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Printed microwells with highly stable thin-film enzyme coatings for point-of-care multiplex bioassay of blood samples

Liting Zhang,[a,b] Xiaodan Cao,[a] Lu Wang,[b] Xueyan Zhao,[b] Songping Zhang[c] and Ping Wang*[,b]

a. Biomedical Nanotechnology Center, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, P. R. China

b. Department of Bioproducts and Biosystems Engineering, University of Minnesota, Minnesota 55108, United States

c. National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, P. R. China

*Corresponding Authors: ping@umn.edu

Abstract

A paper-based colorimetric biosensor suited for point-of-care bioassay of blood samples is developed using highly stable enzyme thin-film coatings confined within inkjet printed polymeric microwells. The microwells are developed through a simple one-step inkjet printing of hydrophobic polystyrene on paper, with walls formed by the polymer that fills the gaps inside the paper body. The microwells can also be patterned to be interlinked with printed microchannels for multiplex bioassay. Thin film enzyme coatings confined within the microwells are then constructed, thereby constituting biosensors that work in a way like the traditional microwell plates, yet allow easy colorimetric readouts with naked eyes or portable devices such as smart phones. The efficiency of the paper-based sensor was demonstrated for colorimetric assays of glucose and lactate, both as individual analytes or mixed, as well as samples with red blood cells. Such sensors showed good sensitivities within the concentration ranges of the analytes in human blood (0.5-10 mM), with a visible sensitivity of <0.5 mM detectable for naked eyes for a sample size as small as 1 μL. More accurate digital readouts were shown feasible with computerized scanners or smartphones. The thin-film coating format affords the paper biosensors extended lifetime, and could keep 100% performance over 6 months of storage under room temperature, or up to one month heated at 50^{o}C. That promises refrigeration-free storage of the sensor. The simple preparation, high enzyme stability and the easy-to-use feature of the paper-based sensor promise low-cost and reliable point-of-care multiplex bioassay for biomedical diagnostics.

Introduction

Point-of-care assay refers to diagnostic analysis that can be performed at the immediate locations where patient care is given, that way allows clinical management decisions to be made in a timely manner, which is especially critical to environments of limited resources.[1-5] Accordingly, point-of-care assay requires analytical devices to perform quickly and simply without using sophisticated laboratory equipment. This is fundamentally different from traditional lab-based bioassays, which generally require multiple step sample processing and assays with digital readouts taken with instruments operated by well-trained personnel.

Among the different strategies developed for point-of-care assay, paper-based biosensors with data reading using handheld devices are particularly appealing, because of their low-cost and good compatibility with the widely used colorimetric and electrochemical data acquisition and processing devices.[6-10] Recent research in the area has shown a particular interest in developing microfluidic paper devices to allow multiplex bioassays. Whitesides and coworkers presented the preparation of microfluidic paper devices by using a multiple layer patterning strategy, and coupled with colorimetric detection to simultaneously quantify glucose, cholesterol and total protein in biological fluids.[11-13] Yager *et al.* demonstrated the multi-analyte detection with controlled reagent transport in 2D paper network.[14] Dungchai *et al.*[15] also reported a work on paper-based microfluidic devices with three-electrode system for electrochemical analysis of glucose, lactate and uric acid. Nie *et al.* performed an electrochemical analysis of glucose, cholesterol, lactate and alcohol on paper microfluidic devices using a portable commercial electrochemical reader.[16] While most of those works demonstrate well exciting design concepts, some fabrication of these devices required several time consuming and expensive steps, increasing the cost and precluding large-scale manufacturing.[17]

Considering the reliability of bioassay using portable devices, one critical requirement for successful point-of-care assay is the extended storage lifetime of the sensors, especially when exposed to extreme environment conditions. The glucose assay on microfluidic paper sensor developed by Whitesides and coworkers showed decreased signals after 24 h storage at 23^{o}C,[11] most like due to enzyme deactivation. Cha *et al.* also found a quick functionality loss of glucose testing paper strips when they were stored under elevated temperatures such as 50^{o}C.[18] A more recent study on paper-based biosensors using enzymes entrapped in silica gel showed 50% decline over the first eight days of storage under 4^{o}C.[19] Apparently, short-lived and unreliable sensors may not serve the purpose of point-of-care analysis very well for resource-limited environment when the sensor itself requires expensive low-temperature storage and transportation.

Accordingly we seek paper-based sensors with simplified fabrications and enhanced stability suited for refrigeration-free storage. Our earlier studies have shown that enzymes could be extremely stabilized through thin film coatings by confining enzymes into a compatible environment that offers enhanced molecular interactions.[20-21] Inspired from that, we examine the effect of enzyme stabilization by entrapment with soluble starch in form of thin-film coatings, which offer highly compatible environment for enzyme molecules and promote cross-interaction via formation of hydrogen bond. The starch at the same time serves as a chromogen agent on paper sensor. Such an enzyme coating is then confined within planar microwells with polystyrene barriers inkjet printed onto papers. The performance of the sensor and stability of the enzyme coating are examined with respect to various processing and operational conditions.

Experimental

Materials

Glucose oxidase from *Aspergillus sp*. (GOx, EC 1.1.3.4, ≥ 180 units/mg solid) was provided as a gift from Amano Inc. (Japan). Lactate oxidase from *Pediococcus sp*. (LOx, EC 1.13.12.4, ≥20 units/mg solid) and peroxidase from horseradish (HRP, EC 1.11.1.7, Type VI, 250-330 units/mg solid) were purchased from Sigma-Aldrich (USA). Polystyrene (Mw=2000) purchased from Polymer Source Inc. (Canada). Filter paper (Whatman Grade 1, 460 X 570 mm sheets, typical thickness 180 μm, 88 g/m^2, with pore size 11 μm), human red blood cells and bovine serum albumin were purchased from Sigma-Aldrich (USA). Thermo-inkjet printer HP 5600 and cartridge HP 27 was adapted for printing polystyrene to construct hydrophobic barriers on filter papers. D-(+)-glucose, L-(+)-lactic acid, potassium dihydrogen phosphate, sodium hydroxide, butyl acetate, phenol acetate, methyl salicylate, soluble starch, potassium iodide, 4-aminoantipyrine, and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine were obtained from Fisher Scientific Inc. (USA).

Inkjet printing of microwells

HP thermo ink jet printer (office jet 5600) and ink cartridge (HP 27 black) were applied for polystyrene solution printing. The ink cartridge was pre-cleaned and the sponge was restored. Polystyrene solution (typically 4% wt/v) was injected into the cartridge tank, and the refilled cartridge was installed into the printer. The printed bands forming the wells and channels were controlled to be with an inner diameter of 0.16 inches, outer diameter 0.27 inches (a typical layout of the design is shown in Scheme 1). The printed papers were cured in oven at 100^{o}C for 3 h before being used.

Characterization of microwells

The morphology of printed polystyrene barriers on paper was imaged by scanning electron microscopy (SEM, Hitachi S3500N), after platinum sputter-coating with layer thickness 15 Å. The hydrophobic property of barriers constructed by polystyrene printing was characterized via water contact angle measurement. It was measured under ambient conditions by the sessile drop method with the Kruss Drop Shape Analysis System DSA10.[22] Water used was purified to 18 MΩ·cm with a Type II Laboratory Spectra Pure Water System. Typically water drop of 5 μL was gently lowered and attached to polystyrene printed-paper surface from a 100 μL glass syringe. Contact angles were then measured using a horizontal baseline.

Preparation of sensor detection zones

Typically, GOx functionalized starch coating was prepared by first dissolving 0.5 mg GOx and 1.0 mg HRP in 1 mL deionized water. The apparent GOx activity in the mixed enzyme solution was 120 U/mL as determined by using 4-Aminoantipyrine, and N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)-m-Toluidine as color reagent. Meanwhile, soluble starch (2.0 g) was dissolved in 10 mL buffer (50 mM, monopotassium phosphate-sodium hydroxide, pH=7.3) with heating and magnetic stirring. The solution was cooled in water bath at 20^{o}C before adding 40 mg/mL potassium iodide. Enzyme solutions were then mixed with starch solution with a volume ratio of 1:1. The resulted mixture (typically 2.0 μL) was coated into each printed polystyrene microwell and the GOx functionalized paper was dried with in air at room temperature for 24 h before being used. The preparation of paper sensor for lactate assay was similar, except GOx was replaced with lactate oxidase (LOx). For simultaneous testing of glucose and lactate in blood samples, the mixed enzymes-starch solutions were applied in the detection zones.

Glucose and lactate assays

Different concentrations of substrate solution (glucose and lactate) were prepared by directly dissolving proper amounts of glucose or lactic acid in phosphate buffer with addition of 0.9% NaCl, and the pH of final solution was adjusted to 7.3 by using NaOH solution (2.0 M), if needed. Bioassay tests were initialized by adding 1.0 μL substrate solution into the sensor wells, and the paper sensor was placed between two glass slides to alleviate humidity changes. The upper glass slide was removed after 15 min and paper sensor was left to open air for another 15 min to evaporate the residual water that might interfere with color density readings. Paper sensors were then scanned with a photo scanner (HP 5600, color photo setting, 1200 dpi resolution). The resulting photo was then converted into gray scale and analyzed by using image-J software.

Thermo stability of paper sensor

The bioactive papers were cut into a size of 1.5 × 1.5 cm with the polystyrene wells located in the center, then sealed in plastic bags and incubated at desired temperatures. The paper sensor samples were periodically removed and cooled to room temperature, were then tested with the application of 1 μL of substrate solution (5.0 mM glucose or 2.0 mM lactate). The testing procedure was the same as mentioned above. The thermo stability of paper sensor was evaluated by relative color intensity with the data for fresh samples before heated incubation taken as 100%.

Simultaneous assay of glucose and lactate in artificial blood

Artificial plasma was prepared according to PN-EN ISO 10993-15 standard (g/L in distilled water): 6.8 NaCl, 0.2 $CaCl_2$, 0.4 KCl, 0.1 $MgSO_4$, 2.2 $NaHCO_3$, 0.126 Na_2HPO_4, and 0.026 NaH_2PO_4. Artificial blood samples were prepared by using the pre-prepared artificial plasma with addition of glutaraldehyde pre-treated human red blood cells (4.0%, wt/v), bovine serum albumin (65 mg/ml), and required amounts of glucose and/or lactic acid. Aliquots of the artificial blood sample (typically 5.0 μL) were pipetted into the sampling zones of the double-channel paper sensors for analysis with the final color intensity detected in the detection zones designed for glucose and lactate.

Results and discussion

Inkjet printing of microwells and dispersion of polystyrene inside paper support

As paper is hydrophilic and allows quick dispersion of aqueous samples, it is necessary to develop hydrophobic barriers that will confine the sample (and assay agents) within certain region for assay reactions, or direct the sample to desired locations. Such a confinement could not only enable the paper sensor for multiplex assays, but also improve the sensitivity with application of small amounts of samples.[23-25] Polystyrene is chosen in the current work for its excellent compatibility and solubility in a variety of solvents that allow optimization of the solution properties for optimized printing effect. Whatman grade 1 filter paper with a medium retention size about 11 μm, which is close to the diameter of human red blood cell (7-9 μm) and thus can trap and fix the cells in the sample region, was selected as the support in this study.[26]

To print polystyrene microwells on paper, polystyrene solution needs to penetrate through the

fiber matrix in the printed areas, thereby forming hydrophobic walls once solidified inside the paper that will ultimately contain the liquid samples and assay agents applied to the encircled volume. That requires proper selection of solvents for polystyrene. Indeed, solvent selection is also required to make printable inks for printing on paper surfaces. Ideally the physical properties of the polystyrene solution should match that of regular inks. Surface tension and viscosity are considered two critical parameters for ink preparation. The ideal surface tension is about 25-50 mN/m, and 1~9 cP for viscosity at room temperature for HP cartridge printers.[27] Accordingly, several organic solvents including methyl salicylate, phenyl acetate and butyl acetate that have properties close to that range were selected and examined to formulate polystyrene solutions. Fig. 1 shows surface tension and viscosity of these polystyrene solutions. As a result, both methyl salicylate and phenyl acetate could afford polystyrene solutions with properties in the range required for printable inks, while butyl acetate solutions fell a bit outside the range (low viscosity) when polymer concentration was below 2%.

Polystyrene solutions were then examined for printing effect. All the polystyrene inks prepared with three different solvents produced prints of smooth appearance. When examined with SEM images (Fig. 2), the prints with methyl salicylate and phenyl acetate polystyrene inks generated uniformly distributed coatings on surface of the paper (Fig. 2B and 2C), indicating that majority of the polymer was on the outer surface of the paper and did not penetrate. By contrast, the printing with butyl acetate showed no apparent polymer accumulation on the surface (Fig. 2D), indicating good penetration and distribution of the polymer. Our further tests showed that repeating the printing for 2 or 3 more times with butyl acetate (with a polymer concentration of 4%) would eventually deliver enough polymer material to fill up the open gaps between the

paper fibers, and thereby forming the walls of the microwells inside the paper body. The wettability of the printed area, another factor in addition to void filling contributing to the desired confinement of aqueous samples, was then examined. The water-contact angle obtained from methyl salicylate and phenyl acetate printings was 120 ± 4° and 113 ± 4° respectively (inserts of Fig. 2B and 2C), while that for butyl acetate was 99 ± 3° (insert of Fig. 2D). Since the polymer applied is the same for all the three printings, the higher hydrophobicity of the former two printings further indicated the formation of polymer coatings on surface of the paper instead the desired dispersion inside the paper. Accordingly, butyl acetate was selected as the ideal solvent for preparation of the microwells.

Coating formation and distribution of enzyme

To prepare stable enzyme coatings, water-soluble starch is selected. Our earlier studies have shown that enzymes could be stabilized by over 3 orders of magnitude through thin film coatings by confining enzymes into a compatible matrix, with a molecular level dispersion, that offers enhanced molecular interactions.[20-21] By dissolving the enzyme and starch into the same solution for coating, we may generate similar molecular level entrapment layout, different from those realized with less compatible materials where enzyme clusters are generally expected. The hydroxyl groups of the starch may promote cross-interaction with enzyme molecules via formation of hydrogen bond once solidified, further stabilizing the enzymes.[28] Similar molecular interaction mechanisms were also envisioned for enzyme and synthetic polymer systems[29] and single cage entrapment of enzymes with mesoporous silica material.[30] In order to evaluate the morphology and distribution of the enzyme coatings, a fluorescein isothiocyanate (FITC) labeled bovine serum albumin (BSA) protein was first examined as a model enzyme. Fig. 3A shows the

physical appearance of such starch-protein coatings confined within the printed polystyrene microwells, with the middle coating applied with a drop of water (50 μl) to show the confining effect of the microwell. Images of the coatings obtained using laser scanning confocal microscopy are shown in Fig. 3B-3D. Starch coating without protein did not show obvious fluorescent signal (Fig. 3C), and appeared to be the same as that of blank paper (Fig. 3B). Fig. 3D shows the morphology and distribution of the protein-containing coating, with protein molecules well distributed throughout the starch film matrix, filling up the gaps between fibers of the paper body.

Sensor performance and stability

Sensors were prepared by coating enzyme-starch solutions containing different amounts of GOx or LOx onto encircled zones of the printed microwells. A secondary enzyme responsible for color development, HRP, was also applied along with the oxidases. Sensor performance was examined with glucose and lactate samples of known concentrations to evaluate the effects of quantity of key components (Fig. 4). Both the glucose and lactate sensors appeared to be sensitive to small amount of sample solution (1 μl), showed clearly visible color intensity variations corresponding changes in analyte concentrations (typically with an incremental of 0.5 mM). Color intensity digital readings using computerized scanner and image-J software gave more accurate readouts (Fig. 4B and 4D). Since the applied enzymes function mostly in an immobilized mode with activities much lower than those in an aqueous solution, sensors prepared with different amount of enzymes were examined. It appeared that, for glucose sensor, the addition enzyme above 6 mU/well would have the sensor became insensitive to the content of enzyme (Fig. 4A and 4B). For the lactate sensor, it showed similar observations in that 3

preparations with enzyme loadings above 12 mU/well gave similar color readings throughout the test lactate concentration range (Fig. 4C and 4D). The sensitivity appeared to be high in the low concentration range, and both glucose and lactate assays showed good linearity with the correlation coefficients higher than 0.99 for the range of 0.5 to 3.0 mM, which is ideal for monitoring lactate in normal human blood.[31] It is also noticeable that the ideal linear range can be adjusted by controlling the amount of enzyme. For example, a linear calibration appears in the range of 5-15 mM glucose when the enzyme is applied at a level of 1.5 mU/well, suited for diabetes patients with blood sugar level > 7 mM. The effects of other ingredients essential to the sensing reactions were also examined in a similar way to ensure the sensor performance is not affected by the availability of the reacting agents.

To examine the stability of the sensors, both glucose and lactate sensors were evaluated with respect to storage stability under temperature-controlled environment. As a result, the glucose sensor showed no loss of color sensitivity after 30 days of incubation 50^{o}C, giving the same performance as the fresh sample, as well as those stored at 4 and 23^{o}C (Fig. 5). Our tests with samples stored at room temperature for extended times showed, excitingly, no sensitivity loss after 180 days of incubation. Apparently this is a much better improved stability in comparison to other reported enzyme-based sensors. The paper sensor prepared with sol-gel entrapped enzyme lost 10% sensitivity within 1 day;[17] while the paper sensor prepared by directly adsorbing GOx on cellulose paper quickly lost majority of functionality within 1 day at room temperature, or 6 h of incubation at 50^{o}C.[11,18] Similarly, the lactate sensor prepare in this work also showed high stability, with performance retained 100% over 30 days when stored at 4 and 23^{o}C, or 12 days at 50^{o}C. The lactate sensor eventually lost 50% of its sensitivity after heated 30

days at 50°C. That is less stable than the glucose sensor, presumable due to the low stability of lactate oxidase in comparison to glucose oxidase, since other ingredients applied in the sensors are essentially the same. However, this still showed similar magnitude of stability improvement when compared to other lactate sensors reported previously.[32] Overall, the high sensor stability afforded by the thin film coating approach demonstrate the feasibility of refrigeration-free storage of the sensors.

Simultaneous assay of glucose and lactate of artificial blood sample

To demonstrate the feasibility of preparing sensors for multiplex bioassay, microfluidically channeled double-well sensor was designed. As indicated in Scheme 1, the sensor has two detection zones located at the ends of the paper sensor, and one sampling zone located in the center. The sensing and sampling zones are linked with printed microchannels that can guide the flow of liquid samples driven by capillary force. One improvement from the single well sensor was the application of potassium iodide in the sampling zone instead of the sensing zone. It is assumed that potassium iodide in sampling zone can be quickly dissolved in liquid samples and moved into the detection zones along with the targeted analytes through microchannels. We found that this process improved the color uniformity in detection zones, and thus improved the overall color intensity, mostly as that may exclude the background color generated from the specific conjugation of starch and iodide. The artificial blood sample was prepared by mixing desired amount of analytes, salts, proteins and red blood cells. Aliquots of 5 μl of such sample were applied to the sampling zone. While solvable components migrate with the liquid through the channels to the detection zones, red blood cells will be trapped within cellulose fiber matrix and get retained in the sampling zone. As shown in Fig. 6A, red blood cells retained well within

the sampling zones (center spot of the sensor), while color development was evident in the detections zones. The microchannels remained invisible, further indicating no migration of the cells and the color developed in the detection zones corresponds to targeted analytes.

The calibration curves for the simultaneous determination of glucose and lactate in artificial blood samples are showed in Fig. 6B. The curves for glucose and lactate were basically overlapped, most likely due to the complete of the reaction given by the current design, thereby same amount of analytes produced similar color intensity. The slope of the calibration curve in the linear range (0.5 – 3.0 mM) was 29.2. The sensibility may not as ideal as those achieved with electrochemistry- and nanotechnology-based biosensors assisted with bench-top equipments;[33-36] however, the easy color readouts as well as the high stability can afford the paper sensor great practical usability as a point-of-care analytical device.

As mentioned earlier, one significant advantage of such paper sensors for point-of-care bioassay was compatibility with handheld devices. To demonstrate that, we also tried readouts using a smart phone camera, in a similar way as demonstrated by Delaney *et al.*[37] Reading points were randomly picked up in each detection zone, with outputs set in different colors (red, green, blue, termed herein as RGB reading) were taken and calibrated to analyte concentrations (Fig. 6C and 6D). Obviously, the calibration curves developed from these RGB reading values showed very good linearity over the range of detection, although larger error bars than the results from image scanning as a result of variation in reading point selection, are shown as expected. Due to the color nature developed for the sensor, green and blue readouts showed better sensitivity than red readings. Overall, such a sensor is readily adaptable to smartphone devices, which can ultimately form information and communication networks for remote data sharing and diagnostics. Such an

analytical approach should be ideal for remote and mobile diagnostic applications, particularly in the developing world.[38,39] Toward that, more accurate and powerful reading tools via handheld devices are becoming increasingly available.[40,41]

Conclusions

The current work showed that printed polystyrene microwells could form efficient confinement zones and microfluidic channels for simplified preparation of paper-based biosensors. With the assistance of computer printing software, patterned multiplex bioassay paper sensors can be prepared. The sensor requires only small amounts of sample solution, yet offer usable sensitivity for biomedical diagnostic applications. The starch-based thin film coating developed in this work efficiently stabilized the enzymes, substantially enhanced the reliability of the sensor for extended shelf life, and promised refrigeration-free storage. The color development in correspondence to changes in analyte concentration could be detected by naked eyes with a sensitivity of <0.5 mM, while digitalized reading with computerized images including smartphone readings can provide more reliable and sensitive analysis.

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Captions of figures

Scheme 1. Scheme of double-channel patterned microwells for simultaneous detection of glucose and lactate (Unit is inch).

Figure 1. Physical properties of polystyrene solutions with different organic solvents. (White-fill markers refer to surface tension and black-fill markers refer to viscosity (Diamond: methyl salicylate, Square: phenyl acetate, Circle: butyl acetate)).

Figure 2. Scanning electron microscopic (SEM) images of printed polystyrene barriers with different solvents. A: Blank paper; B: Printing with methyl salicylate; C: Printing with phenyl acetate; D: Printing with butyl acetate. Insets: images of water drops for contract angle measurement of the prints.

Figure 3. Morphology of enzyme coatings within the printed microwells. A: Physical appearance of the coatings, with a water drop (50 μl) applied to the center coating; B: Laser scanning confocal microscopy of blank cellulose paper; C: Starch coating without protein; D: Starch coating with FITC-BSA protein.

Figure 4. Sensor performance as a function of enzyme loading. A: Color development for glucose assay; B: Color intensity reading for glucose assay with different enzyme loadings (Diamond: 1.2 U/mL, Square: 6.0 U/mL, Triangle: 12.0 U/mL, Circle: 24.0 U/mL, Cross: 120 U/mL); C: Color development for lactate assay; D: Color intensity reading for lactate assay with different enzyme loadings (Diamond: 12.5 U/mL, Square: 25.0 U/mL, Triangle: 50 U/mL).

Figure 5. Sensor stability as a function of temperature. A: Glucose sensor; B: Lactate sensor; (Square: 4^{o}C, Triangle: 23^{o}C, Cross: 50^{o}C)

Figure 6. Sensor performance for simultaneous glucose and lactate assay in artificial blood sample. A: Color development in detection zones; B: Calibration curves for simultaneous glucose and lactate assay (Diamond: Glucose, Square: Lactate); C-D: Calibration curves for simultaneous glucose and lactate assay using smart phone (Diamond: red value, Square: green value, Triangle: blue value).

Scheme 1

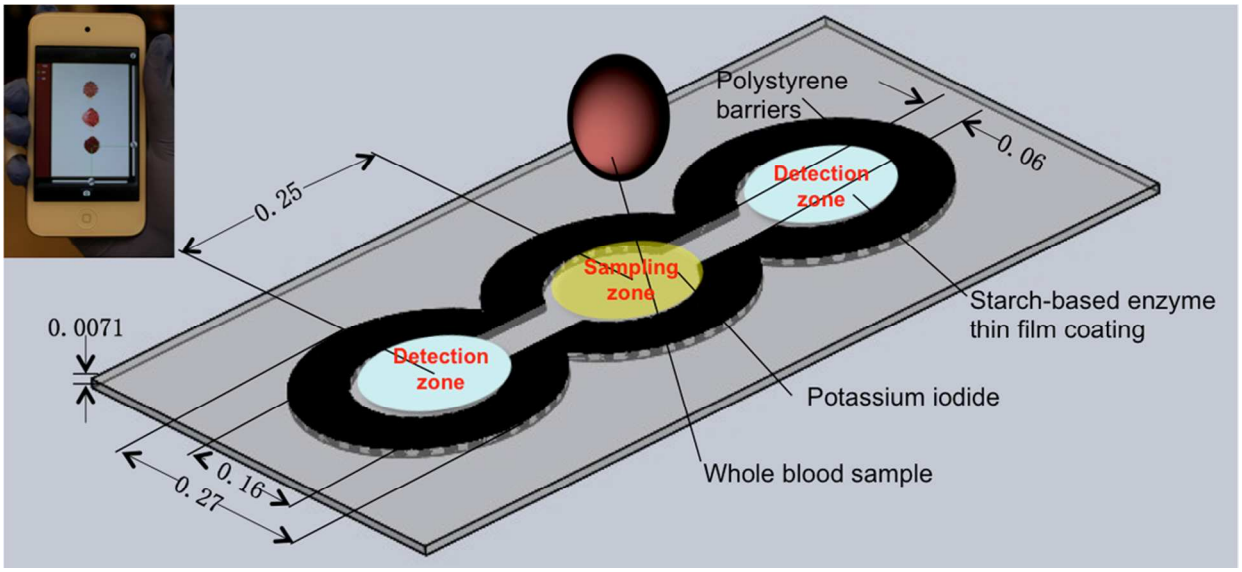

Figure 1

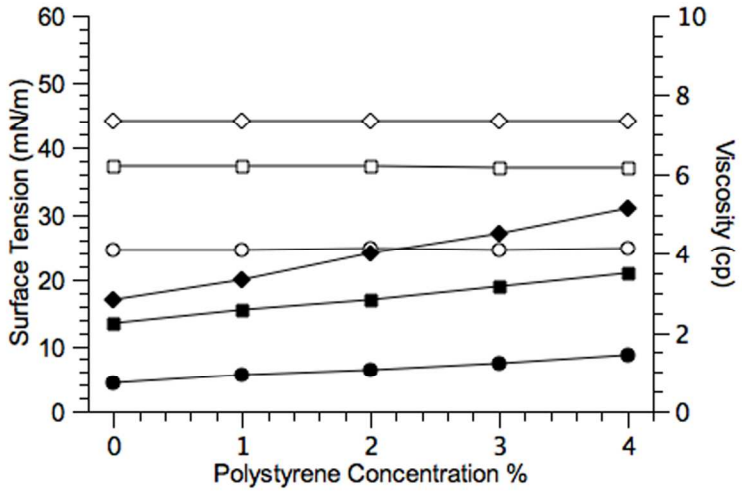

Figure 2

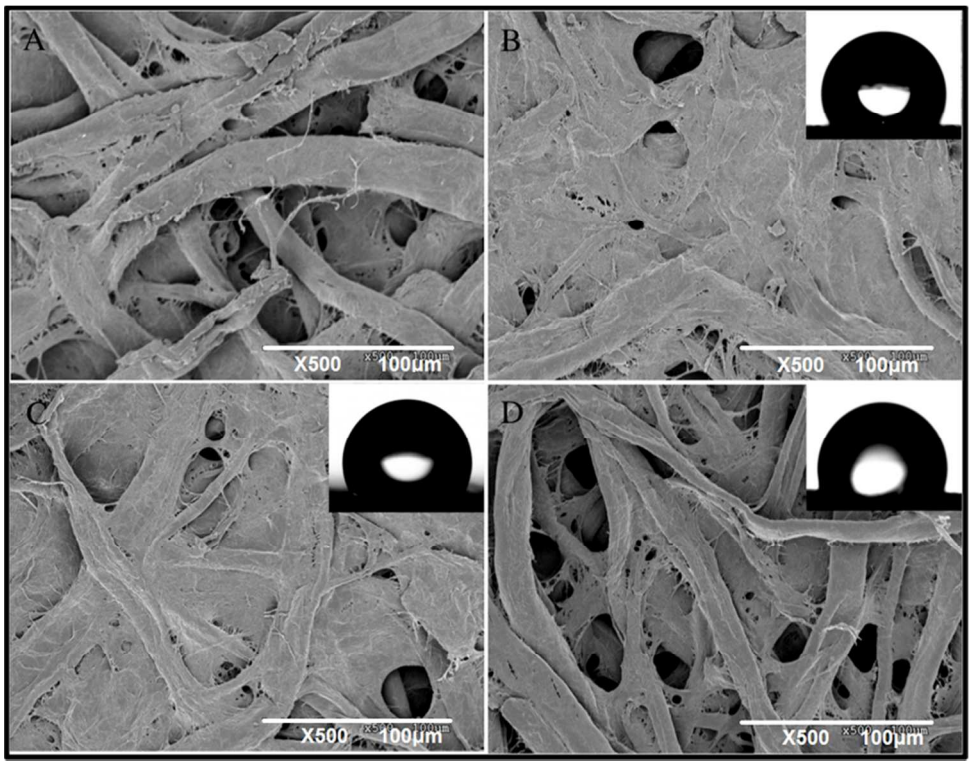

Figure 3

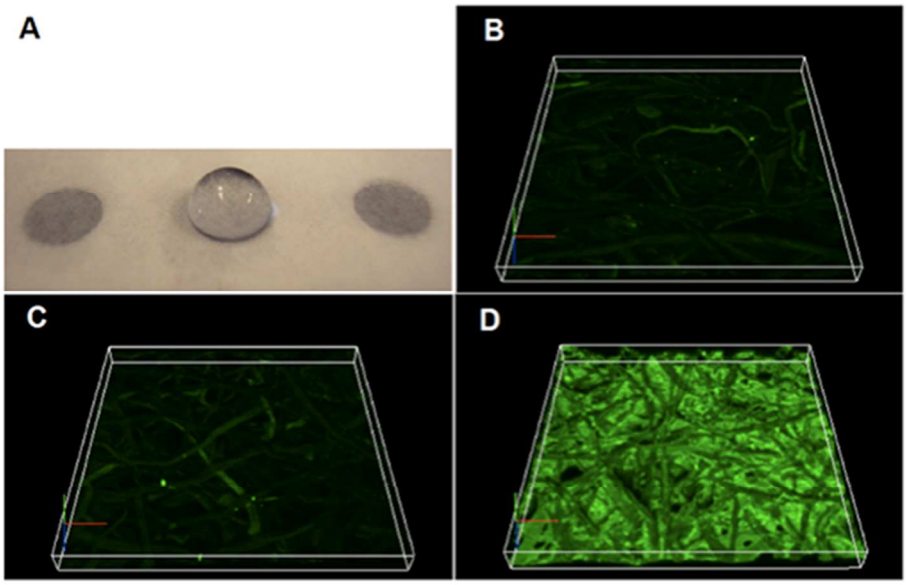

Figure 4

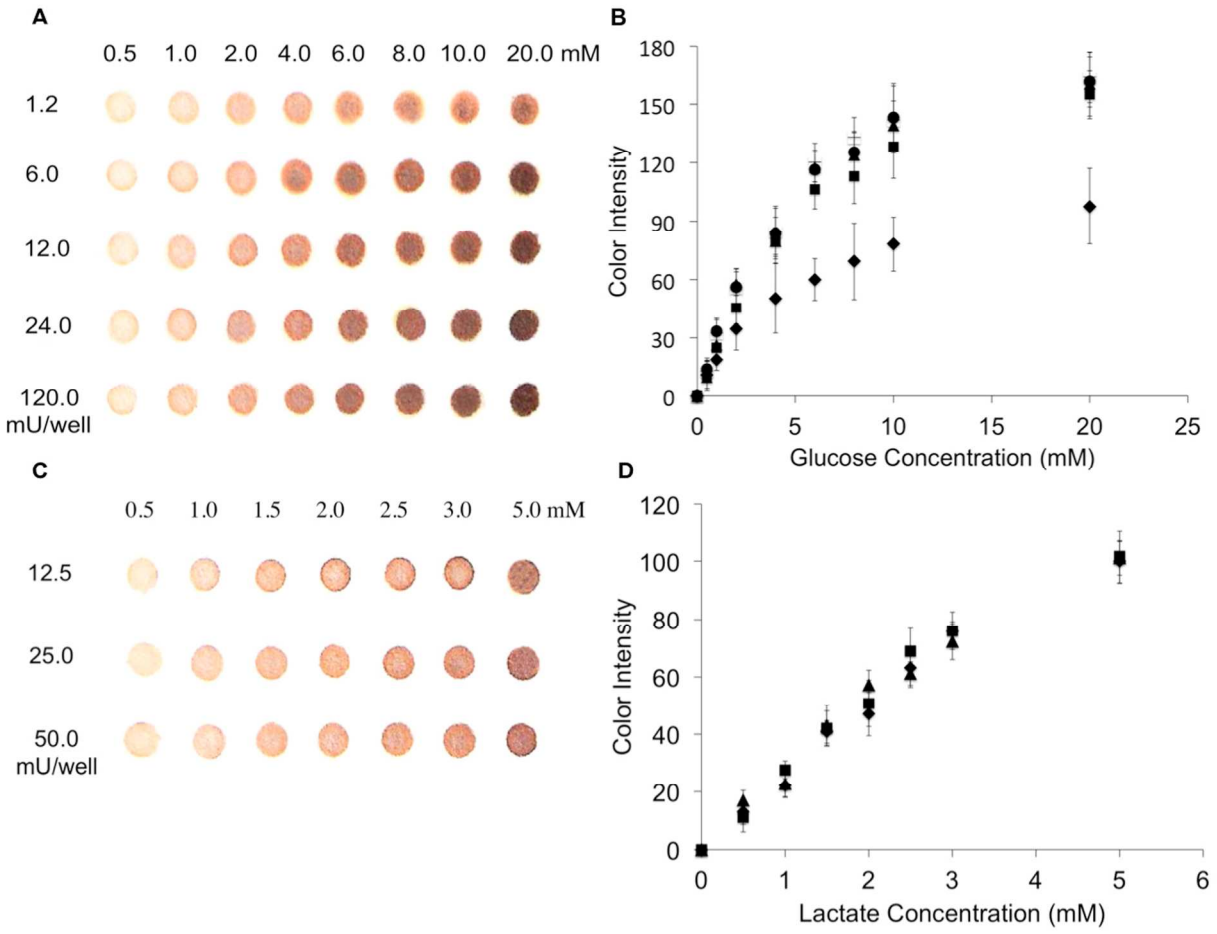

Figure 5

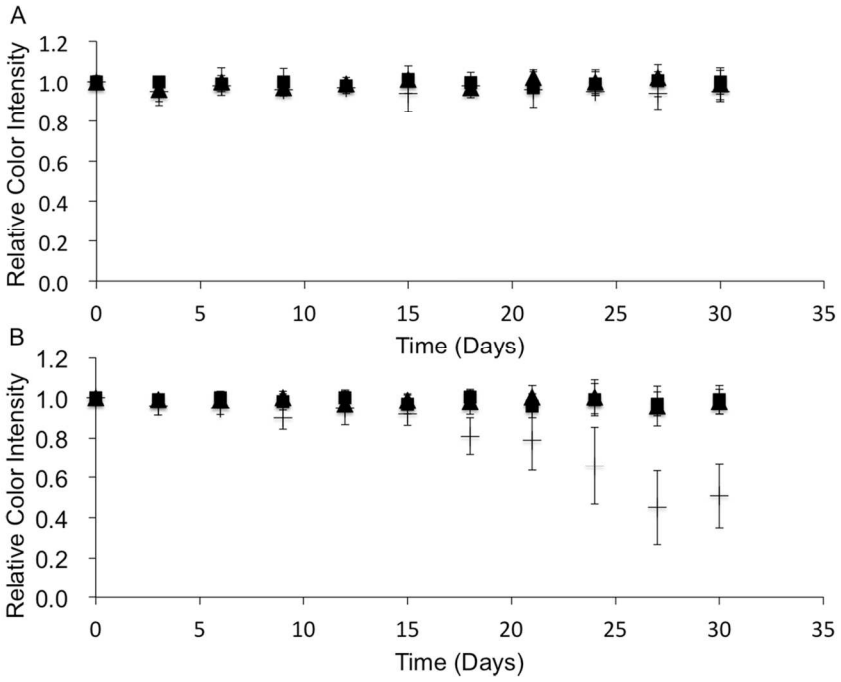

Figure 6