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Abstract
Extracellular protein misfolding and aggregation underlie many of the most serious amyloidoses including Alzheimer's disease, spongiform encephalopathies and type II diabetes. Despite this, protein homeostasis (proteostasis) research has largely focussed on characterising systems that function to monitor protein conformation and concentration within cells. We are now starting to identify elements of corresponding systems, including an expanding family of secreted chaperones, which exist in the extracellular space. Like their intracellular counterparts, extracellular chaperones are likely to play a central role in systems that maintain proteostasis; however, the precise details of how they participate are only just emerging. It is proposed that extracellular chaperones patrol biological fluids for misfolded proteins and facilitate their clearance via endocytic receptors. Importantly, many amyloidoses are associated with dysfunction in rates of protein clearance. This is consistent with a model in which disruption to, or overwhelming of, the systems responsible for extracellular proteostasis results in the accumulation of pathological protein aggregates and disease. Further characterisation of mechanisms that maintain extracellular proteostasis will shed light on why many serious diseases occur and provide us with much needed strategies to combat them.

Keywords
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Roles of Extracellular Chaperones in Amyloidosis

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Abstract

Extracellular protein misfolding and aggregation underlies many of the most serious amyloidoses including Alzheimer's disease, spongiform encephalopathies and type II diabetes. Despite this, protein homeostasis (proteostasis) research has largely focussed on characterising systems that function to monitor protein conformation and concentration within cells. We are now starting to identify elements of corresponding systems, including an expanding family of secreted chaperones, which exist in the extracellular space. Like their intracellular counterparts, extracellular chaperones are likely to play a central role in systems that maintain proteostasis, however, the precise details of how they participate are only just emerging. It is proposed that extracellular chaperones patrol biological fluids for misfolded proteins and facilitate their clearance via endocytic receptors. Importantly, many amyloidoses are associated with dysfunction in rates of protein clearance. This is consistent with a model in which disruption to, or overwhelming of, the systems responsible for extracellular proteostasis results in the accumulation of pathological protein aggregates and disease. Further characterisation of mechanisms that maintain extracellular proteostasis will shed light on why many serious diseases occur and provide us with much needed strategies to combat them.

Keywords: Protein aggregation; receptor-mediated endocytosis; clearance; proteostasis; Alzheimer's disease.

Introduction

Protein homeostasis (proteostasis) refers to all those processes that collectively maintain the levels, structure and function of proteins in living systems. The demands placed upon proteostasis systems become progressively greater with increasing organismal complexity, and in large multicellular organisms, these demands are spread across two major environments, the intracellular and extracellular spaces. Both of these environments impose stresses upon protein structure, such as oxidative stress and fluctuations in temperature and pH. The extracellular space is more oxidising than inside cells[1] and imposes an additional challenge to protein stability in the form of shear stress resulting from the continuous pumping of plasma around the body, which can induce protein unfolding and aggregation.[2-3] Chaperones are key elements in the systems that safeguard against the effects of these stresses, and other influences that negatively impact upon the proteome. Chaperones selectively bind to non-native protein molecules to inhibit their aggregation and some chaperones are able to facilitate the correct folding of proteins into their native conformation.[4] What is becoming increasingly clear is that chaperones also play key roles in the disposal of unfolded/misfolded proteins that are refractory to refolding, and that it is this function that may be especially important in normally protecting against serious disease.

Excessive misfolding/unfolding of proteins can potentially cause pathology by a variety of mechanisms, including loss of biological function, toxic gain-of-function (e.g. cytotoxic oligomers), physical entrapment of other proteins in aggregates, and in the case of large deposits, disruption of tissues. The amyloidoses, and other protein misfolding diseases, result from dysfunctions in proteostasis that lead to an inappropriate accumulation of one or more proteins and associated pathology. Under these conditions, chaperones and other elements of proteostasis may be physically overwhelmed by the quantities of non-native proteins presented to the system and disease results. In the intracellular context, it is now well recognised that the levels of chaperones and the capacity of clearance mechanisms declines with age.[5] This trend, likely to also apply in the extracellular context, may well account for the age-related onset of many of the amyloidoses and related diseases. In the future, the most effective therapies for these diseases will target those processes directly responsible for the underlying causation, rather than treating downstream consequences of this. It is striking then to note that despite the fact that *extracellular* protein misfolding and aggregation is strongly implicated as underpinning the onset of many of the most serious amyloidoses (Table 1), current understanding of those processes controlling proteostasis in the extracellular spaces of the body is at best rudimentary. It is only just over 10 years since the first abundant extracellular chaperone (clusterin) was reported[6-7] and details of how extracellular chaperones protect the body from serious disease is only now beginning to emerge.[8-9]

Table 1. Examples of extracellular amyloid deposition and the co-localisation of extracellular chaperones with amyloid in disease.

Disease	Aggregating protein/peptide	Co-localised chaperones
Alzheimer's disease	Aβ	Clusterin,[10] α_2-macroglobulin,[11] haptoglobin,[12] apolipoprotein E,[13] serum amyloid P[14]
Spongiform encephalopathies	Prion Protein	Clusterin,[15-16] α_2-macroglobulin,[17] apolipoprotein E,[13] serum amyloid P[18]
Familial British dementia	ABri	Clusterin,[19] serum amyloid P,[20] apolipoprotein E [21]
Familial Danish Dementia	ADan	Clusterin,[22] serum amyloid P[20]
Down's syndrome	Aβ	Clusterin,[23] apolipoprotein E,[13] serum amyloid P[24]
Type II Diabetes	Human Islet Amyloid Peptide	Clusterin,[25] apolipoprotein E,[26] serum amyloid P[27]
Haemodialysis-related amyloidosis	β_2-Microglobulin	α_2-macroglobulin,[28] serum amyloid P[28]
Amyloidotic cardiomyopathy	Transthyretin, Immunoglobulin light chain	Clusterin[29-30]
Systemic Amyloidosis	Immunoglobulin light chain	Clusterin,[31] serum amyloid P,[31] apolipoprotein E[21]
Icelandic Type HCHWA-1	Cystatin C	Apolipoprotein E[21]
Myeloma-associated amyloidosis	Immunoglobulin light chain	Apolipoprotein E[21]
Corneal Dystrophies	Keratoepithelin	Clusterin,[32] apolipoprotein E[32]

The expanding family of extracellular chaperones

There is strong evidence supporting the existence of extracellular mechanisms to control proteostasis and to indicate that exposed hydrophobicity targets extracellular molecules for rapid clearance and intracellular degradation,[33-37] however, the precise mechanisms by which this is achieved *in vivo* are not yet known. Exposed hydrophobicity on misfolded proteins is the driving force for the formation of toxic aggregates[38] and is proposed to stimulate aberrant inflammation which often accompanies protein deposition *in vivo*.[39-40] Thus, the discovery of extracellular molecules that preferentially interact with misfolded proteins, be they secreted chaperones (discussed in detail below), receptors (e.g. scavenger,[41-42] toll-like[43-44] and MAC-1[45-46] or elements of protease systems (e.g. tissue plasminogen activator)[47-48] is extremely important. Moreover, the characterisation of how these different molecules interplay in order to maintain proteostasis will potentially shed light on why some amyloidoses occur and provide us with more informed strategies for combating these diseases.

It is well established that "holdase" chaperones are vital in targeting misfolded intracellular molecules for protease degradation or for repair by "foldase" chaperones. While normally intracellular chaperones may be present in the extracellular environment, their abundance is normally extremely low (e.g. Hsp70 is present in blood plasma at < 10 ng/mL).[49] Also, ATP which is needed to fuel intracellular protease systems and foldase chaperones is at least 1,000 times less concentrated outside of the cell than inside.[50] Therefore, the efficient processing of misfolded extracellular proteins is likely to be managed by abundant, normally secreted, ATP-independent mechanisms that are constitutively present in blood plasma, cerebrospinal fluid, interstitial fluid and all extracellular spaces. Three secreted glycoproteins, namely clusterin, haptoglobin and α_2-macroglobulin (α_2M) are known to have ATP-independent chaperone activity *in vitro*.[7,51-52] These structurally unrelated proteins have the ability to stably bind misfolded proteins and thereby inhibit inappropriate protein–protein interactions, prevent aggregation, and maintain proteins in solution. All three extracellular chaperones demonstrate the ability to influence amyloid formation *in vitro*,[53-54] and are found co-localised with clinical amyloid deposits *in vivo* (Table 1). Two other secreted glycoproteins, apolipoprotein E (ApoE) and serum amyloid P (SAP), that are universally found associated with amyloid deposits are also known to influence amyloid formation *in vitro*,[55-56] however, less is known about their ability to stabilise misfolded proteins.

Clusterin

Clusterin (also known as apolipoprotein J/ApoJ) is a highly glycosylated, heterodimeric protein (formed by disulfide-linked α and β subunits) of approximately 60 kDa that is expressed by a wide variety of tissues and found in all extracellular fluids which have been tested. Branched, sialic acid-rich, N-linked carbohydrates contribute 17-27% of the mass of mature human clusterin.[57] This high carbohydrate content has impeded traditional methods for structural analysis such as x-ray crystallography, however, several α-helical regions are predicted by sequence analysis.[58] It has been proposed that amphipathic α-helical regions on clusterin form a molten globule-like binding pocket that is important in mediating its binding to a diverse range of ligands.[59] The concentrations of clusterin in human blood plasma and cerebral spinal fluid (CSF) are 35-105 μg/mL[60] and 1.2-3.6 μg/mL,[61] respectively. Clusterin was named for its propensity to cause cell clustering *in vitro*,[62] however, a large number of diverse biological functions have been proposed including roles in complement regulation,[63] apoptosis[64] and lipid transport.[58] Considering that the ligand binding profile of clusterin is extremely broad, it is plausible that the functions of this protein are equally far reaching. There have been many studies on the importance of clusterin in the pathogenesis and progression of cancer, however, depending on the system clusterin appears to be either pro- or anti-apoptotic.[65] Clusterin is encoded by a single gene that is highly conserved across mammalian species, which supports that its role *in vivo* is of fundamental importance. Supporting a protective role for clusterin, it is up-regulated in experimental models of oxidative stress,[66] shear stress,[67] proteotoxic stress,[68] heat stress[69] and upon exposure to ionizing radiation[70] or heavy metals.[71] Clusterin is also overexpressed in tissue injury and in many serious diseases *in vivo*.[72-73] This includes up-regulation of clusterin expression in several of the amyloidoses

including Alzheimer's disease,[74-75] Down's syndrome[76] and diabetes,[77-78] and also in aging.[65,79]

Clusterin inhibits stress-induced amorphous protein aggregation by binding to exposed regions of hydrophobicity on non-native proteins to form soluble, high molecular weight complexes.[7,80-81] This activity is similar to, but more potent than, the chaperone activity of intracellular small heat shock proteins (sHsps).[80] Like sHsps, clusterin also has a tendency to self-aggregate into high order oligomers. Mildly acidic pH favours dissociation of oligomeric clusterin, thereby increasing the surface hydrophobicity of the molecule and in turn its chaperone activity.[82] Exactly how clusterin is able to stably hold misfolded protein in extremely large yet soluble complexes ($\geq 4 \times 10^7$ Da) remains unknown,[81] but its ability to promiscuously interact with hydrophobic ligands is believed to involve several amphipathic α-helices.[59] Immunoaffinity depletion of clusterin from human plasma markedly increases plasma protein aggregation and precipitation after incubation at physiologically relevant temperature.[82] In plasma subjected to mild shear stress, fibrinogen, ceruloplasmin and albumin have been identified as major endogenous clients for the chaperone action of clusterin,[83] however, the available data suggests that clusterin preferentially binds to hydrophobic regions on proteins regardless of their identity. Thus, it is likely that the detection of endogenous clients in this way was biased towards those proteins that are relatively more abundant and relatively less stable. It has recently been shown that in rats, blood-borne clusterin-misfolded protein complexes are rapidly transported to the liver.[9] Furthermore, the same complexes are preferentially recognised by fucoidin-inhibitable receptors on hepatocytes and are subsequently delivered to intracellular lysosome for degradation.[9] These findings strongly support an important role for clusterin in the targeted delivery of misfolded protein to endocytic receptors for disposal, however, more studies are needed in order to fully characterise this pathway and understand its significance in health and disease. We have proposed that clusterin may be a critical element in a system designed to clear the body of misfolded proteins, and perhaps also proteolytic fragments generated from insoluble protein deposits (Figure 1). The other extracellular chaperones may play similar roles in protecting the body from amyloid and other diseases involving the inappropriate extracellular aggregation and deposition of proteins.

It has been demonstrated that clusterin influences amyloid formation by a large number of peptides/proteins.[53,84-88] While clusterin does not bind to native amyloidogenic proteins nor mature fibrils, the binding of clusterin to prefibrillar species important at the nucleation stage of amyloid formation inhibits their further growth.[53,84] The ability of clusterin to inhibit amyloid formation is dose-dependent but not mono-phasic. At very low ratios of clusterin to fibril forming client protein, amyloid formation can be significantly increased, but decreased at higher ratios of clusterin to client protein.[53] The former effect may be due to the stabilisation of structures that seed amyloid formation. This ratio-dependent behaviour is likely to explain why reports on the *in vitro* effects of clusterin on amyloid cytotoxicity are mixed.[53,88-89] The results of clusterin knockout mice studies have further fuelled debate over whether this protein is protective or harmful. Supporting a protective role, clusterin knockout increases damage after heat-shock,[90] myosin-induced auto-immune myocarditis[91] and post-ischemic brain injury.[92] Moreover,

it has been demonstrated that clusterin knockout mice develop progressive glomerulopathy which is characterised by the accumulation of insoluble protein deposits in the kidneys.[93] This latter result directly implicates clusterin in the clearance of potentially pathological aggregating proteins *in vivo*. In contrast, in amyloid precursor protein (APP) transgenic mice, clusterin knockout reduces fibrillar Aβ amyloid deposition and neurotoxicity.[94] The same result has been shown for ApoE knockout, however, double knockout of clusterin and ApoE resulted in early disease onset and a marked increase in Aβ peptide levels and amyloid formation.[95] Taken together the available data suggests that clusterin and ApoE work synergistically to inhibit the deposition of fibrillar Aβ *in vivo*. The mechanism(s) by which this is achieved are not yet known, however, the formation of complexes between Aβ and clusterin or ApoE is known to affect the rate by which Aβ is cleared from the brain.[96] Of note, the ApoE genotype has been firmly established as a genetic risk factor for sporadic Alzheimer's disease in humans.[97-98] Much more recently, two independent genome-wide association studies of several thousand individuals have identified that polymorphism in clusterin is also a strong genetic risk factor for the same disease.[99-100]

Haptoglobin

Haptoglobin is a secreted glycoprotein that is best known for its role in haemoglobin binding.[101] In humans haptoglobin is expressed as one of three major phenotypes (Hp 1-1, Hp 1-2 and Hp 2-2) depending on the presence of two principal alleles (Hp1 and Hp2) which encode distinct a subunits (α^1 and α^2, respectively). Hp 1-1 consists of a disulfide-linked $(\alpha^1)_2\beta_2$ structure (ca. 100 kDa). However, in Hp 2-1 and Hp 2-2, an additional cysteine residue in the α_2 chain allows the formation of a complex series of various sized disulfide-linked αβ polymers (ca.100 - 500 kDa). As for clusterin, there is no x-ray crystallography data available for haptoglobin. However, haptoglobin shares a high degree of homology with the chymotrypsinogen-like serine protease family[102] and homology modelling has been used to predict its structure.[103] It has been suggested that a large hydrophobic region adjacent to the haemoglobin-binding site is responsible for the chaperone activity of the protein.[104]

Haptoglobin is found in most extracellular fluids and is present in human plasma and CSF at 0.3-2.0 mg/ml[101] and 0.5-2 µg/ml,[105] respectively. In addition to its role in haemoglobin binding, haptoglobin has also been implicated in regulation of the immune system[106-107] and cathepsin B activity,[108] and appears to have pro-angiogenic effects.[109] Haptoglobin is an acute phase protein and is up-regulated during infection, neoplasia, pregnancy, trauma, acute myocardial infarction and other inflammatory conditions.[110] Sequestration of haemoglobin by haptoglobin reduces the amount of free haemoglobin and iron available to catalyse oxidative reactions,[111] and inhibits nitric oxide[112] and prostaglandin synthesis.[113] Thus, the binding of haptoglobin to haemoglobin is a biologically important protective mechanism. Haptoglobin phenotype has been examined for clinical relevance in a number of diseases (reviewed in [110]), however, there is little reported data relating to its significance in amyloid disease. Haptoglobin phenotype is known to be a risk factor for cardiovascular disease in diabetes,[114-115] although, how this

may relate to its chaperone activity remains unknown. One study has reported that haptoglobin concentrations are reduced in the CSF of patients with Alzheimer's disease,[116] however, it has also been reported that haptoglobin CSF levels are not significantly different between Alzheimer's patients and normal control subjects[117] and also that high serum concentrations of haptoglobin are indicative of cognitive impairment.[118] It is unknown whether haptoglobin levels change during human aging, however in horses, foals (12 ≤ months of age) have significantly higher serum haptoglobin levels compared to adults.[119]

Characterisation of the chaperone activity of haptoglobin is so far limited to a few studies. Like clusterin, all three haptoglobin phenotypes inhibit the stress-induced amorphous aggregation and precipitation of a wide variety of proteins *in vitro*.[51,120] However, in contrast to clusterin, lowered pH greatly reduces the chaperone activity of haptoglobin and this corresponds with a reduction in the affinity of haptoglobin for the hydrophobic dye bisANS.[51] The available data suggest that at physiologically relevant pH haptoglobin is more efficient at solubilising stressed proteins that intracellular sHsps, but is less efficient than clusterin.[51] Haptoglobin binds to a range of amyloid forming peptides/proteins and at substoichiometric ratios inhibits amyloid formation by binding to transient prefibrillar species.[54] Complexation with haemoglobin reduces but does not abolish the ability of haptoglobin to inhibit amorphous or fibrillar protein aggregation.[54,104] Haptoglobin polymorphism has been shown to influence the susceptibility to and/or outcome in several diseases, however, so far this does not include any of the amyloidoses.[121] Aside from the co-localisation of haptoglobin with amyloid deposits in Alzheimer's disease (Table 1), there have not yet been *in vivo* studies of the effect of haptoglobin on proteostasis, however, it is known that haptoglobin-haemoglobin complexes are preferentially recognised by the endocytic scavenger receptor CD163.[122] Hp is also known to bind to CD11b/CD18 integrin (Mac-1/CR3), which also binds denatured proteins, the iC3b fragment of complement, and the CD22 B lymphocyte receptor.[123] Thus, it appears feasible that Hp might interact with one or more of these receptors to mediate the clearance and degradation of misfolded extracellular proteins. Therefore, it is tempting to speculate that the formation of complexes between haptoglobin and misfolded proteins may also target them for clearance via a similar mechanism to that implicated for clusterin-client protein complexes.

α_2M

α_2M is a major blood glycoprotein that is also present in most other extracellular fluids. α_2M is a tetramer comprised of four identical subunits that form disulfide-linked dimers, which then non-covalently interact to give the 720 kDa tetrameric quaternary structure.[124] Only limited x-ray crystal data has been obtained for α_2M, however, the extensive structural information available for human C3[125-128] has allowed for detailed prediction of the location of homologous domains within α_2M.[129] α_2M is predicted to contain eight fibronectin type-3 folded macroglobulin domains in addition to an alpha helical TED (thiol ester-containing) domain, a CUB (complement protein subcomponents C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1) domain and a RBD

(receptor binding domain).[127] The concentrations of α_2M in human plasma and CSF are 1.5–2 mg/mL and 1.0–3.6 mg/mL, respectively.[130-131] While α_2M is best known for its role as a broad spectrum protease inhibitor,[131] many other biological functions, including roles in immunomodulation and cancer progression, have been proposed.[132-136] Similar to clusterin and haptoglobin, α_2M has been shown to have a holdase chaperone activity which inhibits amorphous and fibrillar protein aggregation *in vitro*. Depletion of α_2M from human plasma renders plasma proteins more susceptible to precipitation at physiological temperatures.[52] At present it is not known what structural elements are responsible for mediating the binding of α_2M to misfolded proteins. Clusterin is more efficient at inhibiting protein aggregation compared to α_2M, however, given that α_2M is present at very high concentration in human blood plasma, its chaperone activity is likely to have considerable physiological relevance. α_2M retains the ability to trap proteases after binding to misfolded proteins and α_2M-protease-misfolded protein complexes are recognised by LRP;[52] this represents another potential route for the targeted disposal of misfolded proteins *in vivo*. *In vitro* it has already been demonstrated that α_2M facilitates the clearance of Aβ in this way.[137] α_2M inhibits the aggregation of a number of different amyloid forming proteins and protects cells from amyloid-induced toxicity.[54,138] As for the other extracellular chaperones, α_2M does not inhibit fibril elongation or disrupt mature fibrils, but appears to suppress amyloid formation by interacting with transiently formed prefibrillar species that occur early in the aggregation process.[54] Several studies have reported linkage of Alzheimer's disease with markers on chromosome 12.[139] The location of the α_2M gene on chromosome 12 together with evidence supporting a role for α_2M in the clearance of Aβ via LRP[137] have encouraged many researchers to look for disease-associated polymorphisms in the gene, however, the findings of these studies are heavily debated.[140-141] While several independent studies have reported that polymorphism in α_2M is a genetic risk factor for Alzheimer's disease,[142-145] several other studies have failed to show an association.[143,146] There is also vigorous debate about whether mutations in LRP are linked with Alzheimer's disease. [143,147-148]

Other secreted molecules with reported chaperone activity

In recent years *in vitro* chaperone activity has been described for a number of secreted proteins (Table 2). While it is likely that maintaining extracellular proteostasis is the shared role of a number of proteins, many of the proposed extracellular chaperones are currently poorly characterised and the physiological relevance of their chaperone activity remains uncertain. A decade ago it was reported that serum amyloid P (SAP; a 125 kDa member of the pentraxin family) has ATP-independent refolding activity *in vitro*,[149] however, since then there have been no follow on studies. The data collected showed that at a 10 fold molar excess, SAP increased the enzyme reactivation of heat-denatured lactate dehydrogenase by just 25%; this poor efficiency suggests that the refolding activity of SAP may not be physiologically relevant. It is striking that SAP is universally found located with amyloid deposits *in vivo*,[150-153] particularly since its concentration in human plasma is relatively low. *In vitro*, SAP has been shown to inhibit amyloid fibril formation and increase the solubility of Aβ.[56] However, SAP itself is highly resistant to proteases and the binding of SAP to amyloid fibrils protects them from degradation.[154]

While SAP is believed to target some of its ligands to gamma Fc receptors,[155] it is not known to target amyloid for disposal in this way. Furthermore, in SAP knockout mice amyloid deposition is delayed suggesting that it has a pro-amyloidogenic role *in vivo*.[156]

ApoE is a 34 kDa secreted protein that is found in human plasma at approximately 60-120 µg/ml and in CSF at around one tenth of this concentration.[157] In humans there are three common alleles of the ApoE gene designated ε2, ε3 and ε4, which result in three heterozygous and three homozygous genotypes.[158] The ε4 allele of ApoE is a firmly established genetic risk factor for late-onset Alzheimer's disease and the ε2 allele appears to be protective.[97-98] Like clusterin and α_2M, it has been demonstrated that ApoE binds to several different amyloid forming proteins *in vitro*[159-160] and ApoE has been shown to influence amyloid formation in a concentration dependent-manner,[55,161] and to promote clearance of Aβ *in vivo*,[96,162] however, its ability to interact with amorphously aggregating proteins is currently unknown. The ability of ApoE to promote amyloid formation has encouraged some researchers to describe it as a "pathological chaperone",[21] however, like clusterin it is evident that depending on the conditions used, the *in vitro* effect of ApoE on amyloid formation can also be inhibitory. Also of interest is the major high density lipoprotein-associated protein ApoAI which is known to influence Aβ aggregation and toxicity;[163] ApoAI has yet to be examined for a general chaperone activity. Common to the so-called "exchangeable apolipoproteins" (which are capable of moving from one lipoprotein to another) clusterin, ApoE and ApoAI contain a number of amphipathic helices, which mediate the binding of these proteins to lipids and are probably involved in their promiscuous binding to a range of other hydrophobic molecules.[164]

It was reported that the major blood protein fibrinogen (340 kDa) has chaperone-like activity against thermal protein aggregation.[165] The same research group later reported this activity was limited to a minor subclass of fibrinogen known as fibrinogen-420,[166] which contains an additional 236-residue C-terminal domain (α_EC).[167] No explanation was provided for why the results of experiments involving conventional fibrinogen were inconsistent between the two studies. The latter study showed that fibrinogen-420 was able to reduce the thermal aggregation of citrate synthase by around 50% when fibrinogen-420 and citrate synthase were present at equimolar concentrations. No further data was presented for the intact protein, however, recombinant α_EC was shown to have potent holdase-type chaperone activity in a number of different assays. Clearly further studies of fibrinogen-420 are needed before it can be classed together with the known extracellular chaperones.

Another plasma protein of interest in this context is albumin, the major carrier of Aβ in human plasma.[168] Like the extracellular chaperones, albumin is found associated with amyloid deposits *in vivo*[169] and has been shown to inhibit amyloid formation *in vitro*,[170-171] thus a role for this protein in amyloidosis cannot be excluded. However, compared to genuine chaperones, on a molar basis albumin is considerably less effective at inhibiting protein aggregation and for this reason is often used as a negative control protein in chaperone assays.[81,172-173] Moreover, high endogenous concentrations of albumin are not able to prevent protein aggregation when far less abundant chaperone proteins are

depleted from human plasma.[51-52,82] Thus it appears that the chaperone properties of albumin are very limited when compared with those of clusterin, haptoglobin and α_2M. Several other extracellular proteins have been reported to have "chaperone-like" properties, including caseins, secreted protein acidic and rich in cysteine (SPARC) and macrophage inhibitory factor (MIF). All of these proteins have been shown to inhibit amorphous protein aggregation *in vitro*, however, the ability to inhibit amyloid fibril formation has only been demonstrated for caseins. [174-179] SPARC has been shown to inhibit collagen fibrillogenesis and is believed to play an important role in the remodelling of the extracellular matrix[180]; it may also act as an intracellular chaperone for procollagen.[181] SPARC knockout mice develop cataract and abnormal collagen deposition, supporting that the ability of SPARC to act as a chaperone for collagen is important *in vivo*.[182-183] Little is known regarding the ability of SPARC to interact with other client proteins, however, it has been shown that SPARC can prevent the aggregation of heat denatured alcohol dehydrogenase at substoichiometric concentrations.[179] Similarly, macrophage inhibitory factor (MIF) has been shown to stabilise heat denatured malate dehydrogenase and glycogen phosphorylase b.[178] Further studies of the chaperone-like activity of SPARC and MIF are needed before they can be classified together with clusterin, haptoglobin and α_2M, which are known to act against a very broad range of clients.

Table 2. Some less completely characterised putative extracellular chaperone proteins.

Protein	Reported chaperone activity
Serum Amyloid P (SAP)	- Limited ATP-independent refolding activity *in vitro*[149] - Inhibits amyloid fibril formation and increases the solubility of Aβ[56] - Protects amyloid fibrils from degradation.[154] - Universally found located with amyloid deposits *in vivo*[150-153] - In SAP knockout mice amyloid deposition is delayed[156]
ApoE	- Influences amyloid formation *in vitro* [55,161]
Fibrinogen-420	- Prevents thermal aggregation of citrate synthase[166]
ApoAI	- Directly interacts with amyloid precursor protein and inhibits Aβ aggregation and toxicity[163]
Albumin	- Increases the reactivation of chemically or thermally denatured enzymes[184] - Prevents thermal aggregation of sorbitol dehydrogenase and the heat induced inactivation of a number of other enzymes[185] - Binds to Aβ peptide and suppresses amyloid formation[170]
Casein proteins	- Prevent amorphous protein aggregation (induced by thermal stress or reduction) and fibril formation of a number of model proteins[174-177] - Associated with amyloid-like deposits in mammary tissue[186-188]
Secreted protein	- Prevents thermal aggregation of alcohol dehydrogenase

acidic and rich in cysteine (SPARC)	*in vitro*[179] - SPARC knockout mice develop cataract and abnormal collagen deposition[182-183]
Macrophage inhibitory factor (MIF)	- Stabilises thermally denatured malate dehydrogenase and glycogen phosphorylase b *in vitro*[178] - Isolated by Aβ peptide affinity chromatography from rat brain and Alzheimer's disease brain[189]

The emerging importance of extracellular proteostasis in protection from amyloid disease

In the simplest model, two processes contribute to the control of protein concentration; production and clearance. Therefore, the accumulation of proteins at high concentrations can result from two not mutually exclusive possibilities - increased synthesis or reduced protein disposal and degradation (Figure 2). The amyloid hypothesis of Alzheimer's disease proposes that disease pathology is a direct result of an imbalance between Aβ production and its clearance.[190] Indeed the causative mutations in APP and presinilins have been found to increase the production of the aggregation prone $A\beta_{1-42}$ variants of the peptide *in vivo*.[191] This has created immense interest in treating Alzheimer's disease by targeting the production or aggregation of $A\beta_{1-42}$ peptide (see [192] for a review of Alzheimer's drug targets). Indeed, targeting aggregation is a promising strategy to treat amyloidoses such as those associated with transthyretin accumulation.[193] Stabilising native conformations rather than targeting pre-formed oligomers or fibrils is likely to be more successful as all aggregate species will probably exert some detrimental effects. Indeed, Aβ aggregate species ranging from dimers and oligomers through to fibrils have been shown to be toxic.[194-196] Reducing the amount of aggregating Aβ in Alzheimer's disease can also be achieved by targeting the proteases responsible for cleaving APP such as α-, β- or γ-secretase. One prominent example of such a treatment are the gamma secretase inhibitors.[197] The scale of investment in gamma secretase inhibitors has likely run in to the billions of dollars. Reduction of soluble Aβ following treatment with gamma secretase inhibitors looked very promising, however, the fact that APP is only one of several gamma-secretase substrates[198] means that translation into the clinic has been problematic. Most notably, the recent Phase III clinical trial of the gamma-secretase inhibitor Semagacestat resulted in Eli Lilly reporting a worsening of patient outcomes compared to those treated with placebo and that the treatment was associated with an increased risk of skin cancer.[199] However, whether these negative effects are due to loss of gamma secretase activity on other substrates is unknown. In addition, the first generation of anti-aggregation treatments for Alzheimer's disease have been unsuccessful. Tramiprosate, which preferentially binds soluble Aβ and maintains it in a soluble form did not show clinical efficacy in a phase III study.[200] Moreover, the highest concentrations used in a phase II clinical trial of another anti-aggregation drug, Scyllo-inositol, which is thought to bind to Aβ and inhibit its aggregation[201] have been stopped because of high rates of serious adverse events (including nine deaths) among patients;[202] the reason for these negative outcomes is unknown.

Most cases of Alzheimer's disease are sporadic in nature and occur in patients who do not carry mutations that increase the production of $A\beta_{1-42}$. Importantly, recent evidence suggests that it is the clearance of Aβ peptide that is impaired in sporadic forms of Alzheimer's disease rather than altered production levels.[203] The elegant study of Mawuenyega and colleagues shows that average production rates of Aβ do not differ between Alzheimer's disease patients and controls, while the authors estimate that late onset Alzheimer's disease is associated with a 30% decrease in the rate of clearance of both $A\beta_{1-42}$ and $A\beta_{1-40}$.[203] This data is consistent with the idea that in healthy individuals extracellular mechanisms of proteostasis operate to provide efficient clearance of toxic protein species.[8,204] There are a number of potential Alzheimer's disease treatments that target the removal of Aβ via immunotherapy.[192] However, since the role of neuroinflammation in Alzheimer's disease pathology is not clear[205] and given that treatment with an anti-Aβ vaccine (AN-1972)[192] promotes a cytotoxic T cell response and aseptic meningoencephalitis in some patients, the use of immunotherapy in Alzheimer's remains complicated.

An unexplored but logical strategy to reduce Aβ levels in the brain is to exploit the machinery of extracellular proteostasis. However, our current understanding of these mechanisms is largely restricted to knowledge of a small number of extracellular chaperones and cell surface receptors that aid in the specific internalisation of (i) misfolded, aggregating proteins, or (ii) stable complexes formed between extracellular chaperones and misfolded proteins.[9,204] In addition, it is also becoming clear that there are proteolytic systems, such as the plasminogen activator system that will recognise and degrade extracellular protein aggregates.[206] Genome wide association studies of Alzheimer's disease support a role for dysfunctions of extracellular clearance mechanisms in Aβ accumulation; these studies have strongly linked ApoE[159] and clusterin[99] to Alzheimer's disease, in addition to a less striking linkage with α_2M.[145] All three of these proteins can act as extracellular chaperones (see above) and have been shown to bind to Aβ[159,207–208] and to promote its clearance from the extracellular space in cell culture systems.[137,209-210] Relative to control CSF, Alzheimer's disease patient CSF is relatively inefficient at promoting Aβ removal in cell culture studies[211] but this ability can be increased by adding physiologically relevant concentrations of clusterin, haptoglobin and α_2M.[211] A variety of other studies also implicate the extracellular chaperones in Aβ clearance. Aβ in complex with clusterin interacts with the cell surface receptor megalin on mouse teratocarcinoma F9 cells and promotes internalisation and subsequent degradation of Aβ.[209] In addition, ApoE – Aβ complexes are internalised in smooth muscle primary cultures by receptor-mediated endocytosis in a low-density lipoprotein receptor dependant manner.[210] Moreover, α_2M-Aβ complexes are internalised via LRP mediated endocytosis and are subsequently degraded.[137] Importantly, clusterin, α_2M and ApoE have been shown to promote clearance of Aβ *in vivo*.[96,162,212] The removal of radiolabelled Aβ from mouse brain is significantly inhibited by treatment with antibodies against LRP-1 and α_2M.[212] In addition, the rate of $A\beta_{1-42}$ clearance from the mouse brain across the BBB into plasma is increased by more than 80% when it is in complex with clusterin and this is significantly inhibited by anti-megalin antibodies.[96] In the case of ApoE, the clearance of Aβ was isoform dependant with the ApoE ε4 isoform

being the least efficient.[162] Interestingly, genome wide association studies of Alzheimer's disease have also identified a growing list of other genes whose function has been linked with endocytosis (including BIN1, ABCA7, CR1, CD2AP and PICALM).[213] Collectively, these data suggest that extracellular chaperones and endocytosis mechanisms are important for the control of Aβ turnover and that perturbations in the function or concentration of these in humans is likely to be detrimental. Moreover, given that the vast majority of Alzheimer's disease cases are sporadic and are likely to result from defects in clearance rather than the production of Aβ, future therapies for Alzheimer's disease should explore the specific targeting of extracellular clearance mechanisms.

It is likely that clearance mechanisms are also important in other amyloidoses. The build up of β_2-microglobulin in dialysis related amyloidosis is a consequence of its defective clearance.[214] Renal damage can lead to a lowering of the glomerular filtration rate and subsequent increase in plasma β_2-microglobulin concentration. While the rate of synthesis of β2-microglobulin does not vary between haemodialysis patients and controls, the clearance rates are ~20 fold lower in the former.[214] In the case of familial amyloid polyneuropathy (FAP), although there is no published direct measurement comparing transthyretin clearance in FAP patients and controls, it is known that in healthy individuals the amyloidogenic variant of transthyretin ([MET30]TTR) is typically cleared faster than non-amyloidogenic variants.[215] This is consistent with the presence of a system that recognises and rapidly removes non-native protein. The fact that this variant accumulates with age in FAP patients may reflect a gradual breakdown in quality control mechanisms. In some cases the overproduction of a protein, such as immunoglobulin light chain or serum amyloid A protein (SAA) in AL amyloidosis and AA amyloidosis, respectively, is clearly responsible for large scale amyloid deposition. However, amyloid deposits are not static entities or a finite endpoint and can grow or diminish in size if the concentration of the precursor is maintained at a high or low level, respectively (Figure 2).[216] AA amyloid deposits are reported to regress and patient survival is increased in cases where SAA levels remain low.[216] Similarly, although amyloid burden was not measured, reduction of β_2-microglobulin concentration in dialysis related amyloidosis patients improved joint pain, stiffness and ability to perform daily activities.[217] Moreover, reducing the soluble immunoglobulin light chain concentration in AL amyloidosis patients caused reduction of amyloid deposits and improved prognosis in most but not all patients.[218] Therefore, even in cases of vast protein overproduction, therapeutic strategies that increase clearance and help maintain the steady state concentrations of proteins at sub-pathological levels are likely to be of benefit.

A new frontier in amyloid disease therapies?

Current therapies for amyloidoses, are largely limited to treatments to ameliorate the symptoms of disease rather than directly address the underlying causes. Pre-emptive treatments to avoid the onset of disease are so far yet to be realised. Progress in either of these directions will be reliant upon advances in understanding of the cellular and molecular processes that underpin disease onset and progression. Alzheimer's disease is

the single most prevalent and costly amyloid disease impacting upon modern society, and yet even here our level of understanding of the causes is limited. Some of the initial therapeutic strategies have focused on attempting to suppress the production and/or aggregation of $A\beta_{1-42}$, however it has only very recently become apparent that (i) the level of $A\beta_{1-42}$ production is the same in Alzheimer's disease patients and healthy individuals, and (ii) attempts to suppress Aβ production below "normal" levels can have serious side effects (see above). A more "holistic" examination of the systems impacting upon the dynamic levels of disease-relevant proteins (see Figures 1 & 2) is likely to improve the chances of identifying the most appropriate therapeutic targets. In the case of Alzheimer's disease and the other serious diseases associated with inappropriate aggregation and deposition of proteins in the extracellular space (Table 1), the largest gap in knowledge relates to clearance mechanisms, specifically the elements comprising these systems and how they function together to avoid the potentially hazardous expansion of individual protein pools beyond normal safe levels. The available evidence strongly suggests that extracellular chaperones play a pivotal role in extracellular proteostasis, and that a much better understanding of their interactions with both misfolded proteins and cell surface receptors is likely to provide the building blocks critical to design new and effective therapies for many of the most serious amyloidoses.

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Figure Legends

Figure 1. Proposed roles for extracellular chaperones in the maintenance of extracellular proteostasis. Under normal physiological conditions (A) scavenger receptors may directly bind misfolded proteins locally. (B) Circulating extracellular chaperones target and bind to misfolded proteins, maintaining their solubility and facilitating their transport to scavenger receptors. (C) When extracellular proteostasis is disrupted, insoluble protein aggregates can form giving rise to activated proteases (e.g. plasmin). Extracellular chaperones interact with the proteolytic fragments and facilitate their transport to scavenger receptors. In all cases, delivery to scavenger receptors results in the intracellular transport of misfolded proteins to lysosomes for degradation. Reproduced with permission from [9].

Figure 2. Defects in extracellular protein homeostasis results in protein accumulation. (A) Under normal conditions proteins are maintained at a concentration at which is required for their function. The mechanisms of production and clearance are the main proteostasis events that control this protein pool. Proteostasis machinery must be plastic in order to maintain protein concentration during fluctuations in production or clearance. (B)(i) If protein production increases (large black arrow) in the absence of an increase in clearance, or (ii) protein clearance is defective (thin black arrow) this may cause an increase in protein concentration and promote the deposition of the specific protein as amyloid.