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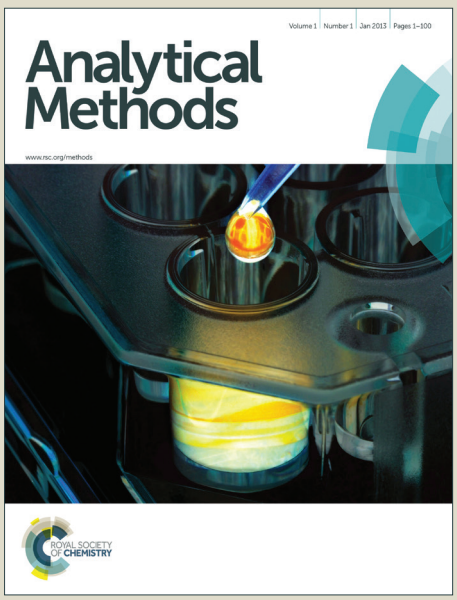

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PAPER

Cotton Fabric as Immobilization Matrix for Low-Cost and Quick Colorimetric Enzyme-Linked Immunosorbent Assay (ELISA)†

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Enzyme-Linked Immunosorbent Assay (ELISA) is a reliable quantification assay used in laboratories around the world. The method itself is time consuming and requires a relatively high volume of reagents. In recent years, microfluidics based on paper platforms has been used extensively to develop devices for point of care diagnosis testing. In this work, cloth was used as a superior alternative to paper (stronger, higher controllable rates for fluid mixing and lower environmental impact) to implement ELISA and quantitatively determine human chorionic gonadotropin. The intrinsic properties of cotton fabric allowed the entrapment of the antibody through the cloth fibers and showed a superior alternative to common immobilization procedures. The cloth-based ELISA was shown to be feasible to detect human chorionic gonadotropin (0 - 140x10^{-6} nmols) via image analysis providing a sigmoid fit (R^2 = 0.983) of the data and limit of detection (LOD) of 2.19 ng/mL. Both the volume of the reagents and the time required for the assay were effectively reduced compared with conventional ELISA. Ultimately, this user-friendly device can potentially be embedded in bandages and gauzes for surgical and clinical settings or in clothing for home care monitoring of elderly and chronic patients.

Introduction

Enzyme-Linked Immunosorbent Assay (ELISA), a detection and quantification assay of substances such as peptides, proteins, antibodies and hormones based on the principle of biorecognition, has been applied widely in industry. The technique presents a satisfactory precision of recognition that allows the identification of several biomarkers including tumor markers. ELISA has been used for the detection of different antibodies and antigens in clinical analysis such as Indirect Competitive (IC) ELISA in order to detect anti-chelated copper monoclonal antibody DF4 in human hair and serum samples[1]. However, each assay requires relatively large volumes of reagent and expensive instruments, in addition to the considerable time required for incubation[2, 3]. ELISA is regarded as a non user-friendly device due to the required instrumentation and the need for a trained technician. Its use in remote locations or home care scenarios is therefore not practical.

In order to improve point of care testing (POCT) and to allow global public health diagnosis, microfluidic devices have been developed to handle a variety of samples such as blood and urine, as well as a range of molecules such as proteins and DNA[4-6]. Microfluidic devices have also been implemented on paper, making bioanalysis inexpensive, disposable and easy to use for clinical analysis[7, 8]. Furthermore, microfluidic devices have been developed for different applications in various aspects of science such as tissue culture and diagnosis[9,10].

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During the last few years, various groups have worked extensively on the development and optimization of microfluidic paper-based analytical devices (µPADs) for various applications. Paper-based analytical devices are expanding, including those used for cancer[11] and diabetes[12]. Recently, paper-based ELISA was also investigated as a simple yet precise and accurate quantitative analytical technique[2, 13]. Others have investigated the feasibility of performing lateral flow immunoassays on cotton thread, since this presents the capability to develop microfluidic systems through the use of knots that can control the fluid mixing[14]. The detection methods used in these low cost-based ELISA devices range from absorbance and fluorescence methods[15] to colorimetric[2, 16]. The visualization of the images on paper-based ELISA were typically carried out using cameras[6]. The colorimetric readouts were then analyzed using image processing software, like ImageJ[2] or Photoshop[17].

In a recent publication Wang *et al.* modified the paper structure by first coating it with chitosan before immobilizing the antibody by cross-linking with glutaraldehyde[13]. In conventional ELISA, antibody is bound to the microtiter plate by the hydrophobic interaction between the plastic plate and the non-polar protein residues. In this study, cloth-based analytical devices (CADs) were proposed as a platform for ELISA in order to reduce the complexity of diagnostic analysis. The simplicity, flexibility and applicability of the CADs have been previously reported[18]. More recently, the feasibility of electrochemical detection using CADs has been demonstrated when measuring lactate in saliva samples[19]. These qualities along with its environmental and user-friendliness set these devices in the forefront of wearable diagnostic technology. Compared to paper, textile-based devices are also more durable and can

provide wicking and mixing of liquids at higher controllable rates[20].

A preliminary study, to embed ELISA on CADs and test its capabilities and required detection times was performed with the human chorionic gonadotropin (hCG) immunoassay. This hormone is produced during pregnancy and hence is widely used for qualitative detection in pregnancy tests. In addition, hCG has increasingly been used for quantitative detection of certain cancers, since its presence at high levels when a patient is not pregnant, may be an indication of a growing tumor[21].

Materials and methods

Reagents

Free-ß-human chorionic gonadotropin ELISA kit was obtained from Alpco Immunoassay Diagnosis, USA, and consists of hCG antigen, enzyme-linked antibody, being the antibody a goat anti whole hCG antibody and the enzyme Horseradish Peroxidase (HRP), tetramethylbenzidine (TMB) and stop solution (1 N HCl). The primary antibody (Anti-hCG beta Mouse monoclonal antibody [5H4-E2]) was obtained from Abcam, Malaysia. The fabric used was a white plain weave cotton cloth (Mirota Batik, Surabaya, Indonesia) washed with soda ash (Na_2CO_3, Sigma Aldrich, USA). The wax used for microfluidic channel patterning was obtained from Jadi Batek Gallery, Kuala Lumpur, Malaysia. All other chemicals and buffers, chitosan (85% deacetylation), glutaraldehyde (2.5% GA), bovine serum albumin (BSA) and phosphate buffer solution (PBS, pH 7.4) were purchased from Sigma Aldrich, Malaysia and used without further treatment.

Experimental

A conventional ELISA calibration was carried out to understand further the assay for the subsequent application on the cloth-based device. The assay was implemented as per the kit protocol, typically using 100 µL of reagent in each step, and the average of absorbance measured at a wavelength of 450 nm (Alpco Immunoassay Diagnosis, USA). For details, see Supplementary Info part 1 and Fig. S1.

The CADs were fabricated using a simple wax patterning technique described elsewhere[18] with a cutter printer and the Graphtec Silhouette software (Silhouette SD, USA). The assay regions or microzones were constructed to resemble the wells of the conventional ELISA in 2 separate rows (one as test containing hCG antibody and another without antibody as control zones) and 6 columns, measuring 3.5 mm in diameter and separated by a distance of 6 mm between each microzones (Fig. S2, Supplementary Info). Different reagent volumes (1, 2, 3 and 4 µL) were pipetted onto the cloth-based ELISA microzones. The color uniformity and assay signal were examined to determine the optimum volume. The antibody immobilization was explored using three different CADs: 1) CAD in which the microzones were modified with 5 µL of chitosan (0.25 mg/mL) prior to antibody immobilization using 5 µL of GA (0.01 M, pH 7.4), 2) CAD where antibody is immobilized with 5 µL of GA, 3) CAD where the antibody is absorbed on the cloth without either chitosan nor GA (Fig. 1). In all devices the hCG primary antibody (3 µL) was incubated for 20 min at room temperature. Then 20 µL of BSA (0.1 M, pH 7.4), acting as blocking buffer, 3 µL of antigen and 3 µL of enzyme-conjugated antibody were added to the microzones with an incubation time of 15 min and a washing procedure (3 times, each with 10 µL of PBS) between reagents. The TMB reagent (3 µL) was then added and allowed to react for 8 min, before finally adding the stop solution (3 µL, 1 N HCl).

The detection of the assay was performed with a camera (automatic settings without flash light and with a distance to the object of 10 - 15 cm) and color images were captured at one-minute intervals from the time when the TMB reagent was added until the addition of the stop solution. The inverted images were converted to grey scale and analyzed using ImageJ software. Gaussian functions were used to determine the normal distribution of the color on the reagent zone. To compensate the difference of the distance from where the image was taken, the reaction zone diameter was set at the known measurement of 3.5 mm. The surface area was then measured for each experiment and the calibration curves were fitted with a sigmoidal function (modified Hill's equation, see Supplementary Information) using Igor Pro 6 (WaveMetrics, Portland, OR, USA). A paired t-test was performed between the immobilized and non-immobilized data. Changes were considered statistically significant at $p<0.05$.

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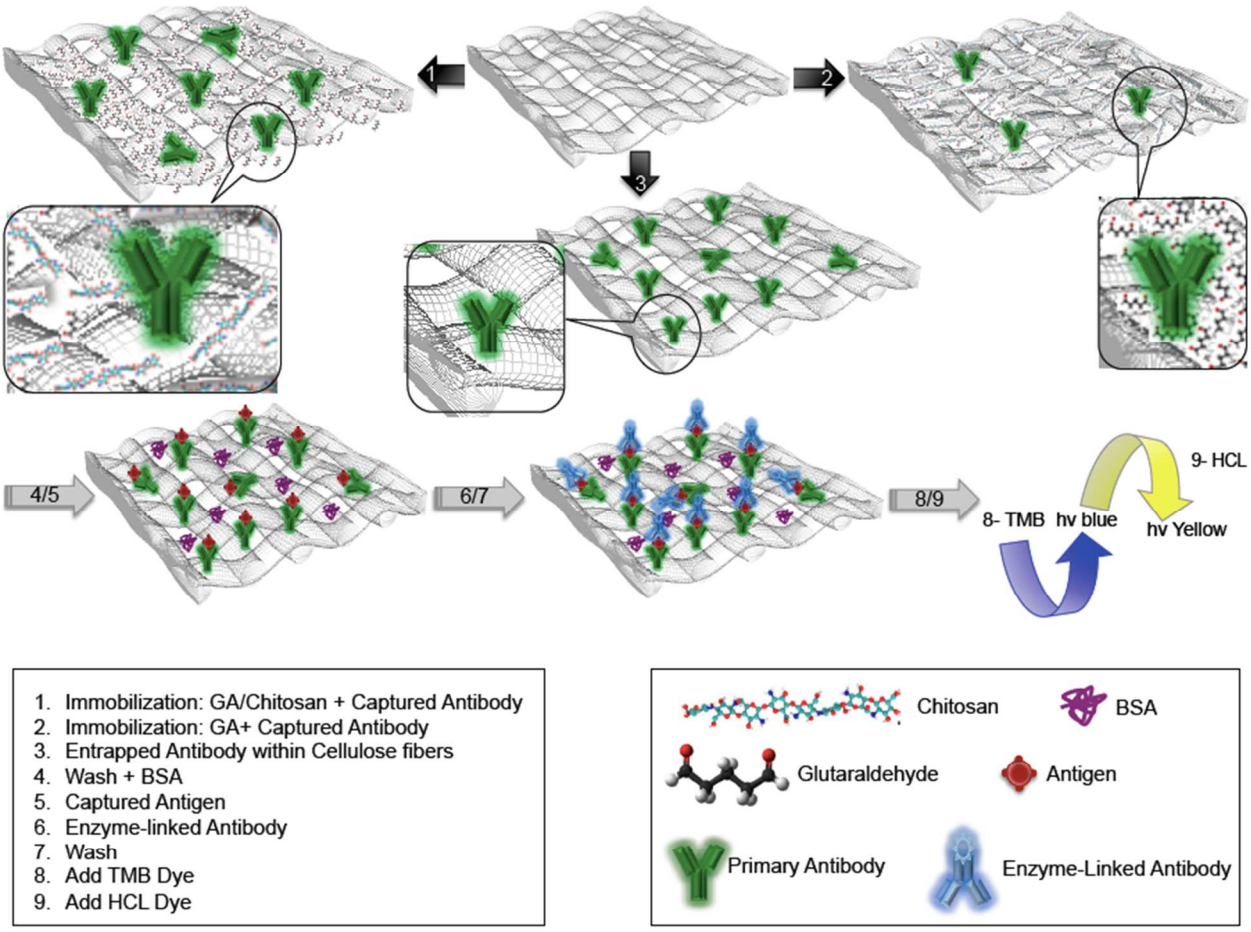

Fig. 1 Scheme of the assembly of cloth-based ELISA device. Three devices were fabricated and the antibody immobilized with different agents: 1) CAD modified with Chitosan and immobilized with GA, 2) immobilization with GA and 3) absorbed (non-immobilized). This was followed by the addition of BSA as blocking agent, antigen and enzyme-conjugated antibody. The addition of the TMB dye produced a blue color as it reacted with the enzyme-linked antibody while HCl stopped this reaction, providing a yellow color for the colorimetric detection.

Results and discussion

Optimization and Analysis

The volume required for the CADs microzones (d = 3.5 mm) was investigated and 3 µL were chosen due to the color uniformity of the results (Fig. 2a). The smallest volumes showed an uneven distribution on the micro zones and inadequate amount of antibody for the assay as observed by the color intensity. When 3 and 4 µL were added, the micro zones showed a competent and uniform distribution of the solution. Since 3 µL was an adequate amount due to its competency and uniformity, this was chosen for the cloth-based assay. Overall the volume and time of the assay required for the cloth-based ELISA were 9 µL and 38 min, in comparison with 450 µL and 110 min in the conventional ELISA, demonstrating the capability of cloth as microfluidic devices (Table S1, Supplementary Info).

During analysis the color intensity was observed to increase as the concentration of the antigen increased (0 to 50 ng/mL). However, assay accuracy was hindered by the reflection of the wet microzones (Fig. 2c-i, 2c-iii). Therefore, the images were converted to grey scale and inverted to analyze the intensity of the lighter area (color area in original picture) as shown in Fig. 2c.

In conventional ELISA, HCl solution is used to stop the enzymatic reaction in order to avoid fluctuating data during the spectrophotometric readings. The camera-captured image allows the analysis of the assay when using TMB dye, without the need for the addition of HCl reagent. In fact the TMB reagent signal is more sensitive than HCl reagent and it is observed to be optimum at 5 min after the reagent addition as shown in Fig 2b.

Antibody Immobilization

Several studies have shown the excellent properties of cotton fabric as a platform for enzyme immobilization when compared with conventional methods since cotton presents high porosity (>95%) and a hydrophilic surface [22, 23]. The results of the immobilization of the primary antibody on the cloth-based ELISA using different immobilization agents are illustrated in Fig. 3 for both detection reagents (TMB and HCl). Both immobilization of primary antibody using GA and by simple absorption on the cloth showed good calibration plots that could be fitted using a sigmoid curve. On the other hand, the CAD modified with chitosan prior to antibody immobilization did not show reliable results. In this case, the hypothesis is that chitosan behaves as a barrier that does not allow the absorption of the

reagents onto the cloth fabric. Chitosan is a linear polysaccharide, the structure of which resembles that of cellulose. Since it contains positive charges, it makes a strong adhesion with negatively charged substrates like cellulose. The hydrogen bonds content and its crystallinity give chitosan its excellent oxygen–barrier and mechanical properties. Other properties include its resistance to grease, to bacteria and in a less extent to water [24]. For this reason, chitosan has been used previously on paper-based ELISA devices to enhance the paper strength when wet and to add functional groups for the covalent immobilization of the antibody [13]. Cotton structure, on the other hand, is arranged in fibers containing multiple cellulose layers coiled in a series of natural springs [25]. This gives cotton its unique properties of strength, durability and absorbency [20]. Furthermore, the electrostatic interactions between the negatively charged cellulose [18] and the common positively charged antibodies provide stronger adhesion between these two [26]. Hence, in this case, chitosan appears to diminish the intrinsic excellent properties of cotton.

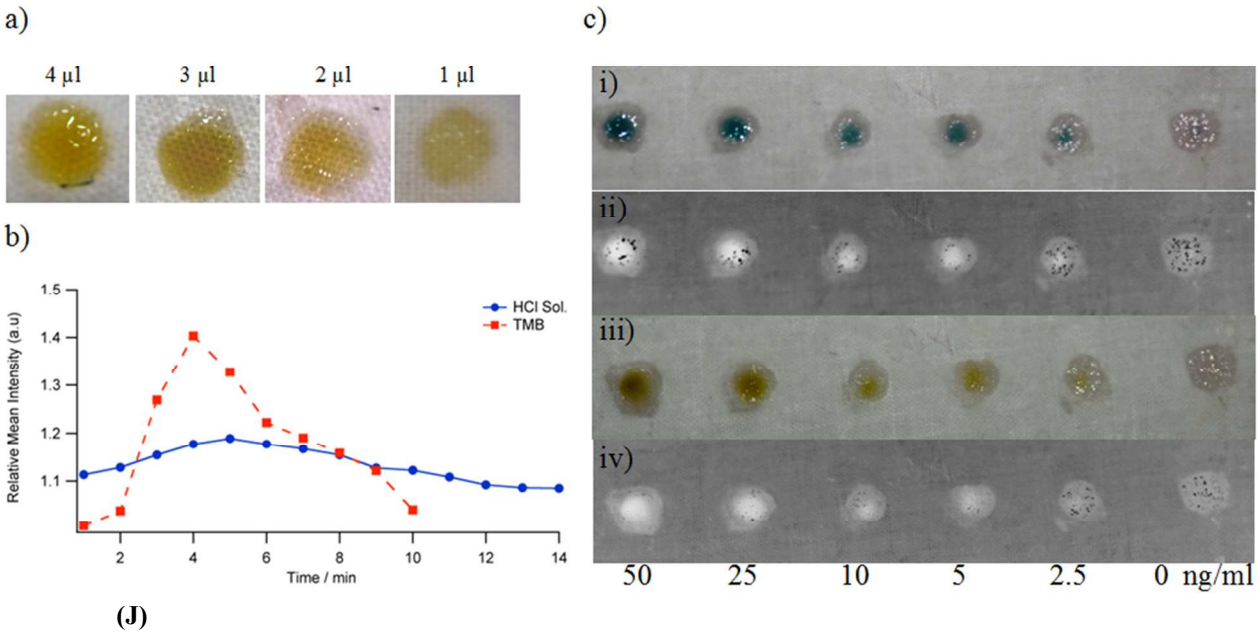

Fig.2 Cloth-based ELISA. a) Microzone volume optimization (V = 1, 2, 3 and 4 µL); b) Color intensity changes over time for both TMB (dashed line) and HCl reagents (solid line) at one-minute intervals for up to 15 min. The maximum intensity was recorded following 5 min from the addition of the reagent; c) The color image of the assay following the addition of TMB solution (blue, i) and its inverted grey scale image [27], the color image of the assay following the addition of HCl solution (yellow, iii) and its inverted grey scale image (iv).

Results showed slightly lower normalize intensity in the case of antibody entrapped by means of the cellulose structure only when TMB was used as the last reagent (p = 0.035), but there was no difference between the immobilization by cross-linking with GA and cellulose entrapment when using HCL as stopping solution (p = 0.509) (Fig. 3a and 3b, respectively). The relative standard deviation (RSD) of five measurements of the same sample at high concentration was < 25% and at low concentration was < 50%. This high RDS is most probably due to the fact that the pictures were taking at different distance from the image each time. Although, further improvement for practical measurement can be made by using scanner instead of camera, we want to demonstrate that it is possible to use this system as a first point diagnosis for home care or remote areas monitoring. Fig. 3c shows the original color intensity of the cellulose entrapment device for both TMB and HCL reagents. In this case both reagents present similar sensitivities, with sigmoid fitting curves of $R^2 = 0.98$ and 0.99 for TMB and HCl, respectively. Overall, the assay showed a linear range between 0 to $20x10^{-6}$ nmols (R^2 =0.996), which is lower to that achieved with conventional ELISA (Fig. S1). This is because of the 2-dimensional nature of the on-fabric colorimetric assay compared to the 3-dimensional nature of conventional microwell-based ELISA. The calibration curves however could be fitted using the Hill equation producing good fit ($R^2 = 0.983$) and hence allowing for extended calibration of the assay. The LOD was calculated as three standard deviation [28] of the blank. For the data obtained with HCL solution, the LOD was calculated to be 163.29 (LOD = 3* 54.43). This corresponds to a concentration of 2.19 ng/mL on the binding curve, or 0.0043 nmols (17.139 kDa) compared with the 9.82 ng/mL obtained with the immunochromatographic assay on thread[14]. These results suggest that cotton, with its coiled structure, provides a superior platform for immobilization of biomolecules than if immobilization agents were used. This could be once more due to the fact that the excellent absorbency properties of cotton are diminished with other chemical agents.

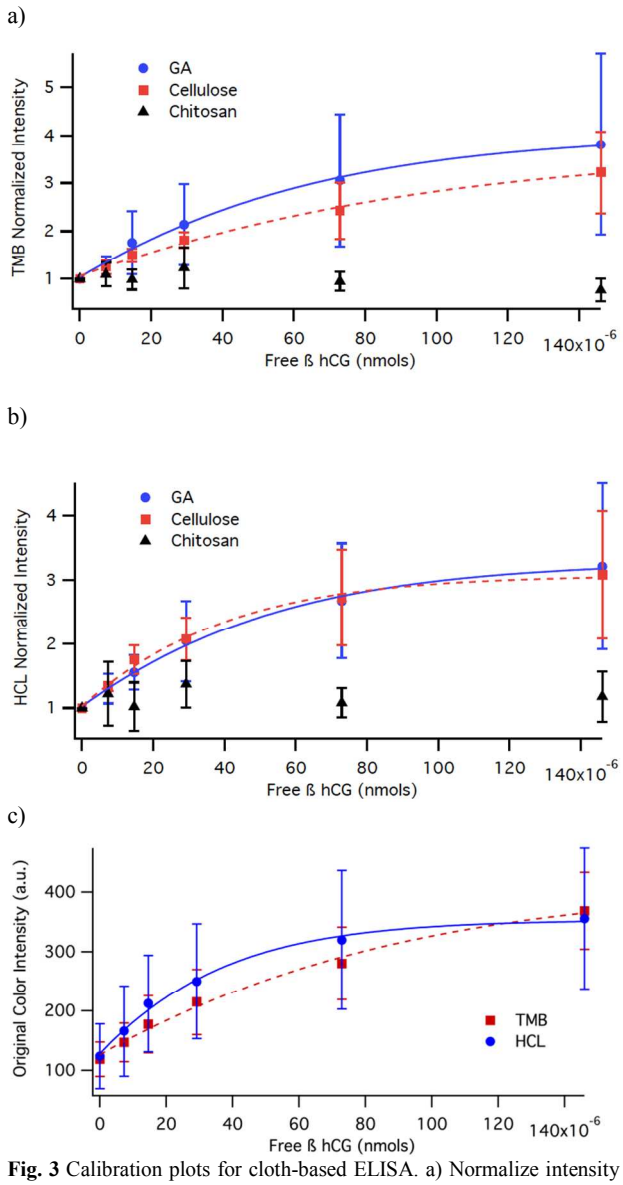

Fig. 3 Calibration plots for cloth-based ELISA. a) Normalize intensity of the microzones following the addition of TMB solution for the cloth modified with Chitosan (black triangle symbols), the GA-immobilized (blue solid line, $R^2 = 0.997$) and cellulose entrapped antibody (red dashed line, $R^2 = 0.983$); b) Normalize intensity of the microzones following the addition of HCl solution for the cloth modified with Chitosan (black triangle symbols), the GA-immobilized antibody (blue solid line, $R^2 = 0.968$) and cellulose entrapped antibody (red dashed line, $R^2 = 0.983$). c) Original color intensity of the cellulose entrapped device for both TMB and HCL reagents. The data is represented as the mean intensity of 5 measurements and the error bars are the standard deviation.

Paper loses its strength when it is too wet and can be easily broken. For these, early researchers are using more resistant papers, such as porouos chromatographic and filter papers which are thicker[2, 29] and require addition of chemicals to modify its properties and strength. Pure cellulose paper still also requires additional reagent to enhance its wet-strength and stability of the immobilized antibodies, e.g. using chitosan[13]. Cotton cloth on the contrary is an environmentally friendly material[30] with excellent absorbency properties[31-34] that does not require modification to improve its physical and chemical properties[35-38]. This adds an advantage over paper devices, since the reagents are reduced. So not only does the method become simpler, but the cost is also reduced. In term of assay performance, our fabric-based ELISA can reach LOD of 2.19 ng/mL. This is worse than conventional ELISA which has LOD of 0.19 ng/ml (see Supporting Information) and Wang's which has attained much better LOD of 0.05 ng/mL[13], yet better than Zhou's thread-based device which has 9.82 ng/mL LOD[14]. However, since our fabric platform requires much less volume of 3 µL compared to 100 µL of the conventional one, its LOD is equivalent to 0.3833 fmol/zone compared to 1.109 fmol/well of conventional ELISA. This is much better than 54 fmol/zone of Cheng *et al*[2]. In addition, our fabric platform can have better miniaturization with 3.5 mm of diameter per each zone, compared to 5 mm of Cheng's[2] and 6 mm of Wang's[13] devices, respectively. Even though by chemical composition paper and cotton fabric are similarly made from cellulose, it is the hierarchical structure of cotton fabric[39] that gives it these additional advantages over paper as a platform for ELISA. The cotton fabric-based ELISA fulfill almost all the criteria outlined by the World Health Organization (WHO) for low-cost diagnostic platforms: affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end-users, or abbreviated as 'ASSURED'[40].

Conclusions

The proposed combination of the conventional ELISA assay on cloth as a diagnosis device has been proven feasible. The ELISA cloth-based device has successfully decreased the volume and incubation time in comparison with conventional ELISA assay. Furthermore, since the immobilization method is not essential for implementing ELISA on clothes due to the characteristic hierarchical woven structures of the fabric and the high absorbability of the cloth, this allows a more economical process compared with previously published ELISA-paper devices. The bulky and more expensive instrumentation required in conventional ELISA is eliminated when using cloth-based ELISA. The proposed platform only requires camera (or scanner) and image software to measure the color intensity of the assay response. This simple detection method and the reduced volume of sample decrease the time required to run the assay and provides an easy-to-use detection assay of common public diseases. The device has the potential to provide fast and accurate results if combined with electrochemical detection.

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