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Preparation, characterization, and in vitro drug release behavior of 6-mercaptopurine-carboxymethyl chitosan

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ABSTRACT

A series of 6-mercaptopurine-carboxymethyl chitosans (6-MP-CMC) were prepared and structurally characterized. Their in vitro drug release behaviors in the buffer solutions containing glutathione (GSH) were investigated. 6-MP-CMC did not release any 6-MP in the media without GSH and containing 2 μM GSH. By comparison, the obvious 6-MP release could be observed within an hour in the media containing 2 mM and 10 mM GSH. And the maximum cumulative release rates were 65.1% and 74.4%, respectively. The buffer pH and the 6-MP content in 6-MP-CMC had obvious influences on the 6-MP release. At 10 mM GSH, the maximum cumulative release rate at pH 5 was higher than that at pH 7.4. 6-MP-CMC in pH 7.4 PBS could self-assemble into nanoparticles. Their mean hydrodynamic diameter determined by dynamic light scattering (DLS) was 155.8 ± 6.0 nm. The particles observed by transmission electron microscopy (TEM) were spherical in shape and had the size of about 100 nm.

1. Introduction

Amphiphilic polymers, consisting of hydrophilic and hydrophobic segments, have attracted the attention in drug carrier for a number of years because of their special physicochemical and morphological characteristics in water (Gaucher et al., 2005; Kataoka, Harada, & Nagasaki, 2001). Of all the amphiphilic polymers, natural polysaccharide derivatives which possess properties such as non-toxicity and biodegradability have been meticulously researched in recent years (Hu et al., 2008).

Carboxymethyl chitosan, a water soluble chitosan derivative, has the increased flexibility of molecular chains in water (Abreu & Campana-Filho, 2005). By hydrophobic modification, the obtained amphiphilic compounds can self-assemble into nanoparticles with a hydrophobic core and a hydrophilic shell. This kind of structure is suitable for loading hydrophobic drugs under mild conditions (Liu, Chen, Liu, & Liu, 2008). For instance, a group of amphiphilic carboxymethyl chitosan derivatives by hydrophobic modification with butyl glycidol ether were prepared and the surface activity and aggregated properties were studied (Sui, Wang, Chen, & Xu, 2004). Linoleic-acid modified carboxymethyl chitosan was synthesized with linoleic-acid groups attached to the amino groups to provide hydrophobic moieties and the efficacy of the self-assembled nanoparticles for the entrapment and release of adriamycin was investigated (Liu et al., 2007). Also, hydrophobically modified O-carboxymethyl chitosan was prepared by the formation of a succinyl linkage between O-carboxymethyl chitosan and cholesterol, which formed self-aggregated nanoparticles (Wang, Liu, Jian, & Zhang, 2007). Furthermore, paclitaxel was chosen as a model drug to assess the potential of the self-assembled nanoparticles as a carrier for anticancer therapy (Wang et al., 2008). In these delivery systems based on the amphiphilic carboxymethyl chitosan derivatives, drugs are physically embedded into the matrixes or adsorbed onto the particle surfaces. The adsorbed drugs instantaneously dissolve when they come in contact with the release media and the drugs entrapped in the surface layers of particles also follow this process. This type of drug release easily leads to burst effect. Meanwhile, the drug release cannot be avoided before their delivery systems reach the site of action. Furthermore, these drug delivery systems have not the stimuli-response to the cell environment, so drug release from their drug delivery systems mainly depends upon the diffusion through the swollen rubbery matrixes.

6-Mercaptopurine (6-MP) is known as a clinically important antimetabolite and antineoplastic drug used in the treatment of human leukemia and many other diseases, such as inflammatory bowel disease, systemic lupus erythematosus, and rheumatoid arthritis. However, the drug is water insoluble, and the free sulfhydryl group can easily form a disulfide bond with the plasma protein. So the drug has a short plasma half-life (0.5–1.5 h) and lower bioavailability (about 16%) (Zacchigna, Cateni, Di-Luca, & Drioli, 2007), and reducing curative effect.

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Table 1
Characteristic data of 6-mercaptopurine-carboxymethyl chitosan.

Samples	SPDP/CMC (molar ratio)	PDP substitution degree (mol/mol)	6-MP content (mg/100 mg)
MP-CMC1	0.5:1	0.14 ± 0.02	9.1 ± 0.3
MP-CMC2	1.0:1	0.25 ± 0.04	12.2 ± 0.4
MP-CMC3	1.5:1	0.39 ± 0.06	19.0 ± 0.5

Covalently bonded disulfides can be formed spontaneously by autoxidation of sulfhydryls, primarily via oxidation upon exposure to air, which can reversibly be cleaved in the presence of reducing agents such as glutathione (2–10 mM) in the cells (Arrick & Nathan, 1984). The disulfide bond is an attractive strategy for intracellular controlled release.

The purpose of this study was to design a novel amphiphilic carboxymethyl chitosan derivative by the hydrophobic modification of carboxymethyl chitosan with 6-MP. It was expected that the compound could form self-assembly nanoparticles in aqueous solution. During circulation, the nanoparticles would be stable; while in the target cells, the nanoparticles would lead to a control release of 6-MP since the disulfide bonds would be reduced to free sulfhydryl groups in response to the relatively higher glutathione levels.

2. Experimental

2.1. Materials

Chitosan (molecular weight, 560 kDa) with a deacetylation degree of 91.13% was purchased from Yuhuan Ocean Biochemical Co. Ltd. (Zhejiang, China). Carboxymethyl chitosan (CMC) was prepared according to the literature (Abreu & Campana-Filho, 2009), its N,O-substitution degree determined by elemental analysis is about 0.70, and the degree of N-substitution is about 0.17, and the viscosity-average molecular weight measured by an Ubbelohde viscometer is 510 kDa. The heterobifunctional cross-linker succinimidyl3-(2-pyridyldithio) propionate (SPDP), glutathione (GSH) and dithiothreitol (DTT) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). 6-Mercaptopurine (6-MP) was purchased from Aladdin Chemical Reagent Co., Ltd.

2.2. Synthesis of 3-(2-pyridyldithio) propionic acid-modified carboxymethyl chitosan (PDP-CMC)

CMC dissolved in 5 mL pH 7.4 phosphate buffer solution (PBS), and SPDP dissolved in 2 mL DMSO was added dropwise to the reaction system. After stirring at room temperature for 2 h, the mixture was collected and first dialyzed (MWCO 14,000) against DMSO for 24 h to remove unreacted SPDP, then against pH 7.4 PBS for 72 h, and finally against deionized water for 24 h. The unreacted SPDP was detected by HPLC. The dialysate was lyophilized to get PDP-CMC. PDP-CMC samples with various degrees of PDP substitution were synthesized. Their substitution degrees to the C-2 amino groups had been estimated by an elemental analysis and shown in Table 1.

2.3. Synthesis of 6-mercaptopurine-carboxymethyl chitosan (6-MP-CMC)

Under argon atmosphere, the acetic acid was added to 5 mL aqueous solution containing 8.5 mg PDP-CMC to adjust the solution pH to 6.5, and a mixed solution of 3.5 mg 6-MP dissolved in 1 mL DMSO was added dropwise to the reaction system. After stirring for 48 h at room temperature, the reaction mixture was first dialyzed (MWCO 14,000) against DMSO for 24 h to remove by-products and the excess of reactants, then against pH 7.4 PBS for 72 h, and finally against deionized water for 24 h to remove salts. The dialysate was lyophilized to get 6-MP-CMC. The other 6-MP-CMC samples with various molar ratios of SPDP/CMC were synthesized in the same method and named in Table 1.

2.4. Structural characterization of 6-mercaptopurine-carboxymethyl chitosan (6-MP-CMC)

FT-IR spectrum analysis: Fourier transform infrared spectroscope (Avator360, Nicolet, MA, USA) was used to determine the chemical structures of PDP-CMC and 6-MP-CMC. Samples were prepared as KBr pellet and scanned against a blank KBr pellet background at range 450–4000 cm^{-1}.

^1H NMR spectroscopy analyses: The ^1H nuclear magnetic resonance (^1H NMR) spectrum was determined on Varian 600 spectrometer (Varian, USA) at 600 MHz. The CMC, PDP-CMC and 6-MP-CMC samples were dissolved in D_2O.

2.5. Determination of drug content

DTT is capable of maintaining thiols completely in reduced state and often used to reduce disulfides quantitatively (Cleland, 1964; Kavimandan, Losi, Wilson, Brodbelt, & Peppas, 2006). The drug content was determined by quantifying the release of 6-MP after reduction of 6-MP-CMC with DTT. Briefly, 10 μL DTT (15 mg/mL) was added to 1 mL purified 6-MP-CMC solution (0.075 mg/mL), the solution were incubated for exactly 15 min and the absorbance was recorded at 325 nm with the UV spectrophotometer. The solution without DTT was used as a control sample. The drug contents were calculated as:

$$\text{Drugcontent(mg/100 mg)} = (\Delta A \times \text{MW}_{6\text{-MP}})/(\varepsilon_{6\text{-MP},325} \times W_{\text{CMC}}) \times 100\% \quad (1)$$

where ΔA are the absorbance differences of samples and control samples; W_{CMC} is the weight of CMC; $MW_{6\text{-MP}}$ is the mole weight of 6-MP; $\varepsilon_{6\text{-MP},325}$ is the extinction coefficient (1.265×10^3 M^{-1} cm^{-1}) for 6-MP at 325 nm. Drug content was also estimated by elementary analysis. The obtained results were very consistent with each other, as summarized in Table 1.

2.6. In vitro drug release

Each of 6-MP-CMC (7.5 mg) was dissolved in 5 mL PBS and the solution was packaged in a dialysis bag. The dialysis bag was put in the release media (15 mL) containing various concentrations of GSH to initiate the release of 6-MP. The medium without GSH was used as a control sample. The release studies were carried out for 17 h at an oscillation condition of 37 °C, 50 r/min. At specific time intervals, 10 μL samples were withdrawn and analyzed with HPLC and the concentrations of 6-MP were determined using the standard curve prepared under same conditions.

Various GSH concentrations (10 mM, 2 mM, 100 μM or 2 μM) were used to determine the GSH dependent release kinetics of 6-MP-CMC. PBS (pH = 7.4) and citrate buffer (pH = 5) were used to study the pH effect on the release.

2.7. Characterization of self-aggregated nanoparticles

Sample preparation: The dry 6-MP-CMC2 polymer was simply dispersed in pH 7.4 PBS to the desired concentration (10 mg/mL).

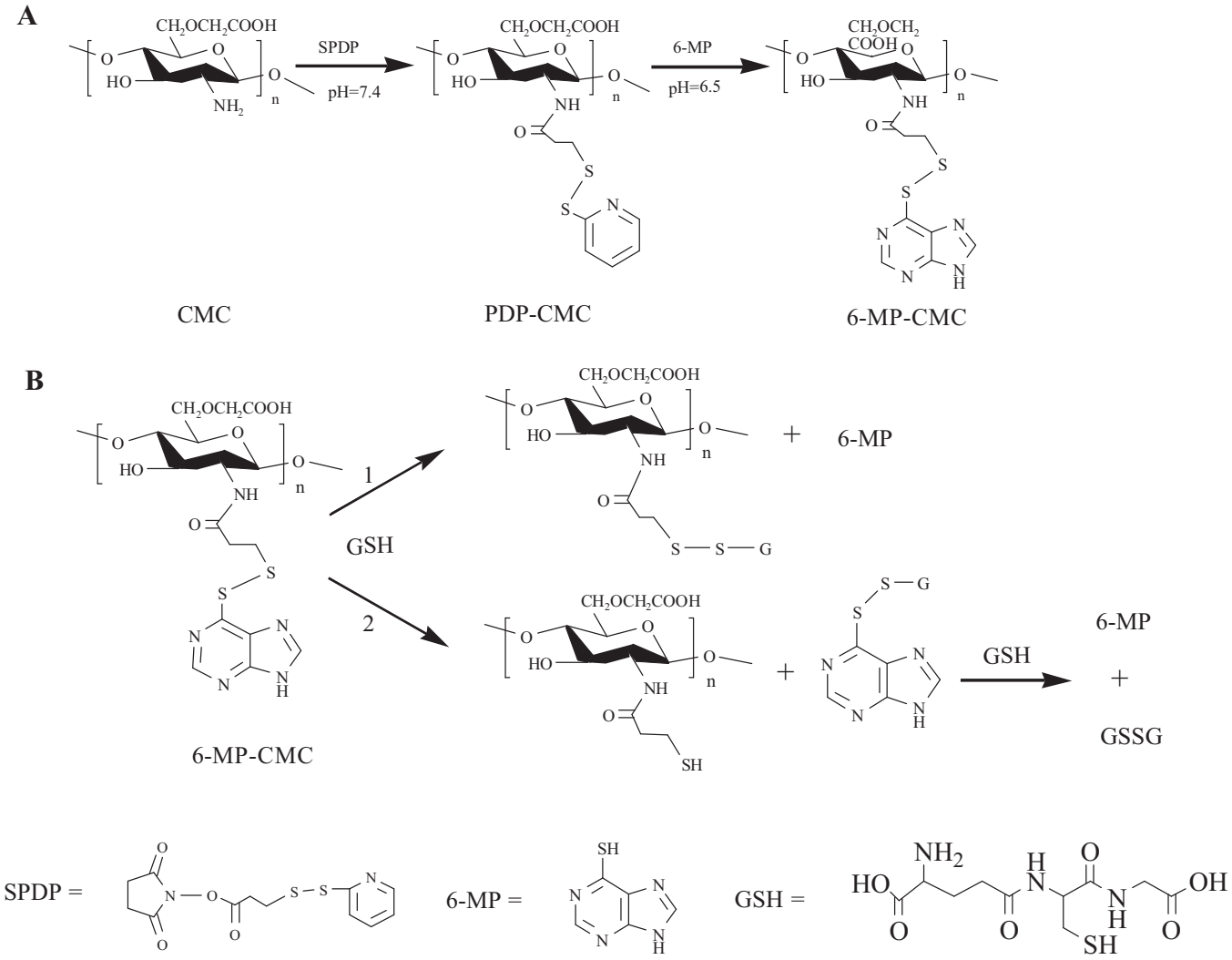

Fig. 1. (A) Schematic illustration for the synthesis of 6-MP-CMC. (B) 6-MP release mechanism of 6-MP-CMC in the presence of excess glutathione.

After oscillated at the condition of 37 °C, 50 r/min for 24 h, the dispersion solution was used for analysis and testing.

Particle size analysis: DLS (Nano-ZS3600, made by Malvern, UK) was used to determine the mean particle size of the self-aggregate sample. All DLS measurements were done with a wavelength of 670.0 nm at 25 °C with an angle detection of 90°. The sample was measured three times, and the average data was calculated.

Morphology observations: TEM (JSM-6700F, made by JEOL, Japan) was used to characterize the morphology of the self-aggregate sample. The sample for TEM analysis was obtained by placing a drop of the colloid dispersion containing self-aggregated nanoparticles onto a carbon-coated copper grid. It was dried at room temperature and then examined using TEM after being negative stained with phosphotungstic acid solution.

3. Results and discussion

3.1. Synthesis and structural analysis of 6-mercaptopurine-carboxymethyl chitosans (6-MP-CMC)

The schematic synthesis of 6-MP-CMC is described in Fig. 1A. CMC was firstly modified with SPDP to obtain PDP-CMC. Then PDP-CMC was linked with 6-MP by a thiol-disulfide exchange reaction (Caruso, Chong, & Chandrawati, 2009) to form 6-MP-CMC. The structures of PDP-CMC and 6-MP-CMC were characterized by FT-IR spectrum and ^{1}H NMR spectroscopy.

The FT-IR spectra of CMC, PDP-CMC and 6-MP-CMC are shown in Fig. 2. The basic characteristics of CMC (see curve a) are showed at:

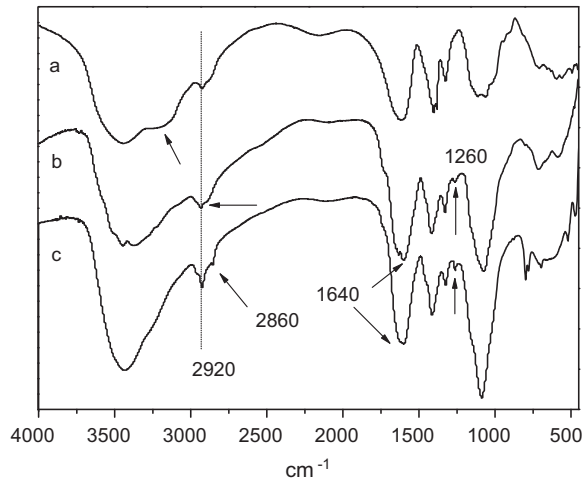

Fig. 2. FT-IR spectra of (a) CMC, (b) PDP-CMC and (c) 6-MP-CMC.

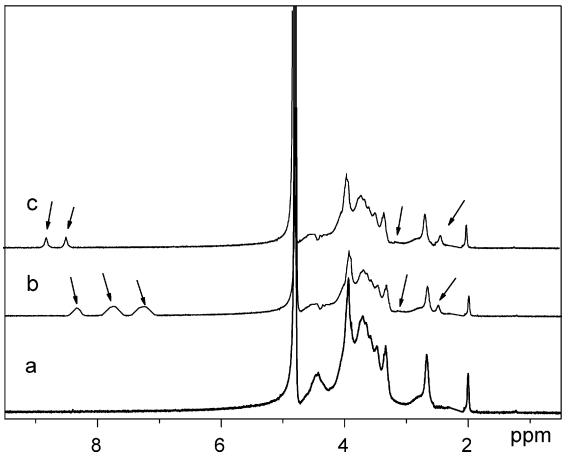

Fig. 3. ^{1}H NMR spectra of (a) CMC, (b) PDP-CMC and (c) 6-MP-CMC.

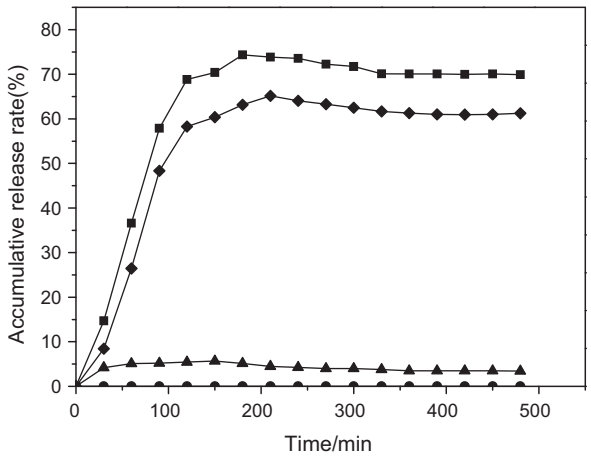

Fig. 4. Release of 6-MP from 6-MP-CMC with 6-MP content of 19.0% in pH 7.4 media with various glutathione concentrations. (■) 10 mM; (♦) 2 mM; (▲) 100 μM; (●) 2 μM.

2920 cm^{-1}(C–H stretch), 1598 cm^{-1} and 1400 cm^{-1}(–COO^- antisymmetric and symmetric stretch), 1028 cm^{-1} (C–O–C stretching vibration in the glucopyranose ring), 1153 and 895 cm^{-1} (specific bands of the β(1 → 4) glycoside bridge), and 3450–3200 cm^{-1} (hydrogen-bonded O–H stretching bands overlapped with the several N–H stretching). In comparison with the CMC spectrum, the increased intensities at 1640 cm^{-1} (amide I band) and 1260 cm^{-1} (C–N stretch) but the decreased intensity at 3200 cm^{-1} (primary amines) in the PDP-CMC spectrum (see curve b) indicated the formation of an amide linkage between CMC and SPDP. In addition, a new absorption band appearing at 2850 cm^{-1} and the increased intensity at 2920 cm^{-1} were corresponded to C–H stretching mode of the methylene in PDP groups. Compared with PDP-CMC spectrum, the purine ring characteristic bands between 795 and 478 cm^{-1} were observed in 6-MP-CMC spectrum, which confirmed that 6-MP was grafted on the CMC.

Chemical structures of the three polymers were determined by ^{1}H NMR spectroscopy. The proton assignment of CMC (see Fig. 3a) is as follows (ppm): 1.999 (CH_3, acetamido group of chitosan), 2.667 (CH, carbon 2 of glucosamine ring), 3.333 (CH, carbon 2 of glucosamine ring with the substituted amino group), 3.475–3.937 (CH, carbon 3, 4, 5 and 6 of glucosamine ring), 4.428 (CH_2, carboxymethyl group), 4.463 (CH, carbon 1 of glucosamine ring). Comparing PDP-CMC spectrum (see Fig. 3b.) with CMC spectrum, the signals at 2.66 and 3.21 ppm were assigned to the two methylene groups. The appearance of the new signals at 7.21, 7.74 and 8.40 ppm was the evidence of pyridyl groups. The almost disappearance of the pyridyl group signals and the appearance of the signals at 8.447 and 8.679 ppm in 6-MP-CMC spectrum (see Fig. 3c) indicated that 6-MP was grafted on CMC by exchanging the 2-pyridyldithio groups.

3.2. In vitro drug release of 6-mercaptopurine-carboxymethyl chitosan (6-MP-CMC)

3.2.1. The glutathione dependent release kinetics

The release profiles of 6-MP-CMC at pH 7.4 PBS containing various concentrations of GSH are shown in Fig. 4. 6-MP-CMC did not release any 6-MP within 3 days in the medium without GSH (data not shown). 6-MP release was also not observed at 2 μM GSH concentration and only less than 10% drug was released even at 100 μM GSH concentration. Comparing to the media containing micromolar GSH, the obvious 6-MP release could be observed within an hour in the millimolar GSH media (2 mM and 10 mM), and the maximum cumulative releasing rates at 2 mM and 10 mM (65.1% and 74.4%, respectively) were reached at 3.5 h and 3 h, and the cumulative release amount increased as the GSH concentration increased. The results demonstrate that the release amount of 6-MP from 6-MP-CMC depends on the concentration of GSH.

GSH is the most abundant natural thiol compounds in the cytoplasm, functioning as a major reducing agent in biochemical processes (Meister & Anderson, 1983). The intracellular GSH concentrations (2–10 mM) are substantially higher than the extracellular level (2 μM in plasma) (Arrick & Nathan, 1984). 6-MP-CMC did not release any 6-MP at 2 μM GSH, while had an obvious release in the millimolar GSH media. This result provides possibilities for intracellular delivery of 6-MP by disulfide-linked 6-MP-CMC carrier. Further, the GSH levels in tumor tissues can be many-fold higher than those in normal tissues (Yeh et al., 2006), thus, 6-MP-CMC should be able to release 6-MP in target cells.

It is worth mentioning that the drug-modified chitosan/carboxylchitosan derivatives as polymeric carriers to improve therapy efficacy have been reported recently. For example, d4T-modified chitosan with a phosphoramidate linkage was reported to improve antiretroviral treatment of d4T (Yang et al., 2009). Docetaxel-modified chitosan was synthesized via carbodiimide coupling reaction, and then its antitumor efficacy and subacute toxicity of docetaxel in vivo were evaluated (Lee, Kim, Lee, & Jon, 2009). Doxorubicin-modified N,O-carboxylchitosan was prepared to release doxorubicin under the hydrolysis of enzyme in the serum (Xie & He, 2006). These chemical modification approaches involve the ester or amide linkages between drugs and polymeric backbones by direct coupling reactions or by incorporation of a spacer arm. These drug releases mainly depend upon the in vivo nonspecific enzyme hydrolysis of the linkage bonds and has not the stimuli-response to the targeting cell environment. Thus, the drug release is difficultly avoided before the polymeric matrixes reach the sites of action.

6-MP-CMC is an asymmetrical disulfide which consists of CMC and 6-MP, this causes the reduction of the disulfide bonds in two possible ways (see Fig. 1B). The first is that GSH is linked to CMC by an exchange reaction with 6-MP, and 6-MP is released in free form. The other may be that GSH is linked to 6-MP by replacing CMC, and 6-MP is released in form of 6-MP-GSH. We proposed that the reduction of the disulfide bonds in 6-MP-CMC mainly depends on the first pathway because of the steric hindrance of CMC. However, as the release of drug proceeds, the free 6-MP in the solution also can link with GSH via an oxidation reaction to form 6-MP-GSH. For this reason, 6-MP-GSH was also monitored during the release studies (RP-HPLC retention time: 6-MP 3.45 min; 6-MP-GSH 4.25 min). 6-MP-GSH can again release free 6-MP by an exchange reaction with

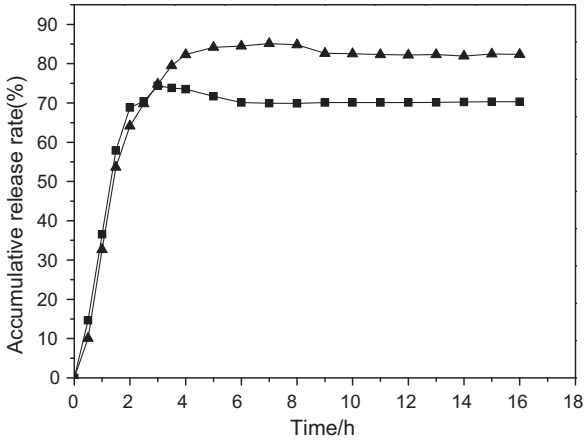

Fig. 5. Release of 6-MP from 6-MP-CMC with 6-MP content of 19.0% in different pH media with 10 mM glutathione concentration. (■) pH 7.4; (▲) pH 5.0.

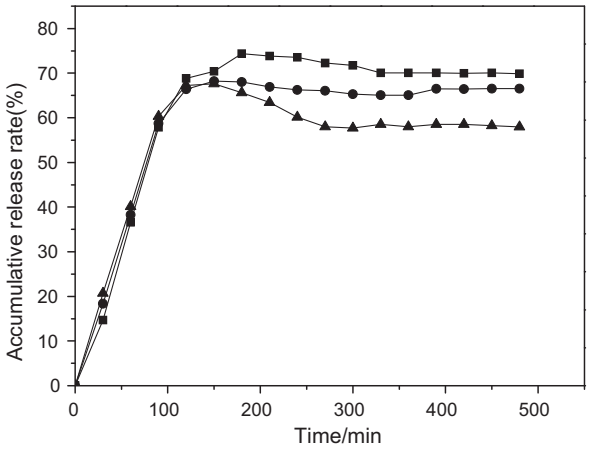

Fig. 6. Release of 6-MP from 6-MP-CMC with different drug contents in a pH 7.4 medium with 10 mM glutathione concentration. (■) 6-MP-CMC3; (●) 6-MP-CMC2; (▲) 6-MP-CMC1.

another GSH molecule and leaving a dimer of glutathione (GSSG) over longer periods of time.

3.2.2. The effect of pH on the release kinetics

The influence of pH of buffer solution on the release behavior of 6-MP-CMC is shown in Fig. 5. A fast release of 6-MP from 6-MP-CMC with 6-MP content of 19.0% was observed in pH 7.4 and pH 5 media with 10 mM GSH. Comparing the release curves at pH 5 and 7.4, it is clear that there was little difference between the drug release at pH 5 and that at pH 7.4 in the first 3 h, but finally the maximum cumulative releasing rate (85.1%) at pH 5 was much higher than that (74.4%) at pH 7.4. The difference may be caused by the following reasons. On the one hand, the hydrophilic property of CMC groups is reduced at pH 5 and the hydrophobic drug groups will be enfolded more tightly by the CMC chains, thus the drug release was a little slower in the first 3 h at pH 5; on the other hand, the reducing activity of GSH is relative to its thiol group with

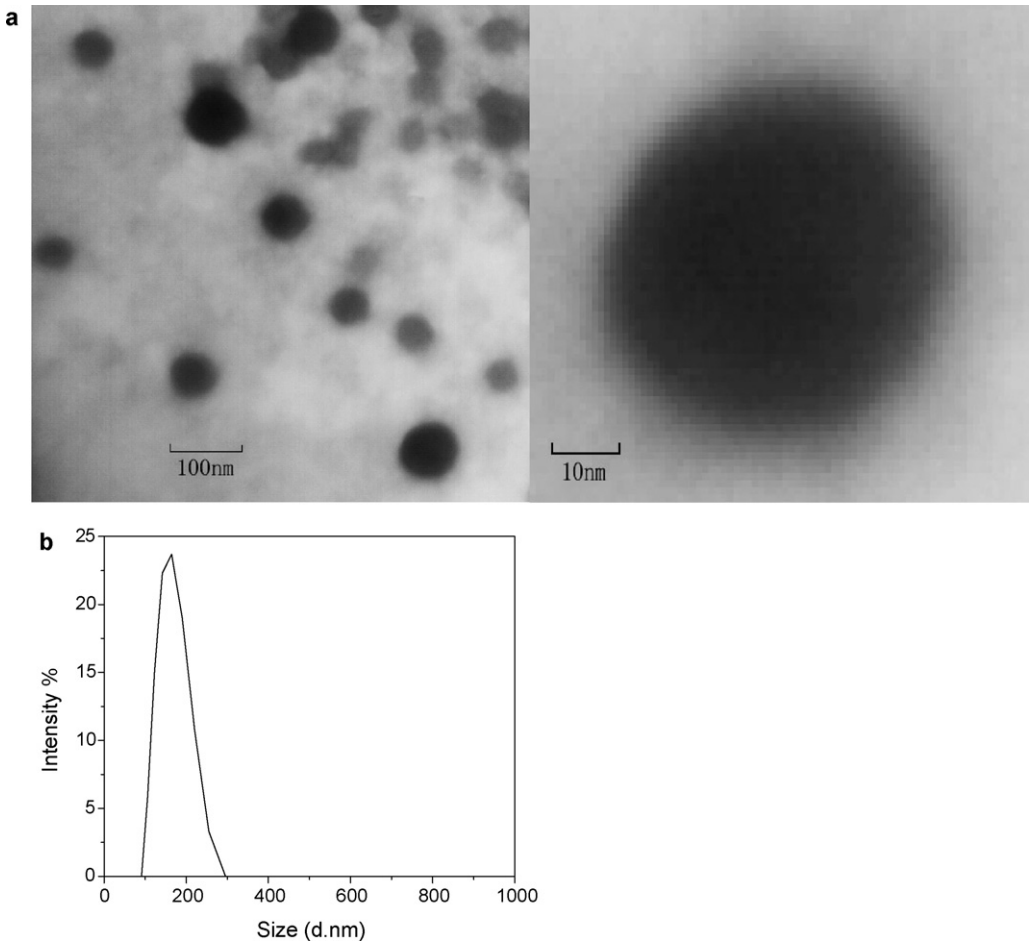

Fig. 7. TEM images (a) and size distributions (b) of self-aggregated nanoparticles prepared from 6-MP-CMC2.

a p*K*a of 8.8 and its thiolate form is more reactive than the thiol (Winterbourn & Metodiewa, 1999), so the reducing activity of GSH is weaker at pH 5.0, and the drug release was slower at the initial. However, as the drug release proceeds, the free 6-MP in the solution is possibly attached to GSH via an oxidation reaction and the oxidation reaction is faster at pH 7.4 compared to pH 5, therefore more MP-GSH is generated at pH 7.4 (more MP-GSH was monitored during the time-period from 3 h to 16 h in the release studies at pH 7.4), and resulting in a higher cumulative release amount of 6-MP in the medium of pH 5.

Many polymeric matrixes exist in an ionized, hydrophilic state at physiologic pH (pH 7.4). After being taken up by the cells via endocytosis mechanism, the polymeric matrixes can become shrunken at lower pH 5.0–6.5 (Aubry, Klein, Martiel, & Satre, 1993) in the endosome and not release drugs. Although some pH-responsive systems also can provide release in the environment, the release is very slow. 6-MP-CMC can rapidly release 6-MP in respond to GSH at pH 5 media containing 10 mM GSH, which shows that 6-MP-CMC has potential of rapid release of 6-MP in response to the cell environment.

3.2.3. *The effect of drug content on the release kinetics*

The effect of composition on the release behavior of 6-MP-CMC is shown in Fig. 6. 6-MP-CMC samples with different drug content (9.1%, 12.2%, 19.0%, w/w) were subjected to in vitro release kinetics studies in a pH 7.4 medium containing 10 mM GSH.

It can be seen from Fig. 6 that the drug cumulative releasing rate was affected by the 6-MP content in 6-MP-CMC. 6-MP-CMC with 6-MP content of 9.1% released almost 67.6% within 2.5 h, 6-MP-CMC with 6-MP content of 12.2% released almost 68.0% within 3 h, whereas 6-MP-CMC with 6-MP content of 19.0% released almost 74.4% in 3 h. These results show that the release rate increased with the increase of 6-MP content in 6-MP-CMC.

3.3. *Morphology and size of self-aggregated nanoparticles*

6-MP-CMC could undergo self-assembly in water solution to form nanoparticles. The mean diameter of these nanoparticles determined by DLS was 155.8 ± 6.0 nm (see Fig. 7b), the nanoparticles were in a nearly monodispersed state with a relatively narrow size distribution. The TEM photograph in Fig. 7a shows these nanoparticles were spherical in shape and the size was about 100 nm which was slightly smaller than the size determined by DLS.The self-aggregation mechanism of nanoparticles may be explained on the basis of the poly-core model proposed by Akiyoshi, Deguchi, Tajima, Nishikawa, and Sunamoto (1997). 6-MP-CMC is an amphiphilic compound consisting of hydrophilic polysaccharide backbone and hydrophobic 6-mercaptopurine moieties. The hydrophobic microdomains are formed by the association of the 6-MP moieties, and 6-MP-CMC backbones coil to form the hydrophilic shells outside these hydrophobic microdomains, thus a minimal energy state is attained in aqueous media. Furthermore, inter- and/or intramolecular hydrogen bonds among tightly packed chitosan backbones also promote the self-aggregation of 6-MP-CMC molecules. In other words, 6-MP-CMC nanoparticle is a non-covalently cross-linked hydrogel structure formed by the hydrophobic association of 6-MP moieties.

4. Conclusions

In this work, novel amphiphilic 6-mercaptopurine-modified carboxymethyl chitosans (6-MP-CMC) were synthesized using a glutathione sensitive disulfide linker for intracellular delivery of 6-MP and their structures were characterized by FT-IR, ^{1}H NMR, and elemental analysis. The 6-MP release from 6-MP-CMC showed dependence on GSH concentration. 6-MP-CMC was stable or only had little release in the media containing micromolar GSH, but could fast release 6-MP at millimolar GSH levels. The buffer pH and the 6-MP content in 6-MP-CMC had obvious influences on the 6-MP release. The maximum cumulative release rate at pH 5 containing micromolar GSH was higher than that at pH 7.4.

The profiles of drug release under the conditions simulating the GSH concentrations likely to be encountered during transit from plasma to the insides of cells showed the 6-MP release from 6-MP-CMC could be avoided at 7.4 buffer containing 2 μm GSH, while the rapid release could be achieved at pH 5.0 medium containing 10 mM GSH. In addition, in aqueous solution 6-MP-CMC could self-assemble into the nanoparticles through the intra- and intermolecular hydrophobic interactions between 6-MP groups. 6-MP-CMC may be useful in the delivery of 6-MP to targeting cells by self-assembling nanoparticle and their further investigations are in progress.

Acknowledgments

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