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ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2015

Impact Factor: 5.64 · DOI: 10.1021/acs.analchem.5b01923 · Source: PubMed

analytical chemistry

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Printed Paper Sensors for Serum Lactate Dehydrogenase using Pullulan-Based Inks to Immobilize Reagents

Balamurali Kannan,[†] Sana Jahanshahi-Anbuhi,[†,‡] Robert H. Pelton,[†,‡] Yingfu Li,[†,§] Carlos D. M Filipe,[*,†,‡] and John D. Brennan[*,†]

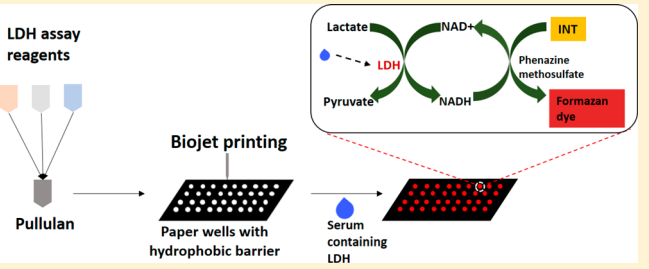
[†]Biointerfaces Institute, McMaster University, 1280 Main St W, Hamilton, Ontario L8S 4L8, Canada

[‡]Department of Chemical Engineering, McMaster University, 1280 Main St W, Hamilton, Ontario L8S 4L7, Canada

[§]Department of Biochemistry & Biomedical Sciences, McMaster University, 1280 Main St W, Hamilton, Ontario L8S 3Z5, Canada

Supporting Information

ABSTRACT: In this study, a paper-based point-of-care (POC) colorimetric biosensor was developed for the detection of lactate dehydrogenase in serum using a nonporous, oxygen impermeable reversibly gelling polysaccharide material based on pullulan. The pullulan could be printed onto paper surfaces along with all required assay reagents, providing a means for high-stability immobilization of all reagents on paper. Serum containing lactate dehydrogenase (LDH) was directly spotted on to the pullulan-coated bioactive paper and provided quantitative colorimetric data that was comparable to that obtained with a conventional plate-reader method. The paper strip was found to be highly stable and could be stored at 4 °C for at least 10 weeks with no loss in performance, as compared to a complete loss in performance within 1 day when the reagents were printed without the stabilizing polysaccharide. The ease of fabrication coupled with the high stability of the printed reagents provides a facile platform for easily manufactured POC sensors.



Lactate dehydrogenase (LDH) is a key biomarker of cancer and organ failure, since these diseases generally result in a significant increase in LDH concentration in biofluids, such as serum.[1−5] Methods for rapid and facile measurement of LDH in serum are highly relevant and useful diagnostic tests, particularly if these methods are suitable for use in remote locations and/or by unskilled users. One of the most widely used assays for monitoring LDH levels involves colorimetric detection of formazan dye using nicotinamide adenine dinucleotide (NAD) as a coenzyme and lactic acid as a substrate.[6] In this assay (Figure 1), LDH catalyzes the conversion of lactate to pyruvate with the simultaneous formation of nicotinamide adenine dinucleotide (reduced form) from NAD. The generated NADH converts iodonitrotetrazolium chloride (INT) to formazan dye, which is bright red in color. The assay is sensitive, but it requires sophisticated equipment such as a plate-reader, multiple sample handling steps and trained personnel for performing the assays, making it unsuitable for resource limited settings. In addition, the reagents have poor thermal and photostability, and thus need to be shipped and stored in the dark at low temperature to avoid degradation, adding to the cost of the assay.[7] Reducing the complexity involved in storing and handling of such reagents, while at the same time removing the need for sophisticated instrumentation, is expected to bring great benefits to resource limited regions where screening is done for large populations with limited facilities and technical expertise.

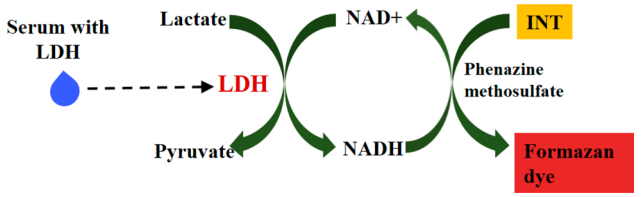


Figure 1. Chemical reactions involved in the assay for the colorimetric detection of lactate dehydrogenase (LDH) in serum.

Paper-based sensing platforms for rapid diagnostics have been widely reported, particularly in the past decade, for applications in various fields such as clinical diagnostics, environmental monitoring and food safety.[8] Hand spotting of reagents directly onto a patterned paper surface is one of the simplest and widely used methods for fabricating paper sensors (e.g., for glucose, bovine serum albumin, and food borne pathogens[9−11]), but is not easily scaled for manufacturing. Newer methods involving printing of reagents can overcome this problem, and have led to a number of different sensors for analytes such as pesticides, bacteria and heavy metals, as reported by our group.[12−15] In our previous work, sol−gel

Received: May 22, 2015
Accepted: August 21, 2015

DOI: 10.1021/acs.analchem.5b01923
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based silica has typically been used as a foundation to generate bioinks that can be printed on paper using piezoelectric inkjet printing technology to produce immobilized biomolecules.[12−15] However, while these materials can be printed, the inks within the cartridges are not very stable and thus printing has to be completed within a few hours to avoid clogging of the printer nozzles. In addition, multipass printing was often required to deposit sufficient material to produce strong signals.

Recently, Jahanshahi-Anbuhi et al. introduced a new technology for the long-term stabilization of labile bioreagents by adding these to an aqueous solution (sol) of the polysaccharide pullulan, and letting the solution gel to form a film or tablet containing the reagents.[16] Upon gelling, pullulan forms a glassy material that is impermeable to oxygen and can be readily dissolved upon adding water. Because of these unique properties, pullulan has been extensively used as a protective agent in the food and drug industry.[17−19] Herein, we investigate the ability to print pullulan solutions containing all necessary assay reagents onto paper surfaces to develop a point-of-care (POC) colorimetric sensor for LDH detection. Optimal ink formulations, the stability of pullulan-entrapped reagents on paper, and the performance of the paper-based biosensors for LDH detection in artificial serum are reported.

EXPERIMENTAL SECTION

Materials. Nicotinamide adenine dinucleotide (NAD), iodonitrotetrazolium chloride (INT), phenazine methosulfate (PMS), lactic acid (LA), lactate dehydrogenase (LDH), and serum replacement were purchased from Sigma-Aldrich. Serum replacement samples contain highly purified, heat-treated bovine serum albumin, heat-treated bovine transferrin, and bovine insulin and were used to avoid biosafety containment issues. Pullulan (desalinized, reagent grade, MW = 200 kDa) was purchased from Polysciences Ltd. Tris (tris-(hydroxymethyl)-aminomethane) buffer was purchased from Bioshop Canada Inc. Water used in this study was deionized using a Millipore Synthesis A10 purification system to a resistance of 18.2 MOhm·cm. All other reagents were of analytical grade and were used as received.

Paper Sensor Fabrication. Whatman No. 1 filter paper was used for all assays. A wax printer (Xerox Phaser 8560N) was used to print hydrophobic wax barriers on a 96 well-plate template (3 mm diameter wells with ∼4.5 mm interwell distance). After printing, the paper was placed into an oven at 120 °C for 2 min to melt the wax through the thickness of the paper, generating an effective hydrophobic barrier. The color reagent mixture (CR mix) was prepared by mixing 100 μL of INT (40 mg/mL in DMSO), 100 μL of PMS (10 mg/mL in water), and 800 μL of NAD (12.5 mg/mL in water). The ink for printing of the paper-sensor strips was prepared by mixing 1000 μL of the CR mix with 1000 μL of 0.4 M lactic acid (in 0.2 M Tris buffer at pH 8.9). Then, pullulan powder was added to reach a final concentration of 8% w/v and mixed thoroughly by vortexing. The final pH of the printing solution was ∼5. A Biodot XYZ3060 automated dispensing unit was used to print and immobilize 4 μL of this ink onto each of the paper-based wells. The Biodot printer can dispense viscous (50 cP) inks at a high-speed with enormous accuracy and precision (CV < 5% according to Biodot technical literature,[20] which was confirmed based on reproducibility of color formation in the paper-based LDH assay, as shown in Figure S1). Manually dispensing such low volumes of the viscous pullulan dispersion into the wells was very difficult and not reproducible (CV > 100%, data not shown). The final concentration of NAD (5 mg/mL), INT (2 mg/mL), PMS (0.5 mg/mL), and lactic acid (0.2 M) in the ink formulation was similar to that reported in the literature for solution based assays.[6] After printing the reagents onto the 96-well paper plate, they were allowed to dry at room temperature, in the dark, for at least 1 h. Control wells were fabricated exactly the same way but without pullulan in the ink-formulation. Figure 2A shows a simplified schematic for the paper-based sensing array.

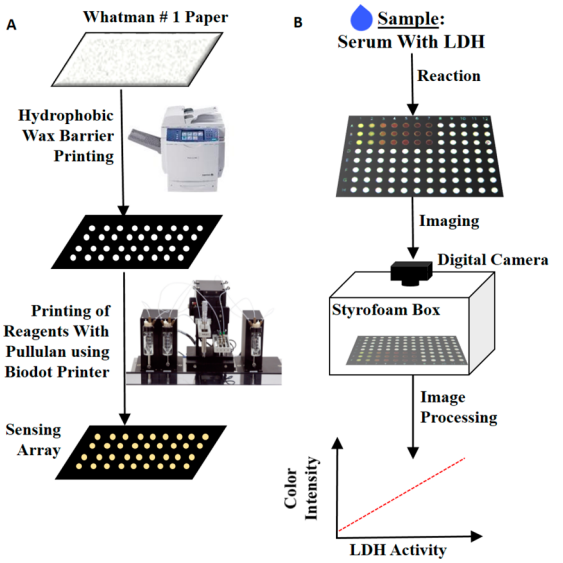

Figure 2. Outline of steps involved in (A) fabricating paper-well based LDH sensors and (B) performing paper-based assays for serum LDH.

LDH Detection in Paper Well-Plates. LDH detection using the paper sensor followed the steps shown in Figure 2B. A series of NADH standard solutions containing 0, 1, 2, 3, 4, and 5 nanomoles NADH per 5 μL were prepared and 5 μL of each solution was spotted onto the paper well-plates in triplicate. The paper well-plate was imaged 5 min after LDH addition using a digital camera (Canon Powershot G11 operated in autofocus mode and without flash). ImageJ[12] software was used to analyze the JPEG images. The ImageJ software uses a 256-bit color scale. The JPEG images were inverted, so that areas that were originally white became black, corresponding to a color intensity of zero and areas that were originally black became white, corresponding to a color intensity of 256. On the basis of this scale, an increase in the amount of red color resulted in an increase in color intensity of the sensor strips. Next, the color intensity was plotted against the nmol of NADH in the standard solutions to obtain a calibration curve. Following generation of the calibration curve, assays were performed by spotting 5 μL samples (serum replacement containing LDH diluted 2-fold with Tris buffer, 0.2 M, pH 8.9) onto fresh paper wells, with samples tested in triplicate. The dilution of serum with basic buffer is required for the assay as the immobilized reagents are printed under acidic pH conditions (pH ∼5) to prevent degradation during storage, while the enzymatic reaction occurs only at pH 7 or greater. The amount of NADH generated per minute was calculated through the color intensity developed after a 5 min reaction time. The LDH activity was calculated through the ratio of NADH generated per unit time (in nmol per minute) to the sample volume (0.005 mL). This gives the LDH activity in

DOI: 10.1021/acs.analchem.5b01923
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mU/mL or U/L. The long-term stability data was generated by storing the paper well-plate in a light-tight container at room temperature (varies between 23 and 26 °C) or in a refrigerator (at 4 °C). The LDH activity was measured after each different sensor storage period had elapsed using freshly prepared LDH in serum, tested at two different concentrations.

Paper-well assays were compared to LDH activity in solution as determined by the plate reader method. In this method, 20 μL of the color reagent mix was added to a clear 96-well plate followed by the addition of 100 μL of 50 mM lactic acid (in 0.2 M Tris buffer pH 8.9). NADH calibration curves were generated by adding 10 μL of a series of standard NADH solutions containing a total of 0, 1.25, 2.5, 3.75, 5, or 7.5 nmol of NADH to the wells. The LDH activity in the serum sample (after the blank subtraction) was calculated by comparing the change in absorbance (Δ*A*) values due to the LDH present in the sample to the NADH standard curve, where LDH activity (U/L) is given by the NADH generated per minute divided by the sample volume.

RESULTS AND DISCUSSION

LDH Detection in Paper Well-Plates and Microwell Plates. Initial studies compared the LDH activity value determined by the paper well-plate assays produced without pullulan with the LDH values obtained by the plate-reader method, with the latter taken to be the accurate value. NADH calibration curves were first generated for a series of NADH standard solutions using the paper sensors without pullulan and microwell plates. Following this, serum spiked with LDH was added to the assay reagents on the paper well plate or in microwells, and the amount of NADH generated per minute from the samples was calculated from the intensity of the developed color using the calibration curve. Figure 3A shows

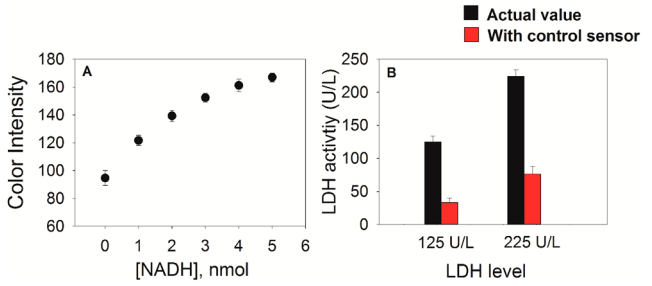

Figure 3. (A) NADH calibration curve using paper-well sensors printed without pullulan. (B) Comparison of the response of the paper-wells printed without pullulan (denoted as "control sensor") to the plate-reader assay (denoted as "actual value") for two samples containing two different levels of LDH (error bars indicate ±1 standard deviation for triplicate measurements).

the NADH calibration curve generated using the paper-based sensors without pullulan (also see Figure S2 for images of the paper well plates). The responses obtained with these paper wells, for two different levels of LDH in serum, were significantly lower than the actual LDH activity obtained using the microwell plate assay (Figure 3B). The low activity measured for the paper-well sensors without pullulan suggests that degradation of one or more of the three labile reagents, namely, NAD^+, INT, and PMS, occurred in the printed reagent mixture. INT and PMS are both quite unstable to temperature and light, respectively, and slowly get converted to a red/purple colored formazan product in the presence of NAD^+.[6] However, we observed that the paper wells did not turn purple over time, indicating that INT and PMS were stable, and thus the NAD^+ likely degraded after immobilization on paper. The cleavage of the nicotinamide ring from NAD^+ at room temperature is well-known, though it is more pronounced in alkaline medium such as that used in our assay (pH 8.9).[21−23]

To avoid degradation of the reagents, we used pullulan as part of the ink formulation, in addition to all the reagents needed to perform the assay. Figure 4A shows that adding

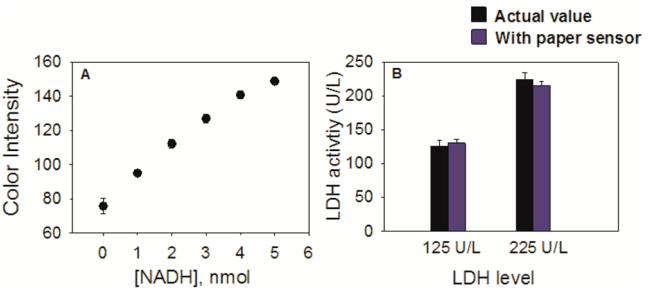

Figure 4. (A) NADH calibration curve using paper sensors where the reagents were printed using a pullulan containing ink; (B) Comparison of the response of the paper-well sensor to the plate-reader assay (denoted as "actual value") for two samples containing two different levels of LDH (error bars indicate ±1 standard deviation of triplicate measurements).

pullulan to the ink formulation produces a lower background signal, but a similar slope for the calibration curve as compared to sensor formed without pullulan (Figure 3A), and improves the accuracy of the paper-well sensor to the point where it is within error of the values obtained from the microwell plate assay (Figure 4B). These results clearly show that pullulan plays a crucial role in protecting the immobilized reagents and in improving the performance of the paper-based sensor. Pullulan films are oxygen impermeable[17] and protect oxygen sensitive reagents when these are entrapped within the films. Once dry, these also provide a protective barrier to hydrolysis. Given that NAD^+ is in the oxidized form, it is likely that the pullulan provided protection from degradation by reducing the exposure of reagents to the surrounding environment, including both water and the cellulose fibers.

Pullulan Location on Paper. All previous studies of pullulan-based reagent stabilization have utilized pullulan bioassay tablets (i.e., pullulan monoliths), which form glass-like materials that dissolve readily when added to water, leading to the rapid release of reagents. There are no previous reports describing the printing of pullulan-containing inks on a paper substrate to form stabilizing films, and thus, it was of interest to determine how the ink was distributed on the paper surface. To determine whether the immobilized pullulan/reagents were present as a thin film on top of the paper surface or instead resided within the pores of paper, we determined the relationship between the film thickness and the amount of pullulan dispensed on a specified area using a previously published method.[24] Using this relationship, it was determined that for the amount of pullulan printed per paper microwell, a pullulan film with a thickness of 62 μm would be formed if all the pullulan resided on top of the paper (see Supporting Information). An optical microscope image of the cross section of the paper sensor wells (Figure 5) shows that there is no significant increase in thickness of the porous Whatman #1 paper (unmodified thickness is 180 μm) when the pullulan

DOI: 10.1021/acs.analchem.5b01923
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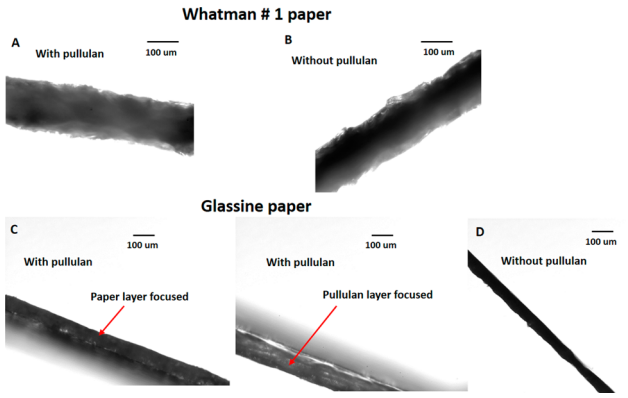

Figure 5. Optical microscope images of the cross sections of pullulan-coated and unmodified paper surfaces. (A) Pullulan printed on Whatman #1 paper; (B) unmodified Whatman #1 paper; (C) pullulan printed on nonporous glassine paper; (D) unmodified glassine paper.

solution was printed and dried (Figure 5A) as compared to the unmodified paper (Figure 5B), while printing of pullulan onto nonporous glassine paper provided an increase in thickness of ~100 μm relative to the unmodified paper (Figures 5C and 5D, respectively). These results show that the pullulan film penetrates into the pores of the Whatman #1 paper, likely filling both macro and mesopores, and that it does not sit on the top of the paper surface. Whatman paper has a porosity of 0.74,[25] and thus we calculated that deposition of 62 μm of pullulan would occupy a thickness of 84 μm of internal pore space, which is much less than the thickness of Whatman #1 paper (180 μm); this calculation shows that the pores can "internally accommodate" all of the pullulan that was printed onto the paper.

Sensitivity of the Paper-Well Sensors. To measure the sensitivity and linear range of LDH detection using the paper sensor, a series of samples (serum replacement) were spiked with different final LDH concentrations and added to the paper-well sensor. The color intensities developed after 5 min of incubation, at each LDH concentration, were plotted against LDH concentration (Figure 6). It was observed that the color intensity changed linearly up to an LDH level of 225 U/L. It should be noted that detection of 225 U/L of LDH is obtained after 2× dilution of the sample with Tris buffer, and thus the

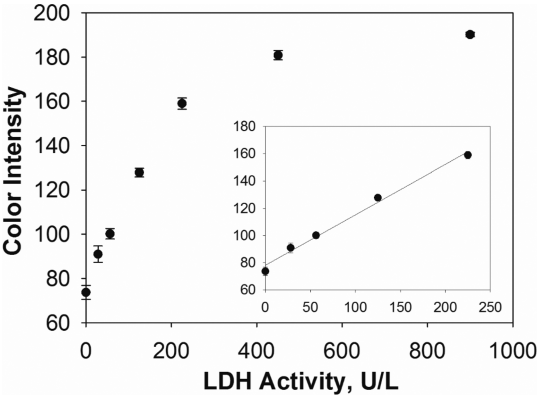

Figure 6. Change in color intensity as a function of LDH activity using paper-well sensors printed with pullulan-containing inks. The inset shows the response of the sensors to LDH activity values over the physiologically relevant range. Error bars indicate ±1 standard deviation of triplicate measurements.

original LDH activity in the serum before dilution was 450 U/L (225 × 2). The dilution factor can be increased to extend the detection to higher LDH levels, which is useful as LDH levels can reach 1000 U/L for some conditions.[1,25] The limit of detection was calculated at 3σ above the background, which corresponds to 13 U/L, which is a much lower than the normal LDH level in human serum (~100−200 U/L).[1,26] On the other hand, the limit of detection using the paper-wells without pullulan was found to be ~80 U/L (Figure S3). These results confirm that the paper sensors without pullulan have two major drawbacks. One is the sensitivity and the other is its inability to detect LDH accurately, as observed from Figure 3. Pullulan-based reagent films address both problems owing to the ability of pullulan to protect the immobilized reagents from degradation, and provide a sensor that has appropriate performance for testing of LDH in clinical samples. Even so, the colorimetric method inherently has false positive reactions under certain conditions, particularly when run under basic pH conditions, as iodonitrotetrazolium salts (INT) will be reduced to formazan in the presence of phenazine methosulfate (PMS) within a few minutes. However, this reaction is much slower than the enzyme catalyzed reaction in the presence of LDH, lactate and NAD^+. Therefore, this will not affect the results if the blank reactions (without LDH) are run as controls.

Stability of Paper-Well Sensors. The stability of paper sensor strips at room temperature (23−26 °C) and at 4 °C was evaluated for the detection of LDH in serum at two different activity levels, one within the normal range (125 U/L) and another out of the normal range (250 U/L). At room temperature, the sensors strips slowly turned purple after 1 week (Figure S4), making measurement of LDH problematic. It was determined that the tetrazolium salt (INT) in the reagent mixture started reducing to formazan when stored at room temperature, even with pullulan present. However, as the background was still relatively low at early storage times, it could be subtracted and thus affected the enzyme detection only after 3 weeks of storage (Figure S5). On the other hand, the paper wells stored at 4 °C did not show any color change even after 5 weeks, and could accurately detect LDH after this storage time (Figure 7). The LDH values are relative (% activity) to the value obtained on day zero. The variation in these values is attributed to the day-to-day variation and the error associated with equilibrating the sensor from 4 °C to the reaction temperature, which could have also affected the value on day zero. Even though there are variations relative to the initial value, our results clearly confirm that the sensor is stable at 4 °C. In solution, the LDH assay mixture was stable for only 1 day, even when stored at 4 °C,[27] showing that the pullulan provides significant enhancements in reagent stability over time. Overall, the above data clearly show that the paper-well sensors have sufficient stability to allow shipping and storage using refrigeration, and can even be shipped and stored at room temperature for a shorter period of time (at least 2 weeks), potentially allowing assays to be done in remote locations with minimal sample handling and cost.

Effect of Reaction Conditions. As is the case for most enzymatic reactions, the colorimetric LDH assay is sensitive to the reaction conditions such as reactant volume, assay time and temperature. The colorimetric readout will vary linearly with volume of reactant and time, as is expected for enzymatic reactions. Thus, it is very important to add a controlled volume of reactant to the paper-based test well. The assay is not sensitive to temperature changes over small ranges normally

DOI: 10.1021/acs.analchem.5b01923
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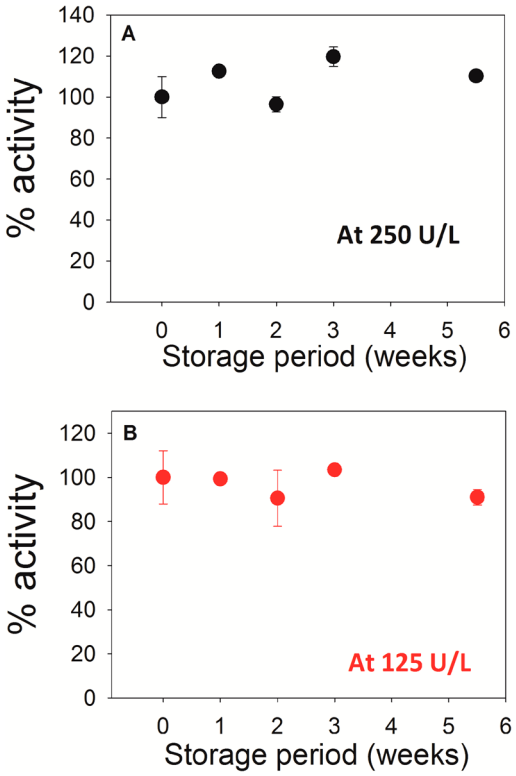

Figure 7. Stability of the paper sensors for the detection of serum LDH at (A) high and (B) low concentrations of LDH for sensors stored at 4 °C. Error bars indicate ±1 standard deviation of triplicate measurements.

found in laboratory settings (±3 °C). For very large changes in temperature (for example, running the test at 37 °C), it would be necessary to obtain a calibration curve under the same conditions.

CONCLUSIONS

In this study, assay reagents were printed within a pullulan-based ink onto paper wells to produce highly stable sensors for LDH activity in serum. All required assay reagents, including the cofactor (NAD^+), substrate (lactic acid) and the color developing agents (INT and PMS) were mixed with pullulan and could be printed onto the paper wells in a highly reproducible manner using an automated bioprinting system, making the production of the sensors amenable to high-speed automated manufacturing. The color development after the addition of LDH could be visualized by the naked eye and quantified by using a digital camera and image-processing software. The LDH levels determined using the paper well-plate sensor were found to be accurate and highly reproducible. The extremely low volume of reagents used in this sensor should significantly reduce the cost associated with serum–LDH detection. The paper-wells could also be stored for short periods (2–3 weeks) at room temperature and for at least 5 weeks when stored in a refrigerator. This eliminates the need for sophisticated lab facilities, for expensive reagent shipment and storage and complex sample handling. As such, the paper-well sensor should be useful for rapid, inexpensive screening of large numbers of samples in resource-limited settings.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01923.

Calculation of film thickness and figures showing reproducibility of printed dots using the biodot printer, image of paper sensor and control sensor, sensitivity of serum LDH detection, images of the sensors strips stored at room temperature for 4 weeks, and stability of the paper sensors stored at room temperature for the detection of serum LDH (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: filipec@mcmaster.ca. Tel: +1-905-525-9140, ext. 27278.

*E-mail: brennanj@mcmaster.ca. Tel: +1-905-525-9140, ext. 20682. Website: www.biointerfaces.ca.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences and Engineering Research Council of Canada for funding through the ***SENTINEL*** Bioactive Paper Network. We also thank the Canadian Foundation for Innovation and the Ontario Ministry of Research and Innovation for Infrastructure funding to the Biointerfaces Institute. J.D.B. holds the Canada Research Chair in Bioanalytical Chemistry and Biointerfaces. R.P. holds the Canada Research Chair in Interfacial Technologies. Y.L. holds the Canada Research Chair in Chemical Biology of Nucleic Acids.

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DOI: 10.1021/acs.analchem.5b01923
Anal. Chem. XXXX, XXX, XXX−XXX