Journal of Applied Polymer Science

Environmental pH-Responsive Fluorescent PEG-Polyurethane for Potential Optical Imaging

Hong Yu Yang,[1] Xiu Mei Zhang,[2] Li Jie Duan,[1] Ming Yao Zhang,[1] Guang Hui Gao,[1] Hui Xuan Zhang[1,3]

[1]Engineering Research Center of Synthetic Resin and Special Fiber, Ministry of Education, Changchun University of Technology, Changchun 130012, China

[2]Department of Radiology, The First Hospital of Jilin University, Changchun 130021, China

[3]Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130012, China

Correspondence to: G. H. Gao (E-mail: ghgao@mail.ccut.edu.cn)

ABSTRACT: A series of environmental pH-responsive block copolymers PEG-polyurethane with tertiary amine groups in the main chain and free carboxyl groups in the side chain were synthesized, including poly(ethylene glycol) (PEG), 1,4-bis (hydroxyethyl) piperazine, 1,6-diisocyanato hexamethylene, and 2,2-dimethylolpropionic acid. The chemical structure, molecular weight, and pH-dependent capacity of pH-responsive PEG-polyurethane were examined by ^{1}H-NMR, FTIR, gel permeation chromatography (GPC), and an acid-base titration. Moreover, the fluorescent dye fluorescein isothiocyanate was conjugated with PEG-polyurethane by the similar polymerization method and the obtained polymer was measured by ultraviolet visible spectroscopy (UV-vis) and fluorescent spectroscopy. The results indicated that the pH-responsive PEG-polyurethane showed a pH-buffering phenomenon and fluorescent imaging in a range of pH values. As a result, we demonstrated its potential application for optical imaging; however, it is believed that more applications in the areas of biomedicine will be possible owing to its free carboxyl acid terminal residues in the stimuli-responsive polymer. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 846–852, 2013

KEYWORDS: nanostructured polymers; polyurethanes; biomedical applications

Received 13 August 2012; accepted 28 November 2012; published online 19 December 2012
DOI: 10.1002/app.38880

INTRODUCTION

Stimuli-responsive polymers have been extensively investigated as smart carriers[1–3] for delivering therapeutic drugs and/or molecular imaging agents to pathological areas with environment sensitivity for biomedical applications.[4–9] Usually, these intelligent polymers can form micro/nanosized delivery systems in an aqueous medium, sometimes conjugate with active-targeting ligands (e.g., antibody, peptides, or nucleic acids) that can target the biomarker of a disease.[10–12] In the other hand, some polymers alone, not conjugated with a targeting ligand, can also target the pathologic area by stimuli-responsive conditions (pH, temperature, redox etc.).[13–15]

Fluorescent imaging agents have also attracted rapidly growing interest due to their high potential in pathological visualization based on imaging technology at the level of cellular and molecular. They are especially attractive for sensing, imaging, biomedical diagnosis, and therapy applications.[16–18] For example, some green fluorescent proteins and nanosized surface-modified quantum dots have already widely investigated as tumor-labeled imaging agents and contributed significantly to the development of biochemistry.[19,20] Among these imaging probes, polymer-based imaging probes[21] are of special interest due to their intrinsic advantages including excellent biocompatibility, low toxicity, long-term stability, and facile conjugation with functional molecules.

Polyurethane was one of the most popular biomaterials due to its enormous diversity of chemical compositions and properties. Especially polyurethane has excellent bio-and blood-compatibility,[22] so that it could be used for medical devices, such as an artificial heart, intra-aortic balloons, pacemaker leads, heart valves, and hemodialysis membranes. However, polyurethane was usually synthesized by diisocyanated and dihydroxyled monomers, and free reactive groups cannot be achieved. As a result, it is very difficult to conjugate or graft other functional agent onto polyurethane. We envisioned that if we introduced carboxyl acid residues into polyurethane, which could be used widely as reactive groups for more biomedical applications.

In this article, a series of pH-responsive PEG-polyurethane block copolymers were synthesized using the double hydroxyl poly(ethylene glycol) (PEG), 1,4-bis (hydroxyethyl) piperazine

Table I. Feed Amounts of Materials and Weight-Average Molecular (M_n) and Polydispersity Index (PDI) of pH-Responsive PEG-Polyurethane

	Mole amounts of feed materials					
Samples	HDI	HEP	PEG	DMPA	M_n	PDI
P1	0.01	0.0014	0.0042	0.0014	4774	1.04
P2	0.01	0.0028	0.0028	0.0014	5026	1.13
P3	0.01	0.0042	0.0014	0.0014	5104	1.06

(HEP), and 1,6-diisocyanato hexamethylene (HDI) through an addition polymerization reaction. PEG was employed in block copolymers to increase the hydrophilic ability in an aqueous medium and piperazine groups were introduced to achieve a pH-buffering capacity of PEG-polyurethane. The resulting PEG-polyurethane was characterized by ^{1}H-NMR, FTIR, gel permeation chromatography (GPC), and an acid-base titration. Moreover, the FITC-conjugated PEG-polyurethane was measured by ultraviolet visible spectroscopy (UV-vis) and fluorescent spectroscopy as a potential optical imaging probe.

EXPERIMENTAL

Materials

Poly(ethylene glycol) (PEG, M_n = 2,000), 2,2-dimethylol propionic acid (DMPA, 98%), 1,6-hexamethylene diisocyanate (HDI, 99%), ethylenediamine (EA, 99.5%), triethylamine (TEA), dimethylsulfoxide (DMSO-d_6, 99.9%) and Fluorescein 5(6)-isothiocyanate (FITC, 90%) were purchased from Aladdin Chemistry Co. Ltd. 1,4-bis (hydroxyethyl) piperazine (HEP) and *N,N*-dimethyl formamide (DMF) were purchased from sigma-Aldrich.

Synthesis of PEG-Polyurethane Block Copolymer

The pH-responsive block copolymer PEG-polyurethane was synthesized by an addition polymerization reaction using PEG, DMPA, HEP, and HDI. The adding materials were carried out at a stoichiometric ratio as listed in Table I. The synthesis route of the pH-responsive block copolymer PEG-polyurethane was shown in Figure 1. In addition, 1 wt % TEA was employed as a catalyst and 5 wt % EA was added as a chain extender during the reaction. The typical reaction procedure was as follows: PEG, DMPA, HEP, HDI, and a catalytic amount of 1 wt % triethylamine were added into a dried 250-mL flask equipped with a magnetic stir bar. Afterward, 100 mL DMF was added into the reaction system. The flask was heated in an oil bath at 70°C under the nitrogen atmosphere, after 6 h, the chain extender EA was added into the system and the reaction was carried out for another 6 h. And the system was cooled down to the room temperature and continued for 12 h. The resulting polymer was precipitated in a seven to eightfold excess of diethyl ether. Then the product was dried under vacuum at room temperature for 72 h. The yields of polymers were over 80%. To prepare fluorescent PEG-polyurethane, moreover, 1 wt % FITC was added simultaneously into the reaction during the synthesis. As a result, FITC was conjugated with polymers through hydroxyl groups of FITC and isocyanate groups of polyurethane.

Characterization of PEG-Polyurethane

The chemical structure of PEG-polyurethane block copolymer was characterized by ^{1}H-NMR using a NMR spectrometer (AVANCE III 400 MHz, Bruker) with DMSO-d_6 as the solvent. The different components in PEG-polyurethane were confirmed by the corresponding proton-peaks from ^{1}H-NMR spectra. The molecular weight and molecular weight distribution of PEG-polyurethane can be measured using a gel permeation chromatography (GPC, Waters), equipped linearly with K-802, K-803, K-804 columns, using $CHCl_3$ as the eluant solution at a flow rate of 1 mL/min. The molecular weight was calculated based on PEG standards. The FTIR spectra of the samples dispersed in KBr pellets were measured on a Bio-Rad FTS 135 FTIR spectrophotometer. Each spectrum was obtained by cumulating 64

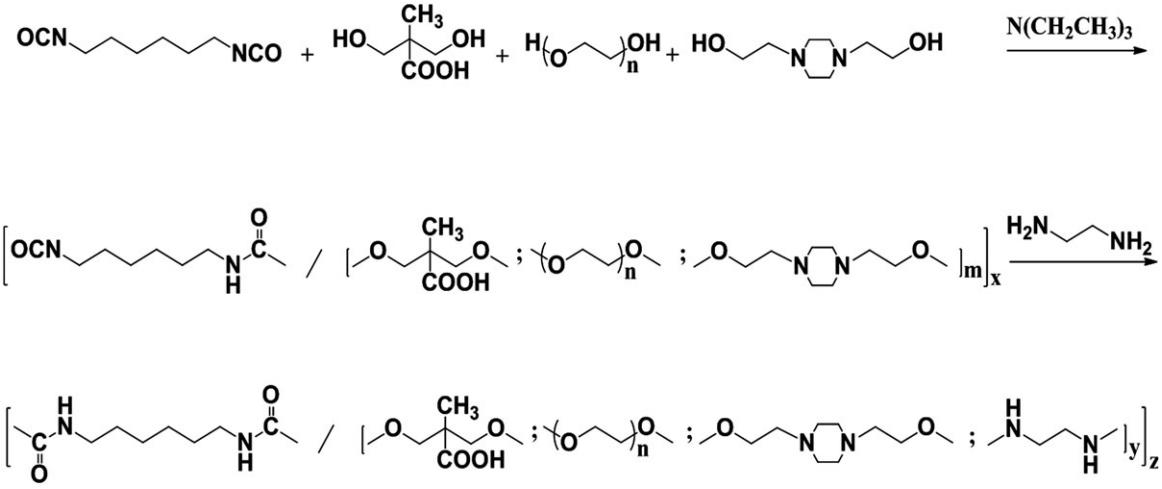

Figure 1. Synthesis scheme of the pH-responsive PEG-polyurethane block copolymer.

Applied Polymer SCIENCE

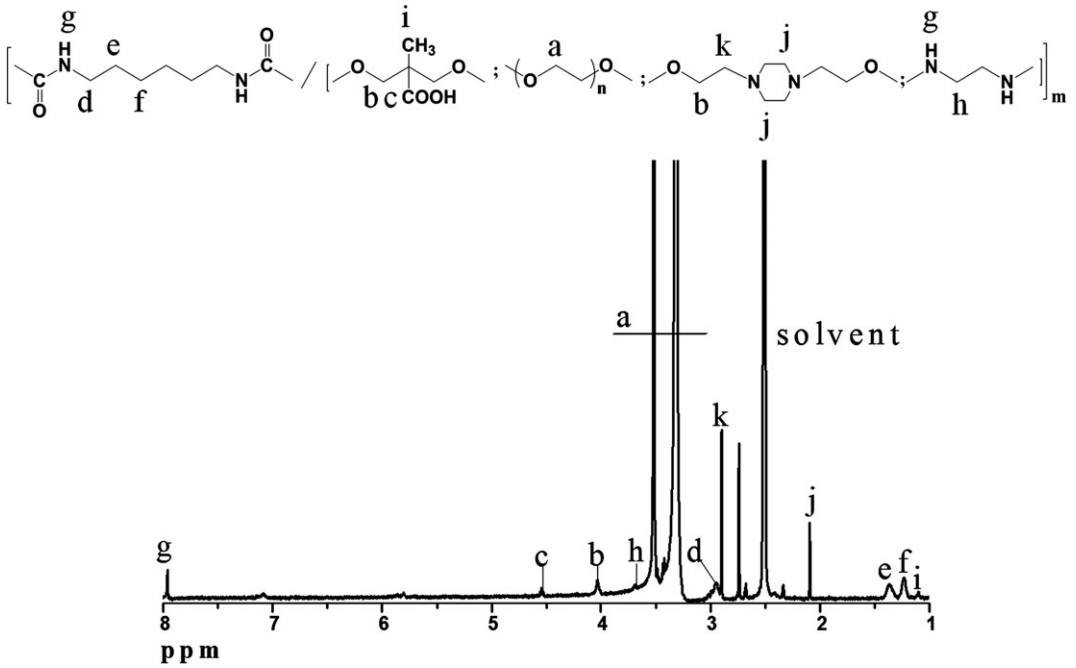

Figure 2. The ^{1}H-NMR spectrum of pH-responsive PEG-polyurethane.

scans. The FTIR spectra of the samples dispersed in KBr pellets were measured on a Thermo Scientific Nicolet iS10 FTIR spectrophotometer.

Acid–Base Titration

The pH-buffering capacity of pH-responsive PEG-polyurethane was measured by an acid–base titration method. Typically, 50 mg of PEG-polyurethane was dissolved in 50 mL of deionized water and titrated to around pH 3.0. By adding 0.01 mL of 0.1*M* NaOH solution drop by drop, the pH value was recorded to obtain the acid–base titration curve.

UV-Vis Spectra

The UV–vis spectra were recorded on a Varian Cary double beam spectrometer using a 10 mm path quartz cell. The wavelength was ranged from 300 to 650 nm using a medium speed scanning. Stock solutions were prepared in DMSO with a concentration of 1 mg/mL.

Fluorescence Spectrometry

The corresponding emission spectrum of pH-responsive PEG-polyurethane with and without FITC was recorded with a SPEX Fluoromax-3 spectrofluorimeter at excitation of 520 nm (obtained from the maximum absorption wavelength). All emission spectra were carried out in 1-cm quartz cuvettes, using λ_{exc} = 519 nm, collecting the emission from 550 to 650 nm (increment of 1 nm), using 1-nm slit widths in excitation and emission (wavelength resolution of 1 nm), and corrected for nonlinear instrument response.

Transmission Electron Microscopy

The transmission electron microscopy (TEM) sample was prepared as follow. The sample was dispersed in an aqueous medium at pH 7.4 and treated by the ultrasonic method. Then a droplet of solution was placed on a copper grid and the excess solution was blotted with a piece of filter paper. The sample was observed on a JEOL 1210 TEM spectroscopy operating at 100 kV and the image was recorded with a digital camera.

Cytotoxicity Evaluation

The cell viability assay was examined using an MTT assay.[23] All the samples with different concentrations were repeated at least four times to obtain the average value. The 2×10^5 L929 fibroblastic cells were seeded on coated metal surface into each well (24 well) at 36.5°C in a humidified atmosphere containing 5% CO_2 for 2 days. DMEM (400 μL) and MTT solution (St. Louis MO, USA) (100 μL) was then added to each well. After additional 4 h incubation at 36.5°C in abovementioned condition, all the solutions in the wells were removed by the vacuum suction. Subsequently, DMSO (400 μL) and a glycine buffer (50 μL, pH 10.5) were added to the wells. The plate was shaken for a few minutes to thoroughly dissolve the dark blue crystals. The solution (100 μL) was then transferred to a 96-well plate and the absorbance was measured at 570 nm by ELISA instrument (SpectraMax M5, Molecular Devices, Korea). The cell viability was calculated by comparing the MTT treated cell solution with the control cell solution.

RESULTS AND DISCUSSION

The successful synthesis of the pH-responsive PEG-polyurethane was confirmed by ^{1}H-NMR spectrometer and the result was shown in Figure 2. The chemical shift in the region of 8.0 ppm (signal g) was associated with the N—H bonds of urethane groups in PEG-polyurethane. The chemical shifts in the region of 3.46–3.52 ppm were attributable to the PEG groups. The chemical shifts at 4.01 ppm and 4.53 ppm correspond to the methylene groups adjacent to the ether bonds of the PEG-polyurethane. The chemical shifts at 2.24 ppm and 2.84 ppm are

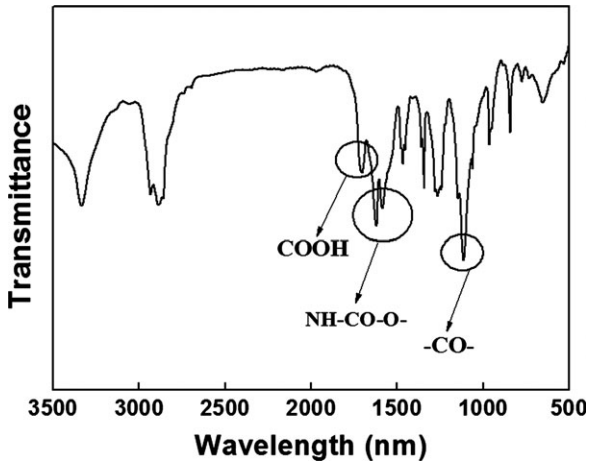

Figure 3. The FTIR spectrum of pH-responsive PEG-polyurethane.

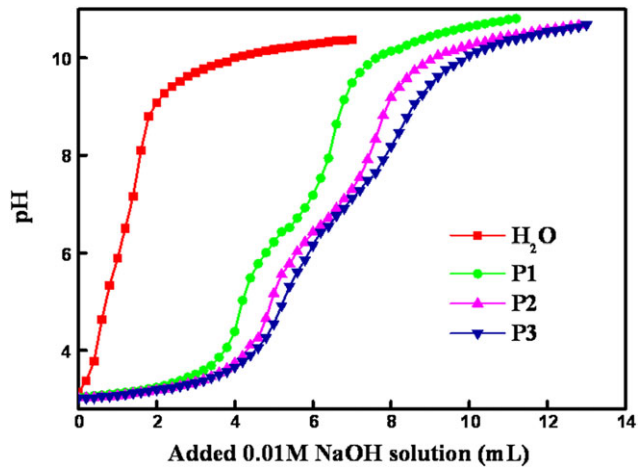

Figure 4. Acid-based titration profiles of PEG-polyurethane block copolymers in an aqueous medium, including (■) H_2O, (●) P1, (▲) P2, (▼) P3. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

associated with the adjacent to nitrogen bonds of HEP in the PEG-polyurethane. The chemical shifts at 1.21 ppm and 1.34 ppm are associated with the remaining methylene protons of PEG-polyurethane. The chemical shift at 1.12 ppm corresponds to the methyl groups of DMPA in the PEG-polyurethane. The ^{1}H-NMR results clearly indicate the existence of pH-responsive polyurethane. FTIR spectroscopy also is a useful technique to confirm the presence of functional groups. As can be seen from Figure 3, the IR spectrum showed PEG-polyurethane. The absorbance at 1760 cm^{-1} is attributed to the carboxyl groups, and it is also worth mentioning that the absorbance at around 1100 cm^{-1} belongs to the stretching vibration of the —CO— groups.[24] At the same time a shoulder band at a lower wave number is attributed to the —NH—COO— groups. The absorbance at 1100 cm^{-1} corresponds to the —C—O—C— stretching vibration of PEG. The number-average molecular (M_n) and polydispersity index (PDI) of the pH-responsive PEG-polyurethane was determined by GPC using PEG standards, shown in Table I.

pH Buffering Capacity of PEG-Polyurethane

The acid-base titration profile of pH-responsive PEG-polyurethane was used to determine the pH buffering range in deionized water. As can be seen from Figure 4, all pH-responsive PEG-polyurethane copolymers were dissolved in ionized water at pH 3.0. The pH buffering capacity can be obtained for PEG-polyurethane with increasing the amount of sodium hydroxide solutions, because of the piperidine amino group containing isolated electron pairs, which could be protonated and deprotonated with changing pH values in an aqueous medium. With

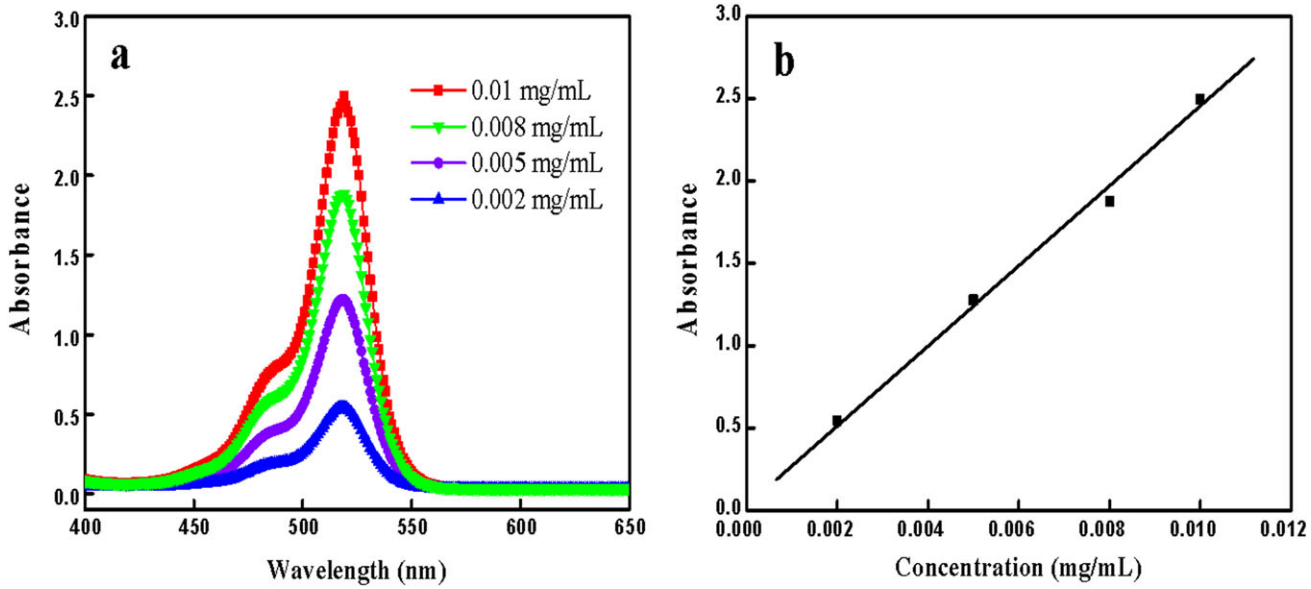

Figure 5. (a) The UV-vis absorbance spectra of FITC with different concentrations in DMSO containing 5 vol% 0.01*M* NaOH solution: (■) 0.01 mg/mL, (▼) 0.008 mg/mL, (●) 0.005 mg/mL, (▲) 0.002 mg/mL; (b) The fitting curve of the maximum absorbance peak value versus the FITC concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Applied Polymer SCIENCE

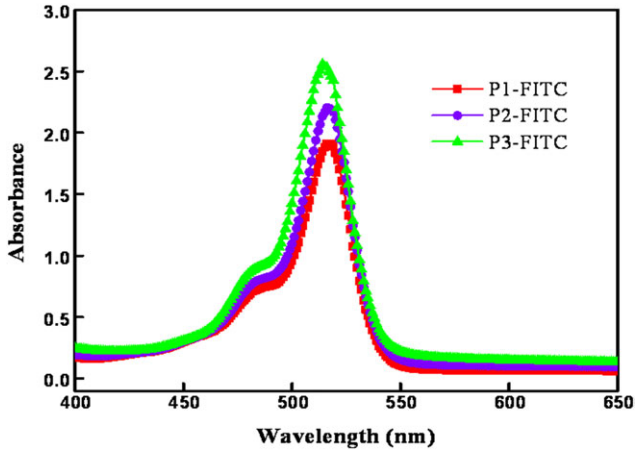

Figure 6. The UV-vis absorbance spectra of PEG-polyurethane-FITC block copolymers in DMSO containing 5 vol% 0.01*M* NaOH solution: (■) P1-FITC, (●) P2-FITC, (▲) P3-FITC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the increase of piperidine components in PEG-polyurethane, the pH buffering capacity would increase much higher than that of water. On the basis of the buffering curves, we have calculated the pK_a of polymers (pK_{a1}=6.83, pK_{a2}=6.89, pK_{a3}=7.02). As a result, the sample P3 showed the highest pH buffering capacity due to the piperidine amino groups in PEG-polyurethane. In addition, the protonation of carboxyl groups would also influence the pH-buffering capacity of PEG-polyurethane.

Concentration Assay of PEG-Polyurethane Containing FITC

In this work, we used a UV-vis spectroscopy to measure the FITC concentrations in synthesized FITC-conjugated PEG-polyurethane block copolymers. To obtain the standard curve, first, FITC dissolved in DMSO containing 5% 0.01*M* NaOH solution with different concentrations. The UV-vis absorbance spectra at FITC with different concentrations were shown in Figure 5(a).

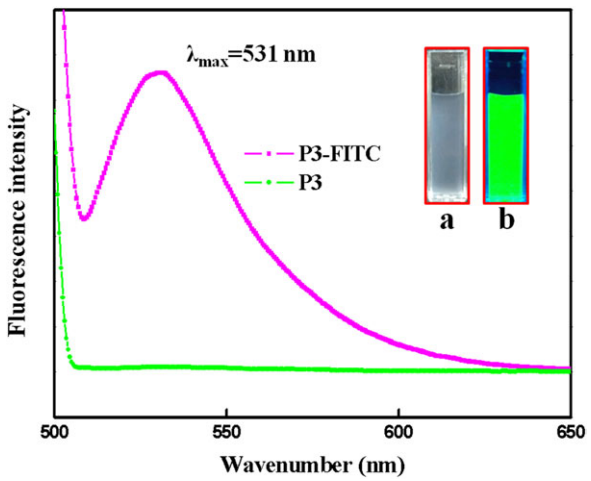

Figure 7. Fluorescence emission spectra of (●) P3 and (■) P3-FITC solutions at pH 7.4 with the excitation of 546 nm, and the optical images of (a) P3 and (b) P3-FITC under a 360 nm UV lamp. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

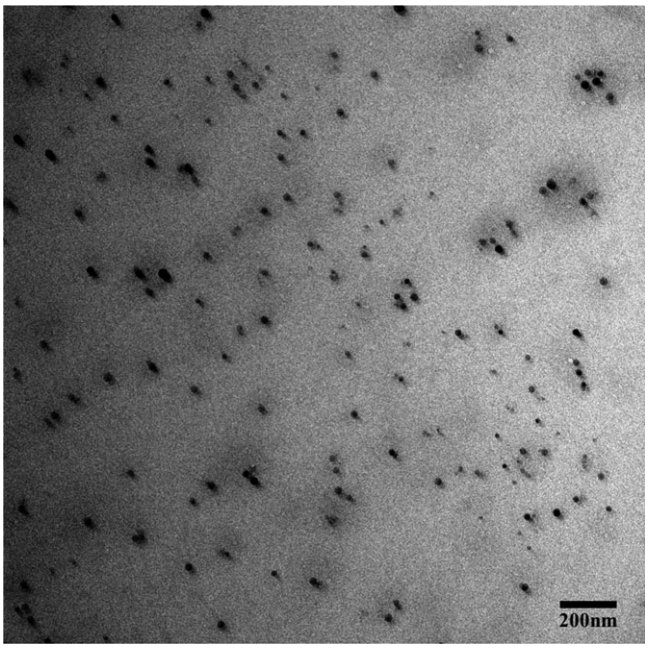

Figure 8. The TEM image of the self-assembly behavior of PEG-polyurethane in an aqueous medium at pH 7.4.

We can obtain the interaction between the maximum absorbance peak value and the FITC concentration in Figure 5(b). As a result, the fitting curve equation could be obtained for $y = 237x + 0.06$ (x: FITC concentration; y: the maximum absorbance peak value). To calculate the FITC concentrations in FITC-conjugated PEG-polyurethane, we measured the UV-vis absorbance spectra of block copolymers in the same solvent with 1 mg/mL of polymer concentration. The result was shown in Figure 6. From observing the highest peak value and calculating the concentration value from to the fitting curve equation, we can measure the FITC concentrations of P1, P2, and P3 corresponding to 0.78%, 0.9%, and 1.0%, respectively.

Fluorescence Property of FITC-Conjugated PEG-Polyurethane

To evaluate FITC-conjugated PEG-polyurethane as an optical imaging agent, the fluorescent propertiy of P3-FITC as an

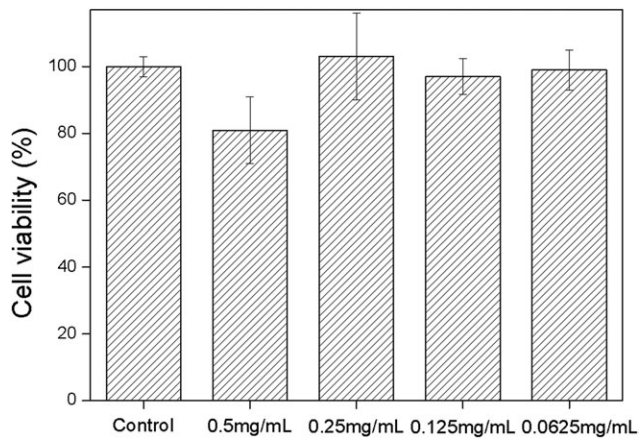

Figure 9. The *in vitro* cytotoxicity evaluation of Fe_3O_4-PEG-PAEA10 on the L929 cell line incubated for 2 days by an MTT assay.

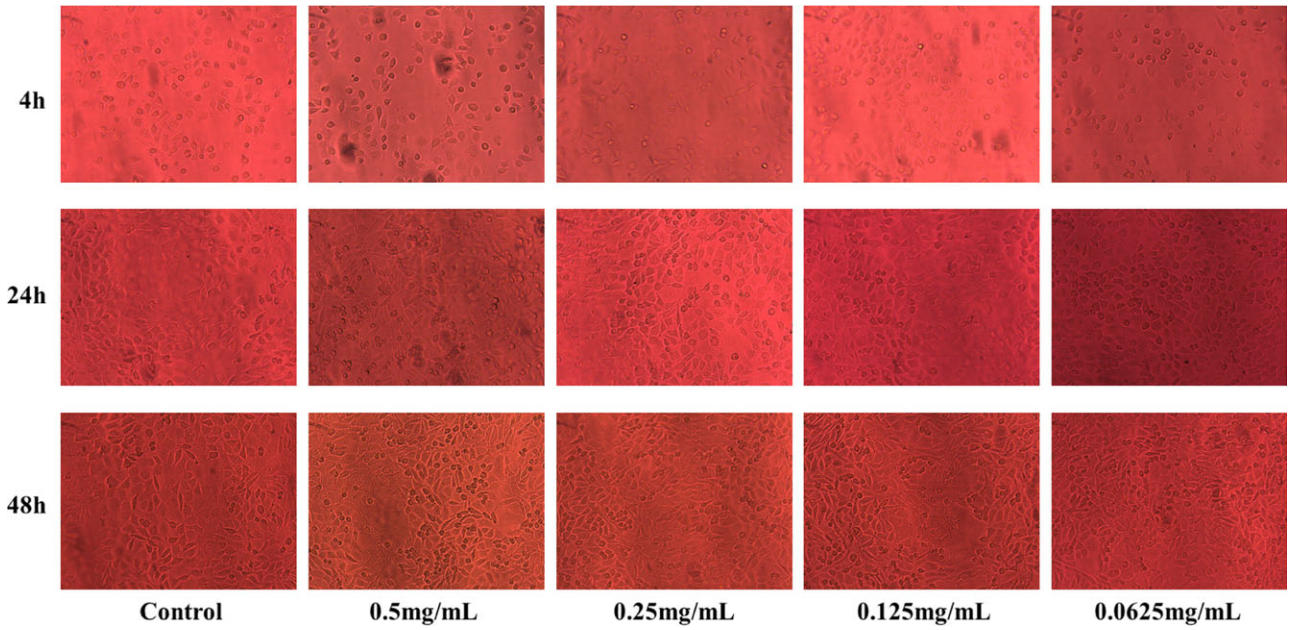

Figure 10. Optical microscopic images of L929 cells in polymer solutions with different concentrations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

elected sample in an aqueous medium was measured by a fluorescence spectrometry. Figure 7 showed the fluorescent emission spectra of PEG-polyurethane with and without conjugating FITC at the excitation of 546 nm. As a result, the maximum emission wavelength was 531 nm for P3-FITC and no fluorescence intensity can be observed for P3. Correspondingly, the images of P3 and P3-FITC were observed in Figure 7(a,b) under a 360 nm UV lamp, respectively, indicating that P3-FITC can be used as a fluorescent imaging probe. In contrast, the sample P3 without conjugating FITC showed no emission lights. Moreover, the self-assembly behavior of PEG-polyurethane in an aqueous medium at pH 7.4 was observed by TEM and Figure 8 showed the morphology images of P3-FITC. Due to the existence of hydrophilic PEG on the main chain, the PEG-polyurethane copolymer can self-assemble into micelles. The well dispersion of particles was observed and the particle size was around 20 nm, indicating that the FITC-conjugated PEG-polyurethane could become a potential nanosized optical imaging probe.

Cytotoxicity

For *in vitro* cytotoxicity test, we demonstrated the evaluation of viability of the L929 cells in the PEG-polyurethane solutions through an MTT assay for 2 days. From Figure 9, obvious toxicity of cells was not observed for PEG-polyurethane solutions with different concentrations, except the cell viability of PEG-polyurethane with 0.5 mg/mL showed a little cytotoxic reaction. The corresponding cell morphologies in PEG-polyurethane solutions with different concentrations after 4, 24, 48 h were observed in Figure 10.

CONCLUSIONS

In summary, a series of pH-responsive PEG-polyurethane block copolymers with tertiary amine groups in the main chain and free carboxyl groups in the side chain were designed and synthesized by an addition polymerization reaction. The results from ^{1}H-NMR and FTIR indicated successful synthesis of the pH-responsive PEG-polyurethane block copolymers. The acid–base titration indicated that the pH-responsive PEG-polyurethane containing piperidine rings have a pH buffering phenomenon in a range of pH values, undergoing the process of ionization and deionization. The results from UV-vis spectra showed that FITC can be conjugated into PEG-polyurethane during the same addition polymerization reaction. Subsequently the fluorescence spectroscopy also showed that FITC-conjugated PEG-polyurethane in an aqueous medium has a maximum emission wavelength of 531 nm. The size of self-assembly polymeric particles in water was around 20 nm by TEM, indicating that the FITC-conjugated PEG-polyurethane could become a potential nanosized optical imaging probe. Therefore, we expect that more biomedical applications would be possible for the pH-responsive PEG-polyurethane owing to its free functional carboxyl groups and simultaneously optical imaging in an aqueous medium.

ACKNOWLEDGMENTS

This research was supported by a grant from National Natural Science Foundation of China (NSFC) (No. 201115148) and Natural Science Foundation of Jilin Province (No. 201115148).

REFERENCES

1. Li, M.; Li, G. L.; Zhang, Z. G.; Li, J.; Neoh, K. G.; Kang, E. T. *Polymer* **2010**, *51*, 3377.
2. Cohen Stuart, M. A.; Huck, W. T. S.; Genzer, J.; Müller, M.; Ober, C.; Stamm, M. *Nat. Mater.* **2010**, *9*, 101.

3. Tanaka, Y.; Gong, J. P.; Osada, Y. *Prog. Polym. Sci.* **2005**, *30*, 1.
4. He, C. L.; Kim, S. W.; Lee, D. S. *J. Controlled Release* **2008**, *127*, 189.
5. Gil, E. S.; Hudson, S. M. *Prog. Polym. Sci.* **2004**, *29*, 1173.
6. Dayananda, K.; He, C. L.; Lee, D. S. *Polymer* **2008**, *49*, 4620.
7. Tan, H.; Chu, C. R.; Payne, K. A.; Marra, K. G. *Biomaterials* **2009**, *30*, 2499.
8. Ruel-Gariépy, E.; Leroux, J. C. *Eur. J. Pharm. Biopharm.* **2004**, *58*, 409.
9. Kim, M. S.; Lee, D. S. *Chem. Commun.* **2010**, *46*, 4481.
10. Gao, W. W.; Chan, J. M.; Farokhzad, O. C. *Mol. Pharm.* **2010**, *7*, 1913.
11. Xu, S. J.; Luo, Y.; Graeser, R.; Warnecke, A.; Kratz, F.; Hauff, P.; Lichac, K.; Haaga, R. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1030.
12. Butsele, K. V.; Cajot, S.; Vlierberghe, S. V.; Dubruel, P.; Passirani, C.; Benoit, J. P.; Jerome, R. *Adv. Funct. Mater.* **2009**, *19*, 1416.
13. Meng, F. H.; Hennink, W. E.; Zhong, Z. Y. *Biomaterials* **2009**, *30*, 2180.
14. Ganta, S.; Devalapally, H.; Shahiwala, A.; Amiji, M. A. *J. Controlled Release* **2008**, *126*, 187.
15. Lee, E. S.; Gao, Z. G.; Bae, Y. H. *J. Controlled Release* **2008**, *132*, 164.
16. Lee, S.; Park, K.; Kim, K.; Choi, K.; Kwon, I. C. *Chem. Commun.* **2008**, *36*, 4250.
17. Chen, K. Y.; Kuo, J. F.; Chen, C. Y. *Biomaterials* **2000**, *21*, 161.
18. Grabowski, C. A.; Mukhopadhyay, A. *Macromolecules* **2008**, *41*, 6191.
19. Akiyoshi, K.; Kang, E. C.; Kurumada, S.; Sunamoto, J. *Macromolecules* **2000**, *33*, 3244.
20. Weissleder, R.; Tung, C. H.; Mahmood, U.; Bogdanov, A., Jr. *Nat. Biotechnol.* **1999**, *17*, 375.
21. Kim, J. H.; Park, K.; Nam, H. Y.; Lee, S.; Kim, K.; Kwon, I. C. *Prog. Polym. Sci.* **2007**, *32*, 1031.
22. Kanyanta, V.; Lvankovic, A. *J. Mech. Behav. Biomed.* **2010**, *3*, 51.
23. Mosmann, T. J. *Immunol. Methods* **1983**, *65*, 55.
24. Ibrahim, M.; Nada, A.; Kamal, D. E. *Indian J. Pure Appl. Phys.* **2005**, *43*, 911.