### Title

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# Understanding and controlling amyloid aggregation with chirality

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## Abstract

Amyloid aggregation and human disease are inextricably linked. Examples include Alzheimer's disease, Parkinson's disease, and type II diabetes. While seminal advances on the mechanistic understanding of these diseases have been made over the last decades, controlling amyloid fibril formation stills represents a challenge and it is a subject of active research. In this regard, chiral modifications have increasingly been proved to offer a particularly well-suited approach towards accessing to previously unknown aggregation pathways, and to provide with novel insights on the biological mechanisms of action of amyloidogenic peptides and proteins. Here, we summarize recent advances on how the use of mirror-image peptides/proteins and D-amino acid incorporations have helped modulate amyloid aggregation, offered new mechanistic tools to study cellular interactions, and allowed to identify key positions within the peptide/protein sequence that influence amyloid fibril growth and toxicity.

The phenomenon of protein aggregation and amyloid formation is associated with over fifty different health disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and type II diabetes (T2D).(1, 2) In these pathologies, abnormal protein aggregates deposit in human tissue and organs, usually adopting insoluble fibrillar assemblies with β-sheet structure.(3, 4) The process of amyloid fibril formation entails a complex mechanistic energy landscape, where disordered and partially folded populations coexist as an equilibrium mixture of aggregation intermediates ranging from monomers, low and high order oligomers, and protofibrils. (Fig. 1) Eventually, these species reach the fibrillary state, which is considered to be the global minimum on the protein folding/aggregation surface,(5–7) although it has been hypothesized that amyloid crystals may be the most stable species, at least in particular cases.(8) It is important to note that the highest energy species may not always be unfolded states, but rather the transition state species (i.e., the nucleus), that nucleate fibril formation, which is a thermodynamically disfavored process. (6) In addition, on-pathway oligomers can nucleate unfolded species, thus representing the transition state leading to fibrils, and therefore higher in energy than unfolded monomer



Dedication

In memoriam Prof. Claude Bernasconi

or pre-oligomeric species. It is also possible that once formed, oligomers do not nucleate other species nor evolve into fibrils (off-pathway oligomers) but rather populate intermediate states with moderately high energy barriers and becoming kinetically trapped in local energy minima.(9)

While amyloid fibrils itself are indeed cytotoxic and were originally thought to be the seminal etiological agent in amyloidogenic diseases,(10, 11) it is believed that aggregation intermediates, usually referred to as protein *oligomers*, are likely the most toxic species in the aggregation cascade.(12–16) However, obtaining detailed structural information on these oligomers is challenging due to their transient, dynamic and heterogeneous nature. High-resolution fibrillary structures of amyloidogenic peptides and proteins are increasingly reported in literature.(17–22) On the contrary, while important advances on the structural understanding of oligomeric entities have been made,(23–28) our understanding of the activity-toxicity relationships of these oligomers remains limited.

## Mirror-image peptides as structural modifiers of amyloidogenic peptides and proteins.

Amyloid β (Aβ) is an intrinsically disordered peptide generated upon the proteolytic cleavage of the amyloid precursor protein (APP).(29) Aβ aggregation is believed to be an important event in the development of AD,(30–32) and altering Aβ aggregation and suppressing Aβ oligomer formation has been pursued and achieved in different ways, both *in vitro* and *in vivo*. Examples include the development of small molecules and peptides that bind and inhibit the proliferation of toxic oligomers,(33, 34) and multiple Aβ oligomer-specific antibodies that have undergone clinical trials.(35) The results, however, have not provided with a successful therapy thus far, leading to over 400 failed clinical trials targeting Aβ aggregation,(36) and with the FDA advisory committee voting against approval of the recently evaluated drug Aducanumab (November 6, 2020). While most of these attempts were focused on delaying and/or preventing Aβ aggregation, recent studies have shown that enhancing fibril formation and bypassing the oligomeric stage may represent a novel alternative to prevent Aβ toxicity,(**37–39) with fibrils potentially acting as a protecting reservoir with the ability to sequester toxic oligomers.(40, 41) With this thought in mind,(42) the Raskatov laboratory recently developed a new strategy to promote Aβ rapid aggregation by generating a racemic mixture of D-Aβ42 and L-Aβ42 (*rac*-Aβ42), resulting in the acceleration of fibril formation and the generation of non-toxic fibrils.(**37) This strategy, which we termed *Chiral Inactivation* (CI), is based on the fundamental principles of molecular stereochemistry, where racemic mixtures often display reduced solubility compared to enantiopure solutions.(43) This effect was also observed in the Aβ40 system, and upon mixing, *rac*-Aβ40 generated structurally different fibrils, with approximately 1/2 of the diameter when compared to L- and D-Aβ40 fibrils, and with enantiopure fibrils displaying a twist which is not present in racemic fibrils.(44) At the molecular level, we believe Aβ-chiral inactivation (Aβ-CI) occurs through formation of a rippled cross-β structure, as firstly termed and hypothesized by Pauling and Corey.(45) In this β-sheet arrangement, in which all-L- and all-D- peptidic units alternate, the L- and D- analogous side-chains are positioned on a 180° staggered conformation to

reduce steric clashing.(Fig. 2) This arrangement was observed by Swanekamp *et al.*, who using L- and D-$(FKFE)_2$ amyloid-forming peptides observed that these peptides do not self-sort but rather assemble into enantiomeric fibrils composed of all-L and all-D peptides alternating L- and D-sequences in a rippled β-sheet orientation.(46) A similar alternating arrangement was reported by Nagy-Smith *et al.*, where enantiomeric mixtures of the MAX1 and DMAX1 β-hairpin peptides *were found* to coassemble in an alternating fashion along the fibril long axis.(*47) By contrast, homochiral β-sheets tend to adopt a pleated configuration, with analogous side-chains adopting an eclipsed conformation (Fig. 2).(*47) Indeed, racemic systems in amyloidogenic proteins are thought to be thermodynamically more stable β-sheet structures when compared to pure L- and D- amyloids, which tend to form pleated β-sheets.(46–51) The acceleration of fibril formation upon racemic mixing for the Aβ system was also recapitulated by the Nilsson laboratory in the model peptide KLVFFAE (which encompasses amino acids 16 to 22 of the Aβ sequence), providing further evidence for the presence of rippled β-sheets by FTIR.(*48) Beyond Aβ, other laboratories have also reported similar results in other systems of interest. For example, the Torbeev laboratory recently showed that the nuclear coactivator binding domain (NCBD) aggregation is enhanced upon L-NCBD and D-NCBD mixing, generating β-sheet-rich structures that are also structurally different from their enantiomeric counterparts. In this study, Garcia *et al.* discovered a novel alternative aggregation pathway for NCBD upon D-NCBD addition to L-NCBD, revealing an otherwise hard to predict amyloidogenic nature.(*52) The Gellman laboratory also observed that phenol-soluble modulin α3 (PSMα3) aggregation was enhanced when L- PSMα3 and D-PSMα3 were mixed.(*53) The resultant racemic fibrils were not toxic, as opposed to enantiomerically pure L- and D-PSMα3, suggesting that an achiral-based mechanism, i.e. membrane disruption, may be underlying PSMα3 toxic actions. In this case, interestingly, the crystal structure of racemic PSMα3 revealed a cross-α structure rather than a cross-β structure, with alternating stacks of L-PSMα3 and D-PSMα3 helices.

## Mirror-image peptides as mechanistic tools to study cellular interactions.

The results discussed above are also an example of how mirror-image peptides can be used as a mechanistic tool to study cellular interactions and how amyloid aggregation may influence cellular toxicity. In this regard, enantiomeric peptides have classically been employed to differentiate between chiral (i.e. receptor mediated interactions) and achiral (i.e. pore formation) mechanisms,(54, 55) although it is important to note that it has recently been reported by the Craik laboratory that, in some cases, the chirality of the phospholipid membrane can also play a role in the modulation of the peptide-membrane interactions.(**56) Results from Dutta *et al.* showed that, when using freshly dissolved synthetic L- and D-Aβ42 peptides, D-Aβ42 had reduced cytotoxicity against rat PC12 (L-Aβ42 reduced cell viability over 50%, D-Aβ42 was found to be non-toxic) and human SH-SY5Y (L-Aβ42 reduced cell viability over 50%, D-Aβ42 cytotoxicity was under 20%) cell lines,(**37) and later reported that L-Aβ42 is internalized in cells about 5-fold more than D-Aβ42.(*57) These results are consistent with a previous study by Ciccotosto *et al.*, where freshly dissolved synthetic L-Aβ42 and D-Aβ42 displayed stereoselective binding to phosphatidylserine present in membranes of mouse cultured cortical neurons, and where

L-, but not D-Aβ42 was toxic and potently blocked long-term potentiation.(58) On the contrary, an earlier study by Cribbs *et al.* found synthetic L-Aβ42 and D-Aβ42 to have similar biological activity in primary cultures of rat neurons.(59) The latter study, however, used pre-aggregated forms of L-Aβ42 and D-Aβ42 when dosing the cultures.

These facts could be pointing towards specific aggregation states as having more influence towards stereospecific interactions with the cellular environment than others, and it may also be indicative of different toxicity mechanisms of oligomeric vs fibrillary forms. More research is needed to better understand those differences. Given that L-Aβ42 and D-Aβ42 had the same oligomerization and aggregation propensity, as expected for mirror-image peptides,(60) the results from Ciccotosto *et al.* and Dutta *et al.* suggest that L-Aβ42 and D-Aβ42 intracellular accumulation and toxicity are linked. Whereas Aβ deposits are Journal Pre-proof mostly extracellular, there is mounting evidence that intraneuronal Aβ plays important roles in AD pathogenicity.(61–63) As such, intraneuronal Aβ may dysregulate calcium homeostasis, disrupt mitochondrial function, trigger increased production of reactive oxygen species and activate pro-apoptotic caspases.(64–66) Neuronal uptake of Aβ may also lead to pathogenic cell-to-cell spreading of the peptide in AD.(67, 68) The preferential interaction of L-Aβ42 vs D-Aβ42 with the cellular environment may be indicative of receptor-mediated interactions as the major contributor of Aβ uptake.(*57) In this regard, a follow-up and recent study by Foley *et al.* using mirror-image segments of the Aβ sequence revealed that Aβ uptake is sequence specific and possibly independent from Aβ aggregation state.(**69) At the same time, it was shown that Aβ uptake was not only dependent on chirality, but also on cellular prion protein ($PrP^C$) expression, with $PrP^C$-dependent L-Aβ40 uptake significantly increased (about 8-fold) over D-Aβ40. Furthermore, following a mirror-image peptide pair (MIPP) approach, in which enantiomeric peptide fragment pairs were employed, we were able to identify amino acids 1–30 of Aβ as sufficient soluble domain for PrP mediated uptake of Aβ. Through this approach, pairs of mirror-image peptidic segments of Aβ were synthesized and dosed to cells that expressed or not PrP. As observed by flow cytometry, the different levels of cell association of the L- and D- peptides clearly pointed towards the amino acid sequence within Aβ responsible for stereospecific interactions with the cellular environment, as well as the domain responsible for the recognition of the peptide by PrP (Fig. 3).

Taken together, these studies highlight the potential of mirror-image peptides towards revealing important biological mechanistic information of amyloidogenic systems, such as interactions with cellular surface receptors and cellular recognition and internalization, otherwise exceedingly challenging to study due to the aggregating nature of the system.

## D-amino acid substituted frameworks to stabilize Aβ conformations.

Amyloidogenic proteins lack a defined and stable folded structure (besides the fibrillary state), with aggregation intermediates often occupying a large and relatively flat energy landscape.(70–73) Strategies to stabilize oligomeric intermediates can thus allow to obtain advanced structural information of these species. In this regard, introducing point chiral mutations within the amino acid sequence of amyloidogenic proteins offers the advantage of keeping most of the peptide/protein properties the same, such as side-chain size,

flexibility, hydropathy, charge, or polarizability, and it allows to focus exclusively in how conformational changes alter the peptide/protein structure. This approach, referred to as *chiral editing*,(73) was followed by the Teplow and Raskatov laboratories to generate focused chiral mutant libraries (FCML) of the Aβ40 and Aβ42 peptides. These FCMLs included D-AA substitutions either in an unbiased standardized (double amino scanning from N- to C-terminus of the peptide) or rationally-designed (introducing D-mutations at selected positions) fashion, allowing to trap and stabilize oligomeric structures otherwise inaccessible due to its rapid aggregation into fibrils.(*74, 75) For example, through a D-amino acid scan (D-AA), Hayden *et al.* were able to obtain structure-assembly relationships of the Aβ40 and Aβ42 peptides, being able to stabilize oligomers of defined molecular weights and pinpointing to specific regions of the Aβ peptide as key modulators of Aβ assembly.(*74) At the same time, work performed by Warner *et al.* (76) showed that introduction of D-Glu at position 22 of the Aβ42 peptide (Aβ42-E22e) delayed fibrillization ~4-fold and increased cytotoxicity ~2-fold, showing that subtle structural changes at this position have an influence on the structure-activity relationships of Aβ42. Interestingly, position 22 of the Aβ sequence holds four familial mutations leading to early onset AD (E22G-Arctic, E22K-Italian, E22Q-Dutch, E22Δ-Osaka),(77) and has been pointed out as a key turn motif for Aβ toxic actions and therapeutic interventions against AD.(78–80) On the other hand, D-AA replacement at position 26 of Aβ42 (Aβ42-S26s), while also delaying aggregation (~8-fold decrease) and stabilizing oligomeric intermediates, led to toxicity inhibition (Fig. 4).(*81) The exact nature of why these mutations result into such an opposite effect is still under study, but as observed by NMR and DFT calculations, D-Ser incorporation at position 26 appears to favor an intramolecular H-bond between Ser26 and Asn27, seemingly disrupting the intermolecular sidechain-to-sidechain Asn27 amide H-bonding, which is thought to be an important fibrillogenic element.(82–84) The latter is an example of how D-AA replacements do not only influence the peptide backbone structure, but can also unveil single amino acid interactions that are critical amyloidogenic elements. Taken together, and as highlighted in Figure 4, these results on the Aβ system show that the thought of oligomers being toxic *per se* is an oversimplification, and that very specific factors underlie the structure-activity relationships of these species. As reported by Zerze *et al.*, the effect of D-AA substitutions in β-hairpin regions of peptides is thought to not follow a monotonic trend on the induced structural changes, but rather to be highly position-specific.(85) Thus, chiral editing represents a powerful strategy to pinpoint the amino acid positions that are key for the amyloidogenic properties of the system. Besides D-AAs incorporated within the Aβ sequence, modulation of Aβ aggregation and toxicity has also been achieved by external chiral agents, such as D-AA-based small peptides and molecules,(86, 87) and nanomaterials,(88) thus representing alternative strategies for therapeutic development.

## D-amino acid mutations to elucidate key amino acids involved in biological processes.

D-AA replacement, or chiral editing, is an equally important tool to understand the mechanisms and structural elements by which D-AA incorporation increases Aβ solubility, given that protein homeostasis and solubility is thought to play an important role in AD

pathogenesis, neurodegeneration, and protein aggregation.(89–92) While most of D-AA mutations tend to increase the peptide/protein solubility and decrease amyloidogenesis,(75, 93) there are cases where the pathology is developed when amino acid epimerization results in decreased solubility. For example, age-related isomerization and epimerization of aspartate and serine residues leads to a decreased solubility and aggregation of the α-crystallin proteins in the eye lens, starting the development of diverse pathologies such as cataracts, cardiomyopathies, and neurodegeneration.(94, 95) Understanding the overall effect that single D-AA mutations have on the physicochemical and structural properties in the peptide/protein of interest can therefore reveal important mechanistic information. For instance, Hua *et al.* performed a series of D-substitutions in insulin to study conformational changes. While a Gly to L-Ser mutation in the B-chain of insulin impaired stability and reduced receptor-binding 2-fold, introducing D-Ser resulted in enhanced stability and a 100-fold receptor-binding impairment, thus allowing the authors to identify key changes required for insulin to undergo the conformational active state necessary for receptor binding,(96) a relevant event in the development of diabetes. This effect has also been observed in hydrogels, where single D-amino acid incorporations in minimalistic models of self-assembling peptides led to drastic changes in the supramolecular structure and paths of assembly of the gels, presumably due to the heterochiral peptide backbone tendency to bend in a turn to maximize non-covalent interactions and exclude water from specific regions.(97)

The aggregating nature of amyloidogenic peptides and proteins make these systems particularly difficult to characterize and study.(98) We believe that using chiral tools as mirror-image peptides/proteins or chiral editing can provide novel mechanistic information leading to the development of amyloidogenic diseases. Here we have summarized how molecular chirality can be used as a tool to either promote oligomer-to-fibril transition, to stabilize oligomeric intermediates (for example, for structural elucidation), to dissect receptor-mediated from receptor-independent interactions, and to probe cellular uptake as well as cell signaling pathways. The discussed results highlight how chirality can be a powerful tool for studying amyloid-forming systems. Concepts presented here should be broadly applicable to many other proteins and peptides, provided they can be made synthetically from D-amino acids at high yield and purity. In this regard, protein size remains a limiting factor; improvement on synthetic methodologies is needed so that larger unnatural D-amino acid bearing proteins can be made in the future.

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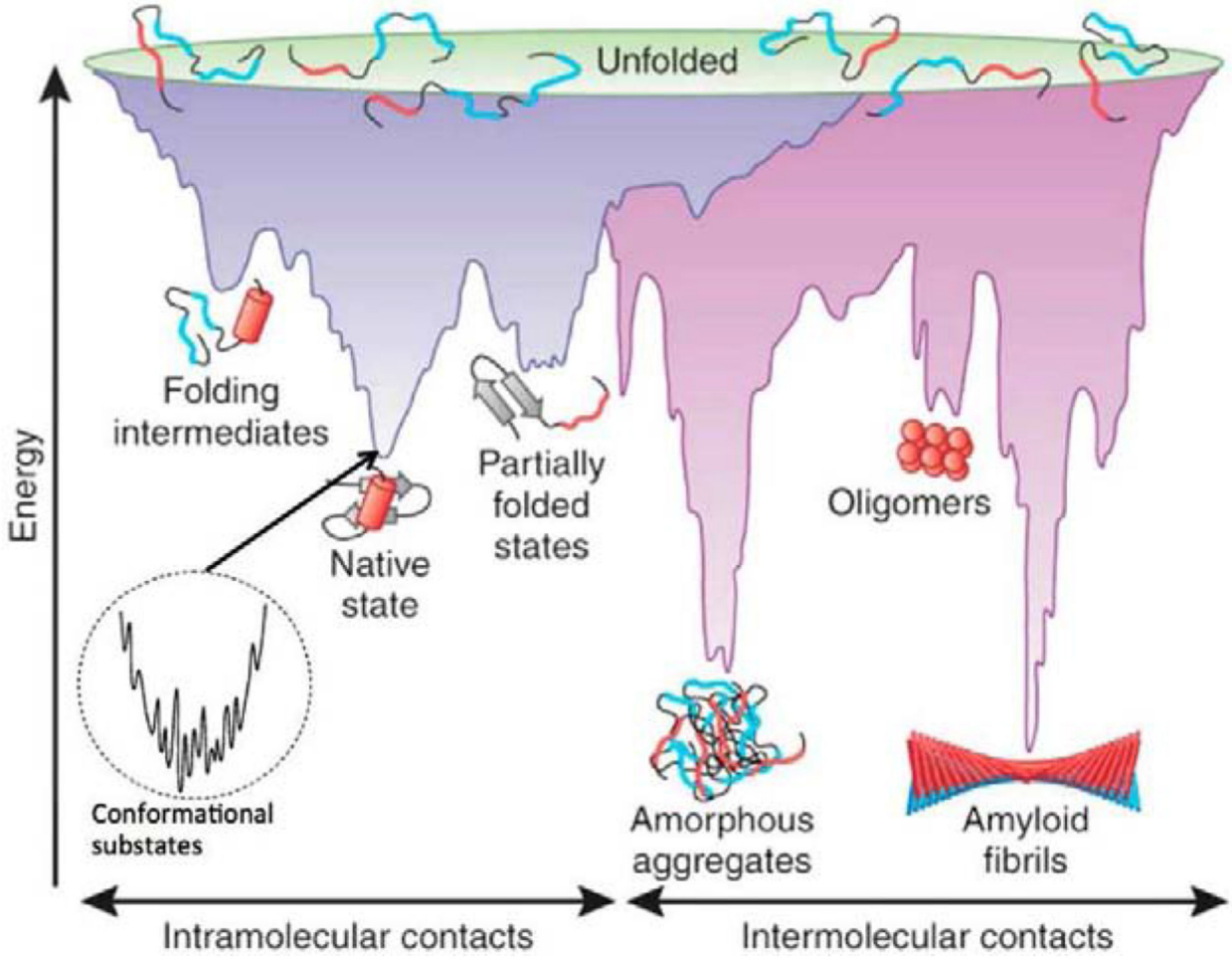


**Figure 1.**

Schematic energy landscape for protein folding and aggregation. The purple-colored landscape shows the folding pathways towards the native state via intramolecular contact formation, while the pink-colored landscape represents the formation of amyloid fibrils via intermolecular contacts. The landscape is represented by the free energy of the protein as a function of some reaction coordinate (planar slices through the 3D surface). Entropy is schematized as width within any particular sub-funnel. Unfolded conformers possess the highest free energies and the largest entropies (top of funnel). Folding occurs as conformers move within (i.e., explore) different regions of conformational space, experiencing progressive decreases in free energy and entropy until the native state is formed. Within the minimum of the native state, a multitude of substrates exist (protein "breathing"). Image reproduced from *Sci. Rep. 2017, 7, 12433*, with permission.

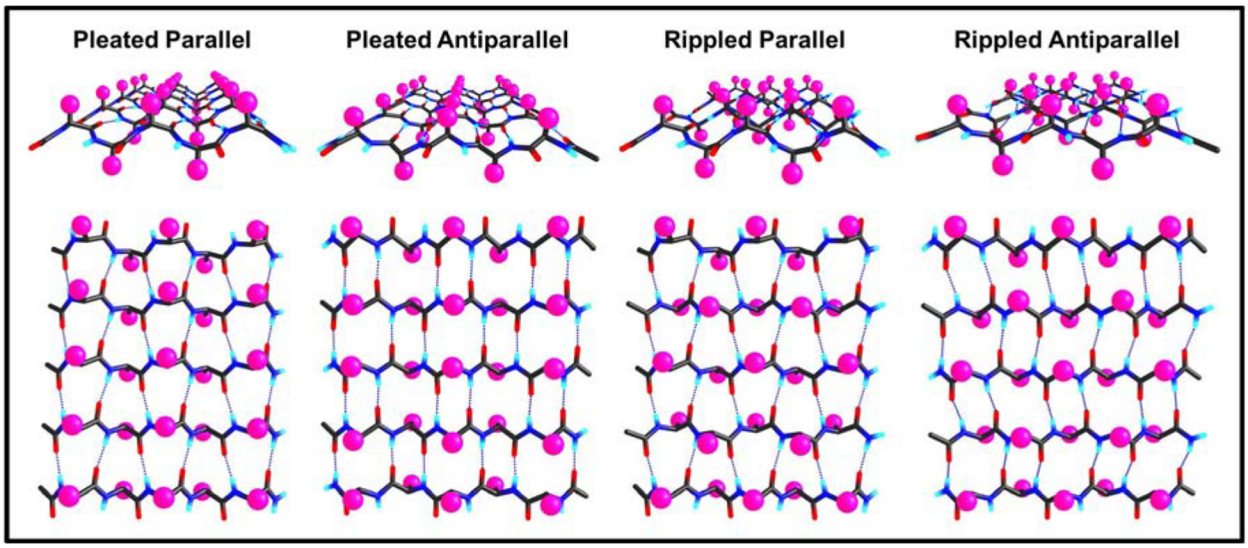


**Figure 2.**
Pleated and rippled cross-β sheets. Models built from Pauling-Corey coordinates, as described in Ref. 48.

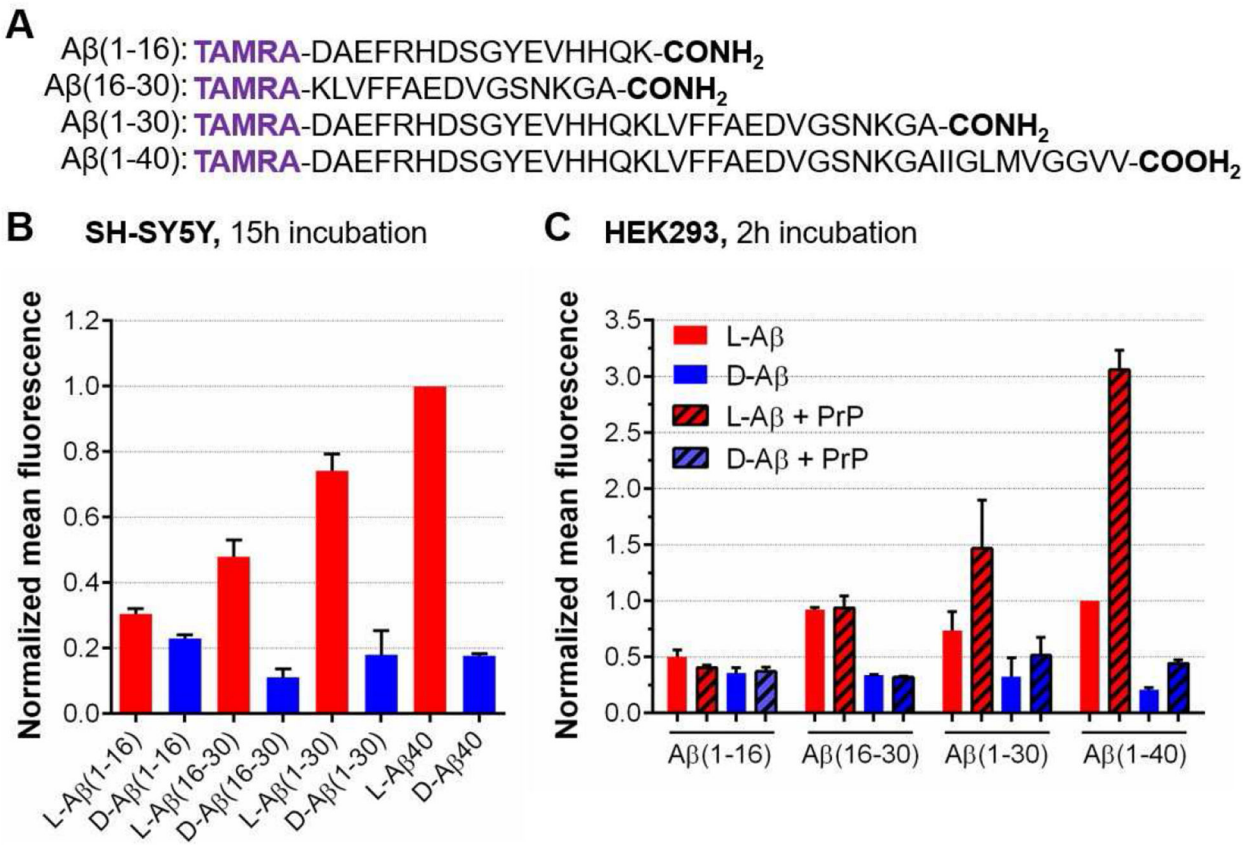


**Figure 3.**

Cellular uptake of the Aβ peptides studied by flow cytometry. (A) Sequence of the mirror-image peptide pair (MIPP) Aβ peptides tested. (B) Mean FACS results in SH-SY5Y cells normalized against L-Aβ40 (5 μM peptide, 15 h incubation). Bars show mean fluorescence with error bars for SD of three biological replicates. (C) Mean FACS results in HEK293T cells with and without $PrP^C$ expression, normalized against L-Aβ40 (5 μM peptide, 2 h incubation). Bars show mean fluorescence with error bars for SD of two biological replicates. Image reproduced with permission from *Proc. Natl. Acad. Sci. U S A* 2020, 117 (46), 28625–28631

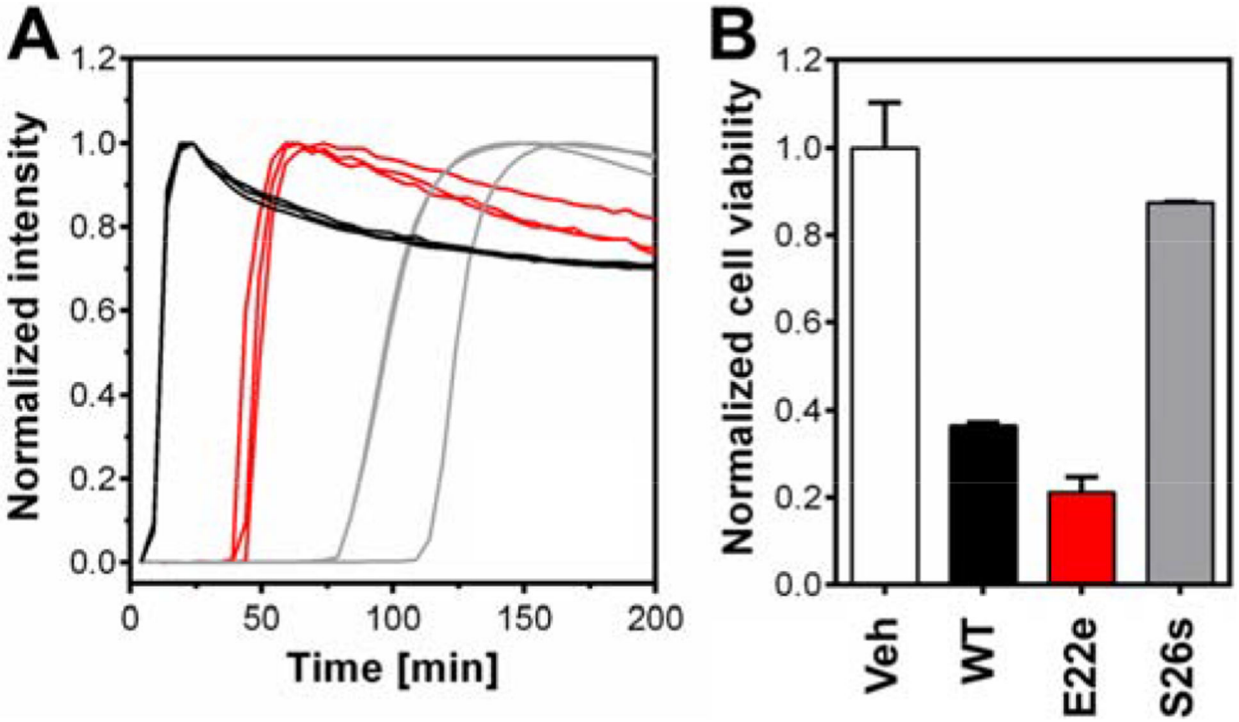


**Figure 4.**

Aggregation-toxicity relationships of Aβ42-WT and chiral variants. (A) ThT fibril formation monitoring showing 3 technical replicates.. Black: Aβ42-WT. Red: Aβ42-E22e. Grey: Aβ42-S26s. (B) Cytotoxicity results in SH-SY5Y cell line after 3-day incubation with Aβ42-WT/E22e/S26s peptides. Veh: Vehicle (cells with media only). Cell viability was determined by WST-1. Error bars show 3 technical replicates. D-amino acid introduced at Glu22 makes Aβ42-E22e more soluble and more toxic, while a D-amino acid introduced at Ser26 makes Aβ42-S26s peptide even more soluble, but non-toxic; neither E22e nor S26s affected the distribution of Aβ oligomers. Data reproduced from *J. Org. Chem. 2020, 85, 1385–1391*, with permission.