A (3-Aminopropyl)triethoxysilane (APTES) and Glutaraldehyde Functionalized Cellulose Surface for Immunoassays

by
William Harley

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Chemical Engineering and Biochemistry and Molecular Biology
(Honors Associate)

Presented May 10, 2023
Commencement June 2023

AN ABSTRACT OF THE THESIS OF

William Harley for the degree of Honors Baccalaureate of Science in Chemical Engineering and Honors Baccalaureate of Biochemistry and Molecular Biology presented on May 10, 2023. Title: A (3-Aminopropyl)triethoxysilane (APTES) and Glutaraldehyde Functionalized Cellulose Surface for Immunoassays.

Abstract approved:_____

Vincent Remcho

The detection of biomolecules is critical for clinical diagnostics but requires specialty labor and high cost. The use of microfluidic paper-based analytical devices (μPADs) for bioassays is simpler and cheaper but has low sensitivity. Improved immobilization of antibodies onto cellulose is demonstrated through surface functionalization with APTES and glutaraldehyde. The nature of this immobilization is explored through Attenuated Total Reflection Fourier-transform infrared spectroscopy (ATR-FTIR spectroscopy) and various washing procedures. The application of this novel immobilization technique is explored through antibodies conjugated with fluorescein isothiocyanate (FITC) in an indirect immunoassay for Rabbit Immunoglobulin G (Rabbit IgG). Compared to the unfunctionalized surface, the functionalized surface improves immobilization of antibodies and has comparable sensitivity in a fluorescent immunoassay.

Keywords: Microfluidics, paper-based analytical devices, immunoassay, surface chemistry

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Honors Baccalaureate of Science in Chemical Engineering and Honors Baccalaureate of Biochemistry and Molecular Biology project of William Harley presented on May 10, 2023.

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Kari van Zee, Committee Member, representing Biochemistry and Biophysics

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Patrick Geoghegan, Committee Member, representing Chemical, Biological, and Environmental Engineering

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

William Harley, Author

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Introduction

Diagnostics are critical to the identification and subsequent treatment of disease, but these diagnostics tend to be intensive, lab-based, and require specialized personnel.[1] Healthcare workers are required to perform the tests and interpret the readout adding obstacles of labor and cost towards receiving clinical diagnostics.[2]

Microfluidic paper-based analytical devices (μPADs) are a popular diagnostic tool for healthcare. They are simpler and cheaper to fabricate than other methods and only require small volumes of fluid lending them suitable for use in resource limited environments, such as developing countries.[2] This makes them promising for point-of-care testing (POCT).[1–3] Additionally, a majority of materials used are biodegradable, abundant, and present excellent compatibility with different chemical applications.[3]

There are various diagnostic mechanisms on μPADs. A common method of detection includes colorimetric assays where color generated by a chemical/biochemical reaction can be measured and correlated with concentration of analyte. Enzymatic reactions are often used to achieve this colorimetric detection. An oxidase oxidizes the analyte to generate hydrogen peroxide which can then be reduced by a peroxidase to oxidize an indicator. The indicator molecule will change color with the change in oxidation state. A common example includes the use of glucose oxidase and horseradish peroxidase with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

When conjugated with a detection molecule, antibodies and aptamers may also be used for analyte detection and share similar methodologies. The detection molecules may be selected to perform a colorimetric or fluorometric reaction. Whereas antibodies are polypeptides that target a given antigen, aptamers are synthetic DNA or RNA sequences designed to also act as receptors for antigens.[4] Both offer high antigen specificity. When compared to antibodies, aptamers have comparable affinities for the similar analytes.[4] They also present many advantages over antibodies including multiple uses through regeneration, preparation at lower cost, and higher thermal stability.[4]

Both antibodies and aptamers are commonly used in sandwich immunoassays. A sandwich immunoassay uses an antibody for antigen capture and a second antibody for antigen detection.[5,6] A primary antibody is immobilized onto a surface and serves to capture the antigen. A secondary antibody is subsequently added and complexes with the captured antigen. This secondary antibody is commonly tagged with a detection molecule (e.g., a fluorophore or an enzyme) to allow for either colorimetric or fluorometric detection of the antigen (Figure. 1). The intensity of this signal can be correlated with concentration of the analyte via a standard curve.

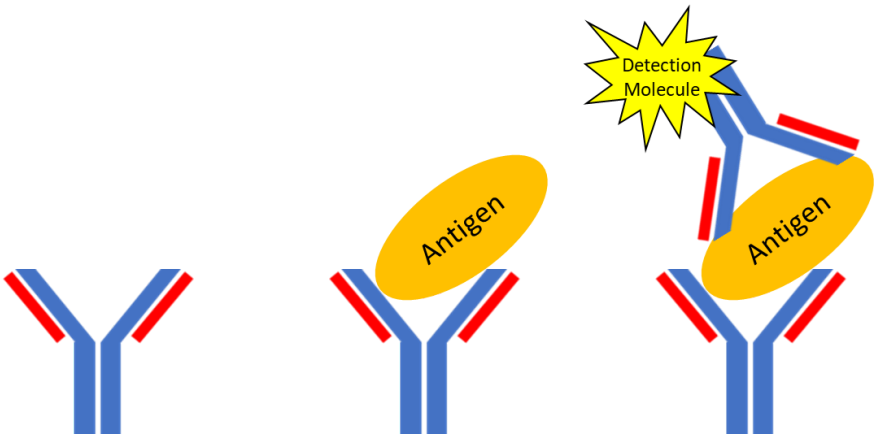

Figure 1. Depiction of a general sandwich immunoassay using antibodies. The primary antibody binds to an epitope on the antigen. A secondary antibody conjugated with a molecule for detection binds to a second epitope on the antigen.

Immunoassays on μPADs were first reported by the Whiteside's group.[2,7] The mechanism included immobilization of an antigen to the paper surface, detection with an antibody tagged with a phosphatase enzyme, and colorimetric detection with a phosphate/nitro blue tetrazolium solution.

Despite the documented advantages of μPADs and versatility of antibodies and aptamers for point-of-care diagnostics, there are notable disadvantages. Tests performed on μPADs tend to be variable and more qualitative than quantitative.[1] Additionally, they have low sensitivity for analyte detection.[1] Particularly for immunoassays, this can be attributed to poor immobilization of antibodies onto paper. Adsorption is the main mode of immobilizing detection molecules such as a capture antibody onto a surface.[8] This interaction is weak and can be easily disrupted leading to decreased sensitivity.

One method to improve the sensitivity of μPADs is covalent immobilization of the biomolecules onto the surface. There are many approaches to achieve this covalent binding. Cellulose surfaces can react with $NaIO_4$ to form aldehyde groups that can form Schiff Bases with biomolecules.[8] Alternatively, linkers can be reacted with the cellulose surface to add functional groups for reaction. Previous literature has used chitosan and glutaraldehyde to immobilize antibodies for a sandwich immunoassay.[3]

There exist many different linkers to covalently immobilize biomolecules to surfaces. A well-documented linker includes (3-aminopropyl)triethoxy silane (APTES).[9] The ethoxy silanes can react with hydroxyls on a surface leaving the more reactive amine group exposed for further linkages. Glutaraldehyde is commonly used to crosslink the amino group of APTES with the amino group of a biomolecule via formation of a Schiff Base.[9] The steps of using APTES and glutaraldehyde to functionalize a surface with exposed hydroxyls is shown in Figure 2. This pair has been documented for use with sandwich immunoassays.[6] The same chemistry can be implemented with aptamers that are modified to include an amino group at their 5' end.[4]

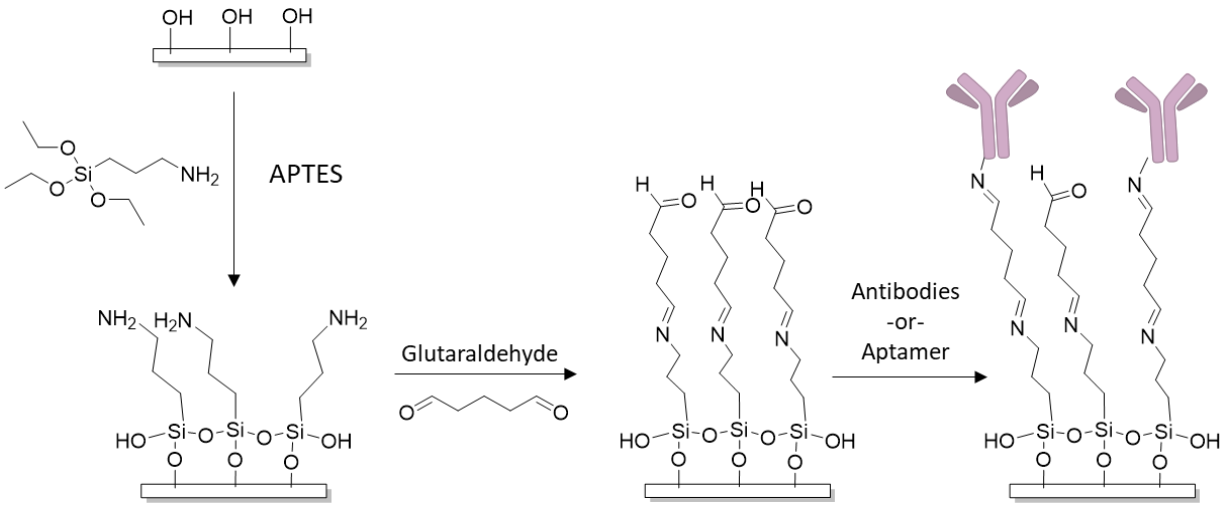

Figure 2. Mechanism of using APTES and glutaraldehyde to functionalize a cellulose surface. APTES can react with surface hydroxyls through an S_N2 reaction resulting in loss of ethanol. Glutaraldehyde reacts with the primary amine to form a Schiff's Base leaving an aldehyde end available for further reaction with a primary amine on a biomolecule.

This method of functionalizing a surface has been demonstrated on silicon, but not yet on a paper-based device. The mechanism proposed in Figure 2 will be used to functionalize the surface of cellulose for application with an immunoassay. The functionalization of the surface and strength of immobilizing antibodies will be characterized as well as its application with an indirect immunoassay as was done by the Whiteside's group.[7] An indirect immunoassay uses a primary antibody for detection of the antigen and a secondary antibody for detection of the primary antibody. Whiteside's group immobilized the primary antibody (Rabbit Immunoglobulin G (IgG)) on paper and quantified detection of this. A general scheme of this indirect immunoassay with the fluorophore fluorescein isothiocyanate (FITC) as a detection molecule can be seen in Figure 3.

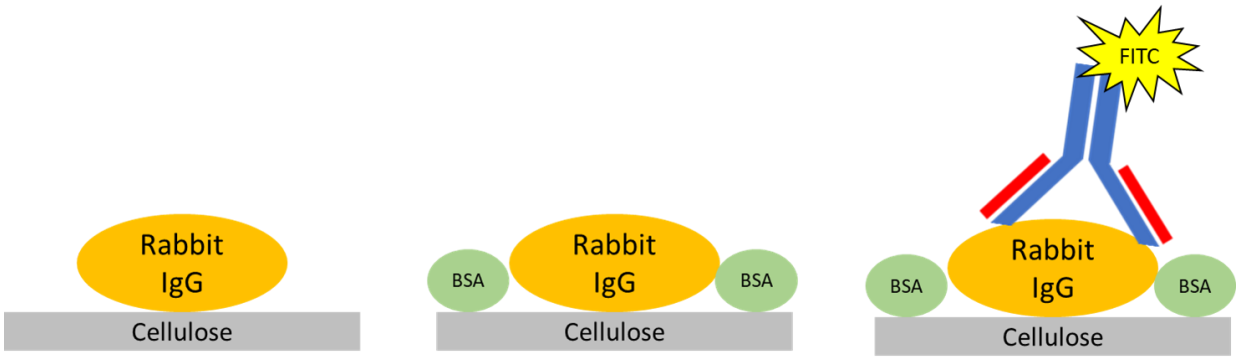

Figure 3. Indirect immunoassay for Rabbit IgG using goat anti-Rabbit IgG conjugated FITC (anti-IgG FITC). Rabbit IgG is first immobilized on the cellulose surface. Remaining reactive sites are blocked with bovine serum albumin (BSA). Anti IgG-FITC are incubated on the surface to bind the antigen for fluorescent detection.

Materials and Device Preparation Methods

Reagents and Materials

Goat Anti-Rabbit IgG-FITC antibody and IgG from rabbit serum were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was purchased from VWR Life Sciences (Radnor, PA). Tween-20, (3-Aminopropyl)triethoxysilane (APTES), and glutaraldehyde were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Ethanol was purchased from Greenfield Global (Toronto, Canada). Toluene was purchased from Thermo Fisher Scientific (Waltham, MA). Polycaprolactone (MW 80,000) (PCL) was purchased from Perstorp (Del Mar, CA).

Deionized water was obtained from a Milli-Q water purification system (Millipore Milli-Q Advantage A10) from EMD Millipore (Burlington, MA, USA) and used for all solutions unless otherwise specified. Glutaraldehyde solution was prepared with a 10 mM phosphate buffer (PB, pH 6, NaH_2PO_4 10 mM) containing 1% (v/v) glutaraldehyde. Washing solution was prepared with 0.05% Tween-20 in 1X phosphate-buffered saline (PBS, pH 7.4, NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2.7 mM). Blocking solution was prepared with 1 % bovine serum albumin (BSA) in 1X PBS pH 7.4 with 0.05% Tween-20. PCL solution was prepared with 4% w/v PCL in toluene.

Whatman grade No. 1 (pure cellulose paper) was purchased from GE Healthcare Worldwide (Shanghai, China). Qualitative Black Filter Paper was obtained from Ahstrom (Helsinki, Finland). Black poster board was purchased from a local general store.

Instruments

Paper was cut with a laser cutter (VLS 3.50, Universal Laser Systems, Scottsdale, AZ, USA). Paper well-plates were read with a SpectraMax Gemini XS microplate fluorometer purchased from Molecular Devices (Sunnyvale, CA, USA).

Functionalization of Filter Paper

The covalent immobilization of APTES and glutaraldehyde onto cellulose filter paper was modeled after prior literature.[10] Solutions are all prepared with Milli-Q water. The cellulose paper was submerged in APTES Solution (10 % (v/v) APTES in 10% (v/v) ethanol) for 1 hr at room temperature while shaking at 85 rpm. The paper was then transferred to a glass petri dish and left to incubate for 24 hr at 100 °C. The cellulose papers were then washed with Milli-Q water for one minute shaking at 85 rpm. This washing was done in triplicate.

The aldehyde groups were introduced to the cellulose surface by submerging the washed papers in glutaraldehyde solution (1% (v/v) glutaraldehyde in PB pH 6 while shaking at 85 rpm at room temperature for 50 minutes. The reacted cellulose papers were then washed with Milli-Q water for one minute shaking at 85 rpm three times. The functionalized papers were stored at room temperature in the dark.

Fabrication of Paper Well-Plate

Poster board and cellulose filter paper were cut using a laser cutter. The parameters for cutting filter paper were: 0.9% power, 3% speed, 250 PPI, 1.3 mm z-axis. The parameters for

cutting poster board were: 1.3% power, 2.5% speed, 250 PPI, 0 mm z-axis. The dimensions are outlined in Figure 4.

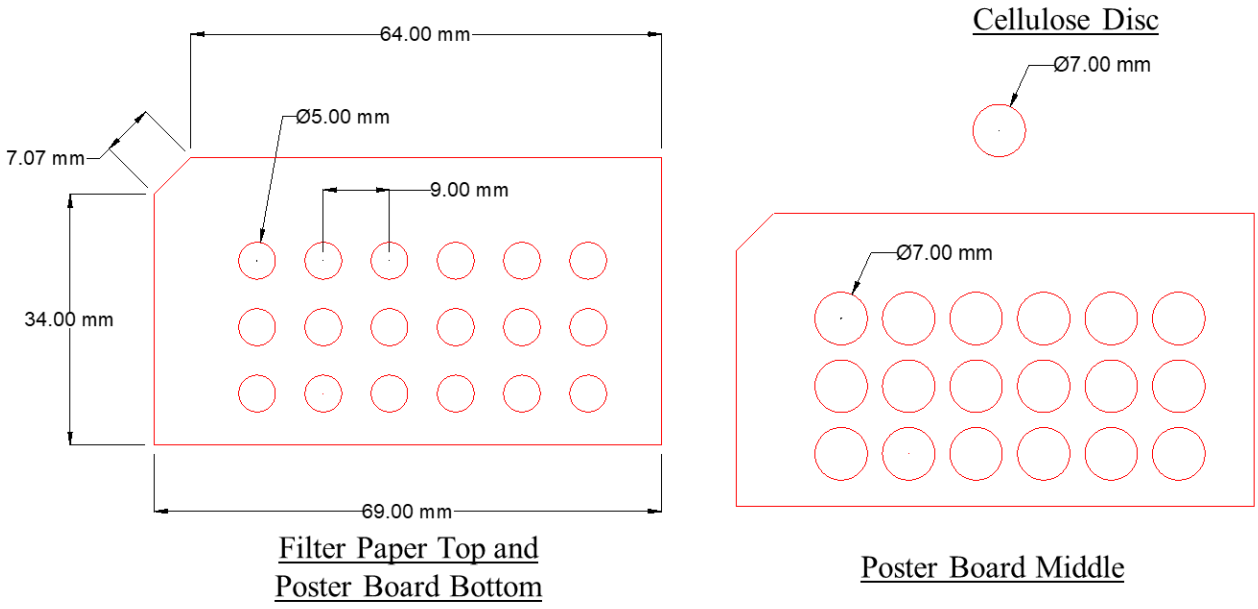

Figure 4. Computer-Aided Design (CAD) drawings used for fabrication of poster board and black filter paper layers of support to hold cellulose disc for the well-plate reader.

The poster board and black filter pieces for construction of the paper well-plate were coated on both sides with a PCL solution (4% (w/v) PCL in toluene) by airbrushing and allowed to dry.

The layers were laminated together with the cellulose discs sandwiched in the middle layer using a thermal laminator (TL1302 thermal laminator, Scotch™ Brand, USA). The layers are indicated in Figure 5.

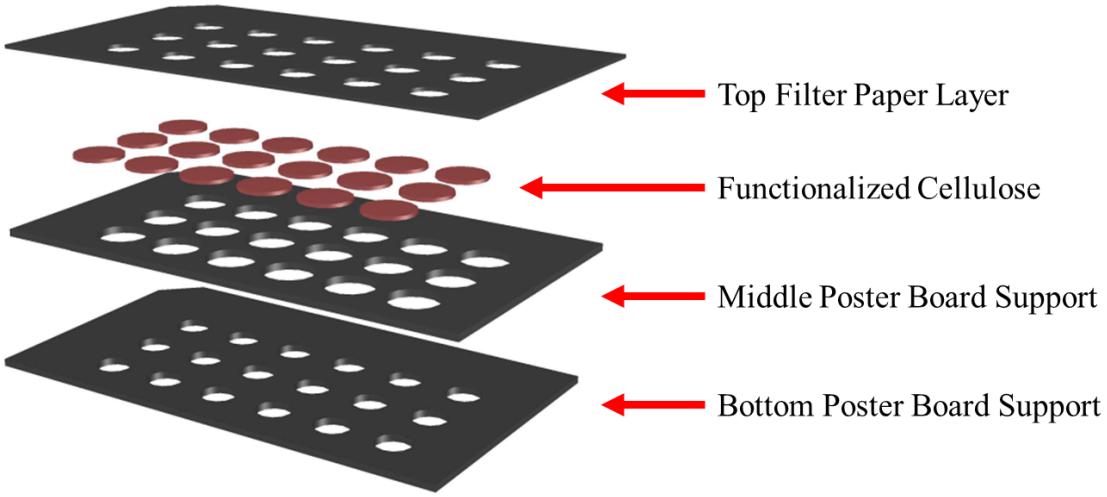

Figure 5. Laminated layers for support to hold cellulose discs in the well-plate reader. The layers are sprayed with 4% (w/v) PCL in toluene using an airbrush on both sides and then laminated together to secure the cellulose discs in the middle.

Biomolecule Immobilization

To immobilize antibodies to the aldehyde group of the APTES-glutaraldehyde surface via an Schiff Base, 3 µL of antibody solution (100 mg/mL Anti- Rabbit IgG in 1X PBS pH 7.4) were deposited in each well and incubated for 10 minutes at room temperature.

Indirect Immunoassay Procedure

The procedure is carried out at room temperature. Rabbit IgG was immobilized onto functionalized cellulose by depositing 3 µL of varying concentrations of Rabbit IgG and incubating for 10 minutes. Unreacted sites were blocked with 3 µL of blocking solution. Rabbit IgG was detected by adding 3 µL of 200 µg/mL of Anti-Rabbit IgG-FITC and incubating for 1 minute. Each well was washed twice with 10 µL of washing buffer by

adding solution to the top of the well and wicking solution with blotting paper underneath as depicted in Figure 6. The wells were dried at room temperature then fluorescence was measured with excitation at 490 nm, emission at 530 nm, and cutoff at 515 nm.

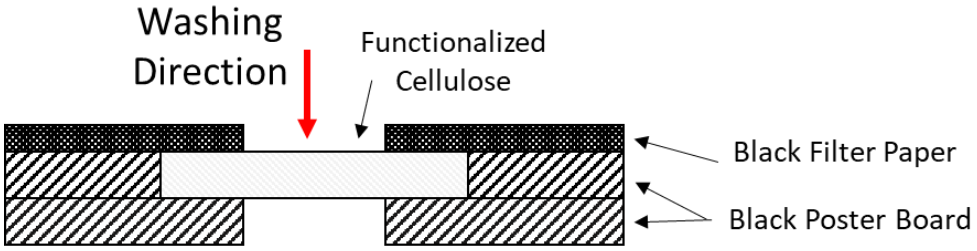

Figure 6. Methodology for vertical washing steps paper well-plate.

Surface Characterization

The presence of specific functional molecules and corresponding reactive groups on the solid-state surface was explored with Attenuated Total Reflection Fourier-transform infrared spectroscopy (ATR-FTIR spectroscopy). Transmission percent was measured between 400 – 4000 cm^{-1}.

Methodology

Optimization of Functionalization Conditions

The initial functionalization of cellulose with APTES and glutaraldehyde was adopted from Moriera et al.[10] Cellulose was incubated in 10% APTES solution (in 10% ethanol) for 24 hours then submerged in 1% (v/v) glutaraldehyde solution for 50 minutes at room temperature. Success of the reaction was determined by the development of a brick red color seen in Figure 7, which indicates formation of a Schiff base between the APTES amino group and glutaraldehyde aldehyde group.[10]

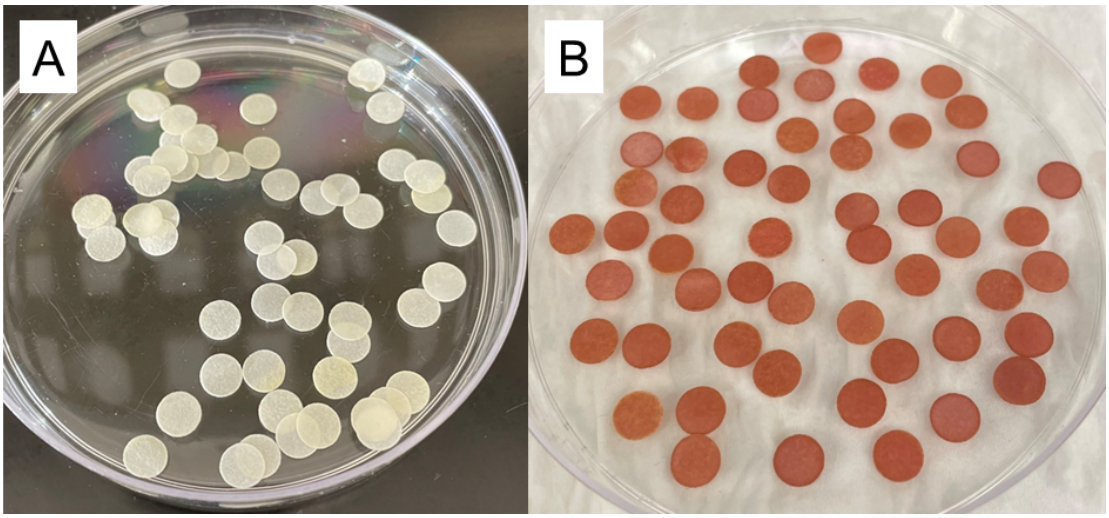

Figure 7. (A) Cellulose paper following 24 hour reaction in 10% APTES solution at room temperature. (B) Room temperature APTES reaction cellulose following 50 minute reaction in 1% glutaraldehyde solution.

To explore the nature of the reaction between APTES and the cellulose surface, reactions for 24 hours with APTES and 50 minutes with glutaraldehyde were repeated at different temperatures and washed with Milli-Q water at different steps. The temperatures explored were room temperature, 30 °C, and 100 °C because silanization at higher temperatures (60-90

°C) disrupt the noncovalent interactions and increase the amount of stronger bonded APTES to the surface. Following the APTES reaction, cellulose papers were washed with Milli-Q water either 1 time for 3 minutes or 3 times for 1 minute each. The results are shown in Figure 8.

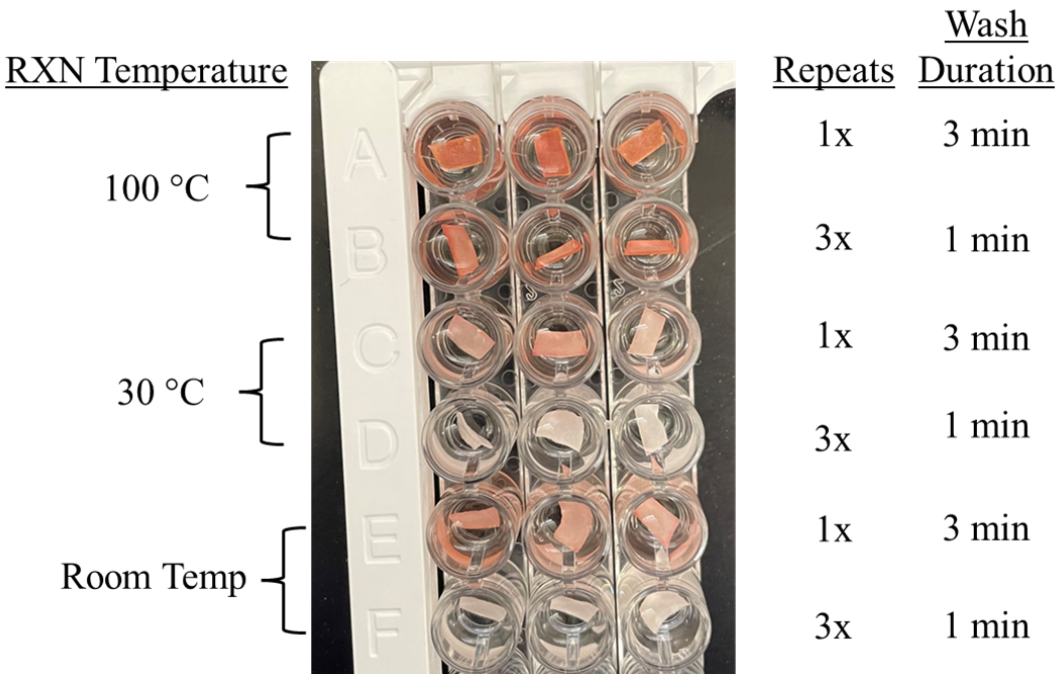

Figure 8. Comparison of APTES reactions at varying temperatures and washing techniques. Following reacting with APTES and washing at different conditions, the cellulose was reacted with glutaraldehyde to develop the red color and indicate extent of removal of APTES from the surface.

When the cellulose papers are washed after reacting with APTES solution at room temperature and 30 °C, there is a decrease in the formation of Schiff Bases with glutaraldehyde as indicated by the decreased intensity in red color. This suggests that washing with water is removing the weakly bonded APTES molecules from the cellulose surface. The washing 3 times for 1 minute each wash results in the most extensive decrease in red color development. The 100 °C reaction shows minimal change in red color intensity

suggesting reacting with APTES at this temperature forms more stable interactions with the cellulose surface.

Surface Characterization with FTIR

The presence of specific functional molecules during functionalization steps and different reaction conditions was explored through FTIR spectroscopy. Spectra were taken following washing of cellulose reacted at 100 °C with APTES and then after washing the paper following reaction with glutaraldehyde. These are shown in Figure 9.

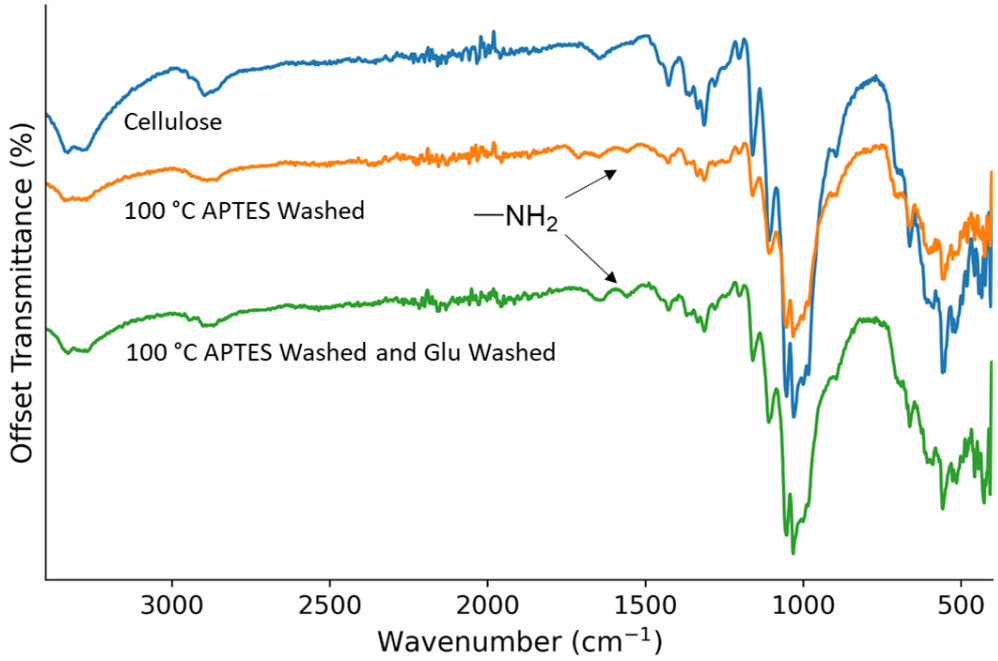

Figure 9. FTIR spectra of untreated cellulose, APTES reacted cellulose at 100 °C, and APTES reacted cellulose reacted with glutaraldehyde. Following each reaction, cellulose was washed in Milli-Q water for one minute three times. The peak at 1566 cm^{-1} indicates a primary amine suggesting immobilization of APTES.

Cellulose peaks are present in all samples. These include OH stretching at 3343 cm^{-1} and CH/CH_2 stretches at 2898 cm^{-1}.[10] Compared to the cellulose control, a peak appears at 1566 cm^{-1}, which can be attributed to primary $-NH_2$ stretches contributed from APTES immobilized onto the surface. A peak characteristic of a Schiff Base is expected between 1614-1690 cm^{-1}; however, there is a peak present at 1640 cm^{-1} on all samples including cellulose which may mask the expected Schiff Base. According to previous literature, the additional peak is characteristic of cellulose at indicating a C=C bond or additional OH stretches.[10,11]

To further establish the lack of strong reaction between APTES and cellulose under room temperature and 30 °C conditions, spectra were taken before and after washing cellulose reacted with APTES at these conditions. These are shown in Figure 10.

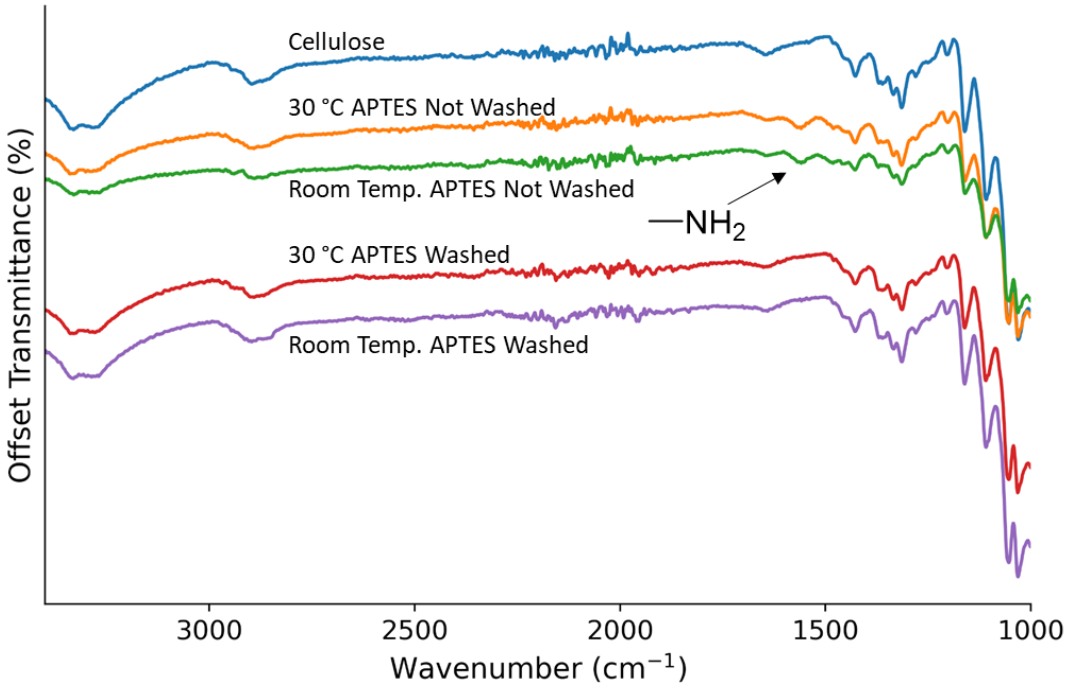

Figure 10. FTIR spectra of untreated cellulose, cellulose reacted with APTES at 30 °C and room temperature before and after washing. The color of traces are as follows: Cellulose (blue), 30 °C reaction not washed (orange), room temperature reaction not washed (green), 30 °C reaction washed (red), and room temperature reaction washed (purple). The peak at 1566 cm^{-1} indicates a primary amine before washing and its disappearance after washing suggests APTES was weakly immobilized and washed away.

Peaks indicative of cellulose including OH stretching at 3343 cm^{-1} and CH/CH_2 stretches at 2898 cm^{-1} are clearly present. Following the reaction and before washing, both surfaces show a peak at 1566 cm^{-1} indicating primary $-NH_2$ stretches of APTES. Following washing with Milli-Q water, both surfaces lose this peak and their spectra mimic that of untreated cellulose. This suggests that APTES was weakly associated with the surface and washed away.

Evaluation of Antibody Immobilization

The antibodies may adhere to the surface through non-covalent interactions as proteins generally attach to interfaces through hydrophobic, electrostatic, and van der Waals forces.[9] This adsorption is random and may result in different orientations of antibodies onto a surface. The strength of interaction between the antibodies and the surface was explored with washing experiments of FITC-tagged antibodies immobilized onto APTES-Glutaraldehyde functionalized cellulose and unfunctionalized cellulose.

The molecule FITC has been used conjugated to antibodies in immunoassays as a method of correlating antigen concentration to intensity of fluorescent signal.[12] Previous work utilized an excitation and emission of 495 and 519 nm with this fluorescent probe.[12] The background signal between the APTES-Glutaraldehyde functionalized cellulose and unfunctionalized cellulose is different as shown in Figure 11. The functionalized cellulose exhibits a strong autofluorescence with a peak around 560 nm. To avoid the large background signal and the cutoff at 515 nm, emission was measured at 530 nm for indication of fluorescent intensity in all experiments.

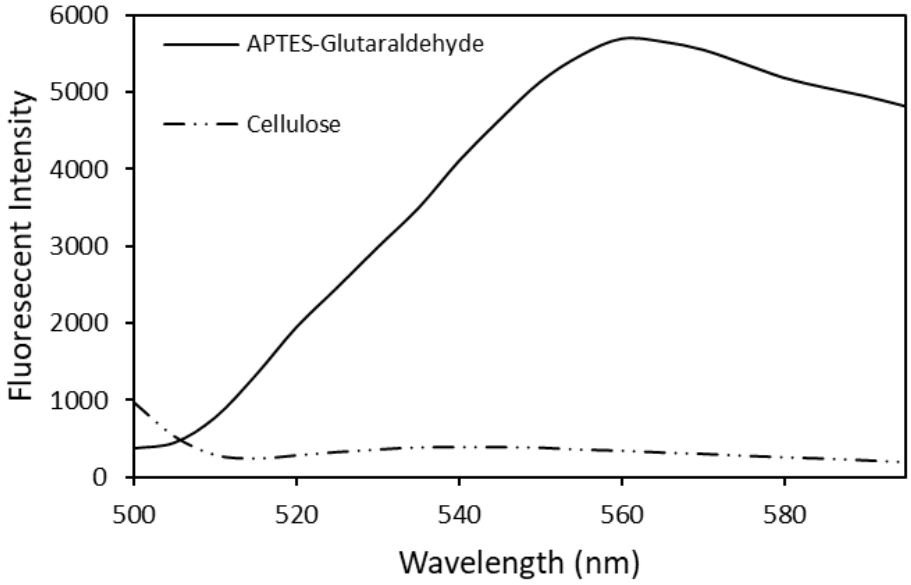

Figure 11. The background fluorescent signals between the APTES-Glutaraldehyde functionalized cellulose and unfunctionalized cellulose is shown for a variety of emission wavelengths at an excitation of 490 nm.

To explore the strength of interaction between the antibodies and the surfaces, both were washed with washing solution containing either 1 M or 0.1 M of NaCl. The fluorescence intensity was measured with an excitation at 490 nm and emission at 530 nm. The results of this experiment are shown in Figure 12.

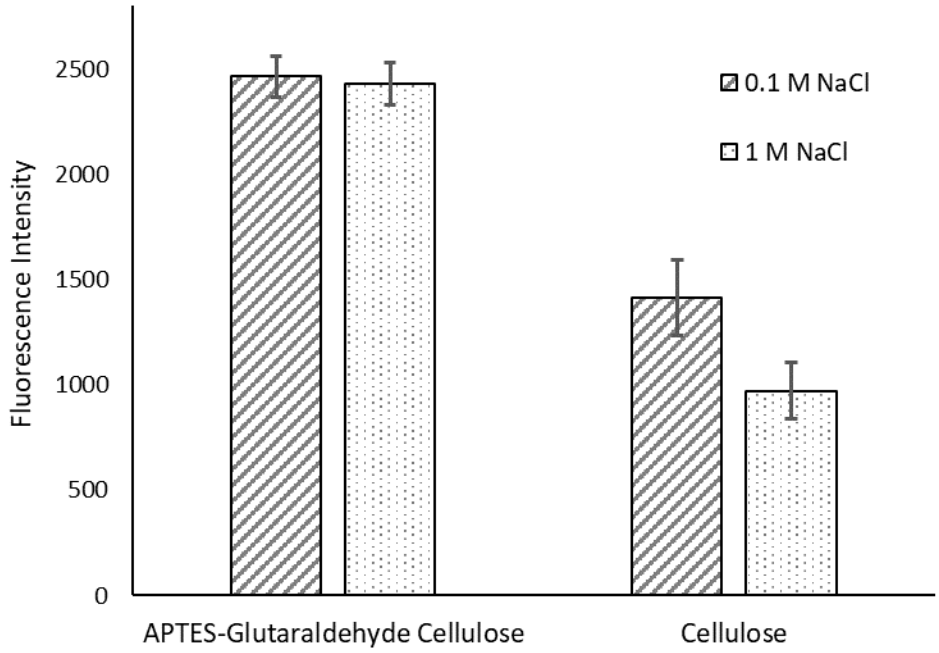

Figure 12. The strength of antibody and surface interaction for functionalized and unfunctionalized cellulose. The immobilized antibody was 100 μg/mL and washed with washing solution containing 0.1 M or 1 M NaCl. Each bar represents three replicates, and the error bars represent one standard deviation.

A one-tailed independent sample t-test suggests that antibodies immobilized onto the APTES-Glutaraldehyde surface did not experience a significant ($p < 0.05$) decrease in intensity between washes ($p = 0.42$). Antibodies immobilized onto the cellulose surface experienced a significant decrease in intensity between washed ($p = 0.0092$). This suggests that antibodies were more stably coupled to the surface functionalized with APTES and glutaraldehyde. This may enhance capacity and sensitivity of a paper-based immunoassay.

In a fluorescent immunoassay, the change in fluorescent intensity can be correlated to concentration of antibodies and therefore bound antigen through a standard curve. Therefore,

it is important to detect a change in intensity against the background signal. There is strong background signal on the APTES-glutaraldehyde functionalized surface in the range of 530-580 nm. To explore the ability to measure changes in fluorescence intensity and the impact of washing on that curve, varying concentrations of FITC-conjugated antibodies were immobilized on both functionalized and unfunctionalized cellulose with the results of these experiments are shown in Figures 13 and Figure 14 respectively. The change in slopes were compared with a one-tailed t-test to determine if the change was significant ($p < 0.05$).

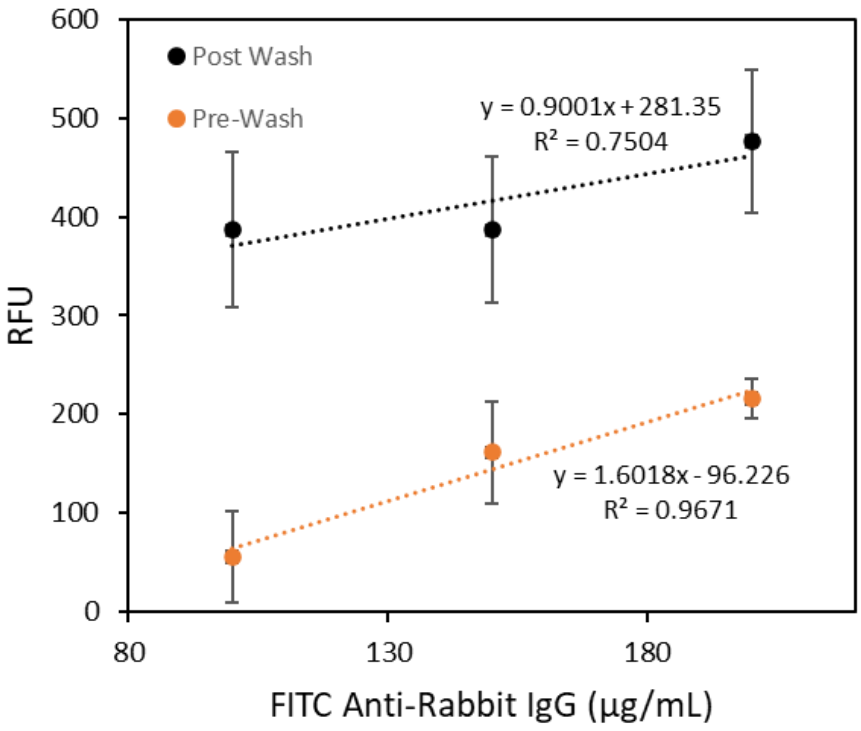

Figure 13. The change in fluorescent intensity is shown for changes in FITC conjugated antibodies immobilized on APTES-glutaraldehyde functionalized cellulose. Error bars represent one standard deviation for n = 3. The slope associated with increasing concentration of FITC conjugated antibodies is not statistically different following washing ($p = 0.37$).

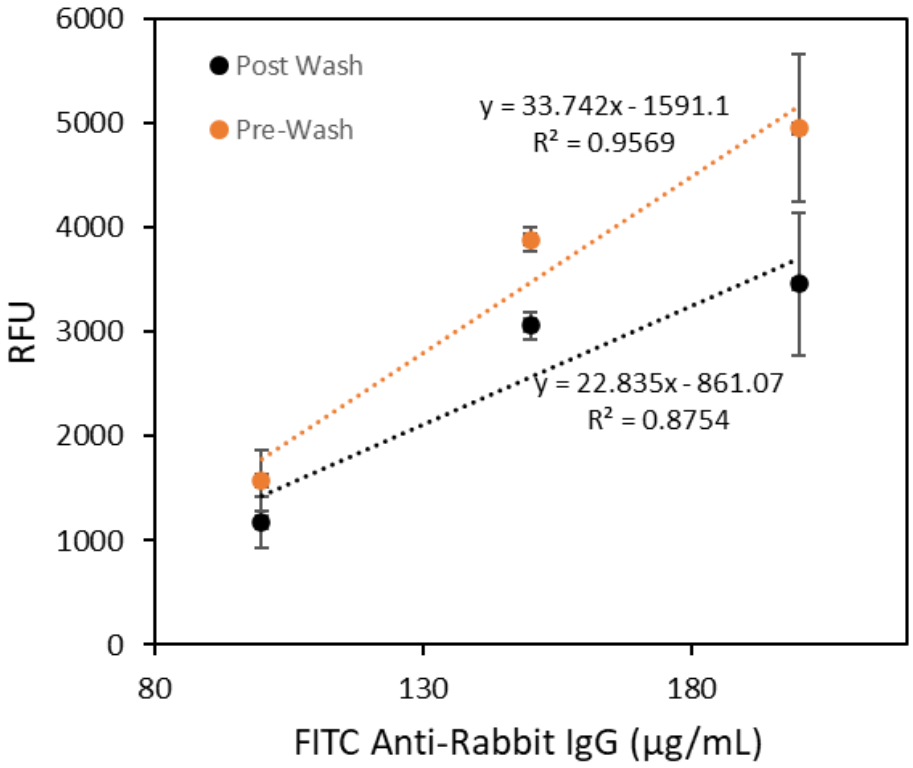

Figure 14. The change in fluorescent intensity is shown for changes in FITC conjugated antibodies immobilized on untreated cellulose. Error bars represent one standard deviation for n = 3. The slope associated with increasing concentration of FITC conjugated antibodies is statistically different following washing ($p < 0.0001$).

The slopes of a linear fit are shown and compared between antibodies not washed and antibodies washed with 120 µL PBS with Tween-20. The change in slope for fluorescent intensity of antibodies on the functionalized cellulose is not significant ($p = 0.37$). The change in slope for fluorescent intensity of antibodies on the non-functionalized cellulose is significant ($p < 0.0001$). This further establishes that the functionalized cellulose surface may enhance capacity and sensitivity of a paper-based immunoassay.

It is important to note the different scales for fluorescent intensity measurements on the functionalized and unfunctionalized cellulose surfaces. The functionalized surface yields a range in fluorescent intensity from 100 to 600, but for the same concentration of FITC-Anti on the unfunctionalized cellulose, the fluorescent intensity ranges from 1000 to 6000. The discrepancy in fluorescence intensity ranges between functionalized and unfunctionalized cellulose surfaces may be attributed to the presence of glutaraldehyde.

Glutaraldehyde has been documented to strongly interfere fluorescent emission through reaction with primary amines and aggregation of molecules.[13] Previous literature that has explored the emission and excitation of glutaraldehyde conjugated to biological films document a peak excitation at 540 nm and peak emission at 560 nm.[14] In this same study, the excitation and emission was not seen following reduction with borane-dimethylamine suggesting that Schiff bases interfere with the fluorescence.[14]

To explore fluorescent interference with anti-IgG FITC, fluorescent measurements were made in the presence and absence of either glutaraldehyde or APTES. To evaluate the effect of glutaraldehyde three samples were prepared: phosphate buffer (PB) pH 6, 100 g/mL anti-IgG FITC in 10 mM phosphate buffer pH 6, and 100 g/mL anti-IgG FITC in 10 mM phosphate buffer pH 6 and 1% (v/v) glutaraldehyde. Fluorescent intensity was measured with excitation at 490 nm and emission at 530 nm. Results are shown in Figure 15.

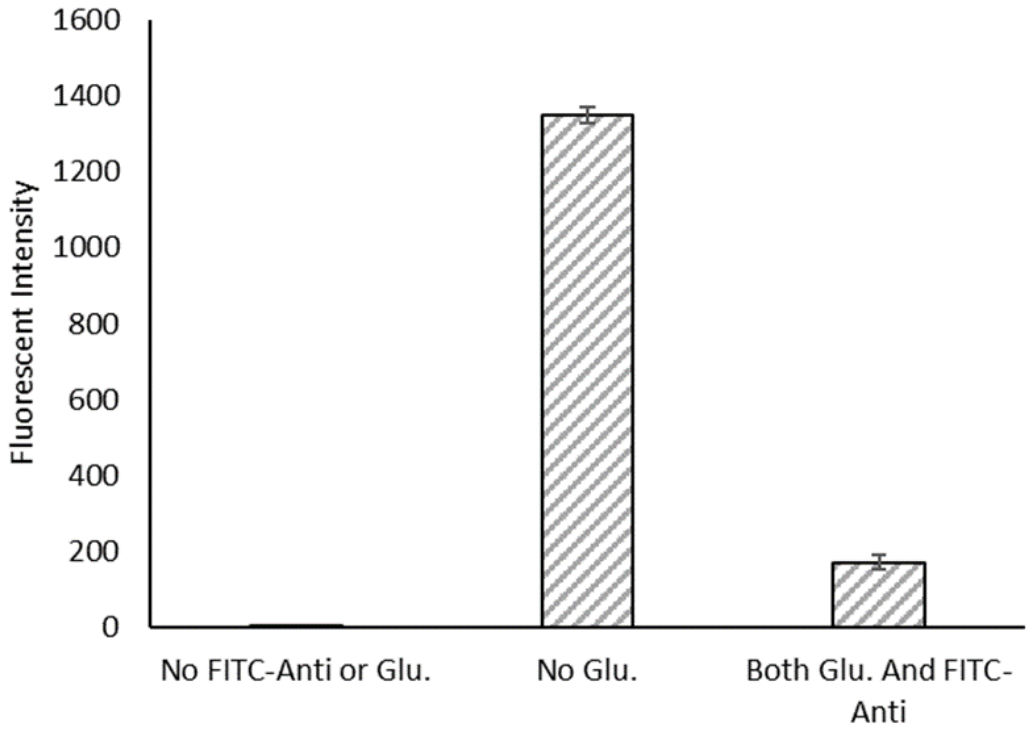

Figure 15. Fluorescent intensity of blank solution (10 mM phosphate buffer pH 6), solution with 100 µg/mL anti-IgG FITC, and solution with both 100 µg/mL anti-IgG FITC and 1% (v/v) glutaraldehyde. Error bars represent one standard deviation with n = 3.

There is a clear reduction in fluorescent intensity when comparing fluorescence without glutaraldehyde to fluorescence in the presence of glutaraldehyde. anti-IgG FITC in solution with glutaraldehyde exhibits a 6-fold decrease in fluorescence intensity. This may suggest that glutaraldehyde partially interferes with the fluorescent signal from anti-IgG FITC. Howeer, it is important to note that the interference does not completely eliminate fluorescent signal.

Indirect Immunoassay

The application of this functionalized surface was demonstrated with an indirect immunoassay for Rabbit IgG. Previous work has demonstrated this assay on μPADs with both colorimetric and fluorescence detection.[7,15] The data is fit with a 5-parameter logistic regression similar to the prior literature. The resulting curve is shown in Figure 16.

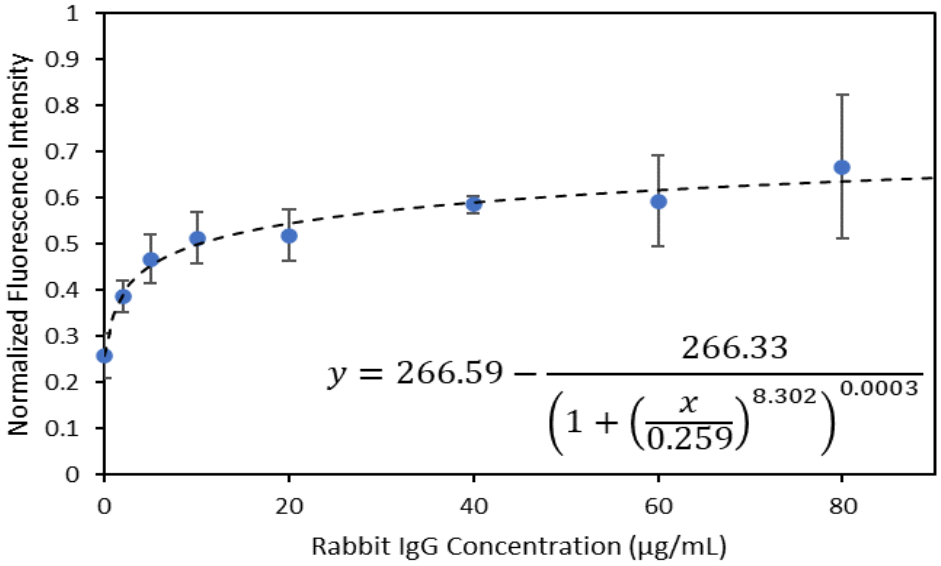

Figure 16. Indirect immunoassay of Rabbit IgG on an APTES-glutaraldehyde functionalized cellulose surface. Error bars represent one standard deviation of n = 4. The data fits a 5-parameter logistic regression.

The same indirect immunoassay was performed on un-functionalized cellulose for comparison with the functionalized surface. The resulting curve is shown in Figure 17.

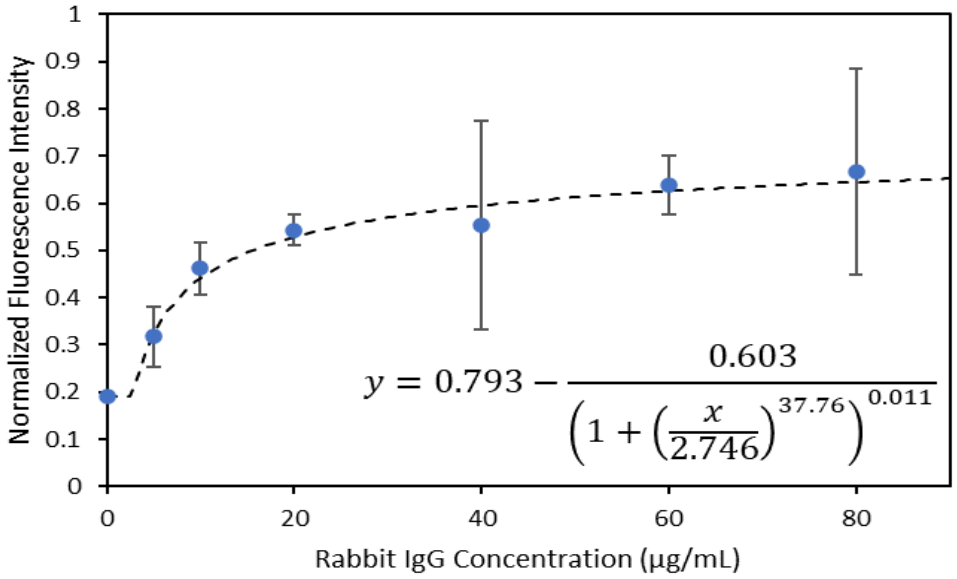

Figure 17. Indirect immunoassay of Rabbit IgG on an un-functionalized cellulose surface. Error bars represent one standard deviation of n =3. The data fits with a 5-parameter logistic regression. Measurements at 2 g/mL Rabbit IgG were removed as outliers.

The linear region (0 – 10 g/mL Rabbit IgG) was plotted and fit with a linear regression to compare the sensitivity of the immunoassay on each surface.[8] These results are shown in Figure 18 for functionalized and unfunctionalized cellulose respectively.

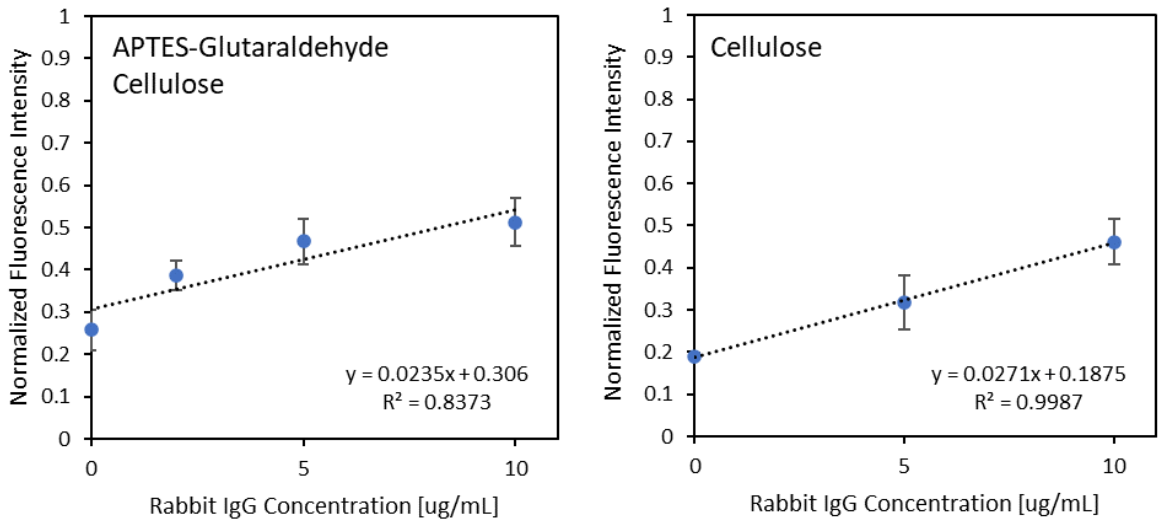

Figure 18. Linear region of indirect immunoassay for Rabbit IgG on APTES-Glutaraldehyde functionalized cellulose and unfunctionalized cellulose. Error bars represent one standard deviation with n = 4 for the functionalized cellulose and n = 3 for the unfunctionalized cellulose.

The slope of the linear fit which correlates the change in fluorescent intensity with a difference in concentration of Rabbit IgG indicates the sensitivity of the immunoassay. The unfunctionalized cellulose has a higher slope suggested higher sensitivity; however, a one-tailed independent sample t-test comparing the slopes indicates that the slopes are not statistically different ($p < 0.05$) and that the functionalized surface has similar sensitivity ($p = 0.493$).

The functionalized surface was predicted to have higher sensitivity due to the formation of a covalent linkage between the antibodies and surface, but it resulted in comparable sensitivity to the unfunctionalized surface. This may be attributed to glutaraldehyde and the Schiff Base greatly reducing the fluorescent intensity of FITC in the immunoassay therefore reducing the ability to distinguish between different concentrations of Rabbit IgG.

Further Directions

There are various avenues for future work with this research. The logical next step includes applying this platform for the detection of antigens through antibody or aptamer pairs for sandwich immunoassays.

Additionally, primary amine groups are present on solvent accessible lysine residues throughout proteins, therefore using this target to covalently immobilize antibodies may result in improper orientation. This would impact binding of the antigen. This can be addressed using heterobifunctional linkers such as (1-ethyl-3-[3(dimethylamino)propyl]-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) / N-hydroxysulfosuccinimide (SNHS) as opposed to glutaraldehyde.[9] EDC and NHS/SNHS can be used to first activate the carboxyl group on a polypeptide then react it with a primary amine such as on APTES.[16] This allows for more targeted orientation of the antibodies because carboxyl groups are located on the fragment crystallizable region of the antibody away from the antigen binding site.[16] Regardless, other papers have demonstrated success with immobilizing antibodies and performing immunoassays with homobifunctional linkers.[3,6]

As outlined previously, the fluorescent interference observed using glutaraldehyde to form a Schiff Base poses an issue to improved sensitivity. This may be addressed through reducing the Schiff Base to an amine using a reducing agent such as sodium borohydride as documented in literature.[9,17] This may also improve stability of the covalent bond between the

linker molecule and the antibody. The fluorescent interference can also be addressed by selecting a fluorescent probe with different excitation and emission spectra to FITC.

It is important to note the utility of other technology to immobilize biomolecules onto a surface as this technology may also be used to immobilize antibodies and improve the sensitivity of immunoassays. Genetic Code Expansion (GCE) allows for engineering of proteins through incorporating non-canonical amino acids (ncAAs) with unique functionality at specific sites within the peptide chain.[18] The ncAA 4-(6-ethyl-s-tetrazine-3yl)phe (Tet2.0) has been demonstrated in bioorthogonal reactions with strained trans-cyclooctane (sTCO) to immobilize proteins to a surface.[19,20] Through site-specific incorporation of the amino acid into an antibody, controlled orientation of immobilization can be achieved and combat the risk of improper orientation. This is hypothesized to improve sensitivity of the immunoassay.

Conclusion

Improved immobilization of antibodies onto a cellulose surface was demonstrated through surface functionalization using APTES and glutaraldehyde. The functionalized surface was characterized using FTIR and various washing procedures. FTIR data suggests that temperature plays an important role in functionalization of APTES to the cellulose surface. The immobilization of APTES-glutaraldehyde on the cellulose surface could be clearly observed through red color development indicative of a Schiff Base, especially with functionalization of APTES at 100 °C. Additionally, the application of this novel immobilization technique was demonstrated using FITC-conjugated antibodies. Antibodies immobilized onto the functionalized cellulose persisted through washing with salt solutions to disrupt non-covalent interactions whereas those on unfunctionalized cellulose were washed away. Additionally, FITC-conjugated antibodies on the functionalized surface maintained a correlation between intensity and concentration of antibodies while those on the unfunctionalized cellulose experienced a decrease in their standard curve. Preliminary results in immunoassays demonstrated with an indirect immunoassay for Rabbit IgG suggest comparable sensitivity to unfunctionalized paper. Fluorescent interference due to glutaraldehyde reduced expected fluorescent intensity which impacted the sensitivity of the immunoassay with FITC. This may be addressed through reduction of the Schiff Base to reduce interference or selecting a different fluorophore with different excitation and emission profiles. Demonstration with a sandwich immunoassay and comparison with other covalent immobilization techniques have yet to be explored but are natural next steps for this work.

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