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Anti-inflammatory and anti-oxidant properties of laccase-synthesized phenolic-*O*-carboxymethyl chitosan hydrogels

Daniela Huber[a], Adrianna Grzelak[a], Martina Baumann[b,c], Nicole Borth[b,c], Gerhard Schleining[d], Gibson S. Nyanhongo[a,*], Georg M. Guebitz[a,c]

[a] Institute of Environmental Biotechnology, University of Natural Resources and Life Sciences Vienna, Konrad Lorenz Strasse 20, 3430 Tulln, Austria
[b] Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria
[c] Austrian Centre of Industrial Biotechnology GmbH, Petersgasse 14, 8010 Graz, Austria
[d] Department of Food Science and Technology, University of Natural Resources and Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria

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ABSTRACT

A bioactive *O*-carboxymethyl chitosan (CMCS) hydrogel crosslinked with natural phenolics with potential application in wound dressings was synthesized using a laccase from *Myceliophthora thermophila* (MTL). The highest degree of cross-linking (49.7%) was achieved with catechol. All the phenolic-CMCS hydrogels synthesized showed excellent anti-oxidant properties with a free radical scavenging activity up to 4-fold higher than in the absence of the phenolics, as quantified by the di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay. In addition, the hydrogels produced showed an anti-inflammatory effect as evidenced by the inhibition of enzymes [myeloperoxidase (MPO), matrix-metalloproteinase-1 (MMP-1) and human neutrophil elastase (HNE)] overexpressed in chronic wounds. Sinapyl-CMCS hydrogels showed an MMP-1 inhibition of 37%. Further, the phenolic-CMCS hydrogels did not affect the viability of the NIH 3T3 mouse fibroblast cell line and were also able to slowly release human fibroblast growth factor 2, reaching 48.3% over a period of 28 days. This study thus shows the possibility of synthesizing multifunctional bioactive chitosan based hydrogels with anti-oxidant and anti-inflammatory properties using natural occurring phenolics as crosslinkers.

Introduction

Hydrogels are 3-dimensional, insoluble, cross-linked polymer networks characterized by the presence of many hydrophilic groups, which enable them to absorb and hold exceptionally high amounts of water. Among their many applications, hydrogels are fast transforming the wound care management landscape, thanks to the pioneering work of Winter [1] in 1962, which demonstrated that a moist wound environment accelerated the wound re-epithelialization process compared to the then traditionally used dry wound dressings. Chitosan, a derivative of chitin from living organisms, is emerging as an extremely versatile polymer that can be processed into different forms such as membranes, sponges, gels, scaffolds, microparticles, nanoparticles and nanofibers for a variety of biomedical applications such as drug delivery, gene therapy and tissue engineering, as well as wound dressing material [2–5]. Although, traditionally, chitosan based polymer synthesis techniques have largely employed toxic chemical cross-linking agents such as glutaraldehyde and formaldehyde [6], this trend is rapidly changing with recent studies successfully demonstrating the possibility of using not only non-toxic, naturally occurring plant derived phenolic compounds as cross-linkers, but also using enzymes as “green catalysts” for the synthesis of chitosan based biomaterials. Enzyme based methods, unlike chemical based methods, have many advantages including specificity, region-selectivity, operating under mild and environmentally friendly reaction conditions, avoiding the use of toxic reagents and also eliminating the need for complicated protection and deprotection steps needed during chemical synthetic methods. As summarized by Hu and Luo [5,6] different phenolic molecules have so far been introduced into the chitosan structure through several strategies, including activated ester-mediated modification, enzyme-mediated methods, and free radical induced grafting reaction, for the synthesis of films, microspheres, particles and hydrogels. Among the enzymes attracting greater attention are laccases (EC.1.10.3.2), multinuclear copper-containing oxidoreductases that catalyze the oxidation of various molecules (phenols,

Abbreviations: CMCS, *O*-carboxymethyl chitosan; MTL, *Myceliophthora thermophila* laccase; DPPH, di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium; MPO, myeloperoxidase; MMP-1, matrix metalloproteinase; HNE, human neutrophil elastase; CS, chitosan; ROS, reactive oxygen species; C, catechol; CA, caffeic acid; E, eugenol; SIN, sinapyl alcohol; TEAC, Trolox equivalent antioxidant capacity; FGF2, fibroblast growth factor 2; DMEM, Dulbeccos's modified eagle medium

* Corresponding author.
E-mail addresses: g.nyanhongo@boku.ac.at, gsnyanhongo@gmail.com (G.S. Nyanhongo).

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aromatic and aliphatic amines, etc) with the concomitant reduction of molecular oxygen to water. The laccase oxidized substrates (reactive species phenoxy radicals, semi-quinones, quinones) couple to the amino groups of chitosan through Michael addition or radical coupling [9–11].

Although laccases have been explored for the synthesis of chitosan based polymers, it is only recently that they have been shown to mediate the synthesis of chitosan based hydrogels [8,9,11]. Our group [14] recently screened a number of laccase substrates, phenolic monomers, for their ability to cross-link chitosan oligomers and produce hydrogels with potential applications as wound dressings. Based on these studies and recent others showing the possibility of synthesizing *O*-carboxymethyl chitosan (CMCS) hydrogels [15,16], we were prompted to explore, for the first time, the possibility of using laccases and naturally occurring phenolics for the synthesis of CMCS based wound dressings. Further, the fact that plant phenolics are well known as excellent radical quenching molecules (antioxidants) [17–19], makes them even more attractive for the synthesis of chronic wound dressings with anti-oxidant and anti-inflammatory properties. Chronic wounds, defined as wounds that do not heal, remaining in the inflammatory phase, are characterized by the persistent release of elevated levels of proteases, reactive oxygen centered species (ROS) and high amounts of exudates [20,21] which prevent wound healing. The proteases degrade growth factors and newly synthesized components of the extracellular matrix (collagen and elastin) [21], while the excessive ROS damage biomolecules and also persistently activate the pro-inflammatory system, thereby creating a vicious cycle that impairs wound healing [22].

Designing a bioactive hydrogel that is able to control proteases, absorb excess exudates, prevent microbial colonization of the wound and quench ROS may help chronic wound healing. Previous studies have already shown the ability of catechol and eugenol to assist in quenching the ROS in chronic wounds [23,24]. Based on this information, this study aims at synthesizing multifunctional, CMCS based, wound dressing hydrogels, using phenolic molecules as cross-linking agents and as bioactive molecules able to control ROS levels and prevent microbial colonization of the hydrogel.

Materials and methods

Materials

Laccase from *Myceliophthora thermophila* (MTL) was purchased from Novozymes A/S (Denmark). *O*-carboxymethyl chitosan (CMCS) with a deacetylation degree of 90% and a carboxylation degree of 84% was from Santa Cruz Biotechnology (Texas,US). Chitopentaose was from CarboSynth Limited (UK). Phenolics (catechol, eugenol, caffeic acid, and sinapyl alcohol), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbonsäure (Trolox), collagenase type I (matrix metallopeptidase 1 – MMP-1) from *Clostridium histolyticum*, elastase (HNE) from human leukocytes, and basic fibroblast growth factor 2 (bFGF-2) were from Sigma Aldrich (US). The mouse cell line NIH 3T3 was from the ATTC (US) and the Dulbeccos's modified eagle medium (DMEM) by Gibco (Thermo Fisher Scientific, US). Myeloperoxidase (MPO) was from Planta (Austria).

Laccase synthesis of hydrogels

The phenolic-*O*-carboxymethyl chitosan (phenolic-CMCS) hydrogels were synthesized by mixing a 5% (w v^{-1}) CMCS solution pH 7,0 and 500 µM of the respective phenolics (catechol-C, caffeic acid-CA) or 2500 µM (eugenol-E, sinapyl alcohol-SIN). The reaction was started by adding 16.7 nkat of MTL and incubated at 25 °C while gently shaking at 50 rpm for 20 min.

FTIR and HPLC–MS analysis of CS and CMCs couplings

Chitosan pentaose (2 mg mL^{-1}) solution was mixed with a 1 mM phenolic solution (catechol, eugenol, caffeic acid, and sinapyl alcohol) and the reaction was started by addition of 16.7 nkat MTL in 100 mM sodium phosphate buffer pH 7.0. After 24 h of incubation, the reaction products were filtered and the samples were prepared for HPLC-time of flight (TOF) mass spectrometry analysis. The coupling products were separated and analyzed using an Agilent HPLC 1260 system (US) with a Hypercarb column (Thermo Scientific, USA) according to the method of Huber et al. [14]. The coupling products of CS and CMCS were further analyzed by FTIR using a Perkin Elmer Spectrum 100 spectrometer (Perkin Elmer Inc., Germany) supplemented with an attenuated total reflection (ATR) sampling device. The absorbance spectra of CS and CMCS were recorded over a wavelength range of 4000–650 cm^{-1} with 10 scans and a resolution of 4 cm^{-1}.

Rheological and structural properties of phenolic-CMCS hydrogels

The rheological properties of the hydrogels were measured using a Malvern Kinexus rheometer (Malvern Instruments Ltd., UK) equipped with a heated plate-plate device (Malvern, CP1/60 SR0036 SS:PL65 C0050 SS). The gelation was monitored at a frequency of 1 Hz and 0.1% strain from the time of starting the reaction (addition of enzyme) at a controlled temperature of 25 °C. The linear viscoelastic region (LVR) was recorded with a strain sweep between 0.001 and 1000% at a frequency of 1 Hz. The frequency sweep was performed from 0.01 to 100 Hz with 0.1% strain.

Swelling properties of the hydrogels

The swelling properties were determined by weighing 1 g of dried phenolic-CMCS hydrogel in a Petri dish and the initial weight of the gel measured. The hydrogels was then immersed in 10 mM phosphate buffered saline (PBS) pH 7.4 for 24 h and weighed again. The swelling in% was calculated using the following equation (Formula (1)):

$$Swelling\ \ efficiency\,[\%] = \frac{W_S - W_D}{W_D} \times 100 \quad (1)$$

Formula 1: Swelling efficiency in% over a period of 28 days.
W_S = weight of the swollen gel, W_D = weight of a dried gel.

Effect of lysozyme on phenolic-CMCS hydrogels

The effect of lysozyme on the phenolic-CMCS hydrogels was investigated adding sterile filtered 0.1 mg mL^{-1} lysozyme to a sterile 10 mM PBS buffer (pH 7.4). A 24 UV sterilized hydrogel was then immersed in the lysozyme-containing buffer in a sterile plastic bag. The hydrogel was then incubated for 28 days at room temperature (18–27 °C) while regularly measuring the weight gain.

Estimating the degree of cross-linking of the phenolic-CMCS hydrogels

A method developed by Moura et al. was used to determine degree of cross-linking of CMCS hydrogels [25]. Two mg of the freeze-dried hydrogels were incubated with 1 mL of ninhydrin reagent and 1 mL of water. The reaction mixture was boiled for 20 min and then cooled on ice to ambient temperature before withdrawing 0.5 mL of the reaction mixtures and stabilizing with 2.5 mL of 50% isopropanol. CMCS alone was used as a control. The reaction mixture was analyzed using UV–vis spectroscopy (Tecan Infinite 200Pro, Switzerland) at 570 nm.

Radical scavenging activity

The anti-oxidant properties were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with slight modifications [26].