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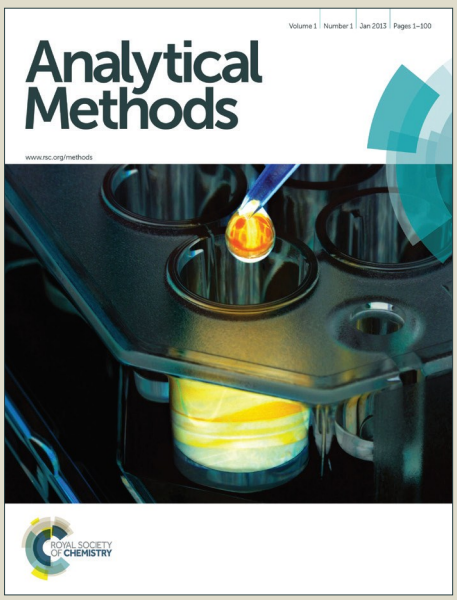

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ARTICLE

A Straw-Housed Paper-based Colorimetric Antibody-antigen Sensor

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Simple and affordable diagnostic tests are in dire need for disease management due to inaccessibility of diagnostic tests in developing countries. Albeit the emergence of paper-based enzyme-linked immunosorbent assay (ELISA) sensors as an attractive alternative due to the assay specificity and simplicity of use, paper-based sensor still suffers from certain limitations such as the requirements of sophisticated instruments or technologies in fabrication or detection. Here, we proposed a simple paper-based ELISA by modifying paper with chitosan and glutaraldehyde to enhance protein immobilization efficiency. Colorimetric results could be obtained in less than half an hour with horseradish peroxidase (HRP) conjugated antibody. The assay was carried out with the use of a drinking straw as an incubation pouch. Quantification using Adobe Photoshop CS2 software was done after documentation using a mobile phone camera. The sensor was found to have a detection limit of 0.5 nM. The approach also holds promise for DNA sensing applications by hybridization.

Keywords: Antibody-antigen interaction; Chitosan; Colorimetric Assay; Glutaraldehyde; Paper based sensor; Point-of-care; scFv

1. Introduction

Paper-based sensors are touted to be an interesting alternative tool for diagnostics due to the low-cost, ease of use, non-refrigerated transportation, availability and portability[1-4]. Paper-based diagnostic was first demonstrated in 1956 for the detection of glucose in urine by Comer[5]. The method garnered a lot of attention for its simplicity and efficiency. Pregnancy test strip is one of the most well-known point-of-care (POC) assays which is widely commercialized using nitrocellulose membranes for protein attachment[6]. To date, paper-based sensors have been widely utilized for many applications including health diagnostics[7-9], environmental monitoring[3, 10] as well for food quality control[11, 12].

Paper-based sensors have been shown to be suitable for enzyme-linked immunosorbent assay (ELISA) based diagnosis of many infectious diseases[13]. Cheng and co-workers [14] designed a 96-microzone paper plate to analyze multiple samples by using rabbit IgG as a model analyte. Alkaline phosphatase conjugated antibody was used to produce colorimetric readouts. Another example was demonstrated by Wang and co-workers[4] whereby chemiluminescence ELISA was performed on chitosan modified paper with wax-screen printing for better microfluidic patterns. The development of paper based sensors has seen several different fabrication techniques as well as detection methods being used[15]. Albeit the high sensitivity of the sensor, sophisticated instruments are usually required either in fabricating the diagnostic tools or in sample quantification. This setup is not feasible for POC assay development especially at areas of limited resources. According to World Health Organization (WHO), diagnostic tools should fulfil the ASSURED criteria which emphasizes on the diagnostic tool to be affordable, sensitive, user-friendly, rapid, robust, equipment-free and able to be delivered[16]. However, cellulose paper has always been associated with low mechanical strength and is unable to immobilize proteins well[17]. This is evident with reports of up to 40% of antibody molecules desorbed from cellulose fibres after absorption[18]. This indicates direct adsorption of antibodies onto cellulose is too weak for permanent immobilization[19]. To address this challenge, we developed a cellulose paper drop immunoassay sensor via crosslinking of chitosan and glutaraldehyde.

The application of chitosan-glutaraldehyde crosslinking is preferred due to the low cost, simplicity of use and rapid reaction[20]. The application of crosslinking between chitosan and glutaraldehyde has attracted vast interest over the years especially for protein immobilization[21-23]. The copolymer of chitosan and glutaraldehyde has high thermal stability[24], making transportation and handling convenient. This allows the resulting copolymer to be widely applied for drug delivery[25, 26], tissue engineering[23, 27] and biosensor development[4, 13].

Chitosan is a favourable biomaterial[28, 29] due to its non-toxicity and biocompatibility properties[30]. Chitosan is a linear biopolymer derived from partial deacetylation of chitin under alkaline conditions. Characterization of chitosan is dependent on the varying degree of deacetylation, resulting in N-acetyl-D-glucosamine and D-glucosamine formation[22]. Despite chitosan appearing to be the second most abundant natural polysaccharide after cellulose, chitosan has several advantages over cellulose. Chitosan is non-toxic, biodegradable and biocompatible giving it wide applications in industries[30-32]. Chitosan is slight positively charged with a pKa value about 6.5 making it suitable to bind negatively charged surfaces[33].

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Glutaraldehyde is one the most widely used crosslinking agent in various fields due to its high reactivity in crosslinking, cost effectiveness, efficiency and commercially available[34]. Also, glutaraldehyde is a common crosslinking agent for chitosan. Glutaraldehyde is a bifunctional molecule having two reactive aldehyde groups for crosslinking, with one aldehyde group at each end of molecule[35, 36]. Crosslinking of glutaraldehyde and chitosan occurs through the formation of Schiff's base between one of the aldehyde group of glutaraldehyde and the amino group of chitosan[37]. The second aldehyde group of glutaraldehyde is therefore still available to crosslink with proteins by reacting with the free amino groups in proteins[36, 38]. Formation of imine bonds (C=N) between –CHO group on glutaraldehyde with the amine group (-NH2) group of chitosan establishes the crosslinking[39]. Taken together, crosslinking of chitosan with protein using glutaraldehyde allows for easy attachment of proteins on cellulose surfaces.

This paper reports on the fabrication process of a paper drop immunoassay sensor for antibody-antigen interaction assay. Our proposed sensor consists of a plastic strip with a cellulose paper disk attached using double-sided tape. The paper disk is modified by chitosan and glutaraldehyde for antigen immobilization. Presence of targeted antibodies could be detected via Protein L HRP that causes a change in colour on the paper. Aside from a qualitative assay, a quantitative analysis of the immunoassay was carried out using Adobe Photoshop CS2 software. In order to allow the application of the sensor in a resource limited setting, we introduce a straw as the washing and incubation pouch to eliminate the need of shakers and pipettors. The combination of the method is able to successfully detect the antibodies present at good sensitivity for POC testing at resource limited settings.

2. Experimental

2.1 Reagents and materials

All antigens (Ubiquitin/Ubi; enhanced green fluorescent protein/eGFP; cherry protein) and antibodies (anti-ubiquitin/anti-ubi; anti-eGFP) were expressed and purified as mentioned in Supplementary Information. Chitosan flakes (≥75% deacetylation) and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, USA). Protein L HRP was purchased from Thermo Scientific Pierce (Hudson, USA). 2, 2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) tablets was purchased from Amresco (Solon, USA). Bovine serum albumin (BSA) was purchased from Nacalai Tesque (Kyoto, Japan). Filter paper 1# was purchased from Millipore (New York, USA). A rigid film binding cover and plastic straws were purchased from a stationery shop. 1 x phosphate-buffered saline (PBS) used in this paper contained 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4 with pH adjusted to 7.4. PBS with 0.1% Tween® 20 (PBST) was used as wash buffer to remove unspecific binding on paper surface due to adsorption. All solutions were prepared using UHQ water prepared by Sartorius Mili-Q water (Gottingen, Germany).

2.2 Pre-treatment of cellulose paper

Cellulose paper disks were produced using Whatman filter paper 1# using a standard paper puncher and immersed in 5µL of 0.25mg/mL chitosan solution in 0.1M acetic acid. Next, 5µL of 2.5% glutaraldehyde cross-linking reagent in 1x PBS buffer (pH7.4) was deposited onto the chitosan modified paper surface. After the paper disk was dried at room temperature (rt), 10µL of PBS buffer was dispensed onto the paper disk surface to remove unbound glutaraldehyde and dust. Excess buffer was removed by a piece of normal tissue paper by contact with the paper disk. The modified paper disk was dried and taped on a plastic strip as demonstrated in Fig. 1.

2.3 Antigen immobilization on modified paper disks

Target antigen (Ubi or eGFP) was diluted in 1 x PBS buffer (pH 7.4) and dropped to the corresponding paper disk on the sensor and dried at rt. Then, 10µL of 1% BSA was applied to the paper disk to block any remaining active sites. Excess BSA was removed by washing with 200µL of 1 X PBST (0.1% Tween) for 2 min and left to dry. Detection of sensor was performed by incubating sensor with target antibody in 1% BSA. After unbound antibody was washed off, the sensor was incubated with Protein L HRP for 2 mins. Finally, the sensor was washed with 1 x PBST for 5 mins with vigorous shaking, followed by incubation in ABTS solution and incubated for 10 mins. Refer to the Supporting Information for detailed protocol.

2.4 Quantification of colorimetric assay of paper-based sensor

Documentation of the colorimetric change on the paper disk by mobile phone camera was done after the paper disks dried. Changes in colour of the paper disk was quantified by Adobe Photoshop CS2 software in grey mode using a fixed quadrant to obtain the mean intensity as described by Martinez and co-workers [2]. Each sample was analyzed three times to get an average mean intensity. Each set of data was run in triplicates. Intensity of the resulting ABTS solution was also analysed by Thermo Multiskan Spectrum spectrophotometer (Hudson, USA) to correlate the data generated by Adobe Photoshop CS2.

3. Results and discussion

3.1 Fabrication of immunoassay paper sensor

The fabrication of the paper sensor is primarily simple and low cost. All the materials are easily obtained and does not require the use of any sophisticated instrument in the production. The backbone of the paper sensor is made of common film binding cover easily available from any local stationery store and was cut into strips. To make sample handling convenient, paper disks were attached to the plastic strip using double-sided tape. The functional paper sensor has a small footprint making transportation and storage easy. Also, introduction of a drinking straw as the incubation pouch for the paper sensor makes sample collection and incubation more convenient without the use of pipettors. The built up of the sensor within the straw house is small allowing space savings for storage and disposal. The vast development in smart phone technology has helped to make sample detection easier with the mobile phone on-board camera for quantification by Adobe Photoshop CS2 software.

3.2 Selection of paper sensor membrane

Selection of Whatman filter paper 1# as the sample pad is due to its high absorbency and good wicking rate as shown in Fig. S-1. These criteria are exceptionally vital for quantitative colorimetric assay on paper sensors. The cellulose materials of the Whatman filter paper 1# that is similar to chitosan structures is able to help

promote binding between the two compounds[40]. As CF6 and Whatman filter paper 1# showed similar patterns, the cheaper Whatman filter paper 1# was used instead of CF6. Another reason CF6 was not used is because the higher sample retention of CF6 which is commonly used as an absorbent pad could increase the background interference of the assay.

3.3 Investigation of chitosan and glutaraldehyde interaction for protein immobilization

Chitosan and glutaraldehyde have been applied extensively for gel formation[41, 42] and protein immobilization[4, 13]. Here, we applied the same principles for protein immobilization on cellulose surface for use as a sensor. The initial design was based on a dipstick whereby samples can be loaded on the reaction zones for detection. All purified proteins applied in this work were determined by SDS-PAGE gel as shown in Fig. S-2. Fig. S-3(a) shows chitosan and glutaraldehyde (C+G) was applied together for efficient eGFP immobilization. The anionic property of cellulose fibre binds well with the cationic chitosan to produce a chitosan layer on the surface of the paper. When glutaraldehyde was added, it will serve as a cross-linker to form covalent bonds between the bound chitosan with the subsequent added protein groups through its aldehyde groups[40]. With the existence of this strong covalent bond, proteins are easily deposited on paper without being washed off. Fig. S-3(b) shows the reduction in eGFP and cherry protein intensity on paper without the chitosan and glutaraldehyde (-C-G) modification. Therefore chitosan glutaraldehyde cross-linking is an effective method for protein immobilization on cellulose surfaces.

3.4 Optimization of chitosan-glutaraldehyde mediated protein immobilization

The amino groups in chitosan have a pKa about 6.5[43, 44]. Theoretically, lower pH values will lead to higher protonation of chitosan amino groups which can contribute to better binding to the negatively charged cellulose paper. This is due to the great influence of buffer pH on chitosan solubility and stability which affects the electrostatic interaction[45]. However, reactivity of glutaraldehyde is higher with the increase of pH[46]. Hence a balance between the lower protonated chitosan amino groups and higher glutaraldehyde activity at higher pH will improve the crosslinking activity. This would explain the little variation in paper immobilized fluorescent proteins intensity from buffer pH 3 until pH 6 (Fig. S-4). However for the purpose of the this sensor, we used buffer pH 4.5 as the optimized working condition because the lower pH will reduce the reactivity of glutaraldehyde and a higher pH will result in a loss of chitosan zeta potential[47].

The optimized molar ratio of chitosan and glutaraldehyde is essential in maximizing the immobilization of protein on cellulose paper. Fig. S-5 shows the intensity of the fluorescent protein was highest when the molar ratio of chitosan and glutaraldehyde was 1:1. Although glutaraldehyde is a divalent cross-linker, it is unable to bind well to cellulose without chitosan. The high affinity between chitosan and cellulose enables the introduction of active amino groups into cellulose fibres because of the similarity between chitosan and cellulose in terms of chemical and molecular structures [40]. Glutaraldehyde is able to bind to paper by crosslinking with the amino groups of chitosan. However, low glutaraldehyde concentrations will greatly reduce the immobilization of proteins even with high concentration of chitosan. This is due to reduced concentration of the glutaraldehyde binding with the protein. We observed a slight increase in the hydrophobicity of the paper surface with varying degrees of chitosan-glutaraldehyde to protein. Solution absorption onto modified paper surface was slightly slower compared to unmodified paper at a higher molar ratio of glutaraldehyde and chitosan. According to Poon and co-workers[48], chitosan surface becomes more hydrophobic when more glutaraldehyde are crosslinked with chitosan. Therefore the use of a minimal amount of chitosan-glutaraldehyde is required to maintain sufficient hydrophobicity.

3.5 Analytical performance of paper sensor

The initial development of this paper was with a dipstick prototype to confirm the specificity and efficiency of the paper-based sensor. Presence of targeted antibodies in the sample was detected via protein L HRP that specifically binds kappa light chains. The HRP catalyses the ABTS substrate to yield a visible green colour product. The change in colour is an easy indicator to show the presence of targeted antibodies in sample. Both paper disks on the sensor turned to dark green colour in the presence of the targeted antibody (anti-eGFP and anti-ubi) while the control disk (C) gave a pale greenish hue. Higher concentrations of the targeted antibody present in the sample resulted in a higher amount of bound Protein L HRP, hence a faster and darker colour formation between ABTS substrate with HRP. The change in colour resulted in a darker shade on the paper when converted into grey mode when analysed with Adobe Photoshop CS2 as presented in Fig. 2A & B. The colour intensity is inversely proportionate to the colour formation on the paper. Pale greenish Control (C) paper disk in grey mode generates higher intensity values. Therefore lower intensities in grey mode were recorded for the samples when compared to the control. These results could be verified by the reading generated by remaining ABTS solutions using a spectrophotometer. The intensity of ABTS solution measured at OD_{405nm} is proportional to the concentration of bound antibody in sample. Thus both the sample bars for ABTS solution gave higher readings compared to controls indicating the presence of targeted antibody in solution.

We also studied the efficiency between modified paper disk and commercial ELISA polystyrene strip by using the same approach as described previously. As demonstrates in Fig. 2D, the polystyrene strip was able to produce darker green hues compared to the sensor. Thus polystyrene strips obtained higher OD with lower background compared to the sensor. Polystyrene strips have higher surface areas for antigen attachment compared to the sensor that is only one sided. More antigens are able to attach to the wall of the polystyrene strip wells compared to the sensor paper disk that eventually produced higher OD_{405nm} values. However, due to only a single side coating of the paper disk, the proteins were more compactly packed on one side of disk surface as opposed in the polystyrene wells. When Protein L HRP was added, the change in colour on the paper disk was fast and notably observed by eye as shown in Fig. 2C. Aside from the surface area of the paper sensor that might contribute to a lower OD_{405nm} values in Fig. 2D, glutaraldehyde toxicity could also be another. Glutaraldehyde is commonly used as the crosslinking agent for chitosan with the formation of Schiff base [37]. Albeit glutaraldehyde crosslinking is efficient and able to improve mechanical strength, it is touted to induce cytotoxicity which might impair the biocompatibility of the crosslinked system[25, 49, 50] even changing protein confirmation at higher concentrations[51, 52]. Hence, a simple test was performed to investigate the effect of glutaraldehyde on the paper disks coated

antigen as discussed in the Supplementary information. As shown in Fig. S-6, glutaraldehyde did alter the antigen conformation as the binding of targeted antibody to antigen dropped half fold after incubation with increasing percentage of glutaraldehyde (concentration 1% to 10%). A reduction in mean intensity at elevated concentrations of glutaraldehyde might be due to changes in surface chemistry of microplate wells which lowered the sorption capacity[48]. Therefore, optimum concentration of glutaraldehyde is important as it plays a crucial role in effective crosslinking. As the crosslinking process requires only mimimum amounts of glutaraldehyde which does not compromise the protein structure, this makes it ideal for use as the crosslinking agent for chitosan. Moreover, cellulose paper modification is necessary to immobilize protein permanently because direct adsorption of molecules onto raw cellulose is too weak[18].

3.6 Feasibility of drinking straw as an incubation pouch

Introduction of a drinking straw as the incubation pouch makes sample collection and incubation much more convenient. Samples such as urine, sweat, or others can be easily collected in straws without the need of a pipettor. In fact, drinking straw is low cost, easily available and portable to be used in field. Coupled with the paper based sensor, diagnostic in the field could be performed without the requirement of specific skills. In order to avoid the carry over protein that might be attached to the drinking straw's wall, a new straw with dimension 2cm x 0.5cm was used for every incubation and washing step as shown in Fig. 3A & 3B. To address this challenge, cross contamination between disks were investigated as they were soaked in the same fluid. Paper disks coated with the specific antigen were incubated with specific antibody within the straws. Fig. 3C shows that cross contamination among the paper disks are less likely albeit soaking in the same liquid. The paper disks only turned into dark green when targeted antibodies were present in the incubation fluid. The negative control gave a pale greenish hue after incubation with antibodies was able to confirm low cross contamination within straw.

We then looked at the detection limit of the instrument free sensor coupled with the straw. In order to verify the detection limit of the sensor housed in a straw, the sensor was incubated with various concentrations of targeted antibodies starting from 0.5nM to 15nM. Paper disks without chitosan-glutaraldehyde modifications were incubated with a range of antibody concentrations prior to the detection limit assay to investigate the direct protein adsorption onto cellulose paper. As shown in Fig. S-7, adsorption of antibody onto unmodified paper was low. The mean intensity obtained from unmodified paper disks (S) was close to the negative (-) paper disks when antibody concentrations were low (0.5nM to 3nM). However, slight adsorption of antibody onto unmodified cellulose paper was detected when antibody concentration was elevated from 5nM to 15nM. Slight adsorption of antibody may lead to false positive result that might interfere with the result of detection limit. However, Fig. S-7 showed with the blocking of 1% BSA, the unspecific binding of antibody due to direct adsorption of protein to paper could be avoided.

Fig. 3D shows the average intensity of the positive control was lowest followed by the sample and with the highest intensity by the negative control. We observed that as low as 0.5nM of antibodies was still detectable in solution with the sensor. However, the pattern of intensity obtained was not linear to the antibody concentration as expected. This might due to the variation in force and number of shaking of the straw housed sensor that could contribute to the pattern obtained. Also, variation in lighting conditions during documentation using mobile phone might also contributed to the inconsistency in intensity pattern. Similar observation was also reported by Oliveira[53] and Martinez in their publications[8]. Oliveira and co-workers[53] printed 96 wells on polyester film by laser-printing to perform immunoassays. They were able to achieve a much lower detection limit at 13fmol with the help of a spectrophotometer microplate reader[53]. The difference in the detection limits observed might be due to the choice of polyester film as the material to fabricate the microzone plate including the high-end detection system with a spectrophotometer microplate reader. The detection limit of the colorimetric information using the mobile phone by Oliveira and co-workers was not reported hence a comparison was not possible. Therefore quantitative analysis using the proposed sensor is not advisable as the sensor is mainly suited for qualitative analysis.

4. Conclusions

Rapid detection of antibody-antigen interaction in low resource settings is feasible with the paper sensor. By using chitosan and glutaraldehyde, cellulose paper can be easily modified to immobilize desired proteins on the surface to perform downstream immunoassay. The modification is easy to carry out and does not require any special reagents or instrumentation. The results obtained via the sensor were distinguishable by eye. The incorporation of a straw in the incubation reservoir of the sensor would allow for easy POC applications. We hope this work along with other paper technology assays reported can help to revolutionize the healthcare of under developed communities in the world.

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Figures:

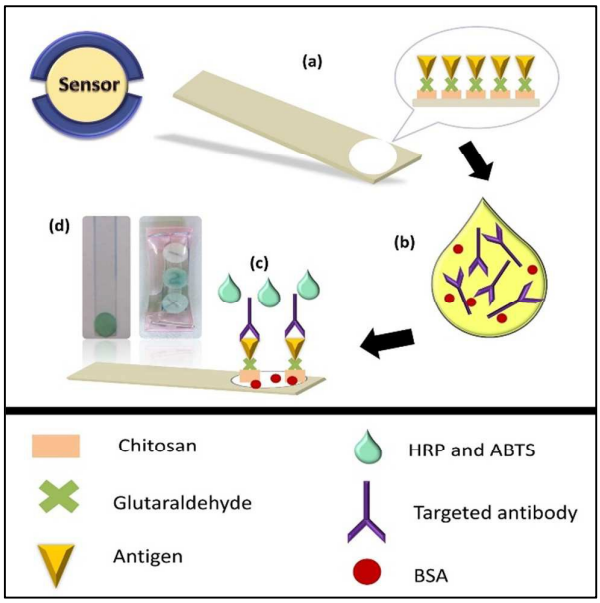

Fig. 1. Fabrication of antigen-antibody paper based sensor. (a) Schematic representation of the sensor construction and sensor immunoassay. (a) Desired antigen was able to immobilize stably to sensor cellulose paper disk with chitosan and glutaraldehyde cross-linking. (b) Sample mixed with BSA solution was dispensed onto sensor. (c) Colorimetric assay was developed in ABTS solution after sensor incubated with protein L HRP. (d) Real sample showing the green colour developed on sensor paper disk when targeted antibody present in sample.

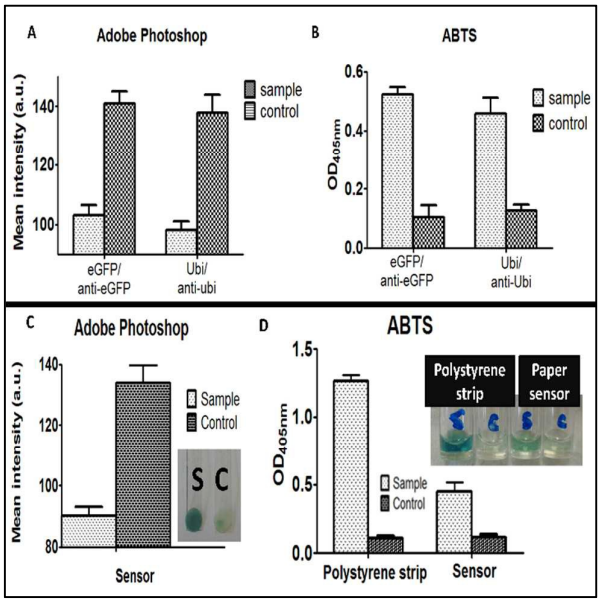

Fig. 2. Quantification of ABTS solution and paper disk after incubated with sample. (A) Adobe Photoshop reading for paper disk. (B) OD_{405nm} reading for ABTS solution. Bar chart for both sample and control when tested with anti-eGFP and anti-ubiquitin (Anti-Ubi) for specific antigens immobilised. Comparison of binding efficiency between ELISA polystyrene strip and modified paper disk. (C) Mean intensity of sensor paper disk by Adobe Photoshop CS2 software after assay developed. (D) Bar chart showing OD_{405nm} reading of ABTS solution for both polystyrene strip and sensor. Each bar represents an average from three repeated experiments and the error bars show one standard deviation.

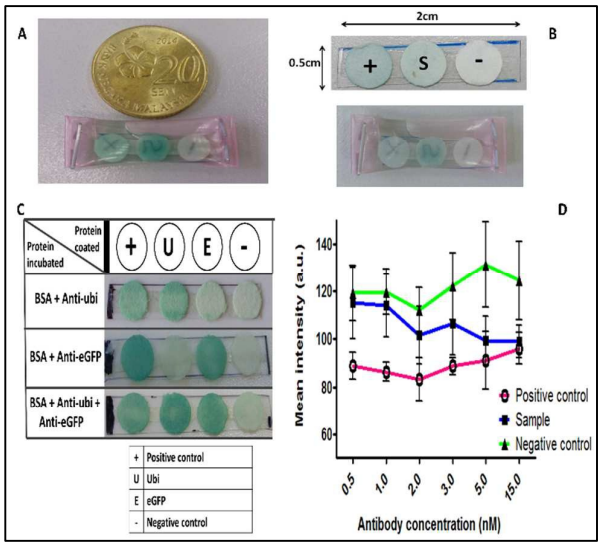

Fig. 3. Introduction of straw as incubation pouch for paper sensor. (**A**) Real sample showing incubation of paper sensor in drinking straw. (**B**) The dimension of sensor was 2 cm x 0.5 cm (length x width) with two ends stapled to avoid the solution from leaking. (**C**) Cross contamination check of paper based sensor with straw. Four paper disks were attached to plastic strip with double sided tape and each were coated with different protein. Positive control (+) was coated with anti-ubiquitin antibody; paper disk labelled (U) was coated with ubiquitin antigen; paper disk labelled (E) was coated with eGFP antigen; paper disk labelled (-) did not coated with anything. Each paper sensor was incubated with different targeted protein and washed within straw. Presence of desired antibody turned the paper disk into dark green. (**D**) Line graph showing the mean intensity of the sensor with the straw pouch for antibody concentration 0.5 nM to 15 nM. Each line represents an average from three repeated experiments and the error bars show one standard deviation.