Luminescence Probes

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Reversible Three-State Switching of Multicolor Fluorescence Emission by Multiple Stimuli Modulated FRET Processes within Thermoresponsive Polymeric Micelles**

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Energy transfer between light-absorbing donors and energy-receiving acceptors occurs on the nanometer distance scale.[1] One of the key issues in designing effective multichromophore luminescent systems is the precise spatial arrangement of the fluorophores. By learning from elegant systems existing in nature, researchers have exploited a variety of artificial nanostructures such as dendrimers,[2] nanoparticles,[3] (multilayered) thin films,[4] and supramolecular assemblies[5] to achieve nanoscale control and accurate location of chromophores, leading to the modulation of luminescence efficiency through the enhancement or restriction of fluorescence resonance energy transfer (FRET) processes. In view of the microenvironment complexity in certain bio-applications such as imaging, biosensing, and clinical diagnosis, it is highly desirable to combine the concept of external stimuli-triggered activation/deactivation of specific emitting fluorophores to achieve higher temporal and spatial detection resolution. Although there are a few examples of luminescent polymeric assemblies and nanoparticles exhibiting two-state switching of luminescence,[6] systems exhibiting both reversible three-state on/off switching of the fluorescence emission and stimuli-responsive tuning of the spatial distributions of FRET donors and acceptors (FRET efficiency) has, to our knowledge, not been accomplished.

Herein we report the fabrication of amphiphilic and thermoresponsive diblock-copolymer-based luminescent micelles exhibiting three-state switchable multicolor fluorescence emission by external stimuli-modulated FRET processes (Scheme 1). The FRET system consists of one type of donor dye and two types of acceptor dyes, and fluorescence emission of the latter two can be switched on and off by changes in pH and light irradiation (UV/Vis), respectively. Such multicolor luminescent polymeric assemblies can act as sensitive ratiometric probes for pH and temperature. Most importantly, the detection sensitivity can be further improved at elevated temperatures because of the closer proximity between FRET donors and acceptors resulting from the thermoresponsive collapse of micelle coronas.

Three polymerizable fluorescent dyes, NBDAE, pH-switchable rhodamine B based monomer (RhBAM; synthesis: Scheme S1, NMR data: Figure S1 in the Supporting Information), and photoswitchable SPMA, were synthesized.[7] Amphiphilic diblock copolymer, P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$, bearing NBDAE and SPMA moieties in the hydrophobic polystyrene (PS) block and RhBAM moieties in the thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) block was synthesized by sequential reversible addition–fragmentation transfer (RAFT) polymerization (Scheme S2, Figures S2 and S3 in the Supporting Information).[8] For comparison, a series of PS-*b*-PNIPAM diblock copolymers with varying combinations of NBDAE, SPMA, and RhBAM residues were also synthesized. The molecular parameters of all diblock copolymers used in this work are summarized in Table S1 in the Supporting Information.

In aqueous solution, P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ self-assembles into spherical micelles, as evidenced from AFM results (Figure S4 in the Supporting Information), which consist of PS cores embedded with NBDAE and SPMA dyes and thermoresponsive PNIPAM coronas embedded with RhBAM dyes.[9] Surface tensiometry measurements at 25 °C revealed a critical micelle concentration (CMC) of $2.3\times10^{-3}\,\mathrm{g\,L^{-1}}$ (Figure S5 in the Supporting Information). The micellar solution at a concentration of $1.0\,\mathrm{g\,L^{-1}}$ exhibits thermo-induced aggregation above 28 °C owing to the well-known lower critical solution temperature (LCST) phase-transition behavior of PNIPAM coronas (Figure S6 in the Supporting Information).[10] Dynamic laser light scattering (LLS) analysis further revealed intensity-average hydrodynamic diameters $\langle D_h\rangle$ of 50 nm and 36 nm for the micellar solution at 25 °C and 35 °C, respectively (Figure S7 in the Supporting Information). A comparison of AFM and dynamic LLS analysis results indicated the shrinkage of the thickness of the micellar coronas from approximately 18 to 11 nm upon heating above the LCST of PNIPAM coronas.

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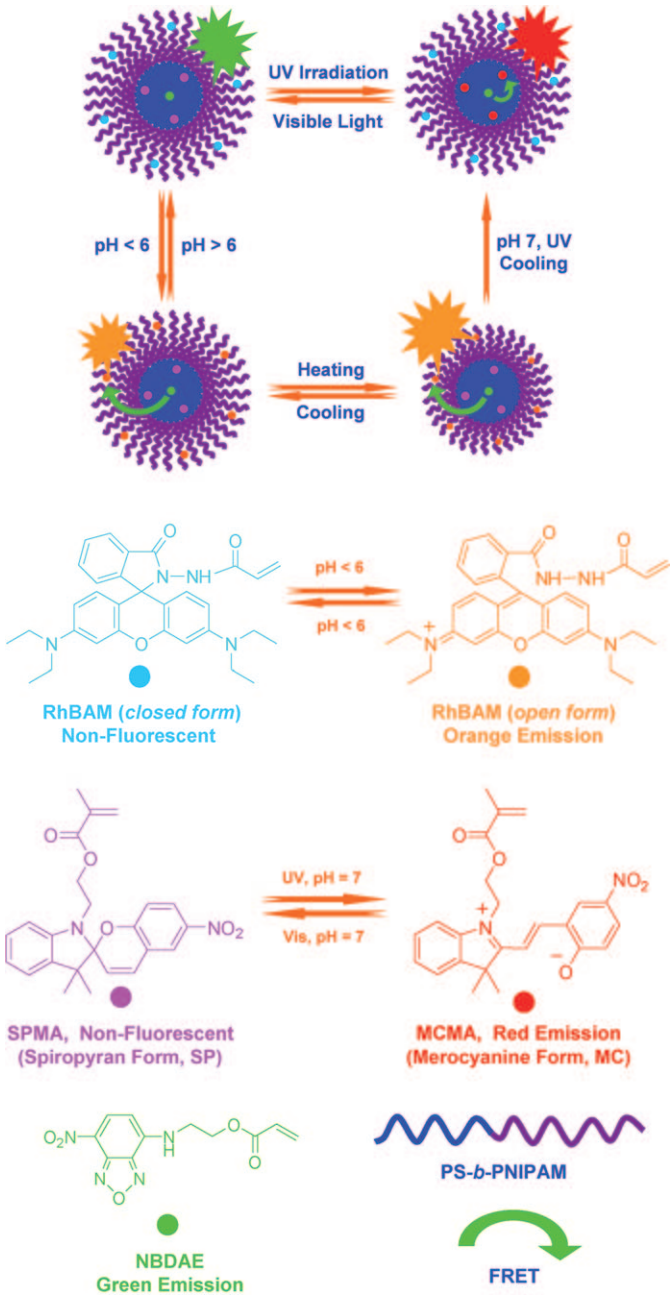

Scheme 1. Construction of a polymeric-micelle-based reversible three-state switchable multicolor luminescent system from amphiphilic and thermoresponsive diblock copolymer P(St-*co*-NBDAE-*co*-SPMA)-*b*-P(NIPAM-*co*-RhBAM). See text for details.

Among the three copolymerized dyes, NBDAE exhibits strong green fluorescence emission at 520 nm, the intensity of which is almost irrespective of solution pH in the range pH 3–9 (Figure S8 in the Supporting Information).[7a] The pH-sensitive RhBAM moiety exists in the spirolactam form above pH 6 and exhibits no fluorescence emission, whereas below pH 6 it transforms into the "open" form and emits strong fluorescence at 580 nm (Scheme 1).[11] Thus, the fluorescence emission of RhBAM residues can be facilely switched on and off by pH variations (Figure S9 in the Supporting Information). The photoswitchable fluorescence emission behavior of SPMA has been well-documented.[6a–c,12] SPMA exists in the colorless spiropyran form under visible light; upon UV irradiation, it transforms from the nonfluorescent form into the merocyanine form (MCMA), which has fluorescence emission at 645 nm (Scheme 1). Considering that the fluorescence emission of both NBDAE and MCMA can be enhanced when located in hydrophobic microenvironment,[7a,12] we choose to copolymerize NBDAE and SPMA moieties into the hydrophobic PS block and pH-sensitive RhBAM dyes into the thermoresponsive PNIPAM block.

Interestingly, the NBDAE fluorescence emission band overlaps well with the absorbance bands of ring-opened RhBAM and MCMA moieties (Figures S8 and S10 in the Supporting Information). Thus, they can be integrated into the diblock copolymer to construct efficient luminescent systems with FRET features.[6b,c,13] P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ possesses one type of donor dye (NBDAE) and two types of potential FRET acceptor dyes, RhBAM and SPMA. The fluorescence emission of the latter two can be switched on and off by pH and light irradiation (UV/Vis), respectively. This novel type of dye combination might lead to the construction of three-state switchable multicolor luminescent systems [NBDAE emission at pH 7 under visible light, NBDAE/RhBAM(open form) FRET system below pH 6 under visible light, and NBDAE/MCMA FRET system at pH 7 upon UV irradiation]. Moreover, the efficiency of the FRET process between NBDAE and ring-opened RhBAM below pH 6 can be facilely tuned by temperature variations by exploiting the thermo-induced collapse of PNIPAM coronas, which results in the closer proximity between FRET donors and acceptors (Scheme 1).

We then explored the pH-modulated luminescence behavior of P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ micellar solution; under the initial conditions, SPMA moieties in the PS block are colorless and non-fluorescent. Above pH 6, only the green fluorescence emission of NBDAE dyes ($\lambda_{em} = 518$ nm, Figure 1 A) is observed, as RhBAM residues exhibit no emission, that is, a FRET process between NBDAE and RhBAM or SPMA does not occur. Upon adjusting to pH < 6, RhBAM moieties convert into the fluorescent ring-opened form, as evidenced from the appearance of a new fluorescence emission band at around 580 nm (Figure 1). Figure 1 C clearly shows that the pH decrease in the range pH 3–6 results in an increase of emission intensity (580 nm) of ring-opened RhBAM, accompanied by a considerable decrease of NBDAE emission intensity at 518 nm. The pH-induced fluorescence emission changes can also be visualized by the naked eye, as evidenced from the transition from green emission at pH 7 to yellow emission at pH 3 (Figure 1 B). The above results indicated the occurrence of a FRET process between NBDAE within micellar cores and ring-opened RhBAM moieties within micellar coronas below pH 6, as the emission of NBDAE residues in diblock copolymer micelles is essentially independent of solution pH (Figure S8 in the Supporting Information). When the solution pH was cycled between pH 7 and 3, we observed repeated changes of emission intensity at

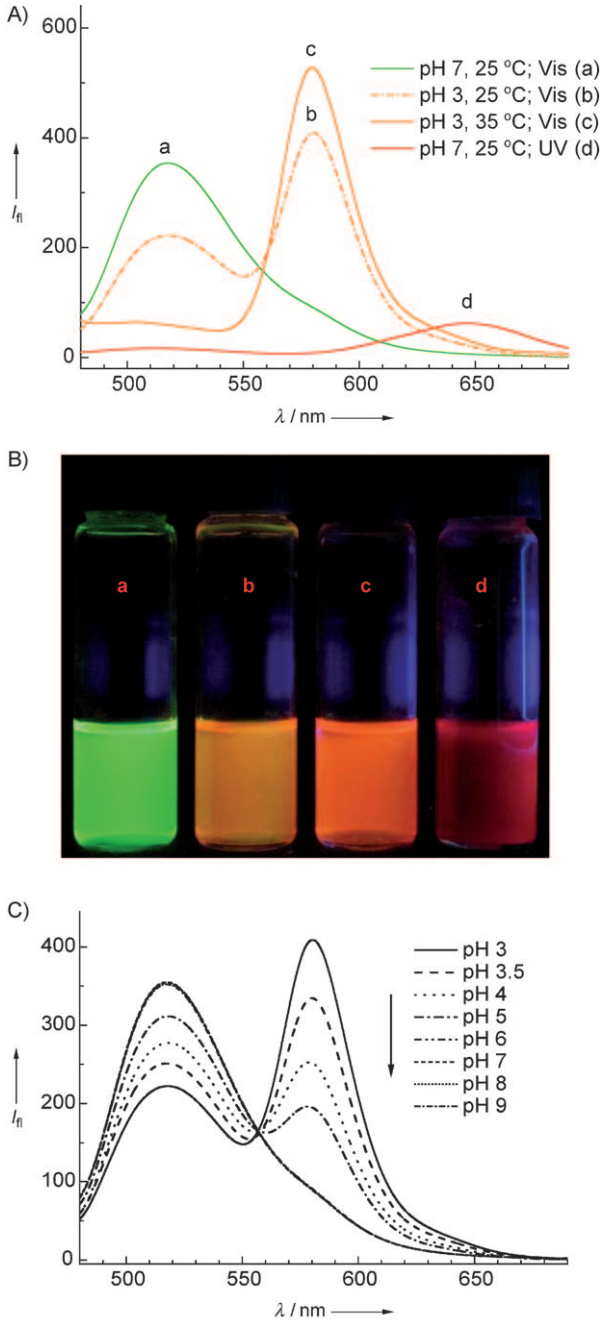

Figure 1. A) Fluorescence emission spectra (λ_{ex} = 470 nm, slit widths for excitation and emission: 5 nm) and B) photograph recorded immediately (unless otherwise stated) under irradiation at 365 nm by a UV lamp for micellar solutions of P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ under various conditions: a) pH 7 and 25 °C; b) pH 3 and 25 °C; c) pH 3 and 35 °C; and d) pH 7 and 25 °C upon UV irradiation for 2 min. C) Fluorescence emission spectra under the same conditions in the pH range 3–9.

580 nm, indicating that pH-induced emission switching is fully reversible (Figure S11 in the Supporting Information).

Under visible-light conditions, the presence of SPMA moieties in the diblock copolymer does not exhibit any appreciable effects on the pH-switchable luminescence behavior, as evidenced from spectrofluorimetric results obtained for dual dye-labeled diblock copolymers, P(St-*co*-NBDAE)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ and P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-PNIPAM$_{60}$, as control samples (Figures S12–S14 in the Supporting Information). The above results indicated that the micellar solution of P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ can act as sensitive ratiometric pH probes in the range pH 3–6 (Figure 1 C and Figure S15 in the Supporting Information). We further employed the thermo-induced collapse of PNIPAM coronas to adjust spatial proximity between NBDAE and ring-opened RhBAM moieties located within micellar cores and coronas, respectively (Scheme 1). As shown in Figure 1 A, B, heating the micellar solution from 25 °C to 35 °C leads to a yellow-to-orange transition of the fluorescence emission. We can clearly observe the considerable increase of RhBAM emission and the dramatic decrease of NBDAE emission at pH 3 and 35 °C, relative to that at pH 3 and 25 °C. This result indicated the occurrence of a more-efficient FRET process for diblock copolymer micelles with collapsed PNIPAM coronas. A more-detailed investigation revealed that emission intensity ratios I_{580}/I_{518} exhibit around a 3.5 times increase in the narrow temperature range 27–32 °C (Figure 2 a and Figure S16 in the Supporting Information). Thus, the diblock copolymer micelles can also act as ratiometric fluorescent thermometers. From Figure S15 and

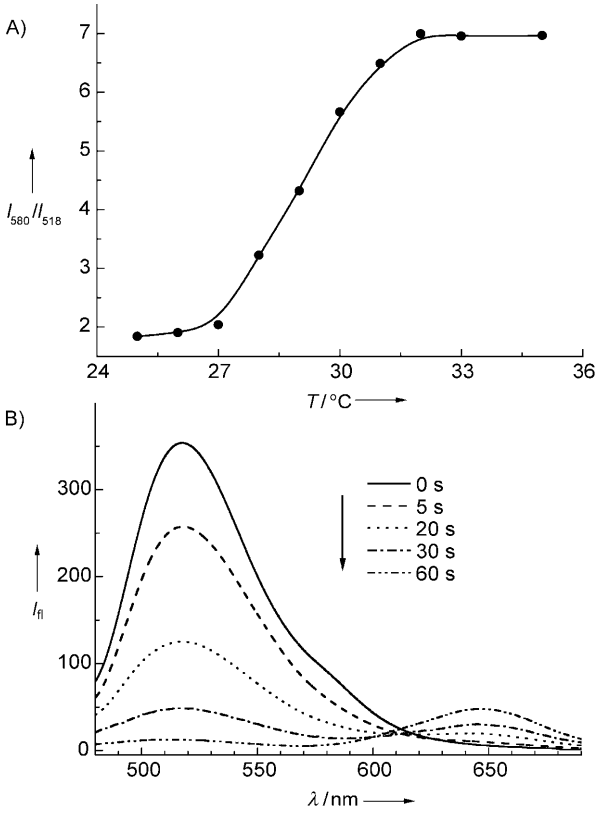

Figure 2. A) Fluorescence intensity ratio changes (I_{580}/I_{518}; conditions as before) as a function of temperature recorded for the micellar solution (pH 3) of P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$. B) Time evolution of fluorescence spectra (conditions as before) recorded for the micellar solution (25 °C and pH 7) upon UV (365 nm) irradiation.

Figure 2a it follows that the diblock copolymer micelles can act as fluorescent ratiometric dual probes for pH and temperature. Moreover, the detection sensitivity of pH can be considerably enhanced at 35 °C, relative to that at 25 °C (Figure S15).

The presence of SPMA moieties in P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ diblock copolymer can add extra features to the modulation of multicolor emission. We further investigated the photoswitchable emission behavior of the diblock copolymer micelles at pH 7 and 25 °C, under which conditions RhBAM moieties exhibit no fluorescence emission; thus, only the green emission from NBDAE moieties can be observed under visible light (Figure 1B). We observed the appearance of a new emission band at around 645 nm upon UV irradiation, the intensity of which gradually increased with irradiation time and stabilized out after 1 min (Figure 2b and Figure S17 in the Supporting Information). Concomitantly, the NBDAE emission intensity at 518 nm exhibits a dramatic decrease. This change indicates the occurrence of a FRET process between NBDAE and the merocyanine form MCMA. Visual inspection of the micellar solution under a UV lamp (365 nm) revealed an abrupt green-to-red transition in fluorescence emission (Figure 1B). It is notable that under UV irradiation at pH 7 and 25 °C, RhBAM residues are stable and exist in the closed form, as evidenced from the control sample only containing NBDAE and RhBAM dyes (Figure S18 in the Supporting Information), which might be ascribed to the chemical structure and the fact that RhBAM moieties are located in a hydrophilic microenvironment.[14] Upon five cycles of alternate UV and visible-light irradiation, the emission intensity at 645 nm can essentially recover to its original value (Figure S17 in the Supporting Information).

In conclusion, we demonstrated the first example of an amphiphilic responsive block copolymer micelle based multichromophore luminescent system exhibiting reversible three-state switching of fluorescence emission (green, yellow, orange, and red) by modulating two independent FRET processes by external stimuli (e.g., pH, temperature, and light irradiation). The reported diblock copolymer micelles can serve as sensitive ratiometric fluorescent dual probes to pH and temperature; moreover, the detection sensitivities can be facilely adjusted through thermo-induced collapse of responsive micellar coronas owing to the closer proximity between the FRET donors and acceptors. This novel type of multicolor luminescent polymeric assemblies augurs well for practical applications in cell imaging, biosensing, and clinical diagnosis.

Experimental Section

Experimental details, including the synthesis procedures, characterization methods, and all relevant characterization data, are available in the Supporting Information.

Preparation of micellar solutions: P(St-*co*-NBDAE-*co*-SPMA)$_{20}$-*b*-P(NIPAM-*co*-RhBAM)$_{60}$ (10 mg) was dissolved in *N*,*N*-dimethylformamide (DMF; 1 mL). Under vigorous stirring, deionized water (9 mL) was added through a syringe pump at a flow rate of 0.2 mL min^{-1}. After the addition was completed, the dispersion was left to stir for another 5 h. DMF was then removed by dialysis (MW cutoff, 14000 Da) against deionized water for 24 h. Fresh deionized water was replaced approximately every 6 h. The micellar solution exhibited no macroscopic phase separation upon standing at room temperature for more than three months, suggesting the formation of stable micelles. All pH adjustments were made by adding aqueous HCl or NaOH.

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