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Modification of carboxymethyl cellulose grafted with collagen peptide and its antioxidant activity

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ABSTRACT

Carboxymethyl cellulose used in wounds has little antioxidant capacity. The aim of the present study was to improve the scavenging ability of carboxymethyl cellulose by modified with collagen peptide. The reaction conditions have been optimized by varying mass ratio of collagen peptide to carboxymethyl cellulose, temperature and reaction time. Antioxidant activities of carboxymethyl cellulose derivatives (CMCC) were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide radicals and the reducing power. The effects of concentration, degree of substitution (DS) and molecular weight on three different radicals scavenging activity and reducing power were examined. Methylthiazol tetrazolium (MTT) assay was used to evaluate the fibroblasts cells cytotoxicity of CMCC. Results showed that the scavenging effects of CMCC increased with the increasing of DS and concentration. This product of CMCC possesses a distinct antioxidant capacity on radicals.

1. Introduction

Carboxymethyl cellulose (CMC) is water-soluble anionic polysaccharide and semi-synthetic derivative of cellulose (Yadav, Rhee, Jung, & Park, 2013), and it is obtained from the reaction of the hydroxyl groups at the 2, 3, and 6 positions of the anhydroglucose units (AGUs) of cellulose with chloroacetic acid (Hiroyuki, 2013). It was implemented in broad range of industrial applications in pharmaceuticals (Bajpai & Anjali, 2003), cosmetics (Lin & Richard, 2002), drug delivery (Agarwal, Alam, & Gupta, 2013), pulp cell regeneration (Chen & Fan, 2007) and wound dressing (Garrett et al., 2008) because of their high water absorption property, gelation behavior, bio-degradability, and biocompatibility.

Carboxymethyl cellulose as wound dressing grants several demands, like close adhesion to the wound bed, having high fluid absorbency and keeping for a long time and could not disrupt or damage the newly formed tissues when removed (Waring & Parsons, 2001). CMC has been shown to provide a moist environment for optimal debridement to support the wound healing process, attributed to its ability to absorb water directly into its fibers (Williams, 1999).

For the treatment of wounds it is also necessary to reduce the elevated concentration of proteases and the high level of reactive oxygen species (ROS) to allow the wound to heal (Trengove et al., 1999; Yager & Nwomeh, 1999; Cullen, Smith, Mcculloch, Silcock, & Morrison, 2002). Reactive oxygen species include the superoxide radical species ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) species, which are now strongly implicated in the pathogenesis of wounds (White & Heckler, 1990; Senel, Cetinkale, Ozbay, Ahc-ioglu, & Bulan, 1997; Waddington, Moseley, & Embery, 2000). Excess ROS cause indiscriminate damage to cellular constituents, such as DNA, lipids and proteins (Mendanha da Cunha et al., 2013), cause destruction to ECM components, such as collagen, proteoglycans and hyaluronan.

It is believed that wounds will only heal when physiological balance is achieved. Therefore, the high level of reactive oxygen species must be reduced. Earlier studies with carboxymethyl cellulose showed little antioxidant capacity against ROS (Moseley, Walker, Waddington, & Chen, 2003). Our aim was to improve the scavenging ability of carboxymethyl cellulose for the application in the treatment of wounds. Thus, we produced a derivative which modified carboxymethyl cellulose with collagen peptide. Collagen peptide has a variety of physiological functions, such as chemotaxis, angiotensin-converting enzyme inhibition, platelet aggregation, reducing osteoclast differentiation, lipid peroxidation inhibition, and defending against oxidative free radical attacks on DNA (Guillerminet et al., 2010; Wang, Zhang, Zhang, Li, 2011; Watanabe-Kamiyama et al., 2010). Collagen has been used to modify substrates to impart hydrophilicity and biocompatibility, such as polyethylene (Bocková, Vojtová, Prikryl, Cechal & Jancár, 2008),

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poly(caprolactone) (Krithica, Natarajan, Madhan, Sehgal & Mandal, 2012) and polypropylene (Zhang, Wang, Feng, Fu, & Xu, 2006). And carboxymethyl cellulose modified with collagen peptide on the in vitro antioxidant activities has yet been reported.

The present study is mainly concentrated on the radical scavenging properties of carboxymethyl cellulose modified with collagen peptide. The scavenging effect on the DPPH radicals, hydroxyl radical and superoxide radical was used to evaluate the antioxidant activities of carboxymethyl cellulose derivatives. The conditions for synthesis were further discussed.

2. Experimental

2.1. Materials

N-Hydroxy sulfosuccinimide (NHS), 2-(N-morpholino) ethanesulfonic acid (MES), 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC) and collagen peptide (M_w = 800) were purchased from Huashun Biological Technology Co. Ltd., Wuhan, China. Sodium carboxymethyl cellulose ($M_w = 7.8 \times 10^3$), potassium ferricyanide, 1,10-phenanthroline, $FeSO_4 \cdot 7H_2O$, H_2O_2 and $K_3Fe(CN)_6$ was purchased from Sinopharm Chemical Reagent Co., Ltd. Shanghai, China. All other reagents were of analytical grade and were used without further purification.

2.2. Synthesis of carboxymethyl cellulose derivatives

First, carboxymethyl cellulose was dissolved under magnetic stirring, then NHS reacted with carboxymethyl cellulose (CMC) at a mass ratio of 1:2 to EDC, and EDC was next added as a percentage of uronic acids available for reaction. After that, collagen peptide with different mass ratio to CMC was added into the mixture after 1 h. This reaction was carried out in MES buffer (pH 6) for 12–28 h at temperature from 25 to 55 °C under constant stirring. Finally, the reaction solution was purified by dialysis for three days. The dialyzed product was finally freeze-dried with lyophilizer to obtain the purified carboxymethyl cellulose derivatives (CMCC).

2.3. Measurement of degree of substitution

The degree of substitution (DS) is defined as the number of carboxyl groups substituted of per repeating structural unit of the carboxymethyl cellulose backbone. In this work, concentration of collagen peptide between 0.001 g/l and 0.05 g/l was linear relation with absorbance at 200 nm by ultraviolet spectrophotometry. And the linear relation was presented as Eq. (1). DS of CMCC was determined by Eq. (2).

$$A = 25.322C + 0.0097 \quad R^2 = 0.99979 \tag{1}$$

$$DS = \frac{11350C}{(800 - 39150C)} \tag{2}$$

where A is the absorbance of collagen peptide, C is the concentration of collagen peptide (Fan et al., 2013).

2.4. Degradation of carboxymethyl cellulose derivatives

Oxidation degradation method (Li et al., 2010) was used to degrade the products to different molecular weights. The oxidation reaction was conducted in a water bath. After CMCC was completely dissolved in distilled water to a final concentration of 2% (w/v), and the solution was pre-heated to the desired temperature, then a series different volume of hydrogen peroxide (30%) was added. After that, the solution was stirred for 20 min under 80 °C to eliminate the residual hydrogen peroxide. The purified product was finally obtained after reduced pressure distillation, washing with absolute ethyl alcohol and freeze-dried with lyophilizer.

2.5. Fourier transforming infrared spectroscopy (FT-IR) analysis

FT-IR spectra of CMCC samples and CMC were performed with a Nicolet 170SX (USA) Fourier transform infrared spectrometer. The test samples were prepared by the KBr-disk method.

2.6. Light scattering measurements

The weight-average molecular weight of the samples was determined by static light scattering. The light-scattering intensities were measured with a modified commercial light scattering spectrometer (ALV/SP-125, ALV, Germany) equipped with an ALV-5000/E multi-digital time correlator and a He–Ne laser (λ = 632.8 nm) in an angular range from 30° to 150° at 10° intervals at 25 °C. The test of CMCC solutions was prepared in 0.1 M NaCl aqueous solution, and made optically clean by filtration through 0.22 μm Millipore filters. The specific refractive-index increments (dn/dc) of CMCC in 0.1 M NaCl aqueous solution were measured on a Optilab refractometer (Wyatt Technology) at 632.8 nm and 25 °C, and were found to be 0.140 g/cm^3.

2.7. Antioxidant activity measurements

2.7.1. DPPH radical scavenging activity

DPPH radical scavenging activity was tested according to the method of Wang Bin (Wang, Yu, Luo, Qu, & Yang, 2010). A volume of 1.5 ml of each sample was added to 1.5 ml of 0.1 mM DPPH in 95% ethanol. The mixed solution was incubated in the dark for 30 min, and the absorbance of the mixture was measured at 517 nm. A control group that samples substituted by ethanol was also prepared. In the blank, ethanol substituted for DPPH solution. The antioxidant activity of the sample was evaluated by the inhibition percentage of DPPH with the following equation:

$$\text{Scavenging activity}\,\% = \frac{A_0 - A + A'}{A_0} \times 100\%$$

where A, A_0 and A' are the absorbance of test sample, the control group and the blank, respectively.

2.7.2. Hydroxyl radical scavenging activity

The scavenging activity of CMCC on hydroxyl radical was measured according to the method of Li et al. (2012). In this system, hydroxyl radical was generated by the Fenton reaction. Hydroxyl radical could oxidize Fe^{2+} into Fe^{3+}, and only Fe^{2+} could be combined with 1,10-phenanthroline to form a red compound (1,10-phenanthroline-Fe^{2+}) with the maximum absorbance at 536 nm. The concentration of hydroxyl radical was reflected by the degree of decolorization of the reaction solution. Briefly, 1,10-phenanthroline solution (1.0 ml, 1.5 mM) and sample (1.0 ml) was added into a screw-capped tube orderly and mixed homogeneously. The $FeSO_4 \cdot 7H_2O$ solution (1.0 ml, 1.5 mM) was then pipetted to the mixture. The reaction was initiated by adding 1.0 ml H_2O_2 (0.03%, v/v). After incubation at 37 °C for 1 h, the absorbance of the reaction mixture was measured at 536 nm against a reagent blank. The reaction mixture without any antioxidant was used as the negative control (A_n), and without H_2O_2 was used as the blank (A_b). The hydroxyl radical scavenging activity (HRSA) was calculated by the following formula:

$$\text{HRSA}\,(\%) = \left[\frac{(A_s - A_n)}{(A_b - A_n)}\right] \times 100\%$$

2.7.3. Superoxide radical scavenging activity

Superoxide radical was generated by UV irradiation of a riboflavin/EDTA solution (Je, Qian, Byun, & Kim, 2007). The reaction mixtures containing 0.5 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO and various concentrations of the sample were irradiated for 1 min under UV lamp at 365 nm. The mixtures were transferred to 50 μl quartz capillary tube of the ESR spectrometer for measurement. Experimental conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; sweep time, 1 min. Superoxide radical scavenging ability was calculated according to the following equation in which H and H_0 were relative peak height of radical signals with and without sample, respectively.

$$\text{Radical scavenging activity}\,(\%) = \frac{(H_0 - H)}{H_0} \times 100\%$$

2.7.4. Reducing power

The reducing power of all samples was determined by the description of a literature report (Wu, Chen, & Shiau, 2003). Generally, each sample dissolved in distilled water (2.0 ml) was mixed with 2.5 ml potassium hexacyanoferrate solution (1%). After 30 min of incubation at 50 °C, 1.5 ml of 10% trichloroacetic acid was added. Finally, 2.0 ml of the upper layer was mixed with 2.0 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ solution, and the absorbance was recorded at 700 nm. The higher absorbance of the reaction mixture indicated the stronger reducing power.

2.8. Cytotoxic assessment using MTT assay

Samples of CMCC were dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) medium and then filtered through a 0.22 μm

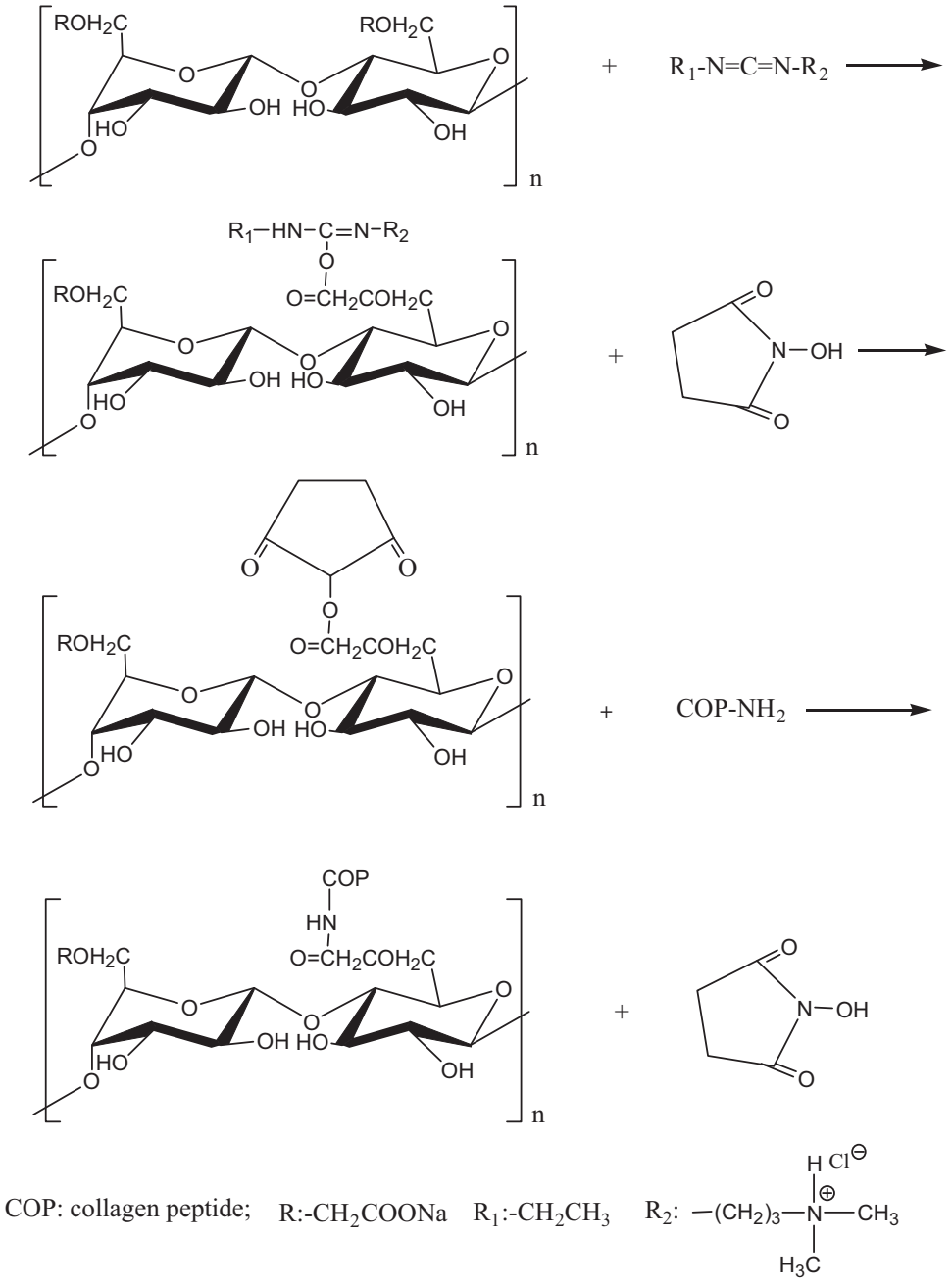

Fig. 1. Reaction scheme for the carboxymethyl cellulose grafting with collagen peptide.

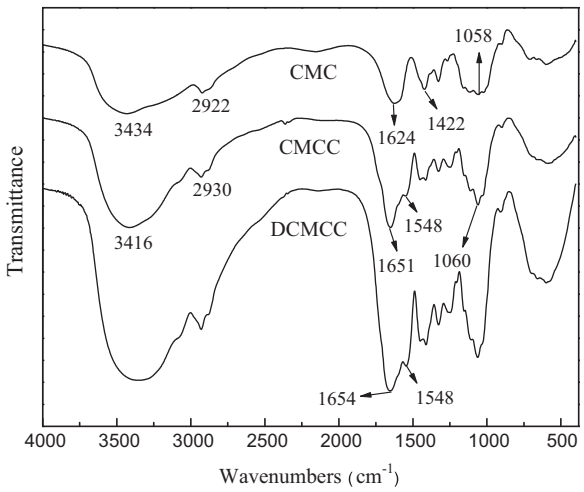

Fig. 2. FT-IR spectra of the carboxymethyl cellulose (CMC), the carboxymethyl cellulose derivative (CMCC, DS = 0.29, $M_w = 1.78 \times 10^4$) and the degradation of CMCC (DCMCC, DS = 0.35, $M_w = 8.2 \times 10^4$).

filter under sterilized conditions (Zhu, Sun, Wang, Luo, & Fan, 2013). The fibroblast cells of rat were cultured at a density of 8.0×10^3 cells/ml on 96-well plates (100 μl/well) in a CO_2 (5%) incubator at 37 °C. After incubation for 24 h, cells were washed with phosphate buffered saline (PBS) and the fresh culture medium was added. Then, 10 μl samples with different concentration were added to 96-well plates. After 22 h incubation, 10 μl of MTT was added to each well, after which the cultures were incubated at 37 °C for an additional 4 h. The cells were washed gently with PBS at pH 7.4 to remove untransformed MTT and sample residues. Finally, 100 μl of DMSO was added to each well to dissolve the MTT formazan purple crystals. Absorbency of the solution was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The relative cell growth (%) was calculated as:

$$\text{Cell viability}\,(\%) = \frac{[\text{OD}]_{\text{test}}}{[\text{OD}]_{\text{control}}} \times 100\%$$

The values were expressed as the mean ± standard deviation ($n = 3$).

3. Results and discussion

3.1. Synthesis of carboxymethyl cellulose derivatives

The synthesis process of carboxymethyl cellulose modified with collagen peptide was shown in Fig. 1. An amide group was formed between the carboxyl group of carboxymethyl cellulose (CMC) and the amino group of collagen peptide using cross-linking agent EDC and NHS. Modification of CMC with collagen peptide was optimized with respect to DS by varying process parameters as showed in Fig. 3. In our experimental design, reaction temperature, mass ratio of collagen peptide to CMC, and reaction time was selected as process parameters. Therefore, 5 levels of reaction temperature (25, 35, 45, 55, and 65 °C), reaction time (12, 16, 20, 24 and 28 h) and collagen peptide mass ratio to CMC (0.4/0.6, 0.6/0.6, 0.8/0.6, 1.0/0.6 and 1.2/0.6) were set in this paper.

3.2. FT-IR

The CMC and CMCC were analyzed by FT-IR spectra in Fig. 2. As can be seen, CMC showed a broad band at 3434 cm^{-1}, which assigned to stretching vibration modes of –OH groups and intermolecular and intramolecular hydrogen bonds. The peak at 2922 cm^{-1} showed the C–H antisymmetrical stretching vibration. Peaks at 1422 and 1624 cm^{-1} were related to the symmetric and asymmetric stretching vibrations of the carboxylate groups (Yadollahi & Namazi, 2013). The absorption peaks between 1000 and 1200 cm^{-1} were ascribed to characteristic of the C–O–C stretching on polysaccharide skeleton (Wu, Chang, & Ma, 2011). In comparison with CMC, the most striking difference of CMCC positioned at 1651 and 1548 cm^{-1}, which corresponded to the amide I and amide II, respectively (Yang et al., 2010). In spectra of degradation of CMCC, the peaks at 1654 and 1545 cm^{-1} showed the amide group still exist. So the degradation of CMCC with H_2O_2 could not change the structure of CMCC.

3.3. Effect of reaction temperature on DS

Fig. 3A showed the relationship between reaction temperature and the DS of CMCC. As can be seen that DS have significant relevance to the reaction temperature. A maximum DS of 0.57 was obtained with 55 °C as the reaction temperature. There was an increase in DS with reaction temperature up from 25 to 55 °C, and it decreased rapidly when the temperature was further increased. It was generally accepted that the activity of reactants and the rate of the reaction increased with increasing temperature. However, the acylation reaction was exothermic, and with the temperature further increased, the reaction direction reversed. So the optimal temperature was 55 °C.

3.4. Effect of reaction time on DS

As well as reaction temperature, the effect of reaction time on DS as showed to be significant in Fig. 3B. DS of CMCC increased rapidly when the reaction time was prolonged from 12 h to 20 h and thereafter it decreased. A maximum DS of 0.47 was obtained with 20 h as the reaction time. Prolonging the time of the reaction was beneficial to the diffusion and absorption of the reactants with the ultimate effect of inducing better contacts between the collagen peptide and CMC (Pushpamalar, Langford, Ahmad, & Lim, 2006). A possible explanation for the decrease may be due to an increased degradation of the polymer when the time was further prolonged (Heinze & Pfeiffer, 1999). It was possible that modified CMC with a comparatively low degree of polymerization may be removed by the procedure of purification. Therefore, it could be concluded that the optimal reaction time was 20 h.

3.5. Effect of mass ratio of collagen peptide to CMC on DS

The effect of mass ratio of collagen peptide to CMC on DS was showed in Fig. 3C. A maximum DS of 0.47 was obtained with mass ratio of collagen peptide to CMC at 1.0/0.6. There was an increase in the DS with the amount of collagen peptide up to 1.0 g and it decreased with further increased. The increase probably was due to the greater effectiveness of the collagen peptide at higher contents in the proximity of CMC molecules. And the decrease might be caused by the increase of the viscosity and density of reaction medium, which might be increase the stereohindrance effect and electrostatic repulsion. As the overall consideration, the optimal mass ratio of collagen peptide to CMC was 1.0/0.6.

3.6. Antioxidant activities of CMCC

3.6.1. The effect of concentration on scavenging activity

Fig. 4 showed the scavenging effect of carboxymethyl cellulose derivative (CMCC) on DPPH, hydroxyl and superoxide radicals at various concentration. The CMCC effectively quenched three different free radicals in a dose-dependent manner. Moreover, CMCC was more active on DPPH and superoxide than hydroxyl radical. At the concentration of 10.0 mg/ml, the inhibitory effect on DPPH,

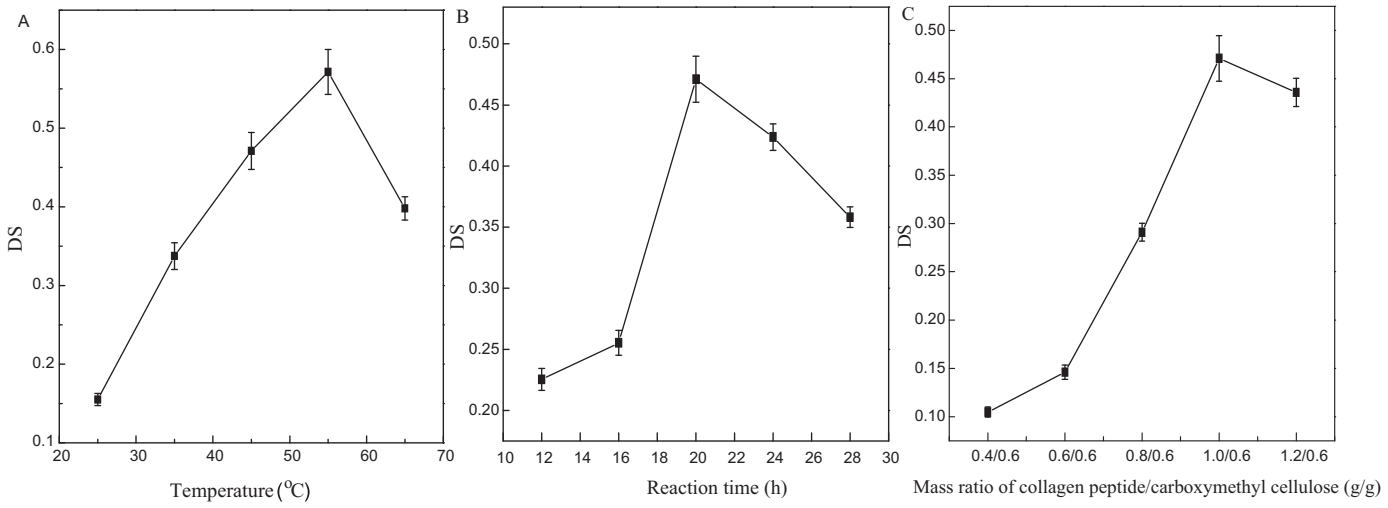

Fig. 3. The effects on DS of carboxymethyl cellulose modified with collagen peptide preparation (A: t = 20 h, collagen peptides/CMC = 1.0/0.6; B: T = 45 °C, collagen peptides/CMC = 1.0/0.6; C: T = 45 °C, reaction time = 20 h).

hydroxyl and superoxide was 55.1%, 36.8% and 44.5%, respectively. At the concentration over 5.0 mg/ml, scavenging ability on DPPH was higher than that of hydroxyl and superoxide. Collagen peptide as positive control was also tested for free radical scavenging effects. The radical scavenging activity of CMCC was a little lower than that of collagen peptide (Fig. 4B). Therefore, these effects were

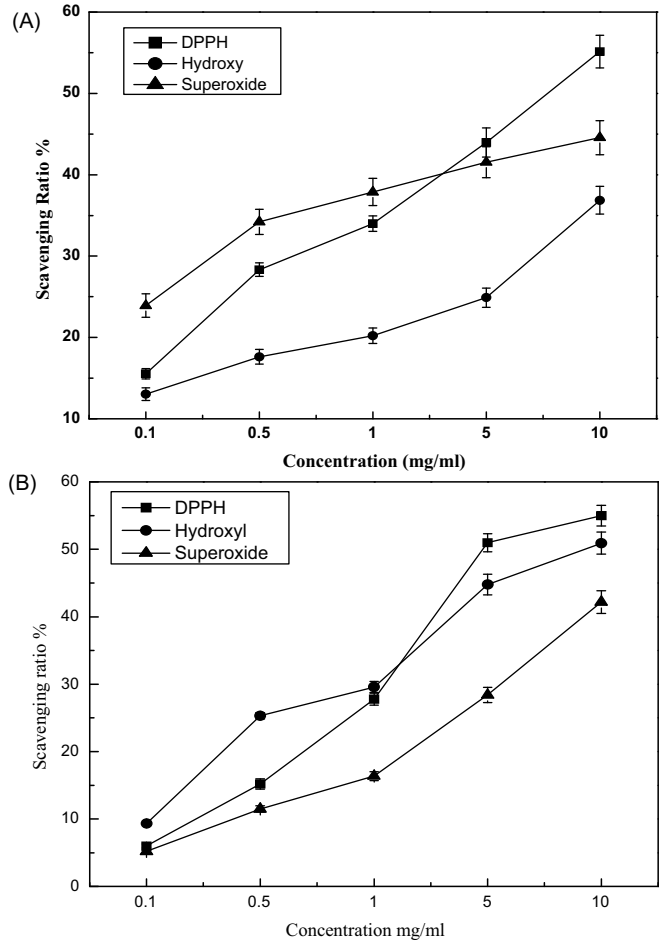

Fig. 4. The effect of the concentration of CMCC (A) and collagen peptide (B) on scavenging activity (CMCC: DS = 0.47, $M_w = 1.78 \times 10^4$, collagen peptide: M_w = 800).

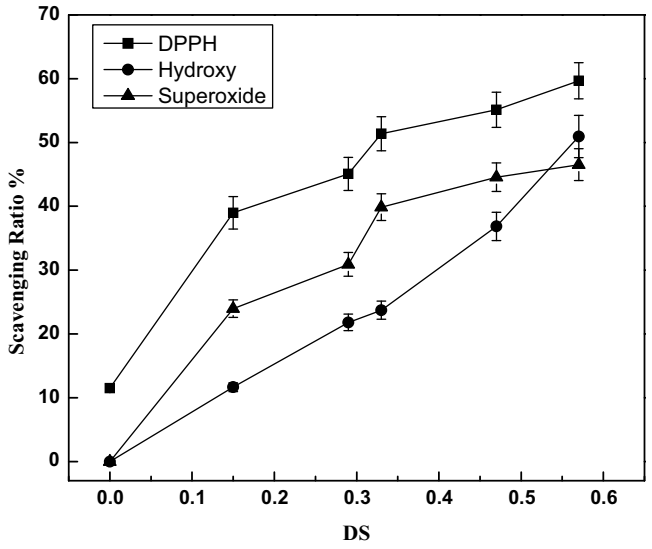

Fig. 5. The effect of the DS on scavenging activity (c = 10.0 mg/ml, $M_w = 1.78 \times 10^4$).

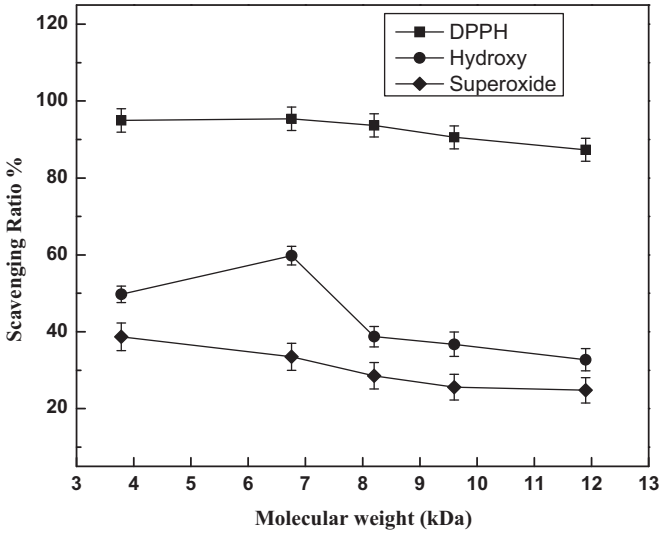

Fig. 6. The effect of the molecular weight on scavenging activity (c = 10.0 mg/ml, DS = 0.29).

most likely due to the modified collagen peptide, as carboxymethyl cellulose itself only showed small scavenging ability of ROS. Moreover, the amino acid residues Lys and Pro, contained in a high amount in collagen peptide, have distinct scavenging capability (Wiegand, Elsner, Hipler, & Klemm, 2006).

3.6.2. The effect of DS on scavenging activity

The scavenging effects on three radicals at varying DS were measured and the results were exhibited in Fig. 5. There was an increase in scavenging effect with the DS up to 0.57. At the DS of 0.57, the scavenging effect of DPPH, hydroxyl, and superoxide radical was 59.6%, 50.9% and 46.5%, respectively. In addition, we can seen that the scavenging effect of CMC (DS = 0) on hydroxyl and superoxide radical was zero, and on DPPH radical was 11.5%. However, when modified CMC with collagen peptide, it exhibited a better ability of scavenging effect, especially on the DPPH radical. DPPH was a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Zhang et al., 2010), and has been widely used to test the free radical-scavenging ability of various samples. Therefore, those results showed that CMCC exhibited better ability of inhibition. Meanwhile, we can concluded that the content of collagen peptide in CMCC has positive correlation with scavenging effect of free radicals.

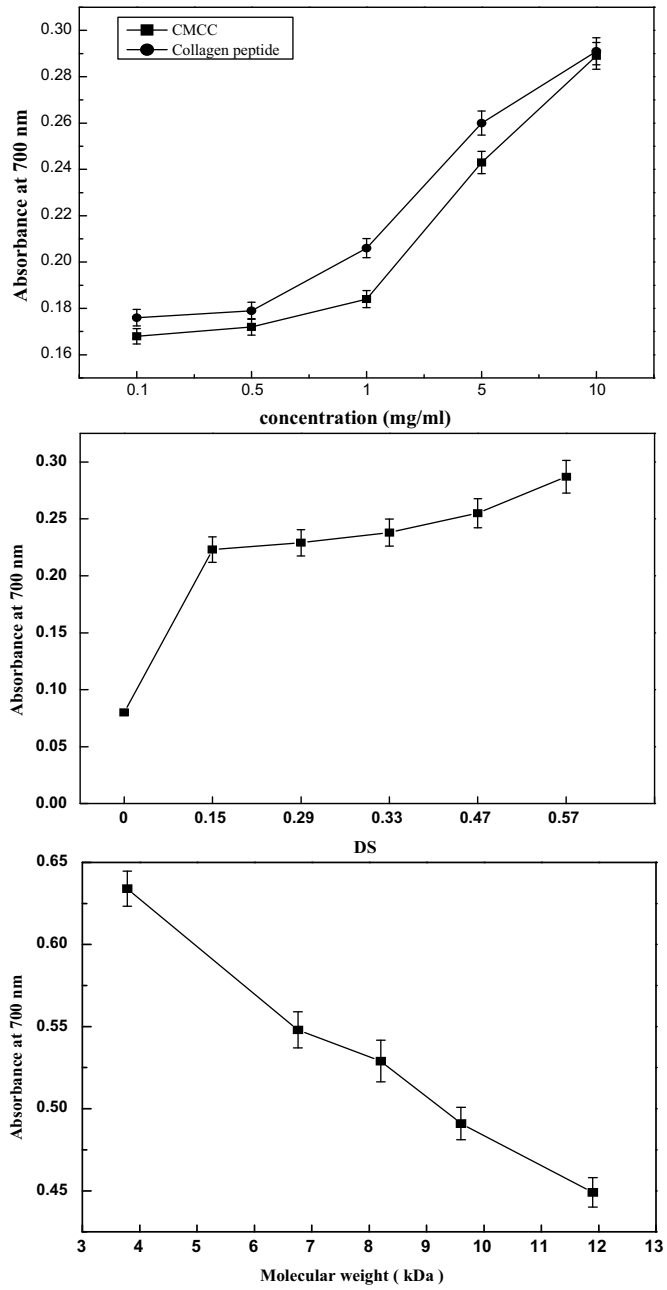

Fig. 7. The reducing power (absorbance 700 nm) of CMCC at different concentration, DS and molecular weight and collagen peptide at different concentration.

3.6.3. The effect of molecular weight on scavenging activity

As can be seen in Fig. 6, molecular weight of CMCC played an important role in antioxidant activities. The scavenging ability of CMCC was better on DPPH than hydroxyl and superoxide radical. In general, the increase in M_w of samples produced reduction in their antioxidant capacities. Fig. 6 showed that CMCC could eliminated DPPH significantly. And the scavenging effect of all the tested samples was over 85.3%, which decreased slowly with M_w increased. Additionally, with the M_w of 6.7×10^3, a maximum scavenging effect on DPPH and hydroxyl radical was 95.4% and 59.7%, respectively. And the maximum scavenging effect of superoxide radical was 38.6% at 3.78×10^3 and decreased with the increase of M_w.

It has been reported that collagen with lower molecular weight (M_w) possibly contained more substrates, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction (Li et al., 2013). In the present study, the M_w was confirmed as the vital factor that determined the antioxidant capacities.

3.6.4. Reducing power

For measurement of the reductive ability, Fe^{3+}–Fe^{2+} transformation in the presence of CMCC samples was investigated. Fig. 7 showed that the reducing power of CMCC increased gradually with the increasing concentration and DS. Compared with the control, the reducing power of CMCC was very closely with collagen peptide. As a whole, at concentration from 0.1 to 10 mg/ml, the reducing power of samples was increased from 0.172 to 0.289. And with DS increased up to 0.57, reducing power increased from 0.08 to 0.287. On the contrary, the lower molecular weight of samples possessed a stronger reductive capability.

3.7. Cytotoxicity of CMCC

The effect of CMCC with different concentration and DS on L929 cells viability was determined by MTT assay (Fig. 8). CMC could not promote L929 cells proliferation and cell viability decreased with

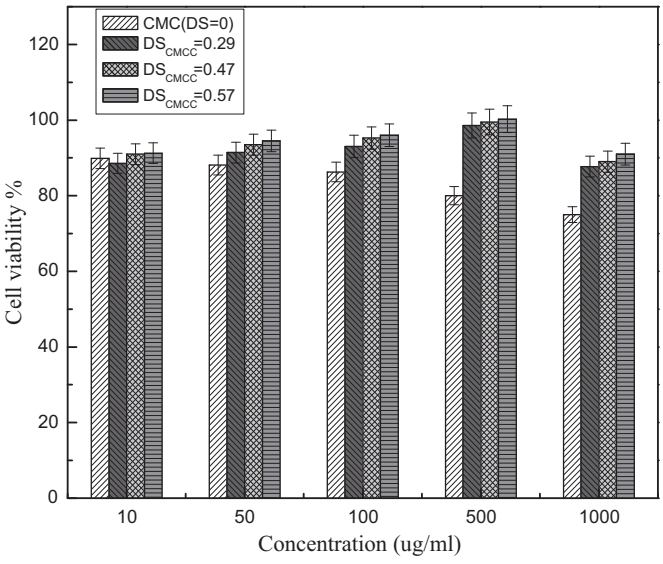

Fig. 8. The effect of the DS and concentration on cell viability of CMCC.

the concentration increased. Compared with the control, CMCC did not appear to promote L929 cells proliferation at 10 μg/ml, and a slight decrease of cell viability was observed at increasing concentration of the samples from 0.01 to 0.5 mg/ml. At the maximal tested concentration of 1 mg/ml, the cell viability was decreased to about 90%. Therefore, cytotoxic effect of CMCC was evaluated on rats fibroblast of L929 cells, and the results showed that CMCC did not show cytotoxic effect on L929 cells.

4. Conclusion

Modified carboxymethyl cellulose with collagen peptide having different DS from 0.10 to 0.57 has been accomplished through investigations into factors affecting the synthesis operation. And the optimal conditions were found: the mass ratio of collagen peptide to carboxymethyl cellulose was 1.0/0.6; the reaction temperature was 55 °C and the reaction time was 20 h. Results obtained concluded that the antioxidant activity of CMCC increased with the increase of concentration and DS. The lower molecular weight of CMCC played a significant effect on antioxidant activity, especially the scavenging effect on DPPH radical. The MTT assay indicated that CMCC was non-toxic to fibroblast cells. The modified products were able to achieve an in vitro reduction of ROS activity. Therefore it seems to be a promising material used for wounds in the future.

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