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Preparation and biological activity of quaternized carboxymethyl chitosan conjugated with collagen peptide

Xiaoming Zhu[a,c], Xiaoyu Zhou[b], Jiayan Yi[b], Jun Tong[b], Huan Wu[b], Lihong Fan[a,b,*]

[a] Hubei Collaboration Innovation Center of Non-power Nuclear Technology, Hubei University of Science and Technology, Xianning 437100, China
[b] College of Chemical Engineering, Wuhan University of Technology, Wuhan 430070, China
[c] School of Nuclear Technology & Chemistry and Biology, Hubei University of Science and Technology, Xianning 437100, China

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ABSTRACT

Tissue repair is a spontaneous process which initiated on wounding. If this complex mechanism is disturbed or impaired, the use of biomaterials might increase the chance of successful healing. In this view, a water-soluble chitosan derivative, quaternized carboxymethyl chitosan (QCMC) was prepared and collagen peptides (COPs) were grafted to the backbone by carbodiimide method. The reaction conditions affecting the degree of substitution (DS) were studied including the mass ratio of collagen peptide to QCMC, reaction temperature and reaction time. The hydrogen peroxide-scavenging activity could be different by changing the DS, concentration and molecular weight. MTT assay was used to investigate the cell viability of the derivative. The results indicated that the introduction of collagen peptide into the QCMC improved its hydrogen peroxide-scavenging activity and cell viability with the DS and concentration increased. Therefore, QCMC conjugated with collagen peptides may prove beneficial to the process of the wound-healing.

1. Introduction

Chitosan is a copolymer consisted of D-glucosamine and *N*-acetylglucosamine, which produced by the deacetylation of chitin. As the only alkaline polysaccharide exists in the nature, chitosan has attracted more and more attention by researchers owing to its multiple bioactivities, such as antimicrobial, anti-tumor and immune-enhancing effects [1]. However, the application of chitosan is often limited by its poor solubility in physiological media. To improve its water solubility, many derivatives have been studied, including quaternization, carboxymethylation and some other ways [2].

Quaternized carboxymethyl chitosan (QCMC) is a novel promising amphoteric polymer. It is often obtained by introducing quaternary ammonium salt on CMC, which has significantly enhanced the antibacterial or antimicrobial activity of CMC. On the other hand, it can also be synthesized by reacting quaternized chitoson with monochloroacetic acid [1,3,4]. In recent years, QCMC has gained more interests of research. The moisture-absorption and -retention abilities of QCMC were studied and the results showed that QCMC had better moisture-absorption and -retention abilities compared with hyaluronic acid (HA) [5]. Cai et al. [6,7] have investigated the flocculating properties of QCMC and its capacity to flocculate chemical oxygen demand COD from printing wastewater. In addition, QCMC shows a better antioxidant activity than carboxymethyl chitosan and it has also been reported that it has therapeutic effect on nonalcoholic fatty liver disease because it can help reducing the chance of inducing insulin resistance or oxidative stress [8,9].

In vivo, cells are surrounded by the extracellular matrix (ECM), a hydrated network of proteins and proteoglycans that provide structure and guidance for the cell in most vital processes [10]. Therefore, for the purpose of improving the cell attachment, growth and differentiation on the biomaterials, such as hydrogels, scaffolds and biofilms, researchers have already attempted conjugated variety of biomaterials with collagen, silk fibroin, gelatin and glycoproteins [11–14]. Collagen, as the main component of the extracellular matrix in the skin, bone, cartilage, tendon and blood vessels, is characterized by a triple helical structure and a repeating sequence of Gly-X-Y [15]. Collagen peptides (COPs) are hydrolyzed from collagen, and it has good biocompatibility, low antigenicity, non-immunogenicity and the ability to promote cell proliferation and attachment [12,16]. Compared with pure collagen, collagen peptide has lower molecular weight so that it could be easier to absorb directly by the human body. Moreover, collagen peptide exhibited

* Corresponding author at: College of Chemical Engineering, Wuhan University of Technology, Wuhan 430070, China. Tel.: +86 27 62773634; fax: +86 27 87859019.
E-mail address: lihongfan2000@hotmail.com (L. Fan).

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excellent aqueous affinities, moisture retention and antioxidant capacity [17]. However, the quaternized carboxymethyl chitosan modified with collagen peptides has not been reported yet. Therefore, through the chemical modification of QCMC with collagen peptides can definitely lead to a new derivative with significant functional properties such as antioxidant activity and promoting cell growth and attachment, which could be applied in the fields of pharmaceuticals and food industry.

Hydrogen peroxide causes toxicity and induces cell death. 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 [18]. In fact, both QCMC and collagen peptide own hydrogen peroxide-scavenging activity. Therefore, the synthesis of QCMC–collagen might significantly enhance the hydroxyl radical-scavenging activity.

In this paper, we aimed to synthesize a chitosan derivative with good hydrogen peroxide-scavenging activity and better interaction between the cells. The results may contribute to finding the application of quaternized carboxymethyl chiotosan modified with collagen peptide in pharmaceutical and food industry fields.

2. Experimental

2.1. Materials

Chitosan (degree of deacetylation = 92.50%) was purchased from Zhejiang Yuhuan Ocean Biochemistry Co. Ltd. (China). Fish collagen peptides (M_w 536) were purchased from Sichuan Mingrang Technology Co. Ltd., Sichuan, China, without further purification. *N*-hydroxy sulfosuccinimide (NHS), 2-(*N*-morpholino)ethanesulfonic acid (MES) and 1-ethyl-(dimethylaminopropryl)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. Preparation of quaternized carboxymethyl chitosan modification with collagen peptides

2.2.1. Synthesis of carboxymethyl chitosan

O-CMC was prepared by our previously reported method with slight modification [19]. In a typical reaction procedure, a certain amount of chitosan was added to NaOH aqueous solution (50%, w/w) in a beaker and the mixture was frozen for 24 h. After this, the alkaline chitosan powders were dispersed with isopropyl alcohol for 1 h under stirring. Then a predetermined amount of monochloroacetic acid was added at 5 min intervals over a period of 20 min. The mixture was washed twice by ethanol after stirred continuously for 5 h at room temperature. The resulting precipitate was dissolved in a large amount of distilled water, and then dialysis against distilled water for 72 h. After dialysis, the product was finally evaporated, collected and dried overnight in an oven at 60 °C.

2.2.2. Quaternization of carboxymethyl chitosan

CMC (10 g) was dissolved in 40 ml of distilled water, and 2,3-epoxypropyl trimethylammonium chloride was added with different molar ratio to glucosamine unit. The mixture was reacted at 80 °C for 8 h with stirring, then dialyzed for 4 days and finally lyophilized to obtain yellow QCMC powder.

2.2.3. Conjugation fish collagen peptides to QCMC

In a typical reaction procedure, QCMC (1 g) was dissolved in 0.2 M PBS (pH 6.0), and then EDC (0.35 g) and NHS (0.15 g) were added into the QCMC solution to activates the —COOH groups for 2 h. After the activation, fish collagen peptide was also dissolved in 0.2 M PBS solution (pH 6.0) and added into the QCMC solution. Magnetic stirring was continuous for 20 h at certain temperature. The final product was purified by dialysis for 4 days. The water was changed three times per day. The dialyzed product was finally freeze-dried with lyophilizer to obtain the purified chitosan derivative. Experiments were conducted under different reaction conditions such as the molar ratio of collagen peptide/QCMC, reaction time and temperature to obtain chitosan derivatives with high degree of substitution.

2.3. Measurement of degree of substitution

The degree of substitution (DS) is defined as the number of amine groups substituted per repeating structural unit of the QCMC. In this work, the DS was measured according to the method of Fan et al. [17], the concentration of COP between 0.001 and 0.05 g/l was a linear relation with absorbance at 200 nm by ultraviolet spectrophotometry. And the standard curve for the linear relation was described as Eq. (1). The QCMC and COP-QCMC samples were also measured with absorbance at 200 nm by ultraviolet spectrophotometry with the concentration of 0.05 g/l. The QCMC sample was the blank control. The DS of COP-QCMC were determined by following Eq. (2).

$$A = 25.322C + 0.0097 \quad (1)$$

$$R^2 = 0.9996$$

$$\mathrm{DS} = \frac{333C}{40 - 799C} \quad (2)$$

where A is the absorbance of COP and C is the concentration of COP [16].

2.4. Degradation of chitosan derivatives

COP-QCMC powder (1 g) was suspended in 50 ml of deionized water, after stirring at 40 °C for 2 h, hydroperoxide of a desired volume was added for predetermined time to yield COP-QCMC of various molecular weights.

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

FT-IR spectra of samples were performed with a Nicolet 170SX Fourier transform infrared spectrometer. The test specimens were prepared by the KBr-disk method.

2.6. Light scattering measurements

The weight–average molecular weight of COP-QCMC was determined with 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-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 COP-QCMC solutions were 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 COP-QCMC in 0.1 M 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.