A Wide Range of pH-Sensitive Fluorescent Probe based on Responsive Double Hydrophilic Block Copolymer for Real-Time Intracellular pH Sensing in Water Samples

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Abstract. In this work, a wide range of pH-sensitive fluorescent probe has been designed by the responsive double hydrophilic block copolymer (DHBCs), which containing the fluorescein (Methacrylic salicylaldoxime, SHMa) and rhodamine (Rhodamine B methacryl hydrazide, RhSAMA) derivatives in the DHBCs. The pH-sensitive fluorescent probe gave blue and red fluorescence emissions at basic pH and acidic pH, respectively. Moreover, the fluorescent probe exhibited changes in fluorescence intensity with an alteration of turbidity of the block copolymer induced by temperature. Furthermore, we have developed a model that combines with the mobile phone for quantitative and qualitative measurement of pH value. The color extract APP for real-time pH measurement in mobile phone was constructed and used to detect the pH of the water samples.

1. Introduction
As a kind of chemical parameter, pH values play an important role in different research fields, such as analytical chemistry, environmental monitoring, biochemistry and food inspection. Generally, pH values can greatly affect the processes of living system in the organisms and influence chemical reactions happened in nature as well. Among the different systems for pH measurement, pH glass electrodes are the most common tools used for pH sensor because of their sensitive to hydrogen ion[1]. However, one of the prominent drawback of glass electrodes is that they cannot perform well in the strong acid or strong alkaline solutions. Moreover, the glass electrodes usually need recalibration in a discontinuous way, which is time consuming. Therefore, it is desirable to develop a novel sensor for the convenient and effective detection of pH at a wide range of value, especially at the extremes of pH range.

Recently, fluorescence sensors have become more and more attracting in the detection of pH because they are easily operable for practical applications and also exhibit high sensitivity and specificity toward different solution mediums [2-4]. According to the recent studies, many fluorescent pH probes based on different materials have been investigated, including semiconductor quantum dots [5], organic fluorescent dyes [6] and noble-metal nanoclusters [7]. But these fluorescence sensors have many shortcomings, such as unfriendly to the ecosystem, undesirable photostability and poor water solubility, which will prevent their further uses in the practice, especially for the environmental studies [8, 9]. It is especially important that the detection of pH-sensitive fluorescent probe requires fluorescence spectrometer, which does not allow rapid real-time detection. The stimuli-responsive block copolymers prepared by dynamic self-assembly have attracted many researchers’ attention in the passed decades[10]. Under specific conditions, stimuli-responsive block copolymers can naturally assemble into different morphology, such as micelles, rods, or vesicles, and then the structure will be disintegrated or reorganized when there is an external stimulus changed, including temperature [11],
pH [12], light irradiation [13], or ultrasound [14]. However, among these stimulus, manipulating temperature to modulate block copolymer assemblies is an adoptable one due to its ready maneuverability, low consumption, timely responsiveness, convenient on/off switch, and no need of introducing external chemicals [11]. Based on this principle, the detection limit of the fluorescent chemical sensor designed and synthesized by Han et al. [15] for detecting aluminum ions and zinc ions at 40 °C was changed from ~5.23 and ~11.99 ppb at 25 °C to ~2.44 and ~8.71 ppb.

In the present paper, the temperature-responsive DHBCs (PEG-b-P(NIPAM-co-SHMa-co-RhSAMa) diblock copolymer) was prepared to monitor the pH in a wide range values, in which the fluorescein (SHMa) (Methacrylic salicylaldoxime) and rhodamine (RhSAMa) (Rhodamine B methacryl hydrazide) in the DHBCs exhibited significant and independent emission alteration over the wide pH values. The final results showed that fluorescein derivatives incorporated in the DHBCs showed blue fluorescence emissions at basic pH and rhodamine derivatives incorporated in the DHBCs showed red fluorescence emissions at acidic pH. As shown in the figure 1, the chemical structures of fluorescein and rhodamine derivatives all perform transitions as the pH values changed. As the DHBCs self-assembles into micelles above the LCST, the quantum yield of fluorescence is obviously intensified and then the fluorescence intensity will be amplified. We have developed a model that combines with the mobile phone for quantitative and qualitative measurement of pH value. Then the color extract APP for real-time pH measurement in mobile phone was constructed and used to detect the pH of the water samples.

![Figure 1. Structure transitions of fluorescein and rhodamine derivatives as a function of pH.](image)

2. Experimental

2.1. Materials
Poly(ethylene oxide) monomethyl ether (PEO113-OH, $M_n = 5.0$ kDa, $M_w/M_n = 1.05$; DP = 113) purchased from Aldrich was used as provided. N-Isopropylacrylamide (NIPAM, 97 %) purchased from Aldrich was recrystallized twice by a mixture of n-hexane and benzene (v/v=2:1) prior usage. 4- (dimethylamino) 2,4-dihydroxyformaldehyde ($\geq 98$ %), Benzohydrazide ($\geq 99$ %), pyridine (DMAP, $\geq 99$ %), dicyclohexylcarbodiimide (DCC, $\geq 99$ %), methacryloyl chloride ($\geq 95$ %), rhodamine B hydrazide ($\geq 99$ %), Azoisobutyronitrile (AIBN, $\geq 98$ %), Ethylenediamine ($\geq 98$ %) and all other reagents purchased from Sinopharm Chemical Reagent Co. were used as received. AIBN was recrystallized from 95% ethanol. Methacryloyl chloride was distilled prior usage. Dichloromethane ($\text{CH}_2\text{Cl}_2$) were dried over CaH$_2$ and distilled immediately before usage. Toluene was distilled over sodium and benzophenone just prior usage. The water used was double distilled deionized water which processed by Millipore system (a specific resistivity of 18.4 M$\Omega$ cm). Other paragraphs are indented (BodytextIndented style).

2.2. Sample Synthesis

2.2.1 Synthesis of SHMa. The SHMa was synthesized according to the previous reported [15]. The
grayish green solid powder (0.90 g, yield: 40%) was obtained. $^1$H NMR (CD$_3$OD, δ, ppm, TMS): 8.48(1H, ArCH=N-), 6.96-7.44(4H, ArH), 5.85(1H, -C(CH$_3$)=CHH), 5.58(1H, -C(CH$_3$)=CHH), 2.04(3H, CH$_3$C-).

2.2.2 Synthesis of RhSAMa. The rhodamine B hydrazide (4.93 g, 0.01 mol) was dissolved in ethanol (20.00 ml) and stirred, then a mixture of ethanol (20.00 ml) and p-hydroxybenzaldehyde (1.22 g, 0.01 mol) was added. The mixture was heated to 70 °C for 1 h, then cooled and filtered to give the crude product. The white solid RhSA (5.43 g, yield: 88%) was obtained after the crude product recrystallized three times in ethanol. The product triethylamine (0.79 g, 7.84 mmol) and RhSA (2.00 g, 7.84 mmol) were dissolved in dichloromethane (30.00 ml) and methacryloyl chloride (0.81 g, 7.84 mmol) was added dropwise in the ice bath. The reaction was incubated at 25.0 °C for 3 h. After that, the solution was washed three times with saturated aqueous NaHCO$_3$ solution. Anhydrous sodium sulfate was used to dry the organic layer. The filtrate was dealt by rotary evaporation to obtain the crude solid which was recrystallized three times in acetonitrile and dried in vacuo to give violet powder solid (3.43 g, yield: 75.0%). $^1$H NMR (CDCl$_3$, δ, ppm, TMS): 8.02(1H, ArH), 7.58(2H, ArH), 7.32(1H, ArH), 6.85-7.15(3H, ArH), 6.50(1H, ArH), 5.78(1H, -C(CH$_3$)=CHH), 5.35(1H, -C(CH$_3$)=CHH), 2.75-4.00(8H, -CH$_2$-), 2.01(9H, -CH$_3$), 1.21(6H, -CH$_3$).

2.2.3 Synthesis of BTPA. The BTPA was synthesized according to the previous reported [15]. Finally the obtained 3.20 g product and yield of 40.9%. $^1$H NMR (CDCl$_3$, δ, ppm, TMS): 7.33 (5H, ArH), 4.64 (2H, ArCH$_2$-), 3.65 (2H, -C(=S)SCH$_2$-), 2.88 (4H, -C(=S)SCH$_2$CH$_2$CO-).

2.2.4 Synthesis of PEO-based MacroRAFT agent. The PEO-based macroRAFT agent was synthesized refer to the previous research[15]. A slightly yellowish powder (4.80 g, yield: 62%) was obtained and named PEO$_{113}$-based macroRAFT agent. GPC analysis demonstrated an Mn of 6.1 kDa and an Mw/Mn of 1.06. $^1$H NMR (CDCl$_3$, δ, ppm, TMS): 7.33 (5H, ArH), 4.60 (2H, ArCH$_2$-), 4.27 (2H, -CH$_2$OCOCH$_2$-), 3.83-3.58 (4H, -CH$_2$CH$_2$O-), 3.54 (3H, CH$_3$O-), 3.38 (2H, -CH$_2$OCOCH$_2$CH$_2$SC(=S)-), 2.82 (2H, -CH$_2$OCOCH$_2$CH$_2$SC(=S)-).

2.2.5 Synthesis of PEG$_{113}$-b-P(NIPAM-co-SHMa-co-RhSAMa)$_{62}$. Dry NIPAM (0.91 g, 8.00 mmol), SHMa (0.10 g, 0.32 mmol), RhSAMa (0.10 g, 0.32 mmol), PEG$_{113}$-based macroRAFT agent (0.42 g, 0.08 mmol), 1,4-dioxane (1.4 g) and AIBN (2 mg, 12 umol) were added into a reaction tube equipped with a magnetic stirring bar. The tube was carefully degassed by three freeze-pump-thaw cycles and then sealed under vacuum. Stirring for 1.5 h at 70 °C in an oil bath, the reaction tube was quenched into liquid nitrogen to stop the reaction. The reaction mixture was diluted with an amount of 1,4-dioxane and the product was precipitated with an excess of cold diethyl ether. The above dissolution-precipitation cycle was repeated for three times. PEG$_{113}$-b-P(NIPAM-co-SHMa-co-RhSAMa)$_{62}$ was obtained as a pink powder in a yield of 63% (0.84 g). The results of the GPC analysis were shown an Mn of 13.2 kDa and an Mw/Mn of 1.09. The degree of polymerization ( DP) of P(NIPAM-co-SHMa-co-RhSAMa) was determined to be 62 by $^1$H NMR analysis). Thus, the polymer was denoted as PEG$_{113}$-b-P(NIPAM-co-SHMa-co-RhSAMa)$_{62}$. The content of SHMa in 0.10 g/L aqueous solution of PEG$_{113}$-b-P(NIPAM-co-SHMa-co-RhSAMa)$_{62}$ was 2.11 μmol/L and Rh6GEMa was 13.23 μmol/L (the content of the SHMa element measured fluorescence intensity at pH 14, and RhSAMa measured fluorescence intensity at pH 1).

2.3. Characterization

Nuclear Magnetic Resonance (H NMR) Spectroscopy. All $^1$H NMR spectra were tested on a German BRUKERAC-P400 type nuclear magnetic resonance ( 400 MHz resonance frequency for 1H) operated in the Fourier transform mode. Solvents used including CDCl$_3$, CD$_3$OD and DMSO-d$_6$.

Gel Permeation Chromatography (GPC). Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) equipped with a RID-20 differential refractive index detector (Japan Shimadzu Company) and a LC20 high performance liquid chromatography pump.
Fluorescence Measurements. Fluorescence signals were measured on a Cary Eclipse luminescence spectrometer (Varian, USA) equipped with a computer and a xenon flash lamp. The slit widths were set at 5 nm for excitation and emitted at 2.5 or 5 nm.

Use Huawei Imagination 6S as a camera, self-developed mobile phone image processing software was used to analysis image.

3. Results and Discussion

3.1. Synthesis of PEG113-b-P(NIPAM-co-SHMa-co-RhSAMa)₆₂

The PEG-b-P (NIPAM-co-SHMa-co-RhSAMa) diblock copolymer was synthesized by the RAFT copolymerization of PEO-based macroRAFT agent, which has a certain fluorescence pH sensing property and is covalently labeled with the dual reactive block SHMa and RhSAMa, respectively. GPC analysis revealed an Mn of 13.2 kDa and Mw/Mn of 1.09 for PEG₁₁₃-b-P(NIPAM-co-SHMa-co-RhSAMa)₆₂. In addition, the SHMa content and RhSAMa content in P(NIPAM-co-SHMa-co-RhSAMa) block were determined to be ~0.45 mol% and ~0.28 mol%, respectively.

3.2. Fluorescence Spectrum Study

The sensor, PEG-b-P(NIPAM-co-SHMa-co-RhSAMa) diblock copolymer, expresses a significant dependence on pH in fluorescence emission spectra. As shown in Scheme 1, it is expected that the structural changes of fluorescein and rhodamine derivatives would affect the fluorescence in polymer probes at various pH. Under alkaline pH conditions, the phenolic hydroxyl groups in SHMa are deprotonated and the structure changes to produce strong blue fluorescence [16]. At the same time, the lactam ring of rhodamine moiety has not been destroyed and maintains its native closed-loop system, which has a weak fluorescence emission intensity. On the contrary, the rhodamine moiety lactam ring is opened and produces a strong pink fluorescence at acidic pH conditions[17]. However, the structure of the fluorescein moiety in SHMa does not change, implying that it does not have fluorescence emission properties.

For investigating the optical properties of the sensor in deionized water, the fluorescence spectroscopy at different pHs was employed (figure 2). Under basic pH conditions, the polymer probe showed a faint absorption band at 728 nm (this faintness means that it is almost undetectable in the mobile phone camera system) and strong fluorescence with blue emission at 470 nm. On the contrary, under acidic and neutral pH conditions, the polymer probe exhibited weak fluorescence at 470 and 558 nm, and strong fluorescence at 728 nm, emitting pink light. The increase in pH resulted in enhanced fluorescence of the fluorescein fraction at 470 nm in the polymer, while the fluorescence intensity at 728 nm gradually decreased. On the basis of these results, the pH-dependent fluorescence change in the polymer can be applied to monitor the pH of the solution.

![Figure 2](image-url)

**Figure 2.** The fluorescence spectra at 470 nm, 588 nm, and 728 nm from pH 1 to 14, respectively.
3.3. The Selectivity Study of Sensor and Reversibility Study

The competition experiments were carried out to investigate the selectivity of sensor over metal ions at pH 3.00 and pH 13.00, respectively. As shown in figure 3, the variation of relative fluorescence intensity after the addition of physiologically ubiquitous metal ions (K⁺, Na⁺, Ca²⁺, and Mg²⁺) exhibits similar tendency at pH 3.00 and pH 13.00, suggesting that the physiologically ubiquitous metal ions in the physiological concentration range does not produce any significant emission changes for pH measurement. Other metal cations, such as Al³⁺, Ba²⁺, Co²⁺, Li⁺, Cu²⁺, Fe³⁺, Mn²⁺, Fe²⁺ and Zn²⁺ also do not interfere with the pH sensing behavior of the probe, revealing that the designed sensor is suitable for high performance pH monitoring.

As shown in figure 4, all signal changes are fully reversible for further investigating the reversibility of the sensor. The reversible cycles of emission changes of the sensor between pH 1 and 14 could be repeated for 10 cycles with a negligible fluorescence bleaching, indicating that the sensor was stable under reversible changes in pH. These results indicated the excellent reversibility of the sensor. All of these afforded significant evidences for, and advantages to, the sensor for application in various environments.

![Figure 3](image1.png)

**Figure 3.** The variation of relative fluorescence intensity after the addition of other metal cations

pH=3 (fluorescence responses 728 nm), (b) pH=13 (fluorescence responses 470 nm)

![Figure 4](image2.png)

**Figure 4.** Change in fluorescence emission intensity when solution pH was cycled between 1 and 14.

3.4. The Temperature Response Behavior of the Sensor

As shown in figure 5, the fluorescence intensities of the sensor at pH 1 and 14 were significantly enhanced with increasing temperature (from 36.0 °C to 40.0 °C) at 728 nm and at 446 nm, respectively. The fluorescence intensity of pH 1 and pH 14 was blown up 2.87 and 1.58 times respectively at 40.0 °C compared with 36.0 °C. Accidentally, the fluorescence only increases when the temperature is
above the LCST (36.0 °C) of the micelles, which is consistent with the critical micelle temperature obtained by the experiment of light transmittance and temperature dependence. In summary, the sensitivity of the detection could be significantly improved by the temperature-induced copolymer self-assembly of the sensor. It can be indicated that the pH response element will be located within the hydrophobic nucleus, which will increase the quantum yield of the fluorescent elements, so as to achieve the purpose of signal amplification.

Figure 5. (a) and (b) Temperature-dependence fluorescence spectra and change relative fluorescence intensity at a wave of 366 nm

3.5. Molds for Qualitative and Quantitative Detection Values of pH
In general, visual inspection of the fluorescence-changing test strip is considered to be its major advantage. Furthermore, mobile phones are almost universally popular and are mostly equipped with high-definition cameras. It is expected to develop a model that combines with the mobile phone for quantitative and qualitative measurement of pH value, based on the pH sensor can produce different color reactions to the acid and alkali environment (Blue fluorescence is alkaline, no fluorescence is neutral, red fluorescence is acidic.). For this purpose, ΔR, ΔG and ΔB of fluorescent samples at different pH values in UV dark box were analyzed to establish the quantitative and qualitative model of acid and alkaline conditions by using PCA and regression analysis (using SPSS 20 software). As shown in figure 6, according to the differences of RGB values at different pH values, the quantitative prediction models of the ΔR, ΔG, and ΔB between after and before the pH reaction are established.

Figure 6. (a) The original image, (b)RGB color difference and (c) extracted RGB value picture of the polymer probe at pH 1-14 under UV (365 nm) dark box.

3.6. The Development of Color Extract APP for Real-Time pH Measurement in Mobile Phone
As shown in figure 7, based on the established quantitative models A and B, the Color Extract APP for real-time pH measurement in mobile phone was developed. APP mainly composed of three
subsystems including the camera system, model input system and data processing system. The camera system can accurately analyze the fluorescence of the sample in the UV dark box. The model input system is convenient for the users to select the appropriate quantitative model according to the actual situation. The data processing system can take the analysis value of the sample RGB of the photographing system into the data numerical model for data processing and display the calculation result.

![Figure 7. Color extract APP for real-time pH measurement in mobile phone.](image)

3.7. Real Water Samples Analysis

As seen in Table 1, the improved method was employed to measure the pH in real water samples for demonstrating the applicability of the method. Four strips from individual batches were examined on three real samples, tap water, pond water and Yangzi River. We set some pH values and measured their specific values with a pH meter, then we use the experimental design method to determine the pH value of the method. The difference between the value of pH meter and this method is very small. The new method established in this experiment is feasible in practical applications and worth promoting.

| Samples         | The value of pH meter | The value of this method | Difference ± SD (%) |
|-----------------|-----------------------|--------------------------|---------------------|
| **Tap water**   | 2.35                  | 2.345                    | 0.213±0.003         |
|                 | 5.90                  | 5.878                    | 0.373±0.005         |
|                 | 9.30                  | 9.324                    | 0.258±0.007         |
|                 | 13.50                 | 13.455                   | 0.333±0.008         |
|                 | 1.80                  | 1.788                    | 0.667±0.008         |
|                 | 6.75                  | 6.780                    | 0.444±0.003         |
| **Pond Water**  | 9.58                  | 9.601                    | 0.219±0.006         |
|                 | 12.90                 | 12.870                   | 0.233±0.009         |
|                 | 1.55                  | 1.561                    | 0.710±0.008         |
| **Yangzi River**| 6.80                  | 6.785                    | 0.221±0.005         |
|                 | 11.50                 | 11.458                   | 0.365±0.007         |
|                 | 13.88                 | 13.905                   | 0.180±0.006         |

4. Conclusion

The temperature-responsive DHBCs (PEG-b-P(NIPAM-co-SHMa-co-RhSAMa) diblock copolymer), in which the fluorescein (SHMa) (Methacrylic salicylaldoxime) and rhodamine (RhSAMa) (Rhodamine B methacryl hydrazide) in the DHBCs were designed. And its good performance for the detection of a wide range of pH (red and blue fluorescence emission under alkaline conditions under acidic conditions) was studied (selectivity sensitivity, time-properties study, reversibility study and temperature sensitivity). Finally, this simple fluorescent sensor with water solubility, excellent biocompatibility and enhanced detection sensitivity was combined with the increasingly electronic communication devices to construct the color extract APP for real-time pH measurement in mobile
phone and used for pH detection of water samples. The data obtained by this simple field program was consistent and showed great potential.

5. References

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