Label-Free Detection of Low Protein Concentration in Solution Using a Novel Colorimetric Assay

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ABSTRACT

A dual pH and temperature sensitive microgel-based etalons were fabricated by sandwiching a "monolithic" microgel layer between two semitransparent, Au layers. The devices exhibit visual color and multipeak reflectance spectra, both of which primarily depend on the distance between the Au surfaces mediated by the microgel diameter. We found that a polycationic polyelectrolyte can penetrate through the Au overlayer to interact with negatively charged microgel confined between Au overlayers. In this submission we report that biotinylated polycationic polymer can penetrate through the Au overlayer of a poly (*N*-isopropylacrylamide)- *co*-acrylic acid (pNIPAm-co-AAc) microgel- based etalon and cause the microgel layer to collapse. The collapse results in a shift in the spectral peaks of the reflectance spectra. We found that the extent of peak shift depends on the amount of biotinylated polycation added to the etalon, which can subsequently be used to determine the concentration of streptavidin in solution at pM concentrations.

Keywords: PNIPAm-based microgel, Photonic materials, Polyelectrolytes, Biosensing

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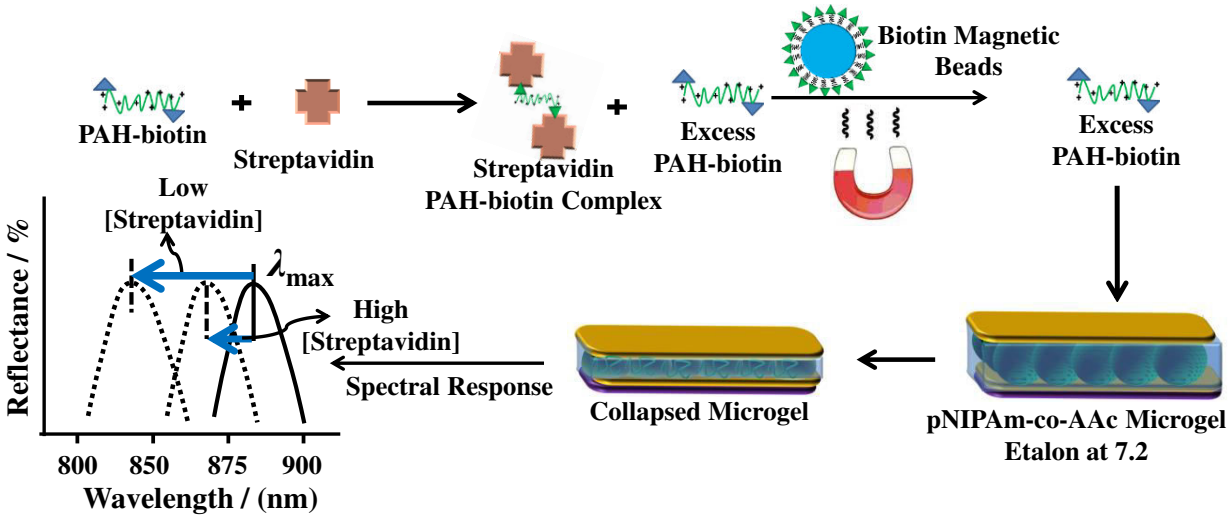


1. Introduction

To properly diagnose and treat disease, low concentrations of DNA and/or protein in e.g., blood, urine, and cerebral spinal fluid, need to be detected. Advances over recent years have allowed increasingly low levels of the biological markers of disease (biomarkers) to be detected (Elghanian et al., 2005; Haes et al., 2005; Hall et al., 2011; Nam et al., 2003; Shiddiky et al., 2012; Wang et al., 2009; Zhu et al., 2010). This has had an enormous impact on the quality of life of individuals as it has allowed for early diagnosis and treatment of disease before symptoms emerge.

In developed countries, receiving a diagnosis and subsequent treatment is relatively facile compared to developing countries. For example, the inability of a patient to reach a hospital for a diagnosis can result in death. As a result, diagnostic devices that are capable of functioning in environments without running water, electricity, and temperature control are being developed. These devices, typically referred to as point-of-care (POC) diagnostics, also need to be reliable, inexpensive, easy to operate/results easily interpreted, and fast (Bonanno and DeLouise, 2010; Yager et al., 2008). In a continuing effort to develop new, more sensitive and selective POC diagnostic devices new materials are being developed.

Currently, polymer-based materials are primarily used for biological applications (Anderson et al., 2004; Holtz and Asher, 1997; Kim et al., 2005; Kim et al., 2006; Langer,1998; Plunkett et al., 2005; Sharma et al., 2004; Tauro and Gemeinhart, 2005). This is partially due to the tremendous chemical diversity of polymer backbones. They are also easily modified with biomolecules, e.g., proteins and DNA using mild reaction conditions (Dong et al., 2007). Of specific interest to this manuscript are responsive polymer-based materials. For example, responsive polymers are able to respond to changes in their environment by undergoing

conformational changes (Gutowska et al., 1994; Kwon et al., 1991; Palankar and Skirtach, 2009; Pelton, 2000; Suzuki and Tanaka, 1990). The most common stimuli include temperature, pH, concentration, ionic strength, light, electric and magnetic field, and redox (Ayano et al., 2012; Palankar and Skirtach, 2009). Usually these responsive polymers exhibit reversibility. That is, once the stimulus is removed from the system, the polymers return back to their initial state (Gutowska et al., 1994; Kwon et al., 1991; Pelton, 2000; Suzuki and Tanaka, 1990). Ideally, the reversible response can be achieved over a large number of cycles without compromising their response to stimuli.

The most extensively studied responsive polymer is poly (*N*-isopropylacrylamide) (pNIPAm). PNIPAm is fully water soluble at T < 32 °C, and exists in water as a fully solvated random coil. At T > 32 °C, pNIPAm "dehydrates" due to favorable chain-chain interactions, and transitions to a dense globule (Wang et al., 1998). This temperature is referred to as the lower critical solution temperature (LCST). Furthermore, the transition is fully reversible. The great advantage of pNIPAm-based polymers is that their transition temperature is close to body temperature, and it can be easily copolymerized with other monomers to acquire additional functionality to stimuli other than temperature (Brazel and Peppas, 1995).

The synthesis of crosslinked pNIPAm-based networks (hydrogels) as well as pNIPAm-based hydrogel particles (nano or microgels, depending on their dimensions) is also well known. Like linear pNIPAm, pNIPAm-based hydrogels and microgels decrease in size, and dehydrate, at T > LCST. Likewise, this transition is reversible. Finally, they too can also be chemically modified such that they are responsive to multiple stimuli (Hendrickson et al., 2010). The most common additional responsivity added to pNIPAm-based microgels is pH. This is typically accomplished by copolymerization of acrylic acid (AAc) into the microgel during synthesis (Hoare and Pelton,

2004; Kim et al., 2004). The pKa value of acrylic acid is ~4.25, i.e., increasing the pH of a solution above 4.25 induces the deprotonation of AAc and thus makes the microgel particles polyanionic in nature. This, in turn, makes the microgel's diameter sensitive to the presence of polycations (Kleinen and Richtering, 2011). For example, Richtering and coworkers (Kleinen and Richtering, 2011) reported the size change of polyanionic poly (*N*-isopropylacrylamide-co-methacrylic acid) pNIPAm-co-MAAc microgels in the presence of polycationic, polydiallyldimethylammonium chloride (pDADMAC). They hypothesized the size change was a result pDADMAC penetration into the microgel structure, resulting in intramolecular crosslinking of the microgel's charged polymer chains.

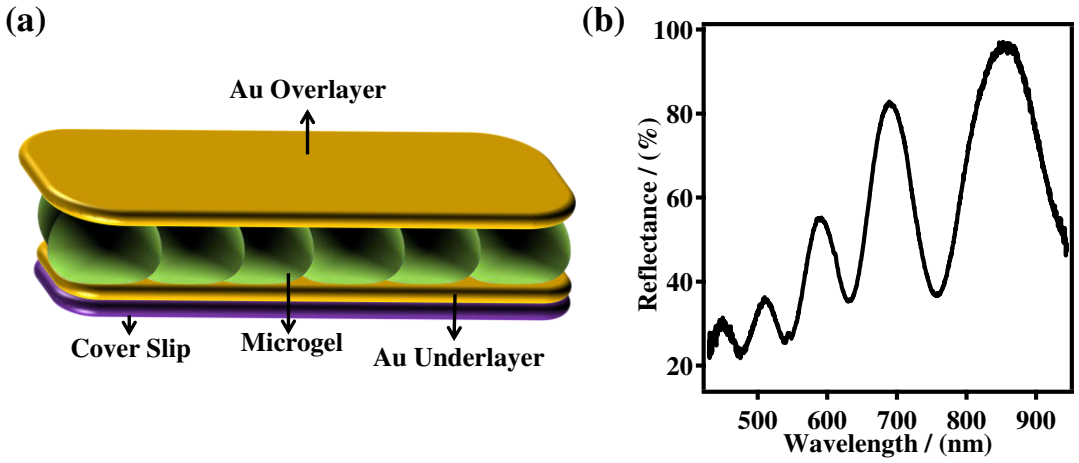


Fig. 1. (a) Basic structure of a microgel-based etalon. (b) Representative reflectance spectrum of a pNIPAm microgel-based etalon soaked in water.

We have shown that pNIPAm microgel-based optical devices (or etalons) can be fabricated by sandwiching pNIPAm-based microgels between two thin Au layers. This is shown schematically in Fig. 1a. These devices show visible color and multipeak reflectance spectra, as seen in Fig. 1b. The position of the peaks in the reflectance spectra is dependent on the distance

between two Au layers and the refractive index of microgel (Sorrell et al., 2011a, 2011b). The position and order of the peaks can be predicted from equation 1:

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

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 shown that these devices change color and peak position with pH, temperature and in the presence of glucose. In each case, the change in the reflectance spectrum is a direct result of the change in the solvation state (and "diameter") of the microgel in the presence of the stimuli. As a result, the distance between the Au mirrors changes leading to a change in λ of the reflectance peak(s) (Sorrell and Serpe, 2012a, 2012b). For example, for sensing glucose in solution, pNIPAm-co-AAc microgels were functionalized with 3-aminophenylboronic acid, which binds glucose. Under the assay conditions, when glucose binds to the microgels the charge density in the microgel-based cavity increases, leading to an increase in the cavity thickness and a concomitant red shift in reflectance spectrum. In this submission, we show that that polyelectrolyte-induced crosslinking (and subsequent collapse) of the etalon's microgel layer can be used for detecting the concentration of streptavidin in aqueous solutions.

2. Materials and Methods

2.1 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%), acrylic acid (AAc) (99%), ammonium persulfate (APS) (98.5%), and poly(allylamine hydrochloride) (PAH, MW=58000) were obtained from Sigma–Aldrich (Oakville, ON) and were used as received. NHS-Biotin ((+)-Biotin N-hydroxysuccinimide ester) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride and sodium hydroxide were 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). Sodium Hydroxide (NaOH, 99.8%) and hydrochloric acid were purchased from Caledon Chemicals (Georgetown, Ontario) and were used as received. 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). EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride), Biotin-modified magnetic particles (average diameter of <50 nm) were purchased from TurboBeads (Magdalenenstrasse, Zurich).

2.2 Procedures

Poly (*N*-isopropylacrylamide-co-acrylic acid) Microgel (pNIPAm-co-AAc) Synthesis

Microgels composed of poly (*N*-isopropylacrylamide-co-acrylic acid) (pNIPAm-co-AAc) were synthesized via temperature-ramp, surfactant free, free radical precipitation polymerization as described previously (Sorrell et al., 2011a, 2011b; Sorrell and Serpe, 2012a). The reaction mixture was comprised of 85% N-isopropylacrylamide (NIPAm) and 10% acrylic acid (AAc)

with a 5% N,N'-methylenebisacrylamide (BIS) crosslinker. The monomer, NIPAm (17.0 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. AAc (2.0 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 resuspension 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.

2.3 Synthesis of Poly (allylamine hydrochloride)-Biotin

PAH-biotin was prepared following a procedure detailed elsewhere (Mak et al., 2003). PAH (40 mg mL^{-1}) solution in water was adjusted to pH 8.1 by addition of 1.0 M sodium hydroxide solution. An aliquot of 50 µL NHS-Biotin (1.51 mg in 50 µL DMSO) was added per mL of PAH solution. The mixtures were gently stirred and incubated for 3 hours at room temperature and then dialyzed for several days against water in a dialysis tube with a MW cut off of 10,000 to remove unreacted NHS-Biotin. The DI water of the dialysis chamber was changed every day. The ratio of PAH's amines to biotin was calculated as 100:1.

2.4 Etalon Fabrication

The details of the paint-on technique used to fabricate microgel-based etalons for this study have been reported elsewhere (Sorrell et al., 2011a). 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.

2.5 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.

2.6 Experimental Setup for Biosensing

The sides of the pNIPAm-co-AAc etalons were sealed using clear nail polish to resist any PAH penetration through the sides of the etalon. After sealing, the assemblies were dried in air

overnight followed by soaking in DI water overnight. Before each experiment, the etalons were placed in a specially designed stainless steel sample chamber and soaked in 30 mL pH 7.2 solution (2 mM ionic strength). The chamber's temperature was controlled using a digital feedback loop. The chamber was sealed with a metal cover with a hole where a pH electrode was introduced to the solution. The center of the cover had a hole where the reflectance probe could be secured and exposed to the etalon. The temperature and pH of the solution were continuously monitored throughout each experiment. The light source's intensity and distance from the assembly was adjusted to result in the highest quality reflectance spectra. The spectrum was recorded in real time and we waited until the spectra ceased shifting before PAH, additional PAH, or PAH-biotin was added to it. Each experiment was repeated at least three times.

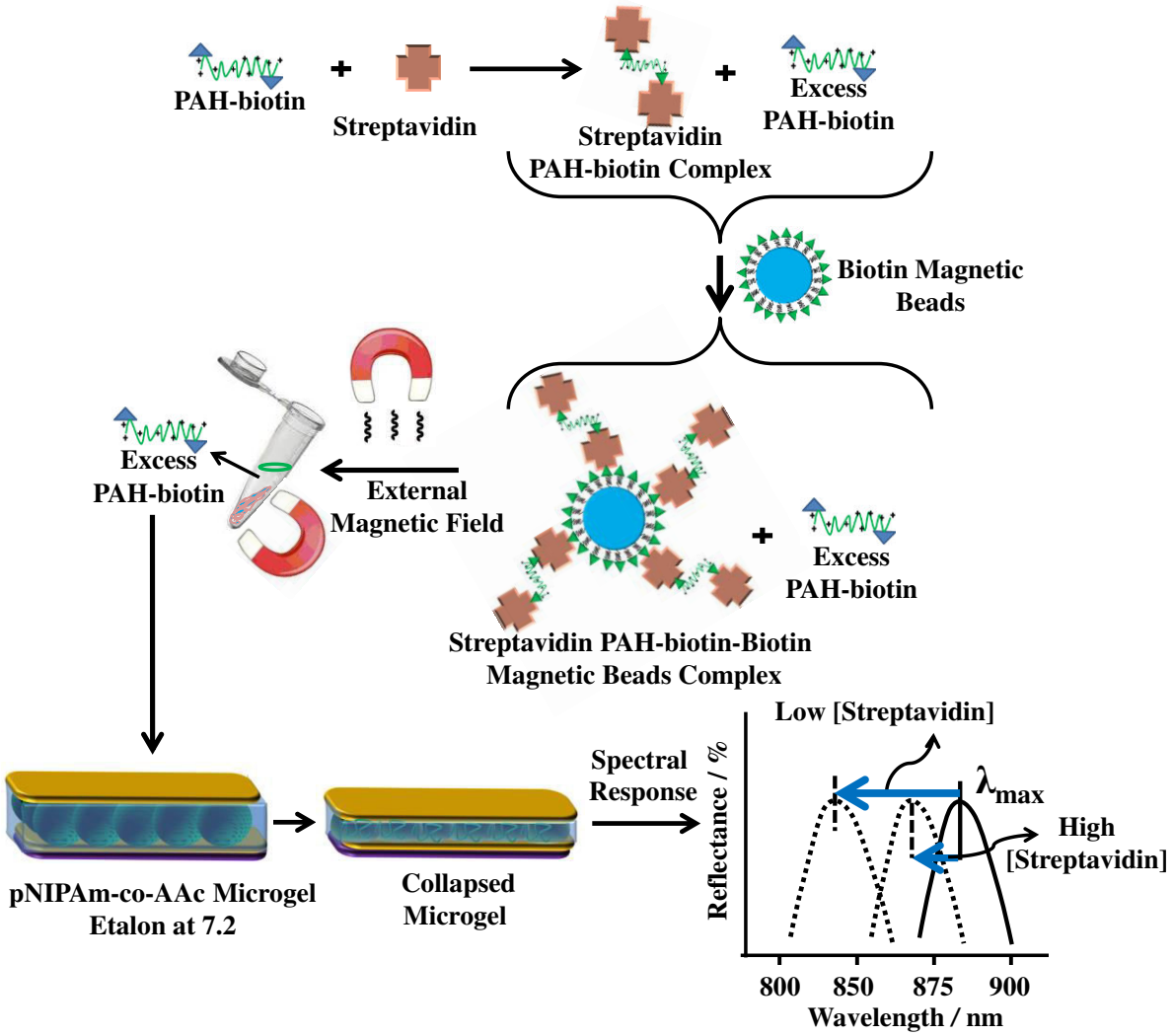
2.7 Assay Conditions and Procedure

13.0 nmoles solution of PAH-biotin was transferred to a microcentrifuge tube, and a given amount of streptavidin was added to yield the desired concentration. The reaction mixture was stirred gently for 2 hours at room temperature. 10 μL of a 30 mg/mL solution of biotin-modified magnetic particles was added to the reaction mixture, gently stirred and incubated at room temperature for another 2 hours. After this time, an external magnet was placed on the tube and held for 2 minutes. The magnet visibly pulled the biotin modified magnetic beads toward the wall of the microcentrifuge tube, with the PAH-biotin:streptavidin bound. The "free" PAH-biotin left in the microcentrifuge tube was subsequently added to the chamber containing the etalon and the reflectance spectrum continuously monitored. A number of controls were performed. For example, we showed that biotin-modified magnetic particles with PAH-biotin:streptavidin bound cannot penetrate through the etalon's Au overlayer and cause the microgels to collapse. We also confirmed that the magnetic particle storage buffer does not have any influence on the position

of the peaks in the reflectance spectra by removing the particles (via magnet) from solution and adding the remaining storage solution to the etalon.

3. Results and discussion

As we mentioned above that the positive polyelectrolyte can penetrate the oppositely charged microgel and reduce the size of the particles by crosslinking (Kleinen and Richtering, 2011), we were interested to study whether the phenomenon of polyelectrolyte penetration through the Au overlayer of etalon to crosslink the confined microgel can be used for sensing biomolecules of interest. The sensing mechanism is shown in Scheme 1. To accomplish this, the polycation, poly (allylamine hydrochloride) (PAH), which is "completely" charged at pH < ~9.0, was modified with biotin. We first showed that unmodified PAH could indeed penetrate the etalon and crosslink the microgel layer. Fig. 2 shows the shift of λ_{max} for the m=3 peak as a function of the amount of PAH exposed to the etalon. Upon addition of 3.22 nmoles PAH, the peak position shifted ~17 nm. It is important to note here that the sides of the etalon were sealed with epoxy, therefore, the PAH must enter the etalon through the Au overlayer. Importantly, the position of the reflectance peak depends on the amount of PAH added to the etalon until the etalon is "saturated" with PAH.



Scheme 1. The proposed sensing mechanism. Streptavidin (the analyte) is added to an excess amount of biotin-modified poly (allylamine hydrochloride) (PAH). The streptavidin-biotin-PAH complex is then removed from solution using biotin modified magnetic particles, leaving behind free, unbound PAH. The unbound PAH is subsequently added to a pNIPAm-co-AAc microgel-based etalon immersed in aqueous solution at a pH that renders both the microgel layer and the PAH charged. As a result, the etalon's spectral peaks shift in proportion to the amount of PAH-biotin that was added. This, in turn can be related back to the original amount of streptavidin added to the PAH-biotin.

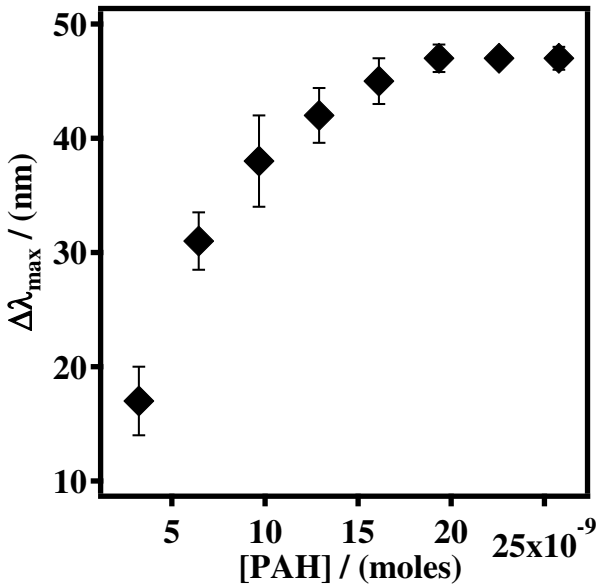


Fig. 2. Cumulative shift of the etalon's m=3 reflectance peak upon addition of PAH solution. The pNIPAm-co-AAc microgel-based etalon was soaked in pH 7.2 solution throughout the experiment, while the temperature was maintained at 25 °C. Each data point represents the average of at least three independent measurements, and the error bars are the standard deviation for those values.

To utilize this phenomenon for sensing protein concentration in solution, we modified PAH with biotin and confirmed that it can also penetrate the etalon and crosslink the microgels. To accomplish this, 1% of PAH's amine groups were modified with biotin by exposure to *N*-hydroxysuccinimide (NHS)- biotin to form an amide bond. The PAH is referred to as PAH-biotin$_{100:1}$.Once the PAH was biotinylated, we added it to the etalon. The data is shown in Fig. 3 which shows cumulative average blue shift of 21 nm with 2.0× 10^{-8} moles of PAH-biotin$_{100:1}$ solution. Successive additions give further blue shifts and follow a linear relationship. From the data, we concluded that the PAH-biotin$_{100:1}$ was able to penetrate the etalon and crosslink the microgels, causing them to collapse resulting in a blue shift of the etalon's spectral peaks.

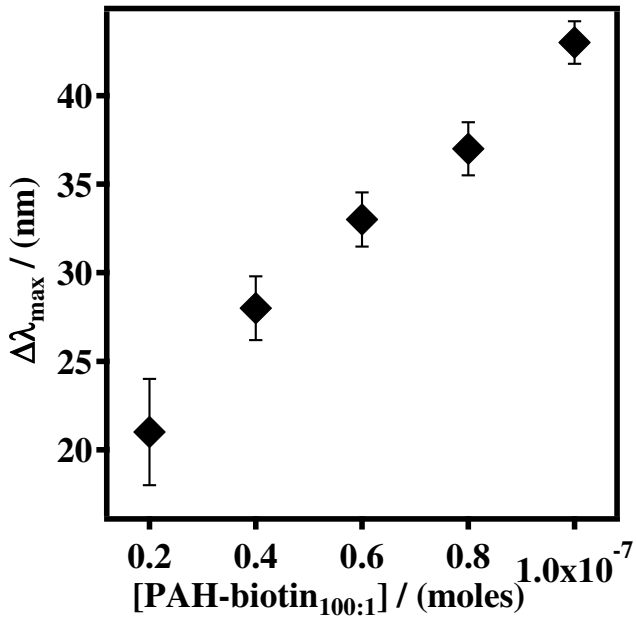


Fig. 3. Cumulative shift of the etalon's m=3 reflectance peak for the addition of PAH-biotin$_{100:1}$ solution to the etalon. The pNIPAm-co-AAc microgel-based etalon was soaked in pH 7.2 throughout the experiment, while the temperature was maintained at 25 °C. Each data point represents the average of at least three independent measurements, and the error bars are the standard deviation for those values.

It is well known that the interaction between streptavidin and biotin is very strong, near covalent ($K_d > 10^{-15}$ M^{-1}) and the kinetics of binding is extremely fast. Therefore, we utilized the above-mentioned PAH-biotin induced spectral peak shifts for sensing streptavidin concentration in solution.

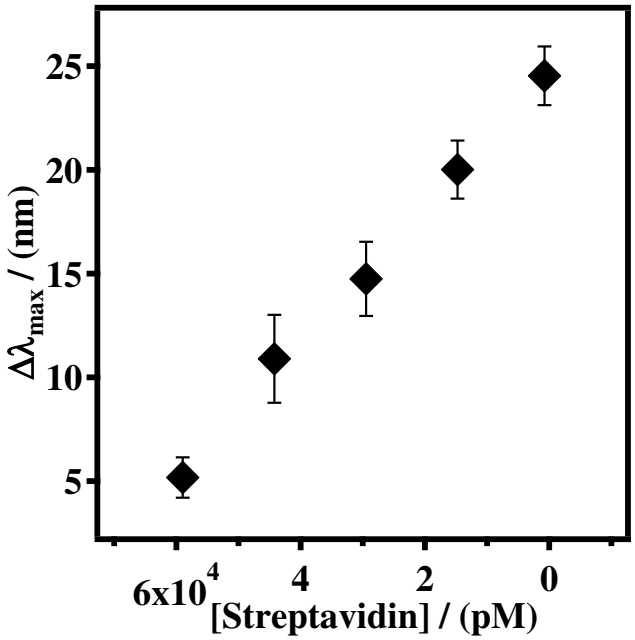


Fig. 4. Cumulative shift of the etalon's m=3 reflectance peak upon addition of amounts of streptavidin to PAH-biotin$_{100:1}$. The pNIPAm-co-AAc microgel-based etalon was soaked in pH 7.2 throughout the experiment, while the temperature was maintained at 25 °C. Each data point represents the average of at least three independent measurements, and the error bars are the standard deviation for those values.

In separate experiments, we exposed 20.0 nmoles of aqueous solution of PAH-biotin$_{100:1}$ to specific amounts of streptavidin such that there was some excess, unbound PAH-biotin$_{100:1}$. Biotin modified magnetic particles were added to the solution, which bound to the PAH-biotin$_{100:1}$:streptavidin complex. An external magnet was used to remove the magnetic particles bound with PAH-biotin$_{100:1}$:streptavidin from the solution. The solution containing the excess, unbound PAH-biotin$_{100:1}$ was subsequently added to the pNIPAm-co-AAc etalon stabilized in pH 7.2 solution maintained at 25 °C. As above, when the PAH-biotin$_{100:1}$ was added to the etalon, it resulted in a blue shift of the etalon's reflectance peaks. The results are shown in Fig. 4. As can

be seen, in this concentration range, the extent of the blue shift depends linearly on the amount of PAH-biotin$_{100:1}$ added to the etalon, which can be easily related to the amount of streptavidin initially added to the PAH-biotin$_{100:1}$. Specifically, a low concentration of streptavidin initially present in solution yields a large amount of excess, unbound PAH-biotin$_{100:1}$ that is added to the etalon, which gives a large etalon response. Alternatively, a high concentration of streptavidin initially present in solution will yield a small amount of excess, unbound PAH-biotin$_{100:1}$ that is added to the etalon, which gives a small etalon response. This point is important to stress -- these devices give larger responses as the analyte concentration decreases. This is contrary to traditional analytical methods used for sensing, which typically exhibit a lower signal:noise as the analyte concentration decreases. Ultimately, we were able to detect streptavidin concentrations in the pM range without any system optimization. By modifying the PAH-biotin concentration, and the PAH-biotin: streptavidin concentration ratio, we feel that the detection limit of the device can be dramatically enhanced.

4. Conclusion:

In summary, we have shown that pNIPAm-co-AAc microgel-based etalons are responsive to the presence of oppositely charged unmodified PAH and biotin midified PAH (PAH-biotin$_{100:1}$). This response originates from the penetration of polycationic polyelectrolytes into the etalon's microgel layer and subsequent microgel crosslinking. As a result, the microgel layer collapses, bringing the etalon's mirrors closer to one another, yielding a blue shift in the etalon's reflectance peaks. We showed that the extent of the spectral peak shift depended on the concentration of PAH and PAH-biotin present in solution, which was exploited to detect the concetration of streptavidin in solution. Ultimately, we were able to detect pM concentration of streptavidin in

solution. This submission represents a significant advance in the sensing field because of its unique response to analyte concentration -- these devices yield better signal:noise as analyte concentration decreases, which is contrary to most other analytical techniques. In the future, we will utilize this sensing scheme for detecting other protiens, and DNA that are considered biomarkers for disease.

5. Acknowledgement:

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