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Cytocompatible injectable carboxymethyl chitosan/*N*-isopropylacrylamide hydrogels for localized drug delivery

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ABSTRACT

Cytocompatible injectable hydrogels with pH and temperature sensitivity based on carboxymethyl chitosan-*graft*-poly (*N*-isopropyl acrylamide)-glycidyl methacrylate (CMCS-PNIPAm-GMA) were prepared by UV crosslinking, and these hydrogels as localized drug carriers for anticancer drug and anti-inflammatory drug were also investigated. The chemical structure of CMCS-PNIPAm-GMA and of their hydrogels was characterized by FT-IR and NMR. The effect of PNIPAm grafting percentage, pH and temperature on the swelling ratio of the hydrogels was studied, demonstrating the pH/temperature-responsive nature of the hydrogels. The morphology of the hydrogels before and after swelling was observed by scanning electron microscope. 5-Fluorouracil and diclofenac sodium as model drugs were encapsulated into the hydrogels *in situ*. Moreover, the effect of pH and temperature on the release of these drugs was discussed. The cytocompatibility of the macromonomer CMCS-PNIPAm-GMA and their hydrogels was studied with dog bone marrow mesenchymal stem cells by using Alamar blue measurement and Live/Dead assay kit. All the results indicated that these degradable injectable hydrogels are good candidates for localized delivery systems of drugs.

1. Introduction

Biocompatible and biodegradable injectable hydrogels formed *via in situ* chemical polymerization or *via* the sol–gel phase transition have gained more and more attention recently (Du, Hamilton, Reilly, & Ravi, 2012; Ko, Shinde, Yeon, & Jeong, 2013; Tan & Marra, 2010). They have been found wide application in biomedical fields, being used as drug delivery systems, cell carriers and scaffolds for tissue regeneration (Delair, 2012; Li, Rodrigues, & Tomas, 2012; Li, Shen, et al., 2012; Nakai et al., 2012; Yu & Ding, 2008). The hydrogel precursors loaded with bioactive molecules such as drugs or growth factors were flowable aqueous solutions before injection, and they rapidly changed into gel under physiological conditions once injected. Therefore, injectable hydrogels containing drugs can be operated in a minimally invasive manner, causing less pain for patients. Following gelation, these matrices become specific-site drug delivery depots for pharmaceutics. Many methods have been developed for fabrication of *in situ* forming injectable hydrogels, such as thermal gelation, ionic complex, self-assembly, chemical crosslinking and photopolymerization (Overstreet, Dutta, Stabenfeldt, & Vernon, 2012; Yu & Ding, 2008). Among these methods, photopolymerization is one of the green techniques, which has good spatial and temporal control over the gelation process under sufficiently mild or physiological conditions (Ifkovits & Burdick, 2007; Zhang, Zhan, & Shi, 2011). Photopolymerization technique has already been employed to synthesize functional polymers and hydrogels for biomedical applications by us (Guo, Finne-Wistrand, & Albertsson, 2011a, 2011c, 2012) and others (Di Biase et al., 2011; Ortiz, Martinez, Valdez, & Duarte, 2010). It has been reported that the UV or visible light could penetrate the surface of the skin to form *in situ* hydrogels after the macromer solution was injected subcutaneously (Anseth et al., 2002; Elisseeff, Anseth, Sims, McIntosh, Randolph, & Langer, 1999; Elisseeff, Anseth, Sims, McIntosh, Randolph, & Yaremchuk, 1999). This means the hydrogels can be formed *in vivo* by transdermal photopolymerization with minimally invasive implantation (Elisseeff, Anseth, Sims, McIntosh, Randolph, & Langer, 1999; Elisseeff, Anseth, Sims, McIntosh, Randolph, & Yaremchuk, 1999;

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Lin, Chen, Moreno-Luna, Khademhosseini, & Melero-Martin, 2013). Moreover, photocrosslinking of water soluble macromonomers can avoid the presence of the unreacted residual monomers and the use of organic solvent in the drug loading process (Ito, 1998).

During the past decades, various naturally and synthetically derived materials were utilized to prepare injectable hydrogels for drug delivery systems (Kim et al., 2012; Rahman et al., 2012). Biopolymers have been extensively used as injectable hydrogels for drug carries due to their excellent biocompatibility and biodegradability (Barbucci, Giardino, De Cagna, Golini, & Pasqui, 2010; Gao et al., 2010; Zhou, Zhang, Zhang, & Chen, 2011). Chitosan is one of the most abundant biopolymers. It contains one amino group and two hydroxyl groups in the repeating glucosidic residue (Bernkop-Schnurch & Dunnhaupt, 2012; Guo, Finne-Wistrand, & Albertsson, 2011b; Hu, Sun & Wu, 2013; Yu et al., 2011). However, chitosan can just dissolve in acid solution, which greatly restricts its application. Various chitosan derivatives have been therefore developed to increase its water solubility at physiological pH condition (Faizuloev et al., 2012; Guo, Yuan, & Gao, 2007b, 2008b). Carboxymethyl chitosan (CMCS) is not only soluble in water, but also has excellent chemical, physical and biological properties including low toxicity, biocompatibility and film, gel-forming capabilities. It has been widely used in biomedical fields such as drug delivery carrier, antimicrobial material, gene delivery system and tissue engineering (Guo, Yuan, & Gao, 2008a; Jayakumar et al., 2010; Upadhyaya, Singh, Agarwal, & Tewari, 2013). Furthermore, it shows good pH and ion sensitivity in aqueous solutions because it simultaneously contains amino groups (—NH_2) and carboxyl groups (—COOH) (Chen, Tian, & Du, 2004; Guo, Yuan, Yao, & Gao, 2007c).

Smart hydrogels or intelligent hydrogels which change their various properties upon environmental stimuli including pH or/and temperature, ionic strength, light, electric field and magnetic field, have found wide application in biomedical and pharmaceutical areas (Ajazuddin et al., 2012; Guo, Yuan, & Gao, 2007a; Qiu & Park, 2012; Schmalz, Schmalz, & Muller, 2012). Among these systems, pH and temperature responsive hydrogels have been most extensively studied because these two factors are crucial to human body and can be easily controlled both *in vitro* and *in vivo* conditions (Carreira, Goncalves, Mendonca, Gil, & Coelho, 2010; Li, Rodrigues, & Tomas, 2012; Li, Shen, et al., 2012; Mao, Bo, & Ji, 2011). Poly(*N*-isopropylacrylamide) (PNIPAm) is one of the most well-known temperature sensitive polymers with a lower critical solution temperature (LCST) at 32 °C around body temperature (Iijima & Nagasaki, 2006; Wei, Cheng, Zhang, & Zhuo, 2009). The combination of CMCS and PNIPAm has been investigated by some researchers (Chen et al., 2010; Zhang et al., 2009) and our group (Guo & Gao, 2007). Zhong's group prepared thermo-responsive core-shell microgels based on PNIPAm and CMCS by emulsion polymerization (Chen et al., 2010). We synthesized interpenetrating polymer networks based on CMCS and PNIPAm by using ammonium persulphate as initiator and *N*,*N*′-methylenebisacrylamide as crosslinker, and studied their controlled release of coenzyme A (Guo & Gao, 2007). However, *in situ* forming injectable hydrogels based on CMCS and PNIPAm have not been reported.

The aim of this work is to develop cytocompatible pH/thermo-responsive injectable hydrogels based on carboxylmethyl chitosan and poly(*N*-isopropyl acrylamide) by photocrosslinking for localized drug delivery. We first grafted PNIPAm onto CMCS and then used ring opening reaction of epoxy group from glycidyl methacrylate (GMA) to introduce double bonds to the CMCS-PNIPAm copolymer. The CMCS-PNIPAm-GMA hydrogels were obtained from the aqueous solution of the macromer CMCS-PNIPAm-GMA *in situ* by photocrosslinking reaction. The chemical structure, swelling behavior, morphology, drug delivery and cell cytotoxicity of the injectable hydrogels were investigated. By developing intelligent injectable hydrogels as localized drug delivery systems from CMCS and PNIPAm, we are taking next step toward the practical applications of these hydrogels.

2. Materials and methods

2.1. Materials

Chitosan (CS, molecular weight 100000–300000) with a degree of deacetylation of 86% and *N*-isopropylacrylamide (NIPAm) were obtained from J&K Scientific Ltd. Monochloroacetic acid, ammonium persulphate ($(NH_4)_2S_2O_8$, APS), glycidyl methacrylate (GMA), photoinitiator 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (I2959), diclofenac sodium (DCS) and 5-flurouracil (5-Fu) were all purchased from Aldrich and were used as received. Sodium hydroxide (NaOH), isopropanol, anhydrous ethanol and acetone were used as received.

2.2. Synthesis of carboxymethyl chitosan

Carboxymethyl chitosan (CMCS) was prepared as described previously (Wang et al., 2010). In a typical procedure, 0.5 g chitosan, 0.85 g sodium hydroxide and 6.25 mL solvent (water/isopropanol = 1/4(v/v)) were added to a flask (50 mL) to swell and alkalize at 50 °C for 1 h. 0.9375 g monochloroacetic acid in 1.25 mL isopropanol was then added to the mixture dropwise. After reacted for 4 h at 50 °C, the mixture was dissolved in 30 mL of water and centrifuged to remove trace amounts of precipitates. The solution was precipitated in 150 mL of anhydrous ethanol. Finally, the product was filtered and rinsed thrice with 70–90% ethyl alcohol to desalt and dewater, and vacuum dried at room temperature. The product obtained was the sodium salt of the CMCS, and the substitution degree of CMCS was 89%.

2.3. Synthesis of CMCS-PNIPAm copolymer

The copolymer of CMCS-PNIPAm was synthesized *via* a free radical polymerization route as shown in Scheme 1. An appropriate amount of CMCS (0.24 g) was first dissolved in 18 mL deionized water under magnetic stirring. After CMCS solution was heated to 70 °C under nitrogen atmosphere, appropriate amount of ammonium persulfate (Table 1) was added. Then the solution was stirred for 10 min before different amount of NIPAm (Table 1) was added. The mixture was stirred for 3 h under nitrogen atmosphere. The solution was then precipitated in acetone. The final product was obtained after centrifuged and dried under vacuum.

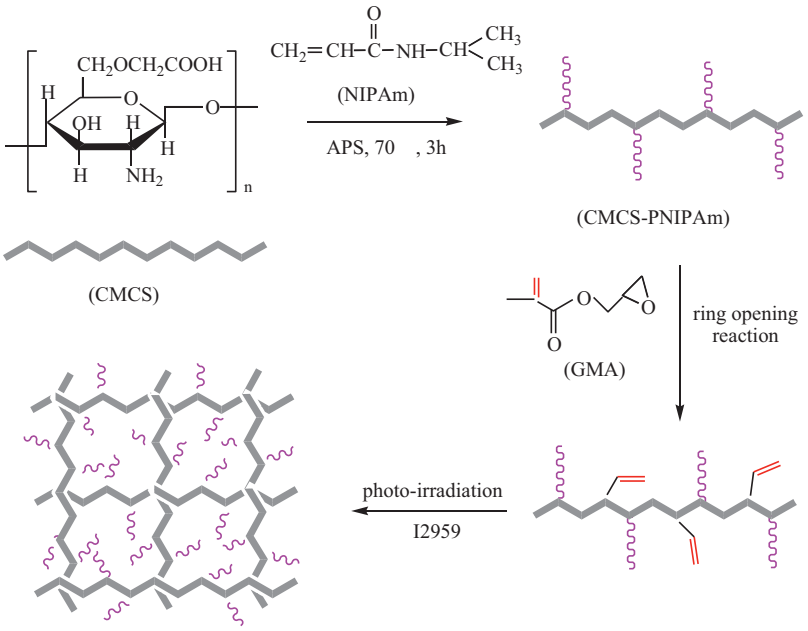
The samples were coded as G1, G2 and G3 which meant the samples with PNIPAm grafting percentage of 80.1%, 134.5% and 172.4%, respectively.

2.4. Synthesis of CMCS-PNIPAm-GMA

In a typical procedure, 0.4 g CMCS-PNIPAm copolymer was dissolved in 20 mL 0.05 M NaOH. 0.1 g of glycidyl methacrylate in 1 mL of isopropanol was then added to the CMCS-PNIPAm solution. After reacted for 6 h at 60 °C under nitrogen atmosphere, the solution was precipitated in acetone and washed until neutral. The final product was dried in a vacuum oven. The G1, G2, and G3 copolymers after grafting with GMA were designated as G1G, G2G, and G3G copolymers, respectively.

2.5. Synthesis of CMCS-PNIPAm-GMA hydrogel

I2959 was used as photoinitiator in this study, as it has been demonstrated to be the least cytotoxic (Bahney et al., 2010). CMCS-PNIPAm-GMA (5 wt%) containing the I2959 (1 wt% of CMCS-PNIPAm-GMA) was first dissolved in deionized water. The solution



Scheme 1. Synthetic route of CMCS-PNIPAm-GMA hydrogels.

was then exposed to UV lamp (Osram Ultra-Vitalux 300-W lamp). The distance between the vial and the lamp was about 80 cm. Photopolymerization was subsequently conducted for 15 min to yield the hydrogels.

The drug loaded hydrogels were prepared in a similar manner as that of the CMCS-PNIPAm-GMA hydrogels, except that the drug was dissolved in the CMCS-PNIPAm-GMA solution before photocuring. After photocrosslinking, the drug was loaded in the hydrogel *in situ*.

2.6. Characterization

FT-IR spectra of CMCS, CMCS-PNIPAm and CMCS-PNIPAm-GMA graft copolymer were recorded on a FT-IR spectrometer (Thermo Scientific Instrument) in the range from 4000 cm^{-1} to 600 cm^{-1}.

^1H NMR (400 MHz) spectra of CMCS, NIPAm, CMCS-PNIPAm and CMCS-PNIPAm-GMA graft copolymer were obtained on a BRUKER Avance spectrometer with D_2O as solvent at room temperature and internal standard (δ 4.79).

The percentage and efficiency of grafting were calculated by the difference of weights before and after grafting reaction according to the following formula: Grafting percentage (%) = $((W_g - W_c)/W_c) \times 100\%$; Grafting efficiency (%) = $((W_g - W_c)/W_m) \times 100\%$; where W_g, W_c and W_m are the weights of purified graft copolymer, CMCS and NIPAm monomer, respectively.

Swelling ratio (SR) of the hydrogels was calculated from swelling measurements. The dry hydrogels were immersed in aqueous solutions of the desired conditions (temperature at 25 °C or 37 °C, pH 2.1 or 7.4) in sealed vials. The hydrogels were withdrawn from the solution after regular periods of time and weighed after the removal of excess surface water with a filter paper, and were then returned to the same vial until swelling equilibrium was established. SR was calculated from $SR = ((W_s - W_d)/W_d) \times 100\%$, where W_s and W_d are the weights of the swollen and dry state samples, respectively.

The morphology of the hydrogels before and after swelling was observed by using scanning electron microscope (FE-SEM, SU-8000, Hitachi, Japan). The hydrogels were freeze-dried with a freeze-dryer (CHRIST) after swelling.

The drug loaded hydrogel disks were placed into a flask containing 50 mL buffer solution. The flask was then put into the constant temperature-shaking incubator at 37 °C or 25 °C with 100 rpm. At designed time points, 2 mL solution was taken out and the same fresh buffer solution was added to keep the same solution volume. The cumulative release of drug was analyzed by monitoring the UV absorption of the drugs (UV absorption of 5-Fu: 267 nm; UV absorption of DCS: 276 nm).

2.7. Cell culture

2.7.1. Mesenchymal stem cell (MSC) isolation and expansion

Dog bone marrow derived MSCs were isolated from femur of 2 weeks old Beagle dog. The femur was collected under sterile condition, stripped of other connective tissue, and then the femoral heads were removed to obtain the marrow. The marrow was suspended in growth medium (low glucose α-MEM (GIBCO), 10% (v/v) fetal bovine serum (GIBCO), 100 U/mL penicillin and 0.1 mg/mL streptomycin) at 37 °C under 5% CO_2. Medium was changed to remove non-adherent cells after 24 h. When the confluence reached 80%, the cells were detached by 0.25% trypsin and passaged at 1:3 dilutions. The cultures were replaced with fresh medium twice a week. Passage 5 (P5) cells were used for proliferation and cytotoxicity study.

Table 1
Feed composition of CMCS-PNIPAm copolymer and PNIPAm graft efficiency and grafting percentage.

Sample name	CMCS (g)	H_2O (mL)	APS (g)	NIPAm (g)	Grafting efficiency (%)	Graft percentage (%)
G1	0.24	18	0.006	0.6	32.0	80.1
G2	0.24	18	0.008	0.8	40.4	134.5
G3	0.24	18	0.01	1.0	41.4	172.4

2.7.2. Cytotoxicity of different CMCS-PNIPAm-GMA polymer solutions

To measure the cytotoxicity of the G1G, G2G and G3G polymer solution, a qualitative viability assay was performed by using a Live/Dead assay kit (Molecular Probes). Briefly, MSCs were seeded into a 96 wells plate at a density of 3000 cells/cm^2, and pre-cultured for 12 h before changing the fresh medium containing 10 mg/mL each polymer solution. Medium containing 0 mg/mL polymer solution served as the positive control. After cultured for 24 h, MSCs were washed with serum-free medium three times and treated with Ethidium homodimer-1 (0.5 mM) and calcein AM (0.25 mM) for 1 h. Cells were observed under an inverted fluorescent microscope (IX53, Olympus, Japan).

2.7.3. Cell proliferation assay of hydrogel extract solution

The MSCs proliferation in medium containing 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL hydrogel extract solution were also investigated. After immersed in medium for 24 h, the extract solution was sterilized by a 0.22 μm syringe filter. MSCs were seeded in 96-well plate at a density of 3000 cells/cm^2, and pre-cultured for 12 h before changing the fresh medium containing a series of different concentrations of hydrogel extract solution. After cultured for 1, 2, 3, 4 and 5 days, MSCs were incubated in medium containing 10% (v/v) Alamar blue dye (Molecular Probes). The plate was then incubated at 37 °C in 5% CO_2 for 4 h. After 4 h incubation, 100 μL medium from each example was read at 560/590 nm in a SpectraMax fluorescence microplate reader (Molecular Devices). Medium containing 10% (v/v) Alamar blue dye served as negative control and medium containing 0 mg/mL hydrogel extract solution served as positive control.

Cell morphology was examined by cytoskeleton and nucleus staining. Phalloidin conjugated FITC (green) was used to visualize F-actin filaments and DAPI (blue) was used to visualize nuclei. Cell morphology was observed under an inverted fluorescent microscope (IX53, Olympus).

2.7.4. Statistical analysis

All data were expressed as means ± standard deviations. To test the statistical significance between experimental groups and control group, Student's *t*-test was used. Statistically significant values were defined as $P < 0.05$.

3. Results and discussion

3.1. Synthesis of the injectable hydrogels by photocrosslinking

The NIPAm was grafted to CMCS main chain by using APS as an initiator. The grafting efficiency was between 32.4% and 41.4%, and the grafting percentage was 80.1%, 134.5% and 172.4% for samples G1, G2 and G3, respectively (Table 1), indicating that the grafting percentage onto the CMCS increased with the increase of the NIPAm concentration. Therefore, we could control the grafting percentage of NIPAm onto the CMCS main chain in a wide range by using different APS amounts in the reaction system. By changing the ratio of CMCS and NIPAm in the copolymer, we could tune the swelling ratio of hydrogels in the later stage. It is well-known that PNIPAm is not biodegradable (Patenaude & Hoare, 2012), and CMCS can be degraded by chitosanase enzymes (Ichikawa, Iwamoto, & Watanabe, 2005). The introduction of PNIPAm into the CMCS would therefore decrease the degradation rate of the hydrogels. In the next step, the GMA was grafted to CMCS-PNIPAm copolymer by ring opening reaction between $-NH_2$ from CMCS and epoxy group from GMA. The double bonds were connected to the CMCS-PNIPAm, and they were used for the photocrosslinking to create the injectable hydrogels in the next step. The drugs can be incorporated into the hydrogels *in situ* before photocrosslinking.

The chemical structure of CMCS-PNIPAm-GMA copolymer and their injectable hydrogels was confirmed by NMR and FT-IR. The ^1H NMR spectrum of CMCS was shown in Fig. 1(a). The peak at 3.9 ppm is attributed to $-CH_3COO^-$ group, indicating that CMCS was formed. The peaks of double bond (CH_2=CH−) at 6.12 ppm and 5.62 ppm, the peak of N−CH− group at 3.89 ppm and the peak of $-CH_3$ group at 1.12 ppm appeared in the spectrum of NIPAm in Fig. 1(b). In curve c, the peaks of double bonds at 6.12 ppm and 5.62 ppm are absent, and the peaks at 3.89 ppm and 1.12 ppm assigned to NIPAm are still present, indicating that the PNIPAm chain was grafted on the CMCS chain. Compared to curve c, curve d of CMCS-PNIPAm-GMA copolymer showed two peaks at 6.56 ppm and 5.56 ppm attributing to the double bonds from GMA and one new peak at 2.11 ppm attributing to $-CH_3$, indicating that GMA was bonded to CMCS-PNIPAm. These double bonds were used to create injectable hydrogels by photopolymerization in the next step.

The IR spectrum of CS showed absorption peaks at 1022 and 1155 cm^{-1} corresponding to vibrational modes of saccharide units in chitosan (Fig. 1 in supporting information (SI)). The peaks of non-modified chitosan at 1647 and 1590 cm^{-1} are assigned to the C=O stretching (amide) and N−H bending (amine), respectively. In curve b of CMCS, a new peak at 1590 cm^{-1} appeared compared to CS which is attributed to carboxylate group (Zhu, Chan-Park, Dai, & Li, 2005), indicating that the CMCS was obtained. In curve c of CMCS-PNIPAm, the peaks at 1620 cm^{-1} corresponding to amide group in NIPAm, and the band at 2973 cm^{-1} attributing to $-CH(CH_3)_2$ groups demonstrated that PNIPAm was grafted onto the main chain of CMCS. The CMCS-PNIPAm-GMA showed a new peak at 1710 cm^{-1} assigned to the ester group of GMA and there is no peak of epoxy group at 906 cm^{-1}, indicating that GMA was connected to the CMCS by a ring opening reaction of epoxy group from GMA with $-NH_2$ or −OH group in CMCS. The IR spectrum of the CMCS-PNIPAm-GMA hydrogel is similar to that of CMCS-PNIPAm-GMA copolymer due to the low content of GMA.

The hydrogels formation was also confirmed by test tube inversion method (Tang & Singh, 2009). The CMCS-PNIPAm-GMA in water is a transparent solution, and it can not flow in the vial after UV-crosslinking (Fig. 2 in SI), indicating that the injectable hydrogel was obtained.

3.2. Swelling behavior of the hydrogels under different pH and temperature

The SR of the hydrogels G1G, G2G and G3G with PNIPAm grafting percentage of 80.1%, 134.5% and 172.4% are shown in Fig. 2. G1G hydrogel showed the fastest swelling speed, and its SR reached to 720% at 80 min in Fig. 2(a). The reason is that CMCS with a large amount of carboxyl groups and amine groups is quite hydrophilic and the electrostatic repulsion between the carboxylate groups ($-COO^-$) at pH 7.4 solution led to the swelling of the hydrogel. G2G and G3G hydrogels showed a SR of 630% and 290%, respectively, meaning that the SR decreased with increasing PNIPAm grafting percentage. The introduction of PNIPAm to the CMCS main chain would result in a large amount of hydrogen bonds between the −NHCO− group and $-NH_2$, −OH group from CMCS, which would reduce the hydrophilicity of the network, and the hydrogen bonds would hinder the swelling of the hydrogel network. So the SR decreased with increasing the PNIPAm grafting percentage. The SR of G1G, G2G and G3G hydrogels with different PNIPAm grafting percentage as shown in Fig. 2(b) and (c) at different temperature and pH showed a similar trend as that in Fig. 2(a).

The effect of temperature on SR of the hydrogels was studied, and the results are shown in Fig. 2(a) and (b). It can be observed that G1G, G2G and G3G hydrogels in 7.4 solution under 37 °C in Fig. 2(b) showed a SR of 260%, 130% and 80%, respectively, and

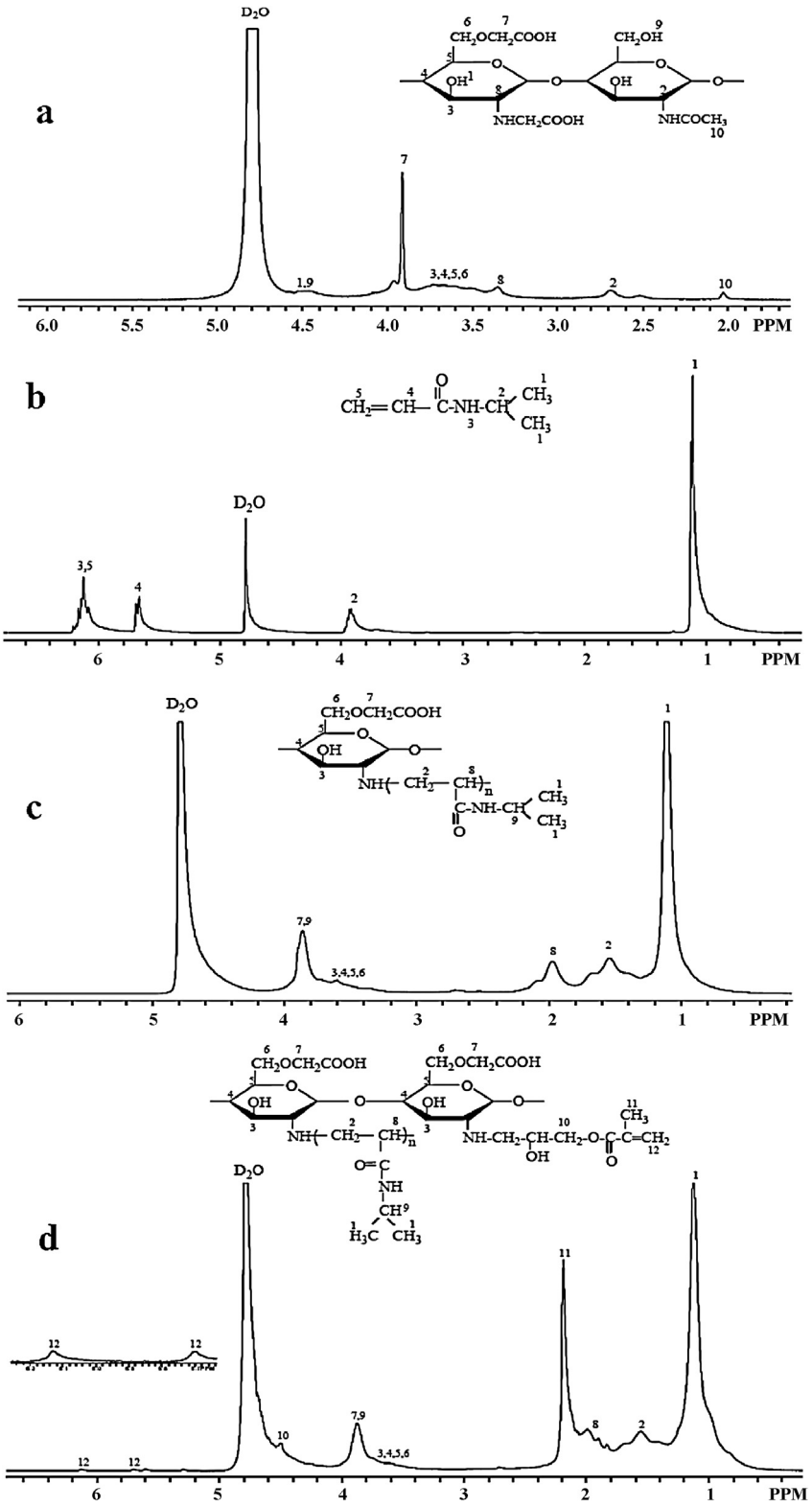


Fig. 1. ^{1}H NMR spectra of (a) CMCS, (b) NIPAm, (c) CMCS-PNIPAm, and (d) CMCS-PNIPAm-GMA.

they are much smaller compared to the SRs in Fig. 2(a) at 25 °C, indicating that the SR decreased dramatically with increasing temperature. This is because the PNIPAm with a LCST of 32 °C in the hydrogels contained hydrophilic —NHCO— group and hydrophobic —$CH(CH_3)_2$ group. At a lower temperature (25 °C), the hydrogen bonds are formed between —NHCO— and water molecules. When the temperature increases to 37 °C, the hydrogen bonds between water and —NHCO— group are quite weak, and the hydrophobic interaction between the —$CH(CH_3)_2$ group is strengthened. As a consequence, the PNIPAm chains are quite hydrophobic and are in a shrinking state. So the SR of the hydrogels is much smaller at high temperature (37 °C).

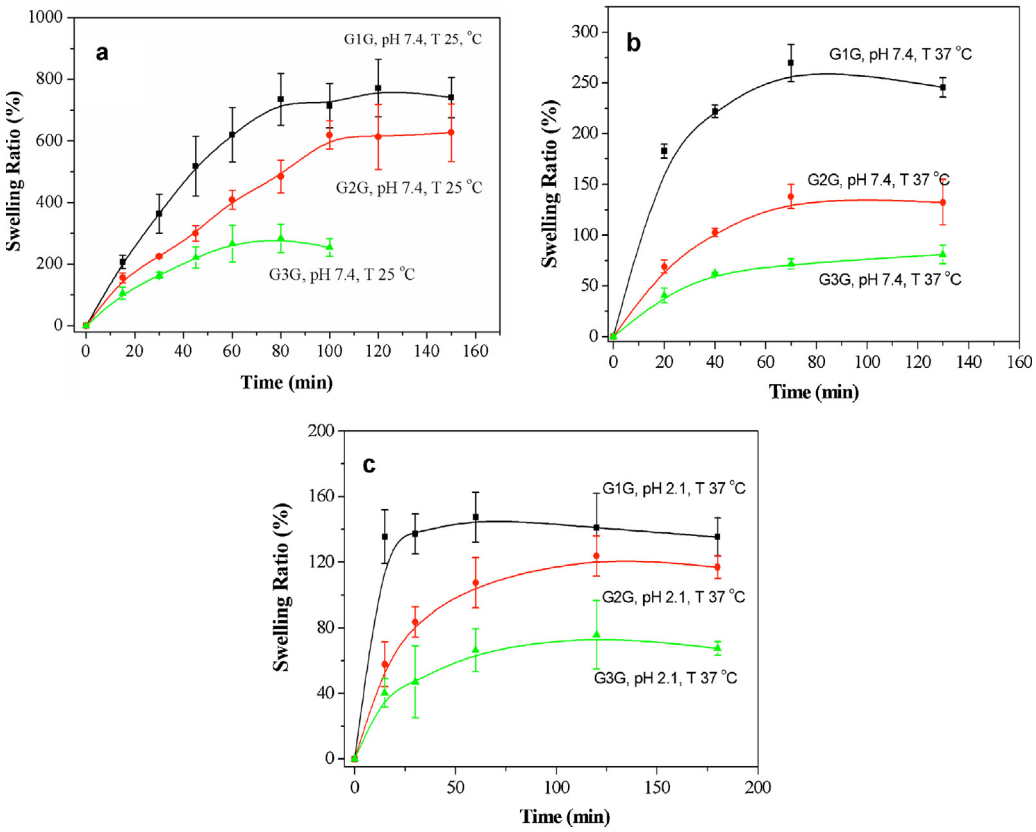


Fig. 2. Effect of temperature and pH on the swelling of CMCS-PNIPAm-GMA hydrogels with different PNIPAm grafting percentage.

The effect of pH of the swelling medium on the SR is shown in Fig. 2(b) and (c). The SRs of G1G, G2G and G3G hydrogels in Fig. 2(c) are 145%, 120% and 72%, respectively. In pH 2.1 solution at 37 °C, the —NH_2 group in the hydrogels changed into —NH_3^+, and the electrostatic repulsion between them leads to the swelling of the hydrogels. On the other hand, the H-bonds between the —COOH, —OH and —NHCO— groups and the hydrophobic nature of PNIPAm at 37 °C restrict the swelling of the hydrogels. As a result of the two effects, the SRs of the hydrogels are smaller in Fig. 2(c) compared to SRs in Fig. 2(b). Furthermore, the differences of SR of the hydrogels between 25 °C and 37 °C are affected by the pH of swelling medium. Generally, the differences of SR at pH 7.4 solution under 37 °C (Fig. 2(b)) are more apparent than that at pH 2.1 solution at 37 °C (Fig. 2(c)). In pH 7.4 solution, the CMCS is in a swelling state and thus the degree of freedom of PNIPAm chains is bigger. The different swelling behavior of these hydrogels would affect their morphologies and their drug release profiles.

3.3. Morphology of the hydrogels

The morphology of the hydrogels before and after swelling was characterized by SEM. The hydrogels G1G, G2G and G3G before swelling (Fig. 3(a)–(c) in SI) generally showed a rather smooth surface, although the roughness increased slightly with the PNIPAm grafting percentage increasing. The G1G, G2G and G3G hydrogels after swelling in pH 7.4 under 37 °C possessed a large amount of holes, and the holes decreased with PNIPAm grafting percentage increasing, which agreed with the SR results as shown in Fig. 2(b). The morphology of the hydrogel G3G in pH 2.1 solution at 37 °C (Fig. 3(g) in SI) showed a large amount of holes with very small diameter. However, the holes of the hydrogels G3G in pH 7.4 solution at 25 °C were quite large (Fig. 3(h) in SI), which was also accordance with the SR results, that is, the G3G in pH 7.4 solution under 25 °C had a much bigger SR about 280% than SR about 70% in pH 2.1 solution at 37 °C.

3.4. Drug delivery properties of the hydrogels

Sustained and localized drug release system not only reduces administration times and undesired side effects of the drug, but also greatly enhances the patients' compliance and comfort. The injectable hydrogel/drug formulation is easily controlled *via* altering the material properties for the release of the drug. Moreover, the pH and temperature also significantly affects the

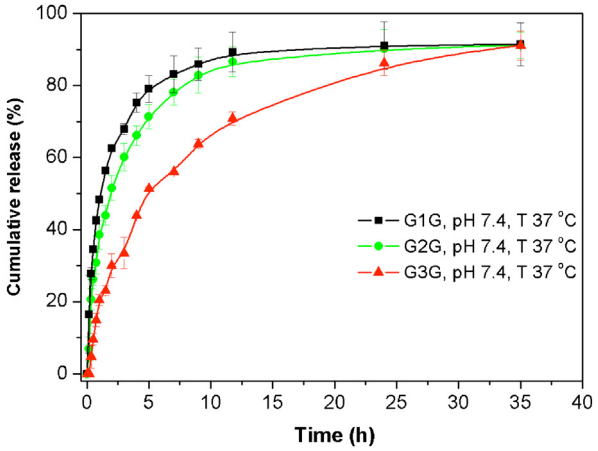


Fig. 3. Drug release curves with 5-Fu in pH 7.4 solution at 37 °C.

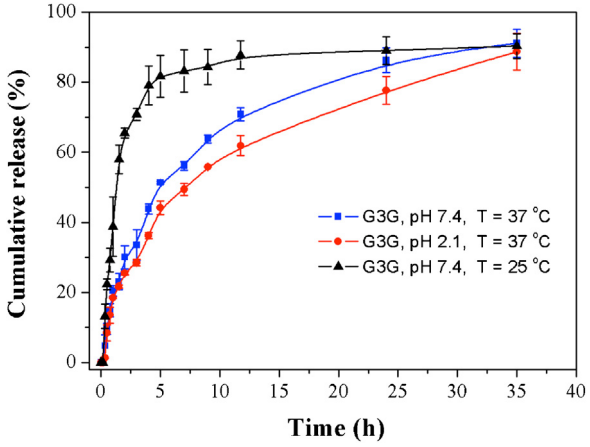


Fig. 4. Effect of temperature and pH on the release of 5-Fu.

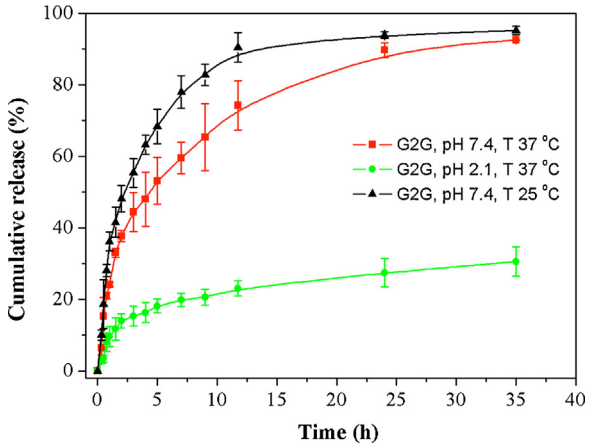


Fig. 5. Release profile of DCS from hydrogel G2G under different pH and temperature.

release of the drug from the pH/temperature-responsive hydrogels. We chose 5-Fu which is a frequently used anticancer drug and DCS which is anti-inflammatory drug as two model molecules, and tested the release behavior of the hydrogels to demonstrate the capacity of these hydrogels as localized drug carrier.

3.4.1. Effect of grafting percentage of PNIPAm on drug release

The effect of PNIPAm grafting percentage on the release of 5-Fu from the hydrogels was shown in Fig. 3. It is obvious that the release order of 5-Fu from the hydrogels was G1G > G2G > G3G, indicating that the higher PNIPAm grafting percentage decreased the release of 5-Fu from the matrix. This can be explained by the swelling behavior of the hydrogels. G3G with highest PNIPAm grafting percentage showed the smallest SR and it had quite small pores (Fig. 3(f) in SI), so the 5-Fu molecule was difficult to diffuse out of the matrix. For hydrogels G1G and G2G, more than 70% of the 5-Fu was released in 5 h. However, only about 45% of 5-Fu was released in G3G, and the release percentage reached to 70% in 15 h, and to 85% in 25 h. In this regard, a constant drug dose for 1 day may easily be achieved by once-daily administration. Moreover, there is no obvious burst release for all the hydrogels, which is another very important property for drug release system.

3.4.2. Effect of temperature and pH on the release of drug

The effect of temperature and pH on the release of 5-Fu was studied and the results are shown in Fig. 4. The release rate of 5-Fu from G3G hydrogel in pH 7.4 solution at 25 °C was about 65% at 2 h, and about 81% at 5 h, which was much faster than that at 37 °C, due to the fast swelling and the bigger SR of the hydrogel at 25 °C. The release of 5-Fu from the hydrogel at pH 2.1 under 37 °C was even slower than that in pH 7.4 solution at 37 °C, which also agreed with the SR as shown in Fig. 2(b) and (c). The PNIPAm is in a shrinking state above the LCST, and it forms a condensed structure of the hydrogels which hinders the release of 5-Fu. The hydrogen bonds between the —NH— group from 5-Fu (Scheme 1 in SI) and —OH, —NHCO— and —COOH from CMCS and PNIPAm is another factor contributing to the slow release from the hydrogels.

Diclofenac sodium (DCS) is one of the most frequently used non-steroidal anti-inflammatory drugs. Because of the short half-life in plasma (1–2 h) and associated adverse effects, it is regarded as an ideal model drug for controlled delivery system. DCS contains —NH— group and —COOH group (Scheme 1 in SI) which can be affected by the pH of the release medium. We encapsulated DCS in hydrogel G2G, and the release behavior was displayed in Fig. 5. The hydrogel showed the fastest release of DCS in pH 7.4 medium at 25 °C due to the fast swelling of the hydrogel. Increasing the temperature to 37 °C decreased the release rate of DCS, which was similar as that of 5-Fu release as shown in Fig. 4. However, the release percentage of DCS from pH 2.1 medium at 37 °C was only 15% at 2.5 h, and it increased to 22% at 12 h and to 27% at 24 h. This could be because the solubility of DCS decreased dramatically as the carboxyl group in DCS in pH 2.1 solution was in carboxyl acid state. Moreover, the hydrogen bonds between the —COOH from DCS and —OH, —NHCO— and —COOH in the network further hindered the release of DCS. This offers the practical application of the hydrogels for oral drug delivery system. The hydrogels would shrink and keep a significant fraction of the drug inside the matrix for 1–3 h in the low pH acid stomach environment, and the hydrogels would swell dramatically and a great deal of the drug was released from the hydrogels when they were transferred to the higher pH intestine condition. In a more practical point of view, these hydrogel materials could bypass the acidity of gastric fluid without releasing substantial amounts of the encapsulated drugs, indicating that they were ideal candidates for localized delivery system of drugs.

3.5. In vitro cytocompatibility of CMCS-PNIPAm-GMA and their hydrogels

3.5.1. Cytotoxicity of CMCS-PNIPAm-GMA polymer solutions

The cytotoxicity of the CMCS-PNIPAm-GMA polymer solutions was determined by Live/Dead assay kit, in which the green color stands for live cells (stained with Calcein AM), whereas the red color stands for dead cells (stained with EthD-1). After cultured for 24 h, dominant live (green) cells were observed in both experimental group and positive control group (Fig. 4 in SI). Those results revealed that the G1G, G2G and G3G are not cytotoxic.

There was no obvious effect of polymer solution on cell morphology after 24 h of culturing. Cytoskeleton staining results revealed that MSCs spread in the presence of the polymer solution, and all the cells showed a spindle-like morphology same as the positive control group.

3.5.2. Cell proliferation assay of G3G hydrogel extract solution

Cell proliferation in medium containing different concentration of G3G hydrogel extract solution was compared with that in normal growth medium at varying time points (Fig. 6). Because the extraction time was 24 h, the degradation of the hydrogels can be neglected. So the main composition of the hydrogel extraction is unreacted macromers and the photoinitiators. The increased fluorescence intensity demonstrated that the cell number had a continuous increase for all the groups. At the first day of culturing, the MSCs in 20 mg/mL hydrogel extract solution showed a higher fluorescence intensity than other groups containing the

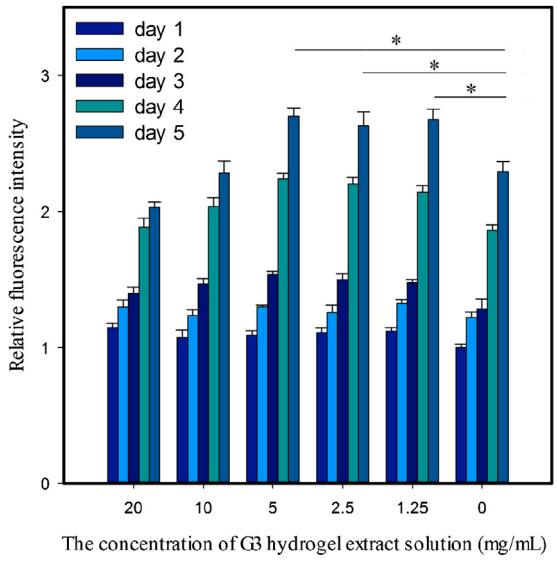


Fig. 6. The MSCs proliferation in the medium containing different concentration of G3G hydrogel extract solution, expressed as percent of fluorescence intensity of 0 mg/mL group at the first day. Mean for $n = 6 \pm$ SD. Error bar indicates standard deviation. $^{*}P < 0.05$.

hydrogel extraction, and it was even higher than that in 0 mg/mL group ($P < 0.05$). The results of second, third and fourth day indicated that the MSCs proliferated in medium containing hydrogel extract solution was even better compared with that in 0 mg/mL group. On the fifth day of culturing, the fluorescence intensity of the 20 mg/mL and 10 mg/mL hydrogel extraction group were slightly lower than that of 0 mg/mL group. However, the ratio of the fluorescence intensity between them was higher than 85%. Moreover, the groups of 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL of hydrogel extract solution showed a promoting effect on the cell proliferation, compared with 0 mg/mL group ($P < 0.05$). During the whole culturing period, the cell numbers of the groups of 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL significantly increased more than 2.4 times ($P < 0.05$), whereas the cell number in 0 mg/mL group increased less than 2.3 times. It was also found that a significant increase of the cell number was observed at days 3–5. This is because the cells firstly need to adjust when they were surrounded by a new environment, and the adjustment will take certain time. After the adjustment stage, cell will enter an exponential growth stage, and cell number exhibits a significant increase during this time.

The cytoskeleton and nucleus were stained to distinguish the effect of G3G hydrogel extract solution on the cell morphology at 1, 3, 5 days. The MSCs had a spindle-like shape in the presence of the different extract solution concentrations of the hydrogels (Fig. 5 in SI). It was obvious that even in the presence of 20 mg/mL of the hydrogels extract solution the MSCs also showed a normal spindle-like morphology compared with the 0 mg/mL group.

4. Conclusions

We demonstrated that the feasibility by employing cytocompatible pH/temperature-responsive injectable hydrogels composed of carboxymethyl chitosan (CMCS), poly (*N*-isopropyl acrylamide) (PNIPAm) and glycidyl methacrylate (GMA) as localized drug delivery system for anticancer drug and anti-inflammatory drug. The injectable hydrogels were synthesized by photocrosslinking of the CMCS-PNIPAm-GMA aqueous solution, and the anticancer drug 5-fluorouracil (5-Fu) and anti-inflammatory drug diclofenac sodium (DCS) were encapsulated in the hydrogels *in situ*. The release of these drug molecules was controlled by the PNIPAm grafting percentage of the hydrogels, pH and temperature of the release medium. The 5-Fu and DCS release from 25 °C solution was much faster than that at 37 °C solution. The DCS release percentage from the G2G hydrogel was 27% in pH 2.1 solution at 37 °C for 24 h, whereas it was 89% in pH 7.4 solution at 37 °C for 24 h, which means the hydrogels could protect the drug in low pH medium (such as stomach) and substantially release them at higher pH condition (such as intestine). The non-cytotoxicity of the prepolymer CMCS-PNIPAm-GMA and their injectable hydrogels was confirmed by mesenchymal stem cell culturing. All these data indicated that these cytocompatible injectable hydrogels have great potentials as localized drug delivery systems.

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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.2013.12.017.

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