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# Functional Comparison of Bioactive Cellulose Materials Incorporating Engineered Binding Proteins

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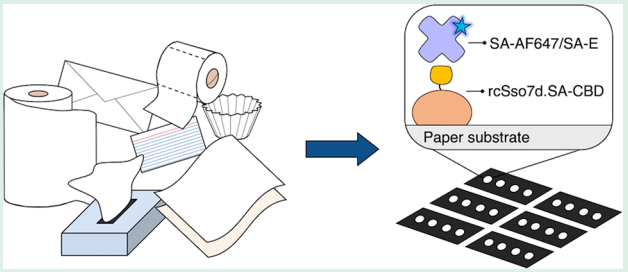
**ABSTRACT:** Whatman No. 1 chromatography paper is widely used as a substrate for cellulose-based immunoassays. The immobilized proteins are used to capture target biomarkers for detection. However, alternative paper substrates may facilitate mass production of immunoassays as diagnostic tests. Here, we assessed the physical characteristics and protein immobilization capabilities of different commercial papers. Some substrates fulfilled our design criteria, including adequate flow rate and sufficient protein immobilization for efficient target capture. This study demonstrates that a variety of paper substrates can be bioactivated and used to capture target biomarkers, enabling development of affordable diagnostic tests from a range of starting materials.




**KEYWORDS:** *cellulose, paper diagnostics, protein immobilization, immunoassay, biosensor*



Point-of-care (POC) diagnostic tests are used for healthcare, food safety, and environmental monitoring applications, particularly in resource-constrained settings. Unlike standard laboratory tests, POC diagnostics must be inexpensive and simple to perform, making them suitable for low-income communities that might lack skilled personnel or adequate infrastructure to operate more complex laboratory tests.[1] To aid in the development of POC diagnostics, the World Health Organization (WHO) established the ASSURED criteria, which states that appropriate on-site diagnostic tests should be (i) affordable, (ii) sensitive, (iii) specific, (iv) user-friendly, (v) rapid and robust, (vi) equipment-free, and (vii) deliverable to those who need them.[2] Novel technologies, such as microfluidic devices, lab-on-a-chip, and immunoassays,[1] aspire to meet these guidelines and bridge the diagnostic gaps[2] that persist in challenging environments through the use of appropriate materials.

Glass, silicon, polymer, and paper are examples of common materials that have been used as substrates in POC devices.[3] In particular, paper is a prime candidate for POC applications due to its inherent physical and chemical properties.[4] Generally comprised of cellulose, Earth's most abundant biopolymer,[5] paper is biodegradable, porous, lightweight, and disposable.[3] Hydrophilic in nature, paper can be patterned with hydrophobic boundaries to define flow paths using several techniques, including photolithography, inkjet printing, polydimethylsiloxane (PDMS) plotting, laser cutting, wax printing, and plasma treatment.[6] Moreover, paper is compatible with various biological samples[7] and can be chemically modified to incorporate desired functional groups.[5]

Numerous studies have proposed ways to enhance paper-based diagnostic tests, including novel fabrication techniques,[8−10] updated detection methods,[9,11] and more stable reagents.[12−14] However, studies have yet to investigate the effect of different types of paper on the binding performance of POC diagnostic assays. Currently, the vast majority of paper-based tests for POC applications use Whatman No. 1 chromatography paper as the substrate.[8,11−13,15−24] This paper is manufactured from high-quality cotton linters and is treated to achieve a high alpha cellulose content (>98%), which is the most stable form of cellulose.[5] Despite its valuable intrinsic properties, Whatman No. 1 is relatively costly and may be difficult to manufacture on the scale needed for the POC diagnostics market. Therefore, an inexpensive and mass-producible paper substrate that yields a detectable binding signal is desired to develop low-cost diagnostic tests.

Many diagnostic test formats depend on the effective immobilization of a binding protein onto the test substrate to capture target biomarkers from patient samples. In recent years, several immobilization approaches have been developed for paper substrates; however, most methods require the chemical activation of the paper, which involves the use of hazardous organic compounds and results in reduced ligand activity.[22,25] Hence, studies have investigated alternative methods of immobilizing proteins to cellulose surfaces. For instance, a method to immobilize peptides on cellulose using a

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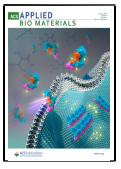

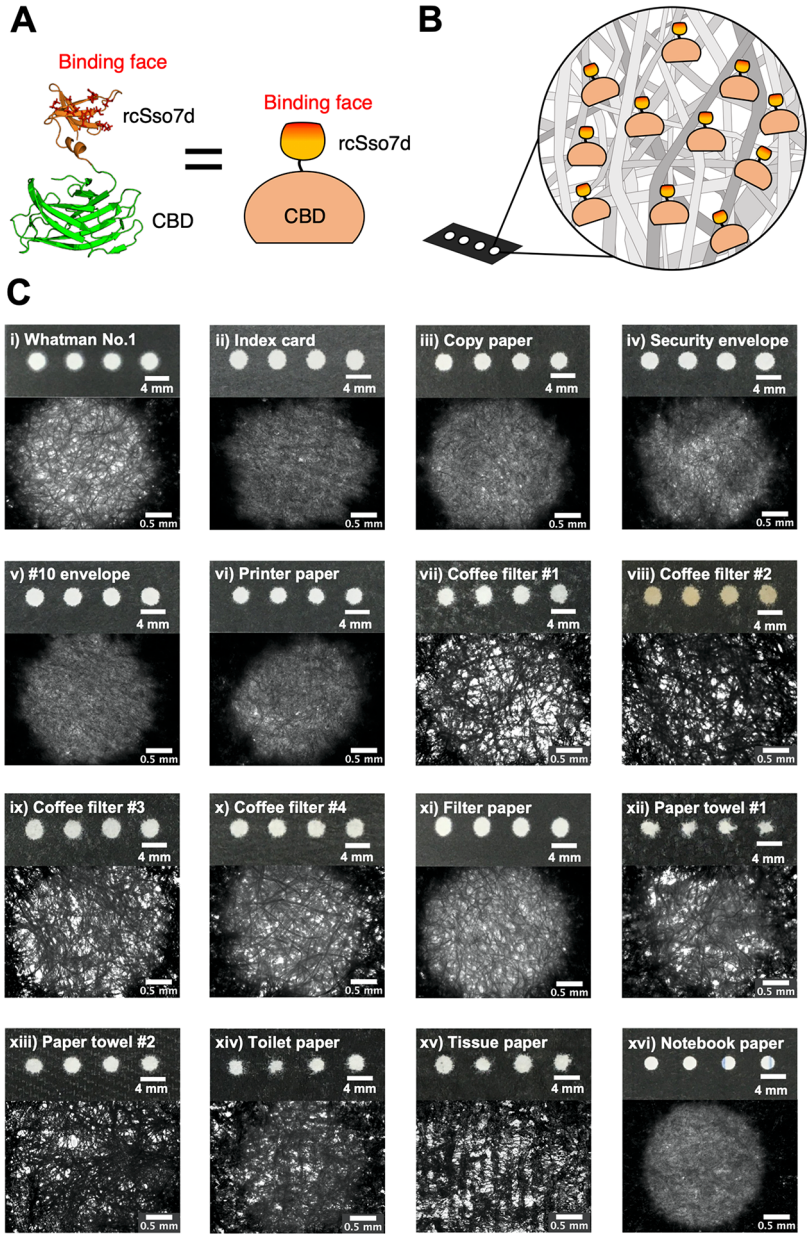


**Figure 1.** (A) Schematic and cartoon representations of the rcSso7d-CBD protein construct, highlighting the amino acids on the binding face (red). (B) Schematic representation of a paper assay strip, depicting the rcSso7d-CBD protein binding to the cellulose strands. (C) Morphology and transmitted light microscope images of the paper test zones: (i) Whatman No.1 chromatography paper; (ii) Staples index card; (iii) Staples 30% recycled copy paper; (iv) Mead security envelope; (v) Staples #10 envelope; (vi) Staples printer paper; (vii) Mr. Coffee coffee filter white (30 ms); (viii) Melitta coffee filter unbleached (30 ms); (ix) BUNN coffee filter white (50 ms); (x) Chemex bonded coffee filter square (100 ms); (xi) Great Lakes filter paper; (xii) Bounty paper towel (90 ms); (xiii) Georgia-Pacific enMotion rolled paper towel (20 ms); (xiv) Georgia-Pacific compact coreless toilet paper (50 ms); (xv) Kleenex tissue paper (15 ms); (xvi) Mead spiral notebook paper (100 ms). All samples were imaged with transmitted light illumination, 4× magnification, and an exposure time of 200 ms unless otherwise noted. Approximate paper test zone areas can be observed in Table 1.

chemical ligation reaction has been described.[26] Moreover, affinity tags like cellulose-binding domains (CBDs), also known as carbohydrate-binding modules (CBMs),[27] can be genetically fused to the capture protein, providing an inexpensive way to immobilize proteins to the cellulose substrate.[20,21,25] Thus far, the application of CBD fusion proteins for diagnostic tests has primarily been limited to Whatman No. 1.[20,21]

Here, we investigated the use of alternative paper substrates in diagnostic tests to surface-immobilize a binding protein and relate a detectable signal to a captured target molecule. We selected a variety of commercially available papers with diverse attributes to assess their performance compared to that of Whatman No. 1. For bioactivation of the substrates, we used an antibody alternative, termed rcSso7d (reduced-charge Sso7d) that had been engineered to bind to streptavidin and that had been genetically fused to CBD.[13,20] We investigated the ability of CBD to bind to the different paper substrates while maintaining the binding activity of the rcSso7d, and we observed that some of the other papers can produce similar specific binding signals compared with Whatman No. 1. We found that some of the tested papers are not ideal for use in


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ACS Appl. Bio Mater. 2021, 4, 392−398

diagnostic devices due to issues such as sluggish flow through the paper, inconsistency of the patterned test zones, and unacceptably high levels of nonspecific binding signals. We demonstrated that the pore size of the papers affects flow rate, as well as protein surface immobilization, and that together both impact binding signal. Collectively, these results can be used to identify alternative paper substrates for use in POC diagnostics.

In this study, we used an engineered binding protein to transform cellulose into a bioactive material. The rcSso7d scaffold has an isolated and well-defined binding face (Figure 1A, red). Mutations can be readily introduced to the rcSso7d binding face to create different variants that specifically bind a diverse range of target molecules.[12,13] In this case, we used a genetic fusion (*rcSso7d.SA-CBD*) to produce a fusion protein consisting of a rcSso7d variant that binds to streptavidin (rcSso7d.SA) and a type 3a cellulose-binding domain (CBD) (Figure 1A; Supporting Information). The CBD, fused to the rcSso7d.SA variant with a Gly-Ser linker,[28] was chosen for its high expression yields in *Echerichia coli*, chemical and thermal stability, and high affinity binding to cellulose.[20] This CBD species immobilized the target-binding rcSso7d to the cellulose fibers, which allowed for a near-complete capture of the target protein from solution, as shown in previous work,[20] without requiring chemical activation of the paper[13] (Figure 1B).

Paper substrates were chosen based on their distinctive characteristics, availability, reduced cost (Table S1), and lack of previously reported data. We selected several commercial office papers (index card, copy paper, security envelope, #10 envelope, printer paper, notebook paper), coffee filters, absorbent papers (paper towels, toilet paper, tissue paper), and an alternative filter paper with similar properties to those of Whatman No. 1. To gain insight into the functionality of these substrates, we printed test zone arrays on each paper using hydrophobic ink to create circular hydrophilic test zones. Figure 1C shows the morphology of the test zones on the different papers and depicts transmitted light microscope images to visualize the cellulose fibers. Some test zones, including those in the paper towels, toilet paper, and tissue paper, had an inconsistent size and were not circular in shape. Because of the differences in the cellulose strands, light transmission varied from paper to paper, making it necessary to tune the exposure time of the microscope depending on the type of paper. The density of the cellulose fibers from the microscope images indicated that the paper towels, toilet paper, tissue paper, and coffee filters had noticeably larger pore sizes than Whatman No. 1. In contrast, the pore sizes of the index card, copy paper, security envelope, #10 envelope, and printer paper were smaller than those in Whatman No. 1.

Adequate flow-through in paper-based assays is an essential requirement in order to obtain test results in a timely fashion, as outlined in the ASSURED criteria. To assess the extent to which samples could flow through each paper, we conducted a preliminary test by applying 20 $\mu$L of water to each test zone and measuring the time required for the liquid to wick through the paper (Table 1). The paper towels, toilet paper, tissue paper, and coffee filters had extremely rapid flow-through, while the office papers had much slower flow through the test zones. The Great Lakes filter paper had similar flow-through time as Whatman No. 1. The flow-through times correlated to the pore sizes observed in the microscope images in Figure 1C, with faster flow-through for larger pores and slower flow-through for smaller pores. The Mead spiral notebook paper was excluded from all studies due to extremely slow flow-through (>10 min), which made this paper substrate infeasible for diagnostic test purposes.

**Table 1. Time for Liquid to Flow Through a Test Zone of Each Paper Substrate**

| paper substrate | shorthand name | approx test zone area[a] | approx flow-through time |
|---|---|---|---|
| Whatman No. 1 chromatography paper | Whatman No. 1 | 6.2 ± 0.4 $mm^2$ | <5 s |
| Staples index card | index card | 6.7 ± 0.4 $mm^2$ | 3 min |
| Staples 30% recycled copy paper | copy paper | 5.8 ± 0.5 $mm^2$ | 2 min |
| Mead security envelope | security envelope | 6.8 ± 0.4 $mm^2$ | 6 min |
| Staples #10 envelope | #10 envelope | 6.9 ± 0.3 $mm^2$ | 6 min |
| Staples printer paper | printer paper | 5.8 ± 0.4 $mm^2$ | 7 min |
| Mr. Coffee coffee filter white | coffee filter #1 | 6.0 ± 0.7 $mm^2$ | <1 s |
| Melitta coffee filter unbleached | coffee filter #2 | 6.2 ± 0.5 $mm^2$ | <1 s |
| BUNN coffee filter white | coffee filter #3 | 6.9 ± 0.4 $mm^2$ | <1 s |
| Chemex bonded coffee filter square | coffee filter #4 | 6.6 ± 0.4 $mm^2$ | <1 s |
| Great Lakes filter paper | filter paper | 5.7 ± 0.4 $mm^2$ | <5 s |
| Bounty paper towel | paper towel #1 | 4.0 ± 0.9 $mm^2$ | <1 s |
| Georgia-Pacific enMotion rolled paper towel | paper towel #2 | 4.1 ± 0.8 $mm^2$ | <1 s |
| Georgia-Pacific compact coreless toilet paper | toilet paper | 4.0 ± 0.9 $mm^2$ | <1 s |
| Kleenex tissue paper | tissue paper | 5.0 ± 1.1 $mm^2$ | <1 s |
| Mead spiral notebook paper | notebook paper | 3.9 ± 0.2 $mm^2$ | >10 min |

[a]The approximate test zone areas of the paper towels, toilet paper, tissue paper, and notebook paper were smaller due to the faster melting rate of the hydrophobic ink when constructing the wells.

To further explore the flow rate through the different papers, food coloring was used to visualize flow through 1 cm wide paper strips. The paper towels, toilet paper, and tissue paper were not included in the test, as they were immediately soaked when placed in contact with the liquid. Figure 2 shows the varying fluid fronts for each type of paper at several time points (30 s, 1 min, 1 min, and 30 s). Some office papers (ii, v, vi) and coffee filters (viii, ix, x) had an uneven and irregular flow, whereas Whatman No. 1 (i), Mr. Coffee coffee filter white (vii), and the Great Lakes filter paper (xi) presented a uniform flow. To some extent, the flow of the copy paper (iii) and security envelope (iv) was also uniform, but some protrusions in fluid fronts were observed. The flow irregularities observed in some papers may be the result of a heterogeneous cellulose fiber network. For instance, papers ix and x, which displayed considerable protrusions, have both areas of higher fiber density and areas of low fiber density that allow for greater light transmission (Figure 1). The office papers exhibited a decreased flow rate compared to Whatman No. 1, which corroborates that the reduced pore size of the office papers can hinder proper liquid flow-through. On the other hand, the Great Lakes filter paper and most of the coffee filters showed higher flow rates than Whatman No. 1, confirming that the larger pore size allows faster fluid flow through the paper.


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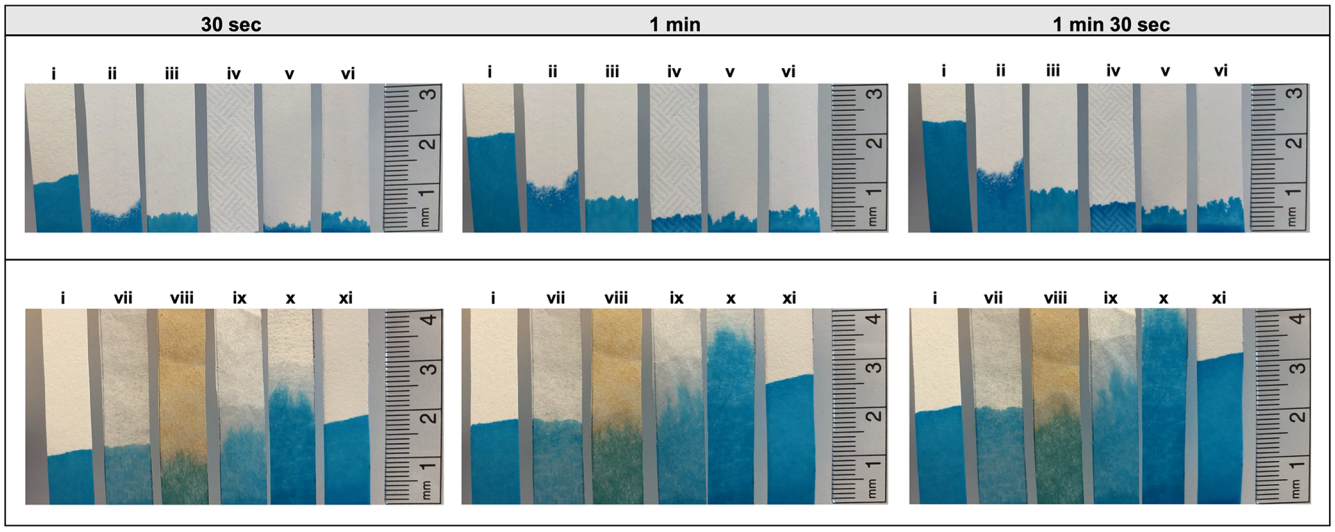


**Figure 2.** Flow profile of the different paper substrates at various time points, imaged after 30 s, 1 min, and 1 min 30 s. (i) Whatman No.1 chromatography paper. (ii) Staples index card. (iii) Staples 30% recycled copy paper. (iv) Mead security envelope. (v) Staples #10 envelope. (vi) Staples printer paper. (vii) Mr. Coffee coffee filter white. (viii) Melitta coffee filter unbleached. (ix) BUNN coffee filter white. (x) Chemex bonded coffee filter square. (xi) Great Lakes filter paper. All paper strips were 1 cm wide.

To test the target-binding activity of the different paper substrates, we used an engineered fusion protein, rcSso7d.SA-CBD, together with a fluorescently labeled streptavidin (SA) conjugate as a model protein analyte. The engineered fusion protein rcSso7d.SA-CBD has a cellulose-binding domain that anchors the protein to the paper and an analyte-binding domain, rcSso7d, which has been engineered to bind streptavidin with picomolar binding affinity.[13] This system allows for the assessment of the binding performance of CBD to the different types of paper and also the binding activity of rcSso7d to streptavidin once the bioactive paper has been prepared.[13,20] The paper substrates were treated with 30 $\mu$M of rcSso7d.SA-CBD to create a high density of binding sites, as determined in previous work.[20] To test binding activity, a 500 nM solution of streptavidin Alexa Fluor 647 (SA-AF647) was allowed to wick through the paper. To compare the amounts of target captured, fluorescence microscopy was used to measure the mean fluorescence intensity (MFI) of each paper, which is proportional to the amount of target bound. The paper towels, toilet paper, and tissue paper were excluded from this and subsequent studies, as their delicate structure and multilayers impeded their use as a diagnostic test. As shown in Figure 3A, the office papers and the Great Lakes filter paper resulted in similar target-binding signals to Whatman No. 1 with a relatively low background, indicating that the binding performance of the rcSso7d and CBD was maintained using these papers. On the other hand, the coffee filters yielded lower target-binding signals compared to Whatman No. 1.

We next sought to conduct paper assays using eosin-conjugated streptavidin (SA-E) instead of SA-AF647 as the binding reagent. Eosin, which can be used as a label for colorimetric polymerization-based signal amplification,[11] a visual technique in paper-based sensing, can also be employed to associate a fluorescence signal proportional to the amount of captured streptavidin. As depicted in Figure 3B, the office papers and most coffee filters had high levels of background signal (gray) in the absence of rcSso7d.SA-CBD, suggesting high levels of nonspecific interactions of SA-E to the paper substrates. This can be attributed to eosin's poor aqueous solubility, which increases nonspecific adsorption to the surface of the paper.[29] Whatman No. 1 had the lowest background signal compared to positive binding signal; however, the Great Lakes filter paper also had a relatively low background signal

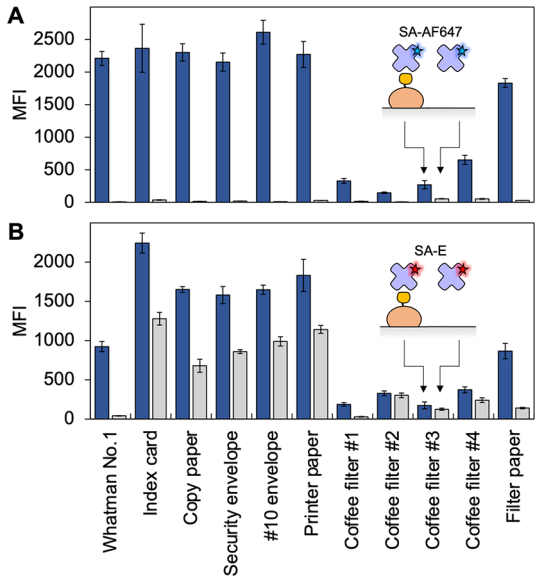


**Figure 3.** Assessment of the target binding performance of the different paper substrates using rcSso7d.SA-CBD with labeled streptavidin to assess specific binding (blue) or with labeled streptavidin alone to assess nonspecific binding (gray). (A) Using SA-AF647. The samples were imaged in the Cy5 channel, with 4× magnification and an exposure time of 80 ms. (B) Using SA-E. The samples were imaged in the Texas Red channel with 4× magnification and an exposure time of 250 ms. Four replicates per sample were analyzed, and the MFI was averaged across all replicates. Error bars represent the standard deviation of the means of four independent replicates. Coffee filter #1, Mr. Coffee coffee filter white; coffee filter #2, Melitta coffee filter unbleached; coffee filter #3, BUNN coffee filter white; coffee filter #4, Chemex bonded coffee filter square; Filter paper, Great Lakes filter paper. Fluorescence microscopy images can be observed in Supporting Information, Figures S1 and S2.


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ACS Appl. Bio Mater. 2021, 4, 392−398

relative to its positive signal. When comparing the background-subtracted signal of the papers with SA-AF647 and SA-E (Supporting Information, Figure S3), the trends are similar, suggesting that the specific binding signal is consistent. Nevertheless, the higher nonspecific binding signal may increase the possibility of obtaining false positives.

Having generated detectable signals in the different types of paper, we then hypothesized that the larger pore size of the coffee filters may have led to a reduced binding signal from insufficient immobilization of rcSso7d.SA-CBD. To determine whether the pore size of the papers affected the binding signal, we quantified the amount of immobilized protein on the paper test zones. Known concentrations of the rcSso7d.SA-CBD binding scaffold were dried onto the test zones of each type of paper to create a standard curve. Experimental samples with different concentrations of rcSso7d.SA-CBD were applied to the test zones, incubated, and washed. The test zones were cut out, and the amount of protein on each test zone was quantified using a micro BCA assay (Supporting Information, Figure S4). While fluorescence microscopy was used to detect SA-AF647 and SA-E, a micro BCA assay can detect immobilized rcSso7d.SA-CBD. Coffee filter #2 reacted with the micro BCA assay reagents even without added protein, so protein immobilization on this paper could not be quantified. As shown in Figure 4, at a reference concentration of 32 $\mu$M, the coffee filters, which had the larger pores, had a lower average amount of protein immobilized onto the test zones. Concurrently, the highest immobilization concentrations were found on papers with smaller pores, including copy paper and #10 envelope. When calculating the amount of immobilized protein per unit area, the Great Lakes filter paper and copy paper displayed the highest efficiency (Supporting Information, Table S2). These findings suggest that the larger pore size not only impacts fluid flow through the paper but also protein immobilization, which may have resulted in the reduced binding signal.

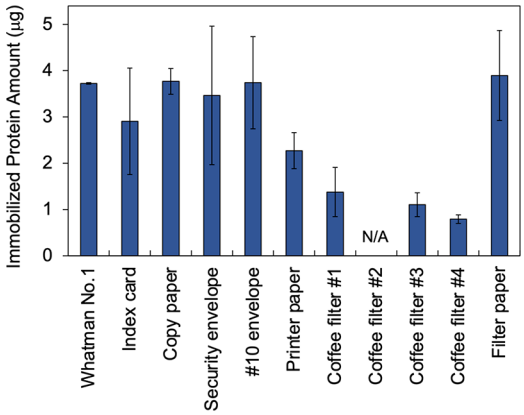


**Figure 4.** Amount of rcSso7d.SA-CBD protein surface-immobilized on each test zone for the different paper substrates. 32 $\mu$M of rcSso7d.SA-CBD was applied to each test zone. Immobilized protein amounts were measured using a micro BCA assay, and absorbance at 562 nm was quantified for each sample. Absorbance values for the experimental samples were correlated against standard curves using a known mass of rcSso7d.SA-CBD evaporated on the test zones. Three replicates were made for each sample, and the protein amount was averaged across all replicates. Error bars represent the standard deviation of the three independent replicates. Results for coffee filter #2 are not available due to interference of the paper substrate to the micro BCA assay reagents. Coffee filter #1: Mr. Coffee coffee filter white; coffee filter #2: Melitta coffee filter unbleached; coffee filter #3: BUNN coffee filter white; coffee filter #4, Chemex bonded coffee filter square; filter paper, Great Lakes filter paper.

In summary, we evaluated the use of alternative paper substrates in diagnostic tests to immobilize a fusion protein and obtain a detectable binding signal. Thus far, Whatman No. 1 chromatography paper has been the most utilized substrate in paper-based diagnostics; however, its cost and difficulty to mass manufacture make it less suitable for resource-limited settings. In this study, we found that the density of the cellulose fibers not only impacts liquid flow-through but also affects adequate protein immobilization, which is essential for rapidly capturing targets. In addition, the feasibility of using alternate paper substrates for diagnostics is contingent on the properties of each paper. Taking into account their morphology, flow profile, and observed binding signal, some papers were deemed nonideal for use in a paper-based assay format. On the contrary, other paper substrates displayed a similar performance to Whatman No. 1, enabling the capture of the target molecule through effective protein surface immobilization. These results are a step toward more accessible diagnostics, enabling those with access to commercial papers and simple equipment to develop their own cost-effective diagnostic tests.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.0c01474.

Detailed materials and methods; supplemental data referenced throughout text (PDF)

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

rcSso7d, reduced-charged Sso7d
rcSso7d.SA, rcSso7d clone engineered to bind to streptavidin
CBD, cellulose-binding domain
BG, background
MFI, mean fluorescence intensity
SA-AF647, streptavidin Alexa Fluor 647
SA-E, streptavidin eosin

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