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INVESTIGATION OF PROTEIN MISFOLDING MECHANISMS IN AMYLOIDOSIS: FROM BIOCHEMISTRY TO THERAPEUTICS

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Abstract

Protein misfolding and aggregation are hallmarks of amyloidosis, a group of debilitating disorders characterized by the deposition of insoluble amyloid fibrils in tissues and organs. This study attempts to examine the biochemistry of protein misfolding in amyloidosis based on three amyloidogenic proteins, namely β-amyloid (Aβ), α-synuclein, and transthyretin (TTR). In vitro biochemical assays, spectroscopic analyses, and electron microscopy were employed to assess various structural transitions from native proteins to toxic oligomers and fibrils. The binding of Thioflavin T and Congo red confirmed the progressive formation of amyloid, while CD and FTIR studies showed a much larger increase in β-sheet content during the aggregation process. The classical fibrillar morphologies were shown by electron microscopy. Toxicity assays indicated that early oligomeric intermediates had a greater detrimental effect on cell viability than mature fibrils. Besides, docking studies coupled with molecular dynamics simulations predicted that curcumin and EGCG are able to bind well to aggregation-prone regions, inhibiting fibril formation and thereby having the potential to be effective therapeutics. Doxycycline showed a moderate inhibitory effect, which may be caused by destabilizing the fibril. These results provide a greater understanding of the contribution of protein misfolding in amyloidosis and advocate for the further use of small molecules as putative therapeutic agents. Future investigations would need to take these compounds through a pharmacological efficacy and safety evaluation in vivo. This work interconnects biochemical mechanisms to clinical practice in regard to amyloid-related diseases.

INTRODUCTION

Protein folding is a basic process that guarantees the structure and biological function of proteins. These proteins must take on their correct three-dimensional arrangement if they are to execute a certain biochemical function in a living organism. The folding process is encoded in the primary amino acid sequence and is facilitated by chaperones and cell quality control systems. But in some pathological or

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stress-induced conditions, proteins can deviate from their native conformations and can accept misfolded or partially folded intermediate states (Shaitan, 2023). These states can in turn lead to protein aggregates that are insoluble and commonly referred to as amyloid fibrils. The disease-causing aggregation of such misfolded proteins marks out this group of diseases known by the collective name amyloidosis (Gomes et al., 2019).

Amyloidosis is a collection of disorders that result from the deposition of insoluble fibrillar proteins either in extracellular or intracellular compartments throughout the body's various tissues and organs. This deposition disrupts normal tissue architecture and thus affects cellular function, leading to organ malfunction and ultimately failure. Amyloidoses, which may be systemic or localized, may arise from the deposition of several precursor proteins (Finkelstein et al., 2022). Pathways widely studied encompass Alzheimer's with β-amyloid, Parkinson's with α-synuclein, systemic light-chain amyloidosis with immunoglobulin light chains, and transthyretin amyloidosis with transthyretin protein. Regardless of diversity among their precursor proteins, all amyloid fibrils subsume a common set of structural and biochemical properties, including cross-β-sheet conformation, resistance to proteolytic degradation, and staining properties when exposed to certain dyes such as Congo red and Thioflavin T (Kuhlman & Bradley, 2019).

The protein misfolding process that leads to aggregation follows a specific sequence of multiple biochemical steps rather than occurring at random. The process starts with protein structure destabilization caused by gene mutations and post-translational modifications and environmental stressors, and age-related oxidative damage. The unstable intermediates experience nucleation, which leads to elongation that generates oligomers and protofibrils before they develop into mature, stable amyloid fibrils (Balasco et al., 2025). The oligomeric species now receive increasing recognition as the most cytotoxic entities, which can break down cellular membranes while interfering with mitochondrial operations and activating apoptosis. The importance of taking early action during the misfolding sequence stands as a key factor in stopping permanent harm from developing (Cocco et al., 2018).

From a biochemical perspective, amyloidosis is the first disorder in which protein folding can be studied under pathological conditions. The molecular mechanisms driving the misfolding and aggregation of proteins are still the subject of ongoing investigations. The questions are: What are the intrinsic structural features rendering certain proteins amyloidogenic? What cellular pathways are unable to block the misfolding process? How do misfolded proteins evade proteostasis mechanisms such as the ubiquitin-proteasome system and autophagy? Resolution of these questions is crucial not just to comprehend disease pathology but also for the planning of effective therapeutic intervention.

Emerging developments in molecular biology, biophysics, and computational modeling have yielded unprecedented knowledge of the mechanisms of protein misfolding. Methods like nuclear magnetic resonance (NMR), cryo-electron microscopy (cryo-EM), circular dichroism (CD), Fourier-transform infrared spectroscopy (FTIR), and molecular dynamics simulations have been used to analyze the structural changes in amyloid formation. In addition, cell and animal models of amyloidosis have allowed pathogenic mechanisms to be investigated and promising drug targets to be identified (Lupas et al., 2021). These include small-molecule stabilizers of native protein conformation, aggregation inhibitors, and compounds that promote cellular clearance of amyloidogenic species.

Therapeutically, protein misfolding disorders are still a daunting challenge because of the heterogeneity and complexity of amyloid diseases. Therapeutic approaches currently in use can be generally classified into four categories: inhibition of fibril formation, stabilization of native proteins, immunotherapy to increase clearance of aggregates, and disruption of preformed fibrils. For example, tafamidis is a stabilizer of transthyretin and is used to treat transthyretin amyloidosis (Nevone et al., 2020). Monoclonal antibodies against β-amyloid have been promising in clinical trials of Alzheimer's disease, but the results are still inconsistent. Furthermore, pharmacological chaperones and proteostasis regulators are being pursued vigorously to restore balance in protein folding in diseased cells.

Notwithstanding these developments, there are still large gaps in our knowledge of the initial events in

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protein misfolding and the specific initiators of amyloidogenesis. In addition, the biochemical heterogeneity of various amyloidogenic proteins requires disease-specific and even patient-specific therapy (Basha et al., 2023). Personalized medicine, based on genomic and proteomic profiling, will likely be central to the future treatment of amyloidosis. The social and economic burden of amyloid diseases is enormous, especially with the increasing incidence of neurodegenerative disorders such as Alzheimer's and Parkinson's in aged populations. The biochemical background of these diseases is not only an academic concern but a public health imperative. There is an urgent need for early detection, improved biomarkers, and effective therapies to alleviate the burden of these progressive and often fatal diseases (Kumar, 2025).

This research aims to investigate the biochemical mechanisms of protein misfolding in amyloidosis, namely to determine the structural intermediates involved in fibril growth and to examine potential therapeutic targets. By integrating spectroscopic techniques, computational simulations, and in vitro assays, this research aims to contribute to the understanding of protein folding disorders and pave the way for novel therapeutic interventions. Special focus will be placed on specifying the role of environmental conditions, genetic mutations, and post-translational modifications to cause amyloidogenesis. In summary, protein misfolding and amyloidogenesis are a core paradigm for the pathogenesis of numerous chronic and lethal diseases (Kumar, 2025). Deciphering the complex biochemical pathways governing these mechanisms is critical to the design of targeted and effective therapies. This work will act as a link between biochemical basic knowledge and clinical translation, with the goal of discovering novel strategies for the prevention and therapy of amyloid diseases.

Methodology

The purpose of this investigation is to discover the underlying biochemical processes of protein misfolding in amyloidosis and evaluate possible therapeutic measures that can modulate these pathologic processes. The investigation brings together laboratory protein research, structural and spectroscopic analysis, cytotoxicity testing, and modeling using computational means to provide an integrated view of amyloidogenesis and inhibition.

1. Selection and Preparation of Amyloidogenic Protein

Recombinant versions of human amyloidogenic proteins identified to be involved in different types of amyloidosis were used in this study. They are β-amyloid (Aβ1–42), α-synuclein, and transthyretin (TTR), all of which have been found to be involved in neurodegenerative and systemic amyloidosis disease. These proteins were produced in Escherichia coli BL21 (DE3) cells using plasmid-based expression systems. After expression, the proteins were purified through affinity chromatography, in this case through His-tag-based purification on a Ni-NTA column. The dialyzed proteins were then purged of impurities or contaminants by dialysis against phosphate-buffered saline (PBS). Protein purity was checked through SDS-PAGE, and concentration was determined through the Bradford assay.

2. Induction of Protein Misfolding and Aggregation

To mimic in vitro conditions favoring protein misfolding and aggregation, the stress-inducing environmental conditions were achieved by incubating the purified proteins. These consisted of incubation at 37°C with 200 rpm agitation, within a physiological pH of 6.5 to 7.4, and the presence of 100 mM sodium chloride to achieve proper ionic strength. Incubation for 72 hours was extended. For the further promotion of the misfolding process, denaturing reagents like urea or sodium dodecyl sulfate (SDS) were added according to the specific structural stability of the target protein (Chiti & Dobson, 2017).

3. Detection and Characterization of Amyloid Fibrils

Amyloid fibril formation was tracked by employing different analytical methods. The Thioflavin T (ThT) fluorescence test was used to identify β-sheet-enriched fibrillar structures. Protein solutions were mixed with ThT dye and incubated, and the fluorescence emission was recorded with a spectrofluorometer at wavelengths of 440 nm for excitation and 485 nm for emission. Kinetics of aggregation were graphed throughout the incubation time. Congo red binding

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assays were also employed for qualitative analysis. Protein aggregates were incubated with Congo red dye, and an absorbance spectrum was recorded over the 400 to 600 nm range. The amyloid fibrils were confirmed by an apple-green birefringence in polarized light microscopy.

To determine secondary structural transitions, CD spectroscopy was conducted in the far-UV region between 190 and 250 nm. This enabled the determination and quantification of β-sheet-rich structures in the protein samples. Morphological characterization of the fibrils was done using transmission electron microscopy (TEM) (González). The samples were placed on carbon-coated copper grids and uranyl acetate stained. Micrographs were captured to evaluate fibril length, shape, and surface characteristics. Further structural proof of amyloid was obtained through the use of Fourier-transform infrared (FTIR) spectroscopy. Spectroscopic analysis within the amide I region of 1625-1645 cm^1 was utilized to view the characteristic β-sheet markers of amyloid fibrils.

4. Analysis of Oligomeric Species

To identify and describe protein aggregation intermediates, Western blotting and SDS-PAGE were performed. After molecular weight fractionation of the protein aggregates, specific antibodies such as anti-Aβ and anti-α-synuclein were employed to detect monomeric, dimeric, and oligomeric forms of the proteins. Size exclusion chromatography (SEC) was used as a complementary technique to separate different sizes of protein aggregates, allowing identification of monomers, oligomers, and protofibrils based on their elution patterns (Rofo et al., 2021).

5. Cytotoxicity Assessment of Aggregates

To ascertain the biological relevance of the protein aggregates that are generated, cell viability assays were carried out with human cell lines. SH-SY5Y neuroblastoma cells and HEK293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and cultured in a humidified atmosphere at 5% CO_2 with 37°C. Cells were treated with various concentrations of the misfolded protein aggregates for 24 hours. MTT assay was employed to determine mitochondrial metabolic activity as a measure of cell viability. The formation of formazan from MTT by viable cells was measured spectrophotometrically, and the values were reported as a percentage of untreated controls. To further determine cytotoxic effects, release assays for lactate dehydrogenase (LDH) were also employed. LDH release from lysed or injured cells was quantified, an estimate of aggregate-induced cytotoxicity and membrane integrity (Pereira et al., 2018).

6. Molecular Docking and Simulation Studies

For exploring candidate therapeutic molecules, which would have the capability of inhibiting amyloid formation or destabilizing established fibrils, molecular docking experiments were performed. A few candidate molecules reported previously to break the amyloid assembly, for instance, curcumin, EGCG, and doxycycline, were used as reference ligands. Using AutoDock Vina software, binding with the native and aberrantly folded protein structure was modeled by these test ligands.

To further confirm ligand-protein interaction stability, a molecular dynamics (MD) simulation was performed via the GROMACS platform. Protein-ligand complexes were simulated under physiologic conditions, and monitoring of parameters including root-mean-square deviation (RMSD), radius of gyration, and hydrogen bond stability during the course of simulation helped in evaluating the conformation stability and binding affinity (Verma et al., 2016).

7. Statistical Analysis

All experimental steps were conducted in triplicate for reproducibility. The derived data were statistically processed using GraphPad Prism and SPSS programs. Results were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by suitable post-hoc tests or Student's t-test was conducted for comparative analysis with a significance cutoff of $p < 0.05$.

Ethical Statement

Because this study is conducted entirely in vitro and no human subjects or live animals are involved, there is no formal need for ethical approval. Nonetheless, all laboratory procedures using recombinant proteins

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were undertaken under Biosafety Level 2 (BSL-2) in conformity with institutional safety protocols.

Summary of Workflow

The method included expression and purification of amyloidogenic proteins, controlled misfolding induction, characterization of fibril and oligomer growth by spectroscopic and microscopic analysis, assessment of cytotoxicities against cultured cells, and computer modeling to study therapeutic inhibition of protein aggregation. This integrated research strategy provides a strong analysis of biochemical and pathological characteristics of protein misfolding in amyloidosis.

Results

The study yielded comprehensive data on the biochemical characteristics of stress-induced amyloidogenic proteins, their structural conversion into amyloid fibrils, cytotoxic activity of misfolded species towards human cells, and inhibitory activity of selected therapeutic compounds. The results offer a clear picture of protein misfolding and its potential regulation.

1. Successful Expression and Purification of Amyloidogenic Proteins

Recombinant forms of β-amyloid (Aβ1–42), α-synuclein, and transthyretin (TTR) were overexpressed in E. coli and purified with affinity chromatography. Each protein was purified and confirmed with SDS-PAGE as single bands consistent with their calculated molecular weights: 4.5 kDa for Aβ, 14 kDa for α-synuclein, and roughly 55 kDa for tetrameric TTR. Bradford assay validated protein yields to be very high at average values of 1.8 mg/mL post-final dialysis.

2. Induction of Protein Misfolding and Aggregation

After incubation under physiologically relevant stress conditions, the purified proteins exhibited evident signs of misfolding and aggregation. Aggregation was most evident after 48 hours, as measured by increased turbidity and the formation of precipitates. β-amyloid and α-synuclein, in particular, exhibited early onset of aggregation compared to TTR, which was relatively stable up to 24 hours of incubation.

3. Thioflavin T and Congo Red Assays Confirm Amyloid Formation

Increased intensity of Thioflavin T fluorescence in Aβ and α-synuclein samples affirmed the presence of amyloid fibrils rich in β-sheets. The intensity of fluorescence increased over time, with Aβ1–42 plateauing after 48 hours, indicating the maturation of the fibrils to be complete. Congo red assays also reaffirmed these observations as absorbance shifts and apple-green birefringence were detected with polarized light microscopy, affirming the amyloid nature of aggregates.

4. Spectroscopic Analysis Reveals Structural Transitions

Circular dichroism spectroscopy indicated a shift towards largely random coil or α-helix forms to β-sheet-enriched conformations in secondary protein structure. CD spectra revealed a typical negative peak at 218 nm in Aβ and α-synuclein after 48 hours, indicating successful amyloid fibril formation. FTIR spectra also established these transformations with intense amide I bands at 1628–1638 cm^{-1}, representing the predominance of β-sheet structure.

5. Morphological Evidence from Electron Microscopy

Transmission electron microscopy gave direct visual evidence of fibril formation. The Aβ1–42 and α-synuclein micrographs showed long, unbranched fibrillar structures with diameters of 8–12 nm and lengths of several micrometers. TTR produced shorter and more broken-up fibrils. The morphology was in line with classic amyloid fibrils, which further supported the biochemical and spectroscopic evidence.

6. Oligomeric Species Identified via Western Blot and SEC

Western blotting demonstrated differential monomeric, dimeric, and oligomeric bands for α-synuclein and Aβ. Oligomeric bands were detected after 12–24 hours of incubation before fibril formation. Size exclusion chromatography also indicated that early incubation samples had soluble oligomers, followed by subsequent fractions eluting in the form of larger aggregates and protofibrils. This

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shows a sequential progression from monomers to cytotoxic oligomers and eventually mature fibrils.

7. Cytotoxicity of Aggregates Confirmed in Cell Lines

Treatment of SH-SY5Y and HEK293 cells with protein aggregates led to a considerable loss in cell viability, particularly with soluble oligomers of α-synuclein and Aβ. The MTT assay revealed a dose-dependent loss in metabolic activity. The cell viability was reduced to 56% in SH-SY5Y cells treated with Aβ1–42 oligomers at 10 μM aggregate concentration. LDH assays verified membrane damage, with an elevated release of LDH from treated cells, corroborating the toxic capacity of the aggregates. TTR aggregates exhibited relatively lesser toxicity, which is consistent with their aggregate kinetics being slower.

8. Molecular Docking and Dynamics Suggest Effective Inhibition

Molecular docking experiments reported high binding affinities of curcumin and EGCG with the misfolded conformations of Aβ and α-synuclein. Binding energies were in the range –8.1 to –9.3 kcal/mol, and major interactions were found at hydrophobic core regions involved in aggregation. Molecular dynamics simulations for 100 nanoseconds revealed stable interactions, negligible RMSD fluctuations, and regular hydrogen bonding, suggesting favorable complex formation and possible inhibition of fibril growth. Doxycycline had moderate binding activity but exhibited promise to destabilize preformed fibrils.

9. Statistical Significance of Findings

All experimental results were processed using proper statistical analysis. One-way ANOVA revealed highly significant differences in ThT fluorescence intensity, levels of cytotoxicity, and structural changes between native proteins, oligomers, and mature fibrils ($p < 0.01$). Post-hoc comparisons verified that oligomers were significantly more cytotoxic than monomeric or fibrillar structures. Reproducibility of the results in triplicate experiments validates the strength of the findings.

Discussion

The results of this work offer valuable information on the molecular and biochemical processes involved in protein misfolding and amyloid formation, and also indicate potential therapeutic approaches. The experimental data confirm the idea that protein misfolding and aggregation into amyloid fibrils are key processes in amyloidosis and other protein-misfolding diseases, including Alzheimer's and Parkinson's diseases.

Successful purification and in vitro misfolding induction of β-amyloid (Aβ1–42), α-synuclein, and transthyretin (TTR) proteins enabled a systematic examination of their structural changes. Thioflavin T and Congo red assays results showed conclusively the progression of native to aggregated forms, with β-sheet-rich structures forming over time (Ciccone et al., 2020). This is supported by earlier work indicating that stressful conditions like low pH, oxidative stress, and metal ion imbalance are responsible for destabilization of native conformations and thus for aggregation.

The differential kinetic formation of the three proteins in question mirrors their different propensities to misfold. Aβ and α-synuclein exhibited much more rapid and extensive formation than TTR, which lends support to the hypothesis that these proteins have inherently unstable conformations or contain key sequences, usually characterized as aggregation-prone regions (APRs), which serve as nucleation sites for fibril formation. These regions have been associated with a number of systemic and neurodegenerative disorders, making them even more biologically relevant (Giorgetti et al., 2018).

Detection of oligomeric species in early stages of aggregation is specifically important. Western blot and size-exclusion chromatography results revealed that such oligomers are formed prior to the mature fibrils and are the probable cause of initiating cellular toxicity. This comes in accordance with the current shift in amyloid research paradigm that increasingly implicates soluble oligomers, rather than mature fibrils, as being the major neurotoxic factors (Shaw et al., 2025).

In our cytotoxicity experiments, oligomeric Aβ and α-synuclein treatment of both neuronal and non-neuronal cell lines caused a severe reduction in cell

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viability and membrane leakage. These observations highlight the key role played by initial misfolded intermediates in inducing cellular damage, largely through pathways that include membrane disruption, disruption of calcium homeostasis, and mitochondrial dysfunction. The relatively lower toxicity of TTR oligomers may account for the late clinical presentation of TTR-associated amyloidoses, e.g., familial amyloid polyneuropathy (Shaw et al., 2025).

The FTIR and CD spectroscopic measurements, which revealed strong β-sheet bands in aggregated samples, supported the biochemical assays. This evidence demonstrates that conversion from native to amyloid conformation is not indiscriminate but along a structural pathway that includes directed secondary structure rearrangements. The electron microscopy photographs also confirmed the fibrillar structure of aggregates and exhibited morphology characteristic of pathological amyloids present in post-mortem tissues of amyloidosis and allied disorders patients (Kardos et al., 2025).

Taken together, these structural studies support the hypothesis that protein misfolding is an ordered and hierarchical process wherein native monomers become oligomers, protofibrils, and ultimately mature fibrils. Interestingly, each step appears to have distinct biochemical and pathological properties with therapeutic variation among these conformers (Ansari et al., 2016).

Our research also tested the activity of natural and synthetic compounds—curcumin, EGCG, and doxycycline—in influencing protein aggregation. The molecular docking and dynamics simulation results showed that curcumin and EGCG had high affinities for binding and stable interaction with Aβ and α-synuclein, implicating that the two compounds are capable of hindering aggregation through stabilization of non-toxic conformations or sequestering APRs. This concurs with available literature to identify the potency of polyphenolic compounds to hinder or reverse amyloid fibril formation (Ansari et al., 2016).

In addition, these compounds' ability to inhibit the formation or disaggregate preformed fibrils, according to in silico and initial in vitro screens, opens doors to early therapeutic intervention in such patients. As a caveat, however, careful attention must be paid to in silico prediction limitations, for which validation shall have to follow in cellular and animal models of detailed kinetic and structural characterizations (Berntsson et al., 2023).

Doxycycline, a tetracycline antibiotic, showed moderate inhibition and is known to operate through a mechanism that is different from the others, quite possibly through destabilization of fibrillar structures rather than through inhibition of initial misfolding. This further points to the necessity for specific therapeutic measures based on the stage of protein aggregation and the protein involved (Lomont et al., 2018).

Notwithstanding these encouraging results, some limitations need to be recognized. In vitro conditions under which this study was performed are highly controlled and do not accurately reflect the intricacies of the intracellular environment, such as the action of chaperones, proteasomal degradation, post-translational modifications, and cellular compartmentalization. Our results thus provide a starting point for understanding aggregation mechanisms, and their further validation in vivo is necessary (Meng et al., 2019). Moreover, the therapeutic candidates screened in this study need to undergo pharmacokinetic and toxicity profiling prior to clinical translation. The blood–brain barrier permeability, systemic bioavailability, and metabolic stability of curcumin and EGCG, for example, are well-known drug development challenges.

Future research should also investigate the pathways by which genetic mutations and environmental factors control protein stability and susceptibility to aggregation. The creation of real-time monitoring assays for aggregation and sophisticated imaging reagents should also continue to advance research into misfolding mechanisms in intact cells and tissues.

Conclusion

This article offers critical information regarding the biochemical processes of protein misfolding and amyloid assembly, which are the basis of amyloidosis and associated diseases. From structural, spectroscopic, and cytotoxic examination, we established that oligomeric intermediate forms of protein species such as Aβ and α-synuclein are highly

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cytotoxic and play a role in cellular dysfunction. The potency of natural molecules like curcumin and EGCG to prevent or reverse aggregation is an exciting therapeutic direction. Yet, additional in vivo studies and clinical verification are needed. In general, elucidation of the stepwise nature of protein misfolding not only expands our insight into disease mechanisms but also informs the design of targeted therapies to forestall or treat amyloid-associated diseases.

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