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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.

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_2, 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 $m\lambda = 2\pi r n_{eff}$, where m is an integer number, r is the radius of the resonator, and n_{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. 1 B). 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 denaturization 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-[Methoxypoly(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

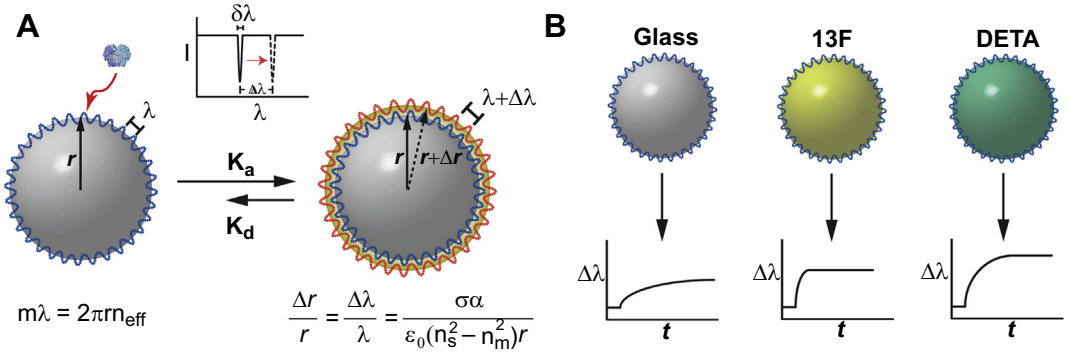

Fig. 1. (A) Schematic representation of WGM biosensing principle. Light evanescently coupled into a glass microsphere of radius r achieves total internal reflectance upon satisfaction of the resonance criteria where n_{eff} is the effective refractive index of the sphere, λ is the resonant wavelength 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, σ, where α is the polarizability of the protein, n_s and n_m are the refractive indices of the substrate and surrounding medium respectively, and e_0 is the permittivity of free space. (B) Silica microspheres are ideal substrates for supporting a wide variety of surface chemistries.