Dyes and Pigments 89 (2011) 236–240

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

A ratiometric hydrophilic fluorescent copolymer sensor based on benzimidazole chromophore for microbioreactors

Liangjun Shen, Ping Zhao, Weihong Zhu*

Key Laboratory for Advanced Materials and Institute of Fine Chemicals, East China University of Science & Technology, Shanghai 200237, PR China

ARTICLE INFO

Article history:
Received 19 January 2010
Received in revised form
4 March 2010
Accepted 16 March 2010
Available online 21 March 2010

Keywords:
Copolymer
Benzimidazole
pH
Chemosensor
Hydrophilic

ABSTRACT

The ratiometric approach is robust and insensitive to factors such as source intensity, photobleaching, or orientation of the patch. A novel ratiometric hydrophilic copolymer of *N*-(1-ethyl-2-(pyridin-4-yl)-1*H*-benzo[*d*]imidazol-5-yl)methacrylamide and 2-hydroxyethyl methacrylate (P(BIPy–HEMA)) containing pyridyl substituted benzimidazole moiety as pH sensor has been developed. Its fluorescence in aqueous system showed two obvious isosbestic points due to two-stepwise protonation. According to the linear curves of I_{462}/I_{423} and I_{536}/I_{462} to pH, such ratiometric pH values could be read directly without external calibration. Considering the hydrophilicity, stability and repeatability in aqueous environment, the fluorescent film with ratiometric characteristic over acidic pH range of 1.7–4.5 makes it high promising for online monitor in microbioreactors.

1. Introduction

The measurement and control of pH are important in chemistry, biochemistry, clinical chemistry and environmental science due to its significant effect on chemical reactions [1]. Optical sensors for pH based on small molecules have been widely developed. Clearly, such small molecular pH sensors may dissolve into medium when they are used to monitor process continuously in a perfused bioreactor system [2]. Consequently, it is preferable to immobilize the molecular sensor on a support via physical doping or covalent bonding method [3]. The chemical incorporation of sensor moieties into polymeric matrix is more performable and efficient from the following considerations: i) avoiding the phase separation and concentration quenching of chromophores, ii) easy miniaturization and fabrication into devices and chip fix onto micro-reactors [4–6], iii) being capable of repeated utilization without any pollution to microbioreactors [7,8].

Since most live actions take place in aqueous, it is a judicious way to introduce hydrophilic characteristic into polymeric sensor for determining a variety of parameters in aqueous biosystem. As the main matrix of contact lens, although poly(2-hydroxyethyl methacrylate) (PHEMA) containing hydroxyl groups in the side chains is insoluble in water, it exhibits excellent hydrophilicity with good film-forming property and high adherence on glass or quartz [9]. Accordingly, PHEMA is an ideal hydrophilic copolymeric matrix for chemosensors in bio-processing. In our previous work, a copolymer containing piperazine substituted naphthalimide was studied as hydrophilic polymer sensor for microreactors at around neutral environment [10]. However, up to date, little research on polymeric sensors has been focused on high acidic range for processes accompanied by acidification, such as gastric pH sensor [11]. With this in mind, herein a kind of hydrophilic fluorescent copolymeric sensor P(BIPy–HEMA) (Fig. 1) incorporating pH-sensitive benzimidazole moiety was synthesized via copolymerization, in which pyridine was introduced to be acted as a proton acceptor in acidic range [12,13]. The push-pull system of 2-(pyridin-4-yl)-1*H*-benzo[*d*]imidazole (BIPy) was selected as fluorescent sensor group due to its high sensitivity to pH via the intramolecular charge transfer (ICT) mechanism. We found that P(BIPy–HEMA) exhibited rather excellent film-forming and hydrophilic properties due to its hydroxyl group in the comonomer of 2-hydroxyethyl methacrylate (HEMA). Its film is sensitively responsive at the expected low pH range of 1.7–4.5 in aqueous system by ratiometric method. According to the linear curves of I_{462}/I_{423} and I_{536}/I_{462} to pH, such ratiometric pH values could be read directly with avoiding several effects, such as source intensity, photobleaching, or orientation of the patch [14].

* Corresponding author.
E-mail address: whzhu@ecust.edu.cn (W. Zhu).

0143-7208/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2010.03.016

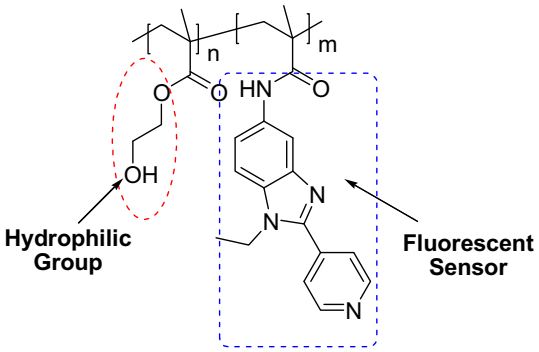

Fig. 1. Chemical structure of copolymer P(BIPy–HEMA).

2. Experimental

2.1. Chemicals and instruments

The starting material of N^1-ethyl-4-nitrobenzene-1,2-diamine was synthesized according to the literature [15]. Methacryloyl chloride and HEMA were used after purified by distillation under reduced pressure. 2,2′-Azoisobutyronitrile (AIBN) was recrystallized from ethanol before polymerization. Other reagents were commercially available and used without further purification.

^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker AV 400 spectrometer. HRMS spectra were obtained using a Waters LCT Premier XE spectrometer. UV spectra were recorded on a Varian Cary 100 spectrometer. Fluorescence spectra were obtained using a Varian Cary Eclipse spectrometer, and the angle of incidence is about 35°. The molecular weight and molecular weight distribution (M_w/M_n) of the resulted copolymer was determined using a Waters 1515 Gel Permeation Chromatography (GPC) with DMF as the mobile phase. The film was obtained by coating a solution of copolymer P(BIPy–HEMA) in methanol (5 wt%) on glass with an average copolymer thickness of about 50 nm. The pH value was adjusted by trifluoroacetic acid (TFA) aqueous solution, and demarcated on a METTLER TOLEDO FE20 pH meter.

2.2. Synthesis

2.2.1. Preparation of 1-ethyl-5-nitro-2-(pyridin-4-yl)-1H-benzo[d]imidazole (**1**)

A mixture of N^1-ethyl-4-nitrobenzene-1,2-diamine (15.0 g, 83 mmol), *iso*-nicotinic acid (10.8 g, 89 mmol) and polyphosphoric acid (PPA, 81.0 g) was stirred at 160 °C for 4 h under the protection of argon. After being cooled to about 100 °C, the reaction mixture was poured into water (150 mL), and adjusted pH to 7.0 with Na_2CO_3. The precipitated solid was filtered, and then dissolved in CH_2Cl_2. The solution was filtered to remove the insoluble impurities and the filtrated solvent was removed by rotary evaporation to obtain a pale yellow powder (4.6 g, yield 20.7%). ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.88 (d, $J = 5.6$ Hz, 2H, pyridyl-H), 8.77 (d, $J = 1.6$ Hz, 1H, phenyl-H), 8.33 (dd, $J_1 = 9.2$ Hz, $J_2 = 1.6$ Hz, 1H, phenyl-H), 7.69 (d, $J = 5.6$ Hz, 2H, pyridyl-H), 7.55 (d, $J = 9.2$ Hz, 1H, phenyl-H), 4.40 (q, $J = 7.2$ Hz, 2H, $-CH_2CH_3$), 1.56 (t, $J = 7.2$ Hz, 3H, $-CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 153.99, 150.36, 143.19, 141.57, 139.75, 136.69, 123.29, 118.49, 115.69, 111.76, 39.99, 14.91. ESI-HRMS: calcd for $C_{14}H_{13}N_4O_2$ $[M + H]^+$ 269.1039, found 269.1042.

2.2.2. Preparation of 1-ethyl-2-(pyridin-4-yl)-1H-benzo[d]imidazol-5-amine (**2**)

To a mixture of **1** (1.5 g, 5.6 mmol), 10% Pd/C (0.25 g) and ethanol (20 mL), an ethanol solution of hydrated hydrazine (1.3 g, 26 mmol in 20 mL ethanol) was dropwise added at 70 °C under the protection of argon. The mixture was refluxed at 80 °C for 4 h. After cooled to room temperature, the mixture was filtered, and the solvent of the filtrated was removed by rotary evaporation. The residue was filtered to give a greenish-yellow crystal (1.1 g, yield 79%). ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.79 (d, $J = 6.0$ Hz, 2H, pyridyl-H), 7.66 (d, $J = 6.0$ Hz, 2H, pyridyl-H), 7.24 (s, 1H, phenyl-H), 7.13 (d, $J = 2.0$ Hz, 1H, phenyl-H), 6.81 (dd, $J_1 = 8.8$ Hz, $J_2 = 2.0$ Hz, 1H, phenyl-H), 4.28 (q, $J = 7.2$ Hz, 2H, $-CH_2CH_3$), 1.49 (t, $J = 7.2$ Hz, 3H, $-CH_2CH_3$).

2.2.3. Preparation of N-(1-ethyl-2-(pyridin-4-yl)-1H-benzo[d]imidazol-5-yl) methacrylamide (monomer **3**)

To a solution of **2** (0.64 g, 2.69 mmol) and CH_2Cl_2 (15 mL) was added methacryloyl chloride (1.5 g, 14.4 mmol). The mixture was stirred overnight at room temperature. The solid was collected by vacuum filtration and washed with CH_2Cl_2. The crude product was purified by column chromatograph on Al_2O_3 column using methanol as eluent to give a light yellow powder (0.8 g, yield 97%). ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 10.10 (s, 1H, –CONH–), 8.96 (d, $J = 6.0$ Hz, 2H, pyridyl-H), 8.34 (s, 1H, phenyl-H), 8.02 (d, $J = 6.0$ Hz, 2H, pyridyl-H), 7.91 (d, $J = 9.2$ Hz, 1H, phenyl-H), 7.79 (d, $J = 9.2$ Hz, 1H, phenyl-H), 5.89 (s, 1H, =C*H*(H)), 5.58 (s, 1H, =CH(*H*)), 4.45 (q, $J = 7.2$ Hz, 2H, $-CH_2CH_3$), 1.98 (s, 3H, $=CCH_3$), 1.42 (t, $J = 7.2$ Hz, 3H, $-CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 167.3, 150.8, 150.6, 142.6, 140.7, 137.9, 134.5, 132.6, 123.7, 120.5, 118.2, 111.3, 111.2, 19.1, 15.5. ESI-HRMS calcd for $C_{18}H_{19}N_4O$ $[M + H]^+$ 307.1559, found 307.1566.

2.2.4. Preparation of copolymer P(BIPy–HEMA)

A solution of monomer **3** (84 mg, 0.246 mmol), HEMA (3.2 g, 24.6 mmol), AIBN (80 mg, 0.487 mmol) and *N*-methylpyrrolidin-2-one (NMP, 5 mL) in a Schlenk tube was subjected to repeated freeze–thaw cycles, then heated at 70 °C for 60 h. After polymerization, the product was precipitated by dropping the reaction solution into ethyl acetate. The crude product was dissolved in methanol and reprecipitated from ethyl acetate. This operation was repeated for 4 times. The resulting solid was dried under vacuum to give copolymer P(BIPy–HEMA) (3.0 g). The weight-average molecular weight of the resulting copolymer determined by GPC was 165,800 with a polydispersity of 5.79. ^{1}H NMR and UV spectroscopic analyses confirmed that the molar ratio of comonomer BIPy to HEMA incorporated in the copolymer P(BIPy–HEMA) was *ca.* 1:150.

3. Results and discussion

3.1. Design and synthesis of P(BIPy–HEMA)

To measure pH with a fluorescent ratiometric method, it is desirable that absorption and emission spectra exhibit obvious shift to the change of pH. In principle, introducing a pH sensing group into a donor-acceptor system may reach this goal. Pyridine is a good proton trapper, and chosen as responsive unit. As known, the pull-push system of benzimidazole group, which shows good fluorescence resulting from ICT transition, is a well-known fluorescent building block in the design of functional materials [16]. As a consequence, it is expected to show ratiometric property with pH changes when incorporating pyridine group to benzimidazole moiety.

In the target copolymer, PHEMA was chosen as the main structure due to its good hydrophilic property. Generally, the fluorescent self-quenching always takes place at high concentration. Here, HEMA is utilized to adjust appropriate chromophore ratio to avoid self-quenching in the copolymer. In general, PHEMA could be prepared by solution, suspension or UV-free-radical polymerization. For the target copolymer of P(BIPy–HEMA),

the method of solution polymerization is preferably selected due to its easy preparation and purification process (Fig. 2). The resulted polymer was well dissolved in methanol and reprecipitated from ethyl acetate. The precipitated solid was collected by filtration and dried in vacuum to obtain pure copolymer P(BIPy–HEMA). To confirm the content or the ratio of fluorophore BIPy incorporated in the copolymer, absorption spectra of comonomer BIPy and copolymer in methanol at the neutral condition were collected. It is indicated that the absorption peak of copolymer P(BIPy–HEMA) located at 305 nm is nearly the same as that of comonomer solution (at 307 nm). Due to the peak at about 305 nm mainly resulted from benzimidazole moiety in P(BIPy–HEMA), the molar ratio (1/150) of comonomer BIPy to HEMA in copolymer could be derived from the standard working curve of absorbance.

3.2. Optical properties of P(BIPy–HEMA) and fluorescence responses to pH

To explore the response of polymer film to acid analyte as polymeric sensor in aqueous biosystem for microbioreactors, titration experiments were carried out in pure distilled water upon adding aliquots of TFA solution. Upon titration with TFA, a stepwise change in both absorption and fluorescence spectra was observed (Fig. 3). A clear isosbestic point at 327 nm was observed, indicative of the formation of monoprotonated polymer P(BIPy–HEMA)–H^+ from pH 4.5 to 2.7 (Fig. 4). While continually adding TFA, the absorption band became flat and moved to longer wavelength, resulting in a new absorption band at 337 nm corresponding to the doubly protonated species P(BIPy–HEMA)–$2H^+$ (Fig. 4) [17].

Moreover, an examination of fluorescence properties of P(BIPy–HEMA) exhibited a similar bathochromic shift with respect to its absorption. The fluorescence spectra were titrated with TFA upon excitation at the isosbestic point of 327 nm in water. Upon adding aliquots of TFA, the emission band at 423 nm (corresponding to P(BIPy–HEMA)) became decreased while a new emission band at 462 nm (corresponding to P(BIPy–HEMA)–H^+, Fig. 4) appeared with an isosbestic point at 440 nm. During the continuous titration of TFA, another emission band at 531 nm (attributing to the formation of P(BIPy–HEMA)–$2H^+$, Fig. 4) was observed with a decrease in the emission intensity at 462 nm, and the isosbestic point moved to 530 nm.

Generally, it is of great interest for developing ratiometric fluorescence sensor due to the self-calibration measurement. For developing a ratiometric fluorescent sensor based on the hydrophilic copolymer, the dependence of intensity ratios upon pH were studied (Fig. 5). Due to the stepwise protonation of P(BIPy–HEMA),

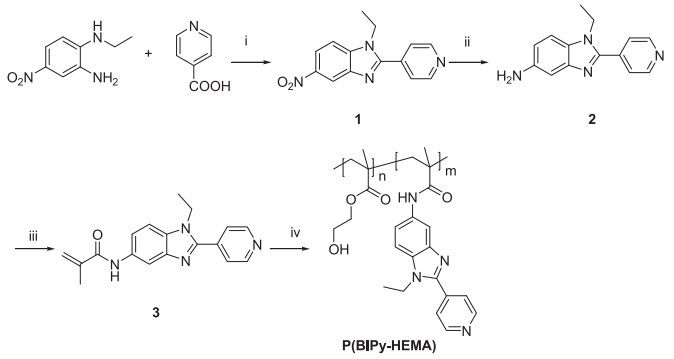

Fig. 2. Synthesis of copolymer P(BIPy–HEMA): (i) PPA, heated at 160 °C for 4 h, yield 20.7%; (ii) Pd/C, NH_2NH_2–H_2O, ethanol, refluxed at 80 °C for 4 h, yield 79%; (iii) methacryloyl chloride, r.t., overnight, yield 97%; (iv) HEMA, NMP, 70 °C for 60 h.

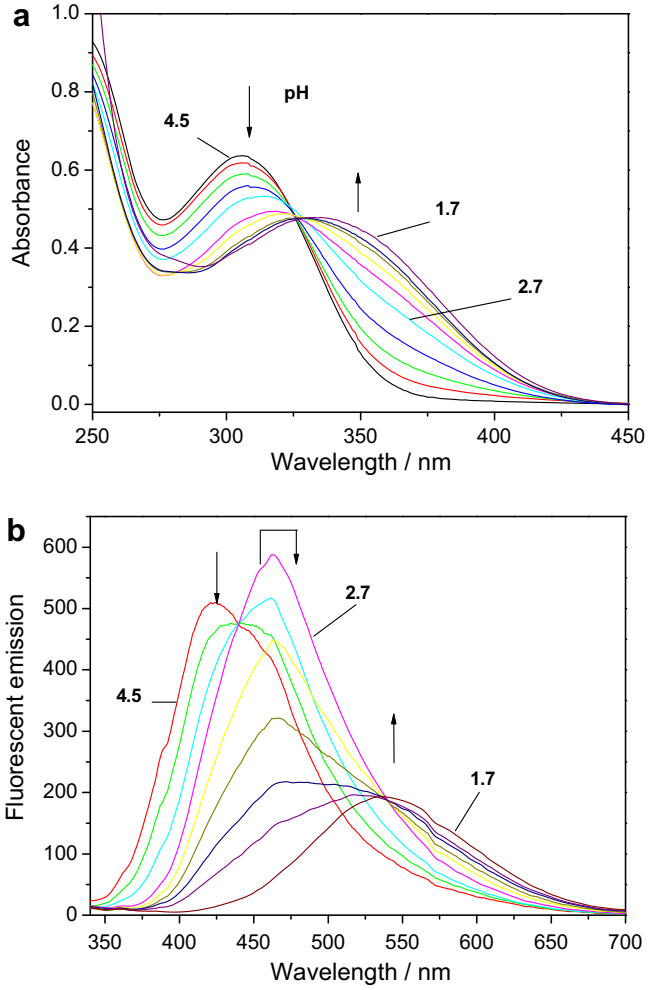

Fig. 3. Changes in spectra of copolymer P(BIPy–HEMA) film in aqueous solution titrated with TFA in the pH range of 1.7–4.5: (a) absorption and (b) fluorescence spectra (excited at the isosbestic point of 327 nm).

it was found that the fitting of I_{462}/I_{423} in fluorescence to pH had two clear sections with good linear fitting (correlation coefficient > 0.998, Fig. 5(a)). Interestingly, the ratio of fluorescence intensity of I_{536}/I_{462} in the specific range (pH < 2.3) also exhibits well linear relative (correlation coefficient > 0.999, Fig. 5(b)).

3.3. Protonation behavior of P(BIPy–HEMA)

Notably from fluorescence spectra, there are two equilibriums of stepwise protonation, that is, single- and double-protonation with evolution of acidic properties for copolymer P(BIPy–HEMA) in

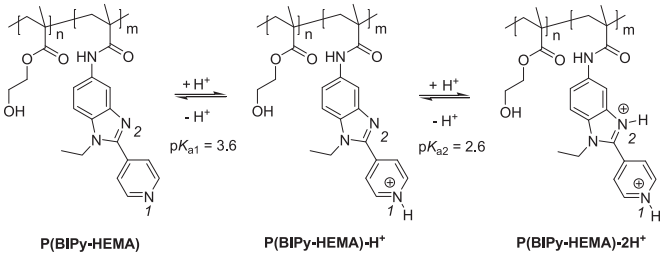

Fig. 4. Fluorescence response to pH through ICT mechanism.

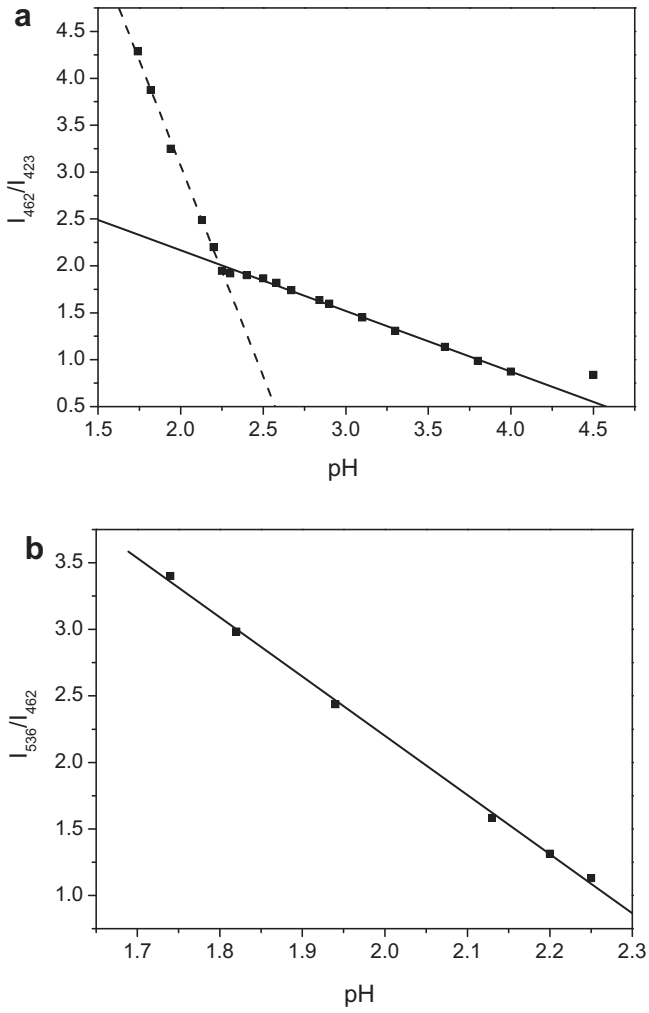

Fig. 5. Fitting curves of fluorescent response of P(BIPy–HEMA) to pH.

aqueous system. The pK_a values of copolymer could be calculated with Equation (1) using the emission titration experiment [18], where I_F is corresponding to the fluorescence intensity of the thin film in its acid form. As shown in Fig. 4, the emission-based pK_a values were found as 3.6 and 2.6 for pyridine-N (N_1, pK_{a1}) and imidazole-N (N_2, pK_{a2}), respectively. Obviously, these pK_a values are lower than that of unsubstituted pyridine and imidazole [19], which might be due to the electron effect of amide group on benzimidazole.

$$pK_a = pH \pm \log[(I_{Fmax} - I_F)/I_F - I_{Fmin}] \quad (1)$$

As a push-pull system, the stepwise protonation of copolymer must have effect on the ICT process. According to pK_a, it can be predicated theoretically that the copolymer undergoes through two steps-wise protonation process on N_1 and N_2 (Fig. 4). Exactly, both absorption and fluorescence spectra of polymer film reveal two-step protonation on N_1 and N_2, respectively (Fig. 4). The ICT band (305 nm) at neutral state red-shifted slightly to 313 nm with a decrease in absorbance, and the emission band also exhibited a bathochromic shift from 423 to 462 nm arising from the protonation of pyridine to result in P(BIPy–HEMA)–H^+ (Fig. 4). Compared with the parent BIPy, the protonation of pyridine pyridine-*N* (N_1) can increase the electronic-pull ability, thus favoring

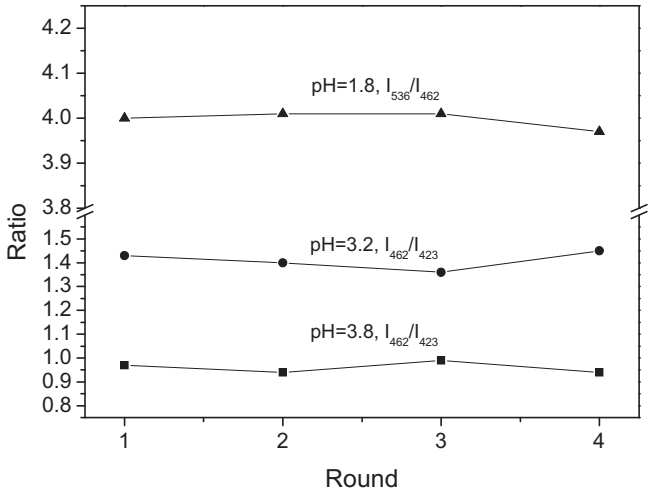

Fig. 6. Repeatability of film in aqueous solution with different pH value (two days per round).

the ICT from amide to pyridyl ring through benzimidazole chromophore. Furthermore, under the continuous addition of acid, imidazole-*N* (N_2) could be also protonated (P(BIPy–HEMA)–$2H^+$) as well for its basic property.

3.4. Hydrophilicity and film-stability of copolymer P(BIPy–HEMA) for real-time monitor

Since the copolymer P(BIPy–HEMA) was designed to work as real-time chemosensor in low-cost microbioreactors, the film should be hydrophilic but water-insoluble as a prerequisite for water environment in bioprocess. As discussed above, the matrix of copolymer P(BIPy–HEMA) containing hydrophilic hydroxyl group exhibits excellent hydrophilicity with good film-forming property. Moreover, due to high polymerization degree, the polymer is well soluble in methanol and ethanol, a little soluble in DMF and DMSO, but insoluble in water. Exactly, the film could be stable with online test in aqueous system for long time. As a good ratiometric fluorescent copolymer sensor for online detecting in microbioreactors, the chemosensor should possess good repeatability. We utilized three pH points to undergo a further experiment, and collected data every two days per round (Fig. 6), which showed that the response at those pH points was capable of keeping good stability in a week, sufficient for general bioprocess.

4. Conclusions

A novel hydrophilic copolymer of P(BIPy–HEMA) was synthesized, and the optical properties were investigated in pure water system titrated by TFA at acidic pH range of 1.7–4.5. Stepwise protonation of BIPy moiety at pyridyl-*N* and imidazole-*N* was analyzed with both absorption and fluorescence spectra. Due to the stepwise protonation of P(BIPy–HEMA), the fitting of I_{462}/I_{423} in fluorescence to pH had two clear sections with good linear fitting. Moreover, the ratio of I_{536}/I_{462} in the specific range (pH $<$ 2.3) also exhibits well linear relation. According to the linear curves of I_{462}/I_{423} and I_{536}/I_{462} to pH, such ratiometric pH values could be read directly with avoiding several factors, such as source intensity, photobleaching, or orientation of the patch. The hydrophilicity, stability and repeatability in aqueous solution make it possible for online monitoring in microbioreactors.

Acknowledgements

We thank the support from NSFC/China, the Program for New Century Excellent Talents in University (NCET-06-0418), Shanghai Shuguang Project (07SG34), and Specialized Research Fund for the Doctoral Program of Higher Education (SRFDP 200802510011).

References

[1] Adhikari B, Majumdar S. Polymers in sensor applications. Progress in Polymer Science 2004;714:699–766.
[2] Wolfbeis OS. Fiber-optic chemical sensors and biosensors. Analytical Chemistry 2004;76:3269–84.
[3] Wolfbeis OS. Materials for fluorescence-based optical chemical sensors. Journal of Materials Chemistry 2005;15:2657–69.
[4] Diao JP, Young L, Zhou P, Shuler ML. An actively mixed mini-bioreactor for protein production from suspended animal cells. Biotechnology & Bioengineering 2008;100:72–81.
[5] Haugland RP. The Handbook. A guide to fluorescent probes and labeling technologies. 10th ed. Eugene, OR: Molecular Probes Inc.; 2005.
[6] Grabchev I, Sali S, Betcheva R, Gregoriou V. New green fluorescent polymer sensors for metal cations and protons. European Polymer Journal 2007;43:4297–305.
[7] Kreft O, Javier AM, Sukhorukov GB, Parak WJ. Polymer microcapsules as mobile local pH-sensors. Journal of Materials Chemistry 2007;17:4471–6.
[8] Kim JM, Chang TE, Kang JH, Park KH, Han DK, Ahn KD. Photoacid-induced fluorescence quenching: a new strategy for fluorescent imaging in polymer films. Angewandte Chemie International Edition 2000;39:1780–2.
[9] Chirila TV. An overview of the development of artificial corneas with porous skirts and the use of PHEMA for such an application. Biomaterials; 2001:3311–7.
[10] Shen LJ, Zhu WH, Meng XL, Guo ZQ, Tian H. A hydrophilic fluorescent polymer containing naphthalimide moiety as chemosensor for microbioreactors. Science in China Series B: Chemistry 2009;52:821–6.
[11] Ryazanova OA, Voloshin IM, Makitruk VL, Zozulya VN, Karachevtsev VA. pH-induced changes in electronic absorption and fluorescence spectra of phenazine derivatives. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 2007;66:849–59.
[12] Zheng MH, Jin JY, Sun W, Yan CH. A new series of fluorescent 5-methoxy-2-pyridylthiazoles with a pH-sensitive dual-emission. New Journal of Chemistry 2006;30:1192–6.
[13] Sun W, Zhou C, Xu CH, Fang CJ, Zhang C, Li ZX, et al. A fluorescent-switch-based computing platform in defending information risk. Chemistry – A European Journal 2008;14:6342–51.
[14] Valeur B. Molecular fluorescence: principles and applications. Wiley-VCH Verlag GmbH; 2001.
[15] Gierczyk B, Leska B, Nowak-Wydra B, Schroeder G, Wojciechowski G, Bartl F, et al. ^{15}N NMR and FTIR studies of 2,4-dinitroanilines and their salts. Journal of Molecular Structure 2000;524:217–25.
[16] Ryazanova OA, Voloshin IM, Makitruk VL, Zozulya VN, Karachevtsev VA. pH-Induced changes in electronic absorption and fluorescence spectra of phenazine derivatives. Spectrochimica Acta Part A 2007;66:849–59.
[17] Shin JM, Cho YM, Sachs G. Chemistry of covalent inhibition of the fastric (H^+, K^+)-ATPase by proton pump inhibitors. Journal of American Chemistry Society 2004;126:7800–11.
[18] Bissell RA, Calle E, de Silva AP, de Silva SA, Gunaratne HQN, Habib-Jiwan JL, et al. Luminescence and charge transfer. Part 2. Aminomethyl anthracene derivatives as fluorescent PET (Photoinduced Electron Transfer) sensors for protons. Journal of the Chemical Society, Perkin Transactions 1992;2:1559–64.
[19] Pozharskii AF, Kuzmenko VV, YuV Kolodyazhnyi, Simonov AM. Relative reactivities of the pyridine and benzimidazole systems in the chichibabin reaction. Chemistry of Heterocyclic Compounds 1972;8:1131–41.