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Development of a Paper-Based Diagnostic for Influenza Detection

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Abstract— **The development of novel paper-based diagnostic tests has surged in recent years, due to the suitability of these tests for use at the point of care. These emerging paper-based tests retain the low cost and ease of use of traditional lateral flow tests, while offering increased sophistication and capabilities that approach those of traditional microfluidic devices. Here, we report on the development of a novel paper-based test for the diagnosis of influenza, commonly known as the flu. Influenza is a ubiquitously occurring infection, affecting 5-20% of Americans and resulting in an average of 23,000 deaths in the U.S., and up to 500,000 deaths globally, each year. Despite its prevalence, the diagnosis of influenza remains unsatisfactory, especially at the point of care. In particular, lateral flow tests for influenza suffer from poor sensitivity and provide only limited information about the infecting flu virus. Point-of-care testing of influenza therefore stands to benefit substantially from improved technology. To this end, we have developed two different versions of a paper-based flu assay, both using computationally designed affinity proteins, or "binders," that bind to the influenza hemagglutinin (HA) protein. One version of the assay utilizes an HA stem-region binder and the other an HA head-region binder. With these assays, we demonstrate the detection of clinically relevant concentrations of recombinant HA and intact influenza virus, as well as the translation of this paper-based system to a two-dimensional paper network (2DPN) folding card device.**

I. Introduction

Every year, influenza (flu) virus infects 5-20% of Americans, accounting for 15 million to 60 million infections [1]. These influenza infections lead to over 200,000 hospitalizations [1] and an average of 23,000 deaths [2] in the U.S. each year. This makes the flu more deadly in the U.S. than HIV and cervical cancer combined [3]. While the flu is especially problematic for young children, pregnant women, the elderly, and people with other health conditions, the flu can also be a severe respiratory disease for otherwise healthy adults. In fact, during the most recent (2013-2014) flu season, adults (18-64 years old) have accounted for the majority of flu-related hospitalizations and deaths [4]. In addition to being a significant health problem, the flu is also a huge financial burden. A detailed study on the economic burden of flu by Molinari *et al.* found that influenza costs the U.S. economy $87B annually, with $10B in direct medical costs, $16B in lost productivity, and $61B in lost economic value due to early death [5].

Despite these grim numbers, the flu is treatable. Antiviral medications such as Tamiflu® are available for flu treatment and reduce the severity of symptoms, shorten the duration of illness, and decrease the risk complications—but only when prescribed early in the course of infection, specifically within the first 48 hours of symptoms [1], [6]. Timely diagnosis of influenza is therefore key for successful disease management.

Given the importance of influenza diagnosis, several lateral flow-based rapid diagnostic tests (RDTs) have been developed for point-of-care (POC) detection of influenza. It has been shown that the correct use of these flu RDTs significantly reduces cost of treatment, time of hospitalization, and erroneous use of antibiotics *versus* antivirals [7]. Despite these benefits, current flu RDTs still suffer from low sensitivity, detecting flu infections only 10-71% of the time [8], resulting in mistreatment, expensive follow-up testing, and often the inability to treat with antiviral medication within the critical 48-hour treatment window. Additionally, all currently available flu RDTs are based upon detection of the internal nucleoprotein and cannot subtype influenza, limiting the resulting diagnostic and epidemiological information. Current flu diagnostic tests are therefore unable to achieve effective early diagnosis for most flu-infected individuals. The key barrier to effective flu diagnosis and disease management is access to accurate, rapid flu testing.

We therefore seek to develop a highly sensitive paper-based assay for POC diagnosis and subtyping of influenza based on the envelope protein hemagglutinin (HA). To do so, we have utilized a combination of our novel two-dimensional paper network (2DPN) platform and novel computationally designed affinity proteins that bind specifically to influenza HA.

We have chosen the paper-based 2DPN platform for this influenza assay due to its ability to perform complex diagnostic testing in a simple, low-cost device. The 2DPN, as reported by others in our laboratory [9]–[13], combines the simplicity of traditional lateral flow tests with the sophistication of microfluidics-based tests to achieve an intermediate format that is highly suitable for POC use. In

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particular, the 2DPN utilizes the shape and spatial arrangement of the paper substrate to control fluid flow and achieve automated, multi-step processing. This multi-step capability can be used to implement additional steps, such as sample pre-processing, signal enhancement, and even simple rinsing, all of which improve signal-to-noise ratios and ultimately the sensitivity of an assay.

We have chosen to use the recombinant affinity proteins due to their advantages over antibodies, which are typically used for protein capture and detection in traditional lateral flow assays. Our recombinant HA binders provide antibody-like affinity but at much lower cost and with increased customizability compared to antibodies, due to the recombinant production of the binder proteins in *E. coli*. We have tested two assay stack formats utilizing two different binders. The first is an HA stem-region binder, which was originally reported by Fleishman *et al.* [14] and has since been optimized by Whitehead *et al.* [15]. The second protein is an HA head-region binder, which is under development with a publication in progress. For the stem-region binder, we have exploited the customizability of its recombinant production by site-specifically biotinylating the binder for immobilization to a streptavidin-coated test region in our paper assay. Using this assay, we demonstrate the detection of clinically relevant concentrations of recombinant HA, with a limit of detection (LOD) of 30 pM. We also demonstrate the translation of this assay to a 2DPN folding card format. For the head-region binder, we employ direct adsorption to the nitrocellulose membrane [16] and demonstrate the detection of both recombinant HA and intact influenza A virus at clinically relevant concentrations using our paper-based system.

II. METHODS

A. Flu Assay with HA Stem-Region Binder

Sets of 3-mm-wide strips were cut from porous nitrocellulose membrane (GE FF80HP) using a CO_2 laser cutter. A nitrocellulose-binding streptavidin mutant (AbCam) was patterned onto the test region of each strip at 1 mg/mL in PBS, and the spotted membranes were stored under desiccation at room temperature at least overnight before use. To run the assay, strips were placed into a 96-well plate pre-filled with the assay reagent solutions and manually moved for each assay step. A cellulose absorbent pad was taped to the top of each set of strips to aid wicking. Each assay consisted of the following steps: 1) 40 µL of biotinylated HA binder (0.1 µM) pre-mixed with recombinant HA (A/California/04/2009 H1N1, Influenza Reagent Resource), 2) 40 µL PBST (PBS + 0.1% Tween-20) wash with 0.1 µM biotin-BSA to block excess streptavidin sites, 3) 20 µL mouse anti-HA detection antibody (0.1 µM, Influenza Reagent Resource), 4) 20 µL PBST wash, 5) 20 µL gold nanoparticle-conjugated goat-anti-mouse secondary antibody (OD 2.5, Arista Biologicals), and 6) 20 µL PBST wash. All assay reagents were diluted in PBST+1% BSA. This assay was performed in replicates of 4 for HA concentrations in the clinically relevant range of 0.2 pM to 3.125 nM, as well as for no-HA negative controls. Test lines were scanned at 600 dpi, and the average green-channel pixel intensity of each test line was quantified.

B. Flu 2DPN with HA Stem-Region Binder

Test membranes for the 2DPN were cut from porous nitrocellulose membrane (GE FF80HP) using a CO_2 laser cutter. A nitrocellulose-binding streptavidin mutant (AbCam) was patterned onto the test region of each strip at 1 mg/mL in PBS, and the spotted membranes were stored under desiccation at room temperature at least overnight before use. All other 2DPN folding card components were fabricated using a CO_2 laser cutter and assembled manually. The folding card was made of Mylar sheets with adhesive layers on one or both sides (Fralock). The sample input pads were made of glass fiber material (Ahlstrom), and wicking pads were made of cellulose (Millipore). To run the 2DPN devices, the assay reagents described in section II.A were applied to the sample input pads, and the folding card was closed to initiate fluid flow and the start of the assay. Exposed adhesive layers in the folding card allowed the card to stay closed with good contact between the input pads and the test membrane. The devices were tested using test samples with 100 nM recombinant HA (A/California/04/2009 H1N1, Influenza Reagent Resource) and no-HA negative controls. After completion of the assay (20 minutes), the devices were scanned at 600 dpi.

C. Flu Assay with HA Head-Region Binder

Sets of 3-mm-wide strips were cut from porous nitrocellulose membrane (GE FF80HP) using a CO_2 laser cutter. The HA head-region binder protein was patterned onto the test region of each strip at 10 µM in PBS. To run the assay, the test strips were assembled in plastic folding cards with cellulose absorbent pads to aid wicking. The devices were placed into a 96-well plate pre-filled with the assay reagent solutions and manually moved for each assay step. The assay consisted of the following steps: 1) 20 µL of HA or live influenza A sample (A/Solomon Islands/3/2006 H1N1, Influenza Reagent Resource), 2) 20 µL PBST wash, 3) 20 µL mouse anti-HA detection antibody (0.1 µM, Influenza Reagent Resource), 4) 20 µL PBST wash, 5) 20 µL gold nanoparticle-conjugated goat-anti-mouse secondary antibody (OD 2.5, Arista Biologicals), and 6) 20 µL PBST wash. All assay reagents were diluted in PBST+1% BSA. This assay was performed in replicates of 4 for HA concentrations in the clinically relevant range of 5 pM to 78 nM, as well as for no-HA negative controls. The assay was also used to test the detection of live influenza A virus at 1.25×10^8 $CEID_{50}$/mL. Test lines were scanned at 600 dpi, and the average green-channel pixel intensity of each test line was quantified.

III. RESULTS AND DISCUSSION

A. Flu Assay with HA Stem-Region Binder

Our novel, paper-based flu HA assay using the stem-region binder (Fig. 1) yielded clear signal for HA spiked into PBST+BSA and no visible signal for the negative controls (Fig. 2). The normalized pixel intensities of the test regions

are plotted in Fig. 2 as the mean +/- SD of n=4 replicates for each HA concentration tested (purple dots). The mean (solid line) +/- SD (dashed line) of the signal from n=4 negative controls are also provided. Overall, this assay yields a statistical limit of detection of 30 pM.

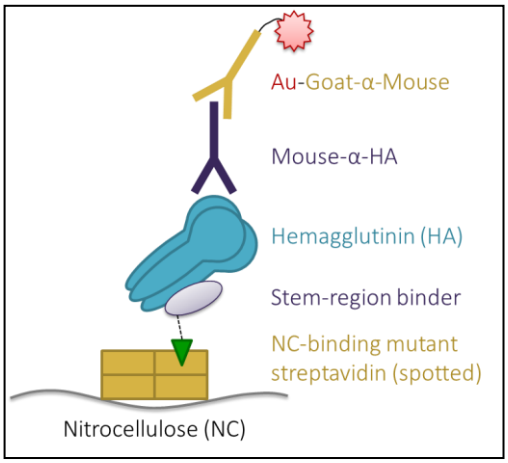

Figure 1. Influenza HA assay using the stem-region binder.

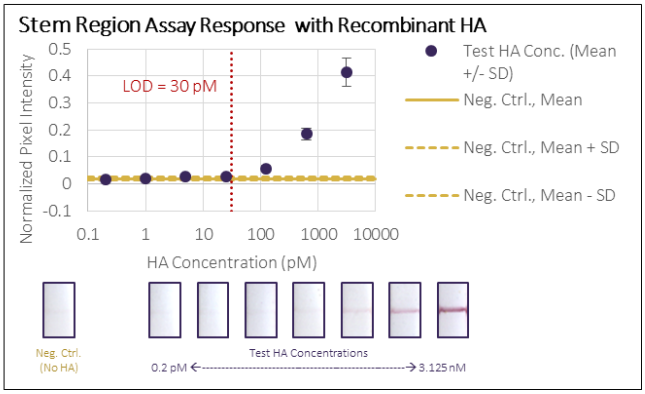

Figure 2. Assay response for recombinant HA using the stem-region binder. The pixel intensities are plotted as the mean +/- SD for n=4 replicates, and a representative image of each test line is shown below the plot.

B. Flu 2DPN with HA Stem-Region Binder

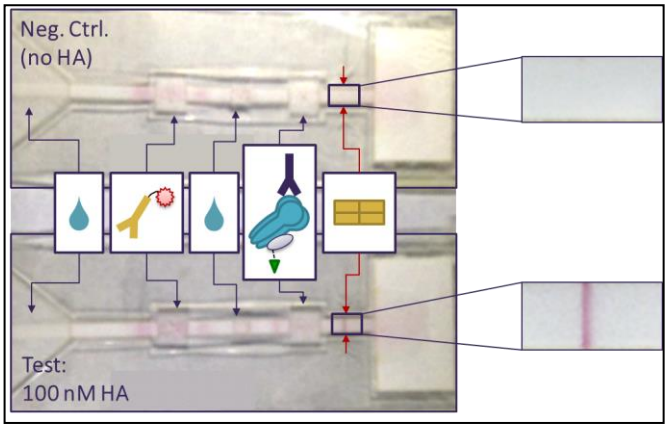

Figure 3. Image of two influenza HA 2DPN folding card devices, one for a negative control sample (no HA) and one for a test sample (100 nM HA). The device components corresponding to the assay stack in Fig. 1 are illustrated on top of the image. A zoomed-in version of each test line is shown on the right.

We successfully demonstrated the translation of our influenza HA assay using the stem-region binder to a 2DPN folding card format, as shown in Fig. 3. These 2DPN devices yielded clear signal for the test samples with recombinant HA and clean negative controls. This demonstration represents the first 2DPN for flu detection and a key step in the development of our paper-based influenza diagnostic. While we used wet reagents for this version of the device, future work will focus on optimizing the 2DPN and including dried reagents in order to further automate device operation and reduce user steps.

C. Flu Assay with HA Head-Region Binder

Our novel, paper-based flu HA assay using the head-region binder (Fig. 4) yielded clear signal for HA spiked into PBST+BSA with no visible signal for the negative controls (Fig. 5A). The normalized pixel intensities of the test lines are plotted in Fig. 5A as the mean +/- SD of n=4 replicates for each HA concentration tested (purple dots). The mean (solid yellow line) +/- SD (dashed yellow lines) of the signal from the n=4 negative controls are also provided. This assay yielded a statistical limit of detection of 150 pM. This assay is also able to detect live influenza A virus (Fig. 5B), verifying its potential as a diagnostic test for the flu.

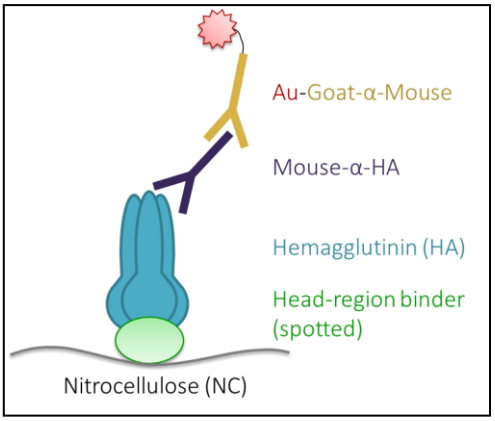

Figure 4. Influenza HA assay using the head-region binder.

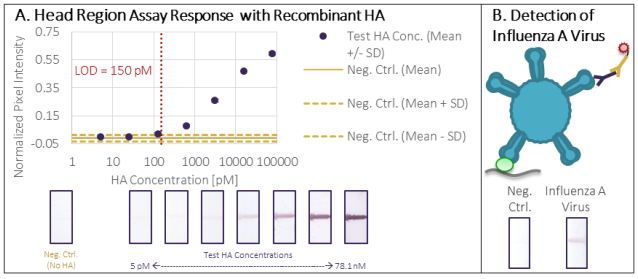

Figure 5. (A) Assay response with recombinant HA using the head-region binder. The pixel intensities are plotted as the mean +/- SD for n=4 replicates, and a representative image of each test line is shown below the plot. (B) Illustration of the assay with intact influenza virus and representative test lines for negative control and test samples, demonstrating the ability of this assay to detect real influenza A virus.

D. Comparison of Assay Formats

Based on the results described above, we observe several trade-offs between the stem-region binder and head-region

binder assay formats. The stem-region binder assay yields a lower limit of detection than the head-region binder assay (30 pM vs. 150 pM), but both LODs fall within the estimated clinically relevant range of 300 fM to 3 nM and require similar levels of improvement to detect the lowest levels of virus in nasal swab samples. Therefore, for either assay, we plan to utilize our 2DPN system to add sensitivity-boosting steps, such as gold enhancement. Additionally, in these current assay configurations, the head-region binder assay requires one less step than the stem-region binder assay, which results in a shorter assay run time. Given these considerations, the HA assay using the head-region binder therefore represents the most promising overall approach and will undergo continued development and optimization.

IV. Conclusion

This work represents a novel demonstration of computationally designed HA protein binders for use in influenza diagnosis. Future work will further optimize the assay to decrease the LOD, improve the automation of the assay in the two-dimensional paper network format, and demonstrate subtyping of influenza based on hemagglutinin. Together, this work represents a significant step in the development of a paper-based influenza test for improved diagnosis and disease management at the point of care.

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References

[1] Centers for Disease Control and Prevention, "Seasonal Influenza (Flu)," 29-Mar-2013. [Online]. Available: http://www.cdc.gov/flu/. [Accessed: 31-Mar-2013].

[2] Centers for Disease Control and Prevention, "Estimates of Deaths Associated with Seasonal Influenza --- United States, 1976--2007," *Morb. Mortal. Wkly. Rep.*, vol. 59, no. 33, pp. 1057–1062, Aug. 2010.

[3] Institute for Health Metrics and Evaluation, "Global Burden of Disease," 2010. [Online]. Available: http://www.healthdata.org/gbd. [Accessed: 31-Jan-2014].

[4] Centers for Disease Control and Prevention, "CDC Reports Flu Hit Younger People Particularly Hard This Season," 20-Feb-2014. [Online]. Available: http://www.cdc.gov/media/releases/2014/p0220-flu-report.html. [Accessed: 30-Mar-2014].

[5] N.-A. M. Molinari, I. R. Ortega-Sanchez, M. L. Messonnier, W. W. Thompson, P. M. Wortley, E. Weintraub, and C. B. Bridges, "The annual impact of seasonal influenza in the US: Measuring disease burden and costs," *Vaccine*, vol. 25, no. 27, pp. 5086–5096, Jun. 2007.

[6] Centers for Disease Control and Prevention, "CDC Recommendations for Influenza Antiviral Medications Remain Unchanged," 10-Apr-2014. [Online]. Available: http://www.cdc.gov/media/haveyouheard/stories/Influenza_antiviral2.html. [Accessed: 16-May-2014].

[7] A. B. Bonner, K. W. Monroe, L. I. Talley, A. E. Klasner, and D. W. Kimberlin, "Impact of the rapid diagnosis of influenza on physician decision-making and patient management in the pediatric emergency department: results of a randomized, prospective, controlled trial," *Pediatrics*, vol. 112, no. 2, pp. 363–367, Aug. 2003.

[8] A. C. Hurt, R. Alexander, J. Hibbert, N. Deed, and I. G. Barr, "Performance of six influenza rapid tests in detecting human influenza in clinical specimens," *J. Clin. Virol.*, vol. 39, no. 2, pp. 132–135, Jun. 2007.

[9] E. Fu, S. A. Ramsey, P. Kauffman, B. Lutz, and P. Yager, "Transport in two-dimensional paper networks," *Microfluid. Nanofluidics*, vol. 10, no. 1, pp. 29–35, Jun. 2010.

[10] E. Fu, P. Kauffman, B. Lutz, and P. Yager, "Chemical signal amplification in two-dimensional paper networks," *Sens. Actuators B Chem.*, vol. 149, no. 1, pp. 325–328, Aug. 2010.

[11] E. Fu, T. Liang, J. Houghtaling, S. Ramachandran, S. A. Ramsey, B. Lutz, and P. Yager, "Enhanced sensitivity of lateral flow tests using a two-dimensional paper network format," *Anal. Chem.*, vol. 83, no. 20, pp. 7941–7946, Oct. 2011.

[12] E. Fu, T. Liang, P. Spicar-Mihalic, J. Houghtaling, S. Ramachandran, and P. Yager, "Two-Dimensional Paper Network Format That Enables Simple Multistep Assays for Use in Low-Resource Settings in the Context of Malaria Antigen Detection," *Anal. Chem.*, vol. 84, no. 10, pp. 4574–4579, May 2012.

[13] G. E. Fridley, H. Q. Le, E. Fu, and P. Yager, "Controlled release of dry reagents in porous media for tunable temporal and spatial distribution upon rehydration," *Lab. Chip*, vol. 12, no. 21, p. 4321, 2012.

[14] S. J. Fleishman, T. A. Whitehead, D. C. Ekiert, C. Dreyfus, J. E. Corn, E.-M. Strauch, I. A. Wilson, and D. Baker, "Computational Design of Proteins Targeting the Conserved Stem Region of Influenza Hemagglutinin," *Science*, vol. 332, no. 6031, pp. 816–821, May 2011.

[15] T. A. Whitehead, A. Chevalier, Y. Song, C. Dreyfus, S. J. Fleishman, C. De Mattos, C. A. Myers, H. Kamisetty, P. Blair, I. A. Wilson, and D. Baker, "Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing," *Nat. Biotechnol.*, vol. 30, no. 6, pp. 543–548, May 2012.

[16] G. E. Fridley, C. A. Holstein, S. B. Oza, and P. Yager, "The evolution of nitrocellulose as a material for bioassays," *MRS Bull.*, vol. 38, no. 04, pp. 326–330, Apr. 2013.