A Novel Label-Free Colorimetric Assay for DNA Concentration in Solution

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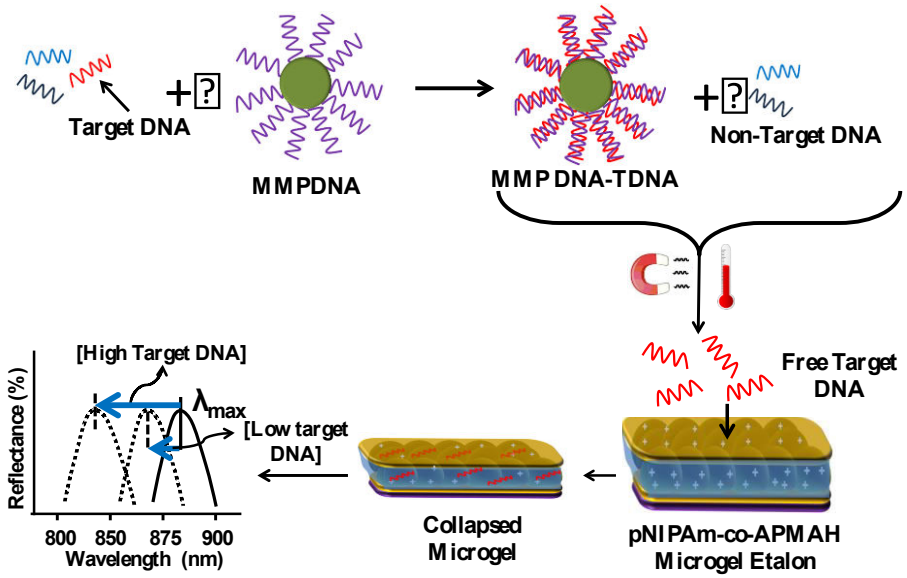
ABSTRACT: Optical devices were fabricated by sandwiching a "monolithic" poly (*N*-isopropylacrylamide-co-N-(3-aminopropyl) methacrylamide hydrochloride) (pNIPAm-co-APMAH) microgel layer between two semitransparent Au layers. These devices, referred to as etalons, exhibit characteristic multipeak reflectance spectra, and the position of the peaks in the spectra primarily depend on the distance between the Au surfaces mediated by the microgel layer thickness. Here, we show that the positively charged microgel layer can collapse in the presence of negatively charged single stranded DNA (ssDNA) due to ssDNA induced microgel crosslinking. The collapse results in a change in the etalon's optical properties, which can be used to detect target DNA in a complex mixture.

Keywords: Poly (*N*-isopropylacrylamide) - based microgel, DNA detection, Responsive polymers, Photonic materials

TOC:



Introduction:

The separation of specific DNA from complex biological samples plays a vital role in disease diagnosis, treatment and in forensic science. Separation of specific target DNA is not

enough though, the DNA of a specific sequence needs to be detected, which may need pretreatment to enrich the concentration of DNA in the sample. While there are number of methods currently available to achieve these goals, new methods are being developed for improved performance as well as for point-of-care (POC) diagnostics. For example, most techniques -- such as polymerase chain reaction (PCR)--are not amenable for POC applications. Furthermore, fluorescence-based DNA assays require high performance equipment for operation, and are costly. Due to these shortcomings, a number of techniques have emerged for the label free detection of low concentrations of DNA, without preconcentration and/or amplification.[1-4]

In this contribution, we present a polymer-based device that can be used for the label-free, colorimetric detection of low concentrations of DNA. The device construct, which is shown in Figure 1, is constructed by sandwiching poly (*N*-isopropylacrylamide) (pNIPAm)-based microgels between two planar Au layers.[5, 6] Poly (*N*-isopropylacrylamide) (pNIPAm) is the most well studied responsive polymer to date. 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.[7] This transition is fully reversible. A number of other responsive moieties can be added during polymerization to make pNIPAm responsive to temperature and other stimuli. In this way, thermoresponsive pNIPAm-based polymers can be made responsive to pH, ionic strength, analyte concentration, light, electric field, magnetic field, and redox chemistry (for example).[8-11]

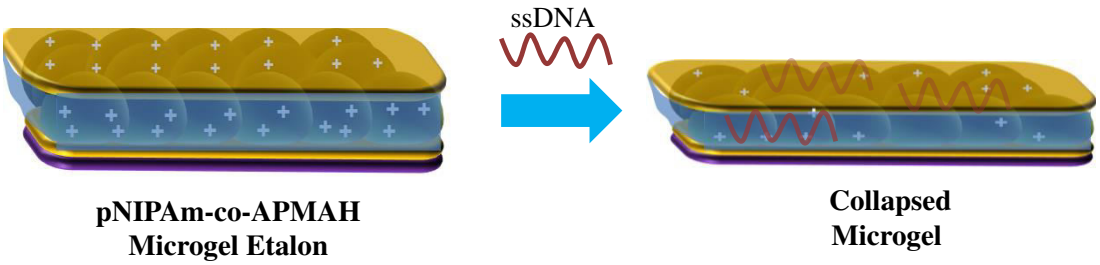


Figure 1: Basic construct of a microgel-based etalon and its response towards ssDNA.

In addition to linear polymers, crosslinked pNIPAm-based polymer networks can be synthesized to form a hydrogel. Furthermore, hydrogel nano and microparticles (nanogels and microgels, respectively) can be synthesized. These materials are highly porous, and water swollen also exhibiting thermoresponsivity with the option of adding additional responsivity as required. The most common additional response is pH, afforded by incorporating a weak acid, e.g., acrylic acid (AAc) into the hydrogel network.[12-14] AAc is a weak acid with a pK_a of ~ 4.25, thus it is charged at pH > 4.25 and vice versa. When the pNIPAm-co-AAc microgels are at a pH > 4.25 they contain multiple charges and are polyanionic.

We have shown that the pNIPAm microgel-based optical devices (or etalons), shown in Figure 1, exhibit visible color and multipeak reflectance spectra. The position of the peaks in the reflectance spectra depends on the distance between two Au layers and the refractive index of microgel layer. The position and order of the peaks can be predicted[15] from Eq. (1):

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

where n is the refractive index of the microgel (dielectric) layer, d is the mirror–mirror distance, θ is the angle of incident light relative to the normal, and m (an integer), is the order of the reflected peak.

We have utilized pNIPAm microgel-based etalons for a number of applications.[16-21] Most important to the work here, we have shown that polycationic linear polymers are capable of penetrating the etalon's outer Au layer to crosslink negatively charged pNIPAm-co-AAc microgels in the cavity.[22] This crosslinking causes the microgels to collapse, changing the distance between the two Au layers, resulting in a shift in the position of the peaks in reflectance spectra. This has been utilized for sensing proteins in solution.[19, 21] In this submission, we show that pNIPAm-co-N-(3-aminopropyl) methacrylamide hydrochloride (pNIPAm-co-APMAH) microgel-based etalons, which are polycationic <pH~10.0 can be fabricated and are capable of detecting negatively charged single stranded DNA (TDNA, sequence: 5′-TTGCTTGGCTTTCAGTTAT-3′) in solution. Furthermore, we are able to utilize this novel system for detecting a specific DNA sequence in the presence of DNA with 2 BP mismatch (2BPMMDNA, sequence: 5′-TTG**G**TTGGCTTT**G**AGTTAT-3′ and 4 BP mismatch (4BPMMDNA, sequence: 5′- TT**CG**TTGGCTTT**G**A**C**TTAT-3′) and complete mismatch (5′-ATAACTGAAAGCCAAGCAA-3′) sequences. The bolded bases indicate the position of the mismatch. This system represents a simple optical detection system to sense target DNA without complex modification or the use of labels. With further optimization, and coupling with a simple electronic readout system, this approach will find application in resource-limited parts of the world for sensing disease biomarkers.

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.[22] 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)

147 The preparation of DNA functionalized MMPs has been reported elsewhere.[23] In
148 short, amino-functionalized MMPs (2.8-μm diameter; Invitrogen) were covalently linked to 5′-
149 thiol-modified oligonucleotides (IDT) with heterobifunctional crosslinker succinimidyl 4-[p-
150 maleimidophenyl] butyrate, SMPB (Pierce Biotechnology, Inc.). First, the MMPs (30 mg mL^{-1},
151 1 mL) were washed twice with 1 mL of anhydrous DMSO in a 50 mL centrifuge tube. A fresh
152 solution of SMPB (50 mg) in DMSO (15 mL) was prepared prior to the reaction (the sample vial
153 was washed with DMSO and collected to avoid sample loss). The SMPB/DMSO solution was
154 added to the magnetic beads, and the reaction between the primary amino group and the *N*-
155 hydroxysuccinimide (NHS) ester of SMPB was allowed to proceed for 4 h with gentle shaking at
156 room temperature. The reaction with SMPB was carried out in the dark. Then, the disulfide
157 bonds in all 5′-thiolated oligonucleotides were reduced by DTT. A 100 μL of freshly prepared
158 0.1 M DTT solution in disulphide cleavage buffer was added to 25 nmol lyophilized DNA in a
159 microcentrifuge tube, wrapped in an aluminum foil and kept standing for 2.5 hrs. After that time,
160 DTT-DNA mixture was passed through NAP-5 column (GE Healthcare Life Sciences, London)
161 and collected into a series of microcentrifuge tubes by adding 1.35 mL of DI water. The location
162 and concentration of DTT reduced DNA was confirmed by UV-visible spectrophotometer and
163 325 μL of 10 μM solution was prepared by coupling buffer. The beads were magnetically
164 separated and washed three times with DMSO (10 mL) and two times with coupling buffer
165 (NaCl (0.2 M), phosphate buffer (100 mM), pH 7.0; 10 mL). Now the DTT reduced 300 μL DNA
166 solution was added to the washed SMPB-activated magnetic beads. The rest of the DTT-DNA
167 solution was kept for calculating the coupling efficiency. The reaction between the maleimide
168 group and the SH group of the DNA was allowed to proceed at room temperature for 2 h under
169 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 coupling efficiency was found to be in the range of 86 % - 95 %. The surface of the DNA-functionalized MMPs 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.[24] 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

192 then placed onto hot plate (Corning, NY) set to 30 °C. A 40 μL aliquot of the concentrated
193 microgels was put onto the substrate and then spread toward each edge using the side of a
194 micropipette tip. The film was rotated 90°, and the microgel solution was spread again. The
195 spreading and rotation continued until the microgels covered the entire substrate and became too
196 viscous to spread further. The microgels were allowed to dry completely on the substrate for 2 h
197 with the hot plate temperature set to 35 °C. After 2 hours, the dry film was rinsed with DI water
198 to remove any excess microgels not bound directly to the Au. Next, the film was placed into a DI
199 water bath and allowed to incubate overnight on a hot plate set to ~30 °C. Following this step,
200 the substrate was again rinsed with DI water to further remove any microgels not bound directly
201 to the Au substrate surface. Then, the film was dried with N_2 gas and placed into the thermal
202 evaporator, and an additional 2 nm Cr followed by 5 nm Au was deposited onto the microgels as
203 an overlayer. After the overlayer addition the Au-microgel-Au structure (or etalon) was soaked
204 in DI water overnight on a hot plate at 30 °C. The assemblies were then rinsed with DI water and
205 dried with N_2 gas and subsequently used for experiments. We point out that the microgels used
206 here were ~650 nm in diameter measured from analysis of differential interference contrast
207 microscopy images. From previous results,[6] we found that the etalon's cavity thickness
208 (defined by the microgel diameter) was ~ 0.6 of the microgels solution diameter. This is the case
209 because the microgels are soft, and sandwiched between the etalon's two Au layers while also
210 being stuck to the Au surface. In this case, we also know that the *visual* color for these etalon's is
211 weak, although the peaks in the reflectance spectra are well defined.

212 **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 in a custom built metal chamber with temperature control. The chamber was built to ensure that the position of the etalon and the light source are fixed, as well as the temperature is well controlled and stable. Before use, the edges of the etalons were sealed using clear nail polish to resist any DNA penetration through the sides of the etalon. After sealing, the assemblies were dried in air overnight. Before each experiment, the etalons were placed in the sample chamber and soaked in 30 mL pH 7.2 solution (2 mM with NaCl). The chamber's temperature was controlled to 25 °C using a digital feedback loop. The chamber allowed the reflectance probe to be inserted such that the device's optical properties could be assessed. The temperature and pH of the solution was continuously monitored throughout each experiment. 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 were stable over time. Each experiment was repeated at least three times.

Separation and Sensing Protocol

Specific volumes (20-150 μL) of target DNA (TDNA) and TDNA mixed with other "interfering" ssDNA (2 and 4 BP mismatch, and complete mismatch, (CMMDNA) with TDNA) were mixed into a microcentrifuge tube. An excess amount (350 μL) of MMPDNA (magnetic micro particles with covalently attached ssDNA complimentary to TDNA) relative to TDNA was added and slowly vortexed for 2 minutes and kept standing for 4h with occasional vortexing for hybridization. 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 TDNA bound to the complementary DNA attached to magnetic microparticles. At that point, all the unbound ssDNA (2BPMMDNA, 4BPMMDNA and CMMDNA) should be suspended in the solution in the microcentrifuge tube, while the MMPDNA-TDNA is stuck via the magnetic field on the centrifuge wall. The supernatant containing the unbound interfering ssDNA was pipetted out and the magnetic particles were washed several times with 1 mL of pH 7.2 solution each time. It was confirmed by UV-Vis that the washing solution doesn't contain any DNA. Next, a specific amount of DI was added to the separated and washed MMPDNA and vortexed to resuspend the MMPDNA-TDNA. The microcentrifuge tube was heated to 80 °C (well above the melting point of TDNA and its complement, which was 49.7 °C) and kept for 5 minutes. While the temperature was maintained, an external magnet was brought close to the wall of the microcentrifuge tube, held for 2 minutes, and the hot supernatant was pipetted out by a micropipette. At that temperature, due to the melting of TDNA from the MMPDNA, all the TDNA should be released into the supernatant while MMPDNA was stuck on the wall by the magnet. The supernatant (with all the TDNA) was cooled and added to the chamber holding the etalon and the optical properties monitored. Since the TDNA was capable of entering the etalon, the spectral peaks shifted in proportion to the

amount of TDNA initially present in the initial sample solution. By varying the concentration of TDNA, supernatant with various concentration of TDNA was collected by the above method and used for sensing. We performed control experiment by mixing 4BPMMDNA and CMMDNA and reacting them with MMPDNA without any TDNA. After the initial magnetic field separation, washing of MMPDNA and heating, we added the extract (which does not contain any TDNA) to the etalon chamber and found no significant shift of reflectance peak. This approach confirms the selectivity of the MMPDNA for TDNA. Other control experiments were performed and we found that MMP storage buffer and reaction buffer do not have any influence on the position of the peaks in the reflectance spectra.

Results and Discussion:

For this approach to work, ssDNA must be capable of penetrating the etalon to collapse the microgels to yield an optical response. To investigate this, we fabricated a pNIPAm-co-APMAH etalon and exposed it to varying concentrations of ssDNA while monitoring the position of the etalons reflectance peaks. We found that the etalon's reflectance peaks all shifted to lower wavelengths upon DNA addition, indicative of microgel collapse. This process usually takes 5 minutes to 1 h; the factors that dictate the response time are unknown, but are under investigation. Shown in Figure 2 is the cumulative shift (relative to the initial peak position) for one reflectance peak. As can be seen, the extent of the peak shift is linearly related to the concentration of the DNA added to the solution, levelling off at high concentrations due to microgel saturation with ssDNA. From the results, we concluded that ssDNA is indeed capable of entering the etalon to crosslink the microgels, and the extent of crosslinking depends on the amount of ssDNA exposed to the etalon.

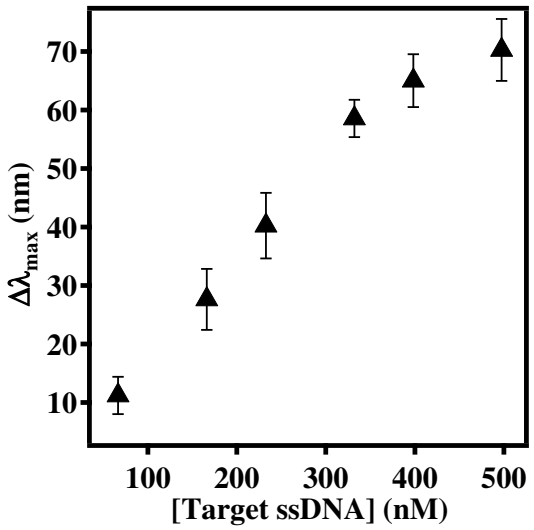
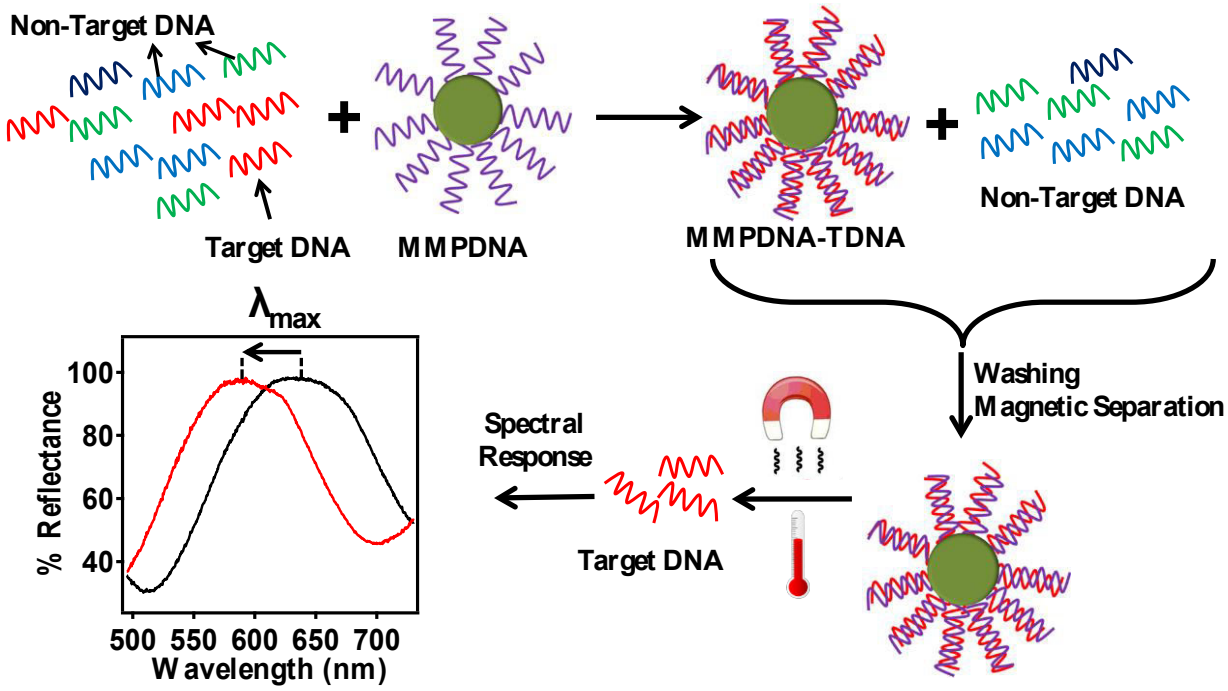


Figure 2: Cumulative shift of a reflectance peak for a pNIPAm-co-APMAH etalon upon addition of increments of target ssDNA solution. The pNIPAm-co-APMAH microgel-based etalon was soaked in pH 7.2 solution throughout the experiment, while the temperature was maintained at 25 °C. Each point in the plot represents the average of at least three independent measurements, and the error bars are standard deviation for those values. A new device was used for each measurement.

With the etalon's response to ssDNA validated, we hypothesized that this system could be used for DNA sensing applications. This approach is detailed schematically in Scheme 1.



Scheme 1: The DNA sensing scheme.

To accomplish this, we synthesized DNA functionalized magnetic microparticles[23] (MMPDNA) as detailed in the experimental section. The thiol modified ssDNA (sequence: 5′-HS-AAAAAA AAAAATAACTGAAAGCCAAGCAA-3′) attached to the MMPDNA was completely complimentary to a target DNA (TDNA) sequence. A known amount of TDNA was mixed with 2BP and 4BP mismatch DNA (2BPMDNA and 4BPMDNA respectively), and complete mismatch DNA (CMMDNA) in a microcentrifuge tube (total solution volume was 400 µL). Here, it is important to note that all the ssDNA were designed to have the same number of bases. A specific excess amount of MMPDNA (350 µL solution which yielded capture DNA that was 2x the amount of TDNA present) was added and allowed to incubate for 4 hours at room temperature. 4 hours was used in these experiments, but shorter incubation times were, and can be used (data not shown). After allowing ample time for hybridization of TDNA to the MMPDNA, an external high pull magnet was brought close to the wall of the microcentrifuge

306 tube and held in place for 2 minutes, until it was visually clear that all the MMPDNA-TDNA
307 was removed from solution. While holding the magnet in place, the supernatant solution was
308 pipetted out, the magnet was removed and the magnetic microparticles were vortexed mildly
309 with additional fresh pH 7.2 solution. The external magnet was again brought close to the
310 microcentrifuge tube and microparticles were separated, supernatant solution extracted and the
311 process repeated again. This was repeated a total of three times to isolate all the MMPDNA-
312 TDNA from the interfering DNA. Then, a specific amount of pH 7.2 solution was added to the
313 clean microparticles and dispersed. At this stage, only the MMPDNA and TDNA complex
314 should be present in solution.
315
316 Once the purification steps were complete, and the MMPDNA-TDNA was in fresh solution,
317 the solution was heated to 80 °C and maintained for 5 minutes. At this temperature the TDNA
318 will be released from the MMPDNA particles (by melting), and while maintaining the solution
319 temperature, a magnet was applied to the tube to capture the MMPDNA and the supernatant
320 solution was removed from the tube, which contains TDNA. When the supernatant was added to
321 the etalon stabilized at 25°C in pH 7.2 solution, the λ_{max} of the reflectance spectrum shifted to the
322 lower wavelength. By changing the initial concentration of TDNA, we were able to observe
323 different shift magnitudes, as shown in Figure 3. The shifts were easily detectable and ranged
324 from 12 nm - 60 nm. We were easily able to detect the concentration of DNA down to the μM in
325 range with no preconcentration and/or amplification of the TDNA concentration. Therefore,
326 preconcentration and/or amplification of the TDNA concentration will only increase the
327 sensitivity of this method.

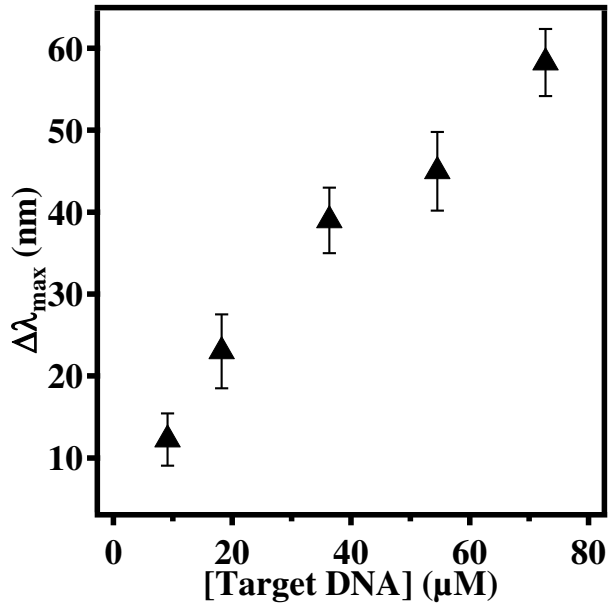


Figure 3: Shift of a reflectance peak for a pNIPAm-co-APMAH etalon upon addition of separated TDNA solution of different concentrations. The pNIPAm-co-APMAH microgel-based etalon was soaked in pH 7.2 solution throughout the experiment, while the temperature was maintained at 25 °C. Each point in the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

Conclusion:

In summary, we have shown that pNIPAm-co-APMAH microgel-based etalons can be made responsive to the presence of ssDNA in solution by exhibiting a blue shift in the peaks of the etalon's reflectance spectrum. This response originates from the penetration of polyanionic ssDNA into the etalon's microgel layer. Once penetrated, DNA interacts with the positively charged microgel electrostatically and crosslinks them. The crosslinking results in shrinking of the confined microgel between Au layers resulting in the peaks shifts, as predicted from Eq. (1). The extent of shift is directly related to the initial concentation of target DNA present in the

sample solution. Ultimately, we were able to detect μM concentrations of target DNA in solution with no TDNA preconcentration, amplification, or other system optimization whatsoever. This method represents a very simple and inexpensive way of sensing DNA without the need for a label. In fact, the devices here cost pennies. Furthermore, the response can be read out colorimetrically, which offers the potential of low cost equipment for running the assay. In the future, we will utilize this sensing scheme for detecting other protien and DNA-based biomarkers for that are specific for disease states.

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