ARTICLE TYPE

Preparation of biodegradable PEGylated pH/reduction dual-stimuli responsive nanohydrogels for controlled release of anti-cancer drug

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A facile and efficient method was developed to prepare the monodisperse biodegradable PEGylated pH and reduction dual-stimuli sensitive poly[methacrylic acid-*co*-poly(ethylene glycol) methyl ether methacrylate-*co*-N,N-bis(acryloyl)cystamine] (PMPB) nanohydrogels with dried particle size below 200 nm via one-step distillation precipitation polymerization as a drug delivery system (DDS) for controlled
10 release of a wide-spectrum anti-cancer drug, doxorubicin hydrochloride (DOX). Under normal physiological media, the nanohydrogels possessed high drug encapsulation efficiency (more than 96%) within 48 h and exhibited good stability with a trifle of premature drug release. However, rapid DOX release was achieved at lower pH or in presence of reductive reagent glutathione (GSH) with cumulative release of more than 85% within 30 h. Furthermore, the nanohydrogels manifested nontoxicity on HepG2
15 cells at a concentration of 10 µg/mL or lower. Grounded on the above excellent characteristics of the nanohydrogels, such as low toxicity, impressive biodegradability, sharp dual responsiveness, adequate drug loading capacity and high drug encapsulation efficiency, the nanohydrogels were supposed to have potential application in the area of cancer therapy.

1. Introduction

20 Over the past decade, hydrogels in the nanoscale range have been developed to meet the demand of various biomedical and pharmaceutical applications,[1-3] especially in controlled drug delivery systems which could decrease adverse side effects and improve therapeutic efficacy of the drugs.[4,5] However, due to the
25 inherent nondegradability of the chemically cross-linked nanoparticles, a series of subsequent adverse results from transdermal and oral administrations, and precisely 'on' and 'off' states of drug release under external stimulations are still severe challenges for researchers in the field of controlled drug release.[6-
30 8] Accordingly, developing biodegradable, biocompatible, relative stable and intelligent nanoparticles with confined scale is crucial to the long-term future of their further applications.

Nanohydrogels possess various advantages in their applications for drug delivery. First of all, they have their own intrinsic
35 properties like macrohydrogels. High water content, desirable stability, large specific surface area and complex three-dimensional network of these nanohydrogels lead to many advantages, for instance, good flexibility, relative high solubility, great drug loading efficiency, enhanced bioavailability and
40 similarity to natural tissues and biological materials.[9] Moreover, they display outstanding nano-dimension effects. This size allows them to get to the lesion place by passive targeting based on the enhance permeation retention (EPR) effect.[10,11] These special effects also make the nanohydrogels cross biological barriers
45 possible and even reach to capillaries.[12] Additionally, the nanohydrogels could be obtained through various polymerization methods[4] and their physico-chemical properties are fairly well contained.[13] Finally, most nanohydrogels are sensitive towards external stimuli, such as pH, ionic strength, redox potential,
50 temperature, light, magnetic field, *etc.,* and undergo changes in their volumes, shapes, molecular charges, ionization states, or aggregation behavior once those environmental changes.[14-16]

For one thing, among those environmental stimuli, pH responsive nanohydrogels have been widely studied and attracted
55 more and more scientists.[14] Since the pH values of the normal physiological environment, tumor extracellular space and some subcellular organelles (endosome and lysosome) are significantly different,[17,18] such micro-environmental differences allow for delivery of drug-loaded nanohydrogels to specific sites in tumor
60 tissues and controlled release of drugs. Due to most tumor tissue exhibit a significantly lower pH compared to the normal tissues, the nanohydrogels containing ionizable functional groups, such as carboxylic and/or amino groups, exhibit large volume changes as the media pH values change through electrostatic interaction, and
65 achieve pH sensitivity to bring about efficient drug loading and release. For another, redox potential difference between the extracellular and intracellular spaces also provides an opportunity for stimuli responsive drug delivery. The glutathione/disulfide glutathione (GSH/GSSG) redox couple is abundant in cells, and
70 the GSH concentration inside cells is hundreds times higher than that of the extracellular compartments.[19,20] Thereby many studies have been focused on introducing disulfide crosslinker (*e.g.* N,N-bis(acryloyl)cystamine) to endow the nanoparticles with

biodegradability in drug delivery systems.[21,22] In the last few years, researches based on pH/reduction dual-stimuli responsive nanohydrogels have already attracted considerable attentions due to their various advantages mentioned above.[23-26]

Many approaches have been designed and developed for the preparation of nanohydrogels recently, as follows: (1) physical self-assembly of polymers; (2) heterogeneous polymerization of monomers; (3) chemical cross-linking of preformed polymers; (4) template-assisted nanofabrication.[4,13] All things considered, physical self-assembly of preformed polymers combining with chemical cross-linking technique is a feasible method to synthesis stable and clean nanohydrogels.[27,28] Among the various combined preparation methods, distillation precipitation polymerization is a novel and favorable technique to prepare monodisperse and stimuli-responsive nanohydrogels based on monomer methacrylic acid (MAA). Bai et al synthesized the uncrosslinked poly(methacrylic acid) (PMAA) nanohydrogels through distillation precipitation polymerization and discussed the growth mechanism and the effect of monomer concentration on the reaction.[29] Huang et al synthesized the crosslinked PMAA nanohydrogels in terms of this method with ethyleneglycol dimethacrylate (EGDMA) as crosslinker.[30] Pan et al prepared the biodegradable chemically crosslinked multi-environmental stimuli responsive PMAA nanohydrogels via distillation precipitation polymerization and investigated their drug delivery performance under a series of environmental stimuli.[23]

In order to enhance the performance of the PMAA nanohydrogels as drug carriers, introducing poly(ethylene glycol) (PEG) appears to be very critical and necessary. It is known to all that PEG is water soluble, nontoxic, and possesses a variety of properties for biomedical and biotechnical applications.[31] It shows excellent biocompatibility, such as prolonged residence lifetime in human body and enhanced EPR effects in tumor site.[32,33] And furthermore, it resists protein adhesion and biological attack, suppresses platelet adhesion and doesn't express antigenic activity.[34-37] PEGylation usually utilizes PEG-based macromonomers to accomplish the surface modification of polymers. Stöver et al prepared the crosslinked poly[methacrylic acid-*co*-poly(ethylene glycol methyl ether methacrylate)] [P(MAA-*co*-PEGMA)] microspheres with average diameter of 0.9-1.5 μm by distillation precipitation polymerization with ethylene dimethacrylate (EDMA) as crosslinker. They only studied the impacts of various factors on the morphology of the microspheres.[38] After that, Huang et al prepared the poly{[poly(ethylene glycol)methyl ether acrylate]-*co*-(acrylic acid)}[P(PEGMA-*co*-AA)] microspheres with EGDMA as crosslinker by distillation precipitation polymerization.[39] Peppas et al. synthesized poly[methacrylic acid-*co*-poly(ethylene glycol) methyl ether methacrylate] [P(MAA-*co*-PEGMA)] hollow microspheres, using N,N′-methylenebisacrylamide (MBAAm) as crosslinker via distillation precipitation polymerization.[40]

Herein, a novel kind of PEGylated dual-stimuli responsive biodegradable poly[methacrylic acid-co-poly(ethylene glycol) methyl ether methacrylate-co-N,N-bis(acryloyl)cystamine] (PMPB) nanohydrogels was designed as a controlled drug delivery system via distillation precipitation polymerization (Scheme 1). The introduction of PEG moieties has several potential advantages, such as prolonged residence in blood circulation, decreased degradation by metabolic enzymes and reduced protein immunogenicity. Most importantly, the disulfide crosslinking bonds based on N,N-bis(acryloyl)cystamine (BACy) could be cleaved by glutathione (GSH) which distributed widely in cells. This property endows the nanohydrogels with reduction-triggered disintegration, biodegradability and improved drug delivery performance. The monodisperse PMPB nanohydrogels could acutely respond to the change of pH and redox potential stimuli independently between the tumor and normal tissues, which provides the possibility for the targeted delivery of drugs to specific tumor sites.
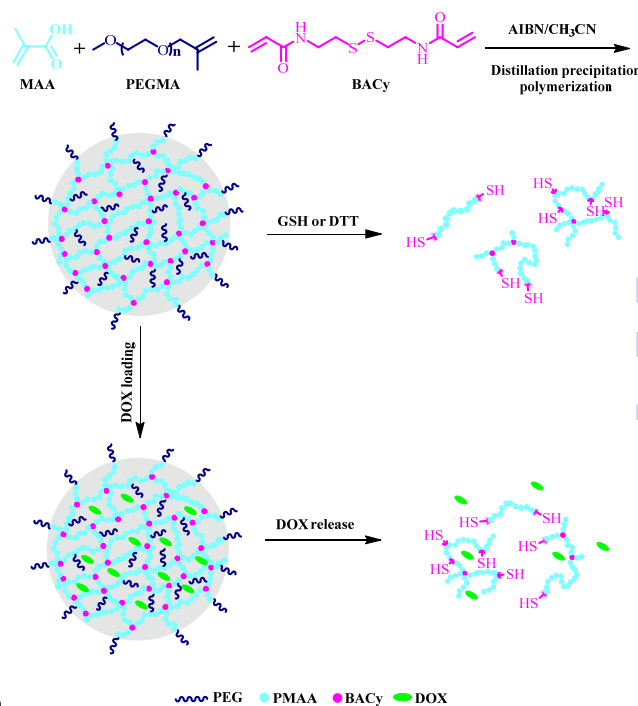
Scheme 1 Schematic illustration of the preparation of the PMPB nanohydrogels, the reduction-triggered disintegration of the nanohydrogels, and their controlled drug release behaviour.

2. Experimental Section

2.1. Materials and reagents

Poly(ethylene glycol) methyl ether methacrylate (PEGMA) (Mn=475) was bought from Aldrich and purified by alkaline alumina column chromatography**.**

Methacrylic acid (MAA) was obtained from Tianjin Chemical Reagent II Co. and distilled under reduced pressure prior to use. 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Tianjin Chemical Co. Ltd. and recrystallized from methanol. Acryloyl chloride was used as recieved from Aihua Chemical Reagent Company. Cystamine dihydrochloride was got from Fluorochem Ltd. DOX, in the form of a hydrochloride salt, was received from Beijing Huafeng United Technology Co. Ltd. Dithiothreitol (DTT) was available from Sun Chemical Technology (Shanghai) Co. Ltd. Glutathione (GSH) was provided by Tianjin Heowns Biochemical Technology Co. Ltd. Analytical grade acetonitrile was obtained from Tianjin Chemical Reagent II Co., dried over calcium hydride, and purified by distillation before utilization. All the other reagents were of analytical grade and used without

any further purification. Double distilled water was used throughout.

2.2. Preparation of PMPB nanohydrogels

The disulfide crosslinker BACy was synthesized according to the procedure reported previously.[41]

The monodisperse PMPB nanohydrogels were synthesized via the distillation precipitation copolymerization of MAA and PEGMA with AIBN and BACy as initiator and crosslinker, respectively. A typical procedure is as following: MAA (0.4050 g, 3.485 mmol), PEGMA (0.1215 g, 0.256 mmol) and BACy (131.6 mg, 0.502 mmol) were dissolved in acetonitrile (40 mL) in a dried 50 mL two-necked flask under ultrasonic bathing for 5 min. AIBN (10.5 mg, 0.064 mmol) was added into the flask after ultrasonic vibration. The flask was then immersed in a heating mantle equipped with a fractionating column, Liebig condenser and a receiver. The reaction mixture was heated to boiling within 30 min. The initially homogeneous reaction solution glimmered blue light and then became a milky white dispersion after boiling for 10 min. The reaction was ended after 20 mL of acetonitrile was distilled off within 2 h. Then the resultant PMPB nanohydrogels were separated by repeating centrifugation (12,000 rpm for 10 min), decantation, and re-suspension in acetonitrile with ultrasonic bathing for three times.

For comparison, the PMPB nanohydrogels with different crosslinking degrees were prepared by altering the feeding ratio of the crosslinker BACy in the copolymerization.

2.3. DOX-loaing

6 mg of dry PMPB nanohydrogels were added into 3.6 mL of 1.0 mg/mL DOX aqueous solution, and then the pH value of the mixture was adjusted to 7.4 with NaOH solution. After that, the mixture was stirred vigorously for 48 h in the dark at room temperature. After the DOX-loaded PMPB nanohydrogels were separated by centrifugation, the DOX concentration in the supernatant solution was analyzed by an UV−vis spectrophotometer at 480 nm. The mass of DOX loaded into the PMPB nanohydrogels was calculated by subtracting the mass of DOX in the supernatant from the mass of the drug in the original solution before drug loading. The drug loading capacity (DLC) and the drug encapsulation efficiency (DEE) were calculated by the formulas as shown below:

$$DLC \ (wt\%) = (Mass \ of \ DOX \ in \ nanohydrogels/Mass \ of \ nanohydrogels) \times 100\%$$

$$DEE \ (wt\%) = (Mass \ of \ DOX \ in \ nanohydrogels/Mass \ of \ feeding \ DOX) \times 100\%$$

2.4. *In vitro* release

The *in vitro* release behavior of the DOX-loaded nanohydrogels with different crosslinking degrees was investigated under different pH environments with or without GSH. For the release of DOX, the DOX-loaded nanohydrogels were dispersed in 10 mL of phosphate-buffered solutions (PBS) with different pH values (pH 5.0, 6.5, or 7.4) and transferred into dialysis bags with a molecular weight cutoff of 14 000. The drug release was assumed to start as soon as the dialysis bags were submerged into 130 mL of PBS at 37 °C. At given time intervals, 5 mL of aliquot was taken out to measure the DOX concentration in the dialysate with UV−vis spectrophotometer. Besides, 5 mL of corresponding

fresh PBS solution was added after each sampling to ensure the total volume of the buffer solution constant. The cumulative release of DOX from the DOX-loaded nanohydrogels was calculated by the following equation:

Cumulative release (%) = (mass of DOX in dialysate and total withdrawn solution/mass of DOX in nanohydrogels) × 100%

2.5. Cell toxicity assays

An MTT assay was performed to evaluate the cytocompatibility of the PMPB nanohydrogels with HepG2 cells. DOX, a clinical anti-cancer drug, was chosen as a model drug. Cells were seeded in 96-well plates at densities of 1×10^4 cells per well and incubated for 24 h, and then the pure nanohydrogels or the DOX-loaded nanohydrogels of given concentration were added to the cells. After the cells were incubated for another 24 h, the culture medium was removed and the cells were continued to incubate in a certain amount of MTT reagent for 2 h. Then the cell bound dye was dissolved with 100 μL of DMSO in each well. The absorbance of each well was recorded on a microplate reader at a certain wavelength. The data were presented as mean values of six measurements.

2.6. Characterization

The morphology of the PMPB nanohydrogels was characterized on a JEM-1200 EX/S transmission electron microscope (TEM) (JEOL, Tokyo, Japan). The samples were dispersed in water with the aid of ultrasonication for 1 h and then deposited on a copper grid covered with a perforated carbon film.

The FT-IR and UV-vis spectra of the nanohydrogels were assessed using a Nicolet 8210 Fourier transform infrared spectrometer (Nicolet Instrument Inn) and a Lambda 35 UV−vis spectrometer, respectively.

The mean hydrodynamic diameter and size distribution of the nanohydrogels were measured by a dynamic light scattering system (BI-200SM, Brookhaven Instruments) using 135 mW intense laser excitation at 514.5 nm at a detection angle of 90° and room temperature. The pH values of the dispersed solutions were adjusted with HCl or NaOH solution.

3. Results and Discussion

3.1. Preparation of PMPB nanohydrogels

Scheme 1 illustrates the preparation of the PMPB nanohydrogels, the reduction-triggered disassembly of the nanohydrogels, and their application for controlled drug release. Owing to the existence of disulfide bonds, the nanohydrogels could be disintegrated into water soluble linear polymers under a condition of adequate amount of GSH or DTT.

In order to study the effect of crosslinker concentration on the polymer nanoparticle size and reduction responsiveness, a series of experiments were designed, in which the concentration of crosslinker was varied from 13 to 20 wt% (relative to total monomer amount of MAA, PEGMA and BACy) and the amount of the initiator (AIBN) was maintained at 1.6 wt% corresponding to the total monomers. As the concentration of BACy increased from 13 to 20 wt%, the average particle size of the nanohydrogels increased from 100 to 175 nm (Fig. 1). This result can be explained by the compromise of two opposite effect factors, the rising number of the initial nuclei in the early

stage of polymerization and the increasing of the monomer conversion rate, and the latter is the dominant factor. Compared with the P(MAA-*co*-BACy) nanohydrogels,[23] the particle size increasing tendency the PMPB nanohydrogels in the present work was so pronounced, possibly due to the steric effect of the long-chain PEGMA and the resulting hydrogen bond interactions.
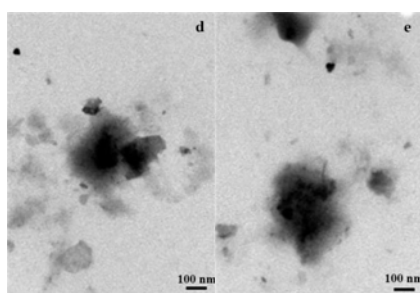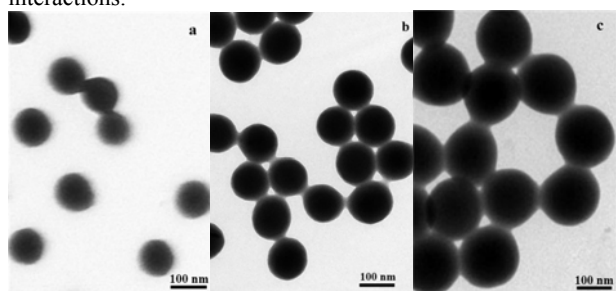

Fig. 1. TEM images of the nanohydrogels prepared with different feeding ratios of the crosslinker (a) 13 wt%, (b) 17 wt%, (c) 20 wt%; disintegration of the nanohydrogels (1.5mg) in presence of reductant (10 mg): (d) GSH or (e) DTT, and digital photographs of the nanohydrogels before and after disintegration with GSH or DTT.

Moreover, a series of experiments with different ratios of PEGMA and MAA was designed to investigate the effects of PEGMA feeding amounts on the nanohydrogels morphology. Increasing the PEGMA amounts from 24 to 63 wt% (relative to MAA), the particle size of the nanohydrogels decreased at first and then increased, and the particle size distribution gradually became wider. Based on the above experiments, the sample with 30 wt% or lower PEGMA was spherical in shape and uniform in size. Thus 30 wt% was selected as the experiment feeding ratio of PEGMA.

To observe the disintegration behavior of the nanohydrogel, the nanohydrogels (1.5 mg) and the reductant (10 mg) were stirred for 48 h at 6.0 mL pH 7.4 water. In the experiment, the milky emulsion rapidly became a clear solution within 1 h upon the addition of DTT, but the nanohydrogels were disintegrated slowly with the addition of GSH. The fragments after

disintegration can be rarely seen as in the TEM images, shown in Fig. 1d and Fig. 1e. Moreover, the nanohydrogels were dispersed in water at pH 7.4 without any reductant for comparison with their states in presence of reductant (GSH or DTT) at the same pH (bottom of Fig. 1). Under the reductive media, the disulfide linkages in the nanohydrogels were cleaved, and the resultant linear polymers were dissolved into water. Thus the dispersion of the nanohydrogels changed into transparent solutions. It also verified the decomposition phenomenon of the nanohydrogels in presence of GSH or DTT, as reported previously.[23,24] Furthermore, the stability of the nanohydrogels was investigated through DLS measurements. The distribution of the nanohydrogels in pH 7.4 PBS solution was uniform, and the hydrodynamic diameter of the nanohydrogels had a small increase as time went on and finally stabilized at around 430 nm after 30 h or more, as shown in Fig. S1. This result indicates that the nanohydrogels were stable under normal physiological media.

Table 1. The recipes and characterization data of PMPB nanohydrogels prepared with different crosslinker contents.

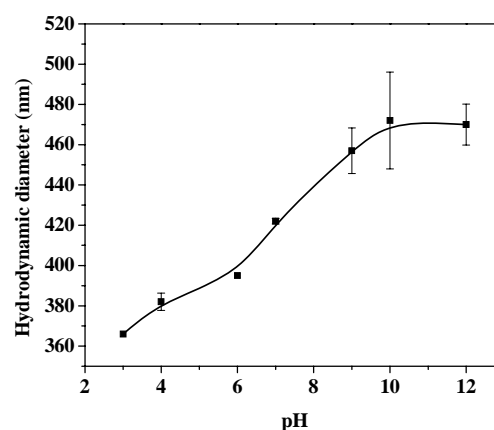| Samples	MAA mg	PEGMA mg	BACy mg	AIBN mg	D_{TEM} nm	D_{DLS} nm
PMPB-c-13	280	84.0	54.6	7.3	100	509
PMPB-c-17	260	78.4	68.0	6.8	121	473
PMPB-c-20	405	121.5	131.6	10.5	175	430

Fig. 2. pH dependence of the average hydrodynamic diameter of the PMPB nanohydrogels.

The sizes of the PMPB nanohydrogels prepared with different BACy feeding ratios in the range of 13-20 wt% were measured by TEM and DLS techniques, and summarized in Table 1. Their size distributions were also obtained by DLS measurements at pH 7.4. The nanohydrogels were monodisperse by TEM analysis as shown in Fig. 1 and this characteristic was further validated by DLS measurements (PDI < 0.05). The hydrodynamic diameters of the resultant PMPB nanohydrogels were obviously much bigger than those from the TEM observation. This suggested that the PMPB nanohydrogels were in highly swollen state and had good hydrophilism in aqueous solution. The regular pattern of nanohydrogels size with different crosslinking degrees, which was investigated by TEM and DLS respectively, were completely opposite. The PMPB nanohydrogels with the lowest crosslinking degree showed the smallest size in dried state (TEM) but the

biggest size in swollen state (DLS). This may be due to the great shrinking effect of the nanohydrogels with high crosslinking degree in aqueous solution.

Furthermore, the pH sensitivity of the nanohydrogels was also studied by DLS measurements. With the increase of the media pH value from 3 to 12, the hydrodynamic diameter of the nanohydrogels (PMPB-c-20) raised from 366 nm to 470 nm (Fig. 2), which confirmed that the nanohydrogels were pH-responsive. Under lower pH values, more hydrogen bonds were formed among PEG brushes and MAA units, resulting in desolvation and collapse of the networks. The electrostatic repulsion between the carboxylate anions became more and more significant as pH increased, and this may be the principal reason for the expansion of the nanohydrogels in the high pH media.[42] Unusually, there was an unstable state at pH 3 or lower pH, which may be due to the neutralization.[43]

and PMPB nanohydrogels with different crosslinking degrees are given in Fig. 3. The strongest absorbance peak at 1710 cm^{-1} is assigned to the stretching vibration of the carboxylic acid groups. The absorbance bands at 1650 and 1545 cm^{-1}, the stretching vibration of carbonyl from the amide bond (amide I band) and in-plane bending vibration of N-H (amide II band) respectively revealed the existence of amide which was introduced from BACy (Fig. 3a). The absorbance peaks at 2990 and 2930 cm^{-1} are corresponded to the asymmetrical stretching vibrations of –CH$_3$ and –CH$_2$, respectively. Additionally, the absorbance at 2930 cm^{-1} is strengthened, resulting from the introduction of PEGMA (Fig. 3a). The characteristic absorbance peak for the –CH$_2$CH$_2$O– units introduced by PEGMA was observed at 1110 cm^{-1}. As shown in Fig. 3b, the amide I and II absorbance bands were enhanced with increasing the feeding ratio of the crosslinker BACy.

3.2. Drug-loading and controlled release performance

DOX, a wide-spectrum antitumor drug, was served as a model drug for the investigation of the potential application of the PEGylated pH/reduction dual stimuli-responsive biodegradable nanohydrogels for drug delivery. For the above experimental purpose, we studied the drug loading and controlled release behavior of the PMPB nanohydrogels which acted as drug delivery vehicles as illustrated in Scheme 1.

To investigate the DOX loading performance of the nanohydrogels with different crosslinking degrees, a set of experiments were designed, with the feeding ratio of DOX and the carriers of 60% or 120%. The drug encapsulation efficiency (DEE) of the three samples with different crosslinking degrees was higher than 95% under the two cases and the drug loading capacity (DLC) was near to the feeding ratios, respectively. It can be seen that the DEE of such kind of nanohydrogels was considerable and outperformed most other similar nanoparticles.[23,25] This may be resulted from the tremendous electrostatic interaction between the carboxyl groups of MAA units and the amine group of DOX. Besides, the molecular size of DOX is large enough for the nanohydrogels with network structure to entrap it.

Recently, a high drug-loading capacity of 208.0% was reported by Yang's group with PMAA-based folic acid-conjugated pH/temperature/redox multi-stimuli responsive polymer microspheres.[44] However, the loaded DOX in the PMPB nanohydrogels with higher DLC could be partially washed off with pH 7.4 PBS in our experiments, although the DLC increased with increasing the DOX feeding ratio. Furthermore, the DLC and DEE values from media with pH values of 6.5 and 5.0 were slightly lower than those from the pH 7.4 media, due to the acidic solubility of DOX. It indicated that the electrostatic interaction between the drug and carriers was not so stable to avoid the leakage of drug from the carriers during blood circulation. Thus the drug-loading was conducted with the feeding ratio of DOX and the PMPB nanohydrogels of 60% in pH 7.4 PBS in the following experiments, and washing with 5 mL pH 7.4 PBS for two times after drug-loading.

The pH responsive controlled release performance of the three PMPB nanohydrogels with different crosslinking degrees was compared without any reducing agent. Under pH 5.0 and 7.4, the release behavior of the sample PMPB-c-13 showed minor distinction. Since the expansion ratio of the sample PMPB-c-13
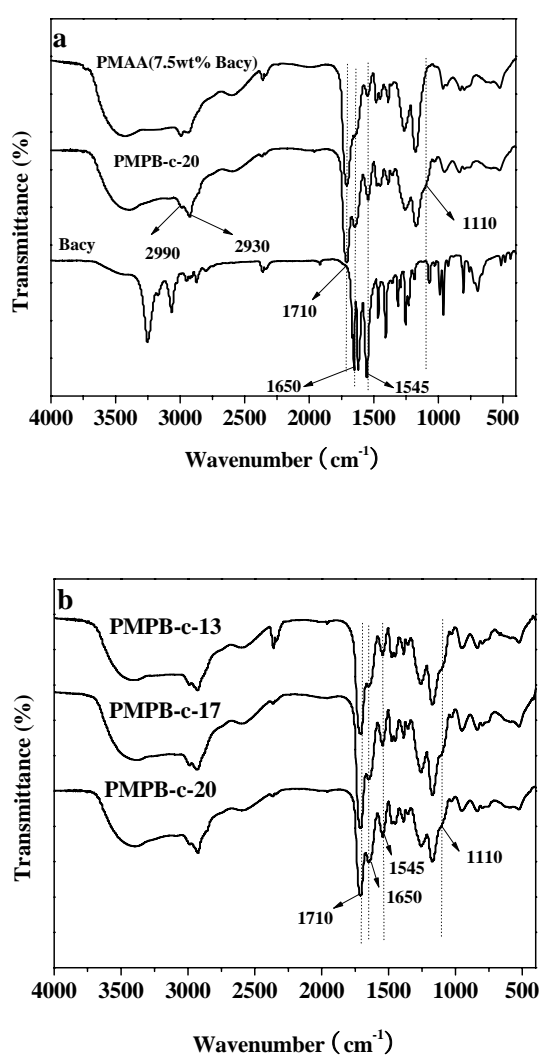
Fig. 3. FTIR spectra of (a) BACy, BACy-crosslinked PMAA and PMPB-c-20 nanohydrogels, (b) PMPB nanohydrogels with different crosslinking degrees.

FT-IR spectra of the P(MAA-*co*-BACy) nanohydrogels, BACy

was the largest among the three samples in water, the interaction between DOX and the nanohydrogels was seriously impaired and this allowed DOX to be released more easily. As the diameter of the nanohydrogels increased, the pH sensitivity became more and
5 more obvious, as shown in Fig. 4a. The sample PMPB-c-20 (1.6 wt% AIBN initiator concentration, 20 wt% BACy crosslinker concentration and 30 wt% PEGMA relative to the MAA monomer) exhibited the highest cumulative release of 42% at pH 5.0 and the lowest one of 14% at pH 7.4, indicating the best pH-
10 responsive controlled release performance. Thus the sample PMPB-c-20 was chosen to study the controlled drug release behavior of the nanohydrogels on the basis of the foregoing consideration. In this part of the drug release experiment, GSH has been selected as reducing reagent for a couple of reasons
15 listed below. The difference between DTT and GSH in chemical structure leaded to some discrepancies during drug releasing. This is principally because that the amide and carboxyl group of GSH could be an effective substitute for the amide group of DOX and interact with the carboxyl group of the nanohydrogels via
20 electrostatic interaction.[23] In addition, the glutathione/disulfide-glutathione (GSH/GSSG) redox couple is occurring widely in cells and DTT is just one kind of synthetic compounds and undetectable in living tissues.[19]
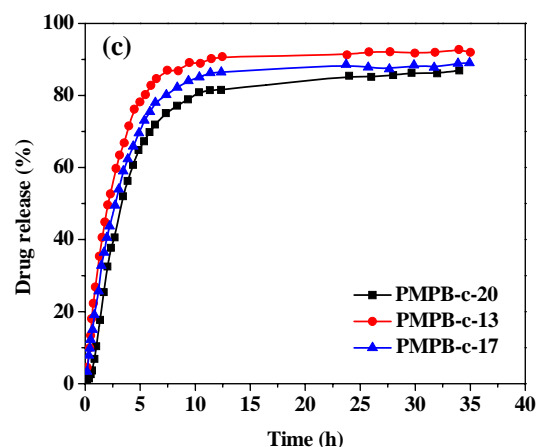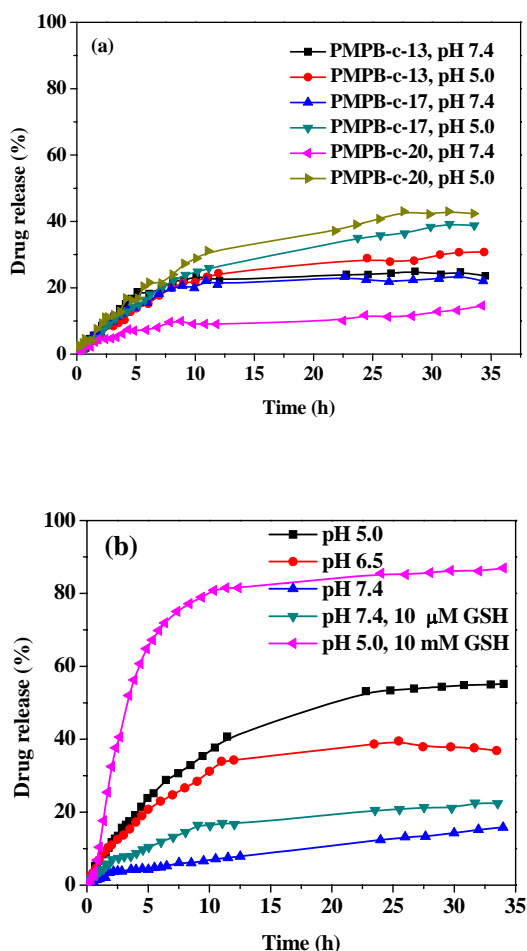

Fig. 4. The pH and reduction dual-stimuli-responsive DOX release profiles of (a) the DOX-loaded PMPB nanohydrogels under pH 5.0 or 7.4 without GSH, (b) the DOX-loaded PMPB-c-
30 20 nanohydrogels under pH 5.0, 6.5 or 7.4 and different contents of GSH, (c) the DOX-loaded PMPB nanohydrogels with different crosslinking degrees under conditions of pH 5.0 and 10M GSH.

The pH and reduction dependent release performances of the nanohydrogels were assessed at pH 5.0, 6.5 or 7.4 with different
35 contents of GSH and the temperature was maintained at 37 °C throughout the experiment. The drug release profiles of the PMPB-c-20 nanohydrogels under certain experimental conditions were shown in Fig. 4b. The DOX released tardily from the nanohydrogels in pH 7.4 PBS (mimicking the blood media)
40 without any reducing matter (GSH), the drug release curve flattened after released for 30 h, and the accumulative release reached 15 %. Moreover, the percentage of total release amount had a minor increase in the presence of 10 μM GSH (correspond to the extracellular concentration of GSH) at pH 7.4 and rose to
45 about 20 % within 34 h. This suggested that the nanohydrogels had good stability with lightly premature release under a physiological pH condition. Under conditions of changing the pH values of the buffer media, the DOX release amount approximated to 13 %, 40 % and 55 % within 24 h at pH 7.4, 6.5
50 and 5.0 respectively. As seen in Fig. 4b, the lower pH was adopted, the greater the DOX cumulative release became. At lower pH, the amine group of DOX turned into quaternary ammonium via protonation, in the same instant the number of hydrogen ions increased,[45] and DOX was more soluble in acidic
55 solutions.[46] This resulted in a weakening of the electrostatic interaction between DOX and the nanohydrogels, meanwhile the DOX molecules could get rid of the bounds and dissolve into the dialysate.

The reduction-induced drug release behavior of the
60 nanohydrogels had also been studied at relatively low pH (pH 5.0). The DOX was given off reasonably faster at a pH of 5.0 and in presence of 10 mM reductant GSH, as compared to the DOX release performance under a condition of pH 5.0 without GSH. Furthermore, the DOX release amount was running at about 86%
65 with 10 mM GSH and almost twice as high as the value at the same pH and period without any GSH. The rate of the DOX release speeded up in presence of GSH at a low pH, largely

because of the cleavage of the disulfide bonds and the decomposition of the entire nanohydrogels, together with the contribution from the weakening of the electrostatic interaction and the charge exchange. The charge exchange aforesaid was
5 operated and the protonated amide group of GSH could replace the DOX which interacted with the nanohydrogels through the electrostatic interaction.[23] With the common-effect of pH and GSH, these drug release experimental results ensured the quick DOX release rate in cancer cells.
10 Beyond the investigation of the pH and reduction stimuli-responsive characters of the nanohydrogels, the drug release behavior of the nanohydrogels with different crosslinking degrees were investigated in presence of 10 mM GSH under pH 5.0. As Fig. 4c suggests, the cumulative DOX release became a little bit
15 higher with lower crosslinking degree of the nanohydrogels. Even though the nanohydrogels with relatively higher crosslinking degree could partly degrade into linear polymer chains, there were still a few relatively large fragments of the ruptured nanohydrogels which remained the strong electrostatic interaction
20 with DOX molecules.

Table 2. The releasing parameters fitted from the Higuchi and Korsmeyer-Peppas models.

Models	Parameters	pH 5.0 with 10 nM GSH	pH 5.0	pH 6.5	pH 7.4	pH 7.4 with 10 μM GSH
Higuchi	k	2.143	1.411	7.226	0.3570	0.5348
	R^2	0.7413	0.9756	0.8984	0.9846	0.9555
Korsmeyer-Peppas	n	1.536	0.7265	0.5989	0.6800	0.5676
	R^2	0.9820	0.9488	0.9489	0.9238	0.9714

25 The release mechanism of the drug delivery was analyzed with the Higuchi kinetics[47] and the Korsmeyer–Peppas semiempirical equation[48] (Fig. S2, SI) and the corresponding model parameters were summarized in Table 2. In the equations, k denotes the drug release rate constant and n denotes the diffusional exponent, and
30 they are obtained from the linear regression analysis. R^2, as the coefficient of determination, represents the interpretative extent of the variable x to y. The intercept of the linear equation (Higuchi equation) could account for the degree of initial burst release, and this rule pertained to the diffusion-controlled release
35 circumstance. The plots at pH 5.0 and in presence of 10 mM GSH based on the Higuchi model of the nanohydrogels resulted in an undesirable straight line with a poor R^2 value of 0.7413, a slope of 2.143 and a positive intercept on the y axis. Leaving aside the fact that the slope of the line was more than 1, it demonstrated
40 that the drug release process exhibited initial burst release phenomenon and anomalous release behavior (non-Fickian release).[49,50] For the Korsmeyer–Peppas model, the exponent was used to determine the model parameter for those cumulative drug release < 60%. The n value of the Korsmeyer–Peppas plot under
45 above conditions was 1.536 with an R^2 of 0.982. This result further validated that the release process was of the Super Case II transport type, controlled primarily by the swelling of the drug-loaded nanohydrogels, and this pattern regularly exhibited a long period of increased swelling at the relaxing front.[51,52] The release
50 performance and the corresponding model parameters were likely to be similar at pH 5.0 and pH 6.5, and the relaxational swelling

was a dominant controlling factor at pH 5.0 during drug-releasing. For a change, the plot for the Higuchi equation at pH 7.4 with or without GSH had a decent linear relationship between the square
55 root of time and the cumulative drug release. The slope of the line was less than 1, it indicated that the release mechanism was the non-Fickian diffusion,[49] which was also confirmed by the Korsmeyer–Peppas equation. According to the kinetic analytic results, the Korsmeyer–Peppas model was more applicable to the
60 drug release system of the pH/reduction dual stimuli-responsive biodegradable nanohydrogels; the release process was more likely to be controlled by more than one force and involved the superposition of various mechanisms.
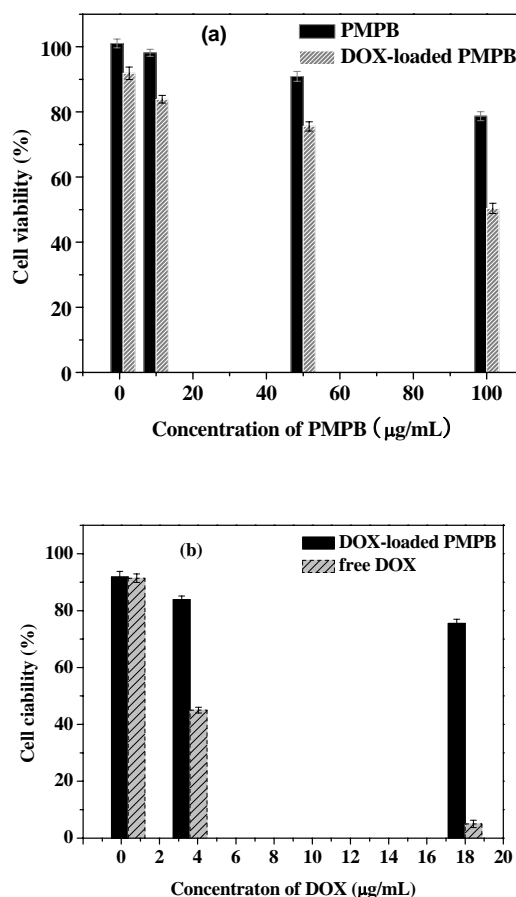

65 **Fig. 5.** Cell viability assay in HepG2 cells of (a) PMPB and DOX-loaded PMPB nanohydrogels with DLC of 60%, and (b) DOX-loaded PMPB nanohydrogels and free DOX by reference to the concentration of DOX. Cell viability (%) was determined by
70 the MTT assay.

3.3. Cell toxicity assays

Concerning the further application of the PMPB nanohydrogels in biomedical fields, the cytocompatibility of the nanohydrogels was evaluated on HepG2 cells through a classic MTT cell assay. The
75 result of the cell toxicity assays is shown in Fig. 5a and normalized against a blank value which the cell was cultured without the nanohydrogels. The nanohydrogels were relatively non-toxic (with the cell viability of 90.9% - 101.0%) up to a concentration of 50 μg/mL. As the nanohydrogels concentration
80 continued increasing to 100 μg/mL, the cell viability turned into

78.7%. Comparatively, the DOX-loaded nanohydrogels showed a bit higher toxicity below 50 μg/mL and exhibited strong toxicity against HepG2 cells under a concentration of 100 μg/mL. To estimate the potency of the nanohydrogels as appropriate drug carriers, a comparison of the drug-loaded nanohydrogels and free DOX was investigated by reference to the concentration of DOX at pH 7.4. The cytocompatibility of the DOX-loaded nanohydrogels was fairly better than that of free DOX as shown in Fig. 5b, and exhibited much lower cytotoxicity at high concentration of DOX dosage. This could be interpreted as the unmet needs of GSH and relative high pH condition. In the case, the DOX-loaded nanohydrogels could just kill a small number of HepG2 cells with higher GSH concentration. It is generally known that GSH exists with relatively low concentration in normal cells. So it is expected that the drug-loaded nanohydrogels may do less harm to normal cells with relatively low GSH concentration, due to the low DOX release ratio. These results also suggested that such kind of nanohydrogels had low toxicity and was capable of playing the role of a drug carrier.

In a recent work, Wang's group have prepared redox/pH dual stimuli-responsive poly(methacrylic acid) (PMAA)-based nanohydrogels with similar dimension to the PMPB nanohydrogels in the present work, except for the PEG brushes. The authors revealed that the fast internalization of nanohydrogels and rapid release of DOX inside cells.[23] Another relative experiment result of Yang's group also got the similar conclusion and confirmed that most of the drug carriers could be transported into cells and rapid release of DOX.[44] Thereafter, it is expected that the PMPB nanohydrogels developed in the present work could also transport DOX into cells efficiently via endocytosis and kill the model cells effectively.

Conclusions

Biodegradable pH/reduction dual stimuli-sensitive PMPB nanohydrogels have been successfully synthesized via facile and clean distillation precipitation polymerization. The introduction of PEGMA could relatively decrease the cytotoxicity of the polymeric nanoparticles. The result of TEM showed that the nanohydroges were uniform sphere in shape and the size of them was all below 200 nm, which offered promise for their potential application as drug delivery carrier. The drug loaded in the biocompatible PMPB nanohydrogels, with little leakage during blood circulation, could be released rapidly upon the acidic media with GSH, mimicking the tumor microenvironment. Meantime, the biodegradability of this type of nanohydrogels was remarkable in presence of adequate GSH under lower pH, the nanohydrogels destroyed and degraded into short polymeric chains and small fragments, and made them excrete from the body easier in shorter period. The independent pH and reduction sensitivity of the DOX-loaded nanohydrogels ensured a particular controlled drug release performance.

Acknowledgments

Notes and references

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