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Pharmaceutical acetylation can modulate the amyloidogenicity of human serum albumin

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Human serum albumin (HSA) is an abundant carrier protein present in the blood plasma manifesting affinity for drugs and ligands. The bindings of several drugs can cause changes in the structural conformation of HSA that may affect its function. HSA is also known to *in vitro* form amyloid-like aggregates with fibrillar morphology as observed under TEM. Earlier, we showed that the HSA amyloid-like aggregates display self-seeding potential and detergent stability and the dimers of HSA, which are preferable for clinical applications due to their longer circulatory life, can also form amyloid-like aggregates. As aspirin, a commonly prescribed drug, was previously documented to acetylate HSA at one of its lysine residues, here, we examined if acetylation has any effect on the *in vitro* amyloid-like aggregation of HSA. We show that HSA acetylated *in vitro* using acetylsalicylic acid manifests relatively reduced levels of amyloid-specific properties such as turbidity, Thioflavin-T-positive aggregation, β-sheet content and stability against an ionic detergent. Also, TEM imaging shows that the acetylated HSA forms relatively less aggregates and with smaller sizes whereas, the aggregates of HSA are more abundant and larger in sizes with fibrillar morphology which further supports that acetylation can attenuate the amyloid-like aggregation of HSA.

Keywords: Acetylation, Human serum albumin, Sarkosyl, Thioflavin-T

Human serum albumin (HSA), a predominant plasma protein in the blood, is a globular protein with a predominantly alpha helical structure[1]. HSA binds to and aids in the transport of several endogenous metabolites such as bilirubin and fatty acids and also exogenous molecules including nutrients and drugs. HSA also helps in the maintenance of osmolarity and manifests antioxidant properties[2]. Changes in the physiological levels of HSA such as, hyperalbuminemia as well as hypoalbuminemia can be a symptom of liver pathologies. HSA is also used as an adjunct with other therapies to treat critical conditions under certain circumstances like acute lung injury in patients with hypo-proteinemia[3].

The sequence of the functional HSA consists of 585 amino acids with a single tryptophan residue present at 214. As part of its secondary structure HSA contains about 67% α-helices, which are divided into three domains with each domain consisting of two sub-domains A and B. Structure of HSA has two high affinity drug binding sites. Of these, the site-1 is a hydrophobic pocket formed in the subdomain A of the second domain and it is a characterized binding site for drugs such as warfarin and acenocoumarol[4]. The entrance of the pocket has positively charged residues and the hydrophobic pocket involves a lone tryptophan at position 214. Because of its high affinity for the drug binding, the pharmacokinetics of HSA and the accompanying alterations in the conformation of the HSA molecule have been of considerable interest[5]. Changes in the conformation of HSA due to the binding of drug molecules including ciprofloxacin and tofacitinib have been well characterized[6,7].

Acetylsalicylic acid, or aspirin, is a commonly prescribed drug molecule which is known to interact with lysine residues of HSA *in vitro* as well as *in vivo*[8,9]. Previous studies from patients have reported that the ingestion of therapeutic doses of aspirin can cause permanent acetylation of HSA and coherent observations have also been made *in vitro* under physiological conditions[9,10]. In fact, previous studies have shown that the aspirin mediated modification of lysine-199 of HSA affects its esterase activity as well as its binding to several other drug molecules[11,12].

Several proteins are involved in causing amyloidosis diseases in humans and some can also

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form amyloid-like aggregates *in vitro* that typically display beta-sheet rich conformation[13-18]. Amyloid proteins can cause cytotoxicity *via* several mechanisms such as by blocking proteosomes and causing oxidative stress[19-21]. Notably, post-translational modifications including acetylation have been known to modulate the amyloid-like aggregations of several proteins including the TDP-43 protein involved in the amyotrophic lateral sclerosis (ALS) disease and the Tau protein involved in the Alzheimer's disease[22-24]. HSA protein has been previously shown to form amyloid-like fibrillar aggregates *in vitro*[25,26]. In this study, we have examined the effect of acetylation on the amyloid-like aggregation of HSA using thioflavin-T binding assay, detergent stability assay, β-sheet content estimation and TEM imaging.

Materials and Methods

Materials

HSA protein expressed recombinantly in *Saccharomyces cerevisiae* was purchased from Sigma, USA. Thioflavin-T, Sodium dodecyl sulphate (SDS) and N-Lauroylsarcosine sodium salt (sarkosyl) were procured from Sigma–Aldrich, USA. Acrylamide, Agarose, Sodium salicylate, acetyl salicylic acid (aspirin), Coomassie brilliant blue, β-mercaptoethanol, sodium chloride, sodium phosphate dibasic and monobasic were purchased from Himedia, India. Methanol was purchased from SRL, India.

Aspirin-mediated acetylation of HSA

Acetylation of 15 mg/mL HSA was carried out using a working concentration of 1.5 mM aspirin prepared from a stock solution of 10 mM concentration and it was incubated for 24 h at 37°C, after which the unreacted aspirin was removed by dialysing against 10 mM phosphate buffer saline containing 0.01 M salicylic acid for 12 h at 4°C. This was followed by dialysing against 10 mM phosphate buffer twice for 12 h each at 4°C to make sure all the free aspirin was removed. This acetylated HSA was checked for its concentration and used for further experiments[10].

SDS-PAGE

HSA protein after the acetylation reaction was analyzed on 10% SDS-PAGE to eliminate the possibility of any protein degradation before usage in further studies. The wild-type HSA protein was also electrophoresed for comparison. Samples were either treated with non-reducing or reducing Laemmli sample buffer before the electrophoresis[27].

Amyloid-like *in vitro* aggregation of HSA

10 mg/mL of acetylated HSA orwild-type HSA proteins were solubilised in 10 mM sodium phosphate buffer (pH 7.4) with 50 mM NaCl. For inducing the amyloid-like aggregation, HSA or acetylated HSA was incubated at 65°C up to 50 h without shaking. After the 24 h or 50 h of incubation, assays to assess the formation of amyloid-like aggregates were performed[28].

Turbidity assay

Turbidity as a measure of protein aggregation was assessed by recording absorbance of the HSA samples at 350 nm at various time-points of incubation from 0 to 50 h[29].

Thioflavin-T binding assay

Thioflavin-T (ThT) dye is known to bind selectively to amyloid aggregates which significantly alters its fluorescence properties[30]. 70 µL of aggregated sample was added with 2 µL of ThT (stock concentration: 30 mM) and ThT fluorescence spectra were recorded in Perkin Elmer Enspire multimode plate reader. The ThT fluorescence excitation spectra were recorded from 250-460 nm wavelengths by fixing the emission at 495 nm. Then, the emission spectra were recorded from the wavelength 460-600 nm after fixing the excitation wavelength at 450 nm.

Visualization of Thioflavin-T-positive aggregates by fluorescence microscopy

For visualization of the ThT-stained HSA or acetylated HSA protein aggregates, 10 µL of HSA or acetylated HSA samples incubated at 65°C for 24 h or 50 h were observed under 10x objective lens using the GFP filter of Leica DM2500 fluorescence microscope[31]. Fluorescence images were further processed for colour using the ImageJ software[32].

Far-UV CD spectroscopy

Proteins undergo changes to β-sheet rich structural conformation upon amyloid-like aggregation[17,29]. To analyze the secondary structure of HSA or acetylated HSA before and after aggregation, the samples were transferred to a quartz CD cuvette of path length 2 mm and the far-UV CD spectra were recorded from wavelength 260-190 nm using Jasco 1500 spectropolarimeter. Freshly prepared HSA and acetylated HSA monomers dissolved in the aggregation buffer or their aggregated versions after 50 h incubation were used to record far-UV CD spectra. The results were expressed as mean residue ellipticity $[\Theta]_{MRE}$. Next, relative secondary structural

contents were estimated using the online secondary structure prediction server Dichro Web using the algorithm CONTIN and the set SP175 optimized for the 190-240 nm range[33].

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)

Aggregations of prion and amyloid proteins confer stability against disaggregation by ionic detergents such as SDS and sarkosyl[18,25,34,35]. For examining their stability against sarkosyl, HSA and acetylated HSA aggregates were first treated with 1% sarkosyl for 10 min at room temperature and then they were mixed with non-reducing Laemmli sample buffer devoid of any detergent followed by electrophoresis on 1% agarose gel. Then, the agarose gel was stained using Coomassie dye for visualization of the protein monomers and their aggregates.

Transmission electron microscopy (TEM)

A high-resolution transmission electron microscope (JEOL, JEM 2100, U.S.A), operated at 200 kV was used to acquire the electron micrographs of HSA and acetylated HSA protein aggregates. The protein aggregate suspension was dropped on a Holey carbon-coated grid and then air dried for 10 min. Then, the protein sample was negatively stained for 20s with 2% uranyl acetate and allowed to dry overnight before capturing the TEM images.

Results and Discussion

Acetylation of HSA affects its amyloid-like aggregation

For examining the effect of acetylation on the amyloid aggregation of HSA, we first acetylated HSA by incubating HSA with aspirin at a molar ratio of 1:1.5 (protein: aspirin) for 24 h at 37°C and removed the excess of unbound aspirin by dialysis[36]. After dialysis, the HSA and acetylated-HSA proteins were examined using 10% SDS PAGE under both reducing and non-reducing conditions. We found that the acetylated HSA retained structural integrity similar to that of the un-acetylated HSA protein as both forms of the protein showed similar slower mobilities under the reducing conditions compared to the non-reducing conditions which suggests of opening of their structures in presence of a reducing agent *via* the reduction of their disulfide bonds (Fig. 1A). Notably, the HSA protein contains 17 disulfide bridges in its structure.

To study the effect of the lysine acetylation on the amyloidogenicity of HSA, we incubated the acetylated HSA at the optimal aggregation temperature of 65°C for 50 h and then examined the formation of amyloid-like aggregates. For this, we first examined the turbidity of the acetylated and non-acetylated HSA aggregates which can report on their aggregation status. The turbidity of the HSA aggregates, as recorded by absorbance at 350 nm, was higher than the aggregates of the acetylated HSA both at 24 h and 50 h of incubations (Fig. 1B). Next, we examined the aggregation status of HSA and acetylated HSA samples by recording the emission and excitation spectra of the amyloid binding dye, thioflavin T (ThT) upon addition to these protein samples. When excited at 450 nm, the HSA sample showed increased ThT emission fluorescence at 495 nm thereby indicating the formation of amyloid-like aggregates (Fig. 1C). In comparison to the HSA sample, the ThT fluorescence emission at 495 nm was significantly reduced in the acetylated HSA sample (Fig. 1C). Likewise, the acetylated HSA sample also manifested lesser fluorescence intensity at 450 nm in the ThT excitation spectra (Fig. 1C). Taken together, the data suggest that acetylation decreases the amyloid-like aggregation of HSA.

Acetylated HSA forms smaller thioflavin-T-positive aggregates with diminished detergent stability

Next, we examined the ThT-stained aggregates of HSA and acetylated HSA under the GFP filter of the fluorescence microscope to visualize the ThT-positive fluorescent protein speckles as an indication for amyloid-like aggregation[31]. For this, samples of the acetylated and non-acetylated HSA proteins after incubation at 65°C in aggregating conditions, were transferred on a glass slide and images were taken using 10x objective lens under GFP filter. After 24 h of incubation, we were able to detect green fluorescent speckles in the HSA samples where the majority of the species frequently observed were with irregular margins and relatively bigger sizes. On the contrary, in the acetylated HSA sample, we observed relatively lesser number of ThT-positive speckles which were also of relatively smaller sizes (Fig. 2). After 50 h of incubation, an increase in the sizes of the aggregates in both the HSA and acetylated HSA samples were observed. However, the HSA samples manifested clustered and relatively bigger size fluorescent speckles compared to the acetylated HSA samples. This data is consistent with the observed reduction in the intensity of the ThT emission and excitation fluorescence for the acetylated HSA

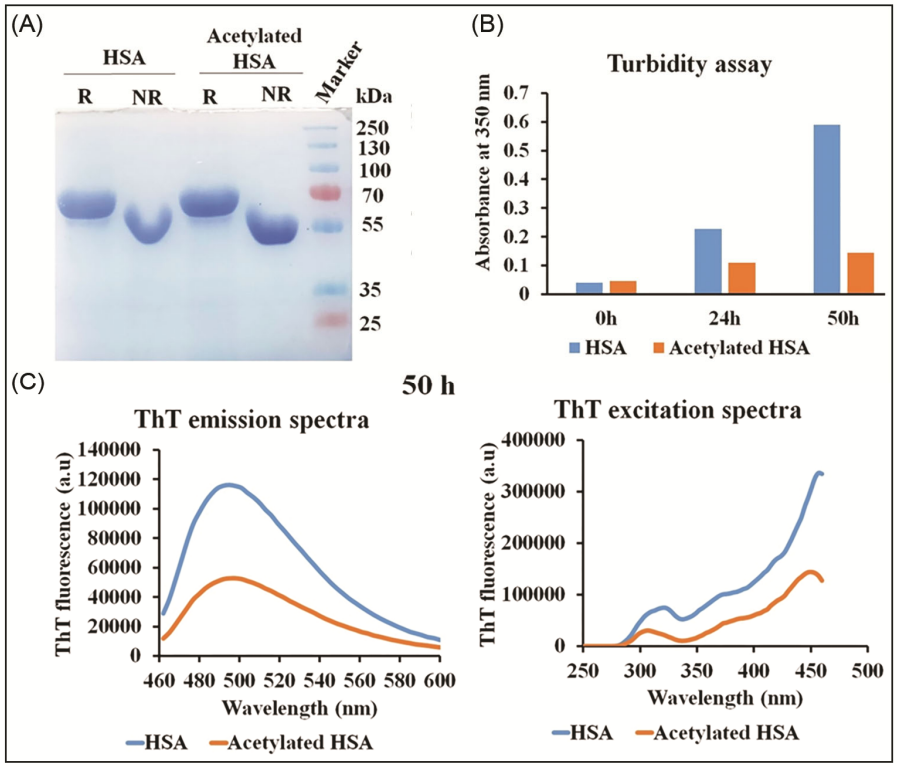

Fig. 1 — Acetylation of HSA affects its amyloid-like aggregation. (A) 10% SDS-PAGE of HSA and acetylated HSA monomers was performed under reducing (R) and non-reducing (NR) conditions; (B) The turbidity of the HSA and acetylated-HSA samples incubated under aggregation conditions was examined by measuring absorbance at 350 nm; (C) Amyloid-aggregation in HSA and acetylated HSA was induced by incubation for 50 h at 65°C followed by addition of thioflavin-T (ThT) dye before recording the ThT emission spectra (excitation: 450 nm) and the ThT excitation spectra (emission: 495 nm)

relative to the non-acetylated HSA (Fig. 1C) and supports that acetylation mitigates the amyloidogenicity of HSA.

Amyloid protein aggregates are known to show enhanced stability against ionic detergents including SDS and sarkosyl[18,25,34,35]. Previously, the aggregates of HSA were also found to display stability to sarkosyl[25]. Therefore, we examined the relative stabilities of HSA and acetylated HSA amyloid-like aggregates in presence of sarkosyl at room temperature using semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)which can analyze protein aggregates of even more than 3000 kDa molecular weight[18,25,34,35]. The non-aggregated samples of both the HSA and acetylated HSA, as expected, displayed only monomers when analyzed on SDD-AGE using 1% sarkosyl treatment at room temperature and electrophoresis on 1% agarose gel. Consistent with our previous observations[25], the HSA aggregates retained larger sizes and majority of the protein failed to enter the gel and was retained in the loading well thereby suggesting presence of very high molecular weight 1% sarkosyl-resistant aggregates. In addition, a fraction of HSA aggregates were broken to high molecular weight aggregates that were electrophoresed in the gel as faint smear thereby also suggesting that the HSA aggregates are resistant to complete disaggregation by 1% sarkosyl treatment at room temperature (Fig. 3). In contrast, we observed that the acetylated HSA aggregates were not trapped in the loading well and rather were able to enter the gel and showed some sarkosyl-stable aggregates of moderate sizes in addition to manifesting a prominent monomeric protein band which suggests of relatively weaker stability to the 1% sarkosyl treatment as compared to the HSA aggregates (Fig. 3). Taken together, the data support that the acetylation of HSA causes a decrease in the ionic detergent stability of the *in vitro*-made aggregates thereby indicating a mitigation of the amyloid nature of the acetylated HSA aggregates.

Acetylated HSA aggregates manifest relatively lesser β-sheet content

As amyloid-like aggregations of several proteins cause transformations in their secondary structures to

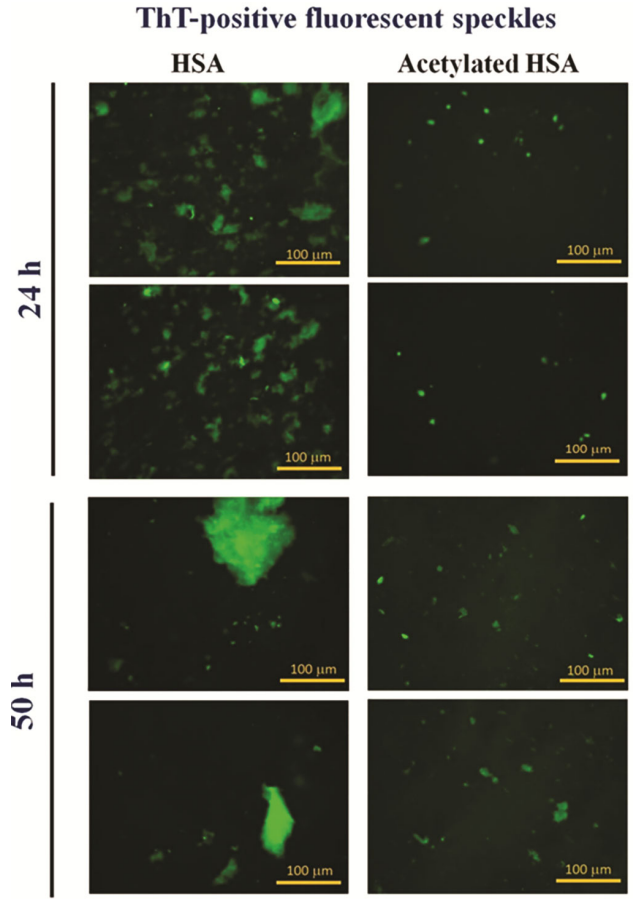

Fig. 2 — Fluorescence micrographs of thioflavin-T-positive speckles of HSA and acetylated HSA aggregates. Thioflavin-T (ThT)-positive amyloid-like aggregates were examined using GFP filter of a fluorescence microscope under 10x objective lens after 24 h or 50 h of incubation of the HSA and acetylated HSA samples under aggregating conditions. The sizes of the ThT-positive speckles in the aggregated HSA samples are comparatively bigger than those from the aggregated acetylated HSA samples

a predominantly β-sheet rich conformation[16,17,35], therefore by recording the far-UV CD spectra we next analysed if the aggregates of HSA and the acetylated HSA differ in their relative β-sheet contents. Similar to the previous reports, the far-UV CD spectrum of the monomeric HSA showed two characteristic negative peaks at 208 nm and 222 nm thereby indicating a predominantly alpha helical secondary structure[37] (Fig. 4A). The far-UV CD spectrum of the monomeric acetylated HSA also displayed two characteristic peaks at 208 and 222 nm and a similar overall shape but with altered amplitude compared to the non-acetylated HSA far-UV CD spectrum (Fig. 4A). The far-UV CD spectra of the aggregated HSA and the aggregated acetylated HSA manifested differences in the trend compared to their respective

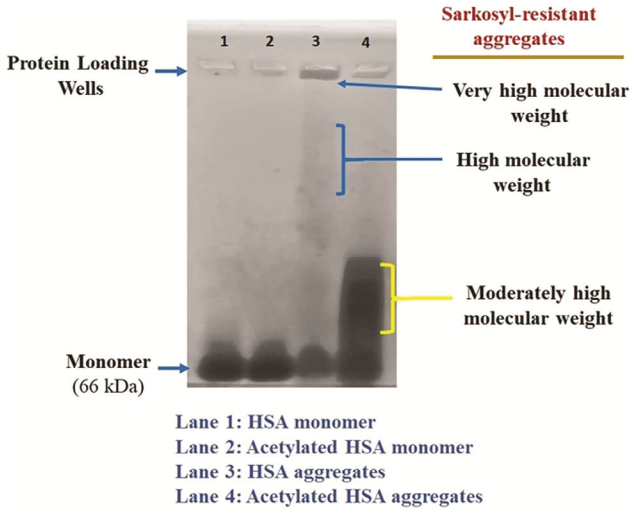

Fig. 3 — Assessment of detergent stabilities of the HSA and acetylated HSA aggregates by SDD-AGE. Relative stability of the pre-formed aggregates of the HSA and acetylated HSA proteins against the ionic detergent sarkosyl was assessed by using semi denaturing detergent agarose gel electrophoresis (SDD–AGE). The samples were treated with 1% sarkosyl for 10 min at room temperature and then electrophoresed on 1% agarose gel and visualized for protein by Coomassie staining. Lane 1: HSA monomer (70 µg protein); Lane 2: Acetylated HSA monomer (70 µg protein); Lane 3: HSA amyloid (240 µg protein); Lane 4: Acetylated HSA amyloid (240 µg protein)

monomeric proteins. The shape of the aggregated HSA far-UV CD spectrum was observed to be similar to as reported previously[37]. Notably, there was a marked difference between the shapes of the far-UV CD spectra of the aggregated HSA and the aggregated acetylated HSA samples (Fig. 4A). Furthermore, when these spectra were used for secondary structural predictions using Dichro Web, the relative contents of the α-helix and β-sheet in the monomeric HSA and the monomeric acetylated HSA were found to be different indicating alterations in the protein conformation upon acetylation (Fig. 4B). Acetylation of lysine residues in proteins can cause the net charge on the protein molecule to be more negative and charge-charge repulsions can affect the folding and structure of an acetylated protein. The observed difference in the secondary structural content of the acetylated HSA relative to the non-acetylated HSA suggest some perturbation in the charge balance in the protein structure upon acetylation. Notably, the aggregates of HSA manifested relatively higher percentage of β-sheet secondary structure when compared with the aggregates of the acetylated HSA thereby suggesting that acetylation reduces the amyloidogenicity of HSA (Fig. 4B).

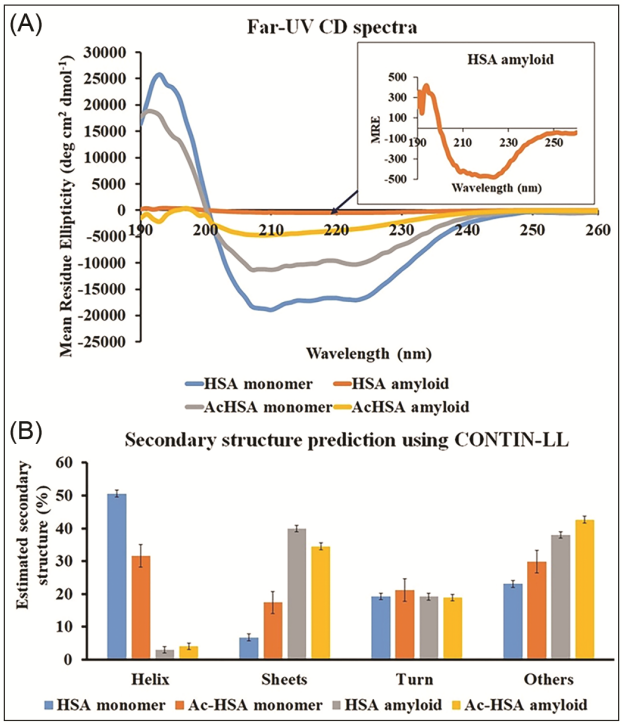

Fig. 4 — Far-UV CD spectra of monomeric and aggregated HSA and acetylated HSA. (A) Far-UV CD spectra of the monomeric HSA and the acetylated HSA freshly solubilized in the aggregation buffer were recorded immediately and the results have been depicted in terms of mean residue ellipticity $[\Theta]_{MRE}$. Pre-formed HSA and the acetylated HSA aggregates from the 50 h of incubation under aggregating conditions, were diluted in the aggregation buffer and their far-UV CD spectra were recorded. The far-UV CD spectrum of the HSA amyloid has been magnified in the inset to clearly show the shape of the spectrum;(B) Secondary structure predictions using Dichro Web prediction tool from the far-UV CD spectra from panel A are shown. Error bars represent standard deviation (n=3)

TEM images manifest smaller and some with globular morphology acetylated HSA aggregates

Transmission electron microscopy (TEM) has been widely used for studying the morphological and structural features of the amyloid fibrils[38]. Thus, we examined if the aggregates of HSA and acetylated HSA differ in their morphological features. For this, the HSA and the acetylated HSA samples incubated for 24 h under optimal temperature of 65°C were examined under TEM after negative staining with uranyl acetate. Consistent with the earlier reports, we observed elongated and fibrillar morphology in the aggregated HSA sample thereby confirming of its amyloid-like aggregation (Fig. 5)[26]. In contrast, when we examined the acetylated samples in TEM, the aggregates were sparse, lacked fibrillar morphology and were significantly smaller in dimensions (Fig. 5).

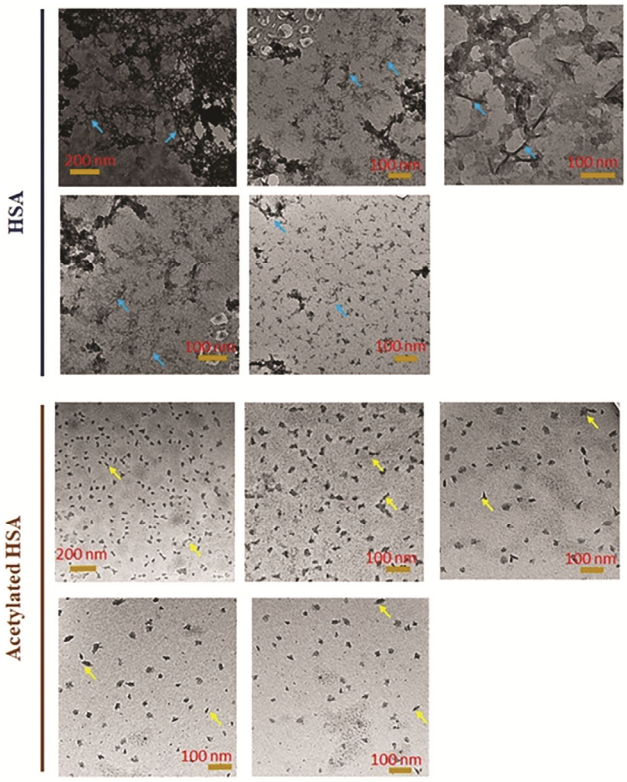

Fig. 5 — TEM micrographs of HSA and acetylated HSA aggregates. Amyloid aggregations of HSA and acetylated HSA were induced by incubating at 65 °C for 24 h and the samples were examined under TEM. The TEM images of the HSA amyloid aggregates negatively stained with uranyl acetate show larger aggregate with fibrillar morphology (indicated by blue arrows). In contrast, the acetylated HSA sample lacks fibrillar species and rather it manifests aggregates that are short, irregular and raft-like (indicated by yellow arrows)

Taken together, the TEM imaging data confirm that acetylation modulates and decreases the *in vitro* amyloidogenicity of the HSA protein. This decrease in the amyloidogenicity of the acetylated HSA may be due to the structural changes in the monomeric protein upon acetylation, as revealed by the far-UV CD spectroscopy, that may make it less aggregation-competent or due to the unmasking of the negative charges upon the neutralization of the positive charge on the lysine residue after the acetylation of its side-chain which would cause inter-molecular repulsions during the amyloid aggregation or due to a combination of both of these effects. This study adds to the widely ongoing *in vitro* and *in vivo* studies on model proteins for structure-function and role in diseases[39,40].

Conclusion

In physiological conditions, the microenvironment can cause considerable changes in the native structure

of HSA. As HSA acts as a drug-carrier, examining the effects of drug molecules on its structure or its propensity to aggregate are important towards its applications. Earlier, HSA has been shown to be acetylated in patients taking the aspirin drug and HSA can also get acetylated *in vitro*. In the current study, we examined the effect of acetylation on the amyloidogenicity of HSA and found that the acetylation of HSA can reduce the *in vitro* amyloid-like aggregation of HSA as evident by the lesser extent of thioflavin-T binding, lesser β-sheet content, reduced ionic detergent stability and relatively smaller size aggregates as visualized by TEM imaging.

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Conflict of interest

All authors declare no conflict of interest.

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