ON-CHIP PROTEIN ASSAYS USING MICROBEAD ARRAYS: AN INTEGRATED SYSTEM FOR SALIVARY-BASED CLINICAL DIAGNOSTICS

W.H. Henley, P.D. Dennis, E.A. Oblath, J.P. Alarie, and J.M. Ramsey
The University of North Carolina at Chapel Hill, USA

ABSTRACT

A microfluidic device containing arrays of microwells filled with microbead sensors was used to perform protein assays in a saliva sample. Techniques for the fabrication of fully integrated arrays of microwells within polydimethylsiloxane (PDMS) microchannels were demonstrated. Subsequent microwell loading with stochastic arrays of encoded microbeads was performed and the devices were used for multiplexed immunoassays. Low femtomolar limits of detection (LOD) for cytokines indicative of pulmonary inflammation have been demonstrated. Device fabrication and optimization experiments for 900-well microbead array devices demonstrating rapid, highly sensitive protein assays are presented.

KEYWORDS: Immunoassay, Cytokine, Protein Assay, Microbead array, Salivary diagnostics

INTRODUCTION

We have developed an easily fabricated microfluidic system with an integrated array of microbeads for the detection of cytokines indicative of inflammation in saliva by a sandwich immunoassay. Immunoassays require minimal sample pretreatment yet provide highly specific detection for analytes in complex biological matrices.[1] While single immunoassay platforms can be clinically useful, the simultaneous analysis of multiple analytes leads to more confident diagnosis without increasing analysis time. Previously reported microfluidic devices utilizing arrays of physically adsorbed antibodies typically require large areas and long incubation times for sensitive detection.[2-3] Immunoassays using antibodies covalently bound to microspheres often demonstrate higher performance than planar versions as the larger surface area to volume ratio provides a greater number of detection antibodies per unit volume.[4-5] The microbead array format also reduces false positives and negatives using redundant sensing elements. Previously reported work by the Walt Laboratory illustrated the advantages of microbead array-based sensors embedded within the distal tip of a fiber optic bundle for multiplexed immunoassays with saliva samples, but manual sample preparation and processing was required before and during analysis.[6-7] We report upon the integration of these sensors into a microfluidic format including all necessary reagent handling to reduce user performed steps, improve reproducibility, and make these assays more amenable to a point-of-care setting. We demonstrate the complete integration of the microarray into the PDMS device and show the detection of several cytokine analytes including VEGF and IL-8 proteins with LOD well below physiologically relevant concentrations.

EXPERIMENTAL

Optimization experiments were performed using 3.1-µm-diameter microbeads that were provided by the Walt group at Tufts University and prepared as previously reported.[6] Briefly, four sets of microbeads were first encoded at four different levels by staining with four different concentrations of Europium (III) thenoyltrifluoroacetonate trihydrate, and each set of beads was functionalized with one particular cytokine capture antibody. In this manner, it was possible to determine the antibody label of any particular bead in a stochastic array of mixed bead types by examining the fluorescence signal intensity of the Europium dye. Applicability of the device to commercially available systems was demonstrated with multiplexed experiments using microbeads from a custom Bio-Plex Pro assay kit purchased from Bio-Rad (Hercules, CA).

Microchips (Figure 1) were fabricated from a patterned PDMS layer bonded to a glass microscope slide. Patterning of the PDMS layer was achieved by a soft lithography technique using secondary molds. The primary master mold was formed from a glass substrate that was first patterned with the microfluidic channel network using standard photolithography and wet chemical etching. An array of microwells was then milled within the channel using a Helios 600 Nanolab Dual Focused Ion Beam System. Each cylindrical microwell measured approximately 3-µm deep and 2.75-µm in diameter, and each array consisted of 900 microwells spaced in a square grid with center-to-center spacing of 8 µm. Secondary PDMS molds were cast from the glass primary mold. After treatment with trichloro(1H,1H,2H,2H-perfluorooctyl)silane, the secondary mold was used to cast the PDMS layers used for the microfluidic chip. Holes were punched at the terminus of each microchannel to form fluid reservoirs, and the PDMS layer treated with atmospheric oxygen plasma and irreversibly bonded to a glass slide.

Equal populations of encoded antibody-functionalized microbeads were mixed in phosphate buffered saline (PBS) solution containing 0.1% poly(ethylene glycol) with ~ 10 kDa molecular weight (PEG 10k) to form a slurry. The chip was filled with the slurry using applied vacuum while the area of the PDMS layer containing the microwell array was repeatedly pressed and released using mechanical force such that it contacted the glass side of the chip and pushed the microbeads into the microwells. Fresh PBS/PEG10k buffer was flushed through the chip to remove free microbeads, the reservoirs were sealed, and the chip was stored at 4 °C until used.

Microchips were filled with PBS/PEG10k buffer. Pinch-type valves could be used in coordination with vacuum applied at the waste reservoir to direct the different solutions into the channel containing the microbead array. Solutions containing cytokines (the sample), biotinylated secondary (detection) antibodies, and streptavidin-Alexa Fluor 488 complex were incubated with the microbead array for 30, 15, and 10 minutes, respectively. The chip was washed with buffer and imaged with a fluorescence microscope to determine bead encoding and detection dye intensity for each bead.

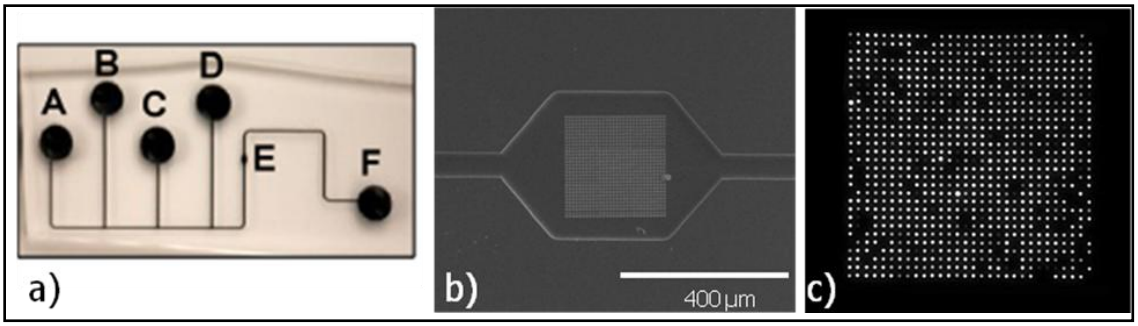

Figure 1: a) A photograph of the chip layout is shown with reservoirs for buffer (A), dye (B), secondary antibody (C), sample (D) and waste (F) filled with black dye. The microwell array is located with the channel (E). b) SEM image of the microwell array and c) fluorescence microscopy image of fluorescing microbeads in the array are shown.

RESULTS AND DISCUSSION

Microbead loading into the microwell arrays was easily accomplished using externally applied mechanical force. The elastomeric properties of PDMS substantially reduce the required bead and microwell dimensional tolerances relative to a rigid material, allowing machine-automated bead loading with excellent retention. Microwell occupation typically ranged from 90-100%, and we have observed no bead loss from the array under tested chemical and physical stresses including sonication. The loading of microbeads into the microwell arrays of previously assembled, bonded chips provides a degree of versatility as bead sets tailored to different applications can be machine-loaded at the point-of-care into a standardized chip.

Treatments to improve the biocompatibility of glass and PDMS surfaces were investigated for use with saliva samples containing mucins. Passivation agents evaluated included static coatings of covalently bonded silanols as well as dynamic coatings of various concentrations of hydrophilic polymers, commercially available blocking buffers, and bovine serum albumin. PBS containing 0.1% PEG 10k was found to be easily implemented and gave the most consistent results.

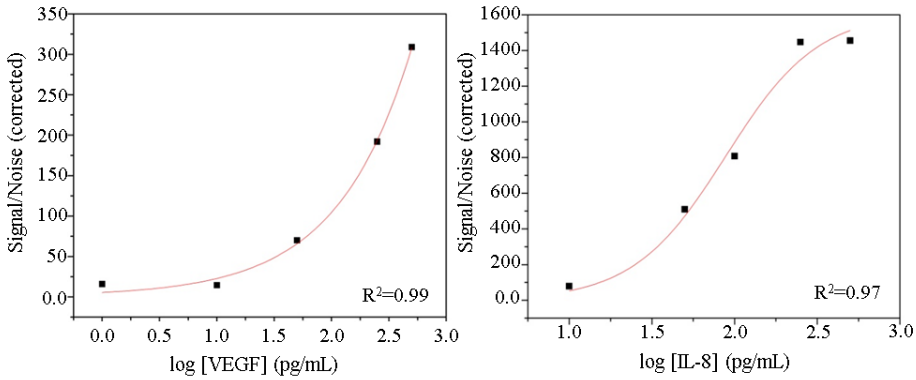

Figure 2: Regression curves used to calculate theoretical LOD for VEGF and IL-8

Sample incubation times, temperature, and reagent concentrations were optimized to improve signal to noise (S/N) values and avoid cross reactivity among different bead types at biologically relevant concentrations. Incubation times for cytokine standards were evaluated across a range of 1 to 120 minutes. Signal intensities more than doubled when cytokine incubation times were increased from 1 minutes to 20 minutes, but only an additional ~17% increase in signal was seen after 60 minutes. Signal improvements of greater than 10% were seen at 37 °C compared to assays conducted at 24 °C (room temperature). Secondary antibody (anti-VEGF) concentration was examined over a range of 0.5-5 µg/mL and concentrations >1 µg produced comparable results; therefore concentrations of 3 µg/mL were used for further studies. Streptavidin-Alexa Fluor concentration was studied between 0.5 and 40 µg/mL. High concentrations of dye produced higher absolute intensities but at the cost of additional noise. A concentration of 20 µg/mL was determined to be optimal for this system.

Optimized conditions were used to determine LOD for cytokines in buffer and saliva supernatant. Concentrations ranging from 10–500 pg/mL were assayed for each cytokine, and signal to noise values were used to generate regression curves (Figure 2). The theoretical LOD was determined by extrapolation to a S/N value equal to 3. Four-parameter logistics fits were used to determine the LOD values shown in Table 1. The addition of sample matrix (saliva supernatant) increases the LOD for the cytokines due to non-specific binding caused by mucins and glycoproteins; however, the LOD values for cytokines in this matrix are still approximately two orders of magnitude below physiologically relevant concentrations.[8]

Simple multiplexed detection of cytokines at physiologically relevant concentrations (500 pg/mL) was explored using commercially available microbeads arrayed in microfluidic chip. Anti-VEGF, anti-IL-8, and anti-TNF-α (control) were purchased from Bio-Rad and used with streptavidin-phycoerythrin dye as recommended by the manufacturer. Increased reagent incubation times (sample – 60 minutes, secondary antibody – 30 minutes, and dye – 20 minutes) were used to compensate for the lower sensitivities observed with these beads. All chips were loaded with a mixture of the three bead types. Control assays (no cytokines present) and assays where only one cytokine was present in solution were compared to multiplexed assays. Arrays were imaged before and after the addition of the dye to negate bead autofluorescence. Net signal intensities were determined by subtracting the average control bead (anti-TNF-α) intensity. No bead type showed significant signal when non-conjugate cytokines were present, but each bead type gave considerable signal when either its conjugate cytokine or both cytokines were present in solution, indicating successful multiplexing for this system.

Table 1. Cytokine LOD values

Cytokine	Matrix	LOD (pg/mL)	LOD (fM)
VEGF	Buffer	0.3	15
VEGF	Saliva supernatant	2.5	130
IL-8	Buffer	1.2	150
IL-8	Saliva supernatant	3.9	500
TIMP-1	Buffer	45	1570
EGF	Buffer	14	2400

CONCLUSION

We have achieved ultra-low LOD for cytokines found in saliva with an assay time < 1 hour using an easily fabricated and disposable microfluidic device. Multiplexed assays for VEGF and IL-8 at a biologically relevant concentration of 500 pg/mL indicate successful multiplexing is possible for these cytokines.

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CONTACT

*J.M. Ramsey, tel: +1-919-9627492; jmramsey@email.unc.edu