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Salting Up of Proteins at the Air/Water Interface

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Supporting Information Placeholder

ABSTRACT: Vibrational sum frequency generation (VSFG) spectroscopy and surface pressure measurements are used to investigate the adsorption of a globular protein, bovine serum albumin (BSA), at the air/water interface with and without the presence of salts. We find at low (2 to 5 ppm) protein concentrations, which is relevant to environmental conditions, both VSFG and surface pressure measurements of BSA behave drastically different than at higher concentrations. Instead of emerging to the surface immediately, as observed at 1000 ppm, protein adsorption kinetics is on the order of tens of minutes at lower concentrations. Most importantly, salts strongly enhance the presence of BSA at the interface. This "salting up" effect differs from the well-known "salting out" effect as it occurs at protein concentrations well-below where "salting out" occurs. The dependence on salt concentration suggests this effect relates to a large extent electrostatic interactions and volume exclusion. Additionally, results from other proteins and the pH dependence of the kinetics indicate that salting up depends on the flexibility of proteins. This initial report demonstrates "*salting up*" as a new type of salt-driven interfacial phenomenon, which is worthy of continued investigation given the importance of salts in biological and environmental aqueous systems.

Introduction

Proteins, which are ubiquitous in biological systems, are also enriched at the sea surface microlayer, the topmost layer of the ocean surface, and present within sea spray aerosol, despite their ultralow concentrations in the ocean.[1-4] Adsorbed proteins at the air/sea water interface play important roles. For example, enzymes at the interface have been suggested to alter the surface properties of sea spray aerosol,[5,6] and surface proteins can also stabilize foams and bubbles.[7-9]

To provide physical insights into protein adsorption at the air/salt water interface, we combine VSFG with surface pressure measurements to study BSA at low bulk concentrations (2 to 5 ppm) at the air/ salt water interface. We demonstrate a "salting up" phenomenon whereby certain proteins partition to the interface within tens of minutes to a few hours, in the presence of salts, but not in their absence. These results show for the first time that ions play an important role in the dynamics or protein adsorption at the air/water interface.

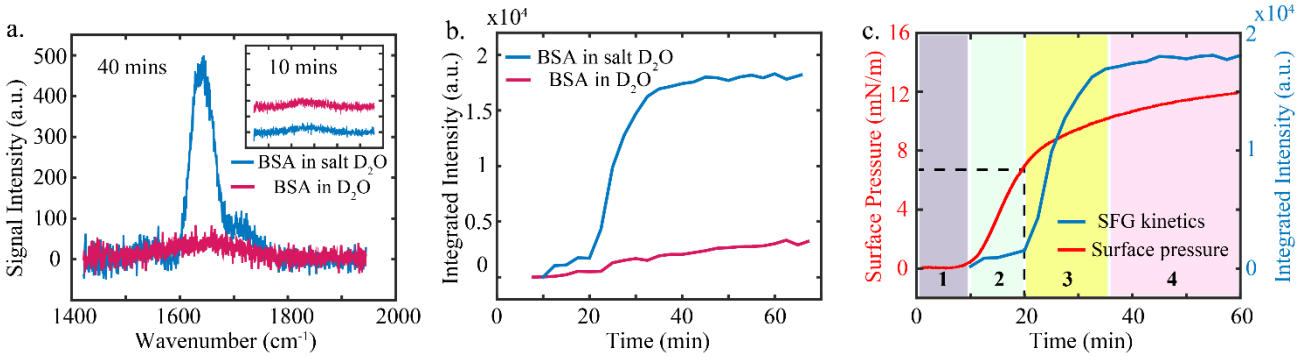


Figure 1. VSFG spectra of BSA monolayer at air/liquid interface. (a) Static VSFG spectra of 3 ppm BSA in salt water and in pure water collected 40 minutes

minutes after the solutions are loaded and the purple spectrum is offset for clarity. (b) Kinetic studies of VSFG signal. VSFG response of BSA at the air/salt water interface undergoes a steep increase at around 20 minutes, whereas no such change is seen at the air/water interface. (c) Adsorption kinetics at the air/salt water interface. Under the same sample condition, surface pressure and SFG show different adsorption kinetics. The dashed lines mark the surface pressure value when the VSFG signal begins to increase. The *colored* areas represent different stages of adsorption (1 to 4), as described in the main text.

Protein concentrations in bulk sea water are found to be at much lower than the corresponding concentration compared to the sea surface microlayer.[10–13] What then drives proteins to the air/liquid interface? To answer this question, air/liquid interface sensitive techniques are needed. VSFG, as a second order nonlinear optical technique, has inherent interface selectivity, and has become a useful spectroscopic tool to detect proteins at air/liquid interface *in-situ*[7,14–18]. Most of the previous VSFG experiments have focused on high protein concentrations (~1000 ppm), and not at low environmentally relevant concentration (~2 ppm).[7,17] Additionally, although the effects of salts on protein behavior in bulk solutions have been extensively studied,[19–23] the influence of relatively high concentrations of salts on proteins at the air/water interface has hitherto not been demonstrated. Most previous studies used buffer solutions, which are at relatively low ionic strength.[15,17,24–26]

Experimental Section

The VSFG spectrometer used in this experiment has been described in previous publications.[27–29] All spectra are collected using SSP polarization (polarization of signal, visible input, and infrared input beams, respectively). Salt solutions with concentrations of ~35 g/L (salinity of sea water) are prepared by dissolving sea salt (Sigma Aldrich) in D_2O and H_2O for VSFG and surface pressure measurements, respectively. The protein solutions are freshly prepared before each experiment. Details about sample preparation can be found in supplemental information.

Results and Discussion

VSFG spectra of BSA show very different behavior at the air/salt water interface compared to the air/pure water interface (Figure 1a). As seen in Figure 1a, there is a VSFG feature from BSA at the air/salt water interface, whereas the spectrum for the air/ pure water interface is featureless. The small bump in the red trace is due to the nonresonant signal of D_2O (Figure S1). The main peak centered at 1650 cm^{-1} in Figure 1a is attributed to the amide I band of α-helices.[18,30,31] Overall, these spectra indicate that salts influence the appearance of the VSFG signal from BSA at the interface. We note the VSFG signal probes interfacial BSA only, and not the bulk BSA due to the electric field induced $\chi^{(3)}$ term,[32–43] because of the large ionic strength (I=0.71 mol/L) used in this study.

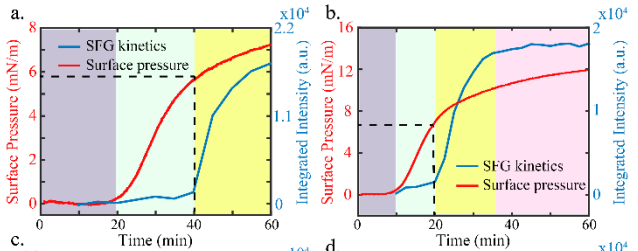
The effect of salts on the VSFG signal from BSA is dynamic. While the spectra show a strong amide I band response at 40 minutes after BSA salt solution is loaded (Figure 1a), at earlier times (after 10 minutes) there is no VSFG signal (inset, Figure 1a). This result suggests the absence of ordered BSA molecules at the interface following the introduction of BSA solution.

To further elucidate the kinetics of the VSFG signal obtained from BSA, we performed time-dependent VSFG measurements on both air/ pure water and air/ salt water interfaces. Figure 1b shows the integrated VSFG intensity of the amide I band as a function of time. From this VSFG kinetic study, we observe that only air/salt water interface shows the emergence of a strong signal and the evolution of protein adsorption. The kinetics of this adsorption process show the following: At first there is an induction period (1 and 2 in Figure 1c), followed by a rise in signal (3 in Figure 1c) and then by saturation of the signal (4 in Figure 1c). This behavior is not observed when BSA is in pure water, nor in systems of salt water without BSA (Figure S2). It is important to note that there were no changes in spectral lineshape and frequency for the amide I band.

There are two possible explanations for these observations. In the first scenario, at low concentrations, few BSA molecules adsorb at the surface because of slow diffusion of BSA from the bulk solution. The diffusion is accelerated by ions, which drive BSA to the interface. Once at the interface, the adsorbed interfacial proteins rearrange into ordered structures and generate VSFG signals. A second scenario is that BSA molecules are adsorbed at the interface from the start, but the proteins need time to rearrange into ordered structures, which leads to the VSFG signal, and that ions play an important role in the protein rearrangement at the interface.

To test these two scenarios, we performed surface pressure measurements. Unlike VSFG, surface pressure measurements are sensitive to the presence of surface species, but rely little on the orientation, conformation or ordering.[44] Thus, we can disentangle the dynamics of surface adsorption from changes in structural/conformational and/or ordering. The surface pressure measurement exhibits a qualitatively similar, but quantitatively different time dependence when compared to VSFG kinetics (red line, Figure 1c): The surface pressure measurement only has a 10-minute induction period, while for VSFG it takes 20 minutes before the signal rapidly rises at the same experimental condition. Following the induction period, surface pressure increases sharply until it reaches a bending point, where there is a much slower rate of increase in the surface pressure. However, only around the time point that the surface pressure reaches a bend in the curve, the VSFG signal start to rise.

Because the surface pressure change reflects the amount of BSA molecules adsorbed at the surface, it means that at 10 minutes after BSA solution is



added, the surface coverage of proteins drastically increases at air/salt water interface. However, because the VSFG signal only starts to increase at 20 minutes, when many BSA molecules are present at the interface, this additional lag phase in VSFG kinetics indicates that rearrangement and ordering of BSA occurs during this period. Therefore, these results support the hypothesis in the first scenario, i.e. there is adsorption of BSA molecules at the interface followed by rearrangement at the interface. In contrast to salt water conditions, proteins in pure water show no surface pressure change within 60 minutes (Figure S4), which is consistent with the VSFG results.

There is another interesting kinetic effect observed. The 20-minute induction period in the VSFG data roughly matches the bend in the surface pressure curve (Figure 1c). At the point of the bend in the surface pressure, the surface concentration is around 0.82 mg/m^2, corresponding to a surface coverage of 90%. (Figure S6)[45] At such a high surface coverage, protein molecules are more likely to interact with one another leading to ordered structures. Therefore, we propose that the surface coverage of BSA needs to reach a critical value (and concomitant with that a critical surface pressure) to undergo any structural rearrangement and/or ordering at the interface.

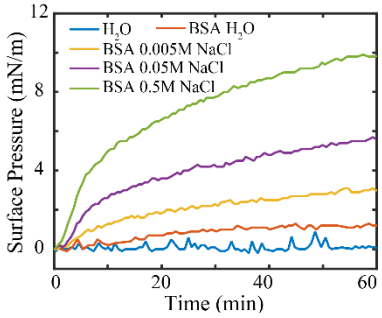


Figure 4. Surface pressure of 5 ppm BSA in different NaCl concentration solution.

To determine the role of surface coverage in the structural rearrangement of BSA at the interface, we measured the VSFG and surface pressure kinetics at three other protein concentrations: 2, 4, and 5 ppm (Fig. 2). If there is a critical surface coverage, the time for the VSFG signal to appear should correlate with the similar surface coverage (pressure) for different bulk protein concentrations. In Figure 3, these four concentrations have different induction times and different duration of all periods for both surface pressure and VSFG signal kinetics. However, VSFG signal begins to increase approximately at the bend in the surface pressure curves for all concentrations, which corresponds to a critical surface pressure of between 5.8 to 6.8 mN/m. At this surface pressure, the corresponding surface concentration is 0.82±0.02 mg/m^2, and surface coverage is 90%±2%. (Figure S6) Thus, from these results we can conclude that 90% is the critical surface coverage, which triggers protein interactions for structural rearrangement and/or ordering.

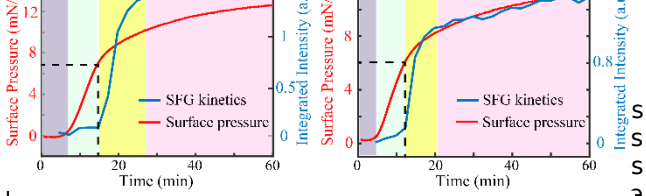


Figure 2. Adsorption kinetics of BSA at the air/salt water interface at different

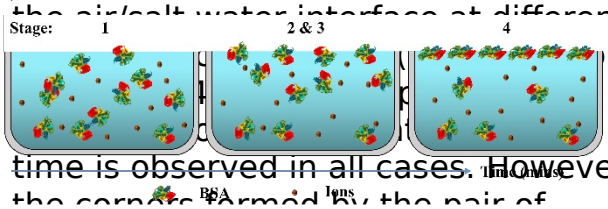


Figure 3. A schematic of the different stages of adsorption kinetics for BSA at the air/salt water interface.

time is observed in all cases. However, before the VSFG signal appears,

up-effect driven by ions.[9] As the surface coverage reaches ca. 50% (Stage 1), it causes the surface pressure to increase[47]. In Stage 2, BSA molecules continue to accumulate at the surface until the surface pressure reaches a value of ca. 6 mN/m, (c.a., 90% surface coverage), which triggers a structural rearrangement and/or ordering that leads to the VSFG signal. In Stage 3, the drastic increase in the VSFG signal reflects BSA reorientation and ordering process at the interface. Both surface pressure and VSFG signal reach an equilibrium (or slowly changing) state in Stage 4.

This four-stage model can be applied to all concentrations investigated here. The induction time is closely related to the BSA concentration - the higher the concentration, the shorter the induction time (the induction times for 2, 3, 4 and 5 ppm are 40, 20, 15, and 12 minutes, respectively). This concentration dependence can be explained by diffusion process that drives the protein to the interface.[8] At higher concentrations, more BSA molecules diffuse to the interface taking less time to reach a critical surface coverage.

In order to get more insights about the mechanism of “salting up” effect of BSA, we performed a salt concentration dependent experiment. The surface pressure kinetics of 5 ppm BSA in three different NaCl concentration solutions, which are 0.5 M, 0.05 M and 0.005 M, are measured, while pure water and BSA water solution are used as control. As shown in Figure 4, Because the onset of salting-up occurs at 0.005M NaCl (0.005 M ionic strength), it suggests that the electrostatic screening is a key driving force for salting-up. For example, the salt ions in solution successfully build a screen layer with a Debye length similar or shorter than the diameter of BSA, thereby screening charges on BSA and reducing the interaction between protein and water to allow BSA molecules to partition to the surface. However, BSA solution with higher NaCl concentration shows larger surface pressure value at equilibrium which indicates larger BSA surface coverage. We believe that the dependence on salt concentration is originated from the volume exclusion in “salting out”. For “salting out”, hydrated ions strongly interact with their first hydration shell which produce large energy barrier to interact with proteins. As a consequence, hydrated ions are excluded from protein molecules in the bulk solution and when more hydrated ions are added, it leads to protein

aggregation and the “salting out’ effect occurs and a reduction of excluded volume at the protein surfaces. Thus, for salting up, when adding more hydrated ions to the solution, aside from electrostatic screening effects, it can also induce more excluded volume at protein surfaces and driving protein to emerge onto surface and aggregate.

To test if “salting up” effect is a general phenomenon to all proteins, we performed the surface pressure measurement on lysozyme and *Burkholderia cepacia* lipase with the same molarity as BSA. The concentration of NaCl is set to be 0.5 M. Interestingly, we find the surface pressure for both lysozyme and lipase solutions does not change after 60 min (Figure S7) which means the protein molecules stay in the aqueous phase. There are two general difference between BSA and the other two proteins: first, it is known that lysozyme and lipase both have rigid structures,[48,49] while BSA is known as a “soft” protein and has low internal stability;[50] second, in NaCl solution (pH=7), BSA is negatively charged while lysozyme and lipase are positively charged. Both differences could cause the observed distinction. To test whether it is the sign of the charge on protein surface, negative or positive, which effects adsorption dynamics, we performed a control experiment of 5 ppm BSA in 0.5 M NaCl solution with pH=3, which is below the isoelectric point of BSA, and therefore BSA is positively charged. The result shows the surface pressure of BSA also increased indicating “salting up” still exists (Figure S8). Thus, despite that electrostatic screening is critical in “salting up”, the type of charges does not play an important role. On the other hand, it is likely that structural flexibility of proteins influence “salting up”. One possibility is that under different environmental conditions, BSA structure changes which could contribute to the differences in the observed surface adsorption kinetics. To test this possibility, we performed ATR-FTIR measurements to probe a thin bulk layer of BSA solution. We found based on the amide-I band, under various salt concentration, the BSA structure is unchanged. (Figure S10). Based on ATR-FTIR results, we found BSA retain its α-helix structures in D2O. The mechanism of how salt ions change protein structures to drive them to the surface subjects to future MD simulation study.

In summary, we observe the accelerated emergence of BSA at air/liquid interface due to the presence of salts, which can be due to a new type of “salting up” effect. It is well known that salts impact protein behavior in bulk solution, with “salting in” and “salting out” effects.[51–53] Our observations lead to a conclusion that while both “salting up” and “salting out” depends on volume exclusion induced by hydrated ions, there are additional mechanistic difference between them, e.g., “salting up’ also depends on flexibility of protein structures. The mechanistic difference is supported by that the phenomenon of “salting up” is not related to the formation of aggregates in the bulk as observed for “salting out”. Experiments performed using dynamic light scattering (DLS) showed no protein aggregation even under much higher protein (1000 ppm) and salt concentrations (Figure S5), which suggests that “salting up” occurs at a different condition than the one that drives “salting out”. One way to understand this substantial difference is that lower protein concentrations may be needed to reach to a critical point for rearrangements in 2D surface compared to the 3D bulk environment.

Conclusion

This study demonstrates for the first time that salts play a pivotal role in BSA adsorption dynamics at air/aqueous interface, which we term as the “salting up” effect. The mechanism of “salting up” lies in the electrostatic screening, structural change of BSA and volume exclusion induced by the hydrated salt ions. Initial mechanistic studies indicate that “salting up” is specific to certain types of proteins that have “soft” structure. Because this study focuses on environmental chemistry relevant conditions of concentration and ionic strength, this new salting-type effect has been uncovered for the first time. While an extensive survey of various salt is out of the scope of this initial report, these initial results lead to further studies that can be performed to better understand mechanistic details of the impact of salts on protein adsorption kinetics. For example, relating these studies to the Hoffmeister series.[54] Further investigation of the salting up effect, using different experimental (e.g. x-ray scattering[55] and VSFG microscopy[56–58]) and computational methods,[59] is warranted.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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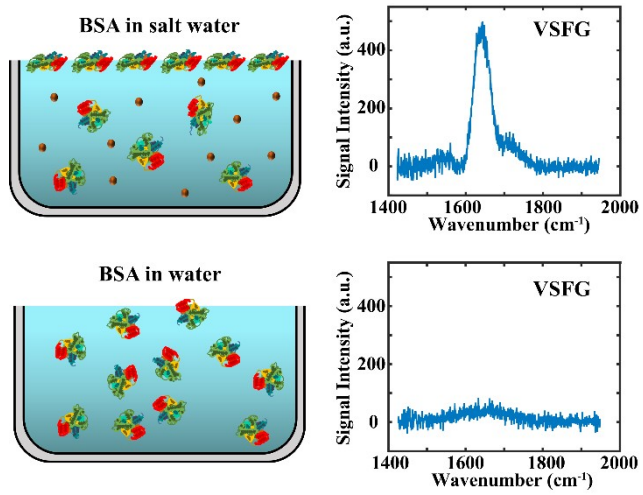
BSA in salt water
VSFG
Signal Intensity (a.u.)
400
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Wavenumber (cm-1)
BSA in water
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Signal Intensity (a.u.)
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Wavenumber (cm-1)