Inquiro, the UAB undergraduate science research journal

Volume 2012 | Number 6 Article 21

2012

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research paper

Development of Dot Array Biosensor using Dip-Pen Nanolithography of Polyacrylamide Ink

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Abstract

The general goal of this project was to develop an acrylamide-based biosensor that may be used to capture and detect biomolecules, such as the cardiac protein troponin T. This paper demonstrates the ability to print nanodots of polyacrylamide hydrogel on a silicon dioxide substrate using dip-pen nanolithlography. Solutions of 3-30%T (w/v) acrylamide monomer were polymerized by adding 3%C (w/w) N,N'-methylene-bis-acrylamide. The addition of polyethylene glycol into the polymer solution resulted in greater stability of the nanodots. Optimal printing conditions were determined by considering the visible gelation time, initial and final viscosity, and porosity of polyacrylamide gel. Individual dots were examined using optical and atomic force microscopy. Fluorescent nanodiamond particles were incorporated into microdots of polyacrylamide on glass slides. It is anticipated that the captured protein will result in a variation in fluorescence, which can be measured to determine the concentration of protein detected. The presence of nanodiamond was confirmed by Raman spectroscopy and fluorescent microscopy.

Introduction

Elevated levels of cardiac troponin T within the bloodstream is associated with heart failure. Detection of high levels of troponin T is currently considered the golden standard to diagnose patients who have experienced symptoms of a heart attack or other cardiac injury [1]. Currently, many available troponin test immunoassays use antibody-antigen bonding, which is constrained by poor stability, high cost, and lack of portability. The purpose of this research is to create a biosensor from the polymer polyacrylamide. Polyacrylamide gels are predominately used for gel electrophoresis, which is a common method for separating proteins, and are nontoxic, water-absorbent, and nonreactive toward proteins [3].

The biosensor would use a molecularly imprinted polymer (MIP) approach for protein capture. For this, the polymer is constructed to have specific recognition sites for a certain template molecule, which would be troponin T [4]. To improve access to the recognition sites on the MIP, the polymer will be printed as nanoscale dot array using dip-pen nanolithography (DPN). A cantilever with a DPN tip that is roughly 100 µm across is dipped into an "ink," which can then be printed in any pattern on a substrate [5]. A biosensor printed in the form of a dot array using DPN will allow for a high surface area-to-volume ratio, which will facilitate the binding of protein into the recognition sites. The concentration of protein captured will be measured using fluorescent nanodiamond (ND) particles incorporated uniformly within the polymer. The captured protein will lower the fluorescence of nanodiamond, which can be calculated to determine the amount of protein.

This paper focuses on the work involved in determining the optimal chemical ratios and conditions for printing polyacrylamide hydrogel dots on a silicon oxide substrate using DPN. The variables considered include chemical concentrations, gelation times, viscosity, and porosity of the final polymer created both with and without the addition of ND.

Materials & Methods

Polyacrylamide gels were synthesized by a radically initiated vinyl addition polymerization using the following reagents: acrylamide, N,N'-methylene-bis-acrylamide (bis), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), polyethylene glycol 8000 (PEG), 2 wt% nanodiamond (ND) (aq); (0-0.2 µm and 0.35-0.5 µm), and Milipore water [3]. For initial experiments, polymer concentrations ranging from 3-30%T (=100*grams bis + acrylamide / mL water) with 3%C (=100*grams bis / grams bis + acrylamide) were analyzed to observe initial viscosities and changes in onset of polymerization. Gel volumes of 5 mL, 1 mL, and/or 0.5 mL were created in 5 mL capped glass vials by adding the appropriate amounts of monomer, initiator, and catalyst into solution.

To prepare monomer solutions, acrylamide and bis were dissolved in water. Both monomer and 15% (wt/vol) APS solutions were prepared fresh daily to prevent chemical degradation and degassed in a vacuum dessicator for 15 min at 200 torr to remove excess oxygen from the environment. Initiator solution and catalyst were pipetted directly into the monomer solution in the ratio of 1 mL water: 5 µL APS solution: 1 µL TEMED. Visible gelation times were measured from the addition of TEMED until the solution exhibited resistance to movement when vial was shaken.

For DPN, roughly 1 µl of gel solution was placed in the appropriate inkwell. A DPN tip was dipped into the ink, blotted on the substrate 2-3 times, and then printed on a silicon dioxide (SiO_2) substrate. The dots were printed in grids of 6 dots by 6

dots, where the dots were 11 µm apart with a 1 second dwell time. The DPN chamber was flushed with nitrogen gas to remove oxygen from the environment when printing.

viscous. To imitate the DPN printing process, 0.2 µl of gel solution was pipetted as dots with roughly 5 mm diameters onto glass slides. However, when dots of 15%T and 3%C pre-

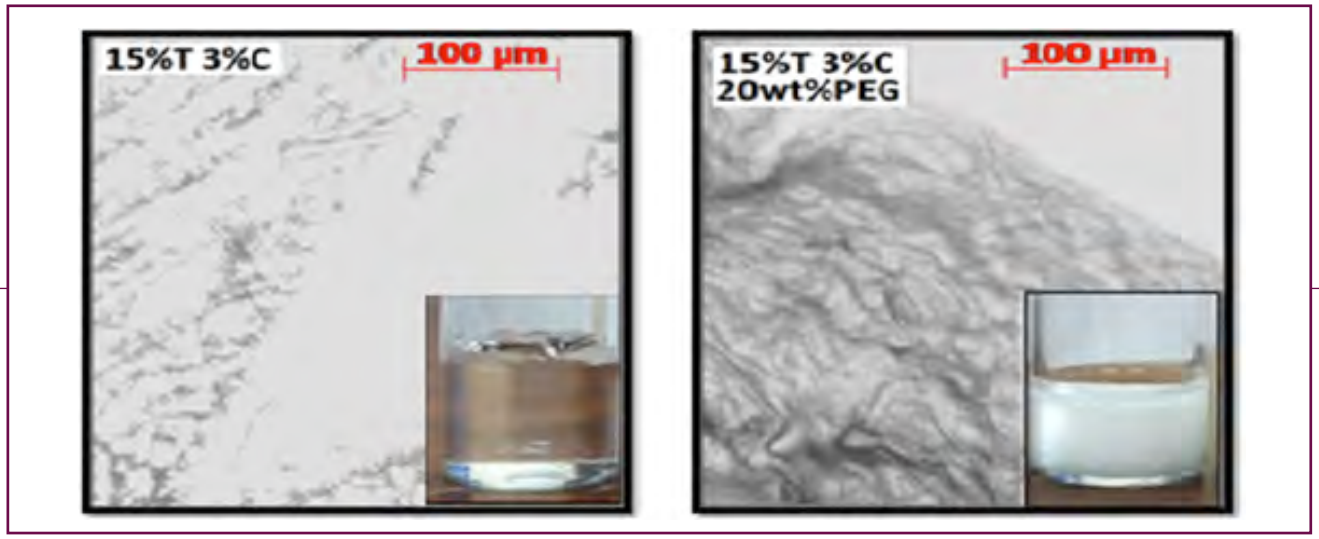

Figure 1. Images of polyacrylamide gel dots on glass slides: 15%T 3%C (left), 15%T 3%C 20wt% PEG (right)

Results & Discussion

Onset of Polymerization

Various %T and %C gels were created based on monomer concentrations from Ahern & Garrell (1988) that formed clear polyacrylamide gels and showed visible gelation within 15 min following the addition of TEMED [6]. Gels created from 3-8%T were found to have a soft, stringy consistency that would not be rigid enough to hold an MIP site. Gels created with 30%T 8%C released excessive heat during the polymerization process and created patterns of white swirls throughout the gel due to a high concentration of bis within the gel [7]. Based on the appearance and gelation times of polymer prepared with various concentrations of monomer, it was believed that a 15%T 3%C gel would be clear, rigid, and stable enough to provide the necessary gel flexibility for imprinting.

The main concern regarding printing polyacrylamide was that before polymerization, the solution would not be viscous enough for DPN printing, and following polymerization, it would be too

polymerized gel solution were created, they lacked the stability to hold their original positions on the slides. Increasing the viscosity of the gel solution was believed to help the dots remain in their pipetted locations. The porogen PEG was added into the gel solution to increase the initial viscosity of the pre-polymerized solution. The addition of PEG would be beneficial for the biosensor as well, since its formation of pores within the gel would allow for greater access to MIP sites.

Gels created with 5-20 wt% PEG showed a higher initial viscosity allowing for greater stability of polymer dots with increasing concentrations of PEG. The viscosity of pre-polymerized gel solution of any %T and %C solution was determined to be approximately 0.84-1.12 cp, which was not viscous enough for printing by DPN. As shown in Figure 1, the polymerized gels with 20 wt% PEG had a rough surface structure and a white color. The gel without PEG did not fully polymerize because the water quickly evaporated, causing the acrylamide and bis to crystallize out of solution. The white PEG powder dissolved

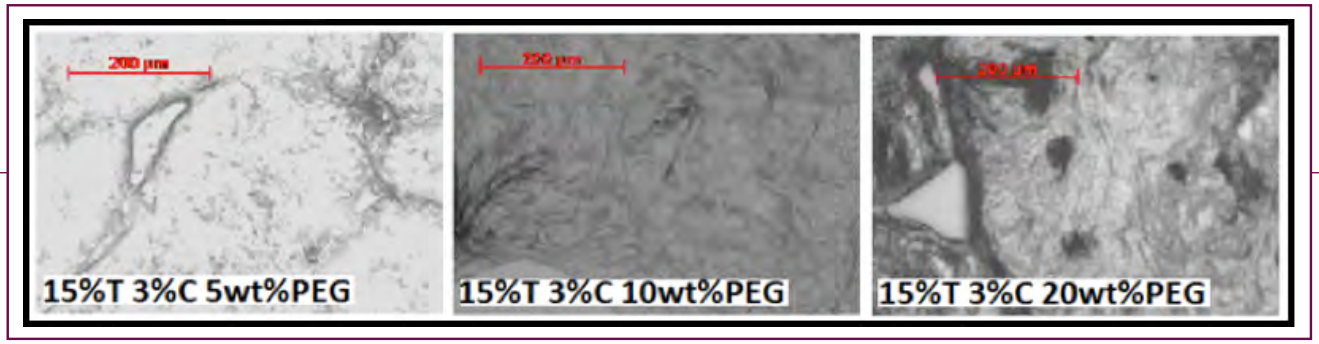

Figure 2. 15%T and 3%C dots with 5-20 wt %PEG. The gel with 20 wt% PEG showed a well-defined and stable structure on the glass slide.

clearly into the monomer solution; however, at the moment of visible gelation, the PEG re-crystallized out of solution and became caught within the gel structure, turning the polymer a bright white color. Pre-polymerized solutions of 20%T and 5%C solution with 20 wt% PEG and 0.1 wt% ND were later measured to be 25 cP, which were viscous enough for printing.

It was known that for DPN printing, longer gelation times are necessary, since the printing process takes a minimum of 20 min. To accomplish this, reduced amounts of TEMED were added to the gel, and the visible gelation was timed. It was noticed during the first successful DPN printing attempt that the dots began to evaporate as time passed. To prevent this, 20%T and 5%C gels were used to allow a stronger, more rigid gel to be formed [3]. A set of experiments with gel created with decreasing amounts of TEMED were performed and lower amounts of TEMED resulted in longer visible gelation times.

Printing Polyacrylamide Inks

For the first DPN attempt, a 15%T and 3%C solution consisting of only acrylamide and bis in water was used. It was tested without the addition of APS or TEMED to avoid the solution polymerizing during the printing process. However, by the time the tips were raised, brought to the substrate, and lowered, the ink had evaporated from the surface of the tip and did not print. Instead a crumbly material of acrylamide and bis, which had crystallized out of solution, fell from the tip on to the substrate. Next, the addition of PEG was considered, since gels created with PEG showed a higher viscosity. Solutions of 15%T and 3%C with concentrations ranging from 5-20 wt% PEG were created. Appropriate amounts of 15% (wt/vol) APS and TEMED were added, and then the solution was immediately pipetted onto glass slides to imitate a large-scale system of DPN dots on the SiO_2 substrate. Images taken of the pipetted dots following polymerization are shown in Figure 2. The 5 wt% and 10 wt% PEG dots tended to slide from their pipetted location on the glass slide before the onset of gelation. Only the 20 wt% PEG solution remained in the exact position where it was pipetted and showed a well-defined surface structure.

A solution of 15%T and 3 %C with 20 wt% PEG and the appropriate amount of 15% (wt/vol) APS was printed. During printing, the ink remained on the tip when it was moved to the substrate and the solution printed well. The printing process was well-controlled and produced similarly sized dots. Close to 252 dots or 6x6 squares were printed before it was necessary to re-dip the tip. Three days after printing, AFM images of the surface of the dots were taken, and as shown in Figure 3, the defined surface structure of roughness of a single DPN printed dot shows that some polymerization had occurred.

Immediately after the printing process, optical microscopy images of the 15%T and 3%C DPN dots confirmed that the dots held their printed positions and were printed with uniform consistency. Figure 4 shows a square of 36 dots a few hours following printing. When the same dots were examined two weeks following printing, it was noticed that the dots had moved slightly from their original printed locations. Additionally, smaller dots or some sort of debris had accumulated around the printed dots. It was visually determined that the diameters of the dots had decreased, possibly due to the evaporation of the water leading to incomplete polymerization.

It was noticed that 0.2-µl dots of polyacrylamide of 15%T and 3%C with 20 wt% PEG on glass slides were not fully polymerized as was earlier believed based on their defined surface structure. However, when a drop of Millipore water was added directly on top of the dot, the entire dot immediately dissolved. This lack of polymerization was attributed to the evaporation of water from the monomer solution when it was originally dotted onto the glass slide. The gels created inside closed vials fully polymerized and did not dissolve in the presence of water. A solution with higher concentration of acrylamide and PEG will help to prevent the water evaporation and lead to the formation of a more rigid gel structure [3].

Addition of Nanodiamond

Nanodiamond (ND) was then added to the printing solution to determine its affects on the gel. The ND solution that was pipetted directly into the monomer solution contained 5 carats in 50 mL water. The nanodiamond size was between 0.25-0.5 µm, with an average diameter of 0.375 µm. The addition of ND turned the gel a white color, since the ND solution itself was white and did not dissolve into the monomer solution but was instead uniformly dispersed. Gels created with 0.2 wt% ND were found to exhibit delayed gelation time by 15 min, impeding the polymerization reaction. As seen in Figure 5, 0.2 wt% ND within the polyacrylamide gel was seen as small dark spots using optical microscopy. When focusing the microscope, the ND on the surface of the gel would reflect white, while the ND further within the gel would appear black.

Optical microscopy images were taken of polyacrylamide dots pipetted onto a glass slide to determine if the ND within the gel would fluorescence (470 ± 20 nm excitation, 525 ± 25 nm emission). In Figure 6, it is shown that ND, without silicon-vacancy defects, readily fluoresced. The fluorescent locations matched several of the black and white flecks of ND in the brightfield image. This confirmed that ND did indeed fluoresce and additional ND particles that were not visible under the brightfield image could be observed through the emission filter. Polyacrylamide ink with ND was attempted to be printed by DPN. A concentration of 20%T and 5%C was used with 20 wt% PEG and 0.1 wt% ND. The higher concentration of monomer and crosslinker was used to prevent water from evaporating as quickly from the inkwell and the printed dots. Both TEMED and APS were added to the ink so that a more fully polymerized gel could be formed. To delay the onset of polymerization, only a

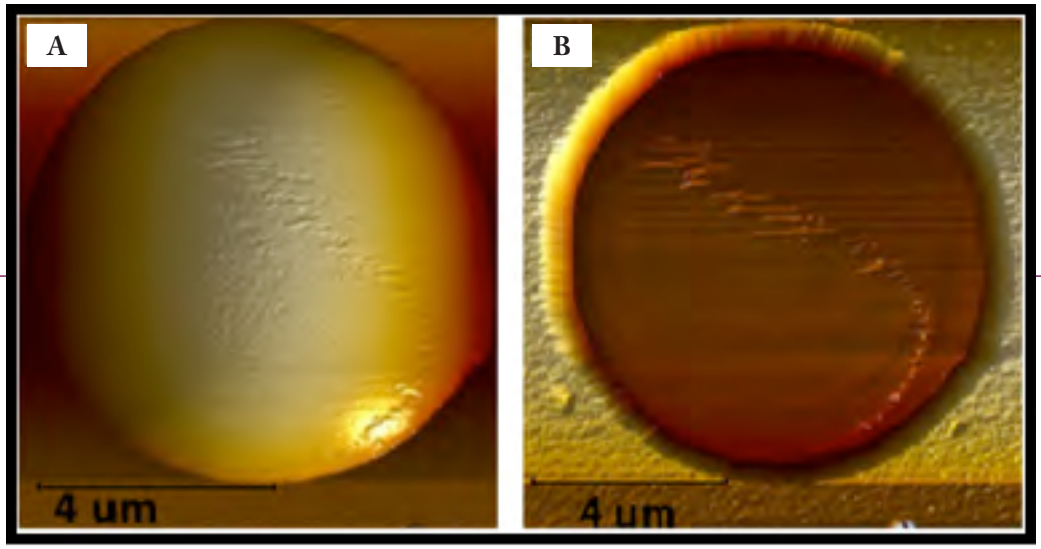

Figure 3. AFM image of a 15%T 3%C single DPN dot. The defined structure showed some polymerization occurred after the DPN printing process. A. Surface Image. B. Phase Diagram.

ratio of 1 mL water: 0.1 µL TEMED was used, instead of the usual 1 mL water: 1µL TEMED ratio. This allowed for approximately 30 min before visible gelation, which was enough time for printing. It was observed that 6x6 squares of dots could be printed without re-dipping the DPN tip (see Figure 7). The printed dots looked similar in size, shape, and structure as the DPN printed dots without ND.

When the fluorescent images of the printed dots were observed, no ND was detected within the dots. Optical microscope images of the inkwell from where the dots were printed did show the presence of ND as did the microchannels that the ink traveled through prior to contacting the DPN tip. The channel which the DPN tips are dipped into was slightly tapered compared to the rest of the microchannel. It appeared that there was ND in the microchannel, but not nearly as high a concentration of ND within the tapered channel as shown by the lack of fluorescence (Figure 8). This may have occurred because the smaller channel blocked the ND due to aggregation at the tapering site.

Raman spectroscopy was performed on DPN printed dots with and without ND; however, both spectra looked similar. No ND peak was observed at 1332 cm^{-1} for the DPN printed dots, but it was observed for a gel film of 15%T 3%C with 0.1 wt% ND, as shown in Figure 6. Interestingly, no ND was observed for a liquid solution of 2 wt% ND in water either. These spectra should be retested when the Raman instrument has been realigned properly. The next attempt at printing ND should involve a higher concentration of ND and use smaller-sized ND (0-0.2 µm).

Conclusion

In this study, an array of polyacrylamide nanodots was successfully printed on a silicon dioxide substrate using dip-pen nanolithography. This work helps demonstrate initial steps in creating a sensor that may be used to capture and detect molecules and other proteins, such as cardiac troponin T. It was shown that polyacrylamide dots with polyethylene glycol could be reproducibly printed, and 252 dots could be printed without re-dipping the printing tip. Nanodiamond particles were added to the polyacrylamide dots on a glass slide and confirmed to be uniformly dispersed using optimal microscopy. Additionally, the nanodiamond within the polyacrylamide fluoresced under a 525 ± 25 nm filter. Future work will involve adding a greater concentration of smaller nanodiamond particles and forming protein recognition sites within the printed dots. Once created, this type of molecularly imprinted biosensor could be used for rapid detection of proteins and other large biological molecules related to diseases or illnesses.

Acknowledgments

The authors thank Sonal Singh for assistance with dip-pen nanolithlography. We are grateful to Jared Ballinger and Jeffrey Montgomery for assistance with Raman spectroscopy. This work was supported by the National Science Foundation (Grant Number NSF-DMR-0646842), Research Experiences for Undergraduates (REU) Award to the University of Alabama at Birmingham.

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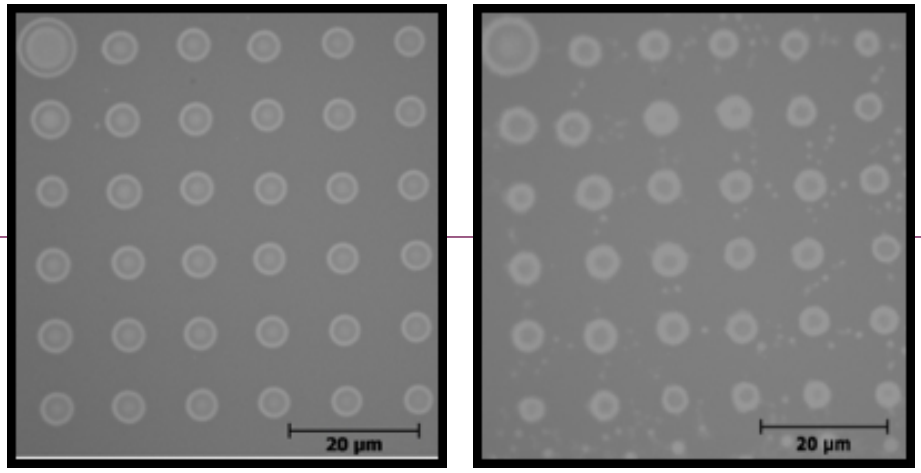

Figure 4. Image of a square of 15%T 3%C DPN dots. After printing (left), dots remain in their printed locations. Two weeks later (right), dots remain in printed locations but show signs of water evaporation.

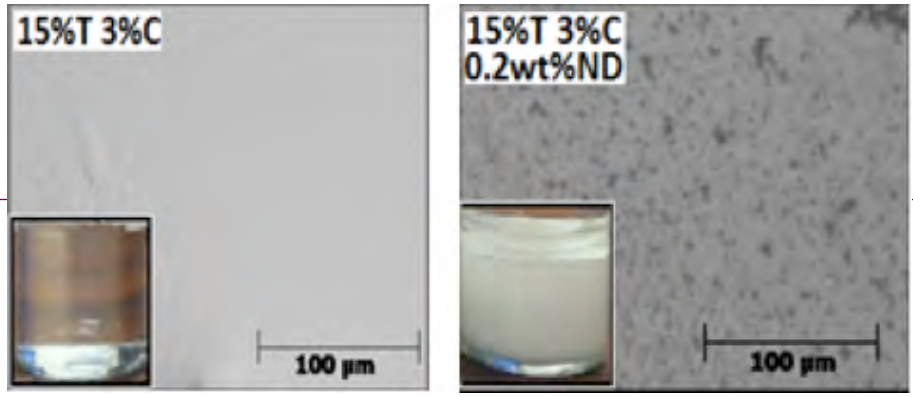

Figure 5. Gel films on glass slides: 15%T 3%C (left), 15%T 3%C with 0.2 wt% ND (right). The ND appeared as black dots in the polyacrylamide gel and was uniformly dispersed.

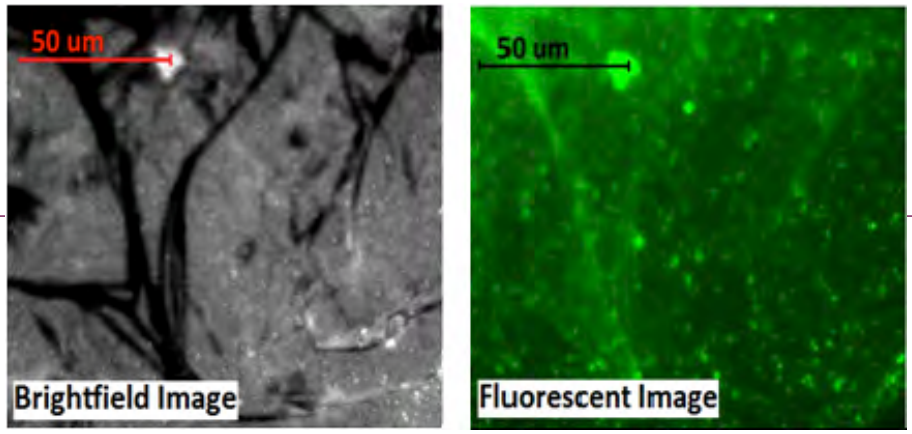

Figure 6. Gel of 15%T and 3%C with 20 wt% PEG and 0.1 wt% ND on a glass slide: brightfield image (left), fluorescent image (right). This shows that the ND fluoresced (470 ± 20 nm excitation, 525 ± 25 nm emission).

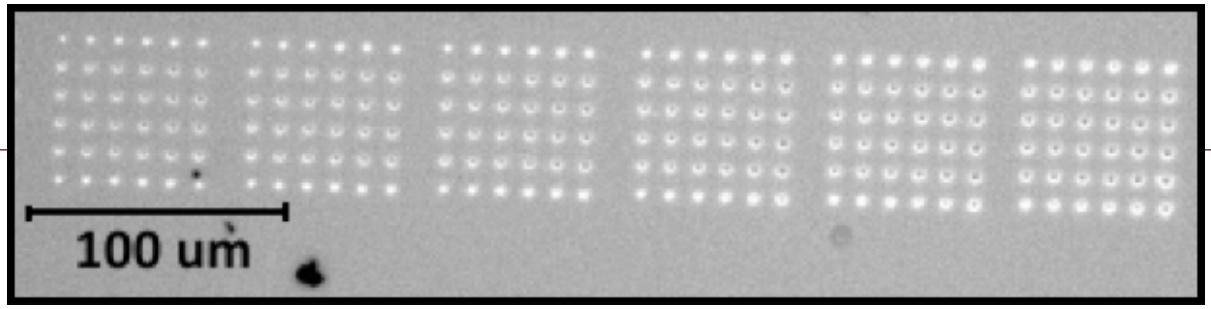

Figure 7. 6x6 squares of DPN printed polyacrylamide dots of 20%T and 5%C with 20 wt% PEG and 0.1 wt% ND.

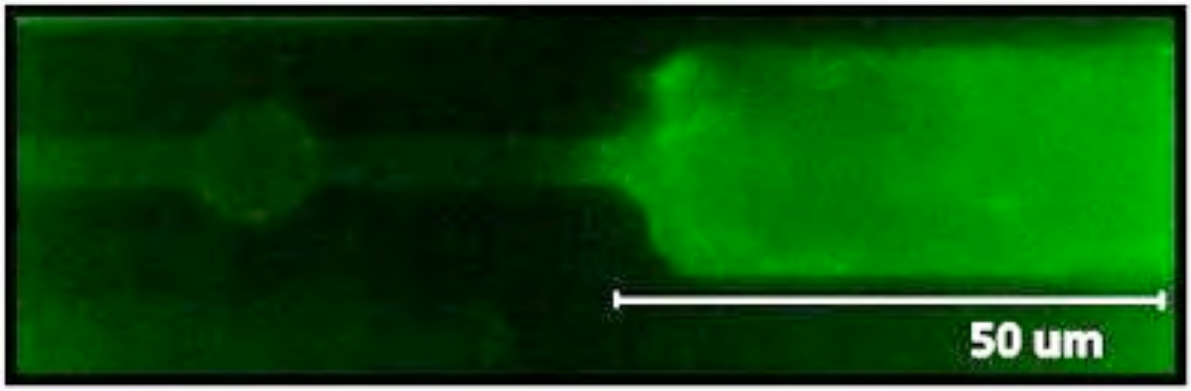

Figure 8. Fluorescent image of microchannels with 20%T, 5%C, 20 wt% PEG, 0.1 wt% ND ink. The tapered microchannel blocked the ND from reaching the DPN dipping location.

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