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Investigating low volume planar surface fluorescent immunoassays with QDs for spatial and spectral multiplexing

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ABSTRACT

The simultaneous detection of two analytes, chicken IgY (IgG) and Staphylococcal enterotoxin B (SEB), in a single spot of a planar surface fluorescent immunoassay was demonstrated using luminescent semiconductor quantum dot nanocrystal (QD) tracers. This spectral multiplexing is made possible by employing one laser to serve as the excitation source for detecting two distinct QD signals from different analytes. Spatial multiplexing – detection of two analytes in different regions of the planar surface – was also demonstrated using fluid handling constructs that allowed for very low solution volumes when performing the assay. The limits of detection for detecting both chicken IgG and SEB within a single spot were 25 ng/mL and 1.6 ng/mL, respectively. A novel slide reading detection platform involving evanescent wave excitation of a planar surface was created in-house for assay measurement. This platform is potentially more amenable to a point-of-care (POC) environment than conventional slide readers because of its modular design architecture and lack of moving parts. The result is a platform which is simple to repair using primarily off-the-shelf consumer components.

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1. Introduction

Antibodies demonstrate the highest levels of specificity and affinity compared to other synthetically produced molecules such as aptamers and molecularly imprinted polymers, so they are often used for conducting immunoassays [1]. It has been demonstrated that up to nine antibodies can be spotted within a single well of a 96-well microtiter plate in a microELISA for detection of multiplex analytes simultaneously by spatially separating the sandwich ELISAs within a single well [2,3]. However, five of these nine assays were statistically different when compared to individually-run assays. Another approach to multiplexing for optical-based sensing relies on spectral resolution within a single spot or location, however, this can be limited by the inherent properties of the detection labels, for example broad overlapping emission profiles among common organic dye labels. [1,4]. In order to circumvent this problem and avoid creating complicated assays, luminescent semiconductor quantum dot nanocrystals (QDs) can be used because of their distinct, very narrow emission profiles. QDs are made up of nanometer-sized semiconductor materials and their size dictates their inherent and unique optical and electrical properties [5]. QDs are valuable for simplifying immunoassay platform design because only one excitation source is needed to excite multiple colored QDs. A variety of different QDs can be excited using the same excitation wavelength because they have broad excitation bandwidths, yet they have narrow emission bandwidths, with the emission wavelengths very specific to the type and size of QD core material [6]. QDs have been applied for various sensing purposes. In one example, Wegner et al. demonstrated spectrally-multiplexed sandwich immunoassays with a simple readout using two QDs for diagnosis of low-volume serum samples [7]. Also Geißler et al. achieved a six-color biosensor (using various dyes, none of which are QDs) to detect five different antibody binding events, which may be relevant for distinguishing small-cell from non-small-cell lung carcinoma [8]. This is an example of an homogeneous in-solution assay that achieved spectral multiplexing within a single sample and demonstrated the need for deconvolution of spectral overlap or cross-talk.

The goal of this work was to design a detection platform capable of detecting multiple QDs and therefore analytes simultaneously using an immunoassay, in a way that is amenable to a point-of-care (POC) environment. In order to accomplish this, we first developed

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low-volume, spatially-multiplexed planar surface fluorescent immunoassays and optimized them using Cy5 dye for detection. To build upon these initial studies and our previous success of spectral multiplexing using a 96-well plate, two different QDs were used to detect two proteins within a single spot of a planar surface fluorescent immunoassay. The analytes used here and in previous studies were chicken IgG and Staphylococcal enterotoxin B (SEB) because they have been shown to be highly selective and therefore have no cross-reactivity [1,9]. SEB, a toxin implicated in food poisoning, is an analyte of public health concern and is used to challenge many sensing platforms [10–12]. Consequently, use of this analyte allows for comparison of performance characteristics of the developed platform to those already published. The Gene Pix 4000B slide scanner used for imaging planar surface fluorescent immunoassays using Cy5 dye is not suitable for investigation of QDs because the excitation wavelengths and emission filters cannot be tuned. As a result, an in-house developed detection system involving an evanescent waveguide was used to image the slides using QD tracers. This technique marks the first step in transitioning the planar surface fluorescent immunoassay from a traditional bench-top laboratory technique to a field-deployable device.

2. Materials and methods

2.1. Materials

Staphylococcal enterotoxin B (SEB) and affinity purified rabbit anti-SEB (Rb-anti-SEB) were purchased from Toxin Technology Inc. (Sarasota, FL). Rabbit anti-chicken IgY (Rb-anti-chicken IgG) and chicken IgG were purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA). Phosphate buffered saline (PBS); phosphate buffered saline with Tween (PBST); methanol, potassium hydroxide; toluene; ethanol; dimethyl sulfoxide (DMSO), (3-mercaptopropyl)triethoxysilane; 4-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS); J. Melvin Freed Brand Microscope Slides, Plain; Biotin, and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (St. Louis, MO). Millipore Amicon® Ultra centrifugal filter devices 100 kDa were obtained from Millipore Corporation (Billerica, MA). Zebra™ desalt spin columns were purchased from Pierce Biotechnology, Inc., part of Thermo Fisher Scientific Inc (Rockford, IL). Amersham™ Cy™5 monoreactive dye was obtained from GE Healthcare Bio-Sciences Corp. The quantum dot nanocrystals (QD) eF605-maleimide were a gift from our collaborators eBiosciences (San Diego, CA), along with their proprietary Conjugation and Purification Buffers. Doubly distilled water (ddW) was used throughout the experiments and was prepared in house using a Barnstead Nanopure Diamond™ water purification system (Dubuque, IA). The 800 nm QDs (Qdot® 800 ITK™ Streptavidin (SA) Conjugate Kit) were supplied by Invitrogen (Grand Island, NY). An 800 nm bandpass filter (85% transmission, 12.5 mm diameter, >93% transmission) and a 605 nm bandpass filter (15 nm bandpass, 12.5 mm diameter, >93% transmission) were obtained from Edmund Optics (Barrington, NJ). Impact-Resistant Polycarbonate was obtained from McMaster-Carr (Robbinsville, NJ). A 438 nm PN 156 1 W laser was obtained from Hangzhou BrandNew Technology Co., Ltd. (Zhejang, China). A Mead Deep Sky Imager PRO III CCD camera was obtained from Adirondack Video Astronomy (Hudson Falls, NY). Clear acrylic was obtained from Piedmont Plastics (Elkridge, MD). Fluid handling chips were designed in CorelDraw X4 (Corel Corp., Ontario, Canada) and micromachined using a computer controlled Epilog Legend CO_2 65 W laser cutter (Epilog, Golden, CO). 3M 9770 adhesive transfer tape was used to hold together the layers of the planar surface fluorescent immunoassay. A REGLO Digital pump, appropriate connectors, and tubing were obtained from Ismatec (Wertheim, Germany). Appropriate connectors for the tubing were obtained from Cole Parmer (Vermon Hills, IL). An Amersham Biosciences Ultrospec 2100 Pro UV/Visible Spectrophotometer (GE Healthcare, Piscataway, NJ, USA) was used to determine the final concentration of the biotinylated Rb-anti-SEB. CCD image intensities were analyzed using ImageJ software, developed and distributed freely by NIH (http://rsb.info.nih.gov/ij/download.htmL). Data were analyzed using Microsoft Excel (Microsoft, Redmond, WA). A GenePix 4000B Microarray Scanner (Molecular Devices; CA) was used to image the slides and GenePix Pro, the acquisition and analysis software, was used to capture the signal from Cy5 for spatial separation and low volume assays only.

2.2. Fluid handling constructs – Epilog printer templates

In this study, novel stencils were created to allow for patterning of capture antibodies onto the surface and performing sandwich assays. A multi-layer system was designed and fabricated in-house to allow the patterning of protein arrays and the performance of protein assays. The system was designed in CorelDRAW 14 (Corel Corp., Ontario, Canada) and then laser-machined from various materials, including vinyl, polycarbonate (McMaster-Carr), and acrylic (Piedmont Plastics) using a computer-controlled Epilog Legend CO_2 65 W laser cutter (Epilog, Golden, CO). A schematic and photographic representation of the multi-layer system is shown in Fig. 1, where A represents the system used for patterning antibodies onto the surface and B represents the system used for performing the assay. The channels that were used for patterning and conducting the assay were made of vinyl and had dimensions of 50 mm × 1 mm and 18 mm × 1 mm, respectively and a thickness of 0.25 mm (Fig. 1). Double-sided tape was placed on one side of the vinyl and acrylic templates to allow for manual application onto microscope glass slides (25 mm × 75 mm × 1 mm). Templates were cleaned using Kimwipes® disposable wipers and ddW to remove debris prior to application.

2.3. Surface functionalization

A variety of immobilization chemistries were initially tested for antibody immobilization to the planar glass surface. The best-performing chemistry, thiol-silane with a GMBS cross-linker, was selected for further study (described in more detail in the supplementary information). In order to create this immobilization chemistry, the first step was to score plain microscope glass slides using a diamond-tip tool to create a label on the edge of the slide to allow for subsequent identification. The slides were cleaned using Kimwipes® disposable wipers and ddW. Slides were placed in a slide holder and immersed in 10% KOH in methanol solution (5 g KOH in 50 mL methanol, which was mixed using a magnetic stirrer). Following incubation at RT for 30 min, slides were rinsed in DI water and ddW, dried, and placed in a new slide holder. Slides were placed in a 2% silane solution prepared in toluene (1 mL silane in 50 mL toluene) and allowed to incubate for 1 h at RT. Slides were rinsed with toluene using a glass pipette. A 2 mM solution of GMBS cross-linker was prepared (12.5 mg was placed in 250 μL dimethyl sulfoxide; DMSO) and vortexed and then mixed into 50 mL ethanol. Slides were immersed in this solution for 30 min at RT. The patterning template had to be attached to the slide surface and exposed to the patterning antibody within 2 h in order to ensure that the chemistry remained active for covalent binding. In order to manually force out air bubbles between the slide and the vinyl patterning template, a protective layer of polycarbonate was placed on top of the template and a plastic tool was used to apply pressure on the surface in a sliding motion. The next template layer (polycarbonate) was assembled and air bubbles were removed using this same