Polymer-Based Devices for the Label-Free Detection of DNA in Solution: Low DNA Concentrations Yield Large Signals

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ABSTRACT: Poly (*N*-isopropylacrylamide-co-N-(3-aminopropyl) methacrylamide hydrochloride) microgel-based optical devices (etalons) have been shown to change their optical properties in the presence of single stranded DNA. We hypothesize that this is due to the negatively charged DNA penetrating through the Au overlayer of the etalon, resulting in crosslinking and collapse of the positively charged microgels. We have shown that this technology is capable of detecting μM concentrations of target DNA in solutions containing 2 and 4 base pair mismatch sequences without the use of labels. Furthermore, the device's response increases as the concentration of DNA decreases, which is unique for sensing strategies. We point out, that coupling this transduction mechanism to DNA amplification strategies could result in extremely low detection limits.

Keywords: Stimuli responsive polymers, DNA sensing, poly (*N*-isopropylacrylamide)-based microgels, Microgel-based etalons

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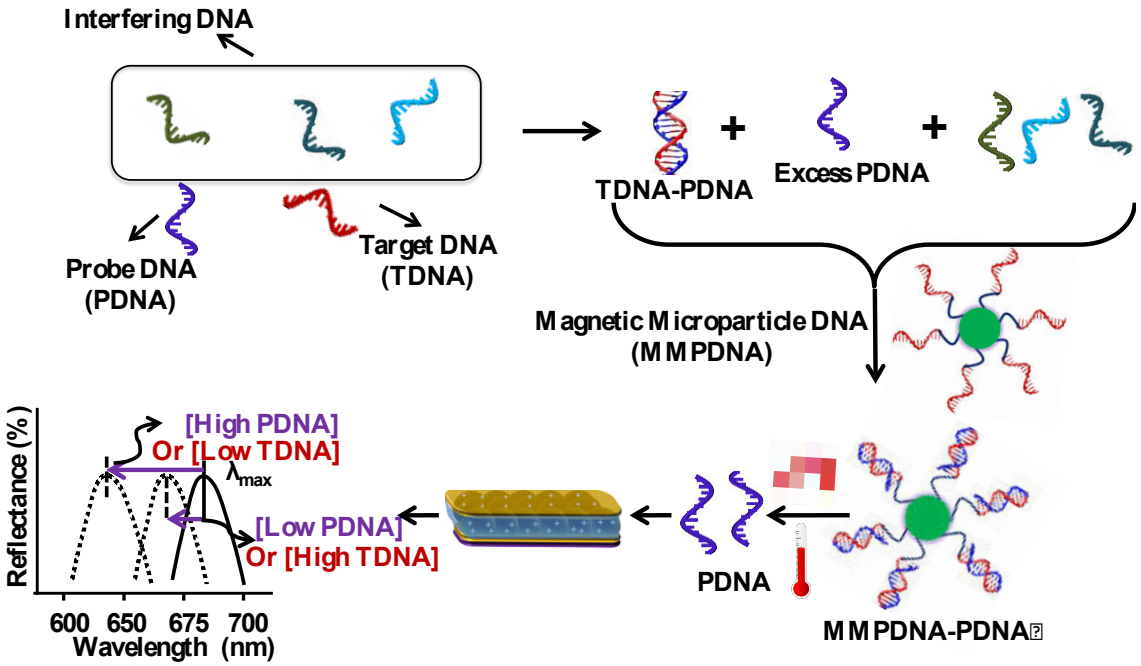


Introduction:

Detection of DNA, proteins and other biomolecules in body fluids offers the possibility of early disease diagnosis and hence the chance to improve quality of life.[1,2] Conventional detection approaches require experienced technicians to utilize sophisticated instrumentation, which are typically expensive and time consuming. To offset these barriers to diagnosis, point-of-care (POC) diagnostic devices are being developed. These devices should be easy to use, inexpensive, and provide quick results. Furthermore, the results of the assay should be easy to determine without expensive equipment. Therefore, the ideal POC diagnostic device will allow the user to readout the results by the naked eye. Hence, colorimetric approaches have received considerable attention, especially those that produce a signal without the use of chromophores and/or fluorophores as labels.

One way to provide a colorimetric signal without the use of the labels indicated above is to use photonic materials. Photonic materials exhibit visual color as a result of the periodic arrangement of at least two components (e.g., colloids) with different refractive indices interacting with light to yield constructive/destructive interference and hence color.[3-6]

Poly (*N*-isopropylacrylamide) (pNIPAm) is the most well studied responsive polymer to date.[7,8] It has attracted enormous attention due to its lower critical solution temperature (LCST). That is, pNIPAm is water soluble and swollen (existing as a random coil) at temperature < 32 °C, which collapses (into a globular state) above 32 °C.[9] This transition is fully reversible. A number of other functional groups can be added during polymerization to make pNIPAm responsive to other stimuli (in addition to temperature). [10-12] In this way, thermoresponsive pNIPAm-based polymers can also be made responsive to pH, ionic strength, analyte concentration, and light.[13-16] In addition to linear polymers, crosslinked pNIPAm-based

polymer networks can be synthesized to form hydrogels or hydrogel colloids, i.e., micro and nanogels. They too are thermoresponsive, and are highly porous, water swellable and additional functionality can be incorporated easily during synthesis. The most common functionalities added are acidic and basic groups, with one of the most common being acrylic acid (AAc). Depending on the pH of the solution these gels can exhibit a negative, positive, or neutral charge.

Our group has developed colored (photonic) materials by sandwiching a layer of pNIPAm-based microgels between two thin Au (metal) layers,[17] as depicted in Figure 1a. The color of the devices depends on the distance between the two metal layers, according to Eq. (1)[18]:

$$\lambda = 2nd\cos\theta / m \qquad (1)$$

where n is the refractive index of the microgel (dielectric) layer, d is the Au–Au distance, θ is the angle of incident light relative to the normal, and m (an integer), is the order of the reflected peak. Since the microgel layer between the two Au layers is responsive to temperature, the distance between the two metal layers can be tuned with temperature. Hence, the color of the device depends on temperature.

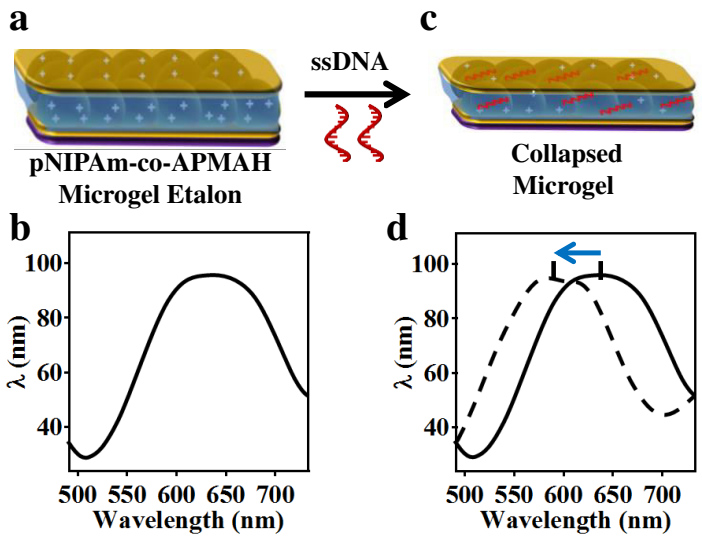


Fig.1 (a) The basic structure of pNIPAm-co-N-(3-aminopropyl) methacrylamide hydrochloride) microgel-based etalon exhibiting (b) a single reflectance peak. Here, the microgels are positively charged in water with a pH < 10.0. (c) After addition of ssDNA, the microgels are crosslinked and collapse, reducing the distance between two Au layers of the device, and (d) the peaks (in this case we show a single peak) of reflectance spectrum shifts to shorter wavelength.

Previously,[19] we have shown that positively charged linear polymers (polycations) could penetrate the outer Au layer of an etalon and crosslink pNIPAm-co-AAc microgels (negatively charged at pH > 4.25). The crosslinking results in microgel collapse, and a concomitant color change and associated shift in the peaks of the reflectance spectrum. This phenomenon was used to determine the concentration of protein in solution.[20]

Experimental Details:

Materials

N-Isopropylacrylamide was purchased from TCI (Portland, Oregon) and purified by recrystallization from hexanes (ACS reagent grade, EMD, Gibbstown, NJ) prior to use. N,N′-methylenebisacrylamide (BIS) (99%), ammonium persulfate (APS) (98.5%), DMSO were obtained from Sigma–Aldrich (Oakville, ON) and were used as received. N-(3-Aminopropyl) methacrylamide hydrochloride (APMAH) were purchased from Polysciences, Inc (Warrington, PA). Sodium chloride was obtained from Fisher (Ottawa, ON). All deionized (DI) water was filtered to have a resistivity of 18.2 MΩ●cm and was obtained from a Milli-Q Plus system from Millipore (Billerica, MA). Chromium (Cr) and Gold (Au) were deposited using a model THEUPG thermal evaporation system from Torr International Inc. (New Windsor, NY). The

annealing of Cr/Au layer was done in a Thermolyne muffle furnace from Thermo Fisher Scientific (Ottawa, Ontario). Anhydrous ethanol was obtained from Commercial Alcohols (Brampton, Ontario). Fisher's finest prewashed glass coverslips were 25×25 mm and obtained from Fisher Scientific (Ottawa, Ontario). Cr (99.999%) was obtained from ESPI (Ashland, OR), while Au (99.99%) was obtained from MRCS Canada (Edmonton, AB). Succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) and Dithiothreitol (DTT) were purchased from Pierce Biotechnology, Inc. All the DNA oligomers were purchased from IDT (Coralville, IA, USA). Amine functionalized magnetic beads (Dynabeads M-270 amine) was purchased from Life Technologies Corporation (Frederick, MD, USA).

Procedures

Poly (*N*-isopropylacrylamide-co-N-(3-Aminopropyl)methacrylamide hydrochloride) (pNIPAm-co-APMAH) Microgel Synthesis

Microgels composed of poly (*N*-isopropylacrylamide-co-N-(3-Aminopropyl) methacrylamide hydrochloride) (pNIPAm-co-APMAH) were synthesized via temperature-ramp, surfactant free, free radical precipitation polymerization as described previously.[19] The reaction mixture was comprised of 90% N-isopropylacrylamide (NIPAm) and 5% N-(3-Aminopropyl)methacrylamide hydrochloride) (APMAH) with a 5% N,N'-methylenebisacrylamide (BIS) crosslinker. The monomer, NIPAm (18.0 mmol), and BIS (1.0 mmol) were dissolved in DI water (100 mL) with stirring in a beaker. The mixture was filtered through a 0.2 μm filter affixed to a 20 mL syringe into a 200 mL 3-neck round-bottom flask. The beaker was rinsed with 25 mL of DI water and then filtered into the NIPAm/BIS solution. The flask was then equipped with a temperature probe connected to a temperature control system, a condenser, N_2 gas inlet (a needle), and a stir bar. The solution was purged with N_2 gas for about

1.5 h, with the stirring set to a rate of 450 rpm, while the temperature was allowed to reach 45 °C. APMAH (1.0 mmol) was then added to the heated mixture with a micropipette in one aliquot. A 0.078 M aqueous solution of APS (5 mL) was delivered to the reaction flask with a transfer pipet to initiate the reaction. Immediately following initiation, a temperature ramp of 45 to 65 °C was applied to the solution at a rate of 30 °C/h. The reaction was allowed to proceed overnight at 65 °C. After polymerization, the reaction mixture was allowed to cool down to room temperature and filtered through glass wool to remove any large aggregates. The coagulum was rinsed with DI water and filtered. Aliquots of these microgels (12 mL) were centrifuged at a speed of ~8500 relative centrifugal force (rcf) at 23 °C for about 40 minutes to produce a pellet at the bottom of the centrifuge tube. The supernatant was removed from the pellet of microgels, which was then resuspended to the same volume (12 mL) of DI water. Centrifugation and re-suspension was repeated five more times to remove any unreacted reagents, linear polymers, and oligomers present with the microgel. After repeated centrifugation pure, concentrated and very viscous microgel pellet was formed and kept in the centrifuge tube for further use.

Synthesis of DNA-Functionalized Magnetic Microparticles (MMPDNA)

DNA functionalized magnetic microparticles were synthesized following a procedure published elsewhere.[21] In short, amino-functionalized MMPs (2.8-μm diameter; Invitrogen) were covalently linked to 3′-thiol-modified oligonucleotides (sequence, 5'- ATA ACT GAA AGC CAA GCA AAA AAA AAA AA/3ThioMC3-D/-3') with heterobifunctional crosslinker succinimidyl 4-[p-maleimidophenyl] butyrate, SMPB (Pierce Biotechnology, Inc.). First, the MMPs (30 mg mL^{-1}, 1 mL) were washed twice with 1 mL of anhydrous DMSO in a 50 mL centrifuge tube. A fresh solution of SMPB (50 mg) in DMSO (15 mL) was prepared prior to the

reaction (the sample vial was washed with DMSO and collected to avoid sample loss). The SMPB/DMSO solution was added to the magnetic beads, and the reaction between the primary amino group and the *N*-hydroxysuccinimide (NHS) ester of SMPB was allowed to proceed for 4 h with gentle shaking at room temperature. The reaction with SMPB was carried out in the dark. Then, the disulfide bonds in all 5′-thiolated oligonucleotides were reduced by DTT. A 100 μL of freshly prepared 0.1 M DTT solution in disulphide cleavage buffer was added to 25 nmol lyophilized DNA in a microcentrifuge tube, wrapped in an aluminum foil and kept standing for 2.5 hrs. After that time, DTT-DNA mixture was passed through NAP-5 column (GE Healthcare Life Sciences, London) and collected into a series of microcentrifuge tubes by adding 1.35 mL of DI water. The location and concentration of DTT reduced DNA was confirmed by UV-visible spectrophotometer and 325 μL of 10 μM solution was prepared by coupling buffer. The beads were magnetically separated and washed three times with DMSO (10 mL) and two times with coupling buffer (NaCl (0.2 M), phosphate buffer (100 mM), pH 7.0; 10 mL). Now the DTT reduced 300 μL DNA solution was added to the washed SMPB-activated magnetic beads. The rest of the DTT-DNA solution was kept for calculating the coupling efficiency. The reaction between the maleimide group and the SH group of the DNA was allowed to proceed at room temperature for 2 h under constant vortex. Next, the DNA-functionalized beads were placed on a high pull magnet (Eclipse Magnetics, Sheffield, UK), the supernatant was removed and preserved, and the beads were washed three times with coupling buffer and then twice with passivation buffer (NaCl (0.15 M), phosphate buffer (150 mM), pH 8.0). The supernatant was used to determine the coupling efficiency by measuring the absorbance at 260 nm and comparing it with that before DNA functionalization. The surface of the DNA-functionalized MMPs (MMPDNA) was passivated by adding a freshly prepared solution (40 mL) of sulfo-NHS-acetate

(100 mg; Pierce Biotechnology, Inc.) in passivation buffer. The passivation process was allowed to proceed for 1h at room temperature with mild shaking. The beads were washed twice with passivation buffer, twice with assay buffer, and stored at 4 °C in storage buffer at a final concentration of 10 mg mL^{-1}.

Etalon Fabrication

The details of the paint-on technique used to fabricate microgel-based etalons for this study has been reported elsewhere.[22] In short, 25 × 25 mm pre-cleaned glass coverslips were rinsed with DI water and ethanol and dried with N_2 gas, and 2 nm of Cr followed by 15 nm of Au were thermally evaporated onto them at a rate of ~0.2 Å s^{-1} and ~0.1 Å s^{-1}, respectively, using a thermal evaporation system by Torr International Inc. model THEUPG (New Windsor, NY). The Cr acts as an adhesion layer to hold the Au layer on the glass. The Au coated substrates were annealed at 250 °C for 3 h followed by cooling to room temperature before use. A 12 mL aliquot of purified microgel solution was centrifuged for 30 min at 23 °C at ~8500 relative centrifugal force (rcf) to pack the microgels into a pellet at the bottom of the centrifuge tube. The microgel pellet contained in the centrifuge tube was vortexed to loosen the pellet and was placed on a hot-plate at 30 °C. A previously coated Cr/Au substrate was rinsed with ethanol, dried with N_2, and then placed onto hot plate (Corning, NY) set to 30 °C. A 40 μL aliquot of the concentrated microgels was put onto the substrate and then spread toward each edge using the side of a micropipette tip. The film was rotated 90°, and the microgel solution was spread again. The spreading and rotation continued until the microgels covered the entire substrate and became too viscous to spread further. The microgels were allowed to dry completely on the substrate for 2 h with the hot plate temperature set to 35 °C. After 2 hours, the dry film was rinsed with DI water

to remove any excess microgels not bound directly to the Au. Next, the film was placed into a DI water bath and allowed to incubate overnight on a hot plate set to ~30 °C. Following this step, the substrate was again rinsed with DI water to further remove any microgels not bound directly to the Au substrate surface. Then, the film was dried with N_2 gas and placed into the thermal evaporator, and an additional 2 nm Cr followed by 5 nm Au was deposited onto the microgels as an overlayer. After the overlayer addition the Au-microgel-Au structure (or etalon) was soaked in DI water overnight on a hot plate at 30 °C. The assemblies were then rinsed with DI water and dried with N_2 gas and subsequently used for experiments.

Reflectance Spectroscopy

Reflectance measurements were conducted in a specially designed sample holder using a USB2000+ spectrophotometer, a HL-2000- FHSA tungsten light source, and a R400-7-VIS-NIR optical fiber reflectance probe all from Ocean Optics (Dunedin, FL). The spectra were recorded using Ocean Optics Spectra Suite Spectroscopy Software over a wavelength range of 350-1025 nm. Measurements were performed in the sample holder, which allows for careful sample positioning, sample stability, solvent injection, and fine temperature control. The light source was always positioned over the center of the etalon.

Experimental Setup for Biosensing

The whole sensing experiment was done at room temperature. Before each experiment, the etalons were fixed in a petri dish with adhesive tap so that the position of the device never changes. The centre of the device was soaked with a few drops of pH 7.2 solution (200 mM with NaCl) and allowed to swell. Now, the light source was adjusted by a clamp to the centre of the

device inserting through the pH 7.2 solution. It was confirmed that the position of the light source is always fixed. Now, the intensity and distance of light source from the assembly was adjusted to result in the highest quality reflectance spectra. Before each measurement, we assured that the reflectance spectrum was stable, i.e., the position of the reflectance peaks ceased movement over time. Each experiment was repeated at least three times.

Separation and Sensing Protocol

Specific volumes of target DNA (TDNA) and probe DNA (PDNA) were mixed into a microcentrifuge tube for hybridization. After 4 hours, an excess amount of MMPDNA (magnetic micro particles with covalently attached ssDNA complimentary to PDNA) relative to PDNA was added and slowly vortexed few times and kept standing for 4h with occasional vortexing. After this time, an external magnet was placed on the wall of microtube and held for 2 minutes. The magnet visibly pulled the magnetic micro particles towards the wall of the microcentrifuge tube, with excess PDNA bound. It is important to note that we added MMPDNA to an excess of PDNA. At that point, TDNA-PDNA double helix should be suspended in the microcentrifuge tube, while the MMPDNA-PDNA is stuck via the magnetic field on the centrifuge wall. The supernatant containing the TDNA-PDNA helix was pipetted out and the magnetic particles were washed three times with buffer. Next, a specific amount of buffer was added to the separated and washed MMPDNA-PDNA complex and vortexed to resuspend the MMPDNA-PDNA. The microcentrifuge tube was heated to 80 °C (well above the melting point of PDNA and its complement MMPDNA) and kept for 5 minutes by mixing with micropipette. While the temperature was maintained, an external magnet was brought close to the wall of the microcentrifuge tube, held for 2 minutes, and the supernatant was pipetted out by a micropipette.

At that temperature, due to the melting of PDNA from the MMPDNA, all the PDNA should be released into the supernatant. The supernatant (with all the PDNA) was cooled and added to the chamber holding the etalon and the optical properties monitored. Since the PDNA was capable of entering the etalon, the spectral peaks shifted in related to the amount of TDNA initially present in the initial sample solution. By varying the concentration of TDNA, supernatant with various concentration of PDNA was collected by the above method and used for sensing. We performed control experiment by using TDNA and allowed them to react with MMPDNA. After the initial magnetic field separation, washing of MMPDNA and heating, we added the extract to the etalon chamber and found no significant shift of reflectance peak confirming the selectivity of the MMPDNA towards PDNA. Other control experiments were performed and we found that MMPDNA storage buffer and reaction buffer do not have any influence on the position of the peaks in the reflectance spectra.

Results and Discussion:

In this contribution, we show that poly (N-isopropylacrylamide) microgel-based photonic materials (etalons) can be used to colorimetrically detect single stranded DNA in solution without the use of labels (i.e., label free). The device is made by sandwiching positively charged poly (*N*-isopropylacrylamide-co-N-(3-aminopropyl) methacrylamide hydrochloride) (pNIPAm-co-APMAH) microgels between two Au layers as shown in Fig. 1a. These devices exhibit typical visual color and characteristic reflectance spectra (see Figure 1b).[23] When the negatively charged DNA is added to the etalon composed of positively charged microgels, the microgels are

crosslinked and collapsed (Fig. 1c) due to electrostatics, and the devices exhibit a spectral shift (Fig. 1d). Shown in Figure 2 is the response of a pNIPAm-co-APMAH etalon stabilized at room temperature in pH 7.2 solution to the addition of varying amounts of "probe" DNA (PDNA, sequence: 5'-TTGCTTGGCTTTCAGTTAT-3'), which is of course negatively charged. As can be seen, the shift in the position of the peaks (in this case one peak) depends on the concentration of PDNA added to the etalon. That is, the more PDNA added to the device, the larger the shift in the peak position. As expected, the response levels off at high concentrations of PDNA due to saturation of the etalon. This observation confirms that PDNA is capable of entering the etalon to crosslink the microgels, and the extent of crosslinking depends on the amount of PDNA exposed to the etalon.

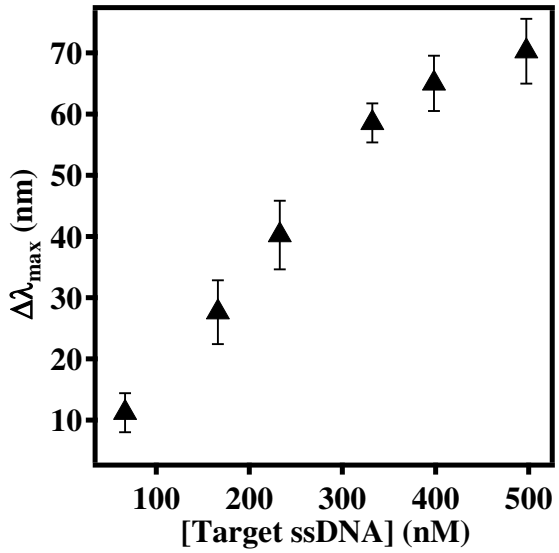
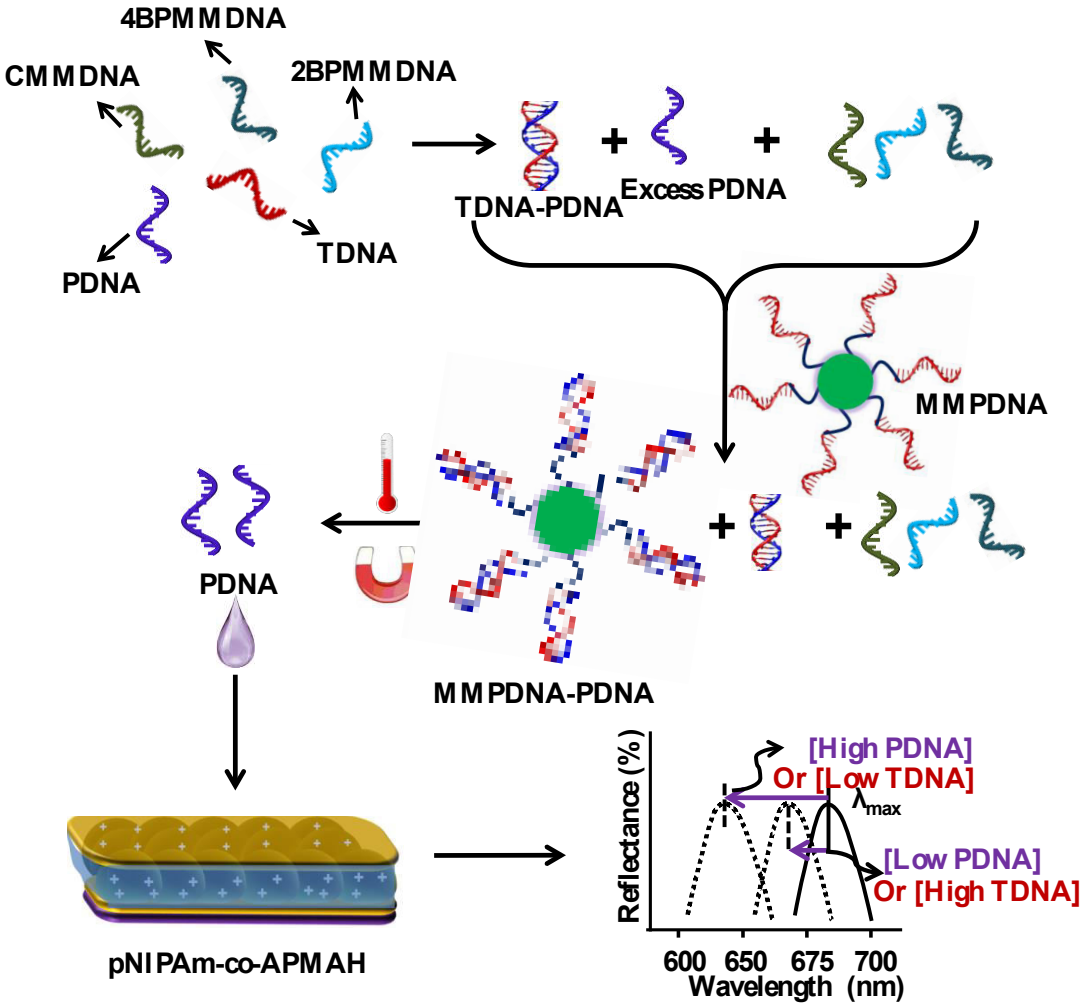


Fig. 2 Shift of a reflectance peak for a pNIPAm-co-APMAH etalon upon addition of increments of PDNA solution. The pNIPAm-co-APMAH microgel-based etalon was soaked in pH 7.2 solution throughout the experiment, while the temperature was maintained at 25 °C. Each point in the plot represents the average of at least three independent measurements, and the error bars are standard deviation for those values.

Here, this phenomenon was exploited to determine the solution concentration of target DNA (TDNA) with a sequence of 5'-ATAACTGAAAGCCAAGCAA-3'. The solutions also contained interfering DNA with 2 bp mismatches (5'-TTGCTT**C**GCT**A**TCAGTTAT-3') and 4 base pair mismatches (5'-TTG**G**TT**C**GCT**A**TC**T**GTTAT-3') (compared to the full complement) and the fully mismatched DNA (5′-GGTTCGAATTGCGTACCGC-3′). To accomplish this, the procedure outlined in Scheme 1 was utilized. Specifically, an excess of PDNA solution (PDNA is fully complementary to TDNA) was added to a given amount of TDNA solution in a microcentrifuge tube (total solution volume was 200 μL). The mixture was allowed to incubate for 4 hours at room temperature with occasional mixing to facilitate hybridization. Following this hybridization step, there will be excess PDNA. Magnetic microparticles modified with the full complement to PDNA (MMPDNA sequence: 5'-ATAACTGAAAGCCAAGCAAAAAAAAAAAA-SH-3') were then added to the mixture and allowed to incubate for 4 hours at room temperature. An excess of MMPDNA was added to the mixture to capture a maximal amount (ideally all) of the excess PDNA. After allowing ample time for hybridization of PDNA to the MMPDNA, the mixture was vortexed and an external magnet was brought close to the wall of the microcentrifuge tube and held in place for 2 minutes, until it was visually clear that all the MMPDNA-PDNA was attracted to the wall of the microcentrifuge tube. While holding the magnet in place, the supernatant solution was pipetted out to remove all PDNA-TDNA complex, the magnet was removed and the magnetic microparticles were vortexed mildly with additional fresh buffer -- this process was repeated a total of 3 times. Then, a specific amount of pH 7.2 solution was added to the clean and isolated

MMPDNA-PDNA complex and dispersed. At this stage, only the MMPDNA- PDNA complex should be present in solution.



Scheme 1. The protocol used for indirectly sensing target DNA (TDNA), by sensing probe DNA (PDNA). An excess amount of PDNA is exposed to a solution containing TDNA and DNA with a completely mismatched sequence (CMMDNA), and DNA with 4 (4BPMMDNA) and 2 base mismatches (2BPMMDNA). The PDNA binds the TDNA completely, leaving behind excess, unbound PDNA in solution. Magnetic microparticles (MMPDNA) that are functionalized with

the complete complement to PDNA are added to the solution to capture the excess PDNA. A magnet is then used to isolate the magnetic microparticles bound with PDNA (MMPDNA-PDNA) from the solution. After washing the MMPDNA-PDNA, the PDNA is recovered by heating the solution to melt the DNA off of the MMPDNA-PDNA, and the excess PDNA is recovered and added to the etalon. In this case, a large spectral shift from the etalon corresponds to a large excess of PDNA, which means a low concentration of TDNA was present in the initial solution. The opposite is true as well -- a low concentration of PDNA left in solution yields a small spectral shift from the device, meaning there was a large amount of TDNA present in the initial solution. This illustrates the strength of the current system, low concentrations of TDNA yield large spectral shifts making the device more sensitive to low DNA concentrations.

Following these steps, the MMPDNA-PDNA particles in fresh solution was heated to 80 °C and maintained for 5 minutes. At this temperature the PDNA will be released from the MMPDNA particles (by melting, the melting point of MMPDNA-PDNA complex is 49.7 °C), and while maintaining the solution temperature, a magnet was applied to the tube to capture and isolate the MMPDNA from the PDNA. While maintaining the elevated temperature and applying the magnet, the supernatant solution was extracted from the tube, which contains only PDNA. The PDNA solution was cooled down and added to the pNIPAm-co-APMAH etalon stabilized at room temperature in pH 7.2 solution, the λ_{max} of the reflectance spectrum was monitored as a function of time.

As shown in Figure 3, the extent of the spectral shift can be related to the concentration of TDNA in the original sample solution. Again, this is due to the etalon responding to the

concentration of PDNA exposed to the etalon, which is *inversely* related to the concentration of TDNA initially present in the tube. This fact has some interesting implications, i.e., low concentration of TDNA results in high concentration of PDNA, which corresponds to a large shift in the position of the peaks of the reflectance spectrum. That is, a large response is achieved for a low TDNA concentration, which is not typical for other analytical techniques, which usually exhibit a small response to low analyte concentrations.

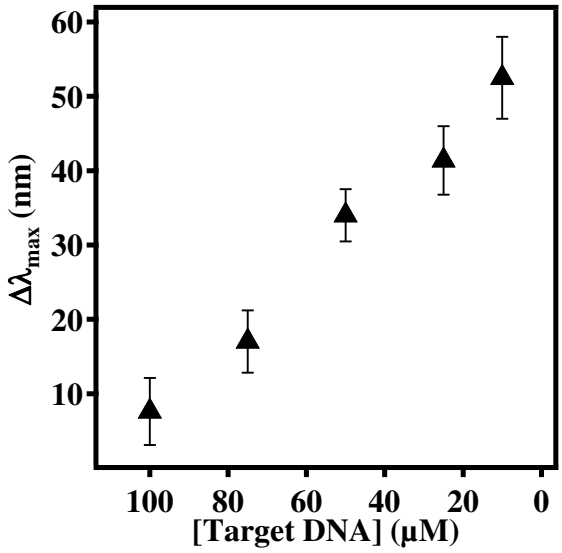


Fig. 3 Shift of a reflectance peak for a pNIPAm-co-APMAH etalon upon addition of separated TDNA solution of different concentrations. The pNIPAm-co-APMAH microgel-based etalon was soaked in pH 7.2 solution throughout the experiment, while the temperature was maintained at 25 °C. Each point in the plot represents the average of at least three independent measurements, and the error bars are standard deviation for those values.

Conclusion:

We were able to show that pNIPAm-co-APMAH microgel-based etalons can be made responsive to the presence of ssDNA in solution. This is due to the negatively charged DNA crosslinking the positively charged microgels in the etalon, causing them to collapse. We showed that the extent of shift is directly related to the concentration of DNA in solution and presented a method to exploit this for detecting the concentration of TDNA in complex mixtures. The strength of this approach is the inverse relationship between device response and TDNA concentration; low concentrations of TDNA in solution yield large device responses. Another advantage is the "offline" sample preparation, which allows for the sensor only to be exposed to the sample once the sample purification is complete. This can avoid many issues associated with nonspecific adsorption, which causes many problems for other sensing protocols. Furthermore, the technology presented here only costs pennies, and has the ability to detect low concentrations of DNA without preamplification steps. While this is the case, coupling this with preamplification steps would dramatically decrease the detection limit of this technology. Finally, further improvement of this method will be carried out for sensing ultralow concentration of DNA and new devices will be fabricated for sensing other proteins and disease biomarkers in near future.

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