Label-Free Biosensors

DOI: 10.1002/ange.200502418

Photobiotin Surface Chemistry Improves Label-Free Interferometric Sensing of Biochemical Interactions**

*Joey C. Latham, Dmitry A. Markov, Henrik S. Sørensen, and Darryl J. Bornhop**

To accurately characterize molecular interactions, determinations should be done in a label-free manner.[1,2] Furthermore, in order to have the capability to fully evaluate the molecular diversity that exists in nature, it would be most advantageous to make these determinations with micro-total-analysis systems (μ-TAS)[3,4] configured for high throughput.[5,6] Yet, conventional detection methods are difficult to implement in μ-TAS and are often volume- or sensitivity-limited, both impediments to performing systems-biology analyses.

Investigators have developed various detection methods for use with microfluidic devices that have promise for label-free nanoscale detection. These include the nanoelectrode,[7] the porous Si (p-Si) sensor,[8,9] the surface plasmon resonance (SPR) detector,[10–13] and backscattering interferometry.[14–16] However, nanoelectrodes foul easily in real-world applications and require multistep manufacturing procedures for the integration into microfluidic chips. The SPR and p-Si methods are sensitive (detection limits of $\approx$10–50 nM for protein interactions[8,9,17]) and capable of sensing label-free biochemical interactions, yet neither technique is inherently compatible with μ-TAS. Integration or immobilization of p-Si into the fluidic network and long solute-diffusion times hinder the use of this method in μ-TAS. Since SPR relies on the excitation of plasmons—collective oscillations of free electrons that occur predominantly in metals—SPR surfaces are coated with a thin metal layer (for example, gold). This makes integration of SPR sensors into plastics challenging and relatively expensive as a result of the deposition process. Additionally, the

[*] J. C. Latham, D. A. Markov, Prof. Dr. D. J. Bornhop
Vanderbilt University
Department of Chemistry
4226 Stevenson Center, Nashville, TN 37235 (USA)
Fax: (+1) 615-343-1234
E-mail: darryl.bornhop@vanderbilt.edu
H. S. Sørensen
RISØ National Laboratory
Department of Optics and Plasma Research
Building 130, Frederiksborgvej 399, 4000 Roskilde (Denmark)

[**] This work was supported by the National Science Foundation, National Eye Institute, BioP graduate school, Applied Biosystems, and NASA. Affiliations include the Vanderbilt Institute of Nanoscience and Engineering, Vanderbilt Institute of Chemical Biology, and Vanderbilt Institute for Integrative Biosystem Research and Education.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

elaborate surface chemistry used to immobilize targets on SPR surfaces has been shown to lose as much as 20% of binding capacity over a 24-hour period.[10] Backscattering interferometry in rectangular channels (BIRC)[15] was recently shown to be applicable to label-free assays, can be used to study reversible molecular interactions in poly(dimethylsiloxane) (PDMS) chips, and is compatible with μ-TAS. Although promising, BIRC has been limited by tedious immobilization chemistry and, owing to less than optimum transduction-scheme concentration detection limits, has bordered on relevance to biological systems.

Herein we address many of these problems through the use of simple and robust surface immobilization chemistry on polymer chips and an improved BIRC instrument based on charge-coupled-device (CCD) transduction and fringe-pattern Fourier analysis. For the first time, it is shown that simple photoactivatable surface chemistry[18,19] can be used for the immobilization of substrates onto PDMS-facilitated, label-free, reversible-binding assays. This simplified approach to surface immobilization is a significant alteration to our earlier binding studies and those methods commonly used for surface activation/immobilization chemistry.[12,20]

A two-step biotin/avidin reaction (Figure 1) with a carbene-generating form of photobiotin consisting of a biotin moiety, spacer arm, and photoactivatable group was used to immobilize target molecules of interest. A solution (7 μL) of carbene-generating photobiotin (0.5 mg mL^{-1}) containing 0.02% Tween 20 and 50% (v/v) acetone was introduced into the PDMS channels and allowed to dry. Irradiation of the photobiotin molecule caused photolysis of the diazirine group, thereby producing an electronically excited np* state. Formation of an excited-state diradical was followed by internal conversion and production of a carbene. The highly reactive carbene was then inserted into the oxidized substrate. As a result, a homogeneous layer of biotin molecules was strongly bound to the channel surface.

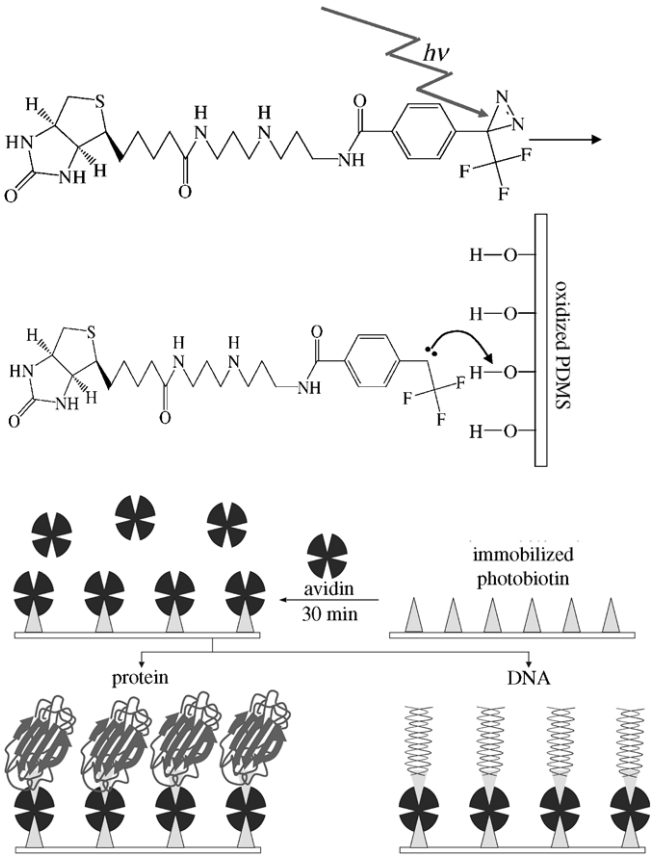

Figure 1. Top: Photobiotin is activated upon exposure to UV light and this promotes insertion into the oxidized PDMS substrate. Center: Avidin binds strongly to the immobilized layer of biotin. The tetrameric nature of avidin signifies that it can simultaneously bind the surface-bound biotin as well as any biotinylated target molecule that passes through the channel (bottom).

A solution of extravidin (0.5 mg mL^{-1}) was injected into the same channels and allowed to incubate for 30 min. Extravidin recognizes the imidazole ring that is *cis* fused to a tetrahydrothiophene ring, and binds to biotin with high affinity (association constant, $K_a = 2.5 \times 10^{11}\,\text{M}^{-1}$).[21] The resulting biotin/avidin complex is very stable to changes in temperature, pH value, and denaturing agents, unlike the lysine/glutaraldehyde/streptavidin/ethanolamine sandwich described in our earlier binding experiments.[15] Fluorescence images (not shown) of fluorescein isothiocyanate (FITC) labeled avidin were used to confirm that the surface chemistry used for immobilization was successful. Thus, biotinylated targets can be immobilized onto the PDMS-molded channel surface to result in a cost-effective, disposable chip that is applicable to the analysis of a variety of reversible biochemical interactions.

As described in detail elsewhere,[14,15,22,23] BIRC (Figure 2) employs a connected light source that illuminates a microfluidic channel, thereby creating a series of high-contrast interference fringes in the backscattered direction that shift with the optical pathlength. Herein we show that the overall performance of BIRC can be improved 100-fold through proper design and implementation of the photo-transduction method.

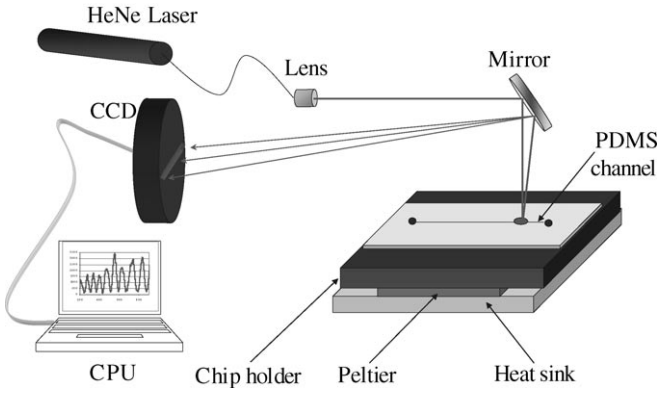

Figure 2. Schematic diagram of BIRC showing the simple optical train consisting of a connected light source, a microfluidic channel, and a phototransducer.

The use of a CCD combined with spatial Fourier analysis[14] for signal transduction, rather than an avalanche photodiode detector, facilitates improved sensitivity and reproducibility, while greatly simplifying alignment. Direction

of multiple fringes onto the CCD array promotes signal averaging and enhanced signal-to-noise (S/N) results. A fringe-pattern Fourier transformation (FFT) algorithm is inherently insensitive to laser-intensity fluctuations[14] and allows spatial phase determination. Since the CCD is a large-area device, three to upwards of eight fringes from the interference pattern can be used, thereby eliminating the need to align the slit-photodetector with respect to $1/e^2$ intensity on a particular fringe.

To evaluate the new immobilization chemistry and transduction method, two reversibly binding pairs were studied, protein A (P_A) linked to immunoglobulin G (IgG) and a 30-mer DNA–DNA pair. P_A, containing four high-affinity ($K_a = 2\times10^8$–$4.54\times10^8\,M^{-1}$) binding sites capable of interacting with the F_c region of IgG from several species, including human and rabbit, was immobilized onto a PDMS chip by using the chemistry shown in Figure 1. The reversible-binding assay of P_A with IgG was repeated five times (Figure 3). The non-

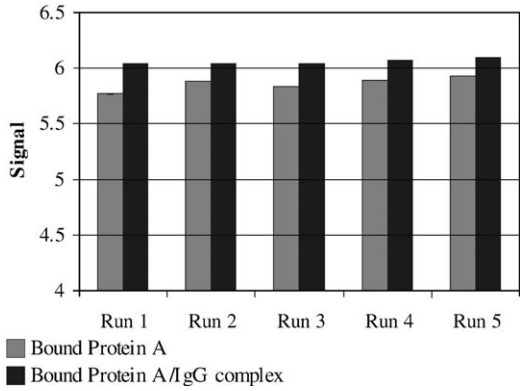

Figure 3. Biotinylated P_A was immobilized on the channel surface. Unreacted P_A was washed out with phosphate-buffered saline (PBS)/Tween 20. A human IgG F_c solution (0.5 mg mL^{-1}) in PBS was introduced into the microfluidic channel and allowed to bind to the P_A for 5 min. The channel was then rinsed with PBS/Tween 20 to remove any unbound IgG. Removal of the bound IgG from P_A was performed by using an acetic acid wash.

complementary fragment, F_{ab}, served as the control and showed no signal (change in refractive index/phase; data not shown) when introduced into the P_A-coated channel. As seen in Figure 3, reversible-binding events can be monitored with an excellent S/N ratio (pooled standard deviation = 0.0017). If it is assumed that complete reaction between the binding pair took place, and on the basis of the target-surface coverage, the 3σ detection limit is 4×10^{-17} mol (40 attomol) in a 500-pL probe volume. These results represent an increase in sensitivity of two orders of magnitude when they are compared to the P_A/IgG experiments performed previously.[15]

The use of microfluidic chips to perform nucleic acid analysis has allowed the detection and discrimination of multiple DNA sequences[24,25] and can eventually facilitate high-throughput screening techniques.[26] As further demonstration of the utility of BIRC, a label-free reversible DNA assay was performed (Figure 4). Immobilization of a biotinylated 30-mer of mActin (5′-ACTCATCGTACTCCTGCTTGCTGATCCACA-3′; $M_W = 9622.5$; melting temperature,

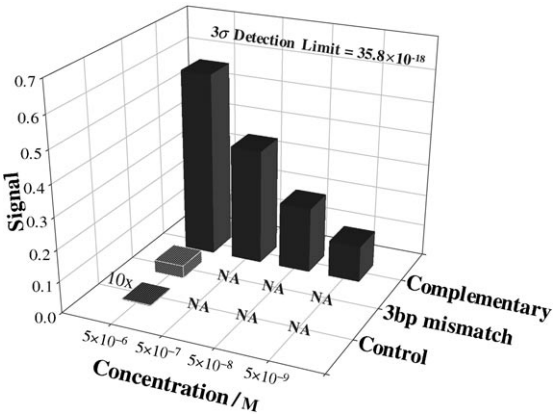

Figure 4. The binding affinity of a complementary DNA strand to an immobilized target over a multiple complementary strand. The complementary strand is also compared to a 3-bp mismatch and control strands at high concentration. All probe strands were allowed 20 min for hybridization. The control signal is shown at ten times its actual signal to allow visualization.

$T_M = 68.7\,°C$) onto the PDMS channel was possible with the biotin/avidin surface chemistry described above. Nonlabeled complementary DNA strands were successively hybridized to the immobilized mActin 30-mer and then removed with an NaOH wash. Binding events of complementary DNA followed exponential growth for BIRC over concentrations ranging from 5 nM up to roughly 500 mM with excellent convergence ($R^2 = 0.988$). A noncomplementary probe strand, according to the Watson–Crick model, served as the control and showed no appreciable signal. If it is assumed that hybridization went to completion and 100 % channel-surface coverage was attained, at 3σ, 36 attomol DNA were quantifiable in a 500-pL detection volume.

Ultimately, it would be desirable to screen for an array of different sequences in a label-free manner to avoid perturbations due to the signaling fluorophore or derivation chemistry. A step toward this requires the determination of the discrimination that is possible with BIRC. We used an oligomer consisting of a three-base-pair mismatch (at positions 5, 15, and 25) from that of the complementary mActin strand as an initial test of specificity. The experiment consisted of immobilizing the mActin 30-mer as noted above and a subsequent repetitive hybridization test with a 5-μM solution of the mismatch strand. Figure 4 shows the typical output from one of the binding evaluations. Three trials of these experiments produced an average signal corresponding to greater than 140 % of that for the control and approximately 7 % of the signal for the complementary strand.

In conclusion, the biotin/avidin surface chemistry significantly simplified substrate preparation, was compatible with PDMS, and facilitated free-solution assays with BIRC. Combination of this new surface chemistry with the CCD FFT transduction has allowed improved detection for P_A/IgG binding by two orders of magnitude.[15] BIRC was also used to monitor DNA hybridization over a wide range of concentrations and allowed discrimination in binding of a three-base-pair mismatch. The use of PDMS microfluidic

chips and the exceedingly simple optical train of BIRC represents a cost-effective platform for label-free molecular-interaction determinations as well as for the investigation of reversible biological interactions within subnanoliter volumes and at the attomole level. The methodology and technology are compatible with large-scale integration and should facilitate high-throughput assays.

Received: July 11, 2005
Published online: December 30, 2005

Keywords: biosensors · interferometry · nucleic acids · proteins · surface chemistry

[1] M. A. Cooper, *Anal. Bioanal. Chem.* **2003**, *377*, 834.
[2] F. E. Torres, P. Kuhnt, D. De Bruyker, A. G. Bell, M. V. Wolkin, E. Peeters, J. R. Williamson, G. B. Anderson, G. P. Schmitz, M. I. Recht, S. Schweizer, L. G. Scott, J. H. Ho, S. A. Elrod, P. G. Schultz, R. A. Lerner, R. H. Bruce, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9517.
[3] C. Hansen, S. R. Quake, *Curr. Opin. Struct. Biol.* **2003**, *13*, 538.
[4] S. K. Sia, G. M. Whitesides, *Electrophoresis* **2003**, *24*, 3563.
[5] J. R. Heath, M. E. Phelps, L. Hood, *Mol. Imaging Biol.* **2003**, *5*, 312.
[6] M. Ferrari, *Nat. Rev. Cancer* **2005**, *5*, 161.
[7] J. Hahm, C. M. Lieber, *Nano Lett.* **2004**, *4*, 51.
[8] V. S. Y. Lin, K. Motesharei, K. P. S. Dancil, M. J. Sailor, M. R. Ghadiri, *Science* **1997**, *278*, 840.
[9] K.-P. S. Dancil, D. P. Greiner, M. J. Sailor, *J. Am. Chem. Soc.* **1999**, *121*, 7925.
[10] R. L. Rich, Y. S. N. Day, T. A. Morton, D. G. Myszka, *Anal. Biochem.* **2001**, *296*, 197.
[11] E. A. Smith, W. D. Thomas, L. L. Kiessling, R. M. Corn, *J. Am. Chem. Soc.* **2003**, *125*, 6140.
[12] J. M. Brockman, A. G. Frutos, R. M. Corn, *J. Am. Chem. Soc.* **1999**, *121*, 8044.
[13] G. J. Wegner, N. J. Lee, G. Marriott, R. M. Corn, *Anal. Chem.* **2003**, *75*, 4740.
[14] D. Markov, D. Begari, D. J. Bornhop, *Anal. Chem.* **2002**, *74*, 5438.
[15] D. A. Markov, K. Swinney, D. J. Bornhop, *J. Am. Chem. Soc.* **2004**, *126*, 16659.
[16] K. Swinney, D. Markov, D. J. Bornhop, *Anal. Chem.* **2000**, *72*, 2690.
[17] T. Sakai, K. Shinahara, A. Torimaru, H. Tanaka, Y. Shoyama, K. Matsumoto, *Anal. Sci.* **2004**, *20*, 279.
[18] S. A. Brooks, W. P. Ambrose, W. G. Kuhr, *Anal. Chem.* **1999**, *71*, 2558.
[19] S. A. Brooks, N. Dontha, C. B. Davis, J. K. Stuart, G. O'Neill, W. G. Kuhr, *Anal. Chem.* **2000**, *72*, 3253.
[20] G. M. Whitesides, E. Ostuni, S. Takayama, X. Y. Jiang, D. E. Ingber, *Annu. Rev. Biomed. Eng.* **2001**, *3*, 335.
[21] A. Chilkoti, P. S. Stayton, *J. Am. Chem. Soc.* **1995**, *117*, 10622.
[22] H. S. Sorensen, H. Pranov, N. B. Larsen, D. J. Bornhop, P. E. Andersen, *Anal. Chem.* **2003**, *75*, 1946.
[23] K. Swinney, D. Markov, D. J. Bornhop, *Rev. Sci. Instrum.* **2000**, *71*, 2684.
[24] A. J. Thiel, A. G. Frutos, C. E. Jordan, R. M. Corn, L. M. Smith, *Anal. Chem.* **1997**, *69*, 4948.
[25] Y. W. C. Cao, R. C. Jin, C. A. Mirkin, *Science* **2002**, *297*, 1536.
[26] F. Vinet, P. Chaton, Y. Fouillet, *Microelectron. Eng.* **2002**, *61–62*, 41.

