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Reduction-sensitive N, N′-Bis(acryloyl) cystinamide-polymerized Nanohydrogel as a Potential Nanocarrier for Paclitaxel Delivery

Linna Yu[a,b,c], Lingping Kong[a,b,c], Junpeng Xie[a,b,c], Wei Wang[d], Chen Chang[a,b,c], Hongli Che[a,b,c] and Mingzhe Liu[a,b,c]

[a]Key Laboratory of Structure-Based Drugs Design & Discovery (Shenyang Pharmaceutical University) of Ministry of Education, Shenyang, P. R. China; [b]Department of Organic Chemistry, School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang, P. R. China; [c]Benxi Institute of Pharmaceutical Research, Shenyang Pharmaceutical University, Benxi, P. R. China; [d]Precedo Pharmaceuticals Co. Ltd., Hefei, P. R. China

ABSTRACT

Novel monomer, *N, N′*-bis(acryloyl) cystinamide (NBACA), was designed and synthesized with L-cystine as row material. By using this NBACA both as the monomer and crosslinker, reduction-sensitive nanohydrogel was prepared in ethanol via distillation–precipitation polymerization. The obtained nanohydrogel can provide a relatively hydrophobic environment and hydrogen-bonding sites inside the gel; therefore, it is suitable for loading hydrophobic drug. When paclitaxel that possess poor water-solubility was used as a model drug, the nanohydrogel represented a high drug-loading capacity, and dispersed well in aqueous solutions. Furthermore, the disulfide-group-containing nanohydrogel exhibited good reduction-sensitive drug-release behavior. The nanohydrogel biodegraded rapidly in a reducing environment, and released approximately 80% of the PTX within 24 h. Cytotoxicity assays showed that the PTX-loaded nanohydrogel exhibited high cytotoxicity against MCF-7 breast cancer cells, while blank nanohydrogels displayed a negligible cytotoxicity.

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Paclitaxel; nanohydrogel; drug delivery; redox stimuli; biomaterials

1. Introduction

Hydrogels have been supposed outstanding candidates as one of the promising carriers in biomedical area due to their unique structure and properties. As cross-linked polymers, the hydrogels can absorb, swell and retain considerable amounts of aqueous fluid through its three–dimensional network [1–3]. In particular, nanohydrogels with a hydrodynamic diameter in the submicron range exhibit high guest–molecular loading capacity and excellent biocompatibility with low side effects, rendering them adaptive for various applications, such as bioimaging and drug delivery systems [4–7]. Moreover, the nanohydrogels can be designed rationally to respond various external stimuli including pH, temperature, light, enzymatic action, and redox gradients, thus enabling environmentally sensitive drug release [8–12].

Among these stimuli-responsive nanohydrogels, reduction-sensitive nanohydrogels have received increasing attention, and widely used in targeted cancer therapy due to the difference in redox potential between normal cells and cancer cells [13,14]. In general, disulfide bonds are employed to achieve a reduction-triggered drug release, since it can be biodegraded responding to reduction signal molecule such as glutathione [15,16]. So far, several reduction-sensitive nanohydrogels have been reported, in which the monomer or backbone structure was derived from hydrophilic component including methacrylic acid [17], dextrin [18], chitosan [19], alginate [20], hyaluronic acid [21], sorbitan [22], etc. We previously reported *N, N′*-dimethacryloylcystine crosslinked nanohydrogels [23]. Therefore, these nanohydrogels are preferred to carrying hydrophilic drug such as cationic doxorubicin (DOX). However, most of the drugs are hydrophobic and poorly soluble in water. Thus, the introduction of hydrophobic segments at the nanohydrogels is a useful approach, although reduction-sensitive nanohydrogels carrying hydrophobic drugs reported previously are limited [24–27].

In this study, a novel *N, N′*-bis(acryloyl) cystinamide (NBACA), was designed and synthesized with L-cystine as row material. By using this NBACA both as the monomer and crosslinker, reduction-sensitive nanohydrogel was prepared for hydrophobic drug, paclitaxel (PTX). Although PTX is one of the most successful anti-cancer drugs [28], the water solubility is very poor (0.3 μg/mL) due to many hydrophobic group in the molecule [29,30]. The reduction-sensitive nanohydrogels developed in this study not only can create a relatively hydrophobic

CONTACT Mingzhe Liu mz_liu03@163.com Department of Organic Chemistry, School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, P. R. China

environment, but also can provide hydrogen-bonding sites for the PTX owing to its electroneutral amide group and polyethylene backbone inside the gel. Besides, a facile one-step synthesis of nanohydrogels in ethanol was achieved by using distillation–precipitation polymerization (D-P polymerization). Comparing with other methods, the D-P polymerization does not need additional surfactant, excipients or stabilizers and the process is easy to scale up [31]. Furthermore, the disulfide group inside the nanohydrogels can degrade in reducing environments; hence, a redox-triggered delivery of PTX is also expected.

2. Experimental

2.1. Materials

L-cystine was purchased from Dipper Biotechnology Co., Ltd. (Shanghai, China). Acrylic chloride, aqueous ammonia (28 wt%), paclitaxel (PTX), glutathione (GSH), 2,2-Azobisisobutyronitrile (AIBN), and dithiothreitol (DTT) were purchased from Sun Chemical Technology (Shanghai, China) Co., Ltd. The AIBN was used after recrystallization in ethanol. Methanol, tetrahydrofuran (THF) and diethyl ether were purchased from Hengxing Chemical Preparation Co., Ltd (Tianjin, China). Triethylamine and thionyl chloride were purchased from Damao Chemical Reagent Factory (Tianjin, China). Methanol and THF were dried with CaH_2 and distillated before use.

2.1.1. Measurements

The Fourier transforms infrared (FTIR) spectra of NBACA monomer, NBACA nanohydrogel and drug-loaded NBACA nanohydrogel (PTX@nanohydrogels) were performed on the Nicolet IS10 spectrometer. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were obtained with a Bruker Advance spectrometer. The morphology of the nanohydrogels was characterized by using scanning electron microscopy (SEM; Hitachi S-3400 N). The hydrodynamic diameter and size distribution of the nanohydrogels were measured by dynamic laser light scattering (DLS; Malvern, Zetasizer Nano ZS90) at a concentration of 1 mg/mL in ultrapure water. Transmittance of the nanohydrogels suspension and the amount of the drug in ethanol were measured by an UV-visible spectrophotometer (SHIMADZU, UV-2700).

2.2. Synthesis of N, N′-bis(acryloyl) cystinamide monomer

The *N, N′*- bis(acryloyl) cystinamide (NBACA) was synthesized as follows: Firstly, L-cystine (10.0 g, 41.6 mmol) and thionyl chloride (8 mL, 110.3 mmol) were slowly added into anhydrous methanol (150 mL, 3.7 mol) at 0°C, followed by returning to room temperature. The solution was kept refluxing for 6 h at 60°C, and then evaporated to remove excess methanol. The resulting crude product was washed and reprecipitated from methanol/diethyl ether (1/8, *v/v*) to give L-cystine dimethyl ester dihydrochloride as white solid (yield: 92.8%). Next, dry L-cystine dimethyl ester dihydrochloride (6.0 g, 17.6 mmol) was dissolved in anhydrous THF (50 mL) followed by the addition of triethylamine (8 mL, 57.6 mmol). Subsequently, acryloyl chloride (5 mL, 61.8 mmol) was added dropwise to the solution at −10°C. The reaction was continued with stirring for 6 h at room temperature. After the evaporation, the resulting precipitate was dissolved in CH_2Cl_2 (100 mL) and thoroughly washed with distilled water (30 mL), 5% $NaHCO_3$ (30 mL) and saturated NaCl (30 mL) for three times, respectively. The organic phase was dried with anhydrous Na_2SO_4, and then filtered followed by the evaporation. The resulting crude product was recrystallized from acetone/petroleum ether to afford *N, N′*- bis(acryloyl) cystine dimethyl ester as a white solid (yield: 38.0%). Finally, *N, N′*-bis (acryloyl) cystine dimethyl ester (374 mg, 0.99 mmol) and 28% aqueous ammonia (3 mL) were mixed and reacted with stirring for 6 h at 0°C. The reaction solution was evaporated and then dried in vacuum at 40°C. The crude product was dissolved in methanol followed by the filtration. After evaporation of the filtrate, the residue was dried to afford the NBACA monomer as a white solid (yield: 67.9%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.42 (d, J = 8.3 Hz, 1H, -NH-C-), 7.55 (s, 1H, -C-NH2), 7.19 (s, 1H, -C-NH2), 6.31 (dd, J = 17.1, 10.2 Hz, 1H, -CH = C-), 6.10 (dd, J = 17.1, 2.1 Hz, 1H, -C = CH2), 5.61 (dd, J = 10.2, 2.1 Hz, 1H, -C = CH2), 4.59 (td, J = 8.7, 5.1 Hz, 1H, -CHCO-), 3.14 (dd, J = 13.4, 5.0 Hz, 1H, -SSCH2-), 2.90 (dd, J = 13.4, 9.0 Hz, 1H, -SSCH2-). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.0, 164.9, 131.8, 126.1, 52.2, 41.2.

2.3. Synthesis of NBACA nanohydrogels

NBACA nanohydrogels were synthesized via distillation–precipitation polymerization method. The typical synthesis procedure was as follows: *N, N′*-bis(acryloyl) cystinamide (75 mg, 0.22 mmol) and AIBN (11 mg, 0.067 mmol) were dissolved in anhydrous ethanol (40 mL) with ultrasonic agitation for 10 min. The reaction mixture was heated to 83°C (the temperature of water bath) and kept for 30 min. The reaction was stopped until about 18 mL of ethanol was distilled from the reaction mixture. After repeating centrifugation (8000 rpm, 10 min) and redispersion (ethanol, 10 mL) cycle for three times, the

precipitates were lyophilized to afford nanohydrogels as white powders.

2.4. Reductive degradation of nanohydrogels

The reductive degradation of NBACA nanohydrogels was monitored by measuring the transmittance change of the nanohydrogel suspension. Lyophilized nanohydrogels (4 mg) were dispersed in 10 mL of phosphate buffer solution (PBS, pH = 7.4) in the presence or absence of reductant (GSH or DTT, 10 mM) respectively. Subsequently, the mixture was placed in a shaking bed (37°C, 150 rpm). At defined time points, 80 μL of the solution was collected and the transmittance was measured by UV-visible spectrophotometer at 630 nm.

2.5. Drug loading assay

PTX was used to prepare drug-loaded nanohydrogels. Typically, 6 mg of PTX and 10 mg of lyophilized empty nanohydrogels were dispersed and ultrasonicated in 10 mL of ethanol for 10 min, and then continuously stirred for 24 h at room temperature. After centrifugating (8000 rpm, 10 min) and removing the supernatant, the resultant precipitate was then lyophilized to afford PTX-loaded NBACA nanohydrogels. To determine the drug-loading content in the nanohydrogels, 1.5 mg of lyophilized PTX-loaded NBACA nanohydrogels were dispersed in 14 mL of ethanol and then stirred for 24 h. Subsequently, the suspension was centrifuged (8000 rpm, 10 min), and the amount of PTX in the supernatant was determined by UV-visible spectrophotometer at 227 nm using a standard curve. The drug loading capacity (DLC) and drug encapsulation efficiency (EE) were calculated by following equations:

$$\mathrm{DLC}(\%)=\frac{\text{weight of PTX in nanohydrogels}}{\text{weight of PTX}-\text{loaded nanohydrogels}}\times 100\%$$

$$\mathrm{EE}(\%)=\frac{\text{weight of PTX in nanohydrogels}}{\text{initial weight of PTX}}\times 100\%$$

2.6. In vitro drug release

The PTX-release behavior was evaluated under different reducing conditions by dialysis method using a dialysis bag (MWCO = 3500) [32]. Typically, 10 mg PTX-loaded nanohydrogels were dispersed in 10 mL PBS (pH = 7.4) buffer containing 1.2 mM of sodium dodecyl sulfate. The sample was then transferred into a dialysis bag followed by dialyzing against 150 mL of the PBS buffer with or without the presence of GSH or DTT (10 mM) and gently shaken (160 rpm) at 37°C. At a predetermined time, 2 mL of the solution was removed and the volume of reservoir was held constant by adding 2 mL fresh buffer medium. The sample solution was extracted with 10 mL CH_2Cl_2 for three times. The CH_2Cl_2 phase was collected, and the solvent was evaporated under reduced pressure. Subsequently, the residue was redissolved with 1 mL ethanol, and the amount of the released PTX was measured three times by UV-visible spectrophotometer at 227 nm.

2.7. Cell culture

The MCF-7 cells were routinely cultured in Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified incubator with 5% CO_2.

2.8. Cytotoxicity assay

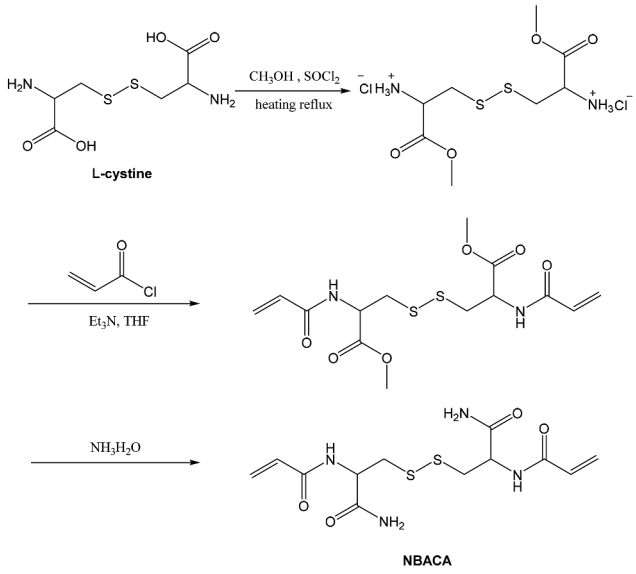
MCF-7 cancer cells were seeded into 96-well plates at a density of 3000 cells/well. After overnight incubation, the cells were incubated in the presence of different samples for 72 h at 37°C. Blank NBACA nanohydrogels and PTX-loaded nanohydrogels and free PTX were diluted with culture medium, respectively, to obtain various concentrations of samples. After incubation, cell viability was investigated by a standard MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assay.

3. Results and discussion

3.1. Synthesis and characterization of NBACA monomer

The *N, N′*-bis(acryloyl)cystinamide (NBACA) monomer was synthesized in three steps (Scheme 1). Firstly, L-cystine dimethyl ester dihydrochloride was prepared by the esterification of L-cystine and methanol in the presence of thionyl chloride. In the second step, *N, N′*-bis (acryloyl) cystine dimethyl ester was synthesized from the amidation reaction of acryloyl chloride with the L-cystine dimethyl ester dihydrochloride in the presence of triethylamine. Finally, NBACA was synthesized by ammonolysis of *N, N′*-bis(acryloyl) cystine dimethyl ester in 28% aqueous ammonia.

The chemical structure of NBACA monomer was confirmed by FTIR, ^{1}H NMR and ^{13}C NMR. As shown in Figure 1, the typical amide band (1656.4 cm^{-1}) was observed clearly. Furthermore, the FTIR spectra also showed peaks at 1625.8 cm^{-1}, 1311.5 cm^{-1}, 961.4 cm^{-1} and 803.9 cm^{-1} due to C = C stretching, bending vibration of C-H on the C = C, respectively. ^{1}H NMR spectrum of *N, N′*-bis



Scheme 1. Synthesis of *N, N'*- bis(acryloyl) cystinamide monomer.

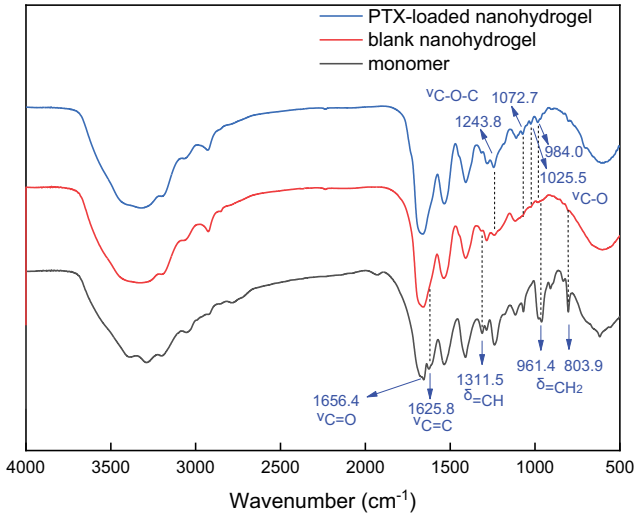


Figure 1. FTIR spectra of NBACA monomer (black), blank nanohydrogel (red) and PTX-loaded nanohydrogel (blue).

(acryloyl) cystinamide is shown in Figure 2. The signals of N-H protons in amide groups were observed at 8.4, 7.5 and 7.2 ppm, and the characteristic signals of C-H protons in the C = C bonds were observed at 6.3, 6.1 and 5.6 ppm. The signals of the C-H protons in methylene at 4.6 ppm and the methylene C-H protons at 3.1 and 2.9 ppm were also observed separately. The ^{13}C NMR spectrum of NBACA monomer showed six different signals corresponding to six nonequivalent carbons (Figure 3). The signals at 41.2 and 52.2 ppm are assigned to the carbon of methylene and methine, respectively. In addition, signals at 126.1 and 131.8 ppm are due to the two carbons of C = C bonds, and the carbons from amide groups showed the signals at 164.9 and 172.0 ppm.

3.2. Synthesis and characterization of NBACA nanohydrogels

NBACA nanohydrogel was synthesized via D-P polymerization by mixing the NBACA and AIBN in ethanol. The resulting nanohydrogels could be dispersed well in aqueous solution (Figure 4(a)). The nanohydrogel was further characterized by using SEM and DLS. As shown in Figure 4(b,c), the average diameter measured by DLS (361 nm) was larger than that determined by SEM (180 nm), indicating that the NBACA nanohydrogels may swell in aqueous solution. Besides, the chemical structure of nanohydrogels was analyzed by FTIR. As shown in Figure 1, comparing with the spectrum of NBACA monomer, the amide band (1656.4 cm^{-1}) was retained in that of the nanohydrogels, whereas the absorption peaks from double bonds (δ_{C-H} 1311.5 cm^{-1}, $\nu_{C=C}$ 1625.8 cm^{-1}, $\delta_{C=C-H}$ 961.4 cm^{-1}, 803.9 cm^{-1}) disappeared. This indicated that NBACA nanohydrogels were successfully prepared by the polymerization of NBACA monomers.

3.3. Reduction-sensitive degradation of NBACA nanohydrogels

The reduction-sensitive behavior of NBACA nanohydrogels was investigated by monitoring transmittance change of nanohydrogel suspension with UV-visible spectroscopy, in which DTT and GSH were used as reductant. As shown in Figure 5, almost no transmittance change in nanohydrogels suspension was observed under no reductant condition; however, after the addition of DTT, the transmittance of suspension increased about 50% within 15 min, indicated that the nanohydrogels rapidly degraded. Upon the addition of GSH, the transmittance of the nanohydrogels also increased significantly after 2 h, whereas the degradation rate was slower than that in the presence of DTT. This may be due to the difference in steric hindrance between the two compounds, the DTT is more readily accessible to the disulfide bond than GSH.

3.4. Drug-loading studies

In order to evaluate the hydrophobic drug-loading property of the nanohydrogel, PTX was used as a model drug, and the drug loading capacity and entrapment efficiency were determined by UV-vis spectrometer. As the PTX dosage increased from 1 mg to 4 mg, the DLC of the nanohydrogels increased from 1.28% to 24.81%, and the

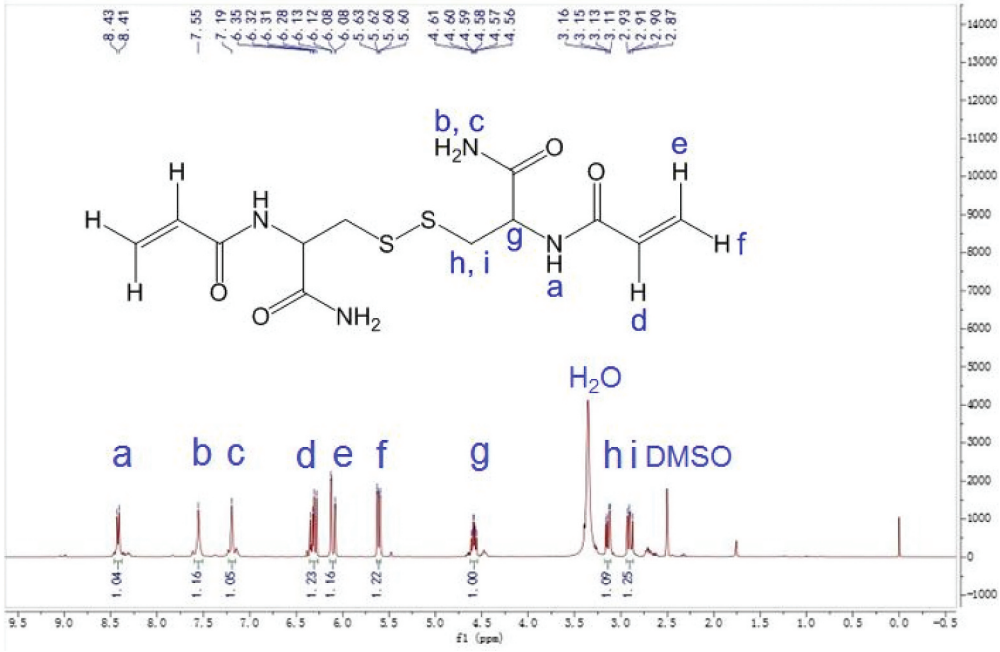


Figure 2. ^{1}H NMR spectrum of *N, N′*-bis(acryloyl) cystinamide in DMSO-d_6.

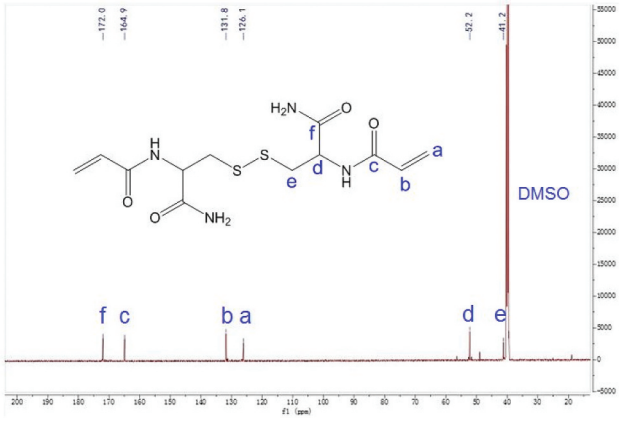


Figure 3. ^{13}C NMR spectrum of *N, N′*-bis(acryloyl) cystinamide in DMSO-d_6.

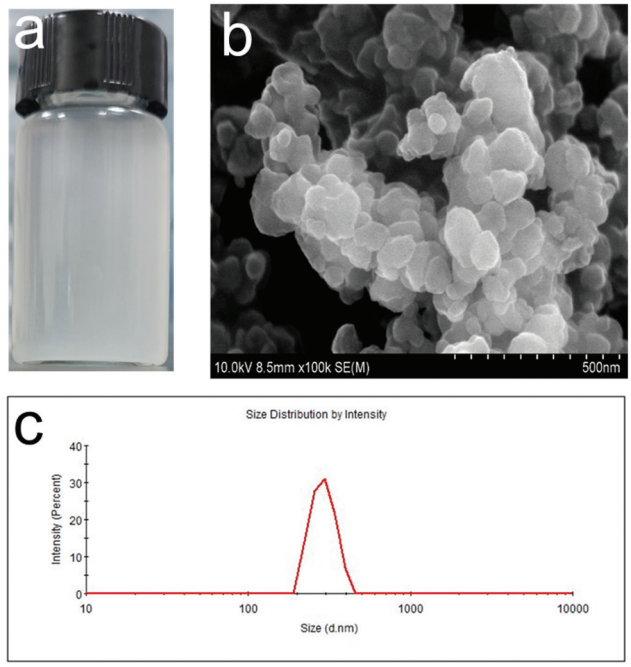


Figure 4. (a) Optical image of NBACA nanohydrogels suspension in ultrapure water. (b) SEM image and (c) particle size distribution of NBACA nanohydrogels.

EE increased from 6.49% to 41.06% (Table 1). The drug-loading capacity (24.81%) obtained for the NBACA nanohydrogels, is higher than that of some other nanogel carrier [25], indicating the potential of these nanohydrogels as a hydrophobic drug carrier. Probably, the relative hydrophobic environment due to the polyethylene backbone as well as the role of hydrogen bonding donor/acceptor of electroneutral amide group inside the nanohydrogels enabled better drug loading content. In addition, FTIR spectrum of the drug-loaded nanohydrogel was also investigated. As shown in Figure 1, all characteristic bands of blank nanohydrogel could be observed in the PTX-loaded nanohydrogel. This result indicated that there was no chemical interaction between PTX and nanohydrogel. In contrast, in the spectrum of PTX-loaded nanohydrogel, additional weak characteristic peaks due to ester group (1243.8 cm^{-1} and 1072.7 cm^{-1}) and C-O stretching peak due to hydroxyl group (1025.5 cm^{-1}, 984.0 cm^{-1}) were found. These

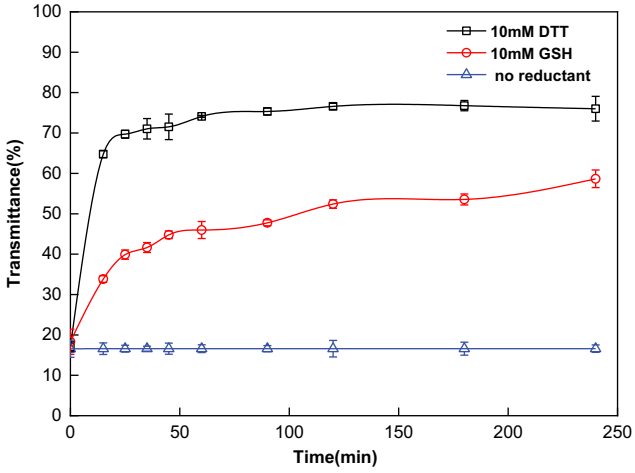


Figure 5. Reduction-sensitive degradation of nanohydrogels in PBS buffer (pH = 7.4) at 37°C under different reducing conditions (10 mM DTT: black line, 10 mM GSH: red line and no reductant: blue line).

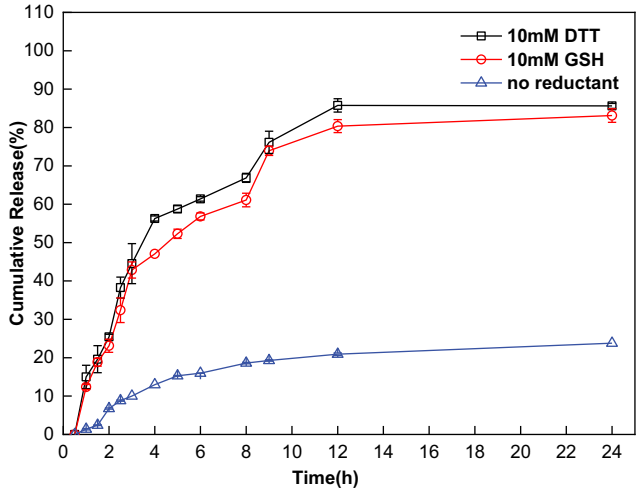


Figure 6. Cumulative release of PTX from nanohydrogels in PBS buffer (pH = 7.4) at 37°C under different reducing conditions (10 mM DTT: black line, 10 mM GSH: red line and no reductant: blue line).

Table 1. Drug loading capacity (DLC) and encapsulation efficiency (EE) of PTX for NBACA nanohydrogels.

Sample	Nanohydrogels (mg)	PTX (mg)	DLC (%)	EE (%)
1	5	1	1.28 ± 0.01	6.49 ± 0.04
2	5	2	8.61 ± 0.07	23.55 ± 0.22
3	5	3	15.42 ± 0.53	30.40 ± 1.21
4	5	4	24.81 ± 0.07	41.06 ± 0.15
5	5	5	11.32 ± 0.10	12.76 ± 0.12

peaks are from the PTX, but not from the nanohydrogel, indicating that PTX is successfully encapsulated in nanohydrogel.

3.5. Release of drug from nanohydrogels

Reduction-triggered release of PTX from NBACA nanohydrogels was also investigated. As shown in Figure 6, approximately 80% of the PTX was released from the nanohydrogels within 24 h under a reducing environment (GSH or DTT). This result suggested that the release of PTX from the nanohydrogel was not only ascribed to cleavage of disulfide bond, but also dependent on its disadsorption from the backbone of nanohydrogels. In contrast, only 25% of the PTX was released under non-reducing conditions, which may be caused by the PTX absorbed on the surface of the nanohydrogel. These results demonstrated that PTX-loaded NBACA nanohydrogels can release the PTX in a reduction-sensitive manner.

3.6. Cytotoxicity assays

MTT assay was performed against MCF-7 breast cancer cells by using blank nanohydrogel, PTX-loaded nanohydrogel. As shown in Figure 7(a), blank nanohydrogels exhibited a negligible cytotoxicity against MCF-7 cells, even at a high dosage, suggesting that the

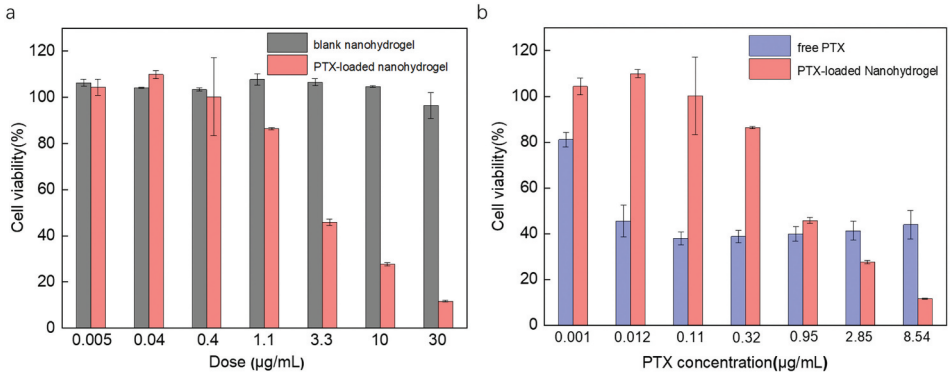


Figure 7. MTT assays for MCF-7 cells treated with blank nanohydrogel, PTX-loaded nanohydrogel, and free PTX, respectively, for 72 h. The same PTX-loaded nanohydrogel (DLC: 22%) was used to compare with blank nanohydrogel (a) and free PTX (b) respectively.

nanohydrogels may be suitable as nontoxic nanocarriers. After treatment with PTX-loaded nanohydrogels, the viability of MCF-7 cells decreased significantly with increasing the concentration of PTX-loaded nanohydrogels. The calculated IC_{50} value of the PTX-loaded nanohydrogels was 3.78 μg/mL, which similar to that of PTX-loaded heparin-alpha-tocopherol succinate (Hep-cys-TOS) nanoparticles (IC_{50} = 0.79 μg/mL) [33]. Besides, although higher cytotoxicity of free PTX than PTX-loaded nanohydrogels was observed in low concentration region; however, the cytotoxicity of PTX-loaded nanohydrogels was higher than that of free PTX in high concentration region, suggesting that aggregation of the free PTX may occurred in high concentration region (Figure 7(b)), which may due to poor solubility of PTX in aqueous media [29]. The cytotoxicity of the PTX-loaded nanohydrogels may be attributed to the fact that the cancer cells uptake the PTX-loaded NBACA nanohydrogels and then release the drugs in a reduction-sensitive manner. Therefore, NBACA nanohydrogels represent a promising drug nanocarrier due to their great biocompatibility and reduction sensitivity.

4. Conclusion

A novel monomer, *N, N*′-bis(acryloyl) cystinamide was designed and successfully synthesized via esterification, amidation and aminolysis reactions. By using the *N, N*′-bis (acryloyl) cystinamide as both a monomer and crosslinking agent, new NBACA nanohydrogels were prepared in ethanol via distillation–precipitation polymerization. The nanohydrogels can provide electroneutral hydrophobic environment and hydrogen bonding sites for hydrophobic drug, thus exhibited a good PTX-loading capacity. Furthermore, the disulfide-bonds-containing nanohydrogel also exhibited promising reduction-responsive drug-release behavior. Cell cytotoxicity results demonstrated that the nanohydrogels showed almost no cytotoxicity, while the PTX-coated nanohydrogels showed strong cytotoxicity in MCF-7 breast cancer cells. Therefore, as a new biodegradable, reduction-sensitive nanocarrier, this type of nanohydrogel could be used for delivering more kinds of hydrophobic drugs as well as PTX.

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Disclosure statement

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ORCID

Mingzhe Liu http://orcid.org/0000-0002-2526-0488

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