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Structure and Function of Stefin B Oligomers – Important Role in Amyloidogenesis

Ajda Taler-Verčič, Mira Polajnar and Eva Žerovnik

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1. Introduction

Many functional proteins act as oligomers. Oligomerization is a well-controlled and regulated process. Those proteins cannot adopt their physiological functions without oligomerization, while the protein misfolding and subsequent unphysiological oligomerization influence the primary protein functions and produce a "gain in toxic function" as the prefibrillar oligomers are toxic for the cells. Misfolding, oligomerization and aggregation are the reasons for the so called conformational diseases. Several of them are neurodegenerative, but some also affect other vital organs. The accumulation of intracellular protein aggregates (various inclusions) and of extracellular protein deposits cause severe cellular degeneration, such as neurodegeneration of affected neurons. Different proteins form rather similar but not identical fibrillar structures, all showing cross-β-structure, where continuous β-sheets run perpendicular to the long axis of the fibrils.

Neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease, prion diseases and many others have in common protein aggregation to amyloid fibrils. Type II diabetes is also an amyloid disease although it is not neurodegenerative. Nowadays it is believed that ordered prefibrillar oligomers or protofibrils may be responsible for cell death and that mature fibrils may even be neuroprotective. Most amyloid prone proteins form different oligomers in the lag phase of the amyloid fibril formation. Conformational diseases (Table 1) are difficult to diagnose in the early stage, because they are usually a-symptomatic during their development. Even when they can be diagnosed in the early stage there is an ethical reason for avoiding diagnosis (i.e. to hesitate letting know the patient or the relatives), because there are still no therapies which would slow down or even stop the progression of this type of diseases. Many different proteins are being studied in order to understand the common molecular mechanism of the conformational disease and to develop

appropriate treatments. Studies use *in vitro* (different spectroscopic methods), *ex vivo* (different cell cultures) and *in vivo* (mouse models) systems to clarify the changes of the protein conformation and the down-stream effects during the whole process.

Conformational diseases (organ/systemic))	Protein
Alzheimer's disease, Down's syndrome (Trisomy 21), Hereditary cerebral angiopathy (Dutch)	Amyloid precursor protein (Aβ 1-42)
Kuru, Gerstmann-Straussler-Scheinker Syndrome (GSS), Creutzfeld-Jacob disease, Scrapie (sheep), Bovine spongiform encephalopathy ("mad cow")	Prion protein
Type II diabetes mellitus (adult onset)	Islet amyloid polypeptide (amylin)
Dialysis-associated amyloidosis	β_2-microglobulin
Senile cardiac amyloidosis	Atrial natriuretic factor
Familial amyloid polyneuropathy	Transthyretin
Reactive amyloidosis familial Mediterranean fever	Serum amyloid A
Familial amyloid polyneuropathy (Finnish)	Gelsolin
Macroglobulinemia	Gamma-1 heavy chain
Primary systemic amyloidosis	Ig-lambda, Ig-kappa
Familial polyneuropathy – Iowa (Irish)	Apolipoprotein A1
Hereditary cerebral myopathy – Iceland	Cystatin C
Nonneuropathic hereditary amyloid with renal disease	Fibrinogen alpha, Lysozyme
Familial British dementia	FBDP
Familial Danish dementia	FDDP
Diffuse lewy body disease, Parkinson's disease	α-synuclein
Fronto-temporal dementia	Tau
Amyotropic lateral sclerosis	Superoxide dismutase-1
Triplet repeat diseases: (Huntinghton's, Spinocerebellar ataxias	Polyglutamine tracts (Huntingtin)
Spinal and bulbar muscular atropy	Androgen receptor
Spinocerebellar ataxias	Ataxins
Spinocerebellar ataxia 17	TATA box-binding protein

Table 1. Diseases of protein misfolding: amyloidoses and non-amyloidoses (reviewed in [1])

Both the amyloid forming proteins involved in certain conformational disease and model proteins are used (Table 2). Stefin B is a model protein for studying amyloid fibril formation.

SH3 domain p 85 phosphatidylinositol 3-kinase	Fibronectin type III phosphoglycenite linase acylphosphatase
HypF N-terminal domain (*E. coli*)	Amphoterin (human)
Apomyoglobin (equine)	Apocytochrome c
Endostatin (human)	Met aminopeptidase
Stefin B (human)	ADA2H
Fibroblast growth factor (*N. viridescens*)	Apolipoprotein CII
VI domain (murine)	B1 domain of IgG binding protein
Curlin CgsA subunit	Monellin
Serpins	

Table 2. Nondisease related amyloid forming proteins/peptides – model proteins (reviewed in [1]) serpins leads to diseases when there are mutations inducing aggregation or change of conformation (e.g. see serpinopathies and the Conformational dementias by *David A. Lomas and Robin W. Carrell* [2].

2. Human stefin B

Stefins are endogenous cysteine protease inhibitors [3], which are ubiquitously expressed in human tissues [4]. They are specific for the papain-family of cysteine proteases and classified as the IH clan in the MEROPS scheme [5]. They are mainly intracellular inhibitors, although have also been found outside the cell in body fluids [6]. They do not have a signal peptide, and they bear a cystatin motif QXVXG, which is the main site involved in binding to target enzymes.

Human stefin B (also termed cystatin B) possesses 98 amino acid residues (Mr= 11 kDa) and no carbohydrate groups or disulphide bridges [7], although it contains one free cysteine [8]. It is an intracellular protein, which tightly and reversibly binds to papain-like cysteine proteases. We will simply call it stefin B from now on.

Stefin B main function is protection against inappropriate proteolysis of lysosomal cysteine proteases [9]. It is an inhibitor of cathepsins B, L, H and S [7, 10, 11]. However, it exerts some additional functions. It was found to interact with five known non-protease proteins (neuro-filament light chain (NFL), brain β-spectrin, RACK-1, human myotubularin related protein 8 (Mtrp) and human T-cell activation protein (Tcrp)) [12]. NFL and β-spectrin are specific to the nervous system. Stefin B (cystatin B) knock-out mice show neurological disorder (loss of the cerebellar granule cells, because of apoptotic bodies, chromatin condensation and some other changes). This suggests that stefin B has an essential anti apoptotic role in the cerebellum [12].

Stefin B is overexpressed in patients with hepatocellular carcinoma and is therefore in combination with some other proteins used as a marker for this disease (it is elevated in the

serum already at the early stage of the disease development and therefore easy to detect) [13]. In different types of cancer (human colorectal cancer [14], gastric cancer [15], esophageal carcinoma [16], prostatic adenocarcinoma [17], bladder cancer [18]…) both lower expression level and higher activity due to higher expression level have been detected.

Stefin B is localized both in the cytosol and in the nucleus [19, 20]. It is expressed in neurons and glial cells in the brain, but with slightly different localizations; in neurons it localizes to the nucleus while in astrocytes it is localized in the nucleus and in the cytoplasm [21]. It inhibits cathepsin L in the nucleus, whose substrates are transcription factors. Interaction with nucleosome – histones H2A.Z, H2B and H3, and cathepsin L in the nucleus has also been reported [19]. Therefore, it likely regulates transcription. Furthermore, stefin B was reported to regulate cell cycle progression into the S phase – entry into the S phase is delayed [19]. Increased expression of stefin B in the nucleus of T98G astrocytoma cells delays caspase-3 and caspase-7 activation and this delay is independent of cathepsin inhibition [22].

Stefin B plays an important role in the immune system. It upregulates the release of nitric oxide from interferon-γ-activated macrophages [23]. The protein is involved in innate immune response to bacterial challenge of the leech *Theromyzon tessulatum* [24]. It has an essential role in protection of the central nervous system from apoptosis [25] and from oxidative stress [26]. Increased level of stefin B was found in the senile plaques of Alzheimer's and Parkinson's diseases and in samples of patients suffering from senile dementia [27].

Stefin B functions are summarized in Table 3.

Loss of functions because of alterations in the cystatin B gene (dodecameric repeat expansions in the promoter region or point mutations in the coding gene) is the cause of progressive myoclonus epilepsy of type 1 also known as Unverricht-Lundborg disease (EPM1) [25, 28]. EPM1 mutans are polymeric and aggregate prone *in vivo* [29]. *In vitro* and *ex vivo* G4R, R68X, [30, 31], G50E and Q71P [32, 33] have been studied. Only G4R mutant folds like the wild type protein [31], all others lack tertiary structure and are partially unfolded [31, 33]. G4R and R68X also form amyloid fibrils [31]. Both G50E and Q71P mutants are more susceptible to cleavages by proteases, because of the partially unfolded structure [33], which likely contributes to loss of function in cells. Stefin B deficiency triggers neurodegeneration by impaired redox homeostasis [26]. G4R and R68X do not have any inhibitory activity, while G50E and Q71P are much less active than the wild type protein [32]. All three missense mutants, except for G4R form rather large but diffuse aggregates in cells when over-expressed, while G4R forms small aggregates similar to those of the wild type protein [32].

Cystatin C, a secreted and extracellular protein, is another cysteine protease inhibitor and mutations in its gene are the cause of hereditary cerebral amyloid angiopathy [34]. Human cystatin C is a risk factor for late onset Alzheimer's disease. The protein co-deposits with amyloid β peptide (Aβ peptide) amyloid plaques in patients with Alzheimer's disease [35]. Moreover, cystatin C binds to both the whole amyloid precursor protein (APP) and to Aβ peptide and it inhibits Aβ peptide amyloid fibril formation *in vitro* [36].

Stefin B functions

inhibition of cathepsins B, L, H and S [7, 10, 11];
interaction with five non protease proteins: neurofilament light chain (NFL), brain β-spectrin, RACK-1, human myotubularin related protein 8 (Mtrp) and human T-cell activation protein (Tcrp) (NFL and β-spectrin are specific to the nervous system) – essential anti apoptotic role in the cerebellum [12];
involved in immune-response to bacterial challenge of the leech *Theromyzon tessulatum* [24];
increased level in the senile plaques of Alzheimer's and Parkinson's and of patients suffering from senile dementia [27];
essential role in some of the neurons in the central nervous system, protecting the cells against apoptosis [25];
protects thymocytes against cell death [37];
cell specific expression [21];
inhibits Aβ peptide amyloid fibril formation in oligomer specific manner [38];
positive/negative progression of cancer [13-18];
upregulation of nitric oxide release from interferon-γ-activated macrophages [23];
found mainly in the nucleus of proliferating cells and both in the nucleus and cytoplasm of differentiated cells;
regulates cell cycle [19];
increased expression in the nucleus delays caspase activation [22];
protecting cells from oxidative stress [26].

Table 3. Stefin B has many different functions.

3. Stefin B oligomers *in vitro* and in cells

Stefin B can adopt different oligomeric states *in vitro* and in cells. On the size-exclusion chromatography (SEC) the wild type protein elutes as a set of well-defined oligomers apart from monomers, dimers, tetramers and even higher oligomers (Figure 1) [39]. Y31 isoform is predominantly dimeric [31], while Y31 P79S mutant is tetrameric [40]. All oligomers can be isolated as separate peaks by SEC and stay stable for weeks at pH 7.0 and 4 °C [38].

SEC results have been confirmed up to decamers by electrospray-ionization mass spectrometry (ESI-MS) [41]. In cells stefin B is present both in monomeric and oligomeric forms. Oligomers size ranges between 10 and 250 kDa. The higher oligomeric species are resistant to 1% SDS and 8 M urea and partially resistant to reducing agents (DTT treatment). The low molecular species comprise monomers, dimers, trimers and pentamers. Stefin B polymers *in vivo* seem to grow by monomer addition and not by domain-swapped dimer addition. The protein binds to many different proteins of various sizes [29]. Already the endogenous stefin B forms small punctate aggregates in cells and after overexpression the aggregates amount increases [30].

Oligomers have been observed for many other proteins, especially the amyloidogenic ones. Oligomeric species appear in the lag phase of the reaction and are usually more stable under
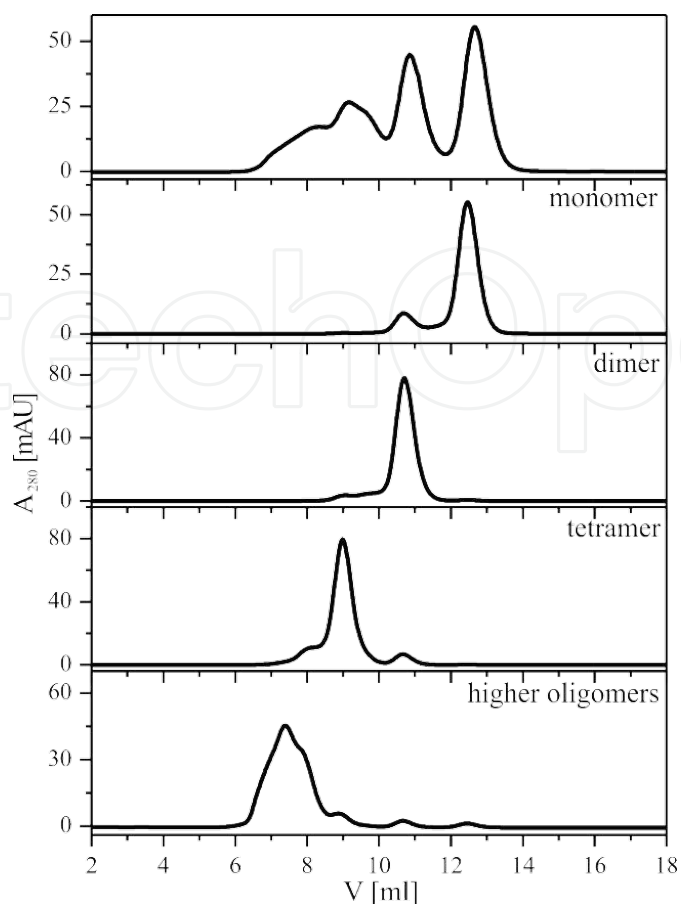
Figure 1. Elution profile of stefin B wild type protein (S3E31 form) at pH 7.0 from SEC. The whole sample (top trace) and separated oligomers as indicated, are shown.

non-amyloidogenic conditions. Size and morphology of those oligomers can be determined by SEC, mass spectrometry, dynamic light scattering and analytical ultracentrifugation. Some examples are given below.

Serpins are serine protease inhibitors also used as a model for studies of amyloid fibril formation. They have key regulatory functions in the inflammatory, complement, coagulation and fibrinolytic cascades [2]. The mechanism of their fibril formation is known in crystallographic details [42, 43]. They form amyloid fibrils through domain-swapping [44]. Serpins preferably oligomerize than interact with other proteins [45]. They form both smaller oligomers and condensed longer polymers [46]. Monomers of some serpins are meta-stable with the rate limiting step representing the transition to the dimer. Once dimers are formed, they can connect to each other to form tetramers or recruit monomers to form trimers and also much longer oligomers [47].

Aβ peptide forms a set of oligomers from monomer to hexadecamer; all even and odd numbered oligomers have been detected by ESI MS [48]. Also for many other amylod forming proteins the whole set of oligomeric species has been observed (insulin [49], β_2-microglobulin [50, 51], SH3 domain [52]…).

4. Inhibitory activity of stefin B oligomers

For a long time it has been thought that monomer is the only active form of stefin B. All other oligomeric forms would serve as a reservoir of monomers or possess additional functions. Now we have shown that all types of oligomers are active against cysteine protease papain (Figure 2). Dimers which are presumably domain-swapped (discussed in the structural characterization part) show the lowest activity in the enzyme to inhibitor 1:2 ratio. They, as well other oligomers – tetramers and higher, are fully active in the enzyme to inhibitor 1:4 ratio (Fig. 2). SEC-isolated oligomers are all >95% pure and the amount of monomer present in other oligomers samples has been tested to show that the inhibitory effect is not due to monomer contamination. Higher oligomers are a mixture of various oligomers, starting from hexamers. The highest peak represents octamers, which are the prevalent species and therefore we propose that the average size of the higher oligomer is an octamer (this was used for determining the molar ratio). The sample of the higher oligomers contains a small amount of all other oligomers and we suspect that some inhibitory effect could be due to this contamination, but possibly not the whole effect. The results are in agreement with the detection of different oligomers in cells [29] and explain that all detected species could retain inhibitory function.
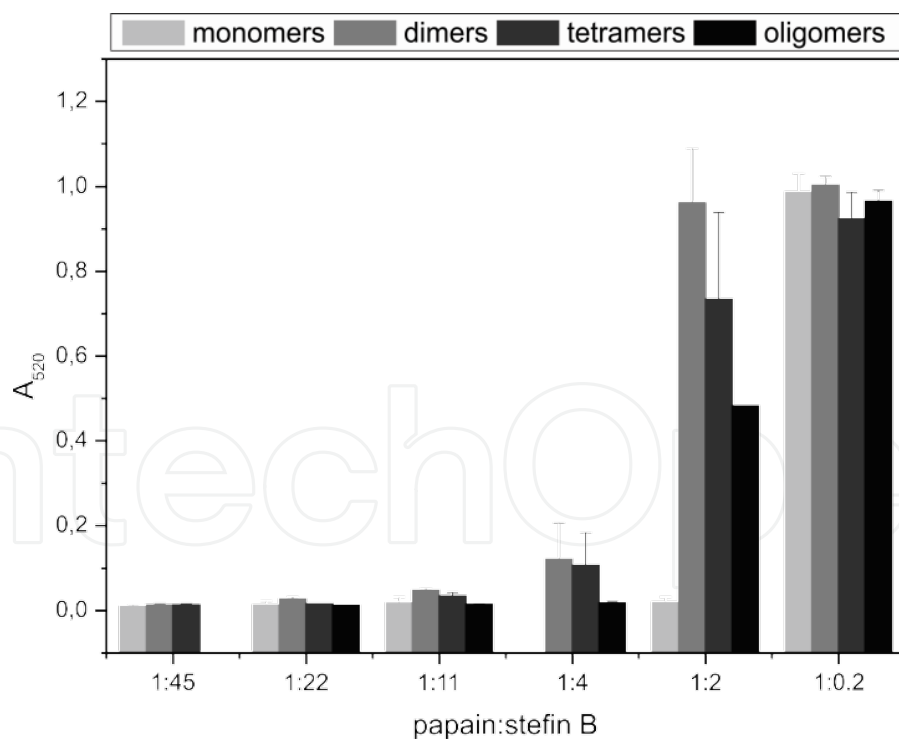

Figure 2. Different stefin B oligomers all exert inhibitory activity against the cysteine protease papain. To evaluate the inhibitory activity of stefin B, BANA test was performed [53]. Stefin B monomers, dimers, tetramers and oligomers were prepared in eight different molar ratios [E]:[I]– 1:45, 1:22, 1:11, 1:4, 1:2, 1:0.2. Higher absorbance means lower inhibitory activity. The average oligomeric state (the highest peak from SEC) in the sample of higher oligomers are the octamers.

5. Structural characterization of different oligomeric states of stefin B

Crystal or solution structure of stefin B monomer and oligomers have been so far characterized from monomer in complex with the target enzyme [54], dimer under amyloid forming conditions [55], tetramer [40] to model of amyloid fibrils [56]. Domain-swapped dimer of stefin A has also been determined by heteronuclear NMR [57].

Stefin B was first crystallized in complex with the cysteine protease papain (Figure 3A) [54]. The structure of the monomeric stefin B consists of a five stranded β-sheet wrapped around a five turn α-helix and with an additional carboxy terminal strand running along the convex side of the β-sheet. This type of the tertiary structure is conserved throughout the cystatin family (Fig. 3A)

High resolution structure of stefin B dimer was determined by heteronuclear NMR [55]. Furthermore, changes within the dimer under amyloid forming conditions were observed and flexible residues were defined. Even under amyloid forming conditions the structure remains largely folded, the main differences are in the flexible loops regions. Prolines on positions 74 and 79 play an important role in the orientations of the loop between strands 4 and 5 (the same loop is involved in "hand-shaking" in the tetramer formation). Four different dimer states have been observed in solution.

When the proline at position 79 was mutated into serine, the tetrameric form of the protein became favourable [40]. This tetramer has been crystallized and its structure determined (Figure 3B) [40]. The tetramer consists of two domain-swapped dimers connected with the so-called "hand-shaking" mechanism to each other. Hand-shaking occurs along with *trans* to *cis* isomerization of the proline at position 74. This proline is conserved throughout the cystatin family. Both domains preserve the fold of the monomer (the same as observed in the complex with papain); each domain is composed of the N-terminal part from one chain (strand 1, the α-helix and strand 2) and C-terminal part from the other chain (strands 3, 4 and 5), between both domains there is a linker region (two peptides, belonging each to one chain).

The structured core of human stefin B amyloid fibrils has been determined (Figure 3C) [56]. Based on the H/D exchange rates the structure could be divided onto protected region (inside the fibril core) and unprotected region (striking out of the fibril). Strands 2, 3, 4 and 5 are protected, while strand 1, α-helix and loops between strands 3 and 4 and 4 and 5 are unprotected. The loop between strands 2 and 3 is the same as the one involved in the domain-swapping mechanism. The fibril core would therefore be made of domain-swapped dimers but with the loop between strands 4 and 5 in position of a tetramer.

6. Initial oligomers on the way to fibril formation

That stefin B forms amyloid fibrils *in vitro* under relatively mild conditions was first shown in 2002 [58] and various solution conditions were probed, fibrils and protofibrils were imaged by AFM and TEM [59]. The fibril formation of stefin B was compared to that of stefin A, a much
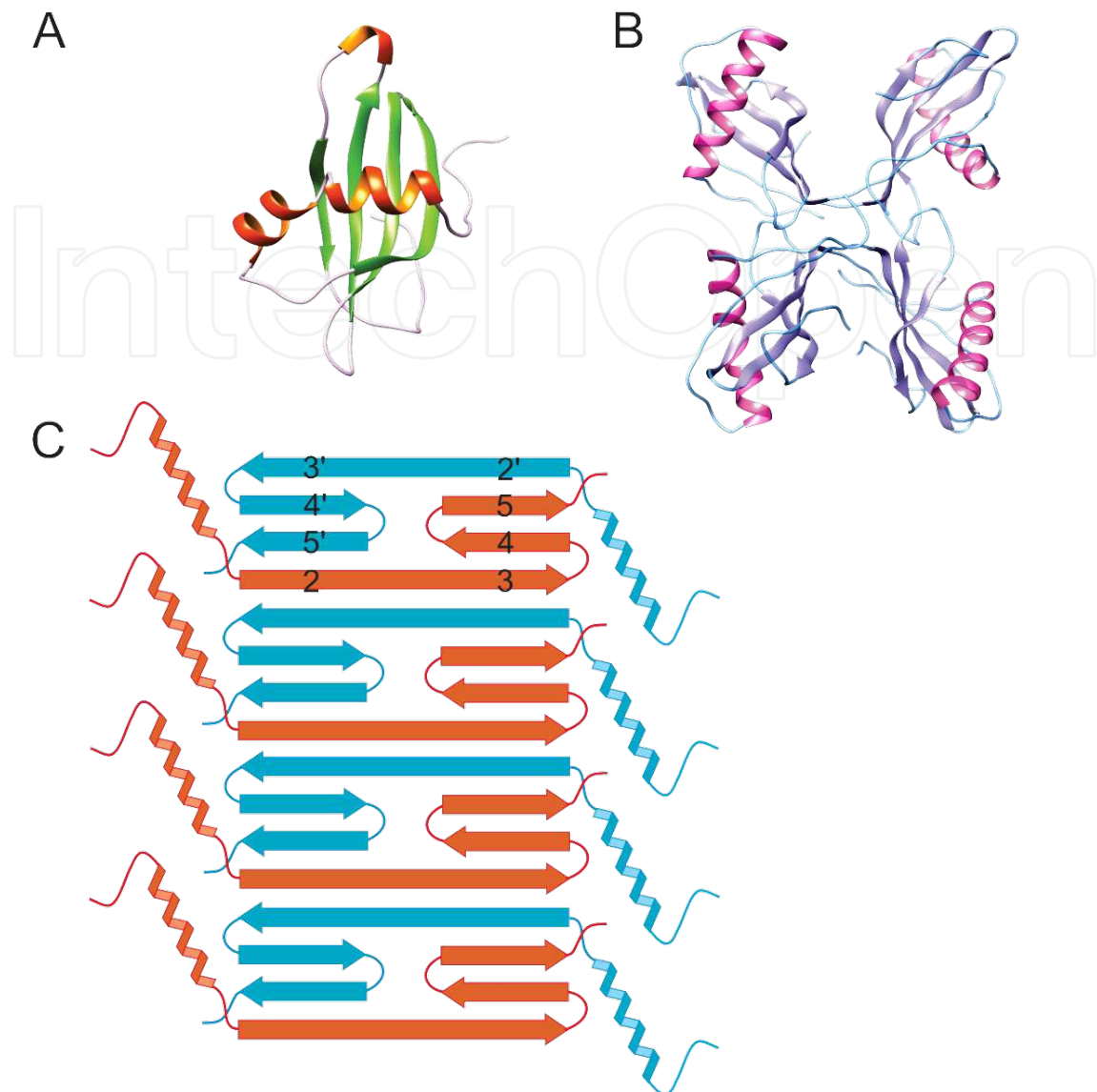
Figure 3. Stefin B structure. (A) stefin B monomer (PDB 1stf) [54], (B) stefin B tetramer (PDB 2oct) [40], (C) secondary structure representation of stefin B fibril [56]

more stable homologue [60], to proline mutants [61], EPM1 mutants [62] and to chimeras between stefins A and B [63].

A model for the mechanism of stefin B amyloid fibril formation has been proposed based on temperature and concentration dependence of the kinetics [64]. Following changes during the lag phase of the reaction by SEC and ESI MS we were able to improve the model of amyloid fibril formation (Figure 4), further explaining the role of various off-pathway oligomers [41]. Dimers seem to be the building block from which fibril formation starts and the fibrils grow [56]. When the process was started from any kind of oligomers, these transformed into dimers [41]. NMR studies have shown that there are actually four types of dimers observed in the lag phase under amyloid forming conditions [55].
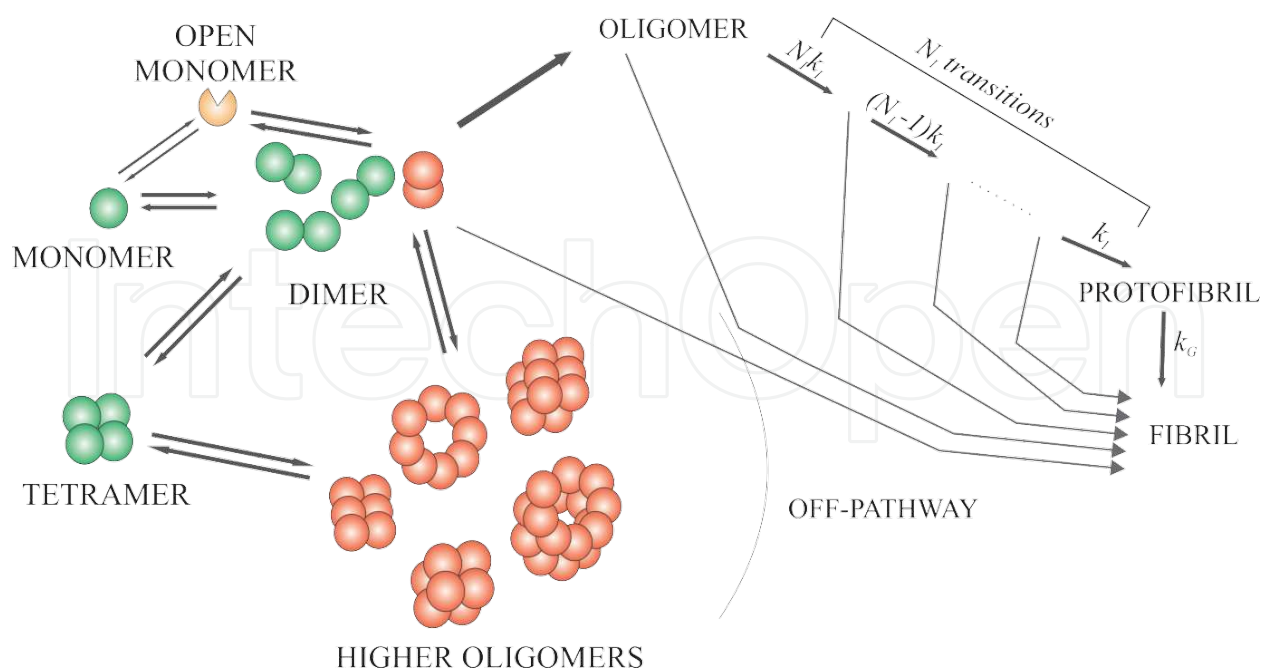
Figure 4. The proposed mechanism for amyloid fibril formation by stefin B [41]. Non-toxic species are coloured green, toxic are coloured red and potentially toxic are coloured orange.

7. Oligomeric state related toxicity

Stefin B higher oligomers are equally toxic to cells as the molten globule state (mutant P74S Y31) [39]. Higher oligomers still have native like CD spectra and increased ANS binding, which would mean that they are properly folded. The toxicity seems to correlate with the ANS binding (ANS binding is indicator of exposed hydrophobic patches in the protein structure) [39]. MTS test has shown that isolated dimers and tetramers are not toxic (Figure 5A), while higher oligomers show some toxicity to neuroblastoma cells (SH-SY5Y). In accordance, low ordered oligomers, monomers, dimers and tetramers do not increase caspase-3-like activity (Figure 5B) [39]. All those results correlate with insertion into lipid membranes, where toxic species insert more effectively than not toxic. Monomers do not get internalized into the cytoplasm when added to the cell medium, while higher oligomers do [39].

Confirming the above results, in another study [65] it was demonstrated that a mutated P74S Y31 form of stefin B, which is molten globule at neutral pH and prefibrillar aggregates at pH 3, which are also in the molten globule conformation, are most toxic.

A growing body of evidence shows that the soluble oligomers formed during amyloid fibril formation exert toxicity and likely cause neurodegeneration [66]. Small soluble oligomers are the cause of synaptic disfunction, whereas large and insoluble aggregates are likely the reservoir of those toxic species [66]. In the case of Aβ peptide a significant correlation has been found between the levels of soluble oligomers and the degree of synaptic alteration, neurode-generation and cognitive decline, while the correlation between insoluble deposits and those

symptoms has not been demonstrated [67]. Non fibrilar dimers or oligomers of α-synuclein could play a major role in Parkinson's disease progression [68]. In accordance with a generic process of amyloid type of protein aggregation, not only oligomers of amyloidogenic proteins related to amyloid diseases exert toxicity, also HypF-N from *E. coli*, SH3 domain of PI3 kinase and some other proteins form oligomers that are toxic to fibroblasts and neurons, whereas amyloid fibrils of the same proteins show only low toxicity [69, 70].

Conformation dependent antibodies distinguish between soluble oligomers [71-73] and amyloid fibrils [74, 75]. Many of those antibodies recognize the generic epitopes regardless of the amino acid sequence and this suggests that different proteins form structurally similar oligomers and fibrils. It was shown that the antibody recognizing Aβ peptide fibrils, recognize also fibrils of transthyretin, islet amyloid polypeptide, β_2-microglobulin and polyglutamine and that the same antibody does not recognize soluble oligomers of those proteins nor the native forms [75]. Antibodies recognizing soluble oligomers also inhibit their toxicity which suggests that soluble oligomers do not share only structural properties but also the mechanism of toxicity [72].
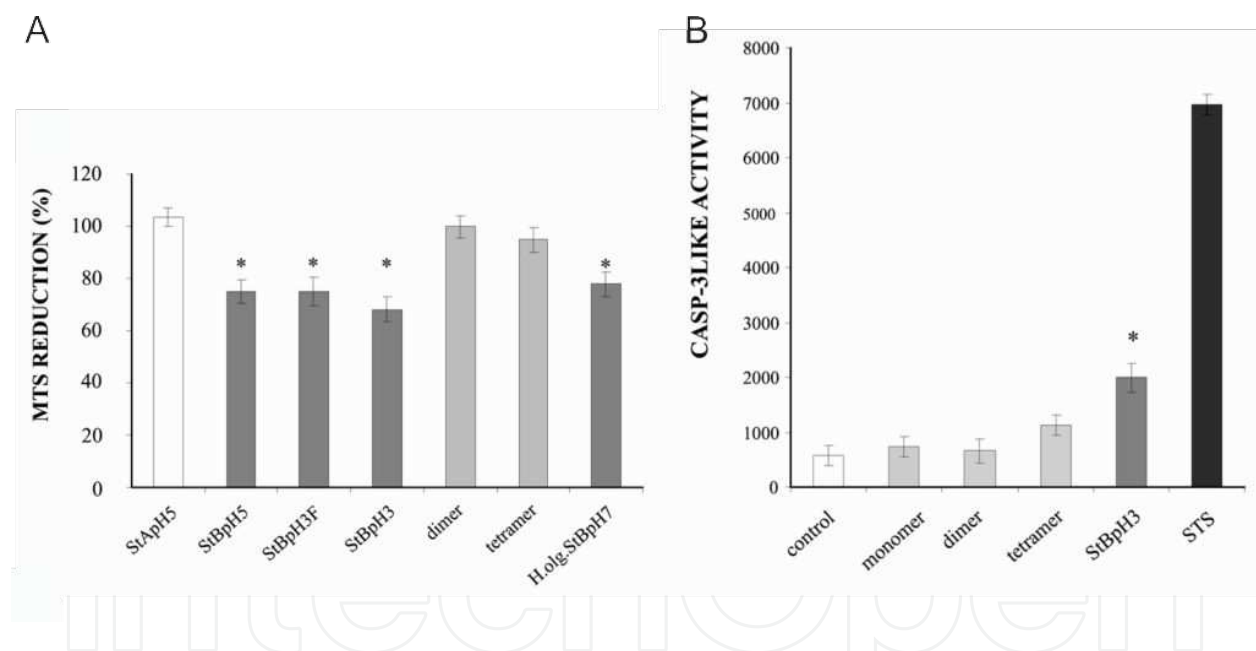

Figure 5. Viability of cells exposed to prefibrillar aggregates and oligomers of stefin B. (A) MTS test-viability of the SHSY5Y cells was measured after exposure for 16 hours to serum free medium, which contained prefibrillar oligomers of stefin B – StBpH3, StBpH5, StBpH3F (filtered), and separated dimers, tetramers and higher-order oligomers of stefin B at pH 7 – StBpH7; each at a final concentration of 44 µM. As a control for the effect of a non-toxic protein, cells were exposed for the corresponding length of time to 44 µM of soluble stefin A prepared at pH 5 (StApH5). All the results are relative to the cells alone, with no added substance or protein in the medium. (B) DEVD-ase activity after exposing SHSY5Y cells to prefibrillar aggregates. 200 µl of concentrated StBpH3 aggregates were added to 800 µL of cell medi‐ um to a final concentration of 44 µM (final pH was checked to be neutral). The cells were treated for four hours with 0.5 µM staurosporine (STS). Statistics-as described [39].

Both oligomers and amyloid fibrils are harmful for the cells; oligomers present mostly the toxic effect, while amyloid fibrils present steric obstacles.

• the disruption of the tissue architecture and functions promoted by the invasion of the extracellular space of organ by amyloid [76, 77]
• the destabilization of intracellular and extracellular membranes by oligomers [78, 79]
• the apoptotic cell death and receptor-mediated toxicity triggered by the oligomer interaction with various neuronal receptors [80]
• the oligomer-mediated impairment of the P/Q-type calcium currents [81]
• the impaired maturation of autophagosomes to lysosome mediated by the oligomer accumulation [82]
• the dysfunction of autophagy, a lysosomal pathway for degrading organelles and proteins [83]
• the oxidative damage-induced disruption of the cell viability promoted by the incorporation of redox metals into amyloid fibrils and subsequent generation of reactive oxygen species [84-88]
• the general disorganization of cellular protein homeostasis associated with the exhaustion of the cell defence mechanisms, such as chaperone system [89, 90]
• proteasome inhibition [91]
• the loss of crucial protein functions and/or gain of toxic functions

Table 4. Cellular degeneration after protein oligomerization/deposition (summarized in [92])

8. Oligomeric state related membrane interactions

Amyloid forming proteins interact with biological membranes and even form pores, which are similar to those formed by the pore-forming proteins [1, 93]. Interactions between stefin B and membranes have been extensively studied [65, 94, 95]. Three different proteins have been studied: wt protein, Y31 isoform and G4R mutant (mutant involved in EPM1 disease). Also some comparisons have been made between native and prefibrillar states of the protein (prepared with incubation at pH 3.3 or 4.8) (Figure 6) [94]. Prefibrillar oligomers may be organized in such a way that they are more amphipatic than the native protein and therefore acquire a higher surface-seeking potential. All forms of the protein insert into acidic lipid membranes, cause permeabilization of unilamelar vesicles and destabilize the membranes [94, 95]. Nevertheless there are some significant differences between them concerning pore formation. The mutant G4R does not form pores, but breaks the membrane very fast (in a few minutes), while both the wt protein and Y31 isoform form pores; wt pores are cation selective (and may not be deleterious for the cell) and Y31 pores are anion selective [95]. Wt sample was separated into different oligomers apart from monomers, into dimers, tetramers and higher oligomers. Monomers, dimers and tetramers insert into lipid membranes with approximately the same critical pressure, while the higher oligomers (average size is around octamers) insert more effectively [65].

The exact mechanism of amyloid induced toxicity is yet controversial; nevertheless the pore formation into plasma or mitochondrial membrane is now the leading theory of pathogenesis. Aβ peptide needs anionic phospholipids for binding and insertion into membranes [96-101]. Monomeric form does not interact with membranes. Amylin (islet amyloid polypeptide) readily forms pores into planar lipid bilayer, preferring negatively charged lipids and furthermore the channel activity is inversely proportional to the amount of negative surface charge in the membrane [102]. Native form of prion protein does not form pores, while the mutant prion protein forms irreversible pores in the lipid bilayer. They claim that the pore formation is the main cause of the prion disease [103]. α-synuclein, a natively unfolded protein, also forms pores into lipid membranes but in contrast to others described it is not known how does the oligomeric form increase membrane permeability without forming discrete channels [104]. In the literature we can find contradictory results what effects amyloid proteins exert on membranes. For the same proteins we can find reports that they affect membranes by a non-channel mechanism and other reports that they affect membranes by the formation of ion channels. Two mechanisms of pore formation into membranes by the amyloid forming proteins are summarized in Table 5.
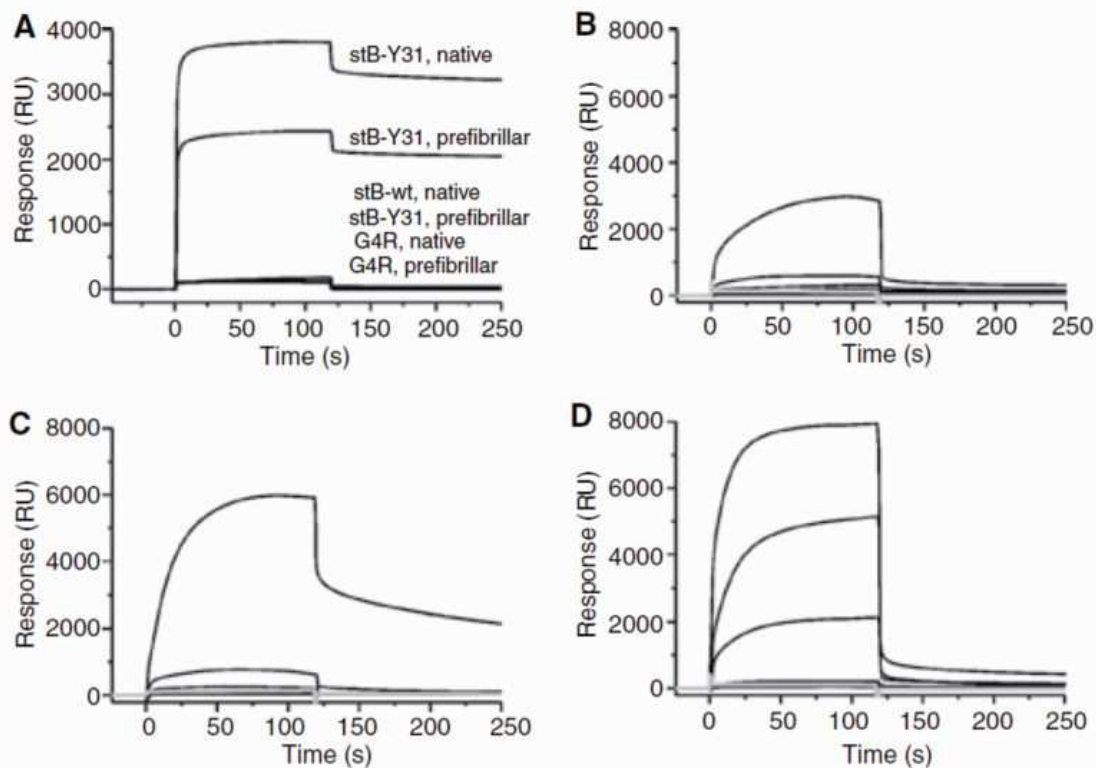

Figure 6. Binding of stefin B variants to liposomes as measured by SPR. (A) Binding to PC LUV. A comparison of binding of 70 µM stefin variants to PC LUV immobilized on the surface of a L1 sensor chip. (B–D) A comparison of the binding of prefibrillar form of stefin variants to PG LUV. The concentration of the protein was 10, 20, 40, 50, 60 and 70 µM (curves from the bottom to the top) in each case. The thick gray line represents binding of native stefin variants at 70µM. (B) StB-wt; (C) StBY31; (D) G4R [95].

Membrane poration followed by nonspecific membrane leakage – increased conductivity of the membranes by a non-channel mechanism	Specific ion transport through ion channel followed by destabilized ionic homeostasis
Aβ40 peptide [105]	Aβ40 peptide [106, 107]
Aβ42 peptide [105]	Aβ42 peptide [108]
α-synuclein [105]	Aβ22-35 peptide [109]
IAPP or amylin [105, 110]	α-synuclein [111]
Polyglutamine [105]	IAPP or amylin [112]
Prion (106-126) H1 [105]	stefin B [95]
SOD1 [113]	

Table 5. The mechanisms underlying globular peptides induced cell dysfunction

9. Copper binding to stefin B and its inhibition of amyloid fibril formation

Divalent metal ions (Cu (II), Zn (II), Fe (II)) are often observed to colocalize with amyloid plaques *in vivo* in much higher concentrations than usually present in the normal environment. This has led to the hypothesis that this metal ions bind to mature fibrils and influence the fibril formation reaction [114].

Indeed it was proven that human stefin B is a copper binding protein [114]. It shows a picomolar affinity to copper at pH 7 and a nanomolar affinity at pH 5. Both the wild type protein and the Y31 isoform bind copper, while the Y31 P79S, which is tetrameric, does not. Monomers and dimers are able to bind copper, while other oligomers are not. Copper binding does not change the conformation of the protein; however it inhibits amyloid fibril formation (Figure 8). Of note it does not prevent aggregation to prefibrillar oligomers, which can be even more toxic to cells. The protein binds two Cu^{2+} ions. Copper binding promotes protein dimerization at neutral pH, while at acidic pH protein undergoes dimerization, already without added copper [114].

Later-on, NMR was used to detect the three possible copper binding sites of stefin B. The first is in the α-helix facing away from the β-sheets together with the loop between strands 4 and 5. The second potential binding site is the C-terminal together with the loop between strands 3 and 4 (this binding site is more likely in the dimeric form of the protein). The third binding site is in the dimer only and is the loop between strands 2 and 3, which is the stretched loop in the domain-swapped dimer together with the few residues at the N-terminal [55].

It was confirmed that copper affects the stefin B fibril formation. In more details, it slows down the elongation phase. It was also shown that the presence of copper destabilizes the protein structure and therefore it was concluded that it preferably binds to the slightly unfolded state of the protein. The final fibril morphology does not differ if copper ions are present or not [55].

Metal ions have different effect on amyloid forming proteins. They can speed up the fibril formation, reduce the lag phase, retard the fibrillization or even stop the process completely [115-117].
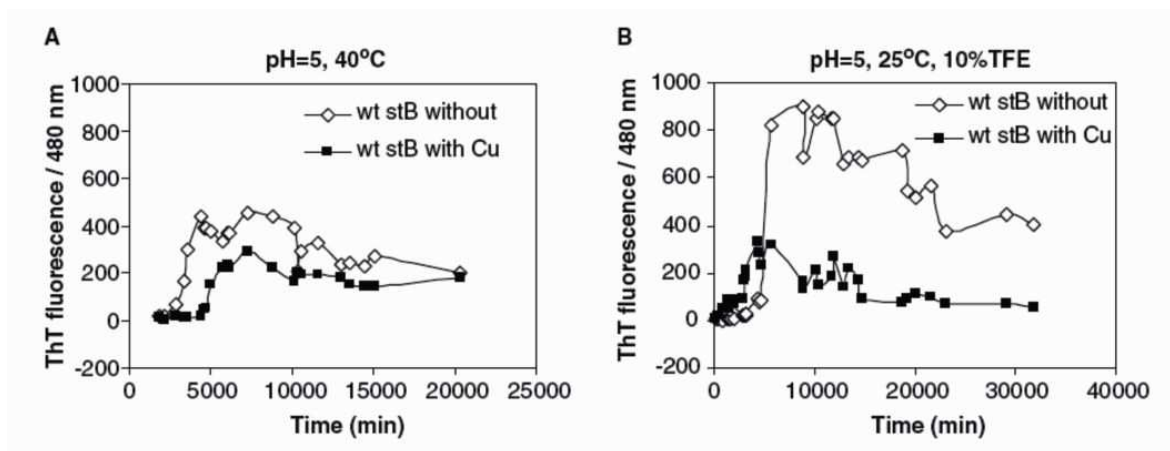
Figure 7. Inhibition of fibrillation of stefin B by Cu^{2+} as probed by ThT fluorescence. Final protein concentration was in all cases 45 µM and final concentration of Cu^{2+} 46 µM, leading to 1 : 1 of protein to Cu^{2+} ratio. (A) Stefin B wildtype (E31 isoform) at pH 5, 40 °C, 0 and 50 µM Cu^{2+} in the buffer. (B) Stefin B wildtype (E31 isoform) at pH 5, 10% TFE, 25 °C, 0 and 50 µM Cu^{2+} in the buffer [114].

Binding of metal ions to prion protein increases proteolysis resistance and structural changes which might play an important role in the conversion process [118].

SOD1 aggregation is enhanced and modulated by Ca^{2+} ions; at physiological pH Ca^{2+}induces conformational changes that increase β-sheet content, they can also divert the aggregation from amyloid fibrils to amorphous aggregates [119]. Cu^{2+} and Zn^{2+} accelerate deposition of Aβ40 peptide and Aβ42 peptide, which results in the amorphous aggregates [120, 121]. Fe^{3+} induces the deposition of fibrillar amyloid plaques of Aβ40 peptide and Aβ42 peptide [120]. Prion protein can directly influence neuronal zinc concentrations [122]. In prion plaques there was found a significant disregulation of copper and manganese, copper was depleted and manganese was enriched [123]. Fe^{3+} and Al^{3+} enhance both formation of mixed oligomers and recruitment of α-synuclein in pre-formed tau oligomers [124]. Copper and selenium inhibit amylin fibril formation, while aluminium and manganese promote it [125]. Copper amylin complex has an anti-aggregating and anti-apoptotic properties, quenching the metal catalysed ROS [126]. Zinc also inhibits amylin fibril formation, furthermore, it favours the formation of amylin hexamers and inhibits the formation of dimers [127].

10. Interaction between oligomers of stefin B and Aβ peptide *in vitro* and in cells-"amateur chaperones"

"Professional chaperones" (heat shock protein family) prevent protein misfolding and subsequent protein aggregation. "Amateur chaperones" bind amyloidogenic proteins and may affect their aggregation process. Both types of chaperones colocalize with pathological lesions of Alzheimer's disease, may be involved in Aβ peptide conformational changes, clearance of Aβ peptide from the brain. Both types of chaperones may be involved in the

aggregation, accumulation, persistence and clearance of Aβ peptide and may therefore serve as a potential targets for medical treatment of those patients [128].

Aβ peptides are produced by proteolytic cleavage of the amyloid precursor protein (APP) by α-secretase, β-secretase and γ-secretase. Cathepsin B, which can be inhibited by stefin B, is likely contributor to β-secretase activity [129]. Cysteine protease inhibitors reduce both Aβ peptide level in the brain and β-secretase activity *in vivo* [129]. Stefin B has an effect on production of Aβ peptides and furthermore it also inhibits Aβ peptide fibril formation [38].

Two isoforms of stefin B (Y31 and wt E31) have been studied. Y31 isoform is predominantly a dimer [31] while the wild type protein exist as mixture of monomers, dimer, tetramers and even higher oligomers [39]. ThT fluorescence and transmission electron microscopy (TEM) have shown that Y31 isoform completely inhibits Aβ peptide fibril formation (Figure 9I). The direct interaction between those two proteins has also been shown by SPR measurements, where concentration dependent interaction has been reported and by ESI MS where the complex between dimer stB and monomer Aβ peptide has been detected (Figure 9II) [38]. Furthermore, isolated oligomers of the wild type protein have been studied and it was shown that only the tetramer inhibits Aβ peptide fibril formation and that the higher oligomers show only a weak inhibition. Stefin B also colocalizes with the Aβ peptide aggregates in cells (shown by confocal microscopy) and with the C-terminal fragment of APP (comprising Aβ peptide sequence) (shown by immunoprecipitation).

Molecule/protein	Type of interaction
stefin B	Inhibits Aβ peptide fibril formation, interaction *in vitro* and in cells [38].
laminin	Inhibits Aβ peptide fibril formation, induce depolymerisation of preformed fibrils [130].
polyphenols	Inhibits Aβ peptide fibril formation [131].
heme	Prevents Aβ peptide aggregation [132].
GroEL	Prevents Aβ peptide aggregation [133].
apolipoprotein E	Slows down the oligomerization of Aβ peptide [134].
myelin basic protein	Inhibitor of Aβ peptide fibrilar assembly [135].
cystatin C	Concentration dependent inhibition of Aβ peptide fibril formation [36].
ferulic acid	Inhibits Aβ peptide oligomer formation from monomers and at the same time accelerates fibril formation from already formed oligomers [136].
S14G humanin	Inhibits aggregation into fibrils and disaggregates preformed fibrils, reduces cytotoxicity effect of Aβ peptide [137].
albumin	Increases the lag phase and decreases the total amount of fibrils [138]
crocetin	Inhibits Aβ peptide fibril formation, destabilizes preformed fibrils, stabilize Aβ peptide oligomers and prevents their conversion into fibril [139].

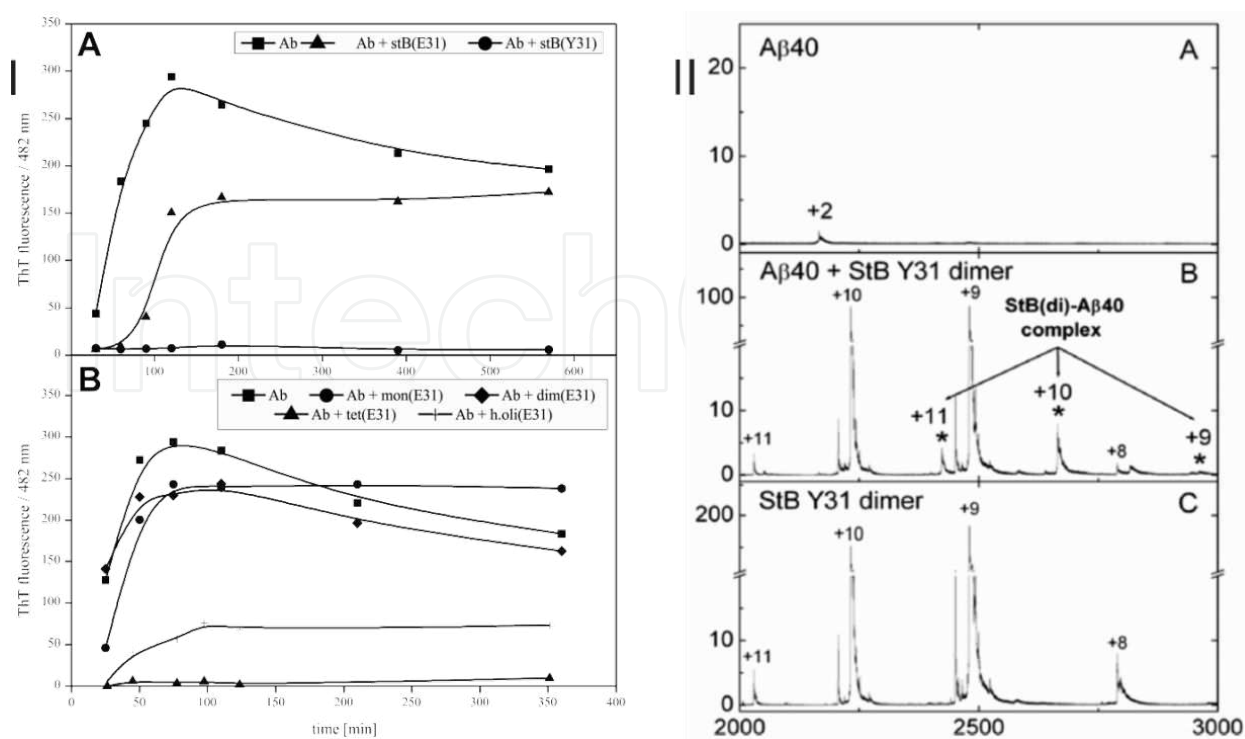**Table 6.** Molecules interacting with Aβ peptide

Figure 8. (I) Inhibition of Aβ peptide fibril formation by stefin B measured by ThT fluorescence and (II) the detection of the complex by ESI MS. (I) The Aβ peptide concentration was 17 µM throughout, pH 7.3, 40 °C. (I, A), Aβ peptide alone, 1:1 molar ratio of Aβ to Y31 stefin B (complete inhibition) and 1:1 molar ratio of Aβ to E31 stefin B. (I, B), Aβ peptide alone, and 1:1 molar ratios to E31 stefin B monomers, dimers, tetramers, and higher oligomers. The protein concentrations of stefin B were 17 µM. (II) Complex detected by ESI-MS. (II, A) ESI-MS spectra of Aβ(1– 40) peptide, (II, C) stefin B dimer (Y31 variant) and (II, B) their mixtures were recorded: (II, A), 2 µM Aβ(1– 40) peptide; (II, B), a mixture of 2 µM Aβ peptide and 2 µM stefin B; and (II, C), 2 µM stefin B. Peaks corresponding to the Aβ peptide-stefin B complex are denoted with an asterisk and numbers above the peaks denote charge state of the ions [38].

In order to show if binding between domain-swapped dimers and Aβ peptide is a generic property, other cystatins have been used. It was shown that only cystatin C dimers inhibit Aβ peptide fibril formation (30% inhibition) while stefin A dimers exhibited no such effect [140].

Several molecules interacting with Aβ peptide have been reported by now (Table 6).

11. Conclusions and perspectives

Stefin B has been so far found as a good model system for studying amyloid fibril formation. It exhibits nearly all features shared with other amyloid forming proteins: it forms mature fibrils under mildly acidic conditions or even at neutral pH at somewhat higher temperature, forms membrane pores and therefore promotes membrane leaking, binds copper ions and its' oligomers are toxic. It is not a model protein only, but also could be termed an "amateur chaperone" affecting Aβ peptide fibril formation both *in vitro* and *in vivo* [38].

We are trying to extend our *in vitro* knowledge to cell cultures to contribute even more to the understanding of conformational disease. It is hoped that new knowledge of protein oligo-

merization and aggregation on the molecular and cellular levels will contribute to the development of new therapeutic strategies for patients with various conformational diseases, including the neurodegenerative ones.

Acknowledgements

Author details

Ajda Taler-Verčič[1,2], Mira Polajnar[1,2] and Eva Žerovnik[1,2*]

*Address all correspondence to: eva.zerovnik@ijs.si

1 Department of Biochemistry, Molecular and Structural Biology, Jožef Stefan Institute, Ljubljana, Slovenia

2 Jožef Stefan International Postgraduate School, Ljubljana, Slovenia

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