DENATURATION OF PROTEINS UPON ADSORPTION TO BUBBLE SURFACES

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ABSTRACT

Foam fractionation is a process that has potential for application in the separation of proteins from aqueous solution because the molecules adsorb to the foam bubbles. However, some proteins are known to denature by unfolding upon adsorption to a gas-liquid interface and, since the utility of unfolded proteins in pharmaceutical applications is low, it is possible that the use of foam fractionation for the enrichment of proteins is compromised through denaturation. In this work, the dynamic surface tensions of samples of aqueous solutions of Bovine Serum Albumin have been measured. One sample was repeatedly foamed by shaking whence the foam was collapsed, causing many cycles of adsorption and desorption in order to give the proteins opportunity to unfold, and the results were compared to a control sample that was spared the pre-foaming treatment. Results showed that the dynamic surface tension of the pre-foamed sample was lower than the control, and therefore demonstrated in a relatively simple experiment that protein denaturation occurred during the foaming process. In addition, the dynamic surface tensions of samples of bovine milk, both controlled and pre-bubbled, have been measured that show a denaturation effect caused by proteins adsorbed to bubbles.

INTRODUCTION

Proteins are amphipathic molecules, which means that they contain both hydrophilic and hydrophobic groups. If a protein molecule is in aqueous solution with a gas-liquid surface present it will act to reduce the Gibbs free energy of the system via two major mechanisms:

1. Protein molecules tend to diffuse towards the gas-liquid surface, whence they adsorb (perhaps overcoming an energy barrier to do so), thereby reducing the surface tension.
2. After adsorption, the protein molecule can rearrange and change its conformational state by unfolding, thereby further reducing the surface tension because more of the hydrophobic group is exposed to the surface.

The evolution of the surface energy as molecules adsorb to the surface, and possibly unfold, occurs over a time-scale of several seconds to several hours, depending on the characteristic times for each mechanism that acts to reduce surface tension. Knowledge of the relative importance of these two mechanisms is relevant to the removal of proteins from aqueous solution by the process of foam fractionation, as will be described next.

The Potential of Protein Foam Fractionation, and Confounding Factors

Foam fractionation is a process in which the concentration of amphipathic species in solution can be significantly enriched because the species adsorb to bubbles which rise up a column to form a foam, which, when collected and collapsed yields a solution that is
higher in concentration of the target species. Foam fractionation was pioneered by Lemlich (1968), and two recent reviews have been written (Stevenson, 2011; Li & Stevenson, 2012) with the former specifically focussed upon applications in biotechnology. A superficial knowledge of foam fractionation would suggest that the unit operation has great potential to remove proteins and enzymes from solution because, although selectivity is poor, it can very effectively and cheaply strip amphipathic species as a precursor to more selective separation processes downstream. For example, it might be thought that foam fractionation would find application in the removal of very high added-value proteins, such as Lactoferrin, from dairy feedstock. The foam fractionation of protein solution has attracted significant research interest with the work of Lockwood et al. (1997) and Brown et al. (1990) being notable.

However when proteins denature by unfolding their practical utility can become seriously compromised, and Clarkson et al. (1999a & b) demonstrated such denaturation can occur when proteins adsorb to gas-liquid interfaces in foam fractionation; Liu et al. (1998) had previously demonstrated the denaturation of enzymes during foam fractionation.

Thus, if foam fractionation does cause significant protein denaturation, the technology is effective for stripping proteins from aqueous solution, but it is not effective as an enricher for separating proteins from solution for further processing. In this paper we will demonstrated a novel experimental technique for easily and quickly assessing the extent of protein denaturation upon adsorption to bubble surfaces by measuring dynamic surface tension of the gas-liquid interface, without having to directly measure changes of conformational state by using either circular dichroism (Lampe et al., 2010) or nuclear magnetic resonance (Mackay et al., 2001).

**Mechanisms of Protein Adsorption and Denaturation**

Amphipathic molecules, such as proteins, adsorb to a gas-liquid interface by potentially two mechanisms (Eastoe & Dalton, 2000):

1. Diffusive adsorption from the bulk solution to the subsurface layer adjacent to the bubble surface. Such a mechanism was well described by Ward & Tordai (1946) who proposed a convolution-type integral that must be solved, generally numerically, to enable estimates of the evolution of surface concentration (commonly known as ‘surface excess’) with time. A numerically efficient method of doing this has been recently proposed by Li et al. (2010).

2. Upon arrival to the subsurface, the proteins may need to overcome an energy barrier by molecular rearrangement into their initial adsorbed state, as described by Graham & Phillips (1979).

However, once adsorbed to the surface, proteins can denature through the unfolding of discrete segments to further decrease the Gibbs free energy. Whilst a detailed description of the mechanism is beyond the current scope, it is noted that Graham & Philips measured the first-order relaxation time of the unfolding process of Bovine Serum Albumin (i.e. the protein employed in the experiments described herein) at a solution surface to be between about two and eight hours.
Static, Dynamic and Equilibrium Surface Tension

There is great confusion in the literature about definition of surface tension (equivalent to surface energy) in the literature. The surface tension is defined as the specific energy caused by forming an interface (and in this case the gas-liquid surface) and has units of force per unit length (or, equivalently, energy per unit area). However, there are three specific types of surface tension that can be defined:

1. **Static Surface Tension.** If the liquid phase is chemically pure, so that it contains no species that can adsorb to the gas-liquid surface, the surface tension is time invariant. Thus it is possible to define a constant static surface tension. For instance, the static surface tension of chemically pure water at 25°C is approximately 72 mN.m⁻¹. A common method of measuring static surface tension is by employing the Wilhelmy Plate apparatus that measures the force exerted by the meniscus of a plate passing vertically through the gas-liquid interface.

2. **Dynamic Surface Tension.** Because amphipathic species that may be contained in solution adsorb to gas liquid interfaces over a period of time, the surface tension does not exhibit a constant (or static) but instead decreases monotonically with time from an initial value of the static surface tension of the solvent. In addition, the finite relaxation time of protein unfolding causes a dynamic surface tension effect. Although the Wilhelmy technique is capable of yielding a time series of forces imparted on the partially-submerged plate, the method has little utility because the precise moment at which the fresh surface is formed is not clear. However, it is almost never used to give dynamic surface tension data, because the distinction between static and dynamic surface tension is generally so poorly understood. A relatively simple method of measuring the dynamic surface tension of a gas-liquid interface is the pendant droplet (or bubble) method (Hansen & Rodsrud, 1991) in which a tension is inferred from the shape of a sessile droplet (or bubble) as it evolves with time by fitting the Young-Laplace equation to images of the droplet (or bubble) in real time. It is this method that will be adopted in the experiments described herein.

3. **Equilibrium Surface Tension.** After the dynamic processes of protein adsorption, rearrangement and unfolding at the interface have concluded (i.e. equilibrium is attained), the interface exhibits an equilibrium surface tension. Because the equilibrium surface tension is approached asymptotically, dynamic surface tension data can be extrapolated to infinite time in order to give a value of the equilibrium surface tension using the method of Makievski et al. (1997) which assumes that surface tension depression is entirely governed by diffusion controlled adsorption to the surface. In practise it is very difficult to directly determine the equilibrium surface tension of a solution using the pendant droplet method because total elimination of evaporation from the liquid phase is problematical (Li et al., 2010). This difficulty is ameliorated by using the pendant bubble method but this is only suitable for transparent liquids (i.e. aqueous solutions of protein, but not bovine milk).

The confusion between the three definitions of surface tension, given above, that has arisen in the literature, is significant. It is often the case that single (i.e. static) values of the surface tension of solutions containing amphipathic species is quoted, whereas, in fact, a time-series of dynamic surface tension values leading to an equilibrium surface tension should instead be reported. For example, there have been two studies on the surface tension of milk reported by New Zealand research groups (Williams et al., 2005; Mukherjee et al., 2005) that have both reported single (or static) values of surface
tension. Of course, the milk-air surface exhibits a dynamic surface tension because proteins adsORB to, and perhaps unfold at, the interface.

In this work, we harness the fact that protein solutions, and milk, exhibit a dynamic surface tension to assess the extent of surface tension depression due to conformational changes of proteins at interfaces, rather than due to adsorption of proteins to the interface. Measurements of dynamic surface tension have previously been used to infer adsorption kinetics (see, for instance Tripp et al., 1995), but this work is novel because it specifically recognises that a depression in surface tension can be caused by both protein adsorption and protein denaturation.

**EXPERIMENTAL METHODS**

A simple, but novel, method of ascertaining the contribution of denaturation to surface tension depression upon protein adsorption to gas liquid interfaces has been developed. Two samples of the same protein solution were taken: The first was used as a control sample of which the dynamic surface tension was measured using the pendant bubble method on a contact angle goniometer (Model OCA 15EC, Dataphysics Instruments GmbH, Filderstadt, Germany) at 23±2°C. Fig. 1 is a photograph of the sessile bubble, the shape of which was tracked to reveal the dynamic surface tension. However, the second sample of protein solution was placed in a plastic jar and was repeatedly foamed by vigorously shaking every ten minutes for three hours, whence the foam was allowed to completely collapse. During this process the solution was kept refrigerated at 4±1°C. This meant that proteins had great opportunity to adsorb to, and potentially denature at, the liquid gas interface. Each dynamic surface tension measurement was conducted in triplicate.

The protein used in these experiments was Bovine Serum Albumin (Roche analytical grade). Solutions were prepared at concentrations of 0.5 and 1.0 g.L⁻¹ in deionised water. The concentrations selected were significantly greater than the critical micelle concentration which is approximately 25 mg/L at pH 5.

The effect of protein unfolding at a gas-liquid interface upon the dynamic surface tension of milk was also investigated. The fat content of milk prevents significant foam formation so 10 ml of milk was vigorously bubbled with humidified air for an hour instead of being shaken as in the case of BSA solutions. The surface tension of milk samples was recorded immediately after the bubbling pre-treatment. Because milk is...
opaque the pendant drop rather than the pendant bubble method was employed. The surface tension of milk was recorded for approximately 16 hours and during this time the droplet volume decreased due to evaporation, although steps were taken to minimise evaporation by humidifying the environment around the droplet.

Two types of milk were selected: “Blue” (i.e. full fat) and “Super-Trim” (i.e. skimmed) milk from the Anchor™ Brand. Both types were Pasteurised and homogenised.

RESULTS AND DISCUSSION

The dynamic surface tension of both the pre-foamed and control samples of BSA solution at a concentration of 0.5 g.l\(^{-1}\) are shown in Fig. 2. The error bars represent the range of experiments conducted in triplicate, with the symbol indicating the arithmetic mean of the data; it can be seen that there was generally excellent reproducibility. Experiments for both samples were conducted for approximately 50 minutes; it can be seen that, even after this extended time period, a value of equilibrium surface tension has not been achieved, in agreement with the relaxation times for denaturation measured by Graham and Phillips (1979).

The initial rate of surface tension depression is similar for the control and pre-foamed samples, suggesting that initial adsorption rates are approximately the same. However, after approximately 200 seconds, the two sets of data diverge with the pre-foamed sample demonstrating an increasingly lower value of surface tension compared to the control sample. This indicates that the pre-foamed sample is able to better stabilise the gas-liquid interface than the control. As a consequence, this shows that the pre-foaming process created protein denaturation by unfolding, and this unfolding could not be emulated when the proteins in the control sample adsorbed just once to the interface within the goniometer itself.

Fig. 2: Dynamic surface tension of BSA solution at a concentration of 0.5 g.l\(^{-1}\)

Corresponding data for dynamic surface tension, but at a concentration at 1.0 g.l\(^{-1}\) are given in Fig. 3. Note that, because the critical micelle concentration is greatly exceeded
in the experiments conducted, one would expect the equilibrium surface tension for the two concentrations to be similar, if surface tension depression was caused only by protein adsorption to the interface, or if the effect of denaturation was independent of concentration. In fact, the surface tension of the 1.0 g.l\(^{-1}\) control sample shows a slightly larger rate of depression than the 0.5 g.l\(^{-1}\) sample, which is consistent with there being a greater initial driving force for protein diffusion to the interface, but the long-time data is indeed similar.

However, the data for the pre-foamed samples at the two employed concentrations exhibit significant differences. At 1.0 g.l\(^{-1}\) of BSA, the long-time surface tension data for the foamed sample is lower than for the control sample, although the difference is less pronounced than that at the lesser concentration. This appears to indicate that the proteins in the pre-foamed sample experience less denaturation by unfolding when the concentration is greater; this is possibly preliminary evidence that a higher bulk liquid concentration provides some kind of protection against protein denaturation, although we appreciate that this is entirely speculative. In addition, the rate of surface tension depression is initially slightly lower in the foamed sample compared to the control sample.

![Fig. 3: Dynamic surface tension of BSA solution at a concentration of 1.0 g.l\(^{-1}\)](image)

Figs. 4 & 5 show dynamic tension for full fat and skimmed milk recorded over approximately 3 hours. It is seen that the pre-bubbled samples in both types of milk demonstrate lower values of surface tension than the control sample throughout the adsorption process, indicating that the bubbling process has caused protein denaturation via the unfolding mechanism. In the case of the skimmed milk (Fig. 5) the rate of surface tension depression is significant even after 3 hours, but this observation is almost certainly due to evaporative effects. However, it is clearly demonstrated that milk exhibits a non-constant dynamic surface tension, and that this must be taken into account when reporting surface tension data.
CONCLUSIONS AND IMPLICATIONS FOR FOAM FRACTIONATION

The purpose of the work reported herein is to demonstrate a simple method for assessing whether foaming or bubbling through a protein containing solution causes denaturation through conformational changes of the molecule at the gas-liquid surface. It has been shown that:

1. For solutions of Bovine Serum Albumin at concentrations significantly greater than the critical micelle concentration, pre-foaming of the solution causes proteins to denature,
and this manifests as a lower dynamic surface tension compared to a control sample that has not experienced the pre-foaming procedure.

2. The pre-foaming process appears to be less successful at causing protein denaturation at the higher concentration compared to the lower, suggesting some type of protective functionality against conformational changes at the higher concentration.

3. For two types of milk, it is similarly observed that pre-bubbling of air through the sample causes denaturation of the proteins contained within.

This work does not attempt to image the conformational state of the proteins adsorbed to interfaces, but is instead intended as a relatively simple method of inferring the degree of protein denaturation that might be caused in a foam fractionation process. The results suggest that molecules of BSA do indeed denature upon adsorption to the surfaces of foam bubbles. However, in the experiments described herein, the proteins undergo repeated adsorption and desorption stages, whereas, in a foam fractionation the cycle occurs only once, and further work needs to be carried out to determine whether there is a dependency upon the number of such cycles.

We regard that possible protective function of a solution with a higher concentration against protein denaturation as interesting. However, the observation has no practical relevance to the design of foam fractionation columns, since the feed is always at a concentration lower than the critical micelle concentration (Stevenson, 2011).

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