International Journal of Pharmaceutics 429 (2012) 158–167

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The effect of Tween® 20 on silicone oil–fusion protein interactions

Nitin Dixit[a], Kevin M. Maloney[b], Devendra S. Kalonia[a,*]

[a] Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, 69 North Eagleville Road, Unit 3092, Storrs, CT 06269, USA
[b] Protein Pharmaceutical Development, Biogen Idec, 14 Cambridge Center, Cambridge, MA 02142, USA

ARTICLE INFO

Article history:
Received 20 January 2012
Received in revised form 1 March 2012
Accepted 5 March 2012
Available online 13 March 2012

Keywords:
Silicone oil/water interface
Protein adsorption
Surfactant
Viscoelasticity

ABSTRACT

There is evidence in the literature that silicone oil, a lubricant, can induce aggregation in protein formulations delivered through prefilled syringes. Surfactants are commonly used to minimize protein–silicone oil and protein–container interactions; however, these interactions are not well characterized and understood. The purpose of this manuscript was to understand the competitive interactions of a fusion protein with the silicone oil in the presence of Tween® 20. An adsorption isotherm for Tween® 20 at the silicone oil/water interface, using silicone oil coated quartz crystals, was generated at 25 °C to identify surface saturation concentrations. A concentration of Tween® 20 providing interfacial saturation was selected for protein adsorption studies at the silicone oil/water interface. The surfactant molecules adsorbed at the interface in a monolayer with a reduced viscoelastic character in comparison to the bound protein layer. A significant reduction in protein adsorption was observed when the surfactant was present at the interface. No desorption of the pre-adsorbed protein molecules was observed when Tween® 20 was introduced, suggesting that the protein has strong interactions with the interface. However, both, Tween® 20 and protein, adsorbed to the silicone oil/water interface when adsorption was carried out from a mixture of protein and Tween® 20.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Silicone oil, chemically a poly(dimethylsiloxane), has been widely used as a lubricant coating in the pharmaceutical drug storage and delivery devices, syringe barrels and vial stoppers, to improve processability during manufacturing and functionality during drug delivery (Smith, 1988). Silicone oil has been implicated to be a risk factor for the development of safe and stable biopharmaceutical formulations stored in syringes/vials because of the susceptibility of proteins to form aggregates or insoluble particulates in its presence (Bernstein, 1987; Jones et al., 2005; Majumdar et al., 2011). Such aggregated species have potential to induce immune response in patients and are clinically unacceptable (Rosenberg, 2006).

Because of their amphiphilic nature, proteins are generally surface active and have a tendency to undergo structural alterations and loss of active structure on adsorption to hydrophobic interfaces (Andrade, 1985; Soderquist and Walton, 1980). Since the silicone oil coating in a drug storage/delivery container presents a hydrophobic interface, the potential of a protein facing instability during storage does exist. Surfactants, especially the nonionic e.g. polysorbate 20 and 80, are often added to protein formulations to prevent or minimize the interface induced damage during purification, filtration, transportation, freeze drying and storage (Nema et al., 1997; Randolph and Jones, 2002).

The effectiveness of nonionic surfactants in stabilizing protein formulations has been widely investigated (Bam et al., 1998; Chang et al., 1996; Mollmann et al., 2005; Thurow and Geisen, 1984; Wang et al., 1995), and different mechanisms have been proposed. These include, competition between the surfactant and the protein molecules for the common interface (Chang et al., 1996; Krielgaard et al., 1998; Mollmann et al., 2005), interaction of the surfactant with protein through hydrophobic sites to prevent the potential surface adsorption of the protein and the resulting denaturation (Bam et al., 1998), and the mechanism of preferential exclusion (Timasheff, 2002). Recently it was shown that the use of polysorbate 20 in protein formulations resulted in reduced silicone oil induced monomer loss (Ludwig et al., 2010; Thirumangalathu et al., 2009). Though the mechanism of surfactant action involved in these studies was speculated to the preferred adsorption of the surfactant over protein to the silicone oil surface, no direct experimental evidence was provided.

A mechanistic understanding of the surfactant effectiveness in influencing protein–silicone oil interactions would help protein formulation scientists in designing formulations with optimum stability against interface induced protein damage, resulting in improved product storage stability. The available studies in the literature reporting the effect of surfactants on the protein–silicone

* Corresponding author. Tel.: +1 860 486 3655; fax: +1 860 486 4998.
E-mail address: kalonia@uconn.edu (D.S. Kalonia).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2012.03.005

oil interactions have used a dynamic liquid/liquid system, consisting of silicone oil based emulsions (Ludwig et al., 2010; Thirumangalathu et al., 2009), which closely mimics the condition where silicone oil droplets are leached out in the bulk solution. With rapidly increasing use of prefilled syringes for the delivery of biologics, syringe manufacturers have devised processes (such as 'baking') to obtain physically stable silicone oil coatings (Romacker and Forster, 2008), which are significantly less prone to leaching silicone oil in the solution. In such a scenario, the area of concern for the protein to adsorb, denature and aggregate would be the solid/liquid interface present at the silicone oil lubricated syringe/water contact areas. As pointed out by Mollmann et al. (2005) very few pharmaceutically relevant proteins and nonionic surfactants have been studied directly at the solid/liquid interface. The reason was attributed to both the lack of surface sensitive techniques and inability to perform in situ analysis, which would help in getting a better understanding of the involved mechanism.

We have shown the utility of quartz crystal microbalance (QCM) in determining protein adsorption to the silicone oil/water interface under different solution conditions (Dixit et al., 2011). Briefly, QCM employs a probe consisting of a thin quartz disc with metal electrodes deposited on both faces. Owing to the piezoelectric properties of quartz and its crystalline orientation, application of an external alternating electric potential through the metal electrodes produces an internal mechanical stress in the crystal, leading to its shear deformation and hence, the vibrational motion of the crystal at its resonant frequency (F) (Buttry and Ward, 1992). This resonant frequency is sensitive to any mass change on the crystal surface, as well as, any change in the viscosity–density of the surrounding environment. In 1959, Sauerbrey derived a relationship between the shifts in the crystal resonant frequency and elastic mass bound to the crystal surface (Sauerbrey, 1959):

$$\Delta F = -\frac{2F_o^2}{A\sqrt{\rho\mu}}\,\Delta m \quad (1)$$

where ΔF is the frequency shift (Hz), F_o is the resonant frequency of the crystal (Hz), Δm is the adsorbed amount (g), A is the active electrode area (cm^2), ρ is the density of quartz (2.648 g/cm^3), and μ is the shear modulus of quartz (2.947×10^{11} $g/cm/s^2$).

It has been described previously that when measurements are made in aqueous solutions, the Sauerbrey equation may not hold as the bound layer of the analyte may be inelastic, where a viscous coupling of the bound layer results in additional resonant frequency shifts with a dampening in the resonant oscillation, manifested in an increase in the series resonance resistance (R) of the quartz crystal (Buttry and Ward, 1992). This requires the measurement of the resistance shift (ΔR) of the crystal besides measuring shift in the frequency (ΔF), which serves as an independent measure of the viscous loading by the bound layer on the crystal surface and helps in differentiating an elastic mass effect from viscosity induced effect (Muramatsu et al., 1995). Measuring ΔR provides information about the physical properties of the bound layers, i.e., if it is rigid or viscoelastic. A layer rigidly coupled to the crystal surface dissipates no energy and results in zero change in the R value. However, a viscoelastic surface layer results in a positive shift in the R value because of the energy dissipation associated with viscous coupling. This viscous coupling results in an additional contribution to the total frequency shift and should be separated from the total frequency shift in order to determine the actual mass adsorbed elastically. In order to achieve this separation, the use of ΔR versus ΔF plot has been reported earlier (Muramatsu et al., 1995; Su and Li, 2005).

Here we describe results from our QCM investigation of the adsorption behavior of a pharmaceutically relevant surfactant, Tween® 20 (polysorbate 20), and a fusion protein at a static silicone oil/water interface.

2. Materials and methods

2.1. Materials

AT-cut quartz crystals with optically flat polished gold/titanium electrodes with a fundamental resonant frequency of 5 MHz were acquired from SRS, Inc. (Sunnyvale, CA). Protein used for these studies was an Fc-fusion protein supplied by Biogen Idec (Cambridge, MA) as a 100 mg/mL frozen formulation in 10 mM citrate buffer (pH 6.0, pI range of approximately 5.2–6.5). Silicone fluid (poly(dimethylsiloxane), trimethylsiloxy terminated; PDMS, 10^6 cSt) was obtained from UCT specialties LLC, Bristol, PA. All other chemicals including, Tween® 20, acetic acid, sodium acetate, hydrochloric acid, sodium hydroxide, hexane, hydrogen peroxide and sulfuric acid were obtained from Fisher Scientific (Fair Lawn, NJ). Deionized water equivalent to Milli-Q™ grade was used to prepare all buffer solutions, which were further filtered using 0.1 μm PVDF filters (Millipore, Billerica, MA). Millipore (Billerica, MA) Amicon ultracentrifugal filters (Amicon ultra-15) with a molecular weight cut-off of 10 kDa were obtained from Fisher Scientific.

2.2. Methods

2.2.1. Sample preparation

Acetate buffer at pH 5.0 with 10 mM solution ionic strength maintained using appropriate concentrations of the buffer species and without the addition of salt, was used. Hydrochloric acid (1 N) and sodium hydroxide (1 N) were used to adjust the pH of the buffer solutions. Prior to analysis, protein was buffer exchanged with the desired buffer using Amicon ultra-15 centrifugal filters with a molecular weight cut off of 10 kDa. Appropriate volume of the stock was diluted to 15 mL with the desired buffer and concentrated back to 0.5 mL or less. This process was repeated at least three times to ensure complete exchange with the buffer. Solution pH of the dialyzed sample was measured using pH meter (UB-5, Denver Instruments, Bohemia, NY) connected to an Orion micro pH electrode (Thermo Scientific, Beverly, MA). The concentration of the protein was determined with a UV spectrophotometer (Cary 50-Bio, Varian, Inc., Palo Alto, CA) using an extinction coefficient of 1.25 $mg\,mL^{-1}\,cm^{-1}$ at 280 nm. The desired concentrations of the samples were prepared with dilution using the same buffer. Solutions of Tween® 20 were prepared either in deionized water or buffer.

2.2.2. Physical stability of the silicone oil coating in the presence of Tween® 20

5 MHz quartz crystals were coated with silicone oil (PDMS) using solvent casting method. The methods of silicone oil coating and details related to crystal handling were described earlier (Dixit et al., 2011). The resonant frequency shift because of the polymer deposition was calculated from the crystal resonant frequency, before and after the polymer coating. The physical stability of the PDMS films formed on the crystals was tested in the presence of surfactant solution using the QCM in flow mode. After establishing a stable baseline with respect to F and R signals using deionized water as the solvent, 0.02%, w/v Tween® 20 solution prepared in the same solvent was injected and the signal shifts were monitored. Once a stable signal was achieved, the system was rinsed with the solvent until there was no further change in the F and R values. The crystal was then removed from the assembly and dried using high purity nitrogen, followed by drying at 100 °C for 15 min. Resonant frequency of the crystal was measured (in air) to calculate

the net frequency change due to polymer removal and/or Tween® 20 adsorption. More details related to system handling are given in the following section. The control experiment consisted of exposing the blank (uncoated) crystal to Tween® 20 in a similar manner, and calculations were performed as described earlier by measuring the pre- and post-adsorption resonant frequencies of the crystal.

2.2.3. Adsorption isotherms for Tween® 20 at silicone oil/water interface

The adsorption of Tween® 20 to the silicone oil/water interface was studied in flow injection mode at 25 °C using QCM (QCM 200; SRS Inc., Sunnyvale, CA). 5 MHz quartz crystals, previously coated with the silicone oil were used in the study. Aqueous solutions of Tween® 20 of varying concentrations viz., 0.002%, 0.004%, 0.007%, 0.01%, 0.02%, 0.1% and 0.2%, w/v were used. Briefly, the solvent of interest (triple distilled water) was made to flow through the system using a single syringe pump (NE 1010X; New Era Pump Systems, Inc., Farmingdale, NY) at a rate of 50 μL/min. After establishing a stable baseline with respect to the changes in F and R values in the solvent, 250 μL of a given Tween® 20 sample was introduced into the system via a sample port. The changes in F and R values for the crystal due to the presence of surfactant were recorded as a function of time using QCM connected to an external computer via RS-232 interface at an interval of 10 s using LabView Standalone software (National Instruments Corporation, Austin, TX). The samples were allowed to remain in contact with the crystal surface until no further changes in the F and R values were observed for equilibrium to get established between Tween® 20 molecules in the solution and that adsorbed to the polymer surface. These shifts in the F and R signals were used to calculate the amount of Tween® 20 bound to the silicone oil/water interface at equilibrium as follows: for each individual experiment, the shifts in the frequency (ΔF_1) and resistance (ΔR_1) produced upon the complete immersion of the dried crystal in water/buffer post stabilization were noted and used to calculate the $(\Delta R_1/\Delta F_1)_{\text{viscous}}$. The change in the frequency (ΔF_2) and resistance (ΔR_2) due to the adsorption of surface active species to the silicone oil/water interface was calculated with respect to the crystal immersed and stabilized in water/buffer. Any viscous contribution to ΔF_2 (caused by the bound analyte layer) was obtained by $(\Delta R_2/\Delta F_2)/(\Delta R_1/\Delta F_1)_{\text{viscous}}$. The remaining fraction was multiplied by ΔF_2 in order to obtain the $(\Delta F_2)_{\text{elastic}}$ and was used to calculate the actual mass bound i.e. the elastic contribution, using Sauerbrey equation (Eq. (1)) taking the crystal constant into account.

The system was then rinsed with the same solvent until no further changes in F and R values were observed. Thus, the difference in the F and R values before Tween® 20 injection and after the system rinse were used to calculate the amount of Tween® 20 that was irreversibly adsorbed to the PDMS surface in the time scale of our studies.

2.2.4. Protein adsorption studies in the presence of Tween® 20

Protein adsorption studies at the silicone oil/water interface were carried out in the presence of Tween® 20 at 25 °C using QCM. The studies were conducted at pH 5.0 and 10 mM solution ionic strength, using a protein concentration of 0.1 mg/mL and 0.02%, w/v of Tween® 20 (1:163; protein to Tween® 20 molar ratio). The experimental scheme of sample insertion in the system involved either, the sequential adsorption mode, where the protein and Tween® 20 were introduced in to the system one after the other, respectively, and vice versa, or the co-adsorption mode, where both the protein and Tween® were added together. A similar methodology has been used earlier for the studies carried out with Tween® 80 and lysozyme adsorption onto hydrophobic and hydrophilic silica surfaces using ellipsometry (Joshi and McGuire, 2009).

2.2.4.1. Sequential mode; Tween® 20 followed by protein (Case I).

The system was first stabilized with respect to the F and R signals with the buffer flowing over the silicone oil coated quartz surface. Tween® 20 at 0.02%, w/v concentration was introduced into the system and allowed to stay in contact with the adsorbent surface with adsorption monitored, until stable F and R values were attained. The system was then rinsed with the buffer to remove any surfactant that was either present in the bulk, or reversibly bound to the surface. Protein was then introduced in to the system and any further changes in the signals were monitored until signal stability was achieved. This was followed by the buffer rinse and signal monitoring. Calculations were performed as described before to obtain the mass of protein adsorbed in the presence of Tween® 20. Studies were also carried out where rinsing of the pre-adsorbed Tween® 20 molecules was avoided before the protein introduction.

2.2.4.2. Sequential mode; protein followed by Tween® 20 (Case II).

A similar procedure as in case I was used with the sequence of sample introduction followed in reverse. The protein adsorption was first monitored, followed by buffer rinse after which Tween® 20 was introduced in the system, followed by a final buffer rinse. Each step was monitored till stability in F and R values was obtained. Amount of protein and Tween® 20 bound was calculated taking into account the F and R values obtained after each sample introduction and solvent rinsing.

2.2.4.3. Co-adsorption (Case III).

Protein and Tween® 20 were mixed together to obtain a solution containing 0.1 mg/mL protein in 0.02%, w/v Tween® 20 and incubated for at least 1 h prior to analysis. The mixture was introduced into the system, previously equilibrated with the buffer, and allowed to stay in the surface contact. Adsorption was monitored with time until stable signals were achieved. The system was then rinsed with buffer to remove any reversibly bound species. Calculations were performed using the F and R values to calculate the total combined mass adsorbed to the silicone oil/water interface.

3. Results and discussion

3.1. Silicone oil coating and its physical stability in the presence of Tween® 20

In a previous study, it was shown that the coatings obtained with high viscosity PDMS were stable in the presence of water (Dixit et al., 2011). Upon exposure of the PDMS coating to a Tween® 20 solution, two outcomes are possible. One, the surfactant molecules can adsorb to the PDMS surface resulting in a frequency decrease (a larger ΔF compared to PDMS coated crystal). Two, surfactant molecules solubilize the PDMS coating which would result in partial or complete removal of the coating. The overall change in ΔF observed will be a result of the change caused by the removal of silicone oil coating and adsorption of the surfactant to PDMS and/or gold surface. Fig. 1 shows the shifts in the resonant frequency of the quartz crystal (measured in air) after the crystals (uncoated or PDMS coated) were exposed to 0.02%, w/v Tween® 20 solution and then dried. For comparison, the frequency shift in crystal resonance because of PDMS coating is also shown. The resonant frequency shift for the uncoated quartz crystal following Tween® 20 adsorption is 3 ± 2 Hz. If the PDMS coating was completely dissolved by the surfactant then ΔF should be of the same magnitude as for the gold surface in the presence of the surfactant. Whereas partially dissolved coating would result in a significant reduction in ΔF compared to the PDMS coating. Bar C in Fig. 1 shows that there is no decrease in ΔF when PDMS coating is exposed to 0.02%, w/v

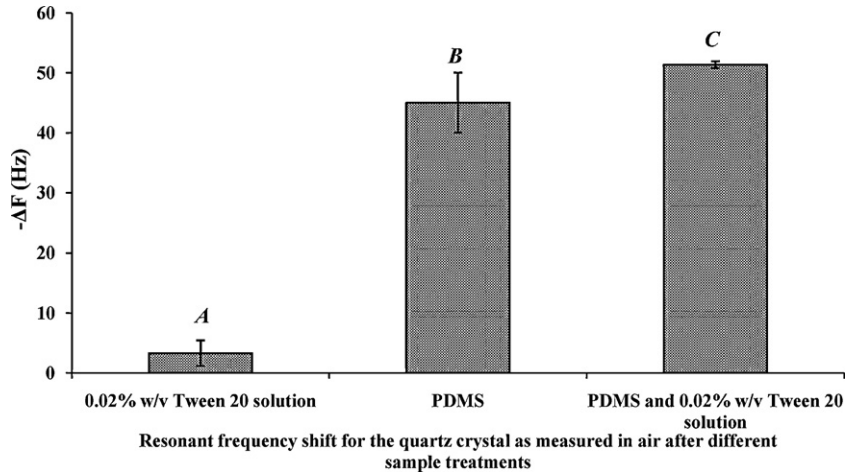

Fig. 1. Effect of Tween® 20 on the PDMS coating. All the frequency measurements were done in air and are in comparison with uncoated (gold plated) quartz crystals. A – frequency shift observed when uncoated crystal was exposed to 0.02% Tween® 20 and dried; B – shift due to PDMS coating and drying; C – shift for the PDMS coated crystal when exposed to 0.02% Tween® 20 and dried ($n \geq 3$).

Tween® 20 for 100 min. This shows that the coating is stable in the presence of the surfactant for the duration of the experiment.

3.2. Kinetics of protein and Tween® 20 adsorption to the silicone oil/water interface

Fig. 2 compares the kinetic profiles of Fc-fusion protein (0.1 mg/mL) and Tween® 20 (0.02%, w/v) adsorption to the silicone oil/water interface at pH 5.0 and 10 mM solution ionic strength. The introduction of the protein into the system causes a drop in the crystal resonant frequency corresponding to protein adsorption at the silicone oil/water interface (Fig. 2A). The adsorption attained a constant value within 30 min as seen by the stability of the frequency signal. Rinsing the system with the buffer causes a <10% of the protein to desorb as seen by the recovery of the frequency shift. However, for the duration of our studies, the protein remains irreversibly bound to the silicone oil/water interface. Such an apparent irreversibility of the protein adsorption on hydrophobic polymeric interfaces by different techniques has been reported in the literature (Andrade, 1985; Brash et al., 1974; Norde and Lyklema, 1978). Similarly, adsorption of Tween® 20 to the silicone/water interface also causes a drop in the crystal resonant frequency; however, the magnitude of the shift is significantly smaller than that observed for the protein. Adsorption of Tween® 20 reached a plateau within 30 min. Introduction of the solvent recovers a small fraction of the frequency shift corresponding to the removal of reversibly adsorbed Tween® 20 molecules. However, a significant amount of Tween® 20 remains irreversibly adsorbed to the silicone oil/water interface over the time span of the experiments. This observation is consistent with previous results on hydrophobic interfaces for different surfactants (Joshi and McGuire, 2009; Liu and Kim, 2009). However, studies on surfaces of varying wettability showed Tween® desorption when rinsed with buffer, suggesting its reversibility (Elwing et al., 1989).

Resistance or dissipation denotes the viscous contribution to the total frequency shift and represents the energy loss associated with adsorption. A rigidly coupled film shows negligible resistance shift due to its elasticity, however, a positive shift in resistance denotes the formation of an adsorbed layer with viscous properties. Fig. 2B shows the resistance shifts upon Tween® 20 adsorption to the silicone oil/water interface. Adsorption of Tween® 20 to the interface causes a significant rise in the resistance signal, and is larger than that seen for the adsorption of the protein. This suggests that the surfactant film is relatively less viscoelastic than the protein film. Once the adsorption is complete, rinsing the system with the solvent leaves a layer of the surfactant molecules which still shows significant viscous properties as seen by the high residual resistance at the end of the experiment (Fig. 2B).

Fig. 3 compares the properties of the adsorbed layers of Tween® 20 and the fusion protein at the silicone oil/water interface with the reference viscous and elastic systems. Deionized water represents a viscous system (frequency shift is purely due to viscous effect; 100% viscous contribution), where the average slope $(\Delta R_1/\Delta F_1)_{\text{viscous}}$ was 0.41 Ω/Hz. The same slope would be zero for a purely elastic system (no energy dissipation associated with the bound layer i.e. $\Delta R_1 = 0$, and frequency shift is purely due to the elastic effect; 0% viscous contribution). The Fc-fusion protein forms a layer which is nearly rigid (4% average viscous contribution to the frequency shift) whereas in comparison, the bound layer of Tween® 20 has significantly higher viscous character (30% average viscous contribution to the frequency shift).

3.3. Adsorption isotherms for Tween® 20 at silicone oil/water interface

The hydrophobic forces that drive the adsorption of surfactants at the air/water interface and subsequent formation of micelles in the bulk are essentially the same as those that promote the adsorption of the surfactant molecules to a hydrophobic solid/liquid interface. However, in comparison to air/water interface, the adsorption to a solid/liquid interface differs as follows: first, the solid surface could be a source of additional interactions such as electrostatics from the ionized surface groups, and second, whereas at an air/liquid interface the penetration of the hydrophobic tail of the surfactant is allowed, it is not permitted on a solid/liquid interface.

Adsorption isotherm is obtained by measuring the depletion of the bulk surfactant as a function of equilibrium bulk concentration and can be used to obtain adsorption affinity, surfactant concentration achieving surface saturation and the orientation of the adsorbed molecules. The adsorption of Tween® 20 at the rigid silicone oil coating/formulation interface as a function of its bulk concentration is shown in Fig. 4. The surfactant concentrations used are both above and below the reported CMC values (0.006%, w/v (Mittal, 1972); 0.007%, w/v (Patist et al., 2000)). The saturation of the interface is observed near 0.007%, w/v bulk Tween® 20 concentration as the mass adsorbed does not change significantly thereafter at higher concentrations. This surfactant

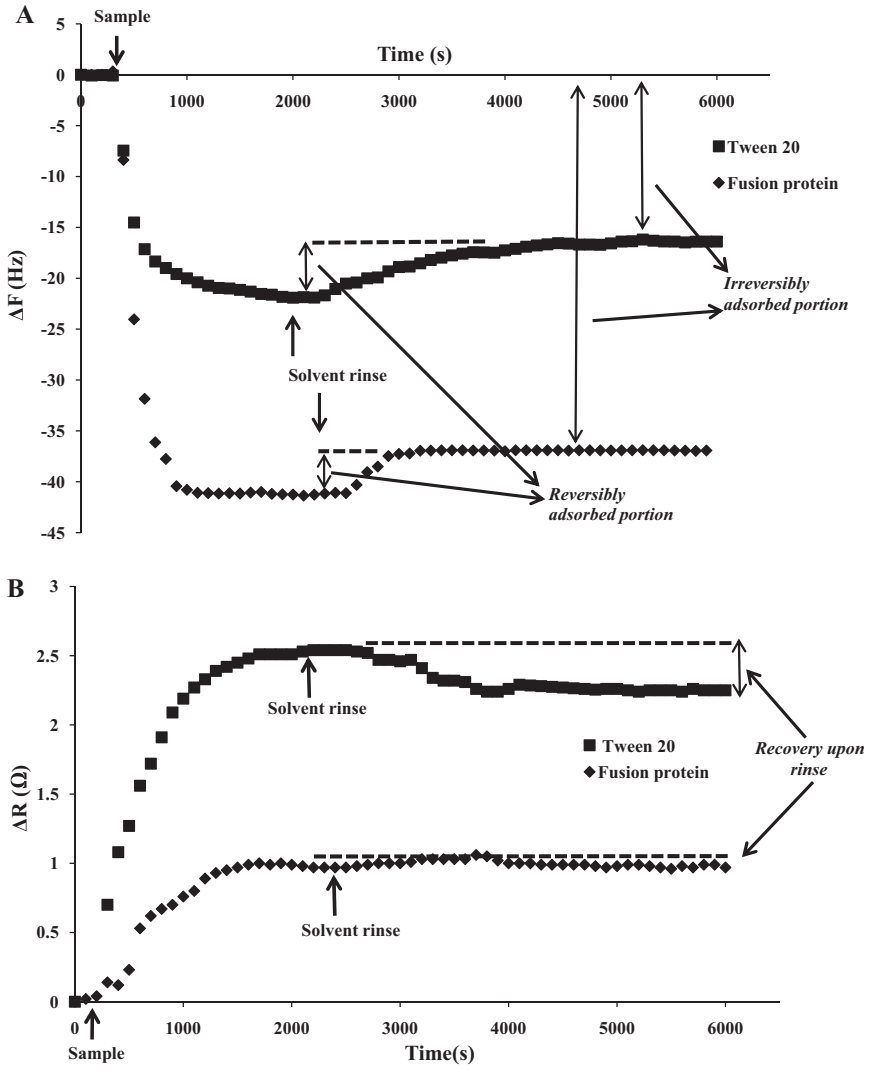

Fig. 2. (A) Time course of frequency shifts observed for the Fc-fusion protein (0.1 mg/mL) and Tween® 20 (0.02%, w/v) adsorption to the silicone oil/water interface at pH 5.0 and 10 mM solution ionic strength. (B) Time course of resistance shifts observed for the Fc-fusion protein (0.1 mg/mL) and Tween® 20 (0.02%, w/v) adsorption to the silicone oil/water interface at pH 5.0 and 10 mM solution ionic strength.

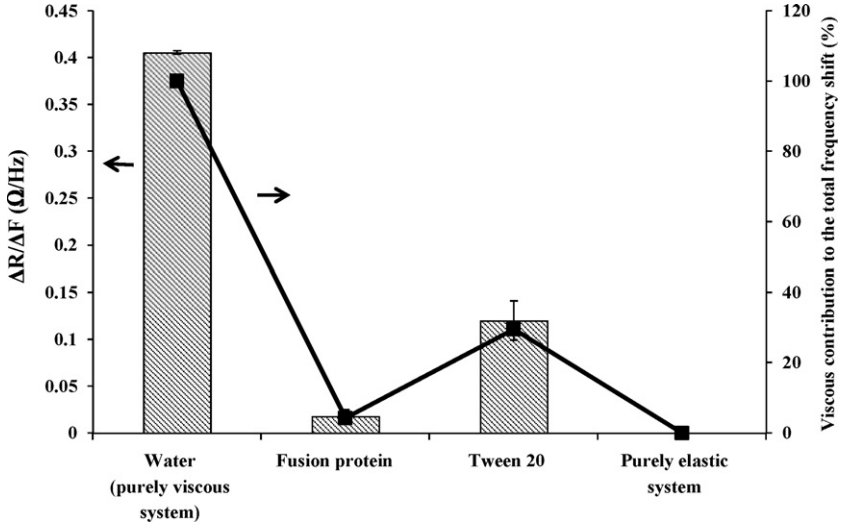

Fig. 3. Viscoelastic properties of the adsorbed layer of the Fc-fusion protein (0.1 mg/mL) and Tween® 20 (0.02%, w/v) to the silicone oil/water interface at pH 5.0 and 10 mM solution ionic strength as determined by the comparison to a purely viscous and a completely elastic system.

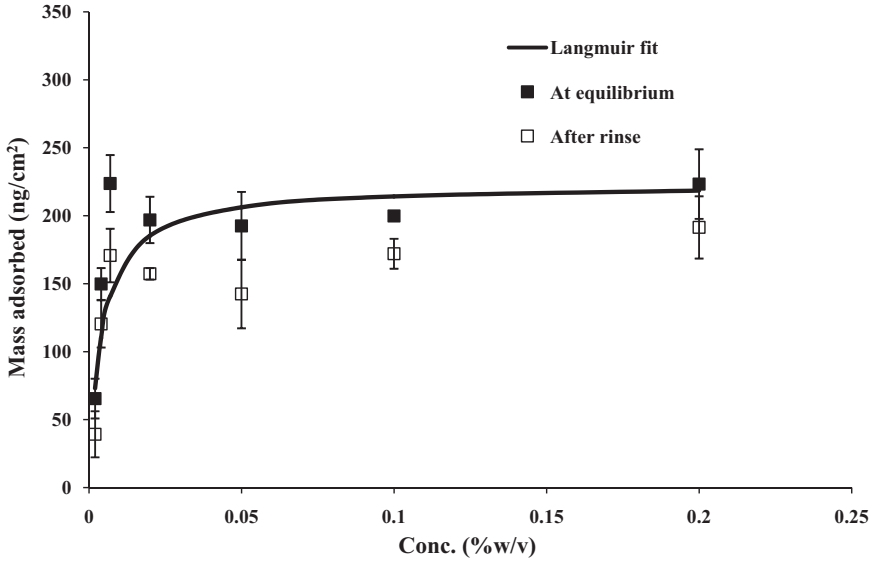

Fig. 4. Amount of Tween® 20 (in water) adsorbed to the silicone oil/water interface at equilibrium (adsorption isotherms) and remaining adsorbed after the rinse as a function of its bulk concentration at 25 °C using QCM. Solid line is a Langmuir fit to the data ($n \geq 2$).

concentration required for the saturation of silicone oil/water interface in the present case is similar as for the saturation of the air/water interface, suggesting that the hydrophobic interactions govern the adsorption of Tween® 20 to the silicone oil/water interface. Fig. 4 shows a type I adsorption isotherm, which is consistent with a Langmuir monolayer adsorption model.

The monolayer adsorption model was utilized to get information about the orientation of Tween® 20 molecules at the interface. Using Eq. (2), and taking the adsorbed mass, the area occupied by each Tween® 20 molecule at the silicone oil/water interface was calculated to be 91 Å^2.

$$\text{Molecular area } (\text{Å}^2) = \frac{10^{16}}{N_A \times m} \quad (2)$$

where N_A is the Avogadro's number and m is the amount of surfactant (in moles) required to form a monolayer on the silicone oil/water interface. Niño and Patino found an average area of 46.5 Å^2 for Tween® 20 monomer at the air/water interface (Nino and Patino, 1998), where the surfactant molecules could be vertically oriented. Our theoretical calculations, taking into account the lengths of the respective bonds of the lauric acid chain (the moiety in contact with the adsorbing surface), give an area of ~40 Å^2 in horizontal orientation. This suggests that Tween® 20 molecules are adsorbed in a loosely compacted layer because of the steric hindrance associated with the bulky oxyethylene moiety. Fig. 4 also shows that the amount of Tween® 20 remaining irreversibly bound to the silicone oil/water interface, after rinsing the system with the solvent, is on average 77% of the amount adsorbed at the equilibrium. In the time scale of our adsorption studies, this irreversibly bound fraction is much smaller than that observed for the protein (Dixit et al., 2011).

3.4. Protein adsorption in the presence of Tween® 20

3.4.1. Case I: protein adsorption following Tween® 20 adsorption

Protein adsorption studies were carried out at pH 5.0 and 10 mM ionic strength because under these conditions the adsorption of the protein was maximum at the silicone oil/water interface (Dixit et al., 2011). Protein bulk concentration of 0.1 mg/mL was sufficient to provide interfacial saturation. Tween® 20 concentration of 0.02%, w/v was selected because it is above its' reported CMC and is sufficient to achieve the saturation of the silicone oil/water interface (Fig. 4). This concentration is also in the range of commonly used Tween® 20 concentration in pharmaceutical industry for protein formulations (0.0003–0.3%, w/v) (Kerwin, 2008). The protein and surfactant concentrations used here did not change the bulk properties (viscosity, density, and viscoelasticity) of the solution to significantly affect the QCM signal.

Fig. 5 shows the adsorption kinetics of Tween® 20 followed by the adsorption kinetics of the Fc-fusion protein at the silicone oil/water interface, as monitored by F and R shifts in the QCM signal. Introduction of 0.02%, w/v Tween® 20 solution led to a decrease in the frequency (because of the surfactant adsorption at the interface) with a simultaneous increase in the resistance (formation of viscoelastic layer at the interface). After obtaining stable signals when no further change in these parameters was observed, the system was rinsed with the buffer which removed the bulk Tween® 20 molecules. Any reversibly adsorbed molecules were also removed during this rinse cycle and can be seen by a frequency increase and resistance shift recovery, both of which reach a plateau after some time. Upon protein introduction in the system, a further decrease in the resonant frequency was observed which indicates the adsorption of protein at the interface. However, the extent of frequency decrease was significantly less than that observed for the protein adsorption at the silicone oil/water interface in the absence of any Tween® 20 (Fig. 2A). This suggests that the presence of pre-adsorbed Tween at the interface leads to a reduction in protein adsorption, but the presence of Tween® 20 did not completely inhibit the adsorption of the protein to the interface.

The resistance signal showed a slight drop when the protein was introduced into the system treated with Tween® 20. This drop could be attributed to the displacement of a fraction of adsorbed Tween® 20 molecules from the surface. On rinsing the system with the solvent, a slight frequency increase can be seen which is attributed to desorption of weakly adsorbed protein molecules. This is supported by the observation that the decrease in the resistance is negligible. It was shown previously in Fig. 2B that the resistance drop after rinsing is negligible for a protein whereas it is significant for the Tween® 20. The final resistance value at the end of the experiment is still significantly higher than that seen with the protein adsorption to the interface alone, suggesting that the protein did not displace Tween® 20 molecules from the interface to a significant extent.

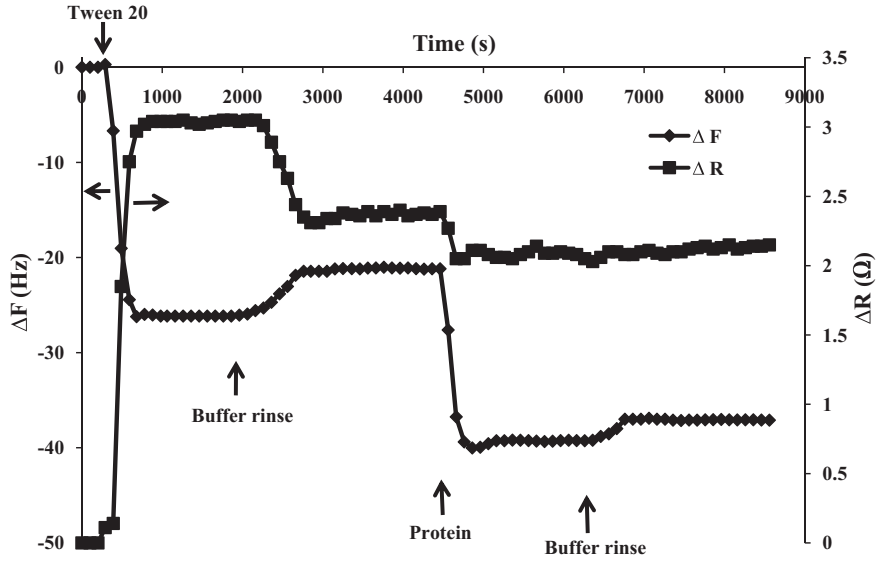

Fig. 5. Time course of frequency and resistance changes as observed for the Tween® 20 and Fc-fusion protein adsorption in sequential mode at pH 5.0 and 10 mM solution ionic strength to the silicone oil/water interface with QCM at 25 °C.

3.4.2. Case II: Tween® 20 adsorption following protein adsorption

Fig. 6 shows the kinetics of protein adsorption at the silicone oil/water interface, which is followed by buffer rinse, and then introduction of Tween® 20. The adsorption of the protein results in a significantly larger resonant frequency decrease and a much smaller crystal resonant resistance increase in comparison to Tween® 20 adsorption to the silicone oil alone (Fig. 2A). After rinsing the system containing the adsorbed protein, addition of 0.02%, w/v Tween® 20 led to a further decrease in the crystal resonant frequency, indicating an increase in the adsorbed mass because of Tween® 20 adsorption. Nevertheless, the magnitude of the frequency decrease was significantly less than that observed for Tween® 20 adsorption to the silicone oil/water interface alone (Fig. 2A). This increase in the adsorbed mass in the presence of pre-adsorbed protein could be attributed to the adsorption of Tween® 20 at the empty sites at the interface and/or on to the adsorbed protein molecules. Despite the fact that the amount of Tween® 20 adsorbed in the presence of pre-adsorbed protein is less compared to Tween® 20 adsorbed at the silicone oil/water interface alone, a high magnitude of resistance increase was observed, which is similar to that observed for Tween® 20 alone. Rinsing the system further, once a plateau in the QCM signals is achieved, led to an increase in the crystal resonant frequency accompanied by a decrease in the resonant resistance, characteristic of desorption of reversibly adsorbed Tween® 20 molecules. Since the frequency shift observed at the end of this experiment was much larger in magnitude than for Tween® 20 adsorption alone to the interface, it suggests that Tween® 20 was not able to displace the protein molecules from the silicone oil/water interface.

It has been previously shown that the ability of a surfactant to displace surface adsorbed protein decreases with an increase in the protein surface residence time at the hydrophobic surfaces

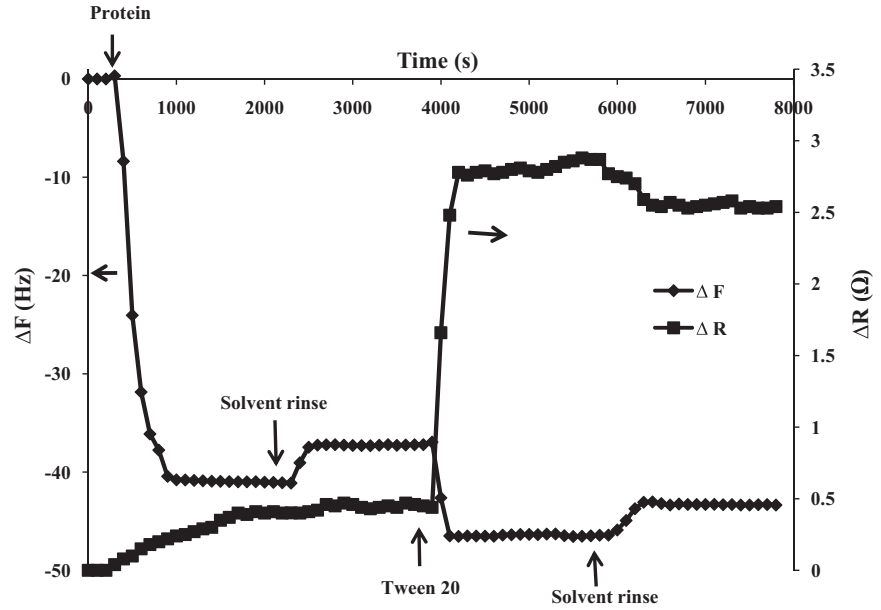

Fig. 6. Time course of frequency and resistance changes as observed for the Fc-fusion protein and Tween® 20 adsorption in sequential mode at pH 5.0 and 10 mM solution ionic strength to the silicone oil/water interface with QCM at 25 °C.

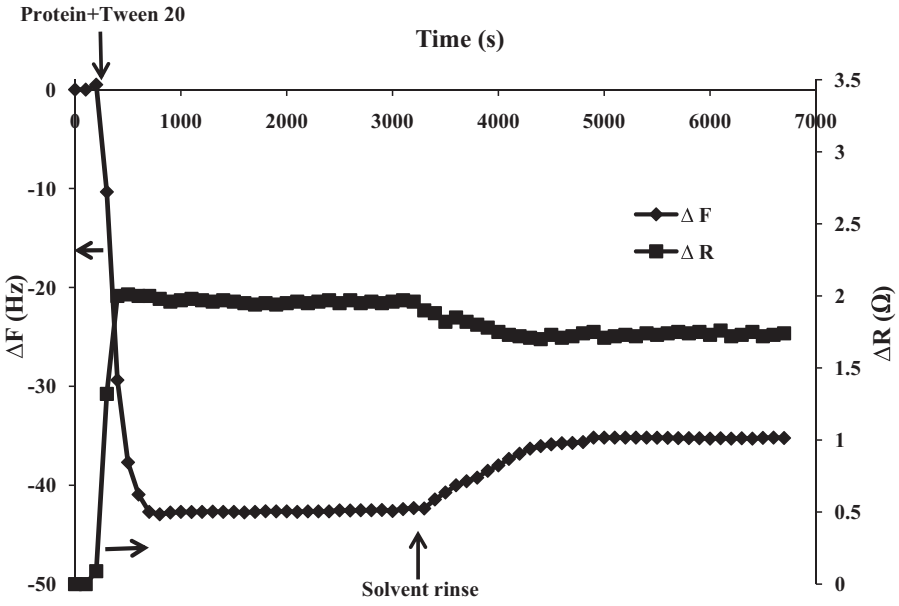

Fig. 7. Time course of frequency and resistance changes as observed for the Tween® 20 and Fc-fusion protein co-adsorption at pH 5.0 and 10 mM solution ionic strength to the silicone oil/water interface with QCM at 25 °C.

(Chen and Dickinson, 1993; Elwing et al., 1989; Rapoza and Horbett, 1990). Since the protein molecules make contacts with a nonpolar interface through hydrophobic residues, the protein conformation can change to minimize the contact of the hydrophobic residues with water. This can lead to multiple contacts resulting in a stronger or rigid adsorption resulting in irreversible binding. Over the time scale of this study, the Fc-fusion protein adsorbs at the silicone oil/water interface irreversibly, which is evident by a low $\Delta R/\Delta F$ ratio and a small change in the resonant frequency after rinsing with the buffer. This rigid binding of the protein could be responsible for the inability of the surfactant to displace the protein from the silicone oil/water interface.

3.4.3. Case III: protein–Tween® 20 co-adsorption

One of the mechanisms by which the nonionic surfactants protect the protein molecule against interface induced damage is by binding to its hydrophobic sites (Bam et al., 1998) and preventing adsorption to the hydrophobic surface. Whether Tween® 20 prevents protein adsorption to the silicone oil/water interface was investigated by studying the adsorption from a mixture of Tween® 20 and the fusion protein. Fig. 7 shows the kinetics of adsorption at the silicone oil/water interface when a solution containing protein (0.1 mg/mL) and Tween® 20 (0.02%, w/v) was added. The adsorption of the surface active species to the silicone oil/water interface causes a decrease in the resonant frequency of the crystal. The magnitude of the shift is similar to the total frequency shift caused by Tween® adsorption followed by protein adsorption to the interface (Case I above; Fig. 5) and is also similar to the frequency shift caused by adsorption of the protein to the interface alone (Fig. 2A). However, the frequency shift is accompanied by an increase in the resonant resistance (~2.0 Ω), which is a characteristic of Tween® 20 adsorption. On the other hand, the frequency decrease at

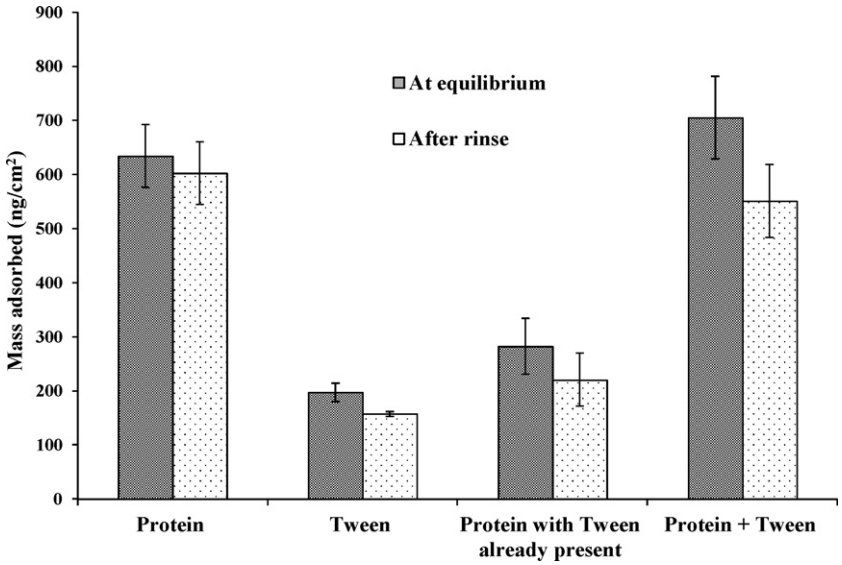

Fig. 8. Comparison of results for the adsorption of Tween® 20 and Fc-fusion protein to the silicone oil/water interface at pH 5.0 and 10 mM solution ionic strength and 25 °C ($n \geq 2$).

equilibrium is significantly greater in magnitude than that observed for Tween® 20 adsorption to the interface alone. This suggests that both Tween® 20 and the protein are adsorbed at the interface when a mixture is used.

3.5. Comparison of adsorption results

In QCM, the adsorbed mass is calculated from the resonant frequency decrease of the crystal. It is not possible to differentiate between the binding species where two components, which can reduce the frequency of the crystal by adsorption, are present together. However in the present case, the resistance shift can be used as a useful indicator to distinguish different adsorbed species. Fig. 8 compares the adsorption of protein and Tween® 20 both at the equilibrium and after solvent rinse of the system. A rinsing step simplifies the interpretation of the adsorption results. A reduction in the adsorbed mass from the crystal surface after a rinse is considered to be the characteristic of the reversibly bound species. The adsorption of both the protein and Tween® 20 was essentially irreversible to a significant extent, with Tween® 20 showing a greater reversibility than the protein. There is a reduction in the mass of protein that binds to the silicone oil/water interface when Tween® 20 was present as a pre-adsorbed species. When the protein is introduced in the system after surfactant adsorption, the extent of protein adsorption does not change significantly whether the system is rinsed or not rinsed with buffer before the introduction of the protein. The results point to the fact that there is a monolayer of surfactant that binds to hydrophobic interfaces in an irreversible manner (Grant et al., 2000; Liu and Kim, 2009), which could prevent/minimize the protein adsorption.

4. Conclusion

Adsorption of Tween® 20 at the solid silicone oil/water interface occurred in a loosely packed monolayer pattern with the bound surfactant layer possessing a significantly greater viscous character in comparison to the bound protein at the same interface. Tween® 20 was found to be effective in significantly reducing the protein adsorption to the silicone oil layer when present as a pre-adsorbed species, however, it was not effective when the surfactant was introduced after the interfacial protein adsorption is complete. Adsorption of both the protein and the surfactant occurred when the adsorption was carried out from a mixture.

Protein adsorbed at the hydrophobic silicone oil/water interface can undergo denaturation. This denatured species can revert back to the solution to cause aggregation in the bulk by combining with similar molecules. In a protein formulation stored in prefilled syringe, this process of protein surface adsorption, denaturation, and desorption can continue to cause increased bulk protein aggregation over the product shelf life (~2 years). Surfactants are added to minimize these protein–silicone oil interactions, however, our knowledge still lacks regarding their mechanism at the interface. Studying the competitive adsorption behavior of protein and the surfactant directly at the silicone oil surface will help us better comprehend these interactions, and to design formulations with optimum stability in the long run. Work is in progress to study the long term adsorption–desorption behavior of this protein at the silicone oil/water interface.

Acknowledgment

The authors are thankful to Biogen Idec for supplying the protein and financially supporting this work.

References

Andrade, J.D., 1985. Surface and Interfacial Aspects of Biomedical Polymers. Protein Adsorption, vol. 2. Plenum Press.
Bam, N.B., Cleland, J.L., Yang, J., Manning, M.C., Carpenter, J.F., Kelley, R.F., Randolph, T.W., 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87, 1554–1559.
Bernstein, R.K., 1987. Clouding and deactivation of clear (regular) human insulin: association with silicone oil from disposable syringes? Diabetes Care 10, 786–787.
Brash, J.L., Uniyal, S., Samak, Q., 1974. Exchange of albumin adsorbed on polymer surface. Trans. Am. Soc. Artif. Intern. Organs 20-A, 69–76.
Buttry, D.A., Ward, M.D., 1992. Measurement of interfacial processes at electrode surfaces with the electrochemical quartz crystal microbalance. Chem. Rev. 92, 1355–1379.
Chang, B.S., Kendrick, B.S., Carpenter, J.F., 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci. 85, 1325–1330.
Chen, J., Dickinson, E., 1993. Time-dependent competitive adsorption of milk proteins and surfactants in oil-in-water emulsions. J. Sci. Food Agric. 62, 283–289.
Dixit, N., Maloney, K.M., Kalonia, D.S., 2011. Application of quartz crystal microbalance to study the impact of pH and ionic strength on protein–silicone oil interactions. Int. J. Pharm. 412, 20–27.
Elwing, H., Askendal, A., Lundstroem, I., 1989. Desorption of fibrinogen and γ-globulin from solid surfaces induced by a nonionic detergent. J. Colloid Interface Sci. 128, 296–300.
Grant, L.M., Ederth, T., Tiberg, F., 2000. Influence of surface hydrophobicity on the layer properties of adsorbed nonionic surfactants. Langmuir 16, 2285–2291.
Jones, L.S., Kaufmann, A., Middaugh, C.R., 2005. Silicone oil induced aggregation of proteins. J. Pharm. Sci. 94, 918–927.
Joshi, O., McGuire, J., 2009. Adsorption behavior of lysozyme and Tween 80 at hydrophilic and hydrophobic silica–water interfaces. Appl. Biochem. Biotechnol. 152, 235–248.
Kerwin, B.A., 2008. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. J. Pharm. Sci. 97, 2924–2935.
Krielgaard, L., Jones, L.S., Randolph, T.W., Frokjaer, S., Flink, J.M., Manning, M.C., Carpenter, J.F., 1998. Effect of Tween 20 on freeze-thawing- and agitation-induced aggregation of recombinant human factor XIII. J. Pharm. Sci. 87, 1593–1603.
Liu, S.X., Kim, J.-T., 2009. Study of adsorption kinetics of surfactants onto polyethersulfone membrane surface using QCM-D. Desalination 247, 355–361.
Ludwig, D.B., Carpenter, J.F., Hamel, J.-B., Randolph, T.W., 2010. Protein adsorption and excipient effects on kinetic stability of silicone oil emulsions. J. Pharm. Sci. 99, 1721–1733.
Majumdar, S., Ford, B.M., Mar, K.D., Sullivan, V.J., Ulrich, R.G., D'Souza, A.J.M., 2011. Evaluation of the effect of syringe surfaces on protein formulations. J. Pharm. Sci. 100, 2563–2573.
Mittal, K.L., 1972. Determination of CMC of polysorbate 20 in aqueous solution by surface tension method. J. Pharm. Sci. 61, 1334–1335.
Mollmann, S.H., Elofsson, U., Bukrinsky, J.T., Frokjaer, S., 2005. Displacement of adsorbed insulin by Tween 80 monitored using Total Internal Reflection Fluorescence and Ellipsometry. Pharm. Res. 22, 1931–1941.
Muramatsu, H., Egawa, A., Ataka, T., 1995. Reliability of correlation between mass change and resonant frequency change for a viscoelastic-film-coated quartz crystal. J. Electroanal. Chem. 388, 89–92.
Nema, S., Washkuhn, R.J., Brendel, R.J., 1997. Excipients and their use in injectable products. PDA J. Pharm. Sci. Technol. 51, 166–171.
Nino, M.R.R., Patino, J.M.R., 1998. Surface tension of bovine serum albumin and Tween 20 at the air–aqueous interface. J. Am. Oil Chem. Soc. 75, 1241–1248.
Norde, W., Lyklema, J., 1978. The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces: I. Adsorption isotherms. Effects of charge, ionic strength, and temperature. J.Colloid Interface Sci. 66, 257–265.
Patist, A., Bhagwat, S.S., Penfield, K.W., Aikens, P., Shah, D.O., 2000. On the measurement of critical micelle concentrations of pure and technical-grade nonionic surfactants. J. Surfact. Deterg. 3, 53–58.
Randolph, T.W., Jones, L.S., 2002. Surfactant–protein interactions. Pharm. Biotechnol. 13, 159–175.
Rapoza, R.J., Horbett, T.A., 1990. The effects of concentration and adsorption time on the elutability of adsorbed proteins in surfactant solutions of varying structures and concentrations. J. Colloid Interface Sci. 136, 480–493.
Romacker, M.T.S., Forster, R., 2008. The rise of prefilled syringes from niche product to primary container of choice: a short history. ONdrugDelivery, 4–5.
Rosenberg, A.S., 2006. Effects of protein aggregates: an immunologic perspective. AAPS J. 8, E501–E507.
Sauerbrey, G., 1959. The use of quartz oscillators for weighing thin layers and for microweighing. Z. Phys. 155, 206–222.
Smith, E.J., 1988. Siliconization of parentral drug packaging components. J. Parenter. Sci. Technol. 42, S3–S13.

Soderquist, M.E., Walton, A.G., 1980. Structural changes in proteins adsorbed on polymer surfaces. J. Colloid Interface Sci. 75, 386–397.
Su, X.-L., Li, Y., 2005. A QCM immunosensor for Salmonella detection with simultaneous measurements of resonant frequency and motional resistance. Biosens. Bioelectron. 21, 840–848.
Thirumangalathu, R., Krishnan, S., Ricci, M.S., Brems, D.N., Randolph, T.W., Carpenter, J.F., 2009. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. J. Pharm. Sci. 98, 3167–3181.
Thurow, H., Geisen, K., 1984. Stabilization of dissolved proteins against denaturation at hydrophobic interfaces. Diabetologia 27, 212–218.
Timasheff, S.N., 2002. Protein–solvent preferential interactions protein hydration, and the modulation of biochemical reactions by solvent components. Proc. Natl. Acad. Sci. U.S.A. 99, 9721–9726.
Wang, P.-L., Udeani, G.O., Johnston, T.P., 1995. Inhibition of granulocyte colony stimulating factor (G-CSF) adsorption to polyvinyl chloride using a nonionic surfactant. Int. J. Pharm. 114, 177–184.