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Biological Imaging and Sensing with Multiresponsive Microgels

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Supporting Information

ABSTRACT: Multiresponsive spiropyran-modified poly(N-isopropylacrylamide)-based microgels were synthesized and their response to temperature; UV, visible, and near-infrared radiation; pH; and Cu2+ was investigated. The responses were a result of the spiropyran groups undergoing a reversible isomerization/reaction from a neutral to a charged form. The isomerization process was investigated via experiment and theory. The two-photon excited fluorescence of the spiropyran-modified microgels was also characterized, and their ability to be used to image live cells was determined. Finally, optical devices (etalons) were fabricated using the spiropyran-modified microgels, and the ability of the devices to change color in response to the above-mentioned stimuli was also investigated. We found that the microgel’s responsivity was retained in the etalon, which yielded color tunable devices and sensors. This investigation illustrates the versatility of pNIPAm-based microgels and microgel-based etalons and showcases the clear utility of such devices for remote actuation, color tunable optics, sensing, and remotely triggered drug delivery systems.

1. INTRODUCTION

The ability of natural systems to sense, respond, and adapt to environmental conditions has both fascinated and inspired philosophers and researchers alike for ages. Inspired by nature, “smart” materials have been developed in the lab that can respond in defined ways to environmental or external stimuli such as temperature, pH, light, magnetic field, electric field, ions, enzymes, and specific organic compounds. Polymeric materials are particularly attractive due to the variety of monomers available that allow their chemistry to be easily tuned. For example, certain monomers can be chosen that result in polymers with specific mechanical properties, biocompatibility, and the ability to swell, shrink, or bend in response to a wide variety of environmental stimuli. Of all stimuli-responsive polymers, temperature-responsive poly(N-isopropylacrylamide) (pNIPAm) is the most well-known and well studied, exhibiting a lower critical solution temperature (LCST) at 32 °C. Consequently, by altering the temperature of pNIPAm in water, its solubility can be tuned; e.g., pNIPAm transitions from hydrophilic (soluble, extended state) to hydrophobic (insoluble, collapsed state) as the LCST is exceeded.

PNIpAm-based polymer networks can also be generated by cross-linking the pNIPAm chains. This can yield macroscopic hydrogels, or hydrogel particles with diameters on the micron to nano scale. Specifically, pNIPAm-based hydrogel particles (microgels) can be synthesized to have diameters on the order of 100 nm to several micrometers. They can be used for sensing and actuation, catalyst supports, drug delivery, and enzyme nanocapsules. In order to expand the utility of the above-mentioned microgels, it is necessary to render them responsive to a number of different stimuli, i.e., individual microgels that can respond to multiple stimuli (multiresponsive). Multiresponsive microgels could open up second-generation applications of microgels with useful combined chemical and physical properties.

Our group’s research program has focused on the development of novel microgels, and investigating their use as sensors, artificial muscles, and drug delivery motifs. In an effort to make our materials even more functional and responsive, we show here that multiresponsive pNIPAm-based microgels could be synthesized by incorporation of spiropyran (SP) into the microgel structure. SP is a unique molecule that exhibits responsiveness to a number of different stimuli. For example, it has been shown that SP can undergo C(spiro)=O bond cleavage upon UV light irradiation, pH changes, ultrasound exposure, mechanical force, electric fields, and in the presence of certain metal ions. Here, we investigate the microgel’s response to ultraviolet (UV) and visible light, pH, temperature, and copper ion (Cu2+), as depicted in Scheme 1a. Specifically, SP’s C(spiro)=O bond can be cleaved by UV light exposure, and at low pH (<4). During the process, SP isomerizes from a colorless, nonplanar, closed and neutral form to the colored, planar, open and charged merocyanine form.
The charged MC structure of SP increases the microgel’s inner osmotic pressure and hydrophilicity, which results in microgel swelling, as shown in Scheme 1a. MC can also be triggered to reform SP by visible light—the SP-MC-SP transition is reversible over many cycles without performance loss. Additionally, Cu²⁺ is able to cleave the C(spiro)−O bond into MC, which can bind Cu²⁺ and cross-link the microgels phenoxide anions with a 2:1 stoichiometry. Finally, the SP-modified microgels are responsive to near-IR (NIR) light. This is a result of SP-to-MC isomerization triggered by exposure to 780−840 nm NIR wavelengths. Interestingly, MC also exhibits two-photon excited fluorescence, absorbing low energy NIR, and emitting visible light at 590 nm. This property is especially important for biological imaging because low energy excitation is gentle on biological tissues and cells. The amount of unreacted AAc was determined by titration with NaOH using methyl red as an indicator—we determined that the unreacted AAc amount was below 3%. These microgels (MG) are denoted as MG-SP (or MC)-a (or -b). We determined the diameter of the microgels via dynamic light scattering (DLS) (Figures S2 and S3), which revealed hydrodynamic diameters of 880 nm for MG, 770 nm for MG-SP-a, and 760 nm for MG-SP-b at 30 °C.

The decrease of the hydrodynamic diameter after the esterification reaction was attributed to the increased microgel hydrophobicity by replacing the AAc with SP. The LCST of the microgels was also determined, and these microgels exhibited an LCST of 32.5 °C for MG and 30.5 °C for MG-SP-a/b in DI water. The decrease of the LCST was also attributed to increased hydrophobicity of microgels by SP modification. Since pNIPAm-based microgels are more sensitive to stimuli near their LCST, most of the following investigations were completed at 30 °C. Transmission electron microscope (TEM) images of the resultant microgels can be seen in Figure 1. From analysis of 30 microgels in the image, we determined that the dried microgels had an average diameter of ~400 nm ±1.5%.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization of SP-Containing Microgels. In order to investigate the effect of chemical structure on SP behavior, two SP monomers (with and without a nitro group) were synthesized in four steps, as detailed in the Supporting Information (SI). The effect of the nitro group on SP isomerization was investigated via experiment and theory. SP containing a nitro group is denoted as SP (or MC)-a, whereas the SP without the nitro group is denoted as SP (or MC)-b (Figure S1). Microgels were synthesized by free radical precipitation polymerization of N-isopropylacrylamide (NIPAm), acrylic acid (AAc), and N,N′-methylenebis(acrylamide) (BIS), following standard protocols (SI). SP monomers were incorporated into microgels via an esterification reaction between SP’s hydroxyl group and the microgel’s carboxylic acid (SI). After the esterification reaction, the pH of the microgel solution changed from 4.2 to 6.8, which is indicative of the acrylic acid being transformed into the ester group. These microgels (MG) are denoted as MG-SP (or MC)-a (or -b). We determined the diameter of the microgels via dynamic light scattering (DLS) (Figures S2 and S3), which revealed hydrodynamic diameters of 880 nm for MG, 770 nm for MG-SP-a, and 760 nm for MG-SP-b at 30 °C.

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2.2. Etalon Construction. Microgel-based optical devices (etalons) were first reported by our group in 2010, since then, they have been shown to be extremely versatile, exhibiting visual color, and multiplex reflectance spectra, which can be tuned as a function of many different stimuli (Scheme 1b). We have also shown that they are able to act as drug delivery devices, and artificial muscles (among other things). In short, etalons are constructed by painting a concentrated microgel solution on a Au-coated glass substrate, followed by copious rinsing with deionized water to remove microgels not directly attached to the Au. Finally, the microgel layer was dried, and a subsequent layer of Au was deposited on the microgel layer. The structure of the resultant etalons was investigated using scanning electron microscopy (SEM), and the images can be seen in Figure S4. The SEM images revealed a single microgel layer, and a structure typical of microgel-based etalons. The optical properties of the devices can be predicted by eq 1

\[ m \lambda = 2nd \cos \theta \]  

where \( m \) (an integer) is the order of a reflected wavelength (\( \lambda \)), \( \lambda \) is the wavelength of reflected light of a given peak order, \( n \) is the refractive index of the dielectric layer, and \( d \) is the distance.
between the two Au layers that is defined by the thickness of the microgel layer (b in Scheme 1b), and $\theta$ is the angle of incident light relative to the etalon normal. Therefore, the positions of the peaks in the reflectance spectra (and hence the device color) depend on the distance between two Au layers and the refractive index of microgels (at a single observation angle). For our devices, the optical properties depend primarily on the distance between the two Au layers.

### 2.3. Temperature Responsivity

To confirm the basic responsivity and function of the etalons, initial experiments focused on characterizing the etalon response to temperature (i.e., thermoresponsivity). Etalons were immersed in water for this investigation; the resulting reflectance spectra are shown in Figure 2. As can be seen, a reflectance spectrum with a peak at 650 nm at 25 °C was observed. When the temperature was increased to 30 °C, the peak exhibited a blue shift of 60 nm, while it exhibited an additional ∼70 nm shift (130 nm total shift) when the temperature was increased to 35 °C. These peak shifts are a direct result of the thermoresponsivity of the pNIPAm-based microgels, which collapse at elevated temperature, decreasing the distance between the etalons two Au layers. This can be predicted from eq 1.

### 2.4. UV Responsivity and Simulation

Since the basic thermoresponsivity of the pNIPAm microgel-based devices was confirmed, the response of the microgels and microgel-based etalons to UV irradiation was subsequently characterized. As can be seen in Figure 3a, when SP is exposed to UV light (∼360 nm), it undergoes an SP-MC isomerization, resulting in a change of the microgel solution color from colorless to pink. Examination of the pink solution with a UV spectrophotometer showed a new absorbance band centered at 540 nm, corresponding to the open MC form of SP (Figure 3b). Exposure to ambient light for 20 min at room temperature caused the peak to disappear (Figure 3b), consistent with the known photolytic reversion to the closed SP form. In order to further understand the adsorption of MC at 540 nm, we used a multiscale theoretical method to predict the light adsorption spectrum of MC. The absorption spectrum was simulated at a B3LYP/6-311+G(d,p) level in the Gaussian 09 package. In our calculations, the basis set 6-311+G(d,p) was used for C, N, O, and H atoms with the B3LYP functional. As shown in Figure 3, the position of the predicted absorption peak (537 nm) is in good agreement with the experimentally observed absorption peak (535 nm). This indicates that simulation at the B3LYP/6-311+G(d,p) level is acceptable. The strong absorption of MC at 537 nm in Figure 3c was attributed to the electronic transition from HOMO (NO-σ and CO-π) to LUMO (NC-π*).

The irreversible isomerization also causes a change of the microgel diameter as a result of the charged MC form transitioning to the neutral SP form. As can be seen in Figure S5, the diameter of the microgels decreased from 810 to 770 nm as a result of the MC-SP isomerization process. We propose that the MG-MC microgels are large in diameter due to their hydrophilicity and the charged state generating an inner osmotic pressure, which results in water entering the microgels and microgel swelling. For comparison, the response of...
pNIPAm-based microgels (without SP) to UV irradiation was investigated at the same conditions, which did not exhibit any size change after UV exposure (Figure S6), indicating that the change in diameter of MG-SP-a resulted from isomerization of SP to MC. The change in diameter of the microgels as a result of the isomerization process can also be characterized using the etalon structure. The MG-SP-a etalon’s reflectance peak exhibits a red shift of 45 nm at 30 °C after exposure to UV light, as shown in Figure 3e. The red shift observed in the reflectance spectrum was attributed to the increase in microgel size as a result of the SP-MC isomerization process. When the etalon was exposed to visible light, the reflectance peak shifts back to its initial position within 2 h. This is due to the isomerization of MC form back to SP form with reformation of the C(spiro)–O bond. The response/reversibility for each etalon was repeated 5 times without a noticeable change in its response. For comparison, etalons fabricated using MG without SP were prepared and tested, which did not exhibit any observable response after exposure to UV light (Figure S7). This further indicates that the shifts in the peaks of the reflectance spectra are a result of SP-MC isomerization. Finally, etalons composed of MG-SP-b microgels were fabricated and tested under the same conditions as above, which did not exhibit any spectral shift after exposure to UV (Figure S8). This result indicates that UV light cannot trigger SP-MC isomerization for MG-SP-b.

In order to understand why SP-a and SP-b behave differently, the activation energy of the C(spiro)–O bond of SP-a and SP-b was calculated using Gaussian 09 at the B3LYP/6-311+G(d,p) level. In their ground states, SP-a and SP-b have similar C(spiro)–O bond lengths (2.26 Å for SP-a and 2.29 Å for SP-b), and SP-a and SP-b need to overcome energy barriers of 12.35 and 15.33 kcal/mol to break the C(spiro)–O bond and generate the MC form, respectively (Figure S9). According to the Arrhenius equation

\[ k = A e^{-\frac{E_a}{RT}} \]  

where \( k \) is rate constant, \( T \) is absolute temperature, \( A \) is pre-exponential factor, \( E_a \) is activation energy, and \( R \) is universal gas constant. The rate constant ratio of SP-a to SP-b is 163, indicating that the nitro group accelerates the isomerization speed by 163. Thus, all of following work was completed using MG-SP-a.

2.5. pH Responsivity. It has been reported that the SP-MC isomerization occurs at low pH,\(^{13} \) generating phenol and quaternary ammonium groups with a color change from colorless to yellow,\(^{17} \) as illustrated in Figure 4a. Therefore, the response of MG-SP-a to pH was also characterized. We showed that, in acidic solution, the SP group of the microgels is in the protonated MC form (MCH) and exhibits a yellow color. After exposure to visible light, the yellow color of microgels disappeared and microgels become colorless, indicating isomerization of the MCH form to the closed-ring SP form. This isomerization process is believed to release a proton (Figure 4a), which can be observed as a pH change. To investigate this further, a certain amount of MG-SP-a was dissolved in water (pH = 4.2 with a molar ratio of SP to H+ = 1.5:1). After 2 h in the dark, the C(spiro)–O bond was broken, and the resultant phenate groups combined with protons to generate MCH with a pH change from 4.2 to 5.3. Once the solution was exposed to visible light for 1 h, the pH of the solution returned to 4.2 (a 10-fold change in H+ concentration). This was attributed to proton release from the isomerization of MCH to SP triggered by visible light. The diameter change of these microgels was also monitored using DLS, which revealed that the diameter of the microgels increased from 770 to 800 nm when MG-SP transitioned to MG-MCH (Figure S10). According to the above results, we hypothesized that etalons composed of MG-SP-a should be pH responsive, swelling at low pH from the charge generation. When the solution pH was decreased from 7 to 3, the observed reflectance peaks shifted 29 nm to higher wavelength (Figure 4b). After that, the etalons were regenerated by exposure to visible light. A total of three individual etalons were investigated, all exhibiting similar properties. An etalon fabricated using MG was also used as a control experiment, which did not exhibit any spectrum shift in response to pH change (Figure S11).

2.6. Cu2+ Responsivity. In a subsequent experiment, the response of MG-SP-a to Cu2+ was investigated. It has been reported that SP-MC isomerization of the small monomers can be triggered by Cu2+, and exposure to visible light can regenerate SP with expulsion of the Cu2+.\(^{50} \) Recently, nanoporous frameworks with SP have been synthesized and used for Cu2+ release.\(^{40} \) The mechanism of Cu2+ response is shown in Figure 5a. In the process, MG-SP-a was dissolved into CuCl2 solution (ratio of SP to Cu2+ is 2:1). After 2 h in the dark, MG-SP-a transitions from colorless to red and the microgels aggregate—this is indicative of the isomerization of the SP form to the MC form. The aggregation is a result of Cu2+ coordinating with the phenoxide anions in a 2:1 stoichiometry. The aggregates can be seen in the bottom of the right tube in Figure 5a. The Cu2+ concentration of the solution was investigated by atomic adsorption spectrophotometry, and the result showed that 35% Cu2+ was incorporated into microgels. After exposure to visible light, 95% of Cu2+ in microgels was released. For comparison, pNIPAm microgels without SP were also tested at the same conditions and were able to absorb 4.5% Cu2+. Figure 5b shows the dependence of the position of the etalon’s reflectance peak as a function of Cu2+ concentration in water. Spectra exhibit blue shifts as the

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**Figure 4.** (a) Reversible SP-MC-SP isomerization triggered by H+ and visible light. (b) Etalon reflectance peak shift as a function of pH; each point is the average from three repeated experiments with a single device, while the error bars indicate standard deviation.
solution concentration of Cu\textsuperscript{2+} was increased. Cu\textsuperscript{2+} acts like a cross-linking site, resulting in deswelling of microgels and blue shifts of etalon spectra. As can be seen in Figure 5b, etalon spectra exhibited a total blue shift of 59 nm, as the Cu\textsuperscript{2+} concentration increases from 0 to 1.2 mmol L\textsuperscript{-1}. Therefore, the device can be used for quantitative analysis of Cu\textsuperscript{2+}. Etalons composed of just MG did not exhibit a significant spectral response to Cu\textsuperscript{2+} (Figure S12).

2.7. NIR Responsivity and Biological Imaging. Lastly, we investigated the response of microgels to NIR exposure (800 nm) and used the microgels for biological imaging (bioimaging). It has been reported that SP can be triggered to transform to MC upon exposure to 800 nm radiation (NIR)\textsuperscript{34}. this wavelength was also capable of inducing fluorescence in the wavelength range from 520 to 640 nm (Figure 6a). In order to use this material for bioimaging, small diameter microgels (SMG) with diameters of 150 nm (determined by TEM (Figure 6b)) were synthesized (see the SI), which was named as SMG-SP(MC)-a. It has been reported that nanoparticles within the diameter range of 100–200 nm can undergo endocytosis, and be taken up by cells\textsuperscript{51}. Once cells take up the SMG-SP-a via endocytosis, the fluorescence images of cells can be obtained due to the fluorescence of MC upon exposure to 800 nm light. Figure 6c,d shows bright-field and two-photon fluorescence images, respectively, of breast cancer cell line (MCF-7) after their incubation overnight in a 1 × 10\textsuperscript{-5} g mL\textsuperscript{-1} SMG-SP-a solution. Under 800 nm two-photon excitation, these cells exhibited red fluorescence due to two-photon excited fluorescence of SMG-MC-a. For comparison, the microscope and two-photon excited fluorescence images of MCF-7 without exposure to SMG-SP-a were obtained, which did not show fluorescence. This clearly shows the ability of SP-MG to be used for biological imaging (Figure S13).

3. CONCLUSION

To summarize, we developed microgels that exhibit responses to multiple stimuli; i.e., they are able to respond by swelling or shrinking in response to exposure to light (UV and visible), solutions of various pHs, and exposure to Cu\textsuperscript{2+}. Etalon devices were fabricated using these microgels, which are capable of changing their optical properties in response to the application of a number of different stimuli. This behavior can be used for sensing applications, as well as for remote actuation and triggered drug/small molecule delivery. Furthermore, we showed that the synthesized microgels have the ability to undergo two-photon fluorescence, which can be exploited for biological imaging applications. This investigation not only showcases the versatility of pNIPAm-based microgel chemistry, and how it can be manipulated to achieve very complex responsivities, but it also shows the versatility of the etalon construct. This chemical/functional diversity, combined with the biological imaging applications, make these materials highly valuable for myriad applications.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.5b04028.

Information on materials used and experimental details, microgel synthesis protocols, chemical structure for SP monomers, dynamic light scattering measurements of microgel diameters, scanning electron microscope images, reflectance spectra for etalon devices, potential energy diagrams for C–O bond breaking, and cell images (PDF)

Video showing the fluorescence of MG-MC-a and its quenching when MC isomerizes to SP (AVI)
Chemistry of Materials

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Notes

The authors declare no competing financial interest.

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■ REFERENCES


