Research Paper

pH-sensitive carboxymethyl chitosan hydrogels via acid-labile ortho ester linkage for potential biomedical applications

Liefeng Hu, Panpan Zhang, Xin Wang, Xu Cheng, Jiejie Qin, Rupei Tang*

Engineering Research Center for Biomedical Materials, Anhui Key Laboratory of Modern Biomaterial Manufacturing, School of Life Science, Anhui University, 111 Jiu long Road, Hefei, Anhui Province, 230601, PR China

ABSTRACT

Hydrogel systems with favorable biocompatibility and biodegradability are of much interest for application in biomaterials and tissue engineering. In this study, a new ortho ester-based acid-labile crosslink agent with dual epoxy end groups was synthesized and crosslinked with carboxymethyl chitosan (CMCS) at different molar ratios to prepare a series of pH-sensitive hydrogels for drug delivery. Doxorubicin (DOX) was then readily loaded into the hydrogels and the in vitro release profiles indicated that the release rate increased rapidly while pH decreased from 7.4 to 5.0, which is consistent with the degradation rate of these hydrogels at corresponding pH conditions. In addition, results from MTT assay and flow cytometry demonstrated that these CMCS-based hydrogels and their degradation products have no cytotoxicity against SH-SY5Y and 293T cells. Therefore, the prepared acid-labile hydrogels could be applied in tumor drug delivery systems and peritumoral implantation therapy by further optimization.

1. Introduction

As soft biomaterials, hydrogels have been used extensively in the area of tissue engineering and drug delivery because of their three-dimensional networks, high water content, flexibility, good biocompatibility and adaptive biodegradability (Deshmukh, Singh, Gunaseelan, Gao, Stein, & Sinko, 2010; Yang, Wang, Cao, Chen, Tang, & Liu, 2014; Yang, Wang, Peng, Fu, Zhang, & Liu, 2012). A number of hydrogels based on natural materials or synthetic polymers have been developed and served as drug carriers since they can absorb a large amount of water-soluble drugs in their three-dimensional polymeric networks (Chen, Meinertzhagen, & Shaw, 1999; Twisk, 2013; Xing et al., 2016).

Generally, hydrogels are formed from the aqueous polymer solution via physical or chemical crosslinking action. Physically crosslinked hydrogels are usually developed by the noncovalent bonds between polymer molecular chains, such as hydrogen bonds, Van der Waals forces, hydrophobic effects and ionic interactions (Hennink & van Nostrum, 2012; Hiemstra et al., 2006; Kontogiorgos, Vaikousi, Lazaridou, & Biliaderis). Physically crosslinked hydrogels can be formed under mild conditions, however, the mechanical properties and strength are relatively weak. On the contrary, chemical crosslinking leads to more stable hydrogels and the mechanical properties and gelation time can be controlled by varying the molar ratio between the polymer and crosslinking agent, but the use of chemical initiators or cross-linkers may induce significant toxicity (Jukes et al., 2009; Peng et al., 2013).

Besides, a variety of chemical crosslinking approaches such as photo-polymerization, free radical polymerization, Schiff-base formation, and Michael-type addition have been employed to prepare stimuli-response hydrogels (Li et al., 2012; Raeber et al., 2005; van de Wetering, Metters, Schoenmakers & Hubbell, 2005). These ‘smart’ hydrogels possess unique sensitivity to biological stimulus including enzyme, pH and thermal, leading to controlled drug release along with the swelling/collapse transitions of hydrogels (Yang, Wang, Cao, Chen, Tang & Liu, 2014). Liu et al. reported a convenient and efficient strategy to prepare a stimuli-responsive hydrogel based on a disulfide-linked 4-armed PEG, and the hydrogels could degrade in the presence of glutathione at low concentration (Yang, Wang, Peng, Fu, Zhang, & Liu, 2012). Okino et al. developed a trans-tissue delivery hydrogel based on a photo-cured gelatin immobilized with gemcitabine. It is demonstrated that the gemcitabine-gelatin hydrogel significantly reduced the tumor volume compared to gemcitabine injection in pancreatic tumor inoculated nude mice (Okino, Maeyama, Manabe, Matsuda, & Tanaka, 2003).

In comparison with intravenous administration by nano-scale drug delivery systems such as nanoparticles, liposomes and micelles, the drug-loaded hydrogels are soft and can attach directly and tightly to the rough surface of the tumor in any shape (Ding et al., 2011; Dong et al., 2009; Konishi et al., 2005; Liu, Meisner, Kwong, Wu, & Johnston, 2009; Konishi et al., 2005; Liu, Meisner, Kwong, Wu, & Johnston, 2009). This unique characteristic of hydrogels makes them an ideal candidate for drug delivery systems and the growing interest in the development of hydrogels indicates that they have the potential to substitute for other delivery systems.

In this study, a new hydrogel system with pH-sensitive properties was prepared by physically crosslinking carboxymethyl chitosan (CMCS) with a dual-epoxy ortho ester crosslink agent, polyethylene glycol (PEG) epoxy bis (allyl carbonate) (EPG). The pH-sensitive hydrogels were loaded with doxorubicin (DOX) to prepare pH-sensitive hydrogels for drug delivery.
2007). These hydrogels can serve as local drug delivery systems that provide stimuli-response controlled and sustained release of chemotherapeutic agents directly into tumor site, resulting in high local drug concentrations as well as reducing systemic side effects (Konishi et al., 2003; Okino et al., 2003; Zhang, Tian, et al., 2016). Although chemically crosslinked polymer hydrogels showed great potential as local drug carriers, these crosslinking methods and modification procedures are relatively complex and expensive (Yang, Wang, Cao, Chen, Tang, & Liu, 2014).

Poly saccharides including chitosan, alginate, dextran, hyaluronic acid and their derivatives have been wildly used for biomedical applications including drug delivery, which are attributed to their nontoxicity, biocompatibility, biodegradability and processability. In order to release loaded drugs under mildly acidic environment, these polysaccharides were usually modified with pH-sensitive groups on their side chains. Bachelder et al. (Bachelder, Beaudette, Broaders, Dashe & Fréchet, 2008) prepared an acid-responsive biodegradable material by modifying dextran with 2-methoxypropene (acetalated-dextran), which was possessed of a favorable toxicity and could encapsulate the hydrophobic and hydrophilic payloads. These polymers degraded via acid catalyzed hydrolysis at tumor environment due to the properties of the acetal bonds. Raja et al. (Raja, Arif, Feng, Zeenat, & Liu, 2017) reported a pH-responsive polymer based on amphiphilic N-acetyl histidine and arginine-grafted chitosan as anticancer drug delivery system for doxorubicin. The N-acetyl histidine and arginine-grafted chitosan nanoparticles exhibited an acidic pH-triggered aggregation and disassembling nature. Yu et al. (2017) reported a temperature- and pH-dual responsive hydrogels by crosslinking CMCS and poloxamer composed of a poly(ethylene oxide)/poly(propylene- oxide)/poly (ethylene oxide) (PEO–PPO–PEO) block copolymers with glutaraldehyde (GA). The results showed that the hydrogels had an excellent potential for application in ophthalmic drug delivery systems. However, these preparation profiles were relatively complicated, and the residual cross-linkers may cause serious cytotoxicity.

In this study, carboxymethyl chitosan (CMCS) was chosen as an initial material to prepare pH-sensitive hydrogels via an acid-labile crosslink agent in a mild condition. CMCS as a derivative of chitosan, is water-soluble, non-toxic, biodegradable and biocompatible, which has been extensively investigated in applications of pharmaceuticals and biomedical materials (Cao, Wang, Cheng, Wang, & Tang, 2017; Kanth, Kajjari, Madalagare, Ravindra, Manjeshwar, & Aminabhavi, 2017; Wang, Wei, Cheng, Wang, & Tang, 2017; Yu et al., 2017). Ortho ester bonds can be hydrolyzed more quickly in response to a mildly acidic condition compared to acetics and ketals, while exhibiting clear kinetic of degradation and excellent biocompatibility (Heller & John, 2004; Heller, Barr, Ng, Abdellauoi, & Gurny, 2002; Heller, 1993; Lai et al., 2014; Wei et al., 2015; Xu et al., 2014). Recently, our group reported several ortho ester-based nano-carriers, whose ortho ester bonds could be hydrolyzed at mildly acidic pH, resulting in accelerated disruption of nano-carriers and controlled drug release (Lai et al., 2014; Wei et al., 2015; Xu et al., 2014; Zhang, Yu, et al., 2016). Herein, a new ortho ester-based acid-labile crosslink agent with dual-epoxy terminal groups (OEDe) was synthesized and interacted with CMCS in aqueous solution at different molar ratios to give pH-sensitive hydrogels, followed by the loading of antitumor drug Doxorubicin (DOX). The chemical structure and micromorphology of these hydrogels were investigated by FT-IR and SEM. Mechanical property and thermo-stability of these hydrogels were also investigated by Simultaneous thermal analysis (STA). Considering that this ortho ester-based crosslink agent can keep stable in neutral condition and rapidly degrade in a mild acid environment, these hydrogels may display appropriate stability under physiological environment (pH 7.4), while rapidly swelling and degrading at pH 5.0, thus leading to a pH-sensitive drug release. In addition, biocompatibility of these hydrogels and their degradation products were investigated using MTT assay and optical microscope observation. Cell apoptosis co-cultured with these hydrogels or their degradation products were investigated by CLSM and flow cytometry.

2. Experimental section

2.1. Materials

Carboxymethyl chitosan (CMCS, Mn ~ 5000, and amino content ~ 1.45%) was purchased from Hanka Biotech (Shandong, China) and used without further purification. The degree of deacetylation is about 85% and the degree of substitution of carboxylation is more than 80%. 3-[4,5-Dimethylthiazol-2-yl]-2,5- diphenyltriazolium bromide (MTT) and diglycero1 were purchased from Sigma-Aldrich. Pyridinium and p-TSA were purchased from TCI (Shanghai, China). Dichloromethane, triethylamine, epichlorohydri9n and methanol were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Tetrabutylammonium Bromide (TBAB) was purchased from Adams Reagent Co., Ltd. Human neuroblastoma cells (SH-SY5Y) and human embryonic kidney transformed cells (293T) were purchased from the American Type Culture Collection (ATCC). Annexin V-FITC Apoptosis detection Kit was purchased from Jiangsu KeyGEN Biotech Corp., Ltd. Other chemicals and solvents were purchased commercially and used without further purification.

2.2. Methods

2.2.1. Synthesis of 4,4′-(oxybis(methylene)) bis (2-(2-(2-(2-methoxyethoxy)ethoxy)-1,3-dioxolane) (OEDe)

4,4′-(Oxybis(methylene)) bis (2-methoxy-1,3-dioxolane) (compound 1) and OEDg were synthesized as described in our previous works (Wei et al., 2015; Zhang, Yu, et al., 2016). OEDe was synthesized as follows. Briefly, in nitrogen atmosphere, OEDg (10.09 g, 0.025 mol), epichlorohydrin (14.059 g, 0.152 mol), sodium hydroxide (6.078 g, 0.152 mol) and TBAB (0.408 g, 0.0013 mol) were added to a flask and stirred continuously at 40 °C for 4 h until the solid component was immersed. After the reaction, the product was extracted with dichloromethane and collected via filtration. The product was then dried with MgSO4 and concentrated to yield 8.13 g (64.94%) of OEDe as yellow oil. 1H NMR (400 MHz, CDCl3, δ, ppm): 2.54-2.60 (m, 2H, CH–O), 2.72-2.80 (m, 2H, CH2–O), 3.07-3.16 (m, 2H, CH2–O), 3.38-3.79 (m, 2H, CH2–O), 4.05-4.20 (m, 4H, 4H, CH2–O), 4.25-4.55 (m, 2H, CH2–O), 5.78-5.90 (d, 2H, CH2–O)), 13C NMR (100 MHz, CDCl3, δ): 44.227, 50.884, 63.844, 65.919, 70.308, 70.351, 70.694, 72.097, 77.362, 115.603. ES1–MS calcd. for (C22H38O13), 510.2; found m/z, 532.2 (M + Na+).

2.2.2. Fabrication of CMCS/OEDe hydrogels

The fabrication process was briefly described as follows: first, 40.00 mg of Carboxymethyl chitosanpowder was added to 2 mL deionized water under magnetic stirring to form a transparent solution. Then, the acid-labile cross-linker (OEDe) was added and stirred for 30 min. The mixture was heated at 60 °C for 3 h to form pH-sensitive CMCS/OEDe hydrogels. The molar ratios of amino groups of CMCS to the epoxy rings of OEDe were set as 1:0; 1:0,5; 1:1 and 1:2. The corresponding hydrogels were designed as control, hydrogel-1, hydrogel-2 and hydrogel-3, respectively. The prepared hydrogel-2 was determined by the solid phase 13C (Bruker Advance III 400 WB).

2.3. Measurements

2.3.1. Morphological observation

Morphological observations were conducted to explore the micro-structural variations before and after degradation. To avoid charging and degradation of the hydrogels (Sun, Li, Stoner, Jiang, Lu, & Rogers, 2011), the hydrogels were lyophilized and then the dry samples were cut off to watch the cross-section after being gold-coated in a sputter coater for 60 s. Micromorphology of each sample was monitored using...
scanning electron microscope (SEM, Hitachi S-4800) at a steady voltage of 30 kV.

2.3.2. Fourier transform infrared spectroscopy (FT-IR)

FT-IR (NEXUS 870, Nicolet Instrument Co. USA) was used to estimate the variations of the epoxy rings and the amino groups by comparing characteristic peaks of OEDe, CMCS and hydrogels. The cream of OEDe and the powders of CMCS and hydrogels were mixed with KBr and then pressed into transparent discs. All samples were scanned from 4000 to 400 cm$^{-1}$ with a scan speed of 2 mm/sec. The FT-IR spectra were normalized and major vibration bands were identified via relative main chemical groups.

2.3.3. Simultaneous thermal analysis (STA)

STA was one of powerful methods to evaluate material thermostability, which can be drawn the thermograms of TG/DTA. To get the Simultaneous thermal analysis (STA) patterns, the gel samples were freeze-dried in a vacuum oven at minus 40 °C for a spell of time and shifted in a desiccator for cooling down to room temperature. The test was carried out under a nitrogen atmosphere with a heating rate of 10 °C/min in the temperature range from 40 to 800 °C.

2.3.4. Rheology analysis

Rheological behaviors of these prepared hydrogels were investigated on a Bohlin Gemini HR nano rheometer (Malvern Instruments Ltd. UK) at 25 °C using parallel plates (2 × 20 mm in diameter). Each sample was placed to the rheometer with adjusting gap in 70 μm and the storage modulus (G') and loss modulus (G'″) were measured. The whole measurement was performed at 1%-10% strain and 1 Hz within the leaner viscoelastic region, and the rheometer of frequency was performed at 0.01–10 Hz.

2.3.5. Extractable gel test

Possible extractable parts of gels were tested before degradation study and drug release. The hydrogel-2 freeze-dried initially was accurately weighted ($m_0$), and then the weighted hydrogels were immersed into a certain amount of PBS solution at different pH values (7.4, 6.5 and 5.0) at 37 °C for 24 h. Separately, a part of products dissolved in PBS at different pH values (5.0 and 6.5) were freeze-dried and the part of products undissolved in PBS at pH values of 7.4 were dried in vacuum oven, and then the freeze-dried products were determined by $^1$H NMR, and the vacuum-dried products were weighted ($m_t$). The crosslinker would randomly and almost react with carboxymethyl chitosan during the crosslinking reaction, and the extractable part could be low crosslinked carboxymethyl chitosan

The weight of extractable products = $m_0-m_t/m_0 \times 100\%$ \hspace{1cm} (1)

2.3.6. X-ray diffraction (XRD) analysis

XRD measurements of the samples were performed by an X-ray diffractometer (XD-3, Purkinje General, Beijing) operating at 36 kV, 20 mA. The scanning rate was 0.02°/min, and the scanning scope of $2\theta$ was 5–90°.

2.3.7. Stability test

To demonstrate if the cross-linker (OEDe) could be instantaneously degraded by self-catalyzed in CMCS aqueous, the prepared hydrogel-2 was placed for 7 d under room temperature. The saved photos were taken by a common camera.

2.3.8. Degradation investigation

In vitro degradation rate of these pH-sensitive hydrogels was determined by measuring their weightlessness in PBS (0.01 M) (Nadin, Khorasani, Kharaziha, & Davoodi, 2017). For this degradation test, samples of hydrogels were lyophilized and sterilized, weighted ($M_0$) under the ultraviolet. Subsequently, each sample was immersed into 10 mL PBS solution at two different pH values (7.4 and 5.0) at 37 °C for incubation. All incubations were done in 50 mL EP tubes and kept in a horizontal laboratory shaker with 120 rpm. At regular intervals, the buffer solution was removed from the EP tube and the remaining gels were freeze-dried and weighed ($M_t$). The buffer was refreshed.

The remaining gel content (%) = $M_t/M_0 \times 100\%$ \hspace{1cm} (2)

Each sample settles three parallel replicates and calculates the

\begin{scheme}
\begin{center}
\includegraphics[width=\textwidth]{fig1.png}
\end{center}
\caption{The synthetic route of OEDe and formation of CMCS/OEDe hydrogels. Reaction conditions: (i) trimethyl orthformate, p-TSA, acetonitrile; (ii) diethylene glycol, pyridinium p-TSA; (iii) epichlorohydrin, TBAB, NaOH/H$_2$O.}
\end{scheme}
Fig. 1. $^1$H and $^13$C NMR spectra of OEDe (400 MHz, CDCl$_3$) (A and B); The solid phase $^{13}$C of CMCS (C) and crosslinked hydrogel (D).
average value.

2.3.9. Swelling measurements

The swelling ratios of hydrogels were evaluated at pH 7.4. The freeze-dried hydrogel-1, hydrogel-2 and hydrogel-3 were cut into square-shaped specimens (2 cm × 2 cm) and accurately weighted \( W_D \), then immersed into 10 mL of phosphate buffer (0.01 M) at 25 °C. At predetermined time, the immersed samples were weighted after the surface water of hydrogels has been removed by the filter papers \( W_S \). The swelling degree of hydrogels was calculated using the following formula.

\[
\text{Swelling degree} = \frac{W_S - W_D}{W_D} \quad (3)
\]

The results are shown as mean and standard deviation.

2.3.10. In vitro release determination

Samples of hydrogel-2 (1.5 cm × 1.5 cm × 0.4 cm) loaded with 1 mg DOX·HCl were prepared by immersing freeze-dried hydrogel into 2 mL of drug solution, and then drug solution was completely absorbed via the properties of swelling. After drug-loading, DOX-loaded hydrogel-2 was cut into same shape and weighted, followed by immersed into 10 mL phosphate buffer (pH 7.4, 6.5 and pH 5.0) and constantly shaken (120 rpm) at 37 °C. At predetermined time intervals, PBS solution was removed, and replaced with 10 mL fresh PBS. The amount of released DOX was measured via a microplate reader (Molecule Devices, USA) at Ex 480 nm and Em 590 nm. All experiments were conducted in triplicate.

2.3.11. In vitro cytotoxicity assay

To investigate the cytotoxicity, these CMCS-based hydrogels and their degradation products were incubated with SH-SY5Y cells and 293T cells prior to observe by a microscope. Considering that the unreacted crosslinker wasn’t quantitatively removed by the process of washing, the crosslinker of OEDe and the degradative OEDe were incubated with SH-SY5Y cells and 293T cells and the degradative OEDe was determined by \(^1\text{H NMR}\). In addition, the cytotoxicity of these samples was evaluated via MTT methods. SH-SY5Y cells were cultured in 96-well and 6-well filled with Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C and 5% CO\(_2\) for 24 h. The medium was then replaced with 200 μL culture mediums containing different contents of hydrogels. The viability of cells in 96-well was evaluated with MTT method and the cells in 6-well were observed by inverted microscope. Cell viability was calculated using the following formula:

\[
\text{Cell viability (%)} = \frac{\text{Absorbance test cells}}{\text{Absorbance controlled cells}} \times 100\% \quad (4)
\]

### Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>CMCS (mg/mL)</th>
<th>M(_1)/M(_2)</th>
<th>Gelation</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>20</td>
<td>0/1</td>
<td>solution</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0.5/1</td>
<td>hydrogel-1</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>1/1</td>
<td>hydrogel-2</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>2/1</td>
<td>hydrogel-3</td>
</tr>
</tbody>
</table>

Fig. 2. Images of inverted hydrogels (A) and freeze-dried hydrogels (B); SEM images of freeze-dried CMCS solution (control) and hydrogels with different ratios of OEDe at different magnifications (C and D).
2.3.12. Cell apoptosis detection

Hydrogels and their degradation products were co-cultured with SH-SY5Y and 293T cells for 24 h. Subsequently, cells were washed twice by phosphate buffered saline (PBS), then, 500 μL Binding Buffer containing 5 μL Annexin V-FITC and 5 μL Propidium Iodide (PI) were added in plates of cells and shake gently. The cell apoptosis was observed using flow cytometric analysis and confocal laser scanning microscopy.

2.4. Statistical analysis

All tests were repeated three or more times. All data were showed as average and standard deviation.

3. Results and discussion

3.1. Fabrication of CMCS/OEDe hydrogels

A series of pH-sensitive hydrogels were prepared by crosslinking CMCS with different ratios of acid-labile cross-linker (OEDe) (Scheme 1). OEDe was synthesized in three steps using glycerol as staring material through transesterification and substitution, which chemical structure was confirmed by 1H and 13C NMR spectra (Fig. 1A and B). The peaks between 8 (ppm) 5.82 and 5.86 in 1H NMR spectra of OEDe were attributed to the special shift of ortho ester proton, and other peaks of OEDe correspond to these shifts as follows, and the 13C NMR spectra also had its own position for each carbon.

CMCS was crosslinked with OEDe at three different molar ratios (1:0.5, 1:1 and 1:2), and the corresponding hydrogels were designed as hydrogel-1, hydrogel-2 and hydrogel-3, respectively. Meanwhile, the uncross-linked CMCS solution was set as the control group (Table 1). The solid phase 13C NMR of CMCS and the crosslinked hydrogel-2 were shown in Fig. 1C and D. The peaks of CMCS appeared on the hydrogels, and the characteristic peaks of C=N bonds were presented in 63.1 ppm and the peaks of the ortho esters occurred at 170.2 ppm, which proved the hydrogels were successfully crosslinked.

From Fig. 2A, it was found that transparent hydrogels (hydrogel-1, hydrogel-2 and hydrogel-3) were obtained via nucleophilic substitution between epoxy rings of OEDe and amino radicals of CMCS, and the degree of crosslinking increased with the increasing feeding ratios of OEDe. After cross-linked, hydrogels could be lyophilized to form porous sponges (Fig. 2B).

3.2. Morphology observation

The morphology of freeze-dried control group, hydrogel-1, hydrogel-2 and hydrogel-3 were studied by SEM at different magnifications, as displayed in Fig. 2C and D. Obviously, the control group exhibited porous and interconnected microstructure, but the structure was disorder and unsystematic. On the opposite, hydrogels crosslinked with OEDe showed a porous network with many regular holes and smooth walls. These characteristics also had an enhanced tendency resulted from the increase of the cross-linking density.

3.3. Fourier transform infrared spectroscopy (FT-IR)

The fabrication of crosslinked hydrogels was then confirmed by FT-IR analysis. The FT-IR spectra of OEDe, CMCS, hydrogel-1, hydrogel-2
and hydrogel-3 were shown in Fig. 3A. For OEDe, a peak was observed at 1797 cm$^{-1}$ due to C=O=C of epoxy rings. However, there is no peak at the same location of hydrogel-1 and hydrogel-2. The new peak appeared at 1128 cm$^{-1}$, which was attributed to C=C=O of five-membered ortho ester rings, and the intensity gradually increased with the increasing ratios of OEDe. The bands at 3057 and 759 cm$^{-1}$, indicated flexible and flexure vibration stretching of C=H in epoxy rings. The broad peak was presented at 1126 cm$^{-1}$, which due to aromatic ether, and a peak of adipose ether was noted at 1062 cm$^{-1}$. These results demonstrated that the epoxy rings of OEDe were easy to react with CMCS. As for CMCS/OEDe hydrogels, the stretching vibration peaks of O=H, C-H and C-N (N=H) were observed at 3423, 2922 and 1602 cm$^{-1}$, respectively. In addition, these peaks deepened and broadened with the increase of ODe contents, which demonstrated that CMCS was successfully crosslinked with OEDe.

The freeze-dried degradation products of hydrogels were also tested by FT-IR (Fig. 3B), and the peaks of ortho esters (1128 cm$^{-1}$) and the crosslinked bonds (1797 cm$^{-1}$) disappeared or partially decreased, indicating that the structures of crosslinked hydrogels were broken.

3.4. Thermo-stability analysis

As shown in Fig. 3C, the TGA curves displayed the degradation rate under high temperature in air and nitrogen. All curves obviously exhibited three weightless steps. In the range from 44 to 142 °C, the weightless rate of control, hydrogel-1, hydrogel-2 and hydrogel-3 reached nearly 8.1%, 5.2%, 4.8% and 0.4%, respectively, which could be ascribed to the loss of crystallographic water from the hydrogel samples. The results also indicated that freeze-dried control group (CMCS solution) contained higher content of crystallographic water than OEDe cross-linked CMCS. The degradation temperatures ($T_d$) of hydrogels (hydrogel-1, hydrogel-2 and hydrogel-3) were observed at 220, 206, and 200 °C, while the temperature of pure CMCS was observed at 234 °C. The weight loss of control, hydrogel-1, hydrogel-2 and hydrogel-3 at 270 °C was 78.8%, 75.4%, 75.2% and 74.5%, respectively. As shown in Fig. 3D, the characteristic sharp peaks represented that the temperature of dehydration ($T_D$) were about 60 °C. The control group was possessed of a lower decomposition temperature than other crosslinked hydrogels. In addition, the weight loss of each sample was in positive correlation with the degree of crosslinking. The derivative thermogravimetry (DTG) results were in accordance with these TGA curves, which further confirmed that cross-linked hydrogels had a higher thermos-stability than un-crosslinked sample.

3.5. Rheological studies

The stability and mechanical properties of the CMCS-based hydrogels could be revealed from rheological characterization. Fig. 4A and B showed the loss modulus ($G''$) and storage modulus ($G'$) of different hydrogels. From the strained rheometer, it was obvious that pure CMCS solution was near to zero, while hydrogel-1, hydrogel-2 and hydrogel-3 performed a very high loss modulus. However, the loss modulus of hydrogel-1 and hydrogel-2 was more stable than that of hydrogel-3, which was due to the excessive crosslinking of hydrogel-3. In addition,
the elastic modulus increased gradually with the increase of the molar ratios of the cross-linker (OEDe), suggesting that the stability of the hydrogels was related to the crosslinking reaction between CMCS and OEDe. As for the rheometer of the frequency, the storage modulus and loss modulus of control group were almost same, and it's very low, which represented that the control group almost had not viscoelasticity and couldn't keep a certain shape. While the storage modulus and loss of hydrogels increased gradually with the increase of the crosslinking density, which confirmed that the viscoelasticity of the hydrogels was related to the crosslinking reaction between CMCS and OEDe. From Fig. 4C, the mechanical properties (sheer stress) of specimens were increased with the increase of OEDe ratios. As shown in Fig. 4D, the decreasing tendency of damping factor (tan δ = G''/G') with increasing content of the cross-linking agent reflected the enhanced elasticity of these hydrogels. All results demonstrated that the crosslinking with OEDe could efficiently enhance the CMCS-based gels' properties.

3.6. Extractable gel test

To comprehensively study the degradation of hydrogels and OEDe, the possible extractable parts of gel were investigated before study of degradation and drug release properties, and the results were shown in Fig. S1 (Supporting Information). Fig. S1A showed that the weightlessness of hydrogel-3 was higher than that of the hydrogel-2 and hydrogel-1 because of the low crosslinked carboxymethyl chitosan. The loss of weight is 29.86% (hydrogel-3), 23.01% (hydrogel-2) and 12.2% (hydrogel-1), respectively, which demonstrated that the appropriate amount of the crosslinker (OEDe) was needed. Fig. S1B theoretically shows the degradation process of the hydrogels cross-linked by OEDe based on the previous works (Qiao, Du, Zhang, Liang, & Li, 2010; Yan et al., 2017; Fu et al., 2017; Zhang, Yu, et al., 2016). From Fig. S1C and D, the degradation products of the hydrogels and OEDe treated at different pH values possessed the characteristic peaks of folic acid, which further confirmed that these hydrogels were acid-labile to mildly acidic conditions.

3.7. XRD analysis

X-ray diffraction is generally used to study crystal lattice arrangements to obtain very useful information on degree of sample crystallinity (Yusof, Illias, & Kadir, 2014; Tripathi, Mehrotra, & Dutta, 2009). As shown in Fig. S2, XRD investigation proved that the pure CMCS exhibited one sharp peak (20.30 °) at 2θ, while the crosslinked hydrogels had one broad peak (19.9 °) at 2θ. Because the cross linker (OEDe) has an amorphous nature, it wouldn't change the main peak of CMCS. Compared to that of contro group (pure CMCS), the crystallinity of

![Image of degradation behaviors of the hydrogels at pH 5.0 and pH 7.4 (A); SEM images of morphological variations of hydrogels at pH 5.0 for 8 h (B); Swelling ratio profiles of hydrogels at pH 7.4 (C); In vitro release profile of DOX from hydrogel-2 in PBS (0.01 M) at pH 7.4, 6.5 and 5.0 under 37 °C, and attached images of DOX-loaded hydrogels after incubation for 168 h (D).](image-url)
crosslinked CMCS/OEDe hydrogels obviously decreased, which was probably attributed to the cross-linkage between the epoxy rings in OEDe and amino groups in CMCS, and the weakened hydrogen bonding formed from amino groups and hydroxyl groups in the polymers. The broad peak of crosslinked hydrogels gradually increased with the increase of crosslinking density.

3.8. Stability test

To demonstrate if the crosslinker could be instantaneously degraded by self-catalyzed in CMCS aqueous, the prepared hydrogels could be preserved and stabilized for a long time, which further demonstrated that the ortho ester bonds were stable in the cross-linked

Fig. 6. In vitro cytotoxicity of degradation products, crosslinker and its degradation on SH-SYSY and 293T cells; the optical images of SH-SYSY and 293T cells after incubation with CMCS, hydrogel-1, hydrogel-2 and hydrogel-3 (A, B, C and D); CLSM images of SH-SYSY and 293T cells co-cultured with control group (E, F), hydrogel-1 (G, H), hydrogel-2 (I, J) and hydrogel-3 (K, L).
hydrogels (Fig. S3).

3.9. pH-triggered degradation

The degradation profiles of these hydrogels were measured in PBS at pH 7.4 and 5.0. As shown in Fig. 5A, these curves (a, b, d) revealed that hydrogels crosslinked with the increasing content of OEDe showed a gradual increase trend in mass losing rate at pH 7.4 during the examined time intervals. The terminal remaining mass of hydrogel-1, hydrogel-2 and hydrogel-3 was 73.39%, 67.22% and 57.61%, respectively.
respectively. At pH 5.0, with the increase content of OEDe, hydrogel-1, hydrogel-2 and hydrogel-3 displayed a gradual rising trend for the mass losing test, and the terminal remaining mass was 66.88%, 59.69% and 40.27% (c, e and f), respectively.

In addition, morphological variations of these hydrogels during the degradation process were further observed using SEM. As shown in Fig. 5B, it could be seen that the surface of hydrogels become coarse and dim after incubated at pH 5.0 for 8 h. Meanwhile, the greater of the crosslinking density, the more surface holes of the degraded hydrogels, which was almost the same with the characteristic of mass remaining.

3.10. Swelling properties

The swelling properties and stability of these hydrogels in physiological pH were vital for drug loading and delivery. The swelling ratio of each sample in pH 7.4 at room temperature (25 °C) was then investigated. As shown in Fig. 5C, the curves showed that all hydrogels were possessed of excellent swelling ability. It took 2 h for hydrogel-1 to reach its maximum swelling ratio peak (14.1), while hydrogel-2 only took 0.5 h to reach its maximum swelling ratio peak (6.7). Besides, the maximum swelling ratio peak of hydrogel-3 was only 5.9. It was obvious that the swelling ratio of each sample was in the following order: hydrogel-1 > hydrogel-2 > hydrogel-3, which was consistent with the degree of crosslinking. Otherwise, hydrogel-1 displayed a sharp decline at swelling ratio after this peak, which may be the partial uncrosslinked CMCS of hydrogels in the low crosslinking degree was redissolved and some of the contained water was overflown. After 8 h, it reached the equilibrium swelling (about 10.0) and was kept in stable state. In contrary, hydrogel-2 and hydrogel-3 reached equilibrium quickly at 2 h (the maximum swelling ratio peaks were pH 6.5 and 5.0) and were kept stable till the end of experiment, which was owned to the higher crosslinking degree of hydrogel-2 and hydrogel-3 than that of hydrogel-1. This tendency indicated that the crosslinking degree could distinctly influence the stability and swelling ability of these CMCS hydrogels. Generally, hydrogel-2 with appropriate degree of crosslinking could lead to preferable swelling ability and relative stability in physiological pH, which was suitable for the application of drug loading and delivery.

3.11. Drug loading and in vitro release

Considering the three dimensional network structure and excellent swelling properties as well as the pH-sensitive degradation, the CMCS hydrogels were suitable for drug delivering. Hydrogel-2 equipped with appropriate mechanical property and degradation was chosen to load a model drug (DOX). DOX was absorbed by the swelling of freeze-dried hydrogels with a high drug loading content and loading efficiency, and DOX-loaded hydrogels were obtained. Fig. 5D showed the release curves of DOX from hydrogel-2 at 37 °C in PBS (0.01 M) at different pH values (pH 5.0, 6.5 and 7.4) during 168 h. It was obvious that in vitro DOX release from hydrogel-2 was pH-dependent, and performed a steady release pattern. The release rate and cumulative amount of DOX increased rapidly with the decrease of pH values. At physiological pH (7.4), only 39% of the loaded DOX was released from the hydrogel even after 7 d. Besides, the release rate increased when the DOX-loaded hydrogels were placed into mildly
an acidic environment. At pH 5.0 and 6.5, the release rate was 40%, 36.2% (24 h), 83.6% and 71.3% (168 h), respectively. Hydrogel-2 was possessed of opportune stability at physiological environments, which was attributed to the crosslinking linkage resulted from OEDe. However, there were still a portion of the loaded DOX would be gradually released with the swelling of hydrogel-2 in neutral solution. On the contrary, the ortho ester bonds of OEDe in hydrogels would be rapidly hydrolyzed at mildly acidic environment such as pH 5.0 and 6.5,
leading to the faster swelling and degradation of these hydrogels. Therefore, the absorbed DOX in hydrogels would be released quickly and completely at acid environment. The pictures of the frame showed the representative images of DOX-loaded hydrogels after incubation for 168 h. It was apparent that the color intensity (red) of DOX-loaded hydrogels was in the following order: pH 7.4 > pH 6.5 > pH 5.0, indicating that the remnant DOX of hydrogels at pH 7.4 was much higher than that at pH 5.0 and 6.5. The CMCS/OEDe hydrogels with pH-dependent degradation and drug release behaviors were expected to be desirable as potential drug carriers.

3.12. In vitro cytotoxicity

To confirm the cytotoxicity of CMCS/OEDe hydrogels, a series of degradation products with different concentrations and the crosslinker were co-culture with SH-SY5Y and 293T cells for 48 h, and then the cell viabilities were measured by MTT assay. Simultaneously, the pure CMCS solution was used as control. The results were shown in Fig. 6A and B. Obviously, all degradation products and OEDe didn’t display any cytotoxicity against SH-SY5Y and 293T cells even at the highest concentration (2.5 mg/mL), suggesting that the degradation products were non-toxic and presented a predictable biocompatibility.

The intact crosslinked hydrogels (hydrogel-1, hydrogel-2 and hydrogel-3) were sterilized and co-cultured with SH-SY5Y and 293T cells for 48 h. Subsequently, the cell morphology and growth status were observed by an inverted microscope, as shown in Fig. 6C and D. It was found that the crosslinked hydrogels didn’t show the cytotoxicity against SH-SY5Y and 293T cells, since the cell adherence and proliferation were unsusceptible during the observation. These results demonstrated that these crosslinked hydrogels and their degradation products displayed negligible cytotoxicity on normal cells and cancer cells, indicating that they could be securely used as anticancer drug carriers.

3.13. Apoptosis analysis

In order to better understand the superiority, SH-SY5Y and 293T cells were co-cultured with CMCS/OEDe hydrogels or their degradation products for 24 h and then stained by an Annexin V-EGFP/PI apoptosis kit. Subsequently, cell viability was qualitatively observed by a fluorescence microscope. As shown in Fig. 6E-L, cells in all groups were almost stained to green by Annexin V, and cells were rarely stained to red by propidium iodide, indicating that most cells were alive after co-culture with hydrogels or their degradation products.

To further demonstrate the non-toxicity of these hydrogels, cell viability was quantitatively analyzed by flow cytometry, as shown in Fig. 7. The results showed that most cells co-cultured with degradation solution or hydrogels were alive. For control group, cell viability of SH-SY5Y and 293T cells reached 94.95% and 94.47%, respectively. Besides, SH-SY5Y cell viabilities were all higher than 90% after co-culture with hydrogel-1, hydrogel-2, hydrogel-3 and their corresponding degradation products. For normal cell lines (293T), cell viabilities were not significantly different with hydrogel-1, hydrogel-2, hydrogel-3 and their corresponding degradation products. For normal cell lines (293T), cell viabilities were all higher than 90% after co-culture with hydrogel-1, hydrogel-2, hydrogel-3 and their corresponding degradation products. For normal cell lines (293T), cell viabilities were all higher than 90% after co-culture with hydrogel-1, hydrogel-2, hydrogel-3 and their corresponding degradation products. For normal cell lines (293T), cell viabilities were all higher than 90% after co-culture with hydrogel-1, hydrogel-2, hydrogel-3 and their corresponding degradation products. For normal cell lines (293T), cell viabilities were all higher than 90% after co-culture with hydrogel-1, hydrogel-2, hydrogel-3 and their corresponding degradation products.

4. Conclusion

In this work, a series of pH-sensitive CMCS/OEDe hydrogels were prepared by crosslinking CMCS with an acid-labile ortho ester-based cross-linker (OEDe) via ring-opening reaction under mild conditions. The micromorphology, mechanical properties and rheological behavior could be controlled readily by altering the feed ratios of CMCS and OEDe. More importantly, these crosslinked hydrogels displayed controlled stability and degradability under different pH values. The introduction of pH-sensitive ortho ester linkage into the hydrogels could make them stable at physiological condition (pH 7.4), while would be possessed of rapid swelling and degradation at mildly acidic environment (pH 5.0). Therefore, DOX was chosen as a model drug and loaded into these hydrogels. In vitro drug release at different pH values demonstrated that the DOX-loaded hydrogels had pH-triggered drug release behavior and the release rate increased with the decrease of pH values. Furthermore, in vitro cytotoxicity tests confirmed the excellent biocompatibility of these hydrogels and their degradative products. All results indicated that CMCS/OEDe hydrogels had great potential in biomedical applications by further optimization.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 51503001), the Research Foundation for Key Program of Education Department of Anhui Province of China (No. KJ2016A030), the Doctor Research Foundation of Anhui University (No. J10113190075), and the Academic and Technology Introduction Project of Anhui University (AU02303203).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.09.004.

References


