Inhibiting Aggregation of β-Amyloid by Folded and Unfolded Forms of Fimbrial Protein of Gram-Negative Bacteria

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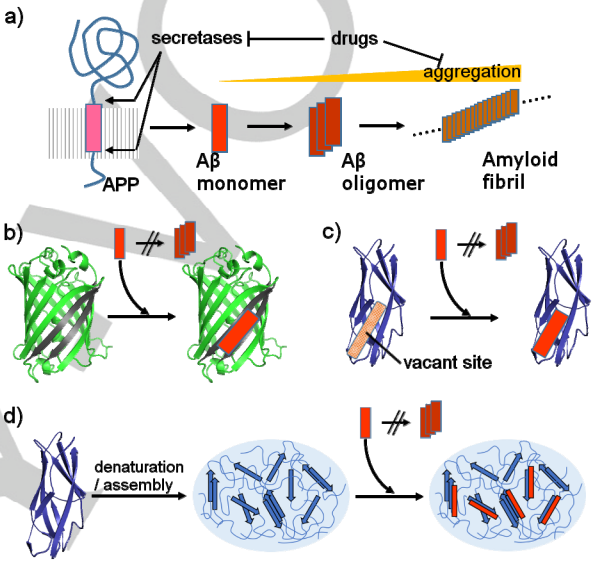
Abstract: Inhibition self-assembly of β-amyloid (Aβ) is considered to be a strategy that can be potentially useful to develop treatment for Alzheimer's disease (AD). We have discovered that a protein unit that is found in the fimbriae of Gram-negative bacteria, which has a vacant site for a β-sheet strand, prevents Aβ oligomerization effectively. Moreover, we found that a soluble but denatured form of this protein shows even higher potency. Our results also demonstrate the applicability of denatured proteins as pharmaceutical material.

Alzheimer's disease (AD), which causes memory and cognitive disorders, is the most frequently diagnosed neurodegenerative diseases. In an aging society, it is estimated that as high as 50% of people over the age of 85 may suffer from this disease, which will present major social and economic risks.[1] Currently, two classes of drugs are on the market to combat AD, namely, cholinesterase inhibitors and an *N*-methyl-D-aspartate receptor agonist.[1] Both approaches chemically modulate neuronal communication and relieve the physical and psychological symptoms of AD patients, especially in the mild to moderate stage. However, the treatment of severe AD is still difficult and more effective drugs including those with different modes of actions have been sought.

Although details of the pathogenesis of AD remain elusive, the β-amyloid (Aβ) cascade is one of the accepted pathways for the development of AD.[2] In this theory, toxic amyloid oligomers (and the fully matured fibrils) are generated by the self-assembly of a 40–42 amino acid peptide, Aβ, which is formed from the amyloid precursor protein (APP) by sequential proteolysis by two secretases (Scheme 1a).[3] Several drugs to prevent[3-4] or modulate[4-5] the APP processing pathway are under clinical trials[3-4] and promising results are awaited. Alternatively, interfering with the aggregation of Aβ is also investigated as an alternative approach, and significant effort has been invested in laboratory-level research,[6] with some molecules now in clinical trials.[1] Such examples range from small molecules[7] to antibodies;[8] arguably, however, it would be beneficial to exploit peptide or protein scaffolds because they provide large surfaces



Scheme 1. a) Formation of β-amyloid (Aβ) from an amyloid precursor protein (APP), which is followed by aggregation to form toxic amyloid fibrils. Both proteolysis of APP and aggregation of Aβ are targets for drug development. b) A previously reported method[9] to inhibit amyloid fibril formation using β-sheet protein surfaces incorporated with Aβ peptide sequences (in gray). c) Our strategy to capture Aβ monomer by using a protein with a vacant β-sheet binding site. This was demonstrated by using a fimbrial protein unit of Gram-negative bacteria. d) The denatured protein material derived from the same protein used in c) showed potent inhibition of Aβ oligomerization.

that can be modified so they effectively sequester Aβ monomer and interfere with its self-assembly. For example, designer proteins,[9] some of whose original β-sheet strands were swapped with Aβ amino acid sequence, were reported to serve as potent inhibitors against Aβ aggregation. In these examples, the incorporated Aβ amino acid sequence on the protein surface binds to Aβ monomer or oligomer to halt its further elongation (Scheme 1b). These successes prompted us to propose an alternative design using proteins as a scaffold for an Aβ aggregation inhibitor. It is known that the aggregation of Aβ peptide is governed by hydrogen bonds, which enable β-sheet formation of the peptidic main chain as well as hydrophobic interactions of the amino acid side chains.[10] Therefore, we hypothesized that a native protein that has a binding domain with β-sheet strands with hydrophobic pockets may be able to suppress the malicious self-assembly of Aβ (Scheme 1c). Here, we report that this design was successfully demonstrated by using a bacterial fimbrial protein; moreover, we found that the protein material obtained from the same protein but in denatured form showed even higher potency against Aβ aggregation (Scheme 1d).

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During the search for protein scaffolds that meet the demands of our design, we were attracted by the unique supraprotein structures[11] of the adhesive fimbriae found on the surface of Gram-negative bacteria. Type I fimbria, which is composed of several 'Fim' proteins, is one such example that has been studied in detail both structurally[11a, 12] and functionally.[13] This external organelle has a lectin module, FimH, at its tip that plays a pivotal role in bacterial adhesion onto the cell surface during colonization.[13b] The pilus part of this fimbria is composed of multiple copies of FimA, which are linked to FimH via FimF and FimG.[14] Each structural unit has an immunoglobulin (Ig)-like fold,[11a] which is formed from two-layered β-sheets (Figure 1). The unique structural property of Fim proteins is that it does not present a self-contained Ig-like fold; instead, one of the peptide strands of the fold is donated from the adjacent protein unit (Figure 1).[11a] The mode of interaction between this donated strand and its acceptor is termed 'donor strand complementation' (DSC).[11a] This interaction forms a highly stable noncovalent bond interaction[15] that has previously been used as a handle for protein purification.[16] Structural analyses[11a, 15, 17] show that DSC is achieved through hydrogen-bond interactions to form a β-sheet as well as through hydrophobic interactions.[15] In this context, we concluded that Fim proteins have ideal structural properties for our design of the Aβ binder. We thus tested these donor strand acceptors as potential inhibitors of Aβ aggregation (Scheme 1c).

In initial attempts, a gene (see the Supporting information, SI) was cloned that encodes the strand acceptor part of FimG of *Escherichia coli* K12 strain (Gene Accession Number: AP009048), termed FimGt[15-16] (Figure 1; 't' denotes 'truncated' to show that it lacks the N-terminal donor strand of its own). When the protein was overexpressed by IPTG induction, FimGt was only found in the insoluble cell debris after lysis of the bacteria. The insoluble proteins were dissolved in 6M guanidinium hydrochloride (GdmHCl) and FimGt was purified by immobilized metal affinity chromatography (IMAC) in denatured form. The protein was then refolded by using a rapid dilution procedure.[16] The refolded FimGt was subjected to gel filtration and three peaks were observed in the chromatogram (Figure 2a), the first and the last of which were collected; the corresponding proteins were termed 'Poly' and 'Mono', respectively, based on the molecular weight estimation from the elution time. Circular dichroism (CD) spectra (Figure 2b) indicated that MonoFimGt has a properly folded structure[15] whereas PolyFimGt showed a similar spectrum to that of denatured FimGt.[15] It was surprising that PolyFimGt remained soluble in the aqueous buffer without any chaotropic agents or detergents in spite of its unfolded structure. Hydrophobic domains in PolyFimGt would be effectively covered by hydrophilic parts of the protein chains to stay soluble in the aqueous buffer. The hydrodynamic diameters of MonoFimGt and PolyFimGt determined by dynamic light scattering (DLS) analysis were 4 and 20 nm, respectively (Figure S1 in the SI), which are consistent with the elution time upon gel filtration. We speculated that two cysteine residues in FimGt (Cys16 and Cys54) would play an important role for the formation of PolyFimGt via random intermolecular disulfide bond formation. However, the C16A/C54A double mutant of FimGt also afforded a peak corresponding to the Poly-form in the gel filtration chromatogram after refolding (Figure S2). Instead, it lost the Mono-form peak. This result indicated that the cysteine residues of FimGt are not essential for PolyFimGt formation but are important to stabilize MonoFimGt.

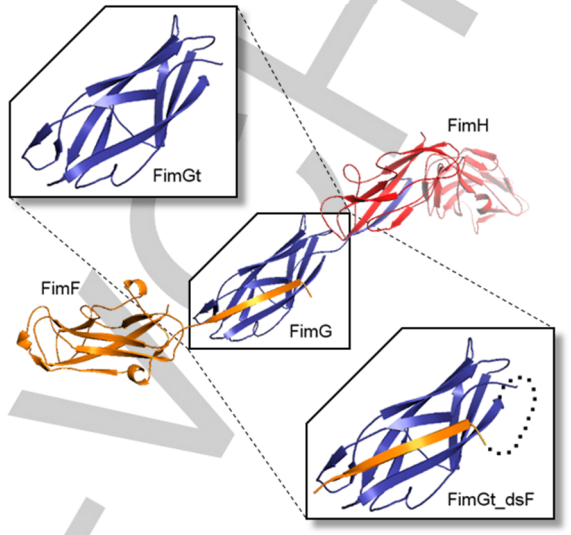

Figure 1. The protein assembly of Type I fimbria through donor strand complementation.[11a] Each unit forms an immunoglobulin-like fold with a strand donation from the adjacent protein unit. The putative folded structures of FimGt and FimGt_dsF used in this study are shown in boxes at the top left and at the bottom right, respectively. FimGt misses the N-terminal strand from the complete FimG. FimGt_dsF has the donor strand of FimF at the C-terminus, which is linked by a $(SGG)_3$ linker (black dotted line).

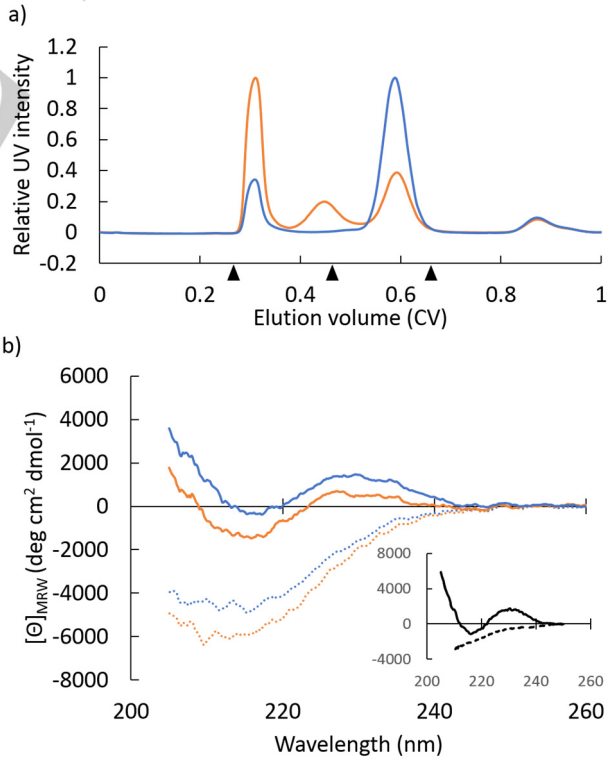

Figure 2. a) Chromatograms of gel filtration for FimGt (orange) and FimGt_dsF (blue) after refolding. The black triangles indicate the elution time of γ-globulin (158 kDa), BSA (66 kDa), and cytochrome c (12 kDa) from left to right. b) Circular dichroism spectra of Mono (solid lines) and Poly (dotted lines) species of FimGt (orange) and FimGt_dsF (blue). The CD spectra of folded (solid line) and unfolded (dotted line) FimGt_dsF reported previously[15] are shown in the inset.

The inhibitory activity of our proteins toward Aβ oligomerization was investigated by using sandwich ELISA assays (Figure 3a)[18] and the results were compared with those of the reference protein BSA.[9b] In this assay, anti-Aβ antibody fixed onto a 96-well plate reacted with working solutions in which Aβ peptide (1-42) was incubated with each tested Fim protein or with BSA. After washing the test samples, the plate was treated with biotinylated anti-Aβ antibody. This biotinylated antibody only binds to oligomerized Aβ because the epitope of monomeric Aβ was occupied by the first antibody fixed on the plate, whereas oligomerized Aβ has several other free epitopes. The fixed biotin was then reacted with the streptavidin–horseradish peroxidase (HRP) conjugate and formation of Aβ oligomer was quantified colorimetrically (Figure 3b) by using the HRP-catalyzing oxidation reaction of *ortho*-phenylenediamine in the presence of hydrogen peroxide.

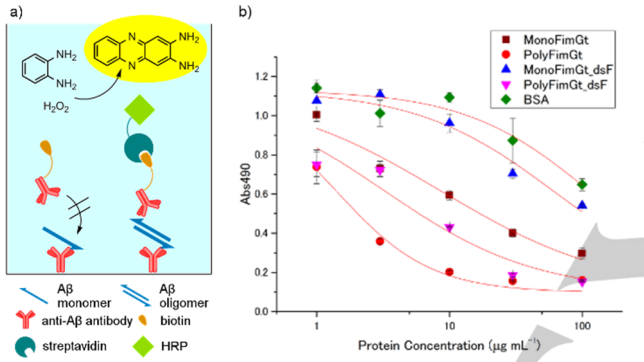

Figure 3. a) The schematic representation of the sandwich ELISA to detect oligomeric Aβ. b) The semi-log plots of Aβ oligomer quantified by ELISA against the concentrations of the various proteins tested. Fitting was performed to determine IC50 values of each protein against oligomerization of Aβ.

As expected, MonoFimGt showed potent inhibition of Aβ oligomerization (IC50 = 8.7 ± 1.6 μg/mL; Table 1), whereas BSA showed only weak inhibition (IC50 = 114 ± 32 μg/mL; Table 1). To examine the effect of the hydrophobic β-sheet groove of MonoFimGt, a self-contained analogue, MonoFimGt_dsF, was also prepared that had a donor strand of FimF at the C-terminus (Figure 1). The hydrophobic β-sheet groove of MonoFimGt_dsF is occupied by the donor strand of FimF.[15] The CD spectra (Figure 2b) and the hydrodynamic diameter (Figure S1) of MonoFimGt_dsF showed similar results to those of FimGt. The IC50 of the self-contained analogue MonoFimGt_dsF is far higher (IC50 = 61 ± 11 μg/mL; Table 1) than that of MonoFimGt. The weak activity of MonoFimGt_dsF when compared with the reference protein BSA is probably due to its β-sheet surface, which was previously reported to interact with Aβ.[9a, 9b] Surprisingly, it turned out that PolyFimGt and PolyFimGt_dsF both showed more potent anti-oligomerization activity, with their IC50 values being 1.4 ± 0.2 and 3.3 ± 0.2 μg/mL, respectively (Table 1). These inhibitory activities are superior to that of the designer GFP mutant (IC50 >10 μg/mL).[9a] The TEM Image of Aβ1-42 after incubation for 30 hr in the presence of PolyFimGt showed no appreciable formation of amyloid fibrils. This also supports inhibition of Aβ oligomerization by PolyFimGt.

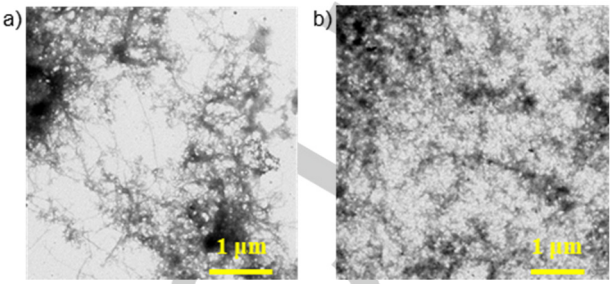

Figure 4. TEM images of Aβ1-42 in the absence (a) and presence (b) of PolyFimGt. Samples were incubated for 30 hr.

Table 1. IC50 values of the tested proteins against formation of Aβ oligomer.

	FimGt		FimGt_dsF		BSA
	Mono	Poly	Mono	Poly	
IC50 (μg mL^{-1})	8.7 ± 1.6	1.4 ± 0.2	61 ± 11	3.3 ± 0.9	114 ± 32

The enhanced potency of MonoFimGt compared with that of MonoFimGt_dsF may come either from the binding of Aβ to a hydrophobic β-sheet groove as we designed (Figure 1) or from partial denaturation to produce a highly potent form of PolyFimGt during the assay. To investigate this, MonoFimGt was first incubated at either 4 or 20°C for 12 h (the same incubation time used in the ELISA experiments) and then subjected to ELISA analysis. If part of MonoFimGt transformed to PolyFimGt during this pretreatment, the incubated sample would be expected to change the CD spectra and particle size, and the activities would be expected to show a dependence on the preincubation temperature. However, the CD spectra and particle sizes did not differ significantly between the samples preincubated at the two temperatures (Figure S3 in the SI). Additionally, when MonoFimGt was incubated at 20°C prior to the ELISA experiment, a comparable IC50 value to that recorded upon incubation at 4°C was observed (Figure S4 in the SI). These results indicate that the high potency of MonoFimGt is not due to its structural conversion into PolyFimGt during incubation at 20°C, but rather to an intrinsically high potent inhibitory activity against Aβ oligomer formation, probably because of its β-sheet groove. It should be noted that when MonoFimGt was incubated at 50°C before the ELISA assays, the IC50 improved (Figure S4) and the CD spectra and particle sizes changed dramatically (Figure S3), indicating its structural shift toward PolyFimGt.

The high potency of PolyFimGt and PolyFimGt_dsF indicate that they have binding sites for the Aβ monomers in spite of their random structures. We think that some parts of the unfolded protein chains serve as 'glue' to keep the unfolded proteins together, with the remaining β-sheet domains serving as binding sites for the Aβ monomer (Scheme 2). Indeed, we have confirmed that dansylated Aβ was coeluted with PolyFimGt in the gel filtration chromatography (Figure S5). We speculate that PolyFimGt possessing incomplete β-sheet domains is in a metastable state that provides higher valence or affinity to Aβ monomer. For example, if multiple sites per protein chain could

bind to the Aβ monomer, the effective concentrations of Aβ binder of PolyFimGt and PolyFimGt_dsF would be higher than that of MonoFimGt, affording lower IC50 values. Although further experiments to corroborate our rationale for the high potency of these proteins are required, these results clearly show that the unfolded protein is more effective for inhibiting Aβ oligomerization than the correctly folded protein.

In conclusion, we hypothesized that a protein scaffold with a β-strand acceptor site is able to inhibit Aβ aggregation and, indeed, demonstrated it using MonoFimGt, which is a designer protein based on that found in the DSC system of the bacterial fimbriae. The structural fold of this protein was stable during the assay and the self-contained analogue, MonoFimGt_dsF, was less potent. These results support the conclusion that the vacant hydrophobic β-strand binding groove effectively binds to the Aβ monomer as expected. Our results thus provide an additional strategy through which to inhibit Aβ aggregation. We anticipate that this approach can be extrapolated to other proteins with similar structures, in particular, Ig-like fold proteins. They include those of human origin, which can be exploited more safely due to their low immunogenicity. Moreover, our method may be applied to the development of drugs that can be used against amyloid-related diseases other than AD.[6] So far our experiments are limited to the molecular assays, but it is important to investigate the efficacy of our protein agents to live cells to move on to the next stage. In that case, we have to take into consideration the interaction of Aβ and our proteins with cellular membranes as they play a pivotal role for the pathogenesis of AD.[19]

In the course of our experiments, we also found that the soluble but denatured form of the same proteins, PolyFimGt and PolyFimGt_dsF, showed even higher potencies. Although the mechanistic details are still unclear, the high inhibitory activity of these protein agents may be because they acquire higher concentrations of β-strand binding sites by their intermolecular self-assembly. We are now investigating the structural factors that determine effective binding to the Aβ monomer and that enable solubilization of these unfolded protein aggregates. We are also searching for other proteins that behave as amyloid inhibitors in their denatured forms. Protein nanoparticles prepared from denatured proteins have previously been reported to act as drug delivery carriers,[20] whereas our proteins inhibit the pathogenesis directly. We hope our results broaden the range of possible applications of denatured proteins in medical applications.

Supporting Information Summary

Experimental section including preparation of Aβ1-42 and dansylated Aβ1-40, expression and purification of proteins and mutants, additional figures of gel flirtation column chromatography, circular dichroism (CD), dynamic light scattering, transmission electron microscopy (TEM), and ELISA experiments.

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Keywords: Alzheimer's disease • Amyloid beta-peptide • Denatured proteins • Fimbrial proteins • Inhibitors

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