Detection of Protein-Small Molecule Binding Using a Self-Referencing External Cavity Laser Biosensor *

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Abstract— **High throughput screening of protein-small molecule binding interactions using label-free optical biosensors is challenging, as the detected signals are often similar in magnitude to experimental noise. Here, 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 with binding affinities or inhibition constants in the sub-nanomolar to low micromolar range. The demonstrated ability to perform detection in the presence of several interfering compounds opens the potential for increasing the throughput of the approach. As an example application, we performed a "needle-in-the-haystack" screen for inhibitors against carbonic anhydrase isozyme II (CA II), in which known inhibitors are clearly differentiated from inactive molecules within a compound library.**

I. Introduction

The development of high-throughput direct binding assays to identify small molecules that interact with protein targets is a critical early component of the pharmaceutical discovery process used to find potential treatments for a variety of diseases.[1] Because small molecules are intrinsically difficult to label without changing their function, there is intense interest in the development of label-free detection technologies.[2] Current label-free assays utilized for the detection of protein-small molecule interactions include isothermal calorimetry (ITC),[3] surface plasmon resonance (SPR)[4] and photonic crystal biosensors (PC).[5] ITC and SPR are capable of quantitative detection of protein-small molecule interaction in low-to-medium throughput applications,[6] while PC biosensor assays have been used to identify small molecule modulators of protein-protein interaction in a high-throughput screening (HTS) manner by incorporating biosensors into standard format microplates.[7] As an extension of the PC biosensor approach, in this work we demonstrate a self-referencing external cavity laser (ECL) biosensor technology for high-throughput and label-free detection of protein-small molecule interactions. The technique is capable of quantitative detection of small molecule binding to immobilized protein targets with accurate referencing, and has the potential for HTS applications through the use of a microtiter plate-based sensor format.

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II. Methods

The self-referencing ECL is a novel optical detection instrument for reading the output of PC biosensors in which an external cavity laser detects the adsorption of biomolecules to the PC with improved resolution when compared to simply measuring the passive resonant reflection spectrum.[8, 9] This is achieved by building an external cavity laser with a broadband semiconductor optical amplifier (SOA) as the gain medium and with narrowband PC resonant reflectors as the wavelength selective elements. The PC biosensor provides optical feedback to the cavity, resulting in lasing at the PCs' peak reflection wavelengths with extremely narrow linewidth.

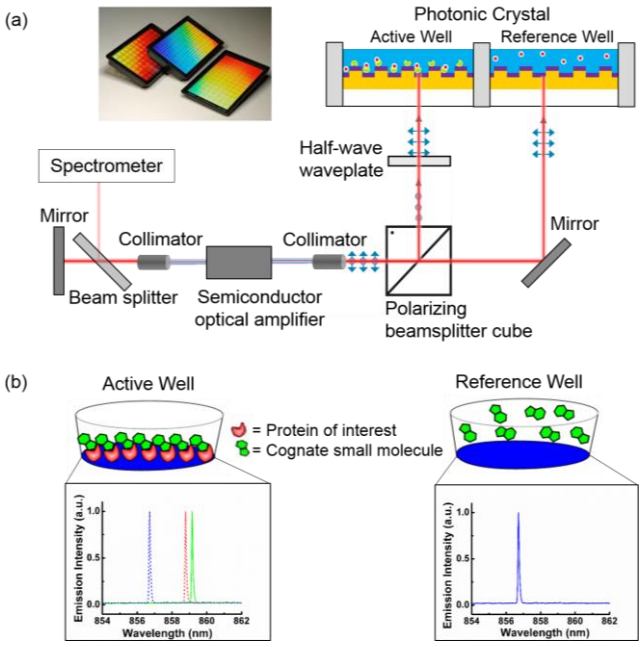

Figure 1. (a) Schematic diagram of the self-referencing ECL biosensor system. Insert: Images of PC biosensor films adhered to the bottom of standard microplates. (b) Biosensor assays used for detection of direct small molecule binding to immobilized protein targets with referencing. Active and reference 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.

Adsorption of biomolecules on PC sensor surfaces modulates the resonant wavelength of the PC, and subsequently tunes the lasing wavelength values (LWV) of the ECL. In this manner, the ECL maintains the high sensitivity of the PC biosensors, while at the same time, achieves greater spectral resolution through the stimulated emission process. The PC sensors are fabricated inexpensively on flexible plastic surfaces using a replica-molding process, and are attached with adhesive to bottomless 96- or 384- well plates for HTS.[5]

In order to achieve accurate referencing, two PC sensors in adjacent wells of a 384-well plate were utilized as wavelength selective elements at the same time. Each sensor selects its own resonant wavelength, so the ECL system can lase at two independent wavelengths simultaneously. Self-referencing was accomplished by designating one well as the "reference" well and the other as the "active" well, where both sensors were prepared identically with exception of the immobilized protein in the active well (Figure 1b). 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 errors. 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. In order to avoid competition between these two simultaneously oscillating modes for stable operation of the ECL system, a pulse-driven bi-stable shutter was used to allow alternate operation of these two modes with a frequency of 0.5 Hz. Kinetic monitoring of the lasing wavelength value is achieved by directing a portion of the lasing emission energy with a beam splitter to a detection instrument such as a spectrometer or interferometer-based laser wavelength meter.

III. Results

To validate the self-referencing technique, we performed a test to demonstrate accurate compensation of environmental fluctuations. 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 0.8 pm over a 20-min time period (Figure 2).

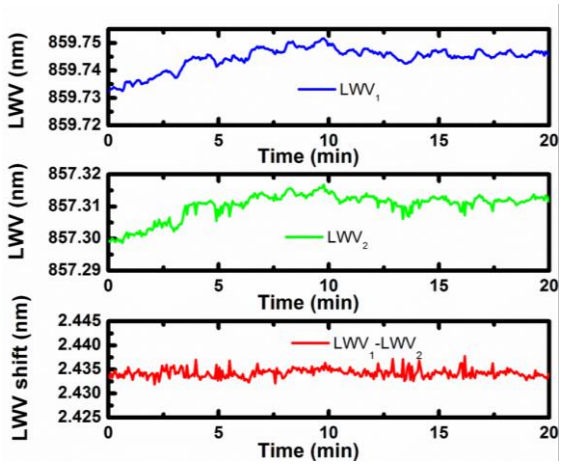

Figure 2. Compensation of environmental fluctuations via the subtraction of LWV of the reference well (green) from the LWV of the active well (blue). The resulting LWV shift (red) has a reduced noise level with standard deviation of 0.8 pm in a 20 minute measurement.

Next, the ECL biosensor instrument was used to study five well-characterized protein-small molecule binding interactions: CA II−dorzolamide (K_D = 1.1 nM),[10] NQO1−dicoumarol (K_I = 1 − 10 nM),[11] XIAP−SM-164 (K_I = 0.56 nM),[12] caspase-3−Q-VD-OPh (IC_{50} < 25 nM)[13] and hLDH-A−N-hydroxyindole-1 (NHI-1) (K_I = 10.8 μM).[14] 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 (Figure 3a) can be readily detected (Figure 3b) despite the pair having the weakest inhibition constant amongst the tested pairs. As shown in Figure 3, the system can also detect the binding interaction of the other four protein-small molecule pairs: XIAP and SM-164 (Figure 3c), CA II and dorzolamide (Figure 3d), NQO1 and dicoumarol (Figure 3e), caspase-3 and Q-VD-OPh (Figure 3f). 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 I), indicating that the observed LWV shift is not due to non-specific aggregation to the sensor surface. Lastly, using

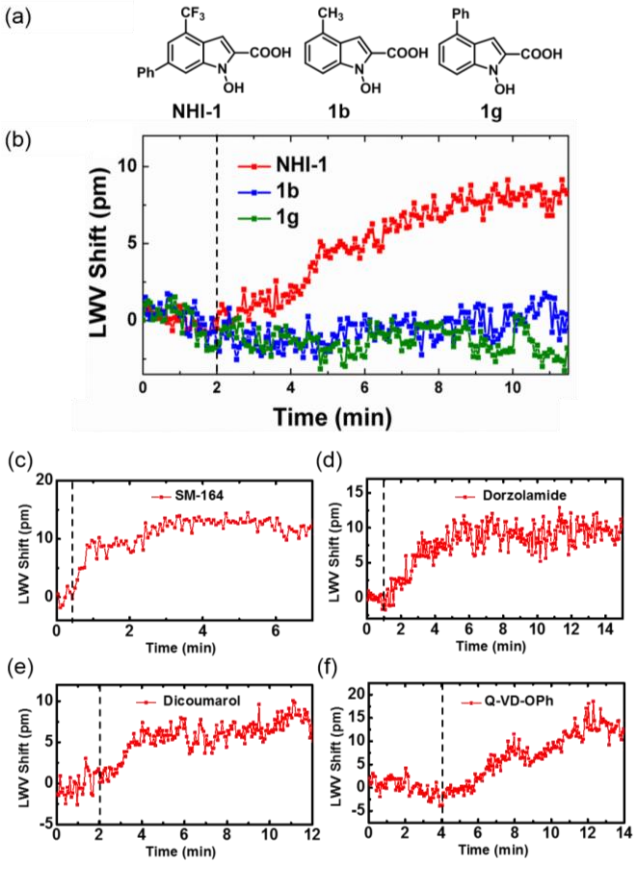

Figure 3. (a) Structure of NHI-1 and its inactive variants 1b and 1g. Observed LWV shift from the binding of 50 μM (b) NHI-1, 1b or 1g to immobilized hLDH-A, (c) SM-164 to immobilized GST-XIAP, (d) dorzolamide to immobilized CA II, (e) dicoumarol to immobilized NQO1 and, (f) Q-VD-OPh to immobilized caspase-3. The vertical dotted line indicates the addition of small molecules to both the active and reference wells.

TABLE I. LWV SHIFT VALUES OF PROTEIN-SMALL MOLECULE BINDING INTERACTION DETECTED ON THE ECL BIOSENSOR[a]

	CA II[b]	NQO1[c]	XIAP[d]	hLDH-A[e]	Casp-3[f]
Dorzolamide	**12.5 ± (1.1)**	-2.4	1.5	2.6	0.3
Dicoumarol	1.2	**11.2 ± (1.0)**	-1.1	-1.5	-0.5
SM-164	-0.9	0.7	**11.5 ± (0.5)**	-0.2	1.4
NHI-1	1.8	2.0	1.8	**14.0 ± (1.0)**	1.2
QVD-OPh	-1.5	1.1	-0.2	0.4	**19.3 ± (1.9)**

[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.

structurally similar but inactive analogues of NHI-1 (1b and 1g, Figure 3a)[15], we were able to demonstrate the specific binding of NHI-1 to immobilized hLDH-A (Figure 3b).

To confirm that the ECL biosensor instrument is sufficiently robust for screening protein-small molecule interactions, multiplexed detection of warfarin binding to immobilized human serum albumin (HSA) (K_D = 1.2 μM)[16] was tested. When the active and reference sensors were exposed to a cocktail of 5 non-binding compounds (Figure

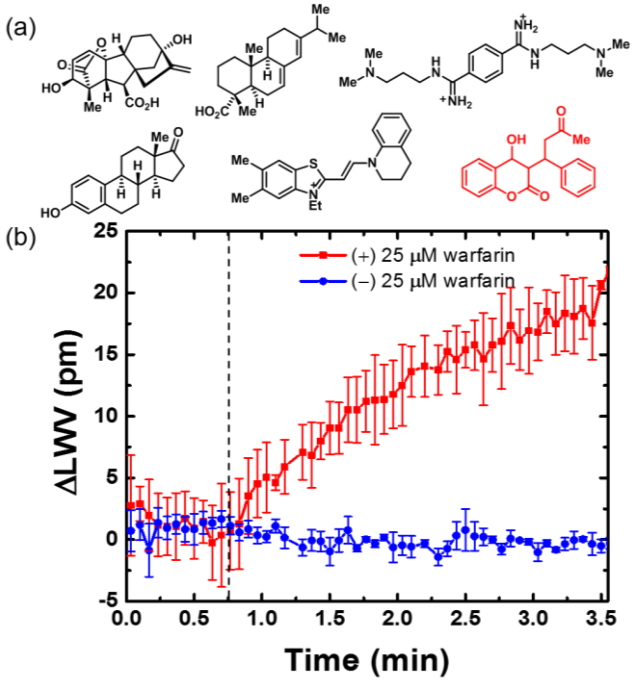

Figure 4. Multiplexed detection of HSA-warfarin interaction. (a) Mixture of small molecules used in the test. Warfarin is highlighted in red. (b) LWV shift due to the binding of warfarin to HSA in the compound mixture. The dotted line indicates the addition of the compound mixture to both the active and reference wells. Values shown are mean of at least three independent measurements; error bars represent standard error of mean.

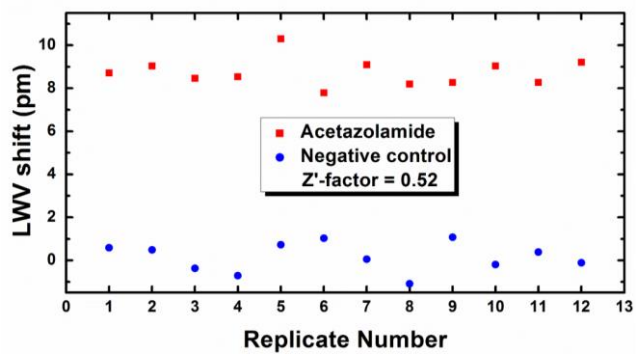

Figure 5. Determination of Z'-factor for the self-referencing ECL biosensor assay with immobilized CA II. 50 μM acetazolamide (5% DMSO) was used as the positive control; 5% DMSO in PBS was used as the negative control.

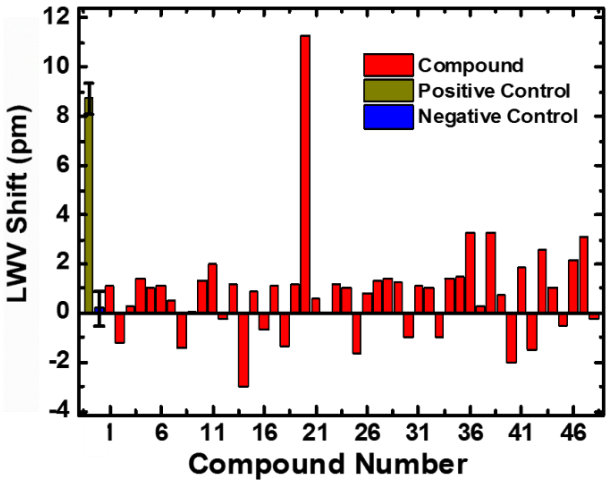

Figure 6. Binding of dorzolamide (compound 20) to immobilized CA II can be detected in the HTS. LWV shift data for all 48 compounds screened at 50 μM with 40 μg of immobilized CA II. 50 μM acetazolamide and 5% DMSO were the positive and negative control respectively. The values shown for the positive and negative controls are mean of at least three independent measurements; error bars represent standard error of mean.

4a), 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 4b) due to its binding to immobilized HSA. This set of data demonstrates the capability of the ECL biosensor to perform multiplexed screening of protein-small molecule interactions.

Finally, to demonstrate the HTS capability of the ECL biosensor assay, a "needle-in-the-haystack" screen for inhibitors against CA II was performed. The Z'-factor of the assay was determined to be 0.52 (Figure 5), indicating that it is sufficiently robust to discern binders from non-binders.[17] A 47-member compound collection obtained from an in-house screening library, plus dorzolamide, was screened at 50 μM (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 Figure 6, dorzolamide is clearly differentiated from the other non-binding compounds, demonstrating the HTS capability of the assay in identifying protein-small molecule binders.

IV. Conclusion

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. The robust label-free assay system was also demonstrated to have the ability to perform multiplexed assays in one well and with the potential for HTS applications. This 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.

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