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Preparation, characterization and feasibility study of dialdehyde carboxymethyl cellulose as a novel crosslinking reagent

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ABSTRACT

The natural biopolymers usually need to be chemically modified by crosslinking reagents to improve their mechanical properties. In the present research, the feasibility of using the dialdehyde carboxymethyl cellulose (DCMC) as a crosslinking reagent was systematically studied. DCMC was prepared by oxidizing carboxymethyl cellulose using sodium periodate. The formation of dialdehyde groups was confirmed by FTIR and the degree of oxidation was determined. The biocompatibility of DCMC was investigated by evaluating its cytotoxicity to L929 fibroblasts and histocompatibility in rat model *via* intramuscular and subcutaneous injection. DCMC-crosslinked carboxymethyl chitosan (DCMC-CMCTS) was prepared and characterized using the glutaraldehyde-crosslinked CMCTS (GA-CMCTS) as control. The result demonstrated that DCMC was non-cytotoxic, biodegradable and biocompatible. The DCMC-CMCTS displayed significantly better thermostability, swelling capacity and cyto-compatibility compared with GA-CMCTS. Our data provided experimental basis for the future application of DCMC as a novel crosslinking reagent.

1. Introduction

Polymeric materials, including synthetic polymers and natural polymers, have received increasing attention in recent years and have been widely applied in the biomedical field (Duncan, 2003; Werle, 2008). Although synthetic polymers have longer history in clinical use, natural biopolymers have gained extensive recognition and application in the past decade due to their unique advantages in biodegradability and biocompatibility (Dang & Leong, 2006; Ko, Sfeir, & Kumta, 2010; Park & Kim, 2010; Peter, Miller, Yasko, Yaszemski, & Mikos, 1998). However, natural biopolymers usually have relatively poor mechanical properties and uncontrollable degradability, which greatly limit their clinical application. Both bioartificial blending and chemical modification methods have been employed to improve the properties of natural biopolymers, in which crosslinking reagents play important roles (Sionkowska, 2011; Wang et al., 2003).

Various synthetic crosslinking reagents have been used in the modification of natural biopolymers, such as glutaraldehyde (GA) (Cheung, Perelman, Ko, & Nimni, 1985), formaldehyde (Madhavan, Belchenko, Motta, & Tan, 2010), polyepoxy compound (Tu et al., 1993) and carbodiimide (Park, Park, Kim, Song, & Suh, 2002). However, the application of these crosslinking reagents has been extremely limited due to their high cytotoxicity and poor biocompatibility (Hao et al., 2011; Nishi, Nakajima, & Ikada, 1995). Therefore, the development of novel crosslinking reagents with good biocompatibility and low cytotoxicity is highly expected.

The carboxymethyl cellulose (CMC), one of the major cellulose derivatives, is an anion linear polymer cellulose ether that is widely applied in many fields such as food, textile, paper-making, painting, pharmaceutics and cosmetics (Guo, Skinner, Harcum, & Barnum, 1998; Heinze & Koschella, 2005; Pal, Banthia, & Majumdar, 2006; Shen, Song, Qian, & Yang, 2010). CMC is nontoxic, biodegradable and has excellent biocompatibility (El-Hag Ali, Abd El-Rehim, Kamal, & Hegazy, 2008; Huangqin & Mingwen, 2008; Jiang, Li, Zhang, & Wang, 2009). It was reported that CMC could be oxidized to form dialdehyde carboxymethyl cellulose (DCMC) by periodate that could cleave the C2–C3 bond of the 1,4-glucan unit and convert the 1,2-dihydroxyl group to paired aldehyde groups with high specificity (Li, Wu, Mu, & Lin, 2011). Considering the similarity of the functional aldehyde groups between DCMC and the synthetic crosslinking reagent GA, which could produce crosslinking with free amino groups of natural biopolymers, it is of great interest to evaluate the feasibility of using DCMC as a novel crosslinking reagent. One most recent study showed that DCMC indeed displays crosslinking effects in stabilizing the microstructure of biological

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tissues (Wang, Wang, Li, Gu, & Yu, 2015). However, the biosafety of DCMC remains unclear and no systematic study of DCMC in the crosslinking and chemical modification of natural biopolymers has been reported.

Carboxymethyl chitosan (CMCTS), a water-soluble chitin derivative, has been reported to have anti-tumor (Zheng, Han, Yang, & Liu, 2011) and wound healing activities (Costain, Kennedy, Ciona, McAlister, & Lee, 1997). The CMCTS has been emerging as a promising biopolymer in biomedical area with enhanced stability, biocompatibility, chelating ability and antimicrobial activity compared with chitosan (reviewed in (Mourya, Inamdar, & Tiwari, 2010)). However, the CMCTS usually needs to be chemically modified to strengthen its dimensional complexity and decrease its solubility in order to expand its biomedical application. Crosslinked CMCTS has been reported previously to be employed in tissue engineering as scaffold or drug delivery carrier (Vaghani, Patel, Satish, Patel, & Jivani, 2012; Wang, Lu, Ao, Gong, & Zhang, 2009). Moreover, considering the more moderate reaction conditions of CMCTS crosslinking compared with chitosan that is only soluble in the aqueous acidic solution below pH 6.5, the CMCTS was chosen in the present work for investigating the feasibility of using DCMC as a crosslinking reagent.

In the present study, DCMC with different degrees of oxidation (D.O.) was prepared and the chemical structure and the D.O. of DCMC were investigated. The cytotoxicity and biocompatibility of DCMC were systematically evaluated. Subsequently, the DCMC was used to crosslink CMCTS. The characteristics and cytotoxicity of DCMC-crosslinked CMCTS (DCMC-CMCTS) were studied. GA, the traditional synthetic crosslinking reagent, was used as control.

2. Materials and methods

2.1. Materials and reagents

CMC (viscosity of the 2% (w/v) CMC in water was ≥1200 mPa s) was purchased from Sinopharm Chemical Reagent Co., Ltd (China). CMCTS (degree of deacetylation was 98% and average molecular weight was 196 kD) was supplied by Biotemed Co., Ltd (China). 3-(4,5-dimethylthiazol-2yl)-2,5, diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin and streptomycin were purchased from Gibco Co. (Grand Island, NY, USA). Sodium periodate, GA and other reagents used were of reagent grade and purchased from Sinopharm Chemical Reagent Co., Ltd (China), unless otherwise indicated.

Adult male Sprague-Dawley rats (200 ± 10 g body weight) were obtained from Qingdao Institute for Drug Control (China). The L929 fibroblast cell line was provided by the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (China).

2.2. Preparation of DCMC

The DCMC was prepared as previously described (Li et al., 2011). Briefly, 2.0 g CMC was dissolved in 100 ml deionized water and stirred for 6 h to get a clear solution. 10 ml sodium periodate solution was added to the CMC solution under stirring. The mixture was stirred at room temperature in darkness for 24 h. Excess ethylene glycol was used to decompose the remaining periodate. The oxidized product, referred to as DCMC, was obtained after filtration, purification and lyophilization. DCMC with different degrees of oxidation (10%, 20%, 40%) was generated by using different concentrations of sodium periodate solution calculated according to the molar ratio of sodium periodate to CMC repeating unit.

2.3. Characterization of DCMC

FTIR spectra were recorded for the determination of the chemical structure of CMC and DCMC. 2 mg of dry sample was pressed into a disc with 200 mg of potassium bromide (KBr). The FTIR spectroscopy was carried out on a Nicolet Nexus-470 Fourier transform infrared spectrometer (Thermo Nicolet, USA) and data analysis was performed using Jwstda-32.

The D.O. of DCMC was determined by hydroxylamine hydrochloride method. 0.5 g DCMC was dissolved in 25 ml distilled water. The pH of the solution was adjusted to 5.0 with NaOH. 20 ml of 0.72 mol/l hydroxylamine hydrochloride (pH = 5.0) was added into the DCMC solution and the mixture was stirred at 40 °C for 4 h. 1.0 mol/l NaOH was used to titrate with the hydrochloric acid produced in the mixture and the consumption of NaOH solution was recorded as V_c (l). CMC solution of the same concentration and pH was used as blank whose consumption of NaOH solution was recorded as V_b (l). The D.O. of the DCMC can be calculated with the following formula:

$$\text{D.O.}\,(\%) = \frac{M\text{NaOH}\,(Vc - Vb)\,/2}{m/211} \quad (1)$$

where M_{NaOH} is 1.0 mol/l, m is the dry weight of DCMC sample, and 211 is the average molecular weight of repeating unit in DCMC. Experiments were performed in triplicate.

2.4. Cytotoxicity of DCMC

The cytotoxicity of the DCMC was evaluated *in vitro* using the cell line of L929 fibroblasts. DCMC with different D.O. was dissolved in DMEM medium (supplemented with 10% fetal bovine serum, 10 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin) to make a concentration gradient (50, 100, 200, 400, and 800 μg/ml). GA with the same AC as DCMC (D.O. = 10%) served as the positive control.

Cells at the logarithmic growth phase were seeded in a 96-well culture plate at a density of 10^4 cells/ml and were incubated for 24 h at 37 °C with 5% CO_2. The culture medium was then replaced with fresh medium containing GA or different concentrations of DCMC. The cell culture was maintained at 37 °C with 5% CO_2 and the medium was replaced every 2 days. MTT assay was performed at 2 d, 4 d, and 6 d. The medium without cells were used as the blank group while the cells treated with no drugs were used as the negative control group. The cytotoxicity was expressed as the percentage reduction of cell viability in terms of relative growth ratio (RGR) and calculated with the following formula:

$$\text{RGR}\,(\%) = \frac{(\text{OD}_1 - \text{OD}_0)}{(\text{OD}_2 - \text{OD}_0)} \times 100\% \quad (2)$$

where OD_0, OD_1 and OD_2 were the average OD of the blank, drugged (either DCMC or GA) and negative control groups, respectively. Experiments were performed in triplicate.

2.5. In vivo *biocompatibility and biodegradability of DCMC*

The animal experiments in present study were carried out in accordance with the ethical guidelines of the Shandong Province Experimental Animal Management Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 36 adult male Sprague-Dawley rats were housed in individual cages and kept under controlled temperature and humidity with free access to food and water. The rats were anesthetized with intraperitoneal administration of pentobarbital sodium (3% in normal saline) at a dose of 1 ml/kg. The dorsal sides of the rats were gently shaved and disinfected with 75% alcohol.