Biosensors and Bioelectronics 24 (2009) 3467–3474

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A PMMA microcapillary quantum dot linked immunosorbent assay (QLISA)

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ARTICLE INFO

Article history:
Received 7 February 2009
Received in revised form 28 April 2009
Accepted 28 April 2009
Available online 6 May 2009

Keywords:
Quantum dots
ELISA
MPO
Inflammation
IBD
Microfluidic
Biosensor
QLISA
PMMA capillary

ABSTRACT

The development of a simple and inexpensive quantum dot based immunoassay for detecting myeloperoxidase (MPO) in stool samples is reported (QLISA). The method developed utilizes readily available polymethylmethacrylate (PMMA) microcapillaries as substrates for performing the sandwich assay. High power (80 mW) and low power (10 mW) UV-LEDs were tested for their efficiency in maximizing detection sensitivity in a waveguide illumination or a side illumination mode. The results obtained indicate that both waveguide and side illumination modes can be employed for detecting MPO down to 15 ng/mL, however the high power LED in a side illumination mode improves sensitivity and simplifies the data acquisition process. The protocol and sensor robustness was evaluated with animal stool samples spiked with MPO and the results indicate that the sensitivity of detection is not compromised when used in stool samples. The effect of the ionic strength of the environment on the fluorescence stability of quantum dots was evaluated and found to affect the assay only if long imaging times are employed. Replacing the buffer with glycerol during imaging increased the fluorescence intensity of quantum dots while significantly minimized the loss in intensity even after 2 h.

1. Introduction

Capillary based assays present several advantages over conventional 96 well plate methods, including small amount of analytes and proportionally less volume of required reagents. The confluence of developments in nano-fluidic handling systems has enabled capillary based microreactors and sensors to be employed in high throughput environments (Meldrum et al., 2000). However, the cylindrical nature of the capillary geometry does pose several challenges in the ability to properly couple and collect light (for colorimetric or fluorometric assays), thus limiting the final sensitivity of capillary based assay detection (Du et al., 2007). Some of these challenges can be addressed by more powerful and sensitive optics and so far capillary assays have achieved sensitivity of femtomolar detection (Halsall et al., 1988) with use of expensive, customized optical systems or electrochemical instruments. In this paper we report the design and implementation of an inexpensive, capillary based assay able to detect myeloperoxidase (MPO) at 100 pM sensitivity in a total volume of 1 μL.

Free standing capillary tubes offer superior simplicity in manufacturing and handling compared to developing a full scale lab-on-a-chip type device. Capillaries have already been used as immunosensors for detecting trace amounts of explosives (Narang et al., 1997, 1998), as high throughput automated genome analysis systems (Meldrum et al., 2000), as drug assays, for example in measuring paclitaxel in blood plasma (Sheikh et al., 2000), and even for the detection of helicobacter hepaticus (Thomas et al., 2007) that causes hepatitis in mice. The examples cited above are mostly immunoassays in conjunction with fluorescence spectroscopy. Immunoassays used for the detection of various biomolecules and biochemicals, rely on the interaction between an antigen and its antibody and possess high specificity depending on the antibody/antigen interaction. This specificity allows development of assays detecting multiple analytes in one capillary (Misiakos and Kakabakos, 1998). Enzyme linked immunosorbent assay (ELISA) is a common bioassay that relies on the antigen–antibody specificity and chemistry, with signal amplification capabilities. In a conventional ELISA technique sensing is mostly accomplished by chemiluminescence (Beumer et al., 1991), although both colorimetric titration (Esterbauer, 1996; Walenga and Fareed, 1994), and fluorescence can be used (Garvey et al., 1987; Savige et al., 1998; Smith and Eremin, 2008). Fluorescence based ELISA has the capability to detect more than one antigen or antibody by multiplexing. Multiplexing, although an elegant way to detect multiple markers, has remained thus far a challenge due to bleed through in the emission bands, the requirement of multiple excitation and emission filter pairs, the low fluorescence life time of fluorophores and the need of high power light sources. In sophisticated flow cytometry (FACS) systems such bleed through has been corrected by software

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doi:10.1016/j.bios.2009.04.043

but this necessitates complicated data analysis and expensive instruments. Recent developments in quantum dots (QDs) enable significant reduction in photo bleaching due to the unique optical properties of these semiconductor nanocrystals. These properties include single excitation maxima irrespective of emission maxima, and narrow emission spectra which allow multiplexing without any bleed through. Quantum dots have found significant applications in biology especially in live cell imaging (Thurn et al., 2007) to follow and understand signaling pathways (Hernandez-Sanchez et al., 2006). Although commercially available QDs are expensive on a per lb basis, the ability to carry out reactions in nano to microliter volumes coupled with moderately sensitive CCD cameras or photon counters can produce a cost effective assay; the raw material costs per unit mass remain low due to the small amount of QDs necessary to carry out the assay.

Both glass and polymer based capillaries have been used to carry out immunoassays. Specifically fused silica (Bange et al., 2005), polystyrene (Misiakos and Kakabakos, 1998), polymethylpentene (Mastichiadis et al., 2002), and polymethylmethacrylate (PMMA) (Petrou et al., 2002) have been used to fabricate capillary biosensors for biomarker detection in volumes ranging from 0.5 to 5 μL. Polymeric capillaries are of particular interest due to readily available functional groups on their surface offering an appropriate substrate for immobilizing antibodies or antigens. Furthermore, recent developments in photochemical methods (McCarley et al., 2005; Wei et al., 2005) of functionalizing polymeric materials provide compelling reasons to choose polymeric capillaries over fused silica or glass capillaries. Several strategies have been devised to detect low concentrations of antigens by solid phase immunoassay in capillaries, primarily focusing on excitation of fluorophores followed by collection of the emitted photons. One such approach takes advantage of the evanescent field at the interface of the polymer/liquid interface for exciting the fluorophores; this particular method requires the material of the capillary to function as a waveguide (Tao et al., 2007). The excellent optical properties of PMMA have allowed use of PMMA capillaries as waveguides and fabrication of sensors to measure optical rotation of chiral molecules (Cox et al., 2006).

We are reporting here a low cost PMMA microcapillary biosensor using QDs as the fluorescent probe for detection of picomolar quantities of analytes, and demonstrating this capability by detecting myeloperoxidase. Our selection of PMMA was based on its optical properties and the capability to selectively functionalize its surface for antibody immobilization. Capillary dimensions of 250 μm I.D. and 2.5 cm long allow us to use a volume of ~1 μL, and these capillaries are commercially available. The high quantum yield of QDs coupled with the ability to excite QDs that emit at different wavelengths with a single UV light guided our choice of reporter probes. The inexpensive capillary based immunofluorescent assay described below was used for detecting and estimating the concentration of myeloperoxidase, an inflammatory marker over-expressed in inflammatory diseases including those of the gastrointestinal tract. Our initial results indicate that it is indeed possible to develop a low cost, robust imunnofluorescence sensor capable of operating with 1–2 μL of analyte and detecting subnanomolar concentrations. The methodology and design used in this paper offer a platform approach for capillary immunoassay development where further improvements in sensitivity can be readily implemented. However, for many clinical applications the sensitivity demonstrated by our data could be adequate to distinguish diseased from healthy individuals. Improvements to increase sensitivity are possible both by chemistry optimization approaches as well as with more elaborate optics. The focus in this paper has been on a low cost easy to deploy assay. The two main reasons for using capillary instead of 96 well plates were; (1) to reduce the volume of sample used and the volume of QD-Ab conjugate from the perspective of economics of the assay, (2) be able to multiplex and (3) be able to detect a biomarker from sources such as swab tests. The reduction in volume allows us to effectively utilize the superior optical properties of QDs without increasing the cost of the assay, and by successfully using QDs we can perform multiplexing with a relatively inexpensive optical set up. Such low quantity sampling methods are not feasible with current ELISA protocols.

2. Materials and methods

Our approach for detecting MPO is a sandwich assay depicted in the flow chart of Fig. 1. Briefly, a polyclonal MPO antibody (pAb) is immobilized on the inner surface of a PMMA capillary (capture antibody). MPO (from the sample of interest) is captured by this pAb and immobilized on the surface. Addition of a QD-mAb complex allows MPO detection by fluorescence.

2.1. Capillary functionalization and MPO assay

PMMA capillaries (pCaps) were selected for the development of this sensitive assay due to the readily available functional groups on the surface of PMMA and its excellent optical properties. Capillaries used in this study were obtained from Paradigm Optics Inc. pCaps (O.D. 500 μm, I.D. 250 μm) were cut into 3 cm long pieces before or after functionalization depending on the experiment and held straight using a custom built spring loaded holder. This eliminated the natural tendency of PMMA capillaries to "buckle". Functionalization of pCaps was carried out by following alkaline ester hydrolysis of methacrylate, a method developed by Bai et al. (2006) for functionalizing PMMA. The method was modified slightly, 1N NaOH at 60 °C was pumped through the pCap using a peristaltic pump (100 μL/min) for 1 h followed by washing with 1× PBS buffer (pH 7.4). This step hydrolyzes the acrylate ester group on the surface of the pCap resulting in COOH termination that is crucial for covalently bonding the MPO antibody to the inner walls of the capillary. A rabbit anti-human polyclonal MPO antibody was purchased from ABD Serotec, Raleigh, NC, USA. Functionalized pCaps were then treated with EDC/NHS (104.7 mM EDC 21.7 mM NHS) (McCarley et al., 2005) for 5 h followed by loading the MPO capture antibody using a concentration of 100 nM. Optimal immobilization of the polyclonal MPO antibody on the inner surface of the pCap was accomplished by incubation at 4 °C for 16 h. Non-immobilized antibodies were then removed from the capillary by washing with a buffer containing 0.1% Tween and 0.03% sodium azide (purchased from Sigma–Aldrich) in 1× PBS at pH 7.4. Subsequently, a blocking buffer containing 2% FBS in 1× PBS buffer was introduced into the capillaries to reduce nonspecific binding of proteins, and excess blocking buffer was washed away with the same wash buffer. The desired analyte, 1 μL of pure MPO (LEE Biosolutions, St. Louis, Missouri, USA) or properly prepared animal sample was then introduced into the pAb immobilized capillaries with the aid of a Hamilton septum adapter and allowed to interact with the pAb for 1 h at room temperature followed by injection of wash buffer at a flow rate of 50 μL/min. Monoclonal anti-human Myeloperoxidase antibody (mAb) conjugated to amine terminated QDs (λ_{em} = 605 nm, from Invitrogen) was used as the reporter molecule. Conjugation of QDs to mAb was carried out by following the protocol provided by Invitrogen. Both MPO and the mAb were purchased from Lee Biosolutions Inc. QD conjugated mAb (QD-Ab) at 100 nM concentration was then introduced into the pCap, incubated at room temperature for 1 h followed by washing with the wash buffer. The intensity of the QD-Ab from the capillaries was obtained by the optical set up shown in Fig. 2b and described in detail below. Several capillaries were imaged at various steps of the process to evaluate and optimize the immobilization