Perspective

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ACS Sens., **Just Accepted Manuscript** • DOI: 10.1021/acssensors.7b00069 • Publication Date (Web): 27 Mar 2017

The mechanistic challenges and advantages of biosensor miniaturization into the nanoscale

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Abstract: Over the past few decades, there has been tremendous interest in developing biosensing systems that combine high sensitivity and specificity with rapid sample-to-answer times, portability, low cost operation, and ease-of-use. Miniaturizing the biosensor dimensions into the nanoscale has been identified as a strategy for addressing the functional requirements of point-of-care and wearable biosensors. However, it is important to consider that decreasing the critical dimensions of biosensing elements impacts the two most important performance metrics of biosensors: limit-of-detection and response time. Miniaturization into the nanoscale enhances signal-to-noise-ratio by increasing the signal density (signal/ geometric surface area) and reducing background signals. However, there is a trade-off between the enhanced signal transduction efficiency and the longer time it takes to collect target analytes on sensor surfaces due to the increase in mass transport times. By carefully considering the signal transduction and reaction-transport kinetics mechanisms governing different classes of biosensors, it is possible to develop structure-level and device-level strategies for leveraging miniaturization towards creating biosensors that combine low limit-of-detection with rapid response times.

Keywords: Biosensors, Miniaturization, Nanoscale, Limit-of-detection, Response time, Signal-to-noise-ratio, Point-of-care, Wearable, Mass transport, Reaction kinetics

Biosensors are devices that detect and/or quantify biologically-relevant analyte for the purpose of disease management and healthcare, food and water surveillance, environmental monitoring, drug development, scientific research, or forensics. These devices are designed to detect a specific analyte or a panel of analytes, where the choice of analyte depends on the application. [1] Centralized and high throughput laboratory instruments, distributed point-of-care diagnostics, and wearable health monitoring biosensors are three broad categories of biosensing instrumentation with potential impact on human health. The laboratory-scale devices are ideally suited for research and clinical applications where highly skilled technicians are available, turnaround times in the order of 24 hours are acceptable, and economy of scale is applicable. The point-of-care devices are applicable to health care and disease management applications where frequent monitoring and fast (in the order of minutes) sample-to-result times are critical, settings where skilled technicians and basic healthcare infrastructure are unavailable, or groups of users or patients that cannot easily access centralized healthcare facilities. Wearable biosensors are applicable to similar settings as point-of-care diagnostics, and they have the additional benefits of operating in real-time or near real-time without being invasive. As a result, they are applicable to real-time health monitoring and go beyond the diagnostics and disease management capabilities of other types of biosensors. Wearable biosensors have generated tremendous interest over the last decade due to the plethora of applications they offer upon integration with smart watches and smart phones[2] offering integrated signal readout, computation and communication capabilities. While the design considerations discussed here are critical for developing the next generation of wearable sensors capable of real-time monitoring of biologically-relevant analytes, their review can be found elsewhere.[3,4]

The potential that life saving and cost saving are achievable through early diagnosis and frequent and accessible health monitoring has placed a significant emphasis on *point-of-care* and *wearable* biosensors. Miniaturization is essential for realizing these two categories of biosensors, and allowing a large number of sensors and sample preparation devices to be integrated in a single lightweight strip, chip, cartridge, or package. This is important for enabling portability and ease of use, achieving multiplexed, parallel, and automated analysis, reducing the manufacturing cost by reducing materials and fabrication costs per device, and facilitating the integration of the sensors and actuators with electronic test and measurement circuits in the same chip, package, or system. In addition to the practical and technological advantages mentioned here resulting from miniaturizing the biosensing *system*, reducing the dimensions of the biosensing *element* has shown to be important for improving the sensitivity of electronic and electrochemical biosensors by increasing the system's signal-to-noise ratio.[5] Miniaturization is an ongoing research trend in the field of biosensing. It is imperative to understand the correlation between miniaturizing the biosensing element and the two most important performance metrics of biosensors: limit-of-detection and response time.

Following a background introduction to the general principles of biosensors, we will discuss the two critical mechanisms involved in determining the limit-of-detection of biosensors: *signal transduction* and *reaction-transport kinetics*. We will then provide examples of strategies that enhance the limit-of-detection of miniaturized biosensors by carefully examining and applying these mechanistic considerations.

General principles of biosensors-biorecognition and signal transduction

As recommended by IUPAC (International Union of Pure and Applied Chemistry), a biosensor is defined as a self-contained integrated device which provides selective quantitative or semi-quantitative analytical information using a *biological recognition element*, which is retained in direct spatial contact with a *signal transduction element* (Figure 1).[6] The biological recognition and signal transduction processes are both important in determining the limit-of-detection of biosensors.

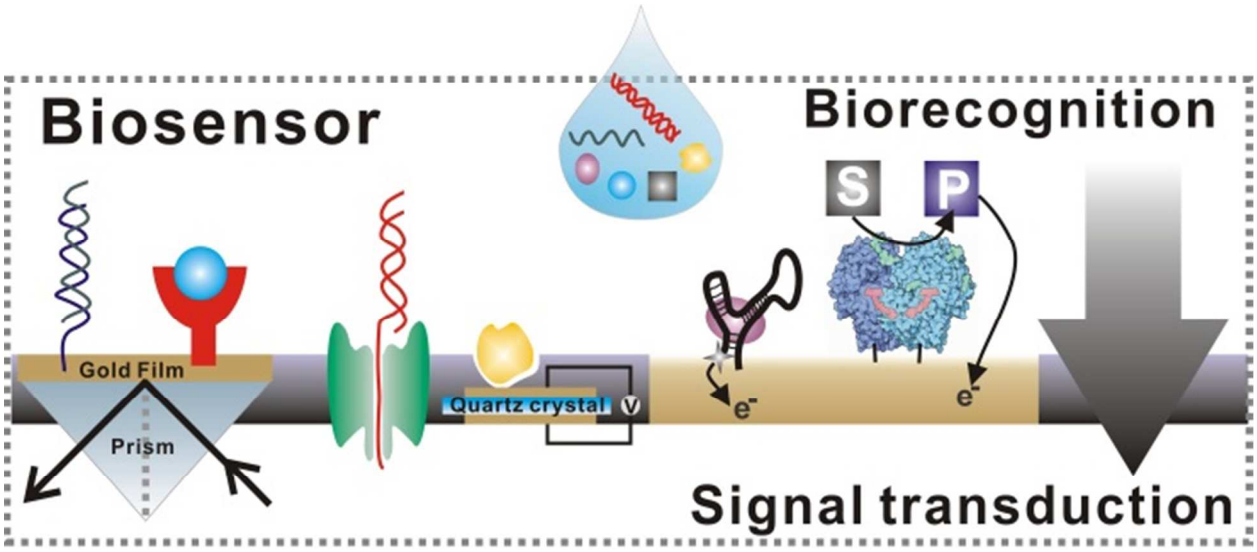

Figure 1 Biosensors combine biorecognition and signal transduction elements for quantitative or semi-quantitative analysis of biologically-relevant analytes. Commonly used biorecognition elements include enzymes for biocatalysis and nucleic acids, protein, or synthetic ligands for bioaffinity-based capturing. Surface plasmon resonance, pore-based sensors, quartz crystal microbalance, electrochemical and electronic biosensors exploit various signal transduction methods.

The biorecognition element translates information from the analyte (*e.g.,* identity, concentration) into a chemical or physical output signal with desired sensitivity and specificity. There are two major classes of biological recognition used in biosensing: biocatalysis and bioaffinity. Biocatalysis is achieved by using one or multiple enzymes that selectively catalyze a reaction involving target analyte as a substrate.[7] The enzyme(s) is typically integrated to an electrode or other transducers to produce quantitative measurements. Catalytic nucleic acids, such as ribozymes (RNAzymes) and deoxyribozymes (DNAzymes) have also been widely adopted as biocatalytic components for sensing metal ions and small molecular targets. As target molecules are typically cofactors, substrates can be identified for generating optical or electrochemical signals. It is also possible to engineer DNAzymes (or RNAzymes) with additional target recognition, regulation, and signal transduction/amplification components, which greatly diversify the biosensing mechanisms.[8,9]

Leveraging on the bioaffinity mechanism is another important approach for achieving biorecognition in sensors. In this case, affinity ligands such as antibodies and nucleic acid probes are used to capture specific target analytes. Emerging ligands including aptamers, peptides, and molecularly imprinted polymers (artificial antibodies) have recently generated much interest due to their ease of production, small molecular weights, and long shelf life. While the production of

antibodies relies on animals or hybridoma cell lines, aptamers, peptides and artificial antibodies are produced through chemical synthesis, and offer lower cost and better batch-to-batch reproducibility. Moreover, as aptamers and peptides are generated *in vitro* using molecular evolution techniques (SELEX for aptamers and phage display for peptides), it is possible to define the sensor specificity using the selection process prior to their integration into the transducer element. [9–12] A limitation of the synthetic ligands is that the classic evolution techniques, such as SELEX, are generally costly and lengthy due to the multiple rounds of selection and the existence of manual steps. The generation of high quality ligands (in terms of affinity and specificity) is also frequently limited by the inert nature of oligonucleotides or peptides towards certain classes of targets, and their insufficient post-selection characteristics. As such, most published aptamer-mediated biosensors are focused on a few well-characterized model targets, including thrombin and ATP. [13] However, with the recent introduction of various modified nucleotides, [14] and the integration of high throughput sequencing techniques and microfluidic systems into the selection process, ligands with the desired functionality can be produced efficiently for almost any target of interest. [10,14] The mechanistic considerations discussed in this paper will focus on biosensors that capture target analytes using bioaffinity elements.

The signal transduction element translates the biorecognition event into a measurable electrical, optical, magnetic, thermal, or piezoelectric signal. In a wide range of biosensors, biorecognition, signal transduction, and signal measurement, *all* occur at a single surface placed at the interface between the transducer and the solution. For example, in bio-functionalized field-effect transistor, [15] electrochemical, [16] surface plasmon resonance,[17] and quartz crystal microbalance biosensors,[18] the changes in the inversion layer charge density, electrochemical current/potential/impedance, refractive index, and resonance frequency are transduced and measured on the same surface housing the biorecognition elements (Figure 1). In addition to these "surface-based biosensors", solution-based homogeneous biosensors have also been designed and widely used for translating the findings of the fields of protein engineering and DNA nanotechnology to practical biosensors, [8,19,20] and to probes for live cell and *in vivo* sensing applications.[21,22] In these assays, biorecognition, signal transduction, and signal measurement do not occur at the same surface. Instead, biorecognition events such as DNA hybridization or aptamer/target complexation occur in solution or at the surface of distributed beads. The changes in biomolecular interactions or conformations are then translated into changes in physically detectable signals (e.g., fluorescence) through homogenous signal transduction events (e.g., the separation between a quencher and a fluorophore). Although, reaction-transport kinetics considerations are critical in designing both surface and solution-based biosensors, we will limit our discussion in the miniaturization challenges surrounding surface-based biosensors.

Miniaturization of biosensors

Miniaturization of biosensors into the microscale has been motivated by the success of the microelectronics industry in doubling the number of on-chip transistors every two years, and lowering the cost per function. Over the past two to three decades, there is tremendous interest in miniaturization of biosensing elements into the nanoscale. This is on one hand motivated by the dimensions of nanomaterials being smaller than cell dimensions and in the order of biomolecules of interest for biosensing such as proteins and nucleic acids. On the other hand, the improved understanding of the optical, electronic, and magnetic properties of nanoscale materials further motivates their use in biosensing systems.

Two processes must be discussed when considering the limit-of-detection of biosensors and its correlation with sensor dimensions – signal transduction efficiency and reaction-transport kinetics. Signal transduction efficiency is related to the minimum number of analytes that must be captured at the sensor surface for obtaining an acceptable signal-to-noise ratio, while the reaction-transport kinetics are related to the time required for that capture to occur. In this section, we will discuss the trade-off between signal transduction enhancement and mass transfer limitations that must be considered in reducing the biosensing lengthscale into the nanoscale. While these considerations are applicable to various classes of biosensors, we limit our discussion of signal transduction considerations to electrical biosensors. The examples and the conclusions discussed here are applicable to other classes of biosensors.

Reaction-transport kinetics considerations in miniaturization of surface-based biosensors

It will be discussed in the following section that reducing the critical dimensions of biosensors into the nanoscale improves their signal transduction efficiency. This means that once the analyte of interest is captured at the sensor surface, it results in an increased signal-to-noise ratio compared to bulk sensors. While this is an important consideration in the design of biosensors, it does not carefully examine the effect of sensor size on the "critical concentration"[23] and response time of biosensors. A set of rules of thumb developed by Squires et al.[23] must be considered to fully understand the implication of sensor lengthscale on its biosensing performance metrics. If analytes are transported to the sensor surface at a rate faster than they can be bound using surface receptors, the sensor is operating under 'reaction-limited' regime. Under this regime, the surface concentration of bound targets can be described by considering first-order Langmuir kinetics as:

$$\frac{b(t)}{b_m} = \frac{\frac{c_0}{K_D}}{1+\frac{c_0}{K_D}}\left(1 - e^{-(k_{on}c_0 + k_{off})t}\right), \quad (1)$$

where $b(t)$ is the surface concentration of bound targets, b_m is the surface concentration of receptors, c_0 is the bulk analyte concentration, k_{on} and k_{off} are the kinetic rate constants for the binding reaction, and $K_D = \frac{k_{off}}{k_{on}}$ is the equilibrium dissociation constant. Assuming that the system has reached steady state, the equilibrium concentration of bound targets will be:

$$\frac{b(t)}{b_m} = \frac{\frac{c_0}{K_D}}{1+\frac{c_0}{K_D}} \quad (2)$$

When having dilute analyte concentrations ($\frac{c_0}{K_D} \ll 1$), the total number of bound targets at a sensor having area A is:

$$N = b_m A \frac{c_0}{K_D} \quad (3)$$

The critical concentration at which *one* target molecule binds the sensor at equilibrium ($N = 1$) is given by:

$$c^* = \frac{K_D}{b_m A} \quad (4)$$

Equation (4) indicates that reducing the sensor area has the implication of increasing the critical concentration. For example the critical concentration of a 2 μm x 10 nm hemicylindrical

nanowire is in the order 10^{-3} M (for a protein with K_D=1 nM) in contrast to a 50 μm x 50 μm micro-sensor having a critical concentration in the order of 10^{-17} M. Detecting target analytes in solutions that are more dilute than the critical concentration of the sensor, requires single molecule detection or patches of sensors. Considering critical concentration for a particular sensor dimension enables sensor designers to estimate the minimum number of sensing elements required in sensor patches for biomolecular analysis. For example, incorporating 10^6 hemicylindrical nanowires in a sensor patch decreases the critical concentration to 10^{-9} M for the abovementioned hemicylindrical nanowire case.

Another important consideration is the response time – the time it takes for the sensor to capture a detectable number of analyte molecules on its surface. If the response time or the time it takes to reach equilibrium is dominated by reaction kinetics, it will be given by:

$$\tau_R = (k_{off} + k_{on}c_0)^{-1} = {k_{off}}^{-1}\left(1 + \frac{c_0}{k_D}\right)^{-1} \quad (5)$$

For dilute analyte concentrations ($\frac{c_0}{k_D} \ll 1$), equation (5) indicates that the response time is inversely proportional to the dissociation rate of target binding. However, if the response time of the sensor is dominated by mass transport, the sensor geometry and size must be carefully designed to achieve the required limit-of-detection in acceptable response times.

Sheehan et al.[24] developed an experimentally validated analytical model to study the effect of sensor geometry and size on the accumulation of biomolecules on the sensor surface. By making the assumption that analytes irreversibly bind to the sensor surface, an assumption applicable to nucleic acid hybridization biosensors, they found an upper limit to the number of molecules accumulated on hemispherical, disk and hemicylindrical sensors as summarized in Table 1. This theoretical model demonstrates that disk and hemispherical biosensors (having radii less than 10 μm) demonstrate a linear increase in the response time as the sensor radius is decreased. This has the implication that it can take days to accumulate 10 DNA molecules on sub-micron disk and hemispherical sensors from 1 fM solutions, a timeline that gets even longer when genomic DNA or proteins, having lower diffusion coefficients are analysed. It should be noted that a different conclusion is applicable to hemicylindrical biosensors. It is found that the critical dimension of hemicylindrical sensors placed with their length parallel to the substrate is the sensor length instead of sensor radius. In this case, the accumulation time is weakly dependant on sensor radius and linearly dependant on sensor length, as a result it may be possible to combine the nanoscale diameter of the hemicylindrical nanowires for enhanced signal transduction efficiency with the increased sensor lengths for decreasing the required accumulation times.

Table 1: The summary of equations describing the number of accumulated molecules as a function of time for hemispherical, disk, and hemicylindrical sensors as presented in reference [24].

Sensor Geometry	Number of Accumulated Molecules, N(t)
Hemispherical	$2\pi N_A c_0 D(at + 2a^2\sqrt{\frac{t}{\pi D}})$
Disk	$4DN_A c_0 at$
Hemicylindrical	$\frac{4lN_A c_0}{\pi}\int_0^\infty \frac{1-e^{-Du^2t}}{J_0(au)^2+Y_0(au)^2}\frac{du}{u^3}$

N_A: Avagadro's number; c_0: Bulk analyte concentration; D: Diffusion coefficient; a: Sensor radius; l: Sensor length, J_0, Y_0: Bessel functions

Signal transduction considerations

In electrical biosensing (electrochemical or electronic), the miniaturization of the sensing electrode or its building blocks are used to increase the system's signal-to-noise ratio. Signal enhancement is achieved in nanogap and high surface area nanostructured electrodes, noise reduction is achieved in field effect transistor-based systems, and a combination of signal enhancement and noise reduction is achieved in nanoscale electrochemical biosensors. In electrochemical signal readout, the miniaturization of the electrode and the electrochemical cell hold advantages over macroscale systems. The background current associated with the charging of the double layer (capacitive current) scales with the area of the conductive portion of the electrode, and can be reduced with reducing the electrode area. The resistive (iR) drop of the system is reduced by shortening the ionic current path in miniaturized electrochemical cells. The reduced time constant of the system resulting from the reduced capacitance and resistance enables performing analysis in systems that involve fast electron transfer kinetics.[5] In addition, the rate of mass transport is enhanced for miniaturized sensors having nanowire and nanosphere geometries that are governed by 2D and 3D diffusion in contrast to 1D planar diffusion observed in bulk electrodes (Figure 2(a)).[25] The combination of enhanced mass transport and reduced capacitive currents in miniaturized electrodes translates to increased signal-to-noise ratio of such bioanalytical systems.

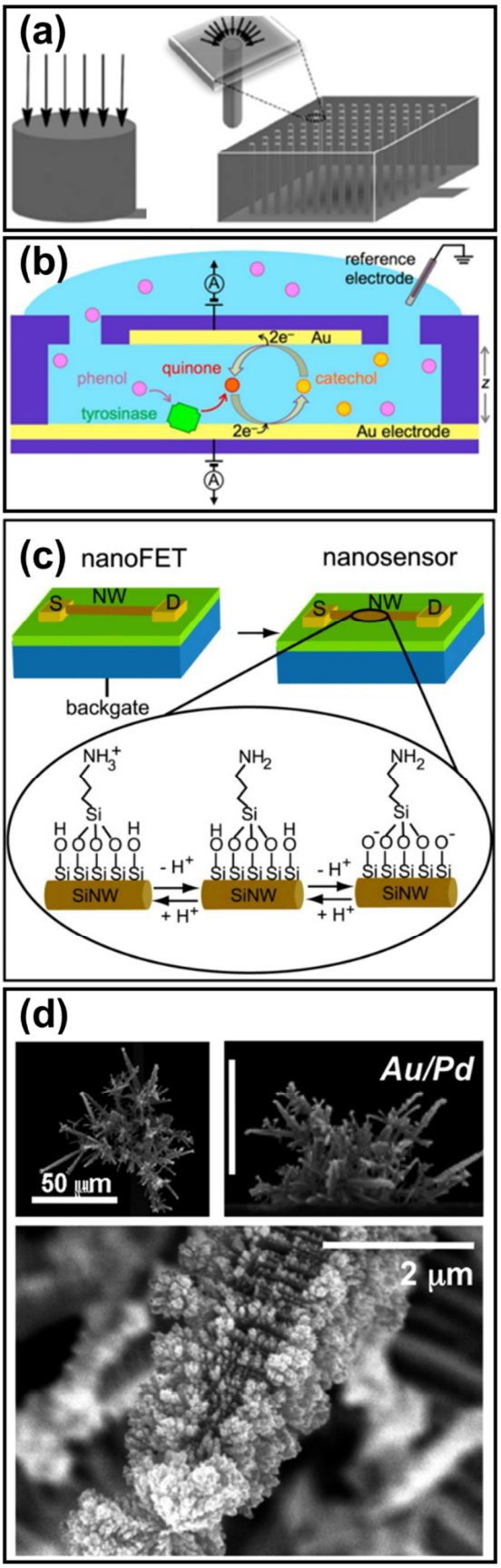
(a)
(b)
reference electrode
A
2e⁻
Au
phenol
quinone
catechol
tyrosinase
2e⁻
Au electrode
z
A
(c)
nanoFET
nanosensor
S
NW
D
S
NW
D
backgate
NH3+
NH2
NH2
Si
H
O
Si Si Si Si Si
SiNW
- H+
+ H+
(d)
50 μm
Au/Pd
2 μm

Figure 2-Miniaturization of biosensing electrodes into the nanoscale enhances the signal-to-noise ratio. (a) micro/nanoelectrodes offer lower capacitive background currents and larger current densities due to radial diffusion (adapted with permission from reference [5], Copyright 2009 The Royal Society of Chemistry); (b) nanogap electrodes increase the redox current magnitude by enabling a single redox reagent to be recycled several times (adapted with permission from reference [26], Copyright 2014 American Chemical Society); (c) nanowire field-effect transistors have dimensions close to the impact volume of biomolecules, making them a candidate for single molecule detection, (adapted with permission from reference [27], Copyright 2001 AAAS); (d) multi-lengthscale electrodes combine the enhanced capture efficiency and probe density of nanoscale biosensors with reduced mass transport times of sub-millimeter sensors (adapted with permission from reference [28], Copyright 2012 American Chemical Society).

Over the past two decades, reducing the inter-electrode spacing into the nanoscale, has been used as means of redox current amplification to generate signals having a sufficient signal-to-noise ratio for electrochemical single molecule analysis. A major challenge in electrochemical *single molecule* analysis is that having a single redox molecule results in n ($n < 4$) electron transfer events. This is too small to be detected with state-of-the-art electrical amplifiers. One strategy for overcoming this challenge is to design miniaturized electrode systems that are separated by nanogaps (Figure 2 (b)). The nanogap separation is important because it allows analytes to rapidly diffuse between two independently biased (reductive and oxidative) electrodes to undergo several thousands of reduction/oxidation processes per second[29]. Ultimately, the adoption of this technology for bioanalytical sensing depends on the ability to (1) successfully couple this readout method with biorecognition strategies that are capable of translating specific biorecognition events into redox signals[26]; and (2) effectively translate the observed electrochemical signal amplification into lowering the system's overall limit-of-detection. It is expected that for such sensors to be able to deliver the limit-of-detection and sample-to-answer times required for point-of-care diagnostic applications, they should be combined with mechanisms and structures that would reduce the time needed to deliver target analytes to the nanogap. These strategies are discussed in the following section.

Miniaturization of semiconductors into the nanoscale have been used in creating "highly sensitive" chemical field effect transistors (FETs). Chemical FETs function by using the changes in the surface charge of the FET gate dielectric layer to modulate the conductance of the semiconductor channel or vary the threshold voltage of the underlying transistor[30]. By modifying the gate surface with ligands, enzymes, membranes, or molecular linkers that offer specificity or selectivity for biomolecules, ions, small molecules, or other biologically-relevant analytes, these systems can be developed into biosensors. Using nanomaterials such as nanowires as the semiconductive building block of chemical FETs allows the depletion/accumulation region to be extended into the bulk in contrast to their planar counterparts having their active zones limited to their surface (Figure 2(c)). Such devices are identified as candidates for single molecule analysis.[27] 2D nanomaterials have also been used as the semiconductive layer in field effect transistor biosensors due to their excellent sensitivity to variations in electronic charge at their surface. Although femtomolar protein detection is demonstrated using these sensors, detecting single *biomolecules* requires the field effect transistor channel lengths to be reduced into the nanoscale to be in-line with the impact dimensions of biomolecules. Nanoscale FET biosensors enable a larger fraction of the channel to be influenced by the electrostatic changes induced by

the single target biomolecule. As with any FET-based system, shortening the channel length could be detrimental to device performance due to the modulation of channel conductivity by the drain-source electric field. This has been addressed in the microelectronics area by decreasing the thickness of gate dielectric and/or increasing its dielectric constant. Similar approaches have been proposed in the field of biosensing. [31]

Nanostructured electrodes with footprints in the micro-scale or macro-scale have demonstrated promise in increasing the sensitivity of electrochemical biosensors due to increased surface area to volume ratio, increased density of biorecognition elements, improved accessibility of biorecognition elements by the target molecules, and higher catalytic activity. In a study focused on nanoporous gold electrodes with pores having diameters in the 20-200 nm range, it was found that the electrode structure in the nanoscale plays a dual role in tuning the analytical sensitivity. When porous electrodes are examined using voltammetry waveforms having the optimal frequency, it is possible to obtain currents that are one order of magnitude larger than those measured using planar electrodes. It is also found that varying the pore size and density is critical for tuning the capture efficiency of biorecognition elements immobilized on porous surface. As a result, porous structures having a smaller density of larger pores yield the lowest limit-of-detection compared to planar and porous structures having a larger density of smaller pores. [32] This result along with preceding findings using fractal[28] and wrinkled[33] surfaces have motivated a trend towards miniaturization of the *building blocks* of electrochemical biosensing electrodes. This multi length scale engineering approach has the advantage that it combines the signal-to-noise ratio enhancement resulting from the nanoscale building blocks of the structure with the lowered response times resulting from the micro to macroscale overall dimension of the sensor, and has been previously reviewed elsewhere.[34]

In situ strategies for reducing the response time of biosensors

In order to take advantage of the enhanced signal-to-noise ratio of biosensors with critical dimensions in the nanoscale, several device-level strategies have been devised to reduce the response time of these biosensors into acceptable ranges (minutes instead of hours or days). These include strategies that employ external forces such as pressure-drive flow, electric field, and thermal gradients (Figure 3a), or internal forces delivered through self-propelled nano-/micro-motors to replenish the analyte-depleted solution (Figure 3b). In addition to methods based on analyte replenishment, creative strategies based on using distributed surfaces for analyte collection are expected to significantly improve the mass transport limitations of biosensors. These strategies along with the challenges involved around their integration into sensing systems are summarized in Table 2.

Active fluidic transport

The problem of long response times affects biosensors embedded in microfluidic systems driven by active pressure-driven flow if the position and dimensions of the sensor and fluidic systems are not carefully designed. For example, in a case where biosensors are placed at the channel edges, a depletion boundary layer evolves around the sensor surface limiting the rate of analyte capture.[23] Increasing the linear velocity of the fluid is achieved by increasing the volumetric flow rate or reducing the channel cross sectional area. Increasing the volumetric flow in a microfluidic channel generates a velocity profile that is largest in the middle of the channel. This means that the undepleted solution is effectively injected into the center of the channel, which in many cases is an ineffective strategy for replenishing the local depletion zones observed at the channel

walls[35]. Furthermore, it has been shown that using flow to increase the analyte flux to the sensor surface becomes ineffective for miniaturized sensors with critical dimensions smaller than 10 μm since these sensors do not significantly deplete the analyte concentration of the bulk solution.[24] A possible solution is to decrease the cross sectional area of the microfluidic channel to flow the undepleted solution through the capture volume of the sensors (Figure 3(a)). Driving the solution containing the target analyte through the bioanalytical sensor is an example of such strategy for placing the analyte in the capture volume of the sensor. This hybrid fluidic/sensing approach has been implemented in plasmonic nanohole array biosensors by using a multi-inlet fluidic system to move the fluid in three dimensions and through the plasmonic nanohole array. It is shown that passing the analyte (in this case isopropanol alcohol) through the sensing array offers higher changes in the resonance shift compared to the case where the analyte is passed over the sensor surface. [36] This strategy is applicable to affinity-based biosensors by carefully designing the pore diameter. On one hand, the pore diameter needs to be small enough to place a large fraction of the target analyte at the capture volume of the sensor. On the other hand, small pore sizes are problematic due to issues related to analyte clogging and system throughput. Another challenge in using this hybrid fluidic/sensing approach as a universal method for enhancing mass transport is its integration with electrical biosensors. These challenges are technical in nature and require the fabrication of membranes that are porous, sufficiently conductive and easily accessed via electrical contacts.

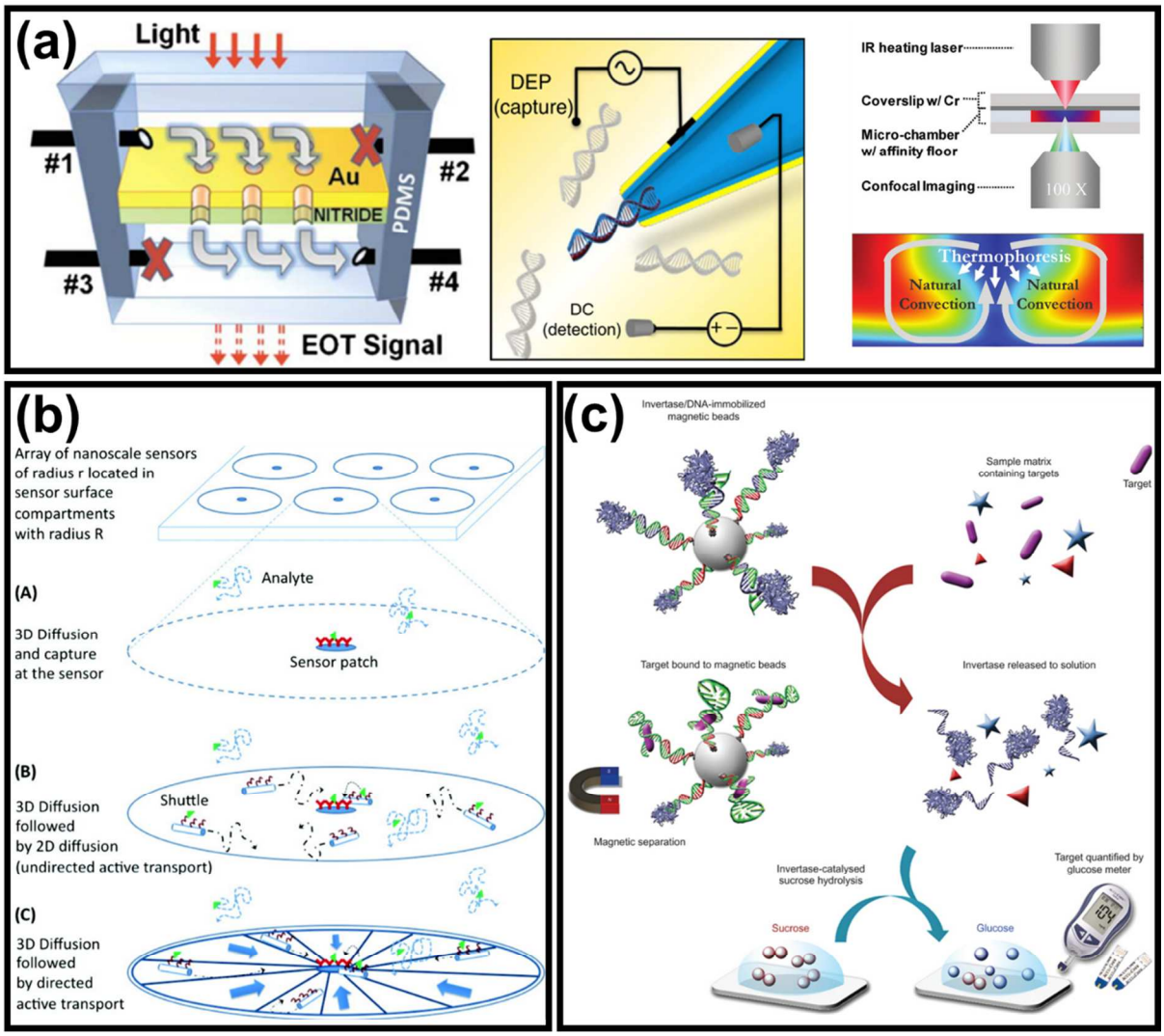

Figure 3-Strategies for reducing the response time of biosensors. (a) external forces, fluidic flow (left), adapted with permission from reference [36], Copyright 2010 AIP Publishing; electric field (middle), adapted with from [37], Copyright 2016 Creative Commons; and thermal gradients (right) adapted with permission from [38], Copyright 2016 The Royal Society of Chemistry; are used to drive analytes to the sensor surface. (b) Nano/micro motors help mass transport through directed or undirected surface diffusion, adapted with permission from [39], Copyright 2010 American Chemical Society. (c) Analyte capture using beads distributed in solution, followed by the release of rapidly diffusing signal transduction reagents, adapted with permission from [40], Copyright 2011Macmillan Publishers Ltd.

Other strategies for alleviating the mass transport limitations of flow-based microfluidic systems are based on (1) removing the depletion layer at the channel walls by introducing fluidic outlets, (2) replenishing the depletion zones at the channel walls by introducing inlets, and (3) introducing spiralling transverse flow or chaotic mixing by creating structural features in the microfluidic channels. [35,41] The inlet/outlet-based strategies (strategies 1 and 2) consider the spatial profile of the depletion zones developed around sensor surfaces, and design precisely positioned inlets or outlets for depletion zone replenishment. This approach has been applied to

microscale sensors coating the entire surface of the microfluidic channel wall. However, it becomes challenging to design and fabricate such systems for manipulating the depletion zones created around sub-microscale sensors. In addition, in systems where there is a limited amount of target molecules, for example, nucleic acid molecules in a diagnostic sample, the outlet method could cause analyte loss. This could be compensated by implementing a system having fluidic feedback, which further adds to the complexity of the system. The approach involving three-dimensional structural features for achieving chaotic mixing in a continuous flow system (strategy 3) could be used as a versatile strategy for improving the mass transport limitations of sub-microscale biosensors. However, strategy 3 has resulted in an enhancement in the analyte capture/charge transfer rate of a factor of 2, which is insufficient for use with nanoscale sensors requiring enhancements at the order of magnitude level[24].

Electric-field based methods

Electrophoresis has long been used for preprocessing charged biomolecules such as nucleic acids. When integrated inside microfluidics, these methods are ideally suited for overcoming the transport limitations of biosensors. Rapid (seconds to minutes) and efficient (up to 800 times) enrichment of DNA inside microfluidics has been demonstrated using a system that balances the velocities generated through electrophoresis and electroosmosis. [42] A vertical stack of two microfluidic channels separated by a porous membrane has been used for this purpose. A DC electric field is used to drive the DNA from a source reservoir to the interface between the microfluidic channel and the porous membrane, and the electroosmotic flow at the channel/membrane interface generates a fluidic velocity in the direction opposite to the velocity caused by electrophoretic forces. This localizes the DNA to a confined volume inside the microfluidic channel. It should be noted that electrophoresis is limited to targets that are inherently charged, and solutions having low ionic strength, which limits its applicability to many practical assays.

Dielectrophoresis (DEP) can be used to overcome some of the limitations of electrophoretic target localization since it relies on the dielectric properties of materials rather than their inherent charge. Electrodeless or insulator-based dielectrophoresis uses dielectric constrictions (e.g. nano constrictions) to locally enhance the electric field and electric field gradients to generate a dielectrophoretic force of sufficient magnitude for manipulating particles having a small effective radius and dielectric contrast. Protein dielectrophoresis has been demonstrated in fused silica-based nano-channels featuring nano constrictions that connect two microfluidic channels biased under different polarities. An enhancement in the dielectrophoretic force in the order of 10^9 is observed using the multi length scale micro channel/nano channel/nano-constriction design, which is translated to a 5 order of magnitude enhancement in protein concentration in 20 s. [43]

Nano/micro constriction-based dielectrophoresis devices have been integrated with hybridization-based electrochemical biosensors with the goal of alleviating the mass transport limitations of these sensors[44]. It is found that adding a conductive electrode in the constriction region has a profound effect on the localization of the electric field and the resultant dielectrophoretic force distribution. Numerical modelling and experimental results obtained from observing the concentration of fluorescently-labelled DNA in micro constrictions suggest that electrodes in the same lengthscale compared to the nano constriction (the same size or up to a factor of 5 larger) are optimal for allowing the force field of the micro constriction to overlap with the electrode area. Using this strategy, DNA capture rate was expedited by a factor of 10

down to the concentration of 10 pM. The inability to detect below this concentration is justifiable by the observation that the pre-concentrated volume is larger than the capture volume of the sensing electrode. This situation could be alleviated by using tall and three dimensional electrodes within the constriction volume.

Nanopore-based biosensors are single molecule analysis systems. These biosensors are limited by mass transport in delivering DNA molecules from the bulk solution into the capture volume of nanopores, which is 8-10 orders of magnitude smaller than the overall sample volume. Dielectrophoresis has been used in nano-pipette based nanopore systems to actively deliver DNA to the capture volume of the pores. The DEP forces generated by biasing the metallized nano-pipettes using AC voltages significantly dominate over diffusive transport 16-19 μm away from the pore position. This force, in addition to the electro-thermal forces caused by Joule heating at the metal-coated nano-pipette, result in 3 orders of magnitude reduction in the limit-of-detection of nanopore based biosensors allowing them to enter the femtomolar limit-of-detection range.[37]

We believe carefully-designed dielectrophoresis-based methods are ideally suited for reducing the long response times encountered in miniaturized biosensors because of their compatibility with microfluidics, applicability to uncharged targets, and the significant enhancements observed in terms of limit-of-detection.

Thermal focusing

Creating temperature gradients in specific locations inside the solution and at the sensor/solution interface has been used to achieve active transport for delivering analytes to sensor surfaces. Natural convection and thermophoretic mechanisms can be used to circulate the fluid and drive analytes towards or away from a heated location respectively. Joule heating[45] and laser-based heating[46] have been used for generating the temperature gradient needed for active transport. In a laser-based system integrated with microfluidics, the laser beam was used to heat the ceiling of a microfluidic channel, where the sensing surface was placed at the channel floor. The localized heating causes a combination of thermophoretic effect driving 200 nm polystyrene particles away from the heated surface, and natural advection circulating the nanoparticles towards the sensing surface. In cases where advection dominates over the thermophoretic effect, and using optimized channel heights (in the order of hundreds of microns), simulation and experimental results show that it is possible to reduce the reaction half-life by more than an order of magnitude. This indicates that methods based on steep temperature gradients (temperature increases in the order of a few degrees applied over micro scale distances) can be exploited for reducing the response time of biosensors.[38]

Micro/nano motors

In molecular shuttles, proteins such as kinesin drive cargo along microtubule tracks for intracellular transport. These bio-motors and their bio-inspired synthetic analogues, self-propelled micro/nano motors, offer a promising solution for alleviating the mass transport limitations of biosensors without relying on external forces.[47] For example, bubble-propelled tubular micro motors, driven by the catalytic oxidation of hydrogen peroxide fuel, has been used to increase the effective diffusion coefficient of tracer micro-particles by over an order of magnitude.[48] Furthermore, in a system containing *bio-functionalized* bubble-propelled micro-motors, a capture enhancement of 2-3 times was observed when collecting protein targets in the

presence of bio-functionalized micro-motors. [49] In order to demonstrate the potential advantage offered by these systems in decreasing the response time of biosensors, theoretical calculations were performed to compare the time required to capture 10 molecules from 1 pM solutions for systems modelled with three different transport mechanisms (Figure 3b) [39]. The first case considers three-dimensional diffusion in solution and capture at the sensor patch similar to the analysis performed by Manalis[23]. The second case considers three-dimensional diffusion in solution, followed by active, non-directional, two-dimensional transport. This case is applicable when molecular shuttles powered by molecular motors are used to transport analyte molecules on sensor surfaces in a worm-like chain, effectively increasing the surface diffusion coefficient. The third case is similar to the second case, except, the active two-dimensional transport is directed towards the sensor patch. This case models a situation where transport tracks are patterned on the surface to direct the movement of molecular shuttles[50] towards the sensor patch. It is noted that in cases where 2D mass transport by surface diffusion is comparable to 3D mass transport by solution diffusion, and analyte dissociation from the surface is negligible, 2D surface diffusion significantly increases the analyte flux to the sensor. The theoretical models demonstrate that the increased surface mass transport offered by molecular shuttles successfully alleviates the mass transport limitations of nanoscale sensors (sensor radius <1 μm) by significantly reducing the time required to accumulate 10 molecules on the sensor surface at a bulk analyte concentration of 1 pM. Interestingly, directed active transport enabled by patterning transport tracks on the surface does not provide a major advantage over non-directed active transport enabled by molecular shuttles.

Solution-based capture and surface-based signal transduction

Another way to overcome the mass transport limitations encountered in miniaturized biosensors is to physically separate the biorecognition and the signal transduction elements. For this purpose, bio-functionalized micro or nanoscale particles are used to capture the target analytes from the solution. This is followed by releasing *rapidly diffusing* intermediate products or *actively driving* the captured targets to the transducer surface or active area. For example, bio-functionalized magnetic beads have been used for performing sandwich-based immunoassays, where molecular recognition initiates a catalyzed gas generation reaction causing measureable pressure changes at the transducer[51]. Functional nucleic acids (aptamers or DNAzymes) immobilized on magnetic beads have been used to quantitatively release a DNA-invertase conjugate in the event of target capture, followed by the catalytic conversation of sucrose to glucose by invertase (Figure 3c). [40] Since glucose is the analyte detected at the transducer surface, the sensor will be operated in a reaction-limited regime, and electrode miniaturization will not present significant mass transport limitations. It is also possible to integrate all the solution-based procedures of this assay onto a lateral flow device, which has been shown to significantly simplify the sensor operation at the point-of-care and reduce the sample-to-answer time.[52] In a digital enzyme-linked immunosorbent assay (ELISA), target capture and labeling can be performed on bio-functionalized magnetic beads distributed in the solution, while detection is performed after driving the beads into femtoliter well arrays by centrifugation. Since, the average distance between magnetic beads in solution is estimated to be 80 μm, a distance that can be traversed by diffusing proteins in <1 minute[53], it is possible to use this approach for overcoming the mass transport limitations encountered by miniaturized biosensors. While this approach uses centrifugation for bead transport, it is possible to use magnetic force for driving the beads into

the sensor's active area. Additionally, while the assay employs fluorescence readout, it can be integrated with electrical readout strategies for reducing the transport times encountered in these systems.

Table 2-The summary of *in situ* strategies used for reducing the response time of biosensors

Category	Strategy	Mechanisms	Challenges
Fluidic	Hybridization of flow channels and sensors	Reducing the diffusion distance that must be overcome by target analyte	-Pore clogging -Throughput limitations -Fabrication challenges
	Strategic inlet/outlet positioning	Localized replenishment of analytes and/or removing depleted solutions	-Design challenges -Analyte loss
	Chaotic mixing	Increasing the 3D mass transport rate	-Achieving sufficient enhancement in analyte capture rate for nanoscale sensors
Force field-based	Electrophoresis	Localizing charged analytes to reduce the diffusion distance using uniform electric fields	-Not applicable to neutral targets -Highly sensitive to solution ionic strength
	Dielectrophoresis	Localizing charged and uncharged analytes to reduce the diffusion distance using non-uniform electric fields	-Overlapping the enrichment volume of the dielectrophoretic system with the capture volume of the sensor
	Thermal focusing	Localizing analytes to reduce the diffusion distance using temperature gradients and the resultant thermophoresis and natural convection	-Heat-induced device and analyte damage -Precise temperature control inside the microchannel environment -Design complexity of the microfluidic system containing sensors and heating elements
Micro/nano motors	Using molecular shuttles	Complementing analyte capture from the solution with an active surface transport process enabled by molecular shuttles such as microtubules propelled by immobilized motor proteins	-Molecular shuttle functionalization -Non-specific adsorption of the analyte onto the molecular shuttle and the adherence of the molecular shuttles on surfaces -Programmable delivery and/or release of the analyte from the molecular shuttle to the sensor surface
	Using self-propelled micro-motors	Increasing the 3D mass transport rate of analytes by the movement of micro-motors such as self-propelled tubular micro-engines having oxygen bubble generating catalytic surfaces (non-functionalized motors) Reducing the effective diffusion distance between the biorecognition elements immobilized on micro-motors and solution-bourne analytes (functionalized motors)	-Functionalized motors: similar challenges as molecular shuttles -Non-functionalized motors: unwanted adherence of motors to surfaces, non-specific adsorption of analytes to motor surfaces, interference of the required fuel (eg. hydrogen peroxide) with the sensing solution and environment
Solution-based capture and surface-based signal transduction	Bead-based biorecognition followed by releasing intermediates	Decreasing the diffusion distance between biorecognition elements immobilized on beads and solution-bourne targets and releasing rapidly diffusing intermediate products	-Unwanted adherence of beads on sensor surfaces -Additional processing required for removing beads -The intermediate product generation could be kinetically sluggish
	Bead-based biorecognition followed by driving the beads to the sensor surface	Decreasing the diffusion distance between biorecognition elements immobilized on beads and solution-bourne targets and using active transport to drive the beads to the sensor surface	-Unwanted adherence of beads on sensor surfaces -Additional devices required for driving the beads to the sensor surface

Conclusions

Miniaturization is critical for realizing point-of-care and wearable biosensors that combine portability, ease-of-use, and low cost implementation with low limits-of-detection and rapid response times. Reducing the dimensions of the sensing electrodes or their building blocks into the nanoscale offers enhanced signal-to-noise ratio in nano-electrode, nanotextured, nanogap, and field effect transistor-based biosensors. However, this enhancement could come at the cost of increasing the biosensor's response time into an unacceptable range. In hemispherical and disk biosensors, the biosensor response time increases linearly with decreasing sensor dimensions. Through structure-level design considerations, it is possible to design the sensor density (in a sensor patch), dimension, and geometry to find an optimal trade-off between signal-to-noise ratio and response time. There are also a number of promising strategies based on external forces – fluidic flow, electric field, thermal gradients – and internal forces – molecular shuttles or self-propelled micro-/nano-motors – for significantly reducing the response time of biosensors. In addition, strategies based on separating the physical locations for biorecognition and signal transduction provide an exciting avenue for enabling biosensors to realize their promise in analyzing clinically-relevant concentrations of target analytes in clinically-relevant times.

Acknowledgements

The authors acknowledge the support from the Natural Sciences and Engineering Council of Canada, Ontario Ministry of Research and Innovation, and the Canada Research Chair program.

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