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Disaggregation of amyloid-like protein aggregates isolated from human cataractous lens

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Crystallins, which represent the major lens protein, play a significant role in ensuring the lens transparency and maintenance of appropriate refractive index of the lens that help in accurate focusing of incident visible light precisely on retina to create clear image perception. Aggregation of lens proteins is known to form the basis of cataract formation. The present study is an attempt to examine the stability of the lens protein aggregates, isolated from human cataract eye lens, against an anionic detergent Sodium dodecyl sulphate (SDS), which is known to disrupt the hydrophobic interaction of protein aggregates. Data that emerged from Congo red (CR), thioflavin T (ThT) and 8-anilino-1-naphthalene sulfonic acid (ANS) binding assay indicated their amyloidogenic nature. A significant reduction in the bathochromic shift of CR λ_{max} and ThT fluorescence emission intensity were observed after treatment of the aggregated proteins with SDS. In the presence of SDS, a significant change in the number and size of the protein aggregates were observed during their morphological analyses under transmission electron microscopy (TEM). Based on the above data it became evident that the hydrophobic interaction plays a crucial role in formation and stabilizing the protein aggregates during cataract formation.

Keywords: Amyloids, Cataract, Crystallin, Disaggregation, Sodium dodecyl sulphate

Vision is one of the most vital senses of living beings, responsible for the visual perception[1]. This vital sense is adversely affected by the onset of the disease called cataract[2,3], which is one of the leading causes of the visual impairment and blindness, all over the world. This disease is relatively more prevailing in developing countries, and the countries with lower socioeconomic status[4-7]. Data of the WHO indicates that nearly 2.2 billion people are living with visual impairment due to cataract and it can only be treated by surgical removal of the eye lens and replacing it with plastic lens, as there is no such drug available for its treatment or prevention[8,9]. Protein constitutes the integral part of living system and their proper structure and function, is essential for organism's survival. Protein aggregates represents one of the major causes for various neurodegenerative diseases such as, Alzheimer's, Huntington's, Spongiform encephalopathy, *etc*[10-12]. These aggregates are found to be highly ordered with abundant β-sheets which are often referred as cross β-sheets. A number of proteins and peptides such as α-synuclein, amyloid β *etc*. are considered to be the major precursors for the formation of toxic protein aggregates. Recent studies have suggested that the existence of amyloid like aggregates and amyloidogenic protein and peptides are present in cataractous eye lens[13-16]. Although some of the lens proteins such as γ-crystallin can form fibrillar structure under *in vitro* conditions, they have not been reported into the cataract eye lens. The major proteins that constitute about 90% of the eye lens are the crystallins[17,18]. They play important role in managing the transparency of the lens, as it refracts the light from outer environment to the retina of the eye, providing a proper vision[19]. When the aggregation of these crystallin proteins in lens occur, it loses its ability to refract the light, and becomes opaque in nature and ultimately leads to the formation of cataract[19]. The aggregates of crystallin can be amorphous or can be highly organized like amyloids, which is still a matter of debate[18]. It is not known that the dominant forces that lead to the aggregation of the lens protein into amyloid like structure is driven by hydrophobic interaction. SDS is known to dissolve the protein aggregates which are mainly formed by hydrophobic interaction. To examine this hypothesis we treated the protein aggregates obtained from

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cataract lens with SDS. In this study we isolated the protein aggregates from surgically removed human cataract lens and used for isolation of water soluble (WS) and water insoluble (WIS) fractions of protein using water extraction method[20]. The insoluble fraction of protein display amyloid like characteristics such as bathochromic shift in λ_{max} of CR, high ThT intensity, high ANS intensity and globular structures under electron microscopy. Presence of SDS significantly reduces the bathochromic shift of CR and ThT, and the particle size was found to be significantly smaller.

Materials and Methods

Materials

The human cataractous eye lenses were collected from the Department of Ophthalmology, Jawaharlal Nehru Medical College and Hospital, Ajmer, Rajasthan, India, in accordance with the ICMR recommendation and Institutional Ethical Committee of Jawaharlal Nehru Medical College and Hospital, Ajmer, Rajasthan, India (Ref. No. 1557/Acad. -III/MCA/2016). Bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), tris-base, disodium phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), potassium chloride (KCl), sodium chloride (NaCl), calcium chloride ($CaCl_2$) were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Sodium azide (NaN_3) congo red (CR), thioflavin-T (ThT), phosphotungstic acid hydrate, sodium dodecyl sulphate (SDS), 8-anilino-1-naphthalenesulfonicacid (ANS) bradford reagent were procured from Sigma-Aldrich Chemical Company, USA. Two hundred mesh Formvar coated copper grids for TEM were obtained from Ted Pella Inc., USA.

Methods

Isolation of human cataractous eye lens proteins

The aggregated protein (amyloids) was isolated from human cataractous eye lenses by using the modified water extraction method[20]. The lenses were collected in cryo vials in 1x DPBS containing 30% *w/v* trehalose and 10% DMSO and stored at −20°C till the further use. The lens samples were thawed to room temperature and washed with ice cold tris-calcium buffer for 30 min on ice. Sample was blot dried and transferred to a centrifuge tube in which tris-EDTA buffer was added and homogenized, following the centrifugation of the sample at 9500 rpm at 4°C for 30 min. Supernatant obtained from this centrifugation is the soluble protein and was stored at 4°C and estimated its protein concentration. Pellet was re-suspended in ice cold pure distilled water and centrifuged at 9500 rpm at 4°C for 30 min. This step was repeated 6 times. All the supernatants were pooled together. Subsequently, 0. 2 M NaCl and 10 mM EDTA was added to this supernatant and incubated for 3 days at 4°C. After incubation sample was centrifuged at 9500 rpm at 4°C for 30 min, pellet was re-dissolved in ice cold water and considered as aggregated protein (amyloids).

Characterization of protein aggregates isolated from cataract eye lens

The isolated protein fractions were characterized by using the below techniques in order to confirm the presence or absence of amyloid aggregates in them.

Congo red (CR) binding assay

CR is the histologic dye possessing the great affinity towards the protein aggregates which are rich in cross-β sheet structures. This dye binds selectively to the amyloidogenic regions of the proteins, providing a red shift in the wavelength of maximum absorption. The stock solution (0. 1% *w/v*) of CR was prepared in distilled water. The analysis of the protein samples were done by keeping the protein and CR concentrations to 250 µg/mL and 35 µM, respectively. The CR absorption spectra for different samples were recorded by a microtiter plate reader in the range of 400-600 nm.

Thioflavin T (ThT) binding assay

The ThT molecule on binding with amyloids provides high fluorescence intensity. For analyzing the samples we had mixed the protein sample with the ThT solution by keeping the concentrations at 250 µg/mL and 20 µM, respectively. The fluorescence spectra were recorded using the JASCO Spectro-fluorometer model RF-5000 in the range of 470-700 nm after excitation at 450 nm, with the slit width of excitation and emission at 10 and 5 nm, respectively, of a quartz cuvette of 1 cm path length.

Intrinsic fluorescence assay

In order to estimate the intrinsic fluorescence of the protein samples, a fluorometer (JASCO Spectrofluorometer FP-8200) was used by keeping the protein concentration as 250 µg/mL. The intrinsic fluorescence spectra of the samples were recorded by keeping the emission range of 290-500 nm, with the excitation at 276 nm and the slit width of excitation and emission as 10 and 5 nm, respectively, of a quartz cuvette of 1 cm path length.

ANS binding assay

The 200 µM stock solution of 8-anilino-1-naphthalene sulfonate (ANS) was prepared by using distilled water, which was then utilized to test the ANS binding to the protein samples by mixing it with the samples such that the final concentrations of the ANS and protein sample were 20 µM and 250 µg/mL, respectively. After mixing the samples were incubated for 10 min at room temperature in dark. The fluorescence spectra of the ANS binding was then recorded by fluorometer (JASCO Spectrofluorometer FP-8200) by keeping the emission range of 400-670 nm, with the excitation at 355 nm and the slit width of excitation and emission as 10 and 5 nm, respectively, of a quartz cuvette of 1 cm path length.

Transmission electron microscopy (TEM) imaging

The transmission electron microscopy (TEM) of the protein samples were performed, in order to obtain the morphology of the protein structure. For the preparation of sample TEM grids, 5 µL of the protein sample was poured onto the formvar coated copper grids and incubated for 1 min. The grid was then blot dried and rinsed in a 50 µL droplet of water and again blot dried. This step of rinsing in water and blot drying was repeated two more times. After the final blot dry the grid was rinsed in a 50 µL droplet of phosphotungstic acid stain. This step was repeated two more times, and after the final blot dry the grid was air dried overnight and then transferred to the grid storage box, and sent for the TEM imaging.

Disaggregation of protein aggregates isolated from cataract eye lens

The solution of 1x critical micelle concentration (CMC) SDS (8.2 mM) was prepared in DPBS buffer. This SDS solution was mixed with the aggregated protein sample, having the protein concentration of 0.7 mg/mL. This mixture was incubated at 37°C for 24 h at static condition. These samples were further analyzed by CR binding assay, ThT binding assay, intrinsic fluorescence, ANS binding assay and TEM.

Results

The protein aggregates isolated from the human cataract eye lenses were examined to confirm the presence of amyloid-like characteristics, by examining their binding affinity to amyloid-specific dyes, such as CR and ThT. As the data shown in the (Fig. 1), it is evident that the insoluble fraction of the isolated sample displays a substantial bathochromic shift of 12 nm, from 483 to 495 nm in the λ_{max} of CR. This significant shift is indicates favorable interaction between the aggregates and CR dye. On the other hand, the appearance of unique shoulder peak at 540 nm indicates the presence of amyloid like structures in the aggregates[21,22]. As CR specifically binds to the cross β-sheets structured protein aggregates to display bathochromic shift in λ_{max}, this observation provides a clue that the isolated protein aggregates from cataract lens might have amyloid like structures. Nevertheless, the CR binding alone is not sufficient for confirmation of the amyloid nature of protein aggregates under *in vitro* conditions. Therefore, to substantiate this observation, the protein aggregates were further tested for its ability to bind with ThT. ThT is another amyloid specific dye that upon binding with amyloid, gives a characteristic fluorescent emission spectral pattern with highest intensity in the range of ≈480-490 nm, after being excited at 450 nm. The intensity of ThT fluorescence is typically found to be proportional to the amount of amyloid present in the sample. The data presented in (Fig. 2) exhibits a significant increase in the fluorescence intensity in the presence of the aggregated lens protein. On the other hand, the soluble fraction of the protein of the isolated from cataract eye lens show relatively lesser ThT fluorescence intensity compared to the insoluble fraction (protein aggregates). The collective data of CR

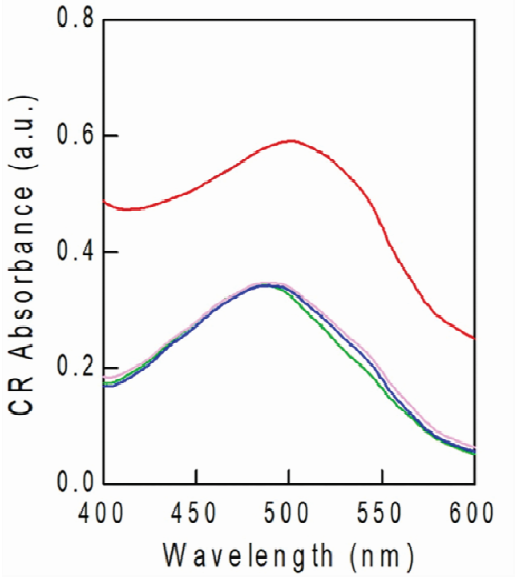

Fig. 1 — Congo red (CR) absorption spectra of the protein aggregates isolated from cataract lens in the presence (blue line) and absence (red line) of SDS. The green and pink lines represent Congo red alone and the soluble protein, respectively

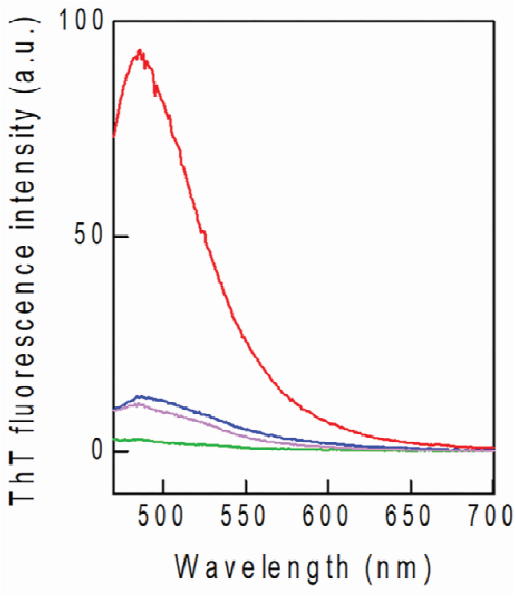

Fig. 2 — Thioflavin T (ThT) fluorescence emission spectra of the protein aggregates isolated from cataract lens in the presence (blue line) and absence (red line) of SDS. The green line represents soluble protein

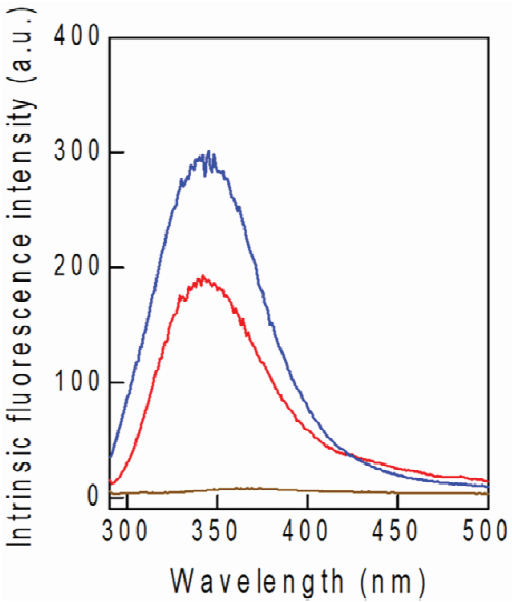

Fig. 3 — Intrinsic fluorescence emission spectra of the protein aggregates isolated from cataract lens in the presence (blue line) and absence (red line) of SDS. The brown line represents the buffer

And ThT binding assays suggest that the protein aggregates isolated form cataract eye lens consists of amyloid-like structures.

Conventionally, amyloid deposits in tissues predominantly consist of fibrillar aggregates, however, a number of non-fibrillar components are also found to be incorporated into the deposits. In addition, many pre-fibrillar states of amyloids are populated with oligomeric structures with high surface hydrophobicity, which are likely to play crucial role in the subsequent aggregation events. Morphologically, the protein aggregates isolated from cataractorus eye lens found be non-fibrillar and possess high surface hydrophobicity. Hence, in order to assess the presence of hydrophobic interactions as a dominant force, the protein aggregates were treated with SDS, which is known for disruption of hydrophobic interactions in protein or protein aggregates. The SDS-treated samples were examined by using CR binding assay and the data is shown in (Fig. 1). It is evident that the red-shift of CR λ_{max} of the SDS-treated sample was found to be significantly reduced, probably due to the dissociation of the protein aggregates in the presence of SDS. This observation provided a clue that the presence of SDS has disaggregated the protein aggregates. In order to further support this observation, we performed ThT binding assay of the SDS-treated samples. The comparative data shown in (Fig. 2), demonstrates significant reduction in the ThT fluorescence emission intensity at 490 nm, indicating reduction in the amyloid-like aggregates in the SDS-treated samples. It is usually observed that any change in the aggregation state could also be reflected through the changing pattern of intrinsic fluorescence of the aggregates. The intrinsic fluorescence intensity depends on the location of trp/tyr residues in the three-dimensional structure of a protein. A substantial conformational changes in the structure of protein lead to change in the location of trp/tyr residues, and hence, they might be exposed to solvents. Such conformational changes can be reflected in their spectral properties. As shown in (Fig. 3), the aggregated lens protein displayed lower intrinsic fluorescence intensity than the SDS-treated sample. In the aggregated lens protein these residues (trp/tyr) are supposed to be internalized and remained separated from the aqueous environment and results in less intrinsic fluorescence intensity. The treatments of the sample with SDS would have led to disaggregation of the aggregates that might have resulted into exposure of the trp/tyr residues to the solvent and exhibiting higher trp/tyr fluorescence emission intensity. Alternatively, the amyloid-like aggregates usually possess higher surface hydrophobicity and its reduction may be an indication of disruption of the aggregates. We performed ANS binding assay to probe the change in the surface hydrophobicity of the protein aggregates in the presence of SDS.

It is believed that reduction in the ANS binding corresponds to decrease in the surface hydrophobicity. Data shown in (Fig. 4) clearly displayed highest ANS emission fluorescence intensity, depicting the abundance of hydrophobic clusters in the protein aggregates. However, the ANS emission intensity was significantly reduced in the presence of SDS, probably due to disruption of the hydrophobic clusters on the surface of the protein aggregates. Finally we examined the morphological features of the aggregates in the presence if absence of SDS using transmission electron microscopy. The data shown in (Fig. 5), evidently depicts the heterogeneous population of protein aggregates in the range of approximately 50 to 500 nm (Fig. 5A). The smaller aggregates found be in the range of 50 to 100 nm where as many large aggregates which would have probably formed by secondary interaction found to be in the range of 200-500 nm. The magnitude of visible aggregates was found to be significantly reduced after the treatment with SDS. As shown in (Fig. 5B), the left-over aggregates were found to be in the range of 20-80 nm, significantly lower than the untreated sample. These observations suggest that the presence of SDS has seriously impacted the intermolecular interaction of protein aggregates formed during cataract formation.

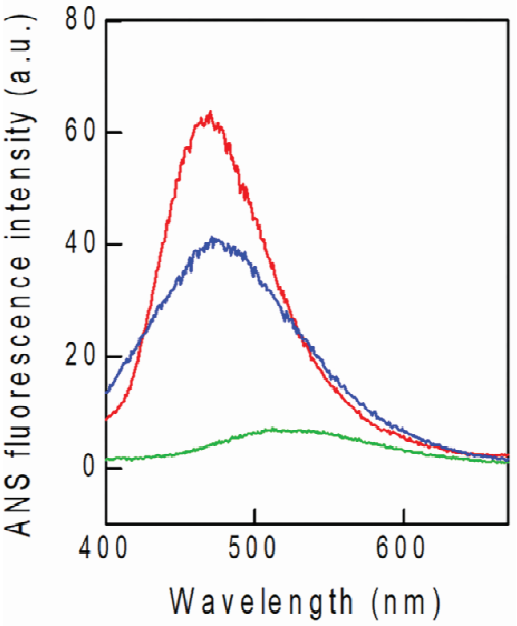

Fig. 4 — ANS fluorescence emission spectra of the protein aggregates isolated from cataract lens in the presence (blue line) and absence (red line) of SDS. The green line represents ANS alone

Discussion

SDS is an anionic surfactant can be used for induction of protein conformational changes. However, the precise mechanism of SDS induced changes on amyloids is not conclusive. SDS is often used as protein denaturant that binds preferentially to proteins, SDS probably interacts with hydrophobic surface of the protein. The effect of SDS on the amyloid-like protein aggregates of cataractous lens was studied in the present study. The goal of the study was to determine the nature of bonding between the protein aggregates isolated from human cataract eye lens. There are several possibilities that have been explored to find a way by which the disaggregation of the protein aggregates can be achieved. In this study, the protein aggregates were treated with SDS using concentration equivalent to 1x CMC (≈2. 3 mg/mL),

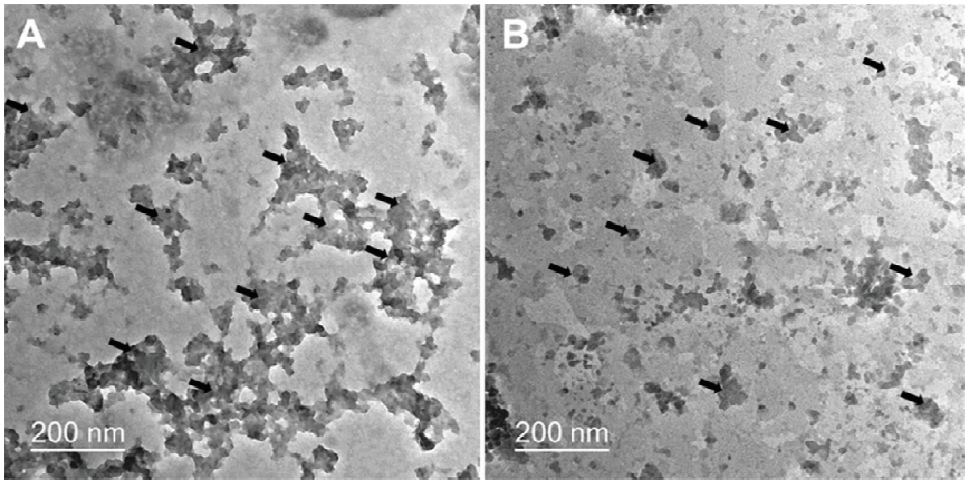

Fig. 5 — Morphological analyses of protein aggregates isolated from cataract lenses. A and B represent the cataract lens protein aggregates in the absence and presence of SDS, respectively

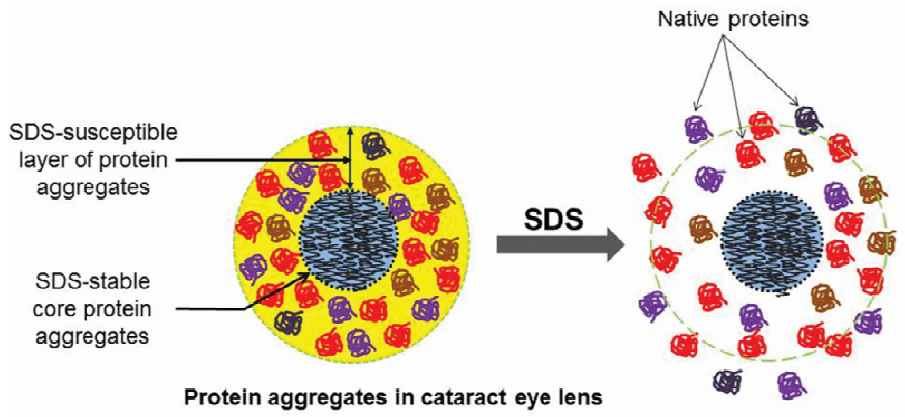

Fig. 6 — Schematic representation of disaggregation of protein aggregates isolated from human cataract eye lens. The inner core remains stable against SDS treatment, whereas the outer layer, which is mainly constituted by random aggregation of native proteins stabilised through hydrophobic interaction. This layer can easily be dissolved in the presence of SDS

and analyzed for its effect by using the amyloid specific dye binding assays, such as CR binding assay, ThT and ANS binding assays, Intrinsic fluorescence, following the electron microscopy for the morphological confirmation. The above data suggested that the disaggregation of protein aggregates in the presence of SDS at the concentration of 1x CMC SDS has been achieved. Mounting evidences suggest that almost all types of protein aggregates whether be it amyloidogenic or amorphous, or salt-induced precipitation, they all are usually stabilized by hydrophobic interactions for their structural stability[23]. However, a number amyloids are shown to highly stable against SDS indicating the presence of interactions other hydrophobic interactions[24]. On the other hand it has also been observed that the presence of SDS induce the formation of amyloid due to it denaturing effect[25]. In pathological specimens, it has frequently been observed that many amyloids co-aggregated with various native proteins probably through the exploration of the hydrophobic interaction[26]. Such proteins in the aggregates (or amyloid plaques) can be simply be disaggregated by treating them with SDS, however, the core amyloid structure might remain unaffected that can further act as seed from recruiting native proteins. In our study, it was observed that the SDS-treatment did not lead to complete dissociation of the protein aggregates. The hydrophobic interaction might be playing crucial role in increasing the particle size of the initial small size amyloid aggregates in eye lens that subsequently might lead to progression of cataract. As evident our study that even in the presence of SDS equivalent to its 1x CMC value, the amyloidogenic parameters and the size of the aggregates was significantly reduced, however, the resultant solution still retained considerable amyloidogenic properties. Based on the existing information and our observation it is obvious that the protein aggregates under investigation possess two layers of aggregation. The outer layer which is often stabilized by hydrophobic interaction, whereas inner core might possess and SDS-stable core amyloid which cannot be disaggregated just by interfering the hydrophobic interaction (Fig. 6). This study demonstrated that the protein aggregates isolated from cataractous eye lens contains two levels of protein interactions, the outer one which are probably stabilized through hydrophobic interactions whereas, the inner one is stabilized through more intense hydrophobic as well as other interactions including hydrogen bonding.

Conclusion

The present study outlines the hydrophobic nature of protein aggregates present in human cataract eye lens. The presence of SDS resulted in significant decreased in CR and ThT binding which is the hallmark characteristic of disaggregation of the protein aggregates. Subsequently, the morphological analysis by TEM also suggests that the size of aggregates were significantly decreased in the presence of SDS. Therefore, the present study clearly draws the conclusion that the protein aggregates are sharing the hydrophobic interactions among them, which might be providing them the rigid structure due to which they became uneasy to dissociate by general means. This study provides additional understanding about the role of hydrophobic interactions during progression of cataract and a rational for designing

novel anti-amyloidogenic approach to mitigate or reducing the progression of cataract.

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Conflict of interest

All authors declare no conflict of interest.

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