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Photonic Crystal Enhanced Cytokine Immunoassay

Patrick C. Mathias, Nikhil Ganesh, and Brian T. Cunningham, *Senior Member, IEEE*

Abstract— **Photonic crystal surfaces are demonstrated as a means for enhancing the detection sensitivity and resolution for assays that use a fluorescent tag to quantify the concentration of an analyte protein molecule in a liquid test sample. Computer modeling of the spatial distribution of resonantly coupled electromagnetic fields on the photonic crystal surface are used to estimate the magnitude of enhancement factor compared to performing the same fluorescent assay on a plain glass surface, and the photonic crystal structure is fabricated and tested to experimentally verify the performance using a sandwich immunoassay for the protein Tumor Necrosis Factor-alpha (TNF-α). The demonstrated photonic crystal fabrication method utilizes a nanoreplica molding technique that allows for large-area inexpensive fabrication of the structure in a format that is compatible with confocal microarray laser scanners. The signal-to-noise ratio for fluorescent spots on the photonic crystal is increased by at least five-fold relative to the glass slide, allowing a TNF-α concentration of 1.6 pg/ml to be distinguished from noise on a photonic crystal surface. In addition, the minimum quantitative limit of detection on the photonic crystal surface is one-third the limit on the glass slide – a decrease from 18 pg/ml to 6 pg/ml. The increased performance of the immunoassay allows for more accurate quantitation of physiologically relevant concentrations of TNF-α in a protein microarray format that can be expanded to multiple cytokines.**

The simultaneous quantitation of multiple proteins promises to aid researchers seeking to understand protein interaction networks and may potentially be clinically useful for diagnosis and prognosis with serum biomarkers[1]. One approach to multiplexed protein detection has been an adaptation of the DNA microarray format to immunoassays. Fluorescence-based protein microarrays have demonstrated detection limits comparable to their enzyme-based counterparts, enzyme linked immunosorbent assays (ELISA), while measuring multiple proteins within each array. These protein microarrays have been adapted and optimized for detection of cancer biomarkers[2] and cytokines[3-5]. Cytokines are a particularly promising class of analytes for multiplexed detection because they rarely act alone and rely on the upregulation or downregulation of multiple cytokines simultaneously to achieve a particular physiological effect. Cytokines are associated with immune responses to infection, but may be associated with non-infectious diseases. Because the immune system is integrated with other physiological systems such as the cardiovascular and gastrointestinal systems and cytokines often act as a signaling system throughout the body, these proteins may be a valuable tool in understanding and diagnosing disease. While protein microarrays on optically passive surfaces such as glass slides have been useful in multiplexed cytokine quantitation, the utility of these arrays can be expanded by a more accurate determination of protein levels as well as lowered limits of detection. In this work, we demonstrate how photonic crystal (PC) surfaces can be used to achieve improved detection sensitivity and more accurate quantification of a representative protein biomarker compared to performing the same immunoassay on a glass surface.

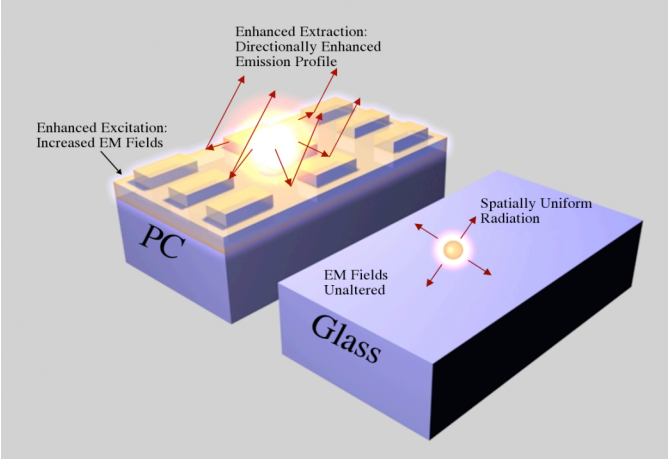

Fig. 1. Schematic of enhanced fluorescence mechanisms observed on the photonic crystal (PC). When the laser excitation wavelength aligns with the PC resonance wavelength, enhanced electromagnetic (EM) fields are observed throughout the PC. This enhanced excitation effect will excite fluorophores more strongly than if they were situated above an optically passive structure such as glass. Furthermore, if the fluorophore emission wavelengths overlap the PC resonance wavelengths, light emitted from fluorophores can be redirected toward the photon detection instrumentation – this is the basis of enhanced extraction.

The PCs used in this work are nanostructures comprised of a periodically modulated low refractive index plastic/SiO_2 surface structure that is coated with a high refractive index dielectric. The purpose of the structure is to provide an efficient optical resonator, as described in previous research[6-9], but summarized briefly here. The periodically modulated dielectric structure of the PC functions as a

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B.T. Cunningham is with the University of Illinois at Urbana-Champaign, Department of Electrical and Computer Engineering (phone: 217-265-6291;; e-mail: bcunning@illinois.edu).

P.C. Mathias is with the University of Illinois at Urbana-Champaign, Department of Bioengineering.

N. Ganesh is with the University of Illinois at Urbana-Champaign, Department of Material Science and Engineering.

reflective optical filter, where only particular wavelength/incident angle combinations interact strongly with the structure, resulting in highly efficient reflection, while all other wavelength/incident angle combinations are transmitted through. Unlike a conventional diffraction grating, only the zeroth-order reflected and transmitted waves can propagate., while higher order modes are cut off. However, the PC supports leaky eigenmodes to which higher diffracted orders can couple through phase-matching. The leaky eigenmodes re-radiate into the reflected direction in phase with the reflected zeroth-order wave, leading to constructive interference. Likewise, the leaky eigenmode re-radiation into the transmitted direction is out-of-phase with the transmitted zeroth-order wave, resulting in destructive interference[10]. This phenomenon is observed under broadband illumination as a highly efficient reflection at a wavelength that fulfills the phase-matching criteria, referred to as the resonant wavelength. The phase-matching condition is also dependent on the incident angle of the external illumination, so each incident angle can have one or more distinct resonant wavelengths. This resonance effect, termed guided-mode resonance, has been exploited to design highly efficient narrowband optical filters[11] as well as label-free optical biosensors[12].

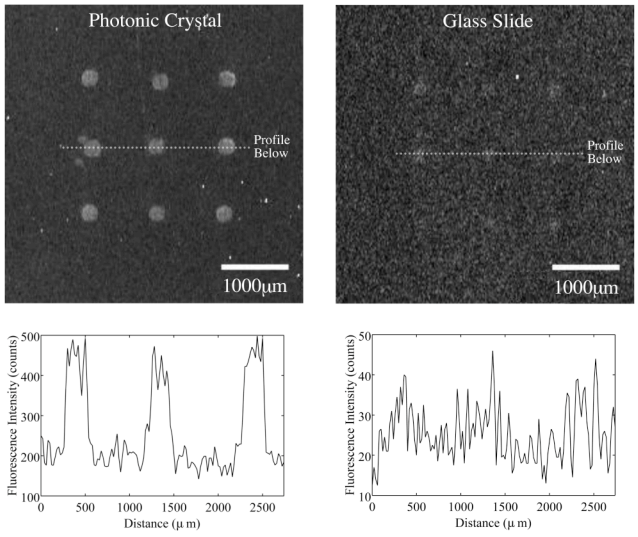

Fig. 2. Fluorescence images and associated line profiles from the PC and glass immunoassays at a concentration of 1.6 pg/ml. The fluorescence images are contrast-adjusted for better visualization of the spots. The PC signal-to-noise ratio is approximately 8 times higher than the ratio for the glass slide immunoassay spots.

The leaky eigenmodes are highly localized within and in direct proximity to the PC surface, and a large energy density is observed at these locations in the form of enhanced electromagnetic fields throughout the structure. The intensity of a fluorophore's emission is proportional to the electric field intensity (which is proportional to the square of the electric field) of the light exciting the molecule, so an enhanced electric field will enhance the excitation of fluorophores close to the device surface. Enhanced excitation works selectively in regions within close proximity to the PC surface due to an exponential decay in the electric field intensity from the PC into the superstrate (region above the PC). Thus enhanced excitation of the PC shares one of the advantages of total internal-reflection fluorescence (TIRF) microscopy because fluorophores close the substrate surface are selectively excited[13]. Rather than utilizing a TIRF microscope, a conventional confocal microarray scanner can be utilized for rapid imaging of large areas. Using PC enhanced excitation, we have demonstrated fluorescence enhancement from the fluorescent dye Cyanine-5[6] and detailed the spectral characteristics of the PC-fluorophore interaction[9] as well as the dependence on distance from the PC on enhanced excitation[8].

While enhanced excitation can be observed when the laser light incident on the PC is spectrally aligned with the resonance wavelength, another enhancement effect can be observed when the fluorophore emission wavelengths overlap the resonant wavelengths of the PC. Enhanced extraction occurs when light emitted by fluorophores couples into leaky eigenmodes and is re-radiated such that it can be easily detected by the measurement instrumentation. Essentially, the PC causes some proportion of photons that would normally radiate through the substrate to be reoriented toward the direction of incident light. Because the same optics that illuminate the sample are responsible for collecting emitted light, this phenomenon allows a higher proportion of the emitted photons to be measured relative to a passive substrate such as glass. PCs are designed to exhibit resonances at an excitation wavelength close to the emission wavelength (typically within 50 nm wavelength); because the resonances continuously span many wavelengths over a range of angles, the eigenmodes will overlap both the excitation and the emission wavelengths. Thus a PC designed for enhanced excitation will perform enhanced extraction as well, and both effects are summarized in Figure 1. The combination of these two effects has been used to enhance the fluorescence from semiconductor quantum dots[7] with a magnification factor of 8x for the excitation effect and 13x for the extraction effect, for an overall sensitivity enhancement of 108x.

In this work, we perform a microspot fluorescence immunoassay for the cytokine TNF-α simultaneously on glass slides and PC surfaces under identical experimental conditions to evaluate the impact of enhanced fluorescence on the assay. The PC used in this work is similar to a combined label-free biosensor and enhanced fluorescence device described previously[6]. While this PC is capable of label-free detection of proteins that could enable spot density quantitation, we focus on the impact of enhanced fluorescence on the signal-to-noise ratio (SNR) of the assay since this can allow more accurate quantitation of protein levels at the lowest concentrations assayed. The optical properties of the PC are explored by computer modeling to predict the magnitude of the enhanced excitation effect, and for comparison with experimental measurements. Using a nanoreplica molding process, PCs the size of microscope slides are fabricated for compatibility with commercial microarray spotters and scanners. A layer of SiO_2 is added to the PC so an identical surface chemistry interaction can be achieved on both the PC and the glass slide. A microspot

immunoassay is performed on both substrates using a fluorescent Cyanine-5 label. By evaluating the immunoassay over a concentration series on glass and PCs, the impact of PC enhanced fluorescence on the assay resolution and detection limit is assessed.

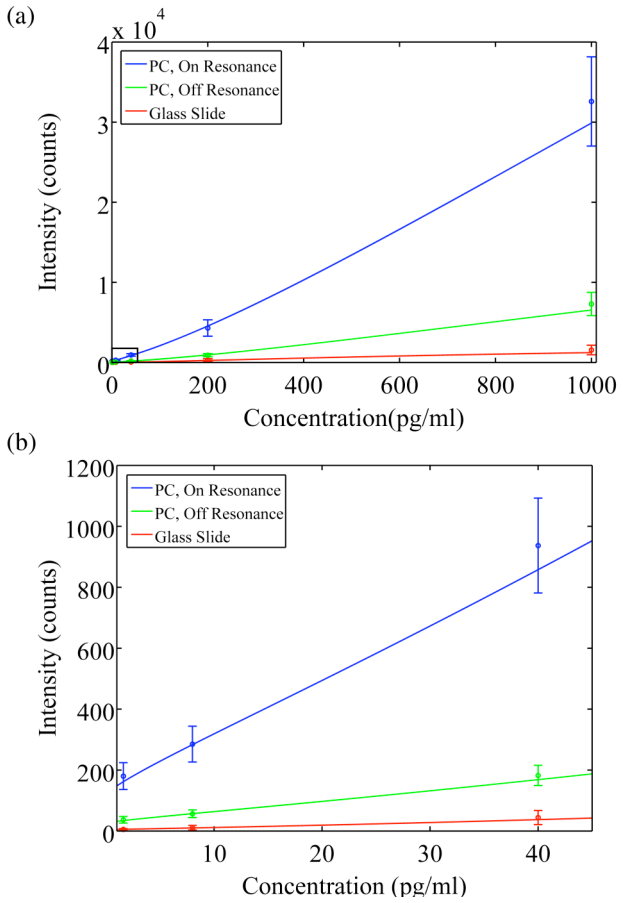

Fig. 3. (a) Net signal intensity as a function of TNF-α concentration for the immunoassay performed on the PC on resonance, the PC off resonance, and the glass slide. The data points and error bars are included with their fitted logistic curves. (b) The data from (a) of the three lowest assay concentrations.

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