Review

CellPress

Biotechnologically engineered protein binders for applications in amyloid diseases

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The aberrant self-assembly of polypeptide chains into amyloid structures is a common phenomenon in several neurodegenerative diseases, systemic amyloidosis, and 'normal' aging. Improvements in laboratory-scale detection of these structures, their clinical diagnosis, and the treatment of disease likely depend on the advent of new molecules that recognize particular states or induce their clearance *in vivo*. This review will describe what biotechnology can do to generate proteinaceous amyloid-binders, explain their molecular recognition mechanisms, and summarize possibilities to functionalize further these ligands for specific applications.

Diagnosis and therapy of amyloid diseases

Amyloid fibrils are fibrillar polypeptide aggregates with a cross-β structure. They are products of a complex self-assembly reaction that involves multiple intermediate states. Fibril structures can also be significantly polymorphic. Amyloid occurs abnormally inside the human body when associated with 'normal' aging or as additional complications of diseases such as type II diabetes or atherosclerosis (Table 1). In some cases, however, amyloid can also be a basis of disease. The most clear-cut cases in this context come from the group of diseases categorized as systemic amyloidosis, which are defined by massive amyloid depositions in multiple organs, such as heart, liver, spleen or kidneys [1]. Amyloid may also underlie numerous pathologies in brain diseases, including Alzheimer's (AD) and Parkinson's (Table 1). The lack of cures for brain amyloid diseases are major unmet medical needs. Treatment of systemic amyloidoses often involves drastic medical interventions such as chemotherapy and liver or heart transplantation. Pharmacological options to inhibit fibril formation directly are scarce [2]. It would be generally desirable to utilize new molecules to improve early diagnosis and facilitate whole-body imaging for monitoring disease and the effectiveness of treatments within living patients.

Considering the development of potential therapies, it is important to know which structural state of amyloid is responsible for disease. Amyloid fibrils, the terminal state of assembly, are directly responsible for numerous pathologies in systemic amyloidosis [1] and cerebral amyloid angiopathy [3] in which large-sized fibril deposits physically distort and impair the affected tissue. This situation differs from many brain amyloid disorders, in which intermediate states of fibril formation exert toxic effects or impair the ordered functions of neurons. Although intermediates may seem, in these cases, to be the prime targets for intervention, it should be kept in mind that the deposition of fibrils as plaques likely contributes to disease. It is also possible that the dissociation or fragmentation of fibrils into protomers or intermediates may promote a local increase in the concentration of amyloid intermediates around neurons, resulting in altered or impaired metabolic activity [4].

In these applications there is great potential for using biotechnology to tailor proteins or peptides that will selectively bind to disease-associated amyloid (referred to here as 'amyloid-binders'). In this review we outline strategies for engineering a protein or peptide scaffold to bind to amyloid with high affinity. We then explain the underlying molecular recognition mechanisms and explore options for functionalizing these binders for applications in amyloid diagnosis, intervention, or basic research.

Strategies to generate binders based on globular protein scaffolds

Amyloid-binding can be achieved using either globular proteins or peptides as scaffolds. Most binders with a globular protein scaffold are based on antibodies, including antigen-binding fragments (Fabs), single-chain variable

Glossary

Affibody: an artificial binding protein that is based on a modified domain (Z-domain) of staphylococcal protein A [5].
Gammabody (grafted amyloid-motif antibody): engineered human heavy-chain variable region (V_H) domain where a short amino acid stretch of an amyloidogenic protein or peptide was inserted into a complementarity determining region (CDR) loop [12].
Intrabody (intracellular antibody): an antibody or an antibody fragment that can bind to its antigen under reducing conditions, such as within the cellular cytosol [11].
Inverse/inverso peptide: D-amino acid analog of an L-amino acid peptide [25].
Nanobody: synonym for the variable (V_HH) domain of heavy-chain antibodies that occurs in camels and some other species [6].
Retro-inverso peptide: D-amino acid analog ('inverse' peptide) of an L-amino acid peptide in which the N-terminal to C-terminal amino acid sequence order is also reversed [40].

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Keywords: protein misfolding; Alzheimer's disease; Parkinson's disease; antibody.

0167-7799/
 http://dx.doi.org/10.1016/j.tibtech.2014.08.004

Table 1. Amyloid fibril diseases and fibril precursors [74,75]

Condition	Fibril-forming polypeptide chain
Neurodegenerative diseases	
Alzheimer's disease	Aβ (β-amyloid) peptide
	Tau protein
Parkinsons's disease	α-Synuclein
Creutzfeldt–Jakob disease	Prion protein
Huntingtons's disease	Huntingtin fragments
Systemic amyloidosis	
AA amyloidosis	Serum amyloid A protein or fragments
AL amyloidosis	Immunoglobulin light chains or fragments
ATTR amyloidosis	Transthyretin or fragments
Conditions commonly associated with amyloid deposition	
Type II diabetes	Islet amyloid polypeptide (amylin)
Atherosclerosis	Apolipoprotein AI
Aging	Transthyretin
	Medin (fragment of lactadherin)
Cardiac arrhythmias	Atrial natriuretic factor

fragments (scFv), and the variable heavy (V_HH) domains from camelid heavy-chain antibodies, sometimes also termed nanobodies (see Glossary). Other protein scaffolds such as protein A-based affibodies have also been used [5].

Biotechnological binders of this type are usually generated by selection from recombinant libraries, but mixed approaches can also be found that combine animal vaccination with phage display [6,7]. Selection provides the possibility to fine-tune particular binding properties. Complementarity-determining region (CDR) diversification and application of subsequent panning rounds can optimize affinity, as was shown for the gantenerumab antibody. This biosynthetic immunoglobulin consists of a crystallizable fragment and a Fab that was originally obtained by phage display from the human HuCAL® Fab1 phage library and subjected to further panning rounds to enable binding of AD Aβ(1–40) peptide fibrils [8]. Another strategy is the application of a competitive selection environment that aims to improve selectivity. This strategy exposes the binder library to two different types of antigens. One type of antigen is immobilized and serves as the target; in other words, the desired binder must recognize this antigen. The other type of antigen (competitor) is soluble in the supernatant, and the desired binder must not cross-react with it. Because the competitor is removed during panning, together with the binders it interacts with, it is possible to select more efficiently binders that recognize the immobilized target antigen but show little interactions with the competitor. This method was used to obtain the V_HH domains B10 and KW1, which discriminate between different conformations of Aβ(1–40) peptide [9,10]. Other selections were adjusted to obtain binders that would serve under specific conditions, such as in the reducing environment of the cellular cytosol. Examples hereof are scFv-based intrabodies, which were obtained through a yeast two-hybrid approach and can bind to Aβ(1–42) baits within the reducing yeast cytoplasm [11].

Rational methods have generated only a few amyloid-binding proteins, such as grafted amyloid-motif antibodies (gammabodies) [12]. Insertion of segments 6–10 amino acids in length of an amyloid-forming protein into the CDR 3 of a variable heavy (VH) domain of a human antibody enabled recognition of the parent protein from which the segment was derived [13]. The great strength of this concept is its versatility and ease of use with different amyloidogenic proteins.

Strategies to generate peptide-based binders

Unlike the case of globular protein based scaffolds, most peptide-based binders have been generated by rational design. The perhaps most widely used method to generate affinity for amyloid is based on observations that peptide fragments from fibril-forming β-strand segments have affinity for their parent protein, similarly to a gammabody. This method was pioneered for Aβ peptide and its highly amyloidogenic KLVFF sequence element [14], but has subsequently been used for many amyloidogenic sites within Aβ and non-Aβ amyloid systems [15,16]. Binders seem to particularly effective if they include specific sequence motifs [17] or aromatic residues [18], or if they are multivalent, as was demonstrated for a branched dendrimer encompassing four KLVFF elements [19].

General problems with the use of peptides inside the body are their short serum half-lives and sensitivity to proteolysis. Many studies addressed this problem by using D-peptides, which are poor protease substrates. D-peptides have been synthesized by conversion of L-peptides into their D-amino acid analogs while retaining their linear amino acid sequences (inverse peptides) [20]. In addition, retro-inverso peptides have been generated, which are inverse (i.e., D-amino acid) peptides in which the N-terminal to C-terminal amino acid sequence order is also reversed [21]. Both methods only require sequence information, which is usually available.

More challenging is the computational design based on complementary binding to an amyloid structure. Only few atomic structures are so far available for full-scale amyloid fibrils or intermediates that would provide a robust basis for such methods. Steric zippers represent a unique solution to this problem because they are crystalline cross-β assemblies of peptide fragments from amyloidogenic proteins [22]. The two cross-β sheets of a steric zipper interact in a self-complementary fashion, usually by interdigitation of their amino acid side chains [22]. Solved atomic structures are now available for many amyloidogenic zippers and have successfully enabled the rational generation of D-peptides with anti-amyloid activity [23].

Peptides obtained by selection procedures are less common, but they do exist. Examples are the p5 peptide, which was obtained by phage-display selection against glycosaminoglycan (GAG) amyloid secondary components [24], as well as the D1 and D3 peptides, which were generated through D-mirror-image phage display [25]. This elegant method uses biotinylated D-Aβ(1–42) peptide as a target such that conversion of the selected peptide into its D-amino acid counterpart enables binding to the natural L-amino acid Aβ and also provides the general advantages of D-peptides.

Specificities and molecular recognition mechanisms

Several biotechnological binders have provided information about their underlying molecular recognition mechanisms.