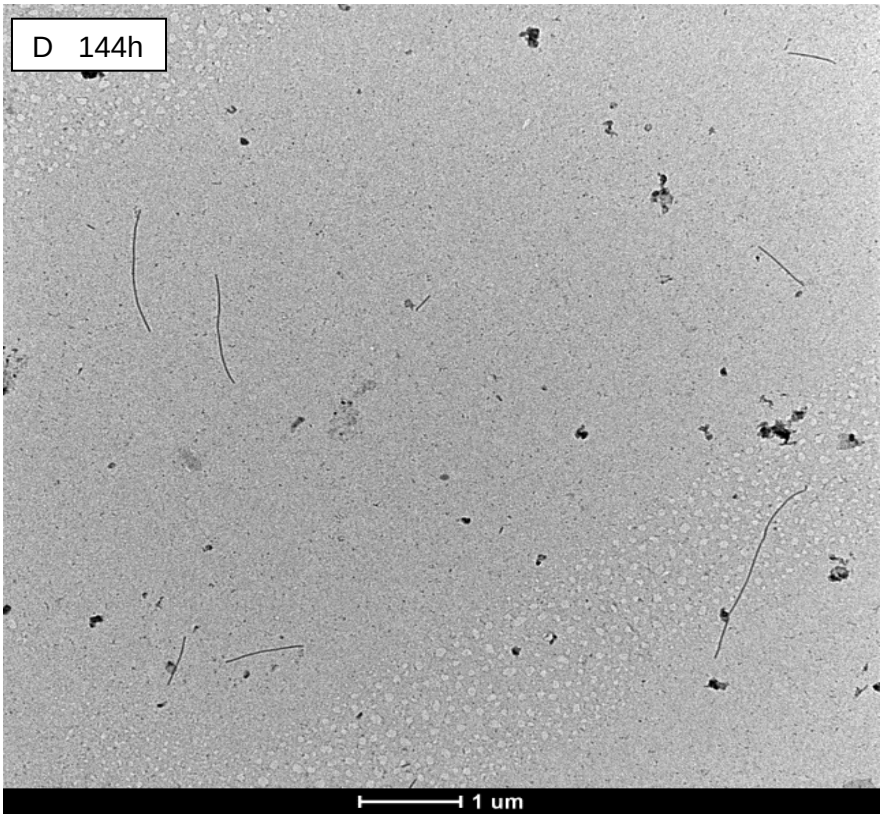

Figure 5.6: Electron micrographs of α_{s2}-CN (5µM) after A 24 h, B 48 h, C 96 and D 144 h incubation at 50°C, showing fibril formation. *Scale bar*, 1 µm.

5.4.2 Effects of EGCG on α_{s2}-CN amyloid fibrils formation

To determine the anti-aggregation efficacy of EGCG toward α_{s2}-CN, fibril formation of freshly dissolved protein (500 µM in phosphate buffer, pH 7.2) was initiated by incubation at 50°C without shaking and monitored over time by Thioflavin T fluorescence. In agreement with previous data (Thorn et al., 2008), a fluorescence increase of α_{s2}-CN alone was observed rising sharply during the early period of incubation and exhibiting no discernible lag phase (Fig 5.7) implying amyloid fibril formation. Incubation of α_{s2}-CN with EGCG leads to a concentration-dependent decrease in the ThT fluorescence, with 1 and 2 µM of EGCG yielding complete abolition of fibril formation by 500 µM α_{s2}-CN (Fig 5.7). The EGCG concentration of 0.05 µM resulted in approximately 50% inhibition of the increase in ThT fluorescence. Furthermore, EGCG incubated alone produced no change in ThT fluorescence (results not shown), indicating that EGCG is neither inherently amyloidogenic nor likely to interact directly with free solvated ThT. These results indicates that EGCG is a very efficient inhibitor of α_{s2}-CN fibril formation, i.e. at 250:1 α_{s2}-CN: EGCG, on a molar basis.

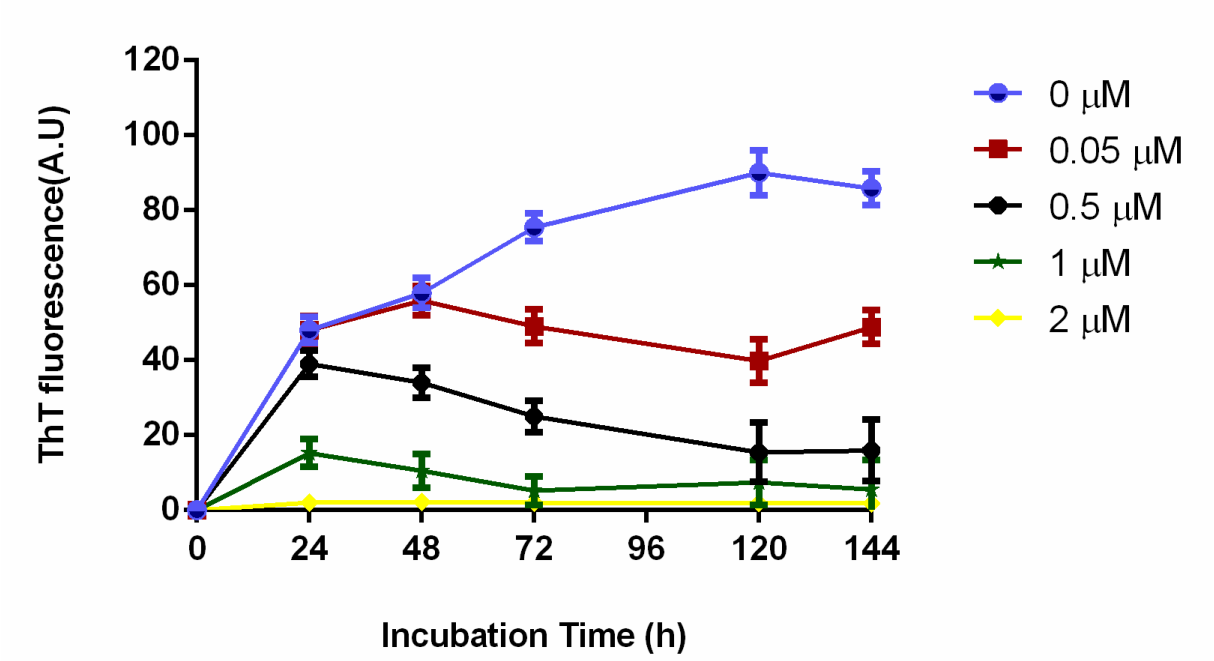

Figure 5.7: The time-dependent fluorescence analysis of fibril formation by α_{s2}-CN in the presence and absence of EGCG. α_{s2}-CN was dissolved in 200 mM phosphate buffer (pH 7.2) incubated at 50°C in the presence and absence of varying concentration of EGCG for 144 hours. Reactions containing EGCG or PBS alone served as controls (results not shown). Error bars represent three independent experiments ±SD.

TEM was used to assess the morphological effect of EGCG on α_{s2}-CN fibril formation (Fig 5.8). TEM micrographs of α_{s2}-CN alone showed mature fibrils (results not shown). In the presence of the lowest concentration of EGCG (0.05 μM) the electron micrograph showed fibrils similar to those formed when α_{s2}-CN was incubated alone under identical conditions and during the same period (Fig 5.8A). For the highest concentration of EGCG, 2 μM, large amorphous aggregates were observed with no evidence of ordered structure which appeared to be assembled from nonfibrillar components (Fig 5.8B).

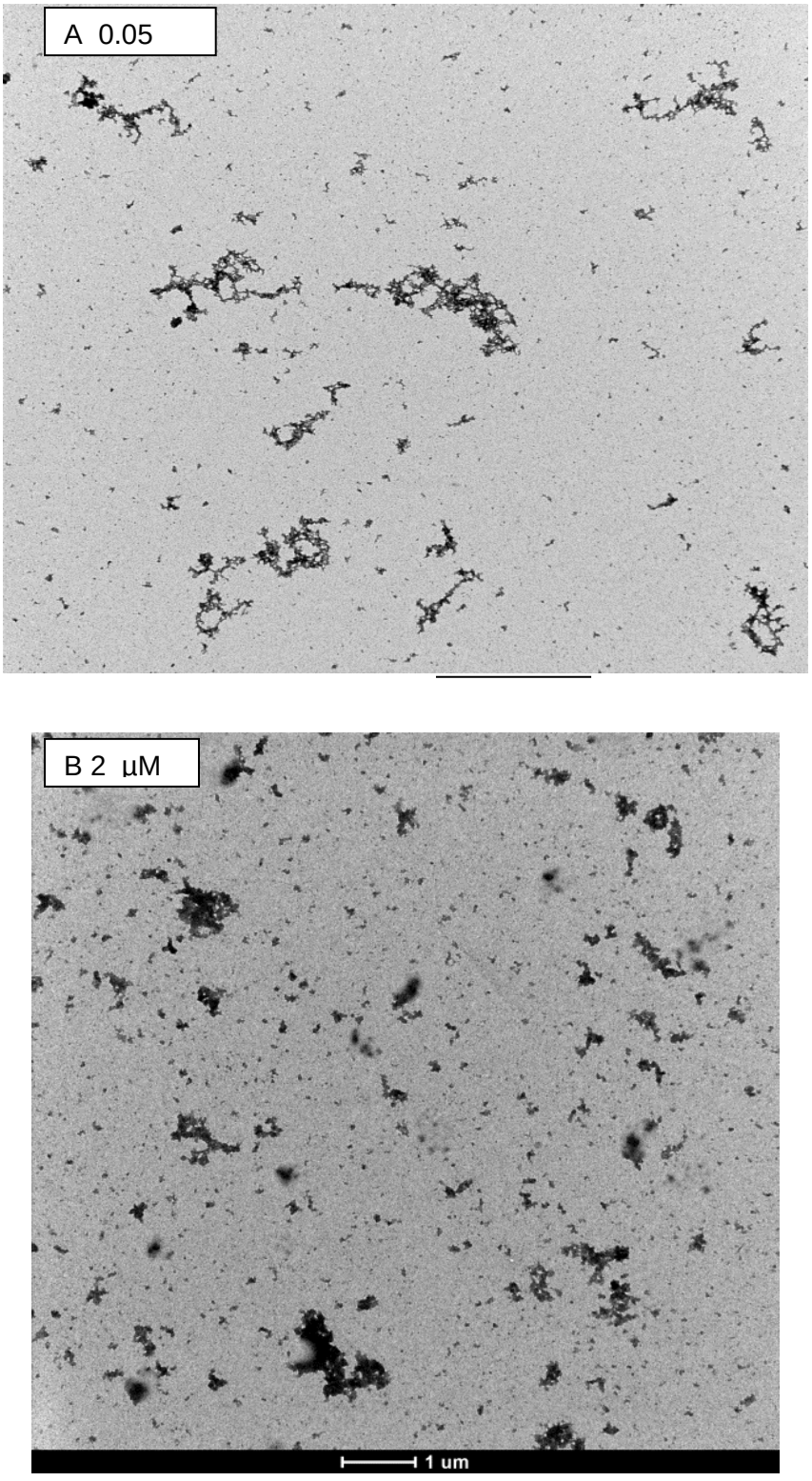

Figure 5.8: Electron micrographs of α_{s2}-CN with varying concentration of EGCG. α_{s2}-CN (50 µM) with (A) 0.05 µM, and (B) 2 µM EGCG after 144 h incubation. Scale bars represent 1 µm.

Finally, to assess the cytoprotective capacity of EGCG, PC 12 cells were treated with α_{s2}-CN pre-incubated at 50°C for 144 h with and without EGCG from the *in situ* ThT fluorescence assay (Fig 5.7). The final concentration of α_{s2}-CN was 5 µM with varying concentrations (0.02, 0.05, 0.5 and 1.0 µM) of EGCG (Fig 5.8). α_{s2}-CN aggregates had a significant decrease in viability of PC-12 cells in a concentration-dependent manner as shown previously (Fig 5.4). When the samples of α_{s2}-CN incubated with EGCG were added to PC-12 cells, there was a decrease in the toxicity of α_{s2}-CN (Fig 5.9) to PC-12 cells. 0.05 µM EGCG did not show any protection to the cells but 0.5 µM EGCG increased cell viability from 47±4% to 70±6% with 1 µM of EGCG showing almost 100% protection of the cells (Fig 5.9). These data corresponded well with the efficiency of EGCG prevention of amyloid fibril formation (Fig 5.7).

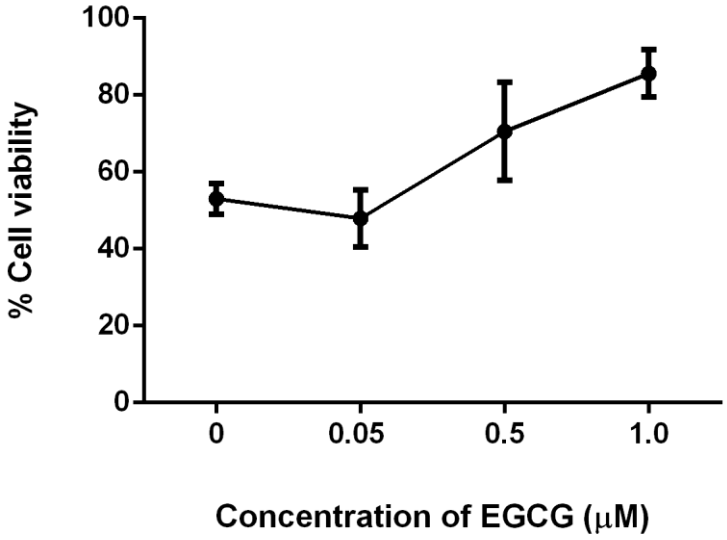

Figure 5.9: Effect of EGCG on αs2-CN induced PC-12 cell toxicity. Different concentrations of EGCG (0, 0.05, 0.5 and 1 µM) were added to α_{s2}-CN (5 µM) dissolved in phosphate buffer (50 mM. pH7.2) incubated for 144h at 50°C. Samples were then added to PC 12 cells containing media and incubated for 48 hours. Cell viability was assessed by MTT

assay. Values are presented as percentage of cell viability compared with control. Results are expressed as mean of three independent experiments ±SD

5.5 DISCUSSION

Thorn *et al.* (2008) showed that α_{s2}-CN readily forms amyloid fibrils under physiological conditions. In the present study, we have extended this findings by showing that fibrils formed by α_{s2}-CN are toxic to PC-12 cells. Furthermore, fibrils formation by α_{s2}-CN is inhibited by a natural antioxidant EGCG thereby preventing the cytotoxicity associated with the fibrils.

Fibril networks with the hallmarks of amyloid are, on occasion, seen extracellularly within the proteinaceous deposits or inclusions of bovine mammary *corpora amylacea* (CA) and electron microscopy has identified fibril-like structures within the cytoplasm in mammary epithelial cells surrounding the CA (Beems et al., 1978, Nickerson et al., 1985). Peptides have been sequenced from the fibrillar CA deposits and they have been identified from α_{s2}-CN (Niewold et al., 1999).

α_{s2}-CN comprises of a mixture of monomers and disulfide-linked dimers (Rasmussen et al., 1992, Rasmussen et al., 1994). The propensity of α_{s2}-CN to assemble into fibrils at physiological pH and temperature raises a question of whether these fibrils are toxic or not to the cells and tissues it comes in contact with? In the present study we have shown that α_{s2}-CN is toxic to PC-12 cells in a concentration-dependent manner. Cell culture studies have shown that oligomers, protofibrils and mature fibrils are toxic, although the level of toxicity of between these species is dependent on the specific fibril-forming protein. For example, oligomers formed by prion protein were highly toxic to cortical neurons whereas the mature fibrils of the same protein demonstrated very little toxicity (Simoneau et al., 2007). However, a similar study of the toxicity of prion oligomers and mature fibrils to SH-SY5Y cells showed that mature fibrils were as toxic as the oligomers (Novitskaya et al., 2006). For Aβ,

oligomers, protofibrils and mature fibrils were toxic to the cells in a similar manner (Chimon et al., 2007, Hartley et al., 1999, Hoshi et al., 2003). In a study of non-disease related proteins, prefibrillar and protofibrillar aggregates were toxic to the cells whereas mature fibrils exhibited no significant toxicity (Bucciantini et al., 2002). Our results indicate that toxicity of α_{s2}-CN is due to prefibrillar species and mature fibrils.

5.5.1 Inhibition of fibril formation by α_{s2}-CN by EGCG

EGCG inhibits amyloid fibril formation by a variety of other peptides and proteins including α-synuclein (Bieschke et al., 2010), SEVI (Hauber et al., 2009), IAPP (Meng et al., 2010), Aβ1-42 and Aβ1-40 (Choi et al., 2001), transthyretin (Ferreira et al., 2009), prion protein (Roberts et al., 2009), human calcitonin (Huang et al., 2012), κ-casein (Hudson et al., 2009), tau and lysozyme (Ghosh et al., 2013). The mechanism that EGCG uses to accomplish this is not clear. It has been proposed that EGCG diverts the normal aggregation pathway into the formation of spherical unstructured amorphous that do not progress further into amyloid fibrils (Ehrnhoefer et al., 2008, Bieschke et al., 2010, Hudson et al., 2009). EGCG, on a molecular level has been proposed to bind to the exposed unfolded regions of proteins thereby blocking the association of aggregation prone regions (Ehrnhoefer et al., 2008). On the basis of the apparent generality of amyloid inhibition by EGCG, the formation of off-pathway aggregates by nonspecific binding of EGCG to exposed backbone sites was proposed to be the mechanism for amyloid inhibition by EGCG. Therefore, we analysed the potential effect of EGCG on fibril formation by α_{s2}-CN. At the lowest concentration of EGCG tested in this study, 0.05 µM EGCG did not provide any protection to the cells even though the ThT fluorescence was reduced to almost 50%. Both 0.5 and 1 µM EGCG showed significant protection to the cells against fibril formation by α_{s2}-CN. These findings are in agreement with studies by Ehrnhoefer *et.al.* (2008) indicating that EGCG redirects αS and

Aβ42 aggregation cascades and thus prevents the formation of toxic aggregates. Moreover, EGCG has shown to have a significant neuroprotective as well as anticarcinogenic effects in cell model systems (Mandel et al., 2005).

In summary, our *in vitro* studies indicate that amyloid fibrils formed by α_{s2}-CN are toxic to PC-12 cells and demonstrate that EGCG is indeed capable of effectively inhibiting fibril formation by α_{s2}-CN, which correlates with a reduction in the cell toxicity of these amyloid fibrils. Our findings highlight the important role that EGCG, a natural ingredient of green tea, may play in the treatment and prevention of fibril-associated diseases including mammary CA amyloidosis which may be associated with fibril formation by αs2-CN.

5.6 ACKNOWLEDGEMENTS

We thank Ms Lyn Waterhouse for assisting with transmission electron microscopic experiments.

6 Conclusions

In chapter 2 (Elias et al., 2014), the computer algorithms; Zipper DB, Zyggregator and Tango identified regions of PAP248-286 (SEVI when in fibril form) that are prone to aggregation. Each computer algorithm used a different method to identify peptide aggregation regions. Of the regions identified, it has been demonstrated that fragments from the central region forms fibrils of similar morphology to SEVI. SEVI and its fragment PAP248-271 formed long unbranched fibril while fibrils formed by PAP257-267 were short and clumped together. The experimental data presented demonstrates the utility of various biophysical methods in determining the structure of SEVI, with the region I267-M271 shown to be crucially important in fibril kinetics by SEVI.

SEVI and its fragments PAP248-271 and PAP257-267 were toxic to neuronal cells but not to confluent epithelial cells suggesting that even though SEVI maybe helping HIV-1 to attach to cells, it might not in itself harm healthy confluent cells. This potentially eliminates one way SEVI may increase HIV infectivity.

In Chapter 3, the enhancement of SEVI and its fragment, PAP248-271, to HIV infection was investigated. PAP248-271 enhanced HIV infection in a similar manner to SEVI. As shown in chapter 2, SEVI does not compromise the integrity of epithelial cells to help HIV to attach to cells; therefore investigations on the enhancement of HIV infection of SEVI and PAP248-271 were extended further by determining whether SEVI and PAP248-271 directed the HIV to a particular coreceptor for entry into the host cells. Infectivity assays showed that both coreceptors, CCR5 and CXCR4 were used by HIV for entry into the cells with the smaller fragment more potent in assisting HIV infection than SEVI.

In Chapter 4, the two studies presented in chapters 2 and 3 were extended further by investigating the ability of clusterin to prevent fibril formation by SEVI thereby preventing cytotoxicity and enhancement of HIV infection associated with these fibrils. Clusterin is found in semen at the concentration of 0.5-15 mg/mL. In an effort to determine the effect of clusterin on SEVI fibril formation, different concentrations of clusterin were added to PAP248-286 and incubated as shown in material and methods section. The results of this study suggest that clusterin prevents SEVI fibril formation and targets the already formed fibrils for degradation, thereby reducing and preventing cytotoxicity of SEVI. In vivo, at least in semen, the ability of clusterin to prevent the formation of SEVI fibrils would thereby effectively abrogate their HIV-1 enhancement activity.

In Chapter 5, the range of amyloid fibrils showing cytotoxicity was extended to α_{s2}-casein by investigating the toxicity of amyloid fibrils from α_{s2}-casein to PC 12 cells. Different morphologies of prefibrillar aggregates were shown depending on the incubation time. The fibrils formed exhibited medium straight structures. The fibrils were toxic to neuronal cells in a concentration dependent manner. The time dependent experiments showed that both the prefibrillar and fibrillar aggregates were toxic to cells. EGCG has shown to be effective in preventing fibril formation by α_{s2}-CN thereby preventing toxicity associated with fibril formation by α_{s2}-CN.

To summarise, SEVI and its fragments PAP248-271 and PAP247-257 forms fibrils of similar morphology at physiological pH and temperature. Fibrils formed by these peptides are toxic to neuronal cells but not to confluent epithelial cells. The fragments showed to be more toxic at a lower concentration compared to SEVI. These fibrils by both SEVI and the fragment enhance HIV infection by helping HIV to attach to both CCR5 and CXCR4 coreceptors. Fibrils formed by α_{s2}-CN are toxic to neuronal as well as confluent epithelial cells. This difference aside, the ease by which both peptides and proteins form fibrils *in vitro* that are

toxic to cells suggests that they might form amyloid fibrils *in vivo* that are toxic to the cells thereby causing harm to the body. It is conceivable that HIV infection via sexual activity would be more likely, if it were not for SEVI-clusterin interactions which potentially inhibit fibril formation. Through the interaction of fibril precursors and fibril inhibitors like clusterin and EGCG accumulation of amyloid fibrils in vivo is prevented.

6.1 Future directions

6.1.1 Cytotoxicity of SEVI to PC 12 cells

Preliminary studies involving the cytotoxicity of SEVI to PC 12 cells have shown that stoichiometric amounts of SEVI reduce cell viability. Additionally SEVI did not reduce cell viability of confluent epithelial (CACO-2) cells. Future studies will explore the resistance of CACO-2 cells to SEVI fibrils. The immediate outcome of this research is a better understanding of how epithelial cells protect themselves from toxicity associated with fibrils. Furthermore toxicity of non confluent epithelial cells needs to be investigated. The results of these studies will be crucial for understanding the impact SEVI has on damaged cells that SEVI would encounter during sexual activities which compromise the epithelial layer and the potential use of therapeutics in the prevention of cytotoxicity of SEVI and other amyloid forming peptides/proteins.

6.1.2 The effect of clusterin *in vivo*

Clusterin has shown to have some chaperone activity by preventing amyloid formation of SEVI *in vitro*. Clusterin has also prevented amyloid formation by variety of species including Aβ, prion protein and apolipoprotein (Hughes et al., 1998, Matsubara et al., 1996, McHattie and Edington, 1999, Oda et al., 1995). The concentration of clusterin is semen (0.5-15 mg/mL), is much higher than the concentration that prevents amyloid fibril formation by

SEVI. Therefore some *in vivo* experiments are needed to determine if this is still the case *in vivo*.

6.1.3 The effect of SEVI fragments on viral infection

SEVI and its fragment PAP248-271 have shown similar activity in their toxicity to neuronal cells, their enhancement of HIV infection as well as the use of the same coreceptors to enter the host cells. The ability of SEVI to promote the interaction between virions and the cell surface is not dependent on the viral glycoprotein and hence not restricted to HIV-1(Münch et al., 2007). Researchers have shown that SEVI enhance infection of other viruses including hybrid xenotrophic murine leukemia-related virus (XMRV) (Hong et al., 2009), cytomegalovirus (Tang et al., 2013), simian immunodeficiency virus (Munch et al., 2013). As there is similarity between SEVI and PAP248-271, more work is needed to determine if PAP248-271 will enhance infection of other viruses including Hepatitis A, B and C viruses, Herpes Simplex Virus and Cytomegalovirus. These experiments can be done by infecting the cells with the test viruses in the presence and absence of SEVI.

6.1.4 Mechanism of toxicity of α_{s2}-CN

α_{s2}-CN has shown to be extremely toxic to both neuronal (PC 12) and epithelial (CACO-2) cells as shown in chapter 2 and 4. CACO-2 cells have shown some resistance to toxicity from fibrils formed by SEVI and its fragments PAP248-271and PAP257-267 as well as RCMκ-CN which are toxic to PC 12 cells (Dehle et al., 2010, Elias et al., 2014). The CACO-2 cell line is the most widely used and best characterized cells of human colonic origin. When grown in culture, the cells exhibit many properties of small intestinal epithelium as they form a polarized monolayer of columnar absorptive cells expressing a brush border on their apical surface with typical small intestinal enzymes and transporters (Artursson, 1991). The cells form dense intercellular junctional complexes resulting in a tight epithelium and thus

presenting a unique barrier (Constanze H, 1999). As fibrils from α_{s2}-CN are toxic to CACO-2 cells while other amyloid forming species are not, a further investigation on toxicity of α_{s2}-CN is of importance.

6.1.5 The effect of on αs2-CN viral infection

α_{s2}-CN easily forms fibrils at physiological pH and temperature. Researchers have shown that other amyloid fibril forming species enhance viral infections. SEVI enhances HIV, XRMV and CMV infections (Munch et al., 2013). β-amyloid enhances Influenza A virus infection (White et al., 2014) while alpha 2-macroglobulin enhances dengue virus (Huerta et al., 2014). It is of great importance to determine *in vitro* whether the amyloid fibrils formed by the milk protein α_{s2}-CN enhance viral infectivity especially viruses that cause gastroenteritis in children i.e. rotavirus, norovirus, adenovirus and astrovirus. Viruses cause about 70% of episodes of gastroenteritis in the paediatric age group (Dennehy, 2011). Gastroenteritis is one of the common causes of morbidity and mortality in children worldwide and accounts for 2 to 3 million deaths per year (Dennehy, 2011). This group depends mainly on milk for their daily food intake therefore the outcome of this research is a better understanding of whether consumption of milk during viral infection has effect on the viral infectivity.

6.1.6 The effect of on αs2-CN tissue mammary glands

Studies have shown that α_{s2}-CN has been isolated from mineralized amyloid-like deposits (corpora amylacea) in bovine mammary tissue. In chapter 2, α_{s2}-CN has shown some toxicity to confluent epithelial cells. Future studies will explore the toxicity of fibrils formed α_{s2}-CN on mammary gland.

7 References

ADESNIK, M. & LEVINTHAL, C. 1969. Synthesis and maturation of ribosomal RNA in Escherichia coli. *Journal of molecular biology,* 46**,** 281-303.

AGUILAR, R. C. & WENDLAND, B. 2003. Ubiquitin: not just for proteasomes anymore. *Current opinion in cell biology,* 15**,** 184-90.

AKARSU, E., PIRIM, I., SELCUK, N. Y., TOMBUL, H. Z. & CETINKAYA, R. 2001. Relation between serum ubiquitin levels and KT/V in chronic hemodialysis patients. *Nephron,* 88**,** 280-2.

AKIYAMA, H., YAMADA, T., KAWAMATA, T. & MCGEER, P. L. 1991. Association of amyloid P component with complement proteins in neurologically diseased brain tissue. *Brain research,* 548**,** 349-52.

AMM, I., SOMMER, T. & WOLF, D. H. 2014. Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. *Biochimica et biophysica acta,* 1843**,** 182-96.

ANDERSON, R. M., MAY, R. M., BOILY, M. C., GARNETT, G. P. & ROWLEY, J. T. 1991. The spread of HIV-1 in Africa: sexual contact patterns and the predicted demographic impact of AIDS. *Nature,* 352**,** 581-9.

ANFINSEN, C. B. 1973. Principles that govern the folding of protein chains. *Science,* 181**,** 223-30.

ARNAUDOV, L. N., DE VRIES, R., IPPEL, H. & VAN MIERLO, C. P. 2003. Multiple steps during the formation of beta-lactoglobulin fibrils. *Biomacromolecules,* 4**,** 1614-22.

ARREDOUANI, M. S., KASRAN, A., VANOIRBEEK, J. A., BERGER, F. G., BAUMANN, H. & CEUPPENS, J. L. 2005. Haptoglobin dampens endotoxin-induced inflammatory effects both in vitro and in vivo. *Immunology,* 114**,** 263-71.

ARRILDT, K. T., JOSEPH, S. B. & SWANSTROM, R. 2012. The HIV-1 env protein: a coat of many colors. *Current HIV/AIDS reports,* 9**,** 52-63.

ARTURSSON, P. 1991. Cell cultures as models for drug absorption across the intestinal mucosa. *Critical reviews in therapeutic drug carrier systems,* 8**,** 305-30.

BAGNERIS, C., BATEMAN, O. A., NAYLOR, C. E., CRONIN, N., BOELENS, W. C., KEEP, N. H. & SLINGSBY, C. 2009. Crystal structures of alpha-crystallin domain dimers of alphaB-crystallin and Hsp20. *Journal of molecular biology,* 392**,** 1242-52.

BAI, Y., ENGLANDER, J. J., MAYNE, L., MILNE, J. S. & ENGLANDER, S. W. 1995. Thermodynamic parameters from hydrogen exchange measurements. *Methods in enzymology,* 259**,** 344-56.

BALCELLS, M., WALLINS, J. S. & EDELMAN, E. R. 2008. Amyloid beta toxicity dependent upon endothelial cell state. *Neuroscience letters,* 441**,** 319-22.

BANDYOPADHYAY, U., KAUSHIK, S., VARTICOVSKI, L. & CUERVO, A. M. 2008. The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. *Molecular and cellular biology,* 28**,** 5747-63.

BARRAL, J. M., BROADLEY, S. A., SCHAFFAR, G. & HARTL, F. U. 2004. Roles of molecular chaperones in protein misfolding diseases. *Seminars in cell & developmental biology,* 15**,** 17-29.

BARRE-SINOUSSI, F., CHERMANN, J. C., REY, F., NUGEYRE, M. T., CHAMARET, S., GRUEST, J., DAUGUET, C., AXLER-BLIN, C., VEZINET-BRUN, F., ROUZIOUX, C., ROZENBAUM, W. & MONTAGNIER, L. 1983. Isolation of a T-

lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science,* 220**,** 868-71.

BEEMS, R. B., GRUYS, E. & SPIT, B. J. 1978. Amyloid in the corpora amylacea of the rat mammary gland. *Veterinary pathology,* 15**,** 347-52.

BERGER, E. A., MURPHY, P. M. & FARBER, J. M. 1999. Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annual Review of Immunology,* 17**,** 657-700.

BHATTACHARYYA, J. & DAS, K. P. 1999. Molecular chaperone-like properties of an unfolded protein, alpha(s)-casein. *The Journal of biological chemistry,* 274**,** 15505-9.

BIESCHKE, J., RUSS, J., FRIEDRICH, R. P., EHRNHOEFER, D. E., WOBST, H., NEUGEBAUER, K. & WANKER, E. E. 2010. EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 7710-5.

BLASCHUK, O., BURDZY, K. & FRITZ, I. B. 1983. Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *The Journal of biological chemistry,* 258**,** 7714-20.

BOGGS, L. N., FUSON, K. S., BAEZ, M., CHURGAY, L., MCCLURE, D., BECKER, G. & MAY, P. C. 1996. Clusterin (Apo J) protects against in vitro amyloid-beta (1-40) neurotoxicity. *Journal of neurochemistry,* 67**,** 1324-7.

BOLDER, S. G., HENDRICKX, H., SAGIS, L. M. & VAN DER LINDEN, E. 2006. Fibril assemblies in aqueous whey protein mixtures. *Journal of agricultural and food chemistry,* 54**,** 4229-34.

BOWMAN, B. H. & KUROSKY, A. 1982. Haptoglobin: the evolutionary product of duplication, unequal crossing over, and point mutation. *Advances in human genetics,* 12**,** 189-261, 453-4.

BRANDES, D. & BERTINI, F. 1964. Role of Golgi Apparatus in the Formation of Cytolysomes. *Experimental cell research,* 35**,** 194-7.

BRENDER, J. R., HARTMAN, K., GOTTLER, L. M., CAVITT, M. E., YOUNGSTROM, D. W. & RAMAMOORTHY, A. 2009. Helical conformation of the SEVI precursor peptide PAP248-286, a dramatic enhancer of HIV infectivity, promotes lipid aggregation and fusion. *Biophysical journal,* 97**,** 2474-83.

BRENDER, J. R., NANGA, R. P., POPOVYCH, N., SOONG, R., MACDONALD, P. M. & RAMAMOORTHY, A. 2011. The amyloidogenic SEVI precursor, PAP248-286, is highly unfolded in solution despite an underlying helical tendency. *Biochimica et biophysica acta,* 1808**,** 1161-9.

BROADLEY, S. A. & HARTL, F. U. 2009. The role of molecular chaperones in human misfolding diseases. *FEBS letters,* 583**,** 2647-53.

BUCCIANTINI, M., GIANNONI, E., CHITI, F., BARONI, F., FORMIGLI, L., ZURDO, J., TADDEI, N., RAMPONI, G., DOBSON, C. M. & STEFANI, M. 2002. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature,* 416**,** 507-11.

BURKEY, B. F., DESILVA, H. V. & HARMONY, J. A. 1991. Intracellular processing of apolipoprotein J precursor to the mature heterodimer. *Journal of lipid research,* 32**,** 1039-48.

BUSINARO, R., FABRIZI, C., FUMAGALLI, L. & LAURO, G. M. 1992. Synthesis and secretion of alpha 2-macroglobulin by human glioma established cell lines. *Experimental brain research,* 88**,** 213-8.

BUTTYAN, R., OLSSON, C. A., PINTAR, J., CHANG, C., BANDYK, M., NG, P. Y. & SAWCZUK, I. S. 1989. Induction of the TRPM-2 gene in cells undergoing programmed death. *Molecular and cellular biology,* 9**,** 3473-81.

CAPULE, C. C., BROWN, C., OLSEN, J. S., DEWHURST, S. & YANG, J. 2012. Oligovalent amyloid-binding agents reduce SEVI-mediated enhancement of HIV-1 infection. *Journal of the American Chemical Society,* 134**,** 905-8.

CARVER, J. A., LINDNER, R. A., LYON, C., CANET, D., HERNANDEZ, H., DOBSON, C. M. & REDFIELD, C. 2002. The interaction of the molecular chaperone alpha-crystallin with unfolding alpha-lactalbumin: a structural and kinetic spectroscopic study. *Journal of molecular biology,* 318**,** 815-27.

CARVER, J. A., REKAS, A., THORN, D. C. & WILSON, M. R. 2003. Small heat-shock proteins and clusterin: intra- and extracellular molecular chaperones with a common mechanism of action and function? *IUBMB life,* 55**,** 661-8.

CASCELLA, R., CONTI, S., TATINI, F., EVANGELISTI, E., SCARTABELLI, T., CASAMENTI, F., WILSON, M. R., CHITI, F. & CECCHI, C. 2013. Extracellular chaperones prevent Abeta42-induced toxicity in rat brains. *Biochimica et biophysica acta,* 1832**,** 1217-26.

CASHIN, K., JAKOBSEN, M. R., STERJOVSKI, J., ROCHE, M., ELLETT, A., FLYNN, J. K., BORM, K., GOUILLOU, M., CHURCHILL, M. J. & GORRY, P. R. 2013. Linkages between HIV-1 specificity for CCR5 or CXCR4 and in vitro usage of alternative coreceptors during progressive HIV-1 subtype C infection. *Retrovirology,* 10**,** 98.

CASTELLANO, L. M. & SHORTER, J. 2012. The Surprising Role of Amyloid Fibrils in HIV Infection. *Biology,* 1**,** 58-80.

CAUGHEY, B. & LANSBURY, P. T. 2003. Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annual review of neuroscience,* 26**,** 267-98.

CHEN, D., MILACIC, V., CHEN, M. S., WAN, S. B., LAM, W. H., HUO, C., LANDIS-PIWOWAR, K. R., CUI, Q. C., WALI, A., CHAN, T. H. & DOU, Q. P. 2008. Tea polyphenols, their biological effects and potential molecular targets. *Histology and Histopathology,* 23**,** 487-496.

CHIANG, H. L., TERLECKY, S. R., PLANT, C. P. & DICE, J. F. 1989. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science,* 246**,** 382-5.

CHIMON, S., SHAIBAT, M. A., JONES, C. R., CALERO, D. C., AIZEZI, B. & ISHII, Y. 2007. Evidence of fibril-like beta-sheet structures in a neurotoxic amyloid intermediate of Alzheimer's beta-amyloid. *Nature structural & molecular biology,* 14**,** 1157-64.

CHITI, F. & DOBSON, C. M. 2006. Protein misfolding, functional amyloid, and human disease. *Annual review of biochemistry,* 75**,** 333-66.

CHITI, F., WEBSTER, P., TADDEI, N., CLARK, A., STEFANI, M., RAMPONI, G. & DOBSON, C. M. 1999. Designing conditions for in vitro formation of amyloid protofilaments and fibrils. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 3590-4.

CHOI-MIURA, N. H., IHARA, Y., FUKUCHI, K., TAKEDA, M., NAKANO, Y., TOBE, T. & TOMITA, M. 1992. SP-40,40 is a constituent of Alzheimer's amyloid. *Acta neuropathologica,* 83**,** 260-4.

CHOI, Y. T., JUNG, C. H., LEE, S. R., BAE, J. H., BAEK, W. K., SUH, M. H., PARK, J., PARK, C. W. & SUH, S. I. 2001. The green tea polyphenol (-)-epigallocatechin gallate attenuates beta-amyloid-induced neurotoxicity in cultured hippocampal neurons. *Life sciences,* 70**,** 603-14.

CIECHANOVER, A. 1998. The ubiquitin-proteasome pathway: on protein death and cell life. *The EMBO journal,* 17**,** 7151-60.

CLARE, D. A. & SWAISGOOD, H. E. 2000. Bioactive milk peptides: a prospectus. *Journal of dairy science,* 83**,** 1187-95.

CLARK, J. I. & MUCHOWSKI, P. J. 2000. Small heat-shock proteins and their potential role in human disease. *Current opinion in structural biology,* 10**,** 52-9.

COHEN, F. E. & KELLY, J. W. 2003. Therapeutic approaches to protein-misfolding diseases. *Nature,* 426**,** 905-9.

COKER, A. R., PURVIS, A., BAKER, D., PEPYS, M. B. & WOOD, S. P. 2000. Molecular chaperone properties of serum amyloid P component. *FEBS letters,* 473**,** 199-202.

COLLARD, M. W. & GRISWOLD, M. D. 1987. Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. *Biochemistry,* 26**,** 3297-303.

COLLINS, K. A. & BENNETT, A. T. 2001. Persistence of spermatozoa and prostatic acid phosphatase in specimens from deceased individuals during varied postmortem intervals. *The American journal of forensic medicine and pathology,* 22**,** 228-32.

CONSTANZE H, H. S., CARL G. REGARDH, ELKE LIPKA, GORDON L. AMIDON, PETER LANGGUTH 1999. Caco-2 versus Caco-2/HT29-MTX Co-cultured Cell Lines: Permeabilities Via Diffusion, Inside- and Outside-Directed Carrier-Mediated transport. *Pharmaceutical Sciences,* 89**,** 63-75.

CORIA, F., CASTANO, E., PRELLI, F., LARRONDO-LILLO, M., VAN DUINEN, S., SHELANSKI, M. L. & FRANGIONE, B. 1988. Isolation and characterization of amyloid P component from Alzheimer's disease and other types of cerebral amyloidosis. *Laboratory investigation; a journal of technical methods and pathology,* 58**,** 454-8.

CREIGHTON, T. E. 1988. Toward a better understanding of protein folding pathways. *Proceedings of the National Academy of Sciences of the United States of America,* 85**,** 5082-6.

CUERVO, A. M. & DICE, J. F. 1996. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science,* 273**,** 501-3.

CYR, D. M., HOHFELD, J. & PATTERSON, C. 2002. Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. *Trends in biochemical sciences,* 27**,** 368-75.

DALGLEISH, A. G., BEVERLEY, P. C., CLAPHAM, P. R., CRAWFORD, D. H., GREAVES, M. F. & WEISS, R. A. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature,* 312**,** 763-7.

DANIK, M., CHABOT, J. G., HASSAN-GONZALEZ, D., SUH, M. & QUIRION, R. 1993. Localization of sulfated glycoprotein-2/clusterin mRNA in the rat brain by in situ hybridization. *The Journal of comparative neurology,* 334**,** 209-27.

DE DUVE, C., PRESSMAN, B. C., GIANETTO, R., WATTIAUX, R. & APPELMANS, F. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *The Biochemical journal,* 60**,** 604-17.

DE SILVA, H. V., STUART, W. D., PARK, Y. B., MAO, S. J., GIL, C. M., WETTERAU, J. R., BUSCH, S. J. & HARMONY, J. A. 1990. Purification and characterization of apolipoprotein J. *The Journal of biological chemistry,* 265**,** 14292-7.

DEHLE, F. C., ECROYD, H., MUSGRAVE, I. F. & CARVER, J. A. 2010. alphaB-Crystallin inhibits the cell toxicity associated with amyloid fibril formation by kappa-casein and the amyloid-beta peptide. *Cell stress & chaperones,* 15**,** 1013-26.

DEMATTOS, R. B., O'DELL M, A., PARSADANIAN, M., TAYLOR, J. W., HARMONY, J. A., BALES, K. R., PAUL, S. M., ARONOW, B. J. & HOLTZMAN, D. M. 2002.

Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 10843-8.

DENNEHY, P. H. 2011. Viral gastroenteritis in children. *The Pediatric infectious disease journal,* 30**,** 63-4.

DESIKAN, R. S., THOMPSON, W. K., HOLLAND, D., HESS, C. P., BREWER, J. B., ZETTERBERG, H., BLENNOW, K., ANDREASSEN, O. A., MCEVOY, L. K., HYMAN, B. T. & DALE, A. M. 2014. The role of clusterin in amyloid-beta-associated neurodegeneration. *JAMA neurology,* 71**,** 180-7.

DICE, J. F. 1990. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. *Trends in biochemical sciences,* 15**,** 305-9.

DIMITROV, D. S., WILLEY, R. L., SATO, H., CHANG, L. J., BLUMENTHAL, R. & MARTIN, M. A. 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. *Journal of virology,* 67**,** 2182-90.

DOBRYSZYCKA, W. 1997. Biological functions of haptoglobin--new pieces to an old puzzle. *European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies,* 35**,** 647-54.

DOBSON, C. M. 1999. Protein misfolding, evolution and disease. *Trends in biochemical sciences,* 24**,** 329-32.

DOBSON, C. M. 2001a. Protein folding and its links with human disease. *Biochemical Society symposium***,** 1-26.

DOBSON, C. M. 2001b. The structural basis of protein folding and its links with human disease. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences,* 356**,** 133-45.

DOBSON, C. M. 2004a. Experimental investigation of protein folding and misfolding. *Methods,* 34**,** 4-14.

DOBSON, C. M. 2004b. Principles of protein folding, misfolding and aggregation. *Seminars in cell & developmental biology,* 15**,** 3-16.

DOYLE, S. M., GENEST, O. & WICKNER, S. 2013. Protein rescue from aggregates by powerful molecular chaperone machines. *Nature reviews. Molecular cell biology,* 14**,** 617-29.

DRAGIC, T., LITWIN, V., ALLAWAY, G. P., MARTIN, S. R., HUANG, Y., NAGASHIMA, K. A., CAYANAN, C., MADDON, P. J., KOUP, R. A., MOORE, J. P. & PAXTON, W. A. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature,* 381**,** 667-73.

DU, Y., BALES, K. R., DODEL, R. C., LIU, X., GLINN, M. A., HORN, J. W., LITTLE, S. P. & PAUL, S. M. 1998. Alpha2-macroglobulin attenuates beta-amyloid peptide 1-40 fibril formation and associated neurotoxicity of cultured fetal rat cortical neurons. *Journal of neurochemistry,* 70**,** 1182-8.

DUGUID, J. R., BOHMONT, C. W., LIU, N. G. & TOURTELLOTTE, W. W. 1989. Changes in brain gene expression shared by scrapie and Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America,* 86**,** 7260-4.

DUNKER, A. K., BROWN, C. J., LAWSON, J. D., IAKOUCHEVA, L. M. & OBRADOVIC, Z. 2002. Intrinsic disorder and protein function. *Biochemistry,* 41**,** 6573-82.

DUONG, T., POMMIER, E. C. & SCHEIBEL, A. B. 1989. Immunodetection of the amyloid P component in Alzheimer's disease. *Acta neuropathologica,* 78**,** 429-37.

EASTERHOFF, D., DIMAIO, J. T., DORAN, T. M., DEWHURST, S. & NILSSON, B. L. 2011. Enhancement of HIV-1 infectivity by simple, self-assembling modular peptides. *Biophysical journal,* 100**,** 1325-34.

ECROYD, H. & CARVER, J. A. 2008. Unraveling the mysteries of protein folding and misfolding. *IUBMB life,* 60**,** 769-74.

ECROYD, H., KOUDELKA, T., THORN, D. C., WILLIAMS, D. M., DEVLIN, G., HOFFMANN, P. & CARVER, J. A. 2008. Dissociation from the oligomeric state is the rate-limiting step in fibril formation by kappa-casein. *J Biol Chem,* 283**,** 9012-22.

EHRNHOEFER, D. E., BIESCHKE, J., BOEDDRICH, A., HERBST, M., MASINO, L., LURZ, R., ENGEMANN, S., PASTORE, A. & WANKER, E. E. 2008. EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nature structural & molecular biology,* 15**,** 558-66.

EHRNSPERGER, M., GRABER, S., GAESTEL, M. & BUCHNER, J. 1997. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *The EMBO journal,* 16**,** 221-9.

ELIAS, A. K., SCANLON, D., MUSGRAVE, I. F. & CARVER, J. A. 2014. SEVI, the semen enhancer of HIV infection along with fragments from its central region, form amyloid fibrils that are toxic to neuronal cells. *Biochimica et biophysica acta,* 1844**,** 1591-8.

ELLIS, R. J. & PINHEIRO, T. J. 2002. Medicine: danger--misfolding proteins. *Nature,* 416**,** 483-4.

EMSLEY, J., WHITE, H. E., O'HARA, B. P., OLIVA, G., SRINIVASAN, N., TICKLE, I. J., BLUNDELL, T. L., PEPYS, M. B. & WOOD, S. P. 1994. Structure of pentameric human serum amyloid P component. *Nature,* 367**,** 338-45.

ENGELMAN, A. & CHEREPANOV, P. 2012. The structural biology of HIV-1: mechanistic and therapeutic insights. *Nature reviews. Microbiology,* 10**,** 279-90.

ENGSTROM, G., HEDBLAD, B., STAVENOW, L., LIND, P., JANZON, L. & LINDGARDE, F. 2003. Inflammation-sensitive plasma proteins are associated with future weight gain. *Diabetes,* 52**,** 2097-101.

ETTRICH, R., BRANDT, W., JR., KOPECKY, V., BAUMRUK, V., HOFBAUEROVA, K. & PAVLICEK, Z. 2002. Study of chaperone-like activity of human haptoglobin: conformational changes under heat shock conditions and localization of interaction sites. *Biological chemistry,* 383**,** 1667-76.

FABRIZI, C., BUSINARO, R., LAURO, G. M. & FUMAGALLI, L. 2001. Role of alpha2-macroglobulin in regulating amyloid beta-protein neurotoxicity: protective or detrimental factor? *Journal of neurochemistry,* 78**,** 406-12.

FARRELL, H. M., JR., KUMOSINSKI, T. F., COOKE, P. H., KING, G., HOAGLAND, P. D., WICKHAM, E. D., DOWER, H. J. & GROVES, M. L. 1996. Particle sizes of purified kappa-casein: metal effect and correspondence with predicted three-dimensional molecular models. *Journal of protein chemistry,* 15**,** 435-45.

FARZAN, M., CHOE, H., MARTIN, K., MARCON, L., HOFMANN, W., KARLSSON, G., SUN, Y., BARRETT, P., MARCHAND, N., SULLIVAN, N., GERARD, N., GERARD, C. & SODROSKI, J. 1997. Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *The Journal of experimental medicine,* 186**,** 405-11.

FENG, Y., BRODER, C. C., KENNEDY, P. E. & BERGER, E. A. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science,* 272**,** 872-7.

FERREIRA, N., CARDOSO, I., DOMINGUES, M. R., VITORINO, R., BASTOS, M., BAI, G., SARAIVA, M. J. & ALMEIDA, M. R. 2009. Binding of epigallocatechin-3-gallate to transthyretin modulates its amyloidogenicity. *FEBS letters,* 583**,** 3569-76.

FINLEY, D. 2009. Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annual review of biochemistry,* 78**,** 477-513.

FITZPATRICK, A. W., DEBELOUCHINA, G. T., BAYRO, M. J., CLARE, D. K., CAPORINI, M. A., BAJAJ, V. S., JARONIEC, C. P., WANG, L., LADIZHANSKY, V., MULLER, S. A., MACPHEE, C. E., WAUDBY, C. A., MOTT, H. R., DE SIMONE, A., KNOWLES, T. P., SAIBIL, H. R., VENDRUSCOLO, M., ORLOVA, E. V., GRIFFIN, R. G. & DOBSON, C. M. 2013. Atomic structure and hierarchical assembly of a cross-beta amyloid fibril. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 5468-73.

FOX, P. F. & MCSWEENEY, P. L. H. 2003. *Advanced dairy chemistry,* New York, N.Y., Springer.

FRANKEL, A. D. & YOUNG, J. A. 1998. HIV-1: fifteen proteins and an RNA. *Annual review of biochemistry,* 67**,** 1-25.

FRENCH, K., YERBURY, J. J. & WILSON, M. R. 2008. Protease activation of alpha2-macroglobulin modulates a chaperone-like action with broad specificity. *Biochemistry,* 47**,** 1176-85.

FRITZ, I. B., BURDZY, K., SETCHELL, B. & BLASCHUK, O. 1983. Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions in vitro. *Biology of reproduction,* 28**,** 1173-88.

FYE LAU, B., ABDULLAH, N., AMINUDIN, N. & BOON LEE, H. 2013. Chemical composition and cellular toxicity of ethnobotanical-based hot and cold aqueous

preparations of the tiger's milk mushroom (Lignosus rhinocerotis). *Journal of ethnopharmacology*.

GALVIN, S. R. & COHEN, M. S. 2004. The role of sexually transmitted diseases in HIV transmission. *Nature reviews. Microbiology,* 2**,** 33-42.

GANEA, E. 2001. Chaperone-like activity of alpha-crystallin and other small heat shock proteins. *Current protein & peptide science,* 2**,** 205-25.

GARCIA-MATA, R., GAO, Y. S. & SZTUL, E. 2002. Hassles with taking out the garbage: aggravating aggresomes. *Traffic,* 3**,** 388-96.

GHOSH, S., PANDEY, N. K. & DASGUPTA, S. 2013. (-)-Epicatechin gallate prevents alkali-salt mediated fibrillogenesis of hen egg white lysozyme. *International journal of biological macromolecules,* 54**,** 90-8.

GOBBETTI, M., STEPANIAK, L., DE ANGELIS, M., CORSETTI, A. & DI CAGNO, R. 2002. Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. *Critical reviews in food science and nutrition,* 42**,** 223-39.

GOERS, J., PERMYAKOV, S. E., PERMYAKOV, E. A., UVERSKY, V. N. & FINK, A. L. 2002. Conformational prerequisites for alpha-lactalbumin fibrillation. *Biochemistry,* 41**,** 12546-51.

GOLDSBURY, C., GOLDIE, K., PELLAUD, J., SEELIG, J., FREY, P., MULLER, S. A., KISTLER, J., COOPER, G. J. & AEBI, U. 2000. Amyloid fibril formation from full-length and fragments of amylin. *Journal of structural biology,* 130**,** 352-62.

GOLDSCHMIDT, L., TENG, P. K., RIEK, R. & EISENBERG, D. 2010. Identifying the amylome, proteins capable of forming amyloid-like fibrils. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 3487-92.

GORRY, P. R., DUNFEE, R. L., MEFFORD, M. E., KUNSTMAN, K., MORGAN, T., MOORE, J. P., MASCOLA, J. R., AGOPIAN, K., HOLM, G. H., MEHLE, A., TAYLOR, J., FARZAN, M., WANG, H., ELLERY, P., WILLEY, S. J., CLAPHAM, P. R., WOLINSKY, S. M., CROWE, S. M. & GABUZDA, D. 2007. Changes in the V3 region of gp120 contribute to unusually broad coreceptor usage of an HIV-1 isolate from a CCR5 Delta32 heterozygote. *Virology,* 362**,** 163-78.

GOTTLIEB, M. S., SCHROFF, R., SCHANKER, H. M., WEISMAN, J. D., FAN, P. T., WOLF, R. A. & SAXON, A. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *The New England journal of medicine,* 305**,** 1425-31.

GRAY, R. H., WAWER, M. J., BROOKMEYER, R., SEWANKAMBO, N. K., SERWADDA, D., WABWIRE-MANGEN, F., LUTALO, T., LI, X., VANCOTT, T. & QUINN, T. C. 2001. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet,* 357**,** 1149-53.

GRIMWOOD, K., ABBOTT, G. D., FERGUSSON, D. M., JENNINGS, L. C. & ALLAN, J. M. 1983. Spread of rotavirus within families: a community based study. *British medical journal,* 287**,** 575-7.

GUYOMARC'H, F., JEMIN, M., LE TILLY, V., MADEC, M. N. & FAMELART, M. H. 2009. Role of the heat-induced whey protein/kappa-casein complexes in the formation of acid milk gels: a kinetic study using rheology and confocal microscopy. *Journal of agricultural and food chemistry,* 57**,** 5910-7.

HAASE, A. T. 2005. Perils at mucosal front lines for HIV and SIV and their hosts. *Nature reviews. Immunology,* 5**,** 783-92.

HAMADA, D. & DOBSON, C. M. 2002. A kinetic study of beta-lactoglobulin amyloid fibril formation promoted by urea. *Protein science : a publication of the Protein Society,* 11**,** 2417-26.

HAMMAD, S. M., RANGANATHAN, S., LOUKINOVA, E., TWAL, W. O. & ARGRAVES, W. S. 1997. Interaction of apolipoprotein J-amyloid beta-peptide complex with low density lipoprotein receptor-related protein-2/megalin. A mechanism to prevent pathological accumulation of amyloid beta-peptide. *The Journal of biological chemistry,* 272**,** 18644-9.

HAMPTON, R. Y. 2002. ER-associated degradation in protein quality control and cellular regulation. *Current opinion in cell biology,* 14**,** 476-82.

HAN, Z., WANG, Z., CHENG, G., LIU, B., LI, P., LI, J., WANG, W., YIN, C. & ZHANG, W. 2012. Presence, localization, and origin of clusterin in normal human spermatozoa. *Journal of assisted reproduction and genetics,* 29**,** 751-7.

HARTJEN, P., FRERK, S., HAUBER, I., MATZAT, V., THOMSSEN, A., HOLSTERMANN, B., HOHENBERG, H., SCHULZE, W., SCHULZE ZUR WIESCH, J. & VAN LUNZEN, J. 2012. Assessment of the range of the HIV-1 infectivity enhancing effect of individual human semen specimen and the range of inhibition by EGCG. *AIDS research and therapy,* 9**,** 2.

HARTL, F. U., BRACHER, A. & HAYER-HARTL, M. 2011. Molecular chaperones in protein folding and proteostasis. *Nature,* 475**,** 324-32.

HARTL, F. U. & HAYER-HARTL, M. 2009. Converging concepts of protein folding in vitro and in vivo. *Nature structural & molecular biology,* 16**,** 574-81.

HARTLEY, D. M., WALSH, D. M., YE, C. P., DIEHL, T., VASQUEZ, S., VASSILEV, P. M., TEPLOW, D. B. & SELKOE, D. J. 1999. Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity

in cortical neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 19**,** 8876-84.

HATTERS, D. M., WILSON, M. R., EASTERBROOK-SMITH, S. B. & HOWLETT, G. J. 2002. Suppression of apolipoprotein C-II amyloid formation by the extracellular chaperone, clusterin. *European journal of biochemistry / FEBS,* 269**,** 2789-94.

HAUBER, I., HOHENBERG, H., HOLSTERMANN, B., HUNSTEIN, W. & HAUBER, J. 2009. The main green tea polyphenol epigallocatechin-3-gallate counteracts semen-mediated enhancement of HIV infection. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 9033-8.

HERMO, L., BARIN, K. & OKO, R. 1994. Developmental expression of sulfated glycoprotein-2 in the epididymis of the rat. *The Anatomical record,* 240**,** 327-44.

HERRMANN, J., SOARES, S. M., LERMAN, L. O. & LERMAN, A. 2008. Potential role of the ubiquitin-proteasome system in atherosclerosis: aspects of a protein quality disease. *Journal of the American College of Cardiology,* 51**,** 2003-10.

HETZ, C., CHEVET, E. & HARDING, H. P. 2013. Targeting the unfolded protein response in disease. *Nature reviews. Drug discovery,* 12**,** 703-19.

HETZ, C. & MOLLEREAU, B. 2014. Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. *Nature reviews. Neuroscience,* 15**,** 233-49.

HIDALGO, I. J., RAUB, T. J. & BORCHARDT, R. T. 1989. Characterization of the Human-Colon Carcinoma Cell-Line (Caco-2) as a Model System for Intestinal Epithelial Permeability. *Gastroenterology,* 96**,** 736-749.

HIND, C. R., COLLINS, P. M., CASPI, D., BALTZ, M. L. & PEPYS, M. B. 1984. Specific chemical dissociation of fibrillar and non-fibrillar components of amyloid deposits. *Lancet,* 2**,** 376-8.

HOCHGREBE, T., PANKHURST, G. J., WILCE, J. & EASTERBROOK-SMITH, S. B. 2000. pH-dependent changes in the in vitro ligand-binding properties and structure of human clusterin. *Biochemistry,* 39**,** 1411-9.

HOLT, C. 1993. Interrelationships of the concentrations of some ionic constituents of human milk and comparison with cow and goat milks. *Comparative biochemistry and physiology. Comparative physiology,* 104**,** 35-41.

HOLT, C. 2004. An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk. *European biophysics journal : EBJ,* 33**,** 421-34.

HOLT, C. & CARVER, J. A. 2012. Darwinian transformation of a 'scarcely nutritious fluid' into milk. *Journal of evolutionary biology,* 25**,** 1253-63.

HOLT, C., CARVER, J. A., ECROYD, H. & THORN, D. C. 2013. Invited review: Caseins and the casein micelle: their biological functions, structures, and behavior in foods. *Journal of dairy science,* 96**,** 6127-46.

HONG, S., KLEIN, E. A., DAS GUPTA, J., HANKE, K., WEIGHT, C. J., NGUYEN, C., GAUGHAN, C., KIM, K. A., BANNERT, N., KIRCHHOFF, F., MUNCH, J. & SILVERMAN, R. H. 2009. Fibrils of prostatic acid phosphatase fragments boost infections with XMRV (xenotropic murine leukemia virus-related virus), a human retrovirus associated with prostate cancer. *Journal of virology,* 83**,** 6995-7003.

HOSHI, M., SATO, M., MATSUMOTO, S., NOGUCHI, A., YASUTAKE, K., YOSHIDA, N. & SATO, K. 2003. Spherical aggregates of beta-amyloid (amylospheroid) show high neurotoxicity and activate tau protein kinase I/glycogen synthase kinase-3beta. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 6370-5.

HUANG, R., VIVEKANANDAN, S., BRENDER, J. R., ABE, Y., NAITO, A. & RAMAMOORTHY, A. 2012. NMR characterization of monomeric and oligomeric conformations of human calcitonin and its interaction with EGCG. *Journal of molecular biology,* 416**,** 108-20.

HUDSON, S. A., ECROYD, H., DEHLE, F. C., MUSGRAVE, I. F. & CARVER, J. A. 2009. (-)-epigallocatechin-3-gallate (EGCG) maintains kappa-casein in its pre-fibrillar state without redirecting its aggregation pathway. *Journal of molecular biology,* 392**,** 689-700.

HUERTA, V., TOLEDO, P., FLEITAS, N., MARTIN, A., PUPO, D., YERO, A., SARRIA, M., SANCHEZ, A., BESADA, V., RAMOS, Y., MARQUEZ, G., GUIROLA, O. & CHINEA, G. 2014. Receptor-activated human alpha 2-macroglobulin interacts with the Envelope protein of Dengue virus and protects virions from temperature-induced inactivation through multivalent binding. *The Journal of general virology*.

HUGHES, S. R., KHORKOVA, O., GOYAL, S., KNAEBLEIN, J., HEROUX, J., RIEDEL, N. G. & SAHASRABUDHE, S. 1998. Alpha2-macroglobulin associates with beta-amyloid peptide and prevents fibril formation. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 3275-80.

HUMPHREYS, D. T., CARVER, J. A., EASTERBROOK-SMITH, S. B. & WILSON, M. R. 1999. Clusterin has chaperone-like activity similar to that of small heat shock proteins. *The Journal of biological chemistry,* 274**,** 6875-81.

ISEKI, E., AMANO, N., MATSUISHI, T., YOKOI, S., ARAI, N. & YAGISHITA, S. 1988. A case of familial, atypical Alzheimer's disease: immunohistochemical study of amyloid P-component. *Neuropathology and applied neurobiology,* 14**,** 169-74.

JAHN, T. R. & RADFORD, S. E. 2005. The Yin and Yang of protein folding. *The FEBS journal,* 272**,** 5962-70.

JAKOB, U. & BUCHNER, J. 1994. Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. *Trends in biochemical sciences,* 19**,** 205-11.

JEHLE, S., RAJAGOPAL, P., BARDIAUX, B., MARKOVIC, S., KUHNE, R., STOUT, J. R., HIGMAN, V. A., KLEVIT, R. E., VAN ROSSUM, B. J. & OSCHKINAT, H. 2010. Solid-state NMR and SAXS studies provide a structural basis for the activation of alphaB-crystallin oligomers. *Nature structural & molecular biology,* 17**,** 1037-42.

JENNE, D. E., LOWIN, B., PEITSCH, M. C., BOTTCHER, A., SCHMITZ, G. & TSCHOPP, J. 1991. Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. *The Journal of biological chemistry,* 266**,** 11030-6.

JENNE, D. E. & TSCHOPP, J. 1992. Clusterin: the intriguing guises of a widely expressed glycoprotein. *Trends in biochemical sciences,* 17**,** 154-9.

JO, E., MCLAURIN, J., YIP, C. M., ST GEORGE-HYSLOP, P. & FRASER, P. E. 2000. alpha-Synuclein membrane interactions and lipid specificity. *The Journal of biological chemistry,* 275**,** 34328-34.

JOHNSON, E. S. 2002. Ubiquitin branches out. *Nature cell biology,* 4**,** E295-8.

JOHNSON, P. R., SWANSON, R., RAKHILINA, L. & HOCHSTRASSER, M. 1998. Degradation signal masking by heterodimerization of MATalpha2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. *Cell,* 94**,** 217-27.

JOHNSTON, J. A., WARD, C. L. & KOPITO, R. R. 1998. Aggresomes: a cellular response to misfolded proteins. *The Journal of cell biology,* 143**,** 1883-98.

JOSEPH, S. B., ARRILDT, K. T., SWANSTROM, A. E., SCHNELL, G., LEE, B., HOXIE, J. A. & SWANSTROM, R. 2014. Quantification of entry phenotypes of macrophage-tropic HIV-1 across a wide range of CD4 densities. *Journal of virology,* 88**,** 1858-69.

KALARIA, R. N., GALLOWAY, P. G. & PERRY, G. 1991. Widespread serum amyloid P immunoreactivity in cortical amyloid deposits and the neurofibrillary pathology of Alzheimer's disease and other degenerative disorders. *Neuropathology and applied neurobiology,* 17**,** 189-201.

KANG, C. Y., HARIHARAN, K., POSNER, M. R. & NARA, P. 1993. Identification of a new neutralizing epitope conformationally affected by the attachment of CD4 to gp120. *Journal of immunology,* 151**,** 449-57.

KAPRON, J. T., HILLIARD, G. M., LAKINS, J. N., TENNISWOOD, M. P., WEST, K. A., CARR, S. A. & CRABB, J. W. 1997. Identification and characterization of glycosylation sites in human serum clusterin. *Protein science : a publication of the Protein Society,* 6**,** 2120-33.

KAUSHIK, S. & CUERVO, A. M. 2012. Chaperone-mediated autophagy: a unique way to enter the lysosome world. *Trends in cell biology,* 22**,** 407-17.

KELLY, J. W. 1998. The environmental dependency of protein folding best explains prion and amyloid diseases. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 930-2.

KIDA, E., CHOI-MIURA, N. H. & WISNIEWSKI, K. E. 1995. Deposition of apolipoproteins E and J in senile plaques is topographically determined in both Alzheimer's disease and Down's syndrome brain. *Brain research,* 685**,** 211-6.

KIM, K. A., YOLAMANOVA, M., ZIRAFI, O., ROAN, N. R., STAENDKER, L., FORSSMANN, W. G., BURGENER, A., DEJUCQ-RAINSFORD, N., HAHN, B. H., SHAW, G. M., GREENE, W. C., KIRCHHOFF, F. & MUNCH, J. 2010. Semen-mediated enhancement of HIV infection is donor-dependent and correlates with the levels of SEVI. *Retrovirology,* 7**,** 55.

KLATZMANN, D., CHAMPAGNE, E., CHAMARET, S., GRUEST, J., GUETARD, D., HERCEND, T., GLUCKMAN, J. C. & MONTAGNIER, L. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature,* 312**,** 767-8.

KNIGHT, J. D., HEBDA, J. A. & MIRANKER, A. D. 2006. Conserved and cooperative assembly of membrane-bound alpha-helical states of islet amyloid polypeptide. *Biochemistry,* 45**,** 9496-508.

KNOWLES, T. P., WAUDBY, C. A., DEVLIN, G. L., COHEN, S. I., AGUZZI, A., VENDRUSCOLO, M., TERENTJEV, E. M., WELLAND, M. E. & DOBSON, C. M. 2009. An analytical solution to the kinetics of breakable filament assembly. *Science,* 326**,** 1533-7.

KRISTIANSEN, M., GRAVERSEN, J. H., JACOBSEN, C., SONNE, O., HOFFMAN, H. J., LAW, S. K. & MOESTRUP, S. K. 2001. Identification of the haemoglobin scavenger receptor. *Nature,* 409**,** 198-201.

KUIKEN, C., FOLEY, B., HAHN, B., MARX, P., MCCUTCHAN, F., MELLORS, J. W., MULLINS, J., WOLINSKY, S. & KORBER, B. 1999. A compilation and analysis of nucleic acid and amino acid sequences. In Human Retroviruses and AIDS. *Theoretical Biology and Biophysics Group*.

KUMITA, J. R., POON, S., CADDY, G. L., HAGAN, C. L., DUMOULIN, M., YERBURY, J. J., STEWART, E. M., ROBINSON, C. V., WILSON, M. R. & DOBSON, C. M. 2007. The extracellular chaperone clusterin potently inhibits human lysozyme amyloid formation by interacting with prefibrillar species. *Journal of molecular biology,* 369**,** 157-67.

KUWAJIMA, K. 1996. The molten globule state of alpha-lactalbumin. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 10**,** 102-9.

LAGANOWSKY, A., BENESCH, J. L., LANDAU, M., DING, L., SAWAYA, M. R., CASCIO, D., HUANG, Q., ROBINSON, C. V., HORWITZ, J. & EISENBERG, D. 2010. Crystal structures of truncated alphaA and alphaB crystallins reveal structural mechanisms of polydispersity important for eye lens function. *Protein science : a publication of the Protein Society,* 19**,** 1031-43.

LAMBERT, M. P., BARLOW, A. K., CHROMY, B. A., EDWARDS, C., FREED, R., LIOSATOS, M., MORGAN, T. E., ROZOVSKY, I., TROMMER, B., VIOLA, K. L., WALS, P., ZHANG, C., FINCH, C. E., KRAFFT, G. A. & KLEIN, W. L. 1998. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 6448-53.

LANSBURY, P. T., JR. 1999. Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 3342-4.

LANSBURY, P. T. & LASHUEL, H. A. 2006. A century-old debate on protein aggregation and neurodegeneration enters the clinic. *Nature,* 443**,** 774-9.

LASHUEL, H. A., HARTLEY, D., PETRE, B. M., WALZ, T. & LANSBURY, P. T., JR. 2002. Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature,* 418**,** 291.

LEONARD, C. K., SPELLMAN, M. W., RIDDLE, L., HARRIS, R. J., THOMAS, J. N. & GREGORY, T. J. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *The Journal of biological chemistry,* 265**,** 10373-82.

LINDQUIST, S. L. & KELLY, J. W. 2011. Chemical and biological approaches for adapting proteostasis to ameliorate protein misfolding and aggregation diseases: progress and prognosis. *Cold Spring Harbor perspectives in biology,* 3.

LORENZO, A. & YANKNER, B. A. 1994. Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 12243-7.

LUHESHI, L. M., HOYER, W., DE BARROS, T. P., VAN DIJK HARD, I., BRORSSON, A. C., MACAO, B., PERSSON, C., CROWTHER, D. C., LOMAS, D. A., STAHL, S., DOBSON, C. M. & HARD, T. 2010. Sequestration of the Abeta peptide prevents toxicity and promotes degradation in vivo. *PLoS biology,* 8**,** e1000334.

MADDON, P. J., DALGLEISH, A. G., MCDOUGAL, J. S., CLAPHAM, P. R., WEISS, R. A. & AXEL, R. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell,* 47**,** 333-48.

MANDEL, S. A., AVRAMOVICH-TIROSH, Y., REZNICHENKO, L., ZHENG, H., WEINREB, O., AMIT, T. & YOUDIM, M. B. 2005. Multifunctional activities of green tea catechins in neuroprotection. Modulation of cell survival genes, iron-dependent oxidative stress and PKC signaling pathway. *Neuro-Signals,* 14**,** 46-60.

MANNINI, B., CASCELLA, R., ZAMPAGNI, M., VAN WAARDE-VERHAGEN, M., MEEHAN, S., ROODVELDT, C., CAMPIONI, S., BONINSEGNA, M., PENCO, A., RELINI, A., KAMPINGA, H. H., DOBSON, C. M., WILSON, M. R., CECCHI, C. & CHITI, F. 2012. Molecular mechanisms used by chaperones to reduce the toxicity of aberrant protein oligomers. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 12479-84.

MARTELLINI, J. A., COLE, A. L., SVOBODA, P., STUCHLIK, O., CHEN, L. M., CHAI, K. X., GANGRADE, B. K., SORENSEN, O. E., POHL, J. & COLE, A. M. 2011.

HIV-1 enhancing effect of prostatic acid phosphatase peptides is reduced in human seminal plasma. *PloS one,* 6**,** e16285.

MATSUBARA, E., SOTO, C., GOVERNALE, S., FRANGIONE, B. & GHISO, J. 1996. Apolipoprotein J and Alzheimer's amyloid beta solubility. *The Biochemical journal,* 316 (Pt 2)**,** 671-9.

MATSUDOMI, N., KANDA, Y., YOSHIKA, Y. & MORIWAKI, H. 2004. Ability of alphas-Casein to suppress the heat aggregation of ovotransferrin. *Journal of agricultural and food chemistry,* 52**,** 4882-6.

MATTSON, M. P. 1995. Degenerative and protective signaling mechanisms in the neurofibrillary pathology of AD. *Neurobiology of aging,* 16**,** 447-57; discussion 458-63.

MATTSON, M. P., CHENG, B., DAVIS, D., BRYANT, K., LIEBERBURG, I. & RYDEL, R. E. 1992. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience,* 12**,** 376-89.

MAYER, M. P. 2010. Gymnastics of molecular chaperones. *Molecular cell,* 39**,** 321-31.

MCDONOUGH, H. & PATTERSON, C. 2003. CHIP: a link between the chaperone and proteasome systems. *Cell stress & chaperones,* 8**,** 303-8.

MCGEER, P. L., KAWAMATA, T. & WALKER, D. G. 1992. Distribution of clusterin in Alzheimer brain tissue. *Brain research,* 579**,** 337-41.

MCHATTIE, S. & EDINGTON, N. 1999. Clusterin prevents aggregation of neuropeptide 106-126 in vitro. *Biochemical and biophysical research communications,* 259**,** 336-40.

MCKNIGHT, A., DITTMAR, M. T., MONIZ-PERIERA, J., ARIYOSHI, K., REEVES, J. D., HIBBITTS, S., WHITBY, D., AARONS, E., PROUDFOOT, A. E., WHITTLE,

H. & CLAPHAM, P. R. 1998. A broad range of chemokine receptors are used by primary isolates of human immunodeficiency virus type 2 as coreceptors with CD4. *Journal of virology,* 72**,** 4065-71.

MEISEL, H. 2004. Multifunctional peptides encrypted in milk proteins. *BioFactors,* 21**,** 55-61.

MENG, F., ABEDINI, A., PLESNER, A., VERCHERE, C. B. & RALEIGH, D. P. 2010. The flavanol (-)-epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils, and protects cultured cells against IAPP-induced toxicity. *Biochemistry,* 49**,** 8127-33.

MICHEL, D., CHATELAIN, G., NORTH, S. & BRUN, G. 1997. Stress-induced transcription of the clusterin/apoJ gene. *The Biochemical journal,* 328 (Pt 1)**,** 45-50.

MILLER, C. J. & SHATTOCK, R. J. 2003. Target cells in vaginal HIV transmission. *Microbes and infection / Institut Pasteur,* 5**,** 59-67.

MORGAN, P. E., TREWEEK, T. M., LINDNER, R. A., PRICE, W. E. & CARVER, J. A. 2005. Casein proteins as molecular chaperones. *Journal of agricultural and food chemistry,* 53**,** 2670-83.

MORIMOTO, A., IRIE, K., MURAKAMI, K., MASUDA, Y., OHIGASHI, H., NAGAO, M., FUKUDA, H., SHIMIZU, T. & SHIRASAWA, T. 2004. Analysis of the secondary structure of beta-amyloid (Abeta42) fibrils by systematic proline replacement. *The Journal of biological chemistry,* 279**,** 52781-8.

MOSERI, A., SCHNUR, E., NOAH, E., ZHERDEV, Y., KESSLER, N., SINGHAL SINHA, E., ABAYEV, M., NAIDER, F., SCHERF, T. & ANGLISTER, J. 2014. NMR observation of HIV-1 gp120 conformational flexibility resulting from V3 truncation. *The FEBS journal,* 281**,** 3019-31.

MUCHOWSKI, P. J. 2002. Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones? *Neuron,* 35**,** 9-12.

MÜNCH, J., RÜCKER, E., STÄNDKER, L., ADERMANN, K., GOFFINET, C., SCHINDLER, M., WILDUM, S., CHINNADURAI, R., RAJAN, D., SPECHT, A., GIMÉNEZ-GALLEGO, G., SÁNCHEZ, P. C., FOWLER, D. M., KOULOV, A., KELLY, J. W., MOTHES, W., GRIVEL, J. C., MARGOLIS, L., KEPPLER, O. T., FORSSMANN, W. G. & KIRCHHOFF, F. 2007. Semen-Derived Amyloid Fibrils Drastically Enhance HIV Infection. *Cell,* 131**,** 1059-1071.

MUNCH, J., SAUERMANN, U., YOLAMANOVA, M., RAUE, K., STAHL-HENNIG, C. & KIRCHHOFF, F. 2013. Effect of semen and seminal amyloid on vaginal transmission of simian immunodeficiency virus. *Retrovirology,* 10**,** 148.

MURPHY, B. F., KIRSZBAUM, L., WALKER, I. D. & D'APICE, A. J. 1988. SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. *The Journal of clinical investigation,* 81**,** 1858-64.

NAIKI, H., HIGUCHI, K., MATSUSHIMA, K., SHIMADA, A., CHEN, W. H., HOSOKAWA, M. & TAKEDA, T. 1990. Fluorometric examination of tissue amyloid fibrils in murine senile amyloidosis: use of the fluorescent indicator, thioflavine T. *Laboratory investigation; a journal of technical methods and pathology,* 62**,** 768-73.

NANGA, R. P., BRENDER, J. R., VIVEKANANDAN, S., POPOVYCH, N. & RAMAMOORTHY, A. 2009. NMR structure in a membrane environment reveals putative amyloidogenic regions of the SEVI precursor peptide PAP(248-286). *Journal of the American Chemical Society,* 131**,** 17972-9.

NARAYAN, P., HOLMSTROM, K. M., KIM, D. H., WHITCOMB, D. J., WILSON, M. R., ST GEORGE-HYSLOP, P., WOOD, N. W., DOBSON, C. M., CHO, K.,

ABRAMOV, A. Y. & KLENERMAN, D. 2014. Rare individual amyloid-beta oligomers act on astrocytes to initiate neuronal damage. *Biochemistry,* 53**,** 2442-53.

NARAYAN, P., MEEHAN, S., CARVER, J. A., WILSON, M. R., DOBSON, C. M. & KLENERMAN, D. 2012. Amyloid-beta oligomers are sequestered by both intracellular and extracellular chaperones. *Biochemistry,* 51**,** 9270-6.

NARITA, M., HOLTZMAN, D. M., SCHWARTZ, A. L. & BU, G. 1997. Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide via cell surface low-density lipoprotein receptor-related protein. *Journal of neurochemistry,* 69**,** 1904-11.

NICKERSON, S. C., SORDILLO, L. M., BODDIE, N. T. & SAXTON, A. M. 1985. Prevalence and ultrastructural characteristics of bovine mammary corpora amylacea during the lactation cycle. *Journal of dairy science,* 68**,** 709-17.

NIELSEN, H. M., VEERHUIS, R., HOLMQVIST, B. & JANCIAUSKIENE, S. 2009. Binding and uptake of A beta1-42 by primary human astrocytes in vitro. *Glia,* 57**,** 978-88.

NIEWOLD, T. A., MURPHY, C. L., HULSKAMP-KOCH, C. A., TOOTEN, P. C. & GRUYS, E. 1999. Casein related amyloid, characterization of a new and unique amyloid protein isolated from bovine corpora amylacea. *Amyloid : the international journal of experimental and clinical investigation : the official journal of the International Society of Amyloidosis,* 6**,** 244-9.

NILSSON, M. R. 2004. Techniques to study amyloid fibril formation in vitro. *Methods,* 34**,** 151-60.

NORMAN, D. J., FENG, L., CHENG, S. S., GUBBAY, J., CHAN, E. & HEINTZ, N. 1995. The lurcher gene induces apoptotic death in cerebellar Purkinje cells. *Development,* 121**,** 1183-93.

NOVITSKAYA, V., BOCHAROVA, O. V., BRONSTEIN, I. & BASKAKOV, I. V. 2006. Amyloid fibrils of mammalian prion protein are highly toxic to cultured cells and primary neurons. *The Journal of biological chemistry,* 281**,** 13828-36.

O'BRYAN, M. K., BAKER, H. W., SAUNDERS, J. R., KIRSZBAUM, L., WALKER, I. D., HUDSON, P., LIU, D. Y., GLEW, M. D., D'APICE, A. J. & MURPHY, B. F. 1990. Human seminal clusterin (SP-40,40). Isolation and characterization. *The Journal of clinical investigation,* 85**,** 1477-86.

OBOROCEANU, D., WANG, L., BRODKORB, A., MAGNER, E. & AUTY, M. A. 2010. Characterization of beta-lactoglobulin fibrillar assembly using atomic force microscopy, polyacrylamide gel electrophoresis, and in situ fourier transform infrared spectroscopy. *Journal of agricultural and food chemistry,* 58**,** 3667-73.

ODA, T., WALS, P., OSTERBURG, H. H., JOHNSON, S. A., PASINETTI, G. M., MORGAN, T. E., ROZOVSKY, I., STINE, W. B., SNYDER, S. W., HOLZMAN, T. F. & ET AL. 1995. Clusterin (apoJ) alters the aggregation of amyloid beta-peptide (A beta 1-42) and forms slowly sedimenting A beta complexes that cause oxidative stress. *Experimental neurology,* 136**,** 22-31.

OLOFSSON, A., OSTMAN, J. & LUNDGREN, E. 2002. Amyloid: morphology and toxicity. *Clinical chemistry and laboratory medicine : CCLM / FESCC,* 40**,** 1266-70.

OLSEN, J. S., DIMAIO, J. T., DORAN, T. M., BROWN, C., NILSSON, B. L. & DEWHURST, S. 2012. Seminal plasma accelerates semen-derived enhancer of viral infection (SEVI) fibril formation by the prostatic acid phosphatase (PAP248-286) peptide. *The Journal of biological chemistry,* 287**,** 11842-9.

PATEL, B. & CUERVO, A. M. 2015. Methods to study chaperone-mediated autophagy. *Methods*.

PEARCE, F. G., MACKINTOSH, S. H. & GERRARD, J. A. 2007. Formation of amyloid-like fibrils by ovalbumin and related proteins under conditions relevant to food processing. *Journal of agricultural and food chemistry,* 55**,** 318-22.

PEPYS, M. B. 1979. Isolation of serum amyloid P-component (protein SAP) in the mouse. *Immunology,* 37**,** 637-41.

PEPYS, M. B. 1988. Amyloidosis: some recent developments. *The Quarterly journal of medicine,* 67**,** 283-98.

PERUTZ, M. F. 1999. Glutamine repeats and neurodegenerative diseases: molecular aspects. *Trends in biochemical sciences,* 24**,** 58-63.

PICKART, C. M. & COHEN, R. E. 2004. Proteasomes and their kin: proteases in the machine age. *Nature reviews. Molecular cell biology,* 5**,** 177-87.

PILCHER, C. D., TIEN, H. C., ERON, J. J., JR., VERNAZZA, P. L., LEU, S. Y., STEWART, P. W., GOH, L. E. & COHEN, M. S. 2004. Brief but efficient: acute HIV infection and the sexual transmission of HIV. *The Journal of infectious diseases,* 189**,** 1785-92.

PLEMPER, R. K. & WOLF, D. H. 1999. Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends in biochemical sciences,* 24**,** 266-70.

POON, S., EASTERBROOK-SMITH, S. B., RYBCHYN, M. S., CARVER, J. A. & WILSON, M. R. 2000. Clusterin is an ATP-independent chaperone with very broad substrate specificity that stabilizes stressed proteins in a folding-competent state. *Biochemistry,* 39**,** 15953-60.

POON, S., RYBCHYN, M. S., EASTERBROOK-SMITH, S. B., CARVER, J. A., PANKHURST, G. J. & WILSON, M. R. 2002. Mildly acidic pH activates the extracellular molecular chaperone clusterin. *The Journal of biological chemistry,* 277**,** 39532-40.

POPOVYCH, N., BRENDER, J. R., SOONG, R., VIVEKANANDAN, S., HARTMAN, K., BASRUR, V., MACDONALD, P. M. & RAMAMOORTHY, A. 2012. Site specific interaction of the polyphenol EGCG with the SEVI amyloid precursor peptide PAP(248-286). *The journal of physical chemistry. B,* 116**,** 3650-8.

PORAT, Y., ABRAMOWITZ, A. & GAZIT, E. 2006. Inhibition of amyloid fibril formation by polyphenols: Structural similarity and aromatic interactions as a common inhibition mechanism. *Chemical Biology & Drug Design,* 67**,** 27-37.

POWERS, J. M., SCHLAEPFER, W. W., WILLINGHAM, M. C. & HALL, B. J. 1981. An immunoperoxidase study of senile cerebral amyloidosis with pathogenetic considerations. *Journal of neuropathology and experimental neurology,* 40**,** 592-612.

PRESTON, B. D., POIESZ, B. J. & LOEB, L. A. 1988. Fidelity of HIV-1 reverse transcriptase. *Science,* 242**,** 1168-71.

PRIYA, S., SHARMA, S. K. & GOLOUBINOFF, P. 2013. Molecular chaperones as enzymes that catalytically unfold misfolded polypeptides. *FEBS letters,* 587**,** 1981-7.

RAMBARAN, R. N. & SERPELL, L. C. 2008. Amyloid fibrils: abnormal protein assembly. *Prion,* 2**,** 112-7.

RASMUSSEN, L. K., HOJRUP, P. & PETERSEN, T. E. 1992. Localization of two interchain disulfide bridges in dimers of bovine alpha s2-casein. Parallel and antiparallel alignments of the polypeptide chains. *European journal of biochemistry / FEBS,* 203**,** 381-6.

RASMUSSEN, L. K., HOJRUP, P. & PETERSEN, T. E. 1994. Disulphide arrangement in bovine caseins: localization of intrachain disulphide bridges in monomers of kappa- and alpha s2-casein from bovine milk. *The Journal of dairy research,* 61**,** 485-93.

REEVES, J. D., HIBBITTS, S., SIMMONS, G., MCKNIGHT, A., AZEVEDO-PEREIRA, J. M., MONIZ-PEREIRA, J. & CLAPHAM, P. R. 1999. Primary human

immunodeficiency virus type 2 (HIV-2) isolates infect CD4-negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo. *Journal of virology,* 73**,** 7795-804.

REKAS, A., ADDA, C. G., ANDREW AQUILINA, J., BARNHAM, K. J., SUNDE, M., GALATIS, D., WILLIAMSON, N. A., MASTERS, C. L., ANDERS, R. F., ROBINSON, C. V., CAPPAI, R. & CARVER, J. A. 2004. Interaction of the molecular chaperone alphaB-crystallin with alpha-synuclein: effects on amyloid fibril formation and chaperone activity. *Journal of molecular biology,* 340**,** 1167-83.

ROAN, N. R., MUNCH, J., ARHEL, N., MOTHES, W., NEIDLEMAN, J., KOBAYASHI, A., SMITH-MCCUNE, K., KIRCHHOFF, F. & GREENE, W. C. 2009. The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection. *Journal of virology,* 83**,** 73-80.

ROAN, N. R., SOWINSKI, S., MUNCH, J., KIRCHHOFF, F. & GREENE, W. C. 2010. Aminoquinoline surfen inhibits the action of SEVI (semen-derived enhancer of viral infection). *The Journal of biological chemistry,* 285**,** 1861-9.

ROBERTS, B. E., DUENNWALD, M. L., WANG, H., CHUNG, C., LOPREIATO, N. P., SWEENY, E. A., KNIGHT, M. N. & SHORTER, J. 2009. A synergistic small-molecule combination directly eradicates diverse prion strain structures. *Nature chemical biology,* 5**,** 936-46.

ROBERTS, J. D., BEBENEK, K. & KUNKEL, T. A. 1988. The accuracy of reverse transcriptase from HIV-1. *Science,* 242**,** 1171-3.

ROBEY, W. G., SAFAI, B., OROSZLAN, S., ARTHUR, L. O., GONDA, M. A., GALLO, R. C. & FISCHINGER, P. J. 1985. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. *Science,* 228**,** 593-5.

RONNBERG, L., VIHKO, P., SAJANTI, E. & VIHKO, R. 1981. Clomiphene citrate administration to normogonadotropic subfertile men: blood hormone changes and activation of acid phosphatase in seminal fluid. *Int J Androl,* 4**,** 372-8.

ROSENBERG, M. E. & SILKENSEN, J. 1995. Clusterin: physiologic and pathophysiologic considerations. *The international journal of biochemistry & cell biology,* 27**,** 633-45.

ROSS, C. A. & PICKART, C. M. 2004. The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative diseases. *Trends in cell biology,* 14**,** 703-11.

ROUSSEAU, F., SCHYMKOWITZ, J. & SERRANO, L. 2006. Protein aggregation and amyloidosis: confusion of the kinds? *Current opinion in structural biology,* 16**,** 118-26.

ROYCE, R. A. 1997. Sexual transmission of HIV (vol 336, pg 1072, 1997). *New England Journal of Medicine,* 337**,** 799-799.

RUSERT, P., FISCHER, M., JOOS, B., LEEMANN, C., KUSTER, H., FLEPP, M., BONHOEFFER, S., GUNTHARD, H. F. & TRKOLA, A. 2004. Quantification of infectious HIV-1 plasma viral load using a boosted in vitro infection protocol. *Virology,* 326**,** 113-29.

SADRZADEH, S. M. & BOZORGMEHR, J. 2004. Haptoglobin phenotypes in health and disorders. *American journal of clinical pathology,* 121 Suppl**,** S97-104.

SAITOH, T., HORSBURGH, K. & MASLIAH, E. 1993. Hyperactivation of signal transduction systems in Alzheimer's disease. *Annals of the New York Academy of Sciences,* 695**,** 34-41.

SALI, A., SHAKHNOVICH, E. & KARPLUS, M. 1994. How does a protein fold? *Nature,* 369**,** 248-51.

SAMUEL, D., KUMAR, T. K., GANESH, G., JAYARAMAN, G., YANG, P. W., CHANG, M. M., TRIVEDI, V. D., WANG, S. L., HWANG, K. C., CHANG, D. K. & YU, C.

2000. Proline inhibits aggregation during protein refolding. *Protein science : a publication of the Protein Society,* 9**,** 344-52.

SATTENTAU, Q. J. & MOORE, J. P. 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *The Journal of experimental medicine,* 174**,** 407-15.

SATTENTAU, Q. J., MOORE, J. P., VIGNAUX, F., TRAINCARD, F. & POIGNARD, P. 1993. Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *Journal of virology,* 67**,** 7383-93.

SAVAS, M. C., KORUK, M., PIRIM, I., YILMAZ, O., KARAKOK, M., TAYSI, S. & YILMAZ, A. 2003. Serum ubiquitin levels in patients with nonalcoholic steatohepatitis. *Hepato-gastroenterology,* 50**,** 738-41.

SAWYER, L., . HOLT, C. 1993. Caseins are rheomorphic proteins, interpretation of primary and secondary structures of the α_{s1}-, β- and κ-caseins. *J Chem Soc Faraday Trans* 2683-2692.

SCHEIDT, H. A., MORGADO, I., ROTHEMUND, S. & HUSTER, D. 2012. Dynamics of amyloid beta fibrils revealed by solid-state NMR. *The Journal of biological chemistry,* 287**,** 2017-21.

SCHERZINGER, E., LURZ, R., TURMAINE, M., MANGIARINI, L., HOLLENBACH, B., HASENBANK, R., BATES, G. P., DAVIES, S. W., LEHRACH, H. & WANKER, E. E. 1997. Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell,* 90**,** 549-58.

SCHUBERT, D., BEHL, C., LESLEY, R., BRACK, A., DARGUSCH, R., SAGARA, Y. & KIMURA, H. 1995. Amyloid peptides are toxic via a common oxidative mechanism.

Proceedings of the National Academy of Sciences of the United States of America, 92**,** 1989-93.

SHARKEY, D. J., MACPHERSON, A. M., TREMELLEN, K. P. & ROBERTSON, S. A. 2007. Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Molecular human reproduction,* 13**,** 491-501.

SHATTOCK, R. J. & MOORE, J. P. 2003. Inhibiting sexual transmission of HIV-1 infection. *Nature reviews. Microbiology,* 1**,** 25-34.

SHOFFNER, J. M. 1997. Oxidative phosphorylation defects and Alzheimer's disease. *Neurogenetics,* 1**,** 13-9.

SIEVERS, S. A., KARANICOLAS, J., CHANG, H. W., ZHAO, A., JIANG, L., ZIRAFI, O., STEVENS, J. T., MUNCH, J., BAKER, D. & EISENBERG, D. 2011. Structure-based design of non-natural amino-acid inhibitors of amyloid fibril formation. *Nature,* 475**,** 96-100.

SILKENSEN, J. R., SKUBITZ, K. M., SKUBITZ, A. P., CHMIELEWSKI, D. H., MANIVEL, J. C., DVERGSTEN, J. A. & ROSENBERG, M. E. 1995. Clusterin promotes the aggregation and adhesion of renal porcine epithelial cells. *The Journal of clinical investigation,* 96**,** 2646-53.

SIMONEAU, S., REZAEI, H., SALES, N., KAISER-SCHULZ, G., LEFEBVRE-ROQUE, M., VIDAL, C., FOURNIER, J. G., COMTE, J., WOPFNER, F., GROSCLAUDE, J., SCHATZL, H. & LASMEZAS, C. I. 2007. In vitro and in vivo neurotoxicity of prion protein oligomers. *PLoS pathogens,* 3**,** e125.

SIPE, J. D. & COHEN, A. S. 2000. Review: history of the amyloid fibril. *Journal of structural biology,* 130**,** 88-98.

SISTLA, S. 2013. Structure-activity relationships of alphas-casein peptides with multifunctional biological activities. *Molecular and cellular biochemistry,* 384**,** 29-38.

STEFANI, M. & DOBSON, C. M. 2003. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *Journal of molecular medicine,* 81**,** 678-99.

STEWART, E. M., AQUILINA, J. A., EASTERBROOK-SMITH, S. B., MURPHY-DURLAND, D., JACOBSEN, C., MOESTRUP, S. & WILSON, M. R. 2007. Effects of glycosylation on the structure and function of the extracellular chaperone clusterin. *Biochemistry,* 46**,** 1412-22.

STRAUSS, S., BAUER, J., GANTER, U., JONAS, U., BERGER, M. & VOLK, B. 1992. Detection of interleukin-6 and alpha 2-macroglobulin immunoreactivity in cortex and hippocampus of Alzheimer's disease patients. *Laboratory investigation; a journal of technical methods and pathology,* 66**,** 223-30.

SULTAN, A., RAMAN, B., RAO CH, M. & TANGIRALA, R. 2013. The extracellular chaperone haptoglobin prevents serum fatty acid-promoted amyloid fibril formation of beta2-microglobulin, resistance to lysosomal degradation, and cytotoxicity. *The Journal of biological chemistry,* 288**,** 32326-42.

SUN, H., CHOW, E. C., LIU, S., DU, Y. & PANG, K. S. 2008. The Caco-2 cell monolayer: usefulness and limitations. *Expert opinion on drug metabolism & toxicology,* 4**,** 395-411.

SUNDE, M. & BLAKE, C. 1997. The structure of amyloid fibrils by electron microscopy and X-ray diffraction. *Advances in protein chemistry,* 50**,** 123-59.

SUNDE, M., SERPELL, L. C., BARTLAM, M., FRASER, P. E., PEPYS, M. B. & BLAKE, C. C. 1997. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. *Journal of molecular biology,* 273**,** 729-39.

SYLVESTER, S. R., SKINNER, M. K. & GRISWOLD, M. D. 1984. A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biology of reproduction,* 31**,** 1087-101.

TAN, S. Y. & PEPYS, M. B. 1994. Amyloidosis. *Histopathology,* 25**,** 403-14.

TANG, Q., ROAN, N. R. & YAMAMURA, Y. 2013. Seminal plasma and semen amyloids enhance cytomegalovirus infection in cell culture. *Journal of virology,* 87**,** 12583-91.

TARTAGLIA, G. G. & VENDRUSCOLO, M. 2008. The Zyggregator method for predicting protein aggregation propensities. *Chemical Society Reviews,* 37**,** 1395-1401.

TAYLOR, J. P., HARDY, J. & FISCHBECK, K. H. 2002. Toxic proteins in neurodegenerative disease. *Science,* 296**,** 1991-5.

TERZI, E., HOLZEMANN, G. & SEELIG, J. 1997. Interaction of Alzheimer beta-amyloid peptide(1-40) with lipid membranes. *Biochemistry,* 36**,** 14845-52.

THALI, M., MOORE, J. P., FURMAN, C., CHARLES, M., HO, D. D., ROBINSON, J. & SODROSKI, J. 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *Journal of virology,* 67**,** 3978-88.

THOMAS, P. J., QU, B. H. & PEDERSEN, P. L. 1995. Defective protein folding as a basis of human disease. *Trends in biochemical sciences,* 20**,** 456-9.

THORN, D. C., ECROYD, H., SUNDE, M., POON, S. & CARVER, J. A. 2008. Amyloid fibril formation by bovine milk alpha s2-casein occurs under physiological conditions yet is prevented by its natural counterpart, alpha s1-casein. *Biochemistry,* 47**,** 3926-36.

THORN, D. C., MEEHAN, S., SUNDE, M., REKAS, A., GRAS, S. L., MACPHEE, C. E., DOBSON, C. M., WILSON, M. R. & CARVER, J. A. 2005. Amyloid fibril formation by bovine milk kappa-casein and its inhibition by the molecular chaperones alphaS- and beta-casein. *Biochemistry,* 44**,** 17027-36.

TREWEEK, T. M., THORN, D. C., PRICE, W. E. & CARVER, J. A. 2011. The chaperone action of bovine milk alphaS1- and alphaS2-caseins and their associated form alphaS-casein. *Archives of biochemistry and biophysics,* 510**,** 42-52.

TRKOLA, A., DRAGIC, T., ARTHOS, J., BINLEY, J. M., OLSON, W. C., ALLAWAY, G. P., CHENG-MAYER, C., ROBINSON, J., MADDON, P. J. & MOORE, J. P. 1996. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature,* 384**,** 184-7.

TROMBETTA, E. S. & PARODI, A. J. 2003. Quality control and protein folding in the secretory pathway. *Annual review of cell and developmental biology,* 19**,** 649-76.

TROUGAKOS, I. P. & GONOS, E. S. 2002. Clusterin/apolipoprotein J in human aging and cancer. *The international journal of biochemistry & cell biology,* 34**,** 1430-48.

TROUGAKOS, I. P. & GONOS, E. S. 2006. Regulation of clusterin/apolipoprotein J, a functional homologue to the small heat shock proteins, by oxidative stress in ageing and age-related diseases. *Free radical research,* 40**,** 1324-34.

UNAIDS 2013. The Gap Report.

UNAIDS, F. S. 1996. Joint United Nations Programme on HIV/AIDS. The HIV/AIDS situation in mid 1996.

UVERSKY, V. N. 2002. What does it mean to be natively unfolded? *European journal of biochemistry / FEBS,* 269**,** 2-12.

VIVEKANANDAN, S., BRENDER, J. R., LEE, S. Y. & RAMAMOORTHY, A. 2011. A partially folded structure of amyloid-beta(1-40) in an aqueous environment. *Biochem Biophys Res Commun,* 411**,** 312-6.

WALSH, D. M., KLYUBIN, I., FADEEVA, J. V., CULLEN, W. K., ANWYL, R., WOLFE, M. S., ROWAN, M. J. & SELKOE, D. J. 2002. Naturally secreted oligomers of

amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature,* 416**,** 535-9.

WEIBEZAHN, J., SCHLIEKER, C., TESSARZ, P., MOGK, A. & BUKAU, B. 2005. Novel insights into the mechanism of chaperone-assisted protein disaggregation. *Biological chemistry,* 386**,** 739-44.

WEISS, R. A. 1996. HIV receptors and the pathogenesis of AIDS. *Science,* 272**,** 1885-6.

WEJMAN, J. C., HOVSEPIAN, D., WALL, J. S., HAINFELD, J. F. & GREER, J. 1984. Structure and assembly of haptoglobin polymers by electron microscopy. *Journal of molecular biology,* 174**,** 343-68.

WELCH, W. J. & BROWN, C. R. 1996. Influence of molecular and chemical chaperones on protein folding. *Cell stress & chaperones,* 1**,** 109-15.

WHITE, M. R., KANDEL, R., TRIPATHI, S., CONDON, D., QI, L., TAUBENBERGER, J. & HARTSHORN, K. L. 2014. Alzheimer's associated beta-amyloid protein inhibits influenza A virus and modulates viral interactions with phagocytes. *PloS one,* 9**,** e101364.

WILSON, M. R. & EASTERBROOK-SMITH, S. B. 2000. Clusterin is a secreted mammalian chaperone. *Trends in biochemical sciences,* 25**,** 95-8.

WILSON, M. R., YERBURY, J. J. & POON, S. 2008. Potential roles of abundant extracellular chaperones in the control of amyloid formation and toxicity. *Molecular bioSystems,* 4**,** 42-52.

WOJCIK, C. & DEMARTINO, G. N. 2003. Intracellular localization of proteasomes. *The international journal of biochemistry & cell biology,* 35**,** 579-89.

WOJTOWICZ, W. M., FARZAN, M., JOYAL, J. L., CARTER, K., BABCOCK, G. J., ISRAEL, D. I., SODROSKI, J. & MIRZABEKOV, T. 2002. Stimulation of

enveloped virus infection by beta-amyloid fibrils. *The Journal of biological chemistry,* 277**,** 35019-24.

WONG, J. H. & NG, T. B. 2006. Limenin, a defensin-like peptide with multiple exploitable activities from shelf beans. *Journal of peptide science : an official publication of the European Peptide Society,* 12**,** 341-6.

WU, L., GERARD, N. P., WYATT, R., CHOE, H., PAROLIN, C., RUFFING, N., BORSETTI, A., CARDOSO, A. A., DESJARDIN, E., NEWMAN, W., GERARD, C. & SODROSKI, J. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature,* 384**,** 179-83.

WYATT, A. R., YERBURY, J. J., BERGHOFER, P., GREGURIC, I., KATSIFIS, A., DOBSON, C. M. & WILSON, M. R. 2011. Clusterin facilitates in vivo clearance of extracellular misfolded proteins. *Cellular and molecular life sciences : CMLS,* 68**,** 3919-31.

WYATT, R., KWONG, P. D., DESJARDINS, E., SWEET, R. W., ROBINSON, J., HENDRICKSON, W. A. & SODROSKI, J. G. 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature,* 393**,** 705-11.

WYATT, R., SULLIVAN, N., THALI, M., REPKE, H., HO, D., ROBINSON, J., POSNER, M. & SODROSKI, J. 1993. Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. *Journal of virology,* 67**,** 4557-65.

YANKNER, B. A. & LU, T. 2009. Amyloid beta-protein toxicity and the pathogenesis of Alzheimer disease. *The Journal of biological chemistry,* 284**,** 4755-9.

YE, Z., FRENCH, K. C., POPOVA, L. A., LEDNEV, I. K., LOPEZ, M. M. & MAKHATADZE, G. I. 2009. Mechanism of fibril formation by a 39-residue peptide (PAPf39) from human prostatic acidic phosphatase. *Biochemistry,* 48**,** 11582-91.

YERBURY, J. J., KUMITA, J. R., MEEHAN, S., DOBSON, C. M. & WILSON, M. R. 2009. alpha2-Macroglobulin and haptoglobin suppress amyloid formation by interacting with prefibrillar protein species. *The Journal of biological chemistry,* 284**,** 4246-54.

YERBURY, J. J., POON, S., MEEHAN, S., THOMPSON, B., KUMITA, J. R., DOBSON, C. M. & WILSON, M. R. 2007. The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 21**,** 2312-22.

YERBURY, J. J., RYBCHYN, M. S., EASTERBROOK-SMITH, S. B., HENRIQUES, C. & WILSON, M. R. 2005a. The acute phase protein haptoglobin is a mammalian extracellular chaperone with an action similar to clusterin. *Biochemistry,* 44**,** 10914-25.

YERBURY, J. J., STEWART, E. M., WYATT, A. R. & WILSON, M. R. 2005b. Quality control of protein folding in extracellular space. *EMBO reports,* 6**,** 1131-6.

ZAMARCHI, R., ALLAVENA, P., BORSETTI, A., STIEVANO, L., TOSELLO, V., MARCATO, N., ESPOSITO, G., RONI, V., PAGANIN, C., BIANCHI, G., TITTI, F., VERANI, P., GEROSA, G. & AMADORI, A. 2002. Expression and functional activity of CXCR-4 and CCR-5 chemokine receptors in human thymocytes. *Clinical and experimental immunology,* 127**,** 321-30.

ZHANG, X., FU, X., ZHANG, H., LIU, C., JIAO, W. & CHANG, Z. 2005. Chaperone-like activity of beta-casein. *The international journal of biochemistry & cell biology,* 37**,** 1232-40.

ZLOKOVIC, B. V. 1996. Cerebrovascular transport of Alzheimer's amyloid beta and apolipoproteins J and E: possible anti-amyloidogenic role of the blood-brain barrier. *Life sciences,* 59**,** 1483-97.