Design considerations of a hollow microneedle-optofluidic biosensing platform incorporating enzyme-linked assays

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Abstract

A hollow metallic microneedle is integrated with microfluidics and photonic components to form a microneedle-optofluidic biosensor suitable for therapeutic drug monitoring (TDM) in biological fluids, like interstitial fluid, that can be collected in a painless and minimally-invasive manner. The microneedle inner lumen surface is bio-functionalized to trap and bind target analytes on-site in a sample volume as small as 0.6 nl, and houses an enzyme-linked assay on its 0.06 mm\(^2\) wall. The optofluidic components are designed to rapidly quantify target analytes present in the sample and collected in the microneedle using a simple and sensitive absorbance scheme. This contribution describes how the biosensor components were optimized to detect in vitro streptavidin-horseradish peroxidase (Sav-HRP) as a model analyte over a large detection range (0–7.21 µM) and a very low limit of detection (60.2 nM). This biosensor utilizes the lowest analyte volume reported for TDM with microneedle technology, and presents significant avenues to improve current TDM methods for patients, by potentially eliminating blood draws for several drug candidates.

Keywords: hollow microneedle, biosensor, therapeutic drug monitoring, optofluidic sensing, enzyme-linked assay

(Some figures may appear in colour only in the online journal)

1. Introduction

Emerging clinical biosensor concepts promise significant improvements to therapeutic drug monitoring (TDM), disease diagnostics, and treatment planning [1–4]. Over the past decades, many clinical biosensing systems have been developed and commercialized for TDM (i.e. glucose monitoring in diabetic patients [1]), early detection of disease (i.e. HIV [2] and malaria [3]) and bodily changes (i.e. pregnancy [4]), and for other diagnostics applications, as summarized by [4]. Most commercial clinical biosensing tools require extracting a biological fluid sample, typically in the form of saliva, blood, or urine; followed by analysis of the sample requiring large sample volumes and being limited to high concentrations of the target compound. While urine and saliva provide non-invasive methods for biological fluid collection, a majority of TDM methods require blood for analysis [4, 5]. For instance,
Microneedle-based technologies are most promising to enable TDM in low sample volumes and for low concentration drug candidates. Microneedles being sub-millimeter needle-like structures pierce through the outer-most stratum corneum layer of the skin in a painless way and allow minimally-invasive collection of ISF. Typically, this is done by collecting ISF using a microneedle device and transferring the sample to an analytical device for drug evaluation [14–16]. For example [16], created pores in the skin with dissolving solid microneedles and withdrew ISF using a pipette, followed by diluting the collected ISF and analyzing it for vancomycin using liquid chromatography-tandem mass spectrometry. A similar approach of using a glass microneedle combined with vacuum to collect ISF and analyzing it for vancomycin using analytical device for drug evaluation [17], where the collected ISF was transferred and analyzed for glucose concentration. In more advanced and integrated lab-on-chip TDM systems [12, 14, 15], have mounted sensing elements to the back of hollow microneedle devices to assess glucose concentrations in ISF immediately after collection. However, except for the solution presented by Jina et al where pre-filled hollow microneedles facilitated the diffusion of glucose from ISF into the back of the microneedle device for sensing, other devices required fluid transfer out of the microneedle for drug quantification. This presents major limitations due to extremely small volumes of accessible dermal ISF (<1 µl mm⁻² of skin [18]) being insufficient for standard analytical techniques.

We developed a microneedle-based lab-on-chip device to trap vancomycin from sub-nanoliter volumes of samples and immediately conduct reaction schemes for drug evaluation inside a hollow microneedle lumen [19]. This device combined many of the key elements required in an ideal TDM system, specifically, being painless, requiring an extremely low sample volume (~0.6 nl), being rapid, lacking a need for sample transfer out of the microneedle, and not requiring bulky laboratory equipment for drug quantification at very low limit of detection. Compared to the previously published work, the work presented here describes and demonstrates in detail: (1) the design considerations for microneedle-optofluidic biosensors, (2) the assessment of the individual components of such a complex system for system design, and (3) the in vitro potential of such a device as a biosensing platform, capable of collecting, trapping, and analyzing biological molecules in sub-nanoliter volumes of sample at nano- to micro-molar concentrations. This study utilized streptavidin-horseradish peroxidase as a model drug to interact with the biotinylated microneedle surface, which was quantified by an enzyme-linked absorbance assay. The biotin-streptavidin platform will facilitate the immobilization of specific recognition elements to adapt the sensor for the detection of a plethora of drug candidates.

2. Methods

2.1. Materials

The following materials were purchased for this study:

- Thiol-polyethylene glycol-biotin (thiol-PEG-biotin, mol. wt. 5kDa, purity >95%) and thiol-methoxy polyethylene glycol (thiol-mPEG, mol. wt. 5kDa, purity >95%) from Nanocs Inc. (Boston, MA, USA); streptavidin (Sav) from Streptomyces avidini (lyophilized powder, mol. wt. 60kDa), and 3,3,5,5-tetramethylbenzidine (TMB, purity ≥95% NT, mol. wt. 240.34 Da) from Sigma-Aldrich (Buchs, Switzerland); streptavidin-horseradish peroxidase (Sav-HRP) conjugate (2.5 mg ml⁻¹, mol. wt. 110kDa) from Invitrogen Corporation (Camarillo, CA, USA); hydrogen peroxide (30% H₂O₂, Perhydrol®) from Merck (Darmstadt, Germany); SU-8 2075 photo-curable polymer resist and the propylene glycol methyl ether acetate (PGMEA) developer from MicroChem (Newton, MA, USA); and polydimethylsiloxane (PDMS, Sylgard 184) from Dow Corning Corp (Midland, MI, USA).

2.2. Hollow microneedles

Single hollow metallic microneedle devices were fabricated according to [20] with modifications, involving a 3-step gold-nickel-gold metal electrodeposition process. Electroplated metal sheets with microneedles were cut into smaller circular pieces (3 mm diameter, and 7.1 mm² area). Hereforth, a ‘microneedle backside’ refers to the backside surface of such a 3 mm diameter circular sheet of gold-coated nickel containing a single out-of-plane hollow microneedle (the side of the metal sheet opposite the protruding microneedle). The surface area of the microneedle backside and inner lumen are 7.1 mm² and 0.06 mm², respectively.

2.3. Microneedle surface functionalization

2.3.1. Surface cleaning. All surfaces were cleaned using UV irradiation at a 170nm wavelength for 10min using a flat excimer Ex-Mini source (Hamamatsu, Japan).

2.3.2. Self-assembled monolayers. Thiol-PEG-biotin and thiol-mPEG conjugates were employed to construct self-assembled monolayers (SAM) on gold surfaces [21, 22]. A 5000Da PEG chain (PEG-5000) was selected due to its ability to reduce non-specific adsorption of proteins in biological fluids [23]. Thiol-PEG-biotin and thiol-mPEG solutions were prepared fresh by dissolving in milliQ water to a concentration
of 1 mM. Thiol-PEG-biotin to thiol-mPEG molar fractions ranging from 0–1.0 (referred to as ‘PEG-biotin solutions’ hereforth) were obtained and tested to determine the optimum surface density of biotin on the gold-coated substrates for Sav binding, as done previously [19]. Droplets of 5 µl from each PEG-biotin solution were incubated on the microneedle backside surfaces for 2 h at room temperature (figure 1(A)). The gold-surfaces were withdrawn from the PEG-biotin solutions, and washed with phosphate buffer. Sav-HRP (2.5 mg ml⁻¹) was diluted with phosphate buffered saline (PBS) to a concentration of 10 µg ml⁻¹ (0.1 µM). Biotin-functionalized microneedle backside surfaces were incubated with 5 µl droplets of 0.1 µM Sav-HRP for 1 h at room temperature, followed by three PBS washing steps. Sav-HRP was quantified using the 3,3′,5,5′-tetramethylbenzidine (TMB) assay, as described below.

2.3.3. Streptavidin-HRP binding. Microneedle backside surfaces functionalized with a SAM of 0.1 molar ratio of thiol-PEG-biotin to thiol-mPEG were incubated with 5 µl droplets of Sav-HRP (concentrations ranging from 1.9–192 µM) for 1 h at room temperature to determine the level of bound Sav-HRP. At the end of the incubation period, the microneedle bases were washed with PBS and assessed for the Sav-HRP levels using the TMB assay.

2.4. TMB assay

The enzyme-linked TMB assay is used to quantify the activity of the enzyme HRP. HRP oxidizes 3,3′,5,5′-tetramethylbenzidine (colorless) in the presence of hydrogen peroxide, changing the color of the TMB solution to blue, which can be detected at a wavelength of 635 nm (as shown in figure 1). The Sav-HRP conjugate is used as a model drug to quantify binding concentrations of molecules to the microneedle surfaces. The absorbance of the TMB oxidation end-product correlates to the concentration of Sav-HRP conjugate present on a surface.

2.4.1. Conventional TMB assay. After binding the Sav-HRP conjugate to the microneedle backside, a 10 µl droplet of the TMB stock solution (0.4 mM TMB, 0.04 mM H₂O₂ in 0.1 M citrate buffer, pH 6.8) was placed on top of the functionalized microneedle backside, and incubated for 10 min at room temperature to allow the HRP to produce the TMB oxidation end-product. After 10 min, a 2 µl volume of the TMB solution was extracted from the microneedle backside surface, and its absorbance at 635 nm was determined using a NanoDrop ND-1000 spectrometer (ThermoFischer Scientific, Waltham, MA, USA).

2.5. Integrating the microneedle to optofluidic sensing device

To create a continuous biosensing system without major and time-consuming moving parts, a surface functionalized microneedle was integrated into an optical sensing unit that acts simultaneously as a microfluidic detection chamber and as an optical waveguide, forming an optofluidic device (OFD; figure 2). A microneedle base was attached to the OFD using an instant-bonding cyanoacrylate adhesive. It was designed to measure in situ the absorbance of end-products from enzymatic reactions that take place on the microneedle surface, using a simple diode laser and a photodetector. The microneedle was attached to the optofluidic transducer made of polydimethylsiloxane (PDMS; figures 2(A) and (B)). Initially, a master was fabricated by photolithography using the negative tone SU-8 photoresist. Then, it was copied by casting of PDMS and thermal cross-linking at 80 °C as described previously [19].

The detection chamber/optical waveguide (hereforth referred to as the ‘OFD waveguide’), which is the main component of the device, was fabricated with dimensions of 150 µm height, 50 µm and 100 µm widths, and 0.1–5 mm lengths. Light was coupled in and out of the OFD using a luminescent diode laser (635 nm, 1 mW), which was coupled to a 4 µm diameter single-mode optical input fiber. A 50 µm diameter multi-mode output fiber was connected to a silicon PIN photodiode (PM100D compact power and energy meter console; Thorlabs, NJ, USA) and was placed 30 µm away from the opposite end of the OFD waveguide to collect the output light from the waveguide at a frequency of 1 Hz. The fluid inlet to the OFD was through the microneedle base, while the outlet was connected to a syringe pump to improve fluid flow control.

The optical properties of the devices were characterized using methyl green, a dye which absorbs light at 635 nm, similar to the TMB oxidation end-product. Methyl green was diluted to an absorbance reading of 2.5 AU 10 mm⁻¹ path length.

2.5.1. Microneedle-optofluidic device performance. Biosensing tests using the microneedle-optofluidic biosensor were conducted as follows:

1. To assess the performance of microneedle backside, a 5 µl volume of Sav-HRP (concentration ranging from 4.8 nM to 57.7 µM) was placed on the microneedle outer surface and gradually pulled into the reaction chamber (microneedle backside and lumen), allowing the Sav-HRP to interact with surface-bound biotin. Similarly, to assess the performance of microneedle lumens only, a 1 µl volume of Sav-HRP was placed on a glass slide, and the microneedle tip was brought in contact with the droplet for 30 s to allow capillary flow of 0.6 nl of Sav-HRP into the microneedle lumen.

2. After 10 min of incubation, the Sav-HRP solution was removed from the microneedle by flushing the device with 20 µl of phosphate buffer (using the syringe pump).

3. A 10 µl droplet of the TMB solution (0.4 mM TMB, 0.04 mM H₂O₂) was placed on the microneedle outer surface. The TMB solution was guided through the Sav-HRP on the microneedle lumen and backside into the waveguide at a flow rate of 10 nl s⁻¹ for 240 s.

4. After 240 s the remaining TMB solution from the microneedle outer surface was removed and replaced
4 by 50 µl phosphate buffer, which was flushed through
the system to terminate the enzymatic reaction and the
biosensing process.

In the case of performing multiple TMB biosensing cycles
in one device, steps 2–3 were repeated (at same or different
TMB concentrations). From the data collected, average
absorbance (at 635 nm) of the TMB oxidation end-product
was determined during the 196 s–205 s time interval to com-
pute the TMB calibration curves in the biosensor.

3. Results and discussion

3.1. Surface functionalization

The well-understood Sav-biotin binding was used to assess
the ability to perform bio-recognition assays on microneedle
surfaces. The direct binding capacity of Sav to biotin was eval-
uated in the biosensing system, compared to the competitive
binding scheme for Vancomycin in the previously published
work [19]. The Sav-biotin interaction has been widely used to
construct functional surfaces due to its high affinity binding
and bond stability, as we previously demonstrated [19]. It
can act as a linking platform to build surfaces with a large
variety of functional moieties, in a step-wise fashion. SAMs
containing thiol-PEG-biotin were formed and optimized on
the gold-coated backside surfaces of microneedle devices to
accommodate optimal binding of Sav. The highest level of Sav
binding was found for surfaces functionalized with a 10 mol%
thiol-PEG-biotin and 90 mol% thiol-mPEG (0.63 ± 0.05 AU),
similar to previously reported values [19]. This was three-fold
greater than the level of Sav binding in a surface functional-
ized with 100 mol% thiol-PEG-biotin (0.19 ± 0.05 AU). As a
result, a 10 mol% thiol-PEG-biotin to 90 mol% thiol-mPEG
was used in all further SAM formations on microneedle back-
sides and lumens.

Microneedle backsides functionalized with thiol-PEG-biotin
were tested for Sav-HRP binding to determine the
specific Sav-biotin binding behavior, including the linear
binding range and binding saturation levels (figures 1(A) and
(B)). A conventional TMB assay was conducted on microneedle
backsides to determine the level of bound Sav (figure 1(C)).
Sav-HRP binding to the biotinylated microneedle backside
was linear up to 38.5 nM at a sensitivity of 8.59 AU µM⁻¹
and a limit of detection (LoD) of 0.83 nM (figure 1(D)). The LoD

\[
\text{LoD} = \frac{3 \times \sigma_{\text{blank}}}{s}
\]

Figure 1. Microneedle surface functionalization. (A) Microneedle devices were functionalized with thiol-PEG-biotin to determine
and optimize the functionalization process and streptavidin binding. The backside of gold-coated single microneedle devices (7.1 mm²
area) were cleaned by UV irradiation (A-1), incubated with PEG-biotin solutions (A-2), and bound with Sav-HRP (A-3) at 22 °C for
1 h. (B) The model-biosensing reaction scheme involved binding Sav-HRP (B-1) and quantifying the bound Sav-HRP level by using the
enzyme-catalyzed TMB oxidation (B-2). (C) The conventional TMB assay parameters were optimized using a NanoDrop UV spectrometer
(1 mm path length). A colorless TMB solution was converted by HRP in the presence of H₂O₂ (C-1) to a TMB oxidation end-product (blue
color) over a 10 min reaction period (C-2) and (C-3), absorbance of which was quantified at 635 nm. (D) The amount of Sav-HRP bound to
the microneedle device surface was quantified using the TMB assay to provide a direct binding curve.
of the optofluidic components are optical fibers, and the position of the air mirrors. Refractive indices through the optical waveguide/detection chamber, locations of the system for analyte concentration determination. (B) Top view of the optofluidic device, illustrating the TMB end-product flow direction through the optical waveguide/detection chamber; locations of the optical fibers, and the position of the air mirrors. Refractive indices of the optofluidic components are \( n_{\text{air}} = 1.0 \), \( n_{\text{water}} = 1.33 \), and \( n_{\text{PDMS}} = 1.41 \).

was calculated from the standard deviation of a blank measurement \( s_{\text{blank}} \) and the measurement sensitivity \( s \).

The measurement sensitivity was determined by the slope of the linear binding range in figure 1(D). The highest Sav-HRP binding was observed at 38.5 nM (0.36 ± 0.05 AU), after which the signal decreased, and then stabilized around 0.15–0.20 AU (figure 1(D)). The highest Sav-HRP binding was very similar to the optimum value reported previously [19]. The decrease and stabilization in the signal may be attributed to effects from the competition between free Sav and bound Sav to the biotinylated surface at high Sav concentration; an effect of the four-fold biotin-binding capability per Sav; and/or the conformational differences in Sav binding to biotin on the surface at concentrations higher than 38.5 nM. The linear Sav binding range presented in figure 1(D) indicated that molecule binding and detection can be performed on the biotinylated microneedle surfaces.

### 3.2. Optofluidic device optimization

The OFD’s sensing characteristics were optimized prior to performing binding and detection of Sav-HRP. A number of parameters of the sensing system, including the integration of air mirrors, and the OFD waveguide width and length were manipulated to enhance the sensitivity of detection, while ensuring that the sensing occurred in the linear detection range for the TMB assay at given reactant concentrations. The OFD waveguide was designed to enhance the interaction between the light and the colored substrate in the sensing chamber. In principle, the coupling of the light at the chamber \( (n_{\text{water}} = 1.33) \) and PDMS \( (n_{\text{PDMS}} = 1.41) \) interface is based on hollow waveguide confinement [24], since \( n_{\text{water}} \) is smaller than \( n_{\text{PDMS}} \). However, a significant optical signal was lost in this form of light coupling (up to 23 dB). The intrinsic optical losses were significantly reduced to 17 dB by engineering air-mirrors along the waveguide to reflect what would be otherwise unrecoverable optical transmissions back towards the OFD waveguide. Air mirrors allowed total internal reflection to reflect back to the OFD waveguide a large amount of incident light. The OFD included self-aligning optical fiber channels and air-mirrors to confine the light in the waveguide and increase the signal to noise ratio (SNR).

During the optimization of the OFD, the optical signal transmission through the citrate flow buffer (at a 10 nl s⁻¹ flow rate) was measured in OFD waveguides of varying dimensions with length and width ranging from 0.1–5.0 mm and 50–100 \( \mu \)m, respectively (figure 3). From the 1 mW diode laser input power at 635 nm that was fed into the OFD waveguide, the mean power of noise from the OFD was ± 50 pW, while the optical output power through the buffer-filled OFD waveguide ranged from 1–443 pW. This provided the biosensor with a high SNR between 44.5–69.5 dB for reliable optical measurements. The optical losses recorded through the buffer-filled OFD waveguide increased linearly as a function of its length. At very short lengths (0.1–0.5 mm), the 50 \( \mu \)m OFD waveguide channel width yielded lower optical losses compared to the 100 \( \mu \)m channels. Beyond 1 mm channel length, the impact of channel width on the optical losses appeared negligible in the studied range.

Methyl green, a dye absorbing light at 635 nm wavelength similar to the oxidation product of TMB, was used to characterize the absorbance in the OFD waveguide due to sensing events. A linear increase in the absorbance of methyl green was observed as a function of the OFD waveguide length for both 50 \( \mu \)m and 100 \( \mu \)m width levels, as expected by the Beer–Lambert law. The absorbance sensitivity determined in the OFD waveguide with a 50 \( \mu \)m width was slightly higher compared to a device with 100 \( \mu \)m width. The 5 mm long and 50 \( \mu \)m wide OFD waveguide yielded the highest absorbance in the linear range for methyl green (2.3 AU). Beyond 5 mm

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**Figure 3.** Optimization of the optofluidic device. The width and length dimensions of the OFD waveguide were modified and tested for intrinsic optical losses using citrate buffer.
in length, absorbance values deviated from linearity for both widths (data not shown). As a result, an OFD waveguide with 5 mm length and 50 \( \mu \)m width was chosen to integrate a surface functionalized microneedle for proof-of-concept biosensing using the Sav-biotin scheme in figure 1(B). This waveguide also provided an extremely low fill volume of \(~40\) nl of TMB end-product for measurement, which allowed for rapid measurement with minimal reagent use.

3.3. Performance of the integrated microneedle-optofluidic biosensor

The microneedle-OFD was first used to assess the binding of Sav-HRP to the entire surface of the microneedle backside (figures 4(A) and (B)). To utilize the functionalized microneedle backside (7.1 mm\(^2\) area), a 5 \( \mu \)l droplet of Sav-HRP with concentrations up to 58 nM was brought into the microneedle base through the microneedle lumen by suction flow generated using a syringe pump. After removal of Sav-HRP from the microneedle backside, a TMB droplet was placed on the microneedle and flowed into the OFD through the microneedle lumen at a rate of 10 nl s\(^{-1}\), providing sufficient contact between the TMB reactants and the HRP bound to the microneedle backside and producing the colored end-product that was measured in the OFD waveguide (figure 4(A)).

The rate of TMB conversion to its oxidation end-product reached a plateau within 2–3 min due to enzyme saturation and activity depreciation. The average absorbance at 200 ± 5 s after the reaction started was used to establish a calibration curve for the binding of Sav-HRP to the microneedle backside (figure 4(B)). Sav-HRP binding and detection in the biosensor yielded a linear detection range from 1.14–38.5 nM at a sensitivity of 10.6 AU \( \mu \)M\(^{-1}\), providing a comparable translation of the conventional TMB assay of microneedle backside surface (figure 1(D)) to the biosensor with about 2/3 reduced measurement time. Given that the volume of the OFD waveguide was \(~40\) nl and the TMB measurement only required \(~3\) min of flow through the microneedle surface at 10 nl s\(^{-1}\), the measurement could be performed with less than 5 \( \mu \)l of the TMB stock solution in the biosensor. The LoD in the biosensor (1.14 nM Sav-HRP, determined using equation (1)) was comparable to the conventional TMB assay (0.83 nM Sav-HRP); while the biosensor yielded a higher sensitivity (10.6 AU \( \mu \)M\(^{-1}\)) compared to the conventional TMB assay for the microneedle backside (8.59 AU \( \mu \)M\(^{-1}\)).

To demonstrate the specificity of Sav binding to the modified microneedle backside surface, a biotin-less self-assembled...
monolayer (100 mol% thiol-mPEG) was incubated with the highest concentration of Sav-HRP (38.5 nM) and assessed using the TMB assay in the biosensor (figure 4(A)). Lack of binding of Sav-HRP to the microneedle backside was evident by the absence of TMB oxidation (baseline result) in figure 4(A).

3.3.1. Biosensing in the microneedle lumen. Having confirmed the functionality of the biosensor, the potential to bind Sav-HRP only inside the microneedle lumen was assessed.

During SAM formation on the microneedle base, the inner microneedle lumen containing an electrodeposited layer of gold was also functionalized with the biotinylated thiol-PEG layer. During Sav-HRP binding, capillary forces were used to fill the microneedle lumen without allowing the sample to come in contact with the backside surface of the microneedle device, ensuring that only 0.6 nl of volume filled the microneedle lumen and interacted with the 0.06 mm² surface area. Significantly higher Sav-HRP concentrations (>0.5 µM) were needed to achieve a detectable signal from the microneedle lumen only due to a significant reduction in the active surface area where biotin molecules were present for biorecognition and binding of Sav-HRP; and the extremely low quantity of Sav-HRP molecules present in the 0.6 nl sample volume at concentrations lower than 0.5 µM. Above 0.5 µM, Sav-HRP bound to biotin in the microneedle lumen in sufficient quantities to allow detection using TMB (figures 4(C) and (D)). Sav-HRP binding in the microneedle lumen followed a linear trend up to 7.21 µM at a detection sensitivity of 0.17 AU µM⁻¹ and a LoD of 60.2 nM. Beyond 7.21 µM, Sav-HRP binding saturated the surface and hindered further binding. The linear Sav-HRP detection range and the LoD in the microneedle lumen was significantly higher than in the microneedle backside, while the detection sensitivity was significantly lower than in the microneedle backside. Nonetheless, the binding and detection of Sav-HRP in a microneedle lumen with only 0.6 nl is the smallest reported biosensing volume used in a microneedle-based assay. In comparison, other microneedle-based technologies required more than 1000 times higher sensing fluid volumes (>1 µl) [14, 15, 17].

A summary of key results from the microneedle-optofluiddic biosensor is presented in table 1.

3.4. Additional utility of the biosensor

The microneedle-optofluiddic biosensor can also be used as an enzyme sensor to study enzyme kinetics and dynamics, and to optimize enzyme reaction conditions. As an example, the relationship between one reactant (H₂O₂) and an enzyme’s turnover was determined by binding 38 µM Sav-HRP to the microneedle (figure 5(A)). A linear dependency of the

Table 1. Performance summary for the microneedle optofluiddic biosensor and the conventional biosensing assay.

<table>
<thead>
<tr>
<th>Detection of Sav-HRP</th>
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<tr>
<td>Functionalized surface</td>
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<tr>
<td>Sample volume</td>
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<td>Sensing area</td>
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<td>Maximum detectable concentration (in the linear range)</td>
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<td>Sensitivity</td>
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<td>Limit of detection</td>
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<td>Time</td>
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Figure 5. Utility of the microneedle-optofluiddic sensor as an enzyme sensor. (A) Response of the HRP enzyme to increasing levels of hydrogen peroxide from 0.02-0.16 mM to determine the optimal reactant concentrations (38 µM Sav-HRP). A calibration curve of TMB absorbance versus H₂O₂ concentration (A-inset) showed the linear range for H₂O₂. (B) Repeatability of the TMB assay measurements in the sensor over multiple TMB cycles (9.6 µM Sav-HRP, 0.04 mM H₂O₂). Peaks represent activity of the HRP to produce the colored TMB end-product, while valleys represent washing of the microneedle surface with phosphate buffer to remove reactants from the system.
The process of developing a proof-of-concept biosensor integrating surface-functionalized hollow metallic microneedles and an optofluidic sensor was demonstrated using the universal Sav-biotin platform that can be adapted to the detection of a myriad of highly relevant analytes such as TDM drugs. Different components of the biosensor have been optimized to obtain a highly performing OFD capable of sensing analytes in volumes below 1 nl. The biosensor was validated in vitro by detecting the direct binding of Sav-HRP to a biotinylated thiol-PEG monolayer on the microneedle surface at extremely low LoDs ranging from 1.1–60.2 nM. Furthermore, this detection was performed in real-time using extremely low sample volumes that are not otherwise possible with conventional assays, which usually require more than 1 µl of sample for analysis. All components of the biosensor provided great flexibility and avenues for optimization of the performance of the sensing system for specific TDM drug candidates and applications. Drug-specific biotinylated ligands may be used to recognize and detect drug candidates in extremely low volumes of biological fluids and bind them to the Sav inside the microneedle lumen. This biosensing platform will pave the way to the development of patient-friendly, painless, minimally-invasive, and point of care diagnostic tools for specific TDM applications.

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