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A label-free optical technique for detecting small molecule interactions

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Abstract

A novel approach for the label-free detection of molecular interactions is presented in which a colorimetric resonant grating is used as a surface binding platform. The grating, when illuminated with white light, is designed to reflect only a single wavelength. When molecules are attached to the surface, the reflected wavelength (color) is shifted due to the change of the optical path of light that is coupled into the grating. By linking receptor molecules to the grating surface, complementary binding molecules can be detected without the use of any kind of fluorescent probe or radioactive label. The detection technique is capable of detecting the addition and removal of small molecules as they interact with receptor molecules on the sensor surface or enzymes in the solution surrounding the sensor. Two assays are presented to exemplify the detection of small molecule interactions with the biosensor. First, an avidin receptor layer is used to detect 244 Da biotin binding. Second, a protease assay is performed in which a 136 Da *p*-nitroanilide (*p*NA) moeity is cleaved from an immobilized substrate. Because the sensor structure can be embedded in the plastic surfaces of microtiter plates or the glass surfaces of microarray slides, it is expected that this technology will be most useful in applications where large numbers of biomolecular interactions are measured in parallel, particularly when molecular labels will alter or inhibit the functionality of the molecules under study. Screening of pharmaceutical compound libraries with protein targets, and microarray screening of protein–protein interactions for proteomics are examples of applications that require the sensitivity and throughput afforded by this approach.

Keywords: Optical biosensor; Small molecule assay; Direct assay; High throughput screening; Microtiter plate; Resonant diffraction

1. Introduction

One of the major challenges currently facing drug development laboratories is screening the interaction of small molecule pharmaceutical candidates against a large host of enzymes and inhibitors (Pandey and Mann, 2000; Leigh Anderson et al., 2000; Patterson, 2000; MacBeath and Schreiber, 2000; De Wildt et al., 2000). Methods that have the ability to simultaneously quantify biomolecular interactions with sufficient sensitivity to measure the attachment and detachment of molecules and molecular moieties with molecular weight less than 1000 Da are of particular importance. The ability to measure biochemical interactions without the use of fluorescent tags or other labels is desirable for these applications, as the label may alter or inhibit the functionality of the small molecule under study. Further, because the number of small molecule candidates generated by combinatorial chemical library methods can be extremely large, it is important to screen the interaction of the library with protein targets in a format that is compatible with high throughput measurement—especially in a format that is compatible with existing laboratory fluid handling infrastructure.

Biosensors have been developed to detect a variety of biomolecular complexes including oligonucleotides, antibody–antigen interactions, hormone–receptor interactions, and enzyme–substrate interactions. In general, biosensors consist of two components: a highly specific recognition element and a transducer that converts the molecular recognition event into a quantifi-

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able signal. Signal transduction has been accomplished by many methods, including fluorescence, interferometry (Jenison et al., 2001; Lin et al., 1997), and gravimetry (Cunningham, 1998).

Of the optically-based transduction methods, direct methods that do not require labeling of analytes with fluorescent compounds are of interest due to the relative assay simplicity and ability to study the interaction of small molecules and proteins that are not readily labeled. Direct optical methods include surface plasmon resonance (SPR) (Jordan and Corn, 1997), grating couplers (Morhard et al., 2000; Bier and Scheller, 1996; Kunz et al., 1995), ellipsometry (Jin et al., 1995), evanascent wave devices (Huber et al., 1992), and reflectometry (Brecht and Gauglitz, 1995).

This paper describes a novel approach for the detection of small molecule interactions in which a colorimetric resonant diffractive grating is used as a surface binding platform of a microtiter plate and a glass slide. The sensor structure, when illuminated with white light, is designed to reflect only a single wavelength. When molecules are attached to the surface, the reflected wavelength (color) is shifted due to the change of the optical path of light that is coupled into the sensor. By linking receptor molecules to the sensor surface, complementary binding molecules can be detected without the use of any kind of fluorescent probe or radioactive label. The detection technique is capable of resolving changes of <0.1 nm thickness of protein binding, and can be performed with the grating surface either immersed in fluid or dried. The readout system consists of a white light lamp that illuminates a small spot of the grating at normal incidence through a fiber optic probe, and a spectrometer that collects the reflected light through a second fiber, also at normal incidence. Because no physical contact occurs between the excitation/readout system and the grating surface, no special coupling prisms are required and the grating can be easily adapted to any commonly used assay platform, such as microtiter plates and microarray slides. A single spectrometer reading is performed in several milliseconds, thus it is possible to quickly measure a large number of molecular interactions taking place in parallel upon a grating surface, and to monitor reaction kinetics in real time.

A separate publication describes in detail the optical theory of the *B*iomolecular *IN*teraction *D*etector (BIND) biosensor, sensor design, materials, and fabrication (Cunningham et al., 2002a). The publication also describes the first biochemical assays performed with the sensor for larger proteins, demonstration of kinetic binding measurements in real time, and a description of how the sensor structure is produced in glass surfaces. Previous work also describes the manufacturing fabrication of BIND sensor structures into continuous sheets of plastic film, and their incorporation into disposable microtiter plates (Cunningham et al., 2002b).

The BIND sensor utilizes a subwavelength structured surface (SWS) to create a sharp optical resonant reflection at a particular wavelength that can be tracked with high sensitivity as biological material is attached to the sensor surface. The SWS consists of an optical grating fabricated from high refractive index materials built upon a substrate of lower refractive index. The purpose of the grating is to couple normally incident light into a zero-order mode that travels parallel to the sensor surface. The interaction between the coupled mode and the optical density of any material placed in contact with the sensor surface manifests itself in a shift in the wavelength of reflected light. An SWS structure may be used as a microarray platform by building a grating surface that is the same size as a standard microscope slide and placing microdroplets of high affinity chemical receptor reagents onto an x–y grid of locations on the grating surface. Likewise, an SWS structure can be built to be the same size as a standard microtiter plate, and incorporated into the bottom surface of the entire plate. When the chemically active microarray/microtiter plate is exposed to an analyte, molecules will be preferentially attracted to locations that have high affinity. Some locations will gather additional material onto their surface, while other locations will not. By measuring the shift in resonant wavelength within each individual microarray/microtiter plate location, it is possible to determine which locations have attracted additional material. The extent of the shift can be used to determine the amount of bound analyte in the sample and the chemical affinity between receptor reagents and the analyte.

Fig. 1 shows the resonant reflectance spectra measured from a plastic-substrate sensor using the readout

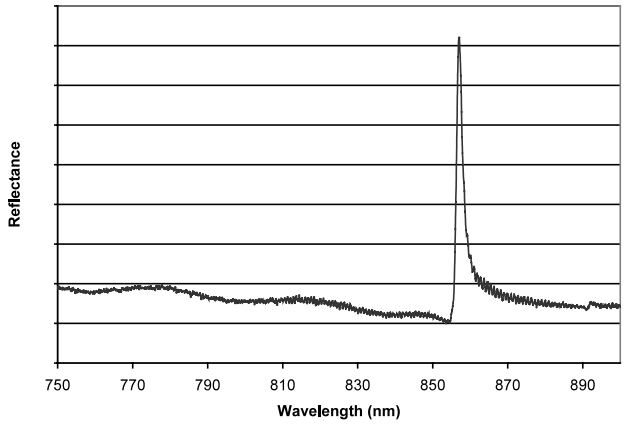

Fig. 1. Reflection efficiency as a function of wavelength for a BIND biosensor illuminated with white light from a collimated fiber source. The sensor surface acts as a perfectly reflecting mirror at the resonant wavelength, while reflected intensity is suppressed at all other wavelengths. The location of the peak resonant wavelength is tracked as biochemical reactions occur on the sensor surface.

instrument described in Cunningham et al. (2002b). The structure behaves as a perfectly reflecting mirror at the resonant wavelength, but does not reflect other wavelengths. By tracking the location of the peak resonant wavelength during the course of a biochemical assay, the binding/removal of material from the biosensor surface can be quantified. For sensors built upon plastic substrates, the resonant wavelength is ~860 nm, while sensors built upon glass substrates utilize a resonant wavelength of ~780 nm. Glass and plastic sensor structures provide similar levels of sensitivity. Their respective fabrication approaches and characterization are described thoroughly in Cunningham et al. (2002a,b).

This paper describes the first application of the sensor to label-free small molecule biochemical assays. For this publication, only one interaction was monitored at once. The results of two experiments are presented. First, a sensor is prepared with an avidin receptor layer to measure the binding of biotin (MW = 244 Da). Second, the removal of a 136 Da *p*-nitroanilide (*p*NA) group from an immobilized peptide by interaction with the caspase (*C*ysteine-requiring *Asp*artate prote*ase*)-3 enzyme is demonstrated.

2. Avidin/biotin binding assay

The purpose of the experiment is to immobilize a large (60,000 Da) avidin molecule to the sensor surface as the receptor for detecting the binding of a much smaller (244 Da) biotin molecule. Four wells of a plastic-substrate sensor embedded into the bottom of a standard 96-well microtiter plate were prepared with a poly–phe–lysine (PPL) surface by incubating with a 1.0 mg/ml solution of PPL (Sigma) in PBS (pH 7.4) for 1 h, followed by washing with PBS 3× and rinsing with water.

Two of the four wells were biotinylated by incubating with a solution of NHS–LC–biotin (Pierce) in TPBS (a reference buffer solution of 0.01% Tween 20 in phosphate buffer solution, pH 8) at 1.0 mg/ml concentration for 1 h at 37 °C. The biotinylated wells were subsequently immobilized with avidin receptor molecules by exposing the NHS–LC–biotin surface to a 1.0 mg/ml solution of avidin (Pierce) for 30 min, followed by thorough rinsing in PBS and water. As shown in Fig. 2, attachment of the avidin receptor coating results in a positive shift of the sensor's peak wavelength value (PWV) of 0.7 nm.

Following the surface preparation protocol, all four wells were filled with pure water, and monitored at 6-s intervals for 15 min to establish baseline stability. The sensor is illuminated through the bottom of the well, and reflected light is also collected through the bottom of the well. Fig. 3 shows the PWV shift as a function of time for the four wells. The wells are exposed to ambient room conditions, without temperature control.

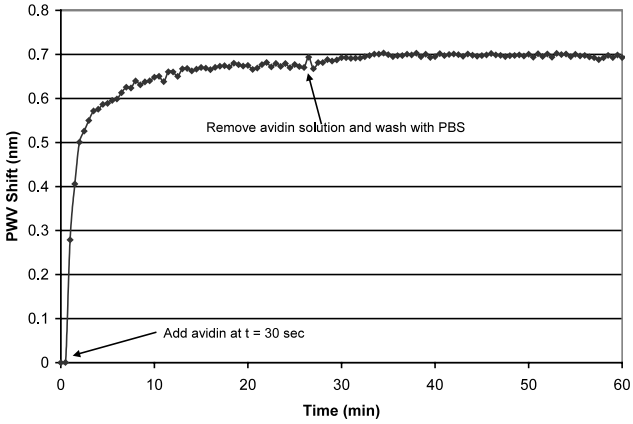

Fig. 2. Peak resonant wavelength as a function of time during the covalent attachment of avidin to the sensor.

Following establishment of the baseline, biotin at a concentration of 4 μM was added to one of the PPL-only wells, and to one of the avidin-immobilized wells. After an incubation period of 5 min, the biotin solution was removed from the wells, and replaced with water. Fig. 4 shows the PWV response as a function of time for all four wells. Fig. 4 shows that the stable baseline for the PPL-surface and the avidin-immobilized surface is maintained when exposed only to water, but that a specific response is observed by the avidin-immobilized surface when exposed to biotin. The bulk refractive index of the biotin solution results in a PWV shift of ~0.005 nm, as measured by the decrease of PWV that is measured when the biotin solution is replaced with water. The nonspecific binding of biotin to the PPL-surface results in a final measured PWV shift of 0.025 nm, while the specific binding of biotin to the avidin-immobilized surface results in a final measured PWV shift of 0.071 nm. The total shift due to specific interaction between avidin and biotin is calculated by subtracting the baseline PWV curve (well D6) and the nonspecific binding curve (well C6) from the specific binding curve (well E6) as shown in Fig. 5. The total shift due to specific interaction is 0.05 nm.

3. Caspase-3 protease assay

The purpose of the caspase-3 protease assay is to demonstrate the BIND sensor's ability to measure the presence and cleavage of small molecules in an experimental context that is relevant to pharmaceutical compound screening.

Caspases are a family of proteases that mediate cell death and are important in the process of apoptosis. Caspase-3, an effector caspase, is the most studied of mammalian caspases because it can specifically cleave

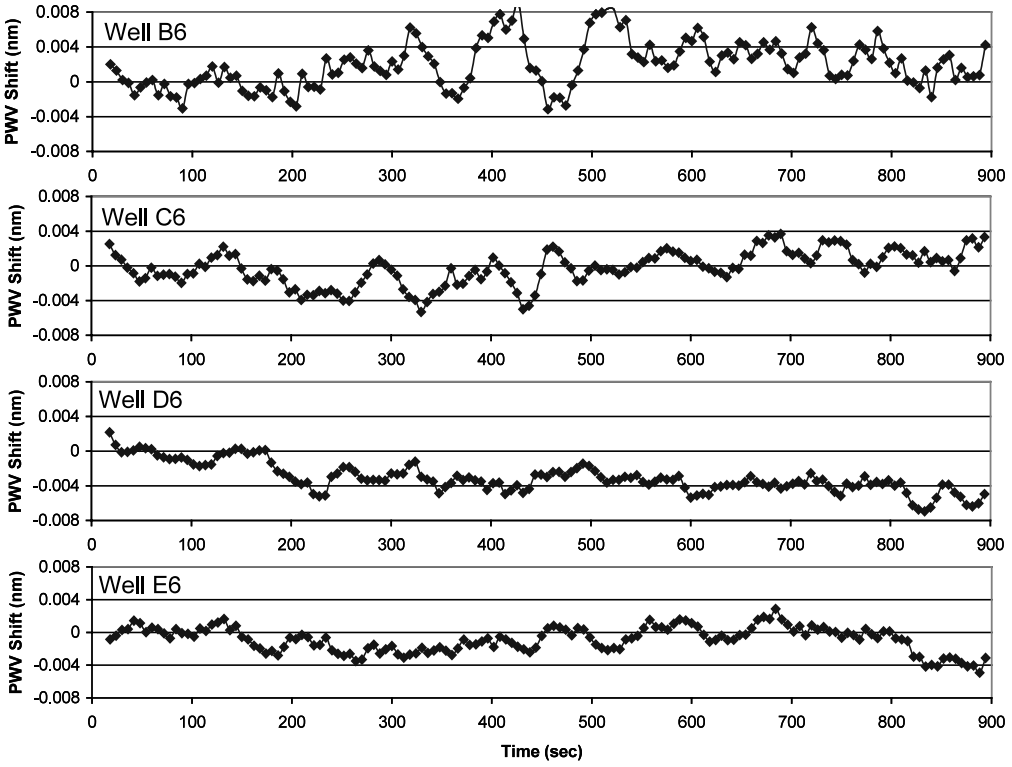

Fig. 3. Peak resonant wavelength as a function of time demonstrating the stability of the peak resonant wavelength measurement. Wells D6 and E6 are immobilized with avidin, and appear to produce a more stable baseline signal than surfaces without avidin. A negative drift is observed in wells D6 and E6 that may indicate slight disassociation of avidin.

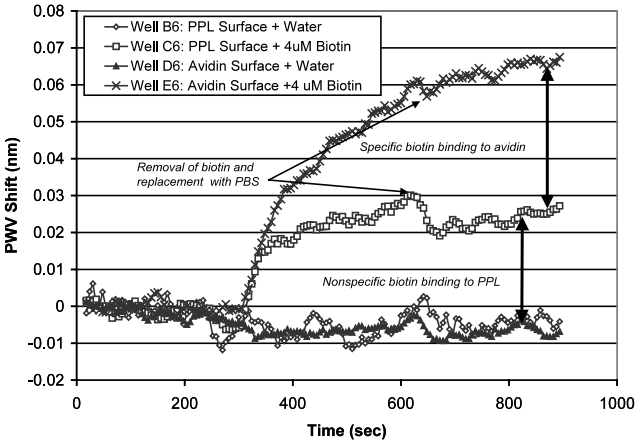

Fig. 4. Peak resonant wavelength as a function of time during the exposure of a 4 μM solution of biotin to the avidin-activated sensor (well E6). A control well (C6) measures the nonspecific binding of biotin to a PPL surface, while the baseline drift stability of an avidin surface and a PPL surface in water are simultaneously monitored.

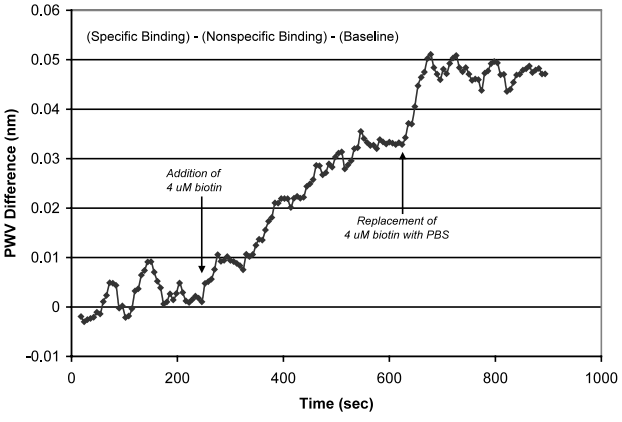

Fig. 5. By subtracting the baseline drift signal (well D6) and the nonspecific binding signal (well C6) from the specific binding well (well E6), the total signal due to specific binding during the course of the experiment can be measured.

most known caspase-related substrates. The caspase-3 assay is based on the hydrolysis of the five amino acid peptide substrate NHS–Gly–Asp–Glu–Val–Asp–*p*-NA (NHS–GDEVD–*p*NA) by caspase-3, resulting in the release of the *p*NA moiety.

$$(\text{NHS–GDEVD–}p\text{NA}) \xrightarrow{\text{caspase-3}} (\text{NHS–GDEVD}) + p\text{NA}$$

The NHS molecule attached to the N-terminal of the GDEVD provides a reactive end group to enable the NHS–GDEVD–*p*NA complex to be covalently bound to the biosensor with the *p*NA portion of the complex oriented away from the surface. Attached in this way, the caspase-3 will have the best access to its substrate cleavage site. A commercially available inhibitor to caspase-3 is Ac–DEVD–CHO. When Ac–DEVD–

CHO is present, caspase-3 will not interact with the substrate molecule, and *p*NA will not be released.

This type of assay is typically performed in solution by measuring increased adsorbance at a wavelength of 405 nm when *p*NA is released from the substrate. A commercially available substrate (Sigma), Ac–DEVD–*p*NA, may be used, where an acetyl group (Ac) is attached to the DEVD N-terminal. Because the Ac group cannot be readily immobilized to a solid support, a new substrate was custom-fabricated for purposes of the experiment. As described above, the custom substrate substitutes an NHS molecule for the Ac group. NHS, in turn, can be covalently bound to an NH_2-activated solid support. In addition, a $(CH_2)_6$ space arm was inserted between the NHS and GDEVD–*p*NA to increase the distance of caspase-3 cleavage site to the sensor surface.

Fig. 6 shows the adsorbance at 405 nm for the cleavage of the commercially available substrate, Ac–DEVD–*p*NA, by caspase-3. As a negative control, Fig. 6 also shows that no *p*NA is released when the Ac–DEVD–CHO inhibitor is present in solution. Fig. 6 also shows the adsorbance at 405 nm for the cleavage of the custom substrate, NHS–GDEVD–*p*NA, by caspase-3. Also shown is a negative control in which no *p*NA is released when the Ac–DEVD–CHO inhibitor is present. This result shows that the custom substrate functions as expected.

While the previous experiment demonstrates that the custom substrate functions properly in solution, it is necessary to demonstrate that it also functions properly when immobilized to the biosensor. As a demonstration experiment, a glass microscope slide was first chemically activated using the same process that will be used to activate the biosensor. Rubber gasket incubation chambers were adhered to the activated slide, so that physically separated −5 mm diameter regions could each hold several hundred microliters of fluid. A fixture was fabricated so the adsorbance of the fluid within the incubation chambers could be measured in a standard microplate reader (Molecular Devices).

The surface binding procedure included activation with NH_2, followed by deposition of a layer of PPL. As described in the literature (Jenison et al., 2001), PPL provides a high surface area polymer layer that can subsequently bind a high concentration of other small molecules. The PPL surface was subsequently reactivated with NH_2, and then functionalized with the NHS–GDEVD–*p*NA substrate. Following thorough rinsing in PBS to remove unbound substrate, we measure the amount of active substrate that is bound to the surface by exposure to caspase-3 (100 ng/ml, 10 ng in total) and measuring the amount of *p*NA that is released into solution.

As shown in Fig. 7, two concentrations (1, 10 mM) of the substrate were used to activate the sensor surface. Both concentrations result in clearly measured release of *p*NA into solution when the surface-bound molecules are exposed to caspase-3. The higher substrate concentration results in a higher concentration of surface-bound substrate.

A BIND biosensor was activated with the same NH_2–PPL–NH_2 procedure that was used for the glass microscope slides in the control experiment. An incubation chamber was sealed over the sensor, which was filled with 100 μl of PBS. At $t = 0$, 100 μl of 10 mM

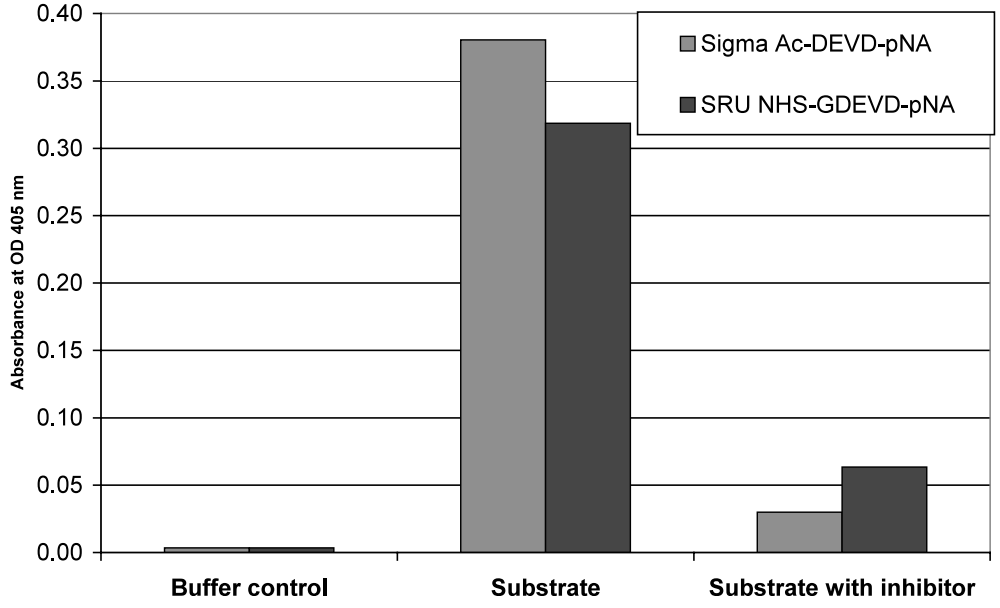

Fig. 6. Four hundred and five nanometer adsorbance measurements comparing the commercially available substrate molecule for testing caspase-3 binding with the molecule custom fabricated for purposes of this experiment. The custom molecule was optimized for attachment to a solid support by including an NHS moiety. Both molecules show similar function with respect to their interaction with caspase-3 and its inhibitor.

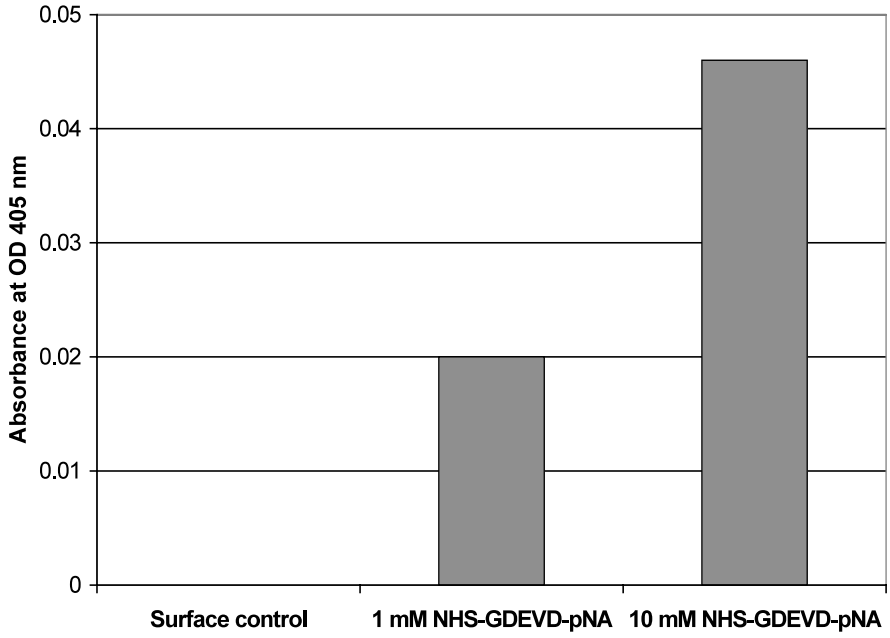

Fig. 7. Measurement of 405 nm adsorbance of flat-bottom glass microtiter wells with the custom molecule immobilized only to the bottom surface. The measurement demonstrates that *p*NA is released from the surface-immobilized molecule when it is exposed to caspase-3.

NHS–GDEVD–*p*NA substrate was added, and the peak resonant wavelength value of the sensor was monitored as a function of time. As shown in Fig. 8, the kinetic attachment of the substrate (MW = 860 Da) to the biosensor surface is observed. The majority of surface attachment occurs in the first minute of substrate–surface exposure, followed by a more gradual saturation. This experiment allows us to confirm by a direct measurement that the substrate molecule is incorporated onto the sensor surface.

Finally, a sensor was prepared by cleaning in 3:1 H_2SO_4–H_2O_2 solution (room temperature, 1 h), followed by silanation (2% silane in dry acetone, 30 s) and attachment of a poly(phenylalanine, lysine) (PPL) layer (PPL, 100 μg/ml in PBS pH 6.0 with 0.5 M NaCl, 10 h). The NHS–GDEVD–*p*NA complex was attached by exposing the sensor to a 10 mM solution in PBS (pH 8.0, room temperature, 1 h). A microwell chamber was sealed over the sensor surface, and cleavage of the *p*NA was performed by addition of 100 μl of caspase-3 in buffer (100 ng/ml, 1 × enzyme buffer, room temperature, 90 min). Following exposure to the caspase-3 solution, the sensor is washed in PBS.

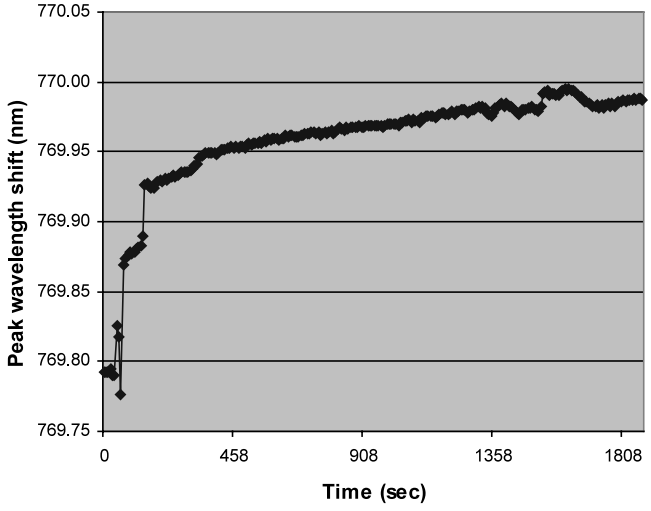

Fig. 8. Peak resonant wavelength as a function of time for the attachment of the 860 Da NHS–GDEVD–*p*NA custom molecule to the PPL–NH_2 activated sensor surface.

The peak resonant frequency of the sensor was measured before attachment of the NHS–GDEVD–*p*NA complex, after attachment of the complex (MW = 860 Da), and after cleavage of the *p*NA (MW = 136) with caspase-3. As shown in Fig. 9, the attachment of the peptide molecule is clearly measurable, as is the subsequent removal of the *p*NA. The *p*NA removal signal of $\Delta\lambda = 0.016$ nm is 5.3 × higher than the minimum detectable peak wavelength shift of 0.003 nm. The proportion of the added molecular weight and subtracted molecular weight (860 Da/136 Da = 6.32) are in close agreement with the proportion of peak wavelength shift observed for the added and subtracted material (0.082 nm/0.016 nm = 5.14).

The results of this experiment confirm that the BIND sensor is capable of measuring small peptides (in this case, a 5-per peptide) without labels, and even detecting the removal of 136 Da portions of a molecule through the activity of an enzyme. Again, the data shown is not temperature compensated or referenced in any way to improve signal-to-noise ratio.

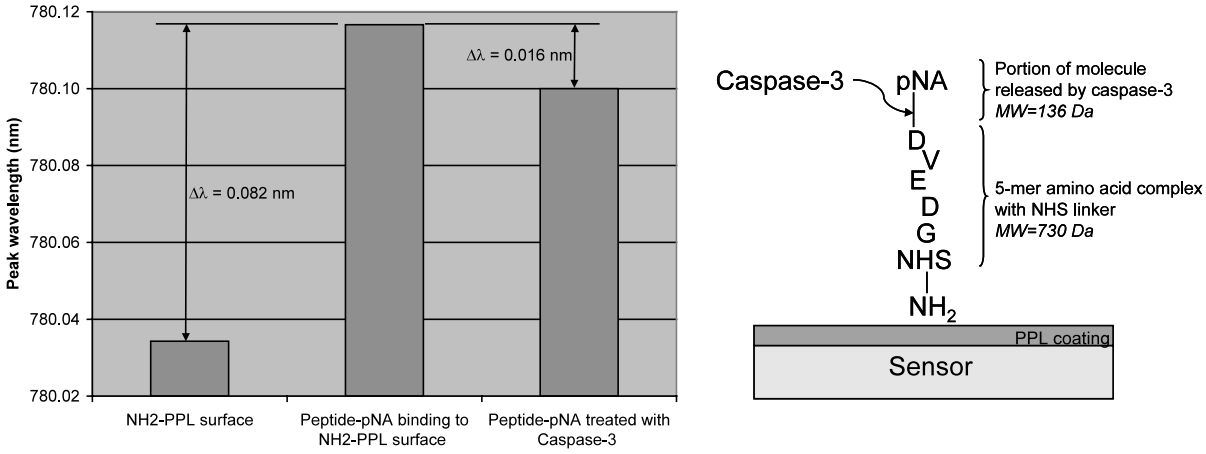

Fig. 9. Comparison of the peak resonant wavelength shift for the attachment of NHS–GDEVD–*p*NA and subsequent removal of the 136 Da *p*NA moiety by caspase-3.

4. Conclusion

A novel approach for the detection of small molecule interactions was described in which a colorimetric resonant diffractive grating surface is used as a surface binding platform. The sensor operates by reflecting an extremely narrow band of wavelengths when illuminated with collimated white light, and detecting shifts in the reflected wavelength when biomolecular material is added or removed from the surface. The sensor can be used as a general-purpose platform for biochemical assays by linking receptor molecules to the grating surface and detecting complementary binding molecules in a test sample without the use of any kind of fluorescent probe or particle label.

Two example assays were performed to demonstrate the use of this phenomenon as a biosensor, and to explore its use for detection of small molecule interactions. The experiments described demonstrate the BIND sensor's ability to detect the binding of a 244 Da biotin molecule to a 60,000 Da avidin receptor molecule, the ability to detect the binding of a five amino acid peptide, and the ability to detect cleavage of 136 Da portions of a bound molecule.

It is expected that this technology will be most useful in applications where large numbers of biomolecular interactions are measured in parallel, particularly, when molecular labels will alter or inhibit the functionality of the molecules under study. Screening of pharmaceutical compound libraries with protein targets, and microarray screening of protein–protein interactions for proteomics are examples of applications that require the sensitivity and throughput afforded by this approach. The sensor can be manufactured in large areas using a plastic embossing process, and thus can be inexpensively incorporated into common disposable laboratory assay platforms such as microtiter plates and microarray slides.

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