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Evidence of conformational changes in oil molecules with protein aggregation and conformational changes at oil–'protein solution' interface

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ABSTRACT

Time-dependent conformational changes of proteins and oil molecules at oil–'protein solution' interface were studied using ATR (Attenuated Total Reflection)-FTIR spectroscopic technique for the case of *Bacillus subtilis* extracellular proteins (*BS*EPs) and bovine serum albumin (BSA) in hexane–'protein solution' system. The IR spectra collected on the protein aggregate – film – formed at the hexane–'protein solution' interface demonstrated time-dependent conformational changes of the proteins through changes in the shapes and positions of the H_2O–'amide I' cross peaks and the amide II peaks as a function of time (0–90th minute). Hexane–protein intermolecular association in the film was evident as the CH stretching vibration peaks of hexane were present along with the amide peaks in all the spectra collected over a period of 90 min. Conformational changes of the hexane molecules, along with that of the proteins, were observed *via* variations (broadening and red/blue shifts) in the CH stretching vibration peaks of the CH_3 and the CH_2 groups of hexane. The red/blue shifts of the CH stretching vibration peaks of hexane were different with *BS*EPs and BSA, further indicating that the conformational changes of hexane molecules being protein specific. As similar to the protein types considered here, at oil–'protein solution' interfaces, conformational changes of the oil molecules appear to be a regular phenomenon.

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1. Introduction

Protein conformational dynamics at oil/water interfaces are of fundamental importance to many processes in biology (e.g., protein–lipid interactions) [1–4], food (e.g., role of milk proteins as emulsifiers) [5,6], and pharmaceutical industries [7,8]. Proteins undergo conformational changes at the oil/water interfaces and such changes often are associated with protein aggregation; within certain cases (e.g., milk proteins such as casein), skin-like patterns form with a dense inner layer immediately at the interface and a diffuse layer away from the interface [9,10]. Numerous studies probing the oil–'protein solution' interfaces have elucidated protein conformational changes by using different techniques such as ellipsometry [11], fluorescence [12] and IR/Raman spectroscopy [13,14]. A number of proteins that exhibit conformational changes and as a consequence aggregate to form a skin-like coat at oil–'protein solution' interfaces are such as apomyoglobin, β-casein, α-casein, lysozyme, bovine serum albumin, κ-casein, β-lactoglobulin, and, many bio-peptides – especially with relevance to their interactions with cell membranes [15,16]. Protein conformational dynamics and aggregation at oil/water interface [17], diffusivity of proteins across an interfacial protein aggregated layer [18], and rheological/elastic behavior of the skin-like aggregates [19] have been studied extensively. Protein aggregation at oil/water interfaces is a complex process and involves van der Waals, hydrophobic and electrostatic interactions, and hydrogen bonding [20,21]. While protein conformational changes at oil–water interfaces are often seen, there are a few evidences that indicate conformational changes of the oil molecules at oil–'water (protein solution)' interfaces [22–27]. The role of the oil/'protein solution' interface on the protein conformational dynamics, specifically accounting for the conformational changes of the oil molecules that are part of the interface has been demonstrated through interfacial properties such as interfacial tension and Laplace pressure [20,21,28]. A number of earlier publications provide evidences of the conformational changes of oil/lipid molecules with emphasis on such changes occurring due to temperature perturbations, adsorption onto different types of solid substrates, and nano-confinement of oil molecules, e.g., the CH stretching vibrations of hydrocarbon molecules such as cyclohexane, ethane and benzene exhibit changes (e.g., red/blue shift) when subjected to temperature variations (reasonably higher than the room temperature) [29–31]. Such

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conformational changes are attributed to hyperconjugative type interactions that involve σ^* antibonding and σ bonding orbitals of the CH of alkanes and the substrate moiety.

Among bio-peptides, some strains of *Bacillus subtilis* have been reported to produce mechanically flexible protein coat, which protects the spores from the toxic molecules [32–34]. Earlier studies suggest that BSA readily adsorbs at oil–water interface and undergoes conformational changes [35]. Here, the stability of the *B. subtilis* extracellular protein (*BS*EPs) and Bovine Serum Albumin (BSA) aggregates – film – at the hexane–'protein solution' interface was studied along with the time-dependent conformational changes of the proteins in the film. Time-dependent conformational changes of the *BS*EPs/BSA were followed using ATR-IR (Attenuated Total Reflection-Infrared) spectroscopic technique. We have adopted an in situ ATR-IR spectroscopic approach (explained in Section 2) which enabled the concurrent estimation of the conformational changes of proteins and hexane molecules in the film. The H_2O–'amide I' cross peaks and the amide II peaks in the spectra collected on the film over a period of time (0–90 min) were analyzed to follow the time-dependent conformational changes of the proteins. From the same spectra, information on the time-dependent conformational changes of the hexane molecules were also obtained by analyzing the changes in the CH stretching (*symmetric* and *asymmetric*) vibration peaks of the CH_3 and the CH_2 groups, and thus, led to the concurrent estimation of the conformational changes of hexane along with that of the proteins molecules in the film.

2. Experimental

2.1. Materials

The extracellular proteins were isolated from 72 h grown bacterial culture of *B. subtilis* 168 strain acquired from American type culture collections (ATCC 6633). The bacteria were grown in Bromfield [33] medium, and isolation of the extracellular proteins from the culture broth was carried out according to the methods described elsewhere [32,34,36]. In brief, the cells were separated from the broth by centrifugation of the broth at 6000 rpm and the *BS*EPs (*B. subtilis* extracellular proteins) were precipitated from the cells-free supernatant using $(NH_4)_2SO_4$ (at 80 wt% saturation). Purification of the precipitated *BS*EPs was carried out by dialyzing (2000 MWCO dialysis membrane) the precipitate against 50 mM Tris–HCl Buffer (10% glycerol, 2 mM EDTA, 50 mM KCl). Lyophilized BSA protein [assay: ≥98% (agarose gel electrophoresis)] powder, HPLC high purity grade n-hexane and ACS reagent grade (≥99.0%) $(NH_4)_2SO_4$ were obtained from Sigma–Aldrich. Reagent used for the estimation of proteins concentrations included Bradford reagents which were obtained from Sigma–Aldrich.

2.2. Droplet size distribution

Hexane-in-water (oil-in-water emulsion – O/W) and hexane-in-hexane (oil-in-oil emulsion – O/O) emulsions were prepared using *BS*EPs and BSA protein solutions (proteins in water, pH 7–7.5) having proteins concentrations in the range of 100–2000 ppm. Hexane was gently added to the protein solution at 1:1 (v/v) ratio in 1.5 mL Eppendorf tubes and at room temperature (25 °C). The tubes were capped, and the two liquids (oil and 'protein solution') were allowed to mix rigorously for 1 min in a vortex shaker (rotation speed of 2000 rpm), which resulted in two types of emulsions: (a) 'protein coated hexane droplets' in water: transparent solution at the bottom part of the tube, designated as sample-1, and (b) 'protein coated hexane droplets' in hexane – a phase with foamy appearance on the top part of the tube, designated as sample-2. Vortexing led to the mechanical dispersion of hexane in the protein solution, resulting in the formation of the hexane–water interfaces, and consequently led to adsorption of the proteins at the interfaces. Extended length gel pipette tips were used to acquire samples (sample-1) from the bottom part of the Eppendorf tubes, whereas, to acquire samples from the top part (sample-2) pipettes with wider opening (tips of the pipettes were sawed-off to ∼5 mm with a blade) were used. Pipettes with wider opening were used to maintain the integrity (foamy appearing phase) of the samples. Droplet size distributions (DSD) of all the sample-1 types were determined using Malvern Zetasizer Nano-ZS, and that of sample-2 by estimating the droplet sizes from the images of the droplets captured through a phase contrast microscope.

2.3. Topology of protein film at hexane–'protein solution' interface

Topological features of the proteins films which encapsulated the emulsified hexane droplets in sample-2 types were studied by observing the sample-2 droplets under a phase contrast microscope. Approximately 500 μL of the sample carefully acquired from an Eppendorf tube was gently placed on a glass slide. The glass slide placed under the phase contrast microscope was encased within a thermostat which enabled the sample temperature being maintained at ∼25° C. A transparent glass window on the top part of the thermostat allowed observation of the droplets under the microscope. The area on the sample that was considered for observation was around the center part (after gaining focus as desired) of the sample, spanning an area of approximately 5 mm × 5 mm, where different sections in the 5 mm × 5 mm window were scanned to acquire the droplet images. The droplets in different sections were continuously monitored for a longer (up to 120 min) duration, during which the droplet images were captured using a Hitachi CCD camera attached to the microscope. Over time, the protein film exhibited its characteristic topological features before rupturing and eventually leaving a residue on the glass slide. By choosing the center part of the sample and scanning within a 5 mm × 5 mm window, the droplets at the periphery of the samples were avoided, where the droplets ruptured irregularly and at a faster rate compared to the droplets that were at the center part of the sample. Complete rupture time (CRT) of a film was recorded as the time from the moment the sample-2 (droplets in oil) types were placed on a glass slide to the time the droplet collapsed due to the rupturing of the film. CRT values were determined for droplets prepared with protein (BSEPs and BSA) solutions having a range (100–2000 ppm) of protein concentrations. For the samples prepared with lower protein concentrations (<400 ppm), the droplets were not stable for longer durations (droplets collapsed at a faster rate as compared to the time available for the measurements of the CRT values), neither in the Eppendorf tubes nor on the glass slides. Therefore, for the samples that were prepared with lower protein concentrations, the droplets were chosen randomly within the 5 mm × 5 mm window, and the CRT values reported here are approximate values and only for reference purpose. The CRT values of the sample-2 droplets, while being in the Eppendorf tubes (in emulsion phase) were also studied. The DSD for the samples in the Eppendorf tubes were determined regularly for a longer duration (>10 days). Until the time the DSD differed from that determined at the beginning (when the droplets were devised) was considered as the CRTs of the droplets in the samples, a measure of emulsion stability.

2.4. Protein adsorption behavior at hexane–'protein solution' interface

Protein adsorption densities at the hexane–'protein solution' interfaces in the sample-2 types were determined as a function of

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