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High-Speed Spinning-Disk Interferometry on the BioCD for Human Diagnostic Applications

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Abstract—**The BioCD is an optical biosensor that uses high-speed spinning-disk interferometry for direct detection of antigen binding in multiplexed immunoassay protein arrays. The high-frequency sampling suppresses the noise floor and enables a metrology-limited height resolution of 1 picometer for measuring surface-bound protein. In assay protocols there is a direct trade-off between multiplex degree and limit of detection. In this paper, we demonstrate quantitative assays and the scaling properties of direct detection on the BioCD in assays of clinical relevance for human diagnostics, including haptoglobin assays and prostate-specific antigen assays in human patient sera.**

I. Introduction

THE BioCD is a biological compact disk that uses high-speed laser scanning interferometry to detect surface-bound mass on spinning substrates [1]. The high-speed detection moves the sampling frequency far from 1/f noise and provides a noise-floor suppression of approximately 50 dB [2]. The interferometric detection is not digital , but rather uses analog detection in the condition of phase-quadrature interferometry [3]. The potential for highly-multiplexed assays in a compact format makes the BioCD an attractive candidate for point-of-care applications [4].

There are several quadrature classes for interferometric protein detection on spinning discs. The micro-diffraction class [1, 5, 6] uses microstructures on the disc in an approach that is closest to digital compact disks, but that converts the digital readout to a sensitive analog signal. The adaptive optical class [7, 8] uses a nonlinear adaptive optical mixer [9, 10] to phase-lock the signal and reference beams. The phase-contrast class of BioCD [11] uses differential phase contrast laser scanning to detect spatial gradients caused by protein binding. The in-line quadrature class of BioCD [3, 12] uses an interference film on a silicon wafer to directly convert protein phase modulation of light into a detectable signal. In all of these approaches, the presence of molecules on the translating disc surface produces phase modulation that is converted to intensity modulation in the far field through interferometric phase quadrature.

In this paper, the application of the BioCD to clinically-relevant immunoassays is presented for human haptoglobin and for prostate specific antigen (PSA). The detection of PSA down to 1 ng/ml *in serum* is, to our knowledge, the first demonstration at this sensitivity level for a cancer biomarker in the complex protein background of serum achieved by an interferometric biosensor.

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II. Quantitative Haptoglobin Assays

The performance of a BioCD is quantified with a haptoglobin immunoassay. A 96-well format for the BioCD is shown in Fig. 1. There are 9 wells for each concentration. ranging from 1 ng/ml to 10000 ng/ml. The disk is 100 mm in diameter composed of a silicon wafer [12]. Each well is defined and separated from the others by a surface-layer of hydrophobic ink. The well diameter is approximately 4 mm with an 8x8 protein pattern printed in the center of each well. The haptoglobin assay is run on a Quadraspec Assay Development Kit (ADK) which consists of an 8x8 pattern of protein A/G and the antibody IgY. Protein A/G binds the Fc portion of antibodies and orients the antibody for nearly 100% biological activity. The IgY spots perform as non-specific reference spots.

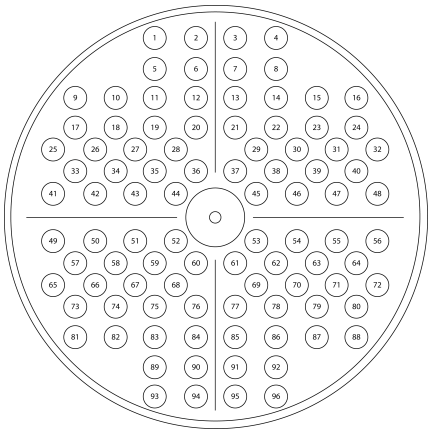

Fig. 1. BioCD 96-well layout with color coded for haptoglobin concentration in an immunoassay to establish a standard curve for detection.

The standard curve for haptoglobin capture is obtained by static incubation for 1 hour. The curve is shown in Fig. 2 from 0.3 ng/ml to 10 ug/ml. Each data point consists of the values from 9 wells, and the error bars are the standard deviation. The curve is a stretched Langmuir function

$$h(nm) = h_{Sat}\frac{[Ag]^e}{(k_D)^e + [Ag]^e} \qquad (1)$$

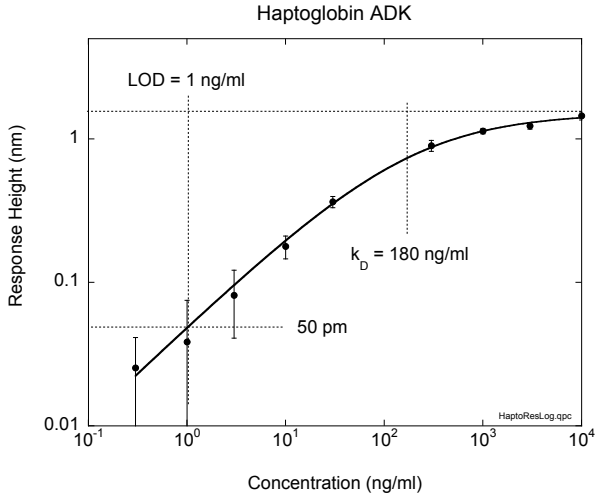

Fig. 2. Standard curve for a Quadraspec Inc. haptoglobin assay development kit (ADK) with protein A/G immobilization.

where k_D is the effective equilibrium constant, h_{Sat} is the original antibody spot height and e is a stretch exponential. The data are fit with a saturation height of 2 nm, an equilibrium constant of k_D = 180 ng/ml and a stretch exponent of e = 0.65. The detection limit in surface height is 50 pm corresponding to a concentration of 1 ng/ml.

Quantitative concentration recovery is obtained from the standard curve by projecting the measured height increment onto the standard curve, then projecting down to the concentration axis. The recovered concentrations are plotted against the applied concentration in Fig. 3. The coefficient of variance (CV) of the recovered concentrations are within 20% between 10 ng/ml and 1000 ng/ml. This combination of performance values gives the performance specification for label-free detection on the BioCD: The limit of detection is approximately 1 ng/ml, with a quantitative dynamic range of 100:1 between 10 ng/ml and 1 ug/ml.

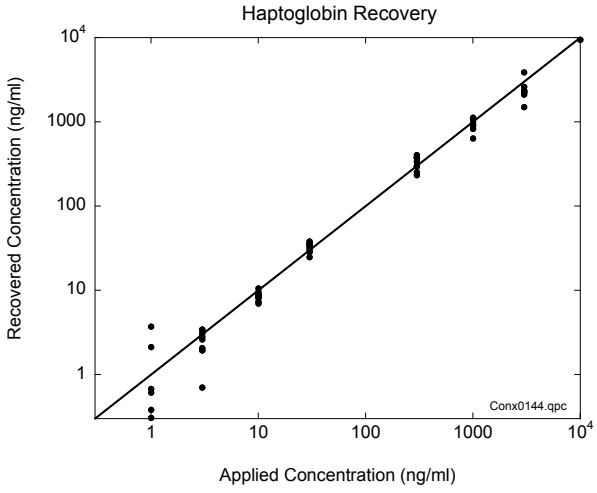

Fig. 3. Haptoglobin concentration recovery from the standard curve. The recovery CV is below 20% in the range from 10 ng/ml to 1000 ng/ml.

III. Prostate-Specific Antigen

Prostate cancer (PCa) accounts for 10% of all deaths from cancer [13]. A major focus of prostate cancer research has been the early detection of PCa using serum biomarkers [14]. The most commonly used biomarker for prostate cancer is prostate specific antigen (PSA), a member of the kallikrein family. PSA in seminal fluid has a concentration of 0.5 mg/mL to 2.0 mg/mL [15, 16]. The early progression of PCa disrupts the basal cell layer and basement membrane in the prostate epithelium, which allows PSA to leak into the peripheral circulation [17, 18]. PSA begins as a 24-amino acid precursor protein called proPSA [19] that is inactive. Seven of the N-terminal amino acids are cleaved by other prostate kallikreins from the proPSA to create the active enzyme that has a single asparagines-linked oligosaccharide and a mass of 33 kDa [20-22]. Additional cleaved isoforms of PSA occur that are called BPSA because they tend to be associated with benign prostatic hyperplasia. These concentrations are decreased in PCa tissue relative to normal prostate [23]. A truncated form of proPSA, called [-2]pPSA, has two extra amino acids relative to the active mature PSA. It is catalytically inactive and circulates as free PSA. Increased concentrations of [-2]pPSA are associated with PCa tissue [24]. Further truncated isoforms of proPSA may also be elevated in PCa tissue [25, 26]. The elevation of the these truncated isoforms is likely to be caused by decreased cleavage of proPSA by hK2 in PCa tissue. These kallikreins hK2 and hK4 may also be useful as markers for PCa [27], as well as the cancer marker Her-2neu.

Most of the PSA that circulates in the peripheral blood is intact and active and forms a 80- to 90-kDa complex with the protease inhibitor alpha1-antichymotrypsin (ACT). This accounts for about 70% to 90% of the PSA in blood [28, 29]. Smaller amounts form complexes with other protease inhibitors. These complexes are "bound" relative to isoforms of PSA that do not form complexes, called free PSA (fPSA). Collectively, the bound and free PSA is called total PSA (tPSA). The fPSA is not bound because of internal cleavages that prevent complexing. The free PSA comprises 10% to 30% of total PSA. The internal cleavages occur by proteases in healthy prostate fluid, and are suppressed in PCa because of disruption of normal function of the prostate tissue. Therefore, the ratio of free to total PSA (fPSA/tPSA, also called the PSA index) is lower in PCa and may be another indicator for discrimination between normal and PCa. A high fraction of fPSA (25% to 95%) is the truncated form [-2]pPSA, which is higher in patients with positive biopsies [24].

Antibodies used in most assays recognize both complexed and free PSA (except for the complex with alpha2-macroglobulin that completely surrounds the PSA) and

hence identify tPSA. While studies in the early 1990s validated the use of total PSA as an indicator of PCa at levels above 4 ng/ml in serum [30], it is not a specific marker to PCa, being normally expressed in high concentrations in healthy prostate tissue. The false negative rate at this concentration threshold is approximately 20% to 50% for men with clinically significant organ-confined PCa [31, 32]. This false negative rate is an area of significant concern and has motivated the search for alternative tests.

A. Endpoint Assay and Scaling

To establish the utility of the BioCD for prostate cancer applications, we performed an end-point assay at a concentration of 10 ng/ml on a single-well BioCD with 25,000 spots [33]. Half the spots were anti-PSA, while the others were IgY reference spots. These two types of spot were arranged in a 2x2 unit cell with specific antibodies and non-specific reference spots on opposite diagonals. The assay was run in a label-free format (printed antibodies binding PSA antigen out of solution) followed by a second sandwich antibody to act as a mass tag and to help discriminate against non-specific binding. The histogram of the spot height increments is shown in Fig. 4 after the sandwich step. The spot height increase is approximately 0.5 nm with a standard deviation of 50 picometers.

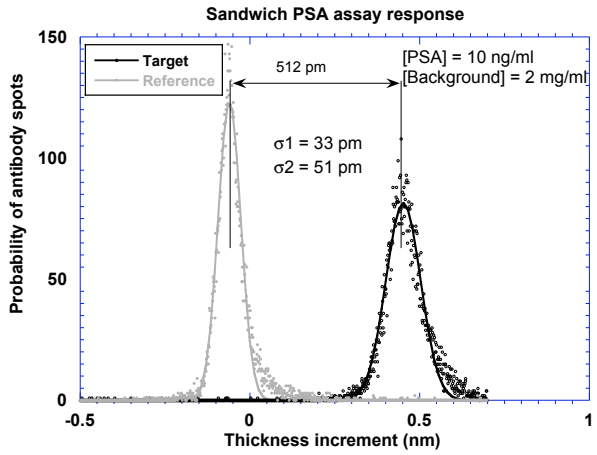

Fig. 4. Histogram of an IgG sandwich assay for 10 ng/ml prostate specific antigen in a 2 mg/ml protein background. The histogram contains the response of over 10,000 antibody spots. The mean surface increment is 0.5 nm for antigen plus second antibody.

The scaling of the limit of detection as a function of the number of antibody spots contained in an assay is calculated by combining the endpoint assay with the standard deviation of the spot height. The results, given in terms of the minimum detectable concentration, are shown in Fig. 5 as a function of the number of spots per assay. The PSA assay is compared against an anti-rabbit assay, and both are given for label-free detection (printed antibody binding an antigen) and for a sandwich (applying a second antibody to bind the antigen). Label-free PSA detection for a single spot is 8 ng/ml, and improves to 300 pg/ml for 1000 spots with a square-root of N scaling. The sandwich assay has a minimum detectable concentration of 30 pg/ml for 1000 spots per assay. Rabbit label-free assays outperform the label-free PSA assay, but the rabbit sandwich is not as sensitive as the PSA sandwich. This is in part due to the 35 kDa size of the PSA antigen, which gives a weaker label-free signal, but the PSA antibodies have a much higher affinity than anti-rabbit antibodies.

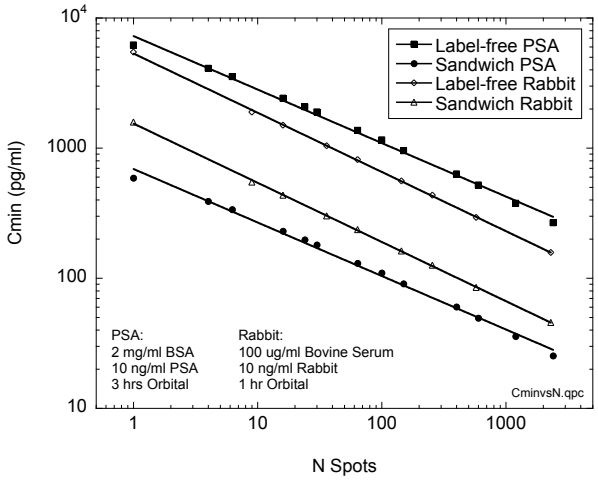

Fig. 5. Limit of detection (LOD) in pg/ml as a function of the number of protein spots per assay for PSA and for a Rabbit positive control. The two sets are for label-free and sandwich detection.

B. Standard Curves and Performance in Serum

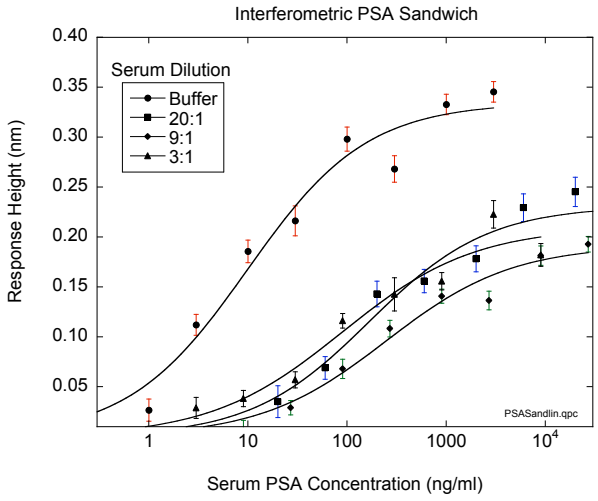

Fig. 6 Standard curves for PSA detection in a serum background. The top curve is for antigen in buffer, while the three lower curves are in increasingly concentrated serum. The 3:1 dilution response is within the same range as the 20:1 dilution, showing good rejection of nonspecific serum protein binding.

Concentration recovery for the PSA assay requires a standard curve in serum, shown in Fig. 6, with PSA spiked into serum dilutions at ratios of 20:1, 9:1 and 3:1. While the buffer performance is better, all serum dilutions generated similar standard curves, demonstrating the ability of the BioCD to compensate for nonspecific serum binding. The ability to perform assays down to 1 ng/ml in the complex protein background of serum is an important advance for interferometric biosensors. While normal ranges of PSA in serum are below 4 ng/ml, disease levels can approach µg/ml concentrations, which are easily in the range of this measurement.

These results show the potential for the BioCD for human diagnostic applications. By trading off number of assays per disc against limit of detection, many levels of assay multiplexing are available.

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