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# Fibrillogenesis of human serum albumin in the presence of levodopa – spectroscopic, calorimetric and microscopic studies

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ABSTRACT

Studying amyloid associated neurodegenerative diseases is an active area of research. Cure for these diseases are still to be discovered. In the present study we have performed comprehensive biophysical and computational experiments showing levodopa not only significantly inhibits heat induced fibrillization of human serum albumin but also disaggregates preformed fibrils. Thioflavin T (ThT) binding assay was used to monitor the fibrillation process of human serum albumin (HSA) at 65 °C in the presence and absence of levodopa. Binding of levodopa was studied using isothermal titration calorimetry (ITC), binding constant was found to be $3.6 \times 10^3$ $M^{-1}$. Thermal stabilization effect of levodopa on HSA was studied using differential scanning calorimetry (DSC). Microscopic imaging techniques were employed to analyze the morphology of aggregates and effect of levodopa on aggregation. Further, molecular docking study was also utilized to decipher the amino acid residues involved in the binding interaction of levodopa with HSA. Levodopa interferes in the Fibrillogenesis of HSA by interacting with the amino acid residues near to drug binding site II on the HSA with the binding constant of the order of $10^3$ and stabilizes the protein. The results are indicative of the potential use of levodopa as a therapeutic agent for the treatment of amyloid diseases.



## 1. Introduction

There are several human diseases that have been identified to be caused by protein deposition both extracellularly and intracellularly [1–3]. These diseases are caused by formation of insoluble amyloids in tissues [4]. The amyloid species are highly ordered and stable, they are not easily cleared off from the system. Amyloids are toxic species. They have direct toxicity through the deposition of huge masses of amyloids that interfere with cell functioning leading to cell death due to macromolecular overloading. Indirect toxicity arises when amyloids trigger a series of lethal events leading to cell death. They interact with membranes leading to permeabilization and cell damage [5,6]. Some important protein deposition diseases include Alzhimers, Parkinsons Type II diabetes and systemic amyloidosis [7]. Neurodegeneration is an important implication of cell death caused by aggregation of proteins as regeneration of neuronal cell function is minimal [8]. Formation of amyloids has been found to be the generic property of polypeptides as proteins that are not associated with any pathophysiological state may also form amyloids when subjected to suitable favoring conditions in vitro [9,10]. Irrespective of the source, amino acid sequence and tertiary structure amyloids have common tinctorial, structural and toxicity properties which suggest the common mode of generation of amyloids [11] that can be targeted for the development of possible common therapeutic agents against amyloid diseases. The identification of small molecules that can attenuate the fibrillation process or disassemble the mature fibrils is an important step in the pharmaceutical development program for aggregation diseases [12–15].

Levodopa is a naturally occurring form of dihydroxy phenylalanine [16]. In humans Levodopa is immediate precursor of dopamine

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[17]. Levodopa can cross the protective blood–brain barrier [18], whereas dopamine could not perform this function. Consequently, in the treatment of Parkinson's disease, levodopa is used to increase the dopamine concentration [19]. Once levodopa has entered the central nervous system, it is converted into dopamine by the enzyme, dopadecarboxylase [20]. Vitamin B is a required cofactor in this reaction [21]. Levodopa has shown to have anti-oxidant properties [22]. Interestingly this compound is also used in attention deficit hyperactivity disorder (ADHD) by improving the sleep disorder and in the treatment of anxiety [23].

Human serum albumin (HSA) is the protein present in the highest concentration in human plasma at normal concentrations between 35 and 50 g/l. HSA has 585 amino acids and is monomeric polypeptide [24]. Although HSA is very stable but it do forms amyloids under favorable conditions. It is the intrinsic property of polypeptide to form aggregates under physicochemical conditions that endorse intermolecular protein association in specific conformation forming amyloids [25]. HSA is widely studied protein and is well characterized as an important carrier molecule. HSA serves as an important model to study protein ligand interactions [26–28]. HSA can be employed as an important system to study amyloid formation and inhibition by small molecules. The HSA aggregation inhibition may serve as important tool in designing anti-amyloidogenic drugs.

## 2. Materials and methods

Human serum albumin (HSA), Thioflavin T (ThT), 1-anilino 8 naphthalene sulphate (ANS), Levodopa of highest purity available were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade. Double distilled water was used throughout the study.

### 2.1. Sample preparation

The stock solution of HSA (100 μM) was prepared in 20 mM sodium phosphate buffer of pH 7.4. The concentration of the HSA was determined spectrophotometerically by a UV–vis spectrophotometer (Perkin Elmer Lambda 25) at 280 nm using $E^{1\%}_{280nm} = 5.3$ for HSA[29]. For the preparation of amyloids, 100 μM HSA solution was incubated at 65 °C in the presence of 50 mM NaCl for 120 h in a circulating shaking water bath [30]. For rest of the studies, HSA was diluted to 10 μM with the same conditions. The stock solution of Levodopa was prepared at the concentration of 2 mg/ml in distilled water.

### 2.2. ThT fluorescence spectroscopic assay

ThT fluorescence measurements were recorded on Shimadzu 5301PC fluorescence spectrophotometer equipped with water circulator (Julabo Eyela). The spectra were recorded from 450 to 600 nm and excitation at 440 nm. The stock solution of ThT was prepared in double distilled water and filtered through 0.2 μm Millipore filter. ThT concentration was calculated using $\varepsilon^{1\%} = 36{,}000\ M^{-1}\ cm^{-1}$ at 412 nm [31]. The excitation and emission slit widths were set at 5 nm. The protein samples (10 μM), in the absence and presence of different concentrations of levodopa (20 μM, 50 μM and 100 μM), were withdrawn at definite time intervals and mixed with ThT so as to obtain ThT to protein molar ratio of 1:1 and the samples were stored for 30 min in the dark at room temperature before taking the readings. All the measured spectra were subtracted from their appropriate blanks. All curves were fitted as we have described previously [32].

### 2.3. ANS fluorescence assay

ANS solution was prepared in distilled water as described previously [33]. HSA samples (10 μM) in the absence and presence of levodopa (100 μM) incubated for 120 h at 65 °C were mixed with 50 fold molar excess of ANS and then the mixtures were kept in dark for 30 min at room temperature. The ANS fluorescence measurements were performed on Shimadzu RF-5301 PC fluorescence spectrophotometer equipped with water circulator (Julabo Eyela). The excitation wavelength for ANS fluorescence was set at 380 nm and the emission spectra were recorded from 400 to 600 nm. Both excitation and emission slits were set at 5 nm.

### 2.4. Circular dichroic measurements in far UV range

Far-UV CD measurements were performed on a JASCO spectropolarimeter (J-815) with a thermostatically controlled cell holder attached to a Peltier with multitech water circulator, at 25 °C, in a cuvette of 0.1 cm path length. The scan speed and response time were set as 20 nm/min and 2 s, respectively. Each spectrum was an average of three scans. The experiments were carried out with HSA (10 μM) in the presence and absence of levodopa (100 μM) incubated at 65 °C for 120 h.

### 2.5. Dynamic light scattering (DLS) measurements

The changes in the aggregation behavior of HSA in the presence and absence of levodopa were determined using DLS. The hydrodynamic radii $R_h$ measurements were performed using a protein concentration of 10 μM at 830 nm on a DynaPro-TC-04 (Wyatt Corporation) instrument, equipped with a temperature controlled microsampler. The wavelength of 830 nm was chosen to obtain optimum scattering and it is good for fluorescence suppression. All the solutions were filtered through 0.22 μm pore size micro-filters (Whatman International, Maidstone, UK). Data were analyzed by Dynamics 610.0.10 software provided with the instrument. The mean hydrodynamic radius ($R_h$) and polydispersity index (Pd%) were estimated on the basis of an autocorrelation analysis of scattered light intensity based on the translational diffusion coefficient, using the Stokes–Einstein equation [34]:

$$R_h = KT/6\pi\eta D_W{}^{25\,^\circ C} \quad (1)$$

where, $R_h$ is the hydrodynamic radius (nm), k is the Boltzmann's constant, T is the absolute temperature (K), η is the viscosity of water and $D_W{}^{25\,^\circ C}$ is the translational diffusion coefficient.

### 2.6. Transmission electron microscopy

TEM images were taken using a Philips CM-10 transmission electron microscope operating at an accelerating voltage of 200 kV. The amyloid fibril formation was assessed by applying 6 μL of HSA (10 μM) incubated at 65 °C for 120hr in the absence and presence of levodopa on a 200-mesh copper grid covered by a carbon-stabilized formvar film. Extra fluid was evacuated after 2 min and the grids were then negatively stained with 2% (w/v) uranyl acetate. All samples were incubated overnight and images were viewed at 10,000×.

### 2.7. Differential scanning calorimetry

DSC measurements were carried out using VP-DSC micro calorimeter (Micro Cal, Northampton, MA). The buffer and protein solutions were degassed under mild vacuum prior to the experiment. Samples were prepared in 20 mM sodium phosphate buffer, pH 7.4. The DSC measurements of HSA (10 μM) in the presence of 1:10 ratio of levodopa were performed from 25 to 90 °C at a scan rate of 0.5 °C/min. Data was analyzed using Origin software