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Preparation and characterization of sodium alginate modified with collagen peptides

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ABSTRACT

Collagen peptides grafted sodium alginate (SA-COP) was prepared by reacting alginate with collagen peptide via amide linkage in presence of 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy sulfosuccinimide (NHS). The reaction conditions affected the degree of substitution (DS) were studied including the mass ratio of collagen peptide to sodium alginate, reaction temperature and reaction time. The hydrogen peroxide scavenging activity could be controlled by adjusting the DS, concentration and molecular weight. MTT assay was used to investigate the cell viability of SA-COP. The results indicated that the SA-COP exhibited better cell viability, and with the DS and concentration increasing of SA-COP, cell viability increased. The improved functionalities of the derivative might be explained by peptides characteristics.

1. Introduction

Sodium alginate is a biocompatible, non-toxic, non-immunogenic and biodegradable natural linear polysaccharide obtained from the various species of brown seaweed (Jayakumar et al., 2009; Yadav, Mishra, Sand, & Behari, 2011; Yang, Xie, & He, 2011). Chemically, it is a block copolymer composed of two different repeating units, (1-4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers which vary in amount and sequential distribution along the polymer chain depend on the biological source, growth, and stationary conditions (Chiaoprakobkij, Sanchavanakit, Subbalekha, & Pavasant,& Phisalaphong, 2011; Gomez, Pérez Lambrecht, Lozano, Rinaudo, & Villar, 2009; Han, Zhou, Yin, Yang, & Nie, 2010; Rowley, Madlambayan, & Mooney, 1999). Due to the physicochemical properties of alginate, such as thermo-stability, colloids, thickening, emulsification and suspension, film forming and balling, and sol–gel transformation (Goh, Heng, & Chan, 2012; Yang & He, 2012), it is widely applied in the fields of wound care and therapeutics (Goh et al., 2012), tissue engineering (Marijnissen et al., 2002), pharmaceuticals (Zhang et al., 2010), and food chemistry (Bierhalz, da Silva, & Kieckbusch, 2012).

Collagen peptides (COP) are hydrolyzed from collagen (Saito, Kiyose, Higuchi, Uchida, & Suzuki, 2009). Collagen is the main component of the extracellular matrix in the skin, tendon, bone, and cartilage and blood vessels, which is characterized by a triple helical structure and a repeating sequence of Gly-X-Y (X and Y are often Pro and Hyp, respectively) (Kobayashi et al., 2011; Krishna, Jha, Jia, & Kiick, 2011). Collagen has low antigenicity, non-immunogenicity, good biocompatibility and biodegradability, and the ability to promote cell attachment and proliferation (Chen, Wang, Chen, Ho, & Sheu, 2006; Chen, Mo, He, & Wang, 2008). While, due to the large molecular weight the solubility of collagen limited its application. Compared with collagen, COP are low molecular weights, which enable easier direct absorption by the human body (Wang, Zhang, Zhang, & Li, 2011). Moreover, COP exhibited excellent aqueous affinities, moisture retention and water-holding capacity (Kobayashi et al., 2011). In addition, COP has a variety of physiological functions, such as chemotaxis, angiotensin-converting enzyme inhibition, platelet aggregation, stimulating osteoblast growth and differentiation, reducing osteoclast differentiation, extending the life of skin cells, lipid peroxidation inhibition, and defending against oxidative free radical attacks on DNA (Guillerminet et al., 2010; Wang et al., 2011; Watanabe-Kamiyama et al., 2010). Therefore, chemical modification of alginate with collagen peptides can lead to new products with significantly functional properties such as antioxidant activity and promoting cells growth applied in the

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fields of pharmaceuticals and food industry. However, the alginate modified with collagen peptides is little reported. Therefore, the purpose of this work was to prepare sodium alginate modified with collagen peptides.

Hydrogen peroxide causes toxicity and induces cell death (Ak & Gülçin, 2008). On the one hand, hydrogen peroxide activates the nuclear enzyme activity leading to cell death; on the other hand, it induces the oxidative degradation of most biological macromolecules such as lipids, proteins or enzymes, carbohydrates and nucleic acids to generate the hydroxyl radical as being the reactive oxygen species (Özyürek, Bektaşoğlu, Güçlü, Güngör, & Apak, 2010). However, the natural alginate there is no hydrogen peroxide scavenging activity. Therefore, the synthesis of alginate derivatives which own hydrogen peroxide scavenging activity is important.

In this paper, we aimed to synthesize an alginate derivative with good hydrogen peroxide scavenging activity and promotion of cell growth. The results may contribute to finding the application of alginate modified with collagen peptide in pharmaceutical, and food industry fields.

2. Experimental

2.1. Materials

Sodium alginate was purchased from Sinopharm group chemical reagent Corp., Shanghai, China. Collagen peptides (M_w 800) were purchased from Sichuan Mingrang biological technology Co. Ltd., Sichuan, China, without further purification. *N*-hydroxy sulfosuccinimide (NHS), 2-(*N*-morpholino) ethanesulfonic acid (MES), and 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC) were purchased from Huashun Biological Technology Co. Ltd., Wuhan, China. All other reagents were of analytical grade and were used without further purification.

2.2. Synthesis of alginate derivatives

In a typical reaction procedure, sodium alginate (0.6 g) was dissolved in 0.2 M MES buffer containing 0.3 M NaCl at pH 6.5 (50 ml), and then EDC (0.38 g) and NHS (0.12 g) were added into the alginate solution step by step. Magnetic stirring was continuous for 20 h at certain temperature. Collagen peptides were added into the alginate solution and continued to magnetic stir for 10 min. The solution was purified by dialysis through a 10000–8000 molecular weight cut-off dialysis tubing for three days. The dialyzed product was finally freeze-dried with lyophilizer to obtain the purified alginate derivative. The dried samples were stored in vacuum desiccators over P_2O_5 for further analysis. Experiments were conducted under different reaction conditions such as the molar ratio of collagen peptide/alginate, reaction time and temperature to obtain alginate derivatives.

2.3. Measurement of degree of substitution

The degree of substitution (DS) is defined as the number of carboxyl groups substituted per repeating structural unit of the alginate backbone. In this work, the 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 described as Eq. (1). The DSs of alginate derivatives were determined by Eq. (2).

$$A = 37.2679C + 0.0280 \quad R^2 = 0.9943 \tag{1}$$

$$\mathrm{DS} = \frac{19800C}{(800 - 78300C)} \tag{2}$$

where A is the absorbance of collagen peptide, C is the concentration of collagen peptide (Saito et al., 2009).

2.4. Degradation of alginate derivatives

Oxidation degradation method (Li et al., 2010) was used to degrade the products to different molecular weights of SA-COP with DS 0.560. At first, SA-COP (1 g) was accurately weight to be dissolved in distilled water (25 ml). Afterwards, hydrogen peroxide (5.2 ml) was added to the above solution. And the reaction was maintained at 40 °C for 4 h. After that, the solution was stirred for 20 min under 60 °C to remove the residual hydrogen peroxide. The purified product was finally obtained after reduced pressure distillation, washing by absolute ethyl alcohol and freeze-dried with lyophilizer. Experiments were conducted under different volume of hydrogen peroxide to obtain different M_w of SA-COP.

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

FT-IR spectra of SA-COP samples and alginate 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 (M_w) of SA-COPs was determined with static light scattering. The light-scattering spectrometer (ALV/SP-125, ALV, Germany) equipped with an ALV-5000/E multi-τ 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 SA-COPs solutions were prepared in 0.1 mol/l NaCl aqueous solution, and made optically clean by filtration through 0.22 μm Millipore filters. The specific refractive-index increments (dn/dc) of SA-COPs in 0.1 mol/l NaCl aqueous solution were measured on an Optilab refractometer (Wyatt Technology) at 632.8 nm and 25 °C, and were found to be 0.140 cm^3/g.

2.7. Hydrogen peroxide scavenging activity

Tested sample (0.1 mg/ml) of 1.0 ml was mixed with 6.0 ml of phosphate buffer (0.1 mol/l, pH 7.4) and 1.0 ml of 40 mmol/l hydrogen peroxide. After 10 min, the absorbance of mixture was measured at 230 nm with UV–vis spectroscopy. The hydrogen peroxide scavenging activity of the tested samples, expressed as percentage inhibition of H_2O_2, was calculated according to the following formula:

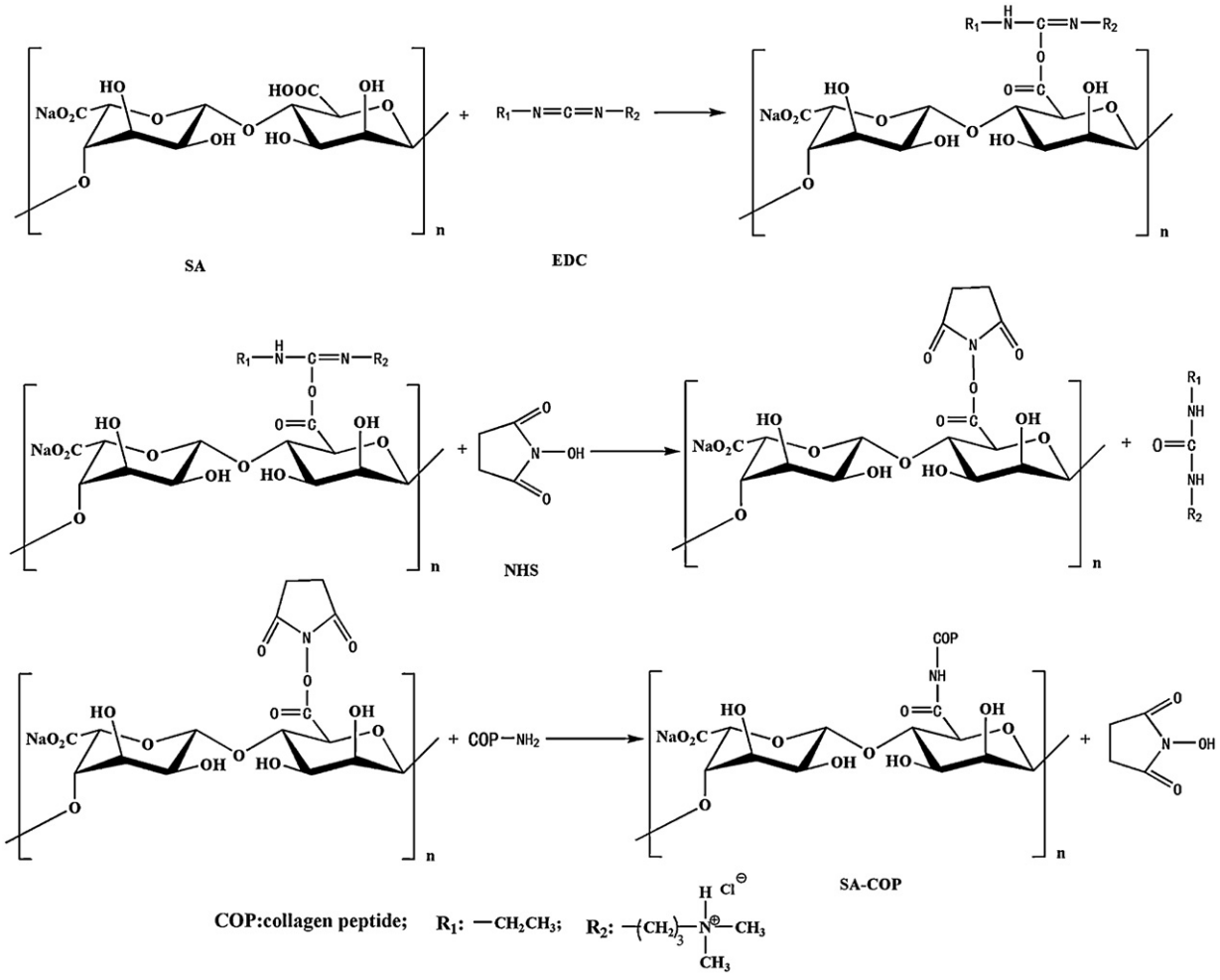
$$\text{Scavenging effect}\,(\%) = \left[1 - \frac{(A_s - A_b)}{A_c}\right] \times 100$$

where A_s is the absorbance value of the tested sample; A_b is the absorbance value of blank sample without hydrogen peroxide; A_c is the absorbance value of control sample without alginate derivatives (Ruch, Cheng, & Klauning, 1989).

2.8. Cell culture

Under aseptic conditions, fibroblasts of rats were put into a culture bottle and incubated in DMEM (Dulbecco's minimum essential medium, Gibco) containing 10% fetal bovine serum (FBS) in the humidified condition of 37 °C and 5% CO_2. Cells were used for experiments when they were three passages old.

The fibroblasts were seeded in a 96-cell plate at 6000 cells/well and cultured for 1 day in 200 μl of DMEM containing 10% FBS. After the alginate derivatives were added for 2 days, the medium was replaced with 200 μl of fresh medium. Then 20 μl of thiazolyl blue (MTT) solutions were added for 4 h. After that, the medium



Scheme 1. Reaction scheme for the alginate modified with collagen peptides.

was removed and 150 μl of dimethyl sulfoxide (DMSO) was added and mixed to dissolve the MTT formazan crystals for 10 min. The absorbance was measured at 492 nm using a microplate reader. The cell viability (%) was calculated according to the following formula:

$$\text{Cell viability}\,(\%) = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A_{sample} was the absorbance in the presence of alginate derivatives and A_{control} was the absorbance in the absence of alginate derivatives (Song, Sun, & Zhang, 2008).

3. Results and discussion

3.1. The synthesis of alginate derivatives

Scheme 1 illustrates the sodium alginate modified with collagen peptide by using EDC and NHS. Sodium alginate first reacted with EDC to form unstable intermediates. Stable NHS-ester was then formed through reaction between the unstable intermediates and NHS. The carboxyl group of alginate was activated by EDC and NHS. The SA-COP was finally synthesized by reacting NHS-ester and collagen peptide. The product and sodium alginate were analyzed by FT-IR spectra in Fig. 1. As can be seen, the alginate showed a broad band at about 3425 cm^{-1}, assigned to stretching vibration modes of O—H groups. The weak peak toward 2930 cm^{-1} was attributed to the C—H antisymmetrical stretching vibration. Two peaks at 1610 and 1414 cm^{-1} were assigned to the C=O and COOH, respectively (Han et al., 2010; Wang, Chen, Jia, Tang, & Ma, 2012). In comparison with sodium alginate, the most striking difference of SA-COP positioned at 1651 and 1555 cm^{-1}, which corresponded to the amide I and amide II, respectively (Yang et al., 2010). The structure of

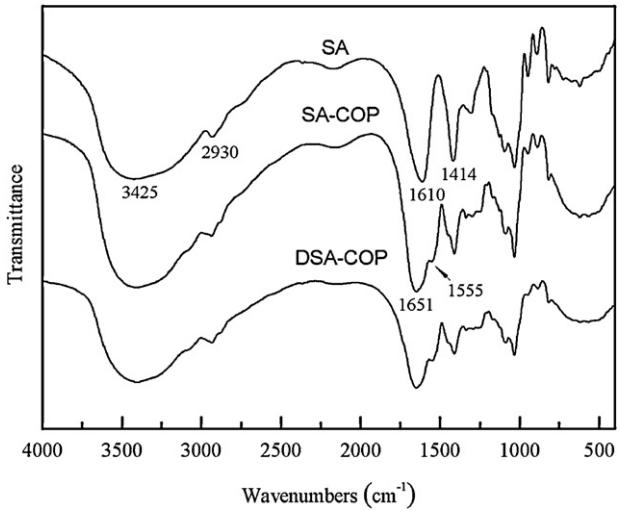


Fig. 1. FT-IR spectra of the alginate (SA), the alginate derivative (SA-COP) and degradation of SA-COP (DSA-COP).

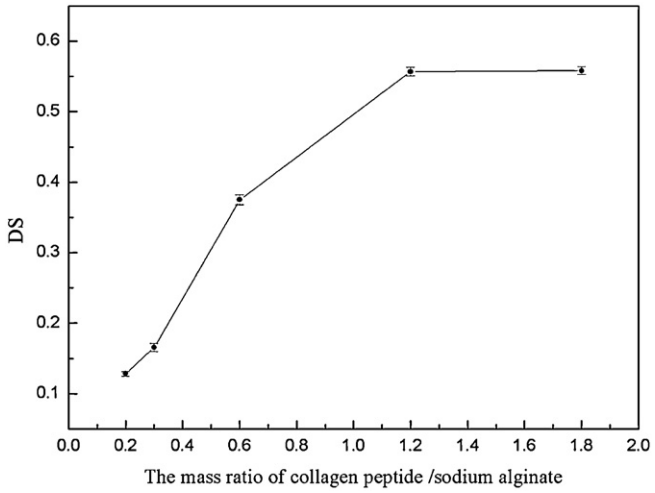


Fig. 2. The effect of the mass ratio of COP to SA on DS (t = 20 h, T = 25 °C).

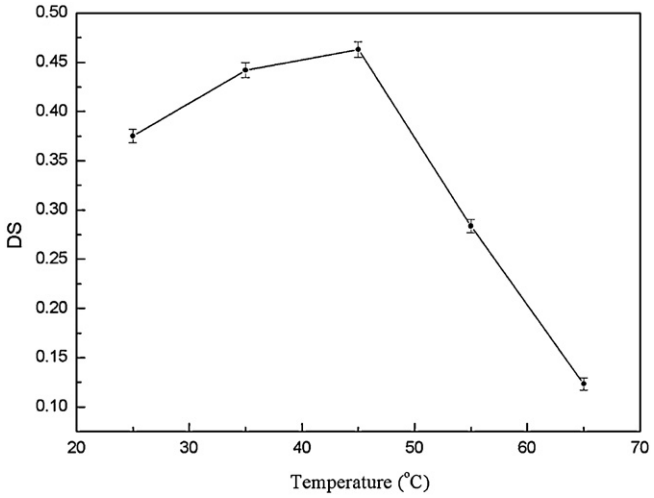


Fig. 4. The effect of the reaction temperature on DS (t = 20 h, $m_{COP/SA}$ = 0.6).

degradation of SA-COP was as the same as the SA-COP. FT-IR spectra have given an evidence of the modification of alginate with collagen peptide. In addition, the degradation of SA-COP with H_2O_2 could not change the structure of SA-COP.

3.2. Optimization of reaction condition of preparing SA-COP

3.2.1. The effect of the mass ratio of COP to SA on DS

As shown in Fig. 2, with the increase of the mass ratio of collagen peptide to sodium alginate from 0.2 to 1.2, the DS of SA-COPs increased obviously from 0.128 to 0.557. When the mass ratio of collagen peptide to sodium alginate further increased to 1.8, the scavenging effect increased slightly to 0.558. This might be due to the stereohindrance effect and electrostatic repulsion, and the reaction was completed into dynamic balance. Therefore, it may be reasonable that the optimal mass ratio of collagen peptide to sodium alginate was 1.2.

3.2.2. The effect of the reaction time on DS

Obviously, when the reaction time increased from 12 h to 20 h, the DS of SA-COPs increased sharply from 0.065 to 0.375 in Fig. 3. However, when the reaction time further increased to 28 h, the DS was decreased to 0.155. The reasons might be as follows: the longer duration of reaction enhanced dissolution and diffusion of the alginate, and made the carboxyl group of alginate be activated completely by EDC and NHS so as to react with collagen peptide efficiently as Scheme 1 described. Nevertheless, with the further extension of time, the NHS-ester could be hydrolyzed. Accordingly, the alginate was not sufficiently modified by collagen peptide, which leaded to the decrease of the DS. Therefore, it could be concluded that the optimal reaction time was 20 h.

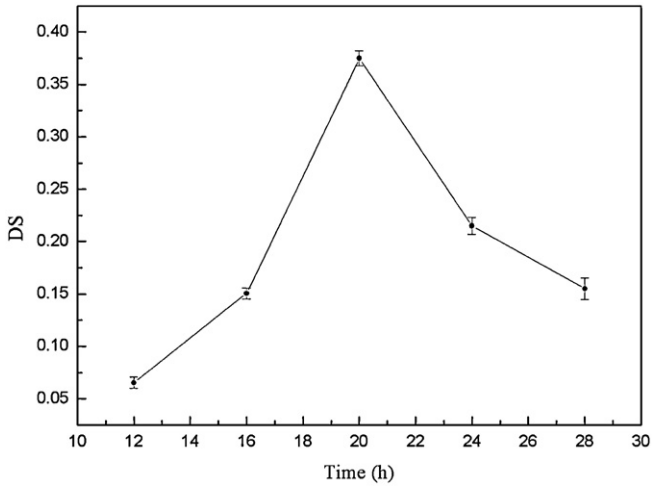


Fig. 3. The effect of the reaction time on DS (T = 25 °C, $m_{COP/SA}$ = 0.6).

3.2.3. The effect of the reaction temperature on DS

As can be seen in Fig. 4, with an increase from 25 °C to 45 °C, the DS increased from 0.375 to 0.463. With further increase from 45 °C to 65 °C, the DS decreased from 0.463 to 0.123. It is reasonable to believe that with increase of the temperature, the activity of reactants and the rate of the reaction increased. Thus, the DS of alginate increased. While, the acylation reaction is exothermic, and with the temperature further increased, the reaction direction reversed so as to decrease the DS. Therefore, the optimal temperature is 45 °C with the highest DS of 0.463.

3.3. Hydrogen peroxide scavenging activity

3.3.1. The effect of DS on hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging effect of sodium alginate was zero in Fig. 5. While, after modification with collagen peptides, the products exhibited better hydrogen peroxide scavenging effect. And with an increase of DS from 0.071 to 0.56, the hydrogen peroxide scavenging effect increased gradually from 1% to 36%. Therefore, it illustrated that the main composition of scavenging hydrogen peroxide was the collagen peptide. And the mechanisms are dependent on unique properties of peptides contributed by their chemical composition and physical properties (Ding et al., 2011). However, further research needs to be carried out at molecular levels.

3.3.2. The effect of the concentration on hydrogen peroxide scavenging activity

Fig. 6 depicts hydrogen peroxide scavenging effect of different concentration of SA-COP. The scavenging effects on hydrogen peroxide increased with the increase of SA-COP concentration. When the concentration of SA-COP was 2.5 mg/ml, the scavenging effects on hydrogen peroxide increased at 42%. It illustrated that the effect of scavenging hydrogen peroxide of SA-COP was concentration related. This is because with the increase of concentration, the content of collagen peptide increased, which is contributed to the scavenging of hydrogen peroxide.

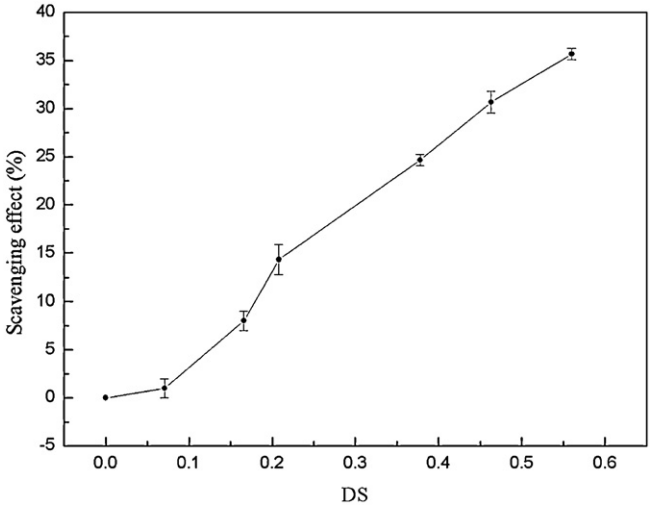


Fig. 5. The effect of DS on hydrogen peroxide scavenging activity (c = 1.0 mg/ml, $M_w = 1.48 \times 10^4$).

3.3.3. *The effect of the molecular weight on hydrogen peroxide scavenging activity*

Molecular weight of SA-COP plays an important role in hydrogen peroxide scavenging activity as shown in Fig. 7. The scavenging effect of SA-COP increased from 15% to 39% as the molecular weight reduced from 1.25×10^5 to 2.43×10^4. However, when the molecular weight further reduced to 0.3×10^4, the scavenging effect decreased to 21%. The reasons might be as follows: on the one hand, with reducing of the molecular weight, the structure of SA-COP is relatively loose and the intramolecular hydrogen bond is weakened. Thus, more amino group and hydroxyl group could react with hydrogen peroxide so as to increase the hydrogen peroxide scavenging activity of SA-COP. It could be the result of the structure of alginate and collagen peptide. On the other hand, with further reduced molecular weight, the part of collagen peptide nearly is degraded to amino acid. Because peptides are better than amino acids (Ding et al., 2011), the changes from peptide to amino acids majorly influenced the hydrogen peroxide scavenging activity of SA-COP. Meanwhile, the structure of alginate is also changed to influence the activity. Therefore, from the results, 2.43×10^4 was the optical molecular weight for hydrogen peroxide scavenging activity of SA-COP.

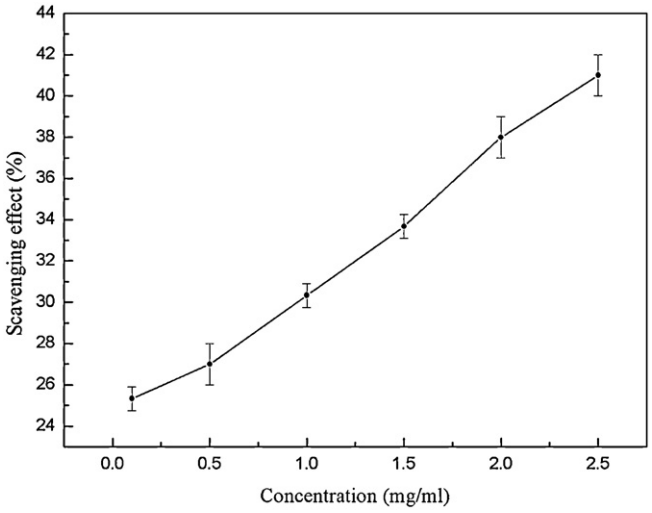


Fig. 6. The effect of the concentration on hydrogen peroxide scavenging activity (DS = 0.463, $M_w = 1.48 \times 10^4$).

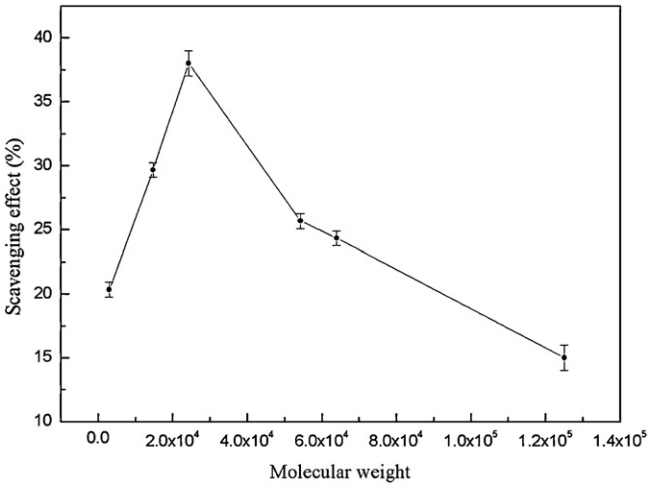


Fig. 7. The effect of the molecular weight on hydrogen peroxide scavenging activity (c = 1.0 mg/ml, DS = 0.463).

3.4. *Cell culture*

As shown in Fig. 8(a), at the same concentration of 1000 mg/l, SA-COPs with different DS of 0.166, 0.378 and 0.56 exhibited better cell viability than alginate. And the cell viabilities increased with the

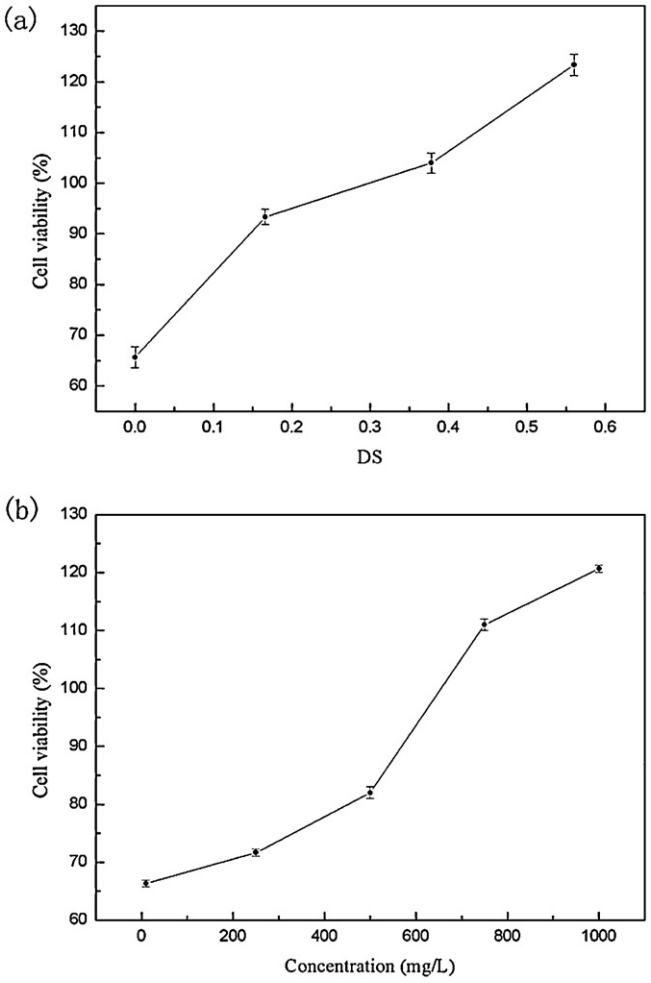


Fig. 8. The effect of the DS (a: c = 1000 mg/l) and concentration (b: DS = 0.56) on cell viability for SA-COP.

increase of DS. The results revealed that the introduction of collagen peptide to alginate was an efficient method to improve cell viability. In addition, from Fig. 8(b), the cell viability of SA-COPs increased gradually with increasing of the concentration. This might be due to the increase of the content of collagen peptide. Although the mechanism of cell viability for the SA-COP and alginate is not completely resolved, it is likely that collagen peptide could stimulate the synthesis of extracellular matrix components, consequently speed up the reconstruction, and promote cell adhesion and proliferation. More work is needed to confirm this hypothesis.

4. Conclusions

In conclusion, a novel alginate derivative was successfully synthesized by modification with collagen peptide, which exhibited good hydrogen peroxide scavenging activity and promoted cell growth. SA-COP with DS value of 0.071–0.560 could be obtained by adjusting the mass ratio of collagen peptide to sodium alginate from 0.2 to 1.8, reaction temperature from 25 to 65 °C and reaction time from 12 to 28 h. And the optimal conditions were found to be as follows: The mass ratio of collagen peptide to sodium alginate was 1.2. The reaction temperature was 45 °C. The reaction time was 20 h. Furthermore, the DS, concentration and molecular weight of alginate derivatives played an important role in hydrogen peroxide scavenging activity. The results indicated that with the increase of DS and concentration, the hydrogen peroxide scavenging activity of SA-COP improved. SA-COP with M_w 2.43×10^4 exhibited better hydrogen peroxide scavenging activity. In addition, the SA-COPs with different DS and concentration were evaluated in terms of cell viability. The results showed that SA-COPs could promote cell growth efficiently. And with the increase of DS and concentration, the cell viability was increased. Therefore, the SA-COP is a promising derivative of alginate which applied in pharmaceutical and food industry fields.

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