

Application of the Suspended Microchannel Resonator as a Biosensing Device to Profiling Signal Transduction in Receptor Tyrosine Kinases

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Introduction

The analysis of molecular interactions is a key area of research in the healthcare, pharmaceutical, and biotechnology fields. Driven by the healthcare industry, millions of dollars are being invested on developing screens to detect various levels of molecular binding. Current methods and tools that have been developed to detect these interactions are high-throughput screens like DNA and antibody microarrays. Most screens require a type of fluorescent labeling or radiolabeling to report the binding of the molecule of interest.

Traditional methods of screening

Fluorescence, a traditional method of screening, is recognized as one of the most sensitive methods of detection in which the emission signal is measured above a low background level. However, this step requires sample preparation and modification of the target molecules, which results in extra time and cost demands. In addition, fluorescent labeling or radiolabeling can interfere with the molecular interaction at the binding site, subsequently leading to false negatives. Fluorescent compounds are also hydrophobic, and in many screens, background binding is a significant problem, creating additional false positives. Hence, a new technology platform has been developed in order to avoid the labeling procedure: the biosensor. To circumvent the need for chemical modification, these label-free detectors have been developed by attaching the detector directly to the capture platform that detects the target molecule (Figure 1). The direct attachment of the detector to the sensing platform eliminates the need for chemical modification by directly measuring the surface binding. The ideal biosensor should be label-free, sensitive, high-throughput, and widely applicable to healthcare.

The biosensor

A biosensor is an analytical device that uses biological molecules to detect other biological molecules or chemical substances. Typically, the detector molecule must be connected to a sensor that can be monitored by a computer, which converts the biological response into an electrical and optical signal. The emergence of biosensors represents the convergence of two disciplines—the specificity of biological systems with the computing power of the microprocessor. A schematic diagram of a typical biosensor is shown in Figure 2.

Biosensors use a variety of techniques, including monoclonal antibodies to detect an antigen, a small synthetic DNA molecule called an oligodeoxyribonucleotide to detect DNA, or a receptor binding to a ligand.

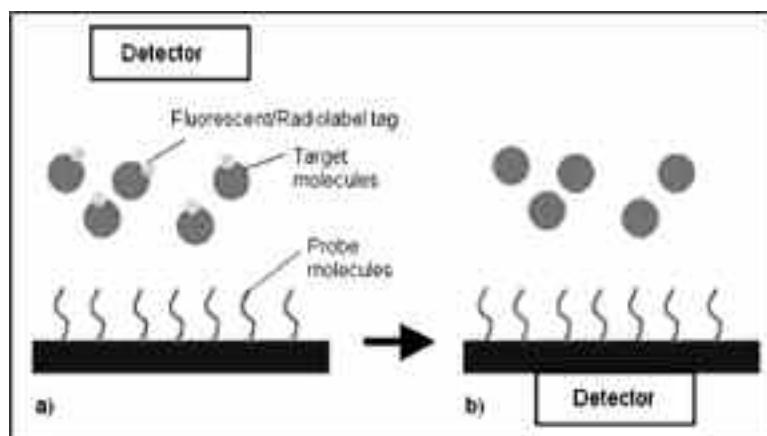


Figure 1. Comparison of traditional screening methods with the novel biosensing method. a) Traditional screening method using a fluorescent or radiolabel tag on the target molecule, with the detector separate from the platform with the probe molecule; b) the biosensor eliminates the tedious step by using a label-free method since the detector is directly to the sensing platform.

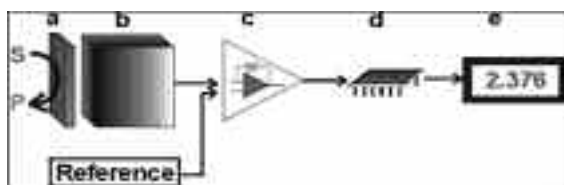


Figure 2. Schematic diagram showing the main components of a biosensor. This device consists of: (a) a biocatalyst that converts the substrate to product; (b) the transducer that determines the reaction and converts it to an electrical; and the signal output is (c) amplified, (d) processed, and (e) displayed.

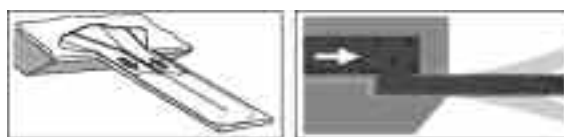


Figure 3. The suspended microchannel resonator, (a) High damping and the large effect mass in liquid plate resonant mass sensors are avoided by using suspended microfluidic channels as resonators; (b) Analytes are detected based on their mass density difference relative to the surrounding solution. The process of microfabrication is described in detail in Appendix A. (Source: Burt et al., 2003).

The future direction of the biosensor lies in the development of “nanosensors” or “microsensors”. The sensor’s sensitivity is in the nanoscale (nano = 10^{-9}). The advancement in this field mainly results from the progress of silicon-based microfabrication.

Common label-free detectors

The quartz-crystal microbalance (QCM) and surface plasmon resonance (SPR) biosensors both have been developed for this case; however, their sensitivity levels still do not match that of fluorescence. QCM and SPR also require large sample volumes (10–1000 μl) and are difficult to scale down.

Quartz-crystal microbalance (QCM).

The QCM technique has traditionally been used to monitor mass or thickness of thin films deposited on surfaces either in a gaseous or in a liquid environment. QCMs are piezoelectric devices fabricated of a thin plate of quartz with electrodes affixed to each side of the plate. They are extremely sensitive mass sensors, capable of measuring mass changes in the nanogram range.

Surface plasmon resonance (SPR).

A common optical detection technique used for biosensor development is optical detection using SPR. SPR is a direct optical immunosensing technique that has been successfully incorporated into an immunosensor format for the rapid and non-labeled assay of various biochemical analytes. Surface plasmon resonance (SPR) sensing has been demonstrated in the past decade to be an exceedingly powerful quantitative probe of the interactions of a variety of biopolymers with various ligands, other biopolymers, and membranes, including protein-ligand, protein-protein, protein-DNA and protein-membrane binding. SPR provides a means not only for identifying these interactions and quantifying their equilibrium constants, kinetic constants, and underlying energetics, but also for employing them in sensitive, label-free biochemical assays. SPR exploits the properties of the evanescent field and relies on the

change in the refractive index of the medium for the signal generation.

Studies on SPR have included the development of the first simple method for absolute quantitative analysis of binding amounts based on SPR response, kinetic, and equilibrium studies. This was done by using SPR of the binding of wild-type and several mutants of streptavidin to biotin-terminated alkythiols immobilized in a self-assembled monolayer on a gold surface. The advantages of optical techniques involve the speed and reproducibility of the measurement.

Limitations on the QCM and SPR.

Although label-free detectors avoid the labeling step, there are drawbacks to these techniques. SPR measurement often requires expensive instruments that are generally larger than is practical for detection. They also require a large sample volume (>100 μl), and are difficult to scale down in size. Most importantly, they are significantly less sensitive than fluorescence screening.

The resonant beam mass sensor

To address these limitations, other devices have been developed: field-effect sensors, integrated optics, and surface stress sensors. The resonant beam mass sensor, though it has been known to be successful in gas sensing environment, is known to result in damping problems due to viscous drag in the fluid environment. This results in a low quality factor in liquid solutions, thus degrading the mass sensitivity and frequency resolution when functioning in fluids. Although resonant beam mass sensors have achieved high mass sensitivity levels and high frequency resolution in gaseous environments, the quality of performance of the device is degraded by the damping and viscous drag (due to large effective mass).

In an effort to circumvent these problems of damping and viscous drag, the fluid that normally surrounds the exterior of the sensor can be localized to the interior of the resonant beam mass sensor, while leaving the exterior of the channel exposed to the environment. Burg et al. have microfabricated a device that has achieved these requirements and can be integrated with conventional microfluidic systems. Their suspended microchannel resonator (SMR) is a unique hollow cantilever design that contains the fluid.

The suspended microchannel resonator (SMR)

The SMR is a device developed as a resonant mass sensor for specific biomolecular detection in picoliter sample volumes. The sensing principle is based on measuring shifts in resonance frequency of a suspended microfluidic channel when molecules accumulate inside the channel walls. This device can be actuated by electrostatic forces and integrated directly with conventional microfluidic systems.

The fabrication process of the suspended microchannel was performed using a polysilicon Damascene process, sacrificial layer etching in hot potassium hydroxide, and bulk micromachining to fabricate suspended microchannels, followed by a planarization with chemical mechanical polishing (CMP). A microfluidic network of poly(dimethylsiloxane) (PDMS) channels was bonded to the chip surface.

The SMR detects specific biomolecules by functionalizing the channel walls with a layer of capture (or probe) molecules. The biomolecules of interest accumulate on the functionalized walls, subsequently reducing resonance frequency. The mass density of the biological molecules is assumed to be greater than the mass density of the fluid.

The device with internal surface A can be modeled as a harmonic oscillator with an effective mass m and resonance frequency f . The relative frequency shift resulting from a small surface mass loading is given as follows:

$$f = 2r\sqrt{\frac{k}{m}}$$

$$f + \Delta f = 2r\sqrt{\frac{k}{m + \Delta m}} = 2r\sqrt{\frac{k}{m}} \sqrt{\frac{1}{1 + \frac{\Delta m}{m}}} = f \sqrt{\frac{1}{1 + \frac{\Delta m}{m}}}$$

Using a Taylor series expansion:

$$f + \Delta f \cong f \left(1 - \frac{1}{2} \frac{\Delta m}{m} \right) + L$$

Dividing both sides by f :

$$1 + \frac{\Delta f}{f} = 1 - \frac{1}{2} \frac{\Delta m}{m}$$

$$\frac{\Delta f}{f} = -\frac{1}{2} \frac{A}{m} \Delta \sigma$$

where $\Delta \sigma = \frac{\Delta m}{A} = \frac{\Delta \rho V}{A}$ represents the ratio of surface area to total mass (the volume was $V \sim 27 \text{ pL}$ and $A = 53000 \mu\text{m}^2$). The equation above shows that for a given detectable frequency shift and a resonance frequency f , the smallest detectable surface mass loading is fully determined by the surface mass loading. The surface mass loading also represents the level of sensitivity. The mass resolution was determined to be $1.4 \times 10^{-17} \text{ g}/\mu\text{m}^2$, which corresponds to a mass sensitivity of $107 \text{ mHz}/\text{pg}$ for small loadings of a water-filled microchannel.

Burg et al. evaluated the mass resolution by measuring frequency response for a $300 \mu\text{m}$ cantilever in the unfilled state (air), filled with isopropyl alcohol, and filled with water (see Figure 4). The sensitivity was determined to be $107 \text{ mHz}/\text{pg}$ for small loadings of a water-filled microchannel. Using this sensitivity together with the 80 mHz noise level and a surface area $A = 53000 \mu\text{m}^2$, the current detection limit is $1.4 \times 10^{-17} \text{ g}/\mu\text{m}^2$ over a $4 \text{ mHz} - 4 \text{ Hz}$ bandwidth.

Burg et al. then demonstrated biomolecular detection by functionalizing the interior channel walls with biotinylated bovine serum albumin (BSA) and detecting frequency changes for the subsequent binding of avidin and biotinylated BSA (Figure 5). They have successfully demonstrated the functionality of the SMR to the biotin/avidin system. They anticipate that the sensitivity level could be even lowered to the single-molecule level ($10^{-19} \text{ g}/\mu\text{m}^2$).

Application of the SMR to profile receptor tyrosine kinase activity

Signal transduction in mammalian cells is mediated by a complex biochemical network of interacting proteins. In order to depict these networks a device is needed to measure the amounts and activities of multiple proteins in a rapid and accurate manner.

Currently, antibody (Ab) microarrays have been developed to regulate the multiplex signal transduction in order to quantify labeled recombinant proteins and proteins in serum.

Surface immobilization methods for the SMR

A variety of methods are commonly used for immobilizing proteins: adsorption, covalent modification, and polymer linkage. The most widely used method relies on nonspecific adsorption of the protein to a solid support. Simple chemical couplings of reactive groups within proteins (amines, acids) have also been used to immobilize the proteins to surfaces comprising complementary reactive groups. Both methods, which require highly purified proteins, often result in randomly oriented and partially denatured proteins. The use of recombinant tags allows proteins to attach to a substrate in a defined orientation, but the interactions of the tags are reversible (e.g., glutathione *S*-transferase, oligohistidine) and are not stable over the course of subsequent assays or require large mediator proteins (e.g., biotin-streptavidin, antigen-antibody). A further disadvantage of these methods is that they are not well-suited to controlling the densities of immobilized proteins. For these reasons, we sought a general method that would selectively immobilize proteins with absolute control over orientation and density and that would not require synthetic modification or purification before immobilization.

Burg et al. have demonstrated the functionality of the biosensor on the biotin/avidin binding system by absorbing BSA on the surface of the Si_3N_4 microchannel.

Burg et al. utilized a biological system (biotin/avidin) with a relatively high binding affinity. However, in order for the biosensor to be used for detecting other biological systems lacking a high binding affinity ($K_d = 10^{-15} \text{ M}$) as the biotin/avidin system, the type of interaction (covalent, ionic, etc.) of binding chemistry must be taken into account. Different levels of binding interactions exist, depending on which molecules are binding and whether it is an antibody-antigen interaction. In our investigation, we have chosen antibody-antigen interaction, specifically the EGF-EGFR binding system.

Because the surface chemistry in binding is different for EGFR and ErbB2 receptor proteins, a different immobilization method must be used in order to immobilize one of the molecules in the antibody-antigen system. Below is a proposed method using covalent attachment of EGFR and ErbB2 on the suspended microchannel resonator.

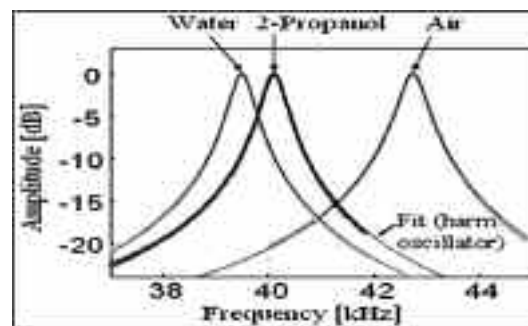


Figure 4. Calibration of the SMR using compounds of known densities: water, isopropanol, and air. Shows the frequency response curves of a $300 \mu\text{m}$ long cantilever.

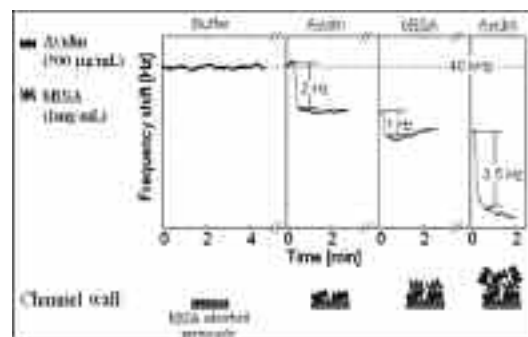


Figure 5. The relative frequency shifts for a 40 kHz SMR. Shows the responses after injection of avidin, biotin, and avidin. Experiment was performed under constant pressure, except during switching (denoted by //).

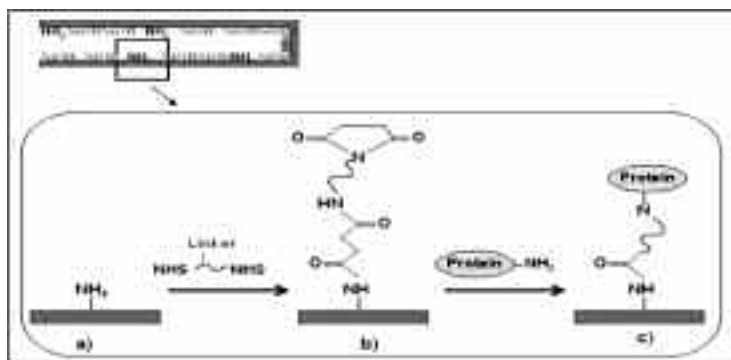


Figure 6. Covalent immobilization method using amide-linkage on a silicon nitride surface. Process shows: a) An amino-presenting surface is commercially treated with available bifunctional linking reagent to b) the coupling with free amino groups. c) The capture protein is then immobilized to the surface at its amino terminus end.

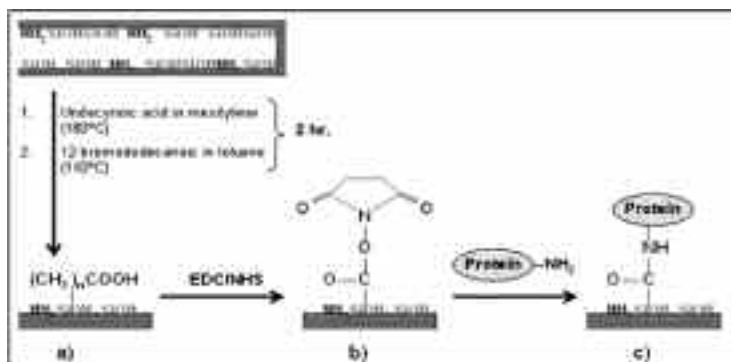


Figure 7. Covalent immobilization method using carboxymethylated monolayer on a silicon nitride surface. (a) Water-soluble EDC mediated activation of a carboxymethylated support; (b) Reactive NHS ester is coupled directly with available amino terminus of the capture protein (or antibody); (c) Protein is immobilized onto the surface.

In order to develop a method to profile abundance of EGFR and ErbB2, monoclonal antibodies specific to EGFR, ErbB2 must first be immobilized on the surface of the SMR. For the biotin/avidin binding system, the biotin was immobilized on the surface by adsorption. However, antibodies can not be conveniently adsorbed on the silicon nitride surface and a covalent attachment method must be used to immobilize antibodies. The SMR surface is composed of silicon nitride (Si_3N_4), a substance known to present two kinds of surface sites—primary amine and silanol groups—each at different concentrations depending on the deposition process. This report proposes two different covalent attachment methods that would not result in interfering with the biological activity of the antigen-antibody binding: amide-linking and carboxymethylated monolayer.

Amide-linking method

The silicon nitride surface contains silanol and amine residues which serve as covalent binding sites. Raiteri et al. used an atomic force microscope (AFM) that is coated with a

Si_3N_4 surface by silanization. Raiteri et al. reported that the sites for silanol ($N_{\text{sil}} = 3\text{-}4.95 \times 10^{18}$ sites/ m^2) and amine ($N_{\text{mi}} = 0.05\text{-}2 \times 10^{18}$ sites/ m^2) for silicon nitride, or 1.64%-28.8% (mean = 15.2%) of the binding sites are amine (Raiteri et al.). These amine groups serve as binding sites for covalent attachments of proteins. Single proteins are anchored to each surface by one short tether per protein, which considerably decreases the risk of interfering with the biological function of the protein. The idea is that the process ends with the molecule covalently bound to the exposed surface by exposing the active site at the free end, thereby reacting with the free environment.

Carboxymethylated (carboxylic acid) support method

In addition to the amide-linking method, proteins could also be immobilized on the surface by termination of carboxylic acid monolayers, as Cricenti et al. immobilized covalent bonds on the surface of flat crystalline silicon and silicon nitride on silicon. The carboxylic group on the surface serve as a coupling site to an amine ($-\text{NH}_2$) group of enzymes or antibodies.

Carboxylic acid monolayers are grown on silicon nitride via a thermal bishydrosilylation and nitrogen alkylation. Figure 7 shows the steps of this carboxylation process.

Profiling levels of EGFR and ErbB2

Once a protein has been successfully immobilized onto the surface by covalent attachment, the levels of EGFR and ErbB2 can be determined using direct detection or microsandwich methods. Nielsen et al. tested both methods, and after considerable testing and optimization, they determined that Ab arrays worked best with microsandwich methods. Although direct labeling achieves higher sensitivity with purified proteins, the microsandwich method is better suited to high-throughput biological screening.

Conclusion

The suspended microchannel resonator has already been demonstrated to profile abundance of the biotin/avidin binding system, yet to expand the application of the SMR to profile receptor tyrosine kinases, the top surface of the SMR must be changed in order to properly immobilize the antibody to the surface. Thus, a covalent immobilization method has been proposed to bind the monoclonal antibodies to the surface.

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