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Laccase-assisted formation of bioactive chitosan/gelatin hydrogel stabilized with plant polyphenols

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ABSTRACT

Laccase-assisted simultaneous cross-linking and functionalization of chitosan/gelatin blends with phenolic compounds from *Hamamelis virginiana* was investigated for the development of bioactive hydrogel dressings. The potential of these hydrogels for chronic wound treatment was evaluated *in vitro*, assessing their antibacterial and inhibitory effect on myeloperoxidase and collagenase. Rheological studies revealed that the mechanical properties of the hydrogels were a function of the enzymatic reaction time. Stable hydrogels and resistant to lysozyme degradation were achieved after 2 h laccase reaction. The inhibitory capacity of the hydrogel for myeloperoxidase and collagenase was 32% and 79% respectively after 24 h incubation. Collagenase activity was additionally suppressed by adsorption (20%) of the enzyme onto the hydrogel. Therefore, the bioactive properties of the hydrogels were due to the effect of both released phenolic compounds and the permanently functionalized platform itself. The hydrogels showed antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

1. Introduction

The different chronic wound types do not share origin or cause, however they feature bacterial infection and high concentrations of matrix metalloproteases (MMPs), myeloperoxidase (MPO) and reactive oxidative species causing excessive degradation of the extracellular matrix (ECM) and the growth factors (Trengove et al., 1999). In healing wounds the MMPs are counteracted by their natural inhibitors (Taylor, Windsor, Caterina, Bodden, & Engler, 1996), while in chronic wounds the ratio proteases/inhibitors is disturbed and most of these enzymes are uninhibited. The protease–antiprotease unbalance is further promoted by MPO-generated hypochlorous acid (HOCl), which from one side inactivates the protease inhibitors and from another triggers the activity of latent MMP (Sorsa et al., 1992). In addition, most chronic wounds are colonized with several bacterial species, *e.g. Staphylococcus aureus* and *Pseudomonas aeruginosa* (Fazli et al., 2009).

Therefore, to stimulate the wound repair a dressing material should simultaneously: (i) control the proteolytic and oxidative enzymes in the wound site, (ii) provide a microorganism-free environment, and (iii) maintain the tissue moisture while absorbing the excessive exudates. Biopolymers with intrinsic antimicrobial and/or healing promoting properties, such as chitosan and collagen/gelatin have been suggested for wound treatment, and though many of them are available on the market (Muzzarelli, 2009a) only few are commercialized as chronic wound dressings. Chitosans of animal and fungal origin are linear and partly acetylated (1→4)-2-deoxy-β-D-glucans with intrinsic antimicrobial properties (Muzzarelli, 1977; Muzzarelli et al., 2012). On the other hand, gelatin (denatured collagen) besides ensuring the cell adhesion and growth (Pulieri et al., 2008), could serve as a competing substrate for several proteases present in the wound site thereby diverting them from digesting the ECM.

Chitosan and collagen/gelatin dressing materials are normally produced in the form of hydrogels according to the widely accepted concept of moist wound healing. The dressing stability in chronic wound application might be compromised by the requirement for low frequency of changing. Thus, these hydrogels need to feature biostability and mechanical strength achievable by additional crosslinking (Moura, Faneca, Lima, Gil, & Figueiredo, 2011). To this end, various chemical cross-linkers have been used, however, their cytotoxicity makes them unsuitable for biomedical

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applications (Fu, Kassim, Parks, & Heinecke, 2001). The search for safe, natural crosslinking agents has brought about the use of genipin due to its low cytotoxicity (Muzzarelli, 2009b) as well as plant polyphenolics, such as proanthocyanidins (PA) (Kim, Nimni, Yang, & Han, 2005) and hydrolysable tannins (HyT) (Shimada, Saitoh, Sasaki, Nishitani, & Osawa, 2006) among others for stabilization of carbohydrate/protein systems (Van Vlierberghe, Dubruel, & Schacht, 2011). In the case of polyphenols, stabilization of carbohydrate and protein matrices is thought to be due to the physical interactions between these compounds and biopolymers. Such natural compound-based approaches can further be upgraded by the application of highly specific enzymatic tools to achieve stable, covalently cross-linked gels. Oxidative enzymes, such as tyrosinase, peroxidase and laccase, are able to promote inter- and intra-molecular coupling reactions in biopolymers and natural phenolics (Mikolasch & Schauer, 2009; Moreira Teixeira, Feijen, van Blitterswijk, Dijkstra, & Karperien, 2012; Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994). For example, laccase (EC 1.10.3.2) would oxidize the phenolic compounds (PC) and tyrosine residues in proteins into reactive quinones, which can further react with nucleophiles such as amino groups from chitosan and gelatin by 1,4-Michael addition or Schiff base formation. On the other hand, PA and HyT are known for their antioxidant capacity, antimicrobial effect, anti-inflammatory and wound healing promoting properties (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). Polyphenolic extracts from *Hamamelis virginiana* (Witch-hazel) rich in PA and HyT are widely used in the therapy of skin diseases (Deters, Dauer, Schnetz, Fartasch, & Hensel, 2001; Lizárraga et al., 2008; Touriño et al., 2008). These extracts are able to protect cells from free radicals inhibiting the proliferation of melanoma cells (Touriño et al., 2008) and exert an inhibitory effect over deleterious chronic wounds enzymes *in vitro* (Díaz-González et al., 2012). The therapeutic superiority of the extracts compared to the isolated single constituents at equivalent doses (Wagner & Ulrich-Merzenich, 2009) was the reason to use as cross-linkers the natural extract instead of the single phenolic substances.

This study aims to generate hydrogel dressings for chronic wound application containing chitosan, gelatin, and natural phenolics further cross-linked by laccase to obtain bioactive and biostable materials with tunable physicomechanical and functional properties. Polyphenolic extract from *H. virginiana* will be oxidized by laccase in a one-step process under mild reaction conditions to covalently crosslink chitosan and gelatin. It is intended that the polyphenols play a dual role in the hydrogel: (i) "passive" – being a structural element, and (ii) "active" – modifying the chronic wound environment by attenuating the deleterious MMPs, MPO and ROS activities, and the bacterial infection.

2. Materials and methods

2.1. Reagents

Gelatin was purchased from Fluka (France) and chitosan KiOmedine-CsU from *Agaricus bisporus* (Mw 90 kDa, degree of deacetylation 85.9%, viscosity 5–200 mPa s (1% solution in 1% acetic acid)) was kindly supplied by KitoZyme (Belgium). Both chitosan and gelatin had pharmaceutical grade. *H. virginiana* (witch hazel) stems were provided by Martin Bauer GmbH (Germany).

Trametes sp. laccase (EC 1.10.3.2, Laccase L603P, 300 U/g of solid (activity measured at 45 °C, pH 4.5, 1 U: μmol of oxidized ABTS/min)) was purchased from Biocatalysts (UK). Collagenase from *Clostridium histolyticum* (1676 U/mg solid, 1 U hydrolyses 1.0 μmol of FALGPA per minute at pH 7.5 and 25 °C in the presence of Ca^{2+}) and N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala (FALGPA) collagenase substrate from Sigma–Aldrich (Spain) were used. Myeloperoxidase (MPO) from human leukocytes (1550 U/mg solid: 1 U will produce an increase in absorbance at 470 nm of 1.0 per minute at pH 7.0 and 25 °C, calculated from the initial rate of reaction using guaiacol as a substrate) comes from Planta Natural Products (Austria). Lysozyme from chicken egg white (dialyzed and lyophilized powder, 100,000 units/mg solid (1 U corresponds to the amount of enzyme which decreases the absorbance at 450 nm by 0.001 per minute at pH 7.0 and 25 °C (*Micrococcus luteus*, ATCC 4698, as a substrate))) comes from Sigma–Aldrich (Spain). All other reagents used were of the highest grade commercially available from Sigma–Aldrich.

Cetrimide agar, Baird Parker agar and trypticase soy broth (TSB) were purchased from Sigma–Aldrich. Bacterial strains *P. aeruginosa*, CECT 110T and *S. aureus*, CECT 86T were provided by the Spanish Type Culture Collection (CECT) and grown in cetrimide and Baird Parker agar respectively.

2.2. Extraction and isolation of PC from H. virginiana

The phenolic extract from *H. virginiana* was obtained as previously reported (Touriño et al., 2008). Briefly, chopped witch hazel stems were incubated in an acetone–water mixture (7:3) for 24 h at room temperature with occasional shaking. The solid was filtered off and the acetone was evaporated at reduced pressure. The remaining solution was defatted with n-hexane and the oligomeric fraction was extracted with ethyl acetate. This organic phase was dried under vacuum, the pellet was dissolved in deionized water, and the solution was filtered through a porous plate. This phenolic extract contains mainly a mixture of small proanthocyanidins (dimers) and hydrolysable tannins (hamamelitannin, pentagalloyl glucose and methyl gallate). Its degree of polymerization was about 1.4 estimated by thioacidolytic depolymerization and HPLC using appropriate molecular standards as described by Torres and Selga (2003) and Touriño et al. (2008).

2.3. Hydrogels preparation

Gelatin solution 2% (w/v) was prepared in 25 mM succinate buffer at pH 4.5 and 60 °C. Chitosan was dissolved in 1% HCl to obtain 2% (w/v) solution, and the pH was adjusted to 4.5 with 1 M NaOH. The solutions of chitosan and gelatin were mixed (C/G) at 2:3 ratio (w/w) at room temperature overnight. Thereafter, 11.25 mL of 1% PC solution (w/v) in 25 mM succinate buffer pH 4.5 was added to 75 mL of the previously prepared C/G mixture 2:3 (w/w). The crosslinking reaction was initiated by the addition of 1.5 mL of laccase solution (2 U/mL) and left to proceed for determined time periods up to 24 h at 45 °C under continuous stirring. The enzymatic reaction was terminated by heating at 100 °C for 2 min and the resulting mixtures were cooled down to −80 °C and freeze-dried. The samples were designated according to the time of the enzymatic reaction. Control mixtures C/G-PC-C and C/G were prepared following the same procedure, but omitting the enzyme, or both PC and the enzyme (Table 1).

2.4. Hydrogels characterization

Rheological measurements were carried out with an ARG2 rheometer (TA Instruments, UK) equipped with electrical heated plates. The samples were analyzed in parallel plate geometry (25 mm diameter) at 45 °C. The liquid samples were transferred to the preheated plate immediately after mixing the corresponding solutions for each test (time $t = 0$) and the measurements started at $t = 60$ s after thermal equilibration. The rheometer was operated in the oscillatory mode. A multiwave analysis program (1 Hz frequency, 3 and 5 Hz harmonics) with controlled 2% strain was used to monitor the crosslinking process. The gelation time was