Materials Science and Engineering C 33 (2013) 2611–2619

Contents lists available at SciVerse ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

The preparation and characterization of novel human-like collagen metal chelates

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ARTICLE INFO

Article history:
Received 15 October 2012
Received in revised form 21 January 2013
Accepted 15 February 2013
Available online 24 February 2013

Keywords:
Calcium
Chelation
Copper
Human-like collagen
Manganese
Metal chelates

ABSTRACT

In order to develop the nutritional trace elements which could be absorbed and utilized effectively, protein chelates were adopted. Calcium, copper and manganese were considered based on their physiological functions, and the new chelates of HLC-Ca, HLC-Cu and HLC-Mn were formed in MOPS or MES buffer and purified by gel chromatography, and then freeze-dried. And they were detected and analyzed by atomic absorption spectrophotometry, ultraviolet–visible absorption (UV–vis) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, fluorescence quenching method, circular dichroism (CD) and differential scanning calorimetry (DSC). The results showed that some chemical reactions happened between HLC and the three metal ions to form new chemical compounds. The thermodynamic parameters, ΔH, ΔG and ΔS, showed that the chelation process between HLC and metal ions was performed spontaneously. Fluorescence quenching spectra of HLC indicated that the quenching mechanism was static in nature. According to the data of DSC, the new chelates were more stable than the free HLC. And HLC-metal complex was non-toxic to the BHK21 cell through MTT assay.

1. Introduction

Calcium, copper and manganese all serve as essential trace elements for human beings and animals. Calcium is central to normal cell processes, because the tight regulation of intracellular calcium is crucial to homeostasis [1]. It is an integrating factor in the generation or diversion of metabolic energy [2] and plays a key role in a variety of neuronal functions, it is also known to control gene expression in skeletal muscle cells or neurons [3,4]. Copper is the third natural trace metal in the human body, followed after iron and zinc [5]. It is required for cell survival and serves as an important catalytic cofactor in redox chemistry for proteins which play some fundamental biological functions in human growth and development, mitochondrial respiration, iron absorption, free radical scavenging and elastin cross-linking [6,7]. Manganese is known to be essential for the development of the brain [8] and transmitted to the brain via both the blood–brain and the blood-cerebrospinal fluid barriers [9]. It is also involved in the metabolism of protein, lipid and carbohydrate, and serves as a cofactor for enzymes such as decarboxylase, hydrolase and kinase [9].

Dietary deficiency of calcium, copper and manganese would affect the growth and development of human beings and animals, and the inorganic salts of these three trace elements were unavailable to absorb or/and increase dose-limiting toxicities [10]. Proteins or amino acids, due to their easy absorption and utility, were developed to be prospective ligands to some ions [11].

Recently, the chelates formed by protein and metal ions have been considered as a potential approach to delivering some important nutrients to consumers at a required quantity so as to avoid the deficiency of metal trace elements [12]. Human-like collagen (HLC), obtained from recombinant *Escherichia coli* containing human-like collagen cDNA, is a recombinant macromolecular protein with a tri-helix repeat structure [13]. Compared with the animal collagen, HLC has several special characteristics which are significantly different from natural collagen from animal tissues, such as good biocompatibility, low immunogenicity, water-solubility, virus-free, little immunogenic reaction, etc. [14,15]. So HLC has been applied in artificial bone, vascular scaffolds and novel hemostatic materials [16,17].

In this research, HLC was severed as a new chelating substrate to bind calcium, copper or manganese. In order to characterize the new chelates of HLC-Ca, HLC-Cu and HLC-Mn, it was important to understand the chemical interactions between calcium, copper, manganese and HLC. This work would provide some important theoretical evidences for the further study and application of chelates.

2. Materials and methods

2.1. Materials

The materials used in this study were as follows: Human-like collagen (HLC), China patent number ZL01116757, was supplied by

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http://dx.doi.org/10.1016/j.msec.2013.02.028

Xi'an Giant Biogene Technology (XABTC) Co. 3-(N-morpholino) propanesulfonic acid (Mops), 2-(N-morpholino)ethanesulfonic acid hydrate (MES) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were all purchased from Sigma-Aldrich Co. (St. Louis, USA). The calcium chloride anhydrous, cupric sulfate anhydrous and tetrahydrate manganese chloride were obtained from Tianjin Chemical Industry Co. (Tianjin, China). Sephadex G-25 was purchased from GE Healthcare Bio-Science AB (Beijing, China). All reactants and solvents used were in analytical grade and double-distilled water was used throughout.

2.2. Preparation of chelates of HLC-Ca, HLC-Cu, HLC-Mn

Dry HLC was dissolved in MOPS buffer solution (pH 7.0, 50 mM) and MES buffer solution (pH 6.0, 50 mM) at room temperature to prepare a fresh solution of HLC at the concentration of 40 μM. The HLC solution was mixed for 30 min with a stirring bar to dissolve protein completely. 250 μL of $CuSO_4$ solution (625 mM) and 540 μL of $MnCl_2{\cdot}4H_2O$ solution (1 M) were dropped slowly to 25 mL and pH 7.0 of HLC solution respectively, and 416 μL $CaCl_2$ solution (1.8 M) was dropped slowly to 25 mL and pH 6.0 of HLC solution. The reaction mixture was stirred for 1 h at room temperature (approximately 25 °C), and then the solutions were filtrated through 0.45 μm filter, next through a gel chromatography equipped with a XK16/100 (mm/cm) Sephadex G-25 column (ATKA purifier, GE Healthcare, US) at a flow rate of 1.5 mL per min to remove the free reagents. The purified chelates were finally freeze-dried using a Freeze Dryer (SIM International Group, FD5-10, US), and the freeze temperature and vacuum degree were -62 ± 0.1 °C and 7 mTorr respectively, when the difference between the control temperature and sample temperature was less than 1 °C, the samples were taken out and stored at 4 °C.

2.3. Ultraviolet–visible spectroscopy

The lyophilized samples of HLC, HLC-Ca, HLC-Cu and HLC-Mn were dissolved in double-distilled water to a concentration of 10 μM and all solutions were filtered with a 0.45 μm filter. The UV–vis absorption through 1 cm-path length quartz cuvettes of these samples was detected with a Unico spectrophotometer (Model 2802pcs, USA) at room temperature (25 ± 1 °C). Before detection, the base line was established with double-distilled water. The spectra were recorded from 200 to 700 nm.

2.4. Fourier transform infrared spectroscopy

The FTIR spectra were obtained from discs which were consisted of approximate 250 mg of potassium bromide (KBr) and 1.5 mg of lyophilized HLC or chelate and compressed under vacuum. An FTIR spectrophotometer (BRUKER EQUINOX-55, Germany) was used in this study. All spectra were recorded within a range from 400 to 4000 cm^{-1}. All measurements were performed in a dry atmosphere at room temperature (about 25 °C). And the results were presented in transmittance units.

2.5. Atomic absorption spectrophotometry

20 μM of chelate solutions was prepared with 5 mL of double-distilled water, and then digested with nitrohydrochloric acid and diluted to 10 mL. The total binding capacity, n_{ion}/n_{HLC} (mol ion/mol HLC), was investigated with an atomic absorption spectrophotometer (SOLAARM6, America). All solutions were filtered through a 0.45 μm filter before use, and all samples were detected in triplicate.

2.6. Fluorescence spectroscopy

The fluorescence spectra were performed with a spectrofluorometer (HITACHI F-4500, Japan) at the temperature of 298, 304, and 310 K respectively. The fluorescence of HLC was recorded at 300–450 nm at an excitation wavelength of 280 nm and with 10.0 nm of the slit widths of excitation and emission. A 2 ml 1×10^{-5} M of HLC was titrated by successive additions of 1 μL stock solution of anhydrous cupric sulfate (1×10^{-2} M) and 2 μL anhydrous calcium chloride and tetrahydrate manganese chloride separately (1×10^{-3} M). All solutions were filtered through a 0.45 μm filter before use, and all samples were detected in triplicate.

2.7. Circular dichroism spectroscopy

The solutions of HLC, HLC-Ca, HLC-Cu and HLC-Mn were prepared separately at the concentration of 10 μM with double-distilled water, and then all fresh solutions were filtered through 0.45 μm filters individually. CD spectra were recorded by a Chirascan spectropolarimeter (Applied Photophysics, Britain) from 190 to 260 nm and all samples were detected in triplicate. A scan speed of 20 nm per min and 1 nm of bindwidth were used. All spectra of the samples were taken using 0.1 cm path length quartz cell at room temperature (approximately 25 °C), and appropriate baseline corrections in the CD spectra were made.

2.8. Differential scanning calorimetry analysis

20 μM of HLC, HLC-Ca, HLC-Cu and HLC-Mn fresh solutions was prepared at room temperature respectively. All samples were degassed by stirring gently under vacuum prior to measurement, and then filtered through a 0.45 μm filter. DSC curves of HLC, HLC-Ca, HLC-Cu, and HLC-Mn were recorded using differential scanning calorimetry (VP-DSC Microcalorimeter, US). The experiments were carried out at a scan rate of 5 °C/min within the range of 30 to 300 °C.

2.9. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction assay

The lyophilized samples of HLC, HLC-Ca, HLC-Cu and HLC-Mn were dissolved in RPMI-1640 medium to the concentration of 50 μM and then filtered through a 0.22 μm filter under sterilized conditions. Baby hamster kidney cells (BHK21) were cultured at a density of 1.0×10^4 cells/mL on 96-well plates (200 μL/well) in a CO_2 (5%) incubator at 37 °C. After incubation for 24 h, 20 μL samples were added to 96-well plates to a concentration of 10 μM (100 μL/well). After incubation for 24 h and 48 h, 20 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 (phosphate buffered saline) at pH 7.4 to remove untransformed MTT and sample residues. 150 μL of DMSO was then 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 (MODEL550, Bio-Rad, USA). The relative cell growth (%) was calculated as:

$$\text{Relative cell growth} = [\text{OD}]_{\text{test}}/[\text{OD}]_{\text{control}} \times 100\%.$$

The values were expressed as the means ± standard deviation ($n=6$).

3. Results and discussion

3.1. Characterization of the new complexes of HLC-Ca, HLC-Cu and HLC-Mn

3.1.1. UV–vis absorption analysis

On the base of UV–vis absorption spectrum, it is easy and reliable to explore the structural changes of the complexes and even to judge whether new chemicals were formed or not [18]. In the present study, the UV–vis absorption spectra of HLC, HLC-Ca, HLC-Cu and HLC-Mn had been recorded.