Carbohydrate Polymers 92 (2013) 989–996

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

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

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ARTICLE INFO

Article history:
Received 1 September 2012
Received in revised form 17 October 2012
Accepted 18 October 2012
Available online 26 October 2012

Keywords:
Chitosan
Chronic wounds
Gelatin
Laccase
Hydrogels
Phenolic compounds

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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http://dx.doi.org/10.1016/j.carbpol.2012.10.045

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

Table 1
Coding and reaction parameters of hydrogels.

Code name	Initial concentration of PC in reaction mixture (μg GAE/g hydrogel)[a]	Laccase incubation time (h)
C/G	No PC	No laccase
C/G-PC-C	25,500	No laccase
C/G-Lacc	No PC	4
C/G-PC-2h	25,500	2
C/G-PC-4h	25,500	4
C/G-PC-6h	25,500	6
C/G-PC-8h	25,500	8

[a] Gallic Acid Equivalents (GAE).

determined using the G' and G'' crossover criterion at 1 Hz, and as the frequency independent crossover of the tan δ curves at the different frequencies. To measure the mechanical properties of the resulting hydrogels, each experiment was repeated for defined reaction times, and the hydrogel was subjected to a stress sweep from 0.01 to 1000 Pa in the oscillatory mode at 1 Hz.

Infrared spectra of the blends were collected over the 3500–800 cm^{-1} range using Perkin-Elmer Spectrum 100 (Perkin-Elmer, Massachusetts, USA) equipped with universal ATR sampling accessory, performing 50 scans for each spectrum.

Scanning electron microscopy (SEM) micrographs of the hydrogels with magnification ×25 were obtained using a FESEM, Quanta 200 FEG (FEI Company, USA).

The release of phenolics from the hydrogels was estimated according to the Folin–Ciocalteu method for the determination of total phenol content in solution (Scalbert, Monties, & Janin, 1989) by monitoring the reaction at 760 nm in a microplate reader (Infinite M200, Tecan, Austria) and the results were expressed in gallic acid equivalents (GAE). Freeze dried C/G-PC hydrogels were incubated in PBS at pH 7.4 and 37 °C for 60 h. At defined time intervals aliquots of the supernatant were removed, the total phenol content determined, and the phenolics release calculated according to Eq. (1).

$$\text{Cumulative release}\,(\%) = \frac{PC_{rel}}{PC_i} \times 100 \quad (1)$$

where PC_i is the total amount of PC and PC_{rel} is the amount of PC released in PBS.

After reaching swelling equilibrium in PBS at 37 °C the hydrogels were removed from the medium, weighed and reintroduced to the medium. Thereafter, 1 mg/ml lysozyme was added. At regular time intervals, the hydrogels were removed from the medium and weighed. The medium was refreshed once a day. The results were compared with those obtained following the same procedure but omitting lysozyme. All experiments were performed in triplicate and the average weight loss value was calculated using Eq. (2).

$$\text{Weight loss}\,(\%) = \frac{W_s - W_{Lys}}{W_s} \times 100 \quad (2)$$

where W_s is the weight of the wet hydrogel at swelling equilibrium in PBS and W_{Lys} is the weight of the sample after lysozyme degradation.

2.5. Assessing in vitro the bioactivity of C/G-PC-2h

In order to discriminate between the bioactivity due to released PC and the bioactivity of the hydrogel matrix itself, hydrogel samples were submitted to an extensive cleaning process to remove the non-covalently bonded PC. The hydrogels were incubated in PBS, changed several times, until no PC release was observed spectrophotometrically. Afterwards the hydrogels were washed with Milli-Q H_2O to remove the salts from PBS, and then the samples were lyophilized. The cleaned hydrogel is refers in the text as hydrogel platform.

The radical scavenging activity of released PC and hydrogel platform was determined separately measuring the decrease in absorbance of 1,1-diphenyl-2-picrylhydrazyl radical (DDPH) at 515 nm (Brand-Williams, Cuvelier, & Berset, 1995). All experiments were carried out in triplicate and the results of the assay were expressed relative to Trolox in terms of TEAC (Trolox equivalent antioxidant capacity) (mM Trolox equiv./g hydrogel).

The inhibitory effect of the blends over MPO activity was measured using guaiacol as a substrate (Capeillere-Blandin, 1998). Hydrogel samples were incubated with 20 μL of MPO 0.325 μM (0.60 U) and 30 μL of guaiacol (167 mM) buffered with 450 μL PBS pH 7.4 at 37 °C. After predetermined incubation times 200 μL of the solution were placed in a 96-well microplate and the reaction was started by adding 22 μL of 1 mM H_2O_2. The change in absorbance at 470 nm was monitored during 5 cycles of 15 s (75 s) using microplate reader Infinite M200 (Tecan, Austria). All measurements were carried out in triplicate and the activity was determined by the rate of absorbance increase per min and expressed as a percentage of enzyme inhibition compared to the control (MPO reaction mixture without sample).

The collagenase activity was measured using FALGPA substrate (Van Wart & Steinbrink, 1981). The collagenase was incubated in presence of hydrogels for different time of incubation. The hydrolysis of FALGPA with collagenase 0.2 μg/mL in 50 mM Tricine, containing 100 mM $CaCl_2$ and 400 mM NaCl, pH 7.4 was monitored spectrophotometrically at 345 nm during 5 min, immediately after mixing the substrate with the enzyme. All measurements were carried out in triplicate. The results were expressed as a percentage of enzyme inhibition compared to the control (sample without hydrogel).

To determine the kinetics of collagenase inhibition, the collagenase was treated with 0.125 mg/ml solution of PC for 3 h at 37 °C. FALGPA solutions (0.125 mM) were used as substrates in the different assays. The hydrolysis of FALGPA was monitored at 345 nm, immediately after the addition of PC incubated collagenase as done in the case of collagenase hydrogel inhibition. The kinetic parameters were calculated from Eisenthal–Cornish-Bowden plot and secondary plots of Kmapp/Vmaxapp *vs* [I] and 1/Vapp *vs* [I].

Sorption studies were performed in 50 mM Tricine, 100 mM $CaCl_2$, 400 mM NaCl, pH 7.4. Hydrogel platforms were incubated with 40 μg/ml collagenase. The protein content in the supernatants was determined at defined time intervals over 24 h by using QuantiPro™ BCA Assay Kit (Sigma).

P. aeruginosa and *S. aureus* were grown on Cetrimide Agar and Baird Parker Agar respectively at 37 °C for 24 h. A microorganism suspension in TSB of each strain was prepared at concentration of 10^4 CFU/mL. Then, the hydrogel samples (25 mg) were incubated under constant shaking with 25 ml of bacterial suspension in 50 mL cap sterile bottles at 37 °C for 24 h. Inoculated medium without sample was used as positive control, whereas as negative control uninoculated medium was used. To determine the inhibition of bacterial growth, at predetermined time intervals, the microorganism suspension (10 μL) was harvested in the agar plate, incubated 24 h at 37 °C and the grown CFU counted afterwards. The measurements were repeated three times for each sample and the average value of inhibition was calculated following Eq. (3).

$$\text{Inhibition}\ (\%) = \frac{CFU_S - CFU_C}{CFU_C} \times 100 \quad (3)$$

where CFU_S are the colonies grown in the bacterial suspension after 24 h incubation with hydrogel samples and CFU_C are the colonies grown in the bacterial suspension after 24 h incubation without sample. All measurements were carried out in triplicate and the mean values for bacteria inhibition are reported.

3. Results and discussion

3.1. Enzymatic cross-linking and functionalization of chitosan/gelatin hydrogels

The laccase-catalyzed gel formation in chitosan, gelatin and PC formulation was evaluated *in situ* by means of rheological measurements. To investigate the influence of PC and laccase alone on the gelation, mixtures of chitosan/gelatin/laccase (C/G-Lacc) and chitosan/gelatin/PC (C/G-PC-C) were monitored as controls. There was a slow evolution of the storage modulus (G') and the loss modulus (G'') for C/G-PC-C, with no gelation observed within 240 min (data not shown). Although it was reported that non-activated PA alone could stabilize C/G membranes by covalent interactions (Kim et al., 2005), under the conditions of the current experiments the enzymatic activation to promote the covalent cross-linking process revealed to be of major importance for the formation of a stable gel network.

Indeed, C/G-Lacc G' showed a slightly steeper increase than G'', meaning a progression of the cross-linking reaction. After reaching the gel point at 50 min, G' increased further to reach a plateau at 160 min and 100 Pa (data not shown) indicating the completion of the cross-linking process. Nevertheless, the laccase effect was rather limited in the cross-linking of chitosan and gelatin due to the low tyrosine content in gelatin (about 0.5%) (Eastoe, 1955). Thus, the cross-linked network is not enough developed to create a stable hydrogel.

Finally, the storage modulus (G'), monitored in the rheological evolution of the C/G with PC and laccase, increased rapidly upon addition of laccase to C/G and PC mixtures, indicating crosslinking between quinones formed by the enzymatic oxidation of PC and the nucleophilic groups present in the biopolymers. The gelation point indicated the formation of an incipient weak elastic network with a low cross-linking density. Gelation took place after 94 min for the C/G-PC system (Fig. 1A). G' increased steadily afterwards due to further increase of the cross-linking density. The loss modulus (G'') also increased due to the increase in the molecular weight caused by the crosslinking reaction. The relative magnitude between storage and loss moduli is given by tan δ ($\tan\delta = G''/G'$), being tan δ the phase angle. Since tan $\delta < 1$, the elastic solid behavior of the samples was predominant over their viscous liquid behavior. The decrease of tan δ confirmed the progressive cross-linking of the hydrogel. G' leveled off after 500 min showing completion of the cross-linking process Beyond this point, G' decreased and tan δ increased corresponding to a failure of the cross-linked structure due to brittleness and excessive strain. The water evaporation observed might have contributed to the failure of the gel network and subsequent loss of hydrogel properties.

Unlike the C/G-Lacc control, the mixture of C/G, PC and laccase showed higher gelation point, probably owing to polymerization reactions of PC (Mita, Tawaki, Uyama, & Kobayashi, 2003) retarding the cross-linking. On the other hand the completion of the cross-linking process after 500 min of enzymatic reaction with G' values higher than 10^5 Pa pointed out a high cross-linking density, thereby confirming that the laccase activation of PC was a major factor driving the biopolymer blends to gelation.

The maximum stress and strain endured by the hydrogel before the network failure were determined on the basis of stress sweeps after different reaction times. The stress and strain increased and decreased respectively with the increase of the laccase reaction time as a consequence of the increased cross-link density of the chitosan, gelatin and PC network (Fig. 1B). The changes of stress and strain were more pronounced during the first 8 h, after which the rate of increase of the oscillatory stress was considerably slower. These results could be explained by reaching the maximum cross-link density after 500 min of enzymatic reaction. Longer treatment time (up to 24 h) did not bring about considerable increase of hydrogel stress. Moreover, the samples prepared in a reaction longer than 8 h were stiff and brittle, and were not considered for further investigations.

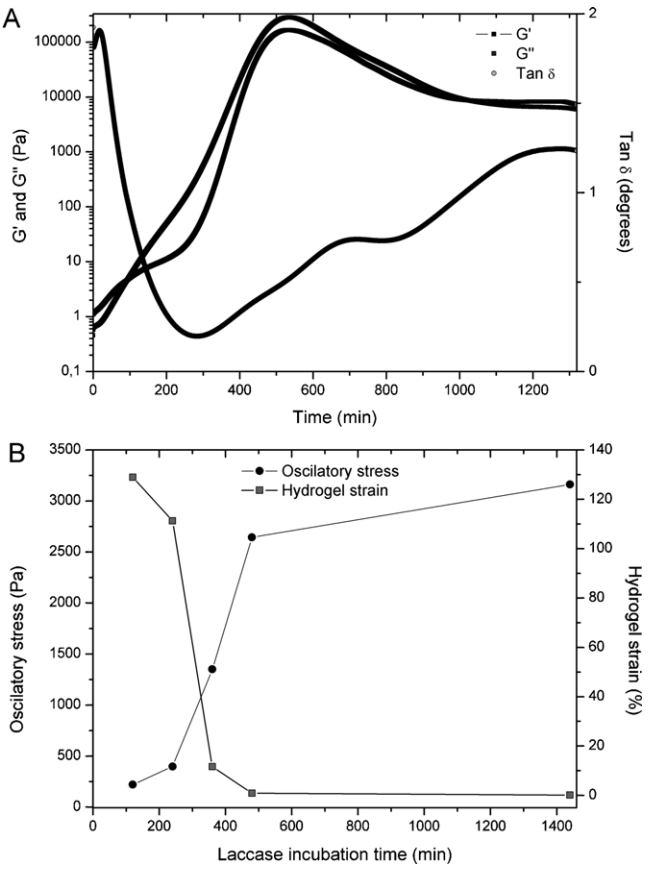

Fig. 1. Rheological characterization of the laccase-assisted cross-linking reaction. C/G (2:3, wt%), laccase (2 U/ml) and PC (1.5 mg/ml) in succinate buffer (25 mM, pH 4.5) at 45 °C. (A) Storage modulus (G'), loss modulus (G'') and tan δ evolution, (B) Oscillation stress sweep–strain curves of C/G-PC hydrogels after different reaction times (2, 4, 6, 8 and 24 h).

The complexity of the used enzymatic cross-linking approach may involve a broad range of molecular interactions and reactions, including electrostatic attractions, hydrogen-bonding, PC polymerization and, above all, the covalent linkages between C/G systems and laccase-activated PC. Clear differences were found in the spectrum of the control sample C/G comparing to sample C/G-PC-8h with the highest cross-link density (Fig. 2). Characteristic bands at 1637 cm^{-1} (amide I) and 1537 cm^{-1} (—NH_2 bending) were observed for both samples. For C/G-PC-8h the peak intensity of unreacted amino groups at 1537 cm^{-1} as well as amide I peak at 1637 cm^{-1} decreased compared to the control, indicating the involvement of these groups in the cross-linking reaction. Additionally, a new peak at 1260 cm^{-1} appeared in the region of 1215–1270 cm^{-1} attributed to C-N stretching of aryl amides formed *via* Michael addition, whereas the peak at 1239 cm^{-1} attributed to alkyl amines decreased. The new bands at 789 cm^{-1}, 837 cm^{-1} and 875 cm^{-1} could be assigned to different pattern substitution of the aromatic rings (Cui et al., 2003). Therefore, the FTIR spectra confirmed that the phenolic compounds from the *H. virginiana* extract were covalently incorporated into the C/G network and the cross-linking occurred most probably and predominantly *via* Michael addition.

The cross-sectional SEM micrographs (Fig. 3) of representative freeze-dried C/G-PC hydrogels revealed porous structures with different pore size and shape depending on the cross-linking

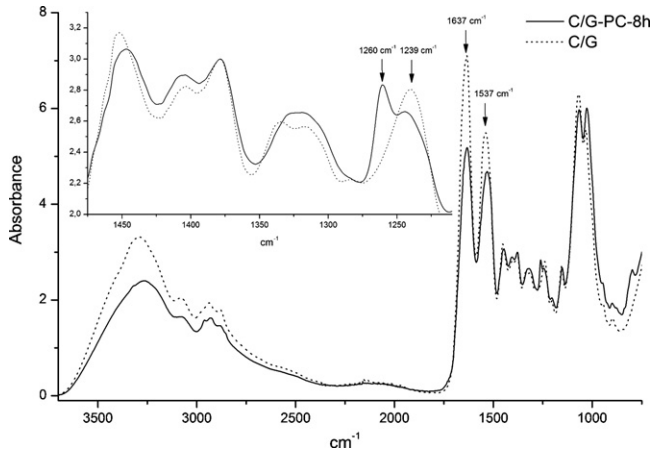

Fig. 2. Normalized FTIR spectra of freeze-dried C/G and C/G-PC-8h samples. The inset image magnifies the region from 1500 cm^{-1} to 1200 cm^{-1}.

density (time of enzymatic reaction). Hydrogen bonding and ionic hydrophobic interactions between PC and biopolymers led to a sheet-like structure of the non-cross-linked control sample (Kim et al., 2005; Wang, Qiu, Cosgrove, & Denbow, 2009). On the contrary, the enzymatically cross-linked hydrogels presented a porous structure with increasing pore size as a function of the extent of cross-linking, also observed in similar cross-linked biopolymer systems (Moura et al., 2011).

3.2. Stability of the hydrogels

The non-cross-linked control sample C/G-PC-C was completely disintegrated after 1 h in PBS, unlike the recently reported 5-days stability and pepsin digestibility of ionically formed chitosan/gelatin/PC films in water (Kim et al., 2005). Similar to C/G-PC-C, the other control sample C/G-Lacc was also completely solubilized after 1 h in PBS, confirming the poor cross-linking density in such system. On the contrary, all enzymatically cross-linked PC-containing hydrogels retained more than 80% of their weight after 60 h in PBS (Fig. 4). Thus, the laccase-assisted cross-linking of the hydrogels with PC was a prerequisite for their stability in physiological conditions. Since the main enzyme in wound fluids that could hydrolyze the β(1→4) glycosidic linkage in chitosan is the lysozyme (Jollès & Muzzarelli, 1999) the hydrogel stability against lysozyme digestion was further evaluated. As expected, comparing the stability of the hydrogels in the presence and absence of the enzyme, faster degradation due to the lysozyme hydrolysis of the chitosan component was observed for all experimental groups (Fig. 4A–C). Surprisingly, the degree of hydrogel degradation by lysozyme was inversely proportional to the laccase incubation time. Usually, the cross-linking should stabilize the hydrogel (Zhang, Qadeer, & Chen, 2011). Samples C/G-PC-6h and C/G/PC-8h were completely degraded by lysozyme after 48 h incubation (Fig. 4D), whereas C/G-PC-2h preserved its structural integrity even after 60 h (Fig. 4A). Considering that the composition of *H. virginiana* extract used in the present study is rich in monomeric and oligomeric phenolic structures (Touriño et al., 2008), these unexpected results could be explained by the capacity of low molecular weight PC to inactivate lysozyme as recent studies revealed (Guzzo, Cappello, Azzolini, Tosi, & Zapparoli, 2011). Shorter laccase application ensures a higher amount of non-covalently loaded low molecular weight PC, while longer incubation time results in higher cross-linking between PC and C/G and further polymerization of PC (Desentis-Mendoza et al., 2006). Consequently, the lower availability of non-grafted, oligomeric PC in the increasingly cross-linked hydrogels would, lead to higher lysozyme degradability.

3.3. PC release

Besides stabilization of the hydrogel matrix, the PC from *H. virginiana* were expected to act as active agents, which controlled release would stimulate the healing process (Hori et al., 2007). The cumulative release of PC from all hydrogels obtained in this study showed two phases: an initial burst release during the material swelling, followed by a sustained release (Fig. 5). Similar quantities of PC were liberated from all hydrogels, nevertheless shorter laccase cross-linking treatment results in slightly higher amount of non-covalently grafted PC and their subsequent release. However, no significant differences between the experimental groups were found as in the case of hydrogels stability. Considering the obtained results for two important parameters of the material for the targeted application, the sample C/G-PC-2h, showing the highest PC release and stability, was selected to study the bioactivity of the hydrogels. Replicates of C/G-PC-2h were submitted to an extensive cleaning process to remove the non-covalently bonded PC in order to distinguish the effect of the permanently modified with PC hydrogel platform. The following experiments were conducted with both C/G-PC-2h and cleaned C/G-PC-2h hydrogel platform (sample further designated as "C/G-PC-2h hydrogel platform" in the text).

3.4. In vitro assessment of the bioactivity of the hydrogels

3.4.1. Radical scavenging activity

C/G-PC-2h hydrogel platform showed considerably higher radical scavenging activity compared to the activity of the released PC, accounting for the low liberation of PC (only 4% in 24 h) from the hydrogel (Fig. 5). As expected, the antioxidant activity of the released PC measured at predefined time intervals (1–24 h) increased gradually with time. The DPPH radical scavenging activity of released PC increased from 1.78 ± 0.01 TEAC (mM Trolox/g hydrogel) after 1 h of hydrogel incubation to 2.10 ± 0.01 TEAC after 24 h hydrogel incubation.

At the same time the activity of the C/G-PC-2h hydrogel platform increased as well progressively from 54.70 ± 9.11 TEAC after 1 h hydrogel incubation to 59.09 ± 2.20 TEAC after 24 h incubation, possibly due to swelling of the hydrogel allowing higher accessibility of DPPH to react with the covalently incorporated in the bulk

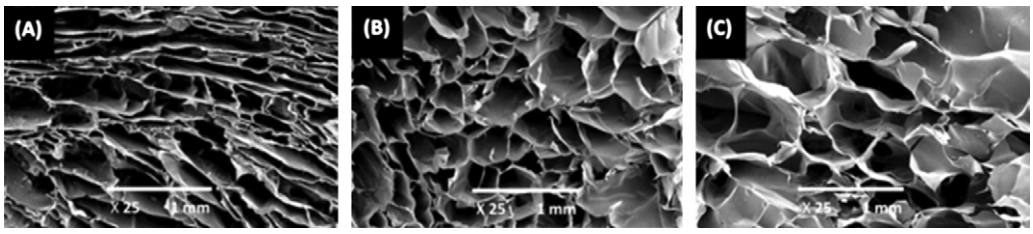

Fig. 3. Scanning electron micrographs of freeze-dried C/G-PC hydrogels. Magnification ×25. (A) C/G-PC-C; (B) C/G-PC-4h and (C) C/G-PC-8h.

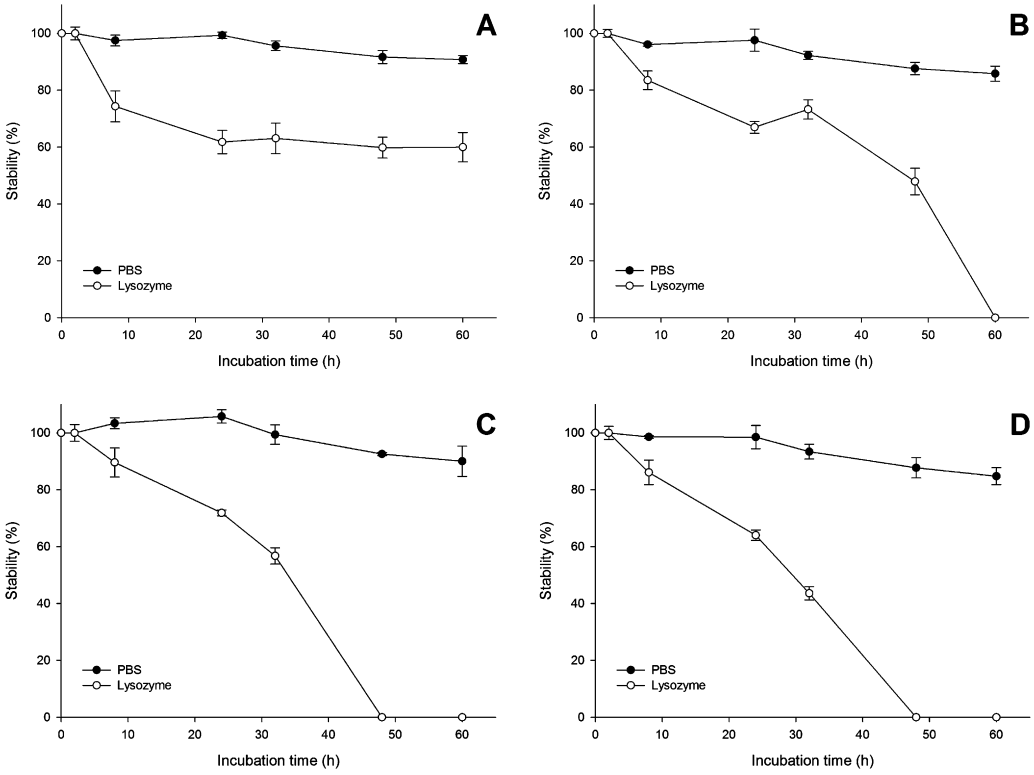

Fig. 4. Stability of hydrogels in PBS and PBS with lysozyme (1 mg/mL) at 37 °C as a function of time. (A) C/G-PC-2h; (B) C/G-PC-4h; (C) C/G-PC-6h and (D) C/G-PC-8h. At regular time intervals, the hydrogels were removed from the medium and weighed. The medium was refreshed once a day. The results are expressed as the average of the hydrogel weight loss (stability) and are mean values from three replicates.

of the hydrogel PC. PC efficiently scavenged ROS both upon release and acting from the biopolymer matrix.

3.4.2. *Inhibition of chronic wound enzymes*

Chronic inflammation leads to the accumulation of MPO-generated HOCl able to oxidize most biological molecules. In addition HOCl triggers the MMPs activation (Fu et al., 2001). The stimulated proteolytic activity contributes to ECM degradation and chronicity of the wounds. It is therefore crucial to control the MPO and MMPs activities in order to provide conditions for healing and ECM reconstruction. The levels of HOCl can by modulated by: (i) MPO substrates diverting the enzyme from generating HOCl, and (ii) HOCl scavengers.

In this study, both C/G-PC-2h and C/G-PC-2h hydrogel platform inhibited the collagenase activity (higher inhibition with C/G-PC-2h) during the first 8 h of incubation, followed by a sustained inhibitory effect up to 24 h. Moreover, the profile of the collagenase inhibition curve in presence of C/G-PC-2h hydrogel platform was similar to the collagenase adsorption curve (Fig. 6A and B) suggesting that enzyme protein adsorption on the hydrogels partially inactivates the collagenase. On the other hand, the higher inhibitory efficiency of C/G-PC-2h sample could be related to the released PC (Díaz-González et al., 2012). A dose-dependent collagenase inhibition was reported for different plant polyphenol extracts. Interaction of the phenolic molecules with the enzymes was suggested to induce conformational changes or blocking of the enzyme active site (Kim, Uyama, & Kobayashi, 2004).

To analyze the inhibition of collagenase by released PC, its activity was studied in presence of different concentrations of PC and FALGPA. The direct-linear Eisenthal–Cornish-Bowden plot describing the kinetics of collagenase inhibition by PC revealed a mixed-type inhibition mechanism.

The kinetic collagenase-PC dissociation constant (Kic) and collagenase-FALGPA-PC dissociation constant (Kiu) (Cornish-Bowden notation) were calculated by secondary plots of Kmapp/Vmaxapp *vs* [PC] and 1/Vapp *vs* [PC] (data not shown) and the Kic and Kiu values obtained were 6.1580 mM and 0.3904 mM respectively. These data suggest that the released PC bind both to collagenase and the collagenase–FALGPA complex probably promoting conformational changes in the enzyme structure and inhibiting its activity (Ganesan, Sehgal, Mandal, & Sayeed, 2011). Mixed-type inhibition may be considered as a consequence of the

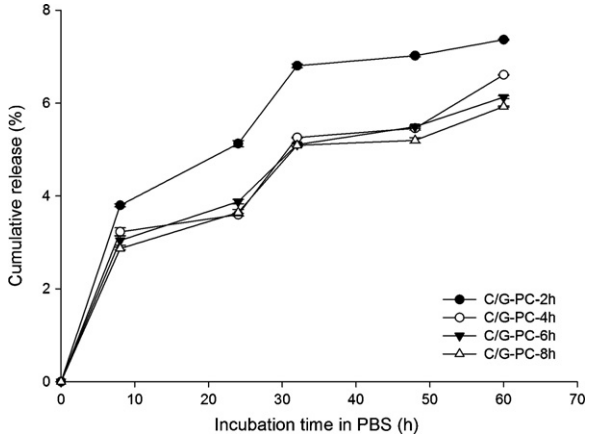

Fig. 5. *In vitro* cumulative release of non-covalently loaded PC from the hydrogels. The samples were incubated in PBS, pH 7.4 at 37 °C for 60 h. At defined time intervals aliquots of the supernatant were removed and the total phenol content determined. The results are averaged from three replicates.

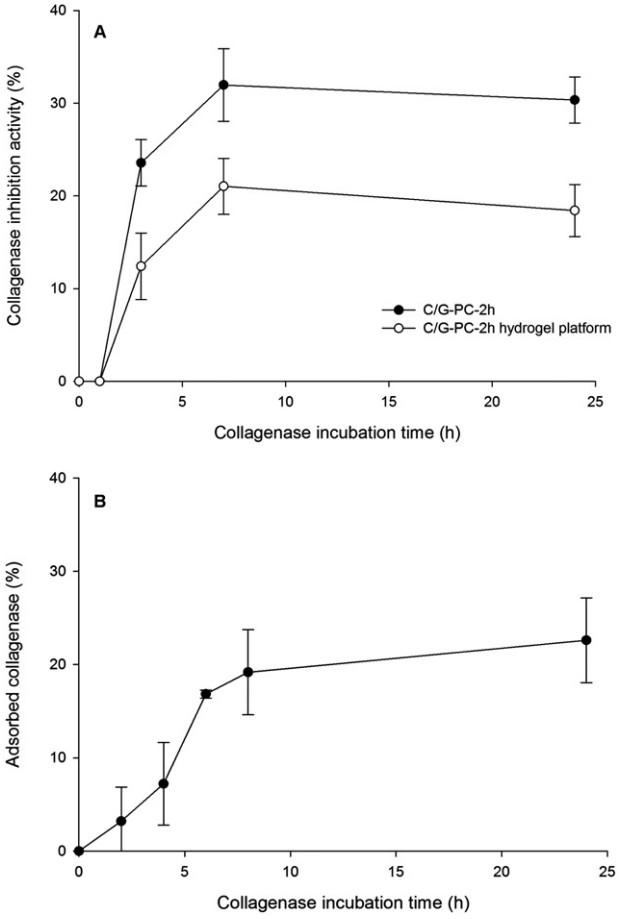

Fig. 6. Inhibition of collagenase activity in the presence of PC-cross-linked hydrogels. (A) The samples and the enzyme (0.2 μg/mL) were incubated in 0.05 M Tricine buffer pH 7.5 (with 0.4 M NaCl and 0.01 M $CaCl_2$, at 37 °C for 3, 6 and 24 h). The reaction was initiated by the addition of FALGPA (300 mM) and the decrease of absorbance at 324 nm was monitored during 5 min to detect FALGPA hydrolysis products. The results represent mean values of three replicates ± standard deviation. (B) Collagenase adsorption on C/G-PC-2h hydrogel platform. The samples and the enzyme were incubated in 0.05 M Tricine buffer pH 7.5 (with 0.4 M NaCl and 0.01 M $CaCl_2$, at 37 °C for 24 h). The protein content in the supernatant was measured at predetermined incubation time using QuantiPro™ BCA Assay Kit. The results represent mean values of three replicates ± standard deviation.

presence of different phenolic compounds in *H. virginiana* extract acting by different inhibition mechanisms (Ganesan et al., 2011; Kim et al., 2004; Madhan, Krishnamoorthy, Rao, & Nair, 2007). These experiments indicate that the proteolytic activity regulation by the developed hydrogels was due both to release of PC and adsorption of the enzyme protein on the biopolymer matrix.

In a next step the MPO inhibitory capacity of C/G-PC-2h and C/G-PC-2h hydrogel platform was measured. The C/G-PC-2h hydrogel platform did not show any MPO inhibition, while the C/G-PC-2h caused about 95% enzyme inhibition within 10 min, further maintained up to 3 h and then followed by a decrease to 79.7% in 24 h (Table 2). Such rapid inhibitory effect is expected considering high MPO inhibition efficiency of *H. virginiana* PC in short time periods (Díaz-González et al., 2012).

In line with the latter, the inhibition of MPO was due to the released PC acting as inhibitors and competing substrates for MPO in the enzyme activity assay using guaiacol. If translated to the chronic wound environment, this combined MPO inhibition and the diversion of the enzyme from its natural cycle would lead to the attenuation of HOCl levels and consequently allow the healing to progress.

Table 2
Myeloperoxidase (MPO) inhibitory activity. C/G-PC-2h was incubated with 0.60 U MPO in the presence of guaiacol (final concentration 10 mM) buffered with PBS pH 7.4 at 37 °C. After predetermined incubation times MPO activity was measured and expressed as a percentage of enzyme inhibition compared to the control (MPO reaction mixture without sample). The results are mean values of three replicates ± standard deviation.

Sample	MPO incubation time (min)	MPO inhibition activity (%)
C/G-PC-2h	10	95.66 ± 0.58
	30	95.93 ± 0.19
	60	95.55 ± 0.28
	180	94.84 ± 0.36
	360	88.00 ± 2.13
	1440	79.70 ± 0.61
C/G-PC-2h hydrogel platform	10–1440	No inhibition

3.4.3. Antibacterial studies

The antimicrobial activity of the enzymatically assembled hydrogels was evaluated against the Gram-positive *S. aureus* and the Gram-negative *P. aeruginosa*. The hydrogels inhibited the growth of both strains after 24 h incubation. The inhibition of *S. aureus* growth was more pronounced than that of *P. aeruginosa* (respectively 84.32 ± 7.56% and 37.06 ± 6.34% for the C/G-PC-2h, and 65.31 ± 2.12% and 23.59 ± 1.03% for C/G-PC-2h hydrogel platform). The tolerance of bacteria for polyphenols depends on the bacteria species and molecular structure of the polyphenols. The extract of *H. virginiana* is rich in epicatechins (EC) which possess antimicrobial activity against *S. aureus* (Masika, Sultana, & Afolayan, 2004) and epigallocatechin gallates (EGCG) that exert antimicrobial activity against *P. aeruginosa* (Xie, Xu, & Liu, 2001) through perturbation of the cell membrane. The higher antibacterial activity observed for C/G-PC-2h was probably due to the release of non-covalently adsorbed PC containing both EC and EGCG. On the other hand, the intrinsic antibacterial properties chitosan are believed to be determined by interactions between its cationic amino groups and the negatively charged microbial cell membranes, resulting in alteration of membrane wall permeability. This mechanism has been reported to be common for both strains, though more efficient on the cell membrane of *S. aureus* (Tao, Qian, & Xie, 2011). Therefore, the antibacterial capacity of the chitosan/gelatin/PC hydrogels is a result of the combined action of the biopolymer platforms, covalently attached PC and released PC.

4. Conclusions

Laccase-assisted coupling between polyphenols of *H. virginiana* and primary amino groups in chitosan and gelatin was used as a versatile functionalization method to obtain stable under physiological conditions bioactive hydrogels. The laccase-initiated oxidation of phenolics resulted in a biopolymer network stabilized by quinones/amino coupling reactions predominantly *via* Michael addition mechanism between the phenolic compounds and the polymers. The phenolic compounds loaded on the hydrogels exerted both: (i) structural function stabilizing the hydrogel, and (ii) bio-activity inhibiting deleterious wound enzymes and bacterial growth to stimulate the wound healing process. The physico-mechanical properties of the chitosan/gelatin-PC hydrogels were dependent on the duration of the enzymatic reaction. Hydrogels stable under physiological conditions and resistant to lysozyme degradation were obtained with relatively short enzymatic reaction time (2 h). The inhibition of the deleterious chronic wound enzymes largely depended on the amount of the released phenolic compounds, being responsible for the inhibition of myeloperoxidase activity acting as enzyme substrates. On the other hand, partial inhibition of collagenase activity was due to both released phenolic

compounds and enzyme protein adsorption onto the hydrogel platform. In addition, the enzymatically assembled hydrogels inhibited the bacterial growth of *S. aureus* and *P. aeruginosa* commonly found in chronic wound.

Acknowledgements

This work was carried out under the scope of the EU project Lidwine (Contract No. FP6-026741). The authors from the Universitat Politècnica de Catalunya and Universitat Rovira i Virgili would like to thank MICINN (Ministerio de Ciencia e Innovación) and FEDER (Fondo Europeo de Desarrollo Regional) for (MAT2011-27039-C03-01) and grants BES-2008-00374 and JCI-2010-06187.

References

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, *28*(1), 25–30.

Capeillere-Blandin, C. (1998). Oxidation of guaiacol by myeloperoxidase: A two-electron-oxidized guaiacol transient species as a mediator of NADPH oxidation. *Biochemical Journal*, *336*(Pt 2), 395–404.

Cui, Y. L., Qi, A. D., Liu, W. G., Wang, X. H., Wang, H., Ma, D. M., et al. (2003). Biomimetic surface modification of poly(L-lactic acid) with chitosan and its effects on articular chondrocytes in vitro. *Biomaterials*, *24*(21), 3859–3868.

Desentis-Mendoza, R. M., Hernandez-Sanchez, H., Moreno, A., Rojas del c, E., Chel-Guerrero, L., Tamariz, J., et al. (2006). Enzymatic polymerization of phenolic compounds using laccase and tyrosinase from *Ustilago maydis*. *Biomacromolecules*, *7*(6), 1845–1854.

Deters, A., Dauer, A., Schnetz, E., Fartasch, M., & Hensel, A. (2001). High molecular compounds (polysaccharides and proanthocyanidins) from *Hamamelis virginiana* bark: Influence on human skin keratinocyte proliferation and differentiation and influence on irritated skin. *Phytochemistry*, *58*(6), 949–958.

Díaz-González, M., Rocasalbas, G., Francesko, A., Touriño, S., Torres, J. L., & Tzanov, T. (2012). Inhibition of deleterious chronic wound enzymes with plant polyphenols. *Biocatalysis and Biotransformation*, *30*(1), 102–110.

Eastoe, J. E. (1955). The amino acid composition of mammalian collagen and gelatin. *Biochemical Journal*, *61*(4), 589–600.

Fazli, M., Bjarnsholt, T., Kirketerp-Moller, K., Jorgensen, B., Andersen, A. S., Krogfelt, K. A., et al. (2009). Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. *Journal of Clinical Microbiology*, *47*(12), 4084–4089.

Fu, X., Kassim, S. Y., Parks, W. C., & Heinecke, J. W. (2001). Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. *Journal of Biological Chemistry*, *276*(44), 41279–41287.

Ganesan, K., Sehgal, P. K., Mandal, A. B., & Sayeed, S. (2011). Protective effect of *Withania somnifera* and *Cardiospermum halicacabum* extracts against collagenolytic degradation of collagen. *Applied Biochemistry and Biotechnology*, *165*(3–4), 1075–1091.

Guzzo, F., Cappello, M. S., Azzolini, M., Tosi, E., & Zapparoli, G. (2011). The inhibitory effects of wine phenolics on lysozyme activity against lactic acid bacteria. *International Journal of Food Microbiology*, *148*(3), 184–190.

Hori, K., Sotozono, C., Hamuro, J., Yamasaki, K., Kimura, Y., Ozeki, M., et al. (2007). Controlled-release of epidermal growth factor from cationized gelatin hydrogel enhances corneal epithelial wound healing. *Journal of Controlled Release*, *118*(2), 169–176.

Jollès, P., & Muzzarelli, R. A. A. (1999). *Chitin and chitinases*. Basel: Birkhäuser Verlag.

Kim, S., Nimni, M. E., Yang, Z., & Han, B. (2005). Chitosan/gelatin-based films crosslinked by proanthocyanidin. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *75*(2), 442–450.

Kim, Y.-J., Uyama, H., & Kobayashi, S. (2004). Inhibition effects of (+)-catechin–aldehyde polycondensates on proteinases causing proteolytic degradation of extracellular matrix. *Biochemical and Biophysical Research Communications*, *320*(1), 256–261.

Lizárraga, D., Touriño, S., Reyes-Zurita, F. J., de Kok, T. M., van Delft, J. H., Maas, L. M., et al. (2008). Witch hazel (*Hamamelis virginiana*) fractions and the importance of gallate moieties electron transfer capacities in their antitumoral properties. *Journal of Agricultural and Food Chemistry*, *56*(24), 11675–11682.

Madhan, B., Krishnamoorthy, G., Rao, J. R., & Nair, B. U. (2007). Role of green tea polyphenols in the inhibition of collagenolytic activity by collagenase. *International Journal of Biological Macromolecules*, *41*(1), 16–22.

Masika, P. J., Sultana, N., & Afolayan, A. J. (2004). Antibacterial activity of two flavonoids isolated from *Schotia latifolia*. *Pharmaceutical Biology*, *42*(2), 105–108.

Mikolasch, A., & Schauer, F. (2009). Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. *Applied Microbiology and Biotechnology*, *82*(4), 605–624.

Mita, N., Tawaki, S.-i., Uyama, H., & Kobayashi, S. (2003). Laccase-catalyzed oxidative polymerization of phenols. *Macromolecular Bioscience*, *3*(5), 253–257.

Moreira Teixeira, L. S., Feijen, J., van Blitterswijk, C. A., Dijkstra, P. J., & Karperien, M. (2012). Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering. *Biomaterials*, *33*(5), 1281–1290.

Moura, M. J., Faneca, H., Lima, M. P., Gil, M. H., & Figueiredo, M. M. (2011). In situ forming chitosan hydrogels prepared via ionic/covalent co-cross-linking. *Biomacromolecules*, *12*(9), 3275–3284.

Muzzarelli, R. A. A. (1977). *Chitin*. Oxford, UK: Pergamon Press.

Muzzarelli, R. A. A. (2009a). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*(2), 167–182.

Muzzarelli, R. A. A. (2009b). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*(1), 1–9.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*(2), 995–1012.

Muzzarelli, R. A. A., Ilari, P., Xia, W., Pinotti, M., & Tomasetti, M. (1994). Tyrosinase-mediated quinone tanning of chitinous materials. *Carbohydrate Polymers*, *24*(4), 295–300.

Pulieri, E., Chiono, V., Ciardelli, G., Vozzi, G., Ahluwalia, A., Domenici, C., et al. (2008). Chitosan/gelatin blends for biomedical applications. *Journal of Biomedical Materials Research Part A*, *86*(2), 311–322.

Quideau, S., Deffieux, D., Douat-Casassus, C., & Pouységu, L. (2011). Plant polyphenols: Chemical properties, biological activities, and synthesis. *Angewandte Chemie International Edition*, *50*(3), 586–621.

Scalbert, A., Monties, B., & Janin, G. (1989). Tannins in wood: Comparison of different estimation methods. *Journal of Agricultural and Food Chemistry*, *37*(5), 1324–1329.

Shimada, T., Saitoh, T., Sasaki, E., Nishitani, Y., & Osawa, R. (2006). Role of tannin-binding salivary proteins and tannase-producing bacteria in the acclimation of the Japanese wood mouse to acorn tannins. *Journal of Chemical Ecology*, *32*(6), 1165–1180.

Sorsa, T., Konttinen, Y., Lindy, O., Ritchlin, C., Saari, H., Suomalainen, K., et al. (1992). Collagenase in synovitis of rheumatoid arthritis. *Seminars in Arthritis and Rheumatism*, *22*(1), 44–53.

Tao, Y., Qian, L.-H., & Xie, J. (2011). Effect of chitosan on membrane permeability and cell morphology of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Carbohydrate Polymers*, *86*(2), 969–974.

Taylor, K. B., Windsor, L. J., Caterina, N. C., Bodden, M. K., & Engler, J. A. (1996). The mechanism of inhibition of collagenase by TIMP-1. *Journal of Biological Chemistry*, *271*(39), 23938–23945.

Torres, J., & Selga, A. (2003). Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography. *Chromatographia*, *57*(7), 441–445.

Touriño, S., Lizarraga, D., Carreras, A., Lorenzo, S., Ugartondo, V., Mitjans, M., et al. (2008). Highly galloylated tannin fractions from witch hazel (*Hamamelis virginiana*) bark: Electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells. *Chemical Research in Toxicology*, *21*(3), 696–704.

Trengove, N. J., Stacey, M. C., MacAuley, S., Bennett, N., Gibson, J., Burslem, F., et al. (1999). Analysis of the acute and chronic wound environments: The role of proteases and their inhibitors. *Wound Repair and Regeneration*, *7*, 442–452.

Van Vlierberghe, S., Dubruel, P., & Schacht, E. (2011). Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review. *Biomacromolecules*, *12*(5), 1387–1408.

Van Wart, H. E., & Steinbrink, D. R. (1981). A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. *Analytical Biochemistry*, *113*(2), 356–365.

Wagner, H., & Ulrich-Merzenich, G. (2009). Synergy research: Approaching a new generation of phytopharmaceuticals. *Phytomedicine*, *16*(2–3), 97–110.

Wang, Y., Qiu, D., Cosgrove, T., & Denbow, M. L. (2009). A small-angle neutron scattering and rheology study of the composite of chitosan and gelatin. *Colloids and Surfaces B: Biointerfaces*, *70*(2), 254–258.

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic & Medicinal Chemistry Letters*, *11*(13), 1699–1701.

Zhang, H., Qadeer, A., & Chen, W. (2011). In situ gelable interpenetrating double network hydrogel formulated from binary components: Thiolated chitosan and oxidized dextran. *Biomacromolecules*, *12*(5), 1428–1437.