Video Article

Covalent Binding of Antibodies to Cellulose Paper Discs and Their Applications in Naked-eye Colorimetric Immunoassays

Yanfen Peng[1], Victor Van Gelder[2], Anburaj Amaladoss[1], Kadamb Haribhai Patel[1]

[1]School of Applied Science, Temasek Polytechnic

[2]University of Applied Sciences Utrecht

Correspondence to: Anburaj Amaladoss at anburaj@tp.edu.sg

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Abstract

This report presents two methods for the covalent immobilization of capture antibodies on cellulose filter paper grade No. 1 (medium-flow filter paper) discs and grade No. 113 (fast-flow filter paper) discs. These cellulose paper discs were grafted with amine functional groups through a silane coupling technique before the antibodies were immobilized on them. Periodate oxidation and glutaraldehyde cross-linking methods were used to graft capture antibodies on the cellulose paper discs. In order to ensure the maximum binding capacity of the capture antibodies to their targets after immobilization, the effects of various concentrations of sodium periodate, glutaraldehyde, and capture antibodies on the surface of the paper discs were investigated. The antibodies that were coated on the amine-functionalized cellulose paper discs through a glutaraldehyde cross-linking agent showed enhanced binding activity to the target when compared to the periodate oxidation method. IgG (in mouse reference serum) was used as a reference target in this study to test the application of covalently immobilized antibodies through glutaraldehyde. A new paper-based, enzyme-linked immunosorbent assay (ELISA) was successfully developed and validated for the detection of IgG. This method does not require equipment, and it can detect 100 ng/ml of IgG. The fast-flow filter paper was more sensitive than the medium-flow filter paper. The incubation period of this assay was short and required small sample volumes. This naked-eye, colorimetric immunoassay can be extended to detect other targets that are identified with conventional ELISA.

Video Link

The video component of this article can be found at https://www.jove.com/video/54111/

Introduction

The point-of-care testing (POCT) diagnostic study is important for the development of new strategies for therapeutics, personalized medicine, and home care[1]. Cellulose papers are widely used as platforms in immunoassays, as they are cheap, accessible, and familiar to users[2]. In addition, the porous structure of cellulose paper possesses the power to drive liquid flow without additional energy impact. Records of paper-based bioanalysis can be found as early as the 20th century, when paper chromatography was first invented in 1952. The most prevalent example is immunochromatographic tests[3], such as pregnancy and diabetes test strips. These tests provide relatively fast assay times and inexpensive analysis[4]. Due to their simplicity, these conventional paper strip tests have been widely used in POCT diagnostics[5].

Detection methods including colorimetric[6], electrochemical[7], and electrochemiluminescence[8] methods have been reported to measure targets in biological samples. In addition to these quantitative methods, a reliable method for immobilizing antibodies on cellulose paper is also important for the development of diagnostic devices. Non-specific adsorption is the main strategy for modifying antibodies on the surface of the paper-based devices[9, 10] to ensure maximum binding capacity to their targets after immobilization. However, a previous study showed that antibodies that are adsorbed onto cellulose paper can desorb from the fibers[11] by 40%. Thus, direct adsorption of antibodies onto cellulose may not provide reproducible results[12]. Covalent immobilization of antibodies that are grafted on the paper surfaces is an alternative method of developing effective paper-based bioassays[13]. Various methods have been reported for the modification of cellulose[14, 15]. Ideally, antibodies should maintain their original functionality after immobilization[12]. Carbonyldiimidazole combined with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate[16]; 1-fluoro-2-nitro-4-azidobenzene through a UV-based activation strategy[17, 18]; a chemoenzymatic strategy based on xyloglucan modification[19]; a 1,4-phenylenediisothiocyanate linking agent[20]; heteropolysaccharide oxidation[21] click chemistry[22]; and cationic porphyrins[23] have been used to covalently immobilize biomolecules on cellulose paper. Chitosan modified paper has been used to develop paper based immunodevices[24-26] since it is abundant and biocompatible[27]. Chitosan is cationic and adheres strongly to anionic cellulose[27]. The capture antibodies are immobilized on the paper through chitosan coating and glutaraldehyde cross-linking. Periodate oxidation is another method for grafting the capture antibodies on the cellulose paper[28]. In this method, sodium periodate is spotted on the paper to convert 1,2-dihydroxyl (glycol) groups in cellulose directly to aldehyde groups. The aldehyde groups are then used to form covalent bonds between polysaccharides and antibodies[28]. Although the fabrication is simple, it is difficult to completely wash out sodium periodate. The unwashed sodium periodate can cause further oxidation of the antibodies that are immobilized on the cellulose paper, affecting the activity and stability of the antibodies. *N*-(3-dimethylaminopropyl)-*N*-

ethylcarbodiimide hydrochloride and *N*-hydroxysuccinimide cross linkers are also used to covalently immobilize antibodies on electrospun poly-L-lactic acid and cellulose acetate nanofibers for the development of nanofiber-based assays[29].

In this study, a silane coupling technique was used to graft amine functional groups on cellulose paper discs. This technique helps to retain the original pore size, wicking, and filtration rate of the cellulose filter papers, allowing maximum vertical flow-through in immunoassays. The silane coupling technique has been widely used in biosensors to functionalize substrate surfaces with secondary amine groups, followed by further modification using biomolecules. The grafting of amine groups on the matrix surface comprises a condensation reaction between -OH groups of the organofunctional silane agents and matrix substrate[30]. The cellulose paper discs were functionalized with amine groups by silane coupling through 3-aminopropyltrimethoxysilane (APS)[31]. This was followed by covalently immobilizing capture antibodies using two different methods. The first method involved binding of periodate oxidized capture antibodies to the amine functionalized cellulose paper discs. The second method used glutaraldehyde as a cross-linking agent to attach the capture antibodies to the amine group-functionalized cellulose paper discs. The presence of capture antibodies was confirmed by rabbit anti-human IgG-fluorescein isothiocyanate (FITC), using a fluorescence molecular imager. The binding activity of rabbit anti-human IgG-FITC to goat anti-rabbit IgG was also evaluated by peroxidase substrate. The effects of various concentrations of sodium periodate, glutaraldehyde, and capture antibodies were investigated. The application test of the immobilized capture antibody was successfully performed through the detection of IgG serum.

Protocol

1. Grafting Amine Functional Groups on Cellulose Paper Discs

1. Prepare one piece of square paper with a dimension of 1 cm × 1 cm, and 100 paper discs made from grade No. 1 cellulose paper with a diameter of 6.0 mm (medium-flow filter paper) using a hole punch.
2. To derive -NH_2 groups on the paper discs, mix 1 ml APS and 10 ml acetone in a 50 ml glass bottle in the fume hood. Add paper discs to the freshly prepared APS reagent mixture, and incubate for 5 hr with orbital stirring (200 rpm) at room temperature[32].
   Caution: Handle APS and acetone in the fume hood.
3. Decant excess solution from the 50 ml glass bottle into an organic waste container.
4. Add 10 ml of acetone to the glass bottle, mix well and decant completely to remove any unreacted APS and other impurities. Repeat this step two times.
5. Spread the paper discs on the paper towel and place in a 110 °C oven for 3 hr. Allow the paper discs to cool. Store the discs in a 50 ml centrifuge tube at room temperature.
6. Use Fourier transform infrared spectroscopy (FTIR) to check the grafting of amine groups on the cellulose square paper, as described below (**Figure 3A**).
   1. Turn on the computer and open the FTIR spectroscopy instrument.
   2. Open the software for FTIR spectroscopy.
   3. Go to 'Measurement → Initialize'. The rectangles for 'BS: KBr', 'Lamp: Infrared' and 'Laser' will turn green when the initialization is finished.
   4. Choose 'Data' below the rectangles, and select '%Transmittance', 'Happ-Genzel', '45', '4.0', and 'Min: 400, Max: 4000' for 'Measurement Mode', 'Apodization', 'No. of Scans', 'Resolution', and 'Range (cm-1)'.
   5. Click 'Measure'.
   6. Select 'Data file' for the background data. Write down the comments.
   7. Click 'BKG' to get the baseline for the background.
   8. Fix the square paper on the film sample holder.
   9. Select 'Data file' for the sample data. Write down the comments.
   10. Click 'Sample' to obtain the spectra for the sample.
   11. Close the FTIR spectroscopy application and turn off the computer.
7. Repeat the above steps (steps 1.1 to 1.6) to prepare amine-functionalized grade No. 113 cellulose square paper and discs (fast-flow filter paper), and obtain the FTIR spectra for the grade No. 113 square paper (**Figure 3B**).

2. Covalent Immobilization of Antibodies on Amine-functionalized Cellulose Paper Discs

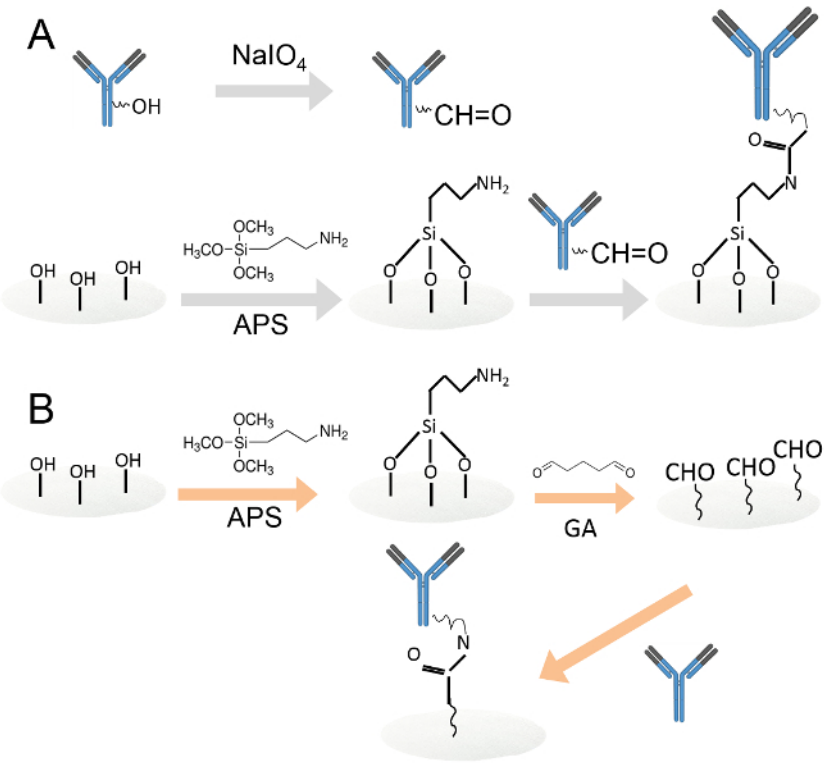

Figure 1. Covalent immobilization of antibodies by two different methods. A. Antibodies immobilized on amine-functionalized cellulose paper discs through periodate oxidation. The carbohydrate residues were oxidized by sodium periodate to produce aldehyde functional groups. Then, the oxidized antibodies were loaded onto amine-functionalized cellulose paper discs. **B**. The antibodies were then immobilized on amine-functionalized cellulose paper discs through glutaraldehyde. The amine functionalized cellulose paper discs were immersed in 0.05% glutaraldehyde solution to introduce aldehyde groups to the paper discs. After washing, antibodies were loaded onto the aldehyde functionalized paper discs. Please click here to view a larger version of this figure.

1. Immobilize antibodies on amine-functionalized cellulose paper discs through periodate oxidation (**Figure 1A**).
    1. Mix 1 µl of 2.5 mM sodium periodate with 2 µl of 0.1 mg/ml rabbit anti-human IgG-FITC and 7 µl of 100 mM pH 5.5 acetate buffer in a 1.5 ml tube, and incubate the mixture in the dark for 30 min.
    NOTE: Follow this volume ratio to prepare more oxidized antibodies if necessary. Change the volume ratio to optimize the concentration of sodium periodate and rabbit anti-human IgG-FITC.
    2. Dilute the above antibody solution with 30 µl of 50 mM phosphate buffer saline (PBS; pH 7.4) to a final volume of 40 µl.
    3. Prepare three amine-functionalized medium-flow filter paper discs and three amine-functionalized fast-flow paper discs (as described in Section 1).
        1. Load 5 µl of the sodium periodate oxidized rabbit anti-human IgG-FITC onto each medium-flow filter paper disc and 8 µl onto each fast-flow filter paper disc. Keep these paper discs in the dark for one hour at room temperature.
    4. Wash each of the paper disc with 0.2 ml of washing buffer (50 mM Tris buffer with 0.15 M NaCl and 0.05% surfactant, pH 7.4). Repeat the wash three times.
    5. Photograph the fluorescence images through a fluorescence molecular imager to identify the presence of antibodies on each cellulose paper disc[32]. Use blank paper discs (treated with the same concentration of antibodies in the absence of sodium periodate) as a control (**Figure S1** to **Figure S3**).
    NOTE: For experimental optimization, change the concentration of one parameter that needs to be optimized and fix the concentrations of all other parameters. A high fluorescence intensity increases the amount of capture antibodies that are immobilized on the cellulose paper discs.

2. Immobilize antibodies on amine functionalized cellulose paper discs through glutaraldehyde (**Figure 1B**).
    1. Add the three APS treated medium-flow filter paper discs and the three fast-flow filter paper discs (described in Section 1) to 2 ml of 50 mM PBS (pH 7.4) that contains 0.05% glutaraldehyde for 1 hr, with orbital stirring at room temperature.
    Caution: Handle glutaraldehyde in the fume hood.
    2. Place three discs each in two 1.5 ml centrifuge tubes. Add 1 ml of deionized (DI) water to each tube and shake the tubes for 10 sec. Remove the water by aspirating with a pipette. Repeat two more times to remove any unreacted glutaraldehyde.
    3. Load 5 µl of 25 µg/ml rabbit anti-human IgG-FITC (capture antibody) onto each aldehyde-functionalized medium-flow filter paper disc, and add 8 µl onto each aldehyde-functionalized fast-flow filter paper disc. Incubate in the dark for approximately 20 min at room temperature. Then, add 10 µl of 50 mM PBS (pH 7.4) to each paper disc without removing the antibodies and incubate for 40 min for the amine aldehyde reaction.

4. Wash the paper discs with 0.2 ml of washing buffer on top of a paper towel. Repeat the wash two times.
5. Photograph the fluorescence images through a fluorescence molecular imager to check for the presence of antibodies on each cellulose paper disc[33]. Use blank paper discs as a control.
   NOTE: In **Figure 4**, '0' stands for the blank paper disc that was treated with the same concentration of FITC-antibody in the absence of glutaraldehyde; in **Figure 5A**, the blank paper discs were treated with glutaraldehyde, but no FITC-antibodies were loaded onto the paper discs.

3. Dry the paper discs (from Sections 2.1 and 2.2) at 37 °C for 10 min.
4. Block the paper discs with 15 µl of blocking buffer (10% skimmed milk powder in 50 mM Tris buffer, pH 7.4, with 0.15 M NaCl) for 10 min at room temperature.
5. Load 5 µl and 8 µl of peroxidase conjugated goat anti-rabbit IgG in PBS (1:10,000) onto medium-flow and fast-flow filter paper discs, respectively. Incubate for 30 min in the dark at room temperature.
6. Wash the paper discs with 0.2 ml of washing buffer on top of a paper towel. Repeat the wash three times.
   NOTE: It is not necessary to remove the washing buffer as the results are not affected by the buffer.
7. Load a 10 µl mixture of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide solution onto each disc.
8. Take images of the paper discs with a digital camera or smart phone after 5 min of incubation.
   NOTE: In **Figure 5B**, '0' stands for the paper discs that were treated with glutaraldehyde, followed by loading antibody-HRP (horse radish peroxidase) conjugate in the absence of FITC-antibody.

3. Paper-based ELISA for IgG Detection

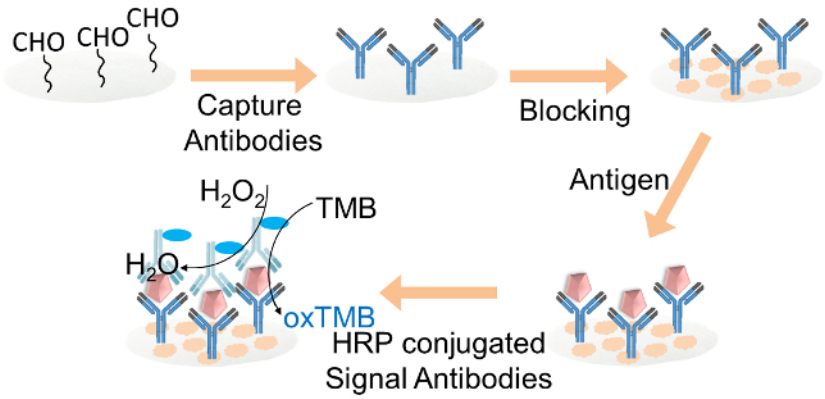

Figure 2. Schematic representation of the paper-based ELISA for IgG detection. Capture antibodies were covalently immobilized on the aldehyde-functionalized cellulose paper discs through glutaraldehyde. The cellulose paper discs were blocked with blocking buffer. Target IgG was then added to the discs, followed by the loading of HRP-conjugated signal antibodies. Finally, the TMB and hydrogen peroxide mixture solution was loaded onto each paper disc for the color readout. Please click here to view a larger version of this figure.

1. Add 5 ml of 0.05% glutaraldehyde solution (prepared in 50 mM PBS buffer, pH 7.4) to a 20 ml glass bottle. Immerse 15 amine-functionalized medium-flow filter paper discs in this solution and keep for 1 hr with shaking at room temperature.
   1. Concurrently, repeat Step 3.1 to prepare another 15 aldehyde functionalized fast-flow filter paper discs.
      Caution: Handle glutaraldehyde in the fume hood.

2. To remove unreacted glutaraldehyde from the paper discs, place the 15 medium-flow filter paper discs in a 15 ml centrifuge tube, and the 15 fast-flow filter paper discs in another 15 ml centrifuge tube. Add 5 ml of DI water to each tube and shake the tubes for 10 sec. Remove the water by aspirating with a pipette. Repeat two times to remove any unreacted glutaraldehyde.
3. Dry the paper discs in a 37 °C oven.
4. Add 5 µl and 8 µl of 0.025 mg/ml mouse IgG-Fc fragment antibodies to each of the medium-flow and fast-flow filter paper discs, respectively, and incubate for 20 min.
5. Add 10 µl of 50 mM PBS (pH 7.4) to each paper disc without removing the antibodies and incubate for 40 min for the amine aldehyde reaction.
6. Wash the paper discs with 0.2 ml of washing buffer on top of a paper towel. Repeat the wash three times.
7. Dry the paper discs in an oven at 37 °C.
8. Block the paper discs with 15 µl of blocking buffer for 10 min at room temperature.
9. Wash each paper disc with 0.2 ml of washing buffer on top of a paper towel. Repeat the wash three times.
10. Run IgG standards.
    1. Load 10 µl of various IgG concentrations (*e.g.*, 0, 10, 125, 250, and 500 ng/ml in PBS) onto each disc in triplicate. Incubate for 1 hr at room temperature.

11. Wash the paper discs with 0.2 ml of washing buffer on top of a paper towel. Repeat the wash three times.
12. Load 10 µl of HRP conjugated mouse IgG-Fc fragment antibodies (1:10,000, 10 mM PBS, pH 7.4), and incubate for 1 hr at room temperature.
13. Wash the paper discs with 0.2 ml of washing buffer on top of a paper towel. Repeat the wash three times.
    NOTE: It is not necessary to remove the washing buffer as the results are not affected by the presence of buffer.
14. Load a 10 µl mixture of TMB and hydrogen peroxide onto each disc.

15. Take images of all paper discs with a digital camera or smart phone after 5 min of incubation.
    NOTE: In **Figure 6A**, '0' stands for paper discs treated with capture antibody immobilization, and the antibody-HRP/TMB solution without IgG serum.
16. Analyze the intensity of each paper disc in the image by Image J.
    1. Convert the images taken in step 3.15 to '.tif' format.
    2. Open 'Image J' software.
    3. Go to 'File → Open', choose the image to analyze.
    4. Choose the shape button 'Oval'.
    5. Go to 'Image → Type → 32 bit'.
    6. Go to 'Edit → Invert'.
    7. Go to 'Analyze → Measure'.
    8. Copy and analyze the data in a spreadsheet.

Representative Results

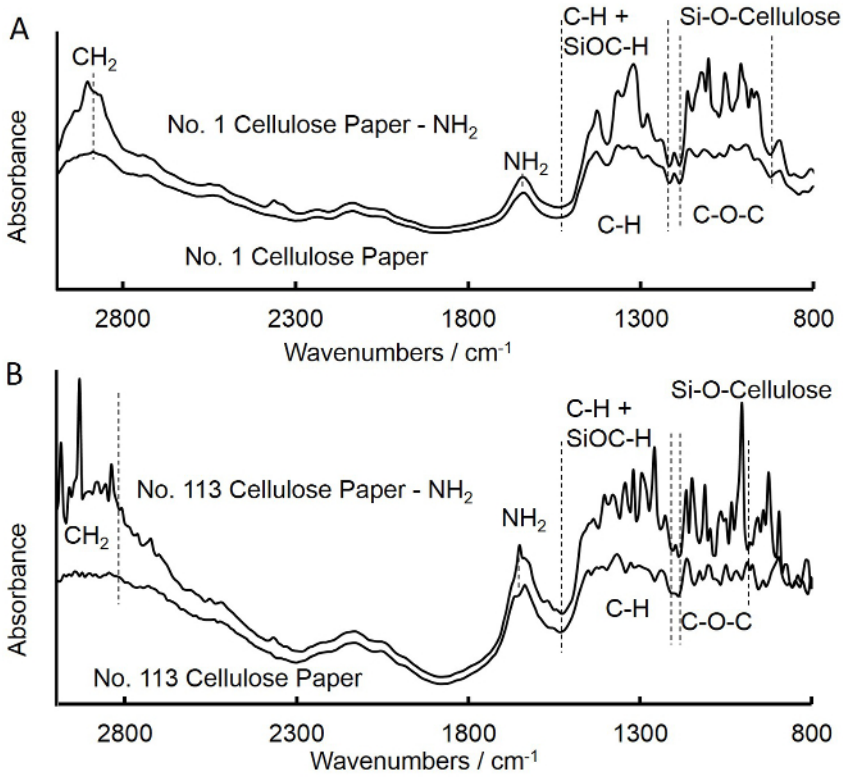

Figure 3. Fourier transform infrared (FTIR) spectra of untreated and APS-treated medium-flow filter square paper (A) and fast-flow filter square paper (B). **A**. The spectra for untreated medium-flow filter square paper was similar to that of APS treated medium-flow filter square paper. The increase in intensities at bands of 902-1,170 cm^{-1} and 1,210-1,500 cm^{-1} for the APS-treated square paper belonged to Si-O-cellulose and C-H deformations of SiOC-H groups, respectively. The increment of the bands at 1,650 cm^{-1} and 2,885 cm^{-1} belongs to the bending of -NH_2 and CH_2 vibrations from the saline propyl moiety, respectively. **B**. The characteristic peaks in the 972 to 1,180 cm^{-1} range were attributed to the Si-O-Si and S-O-cellulose bonds. The peak at 1,003 cm^{-1} was caused by the overlap of the Si-O-Si bond and the C-O stretching of cellulose. All of the results show that APS was successfully grafted onto the cellulose square papers. Please click here to view a larger version of this figure.

The presence of amine functional groups on the cellulose square papers were determined by FTIR. **Figure 3** shows the FTIR spectra of untreated, modified, medium-flow and fast-flow filter square papers. There were three steps involved in the chemical modification of aminoalkyl groups, using APS on the cellulose surfaces. Step one involved the hydrolysis of APS to provide the silanol derivative of APS. Step two involved the adsorption of the hydrolyzed APS derivative onto the cellulose fibers through hydrogen bonding of OH groups from the hydrolyzed APS derivative and the cellulose fibers. Step three involved the condensation of the adsorbed APS derivative, which led to the grafting of the APS derivative onto the cellulose fiber's surface through Si-O-C bonding and the formation of Si-O-Si siloxane bridges[34]. As shown in **Figure 3A**, the increment of the band at 1,650 cm^{-1} was attributed to the bending of NH_2 groups and an increment of the band at 2,885 cm^{-1} corresponding to the CH_2 vibrations of the silane propyl moiety. For the original medium-flow filter square paper, the bands at 902-1,170 cm^{-1} and 1,210-1,500 cm^{-1} corresponded to the vibrations of the C-O-C bonds of glycosidic bridges and CH stretching vibrations. After treating the square paper with APS, the increase in intensity at these two band widths indicates that they belong to Si-O-cellulose and C-H deformations of SiOC-H groups, respectively. Similar to medium-flow filter square paper, the spectra for modified fast-flow filter square paper also increased in intensity at 1,650 cm^{-1} for -NH_2, due to the chemical modification with APS (**Figure 3B**). The strong characteristic peaks in the 972 to 1,180 cm^{-1} range were attributed to the Si-O-Si and S-O-cellulose bonds; the highest peak was at 1,003 cm^{-1} due to the overlap of the Si-O-Si bond and the C-O stretching of cellulose[34]. The characteristic bonds in the wavelength range between 1,188 and 1,510 cm^{-1} for APS treated square paper are caused by the vibrations of the C-O-C bonds of glycosidic bridges and CH stretching vibrations. The CH_2 stretching vibration bonds were shown at 2,800-2,980 cm^{-1} and the highest peak was at 2,932 cm^{-1} for the APS treated square paper. In conclusion, APS can be covalently grafted to No. 1 and No. 113 filter square papers.

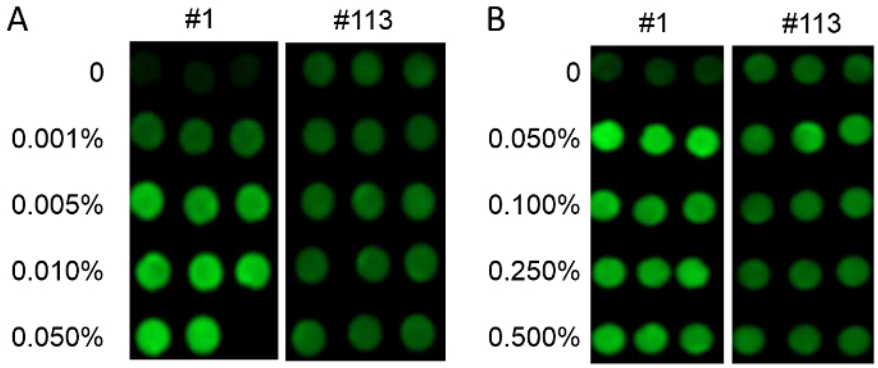

Figure 4. Fluorescence responses to different concentrations of glutaraldehyde in the immobilization of IgG-FITC on the paper discs (Method B). Settings for fluorescence molecular imager: Excitation, 488 nm, emission, 530 nm, resolution, 100 micrometer. **A**: Fluorescence response to 0, 0.001%, 0.005%, 0.01% and 0.050% glutaraldehyde. **B**: Fluorescence response to 0, 0.050%, 0.100%, 0.250% and 0.500% glutaraldehyde. The concentration of IgG-FITC on each disc was 0.01 mg/ml. An increase in the concentration of glutaraldehyde to a maximum of 0.05% resulted in an increased loading amount of IgG-FITC. Concentrations of glutaraldehyde above 0.05% decreased the loading amount of IgG-FITC. Please click here to view a larger version of this figure.

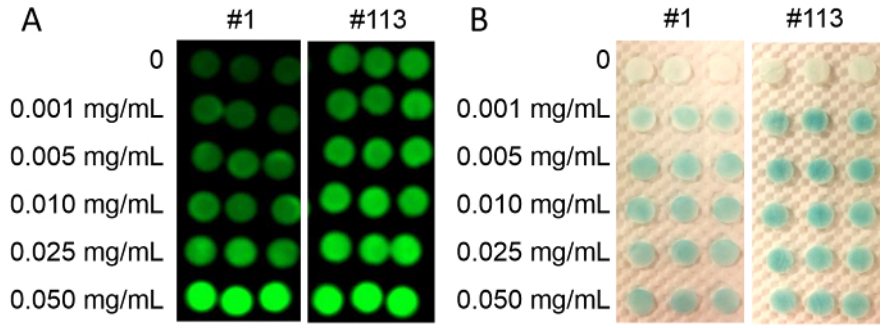

Figure 5. Fluorescence response (A) and colorimetric result (B) of different concentrations of IgG-FITC on the IgG-FITC-immobilized medium-flow and fast-flow filter paper discs (Method B). Settings for fluorescence molecular imager: Excitation, 488 nm, emission, 530 nm, resolution, 100 micrometer. #1 represents grade No. 1, medium-flow filter paper disc, and #113 represents grade No. 113 fast-flow filter paper disc. APS-modified paper discs were treated with 0.05% glutaraldehyde first. Then, different concentrations of IgG-FITC were loaded onto the glutaraldehyde modified paper discs. Increasing the loading concentration of IgG-FITC increased the amount of the immobilized IgG-FITC on the paper discs. However, increased concentrations of immobilized IgG-FITC did not improve the binding capacity to the antigen. Please click here to view a larger version of this figure.

The basic workflow for the covalent immobilization of antibodies on amine-functionalized cellulose paper discs is shown in **Figure 1**. The rabbit anti-human IgG-FITC was covalently immobilized on the cellulose paper discs. The fluorescence from the paper discs indicated the immobilization of antibodies to the paper discs, and the intensity of fluorescence was directly proportional to the concentration of immobilized antibodies. Peroxidase conjugated goat anti-rabbit IgG was then applied to determine the binding capability of the immobilized antibodies. Secondary amine groups and aldehyde groups can react with each other to form a Schiff base, which has been used in bioconjugation[35]. They were also used here for covalent immobilization of capture antibodies. In method A (**Figure 1A**), -NH_2 was introduced to the cellulose paper discs and -CHO was derived from the rabbit anti-human IgG-FITC by oxidizing the Fc region of the antibodies with sodium periodate. In the same way, the effects of various concentrations of sodium periodate and IgG-FITC antibodies were also analyzed. As shown in **Figure S1** and **Figure S2**, sodium periodate from concentrations of 0 to 1 mM and the rabbit anti-human IgG-FITC from 0.016 to 0.16 mg/ml had little effect on the oxidation of 0.08 mg/ml rabbit anti-human IgG-FITC. The amount of antibodies that were covalently bound to the paper discs increased with an increasing concentration of capture antibodies from 0 to 0.075 mg/ml (**Figure S3A**).

A faint color change was observed when the binding capability was determined by the peroxidase assay. This might be due to the use of excess sodium periodate, which reacts with the capture antibodies and causes a loss of binding activity. It is likely that the sodium periodate cannot be completely washed away from the paper discs. This was confirmed by the simple principle that sodium periodate solution provides a yellow color when mixed with purpald solution. Therefore, periodate oxidized antibodies were loaded onto the paper discs and incubated for 1 hour. The paper discs were washed three times with PBS (containing 0.05% tween-20), and then purpald solution was added to these discs. The paper discs showed a purple color immediately, indicating the presence of aldehyde groups from periodate-oxidized antibodies. This purple color changed to yellow within 10 minutes, which confirmed the presence of sodium periodate on the paper discs.

Alternatively, a glutaraldehyde cross-linking method (method B, **Figure 1B**) was used to link the amine groups from the APS-treated paper discs and antibodies. The concentrations of glutaraldehyde (**Figure 4**) and rabbit anti-human IgG-FITC (**Figure 5A**) that were loaded onto the cellulose paper discs were optimized. As shown in **Figure 4**, the fluorescence intensity reached a maximum of 0.05% of glutaraldehyde, which means that the loading of rabbit anti-human IgG-FITC became saturated on the cellulose paper disc at this concentration; an increase in the concentration of glutaraldehyde to 2.5% did not show an increase in the amount of rabbit anti-human IgG-FITC on the cellulose paper discs (**Figure S4**). The fluorescence intensity also increased with increasing concentrations of rabbit anti-human IgG-FITC that were loaded onto the paper discs (**Figure 5A**). A blue color developed immediately upon the addition of the TMB substrate and hydrogen peroxide mixture solution to the paper discs, which contained the peroxidase conjugated detection antibodies (**Figure 5B**). In addition, blocking buffer that contained 10% skimmed milk powder was shown to retain the blocking efficiency after 15 washes (**Figure S5**). Method B was selected to test the application of the immobilized antibodies, as it demonstrated enhanced binding activity to its target. Hence, a 0.025 mg/ml concentration of capture antibodies was used for IgG detection.

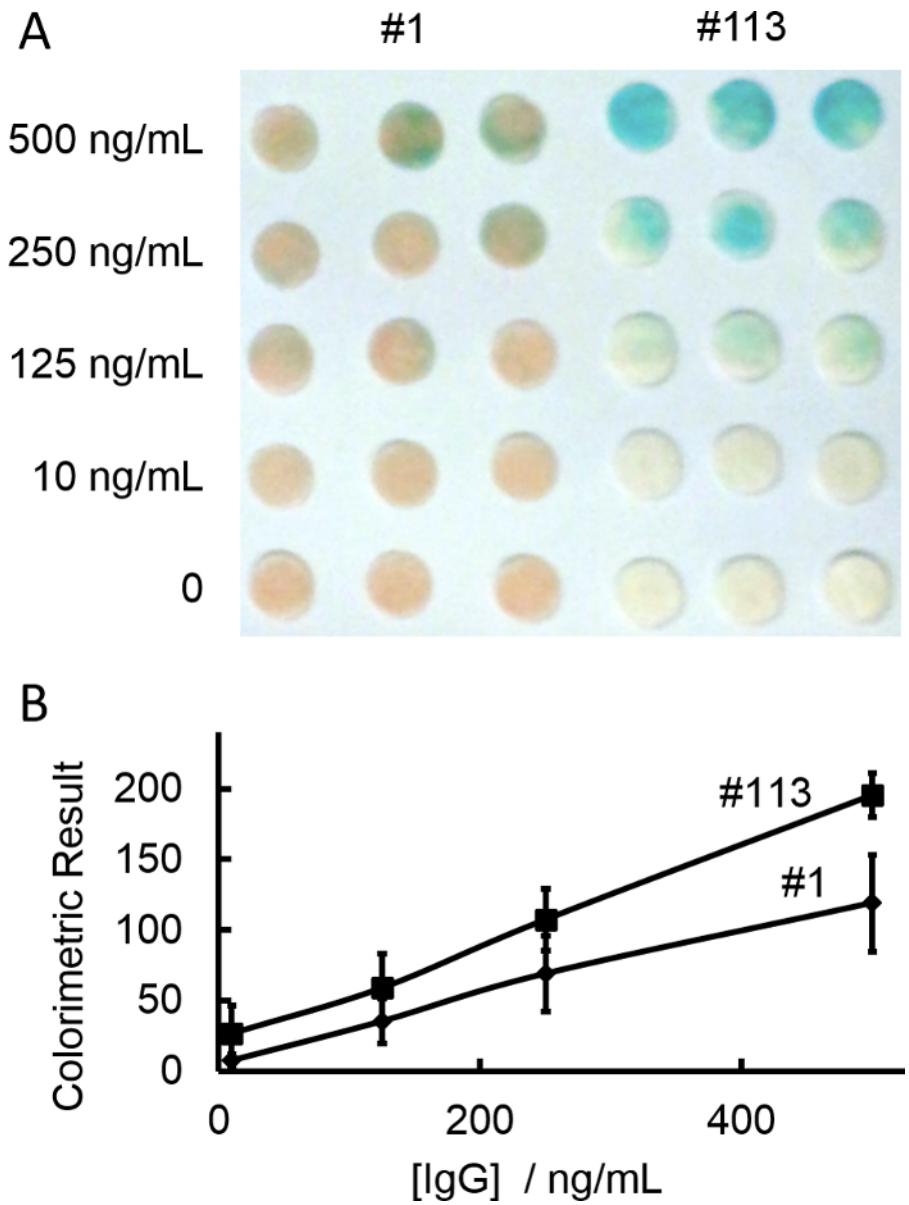

Figure 6. Calibration curves for the determination of IgG by paper-based ELISA. #1 represents grade No. 1, medium-flow filter paper disc, and #113 represents grade No. 113 fast-flow filter paper disc. The upper panel **A** presents the color readout for medium-flow (#1) and fast-flow (#113) cellulose paper discs with different concentration of IgG. The bottom panel **B** presents the ELISA result for different concentration of IgG. Triplicates were used to determine the standard deviation (SD). Please click here to view a larger version of this figure.

Goat anti-mouse IgG-Fc fragment capture antibody, IgG serum, and goat anti-mouse IgG-Fc fragment antibody conjugated HRP were used for the sandwich assay. As shown in **Figure 6** and **Figure S6**, the concentration of IgG serum from 0 to 500 ng/ml had a linear relationship with colorimetric intensity when each step was incubated for 1 hour. In **Figure S6**, the color variation with different concentrations of IgG was obvious for 1 hour of incubation. Yet, the results of the 10 min incubation did not show an obvious change in the color intensity with different concentrations of IgG. Thus, the sensitivity of this paper-based ELISA was suitable for developing practical point-of-care testing.

Figure S1. Fluorescence responses to different concentrations of sodium periodate (Method A). Varying concentrations of sodium periodate were mixed with rabbit anti-human IgG-FITC at a concentration of 0.016 mg/ml and used for the study of antibody oxidation activity. By increasing the concentration of sodium periodate, the loading amount of IgG-FITC increased and reached a maximum at 0.25 mM. It was observed that concentrations of sodium periodate that were higher than this did not increase the amount of immobilized IgG-FITC. Triplicates were used to determine the standard deviation (SD). Please click here to download this file.

Figure S2. Fluorescence responses to different concentrations of rabbit anti-human IgG-FITC (Method A). The concentration of sodium periodate was 0.25 mM in the solution for the antibody oxidation activity study, and the final concentration of rabbit anti-human IgG-FITC on each disc was 0.016 mg/ml. When the concentration of sodium periodate was fixed, the concentration of IgG-FITC had little effect on the oxidation of IgG-FITC. Triplicates were used to determine the standard deviation (SD). Please click here to download this file.

Figure S3. Fluorescence response and colorimetric result from loading different concentrations of oxidized rabbit anti-human IgG-FITC onto the paper discs (Method A). For sodium periodate oxidation, 0.08 mg/ml of IgG-FITC and 0.25 mM sodium periodate were used. The dilution of peroxidase-conjugated anti-rabbit IgG was 1:50,000. Increasing the loading concentration of IgG-FITC on the cellulose paper discs increased the amount of immobilized IgG-FITC, but did not have an effect on target binding. Please click here to download this file.

Figure S4. Fluorescence response to high concentrations of glutaraldehyde in the immobilization of IgG-FITC on the cellulose paper discs (Method B). The concentration of IgG-FITC on each disc was 0.01 mg/ml. Concentrations of glutaraldehyde that were higher than 0.25% did not increase the quantity of immobilized IgG-FITC on the cellulose paper discs. Please click here to download this file.

Figure S5. Effect of number of washing times. A: washing three times. **B**: washing 15 times. Capture antibodies immobilized on the cellulose square paper still had good binding capacity to their targets, even though the square paper was vortexed 15 times. Please click here to download this file.

Figure S6. Paper-based ELISA result for IgG. For the concentrations of IgG from 0 to 500 ng/ml, the results from one hour of incubation are better than the results from 10 minutes of incubation. For high concentrations of IgG, 10 minutes is enough time for incubation. Triplicates were used to determine the standard deviation (SD). Please click here to download this file.

Figure S7. Scanning electron microscopy (SEM) images of medium-flow filter paper at different magnifications. A: 85X and **B**: 20,000X. Field emission scanning electron microscopy (FE-SEM) operating at 5 kV was employed to determine the particle morphology. The cellulose fibers are randomly cross-linked. Please click here to download this file.

Figure S8. SEM images of fast-flow filter paper at different magnifications. A: 100X and **B**: 20,000X. Field emission scanning electron microscopy (FE-SEM) operating at 5 kV was employed to determine the particle morphology. The cellulose fibers are randomly cross-linked. Please click here to download this file.

Discussion

Direct coating of affinity purified goat anti-Mouse IgG-Fc capture antibody on unmodified cellulose paper discs was performed to detect IgG concentrations. The results indicated that, further fixation of the capture antibodies is required for reproducibility. The silane technique was successfully used to introduce amine functional groups to the cellulose paper discs[34]. The concentration of APS affects the immobilization of antibodies. Therefore, the amount of APS in acetone was also optimized. 1 ml of APS in 10 ml of acetone was an optimal concentration for grafting the amine group for the immobilization of antibodies through a glutaraldehyde cross-linking agent. Antibodies were grafted on the cellulose paper discs by periodate oxidation and glutaraldehyde cross-linking methods. Our results revealed that the binding capacity of immobilized antibodies through the glutaraldehyde cross-linking method was better than the periodate oxidation method for medium-flow and fast flow-filter paper discs. Furthermore, capture antibodies, which were immobilized on cellulose paper discs through a glutaraldehyde cross-linking method, retained their function and were stably immobilized even though the paper was subjected to the mechanical stress of 15 washes by vortexing. Ten percent of the skimmed milk powder blocking buffer was found to retain its blocking efficiency after 15 washes (**Figure S5**).

The covalently immobilized antibodies were used to perform a sandwich ELISA with the detection of IgG. IgG at a concentration of 100 ng/ml was detected with the naked eye using this paper disc based assay. The concentration of IgG serum from 0 to 500 ng/ml showed a linear relationship with colorimetric intensity, when each step was incubated for one hour. The immunoassay testing for this paper disc based ELISA required less sample reagent and appeared to be more efficient than conventional immunoassays[36]. The results showed that the sensitivity of fast-flow filter paper disc based immunoassay was higher than that of the medium-flow filter paper based immunoassay. The morphologies of medium-flow and fast-flow filter paper discs that were obtained by field emission scanning electron microscopy are shown in **Figure S7** and **Figure S8**, respectively. As illustrated in these figures, the fibers are randomly cross-linked for both kinds of filter paper discs[25]. The reason for this high sensitivity might be the thickness of the paper disc. For fast-flow filter paper discs the thickness was 420 µm compared to 180 µm for medium-flow filter paper. Thus, more capture antibodies were likely to be immobilized on fast-flow filter paper discs, which would further increase the sensitivity of the assay. At the same time, the pore size for the fast-flow filter paper discs (30 µm) was larger than that of the medium-flow filter paper discs (11 µm). After paper surface blocking, the pore size of the former was still large enough to drive liquid flow without an extra power system. However, the rate of flow for the buffer in the latter was slower. Therefore, fast-flow filter paper was better than medium-flow filter paper in the application of immunoassays.

The reproducibility of the antibodies on the immobilized filter paper discs was evaluated by further incubation of the paper discs with peroxidase-conjugated goat anti-rabbit IgG and detection of the colorimetric result after loading the TMB substrate and hydrogen peroxide mixture solution. The standard deviation was less than 10% for this paper-based device. The stability of rabbit anti-human IgG-FITC on the -NH_2 modified cellulose paper discs was tested for up to two months. The paper discs that were stored at 4 °C maintained their binding activity to peroxidase-conjugated goat anti-rabbit IgG with a slight decrease in their binding activity. However, when the antibody immobilized paper discs were stored at room temperature or 60 °C, they lost their binding activity due to the dryness and high temperature. The captured antibodies could have destabilized and denatured under these conditions.

There are some critical steps in the immobilization of capture antibodies on the cellulose paper discs using glutaraldehyde as a cross-linking agent. Step 1.2 to graft amine groups on the paper discs is a critical step in the successful covalent immobilization of capture antibodies. If this step fails, the entire immobilization process will fail. FTIR was used to determine whether amine groups were successfully immobilized on the paper discs (step 1.6). Second, the grafting of aldehyde groups on the cellulose paper discs is another critical step (Step 2.2.1 and Step 3.1). The antibodies were covalently immobilized on the cellulose paper discs through the formation of a Schiff base. Finally, the step to immobilize capture antibodies on the filter paper discs is important (Step 2.2.3, Step 3.4, and Step 3.5). The amine groups from the capture antibodies reacted with the aldehyde groups from the cellulose paper discs to fix the antibodies on the paper discs. If this step was unsuccessful, capture antibodies would not immobilize on the paper discs and all of the ELISA application tests would be negative. We can use a fluorescence

molecular imager to determine the presence of capture antibodies. Peroxidase-conjugated goat anti-rabbit IgG and peroxidase-based colorimetric reactions can be used to confirm the binding capacity of the immobilized antibodies.

This protocol may encounter some problems, such as a high background to the signal, no signal or a weak signal, and an uneven color readout. The possible causes of these problems and troubleshooting methods for overcoming these problems are discussed below. High background signals usually occur due to a short blocking time. This can be solved by increasing the blocking incubation time, washing the paper discs thoroughly, and avoiding the addition of excess detection antibody conjugate enzyme to remove high background signals. The possible causes include an absence of target antigen, over blocking, insufficient incubation time, and a non-functional detection antibody enzyme conjugate. To overcome these issues, the target antigens must be added and incubated for a suitable time period using a new antibody conjugate and storing it as suggested by the manufacturer. It is advisable to include a positive control for detection. Uneven color readouts are due to the stacking of paper discs during the grafting process. To avoid this problem, the paper discs must be separated during the APS grafting process, and the antigen and detection antibody conjugate enzyme must be spread uniformly on the paper disc at each step.

Although the paper-based assay is a simple and cost effective method, there are limitations. The orientation of antibodies on the substrate surface is critical to the performance of the immunoassay, as the glutaraldehyde cross-linking agent reacts with the amine groups from the Fab region and the Fc region of the capture antibodies. Thus, the immobilized antibodies do not occur in a highly oriented manner. Since there are more amine groups from the Fab region than from the Fc region of the IgG antibodies, the binding activity of the immobilized antibodies is still high enough for their application.

Different surface functionalization methods for cellulose paper were summarized in a recent report[37]. Reagents such as divinyl sulfone; 1,4-phenylenediisothiocyanate; 4-azido-benzenediazonium; epichlorohydrin; 4-azido-a-fluoro-2-nitrocyclohexane; and 4-mercapto-benzenediazonium, could be used to covalently immobilize biomolecules on cellulose paper. Adsorption and entrapment were also utilized to coat the cellulose paper with biomolecules. Compared to the other methods, the combination of the silane technique and the glutaraldehyde cross-linking agent for covalent immobilization of antibodies on cellulose paper is a simple method. In addition, this combination may have little effect on the thermal and mechanical performance of the cellulose paper, allowing the maximum vertical flow-through in immunoassays. This strategy could be extended to immobilize other biomolecules on the cellulose paper.

The methodology demonstrated here could potentially be extended to the detection of any analyte, as long as direct antibodies against it are available. This method can also be used to develop multiplex detection. For example, on a square paper, different areas can be distinguished for various targets. The capture antibodies for the targets can be immobilized through a glutaraldehyde cross-linking agent on their specific area. This can be followed by the ELISA procedure to detect various targets**.** Thus, the proposed method makes the paper-based ELISA a versatile tool for the detection of other targets using the naked eye.

Disclosures

The authors have nothing to disclose.

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