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Application of quartz crystal microbalance to study the impact of pH and ionic strength on protein–silicone oil interactions

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ABSTRACT

In this study, we have used quartz crystal microbalance (QCM) to quantitate the adsorption of a protein on silicone oil coated surfaces as a function of protein concentration, pH and ionic strength using a 5 MHz quartz crystal. Protein adsorption isotherms were generated at different solution pH and ionic strengths. Surface saturation concentrations were selected from adsorption isotherms and used to generate adsorption profiles from pH 3.0 to 9.0, and at ionic strengths of 10 mM and 150 mM. At low ionic strength (10 mM) and pH 5.0 (close to the isoelectric point of the protein), maximum adsorption of protein to the silicone oil surface was observed. At higher ionic strength (150 mM), no significant pH influence on adsorption was observed. QCM could be used as a reliable technique to study the binding of proteins to silicone oil coated surfaces.

1. Introduction

Vials comprise about 50–55% of the sterile packaging systems used for storage/delivery of small volume injectables while syringes account for approximately 25–30% (Sacha et al., 2010). More recently, there has been a surge in the number of injectable biopharmaceutical products delivered through prefilled syringes (Romacker and Forster, 2008). Prefilled syringes offer various advantages such as reduced handling requirements, reduced product contamination and substantial reduction in the cost of manufacturing due to exact dosing that avoids overfill required for traditional vials (Harrison and Rios, 2007; Thorpe, 2005). These advantages have resulted in annual sales of more than 2 billion units worldwide with a growth of 12.8% annually (Harrison and Rios, 2007; Romacker and Forster, 2008).

In both the vials and syringes, lubrication is essential in order to enable component processability during manufacturing and functionality during delivery, which includes the prevention of the rubber stopper conglomeration during filling procedures, ease of plunger movement inside the syringe barrel and reduction of the injection associated friction induced tissue pain (Smith, 1988). Silicone oil, a polydimethylsiloxane, has been used for the past several decades as the lubricant of choice for this purpose. This is due to several advantages such as low surface tension to permit good wetting of most solid surfaces, optimum hydrophobicity to form a water repellant surface, good physicochemical stability and proven biocompatibility. Thus, siliconization of pharmaceutical storage and delivery devices has been a common practice for many years.

Silicone oil has been implicated as a risk factor for protein solutions to aggregate or form insoluble particulates (Bernstein, 1987; Jones et al., 2005). The extent to which this risk may be generalized across protein pharmaceuticals is not clear due to a lack of data that has been published in this area. As such, a fundamental understanding of the factors that influence protein–silicone oil interactions, as well as understanding the nature of the state of the protein at a silicone oil–liquid interface may help better understand this implied risk to protein formulation development, product stability, and therefore overall drug product quality.

As an amphiphilic molecule, proteins are surface active and have a tendency to lose their native structure on adsorption to hydrophobic surfaces (Andrade, 1985; Norde and Giacomelli, 2000; Soderquist and Walton, 1980). The silicone oil/water interface may also affect protein aggregation via interface induced unfolding. In addition to the aggregation problem, the adsorption at the interface becomes an important issue for highly potent low concentration protein solutions (e.g., tuberculin ~ 0.5 μg/mL), where a significant amount of the drug may be lost at the interface and should be compensated for to inject an accurate dose (Mizutani, 1981).

The seriousness of this issue was highlighted by the reports of gradual cloudiness of insulin in multi-dose storage vials, and

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subsequent inability to control blood glucose (Baldwin, 1988; Bernstein, 1987; Chantelau et al., 1986; Chantelau and Berger, 1985). The cloudiness was attributed to insulin particulate formation caused by the contamination of the solution by silicone oil. The silicone oil was used as a lubricant in the disposable syringes used for administration and was released in the solution during dose withdrawal. It was recently reported that the use of siliconized syringes for reconstitution of the drug product resulted in thread-like, gelatinous particles in the solution in less than an hour, which was attributed to the interaction of the protein with silicone oil (Markovic, 2006). Switching to non-siliconized syringes for reconstitution resolved the issue.

Recently, the formulation stability of four different model proteins (lysozyme, BSA, abatacept and trastuzumab) was studied in silicone oil emulsions in the absence and presence of surfactants by measuring soluble protein loss (Ludwig et al., 2010). It was found that the addition of surfactant decreased the adsorption of the proteins on the silicone oil droplets. It has also been reported that agitation has a synergistic effect on the aggregation induced by silicone oil when studied for a model IgG1 antibody as a function of temperature, pH and ionic strength (Thirumangalathu et al., 2009). The aggregation of 4 model proteins, viz., ribonuclease A (RNase A), lysozyme, BSA and concanavalin A (ConA) with silicone oil was studied using changes in the optical density measurements and any structural changes induced by silicone oil using circular dichroism (CD) and second derivative UV spectroscopy (Jones et al., 2005). The aggregation induced in a given protein solution was both pH and protein specific, with more hydrophobic proteins (BSA and ConA) showing more aggregation compared to relatively hydrophilic proteins (RNase A and lysozyme).

A mechanistic understanding of the protein adsorption to silicone oil/water interfaces is critical to the rational use of silicone oil coatings in prefilled syringes. This may help in the optimization of the formulation conditions to reduce interactions at the hydrophobic interface and thus improve storage stability. In order to study the protein silicone oil incompatibilities, generally silicone fluids have been introduced into the liquid protein formulation (Ludwig et al., 2010; Thirumangalathu et al., 2009), which represent a dynamic system, unstable over time unless surfactants are used and hence, may not be considered to be a good model for mechanistic investigation of the protein–silicone oil interactions. An improved approach would be the direct measurement and evaluation of the effect of formulation conditions on the protein–silicone oil interactions at a solid–liquid interface. Such an interface may closely mimic the condition of a lubricated syringe device in contact with the liquid formulation phase. Binding data could then be used in conjunction with stability data under the same formulation conditions to better understand the role of protein–silicone oil interactions during storage stability.

The major challenge lies in the lack of quantitative techniques to determine the amount of protein bound to silicone oil (Ludwig et al., 2010). This is due to extremely low adsorbed amount per unit area and hence, the quantitation of the amount bound requires high resolution and accuracy (Nakanishi et al., 2001). In this paper, we have used quartz crystal microbalance (QCM) to study the binding of a model protein with silicone oil. QCM provides an extremely sensitive, high resolution mass sensing method to study the interactions between the surface and the substrate both in air (Matsuura et al., 1997) and in liquid (Ebara and Okahata, 1994; Okahata et al., 1993; Tanaka et al., 2001). Thus, the binding of even small amount of protein with the silicone oil surface can be detected. The purpose of this work was to investigate the effect of pH and ionic strength on the interaction of a model protein with silicone oil using QCM.

2. Theory

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 which results in the vibrational motion of the crystal at its resonant frequency (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} $g/(cm\,s^2)$).

Thus, the constant terms can be combined together to give a crystal sensitivity constant, C, which is specific to a crystal:

$$\Delta m = C \times \Delta F \quad (2)$$

where $C \approx 17.7$ $ng/(cm^2\,Hz)$ for a 5 MHz crystal, i.e., when 17.7 ng of mass is deposited on 1 cm^2 of area, it will produce a shift of negative 1 Hz in the resonant frequency of the crystal.

Thus, the Sauerbrey equation relies on a linear sensitivity factor and is a fundamental property of a quartz crystal. However, this equation is valid only when the mass attached is uniform and elastic, i.e., the bound molecules are rigidly attached to the surface (Lu and Czanderna, 1984; Marx, 2003), and the film formed could be considered an extension of the thickness of quartz which does not experience any shear force during vibration (Buttry and Ward, 1992).

In liquid-phase measurements, Kanazawa and Gordon (1985) showed that there is a viscous coupling of the solution to the crystal surface which effectively adds a mass component to the oscillating crystal. Thus assuming no slip conditions, the resulting frequency change can be related to liquid properties using the following equation:

$$\Delta F = -F_o^{3/2}\sqrt{\frac{\rho_{liq}\eta_{liq}}{\pi\rho\mu}} \quad (3)$$

where ρ_{liq} and η_{liq} are the density and viscosity of a given liquid, respectively.

The shear wave that generates due to the shear motion of the crystal surface also gets dampened by energy dissipation associated with the liquid. This dissipation of the energy is described by the change in the resistance of the crystal as (Muramatsu et al., 1988):

$$\Delta R = \sqrt{2\pi F_o \rho_{liq}\eta_{liq}}\left(\frac{A}{k^2}\right) \quad (4)$$

where k is an electromechanical coupling factor.

When applying this method for measurements in aqueous solutions, the Sauerbrey equation may not hold as the bound layer of analyte may be inelastic (due to the effect of interfacial liquid properties such as viscosity and density). Viscous coupling of the bound layer will result in an additional shift in the crystal resonant frequency and a dampening in the resonant oscillation which is manifested in an increase in the series resonance resistance (R) of the quartz crystal (Buttry and Ward, 1992). Thus, besides measuring shift in frequency (ΔF), shift in resistance (ΔR) of the crystal also needs to be monitored which serves as an independent measure of the viscous loading by the bound layer on the crystal surface and

helps differentiating an elastic mass effect from viscosity induced effects (Muramatsu et al., 1995). Measuring ΔR provides information about the physical properties of the bound layers, i.e., if it is rigid or visco-elastic. A layer rigidly coupled to the crystal surface dissipates no energy and does not result in any change in the resistance value and hence, the decrease in resonant frequency is directly proportional to the mass in accordance with the Sauerbrey equation. However, if there is a formation of visco-elastic layer on the surface, there will be a positive shift in the R value and Sauerbrey equation may not be valid. In order to determine the nature of the change at the interface responsible for the frequency shift, whether elastic, viscous or both, a ΔR versus ΔF plot has been used (Muramatsu et al., 1995; Su and Li, 2005). Since a purely elastic mass bound to the quartz surface does not result in any energy dissipation, the slope of ΔR versus ΔF graph should be zero; whereas a pure viscous coupling will lead to a linear ΔR versus ΔF relationship resulting in a finite slope. Thus, a system which is viscoelastic should have a slope between the values of slope obtained for a purely viscous and elastic system. Therefore, the behavior of a given system under investigation can be graphically compared on such a plot. Depending upon the closeness of the measured ΔF versus ΔR slope of the system under consideration to the slope obtained for the purely viscous system or to the purely elastic system, it is possible to assess if the attached protein film is viscoelastic.

Besides providing the mass adsorbed on the surface, QCM allows one to study the kinetics of protein adsorption and thus, process of protein adsorption–desorption could be studied in real time. Moreover, this technique does not require any kind of fluorescent or radioisotope labeling of the protein which is not only a time consuming process but also has a potential to denature the protein (Tanaka et al., 2001).

3. Materials and methods

3.1. Materials

Protein used for these studies was an Fc-fusion protein supplied by Biogen Idec (San Diego, CA) as a 100 mg/mL frozen formulation in 10 mM citrate buffer (pH 6.0, pI range of approximately 5.2–6.5). The frozen formulation was thawed, 1 mL aliquots were removed and kept at 2–8 °C. The remaining material was refrozen and stored at −80 °C. Two commercially available silicone fluids (linear chain poly(dimethylsiloxane), trimethylsiloxy terminated; PDMS) of different viscosities, viz., Dow Corning® 360 Medical Fluid (350 cSt; Dow Corning, Inc., Midland, MI) and PS049.5 (10^6 cSt; UCT specialties LLC, Bristol, PA), were obtained. All other chemicals including, acetic acid, sodium acetate, monobasic and dibasic sodium phosphate, o-phosphoric acid, tris(hydroxymethyl)amino methane, sodium chloride, hydrochloric acid, sodium hydroxide, hexane, hydrogen peroxide and sulfuric acid were obtained from Fisher Scientific (Fair Lawn, NJ). L-Histidine was obtained from Sigma–Aldrich (St. Louis, MO). All of the chemicals used were of analytical grade and were used as received. Deionized water equivalent to Milli-Q™ grade was used to prepare all buffer solutions. Millipore (Billerica, MA) Amicon ultra centrifugal filters (Amicon Ultra-15) with a molecular weight cut off of 10 kDa were obtained from Fisher Scientific.

3.2. Methods

3.2.1. Sample preparation

The following buffers were prepared to maintain the solution pH: phosphate (pH 3.0, 7.0 and 8.0), acetate (pH 4.0 and 5.0), histidine (pH 6.0) and tris(hydroxymethyl) aminomethane (pH 9.0). Appropriate concentrations of the buffer species were used in order to maintain 10 mM ionic strength without the addition of salt. For higher ionic strength studies, sodium chloride was added to the buffer to adjust the ionic strength to 150 mM while keeping the buffer strength same. Hydrochloric acid (1 N) or sodium hydroxide (1 N) was 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. 1 mL 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 of 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 $1.25\,(\mathrm{mg/mL})^{-1}\,\mathrm{cm}^{-1}$ at 280 nm. The desired concentrations of the samples were prepared with dilution using the same buffer.

3.2.2. Quartz crystal coating with silicone oil

AT-cut quartz crystals with optically flat polished gold/titanium electrodes with a fundamental resonant frequency of 5 MHz were obtained from Stanford Research Systems (SRS, Inc., Sunnyvale, CA). The crystals were ~2.54 cm in diameter with the upper electrode area of $1.37\,\mathrm{cm}^2$ and lower electrode area of $0.40\,\mathrm{cm}^2$. The lower electrode area is the area of overlap between the upper and lower electrodes, and represents the part of the crystal which is piezoelectrically most active.

Crystals were first cleaned with Piranha solution (1 part of 30% hydrogen peroxide in 3 parts of 95–98% sulfuric acid) and then rinsed thoroughly with deionized water and ethanol, followed by drying with high purity nitrogen. The resonant frequency of the blank crystal was recorded. The PDMS solution was prepared in hexane. For film deposition, solvent casting method was used. A controlled volume of polymeric solution consisting of ~350 ng of the polymer was applied on the larger gold electrode to cover the overlapping electrodes portion. The coated crystals were then dried at 100 °C for at least 2 h. After drying, the resonant frequency of the polymer coated crystal was measured. The difference between the resonant frequencies of the uncoated and coated crystal was determined. The mass of the polymer deposited on the surface was calculated from the shift in the crystal resonant frequency.

In order to test the stability of the PDMS film in an aqueous environment, the coating was exposed to deionized water either in static or in flow mode. In preliminary studies, static mode of water exposure was used where deionized water in sufficient volume was placed on the PDMS coating casted on the crystal surface. After approximately 1–2 h, water was gently lifted from the surface using a micropipette, remaining water was blown away using nitrogen and the crystals were dried at 100 °C for approximately 15 min to remove the residual moisture. The resonant frequency of the dried crystal was measured to determine the net frequency change due to water exposure. Based on the results from static mode, coated crystals were exposed to deionized water in flow mode using the QCM set up described in the following section. Deionized water was passed over the polymer surface at 50 μL/min for approximately 2 h, followed by drying at 100 °C. Resonant frequency was measured to calculate the net frequency change. For reuse, the crystals were treated with hexane to dissolve the polymer coating followed by 0.1 M sodium dodecyl sulfate (SDS) solution to remove any traces of polymer as well as any protein that might have bound to the uncoated portion of the electrode. This was followed by rinsing the crystal with copious amount of water and nitrogen drying.

3.2.3. QCM apparatus

The binding of the protein on silicone oil surface was studied in flow injection mode using a commercially available QCM apparatus (QCM 200; SRS Inc., Sunnyvale, CA). 5 MHz quartz crystals previously coated with the selected silicone oil were used in the study. The coated crystal was mounted in a Kynar® flow cell (SRS, Inc.) using O-rings to clamp the crystal so that the coated side of the crystal faced the liquid while the opposite side made electrical contacts via POGO® pins. The flow cell had an approximate volume of 150 μL for the liquid to make contact with the crystal surface. The fluid enters axially on the center of the crystal and moves radially outwards to the outlet port of the flow cell. In order to drive out any air bubbles that might form during the entrance of liquid inside the chamber, the flow inside the cell was directed against gravity. The stagnation point of the crystal is located at its center, overlapping the area of highest sensitivity. Suitable connections were made using PEEK® tubing (Upchurch Scientific, Inc., Oak Harbor, WA) between the flow cell and solvent syringe through a 6-port injection valve (Upchurch Scientific, Inc., Oak Harbor, WA). The assembly was equilibrated at 25 ± 0.1 °C in a water bath attached to a temperature regulated water circulator. Solvent of interest was flowed through the system using a single syringe pump (NE 1010X; New Era Pump Systems, Inc., Farmingdale, NY) at a rate of 50 μL/min in order to minimize the effect of liquid flow on the QCM signal. After establishing a stable baseline with respect to change in *F* and *R* values in buffer, 250 μL of protein sample was introduced into the system via a sample port. The changes in *F* and *R* values for the crystal were recorded as a function of time using QCM 200 system connected to an external computer via RS-232 interface at an interval of 10 s using LabView Stand alone 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 the protein in solution and that adsorbed on the polymer surface. These shifts in the *F* and *R* signals were used to calculate the amount of protein bound to the silicone oil/water interface at equilibrium. The system was then rinsed with the same buffer until no changes in *F* and *R* values were observed. This rinsing of the system with buffer served two purposes. First, it removed any contribution to the resonant frequency/resistance shift caused by protein solution properties such as viscosity and density, and second, it removed any protein that was reversibly bound to the silicone oil/water interface. Thus, the difference in the *F* and *R* values before protein injection and after the system rinse were used to calculate the amount of protein that was irreversibly adsorbed on the PDMS surface.

3.2.4. Protein adsorption studies

Protein concentrations ranging from 0.001 mg/mL to 1 mg/mL, namely, 0.001, 0.010, 0.025, 0.10, 0.25 and 1 mg/mL were analyzed using QCM at 25 ± 0.1 °C for their adsorption on the silicone oil surface in order to obtain adsorption isotherms. The studies were conducted at pH 3.0, 5.0 and 9.0 at 10 mM and 150 mM ionic strength.

One or more concentrations showing plateau in the mass adsorbed to the interface, i.e., saturation concentrations were picked for adsorption studies on the silicone oil/water interface to generate an adsorption profile as a function of pH in the range of 3.0–9.0 at 10 mM and 150 mM ionic strengths.

4. Results and discussion

4.1. Silicone oil selection

In pharmaceutical packaging industry, silicone oils are generally applied on the device surface (syringes and vial stoppers) in the form of an emulsion, with curing at temperatures as high as 300 °C to get a thin layer of silicone oil (Fries, 2009; Mundry et al., 2000). A similar process was used in these studies to obtain acoustically active thin films of silicone oil on the quartz crystal. The amount used to coat a crystal was calculated based on the surface area of the dimethylsiloxane monomer of 0.42 nm^2 (Fadeev, 2006) and the area of crystal surface (slightly more than 0.40 cm^2). Approximately 350 ng of the silicone fluid (PDMS), which was sufficient to give a few monolayer thick and elastic silicone oil coating on the crystal surface, was deposited. In the preliminary studies, Dow Corning® 360 Medical Fluid (Dow Corning Inc., Midland, MI), which is commercially used for the lubrication purposes in pharmaceutical delivery devices, was used. However, it was found that the films formed were not stable as they were washed from the crystal surface when exposed to water (data not shown). Therefore, a different silicone fluid with higher viscosity (10^6 cSt, PS049.5, UCT specialties LLC, Bristol, PA) was used for the film formation. A higher viscosity fluid is not expected to flow easily and hence, come off the surface due to decreased mobility and increased durability can be achieved. Fig. 1 shows the shift obtained in the resonant frequency of the high viscosity silicone fluid coated crystals before and after exposure to deionized water for 2 h in flow mode. A 50 Hz shift in resonant frequency represented, on average, a 30 molecule thick layer. A similar magnitude of resonant frequencies after exposure to deionized water suggests an insignificant loss of polymer mass from the crystal surface, thus demonstrating the stability of the films obtained with high viscosity PDMS.

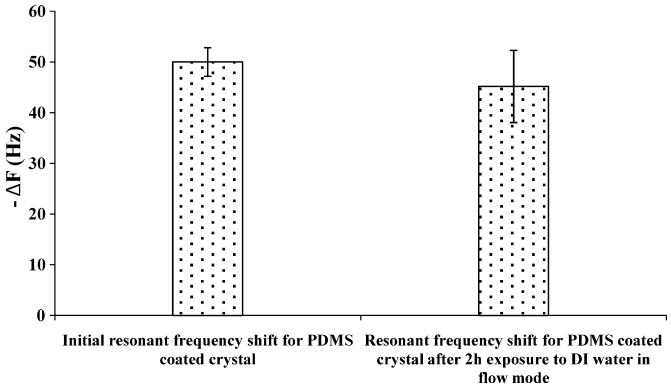

Fig. 1. Shift in the resonant frequency of 5 MHz quartz crystal coated with silicone oil (PDMS) before and after exposure to deionized water in flow mode after 2 h ($n = 6$).

4.2. Adsorption isotherms

Adsorption of the protein to the silicone oil/water interface as a function of its concentration under different solution conditions of pH and ionic strength, was studied using adsorption isotherms. The time dependence of the binding process showed that for all the solutions with protein concentration above 25 μg/mL, the adsorption of the protein attained a constant value within 1 h. Fig. 2 shows an example of the frequency shift (and hence mass increase) obtained as a result of protein adsorption as a function of time.

To characterize the nature of the adsorbed protein film (elastic or viscoelastic), a plot of the resistance shift (ΔR) versus frequency shift (ΔF) was used as given in Fig. 3 (refer to Section 2 for the background). Line A in Fig. 3 represents an elastic mass effect, where ΔR and hence the slope ($\Delta R/\Delta F$) is zero. Line B represents a purely viscous effect obtained from sucrose solutions. Sucrose solution represents a model viscous system. For a purely viscous system, both ΔF and ΔR are expected to be proportional to the square root of the product of viscosity and density values as given by Eqs. (3) and (4), respectively (Kanazawa and Gordon, 1985; Muramatsu et al.,

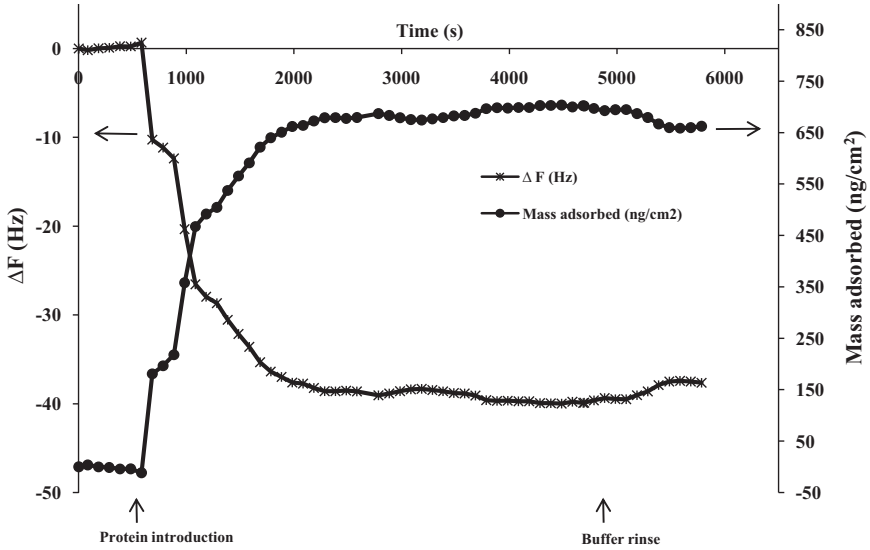

Fig. 2. Typical time course of frequency decrease on adsorption of fusion protein at pH 5/10 mM ionic strength from 1 mg/mL solution to silicone oil/water interface as measured by QCM. The mass increase has been derived from Sauerbrey equation as described in the text. The data points correspond to an interval of 100 s.

1988), and a plot of ΔR versus ΔF should be linear with a finite slope. The resulting ΔR and ΔF values for 0–25% (w/w) sucrose solutions were determined using QCM at $25 \pm 0.1\,^{\circ}C$ in the flow mode. The plot of ΔR versus ΔF for the sucrose solutions was linear ($R^2 > 0.99$) and resulted in a slope ($\Delta R_{suc}/\Delta F_{suc}$) of 0.6788 Ω/Hz (Fig. 3, line B), where the subscript "suc" represents sucrose. Any viscoelastic change at the interface will therefore lie in between lines A and B of the ΔR versus ΔF plot shown in Fig. 3.

To determine the property of the protein film adsorbed to the silicone oil/water interface under a given solution condition, the average of ΔR values of different replicates was obtained and was divided by the corresponding average of ΔF values in order to obtain the $\Delta R_p/\Delta F_p$ value, where subscript p denotes protein. The $\Delta R_p/\Delta F_p$ values obtained were than compared to the $\Delta R_{suc}/\Delta F_{suc}$ value. Thus, the percentage of any viscosity contribution in the total frequency shift observed for protein adsorption can be determined by calculating the percentage of $(\Delta R_p/\Delta F_p)/(\Delta R_{suc}/\Delta F_{suc})$ values. For all the solution conditions studied, $\Delta R_p/\Delta F_p$ values were within 5% of the slope obtained for the purely viscous sucrose solutions. Thus, it is concluded that the protein films formed on the interface were rigid, and Sauerbrey equation can be used to derive the mass adsorbed from the measured frequency shifts.

Fig. 4 shows the isotherms obtained for the protein at pH 3.0 (net positive charge on protein based on p*I* range of 5.2–6.5), 5.0 (close to zero charge) and 9.0 (net negatively charged protein) with 10 mM and 150 mM ionic strength. Each isotherm shows an initial steep rise followed by a plateau. Different parts of the isotherm reflect different interactions that govern the interfacial adsorption (Norde and Lyklema, 1978). The initial part of the isotherm (~10 μg/mL or lower), corresponding to low surface coverage of the protein coverage, is essentially indicative of the affinity of the protein to the silicone layer. In the later portion of the isotherm (~25 μg/mL or higher) as surface coverage increases, protein–protein interactions may also play a role in governing the adsorption. The slopes of the isotherms suggest that under the solution conditions studied, the model protein has a high tendency to interact with the interface. Since the silicone surface is non-ionic, our results indicate that the forces existing between the protein molecules and the silicone surface are primarily hydrophobic in nature. However, the latter part of the isotherms representing the plateau is a strong function of pH and ionic strength of the medium. Since solution conditions govern the net charge on the protein and the magnitude of charge screening, they affect intermolecular protein interactions and hence, the amount adsorbed to the interface. Under all

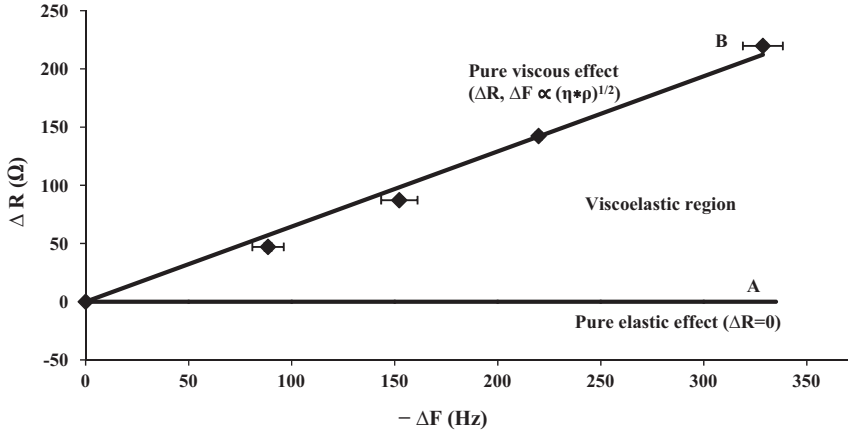

Fig. 3. The ΔR vs. ΔF plot showing an elastic system (line A), a completely viscous system (line B) and the viscoelastic region. Line A is theoretical while line B is the best fit to the experimental data points (♦, $n = 3$) for ΔR and ΔF values which were obtained from 0 to 25% (w/w) sucrose solutions using QCM at $25 \pm 0.1\,^{\circ}C$ as described in the text.

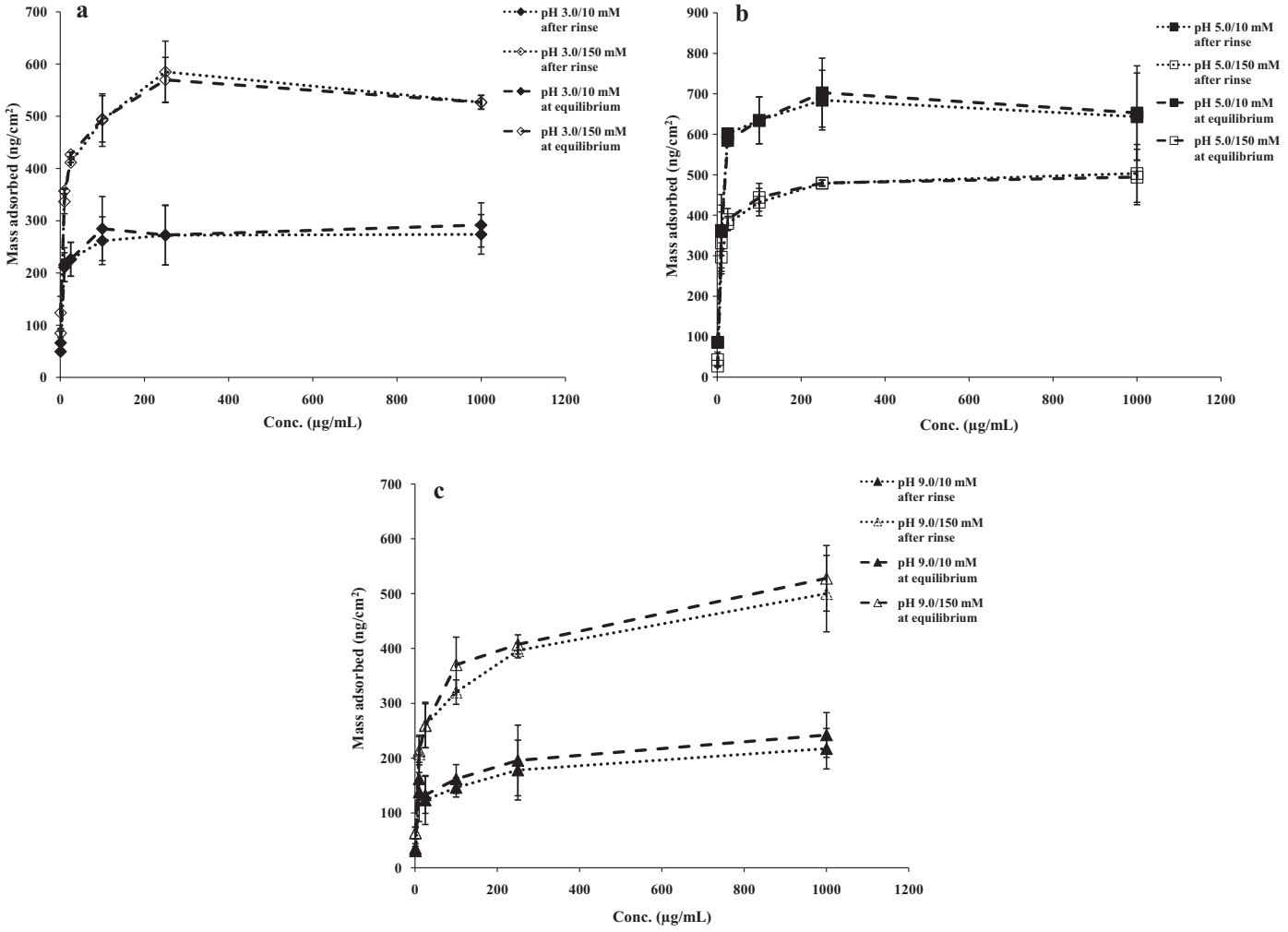

Fig. 4. Amount of protein adsorbed to silicone oil/water interface as a function of its bulk concentration at (a) pH 3.0, (b) 5.0 and (c) 9.0 under 10 mM and 150 mM ionic strength ($n \geq 2$). Both, the amount adsorbed at equilibrium (adsorption isotherms) and after rinsing with buffer are shown.

the solution conditions, isotherms attained plateau above a concentration of 0.1 mg/mL. The only exception being the adsorption at pH 9.0 (150 mM ionic strength), which continued to increase even after 0.1 mg/mL. Such adsorption saturation behavior has been previously observed for different proteins on various interfaces (Elgersma et al., 1990; Koutsoukos et al., 1983; Luey et al., 1991).

In many cases of protein adsorption, the equilibrium relationship between the adsorbed mass of protein and its bulk concentration tends to follow the Langmuir type adsorption behavior (Lee and Kim, 1974; Luey et al., 1991; Tanaka et al., 2001). In such cases, plots of adsorbed mass versus protein concentration showed an initial period of rise with a steep slope followed by plateau at a critical protein concentration and hence, the Langmuir equation was used to fit the data. However, such treatment of data has been argued on the basis that adsorption of protein in most cases is irreversible on the time scale of measurements and hence, a Langmuir-based modeling of the data is inappropriate (Brynda et al., 1986; Haynes and Norde, 1994). The reason for such irreversibility was attributed to the fact that with time, adsorbed protein molecules undergo structural changes with the hydrophobic moiety of protein interacting with hydrophobic interfaces resulting in multi-contact attachments, and hence making the desorption process entropically unfavorable and very slow. On the time scales of our studies, only a slight desorption (<10%) of the interfacially adsorbed protein was observed when the system was rinsed with the buffer following protein adsorption (Figs. 2 and 4), suggesting the irreversibility of adsorption. Although protein desorption under pure buffer environment was not observed, adsorption studies with radio labeled proteins have clearly shown that a dynamic equilibrium exists with protein molecules arriving and leaving the interface at equal rates (Brash and Samak, 1978; Brash et al., 1974). Thermodynamically, such an exchange of protein molecules may be much more likely than a spontaneous desorption in the absence of other molecules in the bulk. Thus at equilibrium, fitting the Langmuir equation to the experimental data for each isotherm is considered to be appropriate. The model fits the experimental data with a high degree of correlation ($R^2 > 0.99$, data not shown).

Despite the fact that the Langmuir equation shows an excellent fit to the data, accurate affinity constants may not be obtained. This is due to the interactions existing between the protein molecules, which have been assumed to be non-existing for Langmuir type behavior to be valid and hence, may require separate treatment of the data. The affinity of the protein to the interface in different solution conditions, however, can still be compared qualitatively based on the initial slopes of the isotherms. Thus, the protein seems to have a high tendency to interact with the interface at pH 5.0 under 10 mM ionic strength, while the affinity is much lower when the adsorption is studied under pH 9.0/10 mM ionic strength solution condition.

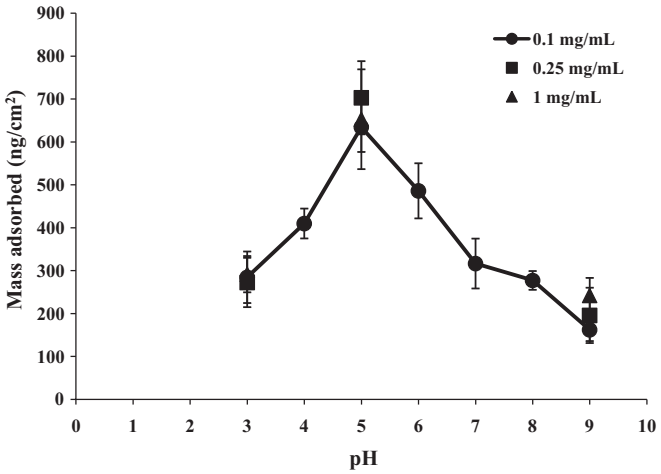

Fig. 5. Mass of protein adsorbed to the silicone oil/water interface as a function of pH at 10 mM ionic strength from 0.1, 0.25 and 1 mg/mL solutions ($n \geq 2$).

4.3. pH effect on adsorption

Fig. 5 describes the amount of protein adsorbed at the silicone oil/water interface as a function of pH at 10 mM ionic strength at 0.1 mg/mL protein concentration. The amount adsorbed at the interface was maximum at pH 5.0. Adsorption of the protein to the surface decreased as the pH was increased or decreased. The charge and its distribution on a protein surface can influence its surface activity (Brash and Horbett, 1987). In previous studies with IgG and albumin adsorption at various interfaces, maximum adsorption was observed at the p*I*, which decreased as the solution pH was changed away from the p*I* (Bagchi and Birnbaum, 1981; Shirahama and Suzawa, 1985). In our study, the maximum adsorption was observed at pH 5.0 which is at close to the lower end of the p*I* range for the molecule (p*I* range 5.2–6.5, determined by IEF gel electrophoresis, data not shown). At this stage the cause of this anomaly is not known. One explanation for this phenomenon could be a shift in the p*I* caused by anion binding to the protein, or a shift in the protein p*I* at the hydrophobic surface. The apparent shift in the p*I* due to ion binding has been observed previously (Longsworth and Jacobsen, 1949). In the isoelectric region, protein molecules carry least net charge and hence the charge–charge repulsions between adsorbed protein molecules are minimized, which leads to a higher amount of protein being adsorbed to the interface. As the pH is shifted away from the isoelectric region either towards the acidic or the basic side, protein molecules attain increasing net charge leading to an enhancement in the protein–protein electrostatic repulsions. These repulsive interactions between the adsorbed molecules lead to a reduction in the adsorbed amount.

Fig. 5 further shows the adsorption data for different bulk concentrations of the protein at 0.25 and 1 mg/mL (both at 10 mM ionic strength) at pH 3.0, 5.0 and 9.0. Increasing the protein concentration in the bulk has no significant effect on the amount adsorbed at the interface. This implies that at low ionic strength the protein adsorbed to the interface at these concentrations has achieved saturation.

4.4. Ionic strength effects

Fig. 6 shows the protein adsorption data at 150 mM ionic strength as a function of solution pH. The data shows that the saturation of the silicone oil/water interface is not achieved at 0.1 mg/mL bulk protein concentration at pH 6.0, 7.0 and 9.0; the amount of protein adsorbed increased further as the bulk concentration was increased. However, the results at 1.0 mg/mL show that

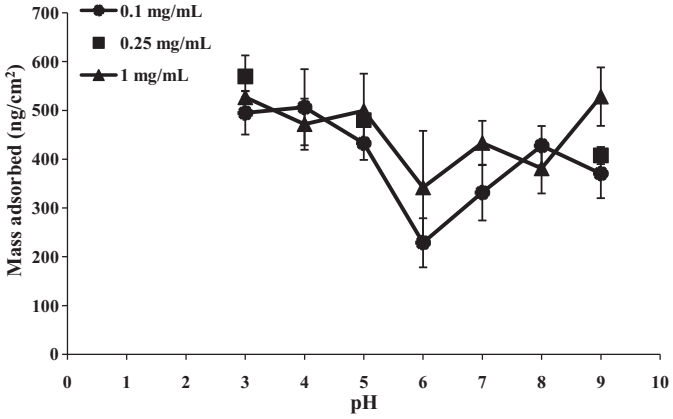

Fig. 6. Mass of protein adsorbed at the silicone oil/water interface as a function of pH at 150 mM ionic strength from 0.1, 0.25 and 1 mg/mL solutions ($n \geq 2$).

within experimental error the amount adsorbed is constant for all pH conditions. This implies that the charge effects are neutralized and the protein adsorption at the interface is not influenced by the solution pH.

Fig. 7 shows comparative adsorption behavior for the protein to the silicone oil/water interface at pH 3.0, 5.0 and 9.0 at 10 mM and 150 mM ionic strengths and a constant bulk protein concentration of 1.0 mg/mL. It is clear from this figure that at low ionic strength the solution pH greatly affects the protein adsorption whereas the pH effect is minimum at 150 mM ionic strength. Both the electrostatic and hydrophobic interactions are important factors affecting the adsorption of the protein to an interface (Haynes and Norde, 1994). At low ionic strength (10 mM), the surface charges on protein molecules act fully and bring a greater contribution to the electrostatic forces. On increasing the ionic strength to 150 mM, the surface charges on the molecules increasingly become shielded, leading to a decrease in the electrostatic intermolecular repulsions. Such effects facilitate and drive adsorption at higher ionic strengths due to decreased lateral repulsions at conditions where the protein carries most net charge (pH 3.0 and 9.0 in these studies). Thus, the results indicate that at low ionic strength electrostatic forces mainly govern the adsorption to the silicone oil/water interface. Since increasing ionic strength has no effect on the entropically driven hydrophobic interactions these forces should dominate the overall adsorption in solutions of higher ionic strength.

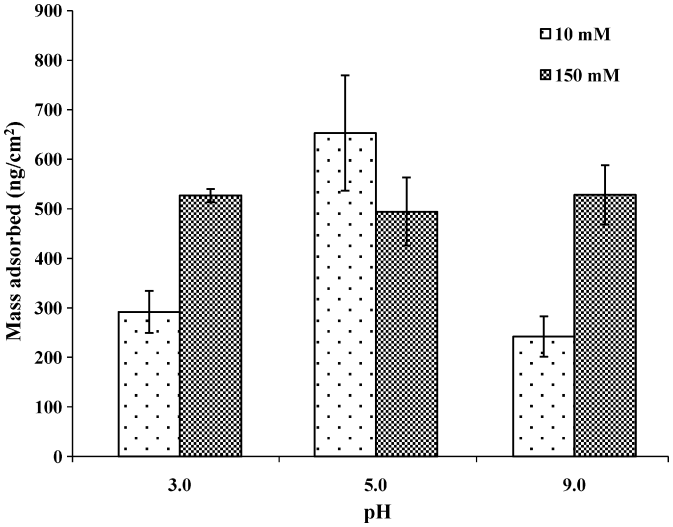

Fig. 7. Comparison of plateau amount of protein adsorbed at silicone oil/water interface for pH 3.0, 5.0 and 9.0 at 10 mM and 150 mM ionic strength ($n \geq 2$).

5. Conclusions

For the first time, QCM was used to monitor and understand the binding behavior of a model Fc-fusion protein to the silicone oil/water interface as a function of solution conditions of pH and ionic strength. At low ionic strength, maximum adsorption occurs near the isoelectric region of the protein while addition of salt to shield the surface charges of the protein leads to pH-independent adsorption. As a whole, the data suggest that both electrostatic and hydrophobic forces are involved in governing the adsorption of the protein to the silicone oil/water interface. Future studies will focus on finding the correlation between protein adsorption to the silicone oil/water interface presented in this manuscript and associated instabilities in protein formulations on long term exposure to such an interface.

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