EXTERNAL CAVITY LASER BIOSENSOR FOR LABEL-FREE DETECTION

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DISSERTATION

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ABSTRACT

Optical label-free biosensors serve as a powerful detection tool to analyze biomolecular interactions, and have been widely used in pharmaceutical drug discovery and biochemical sensing. Desirable performance metrics for such sensors include high sensitivity and fine resolution, which are essential for achieving high-precision detection of small molecules. In this work, we demonstrate a dual-mode external cavity laser (ECL) biosensor to address the desired needs. The utilization of photonic crystal resonant reflector biosensor as mode-selection filter enables high sensitivity, while the stimulated emission process in the laser cavity provides high spectral resolution. Additionally, dual-mode operation of the ECL system enables a self-referencing technique which significantly improves the signal to noise ratio. This advanced instrument enables direct detection of small molecule binding to immobilized protein targets, which is essential for drug discovery process.

We develop a plasmonic ECL to further improve the performance metrics of ECL biosensors. Utilizing surface plasmon resonance (SPR) biosensor as mode-selection filter, the plasmonic ECL demonstrates 3× higher sensitivity than photonic crystal based ECL biosensor. Moreover, compared to that of traditional SPR biosensors, the quality factor of the plasmonic laser biosensor is significantly enhanced, improving the spectral resolution. The enhanced biosensing capability of the plasmonic ECL offers unique opportunity for detecting biologically important molecules and viral particles in extremely low concentration.
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CHAPTER 1. OPTICAL LABEL-FREE BIOSENSOR

1.1. Introduction

Optical label-free biosensors have been powerful detection tools for analysis of biomolecular interactions since the introduction of surface plasmon resonance (SPR) biosensors in 1991.[1-3] Optical biosensors are configured to detect refractive index changes induced by adsorption of biomolecules into the surface-confined evanescent field of a light-coupling transducer surface, and thus are able to perform direct measurement of nearly every type of biological analyte (ranging from small drug molecules, peptides, proteins, nucleic acids, virus particles, bacteria, and cells) in real time. Compared to sensing approaches that utilize a tag molecule (such as a fluorescent dye, nanoparticle, or other family of reporter), label-free detection eliminates the labeling processes that may interfere with the function of a biomolecule. Recent advances in instrumentation and experimental design have led to the development and commercialization of optical label-free biosensor technologies with sufficient sensitivity and resolution for a wide range of useful applications, and have accelerated the development of a tremendous number of label-free assays for biological analytes. Applications of these high-precision label-free assays range widely from life science research, pharmaceutical discovery to medical diagnostics.[1, 4-8]

A characteristic that is shared among nearly all the optical label-free biosensors is that they are comprised of a “passive” optical resonator in which the transducer surface is illuminated with a broad range of wavelengths or a broad range of incident angles.[4] As shown in Fig. 1.1, a narrow sub-set of the incident wavelengths (or angles) is resonantly coupled with
the resonator and subsequently reflected back or transmitted through with greater efficiency than non-coupled wavelengths (or angles). The adsorbed biomaterials on the sensor surface interact with the coupled resonant light, and modulate the resonant coupling condition. Changes in the resonant coupling condition can be translated to a quantifiable signal related to the density of biomaterial present on the sensor surface. For passive optical resonator biosensors, sensitivity and spectral resolution are the two most important parameters that determine sensor performance. The sensitivity, as characterized by the magnitude of transduction signal shift obtained for adsorption of a known surface density of biomolecules, is fundamentally determined by the interaction strength of the electromagnetic field associated with the optical resonator with the adsorbed biomolecules.[9] The sensitivity is often characterized using the bulk refractive index sensitivity $S_b = \Delta \lambda / \Delta n$, the resonant wavelength change $\Delta \lambda$ in response of a refractive index unit (RIU) change $\Delta n$. Resolution, another critical performance criterion, measures the ability to resolve small changes in output response. For resonator-based optical biosensors, resolution is determined by the narrow bandwidth of the resonantly coupled light,

![Figure 1.1](image)

Figure 1.1. (a) Illustration of an optical resonator biosensor that is utilized to detect binding of small molecule to immobilized protein targets on the sensor surface. (b) The adsorbed biomaterials on the sensor surface modulate the resonant coupling condition.
which is typically characterized by the quality factor of the resonance $Q = \lambda/\Gamma$, where $\lambda$ is the resonant wavelength and $\Gamma$ is the full width at half maximum of the resonance.

1.2. Surface Plasmon Resonance Biosensors

Label-free biosensor approaches derived from SPR have been widely used for characterizing biomolecular interactions. SPR is the resonant oscillation of charge density propagating at a metal/dielectric interface, evanescently confined in the perpendicular direction. The simplest geometry sustaining SPR is that of a single, flat interface between a dielectric and metal, as shown in Fig. 1.2a [10]. The dispersion relation of surface plasmon polaritons (SPP) propagating at the interface between the two half spaces is $\beta = k_0 \sqrt{\varepsilon_d \varepsilon_m / (\varepsilon_d + \varepsilon_m)}$ (Fig. 1.2b), where $k_0$ is the free space wave vector, and $\varepsilon_m$ and $\varepsilon_d$ are the permittivity of metal and dielectric material, respectively. The mismatch in wave vector between $\beta$ and the in-plane momentum $k_x = k_0 \sin \theta$ of impinging photons with launch angle $\theta$ can be overcome by patterning the metal surface with a shallow grating of grooves or holes with lattice constant $a$. For the simple one-dimensional grating of grooves depicted in Fig. 1.2c [10], momentum matching takes place whenever the condition $\beta = |k_x + vG|$ is fulfilled, where $|G| = \frac{2\pi}{a}$, and $v = (1, 2, 3, \ldots)$.

The basic principle of SPR sensing is the exploitation of the fact that the spectral position of their resonances depends on the dielectric environment within the electromagnetic near field. When applied to biological sensing, adsorption of molecules on a functionalized metal surface leads to spectral changes of the sustained plasmon modes. Because
electromagnetic fields cannot penetrate into a highly conducting material for distances greater than the skin depth (a skin depth of 13 nm is a typical value for an SPP at a gold-air interface with a free space wavelength of $\lambda = 800$ [11]), the resonantly coupled light resides predominantly outside the metal and in the dielectric surrounding medium, resulting in excellent sensitivity for SPR sensors. Bulk refractive index sensitivity of 7,120 nm/RIU [12] has been demonstrated in the most widely used SPR instruments using the Kretschmann
configuration, and a value of 717 nm/RIU [13] has been achieved in recent work on periodically nanostructured metal films. This high sensitivity has allowed surface plasmon sensors to become established as an analytical sensing technology over the last two decades. However, the high optical loss coefficient of metal leads to low quality factor resonance and a correspondingly wide resonant spectrum, resulting in poor resolution for sensing extremely small changes in adsorbed biomolecular layers.

Besides sensitivity and spectral resolution, selectivity is also a challenging aspect for label-free sensor design. In the case of surface-plasmon-based sensors, this is achieved via functionalization of the metallic surface to ensure only selective binding of the analyte to be sensed. The relative ease of establishing sulfur bonds between gold atoms and organic molecules makes gold a good candidate to ensure selectivity for almost all practical optical sensing applications. In Chapter 4, we discuss the development of gold plasmonic crystal structure and its application as a high sensitivity SPR sensor.

1.3. Dielectric Optical Resonator-Based Biosensors

On the other hand, dielectric-based optical resonators have also been widely used for characterizing biomolecular interactions. Optical resonators can efficiently confine light within a high refractive index dielectric cavity with a high quality factor resonant response, leading to label-free detection with high resolution. Recent demonstrations of passive optical resonator biosensors with high quality factor include whispering gallery mode (WGM) spheres,[9, 14] liquid-core optical fibers,[15] microdisks,[16] and microtoroid resonators.[17] The narrow resonant bandwidth of these approaches enables small resonant wavelength shifts to be
accurately resolved for detection of low concentration analytes, individual nanoparticles, and small molecules. However, with the extremely high quality factor, the coupled resonant light resides primarily within the solid material of the transducer, and the limited interaction between the adsorbed biomaterial and the evanescent electromagnetic field results in reduced sensitivity when compared to plasmonic devices. For example, the bulk refractive index sensitivity of microsphere resonator is $S_b = 30\text{nm/RIU}$,[18] the liquid-core optical ring resonator is reported to have $S_b = 34 \text{ nm/RIU}$,[15] and silicon microring resonator reports $S_b = 163 \text{ nm/RIU}$.[19] In addition to the reduction in sensitivity with increased resonant quality factor, high $Q$ resonators require stringent resonant coupling conditions and tunable laser illumination sources that reduce their robustness and increase their cost relative to low-$Q$ passive optical biosensors.

1.4. Laser Biosensors with Integrated Gain

To meet the challenging requirements for high-precision label-free detection for the most demanding applications, design aspects of an optical resonator biosensor technology include both evanescent field characteristics that maximize sensitivity, and development of sensor structures that are capable of high-$Q$ resonance for achieving high resolution. However, for passive optical resonators requiring external illumination, there is a tradeoff between achieving high sensitivity and generating high quality factor resonance.[20] High sensitivity requires large extended electromagnetic fields from the optical resonator cavity to the surrounding medium, while to obtain a high quality factor resonance, the cavity should be capable of confining the electromagnetic field tightly inside the cavity.
To address the shortcomings of high-\(Q\) ‘passive’ optical resonator biosensors, recent demonstrations of ‘active’ optical resonator biosensors provide a new route to simultaneously achieving high sensitivity and high resolution to obtain detection figure of merit (FOM) metrics that far exceed that of passive optical resonator biosensors, where the FOM is defined as FOM = \(S_b/\Gamma\), in order to simultaneously capture the effects of the magnitude of the refractive index sensitivity and the ability to measure small wavelength shifts. Laser-based optical biosensors utilize a high-sensitivity passive optical resonator with moderate quality factor, and generate their own high intensity and narrow wavelength output though the stimulated emission process. In this way, the two critical performance metrics of sensitivity and resolution are addressed simultaneously. The FOM values of various passive and active optical biosensor technologies are summarized in Table 1.1. In addition to ultrahigh FOM metrics, laser biosensors do not require a tunable laser illumination source, and eliminate the requirement of high precision alignment for evanescent light in/out coupling.[16, 21] In this section, we will discuss recent advances that represent research activity in the field of laser biosensors: the distributed feedback (DFB) laser biosensor, the photonic crystal (PC) nanolaser, the whispering gallery microlaser.[20-26] These systems have been utilized for biosensing or have the potential for biosensing applications. We will describe the laser structures in terms of their optical cavity, gain material, and pump method. The respective sensing mechanisms will also be discussed, and the sensing performance will be evaluated in terms of their sensitivity, resolution, sensor structure fabrication cost, detection instrument configuration, and potential for robust implementation of high throughput assay formats used in biology research.
The DFB laser biosensor, demonstrated by Lu et al. in 2008[21, 27] and subsequently further developed by A.-M. Haughey et al., [28] represents the first report of a label-free biosensing laser. The DFB cavity is based upon a second order Bragg grating that supports a vertically emitting mode by first-order diffraction.[10] A schematic cross-sectional diagram of the DFB laser structure is shown in Figure. 1.3(a). A one-dimensional surface grating formed in ultraviolet (UV)-curable polymer on a flexible plastic substrate is coated with a thin polymer film incorporating organic laser dye. The low refractive index UV-cured polymer layer functions as a cladding layer, upon which a thin film of high refractive index polymer provides vertical light confinement and feedback along the horizontal direction. Doped with laser dye,
the high refractive index layer also contributes to light amplification of the cavity oscillation mode.

To perform a measurement, the DFB structure is optically excited by a short (~10 nsec), high intensity pulse from a frequency doubled, Q-switched Nd:YAG (yttrium aluminum garnet) laser at a wavelength that excites the laser dye (\(\lambda = 532\) nm), and emission from the DFB device is coupled to a spectrometer. Single mode lasing in the \(\lambda = 585-620\) nm wavelength range with a linewidth as narrow as \(\Delta \lambda = 0.09\) nm was demonstrated, as shown in Figure 1.3(b). Altering the refractive index of the media exposed to the DFB laser surface or surface adsorption of biomolecules changes the effective refractive index associated with the resonant mode, and results in modulation of the stimulated emission wavelength. A bulk refractive index sensitivity of \(S_b = 99.58\) nm/RIU was measured, with linear sensitivity for sensing of surface-adsorbed biomolecular layers over a ~14 nm tuning range.
The DFB laser biosensor actively generates its own high intensity narrow bandwidth output and is capable of simultaneously providing high sensitivity and a large dynamic range. The DFB laser biosensor structure is inexpensively fabricated over large surface areas through nanoreplica molding of the grating structure and horizontal dipping to deposit the gain layers.[29] Because the replica molding process is inexpensive and scalable to large areas, the DFB laser biosensor can be incorporated into standard microplate and microfluidic channel formats as demanded in typical life science applications. However, the DFB laser biosensor suffers from several disadvantages. Optical pumping of the structure requires a relatively large and expensive pulsed laser, which is not compatible with portable sensing applications. In addition, laser dye, when used as the gain material, becomes bleached or photodamaged with repeated pump cycles, resulting in gradually decreasing lasing intensity as the device is measured multiple times, although thousands of pulses have been reported for gathering kinetic data that extends for several minutes of a biosensing experiment.[20]

1.4.2. Photonics Crystal Nanolasers

A photonic crystal nanolaser was recently reported for high-resolution refractive index sensing.[26] In this laser system, a nanocavity, formed by a shift in two airholes in a triangular lattice PC slab, confines light into an ultrasmall volume of the order of an optical wavelength. The small modal volume and a high $Q$ factor give rise to high performance lasing: room-temperature and continuous wave (CW) operation was achieved.

The device was fabricated by e-beam lithography upon an epitaxially grown heterostructure with a GaInAsP single-quantum-well. To perform sensing, the nanolaser is
optically pumped by a CW laser with a wavelength of $\lambda = 980$ nm, focused to a 2.5 µm diameter spot on the top surface of the structure. The lasing output from the device was coupled to a multi-mode fiber, and its emission spectrum was analyzed by using an optical spectrum analyzer. The laser peak exhibited a 50dB intensity over the background level and a spectral linewidth of < 26 pm, as shown in Fig 1.4. As a chemically stable refractive index liquid was deposited on the device, a spectral redshift with the refractive index was observed. A maximum refractive index sensitivity of 350 nm/RIU was achieved for the dipole mode. An array configuration using multiple wavelengths was also demonstrated, allowing spectrometer-free sensing. At the time of this writing, a biosensor demonstration of the nanolaser has not been reported, although the device shows strong potential for achieving detection of a small number of adsorbed biological analytes within the active sensing volume. Selective biodetection would require printing of a capture molecule upon the lasing region. In principle, this approach could be multiplexed to enable multiple independent nanolasers to be pumped simultaneously and monitored independently.
1.4.3. Whispering Gallery Microlasers

Optical WGM resonators have emerged as a promising platform for label-free detection of nano-objects.[9, 30] The combination of high \( Q \)-factor and small mode volume enables strong light-matter interaction, and leads to high detection sensitivity, while the narrow resonant linewidth of the WGMs enables high spectral resolution. To extend the detection limit, active resonators with optical gain have been demonstrated for silica microtoroids. Loss compensation mechanisms through integrating rare-earth ions or using intrinsic Raman gain have been explored.[22, 25] The active resonator improves the spectral resolution that is set by the laser linewidth, which can be made much narrower than the resonance linewidth of the cold WGM cavity.

Figure 1.5. Heterodyne detection of single nano-objects using frequency splitting in a whispering gallery microlaser. (a) Schematic of the experimental setup for the WGM microlaser. (b) Schematics showing how the lasing spectrum (middle column) and the beat note signal (right column) change as nanoparticles (blue spheres) bind to the microlaser (left column). Reprinted with permission from [9], © 2011 Macmillan Publishers Limited.
Real-time and label-free biosensing based on the WGM microlaser has been reported by He et al. to detect viruses and single nanoparticles.[25] In the experiments, the WGM microlasers had a toroid-shape structure fabricated from erbium-doped silica with 20 - 40 µm diameter. The fiber-taper coupled microlaser was continuously pumped by a light source at a wavelength of $\lambda \sim 1.46$ µm, and laser emission in the 1.55 µm band was monitored by a photodiode connected to an oscilloscope. Any change in polarizability caused by attachment of nanoscale objects will split the degenerate counter-propagating lasing modes of the WGM microlaser. Thus, nanoparticle adsorption events can be revealed in real time by monitoring the heterodyne beat note of the mixed laser modes. Fig. 1.5 depicts the working principle of nanoparticle detection. The WGM microlaser has a low detection limit arising from its narrow laser linewidth and its self-referencing nature, and can detect individual influenza A virions in air, and polystyrene nanoparticles in water.

Recently, a different physical process was proposed to increase the $Q$-factor/mode volume ($Q/V$) ratio of WGM resonators, where Raman gain-induced loss compensation was utilized in silica WGM resonators for improved detection.[22] This dopant-free scheme was demonstrated to retain the biocompatibility of silica for applications involving biological media.[31] Detection of 20 nm diameter NaCl nanoparticles was demonstrated with Raman gain-enhanced detection using mode splitting. This Raman microlaser provides a dopant-free, self-referenced, and self-heterodyned scheme with a detection limit ultimately determined by the thermorefractive noise. However, there are some shortcomings shared by this Raman microlaser and the WGM microlaser with rare-earth dopants. A particle delivery system with a nozzle and a differential mobility analyzer is needed for the sensed analyte to land precisely on
the active region, where it has the opportunity to be detected. Additionally, the step change in beat frequency goes down as the number of binding events increases, resulting in small dynamic range of detecting ~$10^2$ gold nanoparticles or NaCl nanoparticles with radii of 15-25 nm. This system also requires a pulsed pumping laser, which is not easily compatible with portable sensing applications. The bulk refractive index sensitivity of this device has not been reported, thus it is difficult to directly compare with other sensing approaches.

1.5. External Cavity Laser Biosensors

The laser biosensor systems discussed in Section 1.4 have the gain medium integrated within the sensor to actively generate their own high intensity narrow bandwidth output while retaining high sensitivity. To embed the gain material within the transducer of the laser biosensors, except for the WGM microlaser that utilizes intrinsic Raman gain, requires additional fabrication steps. These approaches all require a large, high power, and expensive optical pumping source. Compared to these devices, the utilization of an external laser cavity to physically separate the optical gain from the sensor surface represents a fundamentally different approach. In this way, the gain is externally provided by a semiconductor optical amplifier using electrical injection. Such a gain separation strategy enables the transducer to be inexpensively fabricated using roll-to-roll fabrication techniques in plastic materials or upon a glass surface using a single lithography step, and also allows the sensor to be operated for long time periods without degradation. The sensor itself can comprise the entire bottom surface of standard microplates, fulfilling the throughput requirement demanded in the life science research applications. This arrangement results in a robust high spectral resolution detection
system that utilizes an electrical pump source, operates in CW mode, while using a sensor structure that can be fabricated inexpensively over large surface areas.

To further improve the performance metrics of the ECL biosensor, a plasmonic external cavity laser biosensor was developed. As aforementioned in Section 1.2, compared with dielectric based optical resonator, plasmonic sensor utilizing SPR offers ultra-high sensitivity, but displays lower quality factor resonance. In the plasmonic ECL, we incorporate optical gain to compensate for the high loss in metal. While the ultra-high sensitivity of SPR sensor is maintained, spectrally sharp resonance is achieved through the stimulated emission process in the laser cavity. Moreover, the plasmonic active laser biosensor extends the detection limit of traditional passive plasmonic resonators by improving the quality factor, offering a unique property to perform accurate detection of biologically important molecules and viral particles in low concentration.

The thesis focuses on the development of ECL biosensor based on photonic crystals and plasmonic crystals, and is organized as follows: In Chapter 2, the physical principle and experimental design of dual-mode ECL biosensor is discussed, with an emphasis on the theory of photonic crystal resonant reflector and the experimental results demonstrating the self-referencing technique. In Chapter 3, the development of dual-ECL with microplate format for high throughput screening is discussed. The detection results of binding between small molecules and a variety of immobilized protein targets are shown, as well as a small scale screening test for inhibitors against a certain protein. Possible approaches to improve the throughput of the system is discussed. In Chapter 4, a plasmonic ECL biosensor is discussed,
in which a plasmonic crystal sensor serves as the transmission filter in the laser cavity. Theoretical explanation of extraordinary optical transmission and experimental characterization of the dispersion diagram are presented. The demonstration of lasing behavior and the refractometric sensing results indicates the great potential for high-precision biosensing applications with the system. A summary is provided in Chapter 5.
References


CHAPTER 2. DUAL-MODE EXTERNAL CAVITY LASER BIOSENSOR

2.1. Introduction

A dual-mode external cavity laser (ECL) biosensor for high-precision label-free detection is demonstrated. [1] In the dual-mode ECL system, two photonic crystal (PC) resonant reflectors are utilized as the sensing surfaces as well as the wavelength-selective mirrors for the laser cavity. The ECL-based sensor system simultaneously emits at two distinct wavelengths corresponding to two different longitudinal cavity modes selected by the two PC reflectors. Through the stimulated emission process, high spectral resolution is achieved without sacrificing the PC sensor’s sensitivity. The dual-mode ECL also addresses a fundamental issue of many types of optical biosensors: label-free optical biosensor detects wavelength shifts which are indistinguishable from the change in resonant wavelength generated by the actual binding event. Sources of noise, including environment fluctuations, bulk refractive index changes as well as non-specific bindings can all modify the resonant wavelength without the presence of biomolecule binding. A self-referencing technology based upon the dual-mode ECL is developed to extract signal of interest and reduce the noise level. The increased signal-to-noise ratio in the dual-mode ECL system enables direct observation of small molecule with great accuracy. In this Chapter, we discuss the physical principle of photonic crystal resonant reflector, the design and characterization of the dual-mode ECL, and the biosensing detection results obtained with the dual-mode ECL system.
2.2. Photonic Crystal Resonant Reflector

The wavelength selective element in the ECL is based on a one-dimensional guided-mode resonance (GMR) filter. One-dimensional or two dimensional PC slabs which possess GMR have been the subject of intense study, representing for a new fundamental optical element for narrow-band filtering, optical switch or sensing applications.[2-4]

The most basic GMR filter is composed of a substrate, a thin dielectric layer with high refractive index and a grating layer, as shown in Fig. 2.1. The GMR represents a 100 % reflective narrow-band spectrally selective resonance. Physically, the resonance phenomenon occurs due to coupling of the externally propagating diffracted fields to the guided modes of a dielectric slab waveguide. In detail, when such a structure is illuminated with an incident light beam, part of the beam directly transmits through the slab and part is diffracted by the grating and coupled into the waveguide layer.[5] For a slab waveguide structure shown in Fig. 2.2a, the

Figure 2.1. Layout of the one-dimensional photonic crystal slab which exhibits guided mode resonance. The structure is composed of a low refractive index ultra-violet cured polymer layer containing periodic surface structure and a high refractive index layer of titanium dioxide. The physical parameters of the device are labeled in the figure.
dispersion diagram for the fundamental transverse magnetic mode (TM$_0$) is solved and plotted in Fig. 2.2b. With the surface grating structure (Fig. 2.2c), when the momentum matching condition is met, this part of the coupled light is then rediffracted out and interferes with the directly transmitted light. At a specific wavelength $\lambda$ and angular orientation $\theta$ of the incident beam, completely destructive interference occurs, resulting in a resonance with zero transmission.

The detailed resonance behavior including far-field diffraction efficiency and near-field electromagnetic field distribution can be accurately simulated by numerical method such as finite-difference time-domain (FDTD) method. Employing a commercial implementation of FDTD, the device depicted in Fig. 2.1 is investigated. The device parameters are grating period $\Lambda = 550$ nm, grating depth $h = 150$ nm, duty cycle = 0.5, and high refractive index layer thickness $t = 105$ nm. The refractive indices of the substrate, high refractive index layer and the surrounding media are 1.45, 2.70 and 1.33, respectively, with the assumption that the materials

![Figure 2.2. Physical principle of guided mode resonance. (a) A slab waveguide structure, (b) calculated dispersion curves of the fundamental guided mode TM$_0$ in the slab waveguide, and (c) A thin slab with surface grating structure.](image)
are lossless and exhibit no dispersion. For incident light polarized perpendicular to the grating lines (transverse magnetic polarization), the transmission and reflection efficiency are obtained, as shown in Fig. 2.3. A sharp reflection peak with 100% efficiency at wavelength $\lambda = 850 \text{ nm}$ with linewidth $\Delta \lambda = 2.5 \text{ nm}$ is observed, implying a resonance with quality factor $Q = \lambda / \Delta \lambda = $

![Normalized Power vs Wavelength](image)

Figure 2.3. Calculated reflection and transmission efficiency for transverse-magnetic polarized light with normal incidence.

![Near Field Intensity Profiles](image)

Figure 2.4. Calculated near field intensity profiles of the resonant mode at 850 nm for (a) one grating period. The field distribution of (b) $|H_z|^2$ field component, and (c) $|E_x|^2$ field component. The color scale associated with each figure represents the intensity of field and is normalized to the unit intensity incident wave.
The narrow-band resonance property of GMR reflectors makes them applicable for a lasing wavelength selective element to select its own resonant wavelength as the lasing wavelength.

Besides the far-field manifestation of the GMR, the near field electromagnetic field distribution of the device is also investigated. The field distribution for TM polarized incidence at the resonant wavelength is presented in Fig. 2.4. The magnetic field distribution in Fig. 2.4b demonstrated the coupled TM waveguide mode in the waveguide. The electric field intensity distribution of \( E_x \) component in Fig. 2.4c presents the enhanced electrical field intensity within the resonator and the exponentially decaying evanescent field into the surrounding medium. The enhanced evanescent field allows strong light-matter interaction to occur on the surface of the PC resonator, making the device applicable for a high sensitivity refractive index sensor. The sensitivity is characterized to be 212 nm/RIU. When a PC device is adapted to a biosensor, as shown in Fig. 2.5, adsorbed biomaterials on the sensor surface interact with the evanescent field and modulate the resonant coupling condition. Therefore, by monitoring the resonant

![Figure 2.5](image.png)

Figure 2.5. The photonic crystal resonant reflector is utilized as a label-free biosensor. When adsorption of biomolecule occurs on the sensor surface, resonant wavelength of the photonic crystal shifts.
wavelength of the PC in real-time, detailed information can be extracted on dynamic mass adsorption, the affinity between the target molecule and its ligand and the dissociation constant of the interaction.

2.3. External Cavity Laser

Tunable ECLs have gained considerable attention for widespread applications in areas such as optical communications, atomic laser spectroscopy, as well as environmental monitoring.\[6, 7\] The ECL system is primarily composed of a laser cavity, a broadband semiconductor optical amplifier (SOA) as gain medium, and an external mode selection filter. With insertion of the mode selection filter into the system, an ECL permits emission of a single mode with an extremely narrow linewidth and wide tuning ranges.\[6\]

![Figure 2.6](image)

Figure 2.6. (a) The schematic plot of the ECL biosensor system. (b) The attachment of biomolecules shifts the resonant wavelength of the PC, and (c) subsequently tunes the lasing wavelength of the ECL.
The narrow linewidth, when the ECL is adapted to an optical label-free biosensor, can boost the spectral resolution for accurate detection, as has been demonstrated in recent developed ECL biosensor technology.[8] In the ECL biosensor system, as illustrated in Fig. 2.6, an SOA provides gain, and a PC sensor serves as a mirror of the laser cavity as well as the sensing surface. The attachment of biomolecules tunes the resonant wavelength of the PC, and subsequently modulates the lasing wavelength of ECL. This arrangement maintains the high sensitivity of the PC, while enables high spectral resolution through the stimulated emission of the laser cavity.

2.4. Design and Characterization of the Dual-Mode External Cavity Laser Biosensor

A fundamental aspect of the ECL laser biosensor, as well as many types of optical biosensors, is the detection of noise signals which are indistinguishable from the signal generated by an actual binding event.[9] A change in the bulk refractive index of the test sample, thermal expansion and temperature fluctuations can all modify dielectric permittivity on the surface and induce signal shifts, serving as noise sources.[9-11] In order to extract the signal of interest and to improve the accuracy of ECL biosensors, we developed a self-referencing label-free biosensor based upon a dual-mode ECL. Dual mode operation of ECL has been studied for its applications in optical communications, atomic laser spectroscopy, and environmental monitoring.[12-16]

In the dual mode ECL sensor system, the two PCs are bonded to the opposite sides of a thin chamber frame, forming a flow cell for test sample. One of the PCs is modified with specific surface chemistry to perform the sensing function, while the referencing PC is untreated.
to serve as a reference sensor. Both PCs serve as wavelength selective mirrors for the external cavity laser cavity, and select two distinct lasing wavelengths corresponding to their own peak reflection wavelengths. The stream of test sample though the flow cell is introduced to both sensor surfaces simultaneously, thus any refractive index change of test sample can induce equivalent shifts on both lasing modes. By performing lasing wavelength shifts subtraction, the system can accurately separate the bio-chemical binding signal from common mode noise signals.

Figure 2.7. (a) SEM image of the PC structure (b) Photograph of the flow cell where sensing and referencing photonic crystal sensors are incorporated as the top and bottom surfaces (c) Schematic drawing of the dual-mode ECL biosensor system. Reprinted with permission from [1], © 2013 American Institute of Physics.
The optical feedback in the dual-mode ECL cavity is provided by the narrowband PC resonant reflectors, as shown in Figure 2.7a. The PC slabs were fabricated using the nanoreplica modeling process (Fig. 2.8) described in our previous publications.[17] In brief, a subwavelength grating structure was replicated from a silicon master wafer to a low refractive index ultra-violet curable polymer with a period of \( A = 550 \text{ nm} \) and depth of \( t = 170 \text{ nm} \). To form a resonant reflectance near 856 nm, a 100 nm thin film of TiO\(_2\) \((n = 2.4)\) was subsequently coated on top of the replicated grating on one PC surface. The referencing PC slab was coated with 104nm TiO\(_2\) thin layer to shift the resonant wavelength to 859 nm when immersed in deionized water (DI). The resonant peak wavelength values (PWV) can be modified by small changes of surface dielectric permittivity induced by attachment of biomolecules on the evanescent field region on the surface. We incorporate these two PC surfaces face to face as top and bottom surfaces of a 3 mm thick flow chamber, as shown in Fig. 2.7b. The flow chamber

![Fabrication process of photonic crystal with nanoreplica molding method.](image)

Figure 2.8 Fabrication process of photonic crystal with nanoreplica molding method.
was fabricated by cutting Poly(methyl methacrylate) (PMMA) into right dimensions. Two cylindrical openings at the ends of the flow cell were tapped and screwed in with 1/16” hose barb adapter connected to tubing as inlet and outlet.

The active medium of the dual-mode ECL biosensor system is a commercially available optical fiber-coupled semiconductor optical amplifier SOA (SAL-372-850, Superlum Inc.) with a center wavelength of $\lambda = 850$ nm and a 3-dB bandwidth of $\Delta \lambda = 40$ nm and with antireflection coating on both surfaces. The output from one end of the SOA is reflected back by a near infrared reflection mirror, while the output light from another end of the polarization-maintaining (PM) fiber was collimated to the flow cell composed of double PCs at normal TM mode incidence. The sensing and reference PCs reflect their own very narrow resonant band of wavelengths, respectively, focused back into the SOA by the same collimator. An active optical resonator with two resonant modes was established with high quality factor though the stimulated emission process. The schematic drawing of the dual-mode ECL setup is shown in Fig. 2.7c. A small portion of the emitting laser light is delivered to a spectrometer with 0.02 nm resolution (HR4000, Ocean Optics). The lasing spectrum, gathered kinetically by the spectrometer at a sampling rate of 2 Hz, was fitted with Lorentzian functions to accurately locate the lasing wavelength value (LWV).

2.5. Self-Referencing Technique

Since the two lasing modes share all optical components of the cavity in our configuration, thermal drift of the gain profile of the SOA will induce wavelength shifts on both the lasing modes thus can be effectively compensated in dual-mode ECL biosensor system. In
addition, the stream of test sample through the flow cell is introduced to both sensor surfaces simultaneously. The detection target will be selectively captured by the immobilized capture agent on the active sensor surface, generating a specific-binding signal. Inevitable thermal drift noise, non-specific binding of the analyte to the sensor surface, along with the slight variations of the bulk refractive index due to the mixing of the analyte solution, will generate equivalent noise signals to the sensing and reference devices. By performing lasing wavelength shifts subtraction, the system can accurately eliminated common-mode error sources, and extract actual biochemical-binding signal on the active sensor.

Dual-mode lasing has been demonstrated in this system. The reflection spectrum of PC filters and the emission spectrum of dual-mode ECL are shown together in Fig. 2.9. Both measurements were taken with sensing and reference PC surfaces immersed in DI water, and

![Graph showing PC resonant reflection spectrum and dual-mode ECL laser emission spectrum](image)

Figure 2.9. PC resonant reflection spectrum and dual-mode ECL laser emission spectrum when both PC sensor surfaces were immersed in DI water, showing a wavelength separation of 3.07 nm. Reprinted with permission from [1], © 2013 American Institute of Physics.
the mode separation is 3.07 nm. In order to characterize the stability of dual-mode lasing operation and the feasibility of self-referencing technique, three experiments were performed. They were environmental fluctuation test, bulk refractive index test, and a protein A/ pig immunoglobulin G (IgG) binding experiment.

2.5.1. Environmental Fluctuation Test

Temperature fluctuations have two main effects on the sensor system: (1) shift the gain profile of the SOA, and (2) modulate the dimensions and the refractive index of the PC materials. The gain profile of the SOA is extremely sensitive to temperature changes. We observed LWV shifts with a fluctuation range of 15 pm even with a commercial SOA temperature control unit with temperature stability of 0.1°C. This large thermal noise makes it necessary to have a reference sensor that shares the same SOA to experience the same wavelength shifts caused by SOA temperature fluctuations. On the other hand, the PC materials used in this work and the test samples are also sensitive to ambient temperature fluctuations due to thermal expansion/contraction (characterized by coefficient of thermal expansion) and thermal-opto effects (characterized by thermal-optic coefficient). We have used rigorous couple-wave analysis computer simulations to study the effect of a small sensor temperature change on the peak wavelength of the PC. For example, a temperature change of 0.05°C results in a LWV shift of 0.1 pm due to the thermal-opto effect of the TiO2 coating, and could result in a LWV shift of 9.0 pm due to the thermal expansion of UVCP. It’s critical to compensate the thermal noise experienced by the PC via an identical reference sensor that is in close proximity to the active sensor and shares the same thermal environment.
In the first test to demonstrate the elimination of environmental fluctuations, laser emission wavelengths for both modes were simultaneously monitored over time, with the measurement results represented in Fig. 2.10(a) and (b). Both of the signals gradually drifted downward, showing the impact of environment. For a 3-minute measurement, the standard deviations for these two signals are 1.4 pm and 1.3 pm, respectively. By subtracting these two signals, a self-referenced signal was obtained exhibiting reduced amount of drift, as shown in Fig. 2.10(c). Furthermore, the standard deviation of the baseline signal fluctuations was reduced to 0.8 pm, demonstrating the reduction of environmental drift.

Figure 2.10. Dual-mode emission wavelengths as a function of time demonstrating the stability of the dual-mode ECL system and the feasibility to eliminate environmental fluctuations. Temporal variation of lasing wavelength values from (a) top PC and (b) bottom PC mode with DI water. (c) Relative laser wavelength shift shows a short-term stability of 0.8 pm for this 3-minute measurement. Reprinted with permission from [1], © 2013 American Institute of Physics.
2.5.2. Bulk Refractive Index Test

In order to verify the common-mode shifts from signal and reference PC filters, we induced bulk refractive-index-induced LWV shifts by the introduction of the solvent dimethyl sulfoxide (DMSO) to DI water with 1% concentration through the inlet into the flow cell. To measure the effect of DMSO concentration on the dual-mode ECL biosensor, lasing wavelength value was initially measured with DI water in the flow cell, establishing two baseline signals for top and bottom sensors. At $t = 3.4$ min, DI water was replaced by 1% DMSO and time

![Figure 2.11. Response plots of laser emission wavelengths during a bulk fluid refractive index experiment demonstrating the capability to correct errors induced by bulk refractive index variations of the analytes. Temporal variation of lasing wavelength values of (a) top PC mode and (b) bottom PC mode with DI water as test sample (before 3.4 mins) and with 1% DMSO solution (3.4 min to 5 min). The introduction of DMSO solution induced 49 pm shifts simultaneously in both the lasing modes. (c) Subtraction of the bottom PC lasing wavelength shift from the top one results in effective elimination of bulk refractive index induced shift and shows a short-term stability of 2.1 pm. Reprinted with permission from [1], © 2013 American Institute of Physics.](image-url)
sequence lasing wavelength data were measured after signal stabilization. We observed a 49 pm upward shifts on both lasing modes, indicating the higher refractive index of the DMSO solution. However, the self-referenced signal did not have clear shift, as shown in Fig. 2.11(c), and had a standard deviation of 2.1 pm. This self-referencing system successfully corrected the common-mode shift caused by the change of bulk refractive index of test sample.

2.5.3. Protein A/ Pig Immunoglobulin G Binding Experiment

To demonstrate the self-referencing technique for detection of biomolecules, protein A and pig IgG bioassay was performed. In the sensor preparation phase, Protein A (Pierce

![Figure 2.12. Kinetic response plots of laser emission wavelengths of Protein A and pig IgG binding experiment. Temporal variation of lasing wavelength values of (a) sensing PC mode and (b) reference PC mode with PBS buffer (before 7.5 min), followed by introduction of Pig IgG solution (7.5 min to 22.5 min) and a wash step. (c) The self-referenced signal was generated by subtracting the binding signal from the non-binding reference signal, indicating only the effect of protein A and pig IgG binding. Reprinted with permission from [1], © 2013 American Institute of Physics.](image)
Biotechnology) with a concentration of 0.5 mg/ml in phosphate-buffered saline (PBS) buffer (Sigma-Aldrich) was introduced to the surface of the detection sensor, and allowed to incubate for 15 min. After rinsing the sensor surface three times with PBS buffer to remove loosely bounded Protein A, a PWV shift of 0.533 nm for the sensing PC was obtained, resulting in a peak reflection wavelength at 855.34 nm. The reference sensor with PWV of 859.45 nm was not functionalized with any biomolecular recognition layer, creating a reference to the Protein A/ IgG binding signal. In the binding experiment, PBS solution was first injected into the flow cell to establish the baseline signals. Pig IgG (Sigma- Aldrich) diluted with 0.01 M PBS to a concentration of 0.5 mg/ml was later introduced into the flow cell and allowed to incubate for 30 min, followed by a wash step to remove any unbounded IgG. Kinetic lasing wavelength value responses of two differential binding signals were obtained for the detection and reference sensors, as shown in Fig. 2.12(a) and (b). The self-referenced signal was obtained by subtracting the non-binding signal from the binding signal, showing a LWV shift of 2.15 nm. Because pig IgG would specifically bind to protein A only, this self-referenced binding signal indicates only the effect of the pig IgG binding, referencing out any environmental fluctuations, bulk refractive index changes, as well as non-specific binding signals.

2.6. Conclusion

In summary, a self-referencing ECL biosensor with dual-mode operation has been demonstrated and characterized. With two PCs assembled in a flow cell format serving as lasing wavelengths selection mirrors and with the semiconductor amplifier providing optical gain, this system achieves high Q-factor resonance and high-sensitivity label-free detection
simultaneously. The ECL system simultaneously emits two distinct wavelengths selected by the sensing and reference PCs. Due to the close proximity and simultaneous exposure to test analytes of both sensors, the reference signal and binding signal share nearly all the common-mode error sources and their subtraction effectively eliminates these noises. We have demonstrated stable dual-mode operation and have explored the feasibility to correct common mode shifts caused by environmental fluctuations, variation in bulk refractive index of test sample by introducing DMSO solution. Protein A and IgG bioassay was performed to further demonstrate the self-referencing technique.
References


CHAPTER 3. DETECTION OF PROTEIN-SMALL MOLECULE BINDING USING A SELF-REFERENCING EXTERNAL CAVITY LASER BIOSENSOR

High throughput screening (HTS) has enabled the identification of small molecule modulators of important drug targets via well-established colorimetric or fluorimetric activity assays. However, existing methods to identify small molecule binders of non-enzymatic protein targets either lack the simplicity (e.g. require labelling one of the binding partners with a reporter) or throughput inherent in enzymatic assays widely used for HTS. Thus, there is intense interest in the development of high throughput technologies for label-free detection of protein-small molecule interactions. In Chapter 3, we describe a novel self-referencing external cavity laser (ECL) biosensor approach that achieves high resolution and high sensitivity, while eliminating thermal noise with sub-picometer wavelength accuracy. Using the self-referencing ECL biosensor, we demonstrate detection of binding between small molecules and a variety of immobilized protein targets, pairs that have binding affinities or inhibition constants ranging from sub-nanomolar to low micromolar. Finally, a “needle-in-the-haystack” screen for inhibitors against carbonic anhydrase isozyme II (CA II) is performed, in which known inhibitors are clearly differentiated from inactive molecules within a compound library.[1]

3.1. Introduction

HTS is a valuable tool for the identification of small molecule modulators of various macromolecular targets and is a critical early component of the pharmaceutical discovery process.[2] Despite the utility of HTS in drug discovery, enzymatic protein targets with colorimetric or fluorimetric activity assay readouts are typically more amenable to this approach.
than non-enzymatic protein targets. Thus, there is an intense need for general binding assays that can be utilized to identify small molecule binders for proteins, not merely enzymatic inhibitors.[3] Current assays utilized for direct detection of protein-small molecule binding include isothermal titration calorimetry (ITC),[4] surface plasmon resonance (SPR),[5, 6] and small-molecule microarrays (SMMs).[7] ITC and SPR are capable of quantitative detection of protein-small molecule interaction in low or medium throughput applications, while SMMs can identify small molecule probes that bind with specific protein targets using a fluorescence-based readout. As an alternative binding assay for high-throughput identification of small molecule binders, an external cavity laser (ECL) biosensor was recently reported.[8, 9] Utilizing a narrowband photonic crystal (PC) resonant reflector[10] as optical feedback in a laser cavity, ECL achieves high spectral resolution through the stimulated emission. The ECL detects the adsorption of biomolecules to the PC biosensors with improved resolution when compared to simply measuring the passive resonant reflection spectrum,[11] and allows the measurement of small wavelength shifts.

Challenges confronting the detection with the ECL biosensor, as well as many types of optical biosensors, are the detection of noise signals that are indistinguishable from the signals generated by an actual binding event. For example, thermal fluctuations of the test sample, thermal expansion/contraction of sensor materials, and non-specific binding can all lead to changes in the measured resonant wavelength that are of similar magnitude to wavelength changes generated by small molecule-protein interactions. Realizing that incorporation of accurate referencing is key to enable direct detection of small molecule binding to immobilized protein targets, herein we describe a self-referencing ECL biosensor detection instrument which
is capable of lasing at two independent wavelengths. The introduction of two-wavelength lasing enables real-time self-referencing by designating one wavelength as a reference for the “active” biosensor. The active and reference resonators share all optical components and thermal environment, and thus share common-mode sources of wavelength shift noise, including thermal drift. Utilizing the reference external cavity mode, the self-referencing ECL eliminates common-mode noise sources to achieve accurate referencing, while simultaneously maintaining high sensitivity and high spectral resolution. As demonstrated herein, using the newly-developed instrument, it is possible to detect small molecule binding to immobilized protein targets with micromolar affinity and to perform HTS.

3.2. Self-Referencing External Cavity Laser with Microplate Format

In order to achieve accurate referencing, two PC sensors in adjacent wells of a 384-well plate were utilized as wavelength selective elements in the ECL cavity at the same time. Each sensor selects its own resonant wavelength, so the ECL system can lase at two independent wavelengths simultaneously. The sensor surface is a one-dimensional PC. The fabrication process of PC biosensors using nanoreplica molding has been described in Chapter 2[12] and is briefly introduced below. An 8-inch silicon wafer with grating structure etched permanently into its surface is used as the master wafer, and the structure is replicated onto a thin layer of UV curable epoxy on a polyester sheet, followed by depositing a thin layer of TiO$_2$ high refractive index material. The flexible substrate with the imprinted PC structure is attached with adhesive onto the bottom surface of standard 96-, or 384-well microtiter plates. In this work, we design the PC with period $\Lambda = 550$ nm, grating depth $h = 170$ nm, and with TiO$_2$ thickness
The PC structure, when covered with water, shows a resonant peak at near infrared (NIR) region with peak reflection wavelength $\lambda = 856$ nm and full-width at half maximum of $\Delta \lambda = 3$ nm.

Figure 3.1. (a) Schematic diagram of the self-referencing ECL biosensor system. Insert: Images of PC biosensor films adhered to the bottom of standard microplates. (b) Biosensor assays used for detection of direct small molecule binding to immobilized protein targets with referencing. Active and reference sensors functionalized with glutaraldehyde are used. The immobilization of protein and the addition of cognate small molecule in the active well both result in shifts in lasing wavelength value of the active sensor. Reprinted with permission from [1], © 2014 American Chemical Society.
The ECL cavity is formed by the PC resonant reflector and a NIR broadband mirror, where the PC serves as the wavelength selective element, as shown in a schematic diagram in Fig. 3.1.[8] Optical gain is provided by a fiber coupled semiconductor optical amplifier SOA (SAL-372-850, Superlum Inc., $\lambda_0 = 850$ nm and a 3 dB bandwidth of $\Delta\lambda = 40$ nm). Light comes from one end of the fiber is reflected against the NIR mirror, while light from the other end of the fiber, after a collimating lens, is directed to a polarizing cube beamsplitter. The incident polarization is adjusted so that the outcoming s- and p-polarized light from the cube have equal intensity. A half-wave plate is then used to switch the s-polarized light to p-polarized light. Both beams, with equal intensity, illuminate two adjacent sensor wells at normal incidence through the bottom of the microplate, respectively, with polarization direction perpendicular to the grating direction. The illuminated regions have an approximately $400\mu m$-diameter. The narrow linewidth reflection of the two PCs is coupled back to the laser cavity and gets amplified by the SOA. An active optical resonator with two resonant modes is established and achieves dual-mode operation with narrow lasing linewidth through stimulated emission process. A small portion of light is directed out from the cavity to a detection system to monitor the lasing wavelength values in real time. Fig. 3.2 shows the dual-mode lasing spectrum of the ECL system, with one well immersed in DI water, and the other well immersed in 10% DMSO solution.

Self-referencing was accomplished by designating one well as the “reference” well and the other as the “active” well, where both sensors were fabricated identically on the same substrate and were prepared identically with exception of the immobilized protein in the active well. Using rigorous coupled-wave analysis computer simulations of the PC sensor
with/without the presence of a 10-nm thick protein layer, we found that the sensitivity for the active and reference sensors are identical. No temperature or environmental control is applied to the detection instrument, sensor, or test samples. Due to the close physical proximity of the active and reference sensors, accurate referencing is achieved to compensate for thermal variations and non-specific binding. Importantly, the active and reference laser cavities share the entire optical system, including the gain medium, optical fibers, and mechanical holding stages, thus any common-mode error that may cause the lasing wavelength to drift will occur to both devices in an identical fashion. Moreover, the side-by-side configuration of the active and reference sensors enables the use of a pulse-driven bi-stable shutter for alternate operation of the two lasing modes with a frequency of 0.5 Hz. This avoids competition between two simultaneously oscillating modes and enables stable operation of the ECL system. Kinetic monitoring of the lasing wavelength value (LWV) is achieved by directing a portion of the

Figure 3.2. Dual-mode lasing spectrum of the self-referencing external cavity laser. Reprinted with permission from [1], © 2014 American Chemical Society.
lasing emission energy with a beam splitter to a detection instrument such as a spectrometer or interferometer-based laser wavelength meter.

To validate the self-referencing technique, tests to demonstrate accurate compensation of environmental fluctuations were performed. By kinetically monitoring the LWVs of the alternating lasing modes, a self-referenced LWV shift was obtained by subtracting the LWV of the reference sensor from the active sensor, resulting in an effectively reduced noise level with a short term standard deviation of \( \sigma = 0.8 \) pm over a 20-min time period (Fig. 3.3). Moreover, the LWV variations of the active sensor have a fluctuation range of 15 pm (Fig. 3.3),

![Figure 3.3](image-url)

Figure 3.3. Compensation of environmental fluctuations via the subtraction of LWV of the reference well (green) from the LWV of the active well (blue). The resulting LWV shift (red) has a reduced noise level with standard deviation of 0.8 pm in a 20 minute measurement. Reprinted with permission from [1], © 2014 American Chemical Society.
demonstrating the importance of the reference sensor. As a proof-of-concept, quantitative detection of biotin binding to an immobilized layer of streptavidin (SA) was performed. Upon establishment of a stable baseline, biotin was added to both the active and reference wells, and their kinetic LWVs were obtained simultaneously (Fig. 3.4). By subtracting the signal of the reference sensor from the active sensor, a referenced LWV shift of 13 pm due to the binding of biotin to SA was observed (Fig. 3.4). The LWV shift as a result of a binding interaction was considerably smaller than the noise introduced by thermal shift and would otherwise be undetectable without the incorporation of the referencing control.

Figure 3.4. Binding of biotin (75 µg/mL) to immobilized SA. LWV shift due to the binding of biotin to SA (red) was obtained by subtracting the LWV of the reference well (green) from that of the active well with immobilized SA (blue). Dotted line indicates the addition of biotin to both the active and reference wells. Reprinted with permission from [1], © 2014 American Chemical Society.
3.3. Detection of Protein and Small Molecule Binding: Materials and Methods

This section discusses the materials and methods utilized in the protein-small molecule binding detection and the small-scale screening test.

3.3.1. Surface chemistry

PC biosensor surface was functionalized first with a polyvinylamine layer (PVA; provided by SRU Biosystems Inc.) using a 10% PVA solution in 1X PBS and incubated at 37°C for 4 h. The wells were then washed thrice with 1X PBS before an additional functionalization with glutaraldehyde (GA). The wells were incubated with a 25% GA solution in 1X PBS for 4 h at 37°C. After 4 h, the wells were washed thrice with 1X PBS.

To immobilize the protein of interest onto the biosensor surface, 40 µL of 1 mg/mL of protein solution was added to each of the active well and incubated overnight at 4°C. Streptavidin (SA, ProZyme), bovine carbonic anhydrase isozyme II (CA II) and human serum albumin (HSA) were diluted in 1X PBS pH 7.4. Lyophilized recombinant human NAD(P)H dehydrogenase quinone 1 (NQO1) was dissolved with 100 mM potassium phosphate buffer at pH 7.4. Purified human lactate dehydrogenase isoform 5 (hLDH5, hLDH-A, mybiosource.com) was diluted in 100 mM sodium phosphate buffer at pH 7.4. GST-XIAP was diluted in XIAP assay buffer. Caspase-3 was diluted in caspase activity buffer (50 mM HEPES, 300 mM NaCl, pH 7.4). After an overnight incubation, the wells were washed once to remove excess unbound protein and kept in their respective buffers at 4°C till they were used. A blocking step was not incorporated in the small molecule detection assay. Since the nonspecific binding observed was
very low even without a blocking step, this step was eliminated for the sake of simplifying the assay protocol.

3.3.2. Protein-small molecule binding assays

The cognate small molecules of interest: dorzolamide, warfarin, SM-122, SM-164 (gift from Prof. Shaomeng Wang, U. Michigan), NHI-1, 1b and 1g (gift from Prof. Filippo Minutolo, U. Pisa) and Q-VD-O-Ph (EMD Millipore) were dissolved in DMSO. Dicoumarol (gift from Prof. David Boothman, UTSW) was dissolved in basic water. The small molecules were added at a final concentration of 50 \( \mu \text{M} \) in 5\% DMSO (1\% DMSO for warfarin) into both the active and reference wells for all of the binding assays. The sensor plate was read for 5-20 min at RT.

3.3.3. Dose response binding assays

Dorzolamide dissolved in 5\% DMSO in 1X PBS was added to both the active and reference wells at five final concentrations of ranging from 200 \( \mu \text{M} \) to 2 nM. The sensor plate with CAII immobilized on the active wells was read for 20 min at RT. Warfarin dissolved in 1\% DMSO in 1X PBS was added to both the control and sensing wells at five final concentrations of ranging from 200 \( \mu \text{M} \) to 2 nM. The sensor plate with HSA immobilized on the active wells was read for 5-10 min at RT.

3.3.4. High-throughput screen

A collection of 48 compounds from an in-house library in 10 mM DMSO was used for the screen, with dorzolamide a known inhibitor of CAII spiked in. The compounds were added to both the active and reference wells and screened at a final concentration of 50 \( \mu \text{M} \) in 5\% DMSO.
DMSO in 1X PBS. Acetazolamide at a final concentration of 50 µM in 5% DMSO in 1X PBS was used as the positive control. 5% DMSO in 1X PBS was used as the negative control. The sensor plate with CAII immobilized on the active wells was read for 20 min at RT to obtain the binding data.

3.3.5. Specific binding assay for HSA and warfarin

A 1% DMSO solution in 1X PBS containing 5 structurally unrelated small molecules in addition to warfarin was added to both sensing well with immobilized HSA and control well at a final concentration of 25 µM. To eliminate the possibility of non-specific binding, the 5 other small molecules were added separately to both the sensing and control wells. The sensor plate was read for 5-10 min at RT.

3.4. Detection of Protein and Small Molecule Binding: Results

The ECL biosensor instrument was used to study five well-characterized protein-small molecule binding interactions: CA II—dorzolamide ($K_D = 1.1$ nM),[13] NQO1—dicoumarol ($K_I = 1 – 10$ nM),[14] XIAP—SM-164 ($K_I = 0.56$ nM),[15] caspase-3—Q-VD-OPh ($IC_{50} < 25$ nM)[16] and hLDH-A—N-hydroxyindole-1 (NHI-1) ($K_I = 10.8$ µM).[17] These protein-small molecule interactions have binding affinities or inhibition constants ranging from sub-nanomolar to low-micromolar, which are typical of most protein-small molecule interactions. The interaction between hLDH-A and NHI-1 (Fig. 3.5a) can be readily detected (Fig. 3.5b) despite the pair having the weakest inhibition constant amongst the tested pairs. As shown in Fig. 3.5, the system can also detect the binding interaction of the other four protein-small
molecule pairs: XIAP and SM-164 (Fig. 3.5c), CA II and dorzolamide (Fig. 3.5d), NQO1 and dicoumarol (Fig. 3.5e), caspase-3 and Q-VD-OPh (Fig. 3.5f). In addition, we were also able to detect the binding of SM-122 to immobilized XIAP ($K_i = 180$ nM)[15] (Fig. 3.6a), and ES936

Figure 3.5. (a) Structure of NHI-1 and its inactive variants 1b and 1g. Observed LWV shift from the binding of 50 $\mu$M (b) NHI-1, 1b or 1g to immobilized hLDH-A, (c) SM-164 to immobilized GST-XIAP, (d) dorzolamide to immobilized CA II, (e) dicoumarol to immobilized NQO1 and, (f) Q-VD-OPh to immobilized caspase-3. The vertical dotted line indicates the addition of small molecules to both the active and reference wells. Reprinted with permission from [1], © 2014 American Chemical Society.
Table 3.1. LWV shift values of protein-small molecule binding interaction detected on the ECL biosensor$^a$

<table>
<thead>
<tr>
<th></th>
<th>CA II$^b$</th>
<th>NQO1$^c$</th>
<th>XIAP$^d$</th>
<th>hLDH-A$^e$</th>
<th>Casp-3$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorzolamide</td>
<td>12.5 ± (1.1)</td>
<td>-2.4</td>
<td>1.5</td>
<td>2.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>1.2</td>
<td>11.2 ± (1.0)</td>
<td>-1.1</td>
<td>-1.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>SM-164</td>
<td>-0.9</td>
<td>0.7</td>
<td>11.5 ± (0.5)</td>
<td>-0.2</td>
<td>1.4</td>
</tr>
<tr>
<td>NHI-1</td>
<td>1.8</td>
<td>2.0</td>
<td>1.8</td>
<td>14.0 ± (1.0)</td>
<td>1.2</td>
</tr>
<tr>
<td>QVD-OPh</td>
<td>-1.5</td>
<td>1.1</td>
<td>-0.2</td>
<td>0.4</td>
<td>19.3 ± (1.9)</td>
</tr>
</tbody>
</table>

$^a$LWV shift values are reported in pm. Values in bold represent the mean of at least 3 independent measurements for the five cognate protein-small molecule pairs with positive binding signal. Values in parentheses indicate standard error of mean. Binding tests were performed using 50 µM of small molecule solution with 40 µg of immobilized protein at RT. $^b$CA II, carbonic anhydrase isozyme II; $^c$NQO1, human NAD(P)H dehydrogenase quinone 1; $^d$XIAP, X-linked inhibitor of apoptosis protein; $^e$hLDH-A, human lactate dehydrogenase-A; $^f$Casp-3, caspase-3. Reprinted with permission from [1], © 2014 American Chemical Society.
to immobilized NQO1 ($K_i = 450$ nM)[18] (Fig. 3.6b) even though they have much weaker affinities to XIAP and NQO1 than SM-164 and dicoumarol. Since the binding interaction is measured in a microplate format, the binding rate between the small molecule and protein target is mainly limited by mass transport, prohibiting the determination of kinetic parameters associated with the binding event. Non-binding signals of the other four non-cognate small molecules can be readily distinguished from the positive binding signal of the cognate protein-small molecule pairs (Table 3.1), indicating that the observed LWV shift is not due to non-

![Figure 3.7. Specific detection of HSA-warfarin interaction. (a) Mixture of small molecules used in the test. Warfarin is highlighted in red. (b) LWV shift due to the binding of warfarin to HSA in the compound mixture. The dotted line indicates the addition of the compound mixture to both the active and reference wells. Values shown are the mean of at least three independent measurements; error bars represent standard error of mean. Reprinted with permission from [1], © 2014 American Chemical Society.]
specific aggregation to the sensor surface. In addition, the small LWV shift observed due to the non-interacting protein-small molecule pairs (Table 3.1) is within $3\sigma$ of the noise generated when only buffer solution was added to both the active and reference wells (Fig. 3.3), demonstrating that the presence of the reference well aids in compensating the noise due to non-specific binding. Lastly, using structurally similar but inactive analogues of NHI-1 (1b and 1g, Fig. 3.5a)[19], we were able to demonstrate the specific binding of NHI-1 to immobilized hLDH-A (Fig. 3.5b).

3.5. Specific Detection of Protein and Small Molecule Binding

To confirm that the ECL biosensor instrument is sufficiently robust for screening protein-small molecule interactions, detection of warfarin binding to immobilized human serum albumin (HSA) ($K_D = 1.2 \, \mu M$)[20] was tested. When the active and reference sensors were

![Figure 3.8. Dose response binding of warfarin to immobilized HSA. Error bars represent the standard error of mean of at least three independent experiments. Reprinted with permission from [1], © 2014 American Chemical Society.](image)
exposed to a cocktail of 5 non-binding compounds (Figure 3.7a), no binding signal was observed in the absence of warfarin, while the addition of warfarin in the cocktail resulted in a LWV shift of 14 pm (Figure 3.7b) due to its binding to immobilized HSA. Importantly, it was determined that the observed shift of 14 pm correlates well with the concentration of warfarin available to bind with HSA, based on further dose response measurements (Fig. 3.8). This set of data demonstrates the specificity of the ECL biosensor for detection of protein-small molecule binding interactions.

3.6. High Throughput Screening

A “needle-in-the-haystack” screen for inhibitors against CA II was performed to demonstrate the HTS capability of the ECL biosensor assay. The Z’ factor was calculated to determine the quality of the screening assay, with σ the standard deviation, and µ the mean, of

![Graph](image)

Figure 3.9. Determination of Z’-factor for the self-referencing ECL biosensor assay with immobilized CA II. 50 μM acetazolamide (5% DMSO) was used as the positive control; 5% DMSO in PBS was used as the negative control. Reprinted with permission from [1], © 2014 American Chemical Society.
Figure 3.10. Binding of dorzolamide (compound 20) to immobilized CA II can be detected in the HTS. LWV shift data for all 48 compounds screened at 50 µM with 40 µg of immobilized CA II. 50 µM acetazolamide and 5% DMSO were the positive and negative control respectively. The values shown for the positive and negative controls are mean of at least three independent measurements; error bars represent standard error of mean. Reprinted with permission from [1], © 2014 American Chemical Society.

Figure 3.11. Dose response binding of dorzolamide to immobilized CA II. Error bars represent the standard error of mean of at least three independent experiments. Reprinted with permission from [1], © 2014 American Chemical Society.
positive and negative controls:[21] \[ Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p + \mu_n|} \]. The \( Z' \)-factor of the assay was determined to be 0.52 (Fig. 3.9), indicating that it is sufficiently robust to discern binders from non-binders.[21] A 47-member compound collection obtained from an in-house screening library, plus dorzolamide, was screened at 50 \( \mu \text{M} \) (1 compound per well) with CA II immobilized to the sensor surface. Each of the 48 compounds was added individually into both the active and reference wells and the LWV shift was measured. As shown in Fig. 3.10, dorzolamide is clearly differentiated from the other non-binding compounds, demonstrating the HTS capability of the assay in identifying protein-small molecule binders. To validate that the “hit” obtained from the screen was real, a range of concentrations of dorzolamide was tested against CA II to obtain dose-response data (Fig. 3.11).

As an initial demonstration of the technology’s capabilities, in the screen with CA II, we performed the tests in serial with the throughput of 3 tests per hour. However, throughput was increased through demonstration of screening 6 compounds at a time within a single well (Fig. 3.7), effectively resulting in a screening throughput of 18 compounds/hour.

It is our intention, with this initial demonstration of the technology, to demonstrate the capability for high throughput, which can be achieved by simple engineering of the instrument and test method. For example, our approach is based on the fact that, in an initial screening to identify small molecule binders to target proteins, we need the LWVs at the initial time point and at the end point to obtain the LWV shifts, rather than LWVs in the whole binding process. Thus, we are able to take initial LWV readings from each well in a microplate to determine the
pre-binding wavelength before introduction of small molecules. After introduction of small molecules, a second scan of the microplate can be used to gather final LWVs for the wells. As each LWV measurement only takes <0.1 seconds, the rate of measurement is limited by the rate of the motion stage that moves the microplate over the optical detection head. Using our current system with a single optical fiber detection head, we estimate that an entire 384-well plate can be scanned in ~3 minutes. Including time for stabilization of the binding interaction, a 384-well plate, screening with 10 compounds/well, with one reference sensor for each active sensor, could screen 1,920 compounds in 15 minutes.

Besides utilizing the end-point reading method, the throughput may be further increased by using multiple SOA in parallel to perform multiple tests at the same time. Although the cost of each SOA is ~$5000, the costs of implementation of an 8-channel detection instrument is consistent with the costs of current HTS instruments.

Figure 3.12. The schematic plot of the external cavity laser system with laser auto-alignment system.
The throughput could be further increased by the utilization of automatic laser alignment system. The schematic plot of the ECL with auto-alignment system is shown in Fig. 3.12. It consists of a photo diode sensor (Thorlabs, PDQ80A), a beam position aligner (Thorlabs, TPA101), two piezo drivers (Thorlabs, TPZ001), and a piezo-actuated 2-axis mirror mount. Together, the systems is used to position and maintain the reflected laser beam from the PC so that it is coupled to the SOA through the right end of fiber, and thus to maintain lasing of the ECL. The coupling of the laser beam to the cavity is controlled by the 45° mirror positioned below the PC microplate. The position of the detector is pre-aligned, so that when the split beam hits the center of the detector, light coupling to the fiber is maximized for lasing. If the laser beam is not coupled into the cavity, the position sensitive detect will measure x and y coordinates of the beam’s position. When combined with the piezo driver, the position signal from the detector is utilized as feedback for the piezo actuator to control the angle of the mirror to center the beam on the detector. The utilization of this auto-alignment system eliminates the need for manual alignment, and also reduces the alignment time to achieve higher throughput.

3.7. Discussion

A self-referencing ECL biosensor system was developed to detect protein-small molecule binding. This optical biosensor system achieves high detection sensitivity and high spectral resolution while being capable of eliminating thermal noise through accurate referencing. The detection instrument and PC biosensor enable the quantitative detection of small molecule binding to immobilized protein targets with various binding affinities. In addition, highly specific detection of protein-small molecule binding was achieved using this
robust label-free assay system. Finally, as an initial demonstration of the HTS capability of this technology, a “needle-in-the-haystack” screen with CA II was performed in serial, and dorzolamide was easily identified among the screening compounds. As demonstrated in this work, protein-small molecule binding can be accurately detected in this robust detection system with no temperature control of the reagents and the sensor, in an HTS-compatible microplate format that minimizes reagent consumption, and with “flat” low density surface chemistry. In light of previous optical biodetection approaches, and SPR biosensing in particular, the ECL-based optical biosensing approach represents substantial technical advance through its ability to use the narrow bandwidth output of the laser to achieve high resolution for detection of small wavelength shifts that occur for small molecule binding interactions with large immobilized proteins.

In the future, we plan to incorporate highly optimized surface chemistry and functionalization techniques to increase the binding response of small molecule binders to the immobilized protein targets. To further enrich the capability of the self-referencing ECL beside identification of small molecule binders, active transport (such as a microplate stirring system) can be implemented to mitigate mass transport limitations of the current setup for measurement of kinetic binding constants. Lastly, with this initial demonstration of the technology, it is our intention to improve the throughput of the current setup to facilitate future HTS endeavors through engineering of the instrument and test method. Higher throughput of the setup can be achieved either via an endpoint reading method or increasing the number of SOAs operating in parallel within the instrument (refer to Section 3.6). With the capability of performing higher throughput assays, this newly developed system offers a sensing platform that can be useful for
the community of chemical biologists and medicinal chemists for the identification and validation of small molecule binders to protein targets in a broad range of biologically significant applications.
References


CHAPTER 4. PLASMONIC EXTERNAL CAVITY LASER BIOSENSOR

To further improve the figure of merit of external cavity laser (ECL) label-free biosensor, we developed a plasmonic external cavity laser biosensor. As discussed in Chapter 1, compared with dielectric based optical resonator, plasmonic sensor utilizing surface plasmon resonance (SPR) offers ultra-high sensitivity, but displays lower quality factor resonance. In the plasmonic ECL, we incorporate optical gain to compensate for the high loss in metal. While the ultra-high sensitivity of SPR sensor is maintained, spectrally sharp resonance is achieved through the stimulated emission process in the laser cavity. Moreover, the plasmonic active laser biosensor extends the detection limit of traditional passive plasmonic resonators by improving the quality factor, offering a unique property to perform accurate detection of biologically important molecules and viral particles in low concentration.

4.1. Introduction

SPR biosensors that measure changes in surface-adsorbed refractive index as biomolecules adsorb onto metal surfaces have been used effectively to analyze biomolecular interactions since the first demonstration in 1991[1, 2]. Due to strong light-matter interaction provided by enhanced near-fields adjacent to a metal/dielectric interface, SPR biosensors demonstrate label-free molecule detection with excellent sensitivity[7], leading to a variety of applications in life science research, pharmaceutical discovery, and medical diagnostics. Building upon the commercial success of SPR biosensors utilizing the Kretschmann configuration, recent work on SPR sensing based on periodically nanostructured metal films has generated intense interest[4, 10-13]. The extraordinary optical transmission (EOT)
effect[15], arising from plasmon interactions between periodic arrays of metallic elements, leads to large field enhancement and high sensitivity to surface-localized refractive index changes. In contrast to the Kretschmann configuration, plasmonic crystal structures do not require physical contact to a coupling prism, enabling detection via measurement of the reflection or transmission spectra.

Extensive effort has been devoted to further enhancing the performance of plasmonic nanostructured sensors for the most challenging biosensing applications, where plasmonic devices with both narrow resonant linewidth and high refractive index sensitivity are required to detect extremely small resonance shifts. Fabrication methods for plasmonic nanostructures have been developed to precisely control their nanometer-scale features[18] and to reduce surface roughness[19], leading to reduced scattering losses and resonant linewidth ($\Gamma$, as measured by full width at half maximum of the resonant transmission spectrum). Novel designs for plasmonic nanostructures that redistribute the plasmon-enhanced electric fields have been demonstrated with the goal of making the resonant field accessible to the surrounding environment[20, 21], so as to achieve greater sensitivity ($S_b$, as measured by the bulk refractive index sensitivity $S_b = \Delta \lambda / \Delta n$, where $\Delta \lambda$ is the shift in resonant wavelength induced by a change in refractive index of liquid on the sensor surface of $\Delta n$). However, due to Ohmic losses of metal, inherent to all passive SPR sensing approaches, the advantage of high sensitivity of SPR sensing is offset by the moderate resolution for sensing small wavelength shifts due to the broad resonance. As a result, several types of high-$Q$ dielectric resonators achieve detection figure of merit (FOM) metrics that far exceed that of SPR, where the FOM is defined as $\text{FOM} = S_b / \Gamma$, in
order to simultaneously capture the effects of the magnitude of the refractive index induced resonant shift and the ability to measure small wavelength shifts. Alternative passive optical resonator approaches that report FOM values greater than SPR include photonic crystals[22], liquid-core optical fibers[23], whispering-gallery mode microspheres[24], and microring resonators[25]. Because increased $Q$ for an optical resonator has generally also resulted in reduced $S_b[3]$, there has been a great deal of research focused on development of active optical resonators that achieve narrow resonant linewidth without sacrificing sensitivity through the process of stimulated emission[3, 5, 8, 9].

In this chapter, we show the experimental demonstration of a plasmonic ECL refractometric sensor, which achieves sharp resonant linewidth associated with external cavity laser emission, while at the same time maintaining the high $S_b$ associated with SPR. [26] In our approach, a plasmonic crystal sensor with high quality factor EOT resonance is utilized as the wavelength-selective element in an ECL cavity. Through the incorporation of broadband optical gain, single-mode lasing at the peak transmission wavelength of the plasmonic crystal is achieved. Variations of refractive index on the plasmonic sensor surface lead to a shift of the transmission peak of the EOT resonance, which subsequently tunes the lasing wavelength of the plasmonic ECL with the same magnitude. The plasmonic ECL performs refractometric measurements by generating lasing wavelength shifts that are easily detected with a laser spectrum analyzer. We demonstrate electrically-pumped, continuous-wave refractive-index tuning of single-wavelength lasing with the plasmonic ECL. Since the lasing emission, mediated by the stimulated emission process, has a significantly narrower linewidth than that of the EOT resonance, the plasmonic ECL improves the spectral resolution of the plasmonic
crystal sensor without sacrificing its high sensitivity, resulting in an overall FOM that is superior to any previously reported approach. The fundamental approach reported here can form the basis of a new generation of plasmonic biosensors for label-free biosensing.

4.2. Plasmonic Crystal

4.2.1. Design and fabrication of plasmonic crystal

The plasmonic crystal, comprised of a periodically nanostructured metal film, was fabricated with a plastic-based nanoreplica molding process on a mechanically flexible substrate[22]. An imprinted epoxy replica of a two-dimensional nanopatterned silicon stamp served as the template for production of the plasmonic lattice. The epoxy substrate had a square array of cylindrical wells with a lattice period of 500 nm, hole diameter of 210 nm and post

![Fabrication process of the plasmonic crystal](image)

Figure. 4.1. Fabrication process of the plasmonic crystal with nanoreplica molding method.
depth of 150 nm. Uniform e-beam evaporation of a 40-nm-thick gold film coated the epoxy surface as well as the bottom of the cylindrical wells, creating a quasi-3D plasmonic crystal. The fabrication process of the plasmonic crystal using nanoreplica molding is shown in Fig. 4.1. The plasmonic crystal therefore consists of a layer of thin gold film with a nanohole array and an underlying gold nanodisk array (Fig. 4.2a). Fig. 4.2b shows an optical image of the sensor surface with a size of 8 mm × 8 mm as defined by the effective area of the silicon stamp. Fig. 4.2c-d present top- and side-views of scanning electron microscope images of the fabricated plasmonic crystal. The top metal thin film and the bottom metal nanodisks are physically separated, with no observable gold coating on the sidewalls. The design of the

![Figure 4.2](image.png)

Figure. 4.2. (a) Schematic illustration of the plasmonic crystal. (b) Optical image of the fabricated plasmonic crystal with a size of 8 mm × 8mm. (c) and (d) Top- and side-view of scanning electron microscope images of the fabricated plasmonic crystal surfaces. Reprinted with permission from [26], © 2014 Optical Society of America.
plasmonic crystal maximizes the strength of plasmon resonance in the desired spectral range. The plasmonic resonances of the periodically nanostructured top metal layer and the bottom gold nanodisks spectrally overlap. The presence of these two resonances results in a significant reduction in the spectral linewidth of the quasi-3D plasmonic crystal, making the device an effective narrowband transmission filter in the near infrared (NIR) part of the optical spectrum.

4.2.2. Resonance modes of plasmonic crystal

We performed polarization-resolved angle-dependent study of the zero-order transmission characteristics of the fabricated device. The device was mounted in a transmission fixture, in which it was illuminated with collimated, linearly polarized white light. The spectral wavelengths and transmission efficiencies of plasmon resonances for \( p \)-polarized illumination as a function of the launch angle, \( \theta \), along the \( \Gamma-X \) axis are mapped in the dispersion diagram, as illustrated in Fig. 4.3a-b. The calculated dispersion of the SPP-Bloch wave (BW) modes is also presented in Fig. 4.3a-b, where the resonant condition is described by the Bragg coupling equation:

\[
|k_\parallel + iG_x + jG_y| = \frac{\omega}{c} \sqrt{\frac{\varepsilon_d \varepsilon_m}{\varepsilon_d + \varepsilon_m}}
\]  

(4.1)

where \( k_\parallel \) is the in-plane wave vector, \( i \) and \( j \) specify the order of the SPP-BW modes, \( G_x \) and \( G_y \) are reciprocal vectors which are inversely proportional to the lattice constant, \( \omega \) and \( c \) are the
Figure 4.3. Experimental transmission spectra as a function of incident angle $\theta$ for white, $p$-polarized illumination along the $\Gamma$-X direction of the plasmonic crystal (a) with air as the background media and launch angle varied from $\theta = 0^\circ$ to $50^\circ$ and (b) with water as the background media and launch angle varied from $\theta = 0^\circ$ to $30^\circ$. Calculated dispersion curves of SPP–BW mode (solid lines) and Rayleigh anomaly (dashed lines) are superimposed. Inset: Schemes of the square lattice structure in reciprocal space. (c) Measured (red dashed curve) and simulated (blue solid curve) transmission spectra for the plasmonic crystal with launch angle $\theta = 16^\circ$. Inset (d) Simulated electric field intensity distribution for the peak at $\lambda = 850$ nm. Reprinted with permission from [26], © 2014 Optical Society of America.
momentum and speed of free-space light, $c_s$ and $c_m$, are the electric permittivity of the adjacent dielectric and the metal.

Along the Γ-X axis, a distinct transmission mode, which manifests itself as the strongest and narrowest plasmon resonance, shifts to longer wavelength as the launch angle is increased. The overall trends of this mode are in good agreement with the simple dispersion relation predicted by Eq. (1), indicating that the resonance originates from the (−1, 0) SPP-BW mode along the gold/air interface. Since the plasmonic crystal investigated in this study is designed to operate as a label-free biosensor in aqueous media, the dispersion of the device when immersed in water was also characterized. The refractive index change of the cover media from air to water resulted in a large red shift of the plasmon resonance modes. The distinct EOT mode associated with (−1, 0) SPP resonance along the gold/water interface was also observed as the strongest and narrowest plasmon resonance, as shown in Fig. 4.3b. The narrow linewidth, combined with the strong transmission intensity of this resonant mode enables the device to perform as a transmission filter to select the lasing wavelength in a laser cavity. In addition, the transmission filter based on this resonance mode is tunable over a wide wavelength range, as the resonance peak can be modulated by changing the launch angle.

Full three-dimensional finite-difference time-domain (FDTD) simulations with appropriate boundary conditions were used to calculate the transmission spectra and the electromagnetic field distributions in and around the metal nanostructures of the device. With a launch angle of 16°, experimental and simulated transmission spectra of the plasmonic crystal in water are shown together in Fig. 4.3c. The calculated near field intensity patterns at the peak
transmission wavelength, as shown in Fig. 4.3d, confirm that the resonance is associated with the excitation of a SPP-BW mode near the gold/water interface of the top nanohole array. Additionally, the near-field distribution suggests the excitation of the localized SPR modes of the bottom nanodisks. The simultaneous presence of plasmon resonances of the nanohole array and nanodisk array creates complex responses that enables strong linewidth narrowing for the observed EOT phenomenon [10, 11, 27, 28]. Besides the high quality-factor plasmonic resonances, the enhanced near-fields associated with the resonance penetrate evanescently into the surrounding media from the gold/water interface. Strong concentration of the electromagnetic field in the dielectric medium allows strong light-matter interactions, suggesting the high sensitivity for refractive index sensing.

4.2.3. Finite difference time domain simulation

To better understand the origins of the resonance modes of the quasi-3D plasmonic crystal (Fig. 4.3), we performed FDTD simulations to model the transmission spectra and the electromagnetic field distributions for three types of plasmonic structures: the bottom gold nanodisk array only, the top gold film with nanohole array only, and the quasi-3D plasmonic crystal structure.

The transmission spectrum of the bottom gold nanodisk array when immersed in water reveals a resonant mode at 870 nm (Fig. 4.4a) with launch angle $\theta = 16^\circ$. The electric field associated with this resonance is concentrated at the edges of the gold disk (Fig. 4.4b), indicating the excitation of localized surface plasmon resonance (LSPR) of the gold nanodisk. The transmission spectrum of the top gold nanohole film, as shown in Fig. 4.5a, exhibits a peak
Figure 4.4. (a) Simulated transmission spectrum for the structure composed of only the bottom nanodisk array. (b) Simulated electric field intensity distributions for the resonance transmission dip. Reprinted with permission from [26], © 2014 Optical Society of America.

Figure 4.5. (a) Simulated transmission spectrum for the structure composed of only the top metal film with nanohole array. (b) Simulated electric field intensity distributions for the resonance transmission peak. Reprinted with permission from [26], © 2014 Optical Society of America.

Figure 4.6. (a) Simulated transmission spectrum for the quasi 3D plasmonic crystal composed of top nanohole array and bottom nanodisk array. (b) Simulated electric field intensity distributions for the resonance transmission peak. Reprinted with permission from [26], © 2014 Optical Society of America.
at 865 nm, associated with intense near-field intensity along the gold/water interface (Fig. 4.5b). This transmission peak arises from the (-1,0) SPP-BW mode and Rayleigh anomaly on the gold/water side of the device predicted by Equation 4.1. This mode red-shifts as the incident angle increases. In our case, with 16° incident angle, the SPP-BW mode of the nanohole array spectrally overlaps with the LSPR of the gold nanodisk. Thus, concurrence of plasmonic resonances of both the nanodisk array and the nanohole array is expected in the quasi-3D plasmonic crystal.

We performed simulations for the quasi-3D plasmonic crystal, and indeed observed a strong peak at 860 nm (Fig. 4.6a) which has nearly the same spectral position as the resonant peaks of both the bottom nanodisk array and the top gold nanohole film. This suggests the simultaneous excitation of both the LSPR of the gold nanodisk and the SPP-BW mode of the nanohole array, and is further confirmed by the near-field distributions shown in Fig. 4.6b. Light transmits through the plasmonic crystal via coupling between these two resonant modes, achieving extraordinary light transmission with high quality factor.

4.3. Plasmonic External Cavity Laser Design

The plasmonic ECL detection instrument is comprised of a semiconductor optical amplifier (SOA), two polarization maintaining (PM) single-mode optical fibers, two NIR mirrors and the plasmonic crystal, as shown in the schematic diagram in Fig. 4.7a. The SOA (SOA-371, Superlum Inc., peak wavelength $\lambda = 850$ nm, and spectral width $\Delta \lambda = 40$ nm) has both edge facets coated with an anti-reflection layer, and is coupled to a single-mode PM fiber.
with a length of 1 m. The output from the fiber on each side, through a collimating lens, is normally reflected against a NIR mirror to form an optical cavity. The plasmonic crystal is mounted on a rotation stage with an adjustable angle, and is inserted into the Fabry-Perot cavity to selectively transmit the narrow range of resonant wavelengths. The transmitted light is efficiently coupled back into the SOA, where it is amplified. Due to the broadband nature of the SOA and NIR mirrors, the peak transmission wavelength of the plasmonic crystal first achieves the threshold condition for lasing, in which the resonant wavelength of the plasmonic crystal is selected.
crystal determines the single-mode lasing wavelength of the ECL cavity. A detection instrument such as a spectrometer or a laser spectrum analyzer may be utilized to quantify the lasing wavelength, and to dynamically monitor lasing wavelength shifts.

As the plasmonic ECL biosensor resonates, a high intensity electromagnetic wave is established on the plasmonic crystal/media interface (Fig. 4.3d). Changes of the bulk refractive index or the attachment of biomolecules will shift the resonant transmission wavelength, and subsequently lead to a lasing wavelength shift in the ECL system. The use of a two meter long optical fiber cavity provides mode spacing of 0.08 pm\[3\], ensuring detection of small wavelength shifts without hopping between modes in a discontinuous manner. While the plasmonic crystal is a passive resonator with moderate quality factor (\(Q\sim100\)), the active laser resonator achieves extremely narrow linewidth through the process of stimulated emission and offers substantially greater spectral resolution. At the same time, the capability of the plasmonic crystal to register changes of refractive index with high sensitivity is maintained.

4.4. Characterization of the Plasmonic External Cavity Laser

The relationship between the laser output power and the injection current is shown in Fig. 4.7b, demonstrating a threshold current of 55 mA. The transmission spectra of the plasmonic crystal and the laser emission spectra of the ECL are plotted together in Fig. 4.7c-d. With a launch angle of \(\theta = 42^\circ\) in air, the plasmonic crystal exhibits a resonance at \(\lambda = 860\) nm, resulting in lasing emission at \(\lambda = 860\) nm (Fig. 4.7c). Similarly, with a launch angle of \(\theta = 18^\circ\) in water, lasing at the peak transmission wavelength of \(\lambda = 862\) nm is demonstrated (Fig. 4.7d). Using a laser spectrum analyzer (721 A series, Bristol Instruments, Inc. absolute accuracy \(\pm 0.2\)
(or $\Gamma (\text{eV}) = 3.62 \times 10^{-5}$ eV). The intense and narrow linewidth emission of the ECL allows the system to resolve extremely small wavelength shifts when used as a refractive index sensor.

4.5. Refractive Index Sensing Using Plasmonic External Cavity Laser

To examine the performance of the plasmonic ECL system as a refractive index sensor, we measured the lasing spectra with the plasmonic crystal immersed in liquid samples of different mixtures of dimethyl sulfoxide (DMSO) and deionized (DI) water. The launch angle was set at 17°, and this specific angle was chosen so that the resonant transmission intensity of the plasmonic crystal is maximized near the peak gain region of the SOA. The concentrations of the DMSO solution in water ranged from 0% to 8%. As we increased the refractive index of liquid on the plasmonic crystal surface, the resonances of the plasmonic crystal shifted to longer wavelengths, which subsequently modulates the lasing wavelength value (LWV) of the plasmonic ECL. The normalized lasing spectra are plotted together in Fig. 4.8a. The linear bulk refractive index sensitivity was measured to be $S_b = 547 \text{ nm/RIU}$ over a dynamic range of 7 nm (Fig. 4.8b). In addition, bulk refractive index tuning of the system demonstrates that single-mode lasing is achieved over a wide range of wavelengths. The full operating range of the system is determined by the gain spectrum of the SOA, which has been selected to provide gain between $\lambda = 830$-870 nm.

To determine spectral resolution and to identify the sensing limitations of the plasmonic ECL, we measured the time-sequence LWVs as we introduced a small refractive index variation
Figure 4.8. (a) Measured lasing spectra of the plasmonic ECL with the plasmonic crystal immersed in DMSO-water mixture solutions with varying compositions at the fixed launch angle of $\theta = 17^\circ$. (b) Bulk sensitivity characterization. Laser emission wavelength shifts as the sensor was exposed to liquid media with different refractive index. A linear fit to the experimentally obtained data reveals a bulk sensitivity of 547 nm/RIU. Inset: Measured response during a refractometric experiment for the plasmonic ECL. The refractive index change was $7.5 \times 10^{-5}$ via the addition of DMSO. Reprinted with permission from [26], © 2014 Optical Society of America.
to the plasmonic crystal. The LWVs were rapidly determined using the aforementioned laser spectrum analyzer, which utilizes a scanning-mirror Michelson interferometer and does not require a separate spectral fitting algorithm to identify the peak position, greatly simplifying the data analysis process. A stable LWV baseline was established when the plasmonic crystal was immersed in DI water. Next, we introduced 0.05% DMSO solution through a flow cell that was attached to the sensor surface. The solutions were introduced to the plasmonic crystal through a flow cell, within which the plasmonic crystal was incorporated as the bottom surface. The flow chamber has a height of 3 mm, width of 9 mm, and length of 18 mm, and was fabricated by cutting Poly(methyl methacrylate) (PMMA) into the desired dimensions. Two cylindrical openings at the ends of the flow cell were tapped and screwed in with 1/16” hose barb adapter connected to tubing as inlet and outlet. The solutions were introduced through the flow cell using a syringe. We measured the time-sequence LWV after signal stabilization, and observed a LWV shift of 23.60 pm, as shown in Fig. 4.8b inset. Since there was no fluid mixing process during this refractometric measurement, the LWV shift was caused by abruptly changing the liquid from DI water to DMSO solution homogeneously in the flow cell. The sensor output noise was quantitatively characterized with the standard deviation $\sigma = 0.62$ pm. The limit of detection (LOD) = $3\sigma/ S_b = 3.38 \times 10^{-6}$ RIU is obtained. With a commonly used “boxcar” technique to average 10 adjacent LWV measurements to reduce noise[29], the LOD is easily further reduced to $1.79 \times 10^{-6}$ RIU. Note that this value is obtained without any form of thermal control or referencing, and that it represents the ability to differentiate refractive index differences with $3\sigma$ resolution (many publications utilize $1\sigma$ values).
4.6. Discussion

As described briefly in the Introduction, a widely adopted metric for comparison between resonant optical biosensors is the FOM. For the plasmonic ECL, a value of FOM = \(2.60 \times 10^4\) is obtained. As listed in Table 4.1, this value is nearly two orders of magnitude larger than the previously reported record high FOMs obtained with a periodically nanostructured plasmonic sensor\([4, 6]\). This extraordinary FOM results from the unique combination of the narrow linewidth of stimulated emission and the high refractive index sensitivity of SPR. Importantly, this method of forming a resonant cavity where the plasmonic sensors interact with optical gain to achieve narrow resonant linewidth is not limited to the plasmonic crystal structure introduced in this paper, but is widely applicable to other plasmonic sensors based on EOT resonances.

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</table>

Table 4.1. Summary of the refractive index sensitivities and FOM values of plasmonic biosensors/ refractometric sensors. Reprinted with permission from \([26]\), © 2014 Optical Society of America.
Recently, several laser-based label-free biosensors, such as the distributed feedback laser[5], the photonic crystal laser[8], and the whispering gallery microlaser[9], have been reported to achieve high spectral resolution by generating a high intensity and narrow linewidth output through the stimulated emission process. Although exciting capabilities have been demonstrated, as listed in Table 4.2, many of these devices require a large optical pumping source and are only capable of operating in pulsed mode. Additionally, degradation of dye, as a result of photo-damage during pulsed measurements, leads to decreased lasing stability and reduced sensor performance. Compared to these devices, the utilization of an external laser cavity to physically separate the optical gain from the sensor surface represents a fundamentally different approach [3, 30, 31]. This arrangement results in a robust high spectral resolution detection system that utilizes an electrical pump source, operates in CW mode, while using a sensor structure that can be fabricated inexpensively over large surface areas. Because SPR sensors deliver greater refractive index sensitivity than dielectric optical resonators, the ability of plasmonic ECL to overcome the low-$Q$ limitation of SPR through a stimulated emission system results in the potential for improved sensor performance compared to previous demonstrated laser biosensors.

In conclusion, we developed a high resolution refractometric sensor based upon a plasmonic ECL that combines the high sensitivity of SPR refractive index sensing with a tunable ECL. To achieve this, we fabricated a polymer-based replica-molded quasi-3D plasmonic crystal sensor, which generates a high quality factor EOT resonance from strong plasmon coupling of the top nanohole array and the bottom nanodisk array. We utilized this spectrally sharp plasmon resonance mode to select and to tune lasing wavelength in the ECL,
resulting in lasing at the peak transmission wavelength. We have demonstrated single-mode operation of the plasmonic ECL system with a large refractive-index tuning rang of 7 nm. The ability to measure refractive index changes with a detection limit of $1.79 \times 10^{-6}$ RIU is demonstrated. As a result of the unique combination of refractive index sensitivity of SPR and narrow linewidth of laser emission, a record high experimental FOM was obtained. With the integration of microfluidic channels and bioaffinity assays, the plasmonic external cavity laser system offers the potential for a novel label-free biosensing approach towards challenging applications, such as detection of small molecule interactions with immobilized proteins, detection of low-abundance proteins in a specimen. Based on the estimated surface mass density sensitivity[32] and the measured spectral resolution, a mass density resolution of $2 \times 10^{-19}$ g/µm$^2$ is predicted for biosensing applications with the plasmonic external cavity laser system. As the technology evolves, we expect to be able to probe small areas with the SOA on the plasmonic crystal surface with an area as low as 1×1 µm$^2$. As a single antibody molecule has a mass of ~

<table>
<thead>
<tr>
<th>Laser system</th>
<th>$\lambda$ (nm)</th>
<th>$S_b$ (nm/RIU)</th>
<th>Pump method, Operating mode</th>
<th>Gain material, External/integrated gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmonic external cavity laser</td>
<td>850</td>
<td>549</td>
<td>Electrical injection, CW</td>
<td>SOA, external gain</td>
</tr>
<tr>
<td>External cavity laser[3]</td>
<td>850</td>
<td>212</td>
<td>Electrical injection, CW</td>
<td>SOA, external gain</td>
</tr>
<tr>
<td>Distributed feedback laser[5]</td>
<td>588</td>
<td>100</td>
<td>Optical pumped, pulsed</td>
<td>Laser dye, integrated gain</td>
</tr>
<tr>
<td>Photonic crystal laser[8]</td>
<td>1600</td>
<td>350</td>
<td>Optical pumped, CW</td>
<td>SQW/MQW, integrated gain</td>
</tr>
<tr>
<td>Whispering gallery microlaser[9]</td>
<td>1550</td>
<td>-</td>
<td>Optical pumped, CW</td>
<td>Er+ ions, integrated gain</td>
</tr>
</tbody>
</table>

Table 4.2. Summary of the refractive index sensitivity and lasing mechanism for various laser biosensors. Reprinted with permission from [26], © 2014 Optical Society of America.
2.5×10^{-19} g, we believe that single antibody molecule detection resolution will become possible with this technology.
References


CHAPTER 5. OUTLOOK AND CONCLUSIONS

A novel external cavity laser (ECL) biosensor technology based on photonic crystal (PC) to detect protein-small molecule interactions is developed. The PC biosensor surface comprises the mode-selection filter of the ECL, providing sufficient sensitivity, while the narrow linewidth emission of ECL enables high resolution to observe direct small molecule binding to immobilized protein targets. The system is built in a high throughput assay format, with an innovative self-referencing strategy that enabled such a demanding assay to be performed without temperature control of the reagents or detection system. We then built off this success and extend the technology, with a plasmonic-based laser biosensing approach that increases signals by ~3x. The application of this system to small molecule discovery of compounds will be the focus of future work.

ECL biosensor, with the enhanced sensitivity, fine resolution and simple instrumentation, represents one of the most significant and active advances in the development of optical label-free biosensors. With the integration of microplates or microfluidic channels and utilization of optimized bioaffinity assays, the ECL biosensor system offers the potential as a high-precision label-free biosensing approach towards challenging applications, such as detection of small molecule interactions with immobilized proteins, detection of low-abundance proteins in a specimen. We envision that progress in ECL biosensor technology will further improve detection abilities of label-free biosensors and allow sensitive, fast, and cost-effective biochemical analysis in both laboratories and in pharmaceutical applications.