



# Combining an optical resonance biosensor with enzyme activity kinetics to understand protein adsorption and denaturation



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## ABSTRACT

Understanding protein adsorption and resultant conformation changes on modified and unmodified silicon dioxide surfaces is a subject of keen interest in biosensors, microfluidic systems and for medical diagnostics. However, it has been proven difficult to investigate the kinetics of the adsorption process on these surfaces as well as understand the topic of the denaturation of proteins and its effect on enzyme activity. A highly sensitive optical whispering gallery mode (WGM) resonator was used to study a catalytic enzyme's adsorption processes on different silane modified glass substrates (plain glass control, DETA, 13F, and SiPEG). The WGM sensor was able to obtain high resolution kinetic data of glucose oxidase (GO) adsorption with sensitivity of adsorption better than that possible with SPR. The kinetic data, in combination with a functional assay of the enzyme activity, was used to test hypotheses on adsorption mechanisms. By fitting numerical models to the WGM sensograms for protein adsorption, and by confirming numerical predictions of enzyme activity in a separate assay, we were able to identify mechanisms for GO adsorption on different alkylsilanes and infer information about the adsorption of protein on nanostructured surfaces.

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## 1. Introduction

The non-specific adsorption of biomolecules onto synthetic interfaces is critical for biomaterials research, where the extent and resulting conformation of adsorbed biomolecules, particularly proteins, determines the biological activity of a surface. It is well established that protein adsorption on biomaterials is a key factor in controlling a host's immunological response to an implant [1]. Protein adsorption has also been recognized as a critical step in the formation of bacterial biofilms [2]. Improving our understanding of the fundamental science of protein adsorption can advance the state of the art in biomaterials, which will lead to improved medical devices with better tissue integration and lower rates of infection.

With the increased utilization of microfluidic systems, the growing importance of photonic (Si, SiO<sub>2</sub>, etc.) sensors [3,4] and the development of many new types of materials with nanoscale features, the problem of non-specific adsorption has taken on a

renewed significance [5]. As the length scales of materials approach those of single biomolecules, it is no longer sufficient to rely solely on theories of adsorption based on ensemble averages of molecular behavior. When dealing with the interaction of single molecules or small numbers of molecules interacting with nanoscale interfaces, it is important to be able to analyze these interactions in a quantitative and non-invasive (label-free) manner. Thus, it is desirable to employ ultrasensitive label free methods that have the potential of probing even single molecule interactions. To date such methods include mechanical biosensors [6], fluorescence, current blockade sensing in nanopores [7], nanoplasmonics [8] and optical microcavity sensing [9–11]. The latter method, in which optical resonances are confined by total internal reflectance in a dielectric cavity (such as a sphere, spheroid, toroid, etc), has proven to be a promising candidate for ultrasensitive detection on engineered surfaces [12–15], especially on modified silica surfaces that are also widely used as substrates for cell culture.

We have addressed this deficiency using an optical microcavity system, where optical whispering gallery mode (WGM) resonances are excited when the wavelength of the incident light meets the resonance criterion  $m\lambda = 2\pi r n_{\text{eff}}$ , where  $m$  is an integer number,  $r$  is the radius of the resonator, and  $n_{\text{eff}}$  is the effective refractive index

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of the resonant light field (Fig. 1A). Light is recirculated within the resonator, enabling an analyte interacting with the evanescent field to be re-sampled multiple times, thus providing the potential for single molecule sensitivity. Biosensors based on the optical microcavity principle have been demonstrated to have limits of detection from the picomolar range to approaching the single molecule regime [10,11]. Although optical microcavity biosensors are relatively new, these limits of detection are already lower than the limits for surface plasmon resonance (SPR) sensors (a few picograms per square millimeter), a sensing technology that is well established and generally considered to be highly sensitive [16]. Given the inherent sensitivity of the method and the fact that resonators are commonly made from silica substrates, it becomes clear that WGM biosensors are an ideal platform for studying surface modification, and more specifically, interactions between biomolecules and modified silica substrates while still satisfying the resonance criteria. This is perhaps most simply realized with alkylsilane monolayers, an important class of synthetic surface coatings used in a variety of biotechnological applications that have been difficult to study as the gold sensing elements used in most SPR systems are limited to Au/thiol chemistry [16]. As biomolecules adsorb to the silane monolayer on the surface of a WGM sensor, the surface dependent binding kinetics of a particular molecule can be quantitatively measured and analyzed (Fig. 1B). The high resolution kinetic data acquired from the sensograms can be used to test models of protein adsorption. Such kinetic analysis becomes particularly relevant for analyzing real-time interactions at biosensor interfaces that cannot be studied using SPR, such as the adsorption of proteins on protein-resistant surfaces like PEG silanes [17]. In addition, the fundamental process of adsorption and denaturation may be different on silanes due to thiols on Au generally being ordered monolayers whereas silanes tend to be disordered monolayers.

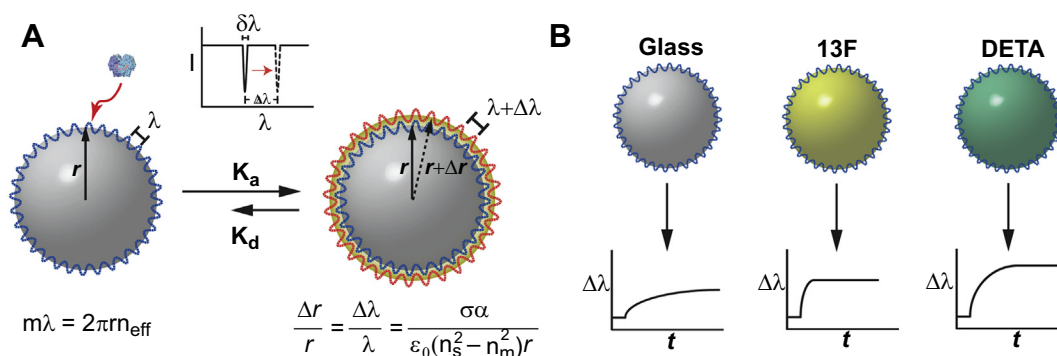
In the present study a custom-built WGM biosensor was used to quantify the kinetics of adsorption of glucose oxidase (GO) on three engineered silane surfaces with a range of different chemical properties. Silane chemistry was used to create three types of self-assembled monolayers (SAMs) on the surface of glass WGM resonators: (3-trimethoxysilyl propyl) diethyltriamine (DETA), 1,1,2,2-perfluorooctyl trichlorosilane (13F), and 2-[Methoxy-poly(ethyleneoxy)propyl] trimethoxysilane (SiPEG). The adsorption kinetics of GO were measured on the three SAMs and the native glass surface at two different solution concentrations. In a previous study biological activity was inferred by measurement of

protein deposition and cell culture on the surfaces [12]. Here we demonstrate direct measurement of the enzyme's catalytic activity on alkylsilane modified surfaces with an off-the-shelf activity assay. The combination of kinetic data from the WGM biosensor and activity information from the assay enabled an unprecedented level of molecular analysis of the protein absorption process.

Even simple biological systems involve many different types of proteins, some of which are not well characterized, so model proteins such as lysozyme [18], xylanase, and glucose oxidase are often used in protein adsorption studies [19]. The enzyme glucose oxidase (GO) was chosen as the model protein for this study because it is widely used in biosensor applications. GO is a well-characterized enzyme and many sensitive activity assays are commercially available. As an example, GO is adsorbed or covalently attached to electrodes to create amperometric blood glucose sensors [20] which have been a critical development in the management of diabetes mellitus [21].

GO is a dimeric glycoprotein that is composed of two identical subunits [22]. The crystal structure of GO from *Aspergillus niger* is available at the Protein Data Bank (1CF3), and its bounding box is 6.0 nm × 5.2 nm × 7.7 nm. Reported values for the molecular mass of GO range from 152 kDa [23] to 186 kDa [24], depending upon the method of purification that was used, and it has an isoelectric point of 4.2 [25]. Previous studies have focused on the effect of different surface chemistries on the structure and activity of adsorbed glucose oxidase [19,26]. Atomic force microscopy (AFM) studies have been performed to determine the size and shape of GO adsorbed on various surfaces, in conjunction with qualitative studies of the kinetics of adsorption [27,28].

In the present work, we demonstrate the utility of the WGM biosensor in combination with an off-the-shelf enzyme activity assay for providing detailed kinetic data for GO adsorption to synthetic surfaces and analyze the data for new mechanistic insights by utilizing numerical models. Five different models were applied to the WGM data in order to elucidate likely adsorption mechanisms for each surface. The models used were based on the Langmuir model [29], which describes site-limited adsorption, and the random sequential adsorption (RSA) model [30], in which steric hindrance is assumed to be the limiting factor in the saturation surface coverage. These basic models have been further developed to model the common case of protein that denatures after adsorption. A Langmuir-like two-stage model was formulated to describe a protein molecule that occupies a given area upon adsorption but then changes its conformation (denatures) to



**Fig. 1.** (A) Schematic representation of WGM biosensing principle. Light evanescently coupled into a glass microsphere of radius  $r$  achieves total internal reflectance upon satisfaction of the resonance criteria where  $n_{\text{eff}}$  is the effective refractive index of the sphere,  $\lambda$  is the resonant wavelength of light, and  $m$  is an integer number. As material, in this case protein, binds to the surface of the microsphere and interacts with the evanescent field, the effective radius of the microsphere increases resulting in a red-shift of the resonant wavelength. The change in wavelength,  $\Delta\lambda$ , can then be used to calculate the average density of adsorbed protein,  $\sigma$ , where  $\alpha$  is the polarizability of the protein,  $n_s$  and  $n_m$  are the refractive indices of the substrate and surrounding medium respectively, and  $\epsilon_0$  is the permittivity of free space. (B) Silica microspheres are ideal substrates for supporting a wide variety of surface chemistries.

occupy a different area on the surface [31,32]. Scaled particle theory was used to derive an RSA-like two-stage model in which the protein was assumed to be spherical and the ultimate surface coverage is limited by the packing density of proteins [32]. These models have been used to interpret the kinetics of fibronectin adsorption by adjusting the rate constants of the model to obtain an optimal fit to experimental data [33]. These four models, respectively, can be summarized as: single-stage Langmuir, single-stage RSA, two-stage Langmuir, and two-stage RSA. A model of two-layer adsorption was also fitted to the data. The fitted parameter values, the quality of fit, and comparison between the modeling results and enzyme activity data were used to draw conclusions about the structure and function of GO on each surface. The combination of whispering gallery mode measurements and extensive quantitative data analysis enabled the detailed explanation of GO adsorption behavior on silane surfaces of varied composition and properties. These results not only are the first detailed investigation of silane modifications directed at low protein concentrations, but also the nanoscale mechanism of denaturation on certain surfaces. Because silicon dioxide is the material of choice in many cell cultures, microfluidic and MEMS devices, this work also gives insight for applications in new biomaterial development.

## 2. Materials and methods

### 2.1. Whispering gallery mode measurements

The instrument used for all measurements was the same as described in Wilson et al. [12]. The instrument was comprised of a laser and detection system, data acquisition system, and a flow cell for sample delivery. A Lucent D2304G distributed feedback (DFB) laser diode with a FC/PC connector, nominal wavelength of 1310 nm and maximum power output of 10 mW was used as the excitation source in all experiments. The laser was mounted on an LDM-4984 butterfly laser diode mount (ILX Lightwave Corp, Bozeman, MT) and controlled with a LDC 3724B single channel current and temperature controller (ILX Lightwave Corp, Bozeman, MT). An HP 331120A waveform generator was connected to the modulation input of the laser controller, and the transfer coefficient was set to 20 mA/V. Under these conditions, a change of 1 V in the modulation signal resulted in a 20 mA change in laser current. Thus, the wavelength emitted by the DFB laser was modulated in a current dependent manner ( $\sim 0.009$  nm/mA). For all experiments a saw-tooth function with a variable peak-to-peak height was used to modulate the laser at a frequency of 100 Hz. The laser output was coupled to an SMF-28e+ optical fiber (Corning Inc., Corning, NY), the end of which was connected to a Thorlabs model PDA10CF InGaAs photodetector. The photodetector was interfaced with a Labview M-series data acquisition card and the signal from the detector was analyzed using a virtual instrument (VI) written in Labview 7.0 (National Instruments, Austin, TX). The data acquisition VI tracked all resonant valleys in the acquired spectrum using a peak fitting algorithm that selected all valleys with a minimum FWHM value set within the VI and determined the position of the valley minimum using a Bessel function. The data acquisition was synchronized with the saw-tooth function created by the function generator such that acquisition began at the minimum and ended at the peak of the function. All resonances were saved, and the highest-Q resonance was chosen for further analysis.

Resonators were fabricated from a single mode optical fiber with a 250  $\mu$ m acrylate polymer coating and 125  $\mu$ m cladding with a 9  $\mu$ m core (SMF-28e+, Corning Inc., Corning, NY) [34,35]. The acrylate coating was first removed using a fiber optic stripper and isopropyl alcohol (iPA). The end of the stripped fiber was then placed in the flame of a nitrous-butane Microflame torch (Azuremoon Trading Company, Cordova, TN). The nitrous-butane flame was used due to the very high temperatures needed to melt the glass and form the resonator ( $>700$  °C). The tip of the fiber was placed in the flame until the glass glowed bright white and began to melt. The surface tension of the molten glass caused it to form into a spheroidal droplet and as the tip melted, the fiber was rotated to ensure that the resonator remained centered on the stalk of the fiber. Resonator radii used for these studies ranged from 125 to 175  $\mu$ m. Waveguides were fabricated by flame drawing a length of SMF-28e+ optical fiber. First, a 1–2 cm section of the acrylate coating in the middle of the length of fiber was stripped and rinsed with iPA. The stripped section of the fiber was mounted between the moving parts of a syringe pump (KD Scientific, Holliston, MA) such that the fiber could be stretched as the glass was softened by the flame from the nitrous-butane torch. The tapered region of the fiber was slowly pulled down to a final diameter of  $\sim 4$   $\mu$ m.

The flow system was comprised of a peristaltic pump and polycarbonate flow cell connected by Silastic tubing (0.76 mm I.D., Dow Corning) and Luer connectors (Harvard Apparatus, Cambridge, MA). The flow cell was fabricated by milling a flow channel in a block of transparent polycarbonate. A shallow channel for mounting the

waveguide was milled perpendicular to the flow channel. A lid was fabricated from polycarbonate sheet and two female Luer connectors with 6–32 tapped ends were threaded into the lid in alignment with each end of the flow channel. The waveguide was placed into the cross channel of the flow cell so that it rested on the channel bottom. The waveguide was sealed in place with Kwik-sil silicon elastomer adhesive (World Precision Instruments Inc., Sarasota, FL) and spliced into the fiber between the laser and the detector. A silane-coated microsphere was aligned so that it was centered in the fluid channel and in contact with the waveguide. The microsphere was then secured into place using Kwik-sil elastomer. A detailed description of the flow cell fabrication and assembly can be found in the [Supporting Information](#).

The flow cell was filled with 50 mM PBS (pH 7.4) and assembled with a 1 mm thick PDMS gasket between the lid and the body. Reservoirs of buffer and protein solution were placed in a water bath to maintain a constant temperature. Buffer and protein solution were recirculated in a closed loop using a peristaltic pump, and 3-way stopcocks were used to switch between the two solutions. Using this system it was possible to switch between the buffer and protein lines without significantly disturbing the flow field. The buffer was circulated through the system until the system had thermally equilibrated and the drift in the baseline signal had stabilized. Solutions of glucose oxidase (Sigma, St. Louis, MO) at 100  $\mu$ g/ml and 10  $\mu$ g/ml were prepared in PBS (pH 7.4). The molecular mass was taken to be 160 kDa as stated in the datasheet provided by the vendor. Adsorption of GO was measured on glass, 13F, DETA, and SiPEG.

Binary files saved by the LabView data acquisition software were analyzed offline using a data analysis algorithm implemented in Python. The algorithm reconstructed the spectral location (in nm) of each resonance over time. The resonance with the lowest FWHM value and a continuous trace over the entire time span was chosen for further analysis. Any drift in the baseline data was corrected by subtracting a linear function from the data. The surface density (mass per unit area) was calculated from the resonance shift [34,36] using the equation:

$$\rho_s = \frac{\Delta\lambda}{\lambda} \frac{(n_s^2 - n_m^2) R}{2n_m \frac{dn}{dc}} \quad (1)$$

A derivation of this equation and the parameter values used in the analysis are shown in the [Supplemental Data](#).

### 2.2. Surface modification of resonators

Glass microspheres and glass coverslip controls for XPS were cleaned in a Harrick model PDC-32G plasma cleaner (Harrick, Ithaca, NY). The chamber was evacuated to a pressure of 300 millitorr, ultrapure oxygen was then purged into the system to a pressure of 800 millitorr, and evacuated again to 300 millitorr. After initiation of the plasma, the pressure in the chamber was adjusted to  $\sim 550$  millitorr. Cleaning was allowed to proceed for 20 min and then the resonators and coverslips were removed for subsequent silane reaction. Solutions of 0.1% silane in toluene were prepared as described previously [12] and transferred to 250 ml Pyrex beakers. The microspheres and coverslips were immersed in the silane solution. Upon completion of the reaction, the microspheres and coverslips were washed 3 $\times$  in dry toluene and dried under ultrapure nitrogen gas. XPS and contact angle goniometry were used to analyze the coverslips and ensure the surfaces were consistent with previous results [37].

### 2.3. Enzyme activity assay

The enzymatic activity of adsorbed GO was measured using the Amplex Red glucose oxidase assay (Invitrogen, Carlsbad, CA). Resonators were soaked in 100  $\mu$ g/ml and 10  $\mu$ g/ml GO solutions for 2 h in a round-bottomed 96-well ELISA plate. After 2 h the resonators were washed 3 $\times$  with PBS. The resonators were then transferred to a new 96-well plate and allowed to soak in 100  $\mu$ l of PBS for 2 h to allow any reversibly bound GO to desorb from the surface of the resonator. After 2 h 50  $\mu$ l of the PBS from each well was discarded. A solution containing 0.1 mM Amplex red, 0.1 mM Glucose, and 0.5 U/ml horseradish peroxidase (HRP) was prepared. 50  $\mu$ l of the Amplex red solution was added to each of the wells containing the resonators and soak buffer. The reaction mixtures were then placed in a Synergy HT multi-mode microplate reader (Bio-Tek, Winooski, VT) and the absorbance at 530 nm was read for each well at 1-min intervals. Standard dilutions of GO at 100 ng/ml, 50 ng/ml, 10 ng/ml, 5 ng/ml, 1 ng/ml, and 0.5 ng/ml were prepared and reacted with Amplex red reagent for all experiments. Total mass of active protein was calculated using the slope of the absorbance values versus time and comparing to the standard dilution curves. The total mass of active protein was then divided by the surface area of the resonator to determine the bound surface density.

### 2.4. CFD analysis of transport in the WGM flow cell

Computational fluid dynamics (CFD) was used to determine the extent of transport limitation in the flow cell of the WGM biosensor. A CFD-ACE+ suite of simulation tools was used to simulate transport in the flow cell as previously described [12]. The diffusion coefficient of GO was assumed to be  $5 \times 10^{-11}$  m<sup>2</sup>/s [23,24]. To simulate the depletion of protein near the resonator surface, an irreversible Langmuir adsorption reaction was defined on the surface of the resonator. The association rate constant and the maximum surface concentration were set to

reproduce the maximum rate of adsorption observed in experiments for each combination of solution concentration and surface modification. The CFD transport analysis was not performed for adsorption on SiPEG because of its significantly lower amount of adsorption.

### 3. Adsorption models

#### 3.1. Langmuir and RSA models

It is well established that single-layer adsorption models can be stated in the general form

$$\frac{d\theta}{dt} = k_a c \Phi(\theta) - k_d \theta \quad (2)$$

where  $\theta$  is the fraction of the surface covered by adsorbed particles,  $c$  is the concentration of protein in solution near the surface,  $k_a$  is the adsorption rate constant, and  $k_d$  is the desorption rate constant. The function  $\Phi$  represents the blocking effect of adsorbed particles. For the Langmuir model the blocking function is simply  $\Phi(\theta) = 1 - \theta/\theta_\infty$  where  $\theta_\infty$  is usually taken to be 1. The blocking function for the random sequential adsorption (RSA) model was not available in analytic form so a fitted polynomial was used to approximate the RSA blocking function [30].

#### 3.2. Adsorption models with post-adsorption transition

Single-layer adsorption models cannot adequately predict the kinetics of adsorption for many combinations of proteins and surfaces. More complex models have been developed to account for these experimental results. Because many surfaces cause proteins to denature upon adsorption, it is common to model adsorption as a two-stage process of adsorption with a post-adsorption transition. This process can be described by the kinetic equations

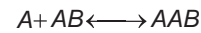
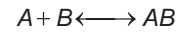
$$\frac{d\theta_\alpha}{dt} = k_a c \Phi_\alpha - k_s \theta_\alpha \Psi_{\alpha\beta} - k_d \theta_\alpha \quad (3)$$

$$\frac{d\theta_\beta}{dt} = k_s \Sigma^2 \theta_\alpha \Psi_{\alpha\beta} \quad (4)$$

Protein adsorbs in state  $\alpha$  and transitions to state  $\beta$  at a rate controlled by the rate constant  $k_s$ . The function  $\Phi_\alpha$  describes the blocking effect for a new particle adsorbing on the surface, while the function  $\Psi_{\alpha\beta}$  describes the blocking for a particle transitioning from state  $\alpha$  to state  $\beta$ . The form of the blocking functions depends upon the assumptions of the model. When the particles are spherical, scaled particle theory (SPT) can be used to derive the following blocking functions [32]. A Langmuir-type blocking function has also been proposed [31] and used to fit the adsorption kinetics of the fibronectin fragment FNIII<sub>7-10</sub> [33]. A detailed description of the two-stage model and blocking functions is shown in the [Supporting Information](#). For this model, enzyme in the initial state was assumed to be active, and enzyme in the denatured state was assumed to be inactive for purposes of predicting the overall surface density of active enzyme.

#### 3.3. A two-layer Langmuir-type model

A two-layer adsorption model was formulated, based on the assumptions of the Langmuir adsorption model. The two-layer adsorption model can be represented by the chemical equations shown in [Scheme 1](#), where  $A$  represents a molecule in solution,  $B$  represents an available adsorption site,  $AB$  represents a single molecule adsorbed on the surface, and  $AAB$  represents a “stack” of two adsorbed molecules. The kinetics of adsorption can be modeled by a set of coupled ordinary differential equations:



**Scheme 1.** Chemical equations for two-layer adsorption model.

$$\frac{d\theta_{AB}}{dt} = k_{a1} c (\theta_\infty - \theta_{AB} - \theta_{AAB}) - k_{d1} \theta_{AB} - k_{a2} c \theta_{AB} \quad (5)$$

$$\frac{d\theta_{AAB}}{dt} = k_{a2} c \theta_{AB} - k_{d2} \theta_{AAB} \quad (6)$$

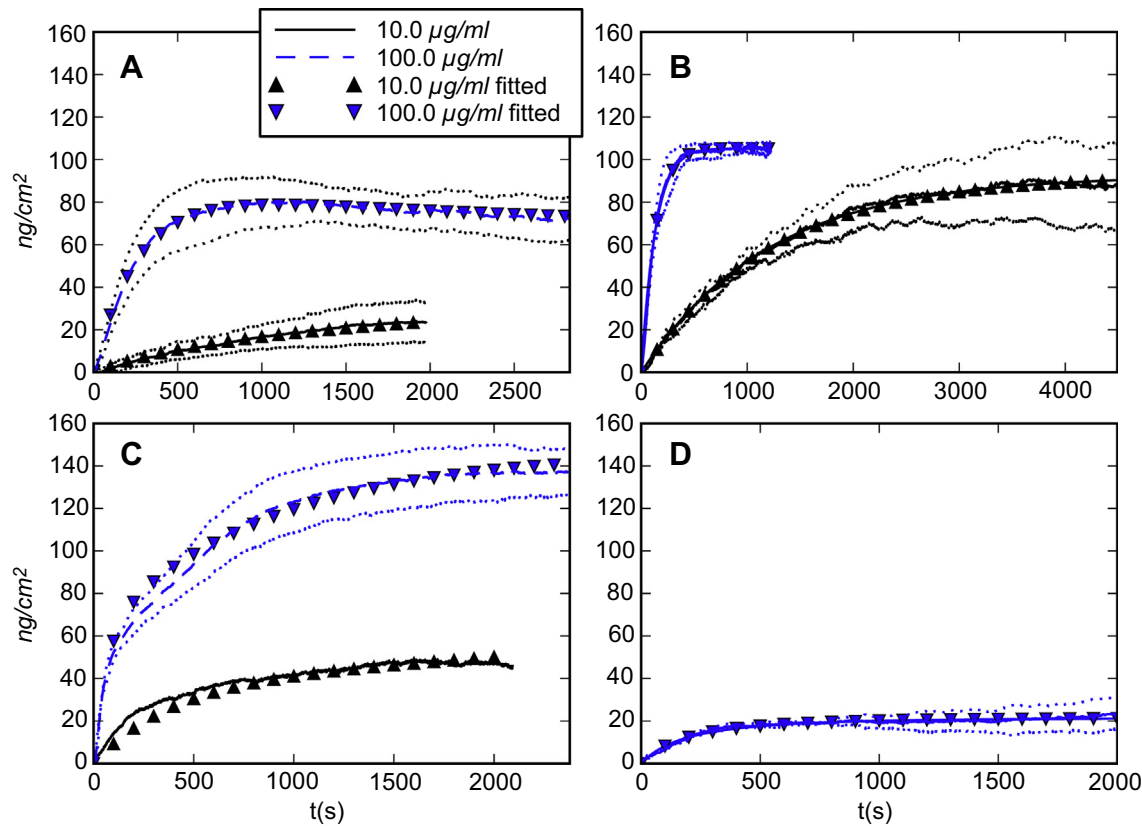
where  $\theta_{AB}$  is the fraction of the surface covered by a single particle and  $\theta_{AAB}$  is the fraction of the surface covered by two layers of particles.  $\theta = \theta_{AB} + \theta_{AAB}$  is the total fractional surface coverage. Although it is straightforward to solve this set of equations analytically, the resulting formulas are complicated, and the analysis of the solutions is beyond the scope of this work.

For the two-layer model, it was assumed that enzyme molecules in the upper layer prevented the substrate solution from interacting with molecules adsorbed in the lower layer. Enzyme in the lower layer was therefore treated as inactive. The activity of adsorbed enzyme in contact with the surface which is not screened by an upper layer depends upon the nature of the surface. It was assumed that hydrophilic surfaces (glass and DETA) had the potential to not induce denaturation upon adsorption, so single-layer protein was assumed to be active for these calculations. The surface density of active enzyme was calculated:  $\rho_{\text{active}} = \rho_{AB} + \rho_{AAB}$ . For 13F (a hydrophobic surface), it was assumed that first layer adsorption induces denaturation and destroys the activity of the enzyme. Only enzyme in the upper layer was considered to be active:  $\rho_{\text{active}} = \rho_{AAB}$ . The surface densities of active protein and total protein predicted by the models were plotted and compared to experimental results.

#### 3.4. Implementation of models and fitting to experimental data

A general single-layer adsorption simulation based on Equation (2) was implemented using the Python programming language. Various blocking functions, such as the Langmuir and RSA blocking functions, could be plugged into the simulation. Another simulation was created based on Equations (3) and (4) that could utilize either the Langmuir or SPT-derived blocking function. Equations (5) and (6) were used to create a two-layer adsorption simulation. The differential equations were solved numerically using the *odeint* routine from SciPy [38,39]. All equations were solved in terms of fractional surface coverage to avoid numerical difficulties that may occur when working with small floating-point numbers.

For comparison with experimental data, the fractional surface coverage predicted by each model was converted to surface density ( $\text{ng}/\text{cm}^2$ ) using  $\rho_i = \theta_i / \sigma_i \cdot M / N_A$ . Third-order splines were used to interpolate each set of experimental kinetic data to the same time points used in the model so that an average kinetic curve could be constructed. Each model was fitted to the average kinetic curve from the WGM biosensor for glass, DETA, 13F, and SiPEG by adjusting the parameters until the best possible fit was achieved, according to the least-squares criterion. The concentration of protein in solution near the surface was assumed to be constant based on the results of the CFD transport analysis. For each surface, average kinetic curves from the two solution concentrations were fitted simultaneously with a single set of parameters using the *leastsq* fitting routine from SciPy, which uses a modified version of the Levenberg–Marquardt algorithm [40]. The quality of fit was quantified by computing the sum of squared errors (SSE) for the



**Fig. 2.** Sensograms and fitted models for GO adsorption onto glass microspheres (A), functionalized with 13F (B), DETA (C), and SiPEG (D). Dotted lines indicate plus or minus one standard deviation for the experimental results. GO exhibited strong affinity for DETA with pronounced concentration dependence on the saturation surface density. Affinity for GO to 13F is also strong, but exhibits less concentration dependence; saturation values at 10 µg/ml concentrations were comparable to those at 100 µg/ml. GO showed less affinity for the glass surface than either DETA or GO, and a strong concentration dependence on the saturation value. Additionally, at 100 µg/ml a clear and reproducible overshoot profile was observed, where the surface densities would reach a maximum value at earlier time points then decrease to a lower plateau value at longer time points.

total surface concentration of adsorbed protein measured by the WGM sensor and the surface concentration predicted by the model. The SSE was divided by the number of time points in the data set to obtain the mean-squared error (MSE) so that the quality of fit could be compared between data sets with different numbers of time points.

4. Results

The WGM sensor was used to establish the kinetics of adsorption and the final amount of adsorbed protein, while the enzyme assay was used to determine the level of activity of the adsorbed enzyme. The conformation state of the adsorbed enzyme was established by comparing the activity level to the total amount of adsorbed protein. Fig. 2 shows the averaged experimental data ( $n = 3$ ) for the adsorption of GO from 10 µg/ml and 100 µg/ml solutions onto native glass resonators (a) and resonators

functionalized with DETA, 13F, and SiPEG (b, c, and d respectively). In order to assess which adsorption model best fit the experimental data from a given protein/surface combination both the quality of the fit of the curve to the kinetic data (MSE) and the model's ability to predict the resultant enzyme activity were considered. Fitting multiple models to the experimental data provided a quantitative means to test hypotheses about the mechanisms of adsorption on each surface. The resulting curve from the model that yielded the best fit (i.e. lowest MSE) as well as best predicted the biological activity of the protein post-adsorption was superimposed over the corresponding data in Fig. 2, and each surface yielded unique adsorption kinetics.

Tables 1–4 show the parameters for each individual model fitted to experimental data for glass, DETA, 13F, and SiPEG resonators (1–4 respectively). Only the RSA and Langmuir models were fitted to the GO on SiPEG data, as the experimental data showed no evidence of a more complex adsorption process on that surface.

**Table 1**  
Parameters for models fitted to GO on glass.

|                    | $k_a(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$    | $k_s (\text{s}^{-1})$                                | $k_d(\text{s}^{-1})$    | $\sigma_\alpha (\text{nm}^2)$ | $\sigma_\beta (\text{nm}^2)$ | MSE  |
|--------------------|------------------------------------------------------|------------------------------------------------------|-------------------------|-------------------------------|------------------------------|------|
| Langmuir           | 1.82e-8                                              |                                                      | 1.15e-3                 | 141                           |                              | 6.15 |
| Langmuir two-stage | 1.35e-8                                              | 1.48e-3                                              | 1.75e-3                 | 122                           | 415                          | 0.73 |
| RSA                | 1.16e-8                                              |                                                      | 1.34e-3                 | 80.8                          |                              | 7.81 |
| RSA two-stage      | 3.80e-9                                              | 2.20e-3                                              | 1.99e-3                 | 31.5                          | 349                          | 1.19 |
|                    | $k_{a1}(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$ | $k_{a2}(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$ | $k_{d1}(\text{s}^{-1})$ | $k_{d2}(\text{s}^{-1})$       | $\sigma (\text{nm}^2)$       | MSE  |
| Two-layer          | 1.85e-8                                              | 5.46e-8                                              | 2.18e-4                 | 4.60e-3                       | 224                          | 3.15 |

**Table 2**

Parameters for models fitted to GO on 13F.

|                    | $k_a(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$    | $k_s(\text{s}^{-1})$                                 | $k_d(\text{s}^{-1})$    | $\sigma_\alpha(\text{nm}^2)$ | $\sigma_\beta(\text{nm}^2)$ | MSE  |
|--------------------|------------------------------------------------------|------------------------------------------------------|-------------------------|------------------------------|-----------------------------|------|
| Langmuir           | 7.20e-8                                              |                                                      | 1.17e-4                 | 247                          |                             | 2.45 |
| Langmuir two-stage | 5.96e-8                                              | 1.14e-2                                              | 6.74e-5                 | 209                          | 291                         | 2.23 |
| RSA                | 3.79e-8                                              |                                                      | 3.81e-5                 | 103                          |                             | 12.8 |
| RSA two-stage      | 1.27e-9                                              | 0.377                                                | 9.73e-3                 | 4.6                          | 175                         | 2.3  |
|                    | $k_{a1}(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$ | $k_{a2}(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$ | $k_{d1}(\text{s}^{-1})$ | $k_{d2}(\text{s}^{-1})$      | $\sigma(\text{nm}^2)$       | MSE  |
| Two-layer          | 7.92e-8                                              | 7.18e-7                                              | 1.51e-4                 | 1.28e-4                      | 496                         | 1.82 |

**Table 3**

Parameters for models fitted to GO on DETA.

|                    | $k_a(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$    | $k_s(\text{s}^{-1})$                                 | $k_d(\text{s}^{-1})$    | $\sigma_\alpha(\text{nm}^2)$ | $\sigma_\beta(\text{nm}^2)$ | MSE  |
|--------------------|------------------------------------------------------|------------------------------------------------------|-------------------------|------------------------------|-----------------------------|------|
| Langmuir           | 3.10e-8                                              |                                                      | 1.83e-4                 | 190                          |                             | 66.1 |
| Langmuir two-stage | 1.15e-7                                              | 9.41e-3                                              | 3.22e-2                 | 161                          | 161                         | 38.7 |
| RSA                | 1.47e-8                                              |                                                      | 2.15e-4                 | 72.6                         |                             | 39.3 |
| RSA two-stage      | 1.19e-8                                              | 0.412                                                | 2.33e-4                 | 28                           | 457                         | 6.47 |
|                    | $k_{a1}(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$ | $k_{a2}(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$ | $k_{d1}(\text{s}^{-1})$ | $k_{d2}(\text{s}^{-1})$      | $\sigma(\text{nm}^2)$       | MSE  |
| Two-layer          | 1.44e-7                                              | 1.26e-8                                              | 1.19e-3                 | 3.42e-9                      | 363                         | 12.6 |

**Table 4**

Parameters for models fitted to GO on SiPEG.

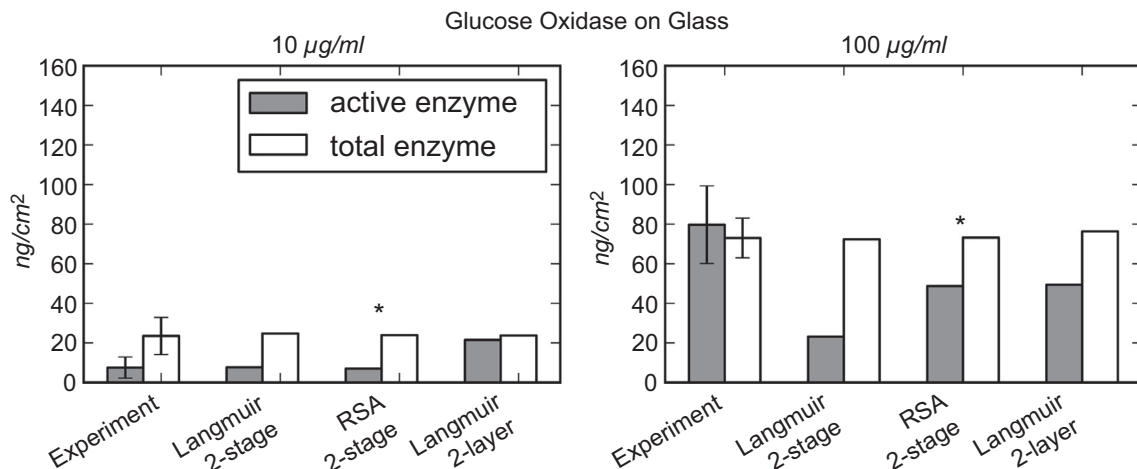
|          | $k_a(\text{cm}^3 \text{ ng}^{-1} \text{ s}^{-1})$ | $k_s(\text{s}^{-1})$ | $\sigma(\text{nm}^2)$ | MSE  |
|----------|---------------------------------------------------|----------------------|-----------------------|------|
| Langmuir | 4.06e-8                                           | 3.67e-4              | 684                   | 0.81 |
| RSA      | 2.18e-8                                           | 2.55e-4              | 482                   | 0.73 |

Figs. 3–5 indicate the surface density of enzymatically active GO on each surface measured by the activity assay and predicted by various models for both concentrations. Enzyme activity predictions for the basic Langmuir and RSA models were not included in the graphs since these models do not allow protein to exist in multiple states. The values for SiPEG were also not included in the figures due to the fact that the enzyme activity assay did not detect any significant amount of active enzyme for either solution concentration.

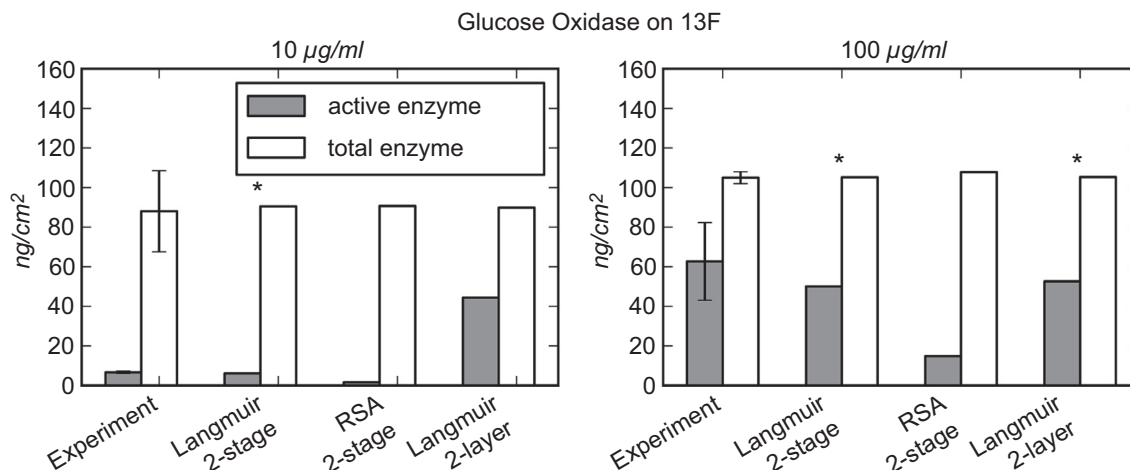
For GO on glass, the two-stage adsorption models fitted the data better than the simple adsorption models (Table 1). The Langmuir-type two-stage model fitted the kinetic data slightly better than the

RSA-type model according to the MSE (0.73 vs 1.19). Given the small difference between the MSE values for these two models, it was necessary to compare their ability to predict enzyme activity as shown in Fig. 3. The enzyme activity data indicated that GO adsorbed on bare glass retained about 25% of its activity at a solution concentration of 10  $\mu\text{g}/\text{ml}$ . When the solution concentration was increased to 100  $\mu\text{g}/\text{ml}$ , almost all the adsorbed enzyme remained active. While both two-stage models predicted the amount of active protein at 10  $\mu\text{g}/\text{ml}$ , at 100  $\mu\text{g}/\text{ml}$  the prediction of the RSA-type two-stage model was significantly closer to the experimentally observed value. The fitted values of  $\sigma_\alpha$  and  $\sigma_\beta$  for these two models indicate that GO spreads out significantly after adsorption and occupies a large footprint on a glass surface. A very low-density layer of adsorbed enzyme may passivate the glass surface, allowing the rest of the adsorbed enzyme to retain its activity after adsorption.

The kinetic data for GO on 13F (Table 2) was fitted well by both of the Langmuir-type models and the RSA two-stage model (MSE of 2.45, 2.23, and 2.3 respectively). The two-layer model yielded the lowest MSE of 1.82, suggesting this mechanism dominates.



**Fig. 3.** Experimental measurements and model predictions of enzyme activity on native glass surface. The asterisk (\*) on the bar indicates the model that fitted the kinetic data with the lowest mean-squared error and best predicted the biological activity of the adsorbed protein.



**Fig. 4.** Experimental measurements and model predictions of enzyme activity on DETA surface. The asterisk (\*) on the bar indicates the model that fitted the kinetic data with the lowest mean-squared error and best predicted the biological activity of the adsorbed protein.

However, the two-layer model greatly over-estimated the predicted activity at a solution concentration of 10 µg/ml, as shown in Fig. 4. The experimental data indicated that almost none of the adsorbed enzyme was active at 10 µg/ml, which was best captured by the two-stage Langmuir and RSA-type models. When the solution concentration was increased to 100 µg/ml, about 60% of the adsorbed enzyme retained its activity. This was reflected in both the two-stage and two-layer Langmuir models, but not the two-stage RSA. The fitted values for  $\sigma_\alpha$  and  $\sigma_\beta$  from the two-stage RSA model were also unrealistic. Thus, it became clear that the Langmuir-type two-stage model matched the experimental data best for GO on 13F. The fitted parameters for this model indicated the area occupied by a molecule in the final state was about 50% larger than that of a molecule in the initial state.

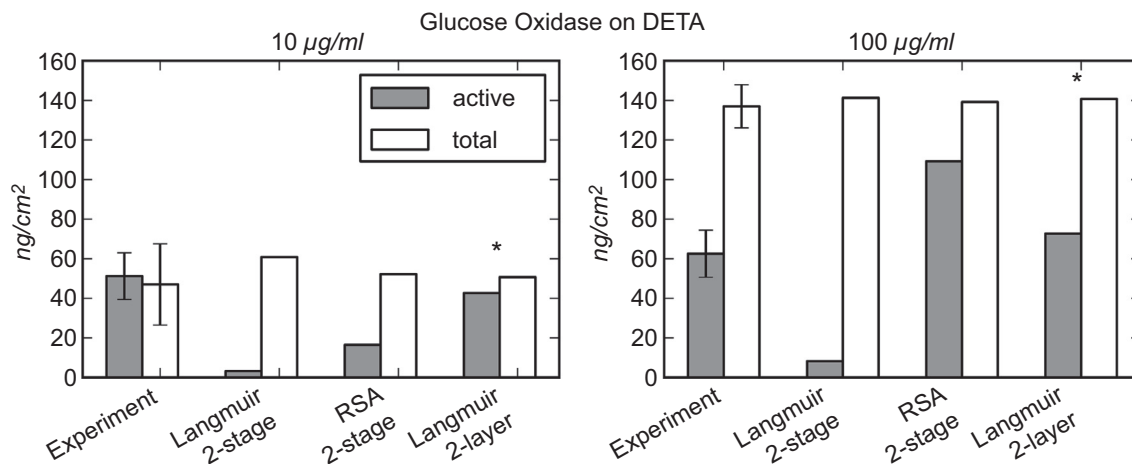
The model fits to the kinetic data for GO on DETA (Table 3) were not as good as the fits to the kinetic data for GO on glass. The RSA-type two-stage model provided the best fit to the kinetic data, as determined by the MSE. It was assumed that the DETA surface did not induce proteins to denature upon adsorption, and this assumption was consistent with the data from the enzyme activity assay at 10 µg/ml, where the adsorbed layer retained ~100% of its expected activity (Fig. 5). When the solution concentration was increased to 100 µg/ml, more than half of the adsorbed GO apparently lost its activity. It is unlikely that this reduction was caused by denaturation, since adsorbed proteins are more likely to retain their native conformation when the rate of adsorption is higher [41]. Further, the fitted parameter values for the two-stage RSA-type model were fairly implausible. The fitted transition rate constant  $k_s$  was two orders of magnitude higher than the transition rate constant for the glass surface, and the surface area occupied by a molecule in state  $\beta$  was 16 times larger than a molecule in state  $\alpha$ . This would mean that not only is DETA a denaturing surface, but a highly denaturing surface, which is contradictory to established protein adsorption theory. An alternative hypothesis for the 50% reduction in activity was provided by the two-layer Langmuir model, wherein a second layer of adsorbed enzyme was formed which prevented the enzyme substrate from reaching the lower layer of enzyme. In this way it was possible to reconcile the low and high concentration data. As can be seen in Fig. 5 the two-stage Langmuir model was able to predict the proportion of active enzyme for the 10 µg/ml data and the 100 µg/ml data within experimental error, while the remaining models clearly did not reflect experimental observation.

For all four surfaces at both concentrations, CFD simulations indicated that the concentration of protein in solution near the surface of the resonator reached 99% of its final value about 20 s after switching from buffer to protein solution. These results demonstrate that transport to the sensor surface does not significantly affect the adsorption kinetics of GO on silane surfaces. This conclusion differs from that of a previous study in which the adsorption of fibronectin was measured at lower concentrations with the same WGM instrument and a significant transport limitation was observed [12]. The molar concentrations of GO used in this study were higher than the concentrations used in the FN adsorption experiments. The initial rate of GO adsorption was also significantly lower than the initial rate of FN adsorption, so the solution near the resonator surface was never depleted of glucose oxidase in the present study.

## 5. Discussion

The combination of direct kinetic measurements from the WGM biosensor and the activity assay provided unique insights into the fundamental mechanisms of protein adsorption for the model protein glucose oxidase on engineered silica substrates. At 100 µg/ml GO adsorbed to the different surfaces according to the following trend (from highest saturation to lowest): DETA > 13F > Glass > SiPEG. At 10 µg/ml the trend observed was slightly different yielding: 13F > DETA > Glass > SiPEG. Furthermore, it can be seen that the saturation values observed on both DETA and glass exhibit strong concentration dependence (differences of ~100 ng/cm² and 60 ng/cm² respectively). GO adsorption onto 13F showed much less concentration dependence with a difference of ~20 ng/cm². It should be noted that despite the sensitivity of the WGM biosensor, it was unable to detect the adsorption of GO on the SiPEG surface at a solution concentration of 10 µg/ml indicating little or no adsorption at that concentration. At 100 µg/ml, adsorbed GO was detected on the SiPEG surface, but at a relatively low surface density (~20 ng/cm²). These results are consistent with the general consensus of previous protein adsorption studies [42] and seems to be a general feature of protein adsorption onto the selected silanes, as was inferred from indirect cell culture experiments done previously [12].

GO adsorbed onto native glass and 13F surfaces retained a significantly higher fraction of its activity at a solution concentration of 100 µg/ml compared to a solution concentration of 10 µg/ml.



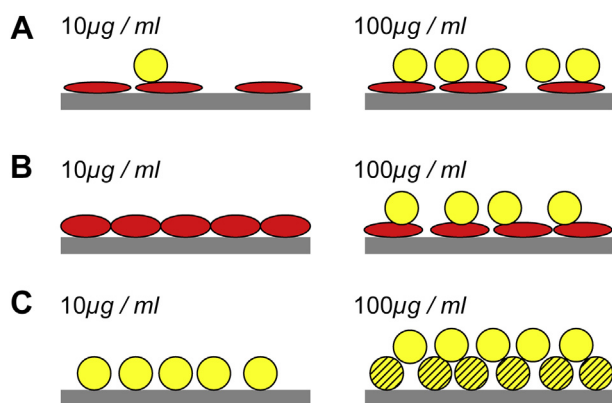
**Fig. 5.** Experimental measurements and model predictions of enzyme activity on 13F surface. The asterisk (\*) on the bar indicates the model that fitted the kinetic data with the lowest mean-squared error and best predicted the biological activity of the adsorbed protein.

According to the conventional theory of protein adsorption, at lower solution concentrations the protein adsorbs at a lower rate and has more time to undergo spreading and denaturation upon adsorption to a surface. However, at higher solution concentrations there is less time for the protein to spread before another protein is adsorbed near it [29]. Thus, the adsorbed species are stabilized by steric constraints (crowding) imposed by neighboring proteins. An alternative hypothesis is that an initial layer of enzyme adsorbed to the surface in a highly spread-out state, resulting in a layer of enzyme with very low surface density and little or no activity that passivated the surface, followed by a second layer of adsorbed enzyme that retained its activity. This hypothesis is illustrated in Fig. 6A for a glass surface and Fig. 6B for a surface modified with 13F. The passivation hypothesis is supported by the fact that two-stage and two-layer models made similar predictions for the activity of enzyme adsorbed onto glass and 13F surfaces from a 100 µg/ml solution. This process could explain the low fraction of active enzyme at 10 µg/ml and the high fraction of active enzyme at 100 µg/ml. Modeling this process would require a model that incorporated both a post-adsorption transition and multi-layer

adsorption. Future work will involve experiments to quantify the extent of protein spreading on strongly denaturing surfaces.

The data presented here suggests an alternative interpretation of the loss of GO activity when adsorbed onto positively charged aminated surfaces from higher solution concentrations. As GO completely retains its catalytic ability when adsorbed from a 10 µg/ml solution, it is clear that (if denatured at all) the protein structure is not perturbed significantly enough to effect its catalytic domain. Thus, it makes sense that protein adsorbed directly to the amine surface from higher concentrations would also remain active. Most theories of protein adsorption maintain that adsorption from higher solution concentrations typically leads to stabilization of protein structure and, thus, retained biological activity. Therefore it is reasonable to postulate an alternate explanation to the observed results. The results of the current study point to the formation of multiple layers of GO, which either further denature the layer beneath it, or prevent the access of substrate to the catalytic domain of the underlying protein layer. A schematic of the two-layer adsorption mechanism is shown in Fig. 6C.

The multi-layer interpretation contrasts with that of Fears and Latour [19], who showed that GO adsorbed from a solution concentration of 1 mg/ml on amine-terminated alkanethiol SAMs formed an adsorbed layer with a ~70% reduction in enzyme activity. Their circular dichroism (CD) data suggested that significant rearrangements in the tertiary structure of GO occurred upon adsorption. Thus it was concluded that the resulting loss in activity was caused by surface-induced denaturation of the enzyme and hence the catalytic domain of GO. Due to sensitivity constraints of the CD method, it was not practical to perform similar measurements at lower solution concentrations. By implementing the ultrasensitive WGM sensor, it was possible to extend these measurements to adsorbed layers from solution concentrations two orders of magnitude lower than possible with CD, and also obtain kinetic binding curves that allowed the fitting of different adsorption models for quantitative comparison. While GO has been used in conjunction with polymeric materials to form engineered multi-layer surfaces, to our knowledge this is the first report of non-specifically adsorbed GO multi-layer formation on engineered silane surfaces. Further experiments are required to verify the multi-layer hypothesis, using techniques such as AFM, SEM and more advanced development of the WGM sensor. It has been proposed that by multiplexing the relatively simple resonance shifting modality used here with techniques such as differential TE/TM



**Fig. 6.** Schematic representation of the protein adsorption mechanism that is most likely to account for experimental observations for the native glass surface (A), a surface functionalized with 13F (B), and DETA (C). Active enzyme is shown as a yellow circle and denatured enzyme is shown as a red oval. Active enzyme that is not accessible to diffusing substrate is shown as a yellow circle with hash lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mode shifting [43], Q-damping, peak-splitting [44], and using multiple wavelength excitation sources [45], it should be possible to obtain a wide variety of information from a WGM resonator (e.g. orientation, geometry, chemical structure) that was previously unobtainable with other methods. In fact, WGM biosensing has the potential to push the limits of our understanding of PA and protein-surface interactions.

The RSA and Langmuir single-layer models fitted the 100  $\mu\text{g/ml}$  SiPEG data set well, as shown in Table 4. The more complex adsorption models were not fitted to this data because the resulting parameters would be unreliable. When a model has too many degrees of freedom relative to the data set, multiple combinations of parameter values may be found that produce the same MSE (non-uniqueness). It is well known that SiPEG surfaces resist protein adsorption, but the lack of enzyme activity on the SiPEG surface was surprising. This may be because the amount of activity was below the detection limit of the assay, or because the enzyme did not retain its activity on SiPEG. Denaturation on SiPEG has not been widely reported, although a study of fibronectin adsorption on similar SiPEG surfaces suggested that denaturation had occurred [12]. Further work utilizing even more sensitive configurations of the WGM biosensor will be useful for answering these questions. By utilizing spheres with smaller radii and shorter wavelengths of excitation light, (near-IR to visible) it will be possible to boost the sensitivity of the biosensor by minimizing the modal volume of the sensor and Q-damping due to the absorption of light into the surrounding water [46]. In this way it will be possible to study protein interactions with PEG and other low-affinity surfaces in detail that have to date been prohibitively challenging.

The insights that were obtained into the mechanisms of adsorption demonstrated the utility of fitting both Langmuir-type and RSA-type models to the experimental data. Each type of model incorporates different assumptions about adsorption behavior. This is especially noticeable with the two-stage models. Although the overall kinetics of adsorption predicted by the Langmuir-type and RSA-type two-stage models may be very similar, the predicted amount of protein in each state could be very different. Fig. 7 shows representative results from the two models when the same parameters were used in each model. The two models predict that similar amounts of enzyme will adsorb, but the Langmuir-type model predicts that the adsorbed enzyme will lose its activity significantly more quickly than the prediction from the RSA-type model. This difference had a significant impact on the overall ability of the models to match experimental data.

Comparing the results from the WGM biosensor to published results is difficult due to the limited amount of data for the adsorption of GO on similar surfaces. The crystal structure of GO from *A. niger* is available at the Protein Data Bank (1CF3), and its bounding box is 6.0 nm  $\times$  5.2 nm  $\times$  7.7 nm [22]. In a previous study, glucose oxidase was adsorbed onto plasma-polymerized thin-films of hexamethyldisiloxane (HDMS) on silica substrates [27]. The native HDMS was modified by exposure to nitrogen and oxygen plasma to produce different surface characteristics, as shown in Table 5. A quartz crystal microbalance (QCM) was used to quantify the saturation surface densities and AFM was used to image the adsorbed protein. At 100  $\mu\text{g/ml}$  the saturation surface densities on HDMS-N and HDMS-O were roughly three times the densities measured on DETA on 13F, respectively. At 10–20  $\mu\text{g/ml}$ , the surface densities measured by QCM and WGM were fairly similar. The QCM and WGM results consistently showed that more GO adsorbed on moderately hydrophilic positively charged surfaces (HDMS-N and DETA) than on hydrophobic surfaces (HDMS and 13F).

The AFM imaging indicated that GO adsorbed on HDMS could be approximated by an ellipsoid with a major axis of 10–14 nm and a minor axis of 6–8 nm. It was found that GO adsorbed to native

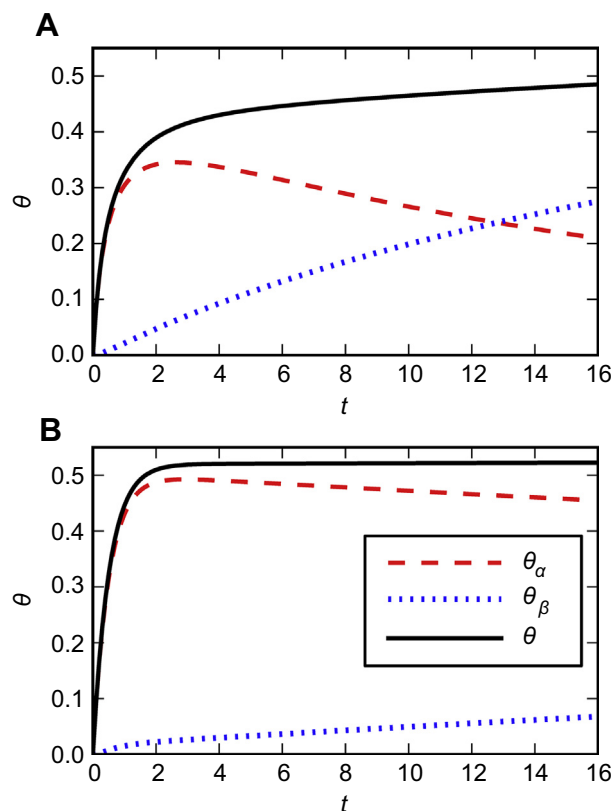


Fig. 7. (A) Kinetics of adsorption for the Langmuir-type two-stage model, and (B) Kinetics of adsorption for the RSA-type two-stage adsorption model.

HDMS surfaces with the major axis normal to the surface (occupying about 40 nm<sup>2</sup>) while GO adsorbed to HDMS-O and HDMS-N with the major axis parallel to the surface (occupying about 80 nm<sup>2</sup>). These dimensions are consistent with previous AFM studies of GO adsorbed on gold which reported that GO is elliptical with a major axis of roughly 14 nm and a minor axis of 8 nm [47]. The parameters fitted to the WGM biosensor data indicate that the area occupied by a single molecule ranges from 30 to 400 nm<sup>2</sup>, which is considerably larger than the area derived from AFM data. The area of an adsorbed molecule was calculated based on the assumption that each molecule occupies a circular footprint and the saturation surface density is limited by sphere packing. If GO has an ellipsoidal shape and does not pack in a dense monolayer, the area predicted by the fitting method will reflect the area of a circle that bounds each adsorbed molecule, rather than the area occupied by the molecule itself.

More generally, numerous studies on protein adsorption have been performed on silane-coated silica substrates using a wide variety of techniques. However, very few of these techniques yield

Table 5  
Properties of surfaces used in this work and in previous measurements of GO adsorption.

| Surface chemistry | Water contact angle | Zeta potential (mV) | Reference |
|-------------------|---------------------|---------------------|-----------|
| Glass             | Wetting             | –25                 | [34]      |
| DETA              | 49 $\pm$ 2°         | 10                  | [35,36]   |
| 13F               | 94 $\pm$ 2°         | –15                 | [37,38]   |
| SiPEG             | 37 $\pm$ 2°         |                     | [36]      |
| HDMS              | >90°                |                     | [21]      |
| HDMS-N            | <50°                | 20                  | [21]      |
| HDMS-O            | hydrophilic         | –40                 | [21]      |

the kind of kinetic data that are required for mechanistic modeling. Previous work utilizing fluorescently-labeled proteins on thin-film modified glass slides allowed real-time measurement of protein binding [48], but was not able to absolutely quantify the amount of protein being detected. Additionally, as this method was not label free, it is difficult to say what the effect of a bulky labeling group was on the rate and end conformation of the adsorbed protein. Optical waveguide lightmode spectroscopy (OWLS) is a label-free technique that allows highly sensitive detection and has been used for studies of PA on silane modified waveguides [49]. However, due to the need for a high refractive index material to confine light, the waveguides are typically made of titanium oxide, which is a fundamentally different material on which to immobilize silanes, thus complicating comparison of results. Additionally the limit of detection for the OWLS technique is  $\sim 0.5$  ng/cm<sup>2</sup> [50], which is far greater than the single molecule detection limit of optical cavity sensors.

Perhaps the most commonly used technique is the quartz crystal microbalance (QCM) system [50–52]. This method has several advantages in that a variety of materials can be supported on the resonator (silanes, polymers, metals, etc.), and optical transparency is not required as in optical methods. However, while limits of detection are very favorable (also  $\sim 0.5$  ng/cm<sup>2</sup>), these still fall short of optical microcavity sensors. Additionally, adsorbed mass is calculated with the Sauerbrey equation. This equation assumes the attachment of a homogenous, rigid layer in order to accurately calculate mass loading [53]. When used for non-rigid adsorbed layers, such as proteins, the Sauerbrey equation can only be used as an approximation. Due to the viscoelastic nature of adsorbed proteins, it is common practice to measure the dissipation of the QCM resonance and calculate the amount of bound water using the Voigt model, thus correcting for the “dry” mass adsorbed to the surface. However, the Voigt model necessarily assumes a single-layer of adsorbed material. Given the complexity of the protein adsorption process presented here, this assumption is not necessarily valid.

## 6. Conclusions

Whispering gallery mode technology has enabled a significant advancement in the ability to understand the nanoscale interactions that drive protein adsorption on silane-modified glass and silica surfaces. A biosensor based on a spheroidal glass WGM resonator was used to quantify the kinetics of adsorption of glucose oxidase on four different types of surface, and five models of adsorption were fitted to each experimental data set. The model fitting allowed additional conclusions to be drawn from the experimental data about the mechanism of adsorption on each surface. The experimentally measured activity of the adsorbed enzyme provided a valuable check on the kinetic fitting results. On glass and 13F surfaces, the experimental data was best fitted by two-stage models that incorporate a post-adsorption transition to model denaturation after adsorption. The two-layer model also fitted the kinetic data for these surfaces well and predicted the activity at 100  $\mu$ g/ml. The adsorption mechanism may incorporate both processes. A thin layer of protein may passivate the surface and allow additional enzyme to retain its activity after adsorption. On a positively charged, moderately hydrophilic surface (DETA), GO retained its activity but formed two layers at higher solution concentrations, limiting substrate access to the lower layer. This comprehensive data set provides new insight into the adsorption behavior of GO, and points to the formation of multiple layers, which either further denature the layer beneath it, prevent the access of substrate to the catalytic domain of the underlying protein layer, or readily desorb after denaturation.

The high sensitivity of the WGM biosensor allowed adsorption measurements to be made on a SiPEG monolayer. With the potential for further improvements in sensitivity and stability, WGM biosensors could be used to understand why nanostructured surfaces like SiPEG resist protein adsorption under experimental conditions but have not shown long-term efficacy in clinical applications. This and other previous work demonstrate the utility of the method for exploring the biocompatibility and adsorption behavior of biopolymers on a wide range of engineered surfaces. The WGM biosensor is especially useful for studying proteins whose function may be impaired by labeling. Optical micro-resonator sensors have the potential to study protein adsorption on a wide range of functionalized silicon and glass surfaces that are of interest to the biomaterials community.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2014.10.002>.

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