Mechanisms of protein aggregation and inhibition

Mohammad Khursheed Siddiqi¹, Parvez Alam¹, Sumit Kumar Chaturvedi¹, Yasser E. Shahein², Rizwan Hasan Khan¹

¹Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh - 200202, India, ²Molecular Biology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Dokki, Cairo, Egypt

TABLE OF CONTENTS
1. Abstract
2. Introduction
3. Protein folding, misfolding and aggregation
4. Mechanisms of protein aggregation
4.1. Reversible association of monomeric proteins
4.2. Aggregate formation by conformationally altered proteins
4.3. Primary nucleation mechanism
4.4. Secondary nucleation mechanism
4.5. 3D domain swapping mechanism
5. Forces involved in folding and aggregation of proteins
6. Factors responsible for protein aggregation
7. Analytical techniques: To study protein aggregation
8. Functional Amyloids
9. Strategies for aggregation inhibition
10. A journey from origin to current scenario of protein aggregation
11. Conclusion and future perspective
12. Acknowledgements
13. References

1. ABSTRACT

Protein and peptid aggregation raises keen interest due to their involvement in number of pathological conditions ranging from neurodegenerative disorders to systemic amyloidosis. Here, we have reviewed recent advances in mechanisms of aggregation, emerging technologies towards exploration, characterization of aggregate structures, detection at molecular level and the strategies to combat the phenomenon of aggregation both in cellular and in vitro conditions. In consistence, we have illustrated almost all factors that influence the protein aggregation both in vitro and in vivo environments. In addition, we have discussed a detailed journey of protein aggregation phenomenon that starts with very first events of protein aggregation. We had also described advancement in current scenarios, present aspects of fibril association to several life threatening disorders and current experimental strategies that are employed to oppose or reverse the amyloid formation.

2. INTRODUCTION

Protein misfolding and its aggregation are emerged as one of the most rousing new edge in molecular medicine as well as in protein chemistry. The contemporaneous curiosity in this topic arises from several considerations; it is believed that the physicochemical features of protein folding may be elucidated; it is also expected to shed light on various human related pathological conditions. Protein misfolding and aggregation usually associated with group of diseases, known as amyloidoses which mesmerize a great deal of recent attention. In amyloidoses, fibrillar aggregates deposit in tissues as intracellular inclusion or extracellular plaques (amyloid). Amyloidoses may be either systemic or localized. Systemic amyloidosis affects multiple organs whereas localized amyloidosis is limited to one organ or tissue type. When such proteinaceous deposit occurs in neuronal cell, results in its degeneration and manifest as neurodegenerative diseases. Neurodegenerative diseases such as Alzheimer’s, Parkinson’s, Amyotrophic lateral Sclerosis, Huntington and other protein aggregate related diseases are categorized as localized amyloidosis (1, 2). Almost all recognized neurodegenerative diseases are associated with accumulation of specific insoluble protein aggregates that are lethal to the cell (3).
Mechanisms of protein aggregation and inhibition

recently recognized as ‘twenty-first century plague (4) and its incident rate is 1% at the age of 60 years and after each five years incidence rate gets double. Currently, 5.3 million people in US are affected with it and are expected to increase up to 13.5 million by 2050 (5). Parkinson’s disease (PD), a disorder of brain, is the second most common form of dementia characterized by difficulty in walking and movement. Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig’s disease, is a neuromuscular disease, affects the nerve that controlling the voluntary muscles (6). Accumulation of insoluble protein aggregate in central nervous system is supposed to be common cause of all neurodegenerative diseases. Still, causative link and effects leading to protein aggregation are largely unknown but ultimately protein aggregates are diagnosed and needs some better comprehensive insights. In this review, we have delineated almost all possible ways or mechanisms by which protein aggregation may occur and in addition, narrated factors and forces that may involve in either aggregate formation or inhibition, both in vivo and in vitro conditions. At last, a journey with full description of protein aggregation has been described in more detail.

3. PROTEIN FOLDING, MISFOLDING AND AGGREGATION

Protein folding is a process in which a polypeptide must undergo folding process to obtain its functionally active three dimensional structure. Anfinsen experiment on ribonuclease A supports this notion which states that the information required to form folded conformation resides in its polypeptide amino acid sequence as the denatured enzyme refolds into native conformation without assistance of any other protein (7). Such studies showed that folding may be initiated by either hydrophobic collapse of non-polar chain of amino acid residues, or formation of secondary structures that provide base for subsequent folding, or formation of covalent bond like disulfide bond.

Before acquiring a biologically active conformation, proteins must undergo through several intermediate structures. Some of these intermediates are more susceptible to form misfolded conformation (Figure 1), a problem attributed to the highly crowded cellular environment (8). Under stress conditions, native proteins are also prone to form misfolded conformers because the energy difference barrier that separates the native and misfolded proteins are too small (9, 10). Remarkably, folding intermediates or misfolded conformers have surface exposed hydrophobic amino acid side chains rather than buried inside as in natively folded proteins (11). Such exposition of hydrophobic residues provides an opportunity for conformers to interact with other molecules especially via hydrophobic interactions, leading to the formation of protein aggregates. Protein aggregation is self-assembly process in which protein molecule with altered, misfolded or partially unfolded conformations associates in specific manner to form higher order structure with low solubility. These structures are often termed as amyloid fibrils. During aggregation, one species can influence the aggregation of another, and also interestingly, different protein species can also co-aggregate together as was for protein containing polyglutamine stretch (12).

Amyloid fibrils are highly ordered structures with characteristic cross β-sheet structural motifs. In cross β-sheet structure, β-sheet structure runs perpendicular to the fibril axis. Amyloid fibrils are 7-12nm in diameter (13). The major β-sheet region of amyloid fibrils is formed by hydrophobic residues (14). Numerous studies have provided some clue that amyloid fibrillogenesis is involves secondary structure transition from α-helix to β-sheet (15,16). Amyloid hypothesis states that amyloid fibrils or its formation acts as causative agent for disease progression or its onset and appears as a major inducement for characterization of amyloid fibrils in vitro (17). More recently, amyloid hypothesis stated that intermediate entities formed during amyloidogenesis are toxic to the neuronal cell (18). In vitro study of amyloids provides links to monitor associated disease progression but certainly also has other applications (19, 20).

4. MECHANISMS OF PROTEIN AGGREGATION

Not only single but several pathways or mechanisms culminate to the protein aggregate formation (Figure 2). These mechanisms are not independent to each other but have several processes in common. In Table 1, different mechanisms utilized by protein to aggregate are summarized in chronological order and subsequent paragraphs deal with some of these mechanisms.

4.1. Reversible association of monomeric proteins

This mechanism states that native protein has an inherent capacity to self-associate in a reversible manner. Native monomer of therapeutic protein insulin molecules were shown to have self-complementary surfaces that force the protein to associate reversibly to form small and larger sized oligomers, and over time these reversible oligomers become irreversible aggregates (21). This mechanism to form aggregate was well reported since 1953, it was said that unfolding of protein is not a necessary condition to form fibrillar structure instead side by side or end to end union of protein molecules brought about aggregation of protein (22).
Mechanisms of protein aggregation and inhibition

4.2. Aggregate formation by conformationally altered proteins

A native protein does not always have tendency to self-associate. Instead, protein with altered conformation or in partially unfolded state have strong propensity to form higher order oligomers. So the initial step in this mechanism is transition from native to non-native structure which distinguishes this mechanism from previous one. Such aggregation prone of protein non-native state may be achieved by stresses such as heat or shear. The condition or excipients that stabilize the native state of protein may be helpful in proposing anti-aggregating agent. Interferon-ϒ (23) and Granulocyte-colony stimulating factor (G-CSF) (24) have been reported to favor this mechanism.

4.3. Primary nucleation mechanism

However, native monomer alone is unable to initiate the process of fibril formation but aggregate of sufficient size favors the formation of larger size aggregate by the addition of monomer to it. This is so called as critical nucleus. This process of aggregation generally exhibits a lag phase during which no visible precipitate appears for a long period of time (perhaps a month) but after such critical period and suddenly, a much larger species appear and accumulate (25). Such process is termed as homogenous nucleation. Another variant of this mechanism is heterogeneous nucleation.
Mechanisms of protein aggregation and inhibition

in which the impurity or contaminant acts as the nucleus. Some examples of impurities are silica particles shed by product vials (26), steel particles shed by a piston pump used for filling vials etc. Generally, amyloid formation occurs via nucleation-dependent oligomerization. This mechanism is widely used to illustrate the phenomenon of protein aggregation.

4.4. Secondary nucleation mechanism

Primary nucleation was supposed to be preceding step of secondary nucleation mechanism. In secondary nucleation mechanism, fibrils of smaller sizes (formed through primary nucleation of critical concentration) act as nucleus for further oligomerization with enhanced propensity. Morphological structure and accessible surface areas of amyloid fibrils affect the kinetics of oligomerization through secondary nucleation mechanism (27).

4.5. 3D domain swapping mechanism

Another striking mechanism for amyloid fibril formation is 3D domain swapping. In this mechanism, identical proteins replace their domain. The result is an intertwined dimer or higher order oligomer, with one domain of each subunit replaced by the identical domain of other subunit. The swapped domain may be an alpha

Figure 2. Schematic presentation showing possible different mechanisms of protein aggregation.
Mechanisms of protein aggregation and inhibition

helix or a β-sheet or an entire tertiary globular domain. Ribonuclease A has been shown to adopt this mechanism to form amyloid like fibrils(28).

5. FORCES INVOLVED IN FOLDING AND AGGREGATION OF PROTEINS

We all are familiar with the process that how polypeptide attain a functionally active native protein conformation through protein folding process. But during folding, amino acid residues which are present far apart in polypeptide chain, come close together (29). Such close proximity of amino acid residues involves different intramolecular interactions that favor the folding of proteins. During folding, these interactions play their role in an efficiently regulated manner. Disturbance to any of these interactions leads the several non-desirable associations of amino acid residues and in turn responsible for the formation of various partially unfolded or misfolded proteins (30). Non-polar side chain of amino acid residues interacts with other non-polar amino acid side chain through hydrophobic interaction. This is supposed to be major driving force for proteins to attain three dimensional conformations (32). In protein aggregation, these interactions are supposed to play major role and not surprisingly, a major portion of research work in field of protein aggregation is devoted towards inhibition of such interaction (33).

6. FACTORS RESPONSIBLE FOR PROTEIN AGGREGATION

Protein aggregation can be induced by wide variety of factors both in vitro and in vivo environments. Physical parameters such as temperature (34), pH (35), co-solvent (36), metal ions (37), freezing and thawing (38), agitation stress (39), surfactants (40) etc, that have potential to partially unfold the protein or increase the propensity of folding intermediates, lead to protein aggregation (Figure 3).

As protein synthesis begins, number of chaperones work to fold the polypeptide properly to be functionally active. Since some chaperone functions are sequence specific, any change or mutation in the polypeptide chain may prevent its binding and abort its function. This inability of chaperone leads to partial folding/ misfolding/ improper folding of protein and consequently results in a conformation which is more prone to aggregation (41). Ability of protein biogenesis system to synthesize defective protein may also be considered as aggregate producing source. These defects may include improper translation, wrong incorporation of amino acid etc.(42).

During ageing, the cellular proteasome ability to eliminate impaired or misfolded proteins is reduced. Therefore, these proteins tend to produce aggregates resulting in fatal consequences. Mutant form(s) of human superoxide dismutase 1 has more exposed hydrophobic surfaces and hence more chaperones are bound, representing the situation where protein homeostasis is not maintained (43).

7. ANALYTICAL TECHNIQUES: TO STUDY PROTEIN AGGREGATION

Protein aggregates possess especially amyloid fibrils of highly ordered structures. These structures made unavoidable requirements of sensitive techniques which are not only merely detect the presence of aggregate fibrils but also to monitor the aggregation kinetics. Some of these techniques are described here and is summarized in Table 2.

As an example, hydrogen bonding in α-helix chain is known to be formed between oxygen of carbonyl group (C=O) and hydrogen of N-H group which determines its cylindrical structure. In β-sheets, intermolecular hydrogen bonding occurs between carbonyl oxygen of one β-sheet to amino hydrogen of another β-sheet which determines its parallel and antiparallel orientation structures. It also occurs between α-helix and occurs when they are at its minimum critical distance (31).
Mechanisms of protein aggregation and inhibition

And such transition of protein secondary structures towards β-sheet enrichment are analyzed by monitoring the change in peak position and ellipticity of Circular Dichroism (CD) spectrum (44, 45). Fourier transform infrared (FTIR) spectroscopy is a measurement of wavelength and intensity of the absorption of infrared radiation (IR) by a sample. In FTIR of protein, strong absorption band appears at 1600-1700 cm$^{-1}$. It occurs due to stretching vibrations of the C=O bond. Since, vibrators exhibit the phenomenon of transition dipole coupling; it should indicate the relationship between the peak position and type of secondary structure. By employing different empirical relationships, different secondary structures have been identified. Non-alpha-non-beta structure, both alpha helix and random coil; and beta sheet structure appear at >1660, 1660-1640, 1640-1620 cm$^{-1}$ respectively (46). These frequencies are closely correlated with different secondary structures of proteins.

Gel electrophoresis is a frequently used protean technique for qualitative and quantitative analysis of proteins. The size of aggregates/proteins detected through gel electrophoresis ranges from 5-500 kDa as shown by separation of amyloid β(Aβ) monomers and oligomers (47). Native gel electrophoresis exploits entirely different principle than in SDS PAGE. Instead of denaturing or reducing the molecule, it separates the molecule as it is. Therefore, aggregates are apparently more sTable in non-denaturing PAGE than in denaturing PAGE as exemplified by Aβ, yeast prion aggregates(48) and oligomer of neuroserpin variants(49). SDS PAGE is quiet useful for studying highly complex protein aggregates. It is frequently used to characterize soluble Aβ aggregates and SDS resistant prion protein aggregates(50).

Thioflavin T, a fluorescent dye, which has been widely used to monitor the presence of amyloid fibrils. It displays a mark increase in fluorescence upon binding to amyloid fibrils with an excitation maximum at around 450nm and emission maximum at around 485nm. It specifically binds to the beta sheet groove structure present in amyloid protein aggregate(51).Another dye, congo red is also used to detect the presence of amyloid fibrils both in tissue sections and in vitro. It produces blue-green birefringence upon complexation with amyloid fibrils. It is also characterized by shift in wavelength of maximal absorption from 490 to 540 nm(52).

Direct visualization of three-dimensional structure of various fibrous systems of biological importance including collagen, keratin and slightly twisted paired helical filaments that act as nucleus for Alzheimer like diseases and morphology of amyloidogenic protein aggregate samples in ambient environment was achieved by Atomic Force Microscopy (AFM). AFM elucidated the basic structural features of protein aggregates at nano meter level like curvature, width, length both qualitatively and quantitatively(53).

X ray diffraction (XRD) is one of the most sensitive techniques for the detection of amyloid fibrils at high resolution. It resolves the cross β-sheet pattern of amyloid. It is easy, fast, non-destructive and used to find the structure of unknown crystals. It gives information about thickness, size, strain and atomic arrangement(54). In XRD, structure is obtained due the interference pattern

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Category</th>
<th>Techniques</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Separative methods</td>
<td>Gel Electrophoresis, Size Exclusion Chromatography, Ultracentrifugation</td>
<td>Assembly size/size distribution</td>
</tr>
<tr>
<td>2.</td>
<td>Spectroscopic techniques</td>
<td>Circular dichroism, UV-spectroscopy, Fourier transform infrared Spectroscopy, Thioflavin T</td>
<td>Structural change to protein, turbidity, scattering, to identify co-existing protein conformers, structural detail, size, structure of aggregates</td>
</tr>
<tr>
<td>3.</td>
<td>Dyes</td>
<td>Congo Red, 1-Anilinonaphthalene-8-Sulfonate (ANS), Bis ANS, Nile Red</td>
<td>Qualitative and quantitative estimation of amyloid formation and Assessment of surface hydrophobicity</td>
</tr>
<tr>
<td>4.</td>
<td>NMR and X ray crystallography</td>
<td>Solid and solution state NMR, X ray diffraction, X ray crystallography</td>
<td>To get insight into the atomic structure of aggregates</td>
</tr>
</tbody>
</table>
Mechanisms of protein aggregation and inhibition

The two dimensional visualization of the protein self-assembly into oligomers, protofibrils and mature fibrils have been facilitated by high-resolution microscopic techniques such as high resolution transmission electron microscopy (HRTEM). HRTEM provides images with high resolution of prefibrillar aggregates, circular species and mature fibrils. It is easy to perform and confirms the fibrillar morphology of amyloid(56). Likewise, field emission surface electron microscopy (FESEM) also used to visualize the three dimensional structure of amyloid fibrils. FESEM provides images with comparatively high resolution but it is not prevalent as other techniques(57, 58).

8. FUNCTIONAL AMYLOIDS

A large number of proteins have been reported that are not associated with amyloidosis but form amyloid fibrils with characteristic morphology, structure and tinctorial properties. These findings support the idea that propensity to form amyloid is an inherent property of polypeptide chain but extent of amyloidogenesis depends on polypeptide sequence. This ability to form amyloid has been exploited by different living systems to perform their normal physiological cycle as some organisms have been shown to convert their endogenous proteins into amyloid fibrils that have beneficial functions rather than disease associated properties(59, 60). The associated roles of functional amyloids are listed in Table 3.

One of the examples of functional amyloids in bacteria *Escherichia coli* is the curli proteins which are used to colonize inert surfaces and mediate binding to host proteins such as fibronectin, laminin, plasminogen, tissue plasminogen activator, and H-kininogen. In addition, the production of melanin in melanocytes is characterized by the presence of intralumenal fibrous striation upon which melanin is formed. This fibrous material is formed from the intralumenal domain of the membrane protein Pmel17, in mammalian system(61).

9. STRATEGIES FOR AGGREGATION INHIBITION

Still it is topic of debate and discussion that whether soluble monomers, or oligomers or larger aggregates are greatly associated with toxicity to the cell that results in various neuron related disorders. In higher eukaryotes, proteins have limited ability to refold into native forms, which increases the possibility of proteins to aggregate. Here, we have described the pathways that prevent the formation or accumulation of aggregates and Table 4 accounts for great details of these approaches.

Inspite of intense research, the mechanism of inhibition is poorly understood but several formulations or drug molecules are proven 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 (62-64). These molecules with the ability to impede the process of aggregation are designed on the basis of concepts that were utilized by the protein to form aggregates. Therefore, such molecules are more potent to hinder the self-assembly process of proteins. Apart from inhibition, molecules which stabilize the native state of proteins and minimize the possibility of protein to aggregate are also reported (Figure 4). This carries a great hope for development of anti-amyloid agents.

Proteasome is a barrel shaped multiprotein complex and believed to be central cellular machinery for degradation of various misfolded and aggregated proteins (Figure 5A)(65). Impairment in proteasome system may results in neuro-degeneration, indicating the crucial role of proteasome for clearance of aggregates(66, 67). The protein which is susceptible to degradation by proteasome machinery is marked by its association with ubiquitin. Another process, autophagy, emerges as major

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Table 3. Functional amyloid with specific functional roles

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> (bacterium)</td>
<td>Curli</td>
<td>Biofilm formation and facilitate binding to host proteins</td>
<td>(119)</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em> (bacterium)</td>
<td>Chaplin</td>
<td>Development of aerial hyphae</td>
<td>(120)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> (fungus)</td>
<td>URE2p</td>
<td>Catabolism of nitrogen</td>
<td>(121)</td>
</tr>
<tr>
<td><em>Neurospora crassa</em> (fungus)</td>
<td>Hydrophobin</td>
<td>Development of aerial hyphae</td>
<td>(122)</td>
</tr>
<tr>
<td><em>Podospora anserina</em> (fungus)</td>
<td>HET-s (prion)</td>
<td>Regulation of heterokaryon formation</td>
<td>(123)</td>
</tr>
<tr>
<td><em>Nephila edulis</em> (spider)</td>
<td>Spidroin</td>
<td>Formation of silk fibers of the web</td>
<td>(124)</td>
</tr>
<tr>
<td><em>Apis mellifica</em> (marine snail)</td>
<td>Neuron-specific isoform of CPEB (prion)</td>
<td>Maintenance of synaptic changes associated with memory storage</td>
<td>(125)</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Intralumenal domain of Pmel17</td>
<td>Forms fibrous striation upon which melanin granules forms</td>
<td>(126)</td>
</tr>
</tbody>
</table>
Mechanisms of protein aggregation and inhibition

Table 4. Anti-amyloidogenic agents and related proteins of study

<table>
<thead>
<tr>
<th>Category</th>
<th>Anti amyloidogenic agents</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols</td>
<td>(-)-Epigallocatechingallate</td>
<td>Huntingtin (127)</td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>Transthyretin (128)</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Myreclin</td>
<td>β-amyloid (129)</td>
</tr>
<tr>
<td></td>
<td>(~)-epigallocatechin</td>
<td>Islet amyloid peptides (130)</td>
</tr>
<tr>
<td></td>
<td>β-gallate</td>
<td></td>
</tr>
<tr>
<td>Chaperones</td>
<td>Alpha S</td>
<td>Casein (91)</td>
</tr>
<tr>
<td></td>
<td>β casein</td>
<td>Casein (91)</td>
</tr>
<tr>
<td>Proteins</td>
<td>Catalase</td>
<td>β-amyloid (101)</td>
</tr>
<tr>
<td></td>
<td>Albumin</td>
<td>β-amyloid (101)</td>
</tr>
<tr>
<td>Dyes</td>
<td>ANS</td>
<td>Casein (91)</td>
</tr>
<tr>
<td></td>
<td>Congo red</td>
<td>β-amyloid (92)</td>
</tr>
<tr>
<td>Surfactants</td>
<td>Commercial (SDS)</td>
<td>Papain (131)</td>
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<tr>
<td></td>
<td>Synthetic (Gemini)</td>
<td>Serum albumin (132)</td>
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<tr>
<td>Sugars</td>
<td>Trehalose</td>
<td>Beta amyloid 40 &amp; 42 (95)</td>
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<tr>
<td></td>
<td>Glycoplymers</td>
<td>β-amyloid (133)</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>Curcumin-gold</td>
<td>β-amyloid (99)</td>
</tr>
<tr>
<td></td>
<td>Gold</td>
<td>β-lactoglobulin (134)</td>
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<tr>
<td>Osmolytes</td>
<td>Trimethylamine N-oxide</td>
<td>P39 cellular retinoic acid-binding protein (96)</td>
</tr>
<tr>
<td></td>
<td>Ecotene</td>
<td>β-amyloid (135)</td>
</tr>
<tr>
<td>Synthetic</td>
<td>2-phenylbenzofuran</td>
<td>β-amyloid (136)</td>
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<tr>
<td>Drugs</td>
<td>derivatives</td>
<td>β-amyloid (137)</td>
</tr>
<tr>
<td></td>
<td>Isoliquiritigenin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>derivatives</td>
<td></td>
</tr>
<tr>
<td>Metal ions</td>
<td>Copper (II)</td>
<td>β-amyloid (94)</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>Single Stranded Synthetic</td>
<td>β-amyloid (138)</td>
</tr>
</tbody>
</table>

contributor for degradation of misfolded and aggregated proteins in the cytosol of mammalian cells(68). It uses specialized double membrane structure that engulfs the substrate to form autophagosomes (Figure 5B). Autophagosomes ultimately fuse with lysosomes that form autophagolysosomes to degrade their contents by acidic hydrolases present in lysosomes(69). Autophagy is regarded as a backup system to complement proteasomal degradation when it is exhausted or overwhelmed of dealing with aggregates. In agreement with this, it also has role in clearance of aggresomes (70, 71).

10. A JOURNEY FROM ORIGIN TO CURRENT SCENARIO OF PROTEIN AGGREGATION

Initially it was thought that protein molecules exist as single entity i.e., they were unable to interact with each other. But later in 1948, a research group showed that protein molecules may associate as it was agreed by evaluating the molecular weight of egg albumin (72). In agreement with this, in 1952, a result was reported that a number of globular proteins like hemocyanin, horse hemoglobin, insulin, fibrinogen, ovalbumin possess the ability to interact with each other when provided with different conditions (73). These conditions may include pressure, salt concentration, heat treatment and were studied by techniques such as electron microscopy, viscosity, electrophoresis, ultracentrifugation, paper electrophoresis, fractional precipitation, streaming birefringence (22). Till now, the causative link for aggregation was unknown but a ray of hope was came when denaturation of proteins was blamed for their aggregation (74). In 1992, this process was further supported by the fact that partial unfolding of protein (e.g. transthyretin) was a necessary requirement for protein to aggregate (75).

Revolution to protein biophysics came into existence when protein aggregates were reported to be associated with diseases (76). This attracts the attention of core group of protein biophysicists to solve the puzzle of protein aggregation and its association with several diseases. Some of these include fibrinogen amyloidosis, lysozyme amyloidosis etc. (41). In addition to this, protein aggregates were also found to be deposited in neurons, leads to their degeneration, a prerequisite condition for dementia or mental retardation. Alzheimer, Parkinson, Huntingtin disease etc. were known to be the pathogenic consequences of neuron degeneration (41). Further research on aggregates leads categorization of aggregates into two either random type-amorphous or well-ordered amyloid structure (77).

Curiosity to know the organization of aggregate, structure, its physical appearance is made possible by utilization of several sensitive techniques to explore it at atomic detail both in vitro and in vivo. In 1997, with the aim to explore its structure, application of synchrotron x-ray diffraction method reveals the common core structure of amyloid aggregate which consist ofhelical array of β-sheets parallel to the fiber long axis, with the strands perpendicular to this axis (78). Apart from its characterization, detection of both amyloid as well as amorphous protein aggregate poses great challenge. In consistence, in vitro detection may include turbidity measurement (e.g. Catalase)(79), Light scattering(e,g.,lgG) (79), Thioflavin T assay(e.g. α-synuclein), and imaging techniques like AFM, Transmission electron microscopy (TEM), Scanning electron microscopy (SEM) etc. In vivo detection may include fluorescence correlation spectroscopy (e,g.amyloid β-peptide) (80), magnetic resonance imaging, positron emission tomography, single photon emission computed tomography, multiphoton microscopy etc. (81). But these techniques have some advantages and disadvantages also. Although Thioflavin T is widely used to diagnose the amyloid fibrils not amorphous aggregates, both in vitro and in vivo, but its sensitivity depends on pH. pH provides positive
Mechanisms of protein aggregation and inhibition

![Figure 4. Schematic illustrations of pathway that inhibit amyloid fibril formation (A)-(E) represent the molecules used to either inhibition or stabilization of protein. (A) Native state stabilization (B) Refolding of polypeptide (C) Diversion from oligomerization pathway (D) Inhibition of fibril elongation by β-sheet breakers (E) Disaggregation of amyloid aggregate.](image)

As mentioned earlier, it was believed that aggregation is simple association of denatured or partially unfolded protein molecules. But intensive research on protein aggregates leads to the development of pathways which result in protein aggregates (Figure 6). A conformation which is different from native one i.e., misfolded conformation is shown to associate more rapidly to form aggregates (83).

During the process of aggregation, initially there were no appearance of visible aggregates but after some critical period, a large clump of proteins were appeared. This mystery was solved by a hypothesis stated that a small preformed aggregate of sufficient size with the ability to nucleate the process of aggregate formation is prerequisite (84). Next to it was the proposition of mechanism of fibril elongation (84). Thereafter, it was also reported that not only change in conformation but also chemical modification to the protein structure is associated with fibril formation (85).

Once the mechanism of formation was explored, then to determine the various factors/conditions (both in vitro and in vivo) which induce or inhibit the aggregation process was emerged as topic of keen interest. In vitro aggregate inducing factors may include temperature, pH, cosolvent, metal ions, surfactants etc. and in vivo may include mutation in polypeptide chain (86), translational error, aging, inability of cellular machinery to clear the aggregate,(87) etc.

Now days, majority of works in field of protein aggregation are carried out to develop or propose an effective formulation/inhibitor which is able to prevent the process of fibril formation. In this context, polyphenols were tested which possess potential to inhibit its formation (88), β-cyclodextrin (a cyclic glucopyranose) and its derivatives inhibits the formation of β amyloid peptide (89). Several other compounds with antioxidant activity were also capable of it (90). Not surprisingly, dyes like ANS (91), Congo red (92), methylene blue (93) etc. which are used to detect presence of protein aggregates, also shown to prevent aggregate formation. Metal ions like Cu (II) (94), sugars like trehalose (95), osmolytes like trimethylamine N-oxide (96, 97), and surfactants (79) also stand in front to abort aggregation process. Nanoparticle (such as copolymeric NiPAM: BAM)(98), synthesized chemicals and drugs also made contributory successful attempts towards inhibition of aggregation process. Plant derived products including Curcumin (99, 100) are claimed for curing protein aggregation. Interestingly, protein molecules itself inhibit the aggregation of other proteins. In this regard, chaperones were initially shown to possess such inhibitory function. Chaperones like Alpha S and β-casein inhibit the casein aggregation (91). But recently, non-chaperone proteins like catalase, pyruvate kinase, albumin, lysozyme, α-lactalbumin, and β-lactoglobulin were also reported to suppress the fibrillation of β-amyloid peptides (101). Intracellularly, cells have been provided with systems which deal with protein aggregates and have capacities to clear the aggregates. Autophagy (102) and proteasome (103) constitute two systems which are vigorously involved in clearance activity inside the cell.

A wide range of scientific disciplines working with protein aggregation and amyloids have been stimulated by their association with several debilitating medical disorders, from Alzheimer’s disease to type II diabetes, and many of which are responsible for major
Mechanisms of protein aggregation and inhibition

Threats to human health and welfare in the modern world. Alzheimer's disease is associated with deposition of β-amyloid and tau in neuronal cells extracellularly and intracellularly, respectively. β-amyloid peptides are generated from amyloid precursor protein by the action of secretases. Hyperphosphorylation of tau protein causes its dissociation from cytoskeletons, a known factor for its deposition. With the aim to screen for an effective compound, different approaches were employed. Some research works are directed towards inhibition of γ-secretase activity (104) and some towards how to inhibit the association of β-amyloid peptide (105) and tau (106).

Although the nature of aggregates is still unclear, but recently it has been shown that there is association of amyloid aggregates with cancer. p53 i.e., tumor suppressor, is frequently mutated in nearly all types of cancers. Such mutations produce conformational alterations which, in several cases are accumulated as intracellular aggregates (107). Such results inferred that cancer could be considered as protein conformation-disease.

Awareness to the deleterious effects of amyloid aggregates is now well known. But beside this, protein oligomers and aggregates are reported to have some positive aspects also. Supramolecular insulin (insulin oligomer) assembly have been proven to be effective in treatment of type 1 diabetes mellitus. Authors have experimentally proved that oligomerization or aggregates of insulin causes sustained release of insulin monomer in blood on injection which has been retained for longer time and has excluded the condition of multiple insulin injections method to cure diabetes (108). Moreover, supramolecular polymeric structure of proteins is also used as drug delivery system. Ulyana Shimanovich et al. (109) have shown that nanofibrils can be used to form protein micro gels and demonstrated the controlled release of encapsulated drugs. Further these gels were found to be nontoxic to human cells and show relatively more efficacy as compared in homogenous solution (110).

Figure 5. (5A) Schematic diagram showing key aspects of ubiquitin-proteasome mediated degradation and (5B) autophagy.
11. CONCLUSION AND FUTURE PERSPECTIVE

Now we are at the level from which we can strongly underscore the relevance that propensity to form fibril is a reflection of its polypeptide sequence, modulated by environmental conditions. Aggregates are formed by the interaction of partially unfolded intermediates containing significant amount of native like structures and rich in β-sheet secondary structures. Both amorphous as well as amyloids possess significant amount of β-sheet structures. These structures require techniques with high resolution and sensitivity to explore its pattern of association and mechanism. Apprehension of kinetics of amyloid formation and pathways may help in designing strategies and approaches that either lead to inhibition or reverse the process of aggregate formation.

It is now more evident that protein aggregates formed in cells are also due to malfunctioning of protein quality control systems like proteasome machinery and autophagy. Insight into the molecular aspects involved behind protein quality control systems may explain the facets of various diseases associated with protein aggregation and hence open way for therapeutic intervention. Further research will be needed to establish the biochemical strategies and genetic manipulation that explore the additional structural components, deposition sites, molecular architectures of protein aggregates. These studies will be a prerequisite for tackling other problems relating to regulation of this process.

Interestingly, beneficial facet of amyloid fibrils to form different nanostructure made them of great value. Nanoparticles or supramolecular polymeric structures formed by amyloid fibrils with remarkable sustained release of monomers may be exploited in near future to treat different human associated diseases. Similarly, protein mircogels as drug delivery systems carry great hope for delivery of therapeutic drugs(110).

There are still many outstanding and critical questions regarding protein aggregation. These include detailed mechanisms of aggregate formation, factors influencing the kinetics of aggregation, nature of molecular interaction and how aggregates are effectively and efficiently prevented, particularly in vivo. Moreover, revealing the pathway that leads to the protein misfolding which is thought as overture to the fibril formation will help in studying normal protein folding and the evolution of protein folding and aggregation. In parallel, co-aggregation of protein molecules raised excellent challenges and has not yet been fully understood.

12. ACKNOWLEDGEMENTS

Facilities provided by IBU, Aligarh Muslim University, Aligarh are gratefully acknowledged. M.K.S is highly thankful to Department of Biotechnology (DBT), New Delhi, for providing fellowship in the form of junior research fellowship. P.A and S.K.C are highly thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi, for financial assistance in the form of junior research fellowship (JRF) and senior research fellowship (SRF), respectively.
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**Key Words:** Protein Aggregation; Protein Misfolding, Amyloid, Review

**Send correspondence to:** Rizwan Hasan Khan, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh-202002, U.P. India, Tel: 91-571-2720388, Fax: 91-571-2721776, E-mail: rizwanhkhhan@hotmail.com