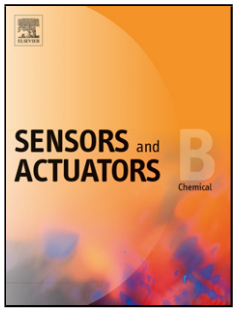

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Sensitive optical detection of clinically relevant biomarkers in affordable microfluidic devices: overcoming substrate diffusion limitations

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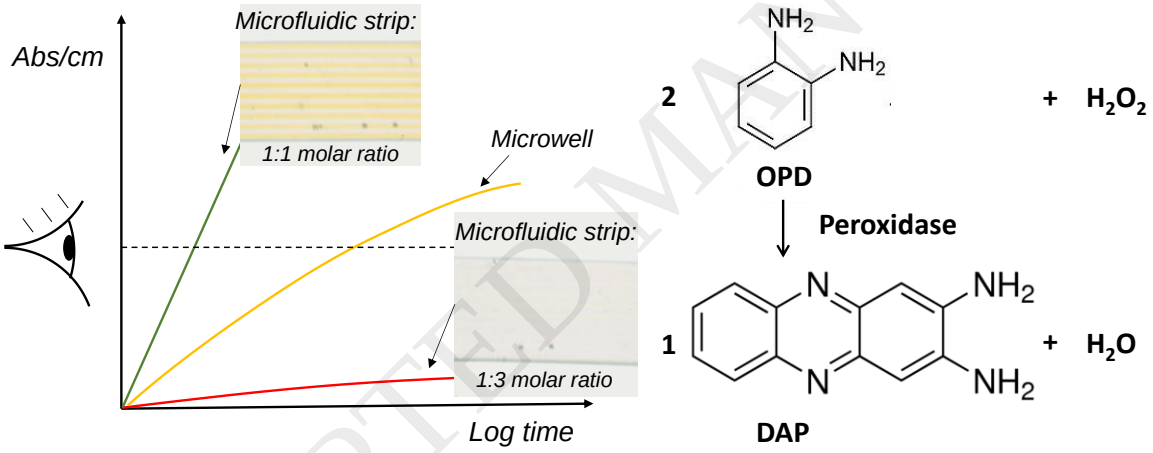
Graphical abstract



Highlights:

- We unravelled the full potential of chromogenic substrates for optical detection biomarkers in microfluidic devices
- Commercial chromogenic substrate formulations are suboptimum tuned to meso-scale systems
- We demonstrated optical detection of clinically relevant biomarkers in microfluidics devices with optimised OPD:H_2O_2 formulation
- Assay times and lower limit of detection for PSA and IL-1β reduced by one order of magnitude

[1] These authors contributed equally to this work.

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 system. 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.

Keywords: microfluidics, colorimetric, immunoassay, biomarker, enzymatic amplification, HRP, OPD

1. INTRODUCTION

The development of affordable diagnostic test 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 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 to 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 nº 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, 10mM was used as IA buffer. The diluent and blocking solution consisted either of SuperBlock (Thermo Fisher Scientific, Loughborough, UK; cat. no 37515) or 1 to 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. To understand the role of diffusion, the procedure was repeated with the enzyme in solution, by spiking each well with the same quantity of EA-HRP.

2.5 Full response curves

Full response curves for PSA and IL-1β were performed in the MCF using different concentrations and molar ratios of OPD:H_2O_2. To obtain the calibration curves, two 30 cm length MCF strips (strips #1 and #2) were coated with 10 µg/ml of human kallikrein 3/PSA capture antibody (CapAb) diluted in phosphate-buffered saline or 20 µg/ml of Anti-Human IL-1β purified (strips #3 and #4) using a single syringe attached with silicone tube for fluid handling (Fig.1C). The MCF strips were incubated for 2 hours at room temperature (20°C) to allow the CapAb to adsorb on the microcapillary surface, and subsequently blocked with 1% BSA (strips #1, #2 and #3) or superblock blocking buffer (strip #4), for 2 hours at room temperature The strips were washed using PBS with 0.05% (v/v) of Tween-20. Each MCF coated strip was trimmed into 30 mm long test strips, and each strip incubated with a serial dilution 0-60 ng/ml of PSA (recombinant human kallikrein 3/PSA) for 20 min (strip #1 and #2) or a serial dilution 0-1 ng/ml of IL-1β for 30 min (strip #3) and 0-5 ng/ml in strip #4. Then, 2 µg/ml of human kallikrein 3/PSA polyclonal biotinylated antibody or 10 µg/ml of Anti-Human IL-1β biotinylated were used as detection antibodies and incubated for 15 min and 10 min, respectively. EA-HRP in a concentration of 4 µg/ml was incubated for further 15 min in coated MCF strips #1 and #2 and for 10 min in strip #3. The enzyme used in strip #4 (IL-1β assay) was High Sensitivity Streptavidin–HRP with 4 µg/ml, incubated for 10 min. Three washing steps were performed afterwards. Subsequently, 1 mg/ml of OPD and 1 mg/ml H_2O_2 (equivalent to molar ratio 1:3) solution was added to strip #1 (PSA assay) and #3 (IL-1β assay) and 4 mg/ml of OPD and 1 mg/ml H_2O_2 (molar ratio 1:1) were added to strips #2 (PSA assay) and #4 (IL-1β assay). The MCF strips were scanned as RGB images with a HP ScanJet G4050 (Hewlett-Packard, CA, USA) flatbed scanner at 2,400 dpi resolution in transmittance mode in intervals of 2–3 min over 30 min of incubation.

2.5 Optical interrogation of MCF strips

For quantitation of colorimetric signal in the microcapillaries, RGB digital images were split into 3 separated channels in Image J (NIH, USA). The blue channel images were used to calculate absorbance values, based on the grey scale peak height of each individual capillary of FEP-Teflon MCF as described elsewhere.[16–18] Absorbance (Abs) was calculated for each individual capillary based on equation (1):

$$\text{Abs} = -\log_{10}\left(\frac{I}{I_0}\right) \quad (1)$$

where I is the grey scale peak height (transmitted light intensity) and I_0 is the maximum grey scale value. The absorbance values were averaged across 10 capillaries for MCF strip.

3. RESULTS AND DISCUSSION

We believe enzymatic conversion using off-the-shelf chromogenic substrates is a solution for the well desirable portability, sensitivity and affordability of POC tests for optical detection of protein biomarkers in the nanomolar to femtomolar range, which represents the core of clinical diagnostics market. However, a key aspect that remains clearly underexplored in miniaturized ELISA tests is the kinetics of enzymatic substrates conversion, which perhaps is the most crucial yet powerful step in a colorimetric or fluorescence ELISA. OPD (MW=108.1 g/mol) is widely used as a HRP chromogenic substrate by the biggest worldwide manufacturers and suppliers of bioanalytical reagents to life sciences laboratories, and typically recommend 0.4 mg/ml of OPD and 0.4 mg/ml of H_2O_2 (MW=34.0 g/mol) or 0.5-1 mg/ml of OPD and 0.3 mg/ml of H_2O_2. Nevertheless, datasheets are usually not detailed enough regarding the OPD:H_2O_2 molar ratio, which is of paramount importance for ELISA detection in miniaturized tests designed to be optically interrogated with less sophisticated readout systems.

In order to optimize OPD enzymatic conversion in our novel ELISA microfluidic platform[17] which used in this study a flatbed scanner for signal readout, we analyzed the stoichiometry of HRP conversion of OPD to 2,3-diaminophenazine (DAP) (Fig. 2A), realizing that two molecules of OPD and one of hydrogen peroxidase (H_2O_2) are necessary for the enzyme to be able to convert OPD (colorless) into DAP (brownish color), which yields a theoretical optimal molar ratio of 2:1. The concentrations of 0.4 mg/ml for both OPD and H_2O_2 as recommended by the suppliers yield a molar ratio of 1:3 for OPD:H_2O_2 in sandwich ELISA assays which, according to Nicell *et al.*[36] has an inhibitory effect in HRP catalytic activity by H_2O_2.

We compared initially the chromogenic sensitivity and dynamic range of a flatbed scanner in respect to DAP detection against a conventional bench-top microplate reader (Fig. 2B). The microplate based detection of DAP was about 256 times more sensitive (0.244 pg/ml) than in a 10-bore MCF produced from Teflon-FEP with a flatbed scanner (62.5 pg/ml). This links to the much shorter light path distance of ~200 µm microcapillaries compared to ~3mm light path distance is a microwell. We noticed, however, the DAP scanning in the MCF presented a much broader dynamic range, equivalent to 5% to 100% of OPD conversion, whereas the dynamic range for microwell is limited to the range of 0.02%-15% conversion. Despite DAP being more sensitive in a microwell plate, it lacks quantitation capability for high rates of OPD conversion, whereas in the small microcapillaries the Lambert-Beer law is valid on a very broad range of equivalent OPD substrate concentrations. We hypothesized therefore that substrate concentration should be dramatically increased in order to achieve much faster and more sensitive immunoassays, should HRP be capable of handling such high concentrations of OPD and/or H_2O_2. Consequently, we tested the effect of both OPD and H_2O_2 concentration in the presence of a constant solubilized ExtraAvidin-HRP (EA-HRP) concentration and noticed HRP conversion of OPD in the commercial formulations is characterized by an inhibition by H_2O_2 (Fig. 2C and 2D).

The initial rates of OPD conversion, $-r_s$ increased with increasing concentration of H_2O_2 (inhibitor, [I]) up to a value of 0.5-1.0 mg/ml (Fig. 2C), beyond which the initial conversion rate started decreasing. This enzymatic behavior is coherent with inhibition by substrate, in this case H_2O_2, and Haldane model[37] mathematically described by equation (2) has shown the lower value for the sum of the squared difference between experimental and model predicted data thus being the best-fitted model in this case:

$$-r_s = -r_{max}\frac{[I]}{K_s + [I] + \frac{[I]^2}{K_i}} \qquad (2)$$

This experiment also allowed determining the ideal concentration of both OPD and H_2O_2, which were 4 mg/ml and 1 mg/ml, respectively, i.e. equivalent to a OPD:H_2O_2 molar ratio of 1:1. Fitting the substrate enzymatic inhibition model in equation (2) a value of 0.07 mg/ml and 0.91 mg/ml were obtained for inhibition constant, K_i with 1.0 mg/ml and 4.0 mg/ml of OPD respectively, indicating a higher degree of enzyme inhibition with the use of lower OPD concentration. With a OPD:H_2O_2 molar ratio of 1:3 used in commercial OPD substrates, the value of $-r_s$ obtained was 0.11 mg/ml.s whereas with 1:1 molar ratio a maximum $-r_s$ value of 0.191 mg/ml.s was obtained which represented a 1.7-fold increase. But the overall impact of using a suboptimum OPD:H_2O_2 molar ratio is much bigger than this value, as there are also

direct implications on substrate depletion and diffusion, limiting the rate of enzymatic conversion which is linked to the development of optical signal.

In Fig. 2D the impact of OPD:H_2O_2 molar ratio is further elucidated. In this experimental set the concentrations were changed for both OPD and H_2O_2 in order to keep initial OPD:H_2O_2 molar ratio constant. This revealed OPD substrate was not responsible for the inhibition, with the values of $-r_s$ increasing as a first order kinetic model for low concentration of OPD and clearly trending to a zero order for higher concentrations of substrate, which is typical of a non-competitive or mixed inhibition kinetic model, mathematically described in equation (3).[38]. Hence, a significant higher conversation rate for reaction with molar ratio 1:1 of OPD/H2O2 is disclosed, which for data points of 1 mg/ml of OPD shows a 2-fold increase in enzymatic conversion rate.

$$-r_s = -r_{S,max}\frac{[S]}{\alpha K_m + \alpha'[S]} \tag{3}$$

where K_i and K_i' are the inhibitor constants representing the inhibition effects and are described below, in equations (4) to (7):

$$\alpha = 1 + \frac{[I]}{K_i} \tag{4}$$

$$\alpha' = 1 + \frac{[I]}{K_i'} \tag{5}$$

$$K_i = \frac{[E][I]}{[EI]} \tag{6}$$

$$K_i' = \frac{[ES][I]}{[ESI]} \tag{7}$$

The kinetic model in equations (3) to (7) considers the inhibitor can bind the enzyme or the enzyme-substrate complex reducing the overall enzyme activity. The kinetic model parameters were found by best-fitting the model to the experimental data using Solver tool in Excel, and are summarized in Table 1. These revealed a significant difference in the maximum rate of substrate conversion, $-r_{s,max}$ shown in Fig. 2D, representing a 3.2-fold increase in maximum rate of conversion of OPD.

It is clear from data in Table 1 that the inhibition has higher impact on enzyme kinetics at a molar ratio of 1:3. As previously discussed, K_m remained approximately constant at both molar ratios tested, with the value of $-r_{s,max}$ being strongly affected by the molar ratio. This reinforces the non-competitive inhibition

model typical of HRP.[38] Based on equation (6) and (7) the smaller the values of both K_i and K_i', the more efficient is the inhibitor binding either the enzyme itself or the enzyme-substrate-inhibitor complex. Comparing the two curves in Fig. 2D, K_i at 1:1 molar ratio was about 280 times higher than the value of K_i at 1:3 molar ratio, suggesting the inhibitor, *I* (in this case H_2O_2) binds directly to the enzyme. For 1:3 molar ratio, the value of K_i obtained was very similar to that of K_i', which again is typical of a non-competitive inhibition,[38] where the reaction is only inhibited by the formation of enzyme-inhibitor complex.

Overall, it appeared that when in excess H_2O_2 inhibits HRP enzymatic kinetics by reducing the enzyme activity, however it does not affect the affinity of HRP to the substrate. This inhibition effect is surpassed by deviating from the molar ratio initially recommended by the manufacturers, i.e. 1:3 to 1:1.

Although molar ratio of OPD:H_2O_2 revealed paramount to the rate of generation of colorimetric signal by affecting HRP kinetics, the diffusion and mass transfer limitations need to be taken into account in respect to the speed and magnitude of colorimetric signal produced, which ultimately controls both speed and sensitivity of the immunoassay. In heterogeneous immunoassays the enzyme is immobilized on the surface and consequently the kinetics of substrate conversion is modified because of mass transfer limitations. Immobilized enzyme reactions present lower initial velocities, due to migration time of substrate molecules to the walls and to possible conformational changes during adsorption or binding process.[39] This is the typical situation in a microtiter plate, where the maximum distance of molecular diffusion is in the range of 3 mm, which can represent several minutes for a medium size molecule. Further experiments with solubilized enzyme in a microwell (Fig. 3A) confirmed the increase in the rate of colorimetric signal generation with the increase of OPD concentration. Nevertheless, experiments with immobilized enzyme (Fig. 3B) revealed not being advantageous to use higher rates of conversion of OPD as this leads to the rapid accumulation of product near the plastic wall and fluctuations in the rate of conversion of OPD which is not beneficial for immunoassays, where typically the end-point or kinetics rate is expected to be linked to the concentration of analyte in the sample. The absorbance values obtained were lower than those for solubilized enzyme as, in one hand, the enzyme immobilization process might interfere with enzyme catalytic activity[40] and, on the other hand, the total enzyme molecules available are dependent on the quantity of molecules bound to the wall. We estimated the turnover number of the HRP enzyme in this specific system by analyzing the velocity rates with the changing of enzyme concentration and, assuming that ExtrAvidin contains two molecules of peroxidase, a values of 1.95×10^6 s^{-1} was obtained.

In miniaturized ELISA platforms such as MCF, diffusion distances are very short and, consequently, an increase in conversion rates of substrate is beneficial. With a maximum diffusion distance of ~100 μm, substrate conversion with HRP will approach the situation shown in Fig. 3A for solubilized enzyme, which ultimately translate into stronger colorimetric signal generated with much shorter incubation times. This feature enhances the signal-to-noise ratios and ultimately improves sensitivity of the assay, assuming the enzyme is working at maximum 'capacity' (activity). Note that when using EA-HRP in a sandwich ELISA, it is known that extravidin binds to the biotinylated antibody, eliminating the problem of catalytic enzymatic activity reduction due to its conformational change.

To illustrate the multiple benefits of the 1:1 molar ratio for OPD:H_2O_2 and higher initial OPD concentration we carried out multiple PSA and IL-1β sandwich ELISAs optimized for the MCF. Fig. 4 shows the effect of OPD incubation on the performance of these two assays. The incubation time required for PSA detection was reduced in 10-fold when compared to the conventional 1:3 molar ratio, and both the signal and sensitivity (or lower limit of detection defined as absorbance of blank plus three standard deviations) were improved from 11.5 to 0.7 ng/ml (i.e. 16x reduction in detection limit). In the case of human IL-1β quantitation the limit of detection was also further improved from 100 to 6 pg/ml. The rate of OPD enzymatic conversion also revealed much faster as the signal obtained with just 3 minutes of OPD incubation was about 3-fold stronger than the signal obtained with 30 minutes OPD incubation with 1:3 molar ratio.

CONCLUSIONS

HRP conversion of a common chromogenic substrate, OPD in a microfluidic device was characterized by H_2O_2 inhibition of enzyme activity, leading to extended incubation times and weak colorimetric signals difficult to detect with less sophisticated optoelectronic components. By increasing the initial OPD concentration from 1.0 to 4.0 mg/ml and OPD:H_2O_2 molar ratio from 1:3 to 1:1 the value rate of enzymatic conversion was increased by 2-fold, which revealed very beneficial for microfluidic devices that contain short diffusion distances. It also avoided the rapid depletion of substrate that characterizes commercial OPD formulations. A number of sandwich assays with PSA and IL-1β detection in fluoropolymer microfluidic MCF strips and a flatbed scanner showed systematically above one order of magnitude increase in sensitivity and/or speed of the assay. This allows mimicking the performance and reliability of sophisticated laboratory detection equipment using low-cost optoelectronic equipment, which is expected to trigger the development of affordable POC tests that fully exploit the low-cost and unique amplification

capabilities of enzymatic ELISA detection with ubiquitous optical detection technologies like smartphones and flatbed scanners. This change in enzymatic substrate molar ratio can be applied to all miniaturized ELISA tests, opening the possibility for a new generation of affordable optical microfluidic POC diagnostic tests.

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Biographies:

Dr Nuno M Reis is a Reader in Bioengineering and Biomedical Innovation at the University of Bath, UK, his research group is particularly focused on the development of transformative microfluidic innovations based on melt-extruded Microcapillary Films (MCFs). He received both undergraduate and Ph.D. degrees in Biological Engineering from University of Minho, Portugal. Previous to Bath, he held a Lecturer position at Loughborough University, UK (2011-2016), and 4.5 years post-doctoral experience at the University of Cambridge, UK (2006-2011). He is named co-inventor and co-author in several international patent applications and peer-reviewed publications specific to the application of MCF to a range of bioanalytical applications.

Dr Ana I Barbosa obtained her PhD in Chemical Engineering from Loughborough University, UK in 2016 where she worked on the development of a novel microfluidic platform, the Microcapillary Film (MCF), for affordable sensitive quantitation of biomarkers. She currently works as a manufacturing scientist for Capillary Film Technology Ltd, a UK based SME developing MCF technology for applications in life sciences and clinical diagnostics. Her research interest is the development of affordable miniaturized design approaches for sensitive quantitation of protein biomarkers without sample preparation need, combining aspects of analytical chemistry with microengineering and biology.

Miss Ana Castanheira graduated in Biological Engineering from the University of Minho, Portugal in 2013. She worked as an immunoassay scientist for Capillary Film Technology Ltd, UK from 2013-2016, where she managed to developed several singleplex and multiplex microfluidic test strips based on Microcapillary Film for ultra-sensitive colorimetric and fluorescence detection of protein biomarkers, including inflammatory cytokines and cardiac biomarkers.

List of Tables

Table 1. Kinetic parameters (non-competitive/mixed inhibition) for the two different molar ratios of OPD:H_2O_2 studied

	1:3 OPD:H_2O_2	**1:1 OPD:H_2O_2**
$-r_{s,max}$ (mg/ml.s)	0.50	1.61
K_m (mg/ml)	8.39	8.39
K_i (mg/ml)	5.53	1561.00
K_i' (mg/ml)	5.32	0.39

Figure captions

Fig. 1 Miniaturized enzymatic assays. (A) Our concept of miniaturized enzymatic assays - immobilized enzymes present similar conversion kinetics to enzymes in solutions, due to small diffusion distance. (B): Microcapillary Film (MCF) - (i) MCF reel (ii) photography of MCF with blue dye inside the 200 µm diameter capillaries. (C) Aspiration of solutions in the microfluidic MCF strips using a 1 ml syringe.

Fig. 2 Aspects of colorimetric detection in MCF and peroxidase inhibition. (A) HRP conversion of OPD chromogenic substrate. (B) Response curves for detection of DAP (colored product) in a miniaturized MCF with a flatbed scanner and in a 96 MTP using a microplate reader. Absorbance values have been normalized by the light path of MCF (0.02 cm) and MTP (0.3 cm) respectively. (C) Initial enzymatic conversion rates for different molar ratios of OPD and H_2O_2. (D) Initial conversion rates of OPD as function of H_2O_2 and OPD concentration. The concentration of EA-HRP was kept constant at 15.6 ng/ml.

Fig. 3 Kinetics of HRP conversion of OPD for varying concentration of chromogenic substrate. (A) EA-HRP in solution. (B) EA-HRP immobilized on plastic surface of a 96 microtiter plate well. Concentration of H_2O_2 was kept constant at 1 mg/ml H_2O_2, as the molar ratio of 1:3 OPD/H_2O_2, and final concentration of EA-HRP immobilized or in solution was kept at 15.6 ng/ml.

Fig. 4 Comparison between two different OPD and H_2O_2 molar ratio in MCF sandwich assays. (A) 4 mg/ml of OPD and 1 mg/ml of H_2O_2 (1:3 molar ratio) makes the PSA MCF assay 10 times faster and with 16x higher sensitivity. (B) IL-1β assay improved sensitivity from 100 pg/ml to 6 pg/ml with 3-fold reduction in assay time. The times shown relate only to the time of incubation of OPD before optical interrogation of the microfluidic MCF strips.♦ 1:1 molar ratio OPD:H_2O_2 and ● 1:3 molar ratio OPD:H_2O_2. Absorbance values have been normalized by the maximum light path distance in the MCF (i.e. 0.02 cm)

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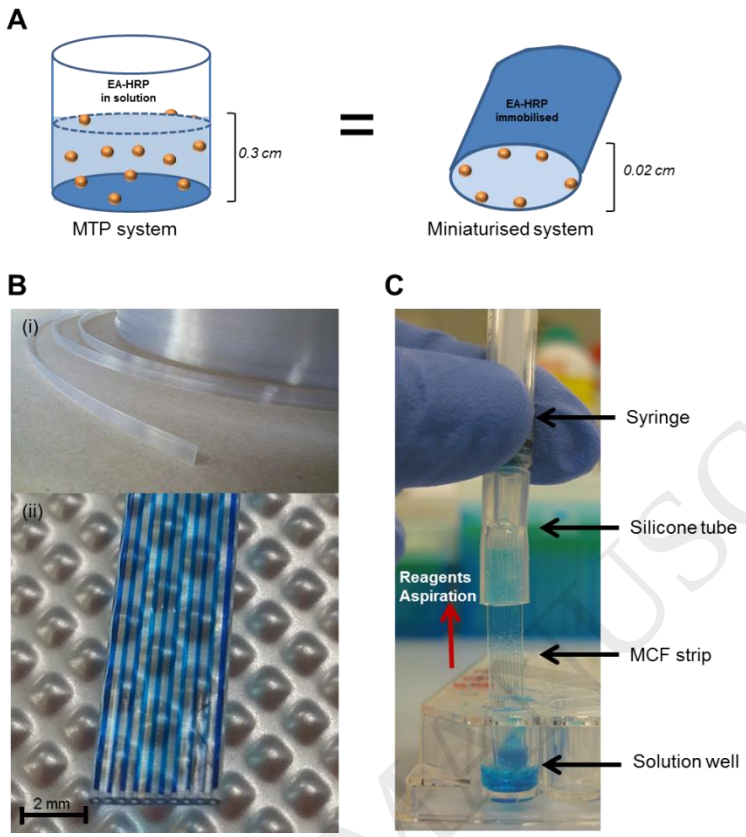
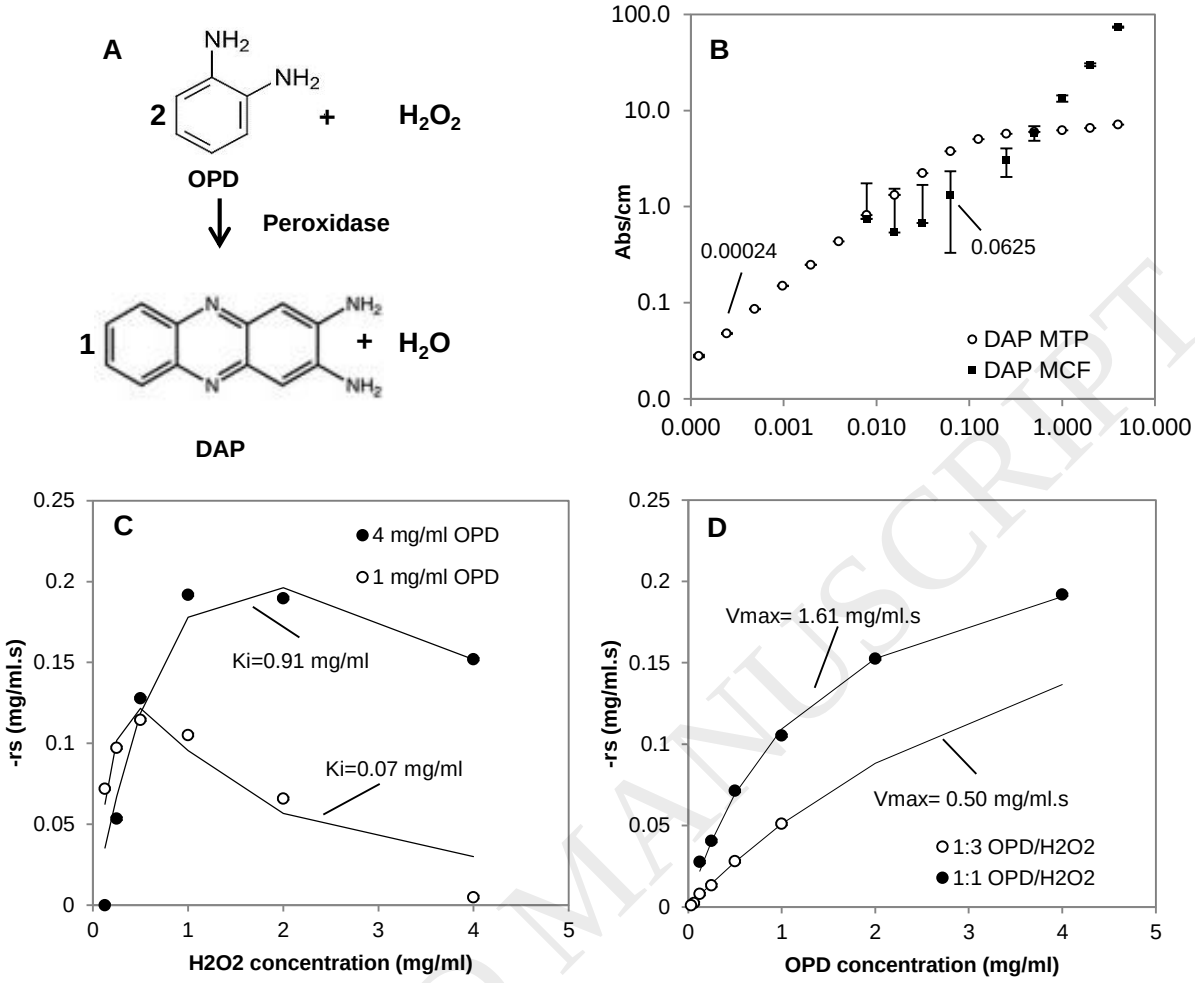


Fig. 1



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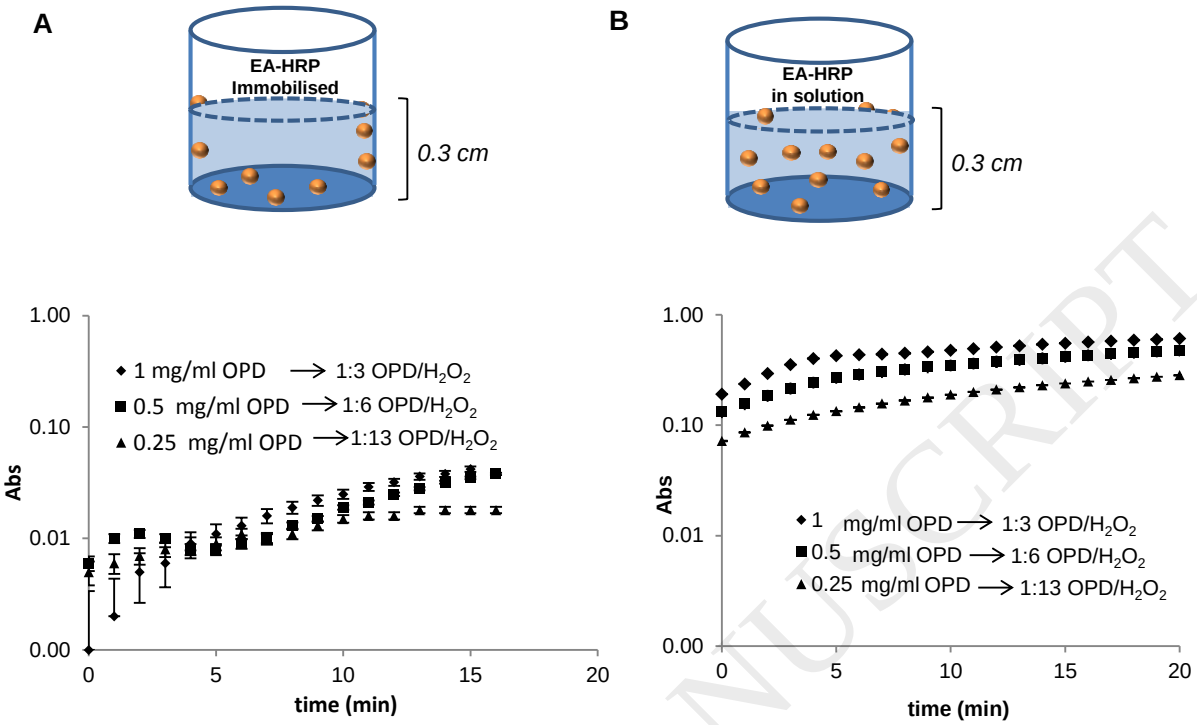

Fig. 3

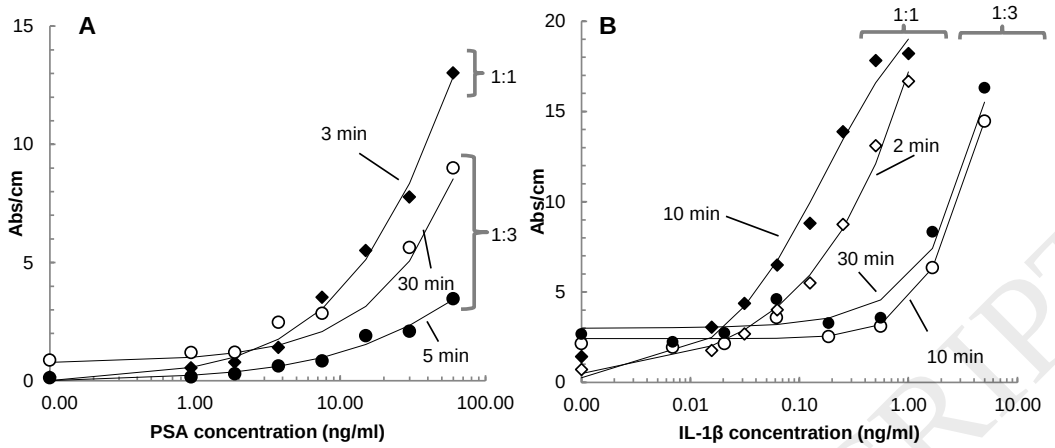

Fig. 4a