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ORIGINAL ARTICLE

Non-enzymatic glycation of α-crystallin as an in vitro model for aging, diabetes and degenerative diseases

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Abstract Alpha crystallin, a small heat-shock protein, has been studied extensively for its chaperone function. Alpha crystallin subunits are expressed in stress conditions and have been found to prevent apoptosis by inhibiting the activation of caspase pathway. Non-enzymatic glycation of protein leads to the formation of advanced glycation end-products (AGEs). These AGEs bind to receptors and lead to blocking the signaling pathways or cause protein precipitation as observed in aggregation-related diseases. Methylglyoxal (MGO) is one of the major glycating agents expressed in pathological conditions due to defective glycolysis pathway. MGO reacts rapidly with proteins, forms AGEs and finally leads to aggregation. The goal of this study was to understand the non-enzymatic glycation-induced structural damage in alpha crystallin using biophysical and spectroscopic characterization. This will help to develop better disease models for understanding the biochemical pathways and also in drug discovery.

Keywords Diabetes · Aging · Alpha crystallin · Presbyopia · Cataract · Lens · Degenerative diseases

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Introduction

α-crystallin is the most abundant and largest of the lens proteins (~18 nm in diameter) consisting anywhere between 30 and 40 subunits with molecular weight ranging from 800 to 1200 kDa (Horwitz 2003). In its native form, α-crystallin consists of two homologous subunits showing 55 % sequence similarity in a ratio of 3:1—αA and αB, with 173 and 175 amino acid residues, respectively. The molecular weight of these two subunits is approximately 20 kDa. αA crystallin is confined to the lens with a small amount in the retina, spleen and thymus (Fort and Lampi 2011; Srinivasan et al. 1992). αB crystallin is ubiquitously present in the lens, retina and the heart, and is expressed under stress and pathological conditions in the spinal cord, muscles, brain and the kidneys (Kannan et al. 2012). Studies show that α-crystallins act as anti-apoptotic regulators and prevent apoptosis under stress conditions, thereby protecting the tissues from damage (Hamann et al. 2013; Lee et al. 2012; Pasupuleti et al. 2010; Reddy and Reddy 2015).

Due to a very low protein turnover, crystallins are considered to be some of the longest-lived proteins in the human body (Craig et al. 1994; Garland et al. 1988; Van Kleef et al. 1975). Because of the long half-life, α-crystallin is prone to irreversible modifications leading to changes in structure and function. The most commonly observed post-translational modifications include photo-oxidization, deamidation, racemization, phosphorylation, acetylation, glycation and age-dependent truncation (Hoehenwarter et al. 2006). Post-translational modifications alter protein–protein interactions and subsequently destabilize and reduce the solubility of native crystallins.

The chaperone function of α-crystallin has been well established and it has also been found to prevent thermal aggregation of other proteins (Borkman et al. 1996; Boyle

and Takemoto 1994; Cherian and Abraham 1995a; Craig et al. 1994; Das and Surewicz 1995; Ganea 2001; Groenen et al. 1994; Heys et al. 2007; Hook and Harding 1996; Horwitz 1992; Jakob et al. 1993; Khanova et al. 2005; Krishna Sharma and Ortwerth 1995; Kumar et al. 2004, 2007; Raman et al. 1995; Raman and Rao 1994; Rao et al. 1993; Rekas et al. 2004; Srinivas et al. 2003; Takemoto and Boyle 1994; Tardieu 1998; Van Montfort et al. 2002; Vanhoudt et al. 2000; Wang and Spector 1995). Over a period of time, α-crystallin undergoes irreversible post-translational modifications of which non-enzymatic glycation is prominent especially in aging and diabetes. As a result, the protein slowly starts losing its chaperone ability and starts to aggregate. Nagaraj et al. have shown that glycation also leads to loss of anti-apoptotic activity of alpha crystallin (Nahomi et al. 2013).

Although α-crystallin has been studied extensively, the quaternary structure of the native protein has not been accurately elucidated. As a result, the locations of protein modifications which are a part of disease pathology are a matter of debate. As the lens is avascular and has no turnover, the modifications that occur in the lens alpha crystallin due to non-enzymatic glycation are permanent (Mukhopadhyay et al. 2010). Reducing sugars react with basic amino acids of proteins to form Schiff's bases which undergo rearrangement to Amadori products and finally form advanced glycation end-products (AGEs). These AGEs lead to loss of protein integrity, increase the hydrophobicity and play an important role in protein denaturation (Monnier 1989, 1990; Monnier and Cerami 1982; Monnier et al. 2008; Njoroge and Monnier 1989; Stevens et al. 1978).

Protein denaturation is usually associated with the formation of aggregates (Hollar et al. 1995; Lindner and Demarez 2009; Meredith 2006). Protein precipitation and aggregation involves the growth of large sized particles and hence is an optimum method for biophysical characterization based on particle size. By studying the light scattering characteristics of protein aggregation, we can hypothesize the effect of glycation on the protein and its role in the decrease of lens flexibility (presbyopia) as well as the formation of a cataract.

Methylglyoxal (MGO) is a glycating agent that is generated non-enzymatically from the oxidation and spontaneous dismutation of intermediates in the glycolysis pathway or enzymatic oxidation reaction catalyzed by peroxidases (Bento et al. 2010; Ray et al. 1994). MGO is known to be toxic and to interfere with cellular mechanisms (Wilker et al. 2001). It has been found to impair functions of mitochondria and also produce reactive oxygen species (Turk et al. 2006). Another source of this dicarbonyl reactive intermediate in the body is deficiency of triose phosphate isomerase leading to elevated dihydroxyacetonephosphate (DHAP) levels observed in congenital hemolytic anemia and other neurodegenerative diseases (Monnier et al. 1993; Rosca et al. 2005). DHAP spontaneously disintegrates to methylglyoxal which acts as a strong agent in the formation of advanced glycation end-products (AGEs). Because MGO reacts rapidly with the proteins, it is reasonable to assume that modification by MGO would be a good in vitro model for studying the long-term effects of glycation on heat-shock proteins.

The main aim of this project was to establish a correlation between the structural, molecular and spectroscopic changes in the protein as it slowly denatures due to aggregation induced by non-enzymatic glycation. This study will in turn help us to understand the importance of studying a glycation model of aging and diabetes while trying to elucidate the pathological mechanisms in various degenerative diseases.

Materials and methods

Methyl glyoxal was purchased from Sigma Aldrich. Fresh calf lenses were obtained from the Brown Packing Co. (South Holland, IL).

Extraction of bovine alpha crystallin

Fresh calf lenses were weighed and then homogenized by stirring in a buffer prepared using 50 mM Tris/0.2 M NaCl/1 mM EDTA/10 mM mercaptoethanol, pH 7.4 at 4 °C. The supernatant was centrifuged at 14,000*g* for 1 h at 4 °C. α-crystallin was isolated from the total soluble lens protein solution by size exclusion chromatography. A total of 30 ml supernatant was loaded on a 1.7 cm × 100 cm CL-6B Sepharose gel filtration column, and using a peristaltic pump, eluted at a flow rate of 1 ml/min and monitored by absorbance at 280 nm. Alpha crystallin elutes first as a single symmetrical peak at approximately 170 ml of the buffer corresponding to an apparent molecular mass of 800 KDa. The isolated α crystallin fractions were pooled and desalted with Ultrafree-15 Biomax-10 K centrifugal filter devices (Millipore Corporation, Bedford, MA) at a speed of 3000 rpm by rinsing three times with water. The purity of the sample was determined by SDS-PAGE with the Pharmacia LKB*Phast System and by mass spectrometry (Mandal et al. 2000).

Sample preparation

Bovine α-crystallin was used as the model protein to study the progression of glycation. Methyl glyoxal (MGO) was used as the glycating agent at a concentration of 10 μM in 1 mg/ml alpha crystallin solution and incubated at 37 °C over a period of 9 h. The aliquots were collected at 0.5, 1,

3, 6, 9 h. The samples were dialyzed against 10 mM phosphate buffer, pH 7.4, lyophilized and reconstituted with 1 ml of MQ water before measurements.

Dynamic light scattering measurements (DLS)

Particle sizes and particle cluster sizes were measured using DLS on a Brookhaven BI-200SM Research Goniometer and Laser Light Scattering System. For a spherical particle diffusing through a solution of viscosity η, the intensity correlation function (g_2) measured through DLS decays with an exponential relaxation rate, $g_2(t) = 1 + \beta\exp(-2\Gamma t)$. The particle size can be obtained from the decay rate via $R = k_B T q^2/6\pi\eta\Gamma$. Here, k_B is Boltzmann's constant, T is the temperature and $q = 4\pi \sin\left(\frac{\theta}{2}\right)/\lambda$ is the scattering vector of the laser light scattered at $\theta = 90°$ and $\lambda = 632$ nm.

For a suspension consisting of a mixture of particles of varying size, the distribution of sizes can be obtained from an inverse Laplace transform. We used the CONTIN software package to obtain particle size distributions in this manner. The accuracy of the distributions was determined based on the polydispersity and the baseline difference from the correlation curve.

Surface hydrophobicity measurements

The surface hydrophobicity of the native and MGO-modified alpha crystallin was studied using a specific hydrophobic probe, 1-anilinonaphthalene-8-sulfonic acid (ANS). Ten microliters of a 10 mM methanolic solution of ANS was added to 1 mL of protein [0.1 mg/mL in 10 mM phosphate buffer (pH 7.4)], and the mixture was incubated for 1 h in the dark at 25 °C. Fluorescence emission spectra were recorded between 400 and 600 nm using an excitation wavelength of 370 nm. The excitation and emission band passes were 5 nm each.

Small angle X-ray scattering measurements

Concentrated samples of alpha crystallin and glycated alpha crystallin have been studied using SAXS. These studies were performed at the 8-ID-I beam line of the Advanced Photon Source at Argonne National labs, IL. The sample in a sealed capillary was placed in the beam line under vacuum. Coherent X-ray photons of energy 7.35 keV were focused using a Kinoform lens placed upstream of the sample. The scattered photons were detected by a PI LCX-1300 direct detection CCD (Princeton Instruments, USA) 4.0 m downstream of the sample. A single camera width spans about 0.3 nm^{-1} in the scattering vector (Q) space. The intensities were measured at 6 overlapping camera widths covering a Q range from 0.1 to 1 nm^{-1}. For a given camera position, 20 frames were collected with an exposure time of 0.2 s for each frame. The shorter exposures of 0.2 s and moving the beam spot to a different location and camera to a new position helps to minimize the radiation damage caused by the X-ray beam. The resulting image pixels were analyzed by a MATLAB GUI to obtain the time-averaged intensity profiles as a function of the scattering vector. The inter-particle distance between the subunits can be determined from scattering vector Q using $Q_{max} = 2\pi/d$; where d = distance between adjacent subunits.

Steady-state fluorescence measurements

The tryptophan fluorescence of unmodified and methyl glyoxal-modified α-crystallin was measured using Hitachi F2500 Fluorescence Spectrometer. The fluorescence emission spectrum of the samples was recorded between 300 and 700 nm using 295 nm as excitation wavelengths maintaining the slit widths at 2.5 nm and PMT voltage of 400 V.

Time-resolved fluorescence measurements

The lifetime profiles for the tryptophan fluorescence decay in unmodified and modified alpha crystallin (10 mM phosphate buffer at pH 7.4) were measured using TimeMaster™ LED system (TM-2000). The samples were excited at 295 nm using a LED light source having approximately 1.5 ns pulse width. The time domain system was used to measure the decay in fluorescence with respect to time. The raw decay data were analyzed using global 1–4 exponential fitting analysis with deconvolution which gives the lifetimes as well as the relative contributions to the total fluorescence at time zero. These data were assessed with a good auto correlation function around zero, weighted number of residuals randomly distributed between +3 and −3, reduced χ^2 values around 0.9–1.1.

Results and discussion

Amplified concentrations of glycating agents in the body cause modifications in glycoproteins as well as heat-shock proteins which is a characteristic feature of aging and diabetic eye diseases (Nagaraj et al. 2012). The incubation of α-crystallin with different glycating agents leads to the formation of aggregates over a period of time which varies between minutes to years. The aggregates are associated with the destruction of native state α-crystallin and formation of AGEs which are initiated by the condensation of basic amino acids and sugars to form Schiff's bases which undergo rearrangement to form Amadori products.

Dynamic light scattering (DLS) uses visible light to measure the time-dependent fluctuations in the scattering intensity to determine the translational diffusion coefficient

(D_T), and hydrodynamic radius (R_H) of a particle (Petta et al. 2008). The protein solutions have shown very good light scattering ability and the particle size of native state α-crystallin was found to be 18 ± 2 nm which was consistent with reports from literature (Augusteyn and Koretz 1987; Horwitz 2003; Regini et al. 2004). The reaction with methyl glyoxal was rapid and the particle size of the aggregates was around 350 nm within 9 h (Fig. 1). With an increase in the particle size, the corresponding diffusivity of the protein decreases. The calculated diffusivity values from the in vitro study agree with the results obtained from the in vitro study to determine the protein diffusivity in rabbit lenses (Nishio et al. 1984; Tanaka and Ishimoto 1977). This study helped us to determine that by measuring the light scattering from the lens and in turn particle size of the aggregates, we can predict the formation of a cataract at a very early stage.

Small angle X-ray scattering (SAXS) was used to measure the correlations between alpha crystallin proteins. SAXS data are very useful in determining the inter-particle spacing and hence help us to understand the packing of α-crystallin in very high concentrations without super-aggregation or crystallization. A recent study has detailed how in concentrated suspensions of alpha crystallin that inter-particle correlations are well described by the structure factor for a hard sphere fluid (Foffi et al. 2014). At low concentrations, alpha crystallin shows a monotonic falloff of intensity with scattering vector q. However, for concentrated suspensions, there is a peak that appears around $q \sim 0.4$ nm^{-1} for concentrations of 100 mg/ml and moves out to around $q \sim 0.5$ nm^{-1} at concentrations around 300 mg/ml with increasing concentration. We measured SAXS from a 250 mg/ml sample of alpha crystallin which showed a strong peak around $q = 0.42$ nm^{-1} (Fig. 2) consistent with Foffi et al. and other research groups (Delaye and Tardieu 1983; Foffi et al. 2014). Upon glycation with 10 μM methylglyoxal, the correlation peak diminishes in intensity and moves to smaller $q = 0.25$ nm^{-1}. This indicates a possible loss of structural integrity of the protein.

The ANS dye is a valuable tool for the detection of protein surface hydrophobicity. Usually, the dye has a low fluorescence yield, which is greatly enhanced on interaction with hydrophobic surfaces. From the experimental data as shown in Fig. 3, we can conclude that hydrophobicity increases with time of glycation. Another important observation during this study is the spectral shape exhibiting 2 peaks. This can be explained based on the fact that the fluorescence of ANS dye is highly solvent dependent. So the polarity of the microenvironment affects the emission maxima of this molecule. Lee et al. have shown that ANS dye shows emission maxima around 550 nm in a highly polar environment and a blue shift of around 50 nm in a less polar environment (Lee and Robinson 1985). This

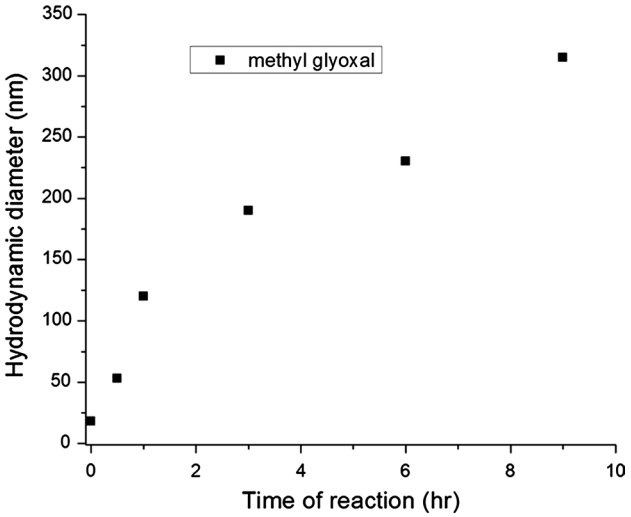

Fig. 1 Increase in hydrodynamic diameter of α with 10 μM methyl glyoxal over a period of 9 h at 25 °C

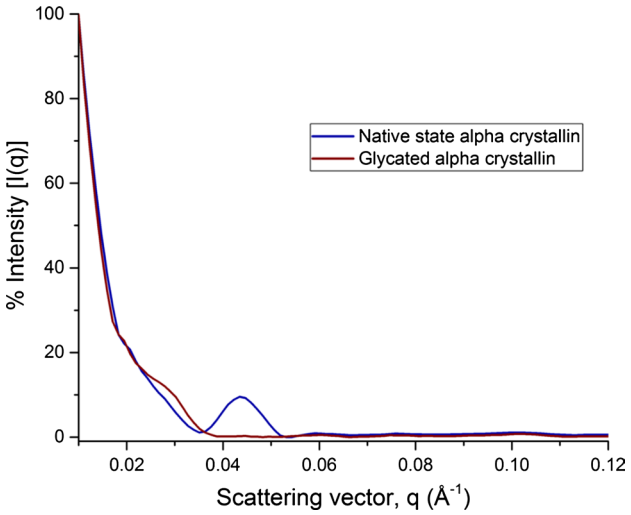

Fig. 2 Small angle X-ray scattering data to measure the inter-particle distances of unmodified and glycated α-crystallin

study shows that the native state α-crystallin, upon glycation unfolds to expose the hydrophobic regions to the surrounding polar environment and due to short-range interactions, forms aggregates. Previous literature shows that the chaperone ability of methyl glyoxal-modified α-crystallin increases initially. However, with increased time of glycation, the protein aggregates, loses its structural integrity and chaperone ability (Cherian and Abraham 1995b; Kumar et al. 2004, 2007). As a result, other lens proteins are easily prone to stress leading to their damage. Our structural studies can explain the loss of chaperone activity reported in literature, thereby establishing the relation between structure and function of alpha crystallin.

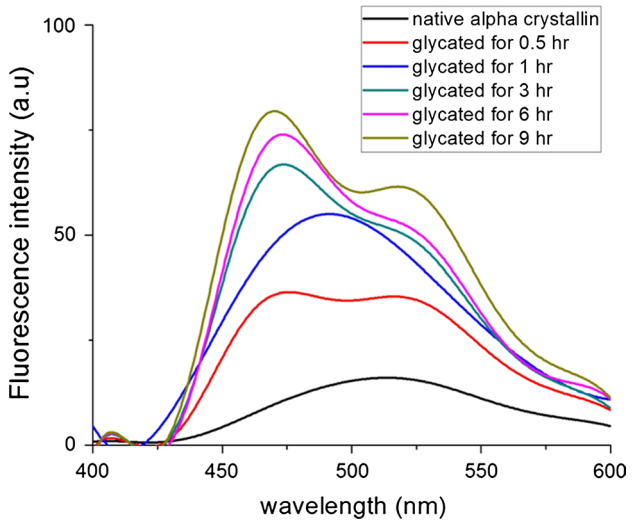

Fig. 3 Change in surface hydrophobicity of the native α-crystallin and 10 μM methyl glyoxal-modified α-crystallin over a period of 9 h at 25 °C

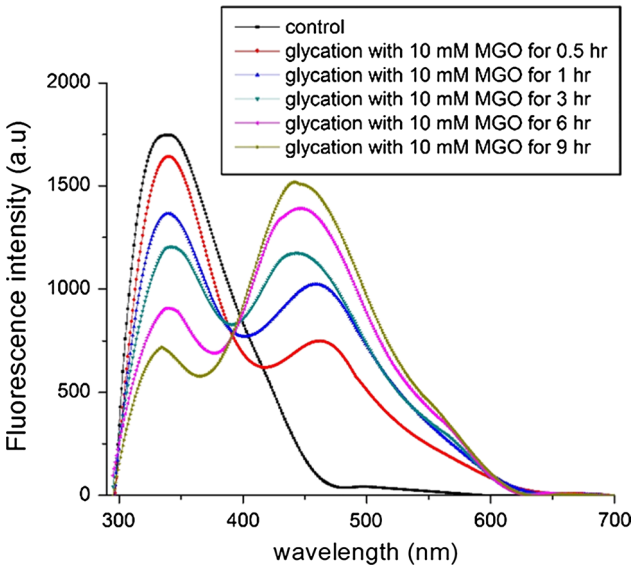

Fig. 4 Change in tryptophan fluorescence of α-crystallin after incubating with 10 μM methyl glyoxal over a period of 9 h at 25 °C

Progressive loss of soluble α-crystallin, associated with increased hydrophobicity and formation of aggregates appears to be responsible for increasing the lens stiffness. Literature suggests that heat-induced denaturation of α-crystallin may be an important factor in the etiology of presbyopia (Heys et al. 2007; Strenk et al. 2005). However, considering the physiological conditions, non-enzymatic glycation seems to be the main culprit. Cataract and presbyopia can produce myopia due to a change in the refractive index of the crystalline lens of the eye. The presence of high molecular weight aggregates and also advanced glycation end-products lead to a change in both the scattering intensities as well as the spectroscopic properties of the lens. Due to change in refractive index of the lens, the angle of the refracted light changes leading to several focal points in close proximity on the retina causing chromatic aberrations.

Tryptophan has a very strong fluorescence in the proteins. The residues which are buried in the hydrophobic core of proteins can have spectra which are shifted by 10–20 nm compared to tryptophans on the surface of the protein (Lakowicz and Geddes 1991). Tryptophan fluorescence can be quenched due to microenvironments. The magnitude of fluorescence intensity can serve as a probe to explain the perturbations occurring in the native state. The wavelength maxima of tryptophan fluorescence shifted on modification indicating a change in the microenvironment which can be confirmed by surface hydrophobicity measurements (Fig. 3). In α-crystallin, tryptophans are not located at the N terminus and hence do not have a free amino group to participate in the Maillard reaction. Dillon et al. have studied extensively on the photo-degradation products of alpha crystallin and showed that they were primarily from tryptophan oxidation (Dillon 1991; Dillon and Atherton 1990; Dillon et al. 1990; Ervin et al. 2001; Finley et al. 1997, 1998; Gaillard et al. 2000; McDermott et al. 1991; Roberts et al. 1991).

Another interesting aspect of tryptophan fluorescence spectral profiles is a shift in the isosbestic point of the spectra. Usually, the presence of an isosbestic point indicates that only two species that vary in concentration contribute to the absorption around the isosbestic point. If a third molecule is participating in the process, the spectra typically intersect at varying wavelengths as concentrations change, creating the impression that the isosbestic point is 'out of focus', or that it will shift as conditions change (Moore 1961). The reason for this is that the different compounds have varying extinction coefficients at one particular wavelength. Tryptophan oxidation products have spectral profiles similar to AGEs with varying extinction coefficients (Borkman et al. 1977, 1980, 1981, 1996; Borkman 1984; Borkman and Lerman 1978; Kurzel et al. 1973; Lerman and Borkman 1978). This helps us to explain the decrease in tryptophan fluorescence with increase in the time of glycation.

Advanced glycation end-products have a characteristic fluorescence and seem to be excited in the spectral region of tryptophan emission. Generation of AGEs can be associated with oxidative stress, tobacco smoking and weakened detoxification of AGE precursors. Dicarbonyl intermediates such as methyl glyoxal cause a lot of structural damage compared to reducing sugars such as glucose. The rate of AGE formation depends on the rate of the formation of highly reactive

Table 1 Changes in tryptophan lifetimes of α-crystallin after incubating with using 10 μM methylglyoxal over a period of 9 h at 25 °C

Time of glycation with methyl glyoxal (h)	Lifetimes, τ (ns) and relative contributions at time zero, A (%)						χ^2 value
	A_1	τ_1	A_2	τ_2	A_3	τ_3	
0	16.1	1.3	53.4	4.3	30.5	9.6	0.97
0.5	12.3	1.5	48.5	4	39.2	9.8	1.11
1	14.7	1.5	44.4	5	40.9	9.8	1.08
3	16.1	1.7	42.3	4.9	39.5	9.8	1.21
6	17.4	1.8	48.2	4.9	34.4	10	1.01
9	18.2	2.3	43.7	3.5	40.2	10	0.99

intermediates due to defective glycolysis pathway and consumption of high sugar diet (Bartling et al. 2011; Simm et al. 2014). The change in AGE fluorescence with decrease in tryptophan fluorescence can be observed in Fig. 4.

Tryptophan (Trp) lifetime measurements can be used to give a rough idea about the changes in the microenvironments. Tryptophan fluorescence is a very valuable tool that can be applied to study folding/unfolding, the effect of environment and solvent exposure on the integrity of the protein. The fluorescence lifetimes of tryptophan can be used to analyze the location of tryptophan and the possibility of short-range interactions. Gaillard et al. have shown that the fluorescence of α-crystallin is predominantly due to the Trp 9 in both A and B chains, while the Trp 60 present in the B chain is buried from exposure (Mandal et al. 2000). However, once the process of glycation begins, it seems that the tryptophans are completely exposed to the surrounding aqueous medium. This can be confirmed from the lifetime measurements as in Table 1, as we do not observe any change in the lifetimes with time of glycation. But, the measurements hint at a possibility that the tryptophan residues undergo a shift in their position once the protein starts to unfold and aggregate leading to the formation of water-insoluble residues.

The Trp residues in peptides generally show fluorescence emission spectra, with a peak around the 340–370 nm region, similar to the Trp residues in aqueous media or in fully denatured proteins (Lakowicz and Geddes 1991; Liang and Liu 2006; Sreelakshmi et al. 2004). However, interaction of peptides possessing Trp residues with proteins would result in diminished exposure of the peptide Trp (as seen in Trp 60) to the aqueous environment with a concomitant blue shift in the fluorescence emission maxima. Simulation studies have shown that, although Trp 60 is exposed to solvent in the α-B subunit, it is buried in the dimer models, located at the interacting interface of the subunits (Guo 2008). Using LCMS and isotopic labeling, Kielmas et al. (2015) have shown that K70, K88, K92, K99, K103, K145, K150 and K166 are the lysine glycation targets on α-crystallin. Gangadharaiah et al. (2010) have reported that R12, R21, R54, R65, R69, R103, R112, R117, R119, R157 and R163 are arginine glycation sites on α-crystallin. Based on these target sites of glycation, we can assume that modifications occur within the vicinity of the tryptophan (Trp 60) exposing it to different microenvironments as they are chemically modified.

Overall, this study helped us to determine changes in spectroscopic and biophysical properties such as particle size and AGE fluorescence characteristic to aging and diabetes. We have examined the effect of protein aggregation on the structure and in turn, function of heat-shock proteins in the lens. While the crystal structure of α-crystallin still remains a controversy, our study helped to lay ground to understand the glycation-induced damage to α-crystallin. Our study will help instigate an awareness among the scientific community in considering a diabetes model while studying the pathological mechanisms involving heat-shock proteins. This may in turn lead to develop more efficient pharmaceutical intervention to suppress the protein aggregation and provide treatment for many serious diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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