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Communication

Comparison of two methods detecting lysozyme adsorption to oil-water interface in the presence of surfactants

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Abstract: To aim of the study was to compare the formation of the adsorbed lysozyme layer at the oil-water interface with two different methods and to scrutinize the possibilities of avoiding film-formation by addition of model surfactants. Surface tension measurements were carried out using pendant drop. An aqueous droplet of 70 µL was formed with a needle (diameter 1.83 mm) in a glass cuvette containing the oil-phase. Film formation was evaluated by withdrawal of the aqueous phase after 10 minutes emersion in the oil phase. Rheological properties were measured by use of a TA AR-G2 rheometer equipped with a double wall ring (DWR) geometry. The system consists of a ring and a Delrin® trough with a circular channel (interfacial areal=1882.6 mm^2). Oscillatory shear measurements were conducted at 0.1 Hz, at 25°C and a strain of 1%. The adsorption of lysozyme to the oil-water interface results in the formation of a flexible protein film. This formation can be prevented by addition of surfactants, in a manner that is dependent on the concentration and the type of surfactant. According to both methods the more hydrophilic surfactants are more effective in hindering lysozyme adsorption to oil-water interfaces. Additionally, the rheological method indicates that the larger surfactants are more persistent in preventing film formation whereas the smaller eventually give place for the lysozyme on the interface. The two methods are complimentary and can both be used to detect the interfacial adsorption of lysozyme and to evaluate the performance of model surfactants in hindering film formation. This will aid in processing of any delivery systems for proteins where the protein will be introduced to oil-water interfaces that could affect the stability of the protein.

Keywords: Lysozyme adsorption; surfactants; interface; surface tension; rheology; viscoelastic multilayer

1. Introduction

The flexibility and aggregation of proteins can cause adsorption to oil-water interfaces and thereby give challenges during formulation and processing.

Protein adsorption to oil/water interfaces occurs in several regimes that can be monitored with several different techniques e.g. surface tension and rheology (1, 2). The first regime (I) is a lag phase, primarily dependent on the diffusion of the protein to the interface (3), where the interfacial tension is unchanged over time. During the second regime (II) the interfacial tension drops, indicating accelerated adsorption of the protein to the interface due to the attractive interaction forces between the proteins already adsorbed to the interface and the proteins in the bulk solution. The end of regime II is marked where a monolayer of the protein is covering the interface (2, 4). A multilayer of the protein is formed during the third regime (III) resulting in a gel-like network structure and viscoelastic behaviour can be detected. The multilayer is attributed to intermolecular interactions and unfolding of the protein towards more favourable conformations in the interface. Only a soft decrease in interfacial tension is detected during the third regime (III) (4). The surface tension method does not contribute to knowledge on the behaviour in the regime (III) whereas the viscoelastic properties have been found useful. Freer et. al. defined the crossover from where the monolayer is fully formed and evolvement of a multilayer starts, crossover from regime (II) to regime (III), as where a drastic increase in G' is observed and G'' levels off (5, 6).

Protein adsorption is a complex process and the presence of surfactants further complicates the system where additional parameters need to be considered. In this study four different surfactants (figure 1) and their ability to alter or prevent protein adsorption is compared using surface tension and rheology.

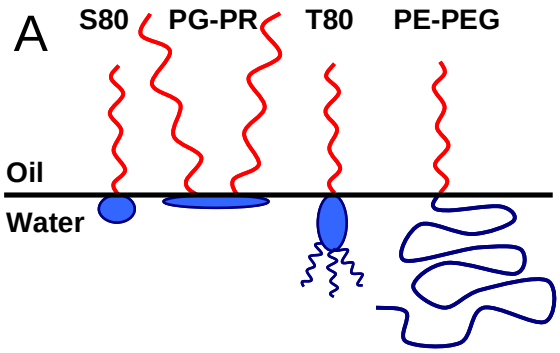

Figure 1: Schematic illustration (not in scale) of the model surfactants used in this study.

2. Experimental Section

2.1. Sample preparation

Lysozyme (from hen egg white, Sigma-Aldrich, Denmark) was dissolved in 10 mM phosphate buffer, pH 7.4 containing potassium-hydrogen-phosphate (KH_2PO_4) and disodium-hydrogen-phosphate

(Na_2HPO_4) both from Merck, Denmark. The surfactants used where: Sorbitan monooleate 80 (S80), polysorbate 80 (T80) and polyethylene-block-poly(ethylene glycol) (PE-PEG) (M_w = 2250 Da with 80% PEG) from Sigma-Aldrich, Denmark, and polyglycerol polyricinoleate (PG-PR) from Danisco, Denmark. Coconut oil, Miglyol 812, Medium chain triglycerol was used as the oil-phase, donated by H. Lundbeck A/S, Denmark. Stock solutions of the surfactants were prepared (S80 and PG-PR in the oil-phase and T80 and PE-PEG in the aqueous phase), which then were diluted to the desired concentration. For S80 and PG-PR the measurement was conducted with the lysozyme solution (0.34 mM) as the aqueous phase and the surfactant solution as the oil-phase whereas for T80 and PE-PEG the lysozyme (0.34 mM) was dissolved in the surfactant solution shortly before the measurement was started and pure oil was used as the oil-phase. All the solutions were placed in a water bath 30 min before measurement to ensure a constant temperature of 25°C.

2.2. Surface tension

Measurements were carried out on a Pendant drop instrument (KRÜSS, Germany). A droplet of the protein solution was formed with a syringe (needle diameter of 1.83 mm). The needle was lowered into a glass cuvette (2x2x2 cm) containing the oil phase and thereafter the droplet was formed. The measurements were conducted at a room temperature (25°C) and the volume of the droplet was kept constant at 70 μL. The interfacial tension is calculated from the shape of the droplet using the Young-Laplace equation:

$$\Delta P = g\left(\frac{1}{R_1}+\frac{1}{R_2}\right) \tag{1}$$

where γ is the interfacial tension, ΔP is the pressure difference over the droplet interface and R_1 and R_2 are radiuses of the width and the length of the droplet, respectively.

2.3. Film formation

The presence of a thin film on the interface between the aqueous droplet and the oil was determined by aspiration of the droplet after 10 minutes exposure to the oil-water interface.

2.4. Double Wall Ring (DWR)

Rheological properties were measured by use of a TA AR-G2 rheometer (TA Instruments-Waters LLC, New Castle, USA) equipped with a double wall ring (DWR) geometry. The system consists of a square-edged ring (platinum-Iridium alloy) and a Delrin® trough (Teflon) with a circular channel. The DWR has an inner radius of 34.5 mm and thickness is 1 mm. The gap was zeroed without the ring attached and then that measuring gap was fixed at 12000 μm. Approximately 18.8 mL of the aqueous phase was placed in the Delrin® trough (inner radius = 31.0 mm and outer radius = 39.5 mm, thus the interfacial areal is 1882.6 mm^2) and the ring was lowered ensuring contact with the surface. Thereafter, 18.8 mL of the oil phase was carefully poured on top of the aqueous phase. The measurement setup of the DWR is depicted in Figure 2.

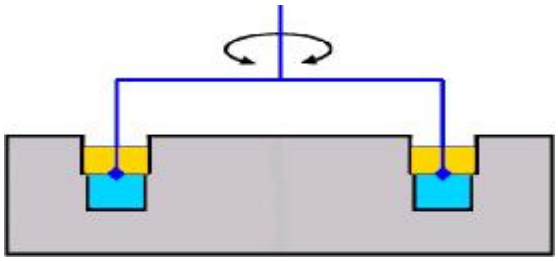

Figure 2: Cross section of the Double Wall Ring geometry (1).

Oscillatory shear measurements were conducted at constant frequency of 0.1 Hz, temperature of 25°C and the strain was set to 1%. Strain sweep was measured at the end of each time sweep to ensure that the strain chosen was within the linear viscoelastic regime where the storage modulus, the elastic response (G') and the loss modulus, the viscous response (G'') are independent of the strain amplitude.

The complex viscosity was used to follow the overall evolvement of the adsorption layer. The complex viscosity includes contributions from both the elastic and the viscous responses and is calculated using equation 1:

$$|\eta^*(\omega)| = \frac{\sqrt{G'(\omega)^2 + G''(\omega)^2}}{\omega} \quad (2)$$

where ω is the angular frequency.

3. Results and Discussion

3.1. Surface tension:

Surface tension gives information on the evolvement of the monolayer, and the time scale of the first adsorption steps. Figure 3A and B show the lowering of the interfacial tension of lysozyme in the presence of various concentrations of T80 or PE-PEG in the short time scale where the regime (I) and (II) are taking place. The monolayer seems to form instantaneously and therefore no lag time, representing regime (I), is detected. The interfacial tension of lysozyme alone starts at 14 mN/m and only falls to 13 mN/m in 10 min. The addition of increased amount of surfactants lowers the end point interfacial tension that is after 10 min measurement and this effect is more pronounced for the T80. Additionally, in the presence of the higher amounts of T80 a drastic decrease of the interfacial tension is detected in the beginning, see figure 3A. Decreased concentration of T80 softens this change in the interfacial tension giving a profile closer to that of lysozyme alone. In figure 3B the effect of the presence of PE-PEG is depicted. The drop in the interfacial tension is not as dramatic when the PE-PEG is used, and the profiles of all the concentrations used resemble more the profile of lysozyme alone, compared to the T80. This could indicate that T80 is more surface active than PE-PEG.

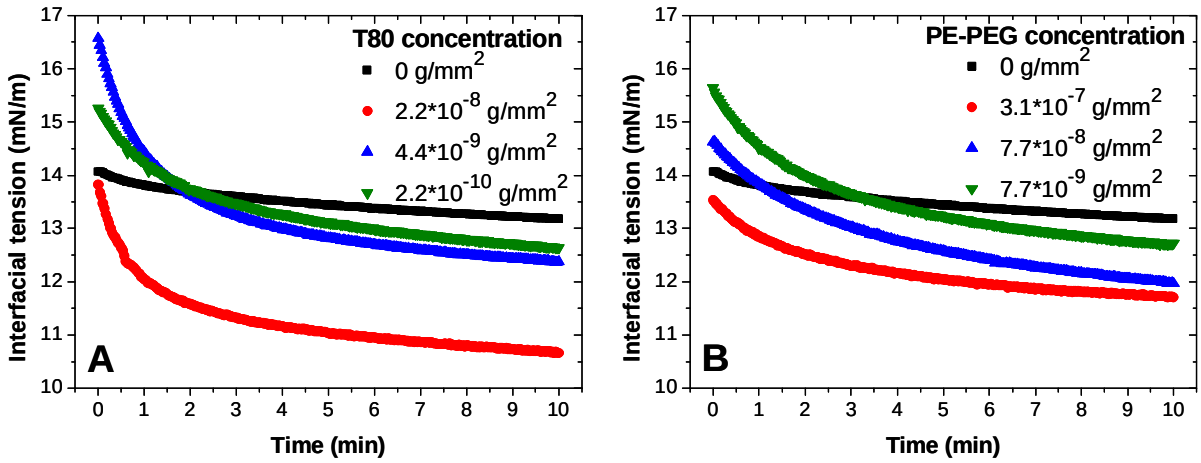

Figure 3: The changes in interfacial tension over time of lysozyme alone compared to the lysozyme in the presence of various amounts of **A)** T80 and **B)** PE-PEG.

The method also offers the opportunity to detect film formation visually (Figure 4 and accompanying movie), even after the short time period of 10 min.

Figure 4: lysozyme 0.07 mM in phosphate buffer after exposure to the oil phase of 10 min.

A film of lysozyme is detected at $2.5*10^{-6}$ g/mm^2 for S80, $1.8*10^{-6}$ g/mm^2 for PG-PR, $1.5*10^{-7}$ g/mm^2 for PE-PEG and $4.4*10^{-9}$ g/mm^2 for T80. T80 is different as the film formed at the lowest concentration is more fragile than for the other surfactants. In figures 3A and B the red curves represent the lowest concentration where no film is detected.

3.2. Double wall ring:

Lysozyme adsorbs quickly to oil-water interface, where the monolayer is formed instantaneously and then develops further to a multilayer (1). Further conformational changes have also been detected up to 24 hours (5, 6). In contrast to the surface tension method, the DWR clearly shows this evolvement of

the multilayer in the longer time scale as an increase in the complex viscosity in figure 5A and B. Various surfactants have been used in order to hinder the adsorption of proteins to oil-water interfaces (7-10), but little is known about the difference between them. The influence of the presence of different concentrations of T80 is depicted in figure 5A, as complex viscosity as a function of time. A 10 fold increase in the concentration of the surfactant delays the adsorption of the protein, but never the less a multilayer is detected within the time frame of the measurement. A 100 fold increase in the T80 concentration is needed in order to fully prevent the protein adsorption as shown in figure 5A. The same phenomenon was detected for S80 (data not shown), however, a 1000 fold increase in the surfactant concentration was needed to prevent the protein adsorption.

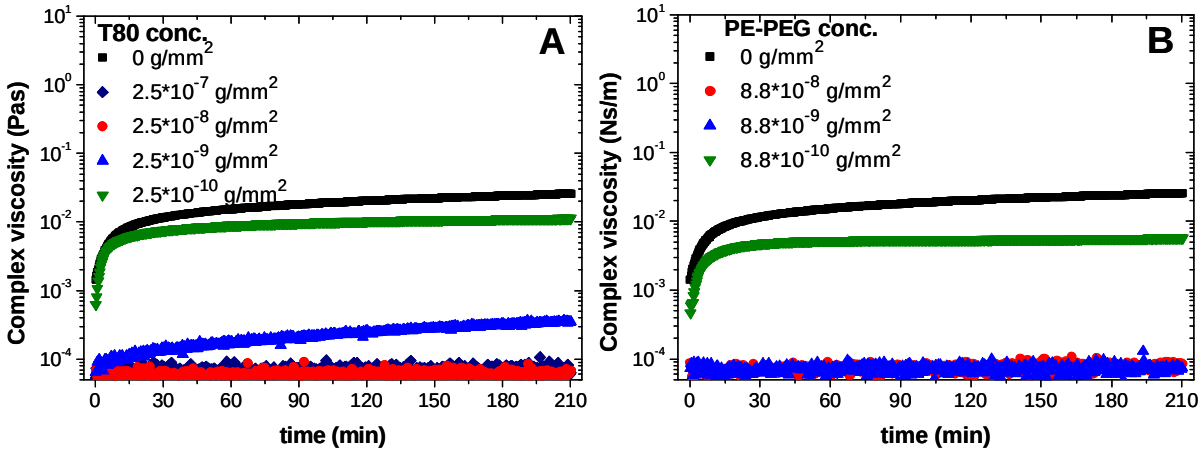

Figure 5: The lysozyme multilayer formation depicted as the complex viscosity (Pas) as a function of time in the presence of various concentrations of **A)** T80 and **B)** PE-PEG.

When the polymeric surfactants (PG-PR or PE-PEG) were used, a 10 fold increase in the surfactant concentration fully prevented the protein adsorption. This can well be seen in figure 5B for the PE-PEG. Increased concentrations of the surfactants decrease lysozyme adsorption and a delayed multilayer formation is seen in the presence of the smaller surfactants, T80 and S80, but not for the polymeric surfactants, PE-PEG and PG-PR, indicating stronger affinity of the polymeric surfactant towards the interface. Thereby larger surfactants seem more effective in preventing the lysozyme adsorption to the interface than the smaller surfactants. However, a multilayer of lysozyme is detected at $2.5*10^{-7}$ g/mm^2 for S80, $8.8*10^{-9}$ g/mm^2 for PG-PR, $2.5*10^{-9}$ g/mm^2 for T80 and $8.8*10^{-10}$ g/mm^2 for PE-PEG and is therefore approximately in the size order of the head groups. This is in good agreement with the results of Szleifer et al that have shown that prevention of protein adsorption by polymers seems to depend on the surface density and not the molecular weight (11). As T80 and S80 are smaller than PG-PR and PE-PEG, the diffusion is faster making the molecules more mobile and therefore the possibility for formation of other structures, e.g. micelles, is higher. Therefore, the small surfactants adsorb to the interface before the protein but eventually give space for the protein and thus causing the delay in the protein adsorption.

3.3. Comparison of the methods:

As discussed earlier the surface tension method gives information on the evolvement of the monolayer, and the first adsorption steps whereas the DWR gives information on the evolvement of the multilayer, and the longer term adsorption events. The various surfactant concentrations are depicted, in figure 6, against G' (value taken after 200 min measurement) as well as the concentrations of the surfactant needed in order to prevent film formation after 10 minutes.

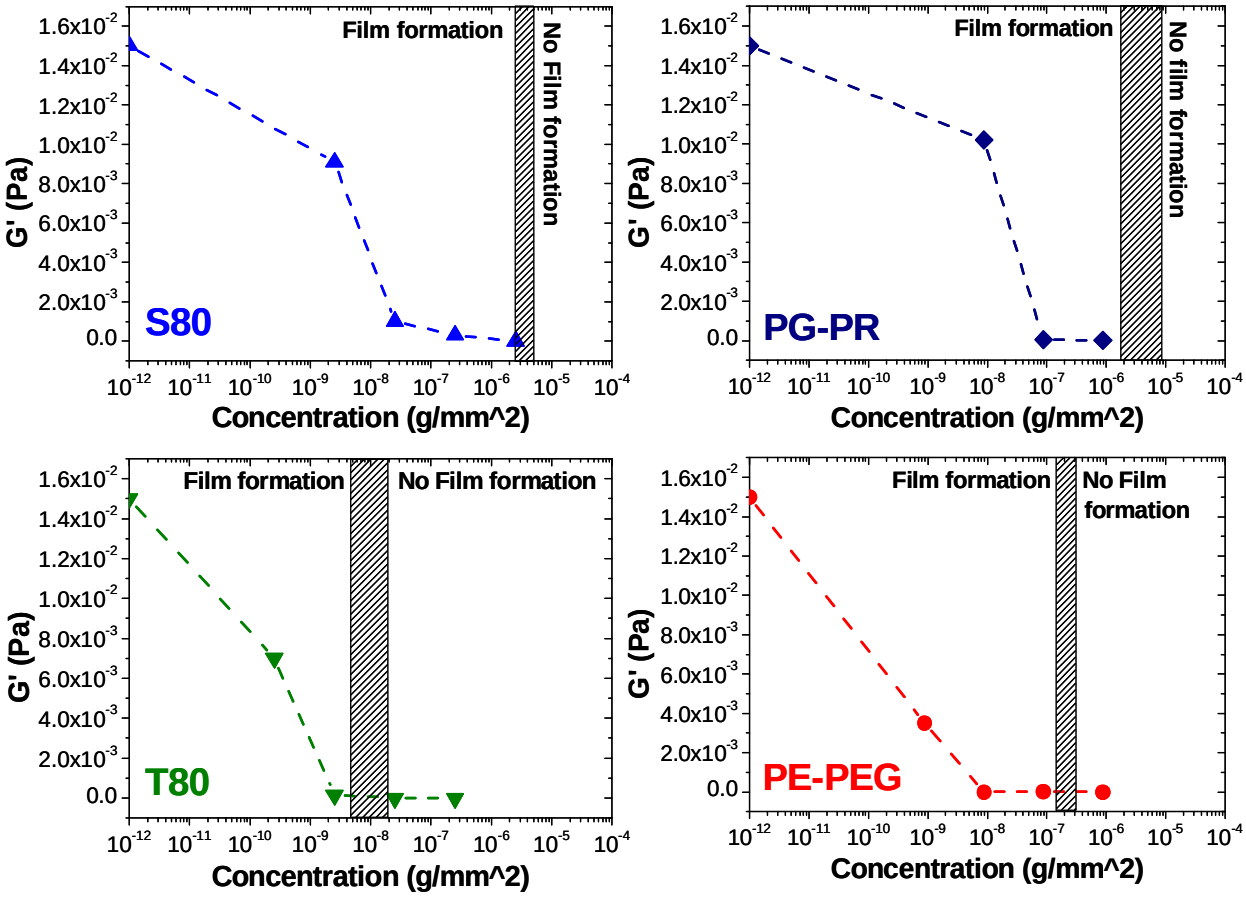

Figure 6: Concentration dependency of G' (determined after 200 min measurement) for lysozyme adsorption in the presence of the different model surfactants compared to the concentrations where the protein film was observed. G' at the concentration of 10^{-12} g/mm^2 corresponds to lysozyme adsorption in the absence of surfactants.

When the concentrations needed for prevention of the multilayer formation for lysozyme the DWR method shows the surfactant in this order: S80>PG-PR>T80>PE-PEG and is therefore approximately in the size order of the head groups. However, when the pendant drop method is used the concentration for prevention of film formation is in this order: PG-PR>S80>PE-PEG>T80.

Both methods show that more hydrophilic surfactants seem more efficient in preventing lysozyme adsorption. The DWR indicates that the larger the surfactant the more efficient whereas the pendant

drop film formation evaluation shows the opposite, however, the differences are less distinct for the pendant drop method.

4. Conclusions

The surface tension measurements give information on the build up of the monolayer where the interfacial rheology is not that strong in the initial step of adsorption. However, the evolvement of the multilayer can be followed by the latter giving information on the later stage of adsorption of lysozyme. Therefore, the two methods are complimentary and the use of both gives detailed information on the adsorption process. When the performance of the surfactants is evaluated the methods show similar results, however, one has to consider the changes in the interfacial area in order to be able to do so. The availability of protein and/or surfactants to adsorb to the interface does play a role in the interfacial adsorption. The surfactants efficiency in prevention of multilayer formation detected by the DWR was PE-PEG>T80>PG-PR>S80 and T80>PE-PEG>S80>PG-PR when observed by the pendant drop method. Both methods show that more hydrophilic surfactants seem more efficient in preventing lysozyme adsorption. However, the DWR indicates that the larger the surfactant the more efficient whereas the pendant drop film formation evaluation shows the opposite. Therefore further studies are needed to be able to conclude on this matter.

Acknowledgements

The authors like to acknowledge TA-instruments for the opportunity to use the Double wall ring system. Additionally, Dennis Isaksen is acknowledged for his skilful work on determining the missing surface tension values and film formation.

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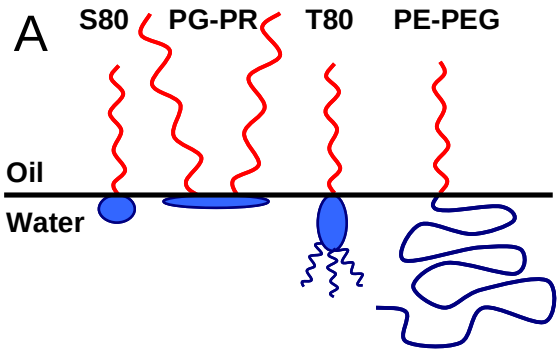

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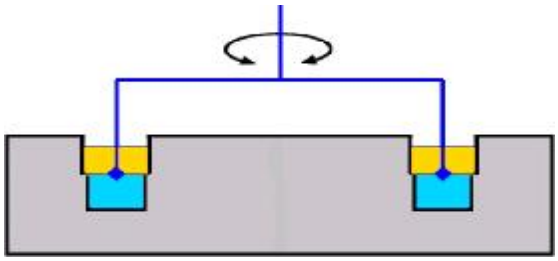

Figure 2: Cross section of the Double Wall Ring geometry (1).

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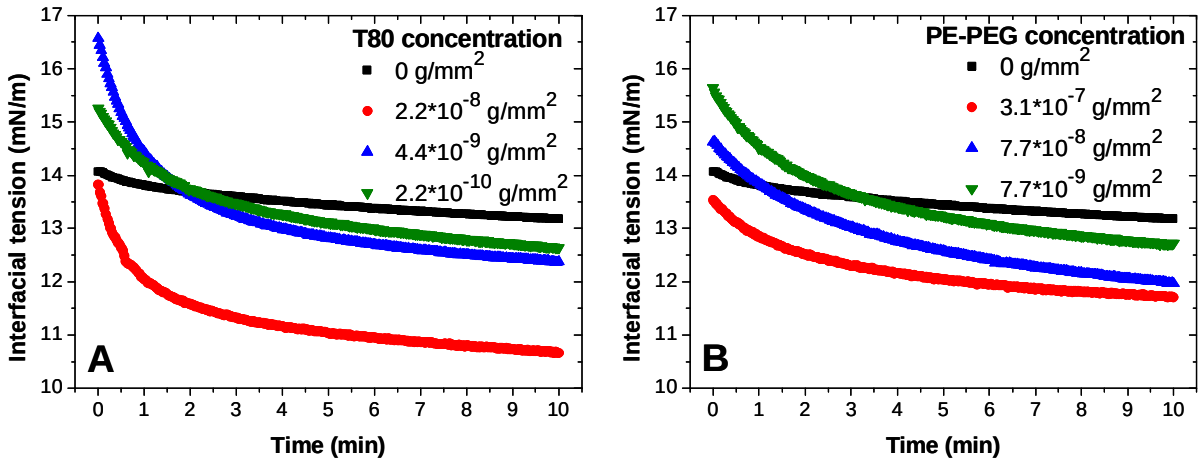

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Figure 4: lysozyme 0.07 mM in phosphate buffer after exposure to the oil phase of 10 min.

A film of lysozyme is detected at $2.5*10^{-6}$ g/mm^2 for S80, $1.8*10^{-6}$ g/mm^2 for PG-PR, $1.5*10^{-7}$ g/mm^2 for PE-PEG and $4.4*10^{-9}$ g/mm^2 for T80. T80 is different as the film formed at the lowest concentration is more fragile than for the other surfactants. In figures 3A and B the red curves represent the lowest concentration where no film is detected.

3.2. Double wall ring:

Lysozyme adsorbs quickly to oil-water interface, where the monolayer is formed instantaneously and then develops further to a multilayer (1). Further conformational changes have also been detected up to 24 hours (5, 6). In contrast to the surface tension method, the DWR clearly shows this evolvement of

the multilayer in the longer time scale as an increase in the complex viscosity in figure 5A and B. Various surfactants have been used in order to hinder the adsorption of proteins to oil-water interfaces (7-10), but little is known about the difference between them. The influence of the presence of different concentrations of T80 is depicted in figure 5A, as complex viscosity as a function of time. A 10 fold increase in the concentration of the surfactant delays the adsorption of the protein, but never the less a multilayer is detected within the time frame of the measurement. A 100 fold increase in the T80 concentration is needed in order to fully prevent the protein adsorption as shown in figure 5A. The same phenomenon was detected for S80 (data not shown), however, a 1000 fold increase in the surfactant concentration was needed to prevent the protein adsorption.

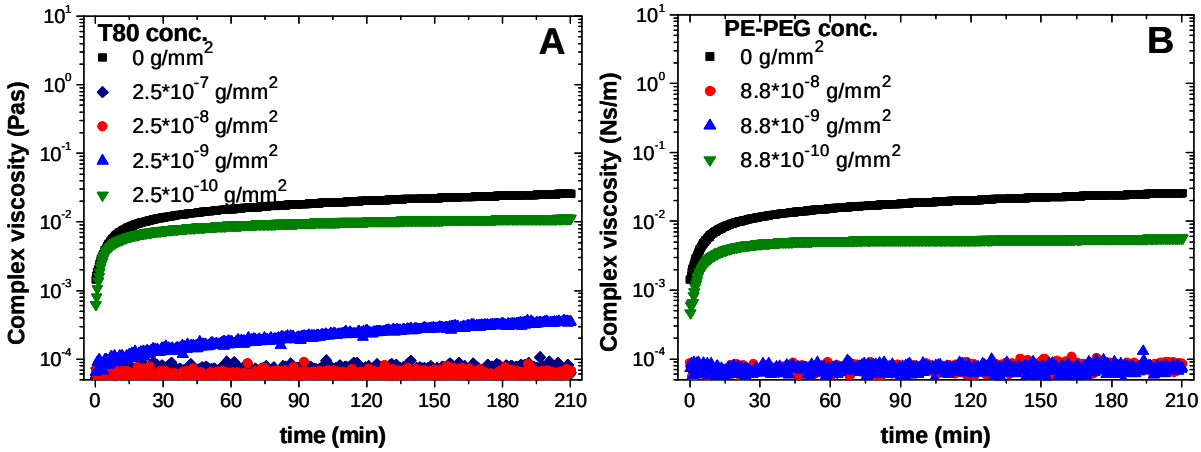

Figure 5: The lysozyme multilayer formation depicted as the complex viscosity (Pas) as a function of time in the presence of various concentrations of **A)** T80 and **B)** PE-PEG.

When the polymeric surfactants (PG-PR or PE-PEG) were used, a 10 fold increase in the surfactant concentration fully prevented the protein adsorption. This can well be seen in figure 5B for the PE-PEG. Increased concentrations of the surfactants decrease lysozyme adsorption and a delayed multilayer formation is seen in the presence of the smaller surfactants, T80 and S80, but not for the polymeric surfactants, PE-PEG and PG-PR, indicating stronger affinity of the polymeric surfactant towards the interface. Thereby larger surfactants seem more effective in preventing the lysozyme adsorption to the interface than the smaller surfactants. However, a multilayer of lysozyme is detected at $2.5*10^{-7}$ g/mm^2 for S80, $8.8*10^{-9}$ g/mm^2 for PG-PR, $2.5*10^{-9}$ g/mm^2 for T80 and $8.8*10^{-10}$ g/mm^2 for PE-PEG and is therefore approximately in the size order of the head groups. This is in good agreement with the results of Szleifer et al that have shown that prevention of protein adsorption by polymers seems to depend on the surface density and not the molecular weight (11). As T80 and S80 are smaller than PG-PR and PE-PEG, the diffusion is faster making the molecules more mobile and therefore the possibility for formation of other structures, e.g. micelles, is higher. Therefore, the small surfactants adsorb to the interface before the protein but eventually give space for the protein and thus causing the delay in the protein adsorption.

3.3. Comparison of the methods:

As discussed earlier the surface tension method gives information on the evolvement of the monolayer, and the first adsorption steps whereas the DWR gives information on the evolvement of the multilayer, and the longer term adsorption events. The various surfactant concentrations are depicted, in figure 6, against G' (value taken after 200 min measurement) as well as the concentrations of the surfactant needed in order to prevent film formation after 10 minutes.

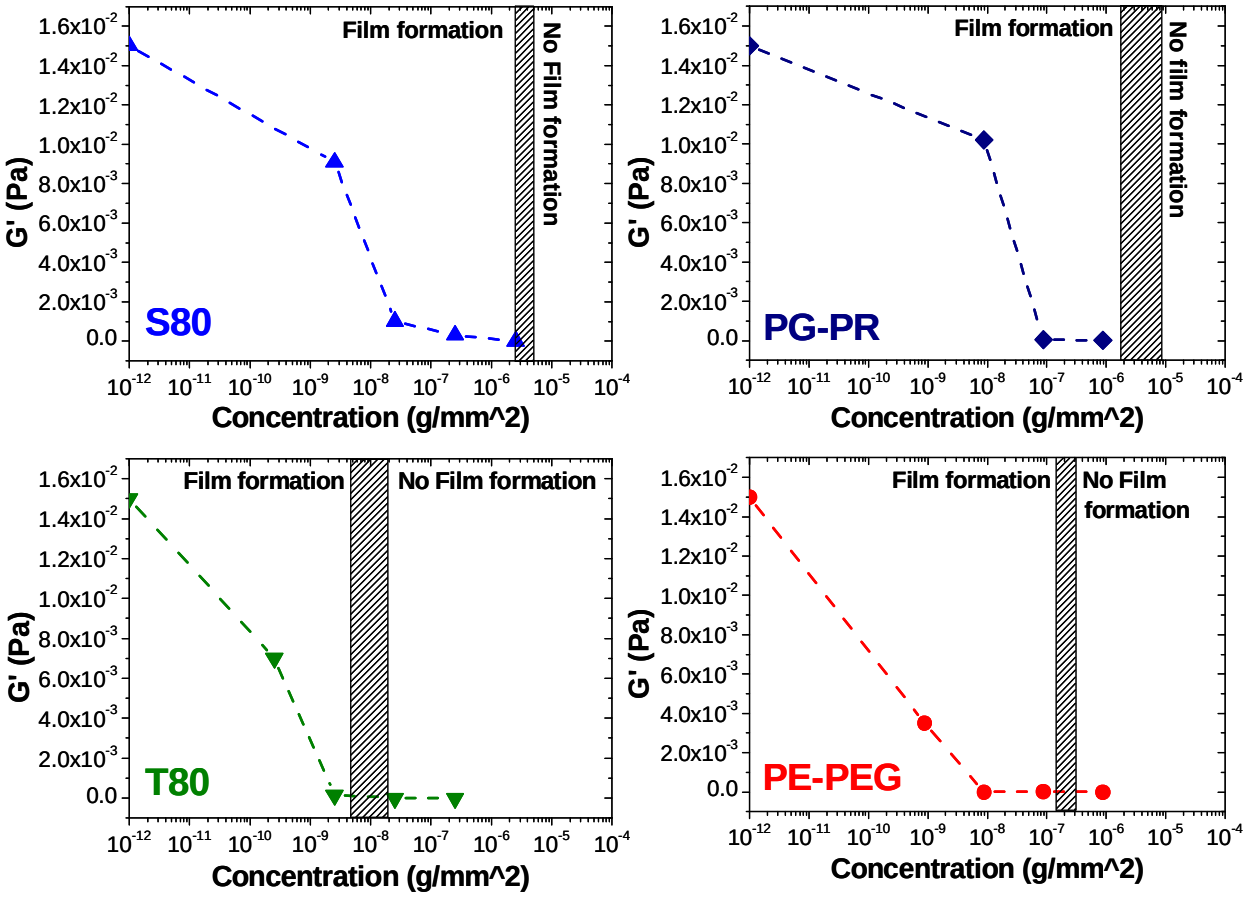

Figure 6: Concentration dependency of G' (determined after 200 min measurement) for lysozyme adsorption in the presence of the different model surfactants compared to the concentrations where the protein film was observed. G' at the concentration of 10^{-12} g/mm^2 corresponds to lysozyme adsorption in the absence of surfactants.

When the concentrations needed for prevention of the multilayer formation for lysozyme the DWR method shows the surfactant in this order: S80>PG-PR>T80>PE-PEG and is therefore approximately in the size order of the head groups. However, when the pendant drop method is used the concentration for prevention of film formation is in this order: PG-PR>S80>PE-PEG>T80.

Both methods show that more hydrophilic surfactants seem more efficient in preventing lysozyme adsorption. The DWR indicates that the larger the surfactant the more efficient whereas the pendant

drop film formation evaluation shows the opposite, however, the differences are less distinct for the pendant drop method.

4. Conclusions

The surface tension measurements give information on the build up of the monolayer where the interfacial rheology is not that strong in the initial step of adsorption. However, the evolvement of the multilayer can be followed by the latter giving information on the later stage of adsorption of lysozyme. Therefore, the two methods are complimentary and the use of both gives detailed information on the adsorption process. When the performance of the surfactants is evaluated the methods show similar results, however, one has to consider the changes in the interfacial area in order to be able to do so. The availability of protein and/or surfactants to adsorb to the interface does play a role in the interfacial adsorption. The surfactants efficiency in prevention of multilayer formation detected by the DWR was PE-PEG>T80>PG-PR>S80 and T80>PE-PEG>S80>PG-PR when observed by the pendant drop method. Both methods show that more hydrophilic surfactants seem more efficient in preventing lysozyme adsorption. However, the DWR indicates that the larger the surfactant the more efficient whereas the pendant drop film formation evaluation shows the opposite. Therefore further studies are needed to be able to conclude on this matter.

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