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3.1 Introduction

There is a continuing demand for fast and simple analytical systems to establish new biosensors for the detection of clinical, biological, environmental, and chemical analytes. As a result, biosensor research has continued to grow over the last twenty years.\(^1\) Generally, a biosensor is described as an analytical device that is used to convert a biological related response into a physicochemical processable signal. Biosensors must show high selectivity, sensitivity, reversibility, and efficient reagent usage.\(^2\)

A biosensor design in general consists of three main parts: a recognition element, a physicochemical transducer, and an electronic part.\(^3\) Several recognition elements or bioreceptors have been used in biosensors including enzymes, whole cells, nucleic acids, and antibodies.\(^4\) Due to their alternative systems and specificity, the most widely adapted recognition elements in biosensors are based on an antibody–antigen interaction, and are known as immunosensors.\(^5\)
Immunosensors, in general, can be classified as competitive or noncompetitive systems. In competitive immunosensors, all reactants are mixed together simultaneously, and a labeled antigen competes with an unlabeled antigen for the same binding site on an antibody. Noncompetitive immunosensors (also known as a “sandwich” immunoassay) proceed in a stepwise manner: first, an antibody for the target antigen is adsorbed to a solid phase. Next, the antibody-coated surface is exposed to a sample that may contain the target antigen. After the adsorption process has stabilized, a multistep washing is done to remove the unbound antigen and a second antibody with a label or a tag is added to enable detection of the antigen. The noncompetitive immunoassay format often offers superior specificity to the competitive format as it uses more than one element for analyte recognition.

Although various modes of sensing transduction have been deployed in immunosensing for efficient biodetection, optical and electrochemical immunosensors are considered to be the most popular immunoassay-based biosensors due to their rapid signal generation and non-destructive operation. Typically, the sensitivity of signal transduction with photonic devices is substantially enhanced by confining the probing light beam into a surface wave to amplify the relevant light–matter interaction. An evanescent field whose intensity decays exponentially as a function of distance from the sensing interface defines the interrogation region of the surface-propagating light wave. Interaction or local change in the evanescent field that occurs following a physicochemical event at the sensing surface is converted into the properties of the optical surface mode to deliver a quantifiable and measured readout. Depending on the particular optical transduction approach, the form of the measured readout includes fluorescence detection, absorbance, spectroscopic shifts, and refractive index changes.

Electrochemical transduction schemes have also been successfully demonstrated to generate immunosensor technologies that are highly selective. The different electrochemical transduction schemes are usually classified by the electrical parameter being measured (e.g., potentiometric, amperometric, voltammetric, capacitative, conductometric, and imped- ance), however, they all share the common advantage that the electrochemical process is interfacial and located next to the electrode surface, which gives better confinement of detectable events to the sensor surface. Among the several electrochemical transduction schemes, electrochemical impedance spectroscopy (EIS) has been proven to increase detection sensitivity and provide an efficient route for bio-sensing.

Recently, sensing with multiple transducing methodologies, where two or more transducers are combined to interrogate a certain kind of binding event, has been gaining in popularity. A combination of multi-sensing transducers can extract different properties, increase selectivity, and provide information that would not be available with a single transduction technique alone. For example, there have been advances in combinations
of conventional electrochemical and spectroscopic measurements, collectively known as spectroelectrochemical methods. Such combinations of optical and electrochemical signals provide information that is difficult to be retrieved with the separation of electrochemistry or optical techniques. In addition to increasing the sensitivity, the capability to tune the optical signal in spectroelectrochemical methods to probe exclusively the Faradaic process of redox activities can potentially provide a superior means to investigate electrochemical processes in molecular adsorbates with extraordinary selectivity by avoiding non-Faradaic components that typically deter conventional electrochemical approaches using electrical signals alone. Also, recent reports have shown that applying a combination of electro-optical measurements leads to an increase in sensitivity of the targeted molecular binding events at the sensing surface and is less sensitive to signals from non-specific binding.

The advances in spectroelectrochemical methodologies have spawned the development of coupled electro-optical sensors. In this chapter, we provide a general overview of two major electro-optical photonic devices developed for immunoassaying applications, namely, the single-mode electro-active integrated optical waveguide (SM-EA-IOW) and the electrochemical surface plasmon resonance (EC-SPR) immunosensors. We believe that an immunoassay-based strategy for direct detection of biological targets based on sandwich bioassays incorporated on highly sensitive electro-active photonic platforms is of great interest. The selectivity of such sensors benefit from the advantages of three highly sensitive and selective features for detection of a target analyte: optical (analytical signal linked to the evanescent field of a surface wave), electrochemical (confinement to the surface and using high sensitive electrochemical methods such as AC impedance) and biological (using a sandwich immunoassay).

3.2 Immunosensing with Single-mode, Electro-active, Integrated Optical Waveguides

3.2.1 Methodology

The sensing methodology using SM-EA-IOW is illustrated schematically in Figure 3.1. The strategy is based on a sandwich immunoassay approach, which starts with the functionalization of the SM-EA-IOW with a capturing antibody (Ab) aimed at a specific target analyte. Once the target analyte is bound to the SM-EA-IOW interface, it promotes the binding of a reporter secondary polyclonal Ab labeled with a redox-active probe that can be electrochemically modulated on the SM-EA-IOW platform to provide the probing optical signal. In the system a polyclonal secondary antibody is labeled with methylene blue (MB) dye. The MB dye functions as a transduction redox probe because it shows a strong optical absorption change upon changes in its redox states. Thus, the analytical signal is both optically and electrochemically locked to
the specific target designed for the antigen detection. It is optically locked by using a specific laser wavelength that is tuned to the absorbance change occurring in the particular redox process. Additionally, it is electrochemically locked by modulating the applied potential at the formal potential of the aimed redox events. Those specific features contribute to reducing undesirable signals from possible interferents existing during the detection process.

### 3.2.2 Sample Preparation

#### 3.2.2.1 SM-EA-IOW Fabrication

A critical step in the fabrication of SM-EA-IOW devices is to obtain simultaneously an extremely high optical transparency that is adequate for propagation of guided waves and an outstanding electrical conductivity for electrical potential modulation. Briefly, a SM-EA-IOW platform on a glass slide was formed by thin film layers of a high-refractive index of alumina, a buffer layer of silica, and a transparent conductive electrode of indium tin oxide. A pair of gratings was fabricated on the glass substrate prior to depositing the multilayer stack, that is used to couple the light beam in and out of the SM-EA-IOW device. Glass substrates with surface-relief gratings were then coated with highly transparent layers (alumina and silica) as described elsewhere. On the top of the guiding layers, an ITO layer was deposited onto the device. The ITO layer was then carefully calibrated and optimized to ensure the high performance of the optical and electrical properties of the SE-EA-IOW devices.
3.2.2.2 Functionalization Protocol of the SM-EA-IOW Interface for an Immunoassay Targeting an Influenza Virus Antigen

Details on the functionalization and characterization protocols for immunoassay assemblies on the SM-EA-IOW surface can be found in the literature.\textsuperscript{33,38–40} Briefly, the build-up of a sandwich immunoassay for detecting the hemagglutinin (HA) protein from the H5N1 avian influenza A virus using the SM-EA-IOW platform was adopted as follows. First, a monoclonal anti-H5 (H5N1) Ab that served as the capture Ab was bound to the SM-EA-IOW interface that was functionalized with APTES. Then, recombinant influenza A hemagglutinin (HA) of the H5N1 influenza virus was injected into the flow cell and allowed to adsorb to the surface-bound capture antibodies. Finally, a MB-labeled polyclonal secondary H5N1 Ab solution was injected into the flow cell to bind to the virus protein species residing on the SM-EA-IOW surface. The presence of bound MB-labeled polyclonal secondary H5N1 Ab was then interrogated with the SM-EA-IOW device.

3.2.3 Experimental Set-up

Figure 3.2 shows a schematic representation of the experimental set-up that was used to control and collect data from the SM-EA-IOW platform.\textsuperscript{41} At the heart of the set-up the SM-EA-IOW platform was mounted...
in a conventional electrochemical flow cell. A set of diffraction-limited optical components was deployed to launch the light beam towards the integrated grating coupler. Electrochemical measurements using the SM-EA-IOW were performed with a standard configuration of three electrodes mounted in a homemade flow cell configuration. A solid state laser source was routed to the input port of the SM-EA-IOW flow cell. The out-coupled light from the SM-EA-IOW flow cell was collected and directed into a photomultiplier detector, which was connected to a low-noise current preamplifier. The optical signal was monitored while a potentiostat was used to control the electric potential applied to the working electrode. In AC potential-modulated absorbance measurements, the collected PMT signal that is electronically processed by a current preamplifier was sent to a lock-in amplifier. An oscilloscope was used to read and record all signal measurements.

3.3 Immunosensing with Electrochemical Surface Plasmon Resonance

3.3.1 Methodology

The sensing methodology using the EC-SPR device is illustrated schematically in Figure 3.342 The strategy is based on a sandwich immunoassay approach similar to the assembly described in Section 3.2.1, which starts with the functionalization of the EC-SPR platform with a capture Ab aimed at the specific target analyte. Once the target analyte species are bound to the SPR interface, they promote the immobilization of a labeled polyclonal secondary antibody with a redox active probe.

Figure 3.3 To create the biosensor interface a glass slide was coated with an adhesion layer followed by a gold film. Then the sensing surface was functionalized with a functional layer to promote the binding of the successive immunoassay assembly. Reproduced from ref. 35 with permission from The Optical Society, Copyright © 2017 Optical Society of America.
3.3.2 Sample Preparation and Functionalization Protocol of the EC-SPR Surface with an Immunoassay Targeting an Influenza Virus Antigen

In order to perform the principle of the EC-SPR sensor, chromium and gold films were deposited on a BK7 glass slide using a DC sputtering technique and functionalized with the sequence of layers as indicated in Figure 3.3 and following known protocols. For detection and quantification of a critical influenza antigen, a monoclonal capture antibody of the targeted influenza antigen was immobilized on the sensor surface. Then the sensing surface was mounted onto an electrochemical flow cell. The next steps for functionalizing and incubating the targeted virus antigen was similar to the one description reported in Section 3.2.2.2.

3.3.3 Experimental Set-up

The gold film served as the working electrode (WE) and was mounted in a micro-electrochemical flow cell with a three-electrode configuration, as illustrated in Figure 3.4. The optical set-up is based on the widely used Kretschmann configuration, where a transverse magnetic polarized light was directed through a prism to excite the surface plasmon resonance. A potentiostat was used to apply and control the electric potential at the working electrode. The optical signal under applied potential modulation was monitored at a fixed angle by a power meter and then recorded by an oscilloscope.

![Figure 3.4](image-url)  
**Figure 3.4** Schematic representation of the EC-SPR set-up with a potentiostat for electrical control, a transverse magnetic polarized laser source for excitation of the plasmon surface wave. A power meter, a lock-in amplifier, and an oscilloscope were deployed for data collection.
3.4 Results and Discussions

3.4.1 SM-IOW-IOW Results

The optical absorbance data on the SM-EA-IOW device for the sandwich immunoassay of the virus H5N1 protein was first collected under CV potential modulation.\textsuperscript{38,39} The results displayed in Figure 3.5 represent the absorbance optical data collected when the SM-EA-IOW device functionalized with the full immunoassay layers was exposed to the HA virus antigen and redox active-labeled secondary Ab. The presence of the virus antigen will promote the binding of the redox active-labeled secondary Ab molecule, then as the applied potential in the CV scans crosses the formal potential of the redox active probe, it triggers an associated optical absorption change. On the other hand, in a case where the virus antigen is absent from the solution the redox active probe signal will be absent. The experimental results in Figure 3.5 confirm the ability of the SM-EA-IOW platform to detect the presence and absence of the virus protein through spectr electrochemical changes in the redox probe.

The CV modulation provides a simplified and obvious identification of the redox process, but using AC impedance modulation on the SM-EA-IOW platform under synchronous detection was shown to improve the signal-to-noise ratio with faster data acquisition time. For device applications, AC voltammetry can serve as a robust transduction scheme to operate the platform as it is not time-consuming and is much more immune to noise due to the frequency filtering capability provided by a lock-in amplifier that reads

![Figure 3.5](image)

**Figure 3.5** Optical absorbance at 610 nm collected in the presence of the HA virus antigen and in the absence of the virus antigen as measured by the SM-EA-IOW platform under CV scans. Reproduced from ref. 35 with permission from The Optical Society, Copyright © 2017 Optical Society of America.
Chapter 3

the modulated AC signal. Additionally, AC voltammetric techniques at a modulation frequency without the virus antigen that maximizes the Faradaic current of the redox probe can be experimentally determined once the resonance frequency has been determined.\textsuperscript{38,39,41} Using such resonance frequency at different DC bias potentials while collecting the optical data with the SM-EA-IOW platform, as shown in Figure 3.6 for different virus antigen concentrations, a plot of the Faradaic current density (\(y\)-axis) against the DC bias potential measured (\(x\)-axis) from the absorbance modulated amplitude displays a peak intensity around the formal potential corresponding to the redox activity of the MB-labeled H5N1 Ab over the functionalized SM-EA-IOW surface. As the DC bias potential is set away from the formal potential of the targeted probe, the analytical signal decreases towards zero. With different concentrations of the virus antigen the peak intensity of the Faradaic current density reported by the redox probe under AC voltammetry showed a proportional relation to the bounded surface concentration of the target antigen, which provides a direct route to the detection and quantification of the virus analyte.

From AC voltammetry, a plot of the corresponding peak intensity of the current density at different virus antigen concentrations can be used to determine the limit of detection. As shown in Figure 3.7, an experimental limit of detection was determined using a standard 3-sigma to be about 4 ng ml\(^{-1}\), for the virus antigen using the SM-EA-IOW platform. The limit of detection result in this experiment surpasses several technologies currently being used.\textsuperscript{45}

\textbf{Figure 3.6} The Faradaic current density from the MB-labeled secondary H5N1 Ab plotted as a function of DC bias potential at different concentrations of the H5N1 virus antigen.
3.4.2 EC-SPR Results

First, under CV potential modulation, the normalized optical intensity displayed in Figure 3.8 represents the normalized optical intensity collected when the EC-SPR device functionalized with the capturing Ab was exposed to the HA virus antigen and MB-labeled H5N1 secondary Ab. Also, Figure 3.8 shows the results of a control experiment when data was collected of the normalized optical intensity using the EC-SPR device functionalized under the same protocol mentioned above but in this case it was exposed just to MB-labeled H5N1 secondary Ab and the target antigen was absent. The results show a reversible change in the optical signal as the modulation potential crosses the formal potential of the MB-labeled H5N1 secondary. The presence of this transition indicates the binding of the redox probe conjugated to the secondary Ab. In contrast, without a HA virus antigen a small linear increase in the optical signal was observed that indicates a small non-specific binding of MB-labeled H5N1 secondary Ab. These results confirm the ability of the EC-SPR sensor to observe the presence of the HA virus antigen through the modulated optical signal using a CV scan.

As discussed previously, to increase the signal-to-noise ratio an AC voltammetric technique can be used under synchronous detection. In order to apply the AC voltammetric modulation, the optimal modulation frequency needs to be determined. To do so, AC impedance measurements can be carried out with the full immunoassay sandwich at the formal potential and several different frequencies. The SPR optical signal $\Delta I_{\text{SPR}}$ was determined by...
subtracting the baseline SPR optical signal from the SPR optical signal collected after the incubation of the MB-labeled polyclonal antibody. Figure 3.9 shows a clear peak centered at about 250 Hz, which was determined to maximize the relevant redox process. Then, following the same idea using the SM-EA-IOW, by employing an AC voltammetry at DC bias potentials ranging around the formal potential while the SPR optical signal is monitored and recorded under synchronous detection at each DC bias potential with different H5N1 virus antigen concentrations, the limit of detection of the EC-SPR sensor can be determined.
As shown in Figure 3.10, a plot of the SPR optical signal $\Delta I_{SPR}$ against the DC bias potential displays a maximum intensity at approximately $-0.17$ V due to the redox activity of mB-labeled h5n1 secondary Ab. Beyond the formal potential of the redox probe, the SPR optical signal $\Delta I_{SPR}$ peak decreases. The peak intensity of the SPR optical signal $\Delta I_{SPR}$ reported by the redox probe is proportional to the bound surface concentration of the hA virus antigen and provides a direct route for hA virus antigen detection.

Using the maximum peak intensity of the SPR optical signal $\Delta I_{SPR}$ for the different bulk concentrations of the virus antigen a calibration curve can be plotted as shown in Figure 3.11 and the limit of detection using a 3-sigma standard was determined to be 300 pM. 

Figure 3.10 The SPR optical signal $\Delta I_{SPR}$ signal from the redox probe versus a different DC bias potential for different concentrations of the H5N1 virus antigen measured under AC voltammetry at a frequency of 250 Hz.

Figure 3.11 The calibration curve based on AC measurements for detection of different concentrations of the HA virus antigen.
3.4.3 Comparative Analysis

The electrochemically-modulated photonic platforms described above successfully showed the remarkable limits of detection that have been reached through a probe that is biologically highly specific. Such results certainly encourage future immunosensing applications of these platform technologies. Still, each methodology has specific advantages and challenges that are worth mentioning:

- The fabrication of the SM-EA-IOW device is challenging, unlike the EC-SPR platform that requires only the standard deposition of a noble metal (Au) film and does not require grating couplers.
- A cleaning and recycling protocol of the sensing interface was successfully established for the SM-EA-IOW platform; in contrast to the EC-SPR device where a covalent bond was formed between the sensing surface and the capture Ab preventing easy recycling procedures.
- Due to the long path of propagation in the SM-EA-IOW, a lower limit of detection for the H5N1 virus antigen was achieved compared to that reached using the EC-SPR sensor.
- A smaller sample volume can be interrogated with the EC-SPR due to the one-bounce and single-spot nature of light interaction with the sample under test.
- Compared to the state-of-the-art, the advances described here have the potential to create new biosensing that has the potential to surpass several technologies currently being used (see Table 3.1).

3.5 Conclusions

The combination of electro-active photonic platforms with a biological immunoassay displaying a redox probe that can be electrically controlled for optical transduction is an important innovation to the biosensing field. Due to the extremely high sensitivity of the photonic devices, an experimentally demonstrated remarkable limit of detection for an influenza virus when using a highly selective probe that is biologically specific through the antibody/antigen binding affinity was achieved. Equally important, the transduction mechanism of the biophotonic devices is also highly

<table>
<thead>
<tr>
<th>Detection technique</th>
<th>Limit of detection (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray photoelectron spectroscopy</td>
<td>1000</td>
</tr>
<tr>
<td>Secondary ion mass spectroscopy</td>
<td>10–100</td>
</tr>
<tr>
<td>ELISA</td>
<td>10</td>
</tr>
<tr>
<td>SM-EA-IOW</td>
<td>4</td>
</tr>
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<td>EC-SPR</td>
<td>16</td>
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</table>

Table 3.1 Limit of detection for several immunoassay techniques.
selective as the monitored analytical signal is optically and electrochemically locked to the probe tailored for antigen detection. The redundancy of these selective factors is expected to minimize unwanted false signals from interferents invariably present during detection with biological specimens. Compared to the state-of-the-art (e.g., PCR, ELISA) the advances described here have the potential to create a new biosensing technology capable of offering substantially shorter detection times with simpler and more cost-effective protocols, which are critical for point-of-care applications in disease diagnostics.\textsuperscript{38}

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\section*{References}


