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New insights into in vitro amyloidogenic properties of human serum albumin suggest considerations for therapeutic precautions

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ABSTRACT

Amyloid aggregates display striking features of detergent stability and self-seeding. Human serum albumin (HSA), a preferred drug-carrier molecule, can also aggregate in vitro. So far, key amyloid properties of stability against ionic detergents and self-seeding, are unclear for HSA aggregates. Precautions against amyloid contamination would be required if HSA aggregates were self-seeding. Here, we show that HSA aggregates display detergent sarkosyl stability and have self-seeding potential. HSA dimer is preferable for clinical applications due to its longer retention in circulation and lesser oedema owing to its larger molecular size. Here, HSA was homodimerized *via* free cysteine-34, without any potentially immunogenic cross-linkers that are usually pre-requisite for homodimerization. Alike the monomer, HSA dimers also aggregated as amyloid, necessitating precautions while using for therapeutics.

1. Introduction

Protein mis-folding results in many human diseases some of which are caused when it leads to amyloid aggregate formation and deposition [1,2]. Amyloids are insoluble, fibrous and well organized protein aggregates having a largely cross-β core structure [1,3]. Some examples of amyloid associated diseases include: Alzheimer's disease, Prion disease, and Huntington's disease, where $A\beta_{42}$ peptide, PrP^{C} protein and mutant huntingtin proteins, are respectively found to be amyloid aggregated and deposited in nervous system [1]. Several proteins from different organisms which are not found, thus far, to cause disease or toxicity, can also form amyloid aggregates in vivo (e.g. mammalian Pmel17 protein, yeast Sup35 & Cyc8 protein, bacterial Curlin protein) or in vitro (Human serum albumin, Bovine serum albumin, endocrine hormones such as ACTH & Oxytocin) [4–8].

Human serum albumin (HSA) which is the most abundant protein in blood plasma, can form amyloid-like aggregates in vitro [6,9]. HSA is an all-α helix, globular protein made of a single peptide chain of 585 amino acids with one free sulfhydryl group at cysteine-34 [10]. It has multiple physiological roles like: as a nutrients transporter & carrier protein in blood for several compounds (e.g.: fatty acids, amino acids, bile salts, metals and hormones etc.) and as a regulator for blood pH & osmotic pressure [11–13]. It has also found applications as a safe and non-immunogenic drug carrier and as a versatile plasma expander, as its administration is considered to be helpful in the treatment of severe hypoalbuminemia during burns, nephritic syndrome, chronic liver cirrhosis and haemorrhagic shocks [14]. Notably, HSA dimers are preferable as a plasma expander as well as a drug carrier due to their increased circulatory life and alleviating effect on the edema associated with unwanted extravasation of the monomeric HSA [14,15].

HSA is found to form amyloid-like aggregates in vitro at physiological pH 7.4 and also at acidic pH but only at elevated temperatures, when present in aqueous solvents [6]. HSA aggregation has been reported at physiological pH at lower temperatures also (25 °C, 37 °C, 65 °C) however in presence of organic solvent ethanol [9,16,17]. HSA aggregates display varying morphologies under different aggregation conditions, possibly due to formation of different structural intermediates during the aggregation pathway

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[6,9]. Unlike a typical amyloid aggregation trend, which follows a lag dependent sigmoidal kinetics, there is absence of any apparent lag period in HSA aggregation [9,18]. Although HSA aggregates conform to several amyloid aggregate-associated properties such as thioflavin-T (ThT) dye binding and Congo red binding, certain important properties such as the self-seeding ability and detergent stability have not been established yet. Amyloid self-seeding is usually assayed in vitro and monitored as decrease in lag period of aggregation, suggesting acceleration of conversion of monomers to aggregates [19]. If HSA aggregates exhibited self-seeding at physiological conditions, any unwanted HSA amyloid contamination in samples used for therapeutics, could potentially seed the circulating HSA monomers into amyloid form, which would be hazardous.

Here we first converted HSA into aggregates exhibiting amyloid-like ThT dye binding and then we examined if the aggregates display amyloid-like detergent stability. Furthermore, we examined self-seeding behavior of HSA aggregates at sub-optimal aggregation temperature, in the view that few previous attempts at optimal aggregation conditions were inconclusive, possibly due to lack of a discernible lag period in aggregation [9,20]. Due to buried nature of the free cysteine-34 in HSA structure, HSA dimers employed for clinical applications have often been obtained using chemical cross-linkers of cysteine, which may potentially be immunogenic [14]. Here we succeeded in homodimerizing HSA *via* disulfide linkage using denaturation in urea and hydrogen peroxide (H_2O_2) induced oxidation. We then examined if HSA homodimers retain the amyloidogenic properties similar to the HSA monomers.

2. Materials and methods

2.1. Materials

Recombinant HSA expressed in *Saccharomyces cerevisiae*, Congo red, Thioflavin-T, N-Lauroylsarcosine sodium salt (sarkosyl), Sodium dodecyl sulfate (SDS), Hydrogen peroxide, 5,5′-Dithio-bis (2-Nitrobenzoic acid) (DTNB, Ellman's reagent) and dialysis membrane were purchased from Sigma–aldrich, USA. Polyvinylidene fluoride membrane (PVDF) and Superose 6, 10/300 GL high performance gel filtration column was obtained from GE Healthcare, USA. Urea, Sodium chloride, Sodium phosphate dibasic and monobasic were purchased from Himedia, India. All other chemicals were also of high purity.

2.2. Amyloid aggregation and detection

HSA protein was diluted in aggregation buffer (10 mM sodium phosphate buffer pH 7.4 containing 50 mM NaCl) to a final concentration of 20 mg/ml and incubated without agitation at 65 °C for 52 h in a water bath to induce aggregation as reported previously [6,9]. Amyloid aggregate formation was assessed by ThT and Congo red binding assays as described below.

2.2.1. Thioflavin-T binding assay

ThT dye has been reported to bind specifically to amyloid aggregates which changes its fluorescence properties significantly [21]. HSA aggregates (20 mg/ml) were mixed with ThT solution (2 mM final) and extrinsic fluorescence emission spectrum of ThT was monitored by excitation at 442 nm and recording emission from 460 nm to 560 nm. An enhanced ThT fluorescence emission intensity at 485–490 nm usually signifies its binding to amyloid-like conformations [21]. Excitation spectra of ThT, after addition to aggregates, were also recorded between 250 and 455 nm after fixing the emission to 495 nm [21].

2.2.2. Congo red binding assay

The dye Congo red can bind to amyloid aggregates with high specificity, which can manifest as a red shift in its absorption maximum (λ_{max}) from 480 nm to 540 nm or increase in absorption intensity in 480–540 nm range [22]. To examine amyloid nature of HSA aggregates, Congo red dye was solubilized in phosphate buffered saline, pH 7.4 and then mixed with ~150 μM HSA aggregates in 1:1 M ratio. The mixture was incubated for 30 min at room temperature and Congo red absorption was recorded from 300 nm to 700 nm [22].

2.3. Semi-denaturing detergent agarose gel electrophoresis (SDD–AGE)

Several amyloid and prion aggregates have been shown to exhibit stability against dis-aggregation by ionic detergents like SDS or sarkosyl (~1–2%), when tested at room temperature [19,23,24]. Amyloid-like detergent stability of the HSA aggregates was examined by SDD–AGE [25]. Briefly, the HSA aggregates were first incubated with 2% sarkosyl at room temperature for 10 min and then mixed with non-reducing Laemmli sample buffer lacking detergent and electrophoresed on 1.5% agarose gel. Protein from the gel was electro-blotted to PVDF membrane and visualized by Coommassie staining [25,26].

2.4. Amyloid seeding

Many amyloid aggregates exhibit lag depended sigmoidal kinetics of formation [18]. Upon addition of pre-formed aggregates (seed), the lag in aggregation can either shorten or completely disappear due to a process termed 'self-seeding', where the monomers are induced to aggregate with rapidity by the seed [27,28]. HSA aggregates (5%) pre-formed at 65 °C were briefly sonicated to break the clumps and added to monomeric HSA (20 mg/ml) dissolved in aggregation buffer of pH 7.4 containing 2 mM ThT. This mixture was then incubated with intermittent shaking at 50 °C for 96 h in Spectramax-M5^e multimode microplate reader (Molecular Devices) and amyloid conversion of the monomer was monitored by recording ThT fluorescence. Aggregation kinetics of an HSA sample incubated without any pre-formed seed was used for comparison. A sample containing only the 5% sonicated seed and lacking any HSA monomer was used as a control.

2.5. Transmission electron microscopy

The electron micrographs of HSA aggregates were acquired using a high-resolution transmission electron microscope (FEI Tecnai, T20G2), operated at 200 kV. A drop of the suspension was placed on a carbon-coated copper grid, dried in desiccator and negatively stained with 0.2% phosphotungstic acid (PTA) for 2 min, before imaging.

2.6. Atomic force microscopy

Atomic force microscopy (AFM) of HSA aggregates obtained from cysteine-linked homo dimers was performed by drop-casting 30-fold diluted aggregates on a freshly cleaved mica sheet. Multimode scanning probe microscope from Veeco Instrument Inc., USA equipped with a Nanoscope IV controller and AFM probe model – Tap190Al (tip radius: ~10 nm, & tip height: 17 μm) purchased from Budget Sensors, were used in tapping-mode at ambient conditions to scan the sample. The cantilever had a resonant frequency of ca. 162 kHz and nominal spring constant of ca. 48 N/m with a 30 nm thick aluminum reflex coating on the back side of the cantilever of the length 225 μm. Imaging was carried out at a rate of 1 Hz and at a resolution of 512 × 512 pixels.

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