Chapter 2

Biosensors

2.1 Overview

The recent trend toward integrated and automated instruments based on bioassays has had an extraordinary impact on the speed and efficiency with which analytical and diagnostic measurements can be made. Bioassays are methods by which one may determine the absolute or relative concentration of a specific biomolecule in a sample; the target molecule is called the *analyte*. The researcher attempting to measure how well a new drug works, the medical laboratory technician testing a patient's blood for disease, and even the biology graduate student manipulating genes in an effort to understand a fundamental cellular function relies on a bioassay to get his or her answer.

Each bioassay is characterized by a method that translates the quantity of analyte present in the sample into a measureable signal. Any device that can uses biomolecular interactions at its surface to report this signal while rejecting unintended, nonspecific signal is called a *biosensor*. It is indeed discouraging to observe in the literature the variety of meanings given to these words; the definitions chosen here are based on those used by the International Union of Pure and Applied Chemistry (IUPAC) [4].

My work focuses on one variety of biosensor–the whispering gallery mode (WGM) optical resonator. I describe the details of this type of device, as well as its fabrication, in Chapter 3. WGM optical resonators have only been used for biosensing applications for less than a decade [5, 6] and the performance and limitations of the technology are still being characterized and debated. It is clear already, however, that

these devices exhibit extraordinary sensitivity and show great promise as a future analytical or diagnostic instrument. This process by which a technology is built into an integrated instrument is slow and laborious, however, and requires an understanding of the many factors that contribute to its performance. The results presented in Chapters 4 through 6 aim to advance this understanding and, hopefully, inform the development of an instrument.

This chapter describes the features common to all biosensors and introduces a range of example technologies: (i) a method for detecting biomolecules, (ii) a method for ensuring only specific detection, and (iii) a method for introducing sample. With these in mind, I introduce the biosensor technologies in use today and explore their advantages and disadvantages. Finally, I discuss what researchers in the WGM biosensing field can learn from these alternatives, and what challenges must be addressed in the ongoing effort to develop a WGM biosensor instrument.

2.2 Specific Detection

A key function of a biosensor is the ability to either ignore signal from molecules other than the analyte or to actively stifle all interactions other than those with the species of interest. Though the latter is the far easier option in nearly all cases, it would be misleading to suggest that either option is, by any means, simple. To reject all interactions other than those with the analyte, the surface of the sensor may be functionalized with a targeting species that will interact only with the analyte.

The interaction between two species that have a particularly high and exclusive affinity for one another is called *specific binding*, and it is due to a variety of factors. It is often depicted as a simple geometric match, with one molecule possessing a "binding pocket" shaped perfectly for its ligand. While this is often the case, a more complete model must take into account the balance between attractive and repulsive forces as well as the overall energy of the bound and unbound systems. The more common type of interaction is called *nonspecific binding*, and it implies only that there is an affinity between two species.

The best example of nonspecific binding is highlighted by the work of Leo Vroman, who spent much of

his career as a hematologist studying how proteins in blood plasma adsorb to surfaces. There is no specific interaction between a protein, such as fibrinogen, and a surface like glass, but it adorbs to a microscope slide nevertheless because the protein is not rigid. Its tertiary structure bends and stretches, sampling a range of conformations. The hydrophobic residues that are typically hidden in the center of the protein to limit their exposure to the polar water molecules in the solvent can induce a restructuring with two effects: (i) these hydrophobic residues can be pressed against the surface (typically less polar than water) to further decrease the strength of their interaction with the solvent and (ii) more of the hydrophilic residues can interact with water due to the higher surface area-to-volume ratio of the pancake-like bound protein [7]. Any generic interaction, including a pure Coulombic one that might draw all positively or all negatively charged species to the sensor surface, can be considered non-specific. Rejecting all signals due to the many possible nonspecific binding methods is, indeed, a challenge.

A popular method to reject this type of undesired adsorption in favor of specific interactions is to simply coat the sensor surface with the targeting species, such that there is no remaining space for other species to occupy. Attaining such high surface coverage is very difficult because of steric effects. An alternative approach is to fill the space between targeting species molecules with a material that actively rejects nonspecific adsorption, e.g., polyethylene glycol (PEG) [8, 9]. It is believed that the high density of hydroxyl groups in PEG produce a structured and stable water layer that would be disrupted (a significant enthalpic penalty) by nonspecific adsorption by other species. A variety of surface treatments have been developed toward a similar purpose [10]. Several have even been incorporated into a molecular architecture that includes the targeting agent [11, 12, 13].

Reversible binding of the analyte to the targeting species can be described using a simple Langmuir kinetic model where [A] is the concentration of analyte, [T] is the surface concentration of unbound targeting species, [C] is the surface concentration of the bound complex, and $[T]_0$ is the total concentration fo the targeting species. The adsorption/desorption reaction

$$A + T \rightleftharpoons C$$

proceeds at a forward (adsorption) rate,

$$\text{rate}_{ads} = k_{ads}[\text{A}][\text{T}], \tag{2.1}$$

and a reverse (desorption) rate,

$$\text{rate}_{des} = k_{des}[\text{C}]. \tag{2.2}$$

The rate of change of the free analyte concentration is

$$\frac{d[\text{A}]}{dt} = -k_{ads}[\text{A}][\text{T}] + k_{des}[\text{C}]. \tag{2.3}$$

At equilibrium, $\frac{d[\text{A}]}{dt} = 0$, and

$$\frac{k_{des}}{k_{ads}} = K_D = \frac{[\text{A}][\text{T}]}{[\text{C}]} \tag{2.4}$$

is the equilibrium dissociation constant. The total concentration of tageting sites [T] is conserves, so the concentration of available sites is $[\text{T}] = [\text{T}]_0 - [\text{C}]$. Therefore, the bound analyte concentration varies with time as

$$\frac{d[\text{C}]}{dt} = k_{ads}[\text{A}]([\text{T}]_0 - [\text{C}]) - k_{des}[\text{C}], \tag{2.5}$$

and asymptotically approaches the steady-state (or “equilibrium") value of

$$[\text{C}]_{\text{eq}} = \frac{[\text{A}][\text{T}]_0}{\text{K}_\text{D} + [\text{A}]}. \tag{2.6}$$

It is simple to show that half of all binding sites are occupied when [A]=K_D. The sensor response as a function of [A] enables measurement of K_D as illustrated in Figure 2.1 for the protein Interleukin-2 [14].

It is worth pointing out that interaction between an analyte and its targeting species can be a delicate function of experimental conditions. Not only is the affinity (K_D) a function of temperature due to the effect of thermal fluctations (or lack thereof) on the probability that two species will be able to bind to one

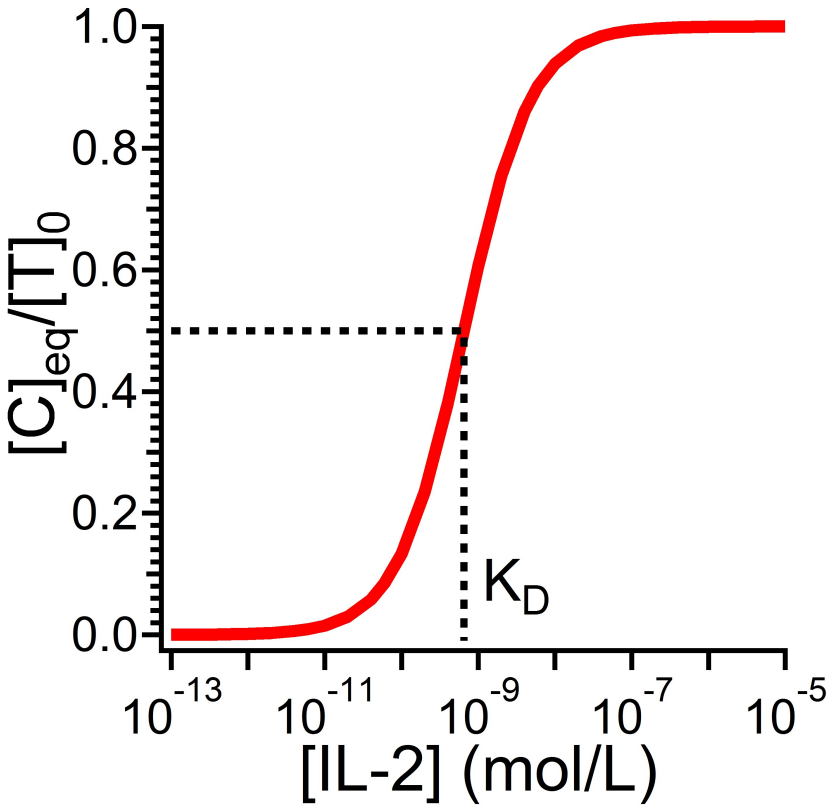


Figure 2.1: An equilibrium binding curve for Interleukin-2 with its T Lymphocyte receptor according to Eq. (2.6). Note the sigmoidal shape whose slope approaches zero in the limit of both high and low analyte concentrations. At low [A], the large relative changes in concetration are still too small in terms of total analyte molocules bound. In contrast, the sensor surface is saturated at high [A] and changes in concentration make little affect little change in sensor signal. K_D is marked at 6.5×10^{-10} M.

another, but the very structure of the species involved often changes with pH, salt concentration, ambient light, and temperature. One should always keep in mind when using biomolecules that they (for the most part) did not evolve for the sole purpose of a bioassay in a research laboratory. They can be unstable, fickle, and uncooperative when it comes to varying experimental conditions.

Targeting Species

The challenge of finding a species to serve as the targeting agent on a biosensor surface has, thankfully, been addressed by the slow and methodical process of evolution. Biomolecules comprise a vast array of species that exist within organisms, including nucleic acids, proteins, and fatty acids. Each species serves a

particular purpose in an organism, and must interact with other biomolecules in order to accomplish it. The problem of finding a targeting species for a given analyte often comes down to finding the complimentary molecule that evolved to interact with it. Consider nucleic acids, which consist of two chains of repeated units called nucleotides. Deoxyribonucleic acids (DNAs) feature the well-known double helix structure in which the two opposing chains form bonds with one another much like the steps in a ladder. There are only four varieties of nucleotides in DNA; the double helix cannot form unless each nucleotide in the sequence is paired with its one complimentary nucleotide on the the opposing strand. Immobilizing a short, single strand of DNA on the surface of the sensor allows only complimentary strands to bind on the device surface with appreciable stability [15]. Much like with a long zipper with only a single tooth unzipped, however, a long target strand could possibly bind a slightly mismatched sequence because the energetic favorability of the bonds that form outweighs the entropic penalty of immobilization.

The principle of complimentary nucleic acid sequences extends beyond this simple example, though. Aptamers can be used where biology has not produced a tailor-made targeting species, even though it provides the architecture to do so. These oligonucleotides are designed with a sequence that will envelop and bind to a particular species [16, 17, 18]. Methods have been established by which a researcher can evolve an aptamer for an arbitrary analyte [19]. Their capability to encode adaptable molecular recognition has even made them appealing tools for gene-regulation platforms [20]. Though the binding affinitiy of aptamers for their analytes varies greatly [21], they can attain K_D values as low as 1 nM.

Perhaps the most popular targeting species for biosensing applications is the antibody. These proteins, also called immunoglobulins, are a part of the body's immune response and feature K_D values of $0.1-100$ nM for their antigens. They consist of four polypeptide chains—two heavy chains and two light chains—connected to one another via sulfide bonds (see Fig. 2.2). As with all proteins, the variations among antibodies that allow analyte-specific interactions are due to the order in which the 20 available amino acids are arranged, and the tertiary structure to which this sequence leads. Figure 2.2 shows the coarse anatomy of an antibody, which features a stem (the "F_C" region) and two arms (the "F_{AB}" regions). At the end of each F_{AB} region is a complementarity-determining region (CDR) that accounts for the source of variation from one antibody

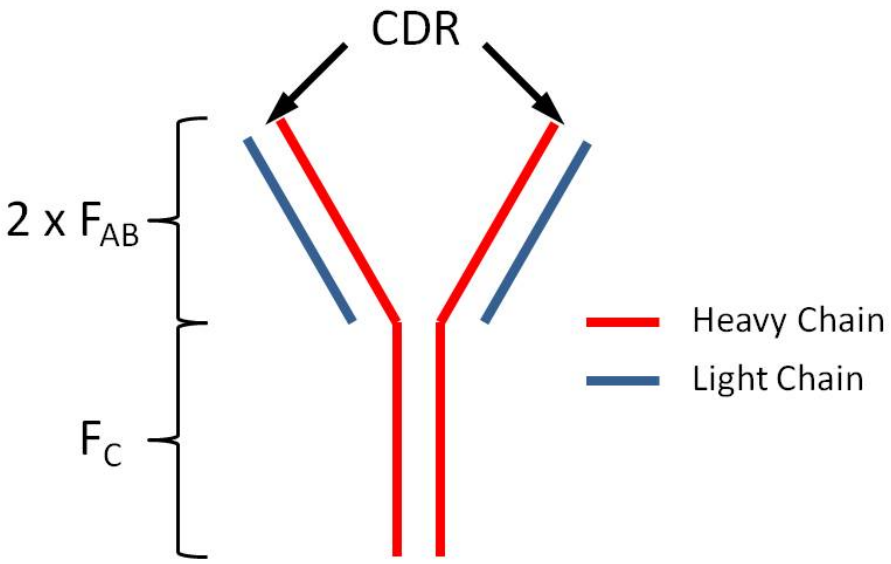


Figure 2.2: This antibody features four polypeptides: two heavy chain (red) and two light chain (blue). Note also the "stem-and-arms" configuration, with one F_C and two F_{AB} regions. The two complementarity-determining regions (CDRs), where analyte binding occurs, are noted at the end of the two F_{AB} regions.

to another and binds to the analyte.

Antibodies exist in *monoclonal* and *polyclonal* varieties. The former refers to antibodies produced when an organism seeks to increase production of a single variant of the antibody by cloning cells that produce only that one type, while the latter refers to a spectrum of antibody variants from a group of cells from different lines. For this reason, monoclonal antibodies are often preferred for sensing applications due to their uniform properties.

Sensor Functionalization Methods

The task of coating a surface with the targeting species of choice is called functionalization. There is a dichotomy among the methods for this purpose: covalent or non-covalent functionalization. The former benefits from stability and the guarantee that your targeting species surface concentration remains constant during an experiment, which makes it possible to use species conservation equations to determine the reaction kinetics of binding and/or desorption. However, covalent functionalization alters the surface chemistry of the sensor and could possibly affect its performance. Non-covalent functionalization methods are less permanent,

but often simpler to implement and less likely to interfere with sensor function. Nonetheless, a covalent method is desirable because it gives control over the orientation of the targeting species not often found in covalent functionalization methods.

Directly attaching the targeting species to the sensor surface, although sometimes possible, is usually unwise because newly formed chemical linkages can affect the molecule's affinity for the analyte. A bifunctional linker molecule is commonly used, one end of which has a moiety to anchor to the sensor surface, and the other has a carefully chosen functional group chosen to react with the targeting species without damaging it. For the gold surface presented by surface plasmon resonance chips (see Section 2.5 below) this linker is often an alkane with a thiol anchor group to react with the gold surface; a maleimide group at the other end of the linker reacts with available cysteine residues on the antibody [22]. The type of coating that results is often referred to as a self-assembling monolayer (SAM) [23].

For the silica WGM biosensor we deal with here, the linker can be an alkane similar to that for a gold surface, replacing the thiol with a trichloro, trimethoxy, or triethoxy silane group that reacts well with acid-treated silica. This type of linker is particularly useful because of the high vapor pressure of the silane, which makes it possible to bind the linker to the silica by vapor deposition, thereby avoiding the damage to and contamination of the ultrasmooth surface of WGM sensors that often accompanies the use of a more harsh liquid-phase environment for functionalization [24]. The bioconjugation chemistry literature provides details of these and other covalent functionalization techniques [12, 13, 11, 25, 26].

Non-covalent techniques rely on physisorption of an anchor molecule, to which either a linker or the targeting species itself may be conjugated. This physisorption, often the result of hydrophobicity of the surface and the anchor molecule, can be exploited to great effect using polymer layers [27, 28], but is not orientation-specific. One elegant, albeit unstable, example is the use of Protein G [29], a protein capable of non-specifically adsorbing to some bare surfaces and maintining its function of binding to the F_C region of any IgG antibody. Figure 2.3 depicts a Protein G-anchored functionalization architecture, including the technique's two main drawbacks: (i) local surface density variations due to the nature of nonspecific adsorption, and (ii) the variability in orientation of the antibodies immobilized by the Protein G molecules.

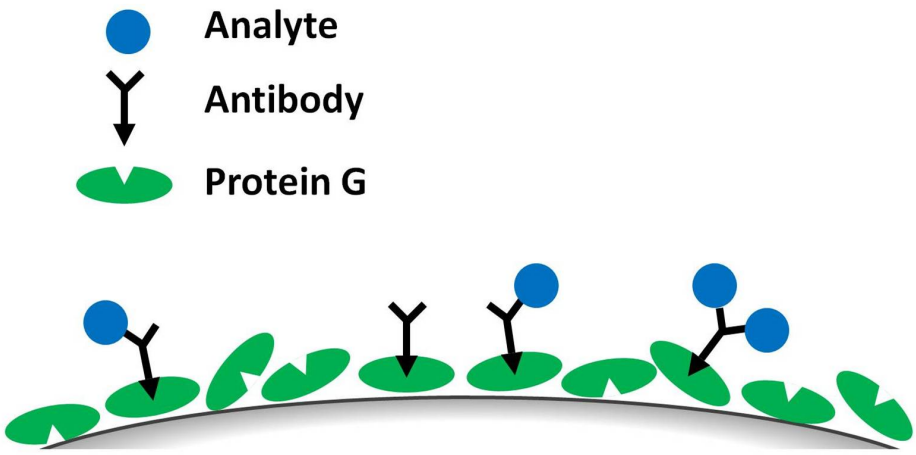


Figure 2.3: The non-covalent functioanlization of a biosensor surface via the non-specific adsorption of Protein G (green) and antibody (black). Exposure to analyte (blue) will lead to binding according to the equilbrium expression in Eqs. (2.1)–(2.4). Note the random orientation of the Protein G molecules as well as the fact that not all such molecules are occcupied by an antibody.

The singular binding orientation available to antibodies that interact with Protein G is an advantage over other surface functionalization methods, some of which result in the highly oriented anchor molecule arrangement but randomly oriented targeting molecules. Nonetheless, sensors using non-covalent functionlization architectures can be unreliable due to the absence of a permanent bond. Fluctuations in solution conditions, temperature, or even just oxidation over time can erode the uniformity of the non-covalent layer.

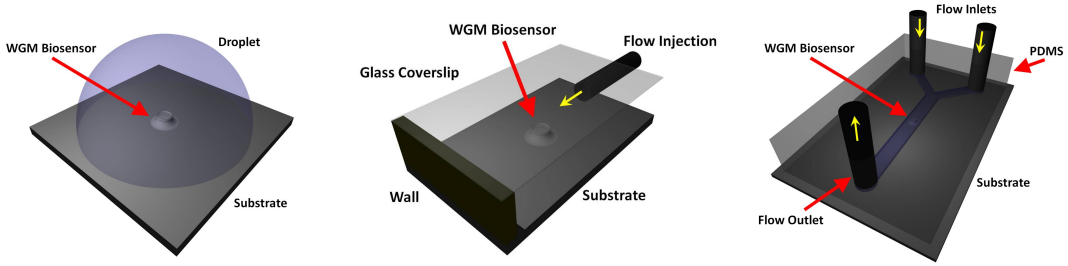
The covalent and non-covalent methods overlap with the use of the well-known complexation reaction between biotin and tetravalent protein avidin. The former is a small molecule known also as vitamin B7, while the latter is a protein commonly found in chicken and other bird eggs. They bind with a $K_D \sim 10^{-15}$ M, making theirs an extraordinarily strong bond that is, at least technically, non-covalent. This pair is an excellent tool for evaluating and characterizing sensors and other assays because it removes all doubt as to whether the species bound to an appreciable extent. Additionally, the utility of biotin and avidin lies in their ability to be conjugated to other species while maintaining their affinity. As such, enzymes, antibodies, and other proteins are available commercially with conjugated biotin or streptavidin. Methods have been developed to exploit this pair for sensor functionalization to impressive ends [24].

2.3 Sample Delivery Methods

As we discuss in Chapter 5, the methods used to introduce sample to a biosensor can significantly affect the data collected by a biosensor. At the very least, a sample delivery system must ensure that the sensor is efficiently exposed to the sample. Two practical constraints are added when working with valuable or rare sample, a common occurrence in medical diagnostics and analytical biochemistry. The first of these is that the delivery system conserve sample as much as possible by limiting the volume required for a measurement. This includes all flow paths and reaction chambers. The second constraint is that the delivery system must minimize the time necessary for a measurement. This second constraint is related to the first in that both aim to improve sample economy, and they represent different tactics for accomplishing this goal.

Combining these two concepts would suggest a small-volume biosensor flow cell as the optimal delivery system. Diffusion alone is an inefficient delivery method for the analyte to the micro- and nanoscale sensors we discuss below if the flow cell is considerable larger than the sensor. Microfluidic devices are a practical solution because they can be repeatably made from conveniently adaptable materials (e.g., polydimethylsiloxane, also called PDMS) with small overall dimensions and micron-scale precision using soft lithography techniques. Their behavior is well characterized [30, 31], partly due to the fact that flow in such small channels with width and height typically less than 500 μm is always laminar. To enter the turbulent flow regime, which is far more difficult to describe analytically, would require impossibly high fluid velocities. In light of the laminar restrictions, mixing of two streams in a microfluidic device is a challenging but realizable feat [32].

Microfluidic devices suffer the drawback of cumbersome pumping systems, high pressures due to the small flow channels, and what can be a slow, serial fabrication procedure. Other, simpler options are available, although they usually do not isolate the system from ambient changes as well as microfluidic devices. For example, extremely sensitive measurements have been made using an open flow cell comprising a substrate for the bottom of the cell, a glass coverslip as the top, and a single wall to connect the two. One or many flow inlets and outlets may be inserted in the absence of walls, and the fluid (typically aqueous) is held in place by capillary forces. The tempation to use a material that avoids the depleting effects nonspecific adsorption onto the flow cell walls, specifically Teflon, is unwise due to the hydrophobicity of that material



(a) Droplet (b) Open Flow Cell (c) Microfluidic Device

Figure 2.4: Methods for delivering sample to a biosensor. (a) The simple batch method, wherein a droplet of solution is placed onto a planar sensor and diffusion delivers sample to the device surface. (b) The open flow cell with flow injection, featuring a substrate and glass coverslip to form the top and bottom. Surface tension prevents the water from draining, requiring that either the top and bottom surfaces be sufficiently wettable *or* the gap sufficiently small. (c) The microfluidic flow cell, a subset of the closed flow cells. These devices are typically made using soft lithography techniques, and their microscale features ensure laminar flow and very little mixing.

and its inability to trap water in the cell. Silica and stainless steel are suitably wettable, however. This sample delivery system, as well as others, is depticted in Figure 2.4. The simplest sample delivery system is the addition of a droplet to engulf a substrate-bound microscale biosensor [33]. The droplet will experience increased evaporation compared to enclosed flow cells, however, and the resulting change in concentration and temperature would likely complicate the interpretation of experimental results.

2.4 Biosensor Performance Metrics

In order to appreciate the strengths and weaknesses of a biosensing technology, one must have a way of comparing it to other technologies. An ideal biosensor would of course be inexpensive, simple to use, and effective. Though cost and ease of use depend greatly on instrument-level design features, one can often get an idea of whether these factors are likely to pose problems in the future. The effectiveness is the most useful tool for comparison of technologies in their developmental stages, however. I will, therefore, focus on the primary figures of merit used in the biosensing community when evaluating device effectiveness: limit of detection (LOD) and dynamic range (DR).

Biosensors may be designed to produce two types of data—transient ("kinetic") data, and endpoint

("equilibrium") data. Transient data shows how the sensor response changes over time and, with careful considerations of mass transfer, can be used to measure the kinetic characteristics of the surface binding reaction (Eqs. (2.1)-(2.4)). Endpoint data records a single point for each experiment to reflect the sensor response after a certain amount of time or after a particular event has occurred. For the most part, this event is when the system reaches a steady-state, which reflects an equilibrium or saturation in the surface reaction occurring at the surface of the biosensor.

The LOD is an intuitive quantity that describes the lowest concentraton at which a biosensor can produce a signal clearly distinguishable from the noise in the measurement. For present purposes, a clearly distinguishable signal is one that has a signal-to-noise ratio (SNR) greater than 1. A SNR value less than 1 implies that the noise overwhelms the magnitude of the signal. A biosensor with a SNR 1 at a concentration of 10 fM can quantatively measure the concentration of sample down to this value, although variability from experiment to experiment may push the LOD higher. For this reason, one must always use multiple trials to demonstrate the true limit of detection for a sensor. Both transient and endpoint data are used to determine the LOD.

The dynamic range of a sensor describes the range of concentrations over which the device can reliably report a concentration perturbation. A plot of the endpoint sensor response (see Figure 2.1) can be used to determine the dynamic range of a sensor with signal X by examining $\frac{dX}{d[\mathrm{A}]}$, an expression formally referred to as the *sensitivity*. The sensor is limited at low [A] by its LOD; at high concentrations the sensor surface may be saturated so that no binding sites remain for adsorption of analyte. In both of these limits, $\left|\frac{dX}{d[\mathrm{A}]}\right| \ll 1$, and even large perturbations in [A] cannot be resolved by the sensor. The signal can change greatly due to such a perturbation if $[\mathrm{A}] \approx K_D$, however. The dynamic range is defined by the concetration window from $[\mathrm{A}]_{lower}$ to $[\mathrm{A}]_{upper}$ inside which a perturbation $\delta[\mathrm{A}]$ produces a sensor response that is distinguishable above the noise σ_{noise}, or where $\delta[\mathrm{A}]\left|\frac{dX}{d[\mathrm{A}]}\right| > \sigma_{noise}$.

An additional consideration is sample economy. As previously mentioned, the sample delivery method often controls sample size, but the physical dimensions of the sensor also play an important role. Small sensors that minimize sample volume are generally preferred in practical applications like medical diagnostics. As

I metion above (see Section 2.3), this is often limited by the sample delivery method, but the actual size of the sensor also plays an important role. Smaller sensors that require less sample are always preferred in practical applications like medical diagnostics. Just imagine a doctor telling you he or she has a test that can tell with 100% certainty whether you have cancer, but that it requires 2 L of your blood. Therefore, it is wise to keep in mind both the size of the device and its ease of integration into a low-volume flow cell when evaluating a biosensor technology.

2.5 Biosensor Technologies

The diverse field of biosensors can be most conveniently organized according to the physical process by which the device translates the adsorption of material into a measureable signal. According to this scheme, there are four predominant categories of sensor: electrical, mechanical, magnetic, and optical biosensors. The discussion that follows will highlight some of the more successful and promising implementations of these technologies. Sensing technologies may be further divided between those that require the analyte or targeting species to be labeled with a particular chemical group or object in order to amplify its interaction with the device and those in which an unlabeled analyte can be detected directly from its interaction with the sensor. For many sensing methods, the detection limit is insufficient without such amplification, but labels may also be needed to distinguish the analyte from other species present in the sample. "Label-free" sensing methods are the focus of much of the research on biosensors because the species of interest very rarely possesses a useful tag naturally. Chemically attaching a label to the analyte in a sample is difficult, costly, and often impractical because of the specificity required in the reaction, especially when the sample is a complex fluid like blood and unintended conjugation reactions are all but unavoidable.

Electrical Biosensors

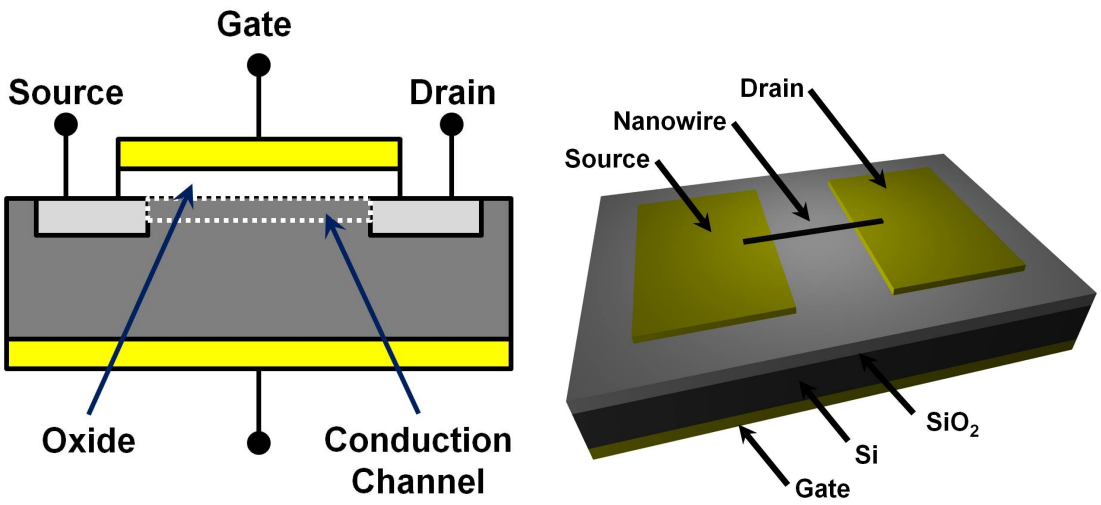
Biosensors that measure how electrical properties of a system change due proximity or contact with analyte have become widespread and enjoy the convenience of using a raw electrical signal, such as current or

impedance, that can be processed directly. This well-represented class of sensors is summarized elsewhere [34, 35]. The most promising among these devices is the nanowire field effect transistors (FET). FETs are devices that monitor the current between two electrical terminals, called the source and drain, embedded in a semiconductor. A second electric field can be applied across two terminals oriented orthogonal to the source to change the concentration of charge carriers (electrons or electron holes) in the semiconductor and gate the current that reaches the drain. Figure 2.5a illustrates this principle.

A FET biosensor is composed of a semiconductor connecting the source and drain electrodes. Charged biomolecules that adsorb to the semiconductor sensor produce an electric field that acts as the gate and changes the charge carrier density within the device. The resulting drain current can be conveniently measured as a reporter for sensing applications. This type of device has been widely applied [34]. Its LOD is generally greater than 1 nM for most biomolecules because the electric field from the bound species only penetrates to a limited depth within the conduction channel. This problem is partly overcome by the use of nanoscale objects to bridge the source and drain such as carbon nanotubes [36, 37] or lithographically defined nanowires [38, 39]. The increased surface area-to-volume ratio for these structures allows the electric field due to adsorbed material to perturb a larger portion of the conduction channel, yielding an LOD low enough to allow for the detection of single virus particles [40]. Figure 2.5b depicts a generic nanowire FET biosensor.

In addition to being a sensitive label-free technology,nanowire FET biosensors can be produced relatively cheaply in parallel with traditional microfabrication techniques that are common in the semiconductor industry. These devices are well suited for incorporation into microfluidic flow cells, and they are sufficiently small to require only microliters of sample for a measurement. While the small sensor size affords a number of advantages, it also poses challenges when integrated in microfluidic systems. Sensing requires molecular interaction with the surface where, in the laminar flow of a microfluidic channel, the velocity is zero. Moreover, the high pressure drop in small channels limits the velocity of the flow and, thereby, the transient response of the sensing system.

The performance of most nanowire FET biosensors decays in the presence of salts. Most biomolecules



(a) FET (b) Nanowire FET

Figure 2.5: Field effect transistors (FETs). (a) A generic FET, including the source and drain with conduction channel between the two. A field applied using the gate can control the density of charge carriers in the conduction channel and change the current measured at the drain. (b) A nanowire field effect transistor (FET) sensor. Biomolecules bound to the surface of the nanowire have a localized electric field that can distort the charge carrier density in the nanowire. Changes in the drain current are used to track how much material has adsorbed to the device.

require a buffered environment to screen interactions that would cause the molecules to precipitate and to stabilize their structure, so a pH-buffered salt solution is often used to dissolve samples of known analyte. Even biofluids like blood or saliva have their own salts and buffering agents for this purpose. These salts screen the electric field of the biomolcule, reducing its effect on the charge carrier population in the conductor [41], thereby limiting the sensitivity in biologically relevant fluids. This negates the field effect provided by the adsorbed material and limits the sensitivity in biologically relevant fluids.

The surface-bound molecular recognition species that enable specific sensing can also diminish the performance of nanowire FET biosensors due to screening effects. Such surface modifications can either promote or retard conduction, depending on the electrical properties of the nanowire. Some functionalization schemes can even modify the charge state of the nanowire permanently [27] and alter the fundamental electrical properties of the device. Identifying methods suitable for nanowire FET sensors remains a challenge.

Recently, however, a clever technique has been used to improve the applicability of these devices [42]. In this method, a two-stage flow system allows the sample to enter a chamber where an immobilized molecular

recognition species can remove the analyte of interest through specific binding. The liquid in the chamber is then flushed and replaced with a low-salt buffer, creating an environment that promotes gradual dissociation of the analyte. This resulting solution is then driven into a second chamber where the nanowire FET sensor is located. The analyte is then free to interact with the sensor in the absence of unwanted species that may have been present in the original sample liquid while also removing the need for functionalizing the surface of the biosensor.

Mechanical Biosensors

Some biosensors use mechanical forces and motion to report the amount of analyte present in a sample. One important example of such a device is the microcantilever [43], which may be used in two modes of detection. First, the static deflection of these devices that results from specific adsorption can be used to measure analyte concentration [44]. Alternatively, the microcantilevers oscillate at a characteristic frequency, much like a tuning fork. This frequency is a function of the shape and material properties of the cantilever, and therefore changes upon adsorption of biomolecules. The deflection and the change in oscillation frequency can be monitored by reflecting a laser off the surface and tracking its position, much the same way as in an atomic force microscope. These label-free devices have been used to specifically sense medically relevant species in complex fluids with a limit of detection of ≈ 1 pM [44]. Their sensitivity limits the applicability of microcantilever sensors, but they can be easily produced using semiconductor processing techniques [45] and are easily functionalized by applying a gold or silica coating that also facilitates laser light reflection.

An alternative mechanical sensor is the quartz crystal microbalance (QCM) [46], which relies on the oscillation of a crystal and the change in resonant frequency as material adsorbs. This oscillation is stimulated electronically, but this frequency change occurs due to the mechanical coupling of bound bimolecules and the crystal surface. As with the microcantilevers, QCMs have LODs of > 1 pM. However, crystal microbalances are available as complete commercial products and are easy to integrate into flow devices. Like microcantilevers, QCMs are very well characterized and easily functionalized via their silicon dioxide surface chemistry.

Magnetic Biosensors

Another variety of biosensor uses changes in the magnetic properties of the system to detect a species of interest. For example, functionalized superparamagnetic nanoparticles can bind specifically to an analyte upon exposure to a sample or injection into an organism. Since these nanoparticles have much slower spin-spin relaxation (T_2) times than the biological species present, they create excellent contrast for magnetic resonance techniques like magnetic resonance imaging (MRI). These particles can be designed for polyvalent binding of the analyte, which amplifies the contrast in magnetic properties by promoting aggregation of particles. These devices are also called magnetic relaxation switches [47, 48] due to the immediate change in magnetic properties of the analyte upon binding with the nanoparticle.

Another common type of magnetic biosensor uses materials whose resistivity changes with applied magnetic field [49, 50]. Application of this magnetoresistive effect to the fabrication of devices with ultrathin ferromagnetic films has led to the development of magnetic random access memory (MRAM), read heads, and other highly sensitive magnetic nanostructures. These devices feature a multilayered architecture, where one layer is a magnetoresistive material and another is typically a layer that enables surface functionalization. Changes in current across these devices can be measured as analyte molecules labeled with ferromagnetic nanoparticles specifically adsorb to the sensor surface and introduce magnetic fields.

One drawback of these technologies is that they require labeled analyte be present in the sample. With exception to iron-containing and other metal-cluster proteins, few biological molecules naturally possess magnetic properties that can be exploited in magnetic resonance or magnetoresistive biosensors. Another disadvantage of magnetic biosensors is the nonspecific interactions that can occur between magnetic nanoparticles or between these nanoparticles are other mateirals. Aggregation in solution or adsorption of clusters onto the sensor surface can lead to false-positive measurements or other artifacts in the signal. Minimizing the size of these particles can help reduce aggregation. Despite these drawbacks, fabrication of magnetic biosensors and the molecular labels they require are typically affordable due to their prevalence in magnetic data storage technologies. They can also be incorporated into microfluidic systems with relative ease, making them useful devices for a limited range of measurements.

Optical Biosensors

Optical biosensors use the interaction between light and matter to report the presence of analyte. These technologies may be divided into two classes. The first uses a form of spectroscopy to isolate the signal due to the analyte, including Fourier transform infrared (FTIR) spectroscopy and ultraviolet-visable (uv-vis) specstroscopy. The most common technique in this class is fluorescence [51], which involves absorption of a photon by a molecule and the subsequent emission of a second photon of lower energy. This emission results from an electron relaxing from an excited state to its ground state. Molecules with electronic structures that allow for both efficient excitation and emission of light in the visible to near-infrared spectrum are referred to as *fluorophores*. Species which exhibit minimal fluorescence when exposed to a particular wavelength of light available for the experiment may be labeled with a fluorophore better suited to that wavelength to enable detection based on the intensity and spectrum of emitted light. The fluorescence signal is isolated using optical filters to eliminate background and excitation light, and collected using a detector (e.g., a photodiode, photomultiplier tube, avalanche photodiode, or charge-coupled device).

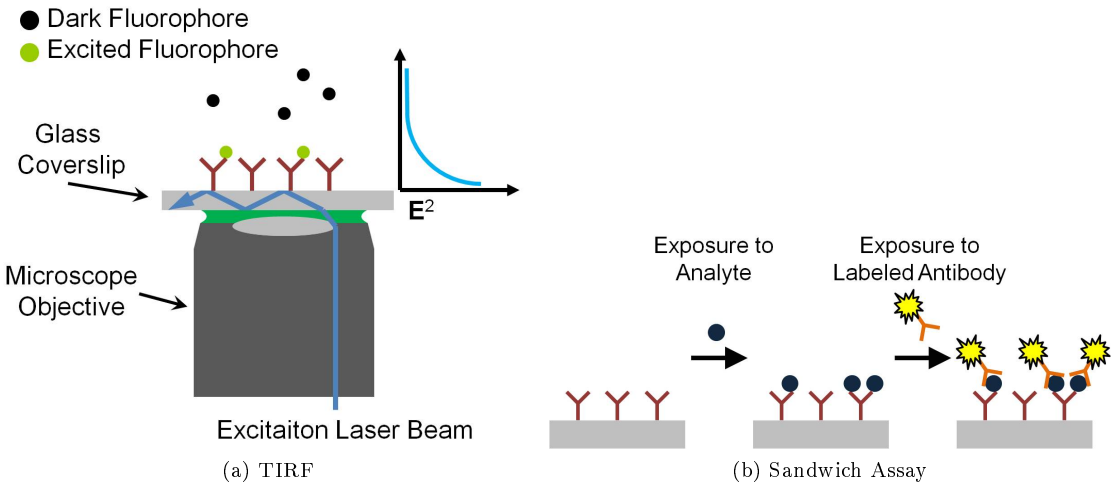
Though widespread in the literature, fluorescence measurements using fluorescently labeled species suffer two key drawbacks. First, attaching a fluorescent tag to the analyte can interfere with the binding reaction that occurs during specific sensing. This is more common for proteins, whose structures can be significantly perturbed by the inclusion of fluorophores, than it is for small molecule analytes. Second, quantitative fluorescence measurements can be inaccurate in the limits of both high and low concentrations of analyte. At low concentrations, the SNR is no longer the dominant factor in determining the LOD. Instead, light from sources other than the fluorophores (i.e., *background* light) may contribute more to the signal than the analyte. Threfore, the signal-to-background ratio (SBR), and not the SNR, often controls the sensitivity of the measurement at low concentrations. At high concentrations, the space between two freely diffusing fluorescently labeled analyte molecules may be sufficiently small so that an excited fluorophore may transfer its energy to a nearby fluorophore. Though it is often described in terms of an emitted photon exciting another fluorophore, the energy transfer involves no radiation. This process is called Förster resonance energy transfer (FRET) and can be used to report proximity between two fluorophores. High concentrations also

lead to frequent interactions between biomolecules present in solution and, for some species, agglomeration. The particles that result may scatter sufficient light to diminish the excitation of fluorophores present or the amount of emitted fluorescence that may reach the detector. Regardless, a deceptively low signal may be recorded for fluorescence measurements at high concentrations.

A common fluorescence technique used for biosensing is the sandwich assay. In this type of experiment, the analyte is selectively bound to a surface by a targeting molecule (such as an antibody), which has been immobilized covalently on the surface of a well or other cell. By labeling the analyte molecule with a fluorescent tag, its surface concentration may be measured via highly sensitive fluorescence spectroscopy. Attaching a fluorophore to the antibody typically affects its affinity for the analyte, however. The sandwich assay avoids this problem by using unlabeled antibody and analyte, and exposing the cell to a fluorescentlylabeled antibody raised specifically for the bound complex. Fluorescence spectroscopy is then used to identify the presence of the antibody-analyte-antibody structure, as shown in Fig. 2.6.

Alternative fluorescence assays range from classical biochemical methods, such as Western blots or ELIZA assays, to new sensor technologies such as total internal reflection fluorescence (TIRF) [52]. In TIRF, one monitors the adsorption of material by measuring the amount of light given off by fluorescent tags attached to the surface-bound analyte molecules. These fluorophores are excited using the evanescent field that results from total internal reflection at the substrate, often a glass prism. Figure 2.6a illustrates this configuration, including the optical path. Fluorophores may only be excited by the evanescent wave, ensuring that only those fluorophores within a short distance (< 100 nm) of the surface will contribute to the emitted fluorescence signal. This technique significantly improves the SBR and, consequently, the sensitivity of the measurement.

The second class of optical biosensor uses changes in the phase of light to report the presence of analyte rather than changes in amplitude (i.e., absorption). A significant benefit of phase shift optical biosensors is that they do not require a label be present in order to detect the resonance. While spectroscopic methods are limited by the weak interaction between light and the analyte (or label) unless a wavelength is used that corresponds to a particular mechanical or electronic resonance which may be excited, phase delay methods



(a) TIRF (b) Sandwich Assay

Figure 2.6: Fluorescence-based biosensor technologies. (a) Total internal reflection fluorescence (TIRF) is characterized by the excitation of fluorescentlytagged species at a surface by an evanescent field that decays exponentially and excites only those fluorophores near the surface. (b) Sandwich assays feature exposure of an antibody-labeled surface to an analyte solution, followed by exposure to a fluorescentlylabeled antibody that binds exclusively to the complex.

require only contrast in the real part of the refractive index, n, to register a change. Moreover, measuring a phase shift is straightforward when using an interferometer, and often significantly reduces the noise of the measurement. Backscatter interferometry exemplifies this strategy [53, 54]. In this method, light is split into two paths; one path is allowed to interact with the sample (the *sample path*) while the other is not (the *reference path*). The two paths are recombined before being sent to a detector. Only when the length of the two paths differ by an integer number of wavelengths will there be constructive interference and the intensity registered by the detector will be equal to that before the split. For any other case, the detector will show a harmonic function in time whose frequency can be related to the difference in path length. Any change to the refractive index along the sample path will cause a phase shift in the light, and backscatter interferometry uses the transient change in frequency that results from combining this phase-shifted light with the reference path light to indicate when analyte is present. This method can detect quantities of material as low as a 30 zeptomoles (30×10^{-21}) [54], however it is ill suited for complex biological samples and requires high concentrations (> 1 pM) of analyte. Regardless, it is still a useful analytical tool as it requires *very* small sample volumes, as low as 3 picoliters, and can easily be incorporated into microfluidic

flow cells.

Surface plasmon resonance (SPR) is a phase shift optical biosensor technology that has become a benchmark in the field over the last decade due to its commercial availability in the form of the Biacore^{TM}and other instruments, well-characterized performance, and adaptable functionalization architecture [55, 56]. This type of biosensor involves using a surface-propagating electromagnetic wave to excite an oscillation in the surface conduction electrons of a material. The plasmon-capable material is typically a metal, such as gold or silver, that can be deposited onto a dielectric, such as silica, and has a complex refractive index $= n + i\kappa$, such that $\kappa > n$. Material that adsorbs to this metal surface alters the local refractive index and, consequently, the local wavelength of the light. A surface wave is produced via total internal reflection (TIR), as shown in Fig. 2.7, at an angle that depends on the refractive index experienced by the surface wave and the incident wavelength. By scanning either incident angle or wavelength, the shift in plasmon resonance can be tracked and the surface binding reaction monitored.

Like all phase shift optical sensing technologies, SPR requires no label on the analyte. The technique is well suited to a flat microfluidic channel geometry for which mass transfer is well understood [57]. It has been used to observe analyte concentrations >1 pM with unlabeled analyte, although a LOD as low as 1 fM has been achieved by attaching plasmonic tags (e.g., gold nanoparticles) to the analyte [58]. Many of the commercial instruments based on this technology feature integrated flow systems and optional automatic sampling mechanisms to enable automated serial measurements. Commercial implementations also often include another advantageous feature—reference channels. In this case a targeting layer is not deposited on one channel so that its signal may be subtracted from other channels to account for non-specific binding. Most commercial SPR chips are prepared with a polymeric coating that is designed to facilitate surface functionalization.

The most significant shotcomings of this technology are the limited sensitivty while using label-free analytes and the high cost. Commercially available SPR instruments commonly cost in the range of $100,000-$300,000. Nonetheless, it is an excellent tool for high-concentration sensing (> 100 fM), and enables the measurement of kinetic rate constants with relative ease. It stands as one of the most reliable and adaptable

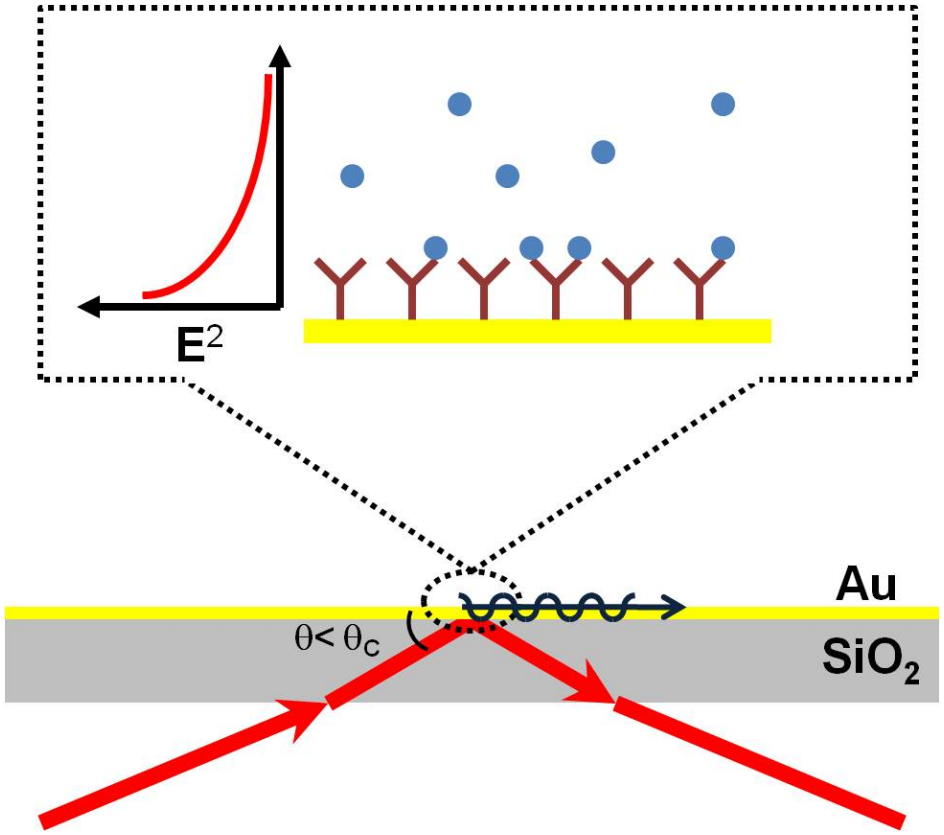


Figure 2.7: Surface plasmon resonance (SPR). Here a surface-propagating wave is generated via total internal reflection in a thin gold film deposited on silica in order to excite a surface plasmon in the metal. Material that adsorbs to the surface shifts the plasmon resonance, which must be compensated for by altering the incident angle of light or the incident wavelength. In this way the surface binding reaction between immobilized targeting species and analyte may be monitored.

tools available today for evaluating biomolecular reaction rates and affinities.

A comprehensive review of label-free optical sensor technologies may be found in Ref. [59]. The work described in this thesis focuses on optical resonators. These devices use resonance to build up intense optical fields and amplify the interaction between light and adsorbing material. The change in refractive index that results from adsorption induces a phase shift in the circulating light and a change in the wavelength required to excite resonance. Description of the fabrication, implementation, and modeling of these devives may be found in subsequent chapters.