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# Macromolecular crowder polyethylene glycol delayed the aggregation of chromium-treated bovine serum albumin

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RESEARCH ARTICLE

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ABSTRACT

The structure of proteins is greatly affected by various interacting xenobiotic and lead to the formation of aggregates. Chromium metal, which was initially considered innocent as a nutrient, has been found to induce some abnormalities in the human body recently. Aggregate formation is associated with the occurrence of pathological conditions such as systemic amyloidosis, cystic fibrosis, *etc*. To have a deeper insight into aggregation susceptibility and structural stability of bovine serum albumin on treating with hexavalent chromium Cr(VI) and the consequences of macromolecular crowding on the native conformation of the protein, the chromium concentration ranged from 0-100 µM where $K_2Cr_2O_7$ was used as the Cr (VI) source. Disruption of native bovine serum albumin (BSA) assembly and formation of aggregates at 50 µM Cr(VI) was unveiled by increased turbidity and fluorescence at 350 nm, reduced intrinsic fluorescence with 10 nm and 20 nm blue shifted enhanced ANS spectra respectively. Significantly enhanced, the ThT fluorescence alone side sigmoidal curve with no lag phase and a 10 nm red shift in congo red spectra sustained conformational changes and indicated aggregation of BSA upon incubation with Cr(VI). Circular dichroism (CD) results showed the disappearance of negative minima at 208 and 222 nm, which confirms the transition of native helical structure to non-native beta sheets. Furthermore, the comet assay showed that Cr-treated BSA aggregates were found to be genotoxic, as an increase in tail length of 11.3 µm had been observed. Crowded microenvironment was mimicked by PEG-4000; a polyethylene glycol, was witnessed to prominently preserve conformational stability of BSA upon treatment with Cr(VI) as all results observed were close to that of native. The decrease in turbidity, fluorescence at 350 nm accompanied by a reduction in 8-anilinonaphthalene-1-sulfonic acid (ANS) and thioflavin T (ThT) fluorescence further verified the inhibition of aggregate formation in the presence of PEG-4000. Furthermore, the increased intrinsic fluorescence, decreased congo red absorption and reduced tail length of 3.4 µm in the comet assay were in co-relation with the above data. The macromolecular crowder PEG-4000 was efficient in delaying the aggregation of Cr-treated BSA, as the kinetics showed a sigmoidal curve with the lag phase. Based on these findings, it could be hypothesized that the native structure was maximally retained in the presence of 100 mg/mL of PEG-4000, demonstrating braking of aggregate formation. It can be established that explicit consideration of macromolecular crowding using a relevant range of inert crowding agents must be a prerequisite for studies concerning intracellular conformational behavior of proteins and enhanced their stability under stress conditions and devising protein formulations with enhanced conformational stability.

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## 1. Introduction

The present phase of knowledge of the role of metals in biological systems indicates that enhanced formation of free radicals and other reactive species can be regarded as a common factor in determining metal-induced toxicity and carcinogenicity [1]. Among all, heavy metals are ubiquitously present, and their level escalates naturally or artificially. Chromium (Cr) is a naturally occurring element ranked 24$^{th}$ in terms of abundance in the earth's crust [2]. It may exist in several chemical forms and valence states in the environment; Cr(0), Cr(III), and Cr(VI) are the most common, but only trivalent and hexavalent forms are important for human health [3,4]. Cr compounds are widely used in various industries and are released in surroundings and, as they are non-degradable, persist in the atmosphere for prolonged periods [5]. The primary route of exposure to Cr compounds is inhalation, oral ingestion of Cr-contaminated food and water, or direct dermal contact with Cr products [6]. Irrespective of the medium of exposure, Cr(VI) is quickly absorbed and enters the blood where it accumulates rapidly and selectively, mainly in erythrocytes and lymphocytes in humans [7]. On the contrary,


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absorbed Cr(III) does not enter blood cells and is rapidly cleared from the blood as transferrin bound, and therefore displays little or no toxicity, this being the reason for selecting Cr(VI) [8].

The widespread use of metals has been associated with multiple organ toxicity and is also known to be key player in some protein disorders, directly or indirectly. Among all biomolecules, majorly blood and plasma proteins are targeted by an extensive range of exogenous and endogenous compounds [9,10]. Bovine serum albumin (BSA) is one of the most widely used and best characterized proteins [11]. It is the most abundant plasma protein in bovines that shares 76% structural homology with human serum albumin (HSA) [12]. It is a single-chain, globular, transporting non-glycoprotein composed of 583 amino acid residues with a molecular weight of 66 kilo Dalton [13]. The primary structure of BSA comprises nine loops composed of 17 disulfide bonds, resulting in three domains (I, II and III) each consisting of two subdomains (A and B); 17 cysteine residues (8 disulfide bridges and 1 free thiol group [14,15]. The well-documented structure of BSA, high bioavailability, low cost, and simple preparation bases provide it for varied use in biochemical research and biological applications. The unique assortment of amino acid residues in the native BSA assembly allows the binding of drugs or bioactive compounds, accounting for its diverse physicochemical characteristics and applications.

Most of the studies concerning the conformational behavior of proteins were lately performed in dilute setting but the intracellular situation is blatantly opposite to that created *in vitro* [16]. On the contrary, cell interiors are extremely congested with compactly packed biological macromolecules that influence the biophysical properties of proteins [17]. This effect exerted by overcrowding of macromolecules is vividly termed as the macromolecular crowding effect which has an inevitable impact on various properties of macromolecules and biochemical processes. It has been examined in depth by adding high concentrations of inert, synthetic, or natural macromolecules, called crowding agents or crowders, to the system in vitro to mimic an *in vivo* scenario [18,19]. The use of proteins as biocatalysts and therapeutic agents is restricted by their inherent instability when subjected to physical and/or chemical stress, and it is extremely desirable to develop and design protein formulation with enhanced stability under unfavorable conditions [20]. The most common chemical modification in this perspective is the attachment of certain additives to the surface of proteins [21]. Proteins can be chemically modified for their advanced in vitro and in vivo to expand their usage which was otherwise hindered [22].

Previously, our lab has considered the effect of macromolecular crowding on the enzyme horse raddish peroxidase [23]. This work deals with effect of hexavalent Cr on the BSA conformation and to study consequences of macromolecular crowding on it. Potassium dichromate ($K_2Cr_2O_7$), a water-soluble Cr compound, was used as source of Cr(VI). It is a common inorganic chemical reagent widely used in industries such as textile manufacture, wood preservation, photography, cooling systems, etc. and as an oxidizing agent in various laboratories. Macromolecular crowding was impersonated by polyethylene glycol (PEG), a polysaccharide and an inert crowding agent. In this study, we had analysed the effect of increasing the concentration of hexavalent Cr on the structure of BSA that drives the model protein towards aggregation. Additionally, the macromolecular crowder PEG was used as an antiaggregating agent BSA treated Cr complex to mimic the *in vivo* conditions.

## 2. Experimental

### *2.1. Materials and instrumentation*

BSA and all fluorescent probes, namely, ANS, ThT, and CR, were purchased from Sigma (St. Louis, U.S.A). PEG-4000, $K_2Cr_2O_7$, monobasic and dibasic sodium phosphate was purchased from Sisco Research Laboratories (Mumbai, India). Monobasic sodium phosphate and dibasic sodium phosphate (pH = 7.4) were used for buffer preparations. The stock solution of 5 mg/mL BSA was prepared by dissolving the protein in 20 mM phosphate buffer at pH = 7.4 and then extensive dialysis was performed in the same buffer to remove impurities. The molar extinction coefficient at 280 nm of 43,824 $M^{-1}cm^{-1}$ was used to determine protein concentration on a single beam Hitachi spectrophotometer using a 1 cm path length [24]. The final concentration of BSA in the experiments in incubated sample was 5 µM unless otherwise mentioned.

### *2.2. Preparation of samples*

BSA was treated with varying concentrations of Cr(VI) ranging from 0-100 µM were prepared in 20 mM phosphate buffer, pH = 7.4 and incubated at 37 °C for 4 hours before performing any measurements were made. To assess the effect of macromolecular crowding, BSA was incubated with 100 mg/mL PEG-4000 for 24 hours and then treated with 50 µM Cr (VI) because the effect of chromium was more pronounced at this particular concentration and all experiments were preceded with that. All experiments were carried out at room temperature. Triplicates of each set were analysed for the results.

### *2.3. Fluorescence measurements*

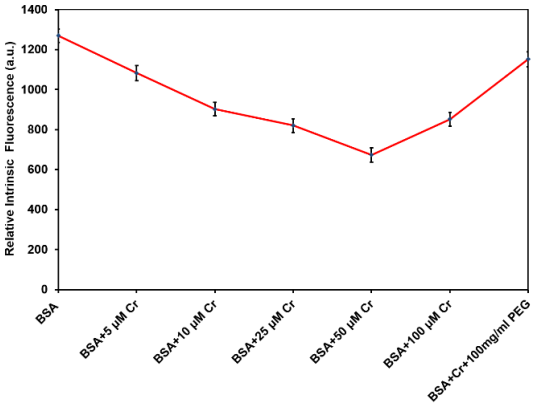
Intrinsic fluorescence spectra were recorded using a 1 cm path length quartz cell on a Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan). Emission was recorded in the range of 300-400 nm and the excitation wavelength was 280 nm [25]. ANS (8-anilino-1-naphthalene-sulfonoic acid) binding was measured by fluorescence emission spectra recorded in the 400-600 nm range with excitation at 380 nm. The ANS concentration was typically 100 molar excess of the protein concentration in the reaction mixture [26]. Thioflavin T (ThT) fluorescence intensity with the following parameters $\lambda_{ex}$ = 440 nm, $\lambda_{em}$ = 460-600 nm, was measured in a 1 cm path length quartz cell for the detection of BSA aggregation [27]. The ThT concentration was 22.5 µM. Rayleigh scattering experiments were performed using a Shimadzu fluorescence spectrophotometer (RF-5301PC) to acquire consistent data. The scattered light intensity was the mean of three samples. The emission spectra of the samples were obtained in the 300 to 400 nm range and the excitation wavelength was set at 350 nm [28].

### *2.4. Absorption spectroscopy*

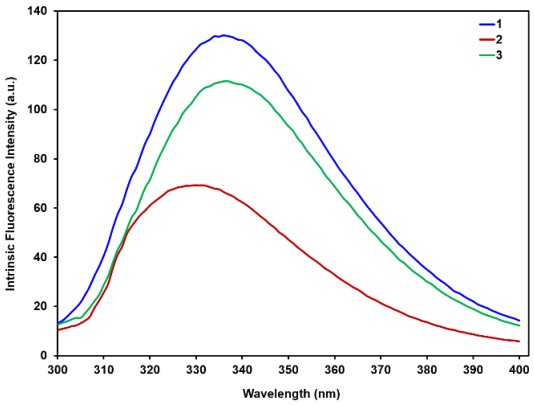
As the absorption peaks can reflect the framework conformation of proteins, UV-visible absorbance was scanned in the ultraviolet range of 150-350 nm to explore the structural changes in BSA. The turbidity was assayed by recording absorption spectra at 350 nm of BSA in dilute and the presence of a macromolecular crowder using a 1 cm path length cell on the Shimadzu UV-1700 Spectrophotometer [29]. Congo red absorbance spectra to inquire the presence of aggregates were logged in the 400-700 nm on a UV-1700 Shimadzu UV spectrophotometer using a cuvette of 1 cm path length after 20 minutes [30].

### *2.5. Circular dichroism (CD) measurements*

The CD spectra of the BSA samples were recorded on an ammonium D-10-camphorsulfonate calibrated J-815 Jasco CD spectropolarimeter in a cuvette of 0.1 cm path length.




(a)




(b)

**Figure 1.** (a) Relative intrinsic fluorescence spectra of native BSA, Cr-treated BSA and BSA-PEG-Cr. (b) Intrinsic fluorescence emission spectra of native BSA (curve 1), Cr-treated BSA (curve 2), and BSA-PEG-Cr (curve 3). The protein concentration was 5 μM and the path length was 1 cm.

The scanning range was between 250-190 nm [31]. Far-UV CD spectra measurements were performed at 25 °C and protein concentration in each incubated sample was 8 μM.

### *2.6. Comet assay*

The isolated lymphocytes were exposed to Cr treated-BSA in a total reaction volume of 1.0 mL of 20 mM phosphate buffer pH = 7.2, and incubated at 37 °C for 1 hour. The reaction mixture was then centrifuged at 720 g, and pelleted lymphocytes were resuspended in 100 μL of PBS and further processed further for the Comet assay while the supernatant was discarded [32].

## 3. Results and discussion

Protein conformations are prone to alterations under a variety of external conditions, such as changes in pH, temperature, chemical denaturants, and metal ions, which then affect the functions of proteins [33,34]. It is well recognized that biological macromolecules evolve and function under crowded environment. Proteins to be biologically operative must have their intact native organization and perform their biological functions in environments crowded with macromolecules, be it extracellular space, cell membrane, or cytoplasm, but most studies have been assimilated through research conducted under dilute conditions [35]. Ample attention is drawn towards in vitro mimicking of macromolecular crowding. Several studies are being done to study its effect on native protein by using concentrated solutions of a crowding agent that was previously safely ignored by biochemists [36,37].

The present study focuses on how the presence of crowding agents influences the conformation and tunes the stability of native BSA in the presence of a metal ion and investigating PEGylation as a potential stabilization strategy against protein aggregation. Binding between metal ions and proteins can elucidate the properties of the metal ion–protein complex, provide useful information on the delivery characteristics of metal ions, and influence the concentration, distribution and conformation of the target protein in the system [38]. Therefore, studies on the binding behavior of Cr(VI) onto BSA not only are helpful for understanding the metabolism and distribution of this metal ion in vivo but also can further elaborate the related effects of Cr(VI) on protein function during the blood transportation process.

### *3.1. Intrinsic fluorescence measurements*

The intrinsic fluorescence emission intensity is interrelated to protein conformation which may notify about its structure and is critically reliant on the fluorescence energy transfer from internal tryptophan residues of protein [39]. It is the sensitive index to survey deviations in the conformation of the protein structure. Structural variations in the tertiary structure of proteins pave the way for environmental changes in aromatic side chains that can be assayed by means of intrinsic fluorescence spectroscopy. Furthermore, alterations in the microenvironment around the tryptophan residue in BSA can modify the emission maxima of the native protein. In addition, the distance and alignment of tryptophan can change the intrinsic fluorescence of BSA on disruption of conformational topology [40]. Figure 1a shows the relative intrinsic fluorescence of BSA in the presence and absence of chromium in a dilute and crowded environment.

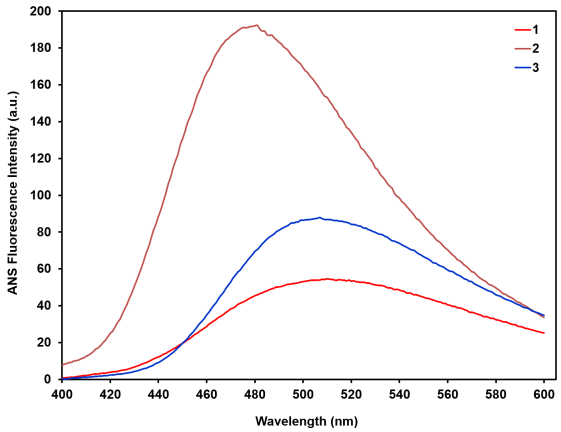



**Figure 2.** ANS fluorescence emission spectra of native BSA (curve 1), Cr-treated BSA (curve 2) and BSA-PEG-Cr (curve 3). The protein concentration was 5 μM.

On increasing the concentration of chromium ions, the fluorescence was further decreased, hinting conformational alterations in native structure of protein possibly due to aggregation [41]. It can be implied that structural changes in BSA occur at 50 μM Cr (VI) undergoing structural modifications from the non-native intermediate state to the ultimately aggregated state, and further experiments were performed with this concentration of chromium. The fluorescence spectra of native BSA (curve 1) showed emission maxima at 340 nm (Figure 1b) [42]. BSA incubated with Cr (curve 2) fluorescence intensity was reduced several folds with a 10 nm blue shift. A substantial decrease in intensity relative to the native protein can be attributed to complete disruption of the native structure leading to aggregation [41]. This decrease in fluorescence intensity can be due to intermixing of aromatic residues after structural unfolding and conformational alterations [43]. Alternatively, BSA pretreated with PEG when incubated with Cr (curve 3) did not exhibit significant changes in fluorescence spectra and the intensity perceived was close to native, hinting toward increased stability of native state at 100 mg/mL PEG. It can be inferred that the aggregation effect of Cr was inhibited in the presence of crowding agent. The preliminary results obtained through intrinsic fluorescence were further analysed employing extrinsic fluorescence assays, ANS and ThT.

### *3.2. 8-Anilino-1-naphthalene-sulfonoic acid (ANS) fluorescence measurements*

ANS fluorescence is a comprehensively used dye for the recognition of hydrophobic patches in the protein structure and exploited to study the course of aggregation [44]. High ANS fluorescence is observed which may be credited to the revelation of hydrophobic clusters to the aggregates' surface due to persuaded unfolding by presence of metal ion or intermolecular interactions that bring upon binding of ANS to hydrophobic groups. It is a valued fluorescent probe widely employed for the analysis and characterization of molten globule and aggregated states that specifically bind to the hydrophobic patches of native proteins [45]. Figure 2 shows the ANS spectra of BSA with Cr in the presence and absence of PEG. The native protein (curve 1) showed slight fluorescence with maxima at 500 nm confirming the intact protein structure where hydrophobic patches remain buried and remain aloof for ANS binding, hence the fluorescence intensity is curtailed. For Cr-treated BSA (curve 2), augmented fluorescence is observed with a 20 nm blue shift attaining the maxima at 480 nm hinting towards high ANS binding to unfolded protein suggesting accessibility of buried hydrophobic residues for dye binding upon unfolding of protein [46]. This blue shift in the spectra with upwelling of fluorescence at increased crowder concentration can be attributed to the point that the enhanced hydrophobicity of the protein atmosphere steered to the augmentation of protein-protein interactions [47]. BSA pre-incubated with PEG when treated with Cr (curve 2) showed no considerable change in ANS fluorescence presenting that not much unfolding occurs and native BSA structure is retained to a significant extent.

### *3.3. Thioflavin T (ThT) fluorescence assay*

The results of ANS fluorescence were established by ThT, the distinct feature of this benzothiazole dye is that it precisely binds to β-sheet aggregates. Enhanced fluorescence is observed during aggregate formation when dye binding sites are created and is followed by interaction between protein side chains with higher amounts of ThT dye [48]. Thus, upon aggregation, ThT molecules come near the side chain of β sheet conformation ensuing stearic interactions between them leading to increased fluorescence, which is a property extensively studied of this dye [49]. As presented in Figure 3a, native BSA (curve 1) exhibited insignificant fluorescence as there were no aggregated structures to bind to ThT dye. A significant increase in fluorescence was observed for Cr-treated BSA (curve 2), indicative of conformational alteration and unfolding that allowed the creation of binding sites for dye molecules to bind to protein. Prominently boosted fluorescence pointed to the structural disorder that leads to aggregation of BSA with Cr [50]. These aggregates have a specific binding site for ThT dye that sterically locks the bound ThT, hence the amplified fluorescence intensity is observed. The increase in ThT emission intensity supported the results and implied structural perturbation in native BSA leading to aggregation after treatment with chromium [51]. For preincubated BSA with PEG when treated with Cr (curve 3), no significant change in fluorescence intensity was perceived, this fluorescence behavior inferred that the presence of a crowder (PEG) protected the native assembly and improved the stability of BSA, thus not allowing ThT to bind in the native state [52].

The kinetics of amyloid formation was studied by monitoring the ThT fluorescence intensity of the preparations at regular time intervals (0-120 hours), as depicted in Figure 3b. ThT fluorescence kinetics of aggregate formation involves distinct phases. BSA treated with 50 μM Cr showed a standardized pattern of amyloid formation, *i.e.*, a sigmoidal pattern without involving the lag phase [53]. In the presence of Cr(VI), the aggregation profile of BSA changed dramatically. However, co-incubation of BSA with PEG and Cr resulted in a reduction of ThT fluorescence. At 100 mg/mL of PEG, the lag phase was found to increase continuously.

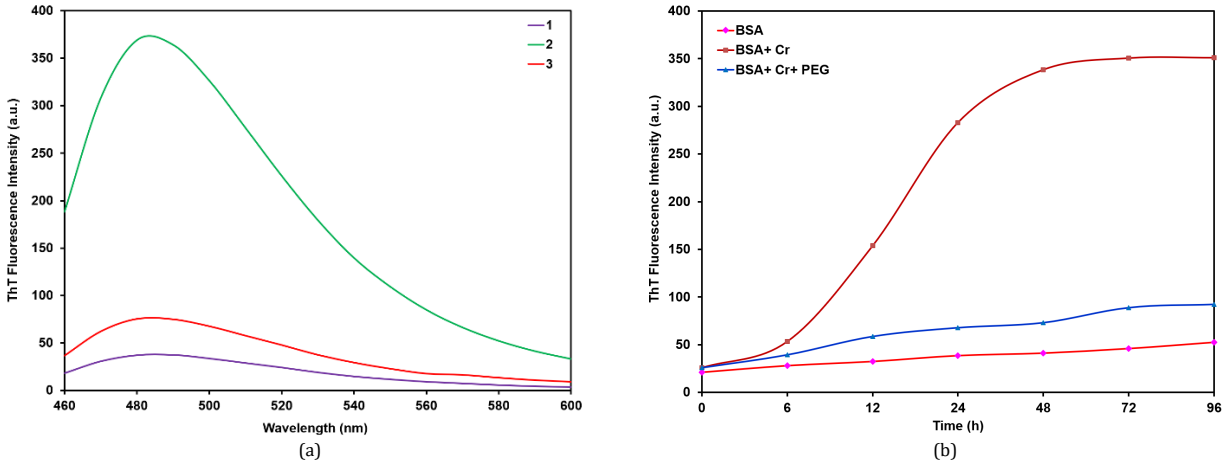



**Figure 3.** (a) ThT emission spectra of native BSA (curve 1), Cr-treated BSA (curve 2) and BSA-PEG-Cr (curve 3). (b) Kinetics of aggregate formation as a function of ThT fluorescence. The protein concentration was 5 μM.

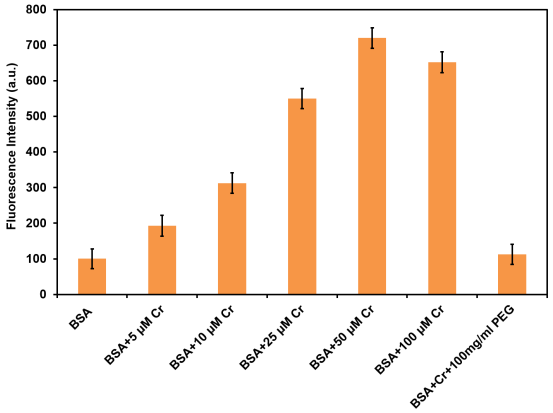



**Figure 4.** Rayleigh scattering measurements of native BSA, Cr-treated BSA, and BSA-PEG-Cr. The protein concentration was 5 μM.

The delay in lag time in the presence of PEG was attributed to the stabilization of the native form as previously reported [54]. This delay in lag time also slows down the apparent growth rate of amyloid formation.

### *3.4. Rayleigh scattering measurements*

Progressive increase in fluorescence intensity due to large particle size upon aggregation of proteins is the characteristic phenomenon of scattering light intensity [55]. Fluorescence intensity at 350 nm is an excellent measure to check the incidence of protein aggregates (Figure 4). Scattering of light intensity for Cr-treated BSA in dilute and crowded scenarios was observed to detect alterations in the native BSA and check formation of aggregates, if any. Light scattering intensity was initially constant, thus showing no indication of aggregation at low Cr(VI) concentration. At 50 μM Cr(VI), notable surge in intensity was observed. The enhanced fluorescence intensity on further increasing Cr concentration, suggests that maximum conformational changes occurred at 50 μM. At this Cr concentration, BSA gets transformed into non-native, ordered aggregates subsequently resulting in maximum light scattering [56]. The decreased fluorescence intensity approaching the native protein was detected in the presence of PEG, suggesting that the native structure of BSA was stabilized to its maximum extent with 100 mg / ml of PEG, indicating the antiaggregatory effect of macromolecular crowding [57].

### *3.5. UV-visible absorption analysis*

The UV-vis absorption spectroscopy technique can be used to investigate the conformation state of a BSA and the formation of metal ion-protein complex with Cr(VI). Here, the UV-visible absorption spectra of native BSA and BSA-Cr complex under dilute and crowded conditions were obtained at 207 nm and are shown in Figure 5. The presented result showed a band in BSA absorbance spectra at 207 nm. The intensity of the peak around 207 nm decreased, signifying that the formation of the Cr(VI)-BSA complex led to the loosening and unfolding of the native framework of BSA. These changes in spectra might arise from the perturbations in the microenvironment around the polypeptide brought upon by the binding of Cr(VI) with BSA. It can be proposed that the formation of a Cr-BSA complex might increase the hydrophobicity of the microenvironment of the chromophore amino acid residues. The peak around 207 nm ($n \rightarrow \sigma^*$) reflects the native organization of BSA which decreased with and shifted to 205 nm [58]. The influence of Cr(VI) on the absorption spectrum of BSA recommended that a conformational change in BSA occurs, which was brought about by the formation of ground-state complexes between the metal ion and protein. Therefore, we can say that the absorption intensity after treatment with hexavalent Cr can be attributed to disturbances in the microenvironment of native BSA that in turn causes structural distortion [59]. However, using PEG as a crowder and then treating BSA with metal ions, the absorbance intensity perceived was approaching the native state, hinting strongly towards the fact that native assembly was retained when PEG came into play.

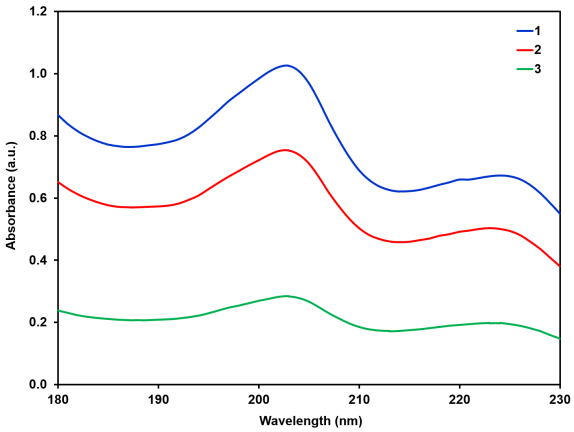



**Figure 5.** UV-vis absorption spectra of native BSA (curve 1), Cr-treated BSA (curve 2), and BSA-PEG-Cr (curve 3). The protein concentration was 5 µM.

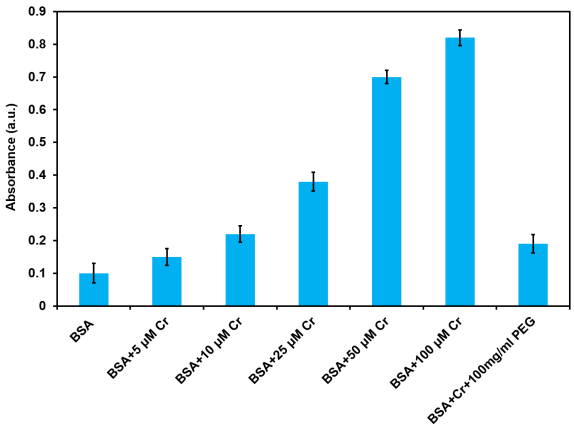



**Figure 6.** Turbidity assay: Absorbance at 350 nm at varying concentrations of Cr (0-100) under a dilute and crowded condition.

### *3.6. Turbidity assay*

The aggregation in BSA under dilute and crowded conditions was measured by quantifying the absorbance at 350 nm (Figure 6). To recreate the *in vivo* set-up like the crowded one *in vitro*, PEG was chosen as a macromolecular crowder, and turbidity analysis was performed to gain insight into the amount of aggregation in protein. In native BSA, there was minimum absorbance, which showed a marked increase in absorbance upon increased treatment with metal. A remarkable increase in absorbance was observed with an increase in Cr concentration. In contrast to this, the absorbance observed for BSA preincubated with PEG was significantly lower compared to BSA incubated with Cr alone This absorption pattern can be justified on the basis of its potential in inducing stability of the native state [60]. It can be implied that macromolecular crowding inhibited the formation of aggregates by protecting the native structure and modulating the stability of protein [61].

### *3.7. Congo red (CR) assay*

Congo red is another diagnostic dye to authenticate the ThT findings and used to monitor formation of aggregates for further corroboration of our findings. Congo Red azo dye (CR) is a symmetrical sulfonated with a hydrophobic center comprising a biphenyl group that is spaced between negatively charged sulfate groups [62]. The addition of Cr to BSA in the presence and absence of PEG produced substantially altered spectra (Figure 7). Native BSA (curve 1) exhibited absorbance intensity at 510 nm, whereas BSA treated with Cr (curve 2) showed considerable increase in absorbance with a 10-nm red shift. This increase in absorption with a red shift could be due to the subsequent formation of a considerable amount of dye-binding moieties formed upon incubation of native BSA with Cr [63]. The distinctive changes in the CR absorption pattern could be a manifestation of β-sheet rich aggregates due to a disrupted and asymmetrical conformation caused by the presence of metal ions [64]. BSA preincubated with PEG-4000 and then treated with Cr (curve 3), displayed a reduced absorbance close to that of the native protein with a substantial rate of deceleration of aggregate formation.

### *3.8. Circular dichroism (CD) spectroscopy*

Far-UV CD analysis is highly sensitive and helps in the determination of any conformational changes occurring in secondary structure of proteins [65]. It provides a direct interpretation of deviations from the native to the non-native state and is one of the most insightful physical techniques for describing the changes that occur in the structure of native proteins [66]. CD spectra of BSA incubated in the absence and presence of PEG were obtained (Figure 8). The native protein (curve 1) confirmed the presence of double minima peaks at 208 and 222 nm characteristic of α-proteins [67]. In Cr-treated BSA (curve 2), a decrease in ellipticity was observed indicating that the alpha helical content was reduced at that particular Cr concentration implying that Cr(VI) bound with the protein amino acid residues of the main polypeptide chain and destroyed their hydrogen bonding networks [68]. Complete loss of both negative peaks and the emergence of a new peak was observed around 218 nm, indicative of β-sheets thereby,

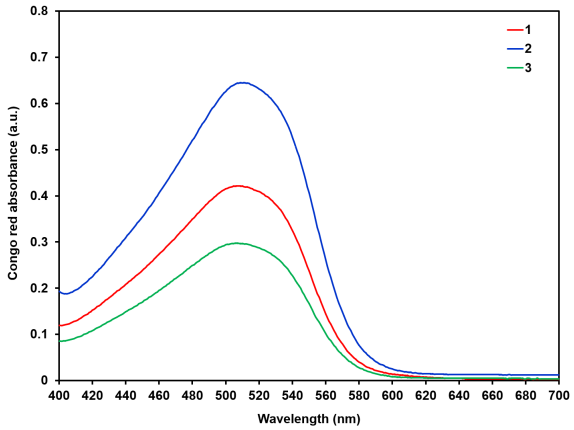



**Figure 7.** CR absorption spectra of native BSA (curve 1), Cr-treated BSA (curve 2), and BSA-PEG-Cr (curve 3). The protein concentration was 5 μM.

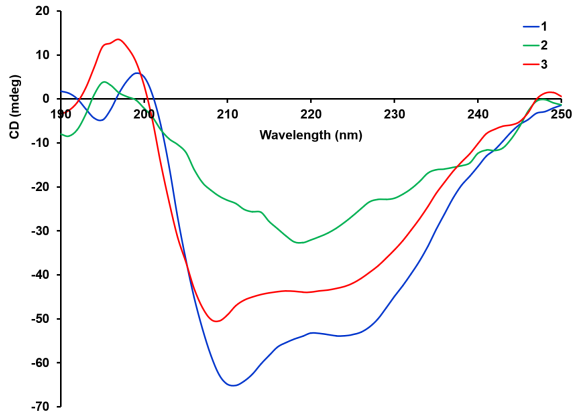



**Figure 8.** Far-UV CD spectra of native BSA (curve 1), Cr-treated BSA (curve 2), and BSA-PEG-Cr (curve 3). The protein concentration was 8 μM.

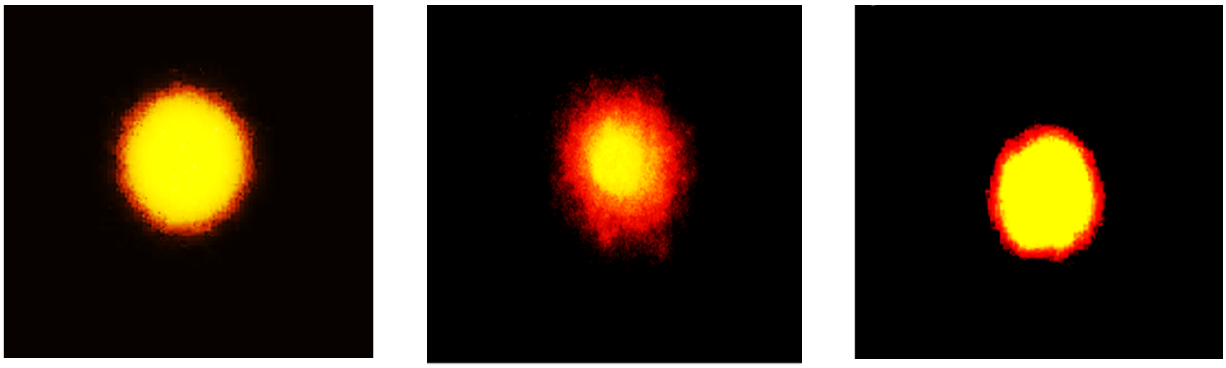
confirming the existence of Cr-induced protein aggregates. This peak shift designated the complete disruption of native helical assembly and the switch to β-sheets which is a distinguishing feature of protein aggregates. For BSA in a crowded environment reflected by PEG (curve 3) spectra was similar to native, no decrease in ellipticity was observed, hinting that alpha helical content was reserved with PEG thereby protecting the stability of native protein [52].

### *3.9. Comet assay*

The comet assay is known to detect nicks or damages in double or single-stranded DNA in very less amounts but with equally high sensitivity. A direct correlation between aggregation and reactive oxygen species burst and its uptake mechanism is demonstrated in many studies [69,70]. The generation of free radicals is the most feasible reason for this DNA damage, as it has earlier been stated earlier, intracellular protein aggregation causes the production of reactive oxygen species, directly or indirectly [71]. It is visibly illustrated in Figure 9 that the BSA-Cr complex is the basis for DNA damage detected from the comet assay by measuring the tail length. The lymphocytes were treated with a metal ion-protein complex in crowding conditions, and the average length of the tail was measured. The image of a control or lymphocyte alone showed a tail length of 1.8 μm, which was very similar to the native BSA, *i.e.,* 2 μm. The tail length of Cr-treated BSA was found to be 11.3 μm implying the occurrence of aggregates, due to which the damage in lymphocyte DNA was found to be maximum. This can be explained in terms of either BSA aggregates directly reaching DNA or indirectly by an increase in the formation of such reactive species that eventually damage DNA [72]. BSA pre-incubated with PEG showed DNA damage of around 3.4 μm in tail length. Aggregates because of their small size might be smashed into the nuclear pore complex and cause DNA damage. These fallouts suggested that the genotoxic nature of aggregates was predominantly observed in Cr-treated BSA compared to that with PEG.

It is reasonable to hypothesize that large PEGs might act as a molecular spacer that minimizes protein-protein interactions that lead to the formation of aggregates and thus PEG-4000 was able to inhibit protein aggregation. These observations collectively suggest that the steric shielding of exposed hydrophobic patches plays an important role in inhibiting protein aggregation and could be helpful in designing stabilizing strategies for multidose protein formulations and has matured into a go-to approach for improving the performance *in vivo* of protein drugs [73-75]. Protein PEGylation could be accompanied by a loss of activity depending upon the number, location, and size of the PEG molecules attached to it [76].

It has been widely established that in living systems, crowding might have dramatically affected biochemical processes such as the constancy of protein structures and enzyme activity [60,77,78]. Additionally, it will be important to check the effect of the mixture of various crowding agents on native proteins to impersonate the actual in vivo situations [35]. Researches support that abnormal aggregation ascends from a key intermediate that may have nonpolar, hydrophobic patches on their surface that are lacking in native state.



(a) (b) (c)

**Figure 9.** Comet assay: Images of (a) lymphocytes treated with native BSA, and (b) Cr-treated BSA, (c) Cr-treated BSA preincubated with PEG.

These intermediates lead to specific intermolecular hydrophobic interactions resulting in aggregation of proteins [79]. Macromolecular crowding is assumed to be responsible for checking the emergence of such species and regulating their concentration.

## 4. Conclusions

Additional research is needed to better understand the toxic effects of chromium and its interaction mechanism with other protein enzymes in the system. Further studies must be performed to highlight the health risk of the Cr-contaminated food and deleterious effects it causes *in vivo*. Thus, there is a dire necessity to design and develop protein formulations with enhanced structural and chemical stability. It is needed to understand the true-life behavior of proteins in their naturally crowded locale through which proteins function, and thus consequences of macromolecular crowding cannot be left unheeded. Many metals have attracted prominent attention in the chemical and pharmaceutical industries; thus, approaches concerning the structural and functional stability of protein will undoubtedly broaden the range of its present applications. More researches should be conducted to study the effect of macromolecular crowding in inhibiting the protein aggregation and to provide molecular insights.

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Conflict of interest: The authors declare that they have no conflict of interest.
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## CRediT authorship contribution statement

Conceptualization: Samra Hasan, Nazim Hussain, Aabgeena Naeem; Methodology: Samra Hasan, Nazim Hussain, Aabgeena; Validation: Samra Hasan, Nazim Hussain, Aabgeena Naeem; Formal Analysis: Samra Hasan, Nazim Hussain, Neha Kausar Ansari, Aabgeena Naeem; Investigation: Samra Hasan, Nazim Hussain; Resources: Aabgeena Naeem; Data Curation: Samra Hasan, Nazim Hussain, Neha Kausar Ansari; Writing - Original Draft: Samra Hasan, Nazim Hussain, Aabgeena Naeem; Writing - Review and Editing: Aabgeena Naeem; Visualization: Samra Hasan, Nazim Hussain, Neha Kausar Ansari; Supervision: Aabgeena Naeem.

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## References

[1]. Jan, A.; Azam, M.; Siddiqui, K.; Ali, A.; Choi, I.; Haq, Q. Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants. *Int. J. Mol. Sci.* **2015**, *16* (12), 29592–29630.
[2]. Langard, S.; Costa, M. Chromium, G.F. Nordberg, B.A. Fowler, M. Norberg, L. Friberg (Eds.), Handbook on the Toxicology of Metals (2007), pp. 487-510 https://doi.org/10.1016/B978-012369413-3/50079-3
[3]. Costa, M.; Klein, C. B. Toxicity and Carcinogenicity of Chromium Compounds in Humans. *Crit. Rev. Toxicol.* **2006**, *36* (2), 155–163.
[4]. Dayan, A. D.; Paine, A. J. Mechanisms of chromium toxicity, carcinogenicity and allergenicity: Review of the literature from 1985 to 2000. *Hum. Exp. Toxicol.* **2001**, *20* (9), 439–451.
[5]. Sharma, P.; Singh, S. P.; Parakh, S. K.; Tong, Y. W. Health hazards of hexavalent chromium (Cr (VI)) and its microbial reduction. *Bioengineered* **2022**, *13* (3), 4923–4938.
[6]. Shin, D. Y.; Lee, S. M.; Jang, Y.; Lee, J.; Lee, C. M.; Cho, E.; Seo, Y. R. Adverse Human Health Effects of Chromium by Exposure Route: A Comprehensive Review Based on Toxicogenomic Approach. *Int. J. Mol. Sci.* **2023**, *24* (4), 3410.
[7]. Coogan, T. P.; Squibb, K. S.; Motz, J.; Kinney, P. L.; Costa, M. Distribution of chromium within cells of the blood. *Toxicol. Appl. Pharmacol.* **1991**, *108* (1), 157–166.
[8]. Wilbur, S.; Abadin, H.; Fay, M.; Yu, D.; Tencza B.; Ingerman, L.; Klotzbach, J.; Shelly J. Toxicological Profile for Chromium. Agency for Toxic Substances and Disease Registry (US), Atlanta (GA), 2012. https://www.atsdr.cdc.gov/toxprofiles/tp7.pdf
[9]. Kragh-Hansen, U. Molecular aspects of ligand binding to serum albumin. *Pharmacological Reviews* **1981**, *33*, 17-53. PMID: 7027277.
[10]. Carter, D. C.; Ho, J. X. Structure of serum albumin. *Adv. Protein Chem.* **1994**, *45*, 153–203.
[11]. Karnieli, O.; Friedner, O. M.; Allickson, J. G.; Zhang, N.; Jung, S.; Fiorentini, D.; Abraham, E.; Eaker, S. S.; Yong, t. K.; Chan, A.; Griffiths, S.; Wehn, A. K.; Oh, S.; Karnieli, O. A consensus introduction to serum replacements and serum-free media for cellular therapies. *Cytotherapy* **2017**, *19* (2), 155–169.
[12]. Arasteh, A.; Farahi, S.; Habibi-Rezaei, M.; Moosavi-Movahedi, A. A. Glycated albumin: an overview of the In Vitro models of an In Vivo potential disease marker. *J. Diabetes Metab. Disord.* **2014**, *13* (1), https://doi.org/10.1186/2251–6581–13–49.

[13]. Yıldız, A.; Kara, A. A.; Acartürk, F. Peptide-protein based nanofibers in pharmaceutical and biomedical applications. *Int. J. Biol. Macromol.* **2020**, *148*, 1084–1097.
[14]. Peters, T. Serum Albumin. *Adv. Protein Chem.* **1985**, 161–245.
[15]. Hirayama, K.; Akashi, S.; Furuya, M.; Fukuhara, K. Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and frit-FAB LC/MS. *Biochem. Biophys. Res. Commun.* **1990**, *173* (2), 639–646.
[16]. Siddiqui, G. A.; Naeem, A. Connecting the Dots: Macromolecular Crowding and Protein Aggregation. *J. Fluoresc.* **2022**, *33* (1), 1–11.
[17]. Ellis, R. Macromolecular crowding: an important but neglected aspect of the intracellular environment. *Curr. Opin. Struct. Biol.* **2001**, *11* (1), 114–119.
[18]. Miklos, A. C.; Sarkar, M.; Wang, Y.; Pielak, G. J. Protein Crowding Tunes Protein Stability. *J. Am. Chem. Soc.* **2011**, *133* (18), 7116–7120.
[19]. Zhou, H.; Rivas, G.; Minton, A. P. Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. *Annu. Rev. Biophys.* **2008**, *37* (1), 375–397.
[20]. Wang, W. Protein aggregation and its inhibition in biopharmaceutics. *Int. J. Pharm.* **2005**, *289* (1-2), 1–30.
[21]. Veronese, F. M.; Mero, A. The Impact of PEGylation on Biological Therapies. *BioDrugs* **2008**, *22* (5), 315–329.
[22]. Frokjaer, S.; Otzen, D. E. Protein drug stability: a formulation challenge. *Nat. Rev. Drug Discov.* **2005**, *4* (4), 298–306.
[23]. Hasan, S.; Naeem, A. The modulation of structural stability of horseradish peroxidase as a consequence of macromolecular crowding. *J. Mol. Recognition* **2021**, *34* (10), https://doi.org/10.1002/jmr.2902.
[24]. Lintner, K.; Fermandjian, S.; St. Pierre, S.; Regoli, D. Proton NMR study of the conformation of bradykinin: pH titration. *Biochem. Biophys. Res. Commun.* **1979**, *91* (3), 803–811.
[25]. Eftink, M. R.; Ghiron, C. A. Fluorescence quenching studies with proteins. *Anal. Biochem.* **1981**, *114* (2), 199–227.
[26]. Hawe, A.; Sutter, M.; Jiskoot, W. Extrinsic Fluorescent Dyes as Tools for Protein Characterization. *Pharm. Res.* **2008**, *25* (7), 1487–1499.
[27]. Hudson, S. A.; Ecroyd, H.; Kee, T. W.; Carver, J. A. The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds. *FEBS J.* **2009**, *276* (20), 5960–5972.
[28]. Hasan, S.; Fatma, S.; Zaman, M.; Khan, R. H.; Naeem, A. Carboxylic acids of different nature induces aggregation of hemoglobin. *Int. J. Biol. Macromol.* **2018**, *118*, 1584–1593.
[29]. Wang, S. S.; Chen, P.; Hung, Y. Effects of p-benzoquinone and melatonin on amyloid fibrillogenesis of hen egg-white lysozyme. *J. Mol. Catal. B: Enzym.* **2006**, *43* (1-4), 49–57.
[30]. Chiti, F.; Taddei, N.; Baroni, F.; Capanni, C.; Stefani, M.; Ramponi, G.; Dobson, C. M. Kinetic partitioning of protein folding and aggregation. *Nat. Struct. Biol.* **2002**, *9* (2), 137–143.
[31]. Greenfield, N. J. Using circular dichroism spectra to estimate protein secondary structure. *Nat. Protoc.* **2006**, *1* (6), 2876–2890.
[32]. Singh, N. P.; McCoy, M. T.; Tice, R. R.; Schneider, E. L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **1988**, *175* (1), 184–191.
[33]. Anand, U.; Jash, C.; Mukherjee, S. Protein unfolding and subsequent refolding: a spectroscopic investigation. *Phys. Chem. Chem. Phys.* **2011**, *13* (45), 20418.
[34]. Liu, Y.; Liu, L.; Chen, M.; Zhang, Q. Double thermal transitions of type I collagen in acidic solution. *J. Biomol. Struct. Dyn.* **2013**, *31* (8), 862–873.
[35]. Zimmerman, S. B.; Minton, A. P. Macromolecular Crowding: Biochemical, Biophysical, and Physiological Consequences. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22* (1), 27–65.
[36]. Minton, A. P. The Influence of Macromolecular Crowding and Macromolecular Confinement on Biochemical Reactions in Physiological Media. *J. Biol. Chem.* **2001**, *276* (14), 10577–10580.
[37]. Jiang, M.; Guo, Z. Effects of Macromolecular Crowding on the Intrinsic Catalytic Efficiency and Structure of Enterobactin-Specific Isochorismate Synthase. *J. Am. Chem. Soc.* **2007**, *129* (4), 730–731.
[38]. Chen, M.; Liu, Y.; Cao, H.; Song, L.; Zhang, Q. The secondary and aggregation structural changes of BSA induced by trivalent chromium: A biophysical study. *J. Lumin.* **2015**, *158*, 116–124.
[39]. Taraska, J. W. Mapping membrane protein structure with fluorescence. *Curr. Opin. Struct. Biol.* **2012**, *22* (4), 507–513.
[40]. Hellmann, N.; Schneider, D. Hands On: Using Tryptophan Fluorescence Spectroscopy to Study Protein Structure. *Methods Mol. Biol.* **2019**, 379–401.
[41]. Ansari, N. K.; Khan, H. S.; Naeem, A. Doxorubicin as a Drug Repurposing for Disruption of α-Chymotrypsinogen-A Aggregates. *Protein J.* **2024**, *43* (4), 842–857.
[42]. Jayabharathi, J.; Thanikachalam, V.; Venkatesh Perumal, M. A study on the binding interaction between the imidazole derivative and bovine serum albumin by fluorescence spectroscopy. *J. Lumin.* **2012**, *132* (3), 707–712.
[43]. Clapp, A. R.; Medintz, I. L.; Mauro, J. M.; Fisher, B. R.; Bawendi, M. G.; Mattoussi, H. Fluorescence Resonance Energy Transfer Between Quantum Dot Donors and Dye-Labeled Protein Acceptors. *J. Am. Chem. Soc.* **2003**, *126* (1), 301–310.
[44]. Schönbrunn, E.; Eschenburg, S.; Luger, K.; Kabsch, W.; Amrhein, N. Structural basis for the interaction of the fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS) with the antibiotic target MurA. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (12), 6345–6349.
[45]. Guliyeva, A. J.; Gasymov, O. K. ANS fluorescence: Potential to discriminate hydrophobic sites of proteins in solid states. *Biochem. Biophys. Rep.* **2020**, *24*, 100843.
[46]. Sancataldo, G.; Vetri, V.; Foderà, V.; Di Cara, G.; Militello, V.; Leone, M. Oxidation Enhances Human Serum Albumin Thermal Stability and Changes the Routes of Amyloid Fibril Formation. *PLoS ONE* **2014**, *9* (1), e84552.
[47]. Kim, W.; Hecht, M. H. Generic hydrophobic residues are sufficient to promote aggregation of the Alzheimer's Aβ42 peptide. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (43), 15824–15829.
[48]. Naeem, A.; Bhat, S. A.; Iram, A.; Khan, R. H. Aggregation of intrinsically disordered fibrinogen as the influence of backbone conformation. *Arch. Biochem. Biophys.* **2016**, *603*, 38–47.
[49]. Khurana, R.; Coleman, C.; Ionescu-Zanetti, C.; Carter, S. A.; Krishna, V.; Grover, R. K.; Roy, R.; Singh, S. Mechanism of thioflavin T binding to amyloid fibrils. *J. Struct. Biol.* **2005**, *151* (3), 229–238.
[50]. Wolfe, L. S.; Calabrese, M. F.; Nath, A.; Blaho, D. V.; Miranker, A. D.; Xiong, Y. Protein-induced photophysical changes to the amyloid indicator dye thioflavin T. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107* (39), 16863–16868.
[51]. Biancalana, M.; Koide, S. Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochim. Biophys. Acta (BBA) - Proteins Proteom.* **2010**, *1804* (7), 1405–1412.
[52]. Siddiqui, G. A.; Naeem, A. Refolding of Hemoglobin Under Macromolecular Confinement: Impersonating In Vivo Volume Exclusion. *J. Fluoresc.* **2021**, *31* (5), 1371–1377.
[53]. Kumar, S.; Walter, J. Phosphorylation of amyloid beta (Aβ) peptides – A trigger for formation of toxic aggregates in Alzheimer's disease. *Aging* **2011**, *3* (8), 803–812.
[54]. Li, S.; Wang, L.; Chusuei, C. C.; Suarez, V. M.; Blackwelder, P. L.; Micic, M.; Orbulescu, J.; Leblanc, R. M. Nontoxic Carbon Dots Potently Inhibit Human Insulin Fibrillation. *Chem. Mater.* **2015**, *27* (5), 1764–1771.
[55]. Li, J.; Uversky, V. N.; Fink, A. L. Effect of Familial Parkinson's Disease Point Mutations A30P and A53T on the Structural Properties, Aggregation, and Fibrillation of Human α-Synuclein. *Biochemistry* **2001**, *40* (38), 11604–11613.
[56]. Kheterpal, I.; Chen, M.; Cook, K. D.; Wetzel, R. Structural Differences in Aβ Amyloid Protofibrils and Fibrils Mapped by Hydrogen Exchange – Mass Spectrometry with On-line Proteolytic Fragmentation. *J. Mol. Biol.* **2006**, *361* (4), 785–795.
[57]. Khan, M. V.; Ishtikhar, M.; Rabbani, G.; Zaman, M.; Abdelhameed, A. S.; Khan, R. H. Polyols (Glycerol and Ethylene glycol) mediated amorphous aggregate inhibition and secondary structure restoration of metalloproteinase-conalbumin (ovotransferrin). *Int. J. Biol. Macromol.* **2017**, *94*, 290–300.
[58]. Chi, Z.; Liu, R. Phenotypic Characterization of the Binding of Tetracycline to Human Serum Albumin. *Biomacromolecules* **2010**, *12* (1), 203–209.
[59]. Ansari, N. K.; Rais, A.; Naeem, A. Methotrexate for Drug Repurposing as an Anti-Aggregatory Agent to Mercuric Treated α-Chymotrypsinogen-A. *Protein J.* **2024**, *43* (2), 362–374.
[60]. Hasan, S.; Isar, M.; Naeem, A. Macromolecular crowding stabilises native structure of α-chymotrypsinogen-A against hexafluoro propanol-induced aggregates. *Int. J. Biol. Macromol.* **2020**, *164*, 3780–3788.
[61]. Hatters, D. M.; Minton, A. P.; Howlett, G. J. Macromolecular Crowding Accelerates Amyloid Formation by Human Apolipoprotein C-II. *J. Biol. Chem.* **2002**, *277* (10), 7824–7830.
[62]. Taboada, P.; Barbosa, S.; Castro, E.; Mosquera, V. Amyloid Fibril Formation and Other Aggregate Species Formed by Human Serum Albumin Association. *J. Phys. Chem. B.* **2006**, *110* (42), 20733–20736.
[63]. Wu, C.; Scott, J.; Shea, J. Binding of Congo Red to Amyloid Protofibrils of the Alzheimer Aβ9–40 Peptide Probed by Molecular Dynamics Simulations. *Biophys. J.* **2012**, *103* (3), 550–557.
[64]. Stathopulos, P. B.; Scholz, G. A.; Hwang, Y.; Rumfeldt, J. A.; Lepock, J. R.; Meiering, E. M. Sonication of proteins causes formation of aggregates that resemble amyloid. *Protein Sci.* **2004**, *13* (11), 3017–3027.
[65]. Adler, A. J.; Greenfield, N. J.; Fasman, G. D. [27] Circular dichroism and optical rotatory dispersion of proteins and polypeptides. *Methods Enzymol.* **1973**, 675–735.
[66]. Sen, S.; Konar, S.; Pathak, A.; Dasgupta, S.; DasGupta, S. Effect of Functionalized Magnetic $MnFe_2O_4$ Nanoparticles on Fibrillation of Human Serum Albumin. *J. Phys. Chem. B.* **2014**, *118* (40), 11667–11676.
[67]. Paul, B. K.; Ray, D.; Guchhait, N. Unraveling the binding interaction and kinetics of a prospective anti-HIV drug with a model transport protein: results and challenges. *Phys. Chem. Chem. Phys.* **2013**, *15* (4), 1275–1287.

[68]. Naeem, A.; Iram, A.; Bhat, S. A. Anesthetic 2,2,2-trifluoroethanol induces amyloidogenesis and cytotoxicity in human serum albumin. *Int. J. Biol. Macromol.* **2015**, *79*, 726–735.
[69]. Tabner, B. J.; El-Agnaf, O. M.; Turnbull, S.; German, M. J.; Paleologou, K. E.; Hayashi, Y.; Cooper, L. J.; Fullwood, N. J.; Allsop, D. Hydrogen Peroxide Is Generated during the Very Early Stages of Aggregation of the Amyloid Peptides Implicated in Alzheimer Disease and Familial British Dementia. *J. Biol. Chem.* **2005**, *280* (43), 35789–35792.
[70]. Jin, S.; Kedia, N.; Illes-Toth, E.; Haralampiev, I.; Prisner, S.; Herrmann, A.; Wanker, E. E.; Bieschke, J. Amyloid-β(1–42) Aggregation Initiates Its Cellular Uptake and Cytotoxicity. *J. Biol. Chem.* **2016**, *291* (37), 19590–19606.
[71]. Yerbury, J. J. Protein aggregates stimulate macropinocytosis facilitating their propagation. *Prion* **2016**, *10* (2), 119–126.
[72]. Husain, N.; Mahmood, R. Copper(II) generates ROS and RNS, impairs antioxidant system and damages membrane and DNA in human blood cells. *Environ. Sci. Pollut. Res.* **2019**, *26* (20), 20654–20668.
[73]. Rodríguez-Martínez, J. A.; Solá, R. J.; Castillo, B.; Cintrón-Colón, H. R.; Rivera-Rivera, I.; Barletta, G.; Griebenow, K. Stabilization of α-chymotrypsin upon PEGylation correlates with reduced structural dynamics. *Biotech & Bioengineering* **2008**, *101* (6), 1142–1149.
[74]. Castellanos, I. J.; Al-Azzam, W.; Griebenow, K. Effect of the Covalent Modification with Poly(ethylene glycol) on α-Chymotrypsin Stability upon Encapsulation in Poly(lactic-co-glycolic) Microspheres. *J. Pharm. Sci.* **2005**, *94* (2), 327–340.
[75]. Inada, Y.; Furukawa, M.; Sasaki, H.; Kodera, Y.; Hiroto, M.; Nishimura, H.; Matsushima, A. Biomedical and biotechnological applications of PEG- and PM-modified proteins. *Trends Biotechnol.* **1995**, *13* (3), 86–91.
[76]. Pasut, G.; Sergi, M.; Veronese, F. M. Anti-cancer PEG-enzymes: 30 years old, but still a current approach. *Adv. Drug Deliv. Rev.* **2008**, *60* (1), 69–78.
[77]. Zhou, H. Crowding Effects of Membrane Proteins. *J. Phys. Chem. B.* **2009**, *113* (23), 7995–8005.
[78]. Totani, K.; Ihara, Y.; Matsuo, I.; Ito, Y. Effects of Macromolecular Crowding on Glycoprotein Processing Enzymes. *J. Am. Chem. Soc.* **2008**, *130* (6), 2101–2107.
[79]. Khurana, R.; Ionescu-Zanetti, C.; Pope, M.; Li, J.; Nielson, L.; Ramírez-Alvarado, M.; Regan, L.; Fink, A. L.; Carter, S. A. A General Model for Amyloid Fibril Assembly Based on Morphological Studies Using Atomic Force Microscopy. *Biophys. J.* **2003**, *85* (2), 1135–1144.