Biophysical Insights into Implications of PEG-400 on the α-Crystallin Structure: Multispectroscopic and Microscopic Approach

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ABSTRACT: Aggregation and precipitation of α-crystallin play a vital role in the cataract development. This study was targeted to delineate the effect of PEG-400 on the structural integrity of α-crystallin employing a multispectroscopic and microscopic approach. Intrinsic fluorescence and UV–vis spectroscopy suggested alterations in the tertiary structure of α-crystallin, namely global transition of native α-crystallin to a non-native form in the presence of PEG–400. Circular dichroism spectroscopy suggested secondary structural transition in a native conformation of α-crystallin in the presence of PEG-400. Loss in the native conformation of α-crystallin is implicated in cataract developments, thus highlighting the clinical significance of this work. Further, a significant increase in ANS fluorescence of PEG-400-incubated α-crystallin (7 days) suggested this non-native form to be molten globule (MG)-like state. Increased Thioflavin T fluorescence (ThT) and congo red (CR) absorbance along with transmission electron microscopy (TEM) confirmed the formation of the aggregates of α-crystallin after prolonged incubation with PEG-400. Insights into PEG-400-induced structural alterations can provide a platform to search for new therapeutic molecules that can combat α-crystallin-directed eye diseases.

1. INTRODUCTION

A cataract is defined as the opacity of a transparent eye lens, which interferes with light transmission to the retina leading to vision impairment. According to the literature, this lethal eye disorder is one of the major causes of blindness across the globe; statistical data demonstrate it to be responsible for nearly 40–80% out of the estimated 45 million blindness cases.1 There are two mechanisms through which cataract can occur, namely, the condensation process; lenticular opacity results from loss of solubility and increased nucleation rate of the crystallins2 or because of unfolding or destabilization of lens crystallins leading to cataractogenesis.3 To maintain the transparency of lens, crystallins are key players. These structural proteins protect the eye lens and because crystallin aggregation and precipitation are associated with cataract, crystallins are of utmost importance. There are three major lens crystallins; α-crystallins and β-crystallins can bind to cell membranes and cytoskeleton, while γ-crystallins, the smallest among the three crystallins, are localized in the nuclear lens and characterized by high protein concentration and low water content.4 α-Crystallin is also called as molecular chaperone constituting a vital factor of stress responses1–7 as it binds to partially unfolded polypeptides maintaining the integrity in a refolding competent state thereby protecting the cell from after effects of irreversible aggregation of proteins.8–10 The expression of αA-crystallin is primarily restricted to the lens with minute amounts found in other tissues,11 while αB-crystallin is ubiquitously expressed and is copious in the brain, heart, and muscle. There are various pieces of literature available that reported the importance of the α-crystallin lens in ocular biology coupled with the physiology of other tissues,11 thus making it the protein of interest. α-Crystallins are multifunctional proteins with diverse biological functions that have generated considerable interest in understanding their roles in health and disease.12 The importance of α-crystallin in cataract can be owed to the fact that the chaperoning ability of α-crystallin is influential in maintaining lens transparency which ultimately plays a role in cataract. The decreased chaperone-like activity of α-crystallin is associated with various types and stages of cataract, highlighting the therapeutic potential of α-crystallin in cataract treatment.13 α-Crystallin serves as the molecular chaperone, acting as a defense, preventing aggregation of damaged/aged lens proteins and enzymes that would otherwise lead to light scattering thereby leading to cataract formation. This chaperone-like

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activity (CLA) of α-crystallin is vital for lens transparency and it is hypothesized that cataract can be slowed down by maintaining optimal or increasing chaperone activity. According to a study, small molecule pharmacological agents from natural sources can prevent the loss of CLA of α-crystallin A-chain (AAC), leading to a delay in cataract formation in preclinical models. Further, it is estimated that if a cataract formation is deferred by 10 years then it can reduce the vision care expense by 50%. A further study also revealed that drug-like synthetic small molecules representing the CAP01023 series specifically increase AAC CLA thereby maintaining the transparency of the eye lens in organ culture experiments of the cataract model.

Any alteration in native conformation of protein leads to altered functionality because a protein must be in its proper conformation to function properly. Cross-seeding of misfolded proteins has pathological implications in protein aggregation disease.14 The protein is functionally active in its native conformation, losing which causes the aggregation of proteins. Protein aggregates are at the heart of many disorders and hence insights into novel therapeutic ways to cure protein aggregate-associated disorders require a sensible understanding of the forces that play a role in protein aggregation and transition of proteins into β-sheet-rich aggregates. Eye drops comprise PEG400 as it is used in ophthalmic solutions for the relief of burning, irritation, and/or discomfort that follows dryness of the eye. With traditional eye drops, only small amounts of the administered drug penetrate the cornea to reach the desired lens tissue because of corneal barriers and dilution caused by lacrimal fluids.15 Thus, PEG400 reaches the lens and hence this is the basis behind this study investigating the effect of PEG400 on the structural integrity of α-crystallin protein. Further, there are in vitro studies also that reported that the intravitreal injection of PEG-400 in rabbits resulted in severe ocular toxicity.16-18 Thus, the rationale behind our study was to investigate the effect of PEG400 on α-crystallin and is the first report of this kind, thus highlighting the novelty of our research.

The present study investigated the effect of PEG-400 on the structural integrity of α-crystallin employing a multispectroscopic and microscopic approach.

### Results

#### 2.1. Intrinsic Fluorescence.

Out of three intrinsic fluorophores, tryptophan has the highest contribution with the other two (tyrosine and phenylalanine) contributing minimally. Intrinsic fluorescence parameters viz. intensity and $\lambda_{\text{max}}$ serve as important markers to detect conformational changes taking place in proteins;19,20 the polarity of the microenvironment around tryptophan plays a pivotal role.20,21 The high reliance of Trp fluorescence on its surroundings is exploited in several studies that elucidate protein conformational changes upon ligand interaction. Native α-crystallin shows a peak corresponding to 346 nm, signifying that the protein is present in its native form (Figure 1A). The integrity of the protein’s tertiary structure can be determined by intrinsic fluorescence spectroscopy. The effect of varying PEG-400 concentrations was evaluated in terms of intrinsic fluorescence. It is obvious from Figure 1A that PEG-400 is inducing conformational alterations in α-crystallin after 7 days of incubation with varying PEG-400 concentrations (1, 1.5, and 2 mM). It is only after incubation of 7 days; significant structural changes were observed. Before day 7, there was no significant change in fluorescence intensity; however, after the seventh day, a drastic change in fluorescence intensity was observed (Figure S1). The concentrations below 1 mM PEG-400 were also analyzed for inducing structural changes evident from changes in fluorescence spectra, but the concentration of PEG-400 below 1 mM does not induce any significant change in α-crystallin (Figure S2). In the presence of 1 mM PEG-400, a significant decrease in fluorescence intensity was observed coupled with no peak shift implying the structural transition in native α-crystallin. Further, in the presence of 1.5 mM and 2 mM PEG-400, a decline in fluorescence intensity was apparent along with a redshift of 2 nm; a maximum decrease in fluorescence intensity observed in the presence of 2 mM PEG-400. Thus, it can be said that PEG-400 induces partial unfolding of the protein, exposing the tryptophan residues to the polar environment. The decrease in fluorescence intensity can also be attributed to the fact that quenching might occur owing to protonated groups such as Asp or Glu which comes in proximity of tryptophan residues upon unfolding. However, it can be concluded from the above data that PEG-400 induces the conformational changes in α-crystallin causing it to become loosely packed, that is, less compact, which exposes the earlier
buried tryptophan residues to water causing a reduction in the fluorescence intensity; with maximum unfolding occurring for 2 mM PEG-400. Thus, intrinsic fluorescence suggests alterations in the tertiary structure of native α-crystallin in the presence of PEG-400.

2.2. UV–Vis Spectroscopy. To validate fluorescence spectroscopy results, UV spectroscopy was also deployed. Figure 1B depicts the UV spectral profile of native α-crystallin and α-crystallin incubated with varying PEG-400 concentrations for 7 days. Changes in the spectral profile were evident, thus implying the fact that PEG-400 induces changes in the tertiary structure of the protein. α-Crystallin in its native form shows a peak at around 280 nm characteristic of the protein in proper conformation (Figure 1B). However, a significant reduction in absorbance coupled with a significant blue shift was evident in the presence of varying PEG-400 concentrations; a blue shift of nearly 8 nm observed for 2 mM PEG-400 is implicative of the maximum alterations in the tertiary structure induced by 2 mM PEG-400. Fluorescence spectroscopy coupled with near-UV spectroscopy suggests that tertiary structural alterations occurred in native α-crystallin upon prolonged incubation with PEG-400. Further, insights into the effect of PEG-400 on native α-crystallin and exposure of hydrophobic patches can be done with the help of ANS fluorescence.

2.3. Circular Dichroism Spectroscopy (CD). CD spectroscopy is a technique that is often exploited to characterize secondary structural alterations in a native protein owing to the binding of a ligand or some other factors. Changes in the secondary structure of the protein are highly sensitive and correspond to changes in far-UV CD spectra. CD spectroscopy was carried out to have an insight into the secondary structure of α-crystallin in the absence and presence of PEG-400 to further investigate the structure of the protein. Figure 2 shows the far-UV CD spectra of native α-crystallin and α-crystallin in the presence of 2.0 mM PEG-400 (8th day). It is quite apparent from the figure that significant changes are taking place in the secondary structure of α-crystallin in the presence of 2 mM PEG-400 when incubated for 8 days. It is only after the 8 day incubation that significant secondary structural alterations are observed in α-crystallin, implying that very minimal secondary structural changes are taking place before this. Thus, these secondary structural alterations in native α-crystallin induced by PEG-400 upon prolonged incubation for 8 days imply that PEG-400 induces the structural transition of native crystallin.

2.4. ANS Fluorescence. ANS fluorescence has been widely used as a probe to monitor the conformational transitions in proteins because of its affinity for partially exposed hydrophobic regions of the protein structure.22 ANS is believed to strongly bind cationic groups of proteins through ion-pair formation.23 Usually, a globular protein in its native conformation possesses a tightly packed, solvent inaccessible hydrophobic core that prevents ANS from binding to it, and hence, no or very little ANS fluorescence is observed for the native protein. Usually, ANS does not bind to the folded or unfolded protein, but its fluorescence intensity increases upon binding to the partially structured folding intermediates.24 To confirm the existence of an intermediate state of α-crystallin in the presence of PEG-400, ANS fluorescence was deployed. Upon binding to the hydrophobic site, it displays a shift in its emission maximum that allowed us to quantify the hydrophobicity of a protein. The exact mechanism of ANS fluorescence is that when a protein loses its native conformation without losing its secondary structure, hydrophobic patches are exposed subsequently to which ANS binds and enhanced fluorescence is observed. Figure 3A depicts ANS fluorescence emission spectra of native α-crystallin in the absence and presence of varying concentrations of PEG-400. There was no significant change in fluorescence intensity of α-crystallin when incubated with PEG-400 below 1 mM, hence concentrations above 1 mM PEG-400 were chosen (Figure 3B). Native α-crystallin shows negligible ANS fluorescence (Figure 3). However, a significant hike in ANS fluorescence is observed for PEG-400-incubated crystallin for 7 days (1, 1.5, and 2 mM). When a protein loses its native conformation, the dye becomes more accessible to hydrophobic patches; ANS goes and binds to these patches thus causing the fluorescence to increase abruptly. Molten globules are known to possess larger surface hydrophobicity and hence more ANS binding, thus, the state observed at day 7 can be regarded as a MG-like state.25 With a high concentration of PEG-400, its effect on crystallin becomes more pronounced which is evident from a large increase in ANS fluorescence (nearly 20 times of native). Thus, it can be said that when incubated for 7 days, PEG-400 destabilized the tertiary structure of the protein without affecting its secondary structure to a large extent (secondary structural alterations were observed only after 8 days incubation), hinting toward the formation of the MG-like state of α-crystallin in the presence of PEG-400; with maximum destabilization taking place for 2 mM PEG-400. Hence, it is quite evident from these observations that PEG-400 induces the formation of an intermediate state of α-crystallin when incubated with PEG-400 for a prolonged period.

2.5. Thioflavin T Fluorescence. Thioflavin T (ThT) is a dye that extensively binds to β-sheets thereby serving as an important marker for aggregate characterization. Post binding of ThT dye with aggregates, there is an increase in ThT fluorescence.26 Figure 4A shows the relative ThT fluorescence intensity of native α-crystallin and 2 mM PEG-400-incubated α-crystallin as a function of the number of days (0–8 days). It is evident from the figure that native α-crystallin does not show any increase in ThT fluorescence up till day 8 while 2 mM PEG-400-incubated α-crystallin shows an increase in ThT fluorescence up till day 8.
fluorescence; a significant increase in ThT fluorescence was observed for 2 mM PEG-400-incubated α-crystallin with day 8 showing maximum ThT fluorescence suggestive of aggregated formation. Figure 4B shows the ThT fluorescence spectra of native α-crystallin (day 8) and α-crystallin incubated with varying PEG-400 concentrations (1, 1.5, and 2 mM).

2.6. Congo Red Assay (CR). The azo dye CR has a high affinity with the β-pleated structure of all forms of amyloid. Thus, to complement our ThT fluorescence observations, CR assay was also used. The results were in line with ThT observations. Figure 5 shows the CR absorption spectra of native α-crystallin and α-crystallin after 8 days of incubation with 2 mM PEG-400. It is evident from the figure that a significant increase in absorbance was observed for 2 mM PEG-400-incubated α-crystallin (8 days) as compared to native α-crystallin (day 8). Thus, ThT fluorescence together with CR assay observations advocated the fact that 2 mM PEG-400 induces aggregate formation in α-crystallin after 8 days of incubation which was further validated by electron microscopy.

2.7. Transmission Electron Microscopy (TEM). The morphological evolution of α-crystallin aggregate formation upon incubation with PEG-400 was monitored using transmission electron microscopy (TEM). Figure 6A,B depicts TEM images of native α-crystallin and 2 mM PEG-400-incubated α-crystallin (eighth day). It is quite evident from the figure that 2 mM PEG-400 induces aggregate formation in α-crystallin. TEM images of 2 mM PEG-400-incubated α-crystallin displays the globular type aggregates. TEM analysis further validates in vitro observations suggesting that aggregate formation of α-crystallin takes place when incubated for 8 days.

3. DISCUSSION
This study delineates the effect of PEG-400 on the structure of α-crystallin which is a key player in maintaining structural integrity. Loss in native conformation of α-crystallin is directly associated with cataract development thus making this study clinically significant. Structural alterations in α-crystallin induced by varying concentrations of PEG-400 were investigated by employing UV–vis and intrinsic fluorescence spectroscopy; changes in UV–vis and intrinsic fluorescence implied that there was a structural transition in native α-crystallin in the presence of PEG-400 to non-native form. CD spectroscopy was employed to analyze secondary structural
changes in α-crystallin in the presence of varying PEG-400 concentrations and it was found that there are significant changes in the secondary structure of native α-crystallin in the presence of PEG-400. Further, ANS fluorescence was employed and it was found that ANS fluorescence is significantly enhanced for PEG-400-incubated crystallin (7 days). This increased ANS fluorescence is suggestive of the fact that PEG-400 induces α-crystallin to molten globule (MG) like state. There are many reports which point out that molten globule leads to aggregation. Further, ThT fluorescence was used to investigate the aggregate formation in α-crystallin in the presence of PEG-400 after prolonged incubation (day 8). There was a marked increase in ThT fluorescence of α-crystallin after 8 days of incubation with varying PEG-400 concentrations (1, 1.5, and 2 mM); the maximum increase observed for 2 mM PEG-400 suggesting maximum aggregate formation. Further, TEM was used to confirm the presence of aggregates and it was obvious from the microscopic analysis that α-crystallin aggregates are formed after 8 days of incubation with 2 mM PEG-400. The importance of this study can also be attributed to the fact that aggregation and denaturation of crystallin are directly linked to cataract development. It should be noted that PEG induces MG like state and aggregates after 7 and 8 days, respectively, so only prolong interactions may affect the structure of the protein. Exposure of PEG-400-induced structural alterations is an advanced study that can provide a platform to search for new therapeutic molecules that can combat α-crystallin-directed eye diseases.

4. MATERIAL AND METHODS

4.1. Materials. α-Crystallin from bovine eye lens (C-4163) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). PEG-400 was bought from Merck chemicals. All the other chemicals used for the preparation of buffers and other reagents were of the best analytical grade available. Double distilled water from a Milli-Q UF-Plus purification system was used for all the experiments.

4.2. Stock Solution α-crystallin and PEG-400. An initial stock solution of 16 μM (9.5 mg/mL) α-crystallin was prepared in 0.02 M Tris buffer, pH 8.1. The concentration of α-crystallin was determined using the extinction coefficient, ε_{280nm} = 0.9 g·cm⁻².²⁷ For PEG-400, the stock solution was made up of 4 mM which was further used for all experiments.

4.3. Intrinsic Fluorescence Spectroscopic Measurements. The fluorescence spectrum was recorded on a Jasco FP-6200 spectrofluorometer (Japan) in a 10 mm path-length quartz cell. The protein was excited at 280 nm, while its emission spectrum was recorded in the range of 300–400 nm. Native α-crystallin was incubated with different concentrations (1, 1.5, and 2 mM) of PEG-400. Each spectrum was the average of at least three scans.

4.4. UV Spectroscopic Measurements. UV absorption measurements of native α-crystallin and α-crystallin in the presence of varying PEG-400 concentrations was recorded using a spectrophotometer Jasco 660 (Jasco, JAPAN) with a cuvette of 1.0 cm path length.²⁸ Each spectrum was an average of three scans.

4.5. ANS Fluorescence Measurements. ANS binding was measured by recording fluorescence spectra exciting the protein at 380 nm with a recording range of 400–600 nm.²⁹ The concentration ANS was taken 100 fold relative to the protein concentration.²³

4.6. ThT Fluorescence. ThT spectra were recorded on Jasco FP 6200 in a 1 cm path length quartz cuvette. The experimental parameters were: excitation was carried out at 440 nm while the recording range was 450–600 nm. The final concentration of α-crystallin was 4 μM while ThT was taken in a ratio of 1:8.

4.7. Congo Red Assay. The CR assay was also used to characterize α-crystallin aggregates by retorting the redshift of CR upon binding with aggregates as per previously published protocols.³⁰ Aliquots were prepared in the presence of different concentrations of PEG 400 (1, 1.5, and 2 mM) with the α-crystallin concentration of 2.5 mg/mL and incubated for 4 h, and, afterward, spectra were recorded in the range of 400–700 nm.

4.8. CD Spectroscopy. CD spectra of free protein and protein in the presence of PEG-400 were recorded making use of a JASCO-J-1500 spectropolarimeter connected with a Peltier-temperature controller in the far-UV region at room temperature under a nitrogen atmosphere with a slit width of 2 nm as per previous studies.³¹ The concentration of protein was 4 μM and PEG-400 concentration was 2 mM. Each spectrum was an average of five scans to remove noise.³²
4.9. Transmission Electron Microscopy. For visualizing the aggregate structure, transmission electron micrographs were collected on a JOEL JEM-2100 (Japan). The experimental apparatus was similar to the ones used in earlier studies.

4.10. Statistical Analysis. All the experiments were performed in triplicates and the data obtained has been expressed in mean ± standard error of the mean (SEM).

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02648.

Relative intrinsic fluorescence of α-crystallin in the presence of 1, 1.5, and 2 mM PEG-400 as a function of number of days (0–8), native protein without PEG-400 is also depicted for a span of 8 days, and relative intrinsic fluorescence as a function of PEG-400 concentrations (PDF)

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Author Contributions
All the authors contributed to the successful accomplishment of this manuscript. A.S. conceived and designed the experiments; A.I. and A.S. performed the experiments; S.A. helped in fluorescence quenching studies; T.M. performed molecular modeling studies; A.S., I.H., M.I.H, F.A., and A.I analyzed the data; A.S., T.M., S.A., and A.I prepared the manuscript.

Notes
The authors declare no competing financial interest.

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REFERENCES

(1) Riaz, Y.; Mehta, J. S.; Wormald, R.; Evans, J. R.; Foster, A.; Ravilla, T.; Snellingen, T. Surgical interventions for age-related cataract. Am. J. Ophthal. 2007, 143, 733–734.
(2) Meehan, S.; Berry, Y.; Luisi, B.; Dobson, C. M.; Carver, J. A.; MacPhee, C. E. Amyloid fibril formation by lens crystallin proteins and its implications for cataract formation. J. of Biol. Chem. 2004, 279, 3413–3419.
(3) Harding, J. J. Cataract, Alzheimer’s disease, and other conformational diseases.Curr. Opin. Ophthal. 1998, 9, 10–13.
(4) Sharma, K. K.; Santhoshkumar, P. Lens aging effects: of crystallins. Biochim. Biophys. Acta, Gen. Subj. 2009, 1790, 1095–1108.
(5) Horwitz, J. Alpha-crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. U.S.A. 1992, 89, 10449–10453.
(6) Ingolia, T. D.; Craig, E. A. Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc. Natl. Acad. Sci. U.S.A. 1982, 79, 2360–2364.
(7) Klementz, R.; Fröhli, E.; Steiger, R. H.; Schäfer, R.; Aoyama, A. Alpha B-crystallin is a small heat shock protein. Proc. Natl. Acad. Sci. U.S.A. 1991, 88, 3652–3656.
(8) Ehrensperger, M.; Gräber, S.; Gaestel, M.; Buchner, J. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. EMBO J. 1997, 16, 221–229.
(9) Jakob, U.; Gaestel, M.; Engel, K.; Buchner, J. Small heat shock proteins are molecular chaperones. J. Biol. Chem. 1993, 268, 1517–1520.
(10) Lee, G. J.; Roseman, A. M.; Saibil, H. R.; Vierling, E. A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. EMBO J. 1997, 16, 69–71.
(11) Srinivasan, A.; Naginiene, C.; Bhat, S.; alpha. alpha A-crystallin is expressed in non-ocular tissues. J. Biol. Chem. 1992, 267, 23337–23341.
(12) Nagaraj, R. H.; Nahomi, R. B.; Mueller, N. H.; Raghavan, C. T.; Ammar, D. A.; Petresh, J. M. Therapeutic potential of α-crystallin. Biochim. Biophys. Acta, Gen. Subj. 2016, 1860, 252–257.
(13) Babizhayev, M. A. Structural and functional properties, chaperone activity and posttranslational modifications of alpha-crystallin and its related subunits in the crystalline lens: N-acetylcarnosine, carnosine and carcinine act as alpha-crystallin/small heat shock protein enhancers in prevention and dissolution of cataract in ocular drug delivery formulations of novel therapeutic agents. Recent Pat. Drug Delivery Formulation 2012, 6, 107–148.
(14) Tripathi, T.; Khan, H. Direct Interaction between the β-Amyloid Core and Tau Facilitates Cross-Seeding: A Novel Target for Therapeutic Intervention. Biochem 2020, 59, 341–342.
(15) Patel, S.; Barnett, J. M.; Kim, S. J. Retinal toxicity of intravitreal polyethylene glycol 400. J. Ocul. Pharmacol. Ther. 2016, 32, 97–101.
(16) Teale, F. W. J. The ultraviolet fluorescence of proteins in neutral solution. Bioch. J. 1960, 76, 381.
(17) Gryczynski, I.; Wiczk, W.; Johnson, M. L.; Lakowicz, J. R. Lifetime distributions and anisotropy decays of indole fluorescence in cyclohexane/ethanol mixtures by frequency-domain fluorometry. Biophys. Chem. 1988, 32, 173–185.
(18) Piston, D. W.; Kremers, G.-J. Fluorescent protein FRET: the good, the bad and the ugly. Trends Biochem. Sci. 2007, 32, 407–414.
(19) Engelhard, M.; Evans, P. A. Kinetics of interaction of partially folded proteins with a hydrophobic dye: evidence that molten globule character is maximal in early folding intermediates. Proteome Sci. 1995, 4, 1553–1562.
(20) Parray, Z. A.; Ahamad, S.; Ahmad, F.; Hassan, M. I.; Islam, A. First evidence of formation of pre-molten globule state in myoglobin: A macromolecular crowding approach towards protein folding in vivo. Int. J. Biol. Macromol. 2019, 126, 1288–1294.
(21) Shamsi, A.; Ahmed, A.; Bano, B. Glyoxal induced structural transition of buffalo kidney cystatin to molten globule and aggregates:
Anti-fibrillation potency of quinic acid. *IUBMB Life* 2016, 68, 156–166.

(22) Amani, S.; Shamsi, A.; Rabbani, G.; Naim, A. An insight into the biophysical characterization of insoluble collagen aggregates: implication for arthritis. *J. Fluoresc.* 2014, 24, 1423–1431.

(23) Shamsi, A.; Amani, S.; Alam, M. T.; Naeem, A. Aggregation as a consequence of glycation: insight into the pathogenesis of arthritis. *Eur. Biophys. J.* 2016, 45, 523–534.

(24) Tripathi, T.; Na, B.-K.; Sohn, W.-M.; Becker, K.; Bhakuni, V. Structural, functional and unfolding characteristics of glutathione S-transferase of Plasmodium vivax. *Arch. Biochem. Biophys.* 2009, 487, 115–122.

(25) Stsiapura, V. I.; Maskevich, A. A.; Kuzmitsky, V. A.; Uversky, V. N.; Kuznetsova, I. M.; Turoverov, K. K. Thioflavin T as a molecular rotor: fluorescent properties of thioflavin T in solvents with different viscosity. *J. Phys. Chem. B* 2008, 112, 15893–15902.

(26) Hatters, D. M.; Lindner, R. A.; Carver, J. A.; Howlett, G. J. The molecular chaperone, α-crystallin, inhibits amyloid formation by apolipoprotein C-II. *J. Biol. Chem.* 2001, 276, 33755–33761.

(27) Ahmed, A.; Shamsi, A.; Khan, M. S.; Husain, F. M.; Bano, B. Probing the interaction of human serum albumin with iprodione, a fungicide: spectroscopic and molecular docking insight. *J. Biomol. Struct. Dyn.* 2019, 37, 857–862.

(28) Hawe, A.; Sutter, M.; Jiskoot, W. Extrinsic fluorescent dyes as tools for protein characterization. *Pharm. Res.* 2008, 25, 1487–1499.

(29) Naeem, A.; Amani, S.; Correction: Deciphering Structural Intermediates and Genotoxic Fibrillar Aggregates of Albumins: A Molecular Mechanism Underlying for Degenerative Diseases. *PLoS One* 2013, 8 (6). DOI: 10.1371/annotation/04f421f-3dfc-466a-8c21-17a7bab1494c.

(30) Shamsi, A.; Ahmed, A.; Khan, M. S.; Al Shahwan, M.; Husain, F. M.; Bano, B. Understanding the binding between Rosmarinic acid and serum albumin: In vitro and in silico insight. *J. Mol. Liq.* 2020, 311, 113348.

(31) Khan, P.; Rahman, S.; Queen, A.; Manzoor, S.; Naz, F.; Hasan, G. M.; Luqman, S.; Kim, J.; Islam, A.; Ahmad, F. Elucidation of dietary polyphenolics as potential inhibitor of microtubule affinity regulating kinase 4: in silico and in vitro studies. *Sci. Rep.* 2017, 7, 9470.

(32) Tripathi, T.; Rahlfs, S.; Becker, K.; Bhakuni, V. Structural and stability characteristics of a monothiol glutaredoxin: glutaredoxin-like protein 1 from Plasmodium falciparum. *Biochim. Biophys. Acta, Proteins Proteomics* 2008, 1784, 946–952.

(33) Shamsi, A.; Abdullah, K. M.; Usmani, H.; Shahab, A.; Hasan, H.; Naseem, I. Glyoxal Induced Transition of Transferrin to Aggregates: Spectroscopic, Microscopic and Molecular Docking Insight. *Curr. Pharm. Biotechnol.* 2019, 20, 1028–1036.