

# DNA Immobilization and Detection on Cellulose Paper using a Surface Grown Cationic Polymer via ATRP

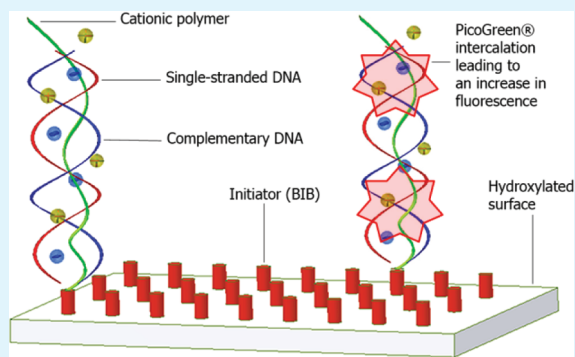
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## S Supporting Information

**ABSTRACT:** Cationic polymers with various structures have been widely investigated in the areas of medical diagnostics and molecular biology because of their unique binding properties and capability to interact with biological molecules in complex biological environments. In this work, we report the grafting of a linear cationic polymer from an atom transfer radical polymerization (ATRP) initiator bound to cellulose paper surface. We show successful binding of ATRP initiator onto cellulose paper and grafting of polymer chains from the immobilized initiator with ATRP. The cellulose paper grafted polymer was used in combination with PicoGreen (PG) to demonstrate detection of nucleic acids in the nanogram range in homogeneous solution and in a biological sample (serum). The results showed specific identification of hybridized DNA after addition of PG in both solutions.

**KEYWORDS:** atom transfer radical polymerization (ATRP), DNA sensors, fluorescence, cationic polymers, oligonucleotides, biomarkers



Synthesis of well-defined polymeric structures using ATRP has been reported in a number of early publications.<sup>1–3</sup> This method is robust and versatile in its capability to yield controlled chain growth and to achieve many different forms of polymers, including hyperbranched polymers,<sup>4–7</sup> star-shaped polymers,<sup>8–10</sup> and block copolymers.<sup>1,11,12</sup> Moreover, several groups have recently reported the use of ATRP method for the “grafting from” of polymer brushes,<sup>13–15</sup> which have great potential in fabrication of medical devices and surface-modified nanoparticles.<sup>16</sup> Compared to the “grafting through” method, “grafting from” polymerizations result in brush copolymers with high molecular weight side chains. In addition, this approach does not require the synthesis of a macromonomer, which is the most difficult step in the “grafting through” process. “Grafting from” is also more favorable than the “grafting onto” method because the latter is limited in achievable grafting density.<sup>17</sup> Grafts from polymer brushes were used as highly defined coatings in various applications ranging from biomedical and bioanalytical applications to biomimetic materials fabrication.<sup>18–20</sup> This includes the use of stimuli-responsive polymer brushes for enzymatic biosensors<sup>21</sup> and ligand modified polymer brushes for integrin specific cell adhesion.<sup>22</sup> However, in this case, we employ these polymer brushes as a platform for the development of a DNA hybridization detection mechanism based on fluorescence intensity change upon the addition of PicoGreen (PG).

The detection of nucleic acids in various solutions, including complex biological environments, has been the focus of many research groups who are trying to develop fast and reliable

detection mechanisms of nucleic acid based disease biomarkers. Among the most significant nucleic acid biomarkers that have recently been discovered are microRNAs.<sup>23–25</sup> The development of simple and effective diagnostic devices based on disease biomarkers such as microRNAs, DNA fragments and proteins have excelled in recent years.<sup>24–27</sup> Many diagnostic assays have been developed that use classical methods of nucleic acid detection which require labeling of the analyte or the probes with fluorophores or other reporter molecules.

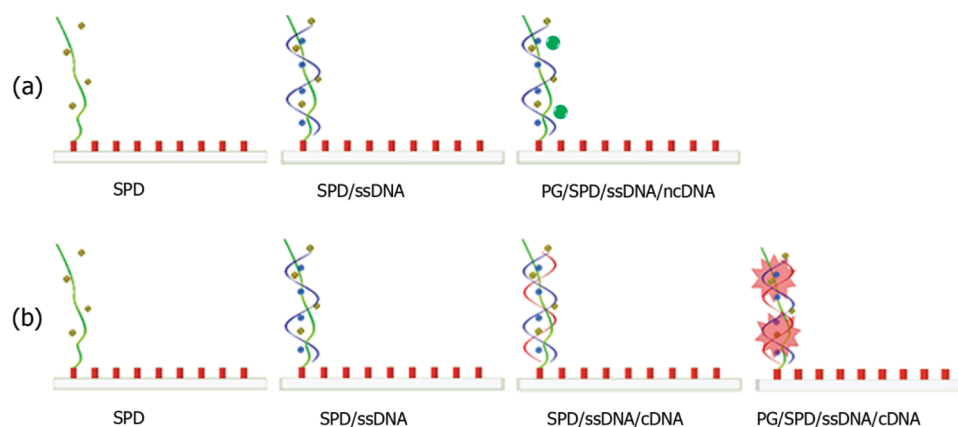
The detection of nucleic acids in solution provides simplicity but lacks the specificity and sensitivity offered by traditional surface based assays especially with regards to detection in complex biological environments, such as serum or blood. New strategies have recently been developed that use cationic polymers to detect nucleic acid hybridization reactions capable of detecting specific sequences of single-stranded DNA molecules.<sup>28–31</sup> In addition, many interesting DNA hybridization transducers have been reported including electrochemical and optical DNA biosensors based on conjugate polymers,<sup>31–33</sup> DNA-derivatized nanoparticles,<sup>34,35</sup> molecular beacons,<sup>36</sup> and bioluminescence techniques.<sup>37</sup> However, these methods, although functional, require the modification of the polymer or amplifier properties. Even though these methods have distinct advantages over conventional procedures of oligonucleotide detection (RT-PCR, Northern blotting,

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**Scheme 1. Schematic Depicting the Steps Leading to the Detection of DNA Hybridization:** (a) Interaction between Surface Polymer (SPD), ssDNA Probe, Noncomplementary DNA (ncDNA), and PicoGreen (PG, Represented by Green Spheres) Yields No Fluorescence and (b) Inclusion of a Complementary Sequence (cDNA) Instead of ncDNA into the System Allows for DNA Hybridization to Occur Leading an Increase in Fluorescence Intensity (Represented by the Red Stars)



etc.),<sup>38</sup> specificity and reproducibility of detection are compromised. In 2004, Dore et al. reported that a cationic water-soluble polythiophene polymer can be used for the detection of nucleic acids at low concentrations. However, the system can only work in hydrogenated Triton X-100 which significantly limits its application in real biological environments, such as blood or serum, where direct detection is required.

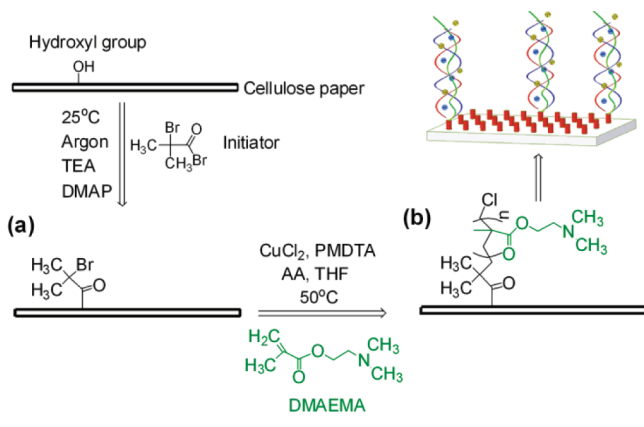
Fluorescent conjugated polymers have been shown to allow the sensitive detection of DNA and RNA in homogeneous solution by complexation of the conjugate polymer to the negatively charged nucleic acids. Because of their unique signal amplification properties, researchers have extensively investigated conjugated polymers as optical transducers in highly sensitive biosensors.<sup>39,40</sup> However, many of these polymeric based biosensors have not been further developed for clinical application and biophysical analysis in clinics because of their complexity and requirement for skilled operatives and specialized equipment.<sup>40–42</sup> Immobilized cationic polymers such as polylysine have also been extensively used to study DNA hybridization detection on various surfaces.<sup>43–47</sup> A study carried out by Costa-Garcia et al.<sup>43</sup> reports the detection of target ssDNA hybridization to a polylysine immobilized ssDNA probe through color change after hydrolysis. However, the detection requires biotinylation of the target sequence which limits its practical application significantly. On the other hand, our method does not require manipulation of the target in any way. By using ATRP, we have more options with regards to polymer structure, size, and charge. To the best of our knowledge, this is the first time anyone has reported the use of a cationic polymer brush grafted from the cellulose paper by controlled ATRP to achieve DNA hybridization detection. The polymer brush is used to immobilize single-stranded DNA (ssDNA) by electrostatic interactions<sup>48</sup> of the amine groups of the polymer to the backbone phosphate groups of the ssDNA. This system is capable of identifying DNA hybridization in serum on the basis of a combination of mechanisms composed of polymer-ssDNA probe interaction, ssDNA-cDNA (cDNA) hybridization and finally, PicoGreen intercalation (Scheme 1).

To grow the polymer brush from the cellulose paper surface, an initiator had to be immobilized onto the surface of the cellulose paper first. This was achieved by immersing the paper in a solution containing 2-bromoisobutryl bromide (BIB, 460

mg, 2.0 mmol, 25 mM), triethylamine (TEA, 222 mg, 2.2 mmol, 27.5 mM), and a catalytic amount of 2-dimethyl aminopyridine (DMAP) in THF (40 mL). By comparison of the hydrophobicity of the initiator grafted paper to that of untreated cellulose paper (control, same reaction conditions but without BIB), it was possible to determine the presence of the bound initiator. Distilled water was carefully pipetted onto either the initiator grafted cellulose paper, or untreated cellulose paper. It was observed that when water drops were added to the untreated cellulose paper, the drops were immediately absorbed. However, when water drops were added to the cellulose paper with the grafted initiator, there was a delay in the time of absorption. This was expected as the initiator (BIB) is less hydrophilic than the cellulose paper, which after immobilization creates a more hydrophobic layer on the cellulose paper surface.

The polymer (SPD) was then grafted from the surface by immersing the initiator modified paper into a reaction mixture containing DMAEMA (6.288 g, 40 mmol), initiator (0.195 g, 1 mmol, excess amount excluding the initiator on the cellulose paper), PMDTA (0.0216 g, 0.125 mmol),  $\text{CuCl}_2$  (0.01675 g, 0.125 mmol), L-ascorbic acid (0.0034 g), and tetrahydrofuran (30 mL) (Scheme 2). Because everything was added in excess, a polymer in solution form (not bound to any surface) was also obtained. This polymer solution was used to estimate the SPD molecular weight by gel permeation chromatography (GPC). Because the polymer solution was obtained from the same reaction flask as the SPD, it was assumed that both polymers will have identical molecular weights. Samples were taken at different time points from the reaction mixture for (GPC) analysis to monitor polymer chain growth over time. The first measurement was taken before the reaction was started (results not shown) in which no polymer peak was seen on the chromatogram. After 30 min of reaction time, the first peak was obtained (Figure 1a). This represented a polymer with a weight average molecular weight ( $M_w$ ) of  $4500 \text{ g mol}^{-1}$ . After 2.5 h, it increased to  $6500 \text{ g mol}^{-1}$  indicating the steady and slow conversion of the monomer into polymer chains. After purification, further characterization was carried out using proton nuclear magnetic resonance ( $^1\text{H NMR}$ ). Important peaks include the tertiary amine methyl group peak (Figure 1b, e at 2.87 ppm), which carry the cationic charge (after protonation in 0.1 M Hydrochloric acid) needed for the

**Scheme 2. Schematic Depicting the Mechanism of Surface Polymerization on Cellulose Paper: (a) Initiator (2-bromoisobutyryl bromide, BIB) Reacted with the Hydroxyl Groups of the Cellulose Paper for 24 h under Argon at Ambient Temperature and (b) Polymer Subsequently Grown from the Surface by ATRP to Obtain a Polymer Brush Composed of a Linear Cationic Polymer**



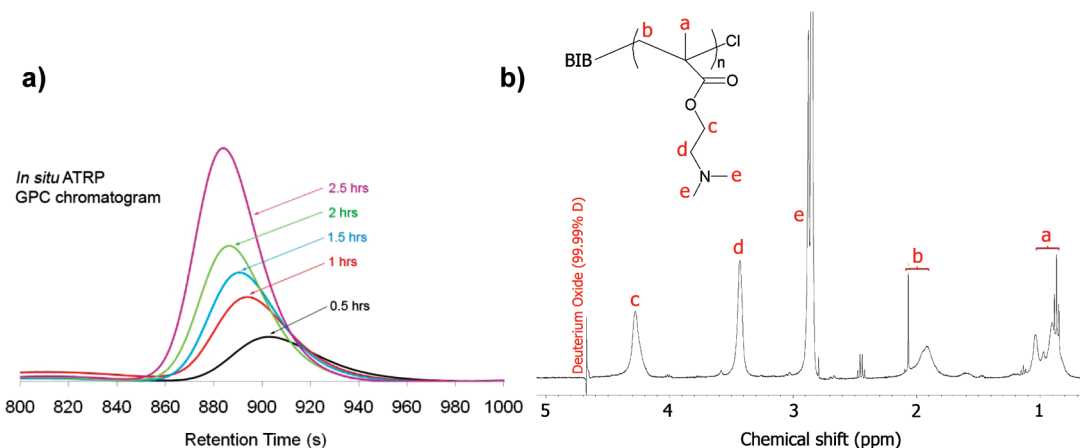
formation of the polymer/DNA complex, and the backbone peaks of SPD (a and b, 0.9 ppm and 1.92 ppm respectively). In order to confirm that the initiator and polymer have been successfully grafted onto the cellulose paper, we carried out energy-dispersive X-ray (EDX) analysis and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) on the untreated cellulose paper, initiator treated paper and polymer grafted paper (See Supporting Information Figures S1 and S2). The results show clear differences between the untreated, initiator grafted and polymer grafted cellulose papers.

We used UV–visible spectroscopy (NanoDrop 2000N) and agarose gel electrophoresis to examine the capability of SPD to form polymer/DNA complexes (Figure 2a). The NanoDrop was designed and optimized for detecting DNA only at the given wavelength (260 nm). The absorbance increases as the amount of DNA detected increases. The polymer binds to the DNA tightly which results in peak shift from the 260 nm and becomes undetectable by the NanoDrop. Any absorbance

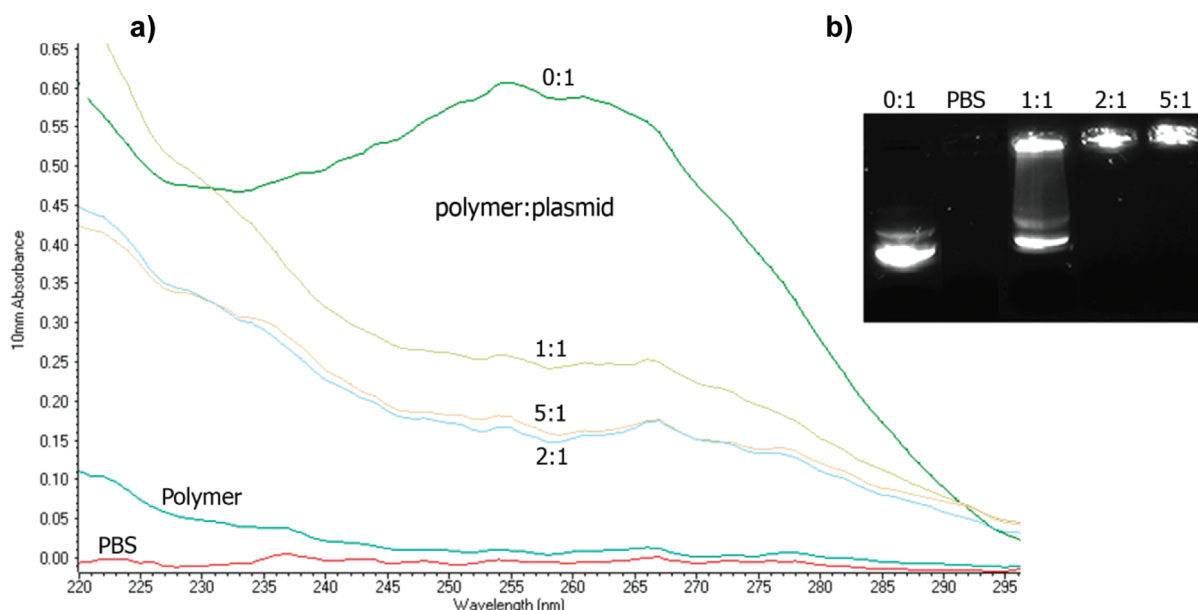
shown in the graph represents DNA that has not been bound to polymer. Optical density measurements displayed in graphical format show a decrease in absorbance when the DNA is bound to the polymer. Interestingly, increasing the polymer to DNA ratio by double or 5-fold did not result in a dramatic decrease in absorbance. A possible explanation for this is that free or uncomplexed polymer gave a false positive increase in optical density. Agarose gel electrophoresis images show normal migration of uncomplexed DNA (polymer:DNA, 0:1) compared with complexed DNA which did not migrate. At a weight/weight ratio of 1:1 (amine/phosphate, N/P, 0.7:1), not the entire DNA sample was restricted from migration, indicating partial complexation at that ratio. Because there was excess polymer in the other ratios, the 1:1 ratio was selected as the optimum for any experiments involving detection using SPD. This is to prevent unspecific binding of target DNA to free amine groups.

After the characterization steps were complete, the detection steps were initialized by binding the ssDNA to SPD. The ssDNA was incubated with the SPD-grafted cellulose paper for one hour at room temperature. This period was sufficient to allow for polymer/DNA complex formation as determined by agarose gel electrophoresis. Unbound ssDNA was rinsed off with PBS. The electrostatic interactions between the polymer and DNA are strong and washing steps do not create enough force to destabilize the bond. The proof is in that PicoGreen (PG) is capable of detecting the ssDNA/cDNA binding (after they form double stranded DNA) which means that the ssDNA has not been washed away in the washing steps. A known amount of complementary sequence (cDNA) or noncomplementary sequence (ncDNA, five mismatches) was then added to the same cellulose paper pieces and allowed to hybridize for 45 min at 42 °C. The addition of ncDNA is unlikely to replace the immobilized ssDNA; this is because the ssDNA probe has already formed stable interactions with the polymer. Subsequently, fluorescence measurements were taken 2 min after the addition of the diluted PG dye solution.

PicoGreen was chosen because it selectively binds double stranded DNA and remains relatively nonfluorescent when unbound.<sup>49</sup> Studies on PG intercalation with DNA reveal that intramolecular dynamic fluctuation is the reason for quenching of PG in its free state.<sup>50</sup> PG has an excitation maximum at 480



**Figure 1.** (a) GPC traces obtained for the chain extension of DMAEMA at different time points starting from 30 min (0.5 h) until the end of the reaction after two and a half hours (2.5 h). GPC analyses was performed by withdrawing 100  $\mu$ L of the excess polymer formed in the reaction pot to characterize the SPD (surface immobilized polymer) at the given time points. (b) <sup>1</sup>H NMR spectrum for the solution form of SPD after purification. BIB represents the 2-bromoisobutyryl bromide ATRP initiator.

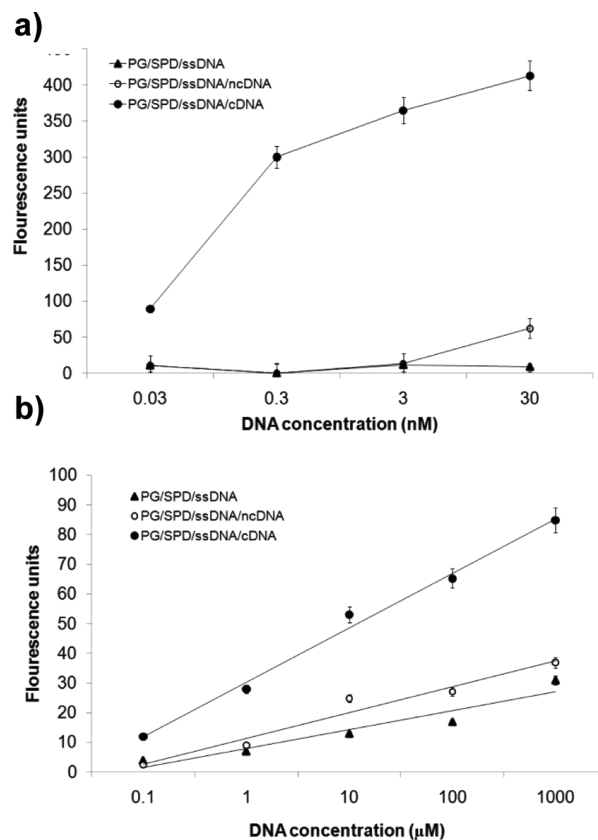


**Figure 2.** UV-visible spectroscopy (a) and agarose gel electrophoresis (b) for various polymer/plasmid ratios. The decrease in absorbance suggests the formation of duplex (complex between the polymer and plasmid) where the best complexation occurred at 2:1. This experiment was carried out using the excess polymer product that was obtained when the polymer was grafted on cellulose paper.

nm and an emission peak at 520 nm. When bound to double stranded DNA, fluorescence enhancement of PG is exceptionally high; little background occurs since the unbound dye has virtually no fluorescence. PG is very stable to photobleaching, allowing longer exposure times and assay flexibility.<sup>51,52</sup>

The significant drawback of most fluorescent intercalators, including PG, is their nonspecific and random extensive interactions within the double stranded DNA groove. The method developed herein, however, allows for selective detection of complementary single-stranded DNA where all noncDNA is removed by repetitive rinsing steps of the SPD-cellulose paper. In other terms, only complementary sequences that perfectly match the immobilized ssDNA sequence will remain on the surface prior to the addition of PG. The binding affinity of the polymer to ssDNA and dsDNA is virtually the same, however, higher N/P ratios (amine groups of polymer to phosphate groups of the DNA) give rise to better binding affinity (as seen in Figure 2). From this it is hypothesized that the interaction between the polymer and ssDNA is slightly stronger than with dsDNA as the latter has more phosphate groups which means the N/P ratio is lower.

Figure 3 shows the difference in fluorescence intensity between hybridized (PG/SPD/ssDNA/cDNA) and unhybridized DNA (PG/SPD/ssDNA/ncDNA). The difference in fluorescence intensity is a clear indicator of PG selectivity to double stranded DNA. This can be seen over a range of concentrations, which go down to 0.03 nM of DNA. The low intensity reading from the ncDNA sample indicate that all unhybridized DNA is being effectively rinsed away. If there was any remaining ncDNA on the surface the reading would have been slightly higher for the ncDNA. This is because PG can also fluoresce when bound to single stranded oligonucleotides but the signal is much less than that with double stranded oligonucleotides. Therefore; the fluorescence intensity of PG is significantly higher in the presence of cDNA than in the presence ncDNA. By comparing the results to the ssDNA alone (PG/SPD/ssDNA), it is clear that the noncomplementary sequence gave a relatively similar response to the ssDNA alone



**Figure 3.** Fluorescence intensity measurements carried out on three cellulose surfaces having the immobilized polymer with either the ssDNA, ssDNA/noncDNA (ncDNA) or ssDNA/cDNA (cDNA) in (a) water or in (b) horse serum. When the cDNA is added, it binds to the ssDNA forming a double stranded DNA and hence the greater fluorescence intensity displayed by PG. The cellulose paper was subsequently washed with water three times to remove excess serum. ( $n = 5$ ) ( $\pm$ SD) (For statistical significance mentioned in text  $p < 0.05$ ).

with fluorescence unit count ranging from 0 to 75 throughout the concentration range. The detection mechanism displayed high sensitivity even at 0.3 nM of DNA concentration. At lower concentrations (below 0.03 nM) there is no statistical difference between the three samples.

We also examined the detection capability of the system in a DNase and RNase free serum sample. In brief, a known amount of cDNA, ncDNA, or PBS was added to horse serum (Sigma Aldrich) at 37 °C. This solution cocktail was then added to a cellulose paper containing the SPD/ssDNA complex after which the PG was added and fluorescence measurements taken. A statistically significant difference in fluorescence intensity between cDNA and other samples can be seen in Figure 3b. Introduction of serum into the system, however, has resulted in  $3.3 \times 10^4$  fold decrease in sensitivity compared to detection in homogeneous solution. The value itself is the difference between the lowest amounts of cDNA that can be detected in both solutions. The lowest value in the homogeneous solution with statistical significance is 0.03 nM (Figure 3a) and for serum it is 1  $\mu$ M (or 1000nM, figure 3b, at 0.1  $\mu$ M there is no statistical significance between values i.e.  $p > 0.05$ ). Serum contains high amounts of large molecules such as proteins in addition to other DNA molecules. Most of these molecules have some sort of interaction with the polymer. Serum proteins tend to form electrostatic interactions with the polymer thus reducing the stability of the ssDNA probe with the polymer. Factors that influence detection sensitivity include accessibility of the cDNA molecule to the immobilized ssDNA probe which is significantly reduced leading to reduction in sensitivity. By removing large proteins and purifying the serum from unwanted large molecules it is anticipated that a significant increase in sensitivity will be observed. Additionally, using a PEG (polyethylene glycol) coated surface will prevent nonspecific binding of serum proteins to the polymer, as PEG has been reported in many studies to prevent nonspecific binding of proteins to polymers by shielding effect.<sup>53</sup> Even though the detection sensitivity is reduced, the specificity of the system to cDNA remained. This allowed us to conclude that detection in a complex biological environment is possible using a surface immobilized polymer.

The current system was tested for single and double mismatches, but we were not able to see similar results as seen in the five mismatch sequence. This means that five mismatch is the current limit of detection. Because the project is in its infancy, we have yet to find a way to improve the system for lower mismatches (i.e., one, two, and three mismatches). Understanding the physical properties of the polymer and its interaction with DNA will help us to find ways to optimize the detection for this application. Additionally, more sensitive instruments will be used to detect differences in fluorescence intensity.

To detect the endogenous microRNA that is upregulated in prostate cancer patients (present at several hundred thousand copies per microlitre in blood),<sup>24</sup> the detection sensitivity and specificity have to be optimized for detection of lower concentrations in serum. For clinical application, sensitivity and specificity will not be enough; the construction of a reliable and robust method of detection will become a crucial part of the application. This is a goal that we are working toward.

In conclusion, we have demonstrated the use of ATRP to graft a cationic polymer from cellulose paper and applied the grafted polymer for a bioanalytical application. The combination of surface grafted polymer and PicoGreen were used to

successfully detect DNA hybridization in the nanomolar range. In addition, the versatility of the mechanism has been demonstrated by detecting a specific complementary sequence in a complex biological environment. It is envisioned that modifications to the system will increase the sensitivity for possible clinical application and disease diagnosis.<sup>54,55</sup>

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Experimental procedures and GPC, FTIR, and EDX results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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