Review

Mechanisms of amyloid proteins aggregation and their inhibition by antibodies, small molecule inhibitors, nano-particles and nano-bodies

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ABSTRACT

Protein misfolding and aggregation can be induced by a wide variety of factors, such as dominant disease-associated mutations, changes in the environmental conditions (pH, temperature, ionic strength, protein concentration, exposure to transition metal ions, exposure to toxins, posttranslational modifications including glycation, phosphorylation, and sulfation). Misfolded intermediates interact with similar intermediates and progressively form dimers, oligomers, protofibrils, and fibrils. In amyloidoses, fibrillar aggregates are deposited in the tissues either as intracellular inclusion or extracellular plaques (amyloid). When such proteinaceous deposit occurs in the neuronal cells, it initiates degeneration of neurons and consequently resulting in the manifestation of various neurodegenerative diseases. Several different types of molecules have been designed and tested both *in vitro* and *in vivo* to evaluate their anti-amyloidogenic efficacies. For instance, the native structure of a protein associated with amyloidosis could be stabilized by ligands, antibodies could be used to remove plaques, oligomer-specific antibody A11 could be used to remove oligomers, or prefibrillar aggregates could be removed by affibodies. Keeping the above views in mind, in this review we have discussed protein misfolding and aggregation, mechanisms of protein aggregation, factors responsible for aggregations, and strategies for aggregation inhibition.

1. Introduction

Folding of a globular protein represents a complex process that involves a series of partially folded intermediates with increasing content of ordered structure and eventually gives rise to a unique 3D-structure, which, for ordered proteins, typically represents biologically functional conformation. For a polypeptide chain undergoing such a self-organization process, there is a competition between folding and misfolding/aggregation; *i.e.*, acquiring a fully folded functional conformation or formation of aggregates (Fig. 1). Protein misfolding in a cell can occur because of a highly crowded cellular environment. To avoid this fate, specialized proteins known as molecular chaperones are called upon for assisting proper folding, prevention of aggregate formation, disaggregation, and unfolding of misfolded species. However, if protein persists in the misfolded state, it is subjected to proteasomal degradation to maintain cell homeostasis. Despite this rather efficient cellular protein quality control, proteins often misfold under various circumstances. For instance, it can be induced by a wide variety of factors, such as dominant disease-associated mutations, changes in the environmental conditions (pH, temperature, ionic strength, protein concentration, exposure to toxins, exposure to transition metal ions, such as Zn^{2+} and Cu^{2+}) [1–7] and posttranslational modifications (PTMs) including glycation, phosphorylation, and sulfation [8,9]. In a similar vein, an increase in the rate of degradation, errors in trafficking, loss of binding partners, interaction with wrong partners, and oxidative damage may also produce aggregation. These factors can act either independently of each other or simultaneously [10]. The resulting misfolded intermediates interact with similar types of intermediates and

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consecutively form dimers, oligomers, protofibrils, and fibrils (or stay in a self-oligomerized form, or form amorphous aggregates). Protein misfolding and aggregation are associated with a group of diseases, known as amyloidosis, which recently have caught a great deal of attention. Amyloid formation is thought to be an intrinsic property of a polypeptide chain in general. Furthermore, it seems that the amyloid β-fold is an evolutionarily conserved structure. In amyloidosis, fibrillar aggregates are deposited in the tissues either as intracellular inclusions or extracellular plaques (amyloid). When such proteinaceous deposition occurs in the neuronal cell, it initiates degeneration of neurons and consequently results in the manifestation of various neurodegenerative diseases shown in. Interestingly, although it is evident now that amyloid fibrils are pathological entities, accumulating evidence indicates that some amyloids might also play functional roles. For example, such functional amyloids participate in melanosome biogenesis, long-term memory formation, and transfer of conformation-based information [11]. Furthermore, it has been discovered that proteins with prion-like domains may play an important role in functional liquid-liquid phase transitions associated with the biogenesis of various membrane-less organelles, including stress granules [10,12].

Mechanism of amyloid fibril formation itself provides clues for the potential approaches for the inhibition of protein aggregation. Therefore, several opportunities for therapeutic interventions of pathological aggregation are available for the treatment of neurodegenerative maladies [13]. For example, the native structure of an ordered globular protein could be stabilized by ligand binding. Monomeric protein/peptide can be sequestered by binding to protein molecule thereby preventing further aggregation. Alternatively, protein aggregates can also be dissolved by an engineered antibody mimetics, antibody proteinssmall, highly stable engineered proteins capable of binding to a large number of target proteins or peptides with high affinity, thereby imitating monoclonal antibodies. Furthermore, blockage of fibril formation, disaggregation of fibrils, and plaque removal are other alternative options available for therapeutic interventions. These approaches might represent potential means for partial treatment and partial reversal of at least some neurodegenerative diseases. To this end, several different types of molecules have been designed and tested both *in vitro* and *in vivo* to evaluate their anti-amyloidogenic efficacies. In this regard, conformational antibodies, affibodies, natural polyphenols, small organic molecules, nano-particles, and nano-bodies have been shown to potently inhibit plaque formation, dissolve oligomers, or pre-fibrillar aggregates and disrupt the amyloid fibrillation of proteins [14–35]. Keeping the above views in mind, in this review we have discussed protein misfolding and aggregation, mechanisms of protein aggregation, factors responsible for protein aggregation, and strategies currently used for aggregation inhibition.

2. Misfolding and aggregation of protein

In vitro and *in vivo* studies have led to the conclusion that it is partially unfolded or misfolded intermediates, which can be accumulated at high concentrations, are particularly prone to aggregation [36–40]. Furthermore, factors that may either increase the hydrophobicity or decrease the net charge of a polypeptide chain or increase its β-sheet contents can give rise to such misfolded intermediates. Misfolded protein has comparatively more solvent exposed hydrophobic patches as compared to the native globular proteins, in which the vast majority of hydrophobic residues are buried inside the rigid core of a protein molecule. The presence of solvent-exposed hydrophobic patches on the surface of misfolded proteins mediates hydrophobic interactions leading to the formation of dimers, oligomers, protofibrils, and finally amyloid fibrils structure (reviewed in [41]) (Fig. 2). Alternatively, such misfolded proteins can stay in various oligomeric forms or can form amorphous aggregates. Amyloid fibrils are highly ordered structures with the characteristic cross-β-sheet structural motifs. In such cross-β conformation, β-sheet structure runs perpendicular to the fibril axis. Amyloid fibrils formed by many proteins are 7–12 nm in diameter [39,42], and the major β-sheet region of amyloid fibrils are typically formed by hydrophobic residues [40,43].

Numerous studies have provided some clues about molecular mechanisms of fibrillogenesis, which typically involves secondary structure transition from α-helical or irregular structure to β-sheet structure [44,45]. The resulting protein aggregates were found to be deposited in neurons, promoting degeneration of neurons, a prerequisite condition for dementia or mental retardation or other neurological dysfunctions in neurodegenerative diseases [46]. Currently, accumulating evidences suggest that amyloid oligomers or amyloid intermediates are more toxic than mature fibrils that comprise the major constituents of the proteinaceous deposits in these diseases. Furthermore, it has been discovered that different types of neurodegenerative
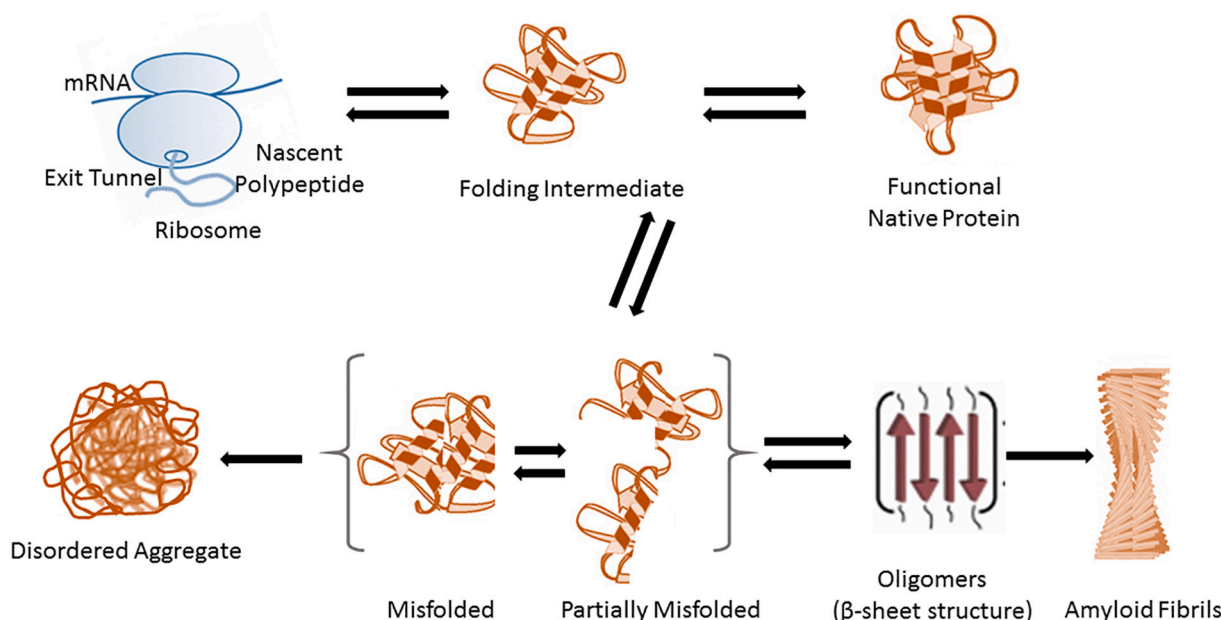

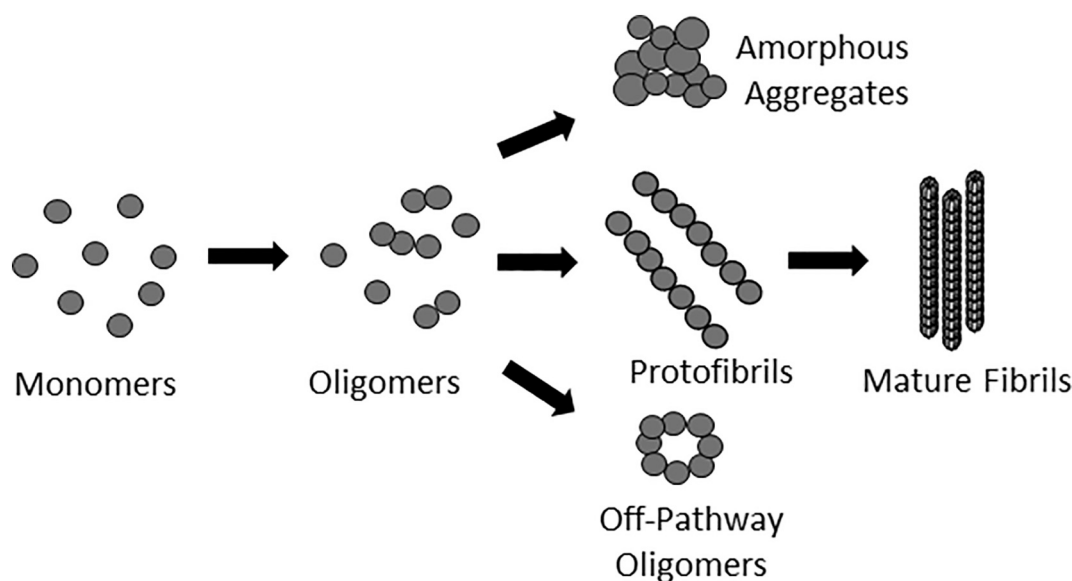**Fig. 1.** Competition between protein folding and aggregation.

Fig. 2. Protein aggregation phenomenon and its associated consequences.

diseases occur *via* different mechanisms [47–52], with the pathological aggregates being further classified into two distinct structural groups: random amorphous structures and well-ordered amyloid fibril structures [53].

Recently, the atomic-level structure of oligomers formed by the amyloid-β Aβ$_{42}$ protein was solved using site-directed spin labeling and electron paramagnetic resonance techniques. Analysis of oligomer structure revealed that each Aβ$_{42}$ molecule forms a single β-sheet structure with three β-strands showing antiparallel arrangement [54-55]. Each of the β-sheet structure consists of four Aβ$_{42}$ molecules which are arranged in a head-to-tail fashion, and these four β-sheets are packed together in a face-to-back arrangement, and the identical segments between different β-sheets are stacked together to form oligomers. An atomic model of an Aβ$_{42}$ amyloid fibril was also solved by solid-state nuclear magnetic resonance (ssNMR) highlighting the difference between reported Aβ$_{40}$ and Aβ$_{42}$ fibrils [56]. Structure of an Aβ$_{42}$ fibril composed of two intertwined protofilaments was determined by cryo-electron microscopy (cryo-EM) complements the ssNMR [57].

The individual subunit forms LS-shaped topology and the dimer interface protects the hydrophobic C termini from the solvent. These prefibrillar oligomers can be converted to fibrils by strand rotation by 90° along the strand direction

3. Mechanisms of protein aggregation

In protein aggregation, misfolded intermediates interact with similar types of intermediates and consecutively form dimers, oligomers, protofibrils, and fibrils. However, as it was mentioned earlier, fibrillation does not represent the only aggregation pathway, and some aggregating proteins can also form various oligomeric structures or can assemble into amorphous aggregates. Several mechanisms of protein aggregation have been proposed, including nucleation-elongation polymerization, the reversible association of native monomers, aggregation of conformationally altered monomers, and surface-induced aggregation. These different mechanisms of protein aggregation can lead to the formation of diverse arrays of fibrillar structures or polymorphisms [58]. Additional polymorphisms could also arise when the same polypeptide chain adopts morphologically different structures.

3.1. Nucleation-elongation and polymerization

The nucleation-elongation process of protein fibrillation is divided into three consecutive steps: lag-phase, exponential phase, and saturation phase. The lag-phase is thermodynamically disfavored, and at this stage, nucleus is formed. The second stage is known as exponential phase, where polymerization and fibril growth occurs. The third stage is described as saturation phase, in which no further elongation takes place and where essentially all soluble species present in the solution are converted into mature fibrils *via* lateral association [59,60] (Fig. 3). The nucleation-elongation polymerization mechanism has been observed for Aβ, α synuclein, islet amyloid polypeptide precursor (IAPP, also known as amylin), and prion aggregation [61].

3.2. Self-assembly of monomeric protein

According to this mechanism, native protein has an inherent tendency to self-associate reversibly because their surfaces are self-complementary in nature, which facilitates interactions with similar complementary monomeric protein to form small reversible oligomers. During the aggregation reaction, these small oligomers form larger oligomers with an increase in protein concentrations, and over a period of time, they can form protofibrils and finally mature fibrils (Fig. 4). Earlier, it was proposed that (partial) unfolding of protein is not the only condition to form amyloid aggregates. In fact, it has been observed that some native monomeric proteins are also capable of associating side-by-side or end-to-end and eventually forms amyloid aggregates [56,62]. For instance, insulin a therapeutic protein easily associates to form reversible oligomers [57,58,63,64]. Similarly, interleukin-1 receptor antagonist (rhIL-1RA) is another type of protein that shows reversible dimerization and trimerization at high peptide concentrations [65].

3.3. Conformationally altered monomeric protein induces aggregation

As aforementioned, native monomers do not always have a high tendency to associate reversibly. Instead, it is a conformationally altered monomeric protein or a partially unfolded or misfolded protein, which has a much higher propensity to form protein aggregates than the native globular protein (Fig. 5). In this mechanism, the first step involves destabilization of the native protein structure by some stress, which includes heat or pH, or pressure, or some other environmental effectors that consequently induce the native monomeric protein to adopt an altered conformation, which is much more susceptible to aggregation. Interferon-γ and granulocyte colony-stimulating factor (G-CSF) are among the examples of proteins that undergo amyloidosis through this
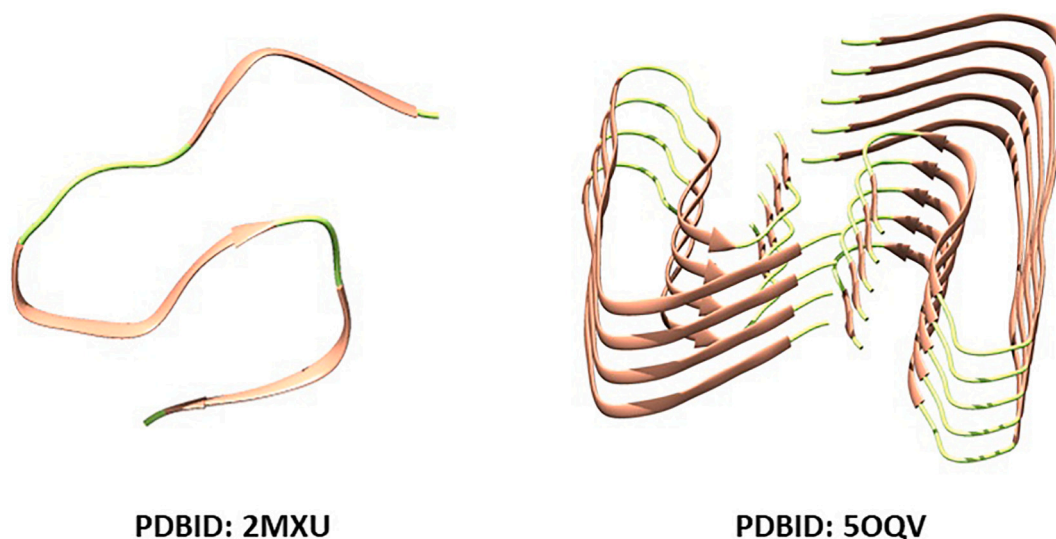
PDBID: 2MXU   PDBID: 5OQV

Fig. 3. Nucleation elongation polymerization.

Native Monomer   **Critical Nucleus**   **Protein Aggregate**

Fig. 4. Self-assembly of monomeric protein.

Monomeric Native Protein   **Surface Available (for interaction) & Air-Liquid medium**   **Partial Unfolding/Adsorption**   **Aggregation of Conformationaly Altered Non-native protein**
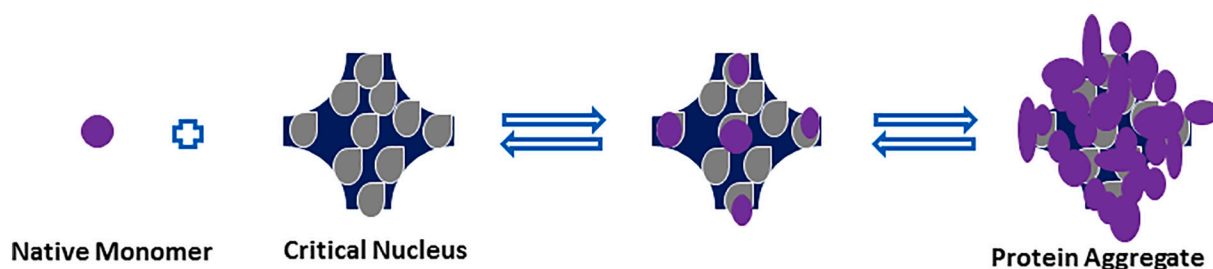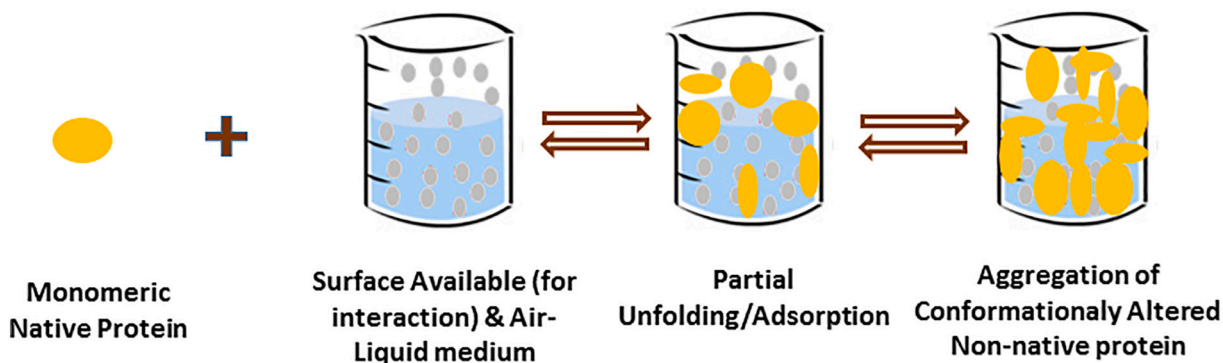
Fig. 5. Aggregation of conformationally altered monomeric protein.

mechanism [66–68]. However, using ^{19}F NMR it was shown that alternative mechanisms are also possible, since IAPP was able to fibrillate without an appreciable build-up of non-fibrillar partially folded intermediates [69].

3.4. Surface-induced protein and peptide aggregation

In surface-induced aggregation mechanism, native monomers bind to the surface of the container or flask or interact with a water-air interface. Once this initial reversible binding event occurs, the monomer undergoes a change in conformation, which facilitates interactions of this misfolded molecule with other monomers, thereby forming aggregates (Fig. 6). Aggregation perhaps may also occur once an altered monomer is released back into the solution. However, it is still not clear

what type of extrinsic factor actually stimulates surface-induced aggregation. Recently using atomic force microscopy (AFM), it was shown that on-surface aggregation takes place at a dilute concentration at which aggregation does not occur in solution [70]. For instance, the experiments were performed with the full-length Aβ protein (Aβ$_{42}$), α-synuclein, and a decapeptide Aβ$_{14\text{-}23}$ [70]. The result revealed that both proteins and peptide fragments showed a dramatic preference for the on-surface aggregation pathway compared to the aggregation in the bulk solution. This mechanism also occurs *in vivo*, because it has been observed that interactions between amyloidogenic polypeptides and cellular surfaces serve as important sources for the manifestation of neurodegenerative disease symptoms [70].
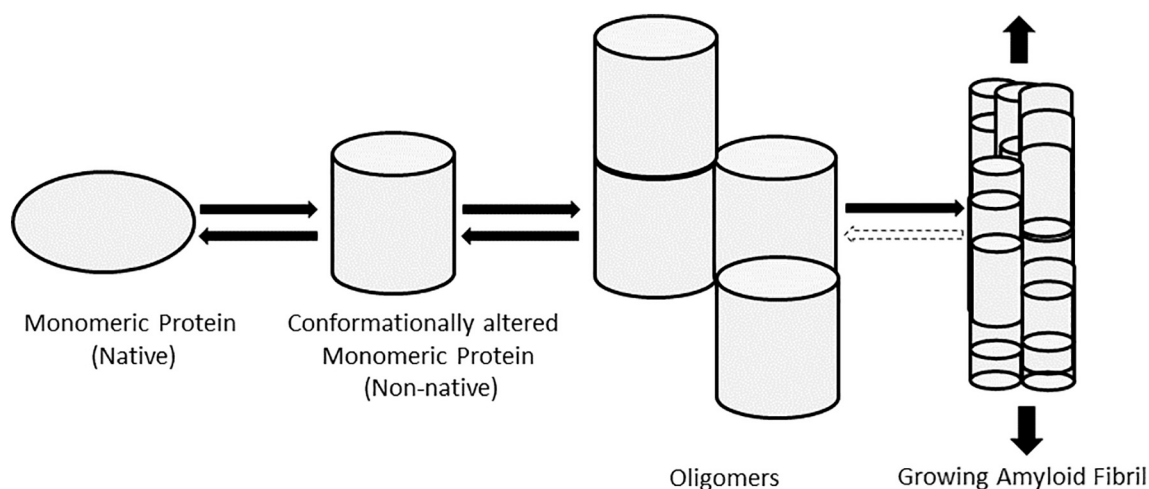
Fig. 6. Surface-induced aggregation.

3.5. Role of membrane in protein aggregation

Studies have shown that membranes also play an important role in amyloid protein aggregation and neurotoxicity binding that culminates in the neurodegenerative diseases [71–73]. In one study, it was shown that the Aβ peptide initially adopts a partially folded structure upon binding to zwitterionic lipid bilayers, and this partially folded species can consequently form amyloid aggregates. Similarly, α-synuclein fibrillation involves formation of a partially folded amyloidogenic intermediate [77], and such intermediate can also be formed at interaction of this protein with the membranes [75,76]. Furthermore, lipid curvature of the membrane was shown to play a role in specific interaction of the membrane with the amyloidogenic proteins. For example, in micelles containing regions of high and low membrane curvature, nontoxic IAPP was preferentially bound to the regions of low curvature, whereas toxic form of this protein preferred interaction with the regions of high curvature [77]. Importantly, this led to modulation of lipid bilayer structure of membrane. For example, several toxic versions of the IAPP peptide were shown to efficiently promote the formation of negative curvature in lipid bilayers, eventually leading to membrane disruption, whereas the nontoxic forms of this protein did not show such possibility [77,78]. Similarly, a recent paper showed that upon binding to cell membrane, Aβ peptide disrupted the membrane structure and dispersed PE lipid components, which are the common constituents of the cell membranes [80]. However, the exact mechanisms of the membrane disruption by amyloidogenic proteins and peptides are not yet fully understood, although several studies have indicated that potential mechanisms might include the formation of transmembrane oligomeric pores [81–84] leading to the non-specific ion permeation, or a detergent-like membrane dissolution [85]. Recently, mechanistic insights into the roles of pathologically thin bilayers that may be easily disrupted during Aβ have also been mentioned [86]. In this mechanism, under pathological conditions, lipids of the membrane can be oxidized, which might facilitate Aβ misfolding and aggregation leading to further disruption of the membrane structure [86]. These observations suggested that various mechanisms related to the interactions between amyloidogenic proteins and membranes might have implications in neurotoxicity and neurodegenerative diseases.

4. Factors involved in protein aggregation

Protein aggregation can be induced by wide variety of factors, including different *in vitro* and *in vivo* factors. *In vitro* environmental factors affecting protein aggregation include temperature [87], pH 88, presence of co-solvent [89], metal ions [90], freezing and thawing

[85,91], agitation stress [92] and surfactants [93]. These factors can partially unfold the protein or increase the propensity of protein to form partially folded intermediates, leading to protein aggregation. On the other hand, during the protein biosynthesis *in vivo*, several chaperones work to ensure the proper and efficient folding of the nascent polypeptide chain. Since chaperone functions are sequence-specific, any changes or mutations in the chaperone polypeptide chain may prevent or modulate its binding to target protein and thereby abort its function as a helper of proper protein folding. This inability of chaperone to fold a polypeptide chain leads to its partial folding or misfolding or improper folding and consequently results in the formation of aggregates [94]. Furthermore, defects in the protein synthesizing machinery (such as improper translation, wrong incorporation of amino acid, *etc.*) might also serve aggregate producers [95]. Distortion of other factors related to normal proteostasis can also serve as aggregate producers. For instance, during aging, the ability of cellular proteasome to eliminate impaired or misfolded proteins is reduced, leading to build up of the misfolded proteins and their aggregation that eventually have fatal consequences.

5. Methodologies for inhibiting the aggregation reaction

Because of the highly crowded cellular environment, proteins have limited ability to refold into their native state, which increases the propensity of proteins to form aggregates. Below, we describe the pathways that can prevent the formation or accumulation of aggregates. Despite intense research, the mechanisms of inhibition of protein aggregation and deposition are poorly understood. However, several formulations or drug molecules have been designed that are proved to be effective against amyloid fibrillation process and have potential to either prevent the aggregate formation or to some extent reverse the process of protein aggregation itself [98–100]. As mentioned above, mechanism of protein aggregation itself tells us about the mechanisms of inhibition therefore, inhibitors designed based on these concepts are potent modulators of the pathological self-assembly of proteins. For example, the native structure of an ordered protein can be stabilized by ligand binding, thereby rescuing protein from partial unfolding and misfolding; aggregates can be cleared by sequestering plasma Aβ by engineered antibody; fibrils can be blocked or disaggregated by small molecule inhibitors, and plaques can be removed by passive immunization All of these approaches provide great hope for the future developments of novel and efficient anti-amyloid agents. Illustrative examples of these strategies are given below.

5.1. Native state stabilization

According to the thermodynamic hypothesis of the protein folding-unfolding equilibrium, molecule that specifically binds to the native state increases the population of this state relative to the populations of other states. Small molecules, or peptides or other proteins that can bind to the native form of a target protein can therefore constrain it from unfolding and aggregation (by increasing the formation of folded population and decreasing the rate of unfolding). One of the examples of successful utilization of this approach is given by the designed stabilizers of transthyretin (TTR), which is a tetrameric carrier of the thyroid hormone thyroxine and retinol-binding protein bound to retinol. The destabilizing mutations in TTR facilitate more facile dissociation and/or misfolding and amyloidogenesis of this protein giving rise to amyloid polyneuropathy (FAP), which is a rare systemic amyloid disease characterized by the endoneurial amyloid deposits, axonal degeneration, and neuronal loss [101]. In addition to FAP disease, misfolding and aggregation of TTR are linked to other amyloid diseases, such as senile systemic amyloidosis (SSA) [94,102] and familial amyloid cardiomyopathy (FAC) [103]. It was proposed that FAP can be treated by stabilizing TTR tetramer by small molecule inhibitors [104]. Based on this hypothesis, tafamidis meglumine (Vyndaq) was designed and synthesized. This TTR stabilizer was shown to efficiently inhibit TTR tetramer dissociation [105] and is used for the treatment of the wild type TTR cardiomyopathy or hereditary TTR-mediated amyloidosis. Phase II/III clinical trials have been successfully completed. Approval of this drug on May 3, 2019, indicated the beginning of a new era in clinical practice, the era of efficient anti-amyloid drugs.

5.2. Antibody and immunization using $A\beta_{42}$ peptide

Monoclonal antibodies raised by passive immunization against critical regions of the $A\beta_{42}$ protein were reported to improve the cognitive function and eliminated other pathological effects of amyloid in transgenic AD mouse model [106,107]. Active immunization with $A\beta_{42}$ in a transgenic mouse model overexpressing human APP also generated anti-$A\beta$ antibodies, which averted $A\beta$ plaque formation in young animals and moderated $A\beta$-related neuropathological changes, and improved the cognitive functions in old animals [100,108]. Therefore, immunotherapy targeting $A\beta$ has been particularly successful for both active and passive immunization. Currently, many antibodies have been designed that are in the late stages of clinical trials [109,110]. These antibodies *in vivo* show the peripheral sink hypothesis, which proposes that the binding of antibodies to $A\beta$ in blood alters the equilibrium and transport of $A\beta$ over the blood brain barrier (BBB), thereby, these antibodies cross the BBB and get into the CNS which might directly interfere with the aggregation process of $A\beta$. Furthermore, it has also been proposed that these antibodies can recognize amyloid fibrils and trigger clearance of plaque *via* the Fc-mediated phagocytosis [11,112]. Although immunotherapy was rather successful in animal models, trials in humans with active $A\beta$ immunization were stopped because of serious side effects including meningoencephalitis [113]. Active and passive immunization strategies with decreased side effects are currently under preclinical and clinical stages [109].

5.2.1. Nanobodies and nanoparticles

It is believed that AD can be treated by small molecule inhibitors. However, some of these inhibitors have an intrinsic drawback, in that they cannot pass through BBB. On the other hand, nano-particles (NPs) exploit the existing physiological mechanisms of passing through BBB including receptors and adsorptive-mediated transcytosis, which can facilitate the transcellular transport of small molecules and other drugs or markers from the blood to brain. Various NPs have been used in the treatment of neurodegenerative disorders [114]. In our earlier review, we have shown that small molecule inhibitor containing NPs, including the curcumin-loaded PBCA NPs decorated with ApoE3 ligands, showed low-density lipoprotein receptor (LDL-R) mediated transcytosis across the BBB and through the SH-SY5Y neuroblastoma cells 115. Inhibition of $A\beta$ mediated toxicity by ApoE3-functionalized nanobodies was evaluated and compared with free curcumin on SH-SY5Y cells. The results indicated a significant reduction (40% compared with free drug at 100 nM $A\beta$) of $A\beta_{42}$-mediated toxicity in cells treated with the nanobodies. This was also accompanied by a reduction of toxic reactive oxygen species formation [107,115]. Similarly, iron-chelating compounds have been recently incorporated into the NPs to facilitate BBB penetration with limited neurotoxicity [116]. These NPs, when conjugated to chelators, showed a unique ability to cross the BBB, chelate metals, and exit through the BBB with their corresponding complexed metal ions. This method is a safe and effective approach for reducing the metal load in neural tissues and thus averts the harmful effects of oxidative damage and its consequences in the brain of AD [108,116]. Recently, nanobodies raised against monomeric ɑ-synuclein inhibit fibril formation and destabilized toxic oligomeric species. Antibody fragments have proved to be effective anti-aggregation molecules. For example, camel single domain antibodies, generally referred as nanobodies, are extremely effective inhibitors of the aggregation reactions of several amyloidogenic proteins, including α-synuclein (α-syn) [109,117], $A\beta$ [110,118]. These antibodies have also been used as effective diagnostic tools for identifying different aggregation intermediates in fibrillation pathways [119]. Most recently, it has been shown that nanobodies could be generated by immunization or non-immunization methods and by synthetic libraries. These antibodies can also potentially inhibit each of the individual species formed on the pathway of fibril formation [120]. Their binding to the particular species effectively blocked fibril formation. Thus, these nanobodies in the future might prove as a promising therapeutic agent in the therapy of neurodegenerative diseases.

5.3. Specific inhibition of amyloid oligomers using conformation-dependent antibodies

In addition to amyloid fibrils, some pre-amyloid conformers accumulate in several neurodegenerative diseases. These amyloid oligomers share common structural features with fibrils and are now thought to be much more toxic entities than fibrils [121–124]. Intensive research is currently focused on creating therapeutics that target general amyloid folds, contrary to targeting specific proteins. In fact, oligomer-specific antibodies, A11, which is specific for amyloid oligomers of different origins, and fibril-specific antibody OC have been developed. These antibodies *in vitro* prevented toxicities of soluble oligomers and fibrils respectively. There is a hope that these antibodies might offer a great opportunity for the therapy of neurodegenerative diseases.

5.4. Inhibition of $A\beta$ aggregation by sequestering monomers

In this mechanism of inhibition, aggregation-prone regions of monomeric amyloidogenic peptides are sequestered by molecules, thereby inhibiting self-assembly or aggregation process of the $A\beta$ protein. In fact, novel topological folds that completely sequester aggregation-prone peptide regions have already been designed by protein engineering technology. For instance, a novel engineered affibody protein (ZAβ3d) has been designed that binds monomeric $A\beta$ peptide with nanomolar affinity [14,15]. This affibody protein ZAβ3d forms a disulfide-linked dimer, which engulfs $A\beta$ hairpin structure by burying aggregation-prone hydrophobic faces within a tunnel-like cavity of affibody dimer and consequently rescues protein from the aggregation process [15,16].

Recently, a small molecule (10074-G5) capable of binding and sequestering the intrinsically disordered amyloid-β (Aβ) peptide in its monomeric, soluble state has been designed. This compound interacts with $A\beta$ and inhibits both the primary and secondary nucleation pathways in its aggregation process [125].

5.5. In vitro inhibition of Aβ aggregation by affibody and dissolution of preformed aggregates

The aforementioned $Z_{Aβ3}$ affibody can not only inhibit the Aβ peptide fibril formation but is also capable of dissolving preformed aggregates of Aβ. For example, treatment of large oligomers (protofibrils) of $Aβ_{42}$ with stoichiometric amounts of affibody led to the dissolution of toxic oligomers. Kinetics analysis revealed that this affibody dissolved protofibrils on a time scale of several hours. Similarly, this affibody also dissolved the preformed fibrils very slowly indicating a high kinetic barrier.

Recently, affibodies drug including β-wrapins molecule has been designed. This affibody has been found to be effective in inhibiting the aggregation reaction of Aβ, α-syn, and IAPP [126–128]. The mechanism of action of β-wrapins drug involves stabilization of amyloidogenic proteins in β-hairpin conformations, leading to prevention of self-assembly process or facilitates the disaggregation of preformed oligomers [126].

5.6. Small-molecule inhibitors of Aβ peptide and α-Synuclein and their possible mechanisms of action

Large numbers of small molecule inhibitors have been reported to inhibit Aβ peptide aggregation. The structure-activity relationships of these inhibitors have also been systematically investigated. These studies revealed that specific Aβ aggregation inhibitors, such as Congo red (CR), chrysamine G (CG), and curcumin, share a similar chemical scaffold. These compounds contain two aromatic groups or inositol groups, which are separated by a backbone of the appropriate length. These two terminal aromatic groups containing linker region interact with different specific sub-regions of Aβ peptide. Determining which sub-region of the inhibitor binds will be the key to future research efforts. Numerous derivatives of CR and chrysamine G, are also effective inhibitors of huntingtin (HTT) aggregation process and act in a dose-dependent manner [129].

Other aggregation inhibitors, such as nordihydroguaiaretic acid and resveratrol are made up of two monolignols that are bridged by a straight or branched alkane chain. These inhibitors inhibit fibril formation by binding along the lateral surface of oligomers, thus preventing amyloid fibril formation laterally rather than preventing their growth by elongation along the fibril axis, indicating that their symmetric structure might be suitable for specific binding of Aβ-form species. Similarly, Aβ, resveratrol has been shown to depolymerize IAPP amyloid fibrils.

Flavonoids are another class of anti-amyloid agents. Analysis of the structures of flavones and their derivatives revealed that they have the same backbone structure, but differ in their inhibitory activities. In this regard, Akaishi et al. [35] have examined the effects of several structurally related flavones on Aβ fibril formation, as well as the structural requirements for anti-amyloidogenic activity. They have found that the 3,4-dihydroxyl group, but not the 3- or 7-hydroxyl groups of fisetin, a flavone, were important for the inhibition of Aβ fibril formation. In a similar vein, coumarin analogs were identified as novel inhibitors of Aβ aggregation. Ortega et al. [35] have found that 3-benzothiazol-2-l-7-diethylamino-4-(1H-tetrazol-5-yl)-chromen-2one was the most effective coumarin-based inhibitor and led to the conclusion that functional groups, such as benzothiazole and triazole, could improve the amyloid inhibitory efficacy. These functional groups recognized and bind amyloid species and inhibited the early and late stages of amyloid aggregation process.

A new class of small molecules based on the chemical structure of apomorphine has been designed that inhibit Aβ aggregation [130]. These molecules were found to inhibit $Aβ_{40}$ fibrillization reaction. Time-dependent electron microscopy studies demonstrated that apomorphine and its derivatives promoted oligomerization of Aβ but inhibited fibrillation process. Preliminary structural activity studies have shown that the 10, 11-dihydroxy substitutions of the D-ring of apomorphine are required for the inhibitory activity because upon methylation of these hydroxyl groups their inhibitory capacity was reduced [130]. Interestingly, the ability of these small molecules to inhibit Aβ amyloid fibril formation stems from their tendency to undergo rapid autoxidation, suggesting that autoxidation products either directly or indirectly inhibit Aβ fibrillization.

Naturally occurring polyphenols are found inabundance in fruits, vegetables, red wine, and tea. Polyphenols inhibit toxic Aβ oligomerization by several mechanisms including enhancing the clearance of Aβ42 monomer, modulating monomer–monomer interactions, and remodeling oligomers to non-toxic forms. Additionally, polyphenols modulate tau hyperphosphorylation and inhibit tau β-sheet formation. These polyphenols can serve as powerful preventive or therapeutic agents for the treatment of AD [131].

Numerous studies in the last decade have shown that (−)-epigallocatechin gallate (EGGG, a polyphenol found in green tea) both *in vitro* and *in vivo* inhibited and/or reduced the deleterious effects of oxygen-derived free radicals, associated with clinical disorders, such as PD, AD, and ALS [132]. Similarly, dietary intake of other polyphenols is also known to attenuate oxidative stress and reduce the risk of AD, PD, HD, stroke, and multiple sclerosis (MS) [133–135].

5.7. Novel inhibitors of protein aggregation

The thermal reversibility and thermal aggregation of human serum albumin (HSA) upon incubation with 3-β-hydroxybutyrate (3BHB) were determined by differential scanning calorimetry and free amine content assay. These results indicated that 3BHB binds the lysine residues of HSA through nucleophilic attack leading to the formation of covalent bonds. The calorimetric results showed that the modification of lysine residue by 3BHB resulted in partial unfolding of HSA and covalent modification of Cys34. Chemical modification of Cys34 led to stabilization of HSA structure and consequently decreased the thermal aggregation and increased thermal reversibility of modified has [136].

In another study, heat induction of HSA fibrillation incubated with different concentrations of SDS was evaluated using a variety of techniques. ThT fluorescence kinetic studies indicated that SDS at low concentrations induced the formation of fibrillar intermediates and where a polar group of SDS interacted with HSA through electrostatic interaction, whereas at high concentrations SDS inhibited fibrillation by solubilizing aggregates mediated by hydrophobic interactions. Contrary to these findings, cell culture studies showed that HSA when incubated with low concentrations of SDS, it inhibited neurite outgrowth of PC12 cells, whereas, at high concentrations, SDS had a lesser effect [137].

Recently, the ability of derivatives of the small organic compound noscapine, derived from the opium poppy, to inhibit fibrillation of the model protein insulin was shown. Several biophysical methods along with computational docking and cell viability studies were used to assess insulin stability and aggregation in order to identify the most potent inhibitor. The best aggregation inhibitor was found to be a phenyl derivative of N-nornoscapine which stabilized native insulin against thermal induced denaturation and also decreased insulin aggregate toxicity against human neuroblastoma SH-SY5Y cells. One important discovery of this study is that inhibitory effects were specific for insulin fibrillation because noscapine compounds did not inhibit fibrillation of other proteins including α-synuclein, Aβ, and FapC [138].

Most recently, the effects of paclitaxel (a polyphenol with a high tendency to interact with proteins) on fibrillation of insulin as a model protein was studied. The effects of paclitaxel on insulin fibrillation were determined by Thioflavin T fluorescence, Congo red absorbance, circular dichroism, and atomic force microscopy. These studies indicated that paclitaxel considerably hindered nucleation, and thereby inhibited fibrillation of insulin in a dose dependent manner. The isothermal titration colorimetric studies showed that the interaction between paclitaxel and insulin is spontaneous which means the reaction is exothermic. Furthermore, it was shown that paclitaxel reduced toxicity

effects of insulin fibrils on PC12 and consequently enhanced their survival [139].

In this study, the inhibitory effect of paclitaxel on lysozyme fibrillation was investigated with respect to thermal and colloidal stability. Fibrillation was monitored by several techniques including ThT fluorescence, circular dichroism, and AFM. Furthermore, paclitaxel-lysozyme interaction was studied using isothermal titration calorimetry and docking. Thermal and colloidal stability was determined by differential scanning calorimetry and zeta-pulse, respectively. In conclusion, authors suggest a model for paclitaxel's inhibitory role through two complementary steps driving to "off-pathway" oligomer formation and attenuation of fibril formation [140].

Recently, the effect of functionalized magnetic core–shell nanoparticles of Fe_3O_4 (MNPs) with β-cyclodextrin (β-CD) on the aggregation/fibrillation of bovine serum albumin (BSA) under diabetic condition known as amyloidogenesis was studied. The BSA amyloidogenesis was significantly inhibited by β-CD-MNPs. Hence, this nanoparticle may find applications for both diagnostic and therapies for the neurodegenerative diseases mediated by glycation/fibrillation under diabetic conditions [141].

A novel study showed that insulin fibrillation was modulated using engineered B-chains containing mutated C-termini. Analysis of data in detail showed that N-terminal region of the B-chain serves as an important regulator of insulin fibrillation, while the C-terminal region of this peptide is crucial for the control of fibrillation, likely serving as an attachment site resulting in the formation of nucleus and protofibril [142].

5.8. Peptide based inhibitors

Most recently, designed a hexapeptide containing a self-recognition component unique to Aβ42 peptide. This hexapeptide mimics the β-strand hydrophobic core region of the Aβ peptide. The peptide is comprised mainly of D-amino acids which enhanced specificity towards Aβ42. Because this peptide contains a C-terminal disruption element, which blocks the recruitment of Aβ42 monomers on to fibrils, thereby inhibits fibril formation with essentially no cytotoxic effects. These data define the peptide-based inhibitor as a potential therapeutics [143].

Most recently, designed small peptidic inhibitors based on the atomic structure of the core of α-syn fibrils. These inhibitors prevented α-syn aggregation in vitro and in cell culture models with binding affinities of 0.5 μM of α-syn fibril seeds. These inhibitors also efficiently inhibited seeding of human patient-derived α-syn fibrils [144].

More recently, designed a series of peptide inhibitors based on the structures of amyloid β (Aβ) fibrils and their amyloid-forming segments, using RosettaDesign software. These authors utilized a chemical scaffold to constrain the designed peptides into β-strand conformation, which significantly improves the potency of the inhibitors against Aβ aggregation and toxicity. Furthermore, these authors have shown that by targeting different Aβ segments, the designed peptide inhibitors can selectively recognize different species of Aβ during fibril formation [145]. All the inhibitors are summarized in Table 1.

6. Concluding remarks and future prospects

Protein misfolding and aggregation are central to neurodegenerative diseases. The misfolded intermediates generally interact with similar intermediates, thereby forming dimers, oligomers, protofibrils, and fibrils (Table 2).

Several mechanisms have been proposed to explain protein aggregation reaction, including a reversible association of native monomers, aggregation of conformationally altered monomers, nucleation-elongation polymerization, and surface induced aggregation. Like protein aggregation, neurodegenerative disorders are multifaceted involving several biological processes, demanding a multiple approaches to treatment. Although, there are still many important

Table 1
Some neurodegenerative diseases associated with protein misfolding and amyloid aggregation.

Neurodegenerative diseases	Aggregating protein or peptide
Alzheimer's disease	Amyloid-β peptide
Spongiform encephalopathies	Prion protein or its fragments
Parkinson's disease	α-Synuclein
Amyotrophic lateral sclerosis	Superoxide dismutase 1
Huntington's disease	Huntingtin fragments
Familial amyloidotic polyneuropathy	Transthyretin mutants

Table 2
Various therapeutic agents against amyloid diseases.

Therapeutics	Target/mechanism
Small molecules	
10074-G5	Inhibit monomeric amyloid β peptide
3APS (Alzhemed)	Inhibit amyloid β aggregation
AL108	Inhibit amyloid β aggregation
Apomorphine	Inhibit amyloid β aggregation
Polyphenols	Inhibit toxic amyloid β oligomerization
Immunotherapeutics	
AAB-001 (Bapineuzumab)	MAb binds to and clears amyloid-β
A11 (antibody)	Specific for amyloid oligomers
OC (antibody)	Fibril-specific
Engineered affibody	
ZAβ3d	Monomeric Aβ peptide
β-Wrapins (affibody drug)	Stabilization of amyloidogenic proteins in β-hairpin conformations
Hexapeptide	Mimics the β-strand hydrophobic core region of the amyloid β peptide

questions pertaining to protein aggregation that remain unsolved, including understanding of the detailed mechanisms by which protein aggregates are formed, factors affecting the kinetics of aggregate formation, nature of the molecular interactions involved in stabilizing these aggregates. Equally important is to understand how one can effectively and efficiently inhibit these aggregates, particularly in vivo. Proteostasis collapse (either by dysfunctional molecular chaperones/ protein degradation machinery or both) is vital part of the process that caused by the pathological misfolded proteins or peptides. A more holistic approach would be required in near future to understand proteostasis in relation to neurodegeneration. Using the principles described in this article as guidelines, and by inventing innovative technologies, novel and efficient inhibitors could be synthesized to battle these deadly neurodegenerative diseases. The combination of several therapy including antibody therapy along with small molecule inhibitors should be explore further for more effective therapeutics for neurodegenerative diseases.

Declaration of competing interest

The authors declare no conflict of interest, financial or otherwise.

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