Counting-based microfluidic paper-based devices capable of analyzing submicroliter sample volumes

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ABSTRACT

In this paper, we report the development of semiquantitative counting-based lateral flow assay (LFA)-type microfluidic paper-based analytical devices (μ PADs) to analyze samples at submicroliter volumes. The ability to use submicroliter sample volumes is a significant advantage for μ PADs since it enables enhanced multiplexing, reduces cost, and increases user-friendliness since small sample volumes can be collected using methods that do not require trained personnel, such as finger pricking and microneedles. The challenge of accomplishing a semiquantitative test readout using submicroliter sample volumes was overcome with a counting-based approach. In order to use submicroliter sample volumes, we developed a flow strategy with a running liquid to facilitate flow through the assay. The efficacy of the devices was confirmed with glucose and total human immunoglobulin E (IgE) tests using 0.5 μ l and 1 μ l of sample solutions, respectively. Semiquantitative results were generated to predict glucose concentrations in the range of 0–12 mmol/l and IgE concentrations in the range of 0–400 ng/ml. The counting-based approach correlates the number of dots that exhibited a color change to the concentration of the analyte, which provides a more user-friendly method as compared with interpreting the intensity of a color change. The devices reported herein are the first counting-based LFA-type μ PADs capable of semiquantitative testing using submicroliter sample volumes.

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I. INTRODUCTION

Microfluidic paper-based analytical devices (μ PADs) have been growing in popularity and employed in areas such as medical diagnostic testing^{1–11} and environmental testing.^{12–16} Some of the key advantages are affordability, disposability, portability, and user-friendliness.^{17–21} User-friendliness is a broad term that typically refers to the capability for μ PAD tests to be easily used and the results interpreted by untrained personnel,²² which enables the tests to be used in low-resource and home-based settings. An important aspect for user-friendly tests is to ensure that untrained users can safely collect and incorporate the samples onto the μ PADs.²² Painless and convenient sample collection methods such as finger pricking²³ or microneedles^{24–26} lead to smaller sample volumes (in the range of 1 μ l–20 μ l) compared to a venous draw.^{23,24,26} To enable multiplexing with these small sample volumes requires that the tests are capable of providing signal readouts using submicroliter sample volumes, and the ability to use lower sample volumes increases the number of tests that can be multiplexed on a single device. The use

of submicroliter sample volumes also reduces the cost of devices since they can use lower reagent volumes and less material. However, it can be challenging to generate semiquantitative signal readouts with low sample volumes. Therefore, there is a need to develop μ PADs capable of semiquantitative analysis using small sample volumes.

Test devices often require that results are either semiquantitative or quantitative to indicate the amount of an analyte that is present in a sample. Colorimetric readouts^{4,16,27–32} can be user-friendly, but quantification based on the color intensity requires the use of external devices, such as a camera and image processing software, or relies on visual detection from the naked eye, which can vary due to the perception of the user.²² To avoid the drawbacks of color intensity measurements, distance-based^{22,33–38} and barcode-based or counting-based test readout^{39–47} methods have been developed for μ PADs. In the distance-based readout method, a colored band is developed along the flow path when the detection reagent interacts with the target analyte, and the length of the band correlates

with how much of the analyte is present and provides a semiquantitative test result. The barcode-based or counting-based test readout techniques provide semiquantitative test results, which involve counting the number of colored segments, zones, or lines. Counting-based readouts were demonstrated with 3D μ PADs^{39,40} and 2D titration μ PADs^{48–50} where selective binding of the analyte and washing were not required. The barcode-based test readout method was primarily tested with lateral flow assay (LFA)-type μ PADs^{41–47} where the target analytes selectively bound at multiple test lines spotted along the sample flow path and the analyte concentration correlated with the number of test lines that changed color. For the LFA-type μ PADs, relatively large sample volumes (25–120 μ l) were used in the tests because a minimum sample volume was required to yield a distinct counting-based test readout using a specific device size.⁴² To generate LFA-type μ PADs that are more broadly user-friendly, the counting-based approach should be adapted to accommodate smaller sample volumes.

There has recently been an increased push to develop μ PADs with miniaturized features.^{28,51–53} Fabrication of LFA-type μ PADs with dimensions smaller than 3–5 mm is challenging, particularly due to the conventional assembly method whereby several different hydrophilic pads are cut and merged.^{42–47} Creating LFA-type μ PADs capable of analyzing small sample volumes will, therefore, require design and fabrication techniques capable of generating the entire device on a single sheet of paper. High-resolution techniques capable of creating miniaturized features in LFA-type μ PADs include recently reported techniques such as shrinkable materials⁵³ and laser cutting,²⁸ which have not yet been adapted to create counting-based LFA-type μ PADs. Another hurdle for the use of small sample volumes in counting-based test devices is the low analyte levels present in small volumes, which makes it challenging to create enough colored segments to produce distinct semiquantitative results. If these challenges can be overcome, counting-based tests can be developed to provide user-friendly μ PADs capable of semiquantitative readouts using small sample volumes and with the added benefits of enhanced multiplexing and reduced costs.

In this study, we design and develop LFA-type μ PADs with a semiquantitative counting-based test readout that enables small sample volumes to be used for user-friendly testing. We demonstrate the proof of concept by performing glucose and human IgE tests using a small volume of a sample solution (0.5 μ l for the glucose test and 1 μ l for the IgE test). We develop a flow strategy involving a running liquid to ensure that submicroliter sample volumes are capable of flowing along the entire assay. In the design of the counting-based test readout, three circular test dots were spotted along the sample flow path using small volumes of a detection reagent (0.2 μ l) for each dot to generate a counting-based colorimetric test readout. The test dots changed their color in the presence of the analyte, and the number of colored test dots successfully correlated to the concentration of the analyte and hence provided a semiquantitative counting-based colorimetric test readout using submicroliter sample volumes.

II. MATERIALS AND METHODS

A. Materials and chemicals

Amersham Protran 0.45 nitrocellulose membrane and cross-absorbed goat antihuman IgE antibody unconjugated (“abT” will

be used herein) were purchased from Fisher Scientific (Hampton, NH, USA). Whatman regenerated cellulose membrane 55 of 0.45 μ m pore size (RC-55) was purchased from VWR International (Mississauga, ON, Canada). Human IgE full length protein (IgE will be used herein), mouse monoclonal antihuman IgE (Fc) antibody conjugated with horseradish peroxidase (HRP) (“abHRP” will be used herein), the goat antimouse IgG unconjugated antibody (“abC” will be used herein), and the TMB-enzyme-linked immunosorbent assay (ELISA) substrate with highest sensitivity were purchased from Abcam Inc. (Toronto, ON, Canada). NaCl, KCl, Na₂HPO₄, KH₂PO₄, Tween-20, bovine serum albumin (BSA), 3,3'-diaminobenzidine (DAB), D-(+)-glucose, sucrose, peroxidase from horseradish (HRP), glucose oxidase from *Aspergillus niger* (GOx), de-ionized water (DI water), and molecular reagent grade pure water were purchased from Sigma-Aldrich (Oakville, ON, Canada). Phosphate buffered saline (PBS) at pH 7.4 was prepared using NaCl, KCl, Na₂HPO₄, KH₂PO₄, and DI water. Complements brand skimmed milk powder was purchased from No-Frills (Oshawa, ON, Canada). Aluminium foil (Handi-foil Corp., Wheeling, IL, USA) was purchased from UOIT central stores (Oshawa, ON, Canada). A roll of positionable mounting adhesive film 568 by 3M™ (Maplewood, MN, USA) was purchased from [amazon.ca](https://www.amazon.ca).

B. Device design and fabrication

The design and final fabricated μ PAD used in the experiment are shown in Figs. 1(a) and 1(b), respectively. The μ PADs were fabricated using a previously reported laser cutting technique,²⁸ where a CO₂ laser beam removes the paper material from foil-backed membranes along the cut line of the design to yield flow barriers throughout the device. The laser cutting technique allows fabrication of smaller features and devices, but a width of 1.5 mm was selected for the detection zone to enable the test results to be read with naked-eye visualization. Whatman regenerated cellulose membranes (RC-55) and Amersham Protran 0.45 μ m nitrocellulose membranes were used in the fabrication of μ PADs for the glucose and human IgE tests, respectively. We use a broad definition of paper to include nitrocellulose, as is now common in the literature.²⁰ Cellulose paper was used for the glucose test since protein binding was not an issue, and it provided faster wicking in comparison with nitrocellulose. For all the glucose tests, RC-55 was used, and for all the IgE tests, Amersham Protran 0.45 was used to ensure consistent flow behavior between tests. The device consists of four zones, which are labeled in Fig. 1(a) as follows: running liquid zone “R,” sample input zone “S,” detection zone (containing test dots “T1-T3” and control dot “C”), and absorption zone “A.” All the zones were patterned on a single sheet of the paper material to eliminate the complexity of merging multiple pads.

C. Operation of the LFA-type μ PAD

To accommodate the smaller sample volumes, we developed a lateral flow strategy to enable the analyte to flow over the readout zones and to provide increased consistency, as shown in Fig. 1. The sample was first pipetted on the “S” zone, and then a running liquid was pipetted on the “R” zone, which allowed the sample to flow over the test dots and control dots. The reagent immobilized on the test dots interacted with the analyte present in the sample

FIG. 1. (a) Schematic of the LFA-type μ PAD designed to analyze small sample volumes. (b) Relative size of the miniaturized μ PAD is shown against a Canadian nickel (five cent coin) and fingertips. (c) Schematic of the μ PAD showing placement of the simulated sample (blue dye) and simulated running liquid (yellow dye). (d) Photographs of the dye test showing the flow of the simulated sample (blue dye) over time for visual confirmation of successful sample flow across the detection zone.

and provided a visual colorimetric signal of analyte detection. The control dot reagent was chosen in such a way so that it can produce colorimetric detection in contact with the sample fluid irrespective of the presence or absence of the analyte.

A preliminary dye test was performed using a μ PAD made out of a nitrocellulose membrane to visually confirm that the sample flowed over the entire detection zone, as shown in Figs. 1(c) and 1(d). A permanent red ink pen was used to make red circular dots that represent the test dots, and a permanent black ink pen was used to make a black dot that represents the control dot. The permanent ink was not washed out by the flowing liquid, which is the same behavior that will be exhibited by the immobilized reagents for the test dots and control dot in analytical devices. A $1\ \mu\text{l}$ volume of a blue dye was used as the sample and pipetted on the “S” zone, followed by pipetting of $10\ \mu\text{l}$ of a yellow dye as the running liquid on the “R” zone, as shown in Fig. 1(c). As the yellow dye was wicked along the device, it caused the blue dye to flow over the detection zone, and after 10 min, all of the blue dye reached the absorption zone “A,” which was visually confirmed, as shown in Fig. 1(d). This preliminary dye test confirmed that the device design required a running liquid to cause the sample fluid to flow over the entire detection zone, which is essential for a sufficient analyte-reagent interaction and consistent flow when using small sample volumes. Users of the test devices should wait until the sample has flowed to the absorption zone before interpreting the test result. An added

benefit of incorporating a running liquid is to diminish the influence of the sample fluid properties on the flow behavior. Since the running liquid dominates the flow behavior, the flow is likely to be more consistent even in cases where the sample fluid properties, such as viscosity, might vary among users.

The devices rely on the progressive consumption of the target analyte to generate semiquantitative results. As the sample flows over the test dots, the target analyte is consumed by interacting with each test dot either through binding or a chemical reaction. Therefore, by counting the number of test dots that change color, the concentration range of the target analyte in the sample can be determined. The concentration range that is revealed by the tests can be assigned by selecting the size of the test dots and the number of them since this will correlate with how much of the target analyte is consumed as the sample flows. Each test dot will consume a specific number of molecules of the target analyte. If it is desirable to use low sample volumes, which have a low number of target molecules, then a low number of smaller test dots should be selected. We selected three test dots to enable us to use low sample volumes while also generating meaningful concentration ranges, since one or two test dots are limited in the number of concentration levels that can be determined. Since the color change of the test dots does not reveal a precise concentration value, but rather correlates to a range, the devices give semiquantitative results (concentration ranges) as opposed to quantitative results (precise concentration values).

Design of the devices will depend on the chemistry involved in the test, the type of the paper selected, and the concentration ranges of interest. The variables that can be adjusted in the design include the type and porosity of the paper used, the number and size of the test dots, the reagent volume and concentration, the sample volume used, and the volume of running liquid used. Since paper has asymmetric porosity,^{52,54} it is important to select a paper type that is compatible with counting-based testing and calibrate the devices to ensure that the dots have effectively reacted with the analyte of interest and are displaying representative color changes. This calibration procedure is performed empirically in this study because of the complexities that arise with prediction of flow in paper-based devices using models such as the widely used Bell-Cameron-Lucas-Washburn imbibition.^{21,55} We perform this calibration procedure in this paper for glucose and IgE testing.

D. Spotting reagents

A common approach in barcode-based lateral flow assays is to spot multiple drops of a reagent side-by-side and create test lines; however, when a single drop of a spotting reagent is placed on a hydrophilic membrane, a circular spot is naturally formed, which is a more simple method to spot the reagents. In our experiments, a single drop of a reagent was spotted on each of the test dots "T1-T3" and the control dot "C" where a specific volume of reagent was used to control the size of the dots (0.2 μ l). The control dot was formed on the membrane by pipetting the reagent as a single drop. The use of a pipette for spotting resulted in some inconsistency in the size of the dots, which is not critical for the control dot but is crucial for the test dots to ensure reproducibility and accuracy of the test result. To overcome the inconsistency associated with pipetting, a syringe pump with microbore plastic tubing containing an opening fitted with a steel sleeve was used to dispense submicroliter reagent volumes and create the test dots, as shown in Fig. 2. The steel sleeve protected the microbore tube from being squeezed by the applied pressure of the fingertips. There are many commercially available drop-wise chemical dispensers, which can be used for similar reagent spotting in large-scale production of μ PADs.

FIG. 2. Spotting submicroliter reagent volumes on the test dots "T1-T3" using a syringe pump, microbore tubing, and fingertips.

E. Glucose test procedure

To demonstrate the efficacy of the miniaturized counting-based μ PADs, a glucose test was performed to determine the level of glucose present in sample solutions made using different concentrations of glucose (0, 3, 6, 9, and 12 mmol/l) with BSA (40 mg/ml) in a PBS buffer. The BSA and PBS buffer were used to generate a solution similar to blood plasma, with proteins present. The glucose levels of 0–12 mmol/l were selected because they represent the range of interest for medical diagnosis.⁵⁶ A 0.2 μ l volume of a DAB solution (10 mg/ml in DI water) was spotted on each of the test dots "T1-T3" using the syringe pump, and a 0.2 μ l volume of a DAB-glucose solution (1:1 ratio of a 10 mg/ml DAB solution and a 20 mg/ml glucose solution made with DI water) was pipetted on the control dot "C." A 0.2 μ l volume of HRP (800 U/ml in pure water) and a 0.2 μ l volume of GOx (800 U/ml in pure water) were each spotted on the sample input zone "S," as shown in Fig. 3(a). The device was allowed to dry at room temperature for 10 min prior to use.

A 0.5 μ l sample was dispensed in zone "S" followed by the dispensing of a 10 μ l volume of DI water in zone "R," which was used as the running liquid for the glucose tests, as shown in Fig. 3(b). As the running liquid was wicked along the device, it caused the sample to flow over the detection zone. The spotted DAB (test dots) changed from a light brown color into dark brown poly(DAB) if exposed to HRP, GOx, and glucose, as shown schematically in Fig. 3(c). In this process, glucose is converted to gluconic acid and hydrogen peroxide by glucose oxidase, and subsequently, the hydrogen peroxide reacts with DAB in the presence of horseradish peroxidase and produces dark brown poly(DAB). Thus, the amount

FIG. 3. Steps for the glucose test: (a) the reagents were spotted on the sample input zone and detection zone of the μ PAD, (b) the test was performed by pipetting the sample solution followed by DI water as the running liquid, and (c) colorimetric detection of glucose over the test dots "T1-T3" (with "T1" and "T2" colored as dark brown as an example of the reaction with glucose).

of glucose in the sample correlates with the amount of hydrogen peroxide produced, and the hydrogen peroxide is consumed progressively as it flows along the detection zone. Therefore, higher concentrations of glucose yielded a larger number of dark brown test dots, and the number of dark brown test dots was correlated to the concentration of glucose in the sample. The control dot changed to a dark brown color regardless of the amount of glucose in the sample because the control dot was composed of a glucose-DAB mixture, and the HRP and GOx eventually flowed to the control dot, as shown in Fig. 3(c). Thus, the control dot changing to dark brown provided confirmation that the fluid successfully reached the end of the detection zone and contained enough HRP and GOx for the reactions to occur.

F. Human IgE test procedure

To demonstrate the efficacy of the miniaturized counting-based μ PADs for immunoassays, a human IgE test was performed to determine the total human IgE present in a sample solution prepared in the PBS buffer with BSA (40 mg/ml), sucrose (1% w/v), and different concentrations of IgE (0, 25, 50, 100, 200, and 400 ng/ml). The BSA, sucrose, and the PBS buffer were used to generate a solution similar to blood plasma, with additional proteins present. In each μ PAD, the test dots “T1–T3” were spotted with 0.2 μ l of “abT” (1 mg/ml), and the control dot “C” was spotted with 0.2 μ l of “abC” (1 mg/ml), as shown in Fig. 4(a). After spotting, the devices were dried in an incubator (HerathermTM Compact Microbiological Incubators, Thermo Fisher Scientific, MA, USA) at 37 °C for 30 min. After drying, the devices were blocked for 15 min in a blocking solution made with 5% (w/v) skimmed milk in the PBS buffer using a lab rocker platform (Bellco Biotechnology, NJ, USA) at medium speed. The blocked devices were washed for 10 min in a washing solution made with 0.05% (v/v) Tween-20 in the PBS buffer using the same rocker platform. The washed devices were dried in the incubator at 30 °C for 90 min, and the dried devices were then ready for the IgE testing.

To perform the IgE test, the sample solutions were mixed with “abHRP” (1 μ g/ml) to yield a new sample solution, which contained IgE bound with the “abHRP” as shown in Fig. 4(a). A 1 μ l new sample solution was placed in the sample input zone “S,” and within 20 s, a 12 μ l volume of the running buffer [the PBS buffer with 2% (v/v) Tween 20] was placed in the running liquid input zone “R,” as shown in Fig. 4(a). As the running liquid was wicked along the device, it caused the sample to flow over the detection zone, where the “abT” (test dots) captured IgE bound with “abHRP,” and the “abC” (control dot) captured the unbound “abHRP” contained in the sample solution, as shown in Fig. 4(b). After 10 min, the sample reached the absorption zone “A” (after flowing over the test and control dots), and any remaining abHRP that did not bind with a test or control dot was washed away from the detection zone and into the absorption zone. A 3 μ l volume of TMB solution was then applied over the detection zone. In the presence of TMB, the IgE bound with “abHRP” at the test dots and the unbound “abHRP” at the control dot changed to a blue color, as shown in Fig. 4(c). The test dots only captured IgE bound with “abHRP”; therefore, the blue color only appeared in the test dots if human IgE was present in the sample solution as it flowed over

FIG. 4. Steps for the IgE test: (a) the new sample solution [a mixture of the sample solution (containing IgE) and “abHRP”] was placed on the sample input zone followed by the placement of the running buffer in the running liquid input zone, (b) IgE-bound “abHRP” was captured at the test dots, and unbound “abHRP” was captured at the control dot as the sample solution flowed over them, and (c) HRP of the “abHRP” turned blue after adding TMB solution on the detection zone (with “T1” and “T2” shown as blue colored as an example).

each test dot. Imaging of the IgE test results was performed approximately 2 min after the TMB was added. The IgE was captured progressively as it flowed over each successive test dot; therefore, higher concentrations of IgE yielded a larger number of blue

colored test dots, and the number of blue colored test dots was correlated to the concentration of IgE in the sample. Unlike the test dots, the control dot captured the unbound “abHRP” in the sample solution regardless of the amount of IgE contained in the sample, and thus, a blue colored control dot provided confirmation that the sample flowed to the end of the detection zone and contained a sufficient amount of “abHRP.”

III. RESULTS AND DISCUSSION

A. Counting-based readout for a miniaturized glucose test

In order to generate a meaningful semiquantitative result from a counting-based test, a calibration was required to ensure that the diagnostically important glucose levels had a distinct number of dots that changed color. This was accomplished by performing several tests while varying the amount of the sample volume used. A larger sample volume contained more glucose molecules, while the capacity for each test dot to consume molecules remained the same. Therefore, even at low concentrations, the use of a larger sample volume resulted in more of the dots changing to a dark brown color. We tested several sample volumes and found that 0.5 μ l yielded the clinically relevant concentration ranges based on the size and number of test dots (3)

that we used on the device. Traditionally, the important diagnostic levels for glucose tests are based on diabetes and hypoglycemia, and glucose concentrations of 0, 3, 6, 9, and 12 mmol/l provide meaningful information about these conditions.⁵⁶ A sample volume of 0.5 μ l was found to generate a distinct number of dots with a dark brown color change for each of the concentrations; therefore, a 0.5 μ l sample volume was used in each of the glucose tests reported in this section.

The tests were performed three times (each one is denoted as a set), and for each set, five μ PADs were fabricated to analyze the different concentrations of the glucose (0, 3, 6, 9, and 12 mmol/l). Figure 5(a) shows photographs of the detection zone for each μ PAD after the glucose tests were completed. A counting of the dark brown dots based on naked eye visualization of Fig. 5(a) reveals that a glucose level of 12 mmol/l corresponded to three dark brown test dots, 9 mmol/l corresponded to two dark brown test dots, 6 mmol/l corresponded to one dark test dot, and 3 mmol/l corresponded to a portion of a test dot with a dark brown color. The control dot “C” changed to a dark brown color in every test, thus confirming that the sample flowed to the end of the detection zone. The results are plotted in Fig. 5(b) to provide a graphical interpretation of the findings. These test results demonstrate that the miniaturized counting-based μ PADs can provide a user-friendly semiquantitative method to monitor the

FIG. 5. (a) Photographs of the detection zones after completion of the glucose tests for three sets of experiments conducted with 0.5 μ l of the sample solution containing various concentrations of glucose (0–12 mmol/l). The test dots changed to a dark brown color if they reacted with glucose; therefore, the number of dark brown dots in each test corresponds to the level of glucose in the sample. (b) The glucose concentrations were plotted against the number of test dots that changed to a dark brown color. For each test dot, the plot distinguished between the progression of two colorimetric states that were observed: a portion of the test dot changed to a dark brown color or the entire test dot changed to a dark brown color.

concentration of biomarkers in relation to the important diagnostic ranges associated with different conditions. The small sample volume requirement will also allow the tests to be integrated with user-friendly sample collection systems such as finger pricking or microneedles.

B. Counting-based readout for a miniaturized IgE test

Similar to the glucose tests, experiments with different volumes of a sample solution were performed, and a sample volume of $1\ \mu\text{l}$ was found to be the best choice to generate a distinct number of test dots with a color change corresponding to the clinical range of IgE concentrations associated with allergic symptoms.^{57–59} According to the literature, in blood plasma, three levels of total IgE are commonly considered in medical diagnosis, normal level: $<48\ \text{ng/ml}$ ($20\ \text{kU/l}$), moderate level: $48\text{--}240\ \text{ng/ml}$ ($20\text{--}100\ \text{kU/l}$), and elevated level: $>240\ \text{ng/ml}$ ($100\ \text{kU/l}$).^{57–59} Each level indicates the probability of allergic symptoms and the probable contribution of allergens in atopic diseases.⁵⁷ The tests were performed three times (each one denoted as a set), and six μPADs were fabricated to analyze each of the different concentrations of human IgE (0, 25, 50, 100, 200, and 400 ng/ml). Figure 6(a) shows photographs of the detection zone for

each μPAD after the IgE tests were completed. A counting of the blue colored dots based on naked eye visualization of Fig. 6(a) reveals that an IgE concentration of 400 ng/ml corresponded to three full blue colored test dots, 200 ng/ml corresponded to two full blue colored test dots, 100 ng/ml corresponded to one full blue colored test dot, and 50 ng/ml and 25 ng/ml both corresponded to less than one test dot changing to a blue color. Distinguishing between 25 and 50 ng/ml was not required, since the clinical level of interest is a concentration below 48 ng/ml. The control dot “C” changed to a blue color in each test, which confirms that the sample successfully flowed to the end of the detection zone. Figure 6(b) provides a graphical interpretation of the findings from the IgE tests.

The counting-based approach is valuable for these total IgE tests because a semiquantitative result provides important test information in a user-friendly test. According to the test results from Figs. 6(a) and 6(b) and based on the clinical range of IgE concentrations noted above, if the blue color is limited to the first test dot “T1,” then it indicates a normal level of total IgE; if dots “T1–T2” change to a blue color, this indicates a moderate level of total IgE, and if the blue color appears in all three test dots “T1–T3,” then it indicates an elevated level of total IgE. This further reinforces the strength of the counting-based approach to provide a

FIG. 6. (a) Photographs of the detection zones after completion of the IgE tests for three sets of experiments conducted with $1\ \mu\text{l}$ of the sample solution containing various concentrations of human IgE (0–400 ng/ml). The test dots changed to a blue color if they bound with IgE; therefore, the number of blue dots in each test corresponds to the level of IgE in the sample. (b) The IgE concentrations were plotted against the number of test dots that changed to a blue color. For each test dot, the plot distinguished between the progression of two colorimetric states that were observed: a portion of the test dot changed to a blue color or the entire test dot changed to a blue color.

semiquantitative readout that is simple to interpret in comparison with the interpretation of the intensity of a change in color.

The IgE testing completed in the laboratory involved premixing of the sample with abHRP and the addition of the running liquid and TMB. A user-friendly μ PAD should avoid the requirement that users premix samples and add precise volumes of fluid to the test. In a future device, these steps could be automated on the μ PADs by predepositing the abHRP onto the sample zone and inclusion of tools that can release a precise volume of fluid, such as a prepackaged dropper or the breaking of a bubble pack integrated on the device.

From the success of the human IgE tests, it can be inferred that similar μ PADs made out of a nitrocellulose membrane (or any paper material with similar protein binding properties) can be developed for other protein biomarkers present in samples. The use of small sample volumes increases the user-friendly aspect of the tests since simple sample collection methods can be used (such as finger pricking and microneedles). Distinct colorimetric detection for the target range of an analyte can be achieved by adjusting the sample volume size, test dot size, number of test dots, and concentration of immobilized antibody spotted in the test dots. Therefore, the counting-based μ PADs presented here can provide a user-friendly way to provide semiquantitative information about a wide range of analytes and over a broad range of concentrations.

IV. CONCLUSIONS

We designed and developed LFA-type μ PADs with a counting-based colorimetric test readout to analyze small sample volumes (in the submicroliter range). The devices were used to provide semiquantitative information about the concentration of glucose and human IgE present in 0.5 and 1 μ l of sample solutions, respectively. The devices successfully provided distinct visual colorimetric detection for varying analyte concentrations where the number of colored test dots correlated to the analyte concentration. Glucose is a nonprotein analyte and IgE is a protein analyte in human blood plasma; therefore, it can be inferred that similar devices can be developed to detect other protein and nonprotein biomarkers. The counting-based μ PADs provide a user-friendly test platform since the counting of colored dots is more straightforward than interpreting the intensity of a color change, and the use of small sample volumes, in the submicroliter range, enables the tests to be used with simple sample collection tools available to untrained users, such as finger pricking and microneedles.

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There are no conflicts to declare.

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