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Microfluidic ELISA Employing an Enzyme Substrate and Product Species with Similar Detection Properties

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Abstract

The requirement for the enzyme label to carry out a chemical reaction directly at the signaling region of the enzyme substrate in order to produce a large change in its detectability places a significant constraint on the scope of enzyme-linked immunosorbent assays (ELISAs). In particular, this requirement limits the kinds of enzyme label-substrate couples employable in ELISAs and prevents their independent optimization with respect to the enzyme reaction and the detectability of the enzyme reaction substrate/product. The detection limit and multiplexing capabilities of the assay are consequently restricted in addition to rendering the technique applicable to a narrow range of assay conditions/samples. Attempting to address some of these limitations, the current article describes a microfluidic ELISA method that does not require the enzyme label to act around the signaling region of the substrate molecule. A highly detectable rhodamine based substrate was synthesized to demonstrate the reported assay which upon cleavage by the enzyme label, alkaline phosphatase, transformed from a monoanionic to a monocationic species both of which had nearly identical fluorescence properties. These species were later separated based on their charge difference using capillary zone electrophoresis in an integrated device yielding a quantitative measure for the analyte (human TNF- α) in our sample. Impressively, the noted approach not only enabled the use of a new kind of enzyme substrate for ELISAs but also allowed the detection of human TNF- α at concentrations over 54-fold smaller than that possible on commercial microwell plates primarily due to the better detectability of the rhodamine dye.

Keywords

capillary zone electrophoresis; ELISA; fluorescence; microfluidics; rhodamine coupling; zwitterionic substrate

1. Introduction

Enzyme-linked immunosorbent assay (ELISA) offers a powerful technique that allows the quantitation of a variety of biological substances such as proteins, peptides, antibodies, hormones, etc., in complex matrices [1,2]. The utility of ELISA in these applications comes from its high specificity based on the antibody-antigen reaction and high sensitivity arising from signal amplification by the enzyme label. In addition, this assay is relatively simple to

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implement and involves only a series of washing and incubation steps prior to its final quantitation, usually by an optical [3] or electrochemical detection method [4]. For these reasons, ELISA is widely accepted as a gold standard for analyzing samples relevant to fundamental biological research, biomedical testing, environmental monitoring and food assessment applications among others [5–8]. Over the years, ELISA methods have been significantly improved through the design of assay surfaces that allow efficient immobilization of the target analyte but minimize non-specific binding of unwanted molecules [9], development of high performance enzyme label-substrate couples [10] and construction of sensitive detection systems [11]. More recently, there has been a concerted effort towards the miniaturization of this technique which has led to further enhancement in its sensitivity as well as a reduction in its sample size and incubation period requirements [12–14].

Remarkably, beyond improving the detection limit for ELISA methods there exists very little literature focused on extending its scope to a broader range of assay conditions/samples. In particular, the kinds of enzyme reactions that are currently employed in ELISA measurements are quite limited and can be broadly categorized as either involving the oxidation of a non-dye to a dye (e.g., many Horseradish Peroxidase catalyzed reactions), or a bond cleaving reaction that involves a bond between an oxygen of a chromophore and some blocking group [15]. In the latter case, the cleavage reaction results in the formation of a much more strongly electron donating oxygen group and dramatically modifies the chromophoric properties of the substrate species. This situation is exemplified by the use of fluorescein phosphate as an enzyme substrate which is moderately fluorescent to begin with but gets converted to the highly fluorescent fluorescein species upon $O-PO_3^-$ bond cleavage by the enzyme label, alkaline phosphatase. The limited number of enzyme reactions employed in current ELISA methods is not surprising however, as the enzyme label-substrate couple involved in the process has to meet some stringent requirements for it to be useful. First and foremost, the enzyme label needs to act at, or near the signaling region of the substrate molecule in order to significantly alter its detectability. This is because without such a change in the substrate detectability it is impossible to determine the occurrence of the enzyme reaction as the substrate and the corresponding enzyme reaction product are both present in the assay medium the entire time. Moreover, it is desirable that either the substrate or the corresponding enzyme reaction product inherently has a high detectability so as to yield a large signal-to-noise ratio in the system. Insofar as the “proto-chromophore” bears little or no resemblance to most biomolecules, this means that enzymes used as ELISA reporters must have extraordinarily broad substrate tolerance. Finally, in order to realize reasonable enzyme reaction periods for ELISAs, typically less than an hour, the enzyme label employed in the technique is required to yield a high turnover number under the assay conditions. Indeed, the most commonly used ELISA reporter enzyme, alkaline phosphatase, has been described by Jencks as “an almost perfect enzyme” for the purpose [16]. Nevertheless, the constraints discussed above significantly limit the kinds of enzyme label-substrate couples available for ELISAs restricting their sensitivity, multiplexing capability as well as their scope for making analytical measurements over a wide range of assay conditions, e.g., extreme pH, temperatures, and/or those involving matrices that tend to interfere with the assay procedures.

To address some of the limitations noted above, we describe the development of a novel microfluidic ELISA method that allows the use of an enzyme substrate which does not undergo any change in its detectability during the course of the enzyme reaction. Instead, the charge state of the substrate species is altered during this reaction period allowing for electrophoretic separation of the enzyme reaction product from the substrate molecules to determine its level in the assay mixture. This level is then shown to directly correlate with the analyte concentration in the original sample establishing the quantitative nature of our assay. In order to demonstrate the noted ELISA method, a highly detectable rhodamine based substrate was synthesized in our current work which upon cleavage of a dianionic phosphate group by the enzyme label, alkaline phosphatase, at a site remote from the chromophore, was transformed from a monoanionic to a monocationic species both of which had nearly identical fluorescence properties. The enzyme reaction was initiated in our system by introducing the substrate solution into an analysis channel that had been previously coated with the antigen-antibody sandwich immunocomplex. To monitor the extent of the enzyme reaction, narrow plugs of this substrate solution were then injected into a separation column after incubation against the assay surface for a chosen period. An electric field was employed to migrate these plugs through the separation column, which allowed the electrophoretic separation of the product molecules from the substrate ones. The peak corresponding to the former species was later quantitated in order to determine the rate of enzyme reaction which correlated well with the analyte concentration in the original sample. Impressively, the noted approach not only enabled the use of a new kind of enzyme substrate for ELISAs but also allowed the detection of human TNF- α at concentrations over 54-fold smaller than that possible on commercial microwell plates primarily due to the better detectability of the rhodamine dye. It must be noted that while capillary zone electrophoresis has been previously coupled to homogenous immunoassays [17], enzyme assays [18] and heterogeneous immunoassays with cleavable tags [19], such integration to broaden the scope or improve the sensitivity of ELISA methods has not been explored to our knowledge which is the focus of the present work.

2. Experimental Procedure

2.1 ELISA substrate synthesis

The reported rhodamine based ELISA substrate for the enzyme label, alkaline phosphatase, was synthesized in our work following the route outlined in Figure 1. Briefly, tribenzylphosphite (compound **1**) was synthesized according to the literature [20] by successively adding two separate solutions of triethylamine and benzyl alcohol both prepared in diethyl ether to a vigorously stirred solution of PCl_3 again prepared in the same solvent at -10°C . The resulting mixture was stirred for 30 min at -10°C and for 15 hrs at room temperature to obtain a pale yellow liquid of compound **1**. In a statistical step, an excess of di-ethylene glycol was de-symmetrized under mild conditions [21] to obtain the protected phosphate tri-ester (compound **2**). Compound **2** was successfully coupled to rhodamine B using dicyclohexylcarbodiimide (DCC) and catalytic dimethylaminopyridine (DMAP) in dichloromethane to obtain compound **3**. The benzyl protecting groups of **3** were removed by solvolysis with trifluoroacetic acid (TFA) containing 5% thiophenol (PhSH) [22]. Substrate **4** was obtained as a chloride salt in chloroform, after treatment with 1 M

aqueous sodium hydroxide followed by 1 M hydrochloric acid. The identities and purities of the above described intermediates, and of compound **4** (the target enzyme substrate) were confirmed by nuclear magnetic resonance (NMR) obtained with a Bruker Avance III 400 and 600 MHz spectrometer. ^1H and ^{13}C chemical shifts were referenced to the residual solvent peak, while the ^{31}P chemical shift was not referenced to any standard.

2.2 Device fabrication

For fabricating the microfluidic devices employed in this work, bottom and cover plates made from borosilicate glass were purchased from Telic Company (Valencia, CA). While the purchased cover plates had both their faces unprotected, the bottom ones came with a thin layer of chromium and photoresist laid down on one of their surfaces. Custom designed photomasks created through Fineline Imaging Inc. (Colorado Springs, CO) were used to pattern the desired channel layout onto the bottom plate using standard photolithographic methods [23,24]. A simple cross-channel network was used in our experiments with all its segments being 100 μm wide and 10 μm deep (see Figure 2). The enzyme reaction was carried out in segment A of this device while the separation of the rhodamine based enzyme reaction product from the enzyme substrate was performed in segment D. The purpose of the other two channel segments (segments B and C) in this network was to aid the injection of the enzyme reaction substrate/product plug into the separation channel. For our current design, the lengths of channel segments A and D were chosen to be 2 and 1.5 cm respectively, while each of segments B and C were made 1 cm long. Following the completion of the photo-patterning process, the photoresist layer was cured in a microposit developer MF-319 (Rohm and Haas) and the chromium layer removed along the channel network with a chromium etchant (Transene Inc.). The fluidic ducts were then etched to the chosen depth using a buffered oxide etchant (Transene Inc.). The protective photoresist and chromium layers on the bottom plate were subsequently removed using the MF-319 and chromium etchant solutions, respectively. Access holes were drilled at the channel terminals using a microabrasive powder blasting system (Vaniman Inc.) to allow the introduction of liquid reagents/sample into the microchannels. Finally, the microfluidic network was sealed off by bringing a glass cover plate in contact with the bottom one in deionized water and allowing the two to bond at 550°C for 9 hrs [25]. Cylindrical glass reservoirs with internal volumes of about 150 μL were affixed over the access holes using a ultraviolet light curable glue (Norland Products Inc.) to serve as sources or sinks for the liquid flow within the microchannels. In order to perform the standard microfluidic ELISAs with a commercial substrate for alkaline phosphatase, microchips with 8 straight channels were prepared using the same fabrication methods as discussed above. The details on the fabrication of these microchips can be found in our previous publications on the topic [26–28].

2.3 Preparation of the ELISA surface

The entire microchannel network was activated for protein attachment by treating with 1 N NaOH (Sigma-Aldrich) for an hour followed by sequentially rinsing with de-ionized water and methanol (Fisher Scientific) for 10 min each. These conduits were then dried at 80°C in a forced-air convection oven, and later derivatized with (3-aminopropyl)triethoxysilane (Sigma-Aldrich) for 1 hr under ambient conditions. The resulting glass surface was subsequently rinsed with methanol, dried again at 80°C, and reacted for an hour with an

aqueous solution of 5% w/v glutaric dialdehyde (Sigma-Aldrich) at room temperature to create a surface that could be covalently attached to the amine groups on a protein molecule [26]. The resulting glutaric dialdehyde coated channel was incubated with a 5 µg/mL solution (prepared in a 0.1 M carbonate buffer, pH 9.6) of mouse anti-human TNF-α, MAb1 (BD Biosciences), then blocked with 1% BSA (prepared in a 0.1 M phosphate buffer, pH 7.4) and 0.1 M lysine (also prepared in the phosphate buffer), and later reacted with the sample for an hour each. Notice that the TNF-α samples used in our experiments were prepared by diluting a stock solution of the analyte with human serum (Sigma Aldrich) in order to ensure the applicability of our assays to specimen of practical interest. Subsequently, the ELISA surface was completed by treating with a 0.1 µg/mL solution (prepared in the phosphate buffer) of biotinylated mouse anti-human TNF-α, MAb11 (BD Biosciences), followed by a 0.2 µg/mL of streptavidin alkaline phosphatase conjugate obtained from Invitrogen again for an hour each. The microchips with 8 straight channels used for performing the standard microfluidic ELISAs were also prepared for an experiment following the same procedure as described above [29]. For the microwell based assays however, commercial plates pre-coated with a human TNF-α capture antibody (Abazyme LLC) were employed. These microwells were also prepared by following the same incubation procedures as in the corresponding microchip based ELISAs beginning with the TNF-α sample incubation step [29]. All incubation periods for our microchip and microwell based assays were chosen to be an hour long with the human TNF-α sample used in them obtained as part of the Abazyme ELISA kit. Overall, the analysis time for the reported ELISA method was identical to that of standard microfluidic and microwell based ELISAs as the separation time needed for the assay was negligibly short relative to the enzyme reaction period. The device preparation time for the microfluidic assays however was 7 hrs longer than that for the microwell based version as the capture antibodies had to be immobilized on the channel surface in the former case while the microwell plates came pre-coated with this particular reagent. For the novel ELISA method described here, a 200 µM solution of the reported rhodamine based enzyme substrate containing 1 mM MgCl₂ (in a 10 mM sodium tetraborate buffer, pH 9.4) was used as the substrate solution. Prior to our experiments, it was established that this level of the substrate species yielded saturation kinetics for alkaline phosphatase under the experimental conditions as was desirable in the quantitation of our assay method. In the case of the standard microfluidic and microwell based ELISAs however, a 1 mM solution of 4-methylumbelliferyl phosphate containing 1 mM MgCl₂ (in a 10 mM sodium tetraborate buffer, pH 9.4) was used for the enzyme reaction as has been reported in our previous work [29].

2.4 Device operation

To enable ELISA measurements using the rhodamine based enzyme substrate reported in this work, the substrate solution was placed in reservoir 1 of the microchip device shown in Figure 2 while filling the remaining reservoirs with a 10 mM sodium tetraborate buffer (pH 9.4). To initiate the enzyme reaction at the channel surface, 0.6 and 1.2 kV were applied to reservoirs 1 and 2, respectively, while electrically grounding reservoirs 3 and 4 for 30 s. This step allowed the introduction of the substrate solution into segment A and also established a flow profile as shown in stage I of Figure 2. The electrical voltages at all the 4 reservoirs were then dropped to zero to allow the enzyme reaction to occur under stagnant conditions.

Following a chosen enzyme reaction period (10-30 min.), 0.6 and 1.2 kV were reapplied to reservoirs 1 and 2, respectively, leaving terminals 3 and 4 electrically grounded for 5 s to reestablish the flow profile shown in stage I of Figure 2. Notice that this washing step had to be just long enough to reestablish the flow profile shown in stage I of Figure 2 but not so long that it purged out a significant amount of the enzyme reaction product generated in channel segment A into waste reservoir 3. In addition, this step also allowed us to wash out the enzyme substrate/product molecules that had migrated into the injection cross and beyond via diffusion during the enzyme reaction period which was determined necessary in our experiments for realizing high efficiency separation of the enzyme substrate/product species as well as ensuring that the enzyme reaction rate estimated by quantitating the separation peaks corresponded to saturation kinetics. Immediately after the washing step, the voltage at reservoir 2 was dropped to zero and that applied at reservoir 1 was raised to 1 kV for 1 s to define an injectable plug for the enzyme substrate/product species (see stage II in Figure 2). This plug was then transported through segment D to enable the desired electrophoretic separation by applying 1.2 and 2.4 kV to reservoirs 1 and 2, respectively, for 20 s keeping the remaining reservoirs electrically grounded (see stage III in Figure 2). It must be pointed out that the no flow condition in the microchannels was achieved during the enzyme reaction period by carefully maintaining the same level of liquid in all the reservoirs in addition to electrically grounding all the 4 channel terminals. Moreover, the choice of a 10 μm deep channel network aided our effort in minimizing liquid flow that could have arisen from any unwanted differences in the hydrostatic heads at the channel reservoirs. The equally detectable substrate and product molecules were observed to separate out based on the difference in their electrophoretic mobilities during their migration through segment D yielding two distinct peaks in the recorded electropherogram. A constant air temperature of 37°C (measured using a thermometer placed close to the microfluidic device) was maintained in our experiments using a heating fan to minimize variations in the enzyme reaction rate caused by fluctuations in the room temperature [26]. The noted electropherograms were recorded using an epi-fluorescence microscope (Nikon) employing band-pass excitation (540-580 nm) and emission (600-660 nm) optical filters. The enzyme substrate and product species were detected in our experiments 0.7 cm downstream of the injection cross in segment D using a 20 $\times$ objective and a photomultiplier tube (Hamamatsu Photonics). The sequence of voltages applied to the different channel terminals in the noted assay were executed using a commercial electrical power supply (LabSmith high voltage sequencer, HAS448 3000) while the process of data acquisition by the photomultiplier tube was controlled with an in-house written LABVIEW program. The microchip and microplate based ELISAs that were carried out with a commercial enzyme substrate in this study were quantitated following procedures reported by our group in previous publications [29,30].

3. Results and Discussion

3.1 Spectral characterization of the enzyme substrate/product

Before proceeding with the quantitation of the reported microfluidic ELISA method, we characterized the spectral properties of the rhodamine based enzyme substrate and its corresponding product employed in this work to establish their similar detectability as was desired in the project. Figure 3 compares the excitation and emission spectra for these two

compounds along with that for rhodamine B prepared in a 10 mM sodium tetraborate solution containing 1 mM MgCl_2 . The concentration for the enzyme substrate and the rhodamine dye was chosen to be 1 μM in the corresponding measurements which were carried out using a Varian Cary Eclipse fluorescence spectrophotometer. The enzyme reaction product used in this study was prepared by taking a 1 μM solution of the enzyme substrate containing 1 $\mu\text{g/mL}$ of streptavidin alkaline phosphatase conjugate (Invitrogen) and leaving the mixture to react for 12 hrs under ambient conditions. It was ensured that practically all of the enzyme substrate was converted into its corresponding product over this period by continually monitoring the fluorescence of the noted solution for another 3 hrs. The change in the solution fluorescence over this 3 hr period was found to be within the noise level of our measurements, and was therefore deemed statistically insignificant. For the spectral measurements, the emission wavelength was set at 580 nm in our spectrophotometer for all 3 compounds when collecting their excitation spectra whereas the excitation wavelength was set at 560 nm when acquiring their emission spectra. Figure 3 shows that under the chosen experimental conditions, the maximum in the excitation and emission peaks for the enzyme substrate occurred around 561 and 580 nm, respectively, while the location of the corresponding peaks for the enzyme reaction product were 560 and 582 nm, respectively. In the case of the rhodamine B dye, the excitation spectrum peaked around 556 nm and the emission spectrum attained a maximum around 573 nm. Overall, the 3 compounds appeared to have a very similar optical signature with the enzyme substrate and the enzyme reaction product in particular having nearly identical fluorescence spectra. In Figure 3(b), we have also included the fluorescence signal measured by our epi-fluorescence microscope for standard solutions of the rhodamine based ELISA substrate and 4-methylumbelliferone (4-MU) in order to better assess our assay results. While a background electrolyte containing 10 mM sodium tetraborate and 1 mM MgCl_2 was used for both these reagents, the fluorescence of the 4-MU species was also measured in the presence of 1 mM 4-methylumbelliferyl phosphate in this electrolyte similar to the situation encountered in an actual ELISA experiment. The readings for the 4-MU solutions were taken using band-pass excitation and emission optical filters that transmitted light in the range of 380-391 nm and 420-480 nm, respectively. As may be noted, Figure 3(b) shows the rhodamine based enzyme substrate to yield a significantly higher fluorescence compared to the 4-MU reagent with estimated limits of detection of around 0.14 μM and 1.53 μM , respectively, in a 10 mM sodium tetraborate buffer containing 1 mM MgCl_2 . Moreover, this detection limit for the 4-MU species increased to about 6.23 μM in the presence of 1 mM 4-methylumbelliferyl phosphate due to an increased background signal as well as a higher noise in the measurements. Combinedly, the noted numbers indicate that the detection limit of the novel ELISA method may be expected to be about 44-fold lower than that for the standard microfluidic ELISAs performed with the commercial 4-methylumbelliferyl phosphate enzyme substrate.

3.2 Assay Quantitation

As noted previously, the performance of the reported microfluidic assay was assessed by quantitating the peak corresponding to the rhodamine based enzyme reaction product recorded in our electropherograms. Notice that this species being monocationic appeared earlier in the electropherograms and was at least 10-times shorter in height than that

corresponding to the enzyme substrate under the chosen experimental conditions. Such disparity in peak height ensured that the concentration of the substrate species in segment A did not drop below 100 μM which was determined to be the value where alkaline phosphatase attained over 95% of its saturation reaction rate. In Figure 4(a), we have included a representative electropherogram showing the enzyme substrate and product peaks for a 0.04 pg/mL TNF- α sample after an enzyme reaction period of 30 min. The figure shows elution times for the substrate and product species to be around 14.1 and 7.8 s, respectively, for a microchip entirely coated with the antigen-antibody sandwich immunocomplex. These elution times were observed to decrease by nearly a factor of 2 however, for an uncoated glass channel indicating a significant suppression of electroosmotic flow (EOF) upon coating of the microchannels with the ELISA reagents. Fortunately, even with such EOF suppression, we did not encounter any challenge in injecting the anionic enzyme substrate species into the separation channel. In fact, this compound was observed to migrate from a high to a low voltage region in segment D at a modest speed of 0.5 mm/s due to the dominance of EOF over its electrophoretic transport rate under the assay conditions. It is also important to point out that because the separation time for the enzyme reaction product in our experiments (~ 15 s) was significantly shorter than the enzyme reaction period in the system (10-30 min), the integration of the separation process to our device did not compromise the accuracy of our analyte concentration measurements as compared to that observed in the standard microfluidic ELISA experiments. We would further like to note that because the separation of the enzyme reaction product from the substrate molecules in our assay was based on electrophoresis rather than chromatography, this process was only marginally affected at worst by the exposure of the channel walls to complex matrices, e.g., serum as in this work, during the ELISA surface preparation steps. Moreover, the buffer used as the background electrolyte for the electrophoretic separation only needs to be compatible with the enzyme reaction conditions relevant to an ELISA and is therefore not anticipated to include any complex matrix components that could interfere with the noted separation. In this situation, the proposed ELISA method should be applicable to most biological samples of practical interest rendering it useful in fundamental research as well as biomedical testing applications.

In Figure 4(b), we have focused on the enzyme reaction product peak in the electropherogram and have followed its change in shape/location for different enzyme reaction periods used in the assay. The figure shows a relatively minor variation in its position for the 5 different enzyme reaction periods ($< 3\%$ RSD) chosen for our assay implying a high reproducibility in its transport rate in our device. Moreover, the peak height/area corresponding to the product species was seen to increase for longer enzyme reaction periods as expected indicating the progression of the enzyme reaction in the system. This trend was quantitatively established in Figure 5(a) which shows the observed variation in the area under the enzyme reaction product peak as a function of the enzyme reaction period in our assays. The peak area in our calculations was estimated numerically by applying the trapezoidal rule for integration on a discretized data set of fluorescence signal acquired at a rate of 100 Hz. The linear trends seen in Figure 5(a) establish that our experiments were indeed run under conditions yielding saturation kinetics for the enzyme label-substrate

couple used in our assays. In addition, the slopes for these lines were observed to increase with an increase in TNF- α concentration in the sample demonstrating the quantitative nature of the reported microfluidic ELISA method. In this situation, the slope of the line associated with a particular TNF- α concentration minus its corresponding value for the blank assay was chosen as the signal (S) yielded by the relevant sample [29]. In Figure 5(b), we have presented the response curve for our TNF- α assays based on this observed signal which has been fitted to a 4-parameter logistic equation [31] of the form

$$S_f = S_{min} + \frac{(S_{max} - S_{min})}{1 + (C/\alpha)^\beta} \quad (1)$$

for further analysis. The parameters S_{min} and S_{max} in Equation (1) denote the minimum and maximum asymptote in S_f (the fitted signal), while α and β refer to the inflection point and Hill's slope in the curve, respectively. Codes written in MATLAB were used to fit the experimental data to Equation (1), which then allowed us to estimate the noise (N) in the system as

$$N = 3 \times \sqrt{\frac{\sum_{i=1}^{i=n} (S_i - S_f)^2}{n - 4}} \quad (2)$$

Here S_i and S_f refer to the experimentally measured signal and that predicted by Equation (1) at any given value of C , respectively, whereas n denotes the number of data points used in the analysis. The factor $n - 4$ appearing in the denominator in Equation (2) is a consequence of the fact that 4-fitted parameters were determined from the raw data set included in Figure 5(b). The limit of detection (LOD) for our microfluidic assay in this situation was determined as the value of C when $S_f = S_{min} + N$ which turned out to be 0.13 pg/mL for the TNF- α sample used in the study. The relevance of this result was established by performing a similar analysis on the data sets obtained from the standard microchip and microplate based ELISAs. The signal for these standard assays were calculated based on the rate of change in the fluorescence signal recorded by the detector with enzyme reaction time minus the corresponding value for the relevant blank experiment. While the LOD for the standard microchip ELISA was determined to be 3.53 pg/mL, the ones performed on a commercial microplate yielded a LOD value of 7.03 pg/mL for the TNF- α species [29]. Interestingly, the standard microchip ELISAs performed in a 10 μ m deep channel yielded a 2 and 2.7-fold lower limit of detection compared to that obtained using the commercial microwell plate version and those performed in a 30 μ m deep channel reported by our group previously [29]. Overall, our experiments showed that the noted approach not only enabled the use of a new kind of enzyme substrate for ELISAs but also allowed the detection of human TNF- α at concentrations over 54-fold smaller than that possible on commercial microwell plates. While this improvement in assay sensitivity is primarily realized due to the better detectability of the rhodamine dye compared to the 4-MU reagent commercially used for the purpose, the separation of the enzyme reaction product from the substrate species in

our assay also played a role in this betterment. In particular, the results included in section 3.1 clearly showed that the limit of detection for 4-MU is compromised by about a factor of 4 when the measurement is carried out in a matrix containing large amounts of the corresponding enzyme substrate 4-MUP. This interference is eliminated in the ELISA method described in this article as the enzyme reaction product is always detected following its separation from the enzyme substrate species. The inclusion of the separation step in our assay interestingly also alleviates issues such photo-induced conversion of the enzyme substrate to the enzyme reaction/other fluorescent products [32] and/or the photo-bleaching of these species resulting from their prolonged exposure to a light source as these signaling molecules now reside in the detection zone only for about a second or less.

To our knowledge, the work described above is the first demonstration of an ELISA method that does not require the enzyme label to act around the signaling region of its substrate species for their successful use in quantitating the immunoassay. This development significantly broadens the kinds of enzyme reactions employable in ELISA systems which may be exploited to improve their sensitivity, multiplexing capability, speed and cost in addition to rendering them compatible with a wider range of experimental samples/conditions. However, these enhanced capabilities come with the need to integrate an electrophoretic separation procedure which adds to the complexity and instrumentation requirements of the assay. While this need somewhat compromises the portability of the assay platform, such units may still be packaged in briefcase-sized boxes similar to previous reports in the literature [33]. The integration of an electrophoresis step however barely prolongs the overall assay time in our approach as the desired separation is completed in < 20 s which is insignificant relative to the incubation and enzyme reaction periods (> 2 hrs) involved in ELISAs. In fact, the ability to employ a more detectable enzyme reaction product may actually allow us to work with shorter enzyme reaction periods without compromising on the assay performance due to an improved signal-to-noise ratio in the measurements. Moreover, this period may be further reduced through on-line pre-concentration of the peak corresponding to the enzyme reaction product during the separation process using a variety of mechanisms reported in the literature [34]. Interestingly, the enzyme reaction and the separation steps involved in our experiment may also be performed on independent devices such as a commercial microwell plate and a capillary electrophoresis unit, respectively, likely without any loss in performance. However, the integration of these two processes on a microchip as demonstrated in this work can significantly simplify the experimental procedures in addition to retaining the benefits of miniaturization such as low sample consumption, shorter incubation period, etc. Lastly, it must be pointed out that while the experiments reported in this article have been performed by maintaining an air temperature of 37°C around the microchip device which included both the ELISA and electrophoretic compartments, this temperature control is only necessary for the enzyme reaction and not the separation process. In this situation, no additional instrumentation is required to thermostat our assay unit beyond what is needed in standard ELISA methods.

4. Conclusions

To summarize, we report the development of a novel microfluidic ELISA method involving the use of an enzyme substrate that does not undergo any change in its detectability during the course of the enzyme reaction. Instead, the enzymatic cleavage reaction at a site remote from the chromophore, and without change of that chromophore leads to an alteration in the charge state of the substrate species during this reaction period which is subsequently exploited to separate the enzyme reaction product from the substrate molecules in an integrated device. The peak area corresponding to the enzyme reaction product recorded in our electropherograms was later shown to directly correlate with the analyte concentration in the original sample establishing the quantitative nature of our assay method. Overall, this approach yielded a 54-fold lower limit of detection for the chosen sample (human TNF- α) as compared to that obtained using a commercial ELISA kit primarily due to the better detectability of the rhodamine dye. But more importantly, the reported assay allowed the use of a new kind of enzyme substrate in ELISA measurements that previously could not be employed in this technique potentially broadening the scope of ELISA methods in general. Interestingly, it may be possible to further improve the sensitivity of the ELISA method described here through on-line pre-concentration of the enzyme reaction product peak during the separation process using techniques such as field-amplified stacking [34]. Alternatively, this approach may be applied to shorten the enzyme reaction period in the system due to an improvement in the signal-to-noise ratio recorded by the electropherograms. We are currently exploiting these possibilities and hope to report our results from the study in a future publication.

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Figure 1. Synthetic route taken to prepare the rhodamine based ELISA substrate for the enzyme label, alkaline phosphatase, as reported in this work.

Figure 2.

Schematics of **(a)** the microchip preparation process and **(b)** the overall assay. **(c)** The three stages included in the sub-figure depict the sequence of voltages that were used to inject a narrow plug of the substrate into segment D following a chosen enzyme reaction period (10-30 min.) and subsequently separate the enzyme reaction product from the substrate species. These species were later detected 7 mm downstream of the injection cross to estimate the extent of the enzyme reaction which was subsequently correlated to the TNF- α concentration in the original sample.

Figure 3.

(a) Spectral characterization of the rhodamine based ELISA substrate and its corresponding enzyme reaction product employed in this work. The spectral profile for rhodamine B has been included to the right for comparison purposes. The dotted lines in the figures indicate the excitation spectrum for an emission wavelength of 580 nm while the solid lines refer to the emission spectrum for an excitation wavelength of 560 nm. (b) Fluorescence signal obtained for different ELISA reporter solutions relevant to this work in a 10 μm deep channel using an epifluorescence microscope system. These solutions were prepared in a 10 mM sodium tetraborate buffer (pH 9.4) containing 1 mM MgCl_2 .

Figure 4.

(a) A representative electropherogram depicting the separation of the rhodamine based enzyme substrate from its corresponding enzyme reaction product employed in this work as recorded 7 mm downstream of the injection cross. The time scale on the x -axis in this sub-figure was set to zero when the injection process was initiated, i.e., at the start of stage II in Figure 2. (b) Series of electropherograms depicting the change in peak height/area corresponding to the rhodamine based enzyme reaction product as a function of the enzyme reaction period chosen for the assay. The number associated with each electropherogram correspond to the enzyme reaction period selected for the experiment.

Figure 5.

(a) Variation in the area under the enzyme reaction product peak recorded in our electropherograms with the enzyme reaction period. (b) Quantitation of the reported microfluidic ELISA and its comparison to standard microchip and microplate based experiments using the kinetic format of the assay. For the y -axis on the left, the assay signal was evaluated as the temporal rate of change in the area under the product peak minus its corresponding value for the blank assay whereas for the y -axis on the right, this quantity was calculated as the temporal rate of change in the recorded fluorescence minus its corresponding value for the blank experiment. The solid lines in the figure correspond to the best fitted curve based on the 4-parameter logistic equation. The fitted parameters (S_{max} , S_{min} , α , β) equal (0.691, 0.0021, 3.53, 1.4) for the reported microfluidic ELISA method, (0.925, 4.13×10^{-3} , 90.3, 1.23) for the standard microchip-based and (0.91, 9×10^{-3} , 280.1, 0.982) microplate-based experiments. The error bars in the experimental data correspond to 1 standard deviation evaluated based on 5 independent measurements.