Sensors and Actuators B 258 (2018) 313–320

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Sensitive optical detection of clinically relevant biomarkers in affordable microfluidic devices: Overcoming substrate diffusion limitations

Ana I. Barbosa[a,1], Ana P. Castanheira[a,1], Nuno M. Reis[a,b,*]

[a] Department of Chemical Engineering, Loughborough University, Leicestershire LE11 3TU, United Kingdom
[b] Department of Chemical Engineering, University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom

ARTICLE INFO

Article history:
Received 1 June 2017
Received in revised form 18 October 2017
Accepted 17 November 2017
Available online 21 November 2017

Keywords:
Microfluidics
Colorimetric
Immunoassay
Biomarker
Enzymatic amplification
HRP
OPD

ABSTRACT

One of the biggest challenges in miniaturization of optical immunoassays is the short light path distance of microchannels/microcapillaries. Protein biomarkers are often presented in circulating blood in the picomolar-femtomolar range, requiring exceptional levels of sensitivity that cannot be met with traditional chromogenic substrates and without sophisticated, bulky detection systems. This study discloses an effective strategy for increasing the sensitivity and shorten the total test time for sandwich ELISAs in microfluidic devices optically interrogated, based on enhancing enzymatic amplification. We found that activity of Horseradish Peroxidase (HRP) in mesofluidic systems is highly limited by diffusion, therefore increasing the concentration of enzymatic substrate in these systems does not translate into an enhancement in enzymatic conversation. The opposite happens in microfluidic systems due to short diffusion distances, however increased concentration of the second enzymatic substrate, hydrogen peroxide (H_2O_2), leads to enzyme inhibition as herein reported. Consequently, we found that the molar ratio of o-phenylenediamine (OPD) to hydrogen peroxide from commercially substrate formulations is not suitable for miniaturized systems. Sandwich ELISA quantitation of a cancer biomarker PSA and human cytokine IL-1β in fluoropolymer microfluidic strips revealed over one order of magnitude increase in sensitivity and 10-fold decrease in incubation time by simply changing the molar ratio of OPD:H_2O_2 from 1:3 to 1:1 and increasing OPD concentration from 1 to 4 mg/ml. This enhancement in enzymatic amplification offers finally the sensitivity required for optical interrogation of novel portable and affordable microfluidic devices with inexpensive and ubiquitous smartphones and flatbed scanners.

1. Introduction

The development of affordable diagnostic tests is highly dependent on the use of low-cost detection systems, which often means lower signal resolution and poor assay performance. Creating tests capable of quantifying biomarkers at very low concentrations whilst maintaining a reduced cost for the test is a fundamental condition for point-of-care (POC) diagnostics industry. Assay miniaturization is one of the main trends in clinical diagnostics, and several studies have succeed in applying microfluidic devices in a range of shapes and detection methods [1–4] for rapid and sensitive detection of different analytes for different clinical situations. This includes infectious diseases [5,6], biomarkers [7] and food allergens [8] to name a few, and typically requires the ability of detecting molecules in the nanomolar to picomolar concentration range. This is achieved with the use of expensive detection equipment that is often incompatible with the user requirements and product specifications for POC tests. An alternative approach that surprisingly remains underexplored is to further potential the natural "amplification" capability of well-established enzymes [9] to yield rapid and sensitive detection, using inexpensive and widespread chromogenic colorimetric substrates and low-cost optoelectronic components [10], such as flatbed scanners [11], smartphones [12,13], and other cost effective readout systems [14].

It appears established within the scientific community that high-sensitivity detection can only be achieved with direct fluorescence labelling of molecules, since fluorophores provide high amplification power required for detecting very low concentrations

* Corresponding author at: Department of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom. Tel.: +44 0 1225 383 369.
E-mail address: n.m.reis@bath.ac.uk (N.M. Reis).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.snb.2017.11.086
0925-4005/

of molecules in biological sample. However fluorescence has main drawbacks in respect to microfluidic POC testing, such as scattering noise, cross-talks, misalignment, autofluorescence of substrate, and low collection efficiency [15].

Our research group has pioneered the application of an extruded, low-cost microfluidic material to immunoassays, based on a fluoropolymer Microcapillary Film (MCF) [16], as shown in Fig. 1B. The hydrophobic surface of Teflon-FEP is ideal for immobilizing immunoassay reagents in the inner surface of the microcapillaries, whereas the refractive index of material similar to that of water allows unique signal-to-noise ratios which favors simple optical detection. This is due to minimal optical refraction at the water:capillary wall interface and thus no distortion is caused by the capillaries when filled with colored aqueous solutions [16]. We have recently reported a 13 min colorimetric prostate cancer antigen (PSA) sandwich assay from whole blood with limit of detection below 1 ng/ml using both a flatbed scanner and a smartphone [13,17]. Also, we have recently reported a multiplex femtomolar detection of four cytokines (IL-1β, IL-6, IL-12 and TNF-α) using colorimetric detection with a flatbed scanner [18]. We believe enzymatic amplification combined with unique characteristics of microfluidic devices is the key to high-sensitivity POC test with low-cost, modest-performance optoelectronic components [19].

In this study we present for the first time our strategy for enhancing enzymatic amplification in colorimetric immunoassay which used optimized Horseradish Peroxidase (HRP) conversion of a very popular chromogenic substrate, *o*-phenylenediamine dihydrochloride (OPD), adapted to microscale enzymatic conversion and enzyme-linked immunosorbent assay (ELISA) detection. HRP is one of the most popular enzymes in ELISA technique for presenting a very high turnover number. We noticed the composition of commercial OPD:H_2O_2 substrate is adapted to standard laboratory systems controlled by diffusion, such as microwell plates [20–22], where HRP performance is sub-optimum. Miniaturization of ELISA in our fluoropolymer microfluidic devices allowed overcoming diffusion limitations, similar to what happens when enzymes are in solution (Fig. 1A). Consequently, HRP enzyme can be used to yield much higher conversion rates of OPD and consequently achieve significant colorimetric signals with shorter incubation times. This resulted in a very large improvement in both assay speed and assay sensitivity, as supported by our experience with PSA and human IL-1β assay development. PSA is the mostly widely used prostate cancer biomarker with a clinical threshold value of 4 ng/ml, above which the patients need to proceed for biopsy examination. PSA values of 0.4–2 ng/ml are the clinical thresholds for disease monitorization after radical prostatectomy and radiotherapy [23–29]. The cytokines clinical threshold values are in the order of pg/ml concentrations [30], which means bioassay devices need to be able to quantitate this concentration range in order to perform e.g. early detection of sepsis [31,32] and infectious diseases [33,34].

2. Materials and methods

2.1. Reagents and materials

2,3-diaminophenazine (DAP) and SIGMAFAST™ OPD (*o*-phenylenediamine) tablets were supplied from Sigma Aldrich Ltd (Dorset, UK). A Human kallikrein 3/Prostate Specific Antigen (PSA) ELISA kit was purchased from R&D Systems (Minneapolis, USA; cat n° DY1344). The kit contained a monoclonal mouse Human Kallikrein 3/PSA antibody (capture antibody or CapAb), a Human Kallikrein 3/PSA polyclonal biotinylated antibody (detection antibody or DetAb) and recombinant Human Kallikrein 3/PSA (standard). Human cytokines reagents were purchased from eBiosciences (Hatfield, UK): IL-1β (cat no: human recombinant protein #14-8018; Anti-Human IL-1β biotin #13-7016; Anti-Human IL-1β purified #14-7018); IL-12p70 (cat no: human recombinant protein #14-8129; Anti-Human IL-12p70 biotin #13-7129; Anti-Human IL-12p70 purified #14-7128); IL-6 (cat no: human recombinant protein #14-8069; Anti-Human IL-6 biotin #13-7068; Anti-Human IL-6 purified #14-7069); and Tumor Necrosis Factor-α (TNFα) (cat no: human recombinant protein #14-8329; Anti-Human TNFα biotin #13-7349; Anti-Human TNFα purified #14-7348). ExtrAvidin-Peroxidase (cat. no E2886) was sourced from Sigma Aldrich Ltd (Dorset, UK) and High Sensitivity Streptavidin-HRP was supplied by Thermo Scientific (Lutterworth, UK; cat no 21130) and used for enzyme detection for IL-1β assay. Phosphate buffered solution (PBS, Sigma Aldrich, Dorset, UK; cat. no P5368-10PAK), pH 7.4, 10 mM was used as IA buffer. The diluent and blocking solution consisted either of SuperBlock (Thermo Fisher Scientific, Loughborough, UK; cat. no 37515) or 1–3% w/v protease-free albumin from bovine serum (BSA, Sigma Aldrich, Dorset, UK; cat no A3858) diluted in PBS buffer. For washings, PBS with 0.05% v/v of Tween-20 (Sigma-Aldrich, Dorset, UK; cat no P9416-50ML) was used. Nunc maxisorp ELISA 96-well Microtiter Plates (MTP) were sourced from Sigma Aldrich (Dorset, UK). The MCF was supplied by Lamina Dielectrics Ltd (Billingshurts, West Sussex, UK).

2.2. Microfluidic strips

The miniaturized platform consisted of a 10 bore, ~200 μm internal diameter fluoropolymer MCF [16] (Fig. 1B) produced by a novel continuous melt-extrusion process [35], which due to its geometrical shape and optical properties can be easily integrated with low-cost and easy access readout system, such as a flatbed scanner.

2.3. Lower optical detection limit

For comparison of MCF and MTP lower detection limit, different concentrations of DAP were detected in the MCF using a flatbed scanner (HP ScanJet G4050) and in the 96 well plate using a Microplate Reader (Epoch, Biotek). A stock solution of 1 mg/ml of DAP (Sigma-Aldrich, Dorset, UK; cat. no. E2886) was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset, UK; cat. no. D8418) and a 1:2 dilution series in PBS was made to complete the calibration curves. Absorbance values were calculated by determining the grey scale peak height for each individual microcapillary in the MCF using ImageJ software (NIH, Maryland, USA), or using the embedded Gen5 data analysis software for microtiter plate (Epoch, Biotek).

2.4. Chromogenic substrate conversion

In order to find the best combination of OPD:H_2O_2 concentration, stock solutions of 4 mg/ml of both OPD and H_2O_2 concentrations were prepared in deionized water and diluted in a 1:2 dilution series. The solutions were placed in a Nunc MaxiSorp ELISA 96-well microwell plate using a matrix arrangement (OPD and H_2O_2 concentrations varied along the rows and columns, respectively). EA-HRP was used in solution in a concentration of 0.0156 μg/ml.

The initial enzymatic rates of HRP conversion of OPD to DAP were determined by testing different concentrations of OPD and HRP using both immobilized and solubilized enzyme. A start solution with 1 μg/ml of EA-HRP was immobilized by overnight incubation in the first well of the first column of the microtiter plate, followed by 1:2 dilution solutions in each column. Then, 1:2 dilutions of 1 mg/ml of each substrate (OPD and H_2O_2) were prepared and placed along the rows in the microtiter plate, reading immediately the absorbance values with a microtiter plate reader.