Synthesis and Characterization of a New Amphiphilic Copolymer Containing Multihydroxyl Segments for Drug Carrier

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ABSTRACT: A new amphiphilic copolymer (copoly-(MR-BMA-HEA-MAA), PRBHM) containing multihydroxyl segments was designed and synthesized for application in drug carrier. PRBHM can be dissolved in water to form aggregates directly with a critical aggregate concentration (CAC) of 0.0138 mg mL^{-1}. The chains of PRBHM can be collapsed into hydrophobic globules when pH decreases from neutral to slightly acid condition (pH = 5.0–7.0) in water. Since the hydrophilic hydroxyl group is independent on pH, PRBHM can keep stable both in neutral and slightly acid aqueous solutions. The hydrophobic small molecules such as 5-(4-(4-vinylbenzyloxy) phenyl)-4,5-dihydro-1,3-diphenyl-1H-pyrazole (PY) can be loaded into PRBHM aggregates via ultrasonic treatment in water, and can be internalized into BEL-7402 cancer cells. The cytotoxicity determination also indicates the good biocompatibility of PRBHM in potential application as a drug carrier. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 2843–2850, 2011

Key words: biomaterials; copolymers; biocompatibility; drug delivery systems

INTRODUCTION

Recently, polymeric aggregates including micelles have attracted considerable attentions in the fields of drug or gene carriers.[1–3] Polymers could be the materials with high biocompatibility and some more important properties such as enhanced permeability and retention (EPR) effect and membrane destabilizing effect. As regards to the antitumor medicines, EPR effect of polymeric carriers would enable drug molecules to target to tumor tissues, which enhances the drug efficiency.[4–8] As Hiroshi Maeda reported, the polymers with a high number-average molecular weight beyond 100,000 would always possess a remarkable EPR effect.[5] The membrane destabilizing effect can prevent drug molecules being decomposed in lysosome, which maintains the drug activity. Generally, such membrane destabilizing effect could be achieved by polymers through two approaches. One is the proton sponge effect,[9–15] which can cause osmotic swelling and vesicle destabilization of some polymers at acidic pH and then allow the drug molecules to be released in cytoplasm. These polymers are mainly cationic aminated species such as poly(ethylenimine) (PEI) and poly (amidoamine) (PAA). The other approach is utilizing the pH-induced conformational change in some anionic carboxylated polymers. Some of them can be collapsed to hydrophobic globules at acidic pH, which possess membrane destabilizing properties.[16–20] The critical pH value of conformational change could be adjusted by copolymerization with other monomers.[17] As such copolymers, the content of carboxylated segments can be varied in a wide range, offering the availability of modification for multifunctions.

The polymeric carrier should be biocompatible to meet the requirements of biological applications. Besides the requirements to the polymers, the toxic solvents should be avoided since the solvent would always remain when preparing the aggregates.[21] However, most organic solvents are used to prepare aggregates, such as dimethylacetamide (DMAC), dimethylformamide (DMF), tetrahydrofuran (THF)

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and dimethyl sulfoxide (DMSO), which are all toxic to cells and harmful to health.[22–26] In addition, the polymeric carrier should be stable in aqueous environments to avoid precipitation. And also in cells, the precipitation of polymeric carrier would influence the drug release process. Generally, the reported anionic carboxylated polymers are the copolymers containing carboxylated units and other hydrophobic units. Those polymers might be lack of sufficient water solubility, for which the aggregates can not always keep stable in water under slightly acid conditions.

In this paper, we have synthesized an amphiphilic copolymer (PRBHM, see Scheme 1), which can be dissolved in water to form micelle-like aggregates[27] directly, to be used as drug carrier. In this copolymer, the fluorescent rhodamine segments enable the polymer to be traced. The carboxylate segments offer the pH sensitivity and membrane destabilizing effect. The butyl segments can enhance the solubility of hydrophobic drug molecules. The hydroxyethyl segments offer the sufficient water solubility. The structure and properties of PRBHM were characterized, and the pH value of conformational change is consistent with that of endosome (5.0–6.5).[16] A hydrophobic small molecule PY was used as a model drug to investigate whether the drug loaded PRBHM aggregates can be internalized by cells. The biocompatibility of PRBHM was also studied by cytotoxicity assay.

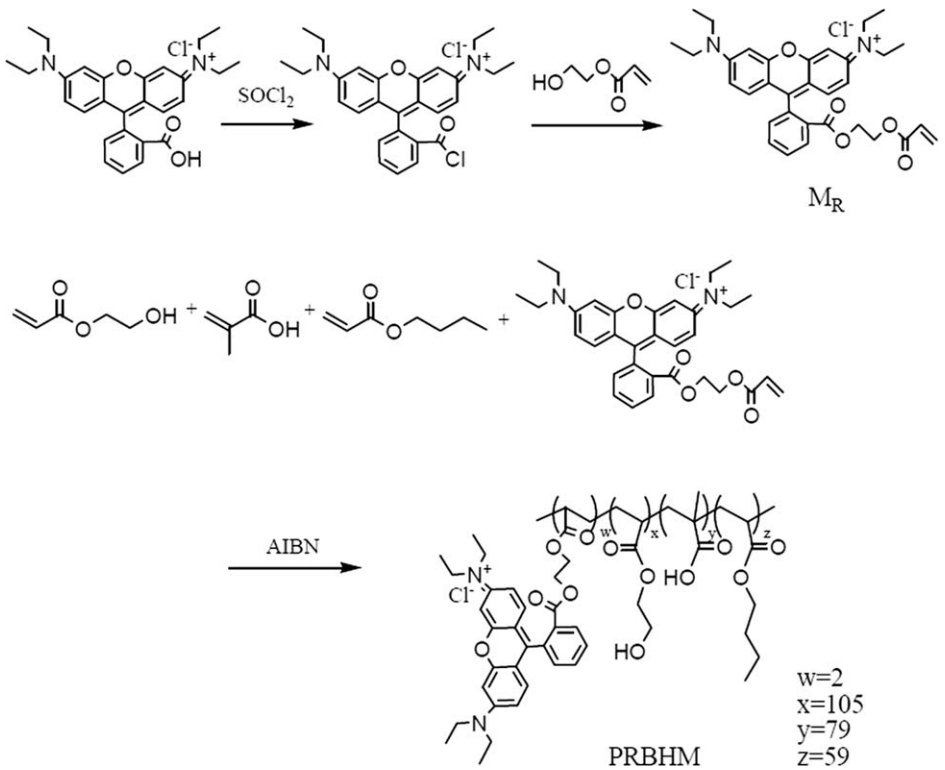
EXPERIMENTAL

Materials

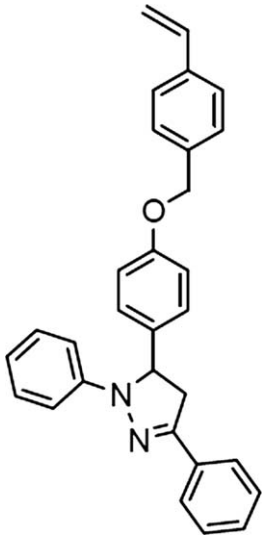
2-Hydroxyethyl acrylate (HEA), methacrylic acid (MAA) and *n*-butyl acrylate (BMA) were purchased from Aldrich and purified twice by passing through a column filled with basic alumina to remove the inhibitor. The initiator 2,2′-azobis (isobutyronitrile) (AIBN, A.R.) was purified by recrystallization with methanol. Rhodamine B (A.R.) and thionyl chloride (A.R.) were purchased from Sinopharm Chemical Reagent Co., Ltd. The small molecule 5-(4-(4-vinylbenzyloxy) phenyl)-4,5-dihydro-1,3-diphenyl-1H-pyrazole (PY, see Scheme 2) was synthesized formerly. Bovine Calf Serum (BCS), Fetal Calf Serum (FCS) and Roswell Park Memorial Institute-1640 (RPMI-1640) media were purchased from Gibco and used as received. The human hepatocellular carcinoma BEL-7402 cells (BEL-7402 cells) were purchased from Chinese Academy of Sciences Cell Bank. All other reagents and solvents were purchased from commercial sources and used as received unless otherwise stated.

Characterizations

^{1}H NMR spectra were measured by INOVA 400 MHz NMR spectrometer using $CDCl_3$ or CD_3OD as solvents at an ambient temperature. The elemental analysis was performed by FT analyzer (1110, Carlo



Scheme 1 The synthetic route of PRBHM.



Scheme 2 The structure of 5-(4-(4-vinylbenzyloxy) phenyl) -4,5-dihydro-1,3- diphenyl-1H-pyrazole (PY)

Erba, Italy). Molecular weight and polydispersity of polymers were measured using gel permeation chromatography (GPC) (1515, Waters, America) with DMF as mobile phase at a flow rate of 1 mL min^{-1} and with column temperature at 30°C. UV-Vis spectra were recorded on a UV-Vis instrument (λ-17, PerkinElmer, America). Fluorescence emission spectra were obtained on a fluorescence spectra photometer (920, Edinburgh Instruments, England). The pictures were obtained on a camera (DMC-FX30, Panasonic, Japan) with an exposure time of 2.5 s. The morphology examination of PRBHM aggregates was performed on a transmission electron microscope (CM120, Philips, Holland) with an accelerating voltage of 100 KV. The sample was prepared by placing a drop of the 0.2 mg mL^{-1} PRBHM solution onto a mesh copper grids and drying the sample in air before measurement. The average diameter of polymer was obtained on a high performance particle size analyzer (5001, Malvern, England). The pH values are obtained on an accurate pH calculator (PHS-3C, Shanghai Precision Scientific Instrument, China).

Cellular uptake and imaging

The fluorescent images of BEL-7402 cells (BEL-7402 cells were incubated for 30 min in 1640 solutions containing 0.02 mg mL^{-1} PY (suspension, A), 0.1 mg mL^{-1} PRBHM (B), and 0.1 mg mL^{-1} PRBHM loaded 3.1×10^{-3} mg mL^{-1} PY (C), respectively.) The human hepatocarcinoma BEL-7402 cells were incubated in FCS-RPMI-1640 solution containing 10% BCS at 37 ± 0.5°C for 25 h to obtain a 70% cell climbing ratio. The media was changed to 200 μL 1640 solution, and was divided into three equally. PRBHM solution, PY suspension and PY loaded PRBHM solution were added into the cell culture to obtain a definite final concentration of 0.1 mg mL^{-1}, 0.02 mg mL^{-1}, and 0.1 mg mL^{-1}, respectively. After 30 min, the solution was replaced to 1640 solution. The fluorescence images were acquired using an Olympus IX-51 inverted microscope equipped with 100 W mercury-xenon arc lamp excitation light source and high speed CCD camera.

Cytotoxicity assay

The BEL-7402 cells were seeded in 96-well plates at a density of 1.3×10^5 mL^{-1}, and incubated at 37 ± 0.5°C for 25 h. Then, the cells were exposed to copolymer aggregates at different concentrations for 24 h. The cytotoxic effects were evaluated using a standard sulforhodamine B (SRB) assay.

Synthesis

Synthesis of Rhodamine B (2-hydroxyethyl acrylate) ester (M_R)

Rhodamine B (2.1 mmol) was dissolved in 1,2-dichloroethane (15 mL), then thionyl chloride (12.7 mmol) was added slowly at room temperature. The reaction mixture was refluxed for 8 h. After the evaporation of solvent in vacuum to obtain rhodamine B acid chloride, 2-hydroxyethyl acrylate (9.6 mmol) was added dropwise to rhodamine B acid chloride solution in dichloromethane and stirred for 24 h at room temperature. The crude product was purified by column chromatography (CH_2Cl_2/MeOH = 30/1, v/v) to obtain 0.9 mmol of product M_R (yield: 42.9%). ^{1}H NMR ($CDCl_3$): δ = 8.31 (d, 1H, J = 7.8 Hz), 7.84 (t, 1H, J = 7.5 Hz), 7.76 (t, 1H, J = 7.7 Hz), 7.32 (d, 1H, J = 7.4 Hz), 7.08 (d, 2H, J = 9.5 Hz), 6.93 (d, 2H, J = 9.5 Hz), 6.81 (s, 2H), 6.38 (d, 1H, J = 17.3 Hz), 6.05 (m, 1H), 5.87 (d, 1H, J = 10.4 Hz), 4.29 (m, 2H), 4.17 (m, 2H), 3.66 (q, 8H, J = 7.2 Hz), 1.34 (t, 12H, J = 7.1 Hz). Anal. calcd. for $C_{33}H_{37}ClN_2O_5$: C 68.68, N 4.85, H 6.46. Found: C 68.47, N 4.79, H 6.31.

Synthesis of Copoly-(M_R-BMA-HEA-MAA) (PRBHM)

HEA (20 mmol), MAA (15 mmol), BMA (15 mmol), M_{RH} (43 μmol), and AIBN (0.5 mmol) were dissolved in 25 mL cyclohexanone in the mole ratio of 40 : 30 : 30 : 0.086 : 1. The solution was degassed using dry nitrogen, and stirred at 60°C for 15 h. The reaction mixture was added dropwise into stirred dichloromethane to precipitate the copolymer. The copolymer was purified with dichloromethane in a Soxhlet extractor until no rhodamine species in the dichloromethane solution could be detected by

UV-Vis instrument and the copolymer was dried to constant weight (4.79 g, yield: 86.3%).

Preparation of PRBHM aggregates

20 mg PRBHM was dissolved in 10 mL phosphate buffer (0.05*M*, pH = 7.4), and the solution was diluted to 50 mL with deionized water to obtain the final concentration of 0.4 mg mL^{-1}. The diluted HCl solution was added into PRBHM solution to adjust the pH value. When loaded the hydrophobic small molecule in the copolymer aggregate, 1.8 mg PY and 9 mg PRBHM were added into 10 mL phosphate buffer (0.05*M*, pH = 7.4), and the suspension was treated with ultrasonic for 30 min. Then it was diluted to 45 mL deionized water and treated with ultrasonic for 12 h. After slowly continuous stirring for 48 h, the PY-loaded PRBHM aggregate solution was obtained by filtration to remove the solid.

RESULTS AND DISCUSSION

Characterization of PRBHM

The content of each unit in PRBHM, except the rhodamine unit, can be calculated from the peak integration ratio of the corresponding protons in ^1H NMR spectra (Fig. 1).[28,29] The resonance at 3.721 ppm (peak 1) is assigned to protons of methylenes in hydroxyethyl segments, the resonance at 4.067 ppm (peak 2, 3) is the characteristic signals of methylene protons in hydroxyethyl segments and butyl segments and the resonances at 1.146 ppm, 1.056 ppm, and 0.940 ppm (peak 11, 12) are ascribed as methyl protons in butyl segments and carboxylate units. The integral areas of peak 1, peak 2,3 and peak 11,12 are 2.00, 3.14, and 3.92, respectively. According to the integral area of these peaks, the ratio of hydroxyethyl segments, butyl segments and carboxylate segments can be calculated as (2.00/2) : ((3.14-2.00)/2) : (3.92/3-(3.14-2.00)/2) = 1 : 0.57 : 0.74. The resonances at δ = 1.393-2.087 ppm (Fig. 1) could hardly be defined more exactly, because the head-to-head or head-to-tail structures randomly exist in PRBHM. The content of rhodamine segments was determined by using the UV-Vis absorption of the corresponding monomer M_R as references.[30] First, the standard ethanol solutions of M_R were tested to obtain the standard linear curve of absorption intensity (I_{abs}, ABS) versus concentration (c_{MR}, $\times 10^{-6}$ mmol mL^{-1}): $I_{abs} = 0.088c_{MR}$-0.007. Then, the concentration of rhodamine segments in the 0.2 mg mL^{-1} ethanol solution of PRBHM was calculated as 13.10×10^{-6} mmol mL^{-1}. Since the average molecular weight of other units is $(1 \times 116 + 0.75 \times 86 + 0.56 \times 128)/(1 + 0.75 + 0.56) = 109.2$ g mol^{-1}, the content of rhodamine segments in copolymer can be calculated as $13.10 \times 10^{-6}/(0.2/109.2) = 7.2 \times 10^{-3}$. Finally, the contents of rhodamine segments, hydroxyethyl segments, carboxylate segments and butyl segments were obtained as following: $w = 2$, $x = 105$, $y = 79$, $z = 59$ (Fig. 1). The number-average molecular weight of PRBHM is 26600 with the polydispersity of 1.8, determined by GPC.

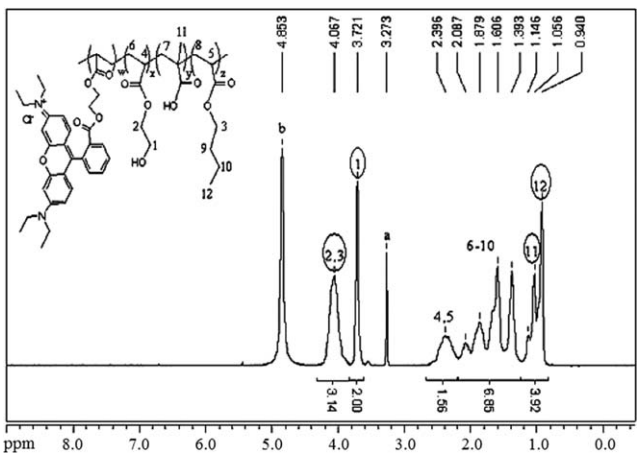


Figure 1 The ^1H NMR spectra of PRBHM with CD_3OD as solvent (peaks a and b are assigned to signals of CD_3OD and water, respectively).

Characterization of Prbhm aggregates

1- effect of concentration

PRBHM can be dissolved in water to form micelle-like aggregates directly. The CAC value can be determined by the concentration dependence of the excitation ratios of pyrene.[31–33] Figure 2 presents the fluorescent excitation spectra of pyrene and the curve of intensity ratio (I_{335}/I_{333}) versus concentration of PRBHM. When the concentration exceeded the CAC value, a number of aggregates formed, and pyrene would be accumulated in the aggregates. The fluorescent property of pyrene was changed when the hydrophilic degree of the environment decreased. The excitation peak at 335 nm shifted left along with the increase of concentration of PRBHM. In that, the CAC value of PRBHM in water was determined by the intersection point of the tangents shown in Figure 2(B), which was 0.0138 mg mL^{-1}.

2- effect of pH

The pH sensitivity of PRBHM aggregate was characterized by the fluorescent emission spectra.[17] By increasing pH, the carboxylic acid groups dissociate to the carboxylate anions and electrostatic repulsions between carboxylates extend the polymer chain.[16,34] On the contrary, in lower pH value, the polymer chains collapse to globule shape due to hydrophilic carboxylic acid groups. The third fluorescent

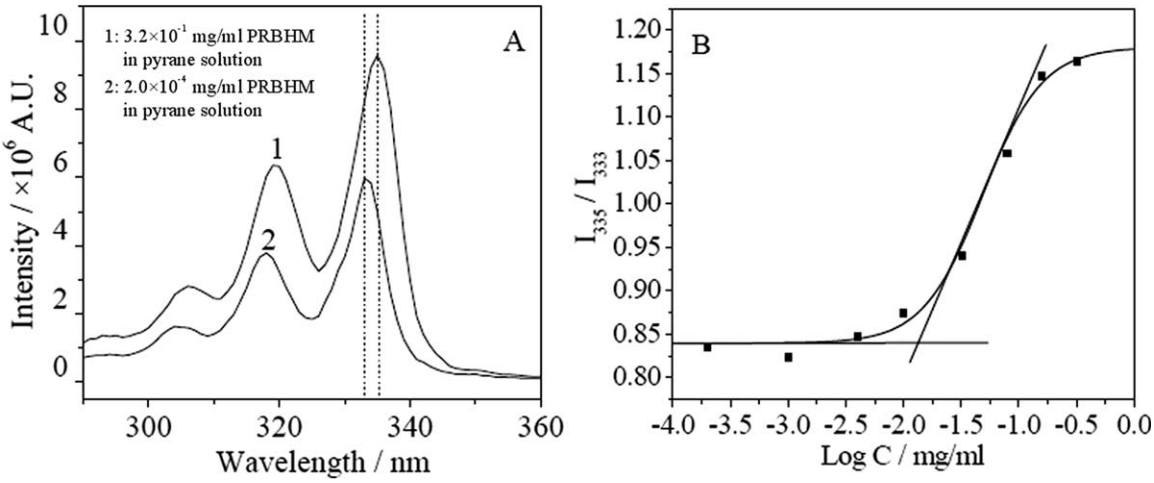


Figure 2 The relationship of excitation intensity of pyrene and concentration of PRBHM (λ_{em} = 390 nm, $c_{pyrene} = 4 \times 10^{-6}$ *M*, pH = 7.0) A: The excitation spectra of pyrene in water solutions containing two different concentrations of PRBHM; B: The curve of intensity ratio I_{335}/I_{333} versus the concentration of PRBHM.

emission peak of pyrene at around 383 nm (I_3) was largely dependent on the hydrophobic degree of environment. Figure 3(A) showed the fluorescent emission spectra of pyrene in different environment. From curve 1 to 3 you can see that the hydrophobic degree of environment was enhanced meanwhile I_3 increased the most remarkably. Figure 3(B) presents the dependence of intensity ratio I_1/I_3 (I_1 is the first emission peak at around 372 nm) on pH. I_1/I_3 turned large while pH increased in the range of 5.0–7.0, which indicates PRBHM aggregates would posses the pH sensitivity in endosome (pH = 5.0–6.5).

Figure 4 shows the TEM image of the PRBHM aggregates prepared in water. It can be seen that the aggregates take a spherical morphology. However, since the TEM image was not obtained under solvent condition, the diameter of aggregates might turn greater when dried to solid. Figure 5 presents the diameter distributions of PRBHM aggregates in water solution. Initially, the average diameter was 290 nm with the dispersion range of 234–526 nm when the pH value equaled 7.0. While the pH decreased to 4.0, the average diameter of the aggregates turned to be 151 nm with a broad dispersion range of 65–940 nm. Such a broad dispersion implies that the hydrophobic degree of PRBHM aggregates increased so that the small diameter aggregates was formed, though they were not stable in aqueous solution, and some of small diameter aggregates would be agglomerated at pH = 4.0. When the pH value of solution decreased to 2.0, the average diameter increased to 1058 nm, which indicates the agglomeration of the hydrophobic aggregates in aqueous solution.

Figure 6 shows the pictures of PRBHM water solution under different pH conditions. The aggregate solution turned turbid when pH decreased from 7.0 to 4.0, just because the large size particles formed. In

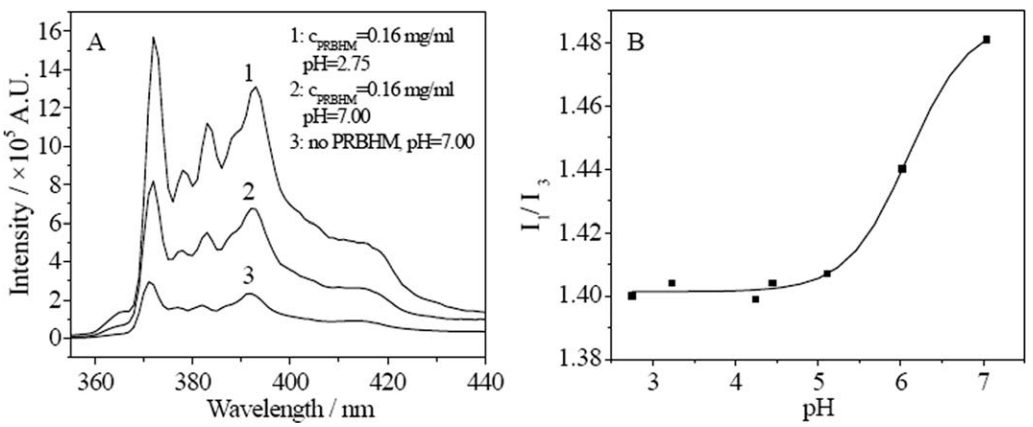


Figure 3 The relationship of fluorescent intensity of pyrene and pH (λ_{ex} = 334 nm, $C_{pyrene} = 4 \times 10^{-6}$ *M*, $C_{PRBHM} = 0.16$ mg mL^{-1}). A: The fluorescent spectra of pyrene in different environments; B: The curve of intensity ratio I_1/I_3 versus the pH value.

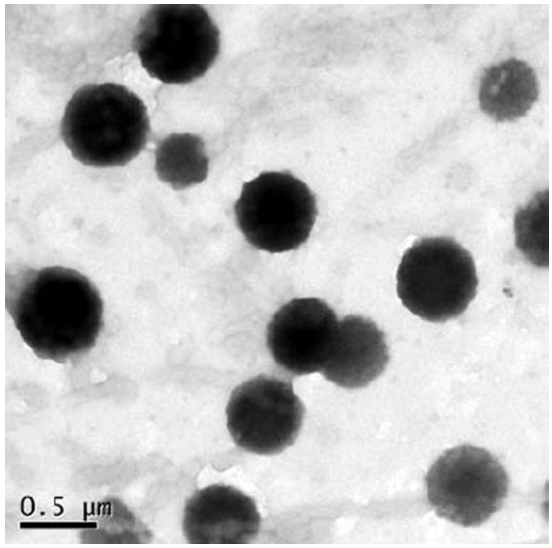


Figure 4 The TEM image of micellar structure prepared from the water solution of PRBHM at pH = 7.4.

PRBHM chains, the hydrophilic hydroxyethyl segments would enhance the stability of aggregate solution both under the neutral and slightly acid conditions. Although the precipitation was found in the PRBHM solution 24 h later, other solutions with the pH $\geq$ 4.0 is keeping stable beyond 60 days.

Cellular uptake and imaging

The cellular experiment was designed to examine whether PRBHM aggregates could load small molecules and be internalized by cells. The hydrophobic small molecule PY can emit intense blue fluorescent light, while PRBHM can emit red fluorescent light.

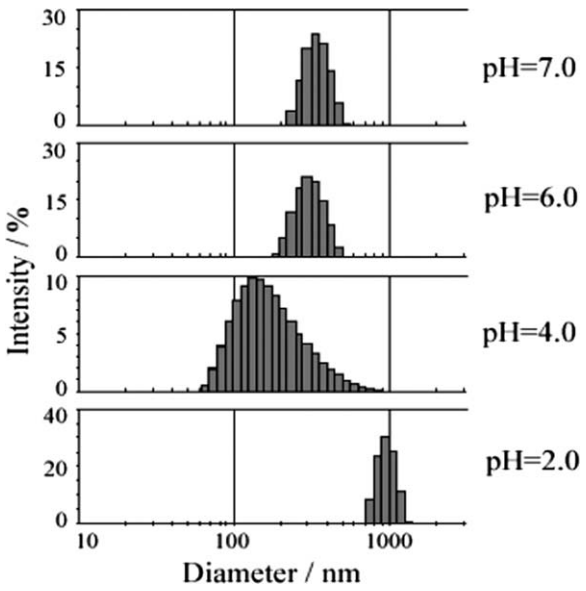


Figure 5 The diameter distributions of PRBHM water solutions (0.16 mg mL^{-1}).

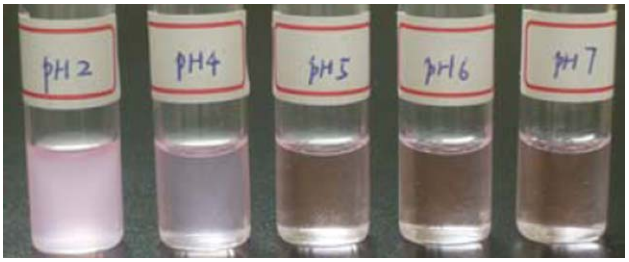


Figure 6 The picture of PRBHM water solutions (0.16 mg mL^{-1}). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The amount of loaded PY was determined by the absorption comparison method similar to the characterization of the content of rhodamine segments in PRBHM. The standard linear curve of absorption intensity (I_{abs}, Abs) versus concentration (c_{PY}, $\times 10^{-3}$ mg mL^{-1}) was $I_{abs} = 0.031c_{MR}+0.003$, and the amount of loaded PY was obtained, which is 3.1 $\times$ 10^{-3} mg mL^{-1} PY in PRBHM solution of 0.1 mg mL^{-1}. Figure 7 presents the fluorescent images of BEL-7402 cells. The fluorescent signal inside cells in the merged images reveals that the fluorescent species can be internalized by cells. We can conclude that PY itself could not be internalized by BEL-7402 cells [see Fig. 7(A)] and PRBHM can be internalized by BEL-7402 cells [see Fig. 7(B)]. PY can be loaded into PRBHM and be internalized by BEL-7402 cells [see Fig. 7(C)]. These results indicate the possibility of PRHBM usage as drug carrier.

Cytotoxicity results

The cytotoxicity of PRBHM was examined using the SRB assay. The BEL-7402 cells were incubated for 24 h in PRBHM solution at different concentrations. The cells were also treated with solutions containing no copolymer as control. Figure 8 presents the cytotoxicity assay results of PRBHM. It suggests that PRBHM alone exhibited almost no cytotoxicity at the concentration less than 12.5 μg mL^{-1} and displayed slightly cytotoxic activity when compared with the control at higher concentrations. As shown in Figure 8, the BEL-7402 cells incubated in the presence of PRBHM kept high cell viability (80%). No significant cytotoxicity was observed even at a high concentration. The toxicological effects may be ascribed to the biocompatible and nontoxic nature of the amphiphilic copolymer. The unique biocompatibility indicates that it may be safe for further biomedical applications.

CONCLUSIONS

A pH sensitive water-soluble copolymer PRBHM was synthesized. The polymer chains of PRBHM can

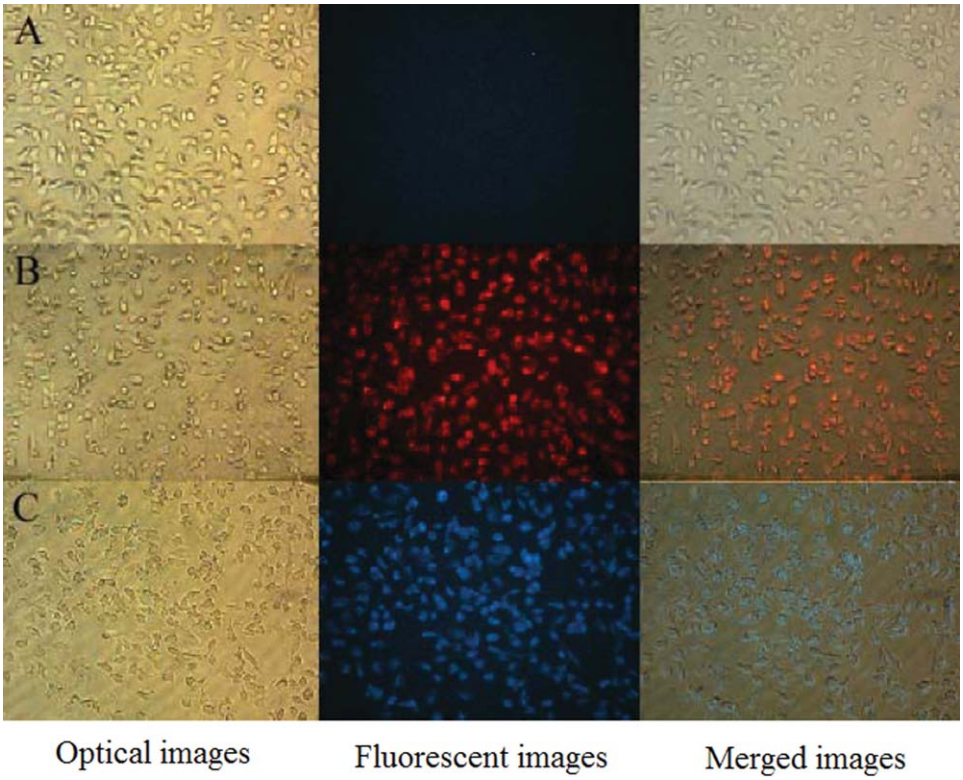


Figure 7 The fluorescent images of BEL-7402 cells (BEL-7402 cells were incubated for 30 min in 1640 solutions containing 0.02 mg mL^{-1} PY (suspension, A), 0.1 mg mL^{-1} PRBHM (B), and 0.1 mg mL^{-1} PRBHM loaded 3.1×10^{-3} mg mL^{-1} PY (C), respectively.) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

be collapsed to form the relatively hydrophobic globules under slightly acid conditions (pH = 5.0–7.0) and can be internalized by some kinds of cells such as the BEL-7402 cell. The hydrophobic small molecules such as PY can also be loaded in PRBHM aggregates as a drug model and be carried into the BEL-7402 cells. The cytotoxicity assay confirmed that PRBHM exhibits no significant cytotoxicity. In addition, PRBHM can be dissolved in water directly to form aggregates, which avoids the potential risk of organic solvents, and PRBHM aggregates can keep stable in both neutral and slightly acid solutions. So this amphiphilic copolymer is regarded as a potential candidate of drug carrier in cancer diagnosis and therapy.

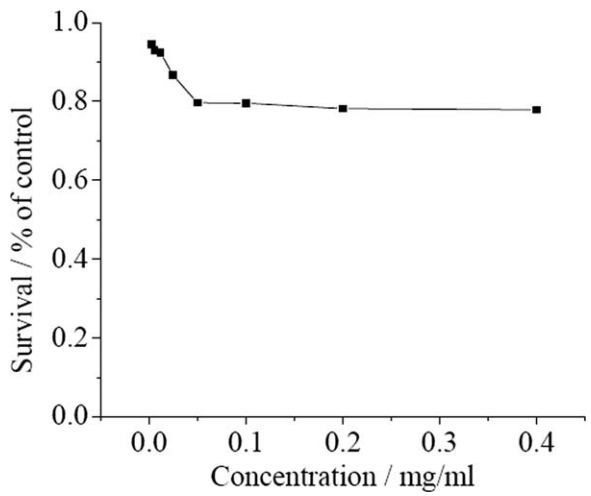


Figure 8 The cytotoxic effect of PRBHM aggregates at different concentrations (BEL-7402 cells were incubated for 24 h before SRB assay).

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