Multifunctional enzymatically-generated hydrogels for chronic wound application

Ivaylo Stefanov†, Sílvia Pérez-Rafael†, Javier Hoyo†, Jonathan Cailloux‡, Orlando O. Santana Pérez ‡, Dolores Hinojosa-Caballero[§], Tzanko Tzanov, †*

†Grup de Biotecnologia Molecular i Industrial (GBMI), Department of Chemical Engineering, Universitat Politècnica de Catalunya – (UPC), Rambla Sant Nebridi, 22, 08222 Terrassa, Barcelona, Spain.

‡Centre Català del Plàstic (CCP) – Universitat Politècnica de Catalunya Barcelona - (UPC-EEBE), C/Colom, 114. Edifici Vapor Universitari, Terrassa 08222, Spain

[§]Unitat de ferides Complexes, Consorci Sanitari de Terrassa

Hospital de Terrassa, Ctra. Torrebonica, s/n, 08227 Terrassa, Barcelona, Spain

[*] Corresponding author

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ABSTRACT

The healing of chronic wounds requires intensive medical intervention at huge healthcare costs. Dressing materials should consider the multifactorial nature of these wounds comprising deleterious proteolytic and oxidative enzymes, and high bacterial load. In this work, multifunctional hydrogels for chronic wound application were produced by enzymatic crosslinking of thiolated chitosan and gallic acid. The hydrogels combine several beneficial to wound healing properties, controlling the matrix metalloproteinases (MMPs) and myeloperoxidase (MPO) activities, oxidative stress and bacterial contamination. *In vitro* studies revealed above 90 % antioxidant activity, and MPO and collagenase inhibition by up to 98 and 23 %, respectively. *Ex vivo* studies with venous leg ulcer exudates confirmed the inhibitory capacity of the dressings against MPO and MMPs. Additionally, the hydrogels reduced the population of the most frequently encountered in non-healing wounds bacterial strains. The stable at physiological conditions and resistant to lysozyme degradation hydrogels showed high biocompatibility with human skin fibroblasts.

INTRODUCTION

Wounds that do not show signs of healing within three to six weeks are described as chronic wounds and require resource-consuming and dedicated nursing care with treatment regimens that are expensive and/or ineffective. Furthermore, the management of these clinically compromised wounds represents a significant cost for the national health systems and has become a major therapeutic challenge due to the ageing population and increasing incidence of conditions that impede wound healing, such as diabetes, obesity and vascular disorders,[1] further worsening the morbidity.[2] Despite not sharing origin or cause, and being clinically and molecularly heterogeneous, the chronic wounds exhibit the following common features - bacterial contamination and high concentration of reactive oxidative species (ROS), myeloperoxidase (MPO) and matrix metalloproteinases (MMPs).[3] In chronic wounds, however, the imbalance between the elevated levels of MMPs and the tissue inhibitors of matrix metalloproteinases (TIMPs) causes an excessive degradation of the extracellular matrix (ECM) and growth factors.[4] Furthermore, the activation of MMPs and the degradation of TIMPs and growth factors are triggered by the MPO. In addition, several bacterial species, e.g. *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) colonize most chronic wounds. On the other hand, it is known that compounds able to chelate the Zn^{2+} from the active centre of the enzyme would inactivate it.[5] This is the principle of action of the most potent MMPs inhibitors – the hydroxamates.[6] Similarly, the thiols are reported as inhibitors of MMPs.[7] Incorporating thiols in the structure of the hydrogels is the initial hypothesis for the MMPs controlling activity of the hydrogels.

Because of the multifactorial nature of the chronic wounds, any single therapeutic intervention is unlikely to have satisfactory healing effect. An efficient therapeutic approach for chronic wound healing should necessarily: i) control the infection-related proteolytic and oxidative enzymatic activities in the wound site, ii) provide microorganisms-free environment, and iii) maintain the tissue moisture while absorbing the excessive exudates.[8] Therefore, chronic wound dressings should combine both antimicrobial and enzyme inhibitory functions coupled to high hydrophilicity. However, the dressing materials that dominate the market[9] are mostly biologically inert hydrogels, whose only functionality is to retain the exudate and maintain the wound moist.[10] In the most advanced designs, the same hydrogels are loaded with antimicrobial agents, e.g. silver,[11] to prevent or heal microbial infection. Towards the development of therapies to treat chronic wounds comprising the aforementioned characteristics, hydrogels with different functionalities have been suggested.[12] Chitosan-based hydrogels alone or in combination with other biopolymers have been widely applied in tissue engineering,[13] regenerative medicine,[14] drug delivery,[15] and wound healing.[16] Chitosan, a copolymer of glucosamine and *N*-acetyl-*D*-glucosamine derived by deacetylation of chitin from fungal or crustacean origin, is a valuable for medical applications polysaccharide due to its biocompatibility, biodegradability, lack of cytotoxicity and intrinsic antimicrobial activity.[17] The major drawback of chitosan-based hydrogels is their poor biostability and mechanical strength that must be enhanced by additional chemical, e.g. glutaraldehyde[18] or carbodiimide[19] crosslinking which often produces toxic residues unsuitable for medical applications.

Alternatively, our group has developed covalently crosslinked gels obtained via enzyme-catalyzed reactions applying mild conditions and avoiding the use of harsh chemicals.[8,20] Modifying the polysaccharide structure can further refine the mechanical and functional

properties of chitosan-based hydrogels. Combining thiolated chitosan (TCS) with polyphenols would increase its mechanical stability,[21] antioxidant and antibacterial activity, and the inhibition efficiency against MPO and MMPs. Upon treatment with oxidative enzymes, such as tyrosinase, peroxidase and laccase, polyphenols undergo oxidative transformation into quinones. These highly reactive species can interact with nucleophilic groups from chitosan, which will additionally stabilize the hydrogel structure via inter- and intra-molecular coupling. Oxidases have been largely used in our group to produce biopolymer-based hydrogels,[8,20] antimicrobial filters,[22] antifouling urinary catheters,[23] and functionalized protein fibers.[24,25] In particular, laccase has been recognized as a 'green enzyme', since it uses O_2 from the air and produces H_2O as the only by-product.[26] The phenolic compound we selected for the current study was the naturally occurring gallic acid (GA) owing to its strong antioxidant activity,[27] demonstrated MPO inhibition and lack of toxicity.[28]

The aim of this work is to develop hydrogel-based dressings in a one-step laccase-catalyzed crosslinking reaction for chronic wounds with tunable physicomechanical and functional properties, which rely on intrinsic antimicrobial and wound repair properties as matrix material (TCS) further upgraded with bioactive molecules (GA) that couple high reactivity with the ability to address specific targets in the biochemical environment of chronic wounds in order to stimulate the healing process. Herein, we suggest that the thiolation of chitosan would have a synergistic effect improving the mechanical properties of chitosan-based hydrogels by disulfide formation, and enhancing the inhibitory capability of chitosan against MMPs by chelation of the Zn^{2+} from their active center.

The resulting hydrogels are expected to: i) absorb the wound exudate and keep the moist environment of the wound, ii) provide a wound environment with controlled ROS, MMPs and

MPO activities, and iii) reduce the bacterial contamination in the wound bed. The efficiency of the developed dressing materials will be evaluated *in vitro* against major factors governing the chronicity in wounds. The morphology, swelling properties and rheological characteristics of the hydrogels together with their stability at physiological conditions will be further examined. Finally, the inhibitory capacity of the hydrogels towards deleterious chronic wound enzymes will be assessed *ex vivo* in the complex environment of a wound exudate collected from a venous leg ulcer.

MATERIALS AND METHODS

Reagents and enzymes

Medical grade chitosan from fungi (15 kDa, degree of deacetylation 87 %) was obtained from KitoZymes (Belgium). Traut's reagent (2-iminothiolane hydrochloride), L-cysteine, sodium phosphate mono- and dibasic, gallic acid, acetic acid, sodium chloride, zinc dichloride, ammonium chloride, ammonium hydroxide, hydrochloric acid, Elman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)), sodium hydroxide, hydrogen peroxide, sodium acetate, sodium carbonate, phosphate buffered saline (PBS), ethylenediaminetetraacetic acid disodium salt (EDTA), 1,1-diphenyl-2-picrylhydrazyl, Bradford reagent (Coomasie Brilliant Blue G), Eriochrome® Black T, Folin & Ciocalteu's phenol reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and guaiacol were provided from Sigma-Aldrich (Spain).

Collagenase from *Clostridium hystolyticum type I* (EC 3.4.24.3, 244.9 U/mg solid: 1 U hydrolyzes 1.0 μmol of furylacryloyl-Leu-Gly-Pro-Ala per min at 25 ºC) and lysozyme from chicken egg white (EC 3.2.1.17, 100000 U/mg where 1 U corresponds to the amount of enzyme which decreases the absorbance at 450 nm by 0.001 per min at pH 7.0 and 25 ºC of *Micrococcus luteus*, ATCC 4698, as a substrate) were purchased from Sigma-Aldrich. Laccase (EC 1.10.3.2, Denilite II Base from *Myceliophthora thermophila* expressed in *Aspergillus oryzae*, 8.6 U/mg where 1 U corresponds to the amount of enzyme which catalyzes the oxidation of 1 μmol ABTS per min at pH 5.5 and 25 ºC) was provided by Novozymes (Denmark). Myeloperoxidase (MPO) from human leukocytes (EC 1.11.2.2, 1550 U/mg solid: 1 U produces an increase of 1.0 absorbance unit per min. at 470 nm at pH 7.0 and 25 ºC, using guaiacol as a substrate) was purchased from Planta Natural Products (Austria). EnzChek Gelatinase/Collagenase Assay Kit was provided from Molecular Probes (USA).

Preparation of the hydrogels

The thiolation of chitosan was carried out in a one-step coupling reaction between Traut's reagent and the primary amino groups of chitosan to obtain chitosan-thiobutylamidine.[29] The coupling reaction allows the functionalization of the biopolymer with free thiol groups, while the established amidine linkages increase its cationic character and potentially its antimicrobial activity.[30] Briefly, chitosan was dissolved in CH_3COOH at 1 % w/v final concentration. Thereafter, the pH was adjusted to ~6.5 with 5 M NaOH and different amounts of Traut's reagent, based on our previous work,[30] were added to prepare chitosan batches with variable degree of thiolation (Table 1). The mixtures were stirred at room temperature under dark for 24 h. Afterwards, the preparations were dialyzed once against 5 mM HCl with 1 % NaCl, twice against 1 mM HCl with 1 % NaCl and once more extensively against 1 mM HCl prior lyophilization and storage at 4 ºC under nitrogen atmosphere until further use.

Table 1. Thiolated chitosan samples (TCS)

Sample code	Reaction ratio chitosan: Traut's	µmols free -SH per g polymer
TCS 1-10	10:1	389 ± 19.2
TCS 1-5	5:1	469.4 ± 26.1
TCS 2-5	5:2	523.4 ± 24.5

The amount of free thiol groups, immobilized on chitosan was assessed using Ellman's reagent.[31] Briefly, 1 mg of the lyophilized material was immersed for 20 min in 500 µL 0.5 M phosphate buffer, pH 8. Afterwards 500 µL of Ellman's reagent (3 mg in 10 mL of the same aforementioned buffer) were added and the samples were left for 3 h in dark conditions. The mixtures were centrifuged during 5 min at 5000 rpm. Then the supernatant was transferred to a microplate reader Infinite M200, Tecan (Austria) and its absorbance was monitored at 450 nm. L-cysteine standards were used for building a calibration curve and non-thiolated chitosan was used as a control sample.

The hydrogels were obtained by laccase-catalyzed crosslinking of TCS and GA at pH 5.5 and 50 ºC. According to the provider's specification, at this temperature the enzyme reaches 80 % of its maximum activity, achieved at 65 – 70 ºC. Briefly, solutions comprising 1.6 mL TCS (7 % w/v), 0.2 mL laccase (2.7 U/mL) and different volumes of 75 mM GA (200, 134 and 67 µL) were prepared. The volume of each mixture was adjusted to 2 mL by adding acetate buffer (0.1 M, pH 5.5) and the mixtures were transferred to a 6-well plate by a syringe equipped with a 21G needle in order to maintain all gel preparation conditions equal. To evaluate the influence of: i) the time of incubation of the hydrogels with laccase (2, 4 and 6 h), ii) the degree of thiolation of chitosan (TCS1-10, TCS1-5, TCS2-5), and iii) the amount of 75 mM GA, different batches of hydrogels (Table 2) were prepared. The aforementioned solutions of TCS and GA without laccase were freeze-dried and used as controls.

Table 2. Samples of thiolated chitosan/gallic acid hydrogels

Sample code	Thiolated chitosan	Gallic acid (mM)	Time of incubation with laccase (h)
2.5TCS1-10	TCS1-10	2.5	2, 4, 6
5TCS1-10	TCS1-10	5	2, 4, 6
7.5TCS1-10	TCS1-10	7.5	2, 4, 6
2.5TCS1-5	TCS1-5	2.5	2, 4, 6
5TCS1-5	TCS1-5	5	2, 4, 6
7.5TCS1-5	TCS1-5	7.5	2, 4, 6
2.5TCS2-5	TCS2-5	2.5	2, 4, 6
5TCS2-5	TCS2-5	5	2, 4, 6
7.5TCS2-5	TCS2-5	7.5	2, 4, 6

To determine their phenolic content, the lyophilized hydrogels (1.4 mg each) were immersed in 70 µL 0.2 N Folin-Ciocalteu reagent, 1.2 mL of Milli-Q water and 120 µL 20 % w/v Na_2CO_3. The mixture was placed in dark conditions at room temperature for 1 h. Afterwards 300 µL of each solution were transferred to a 96-well microplate and the absorbance was recorded at 765

nm. The results were expressed as percentage of free phenol groups remaining after the incubation with laccase.

The polyphenols release was assessed as follows: **t**he lyophilized hydrogel samples (2 mg each) were immersed in 500 μL PBS buffer (pH 7.4) for 24 h at 37 ˚C. Then, the phenol content of 400 μL supernatant was assessed as described above. The results were expressed as the percentage release of polyphenols from the initial concentration of GA, used for the preparation of the hydrogels.

Rheological, structural and morphological characterization of the hydrogels

Rheological characterization of the hydrogels was performed with an ARG2 rheometer (TA instruments, UK), equipped with electrical heated plates. The samples were analyzed in parallel plate geometry (40 mm). The rheometer was set in the oscillatory mode (2 Hz frequency) with controlled strain (2 %) used to monitor the crosslinking process. The blends were placed onto the Peltier plate at 25 ºC and the measurements started after thermal equilibration to 50 ºC. The gelation point was determined by using G' and G'' crossover criterion.

The structural features of the hydrogels after lyophilization were examined with FTIR over 4000-650 cm^{-1} range with Perkin-Elmer Spectrum 100 (Perkin-Elmer, Massachusetts, USA). Each sample was subjected to 64 scans. In order to elucidate the mechanism of crosslinking, the FTIR-spectra of a low molecular weight model compound of TCS, containing both amino and thiol groups namely L-cysteine, was studied.

The lyophilized hydrogels' morphology was studied by scanning electron microscopy (JSM 5610, JEOL Ltd. Japan). Cross-sections of the hydrogel samples were gold-sputtered under

vacuum and micrographs were recorded at 250x magnification. The pore size of the hydrogels was quantified using ImageJ 1.5 software.

In order to determine their swelling equilibrium, the hydrogels were swollen/immersed in PBS, pH 7.4 at 37 ºC up to 24 hours. The samples were periodically removed from the media and weighted until no further change in their weight was determined. Within 6 hours the swelling equilibrium of all samples was reached. The stability of the hydrogels upon enzymatic degradation in physiological liquids was assessed in presence of lysozyme (100000 U/mL).[8] The medium was refreshed and the hydrogels weighted once per day during six days. The results were compared to those obtained following the same procedure but omitting the enzyme. All experiments were performed in five replicates and the average weight loss was calculated, according to the following equation (1):

$$\text{Weight loss (\%)} = 100(W_s - W_{lys})/W_s \qquad (1)$$

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.

The swelling properties of the hydrogels were determined by comparing the weight of the dry lyophilized samples and the weight of the swollen hydrogels after immersion in a physiological solution (PBS, pH 7.4) at 37 ºC for 24 h. After that, the weight of the swollen hydrogels was measured and the results were expressed as percentage of swelling of the hydrogels according to the following equation (2):

$$SW\% = ((W_s - W_d)/W_d)) \cdot 100 \qquad (2)$$

where W_s is the weight of the swollen hydrogel, W_d is the weight of the dry hydrogel and SW% is the percentage of water uptake. Five replicates of all samples were tested and the standard deviations were calculated.

In vitro studies of the inhibitory activity of the hydrogels over deleterious wound enzymes

The inhibitory efficiency of the hydrogels against collagenase was estimated by measuring the increase in fluorescence intensity of fluorescently labeled gelatin (EnzChek substrate) cleaved in presence of collagenase. Briefly, 5 mg of hydrogel samples were incubated with 400 μL 49 U/mL collagenase, dissolved in EnzChek buffer for 24 h at 37 °C. Thereafter 100 μL of the incubated solution were transferred to 96-well microplate and mixed with 80 μL buffer. After adding 20 μL of 250 μg/mL gelatin substrate solution, the measurement started at excitation/emission 493/528 nm. All measurements were performed in triplicate and the results were expressed as collagenase residual activity compared to the collagenase control solution without hydrogel.

A quartz crystal microbalance (QCM) was used to monitor the Zn^{2+} binding capacity and collagenase adsorption on the gels. 5-MHz AT-cut quartz crystals, coated with optically flat polished Cr/Au electrodes were used in QCM200 from SRS Stanford Research Systems. Prior starting the experiments, the quartz crystals were immersed in 6M H_2SO_4 during 5 min, rinsed with ethanol and deionized water, and sonicated during 15 min. Thereafter, the crystals were gently rinsed with milliQ water and dried with nitrogen. The solutions were pumped at a flow rate of 80 μl min^{-1}. At first, the hydrogel was placed on the top of the golden disk and the EnzChek kit buffer was circulated through the cell, until the steady state was reached. Then, this buffer was replaced with a new solution containing EnzChek kit buffer supplemented with $Zn(NO_3)_2$ or collagenase while monitoring the frequency. Once finished, this solution was replaced by the EnzChek kit buffer alone, reaching a new steady state. The difference between both steady states was correlated with the adsorption or binding capacity.

In order to measure the capacity of the hydrogels (3 mg samples) to entrap collagenase and thereby remove it from the wound bed, the hydrogels were incubated for up to seven days at 37 °C in presence of 500 µL 1 mg/mL collagenase solution. Bradford® kit was used to assess the amount of remaining enzyme protein in the solution. Immediately after incubation, 20 µL of each incubated solution were mixed with 280 µL Bradford reagent and the mixtures were incubated for 5 min, prior to monitoring the absorbance at 595 nm. The calibration curve was built after measuring the absorbance of serial dilutions of BSA in presence of equivalent amounts of Bradford reagent.

The MPO inhibition by the hydrogels was measured using guaiacol as a substrate.[32] To determine the impact of the amount of GA, samples (1 mg) prepared with the highest degree of chitosan thiolation and varying the concentration of GA (designated as 2.5TCS2-5, 5TCS2-5 and 7.5TCS2-5) were incubated with 32 µL 0.25 U/mL MPO and 48 µL guaiacol (167 mM) solution buffered with 720 µL PBS pH 7.4 at 37 ºC. After 1 h incubation, 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 the absorbance at 470 nm was monitored during 1 min using a microplate reader. 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 hydrogel sample).

***Ex vivo* studies of inhibitory activity of the hydrogels over deleterious wound enzymes**

Wound exudates were extracted from the UrgoClean® dressing (from Urgo Medical) of a patient with a venous leg ulcer (Hospital de Terrassa (Spain)). After removing the dressing from patient's limb, 1 g specimen containing the exudate, was soaked in 5 mL Milli-Q water for 10

min. Thereafter, the mixture was vortexed for 3 min and centrifuged for 5 min at 10000 rpm. The extracted liquid was collected and centrifuged once again for 5 min at 10000 rpm to remove the sludge from the wound dressing, and finally stored in the fridge at 4 °C for further use.

In order to investigate the MPO inhibition ability of the hydrogels, each hydrogel sample (1 mg) was immersed in 720 µL phosphate buffer (0.1 M, pH 6.5), 48 µL guaiacol and 32 µL of the extracted wound fluid previously diluted 16 times in order to adjust the enzymatic assay conditions. After incubating the mixture for 1 h at 37 °C, the change in absorbance at 470 nm during a 3 min cycle was measured. The results were expressed as a percentage inhibition of MPO. Sample incubated in absence of hydrogel, served as a control and its MPO activity was taken as 100 % at the aforementioned conditions.

For the evaluation of the inhibition efficiency of the hydrogels against MMPs, 400 µL of the chronic wound exudate, previously diluted 8 times with buffer (pH 7.4), was incubated for 24 h at 37 °C in presence of hydrogels with different degree of thiolation and the same concentration of GA. Then, the MMP inhibition by the hydrogels was measured as previously described. Sample incubated without hydrogel served as a control and the activity of MMPs in that sample was considered as 100 %.

Radical scavenging activity

The radical scavenging activity of the hydrogels was determined spectrophotometrically measuring the decrease of absorbance of the DPPH radical at 517 nm. Briefly, 2 mg of each hydrogel were incubated in 60 µM DPPH solution in methanol at 37 ºC in dark conditions for 30 min. All measurements were performed in triplicate. The following equation (3) was used to calculate the antioxidant activity:

$$\text{DPPH inhibition (\%)} = [1 - (A/A_o)] \times 100 \qquad (3)$$

where A_o is the absorbance of the negative control (DPPH solution alone) and A is absorbance of DPPH, incubated with the hydrogels.

Cytotoxicity studies

Hydrogel samples (3 mg each) with different degree of thiolation and the highest concentration of GA (7.5 mM) were previously sterilized under UV-light for 1 hour. The samples were then placed in contact with 1 mL of complete growth medium (DMEM) in 24-well tissue culture-treated polystyrene plate, containing 4.5 x 10^4 cells/well of human skin fibroblasts in a CO_2 incubator at 37 °C for one or seven days. Afterwards, the samples were removed and the growth media withdrawn. Media without hydrogel served as a negative control for 100 % cell viability.

The previously seeded cells were put into contact with the withdrawn culture media and incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 24 h. The cells were further examined for signs of toxicity using an AlamarBlue assay kit (AlamarBlue, Invitrogen). AlamarBlue was diluted in culture medium (10 % v/v) and added to each well after aspirating the culture medium containing the samples. The absorbance at 570 nm was measured with a microplate reader after 4 h incubation at 37 °C, setting 600 nm as a reference wavelength.

Antibacterial activity

S. aureus, E. coli and *P. aeruginosa* were grown on Baird-Parker, coliform and Cetrimide agar, at 37 ºC for 24 h. Suspensions of each strain were prepared at concentration of 10^6 CFU/ml. The hydrogel samples (3 mg each) were incubated with 500 µL of *S. aureus, E. coli* or *P. aeruginosa* suspensions for 24 h at 37 ºC and 450 rpm. Thereafter, different dilutions of the suspensions (prepared in sterile nutrient broth (NB)) were tested for bacterial growth on coliform, Baird-Parker or Cetrimide agar for *E. coli, S. aureus* and *P. aeruginosa*, respectively, for 24 h. The

number of survived bacterial colonies was counted and the bacterial reduction ability of the hydrogels was calculated using the following equation (4):

$$\text{Log reduction} = \log_{10}(B/C) \quad (4)$$

where B and C are the average number of bacterial colonies at 24 h for bacteria incubated without (control sample) and with hydrogel, respectively.

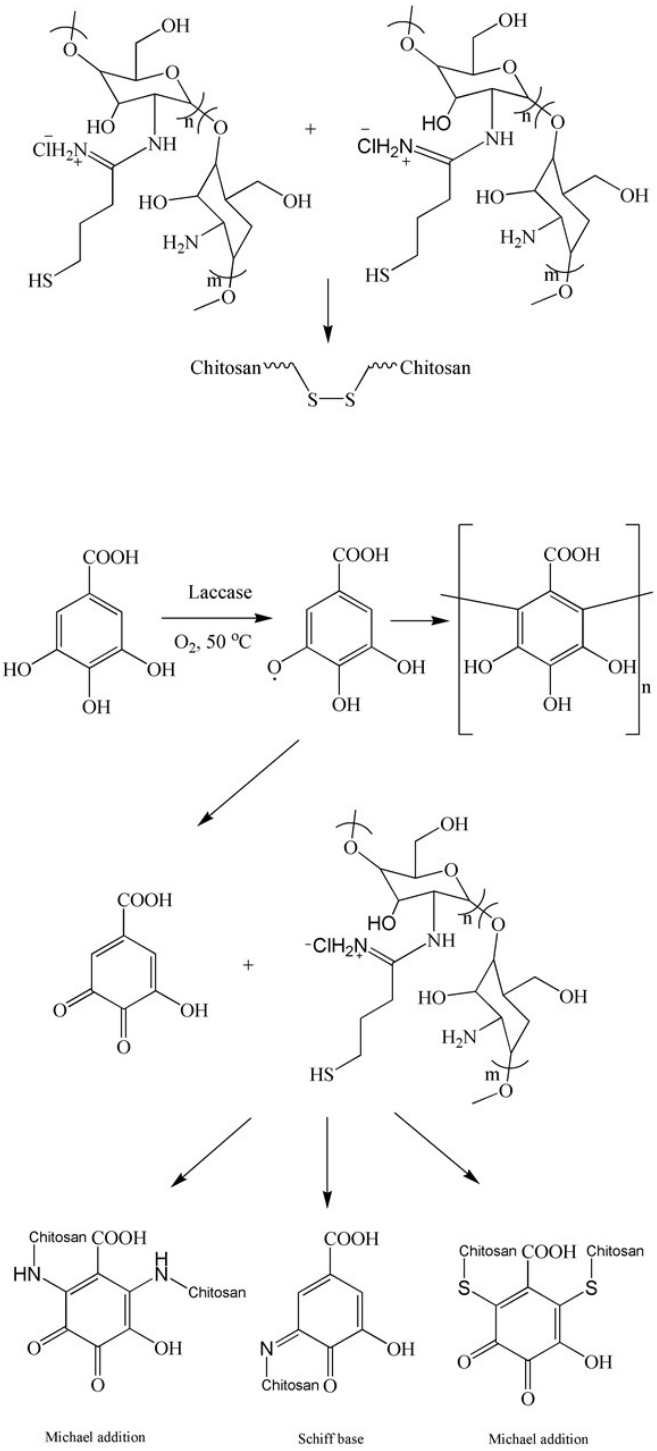
Data analysis

Replicate samples were used in each measurement, and all values are presented as mean values ± the standard deviations (SD). Results were analyzed using Graph Pad Prism version 7.0c (Graph Pad Software, San Diego, CA, USA). Statistical significances were determined using a one-way ANOVA followed by the Dunnett post-hoc test or by the unpaired two-tailed Student's t-test method. P values ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Enzyme-assisted hydrogel formation

TCS/GA hydrogels were prepared via laccase-initiated crosslinking reaction. TCS/GA controls without laccase gelated after at least 8 h of incubation, suggesting that the gelation in absence of enzyme is governed by –SH oxidation by the atmospheric oxygen and –S-S- bonds formation.[29] Controls with non-thiolated chitosan, however, did not gelate even after 24 h of incubation. From these observations one may anticipate that in the formation of the hydrogel matrix both thiol and phenolic moieties were involved. The rationale of the gel formation (Scheme 1) lies in the laccase oxidation of phenols into reactive quinones[33] that further participate in non-enzymatic autopolymerization reactions and crosslinking with nucleophilic groups (-SH and -NH_2) from chitosan, leading to gel formation. Schiff-base formation (for -NH_2) and 1-4 Michael-type addition (for -NH_2 and -SH) are the possible reaction mechanisms between these nucleophiles and the aromatic ring of GA.[34] Disulfide formation upon oxidation of the thiol groups in TCS may also contribute to further crosslinking of the gel as suggested by the gelation of the controls without laccase.



Scheme 1. Crosslinking reactions between TCS and GA leading to hydrogel formation.

To study the nature of the crosslinking reaction between GA and TCS, batches of 7.5 mM GA and TCS with different degree of thiolation were incubated with laccase for 2, 4 or 6 h. The percentage of residual thiol and phenolic groups in the obtained hydrogels was determined in order to elucidate the mechanism of the gelation process (Fig. 1). The similar results for 7.5TCS1-10 and 7.5TCS1-5 samples in 2 h indicate that the crosslinking reaction has a similar initial rate, most probably due to the easy-available thiol groups in these specimens. However, increasing the incubation time, the phenolic groups from GA would react with the thiols groups in chitosan resulting in further crosslinking in the hydrogels containing higher amount of GA. In all cases, the content of free phenolic groups tends to decrease with the increase of the incubation time with laccase (Fig. 1A), supporting the oxidative transformation of the phenolic groups of GA into quinones. Moreover, the interdependence between the degree of thiolation of chitosan and the amount of residual phenolic groups indicates the participation of both groups in the crosslinking reaction. Hence, lowering the thiolation degree of chitosan, the coupling reaction and autopolymerization between the oxidized phenolic moieties is favored[34] in addition to the possible physical interactions between chitosan and GA.[35] Otherwise, in the sample with the highest degree of thiolation, the favored reaction is 1-4 Michael addition between the oxidized GA and the thiol groups, rather than the autopolymerization of the oxidized phenol groups. Another possible reaction mechanism between the oxidized GA and the nucleophilic $–NH_2$ groups could be the Schiff base formation.[23]

In the same way, the amount of free thiol groups in each preparation (Fig. 1B) gradually decreased upon incubation with laccase, which can be explained with their involvement in crosslinking reaction with GA or formation of S-S bonds. The latter reaction, however, is strongly pH-dependent and is more likely to occur at alkaline pHs.[36]

After the first 2 h of incubation with laccase, nearly half of the free thiol groups were reacted independently on the TCS used. After 4 h, around 40 % of the initial amount of thiol groups remained, confirming the continuous crosslinking of the gel network. With the evolution of the hydrogel network structure, after 6 h of incubation with laccase, these changes became less noticeable. In the light of these results, further studies were conducted with TCS/GA hydrogels prepared after 2 h of incubation with the enzyme. In the following experiments, even after this prolonged enzymatic reaction time, the amount of residual free thiol and phenolic groups were found to be sufficient to confer to the hydrogels the necessary for chronic wound application bioactivity.

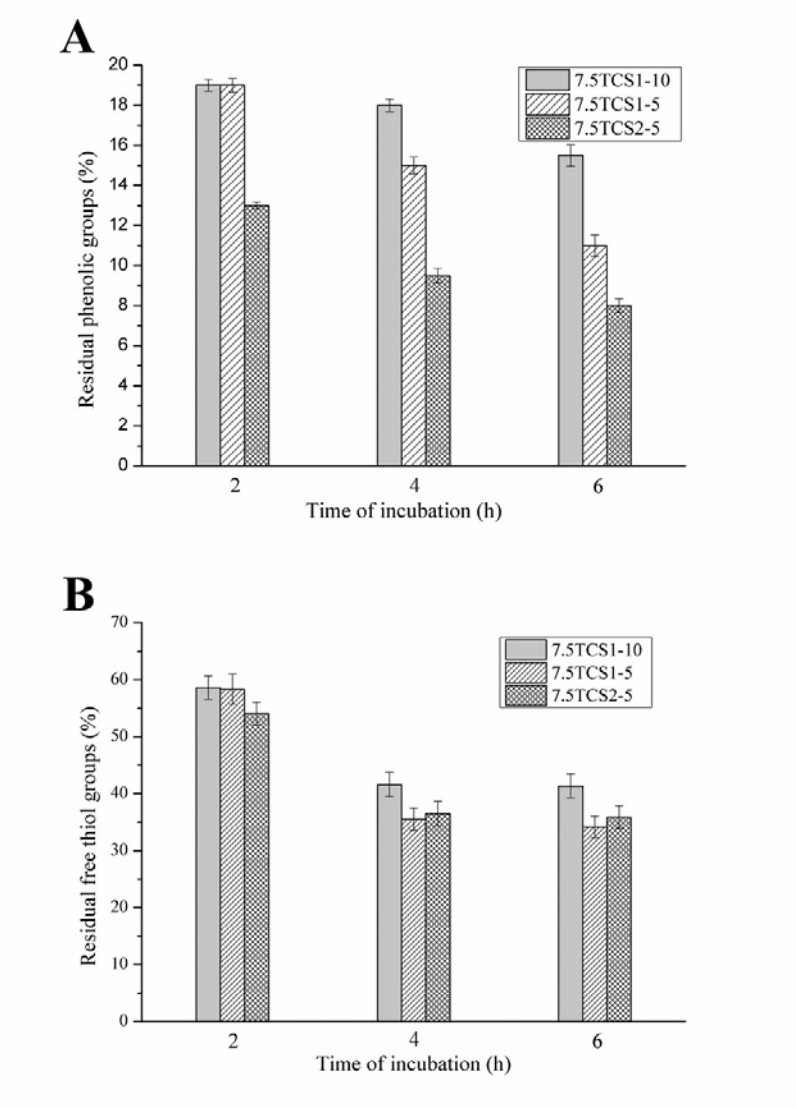


Figure 1. Percentage of residual A) phenolic and B) thiol groups in the hydrogels as a function of the incubation time with laccase. All results are reported as mean values ± SD (n=3) and their statistical significance was calculated using one-way ANOVA ($p<0.05$).

The laccase-assisted TCS/GA hydrogel formation was followed in situ by rheological measurements (Fig. 2), which showed that the gelation point (cross-section of storage (G') and loss (G'') moduli) was reached at different time depending on the amount of thiol groups in TCS. Crosslinking evolution should result in increasing values of G´, and hence mechanically

stable hydrogel network, suitable for wound dressing application.[8] The crosslinking of the TCS with the lowest degree of thiolation (7.5TCS1-10) begun with a parallel evolution of the storage and loss moduli, followed by a sudden rise and steeper increase of the storage modulus, which reached 3.8 Pa after 1200 s. For the same time the storage modulus of 7.5TCS1-5 reached a value of 30.2 Pa, and the sample 7.5TCS2-5 with the highest degree of thiolation ended at 260 Pa. These results confirm that varying the thiolation degree of chitosan the mechanical properties of the resulting hydrogels can be tuned.

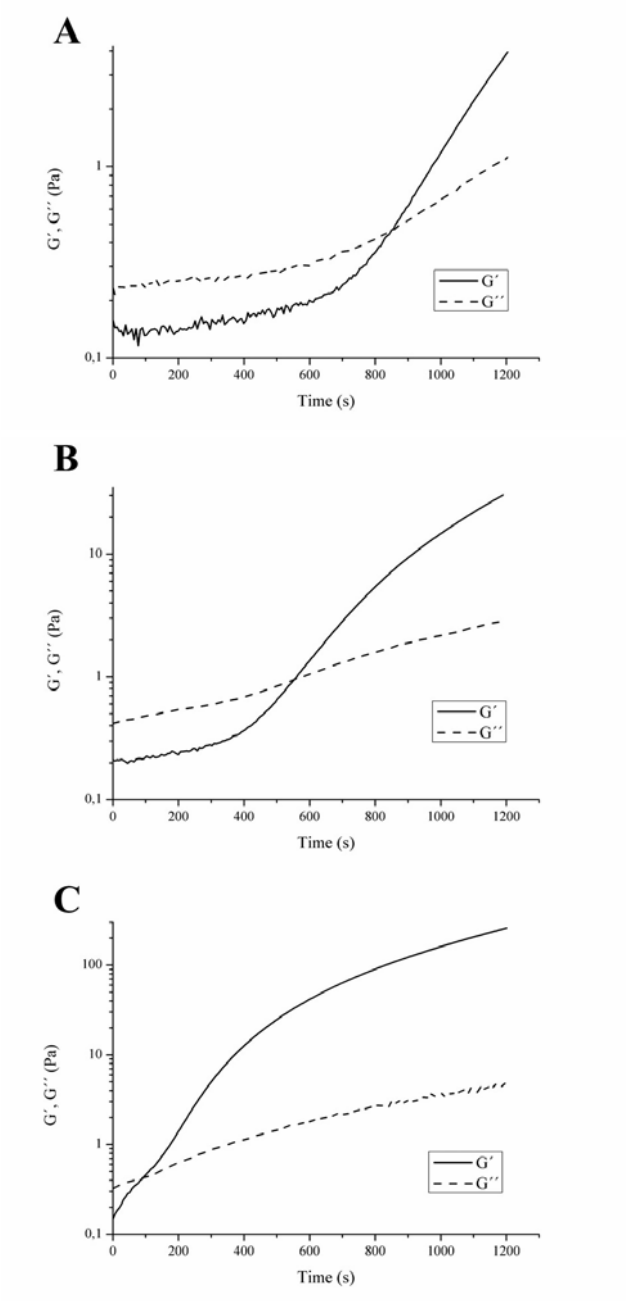


Figure 2. Rheological characterization of the enzymatically crosslinked hydrogels. Storage (G') and loss (G'') moduli vs. time at 50 ºC, laccase (2.7 U/ml), A) 7.5TCS1-10, B) 7.5TCS1-5, C) 7.5TCS2-5.

Some release of polyphenols could be expected from the crosslinked TCS/GA hydrogels due to the presence of unreacted GA. Polyphenols release can be related to the inhibition of the deleterious chronic wound enzymes, in particular MPO,[37] but may negatively affect the biocompatibility of the materials.[38] Burst release of polyphenols was observed in all groups of hydrogels during the initial swelling of the material after 1 hour of incubation in PBS, followed by a slower diffusion over 24 hours (Fig. 3). Expectedly, the highest release of polyphenols was detected for the samples with the lowest degree of thiolation, while the hydrogels with the highest degree of thiolation, where more polyphenols are crosslinked with TCS, showed the lowest release of polyphenols, which is in accordance to the proposed crosslinking mechanism. Such cumulative GA release profile supports our concept for the design of a hydrogel dressing material for chronic wound application. The highest concentration of the active compounds in the wound providing elevated antioxidant activity and inhibitory effect on MPO and collagenase is required upon the application of the dressing on the wound, in order to initiate the healing process.[55] The dressing material with enzymatically tunable degree of crosslinking, could be selected with higher or lower GA release depending on the wound complexity. After the initial burst release, corresponding to the non-covalently incorporated in the structure of the hydrogel phenolics, a minor sustained release was observed due to partial biodegradation of the hydrogel and diffusion of GA from the inner part of the matrix, maintaining the activity of the dressing for longer application periods. On the other hand, the covalently bound to the biomaterial GA, would exerts its bioactivity as long as the material is placed on the wound and is not degraded.

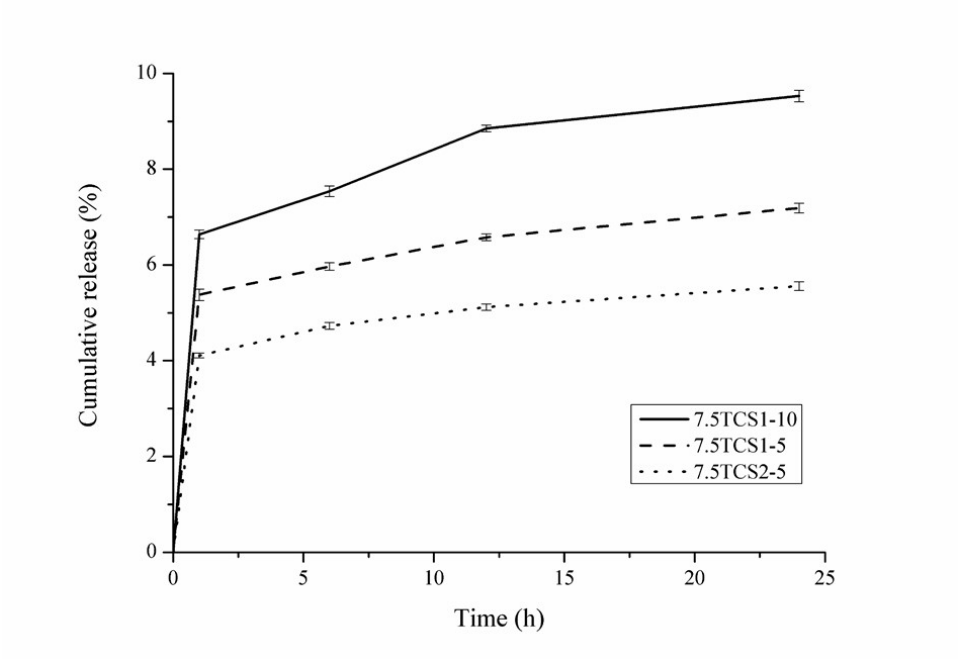


Figure 3. Release of polyphenols from hydrogels with different degree of thiolation over 24 h of incubation in PBS. All results are reported as mean values ± SD (n=3).

FTIR analysis of the hydrogels

The FTIR-spectra of the hydrogels are difficult for interpretation due to the large number of covalent and non-covalent interactions, resulting in overlapping and/or low intensity of the peaks. Nevertheless, clear differences were observed for all samples after the enzymatic crosslinking reaction. The increase of the intensity of the peak at 1407 cm^{-1} in the spectrum[39] of 7.5TCS2-5 (Fig. 4) compared to the TCS2-5 control can be assigned to CH-vibration in the -CH_2S- group resulting from Michael addition reactions between the oxidized GA and the nucleophilic -SH groups of TCS. On the other hand, the characteristic shoulder of -SH stretching

band around 2550 cm^{-1} significantly decreased in the hydrogel spectrum, confirming the involvement of these groups in different interactions.

In order to elucidate the mechanism of crosslinking, we analyzed the FTIR-spectra of L-cysteine (as a rough approximation of a molecule with both $–NH_2$ and –SH groups) enzymatically reacted with GA (inset Fig. 4). Similar to 7.5TCS2-5 decrease of the peak characteristic for –SH groups at 2550 cm^{-1}(Fig. SI.1), confirmed their involvement in Michael addition reaction. The appearance of new weak signals at 2980 cm^{-1} and 1306 cm^{-1}, can be attributed to CH_2-stretching (symmetric and asymmetric) and CH_2-wagging vibrations, respectively. These peaks most probably appeared as a result of $-CH_2S-$ formation. The newly appeared band at 1125 cm^{-1} is due to the stretching vibration of the aromatic ring of GA of the -S-Ar bond. The bands at 776, 846 and 963 cm^{-1} can be assigned to different skeletal vibrations caused by the addition of cysteine to the GA aromatic ring. These findings corroborate the initial hypothesis for Michael addition reaction between TCS and GA in the laccase-initiated crosslinking reaction.

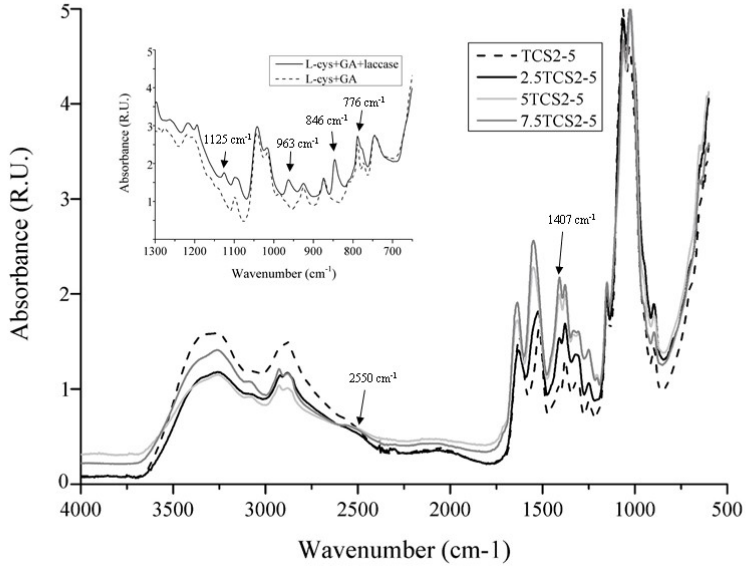


Figure 4. FTIR-spectra of hydrogel sample 7.5TCS2-5 and TCS2-5 control. The inset corresponds to L-cysteine/GA laccase-crosslinked material and its control without laccase in the range of 1300-650 cm^{-1}.

Morphology of the hydrogels

The cross-sectional SEM images (Fig. 5) of representative freeze-dried hydrogels revealed porous structures with pore size and shape depending on the amount of GA used. SEM images of the enzymatically crosslinked hydrogels containing the lowest concentration (2.5 mM) of GA produced porous structure with pores with an average diameter of 135μm. Increasing the concentration of GA resulted in decrease of the pore size, where the hydrogel 5TCS2-5 reached 105 μm and the sample with the highest concentration (7.5 mM) of GA showed the densest network with the smallest (29 μm average diameter) pore size. These gel morphologies are in line with the suggested mechanism for hydrogel formation due to crosslinking reactions between the –SH and $–NH_2$ nucleophiles from TCS and the aromatic ring of GA.

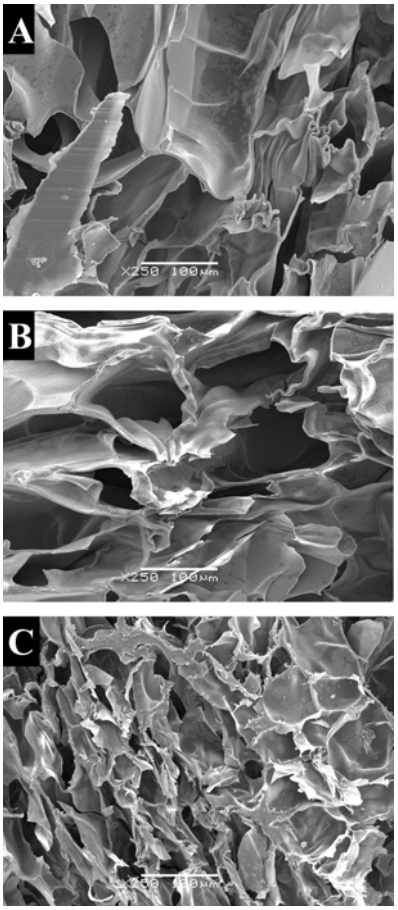


Figure 5. SEM images of lyophilized hydrogels prepared with TCS2-5 and different concentrations of GA: A) 2.5TCS2-5, B) 5TCS2-5, and C) 7.5TCS2-5, after 2 h of incubation with laccase.

The pore size of a hydrogel is a physical characteristic related with the swelling capacity of the hydrogels, i.e. greater degree of crosslinking results in smaller size of the pores and hence less swelling capacity. The crosslinking degree of the hydrogel is also directly related to its stability *in vivo* and susceptibility to degradation by wound enzymes. In addition, the pore size might have impact on drug release profiles since smaller pore sizes will have more sustained and prolonged release. In our hydrogels, GA is the active principle and is incorporated covalently in

the structure of the hydrogel, while the non-covalently reacted GA was burst released at contact of the hydrogel with liquid media.

Swelling ratio of the hydrogels

To promote healing of chronic wounds, the dressing should maintain a moist wound environment while absorbing the excessive exudates.[40,41] The amount of liquid absorbed by the hydrogel is usually a function of the degree of its crosslinking. Accordingly, the impact of factors responsible for hydrogel crosslinking, e.g. the incubation time with laccase and the concentration of GA, on the swelling capacity was evaluated (Fig. 6). Increasing the duration of the enzymatic reaction resulted in the decrease of the hydrogel swelling ratio due to the crosslinking evolution, as supported by the decrease of both phenolic and thiol groups in the material (Fig. 1). The primary function of a hydrogel is to absorb liquids, remaining mechanically stable. Therefore, the combined rheological, morphological and swelling ratio data, together with the amount of residual phenolic and thiol groups indicated that the optimum in terms of structural and functional features hydrogel was achieved upon 2 h of incubation with laccase of the formulation with the highest phenol and thiol content (7.5TCS2-5).

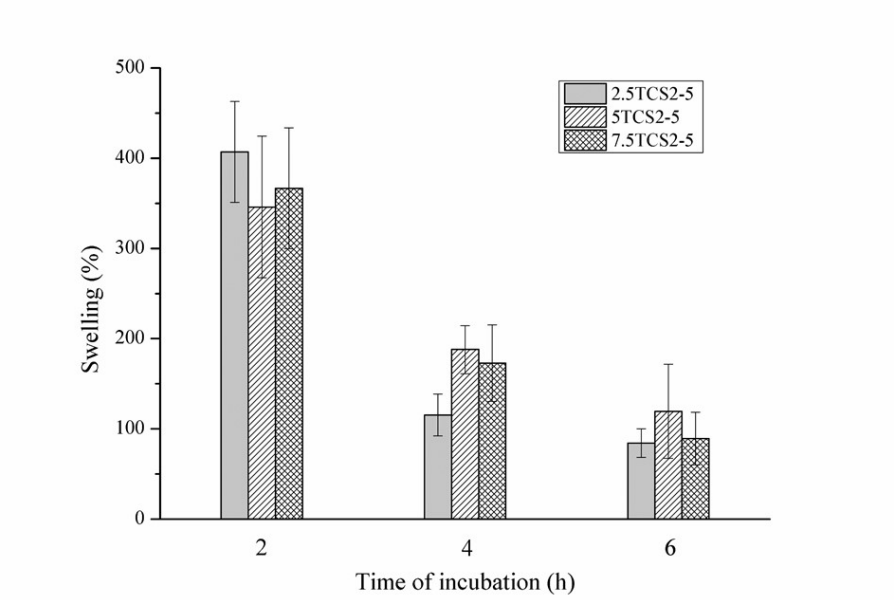


Figure 6. Swelling capacity of the hydrogels prepared with TCS2-5 and varying the concentration of GA after 2, 4 and 6 h of incubation with laccase. All results are reported as mean values ± SD (n=5) and statistical significance was calculated using one-way ANOVA comparing different incubation times ($p<0.05$).

Stability of the hydrogels

One of the required exploitation properties of chronic wound dressings is their biostability during the prolonged, up to six days, application.[20] Thus, we studied the susceptibility of our best hydrogel candidate (7.5TCS2-5) to biodegradation upon elevated lysozyme activity.[42] After six days of incubation in physiological solution (PBS) with lysozyme, 66 % of the initial weight of the gel was preserved (Fig. 7). The enzymatic digestion of the hydrogel did not show significant differences ($p<0.05$) when compared to the gel degradation in PBS without lysozyme. The structural stability of the hydrogel could be maintained due to the covalent crosslinking of the gel

matrix, limiting the access of lysozyme to the susceptible to hydrolysis β-(1–4) glycoside linkages between the monosaccharide residues.

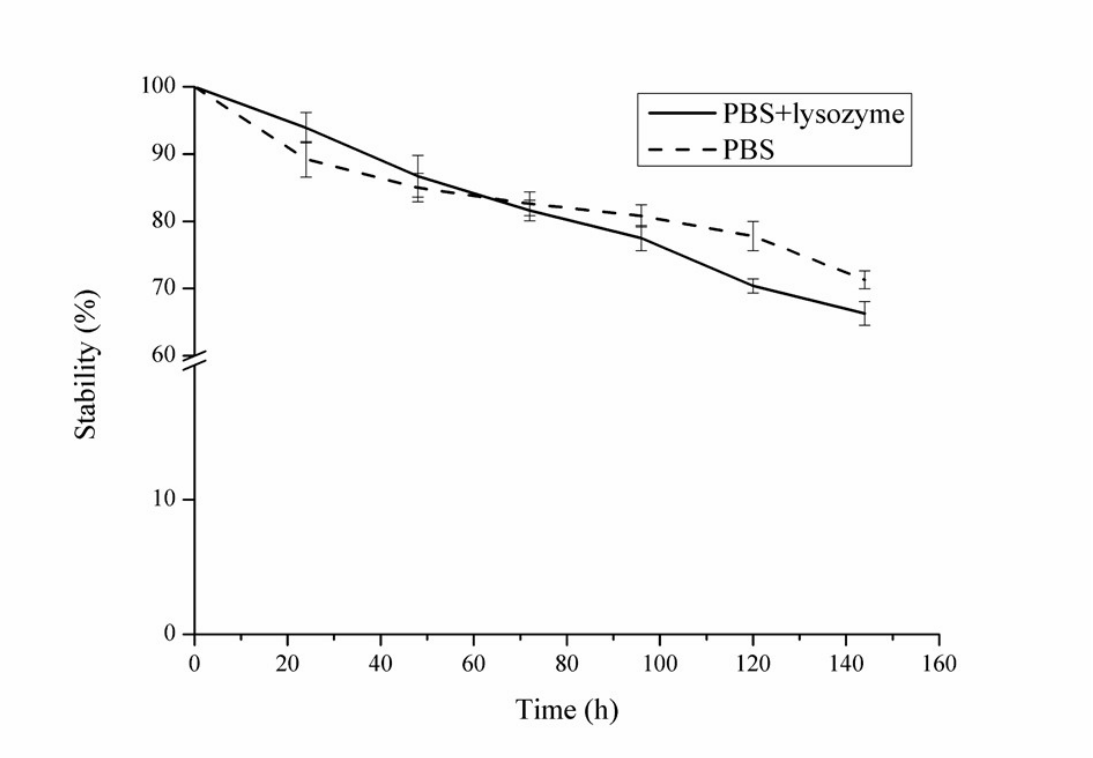


Figure 7. Stability of 7.5TCS2-5 in PBS alone and in presence of lysozyme. All results are reported as mean values ± SD (n=5). and their statistical significance was calculated using two-tailed Student´s t-test method ($p<0.05$).

Zn^{2+} binding capacity

Zn^{2+} ions are cofactors for the activity of bacterial collagenases and MMPs.[43,5] Thus, sequestering Zn^{2+} from the active center of these enzymes by the sulfhydryl groups in TCS would result in their inhibition. Herein, the QCM measurements showed that the hydrogels with the highest degree of thiolation (TCS2-5) present larger Zn^{2+} binding capacity as the GA content

is enlarged. Therefore, Zn^{2+} might be chelated by both the thiol groups of the thiolated chitosan and the crosslinked polyphenols that are capable of complexing different metal ions to a metal-phenolic network.[44]

Radical scavenging activity

Immune cells produce ROS, which in low concentrations provide defense against microorganisms. In chronic wounds, however, the predominant hypoxic and inflammatory environment increases ROS production, which leads to stimulation of proteases and inflammatory cytokines, damages ECM proteins and causes cell death.[45] The application of strong antioxidants reducing ROS to normal levels, was reported to reverse the chronicity and improve wound healing in an animal model.[45] The GA used in our study is largely explored for its antioxidant activity.[46] Additionally, the antioxidant and radical scavenging capacities of thiols have been also demonstrated.[47] Consequently, the DPPH assay revealed radical scavenging activity of all tested hydrogels as a function of the increasing concentration of GA used for their preparation (Fig. 8). The high radical scavenging activity observed might be related to the released polyphenols from the hydrogel matrix. 5TCS2-5 and 7.5TCS2-5 inhibit DPPH nearly 100 % most probably due to the media saturation with sufficient amount of polyphenols.

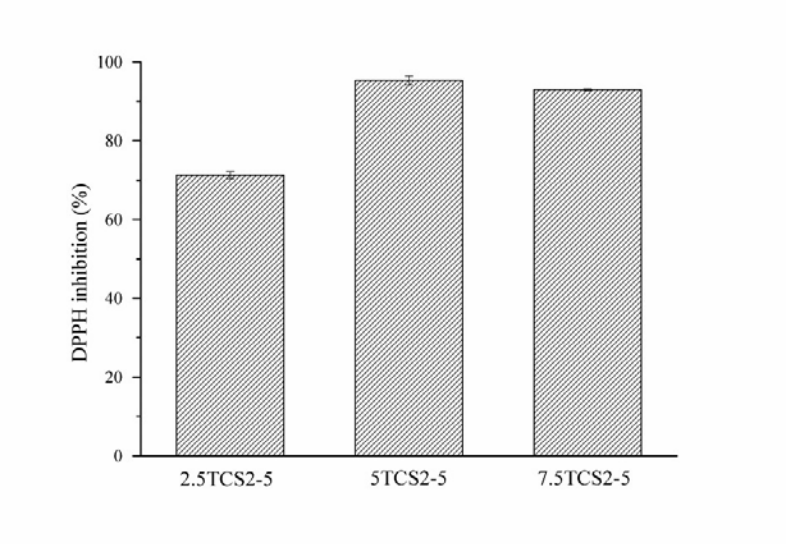


Figure 8. DPPH radical scavenging activity of hydrogels prepared with TCS2-5, different concentrations of GA and incubated for 2 h with laccase for gelation. The error bars indicate the standard deviation of three independent experiments and the statistical significance was calculated using one-way ANOVA ($p<0.05$).

Antibacterial activity of the hydrogels

The antibacterial activity of the hydrogels was assessed against three clinically relevant bacterial strains - the Gram-positive *S. aureus* and the Gram-negative *E. coli* and *P. aeruginosa*[48,49] during 24 h of incubation. The hydrogels inhibited *E. coli*, *S. aureus* and *P. aeruginosa* up to 1.7, 2.2 and 3.1 log, respectively (Fig. 9 and SI.3). As reported, chitosans with low M_w show negligible antibacterial activity.[50] However, a study from our group demonstrated that the modification of low M_w chitosan (15 kDa) with 2-iminothiolane hydrochloride improved its antibacterial properties.[51] Thiolation with Traut's reagent increases the cationic character of chitosan, enhancing its bacterial membrane disturbing capacity.[17] The porous structure of the

hydrogel may also contribute to the retention and eradication of bacteria through a 'trap and kill' mechanism.[52]

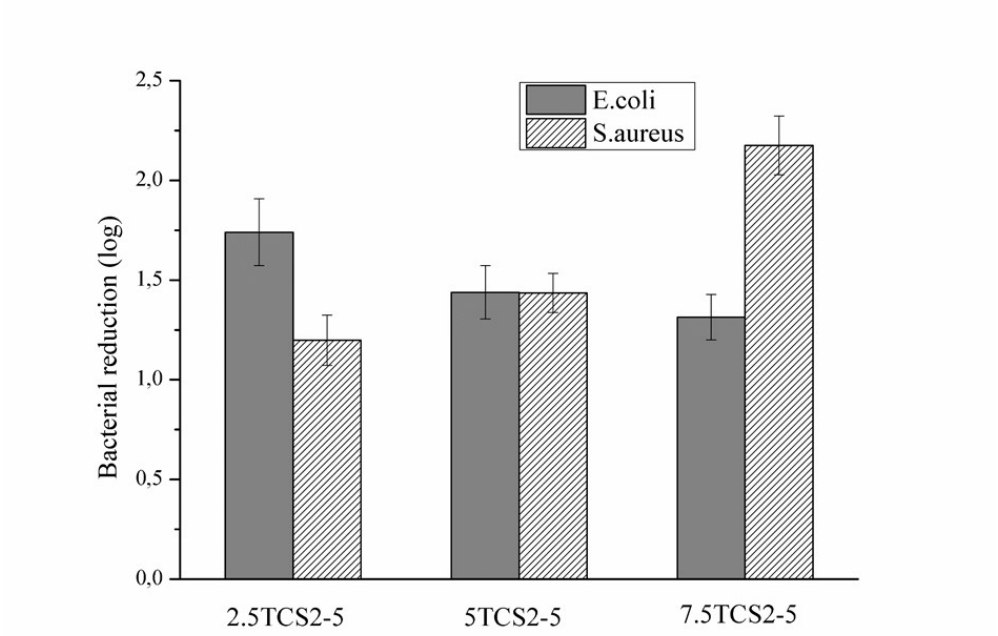


Figure 9. Antibacterial activity of the hydrogels against *S. aureus* and *E. coli*. The hydrogels were prepared with TCS2-5 and different concentrations of GA, and incubated 2 h with laccase for gelation. All results are reported as mean values ± SD (n=3) and their statistical significance was calculated using one-way ANOVA ($p<0.05$).

The increase of the amount of GA in the hydrogel preparation would have a dual positive effect on the hydrogel structure and performance. On the one hand, increasing the amount of GA would provide increased crosslinking of the hydrogel, better stability, and smaller pore size, thus acting as a structural element. On the other hand, GA provides antimicrobial effect to the hydrogels [46]

Two distinct tendencies were observed for the antimicrobial efficiency of the hydrogels against gram-positive or negative strains, also outlined in a previous work of our group.[53] The groups

providing the antimicrobial efficiency of chitosan and thiolated chitosan are respectively $-NH_3^+$ [54] and $=NH_2^+$ [51] that react with the predominantly negative charges on the surface of the bacterial cells. These groups, however, are less accessible in the structure of the crosslinked hydrogels participating also in the crosslinking reactions during hydrogel formation.[8] In Gram-positive bacteria, e.g. *S. aureus*, the interactions of the phenolic groups of GA with the lipid head groups of the lipid membrane, may disturb its integrity and subsequently lead to leakage of cellular components.[55] The minimum bacterial reduction was similar for all tested hydrogels, whereas the maximum of bacterial reduction was almost doubled for the hydrogel with the higher initial phenol content.

In Gram-negative bacteria, such as *E. coli* (Fig.9) and *P. aeruginosa* (Fig. SI.3), the antibacterial effect of the hydrogel is most probably due to the available $–NH_2$ in chitosan, which amount progressively decreases increasing the amount of GA and the possible 1,4-Michael addition or Schiff base crosslinking reactions. Consequently, the antimicrobial effects in the case of Gram-negative bacteria decreased with the increase of phenol content.

Cytotoxicity of the hydrogels

Fibroblasts in the dermis have the functions of: 1) ECM synthesizing cells, and 2) signaling cells by secreting growth factors important for the intercellular signaling and skin repair. However, fibroblasts isolated from chronic wounds have negligible replicative ability.[56] Thus, the biocompatibility of the hydrogel matrices is a crucial characteristic for their application as chronic wounds dressings. The results from cytotoxicity measurements with human foreskin fibroblast cells showed that the samples 7.5TCS1-10 and 7.5TCS1-5 were slightly cytotoxic,

while our best dressing candidate (7.5TCS2-5) showed above 90 % cells survival (Fig. 10), explained by the low release of polyphenols from this hydrogel (Fig. 3).

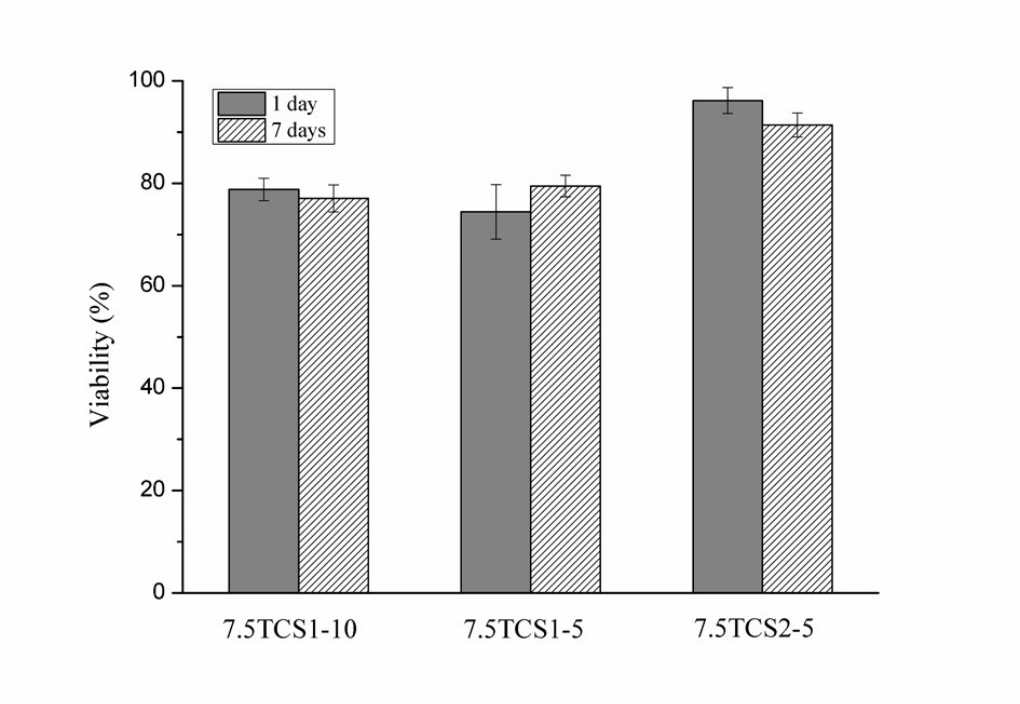


Figure 10. Viability of human skin fibroblasts after one and seven days incubation at 37 ºC with hydrogels with different degree of thiolation. All results are reported as mean values ± SD (n=3) and their statistical significance was calculated using one-way ANOVA ($p<0.05$).

In vitro **and** ***ex vivo*** **inhibition of chronic wound enzymes**

MPO inhibition

MPO originates from polymorphonuclear neutrophils, which are the first cell type activated by the host immune defense against infection in humans.[57] The primary physiological function of MPO is to neutralize invading microorganisms generating hypochlorous acid (HClO).[58] MPO can follow two different catalytic cycles, depending on the substrate availability - halogenation or peroxidase cycle. Recently, our group demonstrated that the polyphenols can act either as

HClO scavenger or can be directly involved in the peroxidase cycle as substrates, thereby inhibiting the chlorination activity of the enzyme.[59] On the other hand, thiol groups have been also reported to inhibit MPO.[60]

The MPO inhibition by hydrogels prepared with different concentrations of GA and the highest degree of chitosan thiolation (TCS2-5) was evaluated *in vitro* and *ex vivo* with venous leg ulcer exudates (Fig 11 A). Varying the concentration of GA in the hydrogels with the highest degree of thiolation (TCS2-5) resulted in up to 95 % MPO inhibition *in vitro* depending on the phenolic content. Despite of the complex composition of the clinically isolated exudates, the hydrogels were able to inhibit between 60 and 90 % of MPO in the *ex vivo* experiments. Most probably, this strong MPO inhibition was due to the burst release of phenolics during the first hour of incubation. Other contributions include the adsorption of MPO onto the hydrogel matrix and subsequent inhibition by the immobilized thiol[60] and phenolic groups.

Collagenase inhibition

The capacity of the hydrogels to inhibit collagenase was studied in terms of amount of enzyme protein removed from the wound bed and retained in the hydrogel and anti-protease activity *in vitro* and *ex vivo*. After incubation with the hydrogels, up to 23 % of the enzyme protein was retained in their structure as a function of the GA content (Fig. SI.4) This effect is correlated with the affinity of proteins to form non-covalent complexes with polyphenols and to covalently interact with quinones.[61,62] Therefore, the presence of polyphenols may contribute to the adsorption of enzyme protein, while the –SH groups could inactivate collagenase chelating the Zn^{2+} from its active center.

Clinically demonstrated inhibition by adsorption of the positively charged MMPs was achieved by the PROMOGRAN™ dressing containing negatively charged oxidized regenerated

cellulose.[63] In our study, the increased positive charge of chitosan after thiolation with Traut's reagent should repel the positively charged MMPs, whereas the negative charge of the carboxylic moiety in GA could contribute for their electrostatic binding.

The presence of MMPs in chronic wound exudates is the main factor for the excessive degradation of ECM. Their suppression is considered as an important factor in wound repair.[64,65] Thus, we studied the collagenase inhibition by the hydrogels *in vitro* and *ex vivo* (Fig. 11 B). The most significant collagenase inhibition (23 % *in vitro* and 68 % *ex vivo*) was achieved by the hydrogel with the highest phenol and thiol content (7.5TCS2-5). These results are consistent with the data for the Zn^{2+} binding capacity of the hydrogels, where 7.5TCS2-5 presented the highest Zn^{2+} binding capacity. Remarkably, a clear dependence of MMP inhibition on the GA content of the hydrogels can be extracted from the *ex vivo* studies.

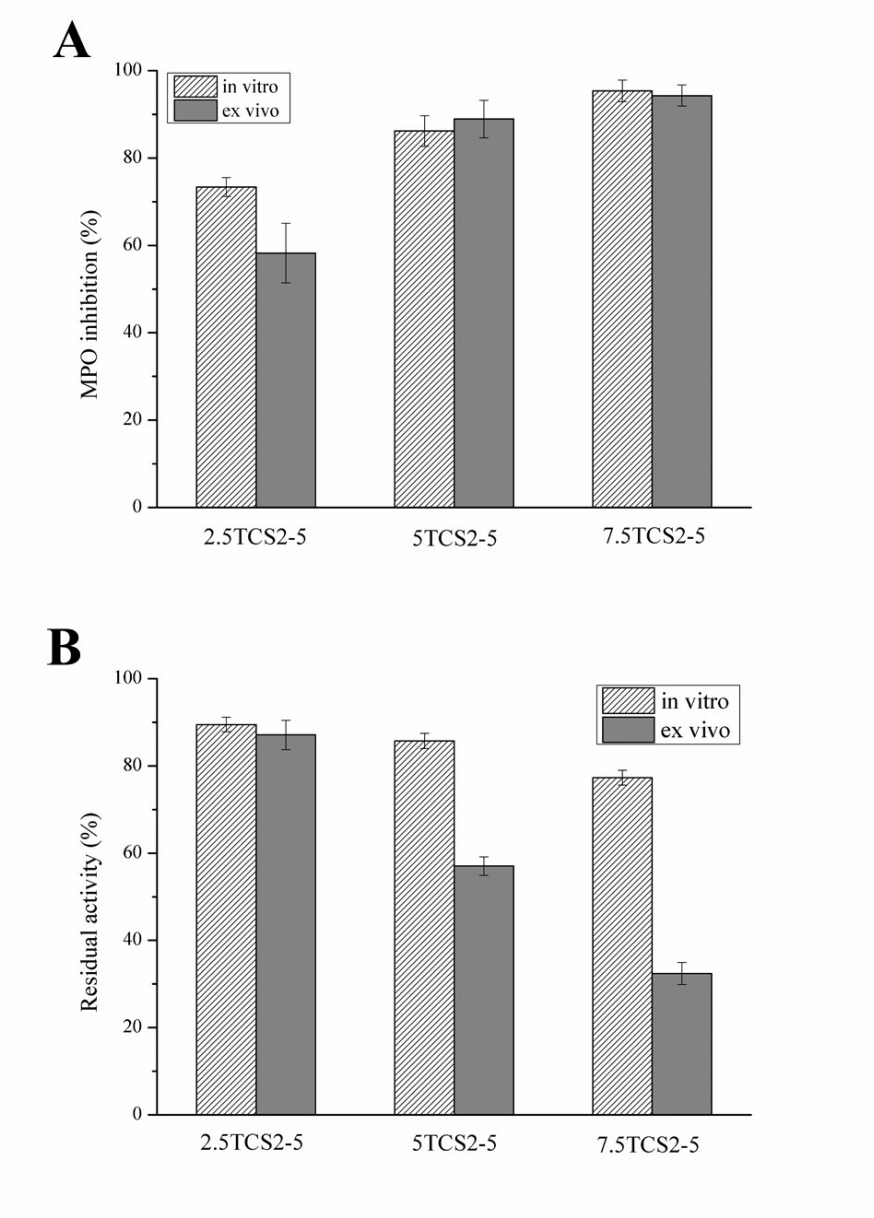


Figure 11. *In vitro and ex vivo* inhibitory efficiency of the hydrogels against A) MPO and B) MMPs. The *ex vivo* experiments were performed by using a clinical isolate, extracted from a patient with venous leg ulcer. The hydrogels were prepared from TCS2-5 and increasing concentrations of GA, incubated 2 h with laccase for gelation. All results are reported as mean values ± SD (n=3) and their statistical significance was calculated using one-way ANOVA ($p<0.05$).

CONCLUSIONS

The increasing incidence of chronic wounds calls for the development of bioactive dressing materials to address the complex oxidative and proteolytic environment of these wounds and promote their healing. Herein, we enzymatically synthesized novel biocompatible and stable in physiological conditions multifunctional hydrogels, which efficiently inhibited oxidative and proteolytic enzymes *in vitro*, scavenged ROS and eradicated clinically relevant *E. coli, P. aeruginosa* and *S. aureus*. The laccase-assisted crosslinking of GA and TCS via quinone/thiol coupling reactions allowed for building of hydrogel networks with tunable mechanical and functional properties. The hydrogels inhibited the MPO and collagenase activities by 90 and 68 %, respectively in *ex vivo* experiments with diabetic leg ulcer exudates. These novel biopolymer dressing materials illustrate an integrated strategy for chronic wound management, upgrading the widely accepted concept for maintaining the moisture environment of the wound with bioactivities to control major factors governing wound chronicity.

ASSOCIATED CONTENT

Supporting Information

QCM sensograms of Zn^{2+} and collagenase absorption in the hydrogels; Protein content in solution; Inhibition of MPO activity-dependence on laccase incubation time; FTIR-spectra of L-cysteine model compound.

AUTHOR INFORMATION

Corresponding Author

* Phone: +34 93 739 85 70 Fax: +34937398225; e-mail: tzanko.tzanov@upc.edu

Notes

The authors declare no competing financial interest

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ABBREVIATIONS

MMPs, matrix metalloproteinases; MPO, myeloperoxidase; TCS, thiolated chitosan; GA, gallic acid; TIMPs, tissue inhibitors of matrix metalloproteinases; ECM, extracellular matrix; DPPH, 2,2-diphenyl-1-pycrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; ROS, reactive oxygen species; DMEM, Dulbecco's modified eagle medium;

NB, nutrient broth; CFU, colony-forming unit; FTIR, Fourier Transform Infrared Spectroscopy; SEM, Scanning Electron Microscopy.

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