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Rapid estimation of the β-sheet content of Human Serum Albumin from the drying patterns of HSA-nanoparticle droplets

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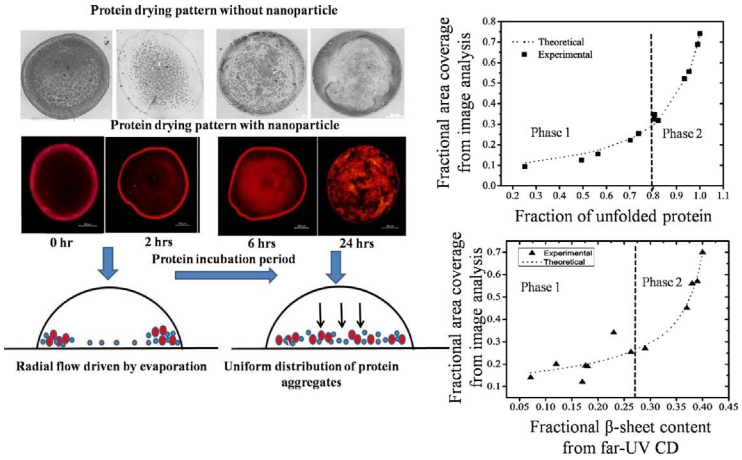
GRAPHICAL ABSTRACT



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ABSTRACT

The formation of specific patterns during evaporation of biofluids e.g. blood, serum etc. can be analyzed for quick identification of several diseases. Human Serum Albumin (HSA) is frequently used as the model protein to study amyloid fibril formation that is responsible for numerous neurodegenerative diseases like Parkinson's disease, Alzheimer's disease etc. In the present study the fibril growth of HSA is characterized by analyzing the drying patterns of the protein solution. The fibril formation of HSA is confirmed by spectrofluorimetry and the β-sheet content is quantified by far-UV Circular Dichroism spectrometry. Examinations of the drying patterns of HSA, in presence of fluorescent nanoparticles, reveals distinctive pattern transformations with increase in the β-sheet content visualized through fluorescence microscopy and FESEM. The fluorescence images of the dried droplet are analyzed using image processing by the color thresholding method. A rapid quantification methodology of the β-sheet content of HSA and the fraction of unfolded protein has been developed by comparing the results of the image analysis with those of the far-UV CD data.

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1. Introduction

Pattern formation during evaporation of suspended colloidal solutions has intrigued numerous disciplines of scientific research. The commonly observed patterns in a dried drop of coffee, known as the "coffee ring effect", has been extensively studied, starting with the pioneering work by Deegan et al. [1]. A ring of suspended particles is produced due to the contact line pinning and subsequent radial outward flow of the interior liquid due to the rapid evaporation of the liquid from the edge [2,3]. In addition to the ring-like structures, a variety of other patterns including spherical or hemispherical cap structures [4,5], pillar-like structures [6], hexagonal and fingering pattern [7,8] have also been observed. The ring structure and patterning is influenced by a variety of factors such as the nature of the constituent particles, that of the solvents, size of the particles, rate of evaporation, volume of the droplet [9], phase transition of solute, relative humidity [10], substrate hydrophobicity [11], etc.

In biomedical applications, the drying patterns of biological fluids, such as, blood, serum [12], tear drops [13–15] are increasingly becoming important tools for disease diagnosis. Based on the coffee ring effect, Brutin et al. first reported regular distinguishable patterns of whole blood drops during evaporation for healthy persons as well as for anemic and hyperlipidaemic persons [16]. The final dried pattern of the blood drop is characterized by the presence of three distinct regions: a central sticking deposit with a network of small cracks, a corona of wide mobile plaques with radially distributed cracks and a fine periphery completely adhering to the substrate. It has been observed that this pattern formation of a dried blood drop depends on various physical conditions like relative humidity (RH), contact angle of the drying droplet etc. [10]. Due to the effect of RH on the contact angle of the blood droplet, the initial evaporation rate is dependent on the RH values. It has been reported that the width of mobile plaques of the corona and the fine periphery region become larger as the RH increases. Moreover, the mobile plaques of the corona are markedly displaced toward the center of the drop leaving a wider deposit consisting of plaques with dark cracks. The evaporation rate at different RH levels strongly influences the final pattern of the drying drop of blood. The drying pattern influenced by biomolecular interaction has been observed by Hurth et al. by using biotin- or streptavidin-coated fluorescent particles. A mechanistic model has been proposed to prove that the biological binding force acts as a major force to form a specific dried structure [17]. Ring like depositions are observed in a drying drop of DNA [18]. At the same time multi-ring pattern formation of DNA solutions due to the continuous pinning – depinning phenomena [19]. The coffee ring effect has been utilized to detect the protein, α-thrombin, at very low concentrations (54 pM in serum) on surfaces of variable wettability [20]. Furthermore, an inhibition of the coffee ring effect has also been observed inside a drying droplet in a bacterial system (*P. aeruginosa*) due to the presence of a biosurfactant [21]. Morphological variations of dried drops of protein (e.g lysozyme, BSA) rings have been monitored under varying solute concentration and evaporation conditions [21,22,23]. The morphological features of dried drops of serum in normal and under different diseases or physiological states have been examined by recording the dynamics of the acoustic-mechanical impedance (AMI) of a drop drying on the surface of a quartz resonator oscillating with ultrasound frequency [24]. Recently, the pattern formation of mixtures of two different proteins, lysozyme and BSA, in native and denatured form have been studied and the images of the dried patterns are measured by fractal dimension calculation [25]. Conformational changes in the form of a coffee ring structure for the Aβ(25–35) system in presence of a lipid membrane system on highly hydrophilic substrates have been reported by Accardo et al. [26]. The pattern formation of the same peptide after fibril formation under different physical conditions has been reported earlier [27].Thus there is a strong and contemporary interest in exploring the drying dynamics and pattern formation of bio-fluids for detection based on feature identification.

Although the previous studies have shown changes in the morphology of protein solutions during evaporation, scant information are available on the drying induced morphological changes and patterns of aggregated protein fibrils. Fibrillation is the process of forming large linear aggregates from misfolded proteins [28]. Many neurodegenerative diseases like Parkinson's disease, Alzheimer's disease, spongiform encephalopathies occur due to the deposition of stable, ordered filamentous amyloid fibrils. The toxic amyloid fibrils, which contain a significantly high cross β-sheet content deposit in the extracellular spaces of various tissues and cause cellular damage [29]. The study of the possible reasons behind amyloid fibrillation and its disparity with various physical properties has resulted in increasing research activities in the last few years.

Human Serum Albumin (HSA) is a globular protein, abundantly available in human blood plasma. It is used as an amyloidogenic model protein due to its tendency towards the formation of aggregates *in vitro* and also due to its physiological importance such as transportation of fatty acids, metal ions and other physiologically important compounds. The native structure of HSA is predominantly α-helical (> 60%) [30]. The process of fibrillation is often favored and triggered under conditions of high temperature, addition of salts, denaturants, metal ions, low pH etc. HSA is extensively used as a model protein for fibrillation studies. The primary characterization of fibrils can be performed by fluorescence microscopy [31] and by spectrofluorimetry using Thioflavin T [31] or Congo Red [32] as a binding agent. The conformational study of proteins is commonly performed by the far-UV Circular Dichroism [33] and FTIR studies [31]. On the other hand the morphological changes during fibril formation are studied by various microscopic techniques such as TEM [33], FESEM and AFM [34]. Among all these processes, the fluorescence microscopy imaging method is more rapid, though it cannot quantify the fibril growth. In this present work we have coupled the fluorescent microscopy technique with the most common technique for conformational study of protein, the far-UV circular dichroism study. The present work reports the specific use of image analysis of the pattern formation during fibrillation of HSA as a quantitative measure of the extent of fibrillation.

It has been reported earlier that in presence of polystyrene nanoparticles of different surface properties, serum albumin leads to distinctive drying patterns [35]. Though qualitatively the images of the dried protein droplets withdrawn at different incubation times can be distinguished, quantitative image analysis of the fibril growth cannot be performed solely from the drying pattern of the droplets containing only proteins. However, we have shown that it is possible to achieve the quantification of fibril growth by analyzing the drying patterns of a droplet containing the protein and nanoparticles. The selection of the nanoparticle is crucial in this case. Devineau et al. have shown that addition of nanoparticles in proteins generates coffee ring structures of the dried droplet that depend on the charge of the nanoparticles as well as the type of the protein. In order to get distinguishable images for different fibril concentrations it is necessary to select a nanoparticle that can generate images with high contrast after complete drying of the droplet. This is possible if the nanoparticles fluoresce intensely when excited at a particular wavelength. At the same time the nanoparticle should be inert in nature, so as not to interfere with the fibrillation process. Additionally an optimum size of the nanoparticle is necessary to obtain clearly distinguishable drying pattern features. In the present work, the patterns of fibrillar HSA, by the addition of polystyrene fluorescence nanoparticles, are quantified and calibrated against the far-UV CD data. The advantage of this process over conventional far-UV CD is that the drying process requires only one microliter volume of sample and the observation can be done under fluorescence microscopy (unlike the conventional process) in a rapid, simple and straightforward manner.