Amyloids here, amyloids there...
What’s wrong with them?

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“Would you tell me, please, which way I ought to go from here?”
“That depends a good deal on where you want to get to” said the Cat.
“I don’t much care where —” said Alice. “Then it doesn’t matter which way you go”, said the Cat.
“—so long as I get somewhere”, Alice added as an explanation.

Lewis Carroll
from “Alice’s Adventure in Wonderland”
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ABSTRACT

Amyloid formation is inherent property of proteins which under certain circumstances can become a pathologic feature of a group of diseases called amyloidosis. There are about 30 known human amyloidosis and more than 27 identified proteins involved in these pathologies. Besides these proteins, there are a growing number of proteins non-related to diseases shown to form amyloid-like structures in vitro, which make them excellent tools for studying amyloid formation mechanisms, physicochemical properties of different amyloid species and the nature of their influence on tissues and cells. It is important to understand the mechanisms by which amyloids interact with different types of cells, as the leading hypothesis in amyloid field suggests that amyloids and especially their intermediate states are the main harmful, toxic species causing tissue and cell degeneration.

Using de-novo synthesized protein albebetin as a model of amyloidogenic protein, we demonstrated that it forms amyloid-like structures under physiological conditions (pH 7 and 37°C). During aggregation it forms 2 different types of intermediate oligomers — cross-β sheet containing and lacking β-sheet oligomers. Only the former induces cellular toxicity in a dose dependent manner. Further aggregation leads to the formation of fully mature amyloid-like fibrils, which are not toxic to the cells during studied period of incubation.

Another model protein in our studies was hen egg white lysozyme, which readily forms amyloid under denaturing conditions (pH 2,2 and 57°C). In contrast to albebetin and many other proteins reported in the literature, we showed that both oligomers and mature fibrils from hen lysozyme affect cell viability. Targeting different mechanisms involved in cellular death, we revealed that oligomers induce slow and apoptotic-like cell death, while mature fibrils cause rapid and mainly necrotic-like cellular death.

One of the important aspects of amyloid studies is to develop measures for inhibiting or re-directing the process of amyloid formation to abolish or neutralize toxic amyloid species. Among the agents having inhibitory or modulatory properties small, phenol containing molecules are widely studied. We investigated the effect of the novel nootropic drug noopept on amyloid formation process of α-synuclein, as this drug is a small dipeptide containing a phenol ring. We showed that noopept is able to modulate amyloid formation process by accelerating it to rapid conversion of α-synuclein into fully mature fibrils, thus eliminating the stage of population of toxic oligomeric species. Using wide range of cytotoxicity assays we showed that amyloid-like fibrils formed in the presence of noopept have no cytotoxic properties. As this medicine is becoming popular and freely available in some countries as a cognitive enhancer, neuroprotective and nootropic agent, further detailed investigations and clinical trials are needed to assess the safety and benefit of noopept in particular for the patients with amyloid related neurodegenerative diseases (such as Parkinson’s or Alzheimer’s diseases).

While in vitro models are useful to study some specific aspects of protein aggregation, their properties and effects on cell viability, it is very
difficult or practically impossible to create an absolutely accurate model of *in vivo* situation. Therefore, it is important to turn to *in vivo/ex vivo* studies to relate the knowledge accumulated from *in vitro* studies to the real situation in the body.

Using human brain hippocampus tissues from individuals with Alzheimer’s disease, we found that besides well-known and widely accepted main pathological hallmark — Aβ peptide deposition, S100A9 and S100A8 pro-inflammatory calcium-binding proteins are also localized in the plaques and in surrounding tissues and very explicitly co-localized with Aβ. Moreover, we found the presence of S100A9 within the neuronal cells, which has not been reported before and can be an important clue for understanding the mechanisms of neurodegeneration. *In vitro* cytotoxicity studies showed that S100A9 protein can efficiently induce cytotoxicity when added exogenously to the neuronal cell culture. These findings suggest that S100A8 and S100A9 proteins play an important role in Alzheimer’s pathology, and potentially can be candidates for the amyloid plaque formation and neurodegeneration. Whether they are associated with inflammatory processes underlying the early onset of disease or produced and accumulated as a consequence of A-beta induced pathology remain to be clarified.

We found that Alzheimer’s disease is not the only pathology associated with A-beta and S100A9 deposition in a form of plaques. Immunohistochemical studies of an aortic valve surgically removed from a patient with aortic stenosis revealed plaque-like structures positively stained with A-beta and S100A9 proteins. These areas are also positively stained with fibril-specific antibodies as well as with Congo red, which also shows very distinct apple-green birefringence under the polarized light. Besides, there is intracellular localization and co-localization of both proteins in interstitial cells throughout the whole fibrous tissue of the valve. The presented case report is the first finding suggesting inflammatory protein S100A9 as well as A-beta peptide as potential candidates for amyloid formation in aortic stenosis valves. We suggest that there is a specific interaction between A-beta and S100A9 during amyloid formation, which can be involved in amyloid-associated pathology in various tissues and organs in the body, which can potentially be caused by inflammatory processes, particularly by its chronic, long lasting forms.
LIST OF PAPERS

This thesis is based on the following papers:

I.

II.

III.

(*) Authors with equal contribution

IV.

V.
**Gharibyan AL**, Narayana VK, Habib A, Sulniute R, Henein MY, Morozova-Roche LA. Inflammatory S100A9 and Aβ amyloids in heart valve of patient with aortic stenosis. (Case report- manuscript 2012)
Contribution to other papers:

**VI**


**VII**


**VII**


**IX**


**X**

INTRODUCTION

Amyloidoses and amyloid proteins

Amyloidoses are a group of diseases associated with abnormal protein deposition, defined as amyloid, in various tissues of the body. The etiology of amyloidoses is very diverse depending on a specific protein aggregation, tissue type and organ localization. There are different classification systems for amyloidosis. An older classification system is based on occurrence of amyloid deposits as primary, secondary and hereditary.

Primary amyloidosis, called also light chain amyloidosis (AL), develops by itself without apparent cause. Commonly affected parts of the body include the heart, lung, skin, tongue, intestines, liver, kidney and spleen.

Secondary amyloidosis develops as a complication of another disease, including multiple myeloma, chronic infections (such as tuberculosis or osteomyelitis), or chronic inflammatory diseases (such as rheumatoid arthritis and ankylosing spondylitis). Parts of the body commonly affected include the adrenal glands, lymph nodes, liver, kidney and spleen.

Hereditary amyloidosis is particularly rare genetic form of disease with 50 % chance of passing the same condition on to the offspring. Often affected parts of the body include peripheral nerves, the nerves of the wrist and the eyes, and kidneys.

Based on tissue and organ affection amyloidosis can be divided into 2 main types — localized and systemic.

In case of localized amyloidosis amyloid protein deposition is organ-restricted affecting single tissue type of the body; while
systemic amyloidosis affects various tissues throughout the body leading to serious changes potentially in any organ.

**Modern classification** of amyloidosis is based on chemical nature of amyloid component of deposits. Current criteria for designation of amyloid protein are the follows: the protein must be the major fibril component of extracellular deposit in the tissue; it should have a cross-β structure on x-ray diffraction analysis, and exhibit affinity for Congo red and green birefringence when viewed by polarization microscopy. Furthermore, the protein must have been explicitly characterized by protein sequence analysis (DNA sequencing in the case of familial diseases) [1]. Typically amyloids are rigid non-branching fibrils with about 10 nm in diameter. Currently 27 human amyloid fibril proteins are stated in nomenclature list (Table 1) with their association to pathologic condition [2]. Amyloid proteins are denoted by prefix “A,” for amyloid, followed by an abbreviation derived from the name of the precursor protein. Besides extracellular amyloid deposits there are several pathologies associated with intracellular protein accumulation (inclusion bodies) which exhibit some of the properties of amyloid fibrils. For example, α-synuclein inclusions, called Lewy bodies in Parkinson’s disease, have fibrillar morphology, predominantly β-structured, but they don’t bind Congo red. These types of inclusions are not considered officially as amyloids, and related pathologies consequently are not stated in classification system of amyloidosis. However in 2004 by the decision of Nomenclature Committee of the International Society of Amyloidosis intracytoplasmic and intranuclear protein aggregates with some similarities to amyloid, considered separately as a list of close relatives of amyloid proteins (Table 2.) [1].
Table 1. Amyloids and their precursors in humans (adapted from [2]).

<table>
<thead>
<tr>
<th>Amyloid</th>
<th>Precursor protein</th>
<th>Systemic or Localized (S/L)</th>
<th>Syndrome or involved tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Immunoglobulin light chain</td>
<td>S, L</td>
<td>Primary Myeloma-associated</td>
</tr>
<tr>
<td>AH</td>
<td>Immunoglobulin heavy chain</td>
<td>S, L</td>
<td>Primary Myeloma-associated</td>
</tr>
<tr>
<td>Aβ₂M</td>
<td>β₂-microglobulin</td>
<td>S, L?</td>
<td>Hemodialysis-associated Joints</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin</td>
<td>S</td>
<td>Familial Senile systemic Tenosynovium</td>
</tr>
<tr>
<td>AA</td>
<td>(Apo)serum AA</td>
<td>S</td>
<td>Secondary, reactive</td>
</tr>
<tr>
<td>AApoAI</td>
<td>Apolipoprotein AI</td>
<td>S</td>
<td>Familial Aorta, meniscus</td>
</tr>
<tr>
<td>AApoAII</td>
<td>Apolipoprotein AII</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>AApoAIV</td>
<td>Apolipoprotein AIV</td>
<td>S</td>
<td>Sporadic, associated with aging</td>
</tr>
<tr>
<td>AGel</td>
<td>Gelsolin</td>
<td>S</td>
<td>Familial (Finnish)</td>
</tr>
<tr>
<td>ALys</td>
<td>Lysozyme</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>AFib</td>
<td>Fibrinogen α-chain</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>ACys</td>
<td>Cystatin C</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>ABri</td>
<td>ABriPP</td>
<td>S</td>
<td>Familial dementia, British</td>
</tr>
<tr>
<td>ALect2</td>
<td>Leukocyte chemotactic factor 2</td>
<td>S</td>
<td>Mainly kidney</td>
</tr>
<tr>
<td>ADan</td>
<td>ADanPP</td>
<td>L</td>
<td>Familial dementia, Danish</td>
</tr>
<tr>
<td>Aβ</td>
<td>Aβ protein precursor (AβPP)</td>
<td>L</td>
<td>Alzheimer's disease, aging</td>
</tr>
<tr>
<td>APrP</td>
<td>Prion protein</td>
<td>L</td>
<td>Spongioform encephalopathies</td>
</tr>
<tr>
<td>ACal</td>
<td>(Pro)calcitonin</td>
<td>L</td>
<td>C-cell thyroid tumors</td>
</tr>
<tr>
<td>AIAPP</td>
<td>Islet amyloid polypeptide</td>
<td>L</td>
<td>Islets of Langerhans Insulinomas</td>
</tr>
<tr>
<td>AANF</td>
<td>Atrial natriuretic factor</td>
<td>L</td>
<td>Cardiac atria</td>
</tr>
<tr>
<td>APro</td>
<td>Prolactin</td>
<td>L</td>
<td>Aging pituitary Prolactinomas</td>
</tr>
<tr>
<td>AIns</td>
<td>Insulin</td>
<td>L</td>
<td>Iatrogen</td>
</tr>
<tr>
<td>AMed</td>
<td>Lactadherin</td>
<td>L</td>
<td>Senile aortic, media</td>
</tr>
<tr>
<td>Aker</td>
<td>Kerato-epithelin</td>
<td>L</td>
<td>Cornea, familial</td>
</tr>
<tr>
<td>ALac</td>
<td>Lactoferrin</td>
<td>L</td>
<td>Cornea</td>
</tr>
<tr>
<td>AOaap</td>
<td>Odontogenic ameloblast-associated protein</td>
<td>L</td>
<td>Odontogenic tumors</td>
</tr>
<tr>
<td>ASEmI</td>
<td>Semenogelin I</td>
<td>L</td>
<td>Vesicula seminalis</td>
</tr>
</tbody>
</table>
### Table 2. Intracellular inclusions with known biochemical composition, with or without amyloid properties. (adapted from [2])

<table>
<thead>
<tr>
<th>Inclusion name</th>
<th>Protein nature</th>
<th>Site</th>
<th>Associated disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewy bodies</td>
<td>α-synuclein</td>
<td>Neurons intracytoplasmic</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>Huntington bodies</td>
<td>PolyQ expanded huntingtin</td>
<td>Neurons intranuclear</td>
<td>Huntington's disease</td>
</tr>
<tr>
<td>Hirano bodies</td>
<td>Actin</td>
<td>Neurons</td>
<td>Neurodegenerative disorders</td>
</tr>
<tr>
<td>Collins bodies</td>
<td>Neuroserpin</td>
<td>Neurons</td>
<td>Forms of familial presenile dementia</td>
</tr>
<tr>
<td>Not specified</td>
<td>Ferritin</td>
<td>Neurons, many Different cells</td>
<td>Form of familial neurodegenerative disorder</td>
</tr>
<tr>
<td>Neurofibrillary tangles</td>
<td>Tau</td>
<td>Neurons intracytoplasmic</td>
<td>Alzheimer disease, fronto-temporal dementia, aging, other cerebral conditions</td>
</tr>
</tbody>
</table>

**Generic property of polypeptide chain**

Besides the proteins involved in diseases, there is a growing number of disease non-related proteins and peptides shown to form amyloid-like structures. There are naturally occurring amyloids in some organisms, mainly invertebrates having certain biological functions, such as well-known curli fibers in *E. coli* which contribute to biofilm formation on the bacteria's membrane surface [3]. The existence of naturally occurring amyloid fibrils are shown also in mammalian tissues [4]. These types of amyloids are called “functional amyloids”. There are also a vast number of proteins shown to form amyloid like structures *in vitro*, when using various denaturing or destabilizing
conditions such as high temperature, extreme pH, agitation, etc. All these proteins having nothing common with each other by their natural states and properties (sequence, secondary, tertiary structures, localization and functions), share common characteristics when aggregated, i.e. cross-$\beta$-sheet cored structure, fibrillar morphology (usually 6-12 nm in diameter), some of them have strong affinity to Congo red, and more often bind another $\beta$-sheet specific dye Thioflavin T, widely used for amyloid studies in vitro. This fact led to a hypothesis that amyloid formation is a generic property of any polypeptide chain [5] (Figure 1).

Figure 1. A general view of some of the conformational states adopted by a polypeptide chain and their interconversions, including $\beta$-structured aggregation and assembly into amyloid fibrils (from Chiti & Dobson, 2006 [6], reproduced with permission of Annual Reviews, via Copyright Clearance Center).
To distinguish between diseases associated amyloids and those produced *in vitro* it is recommended by the Nomenclature Committee to call the latters “amyloid-like” or “amylog” [2]. However, this rule is not strictly followed in the field of amyloid research, and common term “amyloid” is the most often used form for all types of cross-β-sheet protein assembly.

**Suggested mechanisms and conditions of amyloid formation**

Amyloidogenesis is a complex process which begins with structural rearrangement of the native state into a β-sheet conformation. This requires either partial unfolding of globular proteins or partial folding of disordered proteins [7]. This conformation seems to facilitate specific intermolecular interaction such as hydrophobic and electrostatic interaction, which is required for polymerization of protein molecules into amyloid fibrils. Various factors can induce partial unfolding of a protein, among which are mutations, environmental changes (such as pH or temperature) and chemical modifications. However, experimentally it is difficult to detect partially unfolded state, and such a direct evidence is shown only for a few proteins, like transthyretin [8] and β₂-microglobulin [9]. In most of the cases the stability of protein is determinant which is inversely related to fibrillation of the protein [10-12]. For example, the factors destabilizing native conformation of a protein increase its fibrillation propensity and in case of β-lactoglobulin the aggregation propensity is shown to be highest at the concentration of urea corresponding the mid-point of unfolding transition of the protein [13].
While partial unfolding seem to be necessary for amyloid formation, there are evidences showing fibrillation of globular proteins under native conditions, suggesting the initiation of aggregation from a locally unfolded part of a protein, distinct from global unfolding [14].

In case of natively unfolded proteins, such as α-synuclein, amyloid-β, tau and exon 1 region in huntingtin, the process of amyloid formation requires partial folding of these proteins [15-17]. It is interesting to note that globular proteins in fact contain three times more aggregation nucleating regions than intrinsically disordered proteins. It seems that higher β-aggregation propensity is necessary for the formation of highly structured globular proteins [18]. However, although natively unfolded proteins in general have much lower aggregation propensity than globular proteins, their potential to form amyloids are not necessarily lower [19].

Generally, protein polymerization has been described by two basic models, namely, linear (or isodesmic) polymerization and nucleation-dependent polymerization [20-23].

Linear polymerization process can start from any monomeric subunit and each step of monomer addition to any protein species has identical dissociation constant, independent of the size of the polymer [24]. Nucleation-dependent polymerization is described by slow initial step in reaction kinetics followed by rapid polymerization. During the initial step several molecules form a nucleus which serves as a base for addition of sequential monomer molecules with the same rate constant controlling each step for monomer addition and dissociation. This process is differing from isodesmic polymerization on the basis of three criteria: (1) There is a time-dependent lag phase in the formation of the polymer, (2) the lag can be eliminated by the addition of a preformed nucleus (seeding), and (3) there is a critical
concentration representing the monomer in equilibrium with the polymer. A process is considered to be a nucleation–dependent when it fulfills all three criteria, since at least two of the three can be observed in the isodesmic case [25].

In case of amyloid formation the picture appears to be much more complicated. General intrinsic property of any polypeptide chain to form amyloid implies common mechanism of their formation [26]. However in spite of large accumulation of data in the literature the mechanisms of amyloid formation remain unclear, in part due to heterogeneity and the complexity of the early association events. Self-assembly reactions of amyloid fibrils have been generally accepted as a form of nucleation-dependent polymerization [27-30], described by an initial lag phase, where conformational changes of the native state and formation of nuclei (usually oligomeric) is occurring, and no or very little fibrillar structures are determined. This stage is followed by an elongation phase where a large percentage of the starting protein is converted into fibrillar structures by an addition of monomeric or oligomeric intermediates to the preformed nuclei (Figure 2). A common feature among amyloid formation and other nucleation-dependent processes is that the lag phase can be partly or entirely avoided by the addition of seeds [31-33], which are usually fragments of preformed fibrils. By the theories of nucleation-dependent polymerization model originally developed for actin and sickle cell hemoglobin assembly [23, 24] there is a strong concentration dependence of the process with a direct alteration in the size of the “critical nucleus”. However in most of the cases the fibril formation reactions showing features of nucleation-dependent polymerization, the kinetics shows only a weak dependence on initial protein concentration [34-38]. This lead to a conclusion, that the “critical nucleus” is monomeric or very small by size [37, 38].
Figure 2. A schematic presentation of nucleation dependent polymerization of amyloid fibrils.

For some proteins, such as amylin and insulin secondary nucleation pathway has been proposed to be critical for amyloid formation [39-41]. In this case nucleation occurs on the surfaces of pre-existing fibrils.

A vast number of studies show also a very rapid formation of spherical oligomers and/or protofibrils, while the mature fibrils appear upon extended time of incubation [42-44]. This mechanism has been defined as “assembly via oligomeric intermediates” [44-46]. It seems that the formation of pre-fibrillar aggregates in this case is not limited by nucleation event [47-49], and can be considered as a type of isodesmic polymerization [42, 49, 50].

Amyloid structures can be formed at various conditions in vitro. More often the proteins are prone to aggregate under destabilizing extreme conditions, such as low pH, high temperature, or use of denaturants, for example lysozymes [31, 32, 51]. Some proteins can also readily form amyloid fibrils under physiological conditions
(neutral pH and 37°C), like albebetin, α-synuclein, or Aβ peptide [52, 53]. However in vivo conditions for amyloidogenesis remain unclear.

Cytotoxicity of amyloid structures and mechanisms of cell death

Cytotoxicity is one of the key properties of amyloid structures, as many amyloidoses are related to cell and tissue degeneration. However, in spite of large amount of accumulated experimental data, underlying mechanisms of amyloid induced cellular death, as well as particular types of toxic species remain largely unclear and controversial.

General pathways of cell death

Generally, cellular death is divided into two main types — apoptosis (or programmed cell death) and necrosis (or accidental cell death).

Classically apoptosis is characterized by early activation of a cascade of specific proteases, called Caspases (cystein-aspartate proteases), [54, 55], translocation of phosphatidylserines from the inner to the outer leaflet of membrane bilayer [56], chromatin condensation and DNA fragmentation, morphological changes of the cells, like blebbing, and shrinking (Figure 3). Physiologically apoptosis is highly organized process, which allows degrading the cell content and removing by macrophages before the cell’s contents have a chance to leak into the surrounding environment, by this preventing
unwanted inflammatory response [57]. Two main pathways — extrinsic or intrinsic, can trigger apoptosis. The extrinsic pathway is initiated when an apoptotic agent stimulates transmembrane death receptors, such as the Fas or TNF, while the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell [58, 59].

In contrast to apoptosis, necrosis is accidental cell death, caused mainly by mechanical injury of the cell. The cells die rapidly leaking its content in the surrounding area, which causes an inflammatory response [57] (Figure 3).

In recent years it has become evident that the classic description of apoptosis versus necrosis is a simplification of highly complex processes of cell death and survival regulation, and rather a
continuum of death mode with varying contributions of the cellular machinery and mixed features of apoptosis and necrosis can be involved [59]. It has been suggested that this has a protective effect, particularly in the mature neurons in the developed brain in order to maintain the chance of survivability and reversibility of destructive changes until the process of cell death is completed [60-62] (Figure 4).

![Figure 4 Different modes of neuronal death](Reproduced from Leist & Jaattela, 2001, [59]. by permission from Macmillan Publishers Ltd.)

**Toxic amyloid species and their action on the cells**

A bulk amount of studies support the hypothesis that various amyloid species, especially their early intermediates, main cause of tissue degeneration, convincingly showing cytotoxic effect of amyloid
species on various in vitro cell cultures and animal models [63-69]. Toxic properties are not limited by the proteins involved in different amyloid related diseases. Numerous disease non-related proteins were shown to induce cellular toxicity in vitro when aggregated to amyloid-like structures [70-72]. Current leading hypothesis in the field suggests that generic property of proteins to form amyloid, having a common mechanism of their formation, would lead to a common mechanism of their cytotoxic action [26, 73, 74]. While it is largely accepted that the most toxic amyloid species are early soluble oligomeric intermediates, and mature amyloids fibrils are mostly considered harmless or inert [75-80], there are number of evidences showing toxic properties of fibrillar structures [80-90].

In the context of the mechanisms of amyloid induced toxicity a number of studies have shown that prefibrillar amyloid species activate caspases [91] and receptor-mediated signaling pathways associated with apoptosis [71, 78, 92]. In contrast, there are findings that HypF-N amyloid exerts necrotic rather than apoptotic death of NIH-3T3 mouse fibroblasts [74]. The authors have demonstrated that the amyloid activates the extrinsic apoptotic pathways which are followed by intrinsic pathways switching between apoptosis and necrosis, depending on the timing and severity of mitochondria derangement. Indeed, growing evidence has accumulated that the patterns of cell death cannot be simply divided on apoptosis or necrosis due to the overlap and shared signaling pathways between the different death programs [93]. It has been shown that apoptotic and necrotic markers can concomitantly be present in the same cell after cerebral ischemia, indicating that more than one death program may be activated at the same time [94]. A cell may switch back and forth between different death pathways as shown in neuronal cells which exhibited elements of autophagic degeneration upon oncogenic
Ras expression, but showed the apoptosis characteristics upon treatment with TNF-α [95]. However the occurrence of these mechanisms in the human amyloid diseases in vivo remains to be proven.

The role of amyloid fibrils in cellular death also remains unclear. It has been shown, that mature fibrils from Aβ1-40 produced at two different conditions and consequently characterized by different morphologies, exhibit significantly different toxicities in neuronal cells [90]. There is also an evidence that Aβ fibrils bind to the surface receptor complex of microglial cells which leads to activation of intracellular signaling pathways leading to a pro-inflammatory responses [96]. In familial amyloid polyneuropathies the interaction of transthyretin fibrils with RAGE receptors (receptor for advanced glycation end products) were suggested as contributing to cellular stress and toxicity [97]. These indicate that fibrils can act via specific mechanisms, rather than inducing only accidental cell damage.

Most amyloidogenic proteins are characterized by a high heterogeneity and irreproducibility of their amyloid pathways in vitro; indeed, even a slight deviation in sample preparation or storage can change dramatically the final amyloid morphology, a phenomenon which becomes increasingly recognized in the current amyloid research [52, 90, 98]. This in turn can largely affect cytotoxic properties of individual amyloid species, which together with different cell types and variable conditions used in different laboratories, bring to controversy in obtained results.
Model proteins and peptides used in research papers I-III.

Albebetin

Albebetin (ABB) is de novo designed 7.4 kDa protein with 73 amino acid residues. It contains two repeats of αββ- motives forming four stranded β-sheet covered by two α-helices [99, 100] (Figure 5). Short loops connecting the elements of the secondary structure and limiting the number of possible conformations give compactness to this structure.

![Albebetin Model](image)

Figure 5. Albebetin. A. The model of albebetin molecule (created and provided by Anders Öhman); AFM image of amyloid-like fibrils from albebetin (from Zamotin el al., 2006 [72])

This construct demonstrates low immunogenicity, which is associated with its labile tertiary structure, while having well defined secondary structure [101]. 22 charged amino acid residues distributed throughout the whole primary structure and creating a high net charge of -12 at the neutral pH increase ABB solubility. ABB is
characterized by conformational mobility of molten globule type at neutral pH and room temperature due to instability of the molecule conditioned by large electrostatic repulsion. While commonly molten globule state is induced by the additional perturbations or destabilizing conditions, ABB exists in the molten globule state under physiological conditions by definition of its design.

Due to these properties it was initially implied to use ABB as a drug carrier and delivery protein. The biologically active constructs of ABB — N-terminus fused octapeptide LKEKKYSP of human interferon-α₂ (ABB-I) and hexapeptide TGENHR of human leukemia differentiation factor (ABB-DF) [102, 103] showed promising results for usage of ABB as a drug carrier. It has been shown that ABB-I activates thymocyte blast transformation similarly to interferon-α₂ [104], and ABB-DF induces the differentiation and inhibits proliferation of human leukemia cells similarly to molecules of differentiation factor [105, 106]. The fused peptides do not perturb the structure of albebetin and both constructs preserve the molten globule state. Taken in account that molten globules have a big impact in amyloid formation as amyloid precursor state it has been shown that ABB readily assembles into a variety of amyloid structures upon incubation under physiological conditions in vitro [52]. Here we studied cytotoxic properties of main amyloid species of ABB (Paper I).

**Lysozyme**

Hen egg white lysozyme belongs to the family of c-type lysozymes. It is one of the best characterized proteins and its amyloidogenic properties are extensively studied in vitro [32, 51, 107-109]. Human lysozyme, close structural homologous of hen lysozyme,
has been shown to cause systemic amyloidosis in the body as well as forming fibrils \textit{in vitro} [31, 110].

In our research we addressed the questions of cytotoxicity of main amyloid species from hen lysozyme (Figure 6), and showed the possible mechanisms by which different amyloid species cause cell death (Paper II).

![Figure 6. Hen egg white lysozyme. A. Ribbon diagram of hen lysozyme (PDB 2LYZ- source [111] ); B. AFM image of amyloid fibrils from hen lysozyme.](image)

\textbf{α-Synuclein}

α-Synuclein is a 140 amino acid natively unfolded protein abundant in adult brain, the function of which remains largely unknown in normal physiology (Figure 7). One of the functions of alpha-synuclein is the regulation of the size of distinct pools of synaptic vesicles in mature neurons [112]. It has been also shown that α-synuclein being involved in synaptic plasticity increases transmitter release from the presynaptic terminal [113].
Under unknown pathological conditions from its soluble state α-synuclein converts to insoluble fibrillar aggregates (Figure 7B) and accumulates intracellularly in selective types of neurons. These inclusions are called Lewy bodies and are key characteristics of a group of neurodegenerative disorders, called synucleinopathies. These disorders include Parkinson's disease (PD), dementia with Lewy bodies, pure autonomic failure, and multiple system atrophy. Clinically, they are characterized by a chronic and progressive decline in motor, cognitive, behavioral, and autonomic functions, depending on the distribution of the lesions [115]. Upon neuronal death or damage of axons the aggregated species of α-synuclein release into the extracellular matrix. These aggregates can be up-taken by other neurons thus suggesting neuron-to-neuron transmission of the disease [116].
Under certain conditions in vitro α-synuclein can self-assemble into ordered cross-β-sheet amyloid structures similar to the aggregates found in Lewy bodies [15, 17, 117] suggesting that this protein is sufficient to form inclusions [117]. It has been also shown that amyloid species of α-synuclein, similar to amyloids from other proteins, are cytotoxic on studied in vitro cell models [118-120]. The exact sub-cellular mechanisms by which α-synuclein induce cell death, are not clear, however, it seems that at least exogenously added α-synuclein aggregates are up-taken by the cells via endocytosis [116, 118].

**Targeting amyloid formation by small molecules**

Given that amyloid self-assembly remains the main pathological hallmark of many human diseases, studies of revealing external factors which interfere with the process of amyloid formation is promising direction to identify the molecules with potential therapeutic properties [121-123]. A number of small molecules containing aromatic rings, such as polyphenols, are widely studied as potential inhibitors of amyloid formation process in vitro and in some cases they were also shown to have a protective effect in cell culture assays [122, 124, 125]. Other studies have reported that small molecules accelerate amyloid formation [126-128], or convert toxic oligomers into non-toxic amorphous aggregates, as show in particular for α-synuclein remodeling by ECGC [129, 130], and in some cases significantly change the morphology of amyloid fibrils [131, 132]. It has been suggested that π-stacking of planar aromatic rings can contribute to remodeling of fibrillar self-assembly and stability [133, 134]
**Noopept**

Noopept (N-phenylacetyl-L-prolylglycine ethyl ester) is a water soluble proline containing synthetic dipeptide which was designed and selected among series of acyl-proline-containing dipeptides as potential drug with distinct neuroprotective properties [135] (Figure 8).

![Figure 8. Chemical structure of noopept](image)

Its antioxidant and anti-inflammatory properties have been described earlier [136, 137]. Recently it has been shown also improvement of spatial memory and increase in immunoreactivity to Aβ amyloid in a noopept treated Alzheimer's disease mice model [138]. Noopept is about 200 to 50 000 times more potent than piracetam, the best known nootropic, on a dose for dose basis [139]. It produces positive nootropic and cognitive effect in animal models at 0.01 to 0.8 mg/kg concentrations [138, 140, 141]. Currently noopept tablets are freely available in pharmacies in Russia and some other post-SU countries. It is recommended for treatment of cerebrovascular and post-traumatic origin cognitive deficiency in dosages from 10 to 30 mg per day (http://noopept.com).
Given that noopept is a phenol ring containing small molecule, we have chosen it to study the possible interference with α-synuclein amyloid formation and to evaluate the cytotoxic effect of formed structures compared with α-synuclein alone on neuronal cell culture (Paper III).

**Inflammation and Amyloidoses**

All amyloid related diseases in one or another way are associated with inflammatory processes. While in case of many secondary systemic amyloidoses, related to serum amyloid A (AA amyloidoses) there are clear evidences that infections and chronic inflammations are primary causes of disease progression (reviewed in [142]), the impact of inflammation in other amyloid related diseases, particularly in neurodegenerative disorders, such as Alzheimer's, remains the issue of debates. Despite accumulated experimental data suggesting abnormal protein aggregation and accumulation as a primary cause of pathology and consequently tissue degeneration and inflammation, there are however indications about primary role of inflammation as a cause of protein aggregation and disease progression. Among these diseases Alzheimer's disease is the most extensively studied and yet remains probably the most mysterious one.

*Alzheimer's disease*

Alzheimer's disease (AD) is a progressive neurodegenerative disease causing dementia commonly in people greater than 65 years of age and up to 50% of people aged 85 years and older [143]. The
first signs of disease appear in impairment of short-term memory, which indicates to the affection of hippocampal and neocortex areas of the brain. Upon the progression of the disease chemical and structural changes in the brain, including substantial neuronal loss slowly bring to failure of learning and cognitive functions, changes of personality and death. The disease is characterized by three main pathological factors: senile plaques (Figure 9), neurofibrillary tangles (Figure 10) and inflammation. Although the etiology of the disease remains unknown, and the relationship between these three factors are poorly understood, it is widely accepted that plaque formation plays the central role, where the major component is insoluble amyloid form of Aβ peptide, and which occurs in all stages of disease progression [144, 145]. It has been proposed that plaques disrupt the axonal cytoskeleton of neurons [146].

Figure 9. Amyloid plaques. A. Drawings of different stage plaques by Oskar Fischer, 1910, from a brain of a patient with senile dementia (images adapted from Goedert et al, 2009 [147]); B. microscopic images of Aβ plaques from AD brain.

Three morphologically distinct Aβ containing plaques have been identified in both preclinical and end-stage AD [144], namely diffuse
(called also “pre-amyloid”), dense-core and fibrillar plaques. These plaques differentially affect dendritic morphology in both the early and late stages of AD, where progression of dendritic damage is associated with fibrillar and dense-core plaques [144]. It is unclear whether different types of plaques have the same origin and represent just different stages of development, or formed individually (discussed in [148]). However, both the quantity and quality of the plaques show only weak correlation with the disease progression and severity [149, 150]. This fact together with large number of experimental evidences brought to a suggestion, that early prefibrillar, soluble oligomeric species of Aβ, rather than mature fibrils densely packed in the plaques, are the main cause of neurotoxicity and neurodegeneration [64, 151]. It has been shown also that oligomerization of Aβ occurs intracellularly [152-155], accumulation of which leads to synaptic dysfunction and neuronal loss. Given that amyloid-β is a normal metabolite of neurons, and is highly prone to aggregate, it is unclear how healthy neurons control the levels of intracellular oligomeric Aβ in order to avoid neurodegeneration. Nonetheless, there are findings that amyloid plaque formation is not limited by AD pathology, but rather can be a feature of normal ageing with absolutely the same characteristics as in AD [156, 157], and even in younger individuals without any detected sings of dementia [158]. This raises a question about the significance of β-amyloid specifically in development of Alzheimer’s disease.

The second pathological feature of AD is intraneuronal formation of neurofibrillary tangles from hyper-phosphorylated tau protein, referred also as paired helical filaments (PHF) [159, 160] (Figure 10).
Tau is a protein, found mostly in neurons. Its main function is to stabilize axonal microtubule assembly, which is critical for neuronal survival and correct functioning [161, 162]. Although the pathological base for tau phosphorylation and conversion into filaments remain unknown, it has been proposed as initiative factor of AD pathology [158, 163], as the translocation of hyperphosphorylated tau from axonal to somatodendritic compartments prevents its binding to microtubules, instead leads to aggregation into insoluble neurofibrillary tangles, which can disrupt microtubule function [160, 163]. Impaired microtubule function in its turn affects normal axonal transport and synaptic transmission, which can trigger neurodegeneration and potentially the development of AD. Although tau pathology seem to correlate better with AD disease progression than plaques, however, apparently this is not pathognomonic, as it commonly observed in other neurological disorders collectively named “Tauopathies”(summarized in [164]). Interestingly,
phosphorylated pretangle stage tau was observed in majority of the studied brains without any clinically diagnosed neurological disorders, starting from early childhood [158]. These facts reasonably raises a question whether tau pathology can be a cause, a contributing factor or a consequence of Alzheimer’s disease [165], which is equally applicable also for Aβ plaque pathology in this disease. Noteworthy also to mention, that the relationship between these two pathological features remains unknown.

Finally, the inflammation, which is involved in both tau and amyloid plaque pathology, but the role and significance of which in pathogenesis and disease progression remains the issue of debates over a century. The mentioning about involvement of inflammatory processes in AD pathogenesis appeared from the very beginning of AD research. At the same year in 1906 when Alzheimer described the first case of presenile dementia with plaques and tangles, Oskar Fischer described 12 cases of senile dementia with neuritic plaques, and proposed that they can be a result of deposition of a foreign substance which induces a local inflammatory response [166] (about Oskar Fischer and his studies read in [147]). However Fischer could not confirm this idea, as he did not find morphological characteristics of an inflammatory process around the plaques. About eighty years later new findings appeared on the presence of immune-related complement factors, acute-phase proteins, pro-inflammatory cytokines, clusters of activated microglia and reactive astrocytes around amyloid plaques in AD brain [166-169]. These findings led to the concept of “neuroinflammation”, suggesting the involvement of immunological processes in the brain pathology of degenerative origin, and by which completely changing the view of the brain as an immunologically inert organ. This gave rise to an inflammatory hypothesis of AD, as it became clear that the observations of altered
immune processes in AD cannot be ignored. The hypothesis got a support from studies on transgenic animals and human clinical trials, showing that non-steroidal anti-inflammatory drugs can reduce or prevent AD development, as well as epidemiological studies, indicating on lower prevalence of AD among the people for long-term receiving anti-inflammatory therapy (reviewed in [170]). There are also contradicting studies, showing no significant effect of anti-inflammatory drugs and even elevated risk of AD [171, 172]. However, this does not reduce the interest towards understanding the role of inflammation in AD.

As an inflammatory response reactive microglia can produce large amounts of free radicals and other neurotoxic substances which at least shown to induce neuronal cell death in culture [173, 174]. However, neuroinflammation is considered to be a downstream consequence in the amyloid cascade, where amyloid-β activates microglia, initiating a pro-inflammatory reaction and release of neurotoxins, which leads to neurodegeneration [166, 175, 176]. Some studies suggest also, that phosphorylation of tau can be promoted by activated microglia [177]. On the other hand, it has been consistently demonstrated that the neurons themselves are able to produce inflammatory mediators, such as complement, cyclooxygenases, cytokines IL-1, IL-6, and TNF-α, etc. [176]. All these molecules are significantly increased in the AD brain. Therefore it is possible that either neurons themselves complicate the inflammatory reactions in their surrounding and contribute to their own degeneration in AD, or the role of pro-inflammatory mediators in this case is neuroprotective mechanism against local inflammatory reactions [176].

The role of inflammation in AD pathology faces the same question as the role of Aβ and tau, whether it can be a cause of AD, or contribute to the disease progression, or else is a consequence of the
disease, or defensive mechanism of the brain against disease? In any case, long-time chronic inflammatory signal even at low, background level itself can be degenerative.

One thing is clear that AD in fact is heterogeneous disease with multiple “unknowns”, and a cumulative name of presenile/senile dementia, involving many other clinical aspects. Therefore, further focus in solving the puzzle of AD should be directed to find the relationship and missing links between Aβ aggregation, tau phosphorylation and inflammation which would help to understand the base of neurodegeneration and maybe revise or subcategorize the disease into different groups.

**Aortic stenosis**

Aortic stenosis (AS) is a degenerative pathology of aortic valve, prevalent after age of 60 and currently the cause of the majority of the aortic valve surgical replacements. The disease is characterized by narrowing of aortic valve opening during the left ventricular contraction due to the deposition of calcified material into the tissue and reduction of valve motion (Figure 11 A.). On-time diagnosis and treatment (replacement) are very important as the disease progression can lead to heart failure, severe infection and sudden death. In spite of its high prevalence, underlying mechanisms of AS remain largely unknown [178].

Normal function of aortic valve, as well as other cardiac valves is to support unidirectional blood flow through the heart. During systolic contraction of left ventricle aortic valve is opening to allow blood flow from ventricle into the aorta, and closing during diastole to
prevent retrograde flow into the ventricle, when the aorta is filled with blood under the pressure [179] (Figure 11 B,C).

A normal aortic valve is composed of three thin and flexible leaflets (tricuspid), which provide proper opening and closing motions (Figure 11 B, C). However about 1% of overall population congenitally have bicuspid, which is not causing any problem in early life, but considered as one of the risk factor for AS with ageing [181].

The flexibility during opening and closing, and resistance of leaflets to high back pressure during diastole is maintained by complex histological architecture of leaflets, composed of three tissue layers — fibrosa, spongiosa and ventricularis (Figure 12).

Fibrosa layer is exposed to aortic surface, containing densely packed collagen fibers, which tolerate high aortic pressure and prevent backflow. The central core is spongiosa layer composed by loose connective tissue rich in glycosaminoglycans. Following spongiosa towards inflow surface is elastin-rich ventricularis layer [179]. All three layers are populated by valvular interstitial cells,
which are commonly understood to be myofibroblast in nature, with certain similarities to both fibroblasts and smooth muscle cells [182, 183]. The leaflet surface is covered by an endothelial monolayer [179].

![Figure 12](image.png)

Figure 12 Schematic presentation of tissue architecture in aortic valve leaflet. (Adapted from Schoen, 2012 [179] with permission of Annual Reviews, via Copyright Clearance Center).

Mechanical stress, genetic factors and infection/inflammation are considered as key factors for the initiation and development of AS. Calcification of aortic valve occurs intrinsically in the leaflet tissue, beginning from the fibrosa layer (below the aortic surface) and with progression of AS extend deep into the tissue layers, often reaching the ventricular surface.
It has been suggested earlier, that calcific deposits are initiated predominantly in interstitial cells [184], leading to degeneration and passive accumulation hydroxyapatite minerals in death or damaged cells [185]. More recent studies indicate, that calcification is an active biological process associated with inflammation [186]. Chronic inflammatory process was detected in majority of examined AS cases [187]. Mechanical stress–induced activation of endothelial cells increase the expression of surface inflammatory receptors, recruiting monocytes, leukocytes and T lymphocytes to the aortic side (fibrosa layer) of the leaflet. Some studies have shown that macrophages and activated valvular interstitial cells induce excessive level of proteolytic enzymes such as metalloproteases and cysteine endoproteases, as well as pro-inflammatory cytokines which degrade collagen and elastin and remodel extracellular matrix of valvular tissue [188-191]. Activated interstitial cells it turns can transform into osteoblast-like cells leading to calcium deposition [186, 190-192] (Figure 13).

Figure 13. Potential pathways of aortic valve calcification (reproduced from Freeman & Otto, 2005 [192] with permission obtained via Copyright clearance center).
Although it has been shown similarities in underlying mechanisms of atherosclerosis and calcification of aortic valve [193, 194] and supported by some studies on animal models [195], clinical trials have failed to show that a reduction in blood cholesterol slows the progress of AS [196, 197], highlighting the need to better understand this disease.

Several studies showed the presence of amyloid deposits in cardiac valve pathologies [198-200] with high prevalence in AS [200]. Kristen et al, 2010, proposed that amyloid deposition might be depended on degenerative/inflammatory pathology of AS and to a lesser extent is associated with high shear-stress hemodynamics. Moreover, they suggest that it might be a novel amyloid entity or an unusual fragment since none of the most common amyloid proteins have been identified by using a set of well-established specific antisera (the authors used a set of anti-AA, anti-ALλ, anti-ALκ, anti-AHγ, anti-β₂M, anti-ATTR, anti-Fib, and anti-ApoAI antibodies from amYmed) [200].

It has been shown, that localization of Aβ peptides is not limited by brain/CNS and found in large quantities in plasma, platelets, skeletal muscle, and vascular walls [201, 202]. The deposition of these peptides has also been observed in eye degeneration, inclusion body myositis and atherosclerotic vascular disease [202].

Involvement of S100A8 and S100A9 has been shown in many inflammatory and calcification processes, including blood vessel calcification [203-206]. Moreover their amyloidogenic properties have been described recently in calcified inclusion of prostate and in vitro [206] (see below for these proteins).

In our research we hypothesized the possible involvement of Aβ and S100A8/A9 proteins in AS and attempted to find a link between
calcification, inflammation, amyloid development in this degenerative process.

**Pro-inflammatory S100A8/A9 proteins**

S100A8 and S100A9 are Ca\(^{2+}\)-binding “EF-hand type” proteins found only in vertebrates, with molecular masses of 10.8 and 13.2 kDa and 93 and 114 amino acid residues, respectively [207, 208]. They were first named by Moore due to their solubility in 100% saturated ammonium sulfate [209]. Except calbindin D9k, all 22 members of S100 family tend to form homodimers [210]. Some of them, including S100A8 and S100A9, are also able to form heterodimers, which suggests different functions for homo- and heterodimers [207] (Figure 14). Ca\(^{2+}\) and Zn\(^{2+}\) ions are regulating the conformation and stability of S100A8 and S100A9 [211, 212], as well as assembly of S100A8/A9 heterodimers into heterotetrameric and larger complexes [213, 214].

![Figure 14. Structures of S100A8 and S100A9 proteins presented by ribbon diagrams: (A) S100A8 homodimer; (B) S100A9 homodimer; (C) S100A8/A9 heterodimers shown in two projections rotated by 180°; (D) S100A8/A9 heterotetramer calprotectin (Modified from Vogl et al, 2012 [215]).](image-url)
Their ability to form homo- and heterocomplexes in vivo implies their multifunctionality. Indeed, increasing knowledge on these proteins reveals wide and often diverse range of intra- and extracellular functions (reviewed in [215]).

One of the intracellular functions of S100A8 and S100A9 proteins is involvement in cytoskeleton organization via tubulin polymerization [216]. The expression of S100A9 and S100A8 is highly up-regulated in various inflammatory and autoimmune disorders [217-219]. Constituting 40% of neutrophil cytosolic protein they play a key role in the activities of these cells [220]. They are secreted from circulating neutrophils to inflammatory sites during acute phase of inflammatory response. Their pro-inflammatory cytokine-like and chemokine-like activities are shown via activation of the receptor for advanced glycation end products (RAGE) [204, 221-224] and Toll-like receptor 4 (TLR4) [225-227] dependent signaling cascades. On the other hand, the anti-inflammatory properties of S100A8/A9 have been shown in avridine-induced arthritis in rats [228], in the process of wound-healing [229], in removing excess oxidants at inflammatory sites [230]. They are considered also as a distinct class of anti-inflammatory DAMPs (damage-associated molecular patterns) involved in restoring homeostasis [231].

Dual effect of S100A8 and S100A9 has been shown in cancer progression. At low concentrations S100A8/A9 complexes promote tumor cell growth [222, 224] and tumor cell migration [223, 232, 233], while at high concentrations they induce apoptosis on tumor cells [222]. Increased levels of S100A8 and S100A9 was observed in cardiomyocytes and whole hearts in lipopolysaccharide-induced cardiac dysfunction model [204], where S100A8 and S100A9 led to a RAGE-dependent decrease in calcium flux and a RAGE-mediated decrease in cardiomyocyte contractility.
Involvement of S100A8 and S100A9 proteins have been found in calcification of the blood vessels [203], in calcified inclusion called Corpora amylacea both in normal human brain [205] and ageing prostate [206]. Moreover, it has been discovered that S100A8 and S100A9 are able to self-assemble into highly heterogeneous amyloid complexes, including both oligomeric species and highly stable fibrils (Figure 15), found in extracts of prostate corpora amylacea, as well as reproduced in vitro [206]. Recently, S100A8, S100A9 and also S100A12 were found to be increased within cortical neuritic plaques and reactive glia in Alzheimer’s disease brain, and was proposed the participation in the inflammatory processes of the AD pathogenesis [234]. It has been also shown S100a9 gene is significantly up-regulated in the brains of AD animal models (Tg2576 and CT-Tg mice), and of human AD patients. Moreover, S100a9 knockdown were decreasing the memory impairment and neuropathology in AD mouse model [235]. However, the detailed molecular mechanism of these pathological events remains unknown.

Figure 15. S100A8/A9 amyloid fibrils from prostate corpora amylacea extracts. (A) AFM image; (B) stained with amyloid specific dye—thioflavin-T. (Modified from Vogl et al, 2012 [215]).
These facts, together with the ability of S100A8 and S100A9 (and possibly other members of S100 family) to form multiple complexes including amyloid, as well as their multifunctionality urges to focus on identifying the role of these proteins in pathological condition, particularly in neurodegenerative and other amyloid related disorders, as these conditions are closely related to inflammatory processes. In our research we focused on the involvement of these proteins in AD pathology and aortic stenosis (Papers IV and V)
RESULTS AND DISCUSSION

Cytotoxicity is one of the key properties of amyloid species, however, it remains unclear which amyloid species are particularly toxic and by which mechanism they affect cell viability. One of the leading hypotheses in the amyloid field suggests that common property and mechanism of amyloid formation potentially by any polypeptide chain implies a common mechanism of induced cytotoxicity [26, 73, 74]. From this point of view proteins that are not related to any amyloid disease are excellent tools for testing the universality of this hypothesis and for studying the mechanism underlying both amyloid formation and their induced toxicity on cellular level. In our research we used two model proteins – albebetin and lysozyme (Papers I and II).

Paper I. Cytotoxicity of albebetin oligomers depends on cross-β-sheet formation.

In this study we used de novo synthesized albebetin as a model protein, which readily forms amyloid-like structures in vitro under physiological conditions [52], which is beneficial for cytotoxicity studies, as the assembled amyloid structures will not be affected by pH of culture media.

Upon incubation albebetin assembled well-defined and distinct amyloid oligomers of two types, namely, cross-β-sheet containing and not containing oligomers, protofilaments and mature fibrils. Therefore we were able to assess and compare cytotoxic properties of these amyloid species. We have shown that the initial oligomers, containing 10–15 molecules as determined by atomic force
microscopy, do not bind thioflavin-T and do not affect viability of granular neurons and SH-SY5Y neuroblastoma cells. When these oligomers grow to larger species with 30 - 40 albebetin molecules, they develop cross-\(\beta\)-sheet structure and reduce viability of both types of neuronal cells. Neither monomers nor protofilaments or mature fibrils of albebetin displayed cellular toxicity on both neuronal cells. We have suggested that oligomeric size is important for stabilizing cross-\(\beta\)-sheet core, which also seems to be necessary condition.

These findings are in line with and support the current hypothesis about the universality of amyloid formation and toxicity of their early soluble forms. Albebetin was designed to use as a carrier-protein for drug delivery, therefore its amyloidogenic cytotoxic properties require further in depth examination prior subjecting albebetin to the large scale applications.

**Paper II. Lysozyme amyloid oligomers and fibrils induce cellular death via different apoptotic/necrotic pathways.**

Amyloid cytotoxicity was further examined using hen lysozyme as a model protein. Lysozyme is a ubiquitous protein, and its human variant is involved in human systemic amyloidoses [110]. *In vitro* lysozymes are able to form amyloid under destabilizing conditions [31, 32, 51, 107-109]. Here we used pH2.2 and 57°C conditions to produce amyloid structures from hen egg white lysozyme and characterized both oligomeric and fibrillar species by atomic force microscopy and spectroscopic technique. Upon certain periods of incubations well-defined amyloid species were subjected to cellular toxicity assays. We showed that both oligomers and fibrils of hen lysozyme induce a dose (5 - 50 \(\mu\)M) and time-dependent (6 - 48 h)
viability decrease of SH-SY5Y neuroblastoma cells. Using a wide range of cell toxicity assays to target general apoptotic or necrotic features of cell death, we have demonstrated that the oligomers and fibrils act differently on cell viability. Specifically, we showed that fibrils induce rapid decrease of cell viability (detected after 6h of incubation) shown by WST-1 cell viability assay. This effect is associated with cell membrane damage, shown by lactate dehydrogenase release and propidium iodide intake. By contrast, amyloid oligomers induce increasing activity of cellular caspases during 6-24h of incubation; however, cell viability decline was detected only after 48 h of incubation. The viability decrease was accompanied by morphological changes characteristic to apoptotic cells, phosphatidylserine externalization, detected by fluorescent-labeled annexin V binding, as well as lactate dehydrogenase release and DNA fragmentation, stained with propidium iodide. We concluded that amyloid oligomers induce apoptosis-like cell death, while the fibrils lead to rapid necrosis-like death. As polymorphism is a common property of amyloids, we demonstrated that it is not a single uniform species, but rather a continuum of cross-β-sheet-containing amyloids can be cytotoxic.

**Paper III. Neuroprotective and nootropic drug noopept rescues α-synuclein amyloid cytotoxicity**

Identifying molecules which can inhibit or re-direct amyloid formation process is a promising therapeutic prospective.

Number of small molecules containing aromatic rings, such as polyphenols, are widely studied as potential inhibitors of amyloid
formation process *in vitro* and in some cases they were also shown to have a protective effect in cell culture assays [122, 124, 125]. In some cases small molecules are shown to accelerate amyloid formation [126-128], or convert toxic oligomers into non-toxic amorphous aggregates [129, 130], also significantly changing morphology of amyloid fibrils [131, 132]. It has been suggested that π-stacking of planar aromatic rings can contribute to remodeling of fibrillar self-assembly and stability [133, 134].

In this study we used phenol ring containing dipeptide noopept (N-phenylacetyl-L-prolylglycine ethyl ester), with well-known nootropic neuroprotective, antioxidant and anti-inflammatory properties [136-138, 140, 141], to study the possible interference with α-synuclein amyloid formation, which is main pathological hallmark of Parkinson’s disease. We evaluated formed structures in the presence of noopept and their cytotoxic properties on neuronal cell culture.

We revealed that noopept has modulating effect on α-Syn oligomerization and fibrillation, shown by thioflavin-T binding assay, far UV circular dichroism (CD) and atomic force microscopy (AFM) techniques. Noopept does not bind to a sterically specific site(s) in the α-Syn molecule as revealed by heteronuclear two-dimensional NMR analysis, but due to hydrophobic interactions with toxic amyloid oligomers it prompts their rapid sequestration into larger fibrillar amyloid aggregates. Consequently, this process rescues the cytotoxic effect of amyloid oligomers on neuroblastoma SH-SY5Y cells as demonstrated by using cell viability assays, fluorescent staining of apoptotic and necrotic cells and by assessing the level of intracellular oxidative stress. The mitigating effect of noopept against amyloid oligomeric cytotoxicity may offer additional benefits to the already well-established therapeutic functions of this new pharmaceutical,
however, further detailed investigations and clinical trials are needed to assess its safety and benefit, particularly for the patients with amyloid related neurodegenerative disorders.

**Paper IV. Emerging role of inflammatory S100A9 in Alzheimer’s disease amyloid growth and neurodegeneration**

Inflammation is important component of Alzheimer’s disease involved in both tau and amyloid plaque pathology, however, the role and significance of which in pathogenesis and disease progression remains the issue of debates over a century. The presence of immune-related complement factors, acute-phase proteins, pro-inflammatory cytokines, clusters of activated microglia and reactive astrocytes have been shown around amyloid plaques in AD brain [166-169]. Moreover, it has been consistently demonstrated that the neurons themselves are able to produce inflammatory mediators, such as complement, cyclooxygenases, cytokines IL-1, IL-6, and TNF-α, etc. [176]. All these molecules are significantly increased in the AD brain. Therefore it is possible that either neurons themselves complicate the inflammatory reactions in their surrounding and contribute to their own degeneration in AD, or the role of pro-inflammatory mediators in this case is neuroprotective mechanism against local inflammatory reactions [176].

In this study by using sequential staining and stripping immunohistochemical analysis we demonstrated that in AD hippocampus there is significant level of pro-inflammatory S100A9 protein co-localized with Aβ as well as with hyperphosphorylated tau within the plaques. Moreover we found that substantial part of hippocampal neurons is positive to S100A9 both in AD and control
hippocampus, however, the distribution and staining pattern revealed some differences. In the non-demented hippocampus many neurons were evenly stained for S100A9 throughout the whole pyramidal cell layer, granular neurons in the dentate gyrus, and neurons in hilus. In contrast, in the AD hippocampus some pyramidal neurons were positively stained for S100A9 with different intensity from very bright to weak, no neuronal staining was noticed in the dentate gyrus and a fewer weak stained neurons were observed in the hilus.

Immunofluorescence staining of isolated hippocampal and cortical neurons from mice, as well as in human SH-SY5Y neuroblastoma cells confirmed the presence of S100A9 in neuronal cell type in general; indicating that along with other immune related mediators and cytokines S100A9 can also be expressed in neuronal cells.

We also found an interesting inverse correlation between localisation of phosphorylated tau and S100A9 within the neurons in AD hippocampus. While some neurones are immune-positive towards Aβ and phosphorylated tau, repeating the same staining contours within the cells, the other neurons intensively stained for S100A9 displayed very rare immunostaining towards Aβ and no staining with anti-tau antibodies.

As S100A9 was observed also extracellularly within plaques and tissues, we have assessed the effect of exogenous S100A9 on SH-SY5Y neuroblastoma cells and showed that in micromolar concentrations (0.5-20µM) it decreases cell viability in time-dependent manner (during 24 and 48h of incubation). This indicates that the release of S100A9 into extracellular environment can be potentially neurotoxic and cause neurodegeneration in addition to well-established Aβ toxicity.
**In vitro** analysis also showed that being both amyloidogenic Aβ and s100A9 can significantly promote each other’s amyloid assembly and intensify the amyloid growth. This interaction in **vivo** could be one of the possible mechanisms of plaque formation, the role of which should be investigated further, whether this is protective mechanism directed to elimination of toxic pre-mature fibrillar species, or complication of progressing pathology.

As AD is heterogeneous disease with multiple “unknowns”, and a cumulative name of presenile/senile dementia, involving many other clinical aspects, further focus in solving the puzzle of AD should be directed to find the relationship and missing links between Aβ aggregation, tau phosphorylation and inflammation which would help to understand the base of neurodegeneration and maybe revise or subcategorize the disease into different groups.

**Paper V. Inflammatory S100A9 and Aβ amyloids in heart valve of a patient with aortic stenosis**

Aortic stenosis (AS) is a degenerative pathology of aortic valve. The disease is characterized by narrowing of aortic valve opening during the left ventricular contraction due to the deposition of calcified material into the tissue and reduction of valve motion. Surgical valve replacement is the only treatment currently available. In spite of its high prevalence, underlying mechanisms of AS remain largely unknown [178]. It has been suggested, that calcific deposits are initiated predominantly in interstitial cells of aortic valve tissue [184], leading to degeneration and passive accumulation hydroxyapatite minerals in death or damaged cells [185]. More recent studies
indicate, that calcification is an active biological process associated with inflammation [186]. Chronic inflammatory process was detected in majority of examined AS cases [187]. Several studies showed also the presence of amyloid deposits in cardiac valve pathologies [198-200] with high prevalence in AS [200]. However the origin of amyloid proteins remain unknown [200].

Involvement of S100A8 and S100A9 has been shown in many inflammatory and calcification processes, including blood vessel calcification [203-206]. Moreover their amyloidogenic properties have been described recently in calcified inclusion of prostate and in vitro [206]. It is noteworthy, that localization of Aβ peptides is not limited by brain/CNS and found in large quantities in plasma, platelets, skeletal muscle, and vascular walls [201, 202]. The deposition of these peptides has also been observed in eye degeneration, inclusion body myositis and atherosclerotic vascular disease [202]. In this study we examined one case of AS for possible involvement of Aβ and S100A8/A9 proteins in AS and attempted to find a link between calcification, inflammation and amyloid development in this degenerative process.

By using immunohistochemical analysis and Congo red birefringence we have observed the amyloid deposits in the heart valve leaflet of AS patient. Using separate and co-immunostaining for Aβ and S100A9 we suggested that these are two primary candidates to form amyloid in AS. It is the first report about the presence of Aβ and S100A9 inside the tissue of aortic valve. Moreover we have observed the presence and co-localization of the same polypeptides in the interstitial cells within valve leaflet tissues. This supports the notion that increased level of these proteins within the cell may lead to calcification and amyloid depositions, triggering self-perpetuating cycle leading to cell death and tissues degeneration [185]. As pro-
inflammatory S100A9 is a calcium-binding protein, it can play critical role in calcification and transformation of fibroblast-like cells into osteoblast-like cells. *In vitro* analysis also showed that being both amyloidogenic Aβ and s100A9 can significantly promote each other’s amyloid assembly and exacerbate the amyloid growth. S100A9 may also serve as a primary therapeutic target and by reducing chronic inflammatory process the risk of AS can be also significantly decreased.
CONCLUDING REMARKS

By using two model proteins for amyloid formation and cytotoxicity studies we demonstrated that cytotoxic properties can be associated rather with wide range of amyloid structures and the mechanisms of induced toxicity can be different.

- In case of albebetin, the toxicity is clearly correlated with the development the cross-β-sheet in the oligomeric assemblies. The pivotal oligomers, which do not contain cross-β-sheet core, are non-toxic, while the larger oligomers, containing about 30-45 molecules and showing well-developed cross-β-sheet pattern, induce cell death. The initial monomeric albebetin and its protofilaments or mature fibrils do not display toxicity on the neuronal cells. This finding is in line with current point of view, that early soluble aggregates, rather than mature fibrils are the most cytotoxic species.

- In contrast, by using hen egg white lysozyme as amyloid forming protein, we demonstrated that both amyloid oligomers and fibrils are able to decrease the viability of SH-SY5Y cells, however acting via different mechanisms. We concluded that oligomers induce apoptosis-like cell death, while the fibrils lead to necrosis-like death.

As small, aromatic ring containing molecules can dramatically change the course of amyloid formation process, we tested the small, phenol containing neuroprotective drug noopept on α-synuclein amyloid formation and assessed the effect of formed amyloid structures on cell viability.

- We concluded that Noopept stimulates rapid conversion of α-synuclein oligomers into mature fibrils and by this rescues the cytotoxic effect of amyloid oligomers. Noopept also reduces the level of intracellular oxidative stress caused by amyloids.

As inflammatory processes are closely related to degenerative disorders involving amyloid deposition, we studied ex vivo human material from Alzheimer’s brain hippocampus and an aortic valve tissue from a patient with aortic stenosis, to explore the role of pro-inflammatory S100A9 protein.
• We concluded that in Alzheimer’s disease brain hippocampus S100A9 is co-localized with Aβ peptide in plaques.
• Moreover, we found the presence of S100A9 within the neuronal cells, which has not been reported before and can be an important clue for understanding the mechanisms of neurodegeneration.
• In vitro cytotoxicity studies showed that S100A9 protein can efficiently induce cytotoxicity when added exogenously to the neuronal cell culture.
• We also demonstrated that in vitro Aβ and S100A9 can significantly promote each other’s amyloid assembly and intensify the amyloid growth, which can be a possible mechanism of amyloid plaque formation in vivo.
• Whether they are associated with inflammatory processes underlying the early onset of disease or produced and accumulated as a consequence of A-beta induced pathology remain to be clarified.
• For the first time we report about the presence of Aβ and S100A9 inside the tissue of aortic valve.
• The presence of Aβ and S100A9 in aortic stenosis valve tissue both intracellularly and extracellular in form of deposits, as well as their interaction shown in vitro, suggest that these are two primary candidates to form amyloid in aortic stenosis.
• As pro-inflammatory S100A9 is a calcium-binding protein, it can play critical role in calcification and transformation of fibroblast-like cells into osteoblast-like cells.
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