Studying the adsorption of protein at the oil-water interface

Paulina Guzman[1], Natalie J. Medlicott[1], Thomas Rades[1, 2], Lene Jorgensen[2], Stefania G. Baldursdottir[2]

[1]School of Pharmacy, University of Otago, New Zealand, [2]Department of Pharmacy, Faculty of Health and Medical Science, University of Copenhagen, Denmark.

Emails: guzpa535@student.otago.ac.nz; natalie.medlicott@otago.ac.nz; thomas.rades@otago.ac.nz, thr@farma.ku.dk; lej@farma.ku.dk; sba@farma.ku.dk.

Abstract

Objective To characterize the oil-water interfacial adsorption of native and thermally denatured protein using rheology and pendant drop techniques.

Methods Phosphate buffer pH 7 (ionic strength 0.05 M) was used as the aqueous phase and Miglyol 812 (coconut oil; medium chain triglycerol) as the oil phase. Bovine serum albumin (BSA) dissolved in the aqueous phase (0.8 mM and 0.4 mM) was mixed with thermally denatured BSA solutions (0.15 mM) (30 min at 90°C) and interfacial properties were determined.
Oscillatory shear measurements, using a rheometer with double-wall-ring geometry, were conducted at 0.1 Hz and strain of 0.1% (within the linear viscoelastic regime). Interfacial tension measurements were performed using an aqueous drop volume of 50 μL (needle diameter 1.83 mm) which was lowered into the oil phase.

Results BSA formed a viscoelastic film at the oil-water interface. The presence of thermally denatured protein in the bulk aqueous phase delayed the interfacial film formation but gave similar elastic and viscous moduli after 1h. After ten minutes, the surface tension for the native protein alone [7.63±0.25 (mN/m) (0.4 mM) and 9.81±0.29 (mN/m) (0.2 mM)] was higher than for the native protein in the presence of the denatured protein [7.20±0.04 (mN/m) (0.4 mM) and 8.49±0.20 (mN/m) (0.2 mM)]. The magnitude and time course of the rheological properties and interfacial tension suggested that the interfacial behavior of BSA between an aqueous solution and the oil phase was not governed by the native protein concentration alone but depended on the presence of denatured protein.

Conclusion The combination of rheology and pendant drop techniques was shown to be useful for the characterization of the physical behavior of protein at the oil-water interface.

Keywords: BSA adsorption, viscoelastic multilayer, native protein, thermal denatured protein, oil-water interface, interfacial tension, rheology.

1. Introduction

Protein adsorption to interfaces has been reported to affect their physical stability with potential to reduce pharmacological effects and safety when used as therapeutic agents. In oil-water systems, the interfacial adsorption of protein is probably due to its amphiphilic character of the protein molecule. On interaction with the interface the protein may adopt unfolded conformations, change the o/w interfacial properties, and/or interact with the surrounding molecules to form a viscoelastic layer[1] (**Figure 1**). The characteristics of the adsorbed layer may depend on intrinsic properties of the protein such as: molecular weight[2], flexibility[1], size and concentration[3], charge, stability of the native structure, and hydrophobicity[2], as well as external properties of the surrounding environment and presence of other components such as surfactants[3].

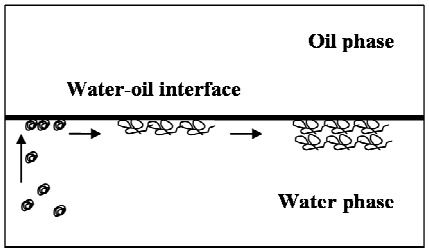

Figure 1: Schematic illustration of protein adsorption to the oil-water interface.

Initial adsorption of protein at the interface results in formation of a monolayer which may further develop into a multilayer. These structures has been previously investigated for some systems using rheology and pendant drop methods[2,4]. In oscillatory shear rheology studies, the multilayer formation is traditionally studied measuring the elastic moduli (G') and the viscous moduli (G'') as a function of time. A crossover point between G' and G'' represents the end of the monolayer formation and the beginning of multilayer formation when a significant increase of G' over G'' can be observed. Finally, the saturation of the interface with protein is represented by equilibrium values of G' and G'' [3]. Recently, Wang et al. (2011) described a methodology to quantify additional parameters in the multilayer, including its strength and elasticity, using strain sweep and frequency sweep steps experiments[5].

The pendant drop method can be used as a complementary technique to obtain information about the initial stages of protein adsorption prior to monolayer formation. Typically in pendant drop experiments an initial rapid decrease in interfacial tension (IFT) occurs as the monolayer is formed. Following this the rate of decrease of IFT slows and a plateau may be reached when a multilayer structure are formed [3].

The aim of this study was to characterize the oil-water interfacial adsorption of native and thermally denatured protein, and to examine how introduction of a small amount of denatured protein affects the interfacial properties of a native protein.

2. Materials and Methods

2.1. Sample preparation

Bovine serum albumin (BSA, A-7906 from Sigma-Aldrich, USA) was dissolved in phosphate buffer (pH 7, ionic strength 0.05M) at 0.15 mM, 0.4 mM and 0.8 mM to give solutions of native BSA. A further solution of BSA at 0.15 mM in phosphate buffer was prepared and heated at 90°C in water for 30 minutes to give heat denatured BSA. Native BSA (0.8 mM and 0.4 mM) and denatured BSA (0.15mM) were mixed to obtain binary mixtures containing final protein concentration of native BSA (0.4 mM and 0.2 mM) and heat denatured BSA solutions (0.07 mM). These solutions were prepared in triplicate. The oil phase was composed of Miglyol 812 (coconut oil; Medium chain triglycerol, donated by H. Lundbeck A/S, Denmark).

2.2. Interfacial rheology using a double wall ring (DWR) geometry

Rheology studies were conducted using a TA AR-G2 rheometer with DWR geometry using a method previously described by Baldursdottir et al. (2011). The system consisted of a square-edge ring (platinum-iridium alloy) and a Delrin® trough (Teflon) with a circular channel (**Figure 2**). The gap was zeroed without the ring attached and then kept constant at 12000 μm. 18.8 mL of each phase were poured into the Delrin® trough (bottom: aqueous phase, top: oil phase). Oscillatory shear measurements were conducted

over time at a constant frequency of 0.1 Hz, strain set at 0.1 % and at a temperature of 25°C (within the linear viscoelastic regime).

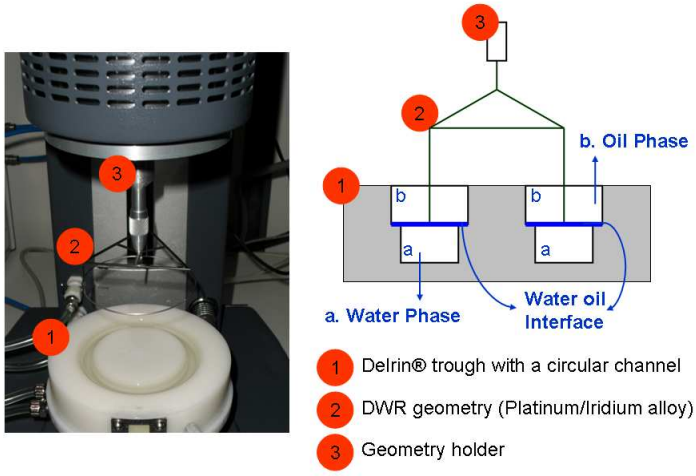

Figure 2. Schematic illustration of the rheometer with DWR geometry.

2.3. Data analysis for rheology measurements

Time sweep, strain sweep and frequency sweep steps were used to investigate and compare strength, viscoelasticity and delay of multilayer formation. The time sweep step was used to investigate the multilayer formation using the crossover values obtained from G' and G'' (N/m) as a function of time. The strain sweep step was used to characterize the thickness of the interfacial film and its resistance to breakdown after 2 h. Frequency sweep step was used to quantify elasticity or solid properties of the film after 2 h. This information was obtained using an interfacial complex viscosity η* (Ns/m) as a function of angular frequency (ω in Hz), where [$\eta^* \propto \omega^{-n}$] and values of the power law exponent (n) close to 1 mean that the final film has more solid-like properties[5]. Interfacial complex viscosity is defined using **Equation 1** [2].

$$|\eta^*(\omega)| = [G'(\omega)^2 + G''(\omega)^2]^{1/2} / \omega \qquad \text{Equation 1}$$

2.3. Pendant drop instrument

Interfacial tension studies were performed using a pendant drop instrument (KRÜSS, Germany) as previously described by Baldursdottir et al. (2010). A drop of protein

solution (aqueous phase) formed at the tip of a syringe (1.83 mm needle diameter) was lowered into a glass cuvette (4 x 2 x 1 cm) containing the oil phase (6.5 mL). The volume of the protein solution drop was held constant at 50 µL and experiments were performed at room temperature (**Figure 3**). To calculate the interfacial tension, the drop profile was obtained from an image to which the Laplace-Young equation was fitted (**Equation 2**).

$$\Delta P = \gamma\left(\frac{1}{R_1} + \frac{1}{R_2}\right) \qquad \text{Equation 2}$$

where ΔP = pressure difference over the drop interface, γ = interfacial tension, R_1 and R_2 = radii of the maximum width and the length of the drop, respectively.[2]

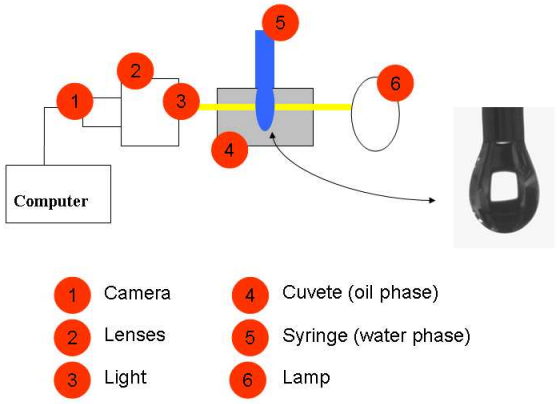

Figure 3. Schematic illustration of pendant drop method.

3. Results and Discussion

3.1. Rheology studies

Solutions of native BSA (0.15 mM) formed a viscoelastic multilayer at the oil-water interface. The denaturation of the BSA (0.15 mM) showed a delayed multilayer formation by more than two hours compared to the native BSA, with the final interfacial layer having a significantly lower G' and G'' (**Figure 4A**).

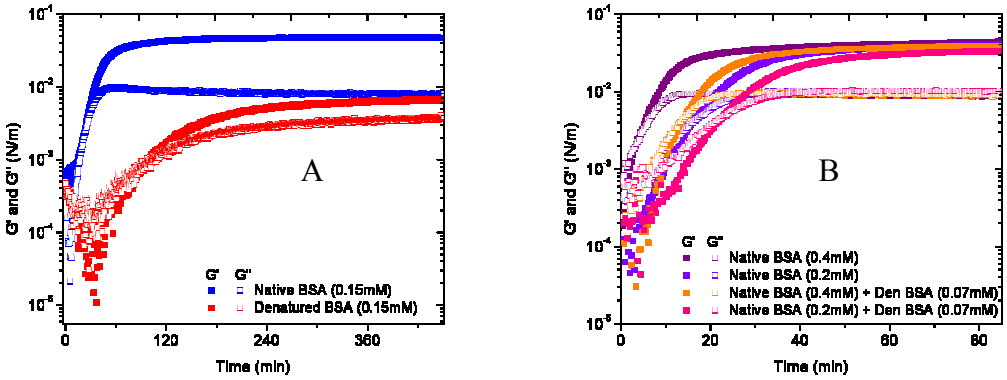

Figure 4. Adsorption of protein to the oil-water interface using a rheometer with DWR geometry. (A) Comparison of multilayer formation between native and denatured BSA (0.15 mM). (B) Native protein alone (0.2 mM and 0.4 mM) and native protein (0.2 mM and 0.4 mM) in the presence of denatured protein (0.07 mM).

BSA is a globular protein where 60% of the amino acids are considered hydrophobic[2]. During thermal denaturation the globular conformation is lost and the hydrophobic groups become more exposed to the aqueous phase than for the native protein conformation[6,7]. The difference observed in multilayer formation could be explained by the changes in protein conformation (globular or extended) and molecular interaction between native and denatured BSA (0.15 mM). One possibility is that denatured protein requires more time than native protein to orientate and adapt its hydrophobic groups towards the oil phase so that it is less likely to remain at the interface. Another possibility is that diffusion of protein molecules to the interface could also be affected by protein conformation with globular BSA (native) able to diffuse more rapidly than the more extended, or less compact form of denatured BSA.

The adsorption of BSA to the oil-water interface was concentration dependent as the multilayer formed more rapidly from a solution with a higher protein concentration (0.4 mM compared with 0.2 mM) (**Figure 4B**). This result was in agreement with previous reports[2,3]. The addition of thermally denatured protein to a solution of native BSA delayed multilayer formation (**Table 1**). However, similar G' and G'' values were reached for both these systems after one hour **(Figure 4B).** After 2 h, the maximum G' value was slightly lower in the presence of denatured protein than for the native protein

alone (**Table 1**). This may be explained if native and denatured protein molecules initially compete to reach the oil-water interface but native BSA forms a stronger molecular interaction than denatured BSA so that it eventually replaces the denatured BSA at the interface. Additionally, the denatured BSA (extended conformation) could interact with native protein in the bulk solution forming different species that delays protein diffusion to the oil phase.

Table 1. Data analysis from time sweep step (crossover time and maximum G' and G'') and frequency sweep step (power law exponent n) measurements

BSA concentration (mM)		Power law	Crossover Time	Maximum	
Native	Thermally denatured	exponent (n)	(min)	G' (N/m)	G'' (N/m)
0.4	0	0.902 ± 0.4%	3.1 ± 0.4	46.7± 4.5	9.3 ± 0.6
0.4	0.07	0.871 ± 2.9%	11.2 ± 3.6 (Δt: 8.1)	41.6± 5.5	9.3 ± 1.0
0.2	0	0.862 ± 0.9%	19.3 ± 2.8	40.0± 2.3	9.8 ± 0.4
0.2	0.07	0.843 ± 0.6%	22.6 ± 0.2 (Δt: 3.3)	35.2± 0.6	9.7 ± 0.4

Strain sweep experiments (**Figure 5**) were conducted to investigate the resistance of the viscoelastic multilayer to breakage when the force applied was increased. The presence of denatured protein slightly lowered G' but had no effect on G''. The force at which the multilayer was disrupted was similar for films formed from either native alone or native with denatured BSA present (native protein concentration of 0.4 mM and 0.2 mM) (approx. 8 mNm). This result suggests that the strength of the multilayer after one hour is an intrinsic characteristic of the protein itself and does not depend on protein concentration or presence of denatured protein in the bulk solution.

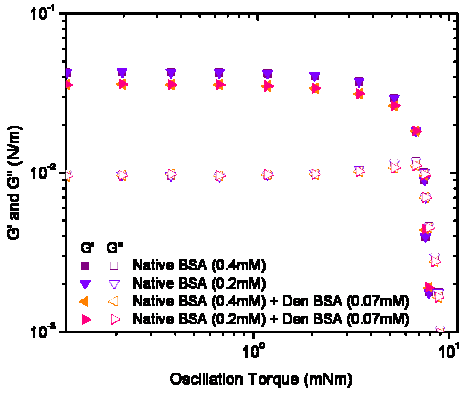

Figure 5. Dynamic moduli as a function of the force applied to the film. The sharp reduction in the moduli indicates film breakage.

Frequency sweep measurements were used to compare solid vs. liquid like properties of the multilayer after 2 h of adding BSA where n close to 1 would result in a more solid like film[5] (Table 1). As tendency, the presence of denatured protein in the bulk solution produces a less solid film than native protein alone. It seems that native (globular) protein interacts to produce a tight film where the entire oil surface is densely covered. In contrast, the presence of denatured protein could produce a softer less densely packed film where areas of the oil surface are not covered by native protein. The extended denatured protein conformation could produce a less tight film than for the native protein alone.

3.2. Interfacial tension

The decrease in the interfacial tension (IFT) was lower for the native protein alone than for native protein in the presence of the denatured protein (**Figure 6**).

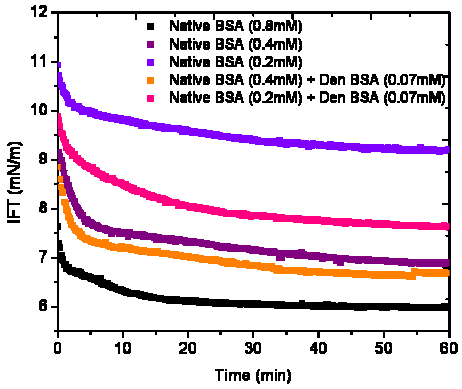

Figure 6. The drop in the interfacial tension over time. Native protein alone (0.2 mM and 0.4 mM) and native protein in the presence of denatured protein (0.07 mM).

Additionally, the reduction of the IFT appeared to be slower for the lower concentrations of the native protein alone (0.2 mM and 0.4 mM) compared to the highest protein concentration (0.8 mM). This is in agreement with previous reports which show that protein adsorption to the oil-water interface is concentration dependent[2,3].

The shape of the curves describing the decrease in the IFT values was also dependent on the addition of denatured protein available in the bulk solution. IFT appeared to decrease more slowly when denatured BSA was added to native BSA at the lower concentration (0.2mM). This may suggest that the molar ratio of native to denatured protein is important. At the concentrations used in this work the molar concentration ratio of denatured to native BSA was 16 % and 25% at native BSA concentrations of 0.4 mM and 0.2 mM BSA respectively. This could support the theory of competition between hydrophobic groups of native and denatured protein at the interface.

4. Conclusions

The combination of rheology and pendant drop methods was shown to be useful for the characterization of protein physical behavior at the oil-water interface. Our results showed that the presence of thermally denatured protein in the bulk solution affected the first steps of protein adsorption which was characterized by delay in the multilayer formation and faster reduction in the IFT than for native protein alone.

Acknowledgement
School of Pharmacy, University of Otago (PhD scholarship for PG).

References

1. A. Bos M, van Vliet T 2001. Interfacial rheological properties of adsorbed protein layers and surfactants: a review. Adv Colloid Interface Sci 91(3):437-471.
2. Baldursdottir SG, Fullerton MS, Nielsen SH, Jorgensen L 2010. Adsorption of proteins at the oil/water interface--Observation of protein adsorption by interfacial shear stress measurements. Colloids Surf, B 79(1):41-46.
3. Baldursdottir SG, Jorgensen L 2011. The influence of size, structure and hydrophilicity of model surfactants on the adsorption of lysozyme to oil-water interface--Interfacial shear measurements. Colloids Surf, B 87(1):96-102.
4. Freer EM, Yim KS, Fuller GG, Radke CJ 2004. Interfacial Rheology of Globular and Flexible Proteins at the Hexadecane/Water Interface: Comparison of Shear and Dilatation Deformation. J Phys Chem B 108(12):3835-3844.
5. Wang L, Xie H, Qiao X, Goffin A, Hodgkinson T, Yuan X, Sun K, Fuller GG 2011. Interfacial Rheology of Natural Silk Fibroin at Air/Water and Oil/Water Interfaces. Langmuir 28(1):459-467.
6. Chi EY, Krishnan S, Randolph TW, Carpenter JF 2003. Physical Stability of Proteins in Aqueous Solution: Mechanism and Driving Forces in Nonnative Protein Aggregation. Pharm Res 20(9):1325-1336.
7. Joubert MK, Luo Q, Nashed-Samuel Y, Wypych J, Narhi LO 2011. Classification and Characterization of Therapeutic Antibody Aggregates. J Biol Chem 286(28):25118-25133.