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Synthesis of protocatechuic acid grafted chitosan copolymer: Structure characterization and *in vitro* neuroprotective potential

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ABSTRACT

Excessive free radicals can cause oxidative damage to human tissues, which results in a variety of diseases. Therefore, the development of antioxidant materials is one of the great projects in biomedical field. In this work, antioxidant protocatechuic acid (PCA) monomers were grafted onto chitosan (CS) backbones to develop a PCA grafted chitosan (PCA-g-CS) antioxidant copolymer *via* the method of free radical-induced grafting reaction. The formation of covalent bonds between PCA and CS were confirmed by FTIR, ^{1}H NMR, XRD and UV–vis. The antioxidant activity of PCA-g-CS was analyzed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical scavenging assays. In addition, the cytotoxicity of PCA-g-CS on neuron-like rat phaeochromocytoma (PC12) cells was evaluated by using MTT assay. The neuroprotective effects against hydrogen peroxide (H_2O_2) and L-glutamic acid (GLU) induced apoptosis in PC12 cells were also investigated. Our results demonstrated that the PCA-g-CS antioxidant copolymer had the ability to scavenge DPPH and hydroxyl radical *in vitro*. Furthermore, the PCA-g-CS was biocompatible and had neuroprotective effects against free radical-induced apoptosis in PC12 cells. This PCA-g-CS copolymer is firstly synthesized for neuroprotection and the results suggest the PCA-g-CS may be a potential antioxidant material in the treatment of oxidative damage related diseases.

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1. Introduction

Reactive oxygen species (ROS) are a serious of free radicals, including peroxide, superoxide, hydroxyl radical, and singlet oxygen [1]. They are normal metabolites involved in the biochemical process of cell electron transport, and have a positive physiological role in tissue homeostasis [2]. However, when in-built antioxidant defense system in body is invalid in some pathological conditions, ROS will be accumulated. The excessive ROS injure relative proteins, lipids and nucleic acids [3], which lead to oxidative damage to normal cells and tissues, and finally cause various oxidative damage related diseases. Therefore, it is necessary to eliminate ROS in time, so as to maintain free radicals homeostasis at a low level.

Antioxidants could protect cells from damage caused by free radicals. However, most of active antioxidants such as glutathione, vitamin C and tea polyphenols, are a kind of small molecular substances that can be easily eliminated by material metabolism. It is difficult to long-termly stabilize in human body, and maintain the antioxidant activity. Therefore, in order to overcome this disadvantage, the incorporation of antioxidants into biodegradable polymer materials is one of viable methods to provide a sustained release of antioxidant during polymer hydrolysis. Chitosan (CS) and its derivatives, are agents that have many excellent physiological properties, including biodegradable, antioxidative, non-toxic, non-immunogenic, antibacterial, and biocompatible [4]. The various properties make it widely used in biomedical applications with many forms, including gels, membranes, nanofibers, micro/nano particles, scaffolds and sponge-like forms [5]. However, chitosan is insoluble in common solvents due to its highly extended hydrogen-bonded structure, which has been a stumbling block in its appropriate utilization. Fortunately, the presence of active amino (C-2), primary hydroxyl (C-3) and secondary hydroxyl (C-6) groups in chitosan contributes to chemical modification, thereby enhancing its intrinsic properties or giving some new properties

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[6]. Recently, chemical modifications of chitosan have attracted a great deal of attention for enhancing or adding the antioxidant activity while keeping the natural backbones of chitosan. Until now, there are three main methods based on different principles to graft natural phenolic antioxidants onto chitosan backbones, including laccase grafting [7–9], esterification/amidation [10–13] and free radical grafting [14–18]. Those new phenolic acid grafted compounds showed desired antioxidant properties and widened applications in food, waste water treatment and biomedicine.

Protocatechuic acid (PCA), as 3, 4-dihydroxybenzoic acid, is a water-soluble monomeric phenolic acid and widely distributed in many traditional Chinese herbal medicines. It can inhibit free radicals from peroxidating lipid and damaging cell membrane, and the antioxidant activity is mainly due to the donation of hydrogen atom (H•) and electron (e) from 3, 4-hydroxyl [19]. Recently, some studies have found that PCA is an effective neuroprotective agent for Parkinson's disease [20], Alzheimer's disease [21], and ischaemic heart disease [22]. Furthermore, it has the potential to be an effective antioxidant due to its strong free radicals scavenging activity in cultured neural cells. For example, our team found that PCA was able to protect against oxidative stress-induced neurotoxicity due to hydrogen peroxide apoptosis in cultured PC12 cells [23]. PCA could also inhibit PC12 cells apoptosis induced by rotenone [24] and MPTP (one of selective nigrostriatal dopaminergic neurotoxins) [25]. The mechanism of the neuroprotective effects in the cellular level might attribute to the inhibition of the oligomerization of α-synuclein [25]. Therefore, based on the summary of the existed studies, PCA may become a desired antioxidant for the treatment of oxidative stress-induced neurodegenerative diseases. However, little attention has been paid to the grafting PCA onto chitosan, and only one research prepared PCA-grafted-chitosan by the carbodiimide mediated coupling reaction, and the purpose was for the development of a novel antioxidant in functional food and an active packing material for food preservation [10,11]. No previous studies have attempted to evaluate the antioxidative and neuroprotective effects of phenolic acid grafted chitosan for neural cells *in vitro*.

In this study, PCA was grafted onto chitosan backbones by free radical mediated chemical crosslinking reaction *via* ester and amide bonds. The chemical structure of prepared PCA-g-CS copolymer was characterized by FTIR, ^{1}H NMR, XRD and UV–vis. The free radical scavenging activity was verified by DPPH and hydroxyl radical scavenging assays. The cell cytotoxicity and its neuroprotective effects against free radical-induced apoptosis in neural cells (PC12 cells) were also evaluated. Our results provided novel information about the antioxidant protection of apoptosis in neural cells by using PCA-g-CS material, and laid a foundation for a prolonged antioxidant activity on chitosan-based nerve repairing materials containing prepared PCA-g-CS component.

2. Experimental

2.1. Materials

Chitosan powder (viscosity = 50 mPa s, 86% deacetylation) was purchased from Jinan Haidebei Marine Bioengineering Co,. Ltd. (China). Chitooligosaccharides (COS) (<5 kDa, 80–85% deacetylation) was purchased from Dalian GlycoBio CO,. Ltd (China). PCA and 1,10-phenanthroline were purchased from Energy Chemical (China). Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), MTT, Calcein-AM, Propidium Iodide (PI) and Hoechst 33342 were purchased from Sigma-Aldrich Inc. (USA). L-glutamic acid (GLU) was purchased from Beijing Hotaibio Science and Technology Co,. Ltd. (China). Other reagents were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. (China). All other materials and reagents used in the study were analytical grade.

2.2. Preparation of antioxidant PCA-g-CS copolymer

The preparation method of PCA-g-CS was mainly from Curcio et al. [18] with some modifications. The method was as follows. Chitosan powder (500 mg) was dissolved in 10 ml of acetic acid aqueous solution (2%, v/v), and 1 ml of H_2O_2 aqueous solution (1.0 M) containing ascorbic acid (54 mg) was added with agitation. High purity nitrogen was then introduced into this reaction system to replace the oxygen. After stirring for 30 min at room temperature, PCA (200 mg) was added into above solution. The reaction was carried out with gentle agitation for 24 h in the atmosphere of nitrogen, to obtain a clear and transparent solution. The solution was centrifuged to remove trace amounts of insoluble impurities, and supernatant was dialyzed against distilled water with dialysis membrane (MWCO = 3500 Da) for 48 h to remove unreacted phenolic acids and other soluble substances. Finally, the dialysate was lyophilized in vacuum freeze-drying equipment (Labconco, USA) for 24 h, to obtain water-soluble PCA-g-CS sample as a pale yellow powder. Native chitosan was prepared as described above without addition of PCA monomers, and the other conditions were consistent with the above steps. The purified PCA-g-CS was detected by high performance liquid chromatography (HPLC, HP1100) to ensure complete elimination of unreacted PCA monomers. The synthesize procedure was shown in Fig. 1.

2.3. Characterization of PCA-g-CS copolymer

The chemical structure of PCA-g-CS copolymer was characterized by Fourier transform infrared spectroscopy (FTIR), Proton nuclear magnetic resonance (^{1}H NMR), X-ray diffraction (XRD) and UV–vis spectrophotometer (UV–vis). The FTIR spectra (EQUINOX55) of samples in KBr pellets were recorded in the frequency range of 4000- 400 cm^{-1} with resolution of 4 cm^{-1}. ^{1}H NMR spectroscopy (Bruker Avance II 400 M) for confirming phenolic protons signal in PCA-g-CS was tested in D_2O (0.5 ml) with operating frequency of 600 MHz. The XRD experiments were carried out using X-ray diffractometer (D/MAX-2400) and the relative intensity was scanned from 5° to 50°. UV–vis absorption spectroscopy (Hitachi UV-4100) was taken for detecting antioxidant phenolic groups in distilled water or aqueous NaOH solution (0.01 M), and the wave number was from 220 to 500 nm.

2.4. Determination of the antioxidant activity of PCA-g-CS

2.4.1. Assay of DPPH radical scavenging activity

DPPH radical scavenging activity of PCA-g-CS was tested by DPPH assay according to the method of Curcio et al. [18] with some modifications. The method comprises the following steps: PCA-g-CS (20 mg) was dissolved into alcohol aqueous solution (80%, 5 ml), then ethanol (5 ml) containing DPPH (200 μM) was added into that solution. The mixture was shaken thoroughly and incubated for 30 min at 25 °C. The absorbance was measured at 517 nm using microplate reader (SPECTRAFLUOR, TECAN, Sunrise, Austria). The native chitosan and COS were as controls and measured in the same condition to evaluate its interference in the DPPH assays. Results were calculated using the following equation:

$$\text{inhibition}\,\% = \frac{A_0 - A_1}{A_0} \times 100\% \quad (1)$$

Where A_0 was the absorbance of the blank solution without samples, A_1 was the absorbance of the samples. Determination of each sample was performed in triplicate.

2.4.2. Assay of hydroxyl radical scavenging activity

Hydroxyl radical (created by Fenton's reagent) scavenging activity was tested according to the method described by Li et al.

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