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Hydrogel microspheres for stabilization of an antioxidant enzyme: Effect of emulsion cross-linking of a dual polysaccharide system on the protection of enzyme activity

Deh-Wei Tang [a], Shu-Huei Yu [b], Wen-Shin Wu [c,d], Hao-Ying Hsieh [b], Yi-Chin Tsai [a], Fwu-Long Mi [c,d,*]

[a] *Department of Biotechnology, Vanung University, Chung-Li 320, Taiwan*
[b] *Department of Polymer Materials, Vanung University, Chung-Li 320, Taiwan*
[c] *Department of Biochemistry, School of Medicine, Taipei Medical University, Taipei 110, Taiwan*
[d] *Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei 110, Taiwan*

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ABSTRACT

Catalase is an antioxidant enzyme abundant in *natural* resources. However, the enzyme is usually inactivated by gastric acid and digestive enzymes after oral ingestion. In this study, carboxymethyl chitosan (CM-chitosan) and hyaluronic acid (HA) conjugate hydrogel microspheres have been prepared by an emulsion cross-linking technique to retain the activity of catalase in simulated gastrointestinal (GI) fluids. Cross-linking reduced the swelling capability and increased the resistance toward hyaluronidase digestion of prepared HA–CM-chitosan hydrogel microspheres. Catalase entrapped in the hydrogel microspheres exhibited superior stability over a wide pH range (pH 2.0 and 6.0–8.0) as compared to the native enzyme. The entrapped catalase was also protected against degradation by digestive enzymes. Following the treatments, the catalase-loaded microspheres, in contrast to native catalase, could effectively decrease the intracellular H_2O_2 level and protect HT-29 *colonic* epithelial cells against H_2O_2-induced oxidative damage to preserve cell viability. These results suggested that the HA–CM-chitosan hydrogel microspheres can be used for entrapment, protection and intestinal delivery of catalase for H_2O_2 scavenging.

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1. Introduction

The gastrointestinal tract (GI tract) is particularly susceptible to reactive oxygen species (ROS) because xenobiotics, toxins, catalase-negative bacteria, mycoplasma, bile acids, and cast-off mucosal cells in the GI tract can generate ROS. Although the enzymes (superoxide dismutase, catalase, and glutathione peroxidase) involved in cellular protection against ROS are present in intestinal tissue, their activity is insufficient to protect the tissue against oxidative stress when ROS exceed the capacity of cellular antioxidant defenses [1]. Accordingly, ROS was found to play an important role in the initiation and progression of a number of *intestinal* diseases, such as inflammatory bowel disease (IBD) [2], ischemic-reperfusion disorders [3] and colorectal cancers [4].

Hydrogen peroxide (H_2O_2) is an extensively studied compound among the ROS generated in the GI tract. It disrupts intestinal epithelial barrier function, produces an increase in paracellular permeability of the epithelium and subsequently leads to trans-epithelial migration of neutrophils across the injured epithelium [5]. Catalase is found in many foods which catalyzes the decomposition of H_2O_2 into water and oxygen, and is a potential antioxidant enzyme served as the body's most potent defense against free radicals. However, the effectiveness of oral administration of the antioxidant enzymes in reducing intestinal oxidative stress is limited because catalase loses its activity in contact with gastric acid and digestive enzymes. Immobilization of catalase in gel beads or hydrogels has been shown to improve the thermal, pH and storage stability of the antioxidant enzyme [6–8].

Hyaluronic acid (HA) is a naturally occurring, biocompatible and biodegradable linear polysaccharide composed of alternating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine with $\beta(1 \rightarrow 4)$ interglycosidic linkage. HA is a mucoadhesive *macromolecule* which can bind to the mucus layers of tissues [9–11]. It also contributes significantly to cell proliferation and migration by interacting with receptors such as CD44 [12,13]. Nevertheless, HA is

* Corresponding author at: Department of Biochemistry, School of Medicine, Taipei Medical University, Taipei City 110, Taiwan. Tel.: +886 287882099; fax: +886 2 2735 6689.
E-mail address: flmi530326@tmu.edu.tw (F.-L. Mi).

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readily dissolved in water and easily degraded by hyaluronidases (HAase). If no treatment is performed, HA is difficult to be used for biomedical and drug delivery applications. Carboxymethyl chitosan (CM-chitosan) is a water soluble chitosan derivative having carboxyl groups on the amino and hydroxyl groups on chitosan. CM-chitosan hydrogels have received increased interest in oral drug delivery due to its pH-responsive properties [14,15]. CM-chitosan has also been used to prepare hydrogel films and nanoparticles incorporating gallic acid, catechin and caffeic acid for antioxidant purposes [16–18]. Because CM-chitosan has a chemical structure partially similar to HA, it can be used to modify the physical and enzymatic digestive properties of HA.

Recently, many studies have focused on the intestinal delivery of bioactive molecules [19–21]. The aim of this study was to prepare HA and CM-chitosan conjugate (HA–CM-chitosan) hydrogel microspheres by an emulsion cross-linking technique. Cross-linking could reduce the swelling ratios and increase enzymatic resistance of HA against HAase degradation. We intended to use the HA–CM-chitosan hydrogel microsphere as a carrier to protect the activity of catalase under simulated GI conditions. The protective effect of the catalase-loaded microspheres against H_2O_2-induced oxidative stress was evaluated by measuring the H_2O_2 level in HT-29 colon epithelial cells and the cell viability. Based on the colonic epithelial model, the intracellular H_2O_2 scavenging activity and cytoprotective effects of the catalase-loaded microspheres could be more precisely identified.

2. Materials and methods

2.1. Materials

Chitosan (Mw $\sim 2.5 \times 10^5$) with a degree of deacetylation of approximately 85% was acquired from Challenge Bioproducts (Taichung, Taiwan). HA (Mw $\sim 3.0 \times 10^4$) was purchased from NOVA Pharma & Liposome Biotech Co., Ltd., Taiwan. Catalase (from bovine liver, 2000–5000 units/mg), hyaluronidase (from bovine testes, 400–1000 units/mg), trypsin (from bovine pancreas, ≧10 000 BAEE units/mg), 2-(*n*-morpholino)ethanesulfonic acid (MES) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich Co. LLC. (USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). All other reagents and solvents used were of reagent grade.

2.2. Synthesis of CM-chitosan

CM-chitosan was synthesized as per a procedure described in the literature with slight modifications [17]. Briefly, chitosan powder (10 g) was suspended in 100 ml of isopropyl alcohol and the resulting slurry was stirred in a 500-ml flask at room temperature. A 25 ml of 10 N aqueous NaOH solution was then gradually added to the stirred slurry. The alkaline slurry was stirred for additional 30 min. Subsequently, monochloroacetic acid (60 g) was added, in five equal portions, at 1 min intervals. The heat was then applied to bring the reaction mixture to a temperature of 60 °C and stirring at this temperature was continued for 3 h. Afterward the reaction mixture was filtered and the filtered solid product (CM-chitosan) was thoroughly rinsed with methanol. The resultant CM-chitosan was dried in an oven at 60 °C.

2.3. Synthesis of HA–CM-chitosan conjugate hydrogels

The HA–CM-chitosan conjugate hydrogels were synthesized as follows. A stock solution of CM-chitosan in water (1.0%, w/v) was prepared by dissolving 0.15 g of CM-chitosan in 15 ml deionized water and stirring for 12 h at room temperature. HA (1.0%, w/v) was dissolved in a buffer solution (50 mM MES, pH 5.0). Subsequently, the NHS/EDC mixture was added to 15 ml of the HA solution to activate the carboxyl groups on the HA backbone. The activated HA solutions were added with appropriate amounts of CM-chitosan solutions at distinct CM-chitosan-to-HA weight ratios (CM-chitosan:HA = 3:1, 1:1 and 1:3). The final concentrations of EDC/NHS mixtures in the polymer solutions were 40 mmol/20 mmol. After thoroughly stirring, the air bubble-free solutions were poured into shallow dishes (with a diameter of 8.5 cm) and continuously reacted for 12 h to obtain cross-linked HA–CM-chitosan conjugate hydrogels. Additionally, CM-chitosan or HA hydrogels were respectively prepared by adding the above mentioned amounts of EDC only or EDC/NHS mixture to the HA or CM-chitosan solution and subsequently reacted for the same period of time. Finally, the cross-linked HA, CM-chitosan and HA–CM-chitosan conjugate hydrogels were thoroughly rinsed with deionized water to remove residual reagents. The hydrogels were air-dried in a dust-free atmosphere to obtain HA, CM-chitosan and HA–CM-chitosan conjugate films for FT-IR analysis, swelling studies and enzymatic degradation.

2.4. Gelation study

To examine the gelation properties for the formation of hydrogels, the viscosity changes in the solutions of HA, CM-chitosan and HA/CM-chitosan blends, after adding above mentioned EDC/NHS mixtures, were monitored during gelation using a viscometer (Brookfield, Model DVII$^+$) [22]. The solutions were maintained at 37 °C and the viscosities were measured using the number 25 spindle at 60 rpm to give the lowest shear conditions. Every typical run shows an initial constant low-viscosity portion followed by a rapid rise in viscosity. The value obtained by extrapolation of the initial linear portion back to the time axis was taken as a measure of time to the onset of gelation.

2.5. FT-IR analysis

FT-IR analysis was conducted by mixing the powder of milled samples (from dried films) with KBr (1:100) to investigate the chemical structures of HA, CM-chitosan and HA–CM-chitosan conjugates. The mixed powder was then pressed into a disk and analyzed by a FT-IR spectrometer (Perkin Elmer Spectrum RXI FT-IR System, Buckinghamshire, England). The sample was scanned from 400 to 4000 cm^{-1}.

2.6. Swelling ability

The swelling ratio of the HA, CM-chitosan and HA–CM-chitosan conjugate films was determined by swelling the dried films in the physiological buffer saline (PBS) at 37 °C. A dried film sample (200 mg) was placed in the PBS solution for a required period of time. Subsequently, the swollen film was taken out and the wet weight of the sample was determined by first blotting the film with a filter paper to remove the adsorbed water on the surface then weighed immediately on an electronic balance. The percentage swelling of the hydrogel films in the medium was calculated as follows:

$$\text{Swelling}(\%) = \frac{\text{Swollen sample weight} - \text{Initial dry weight}}{\text{Initial dry weight}} \times 100\%$$

2.7. Enzymatic degradation

Degradation kinetics were determined by measuring the mass loss rates of the dried films of HA, CM-chitosan and

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