Modulating antioxidant activity and the controlled release capability of laccase mediated catechin grafting of chitosan

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ABSTRACT

Laccase from ascomycete *Myceliophthora thermophila* was used for the oxidation and grafting of catechin (CA) and chitosan (CS). The enzymatic grafting of CA into CS resulted in the improvement of natural properties of chitosan films. The successful grafting of CA to CS was supported by UV-vis and Fourier transform infrared (FT-IR) spectroscopies. The differences in the molecular weight and the degree of deacetylation of the CS affected on the enzymatic grafting rate and thus on the film properties like swelling behaviour, erosion rate, thermal property, tensile strength and breaking strain. The CS films prepared by enzymatic grafting presented high antioxidant activity compared to native CS films. The controlled release profiles of methylene blue and coomassie brilliant blue dye, having different acidity/basicity properties, loaded films were also studied with different release medium. The types of molecule loaded into films, the preparation manner of films and the release medium were decisive parameters affecting the release rate.

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1. Introduction

Biological polysaccharides are receiving a growing interest for the antioxidant and antimicrobial functionalisation of materials in food processing, treatment of fibres and medical/pharmaceutical applications [1–3]. Among the various polysaccharides, chitosan is currently the most studied for wound healing and controlled drug carrying materials with diverse forms such as films, beads, gels, scaffolds and nanoparticles. This is due to chitosan's unique properties of biodegradability, antibacterial activity, non-inflammatory property, non-toxicity, non-antigenic and high charge density [2–4]. Chitosan is composed of 2-amino-2-deoxy-D-glucose (with free amino groups) and 2-acetamido-2-deoxy-D-glucose (with acetylated amino groups) units linked through β -(1 → 4) bonds and it is obtained from the alkaline deacetylation of chitin. The

properties of chitosan depend mostly on its molecular weight and degree of deacetylation (DDA) [5,6]. The deacetylation of chitosan is a process of hydrolysis of acetamide groups in chitin and normally conducted by severe heterogeneous alkaline treatments due to the resistance of such groups imposed by the trans arrangement of the C2-C3 substituent in the sugar ring [7]. The majority of the biological properties of chitosan is related to its cationic behaviour due to protonation of the amino groups in aqueous acidic solutions. Therefore, the DDA is the most important parameter on the biological activities of chitosan like biodegradability, biocompatibility, mucoadhesion, hemostatic, adsorption enhancer, anticholesterolemic, and antioxidant [6,7].

For wound treatment materials, the reduction potential of reactive oxygen is fundamentally necessary as much as an inhibition capacity to microorganism growth. The reactive oxygen that exists in the pathogenesis of wounds and injuries causes oxidative damages to proteins, nucleic acid, lipids as well as the depletion of mitochondrial DNA from human skin [8–10]. Chitosan has been reported as an effective antimicrobial agent showing high inhibition rate against to the microorganism growth [3]. Chitosan possess an antioxidant function but its activity level is very poor due to the lack of a H-atom donor to serve as a good chain breaking antioxidant, especially in the case of medium and high molecular weights

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chitosan [11]. Flavonoids are well known natural phenolic antioxidants and are chemically applied to many substrates to improve their antioxidant activity [10,12,13]. The antioxidant capacity of flavonoids is very interesting and helpful for human health as they can act as anti-carcinogens, anti-inflammatory, and inhibitors of platelet aggregation in 'in vivo' and 'in vitro' studies [10]. In the presence of oxidative enzymes, flavonoids are the target of oxidation and form highly reactive o-quinones that can further couple with the free amino groups in chitosan [11,12,14,15].

Laccase (benzenediol: oxygen oxidoreductase: EC 1.10.3.2) is one of the most promising oxidase enzymes used for biotechnological applications in a variety of industrial fields including pulp and paper, textile, food, biosensor, biofuel and pharmaceutical industries [16–18]. Laccase is able to catalyse the oxidation of aromatic substrates like *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines, as well as some inorganic ions coupled in the presence of molecular oxygen which is reduced to water during catalysis reaction [11]. In this research, we used laccase as an oxidative catalyst to modify chitosan films by grafting a strong antioxidant, catechin. Two types of chitosan with different degrees of deacetylation were used to verify the reaction degree of coupling between phenolic flavonoids and free amino groups in chitosan with laccase mediation. Chitosan films were further characterized by measuring tensile strength, thermal behaviour, swelling rate and antioxidant activity. Additional bioactive molecules were incorporated at chitosan films and their loading and release behaviours were studied.

2. Experimental

2.1. Materials

Two different chitosan compounds were studied for film formation. One was obtained from chitin (*Paralomis granulosa* exoskeleton) as described elsewhere with minor modifications [19,20] and the other was a commercial product purchased from Sigma-Aldrich. The successive standard deacetylation procedures of chitin was performed in aqueous 50% NaOH (w/w) solution under nitrogen atmosphere, reflux and for 3 h. Catechin and other chemical reagents were purchased from Sigma Aldrich and used without any further purification. Laccase from the ascomycete *Myceliophthora thermophila* (Novozym® 51,003) was supplied by Novozymes Bagsvaerd, Denmark.

2.2. Intrinsic viscosity determination

Chitosan solutions of different concentrations were prepared in 0.2 M acetic acid and 0.1 M sodium acetate solution at pH 5 and the viscosity measurement was conducted at 30 °C. The flow times of solvent (t_0) and chitosan solutions (t) were recorded and the specific viscosity ($\eta_{sp} = t - t_0/t_0$) and the reduced viscosity ($\eta_{red} = \eta_{sp}/c$) were obtained. The intrinsic viscosity $[\eta]$ was calculated graphically by extrapolating the curve of reduced viscosity versus concentration to zero concentration. The intrinsic viscosity corresponds to the intercept with y-axis obtained [21,22].

2.3. Determination of DDA and molecular weight of chitosan from NMR spectroscopy

The DDA of chitosan was obtained from the ^1H NMR spectrum, calculated from the relationship of the acetyl group hydrogen signal and the combined area of peaks corresponding to hydrogen in carbons 2–6 [23,24]. ^1H NMR spectra were acquired on a Bruker 300 MHz NMR AvanceIII 300 spectrometer with a Ultrashield 7.05 T superconductor magnet.

11 mg of chitosan was dissolved in 0.7 mL of 1% DCl in deuterium oxide (D_2O) and the experiments were run at 70 °C. 3-(Trimethylsilyl)propionic acid- D_4 sodium salt was used as the reference and the deuteration degree was set a minimum 98% for the calibration of NMR spectra.

The molecular weight of chitosan was calculated by the Mark–Houwink–Sakurada (MHS) equation (Eq. (1)). The relationship between the molecular weight and viscosity are given below: where $[\eta]$ is the intrinsic viscosity, M is the viscosity-average molecular weight, and K and α are the constants that depend on the DDA, the solvent and temperature. When 0.2 M acetic acid/0.1 M sodium acetate solution (pH 4.4) is used as solvent and measurements are performed at 30 °C, K is $0.104 \times 10^{-3} \text{ mL/g}$ and α is 1.12 for DDA of 69% and K is $6.59 \times 10^{-3} \text{ mL/g}$ and α is 0.88 for DDA of over 90% [21].

$$[\eta] = KM^\alpha \quad (1)$$

2.4. UV–vis spectrophotometer analysis

Solutions of 2% (w/v) chitosan compounds were prepared in a 1% (v/v) acetic acid solution. The prepared solutions were further filtered with 0.8 μm pore size of filter paper under air pressure. A 0.02 M catechin solution was prepared in sodium acetate buffer (pH 5). Chitosan and catechin solutions were firstly mixed to the same volume ratio and the final concentration of the solutions were set to 1% and 0.01 M, respectively. $167 \times 10^{-7} \text{ kat}$ of laccase was added to initiate the enzymatic catalysis and the polymerisation of catechin and the grafting of poly(catechin) into chitosan molecules were sequentially occurred in one bath process. To verify the rearrangement of molecules by enzymatic catalysis, UV–vis spectra were collected at room temperature before and after addition of laccase. The spectra were collected at certain time interval until reached 6 h of incubation time. The simply mixed solution of chitosan + catechin without laccase (CS + CA) was also analysed with spectroscopy and their spectra were compared with spectra of chitosan + catechin with laccase (CS + CA + LAC).

2.5. Chitosan film formation

Chitosan films were prepared by a casting/solvent evaporation technique [25–27]. Films were formed in Petri dishes (9 cm in diameter) with 20 mL of 1% CS solutions then oven dried at 47 °C for two days. Neutralization was carried out by immersion of dried films in a 1% (v/v) NH_3 solution for two hours. They were then rinsed with distilled water several times. The modification of CS was carried out by enzymatic grafting of polymerized CA into CS molecules using laccase as described in 2.4. After termination of laccase oxidation in 24 h, the laccase and chitosan conjugates were removed from reaction solution by centrifugation as described in the literature [11]. A 20 mL of supernatant was taken and casted onto 9 cm diameter Petri dishes then oven dried at 47 °C for two days. After neutralization of dried films, they were then rinsed with distilled water until no colour released.

2.6. Characterizations of chitosan films

2.6.1. Thermal analysis

The thermal behaviour of chitosan films was assessed using a 4000 Perkin-Elmer differential scanning calorimeter (DSC). Measurements were performed in two cycles, the first cycle was heated till 200 °C to remove all the evaporable compounds, then cooled till 30 °C. In the second cycle, the sample was heated from 30 °C to 400 °C with a heating rate of 3 °C/min under nitrogen gas passing at a flow rate of 20 mL/min. Samples weighted $8 \pm 0.5 \text{ mg}$.

2.6.2. Fourier transformed infrared (FT-IR) spectroscopy analysis

A Perkin Elmer FT-IR 100 spectrophotometer was employed to record the IR spectra of samples i.e. CS, CS and catechin, and CS functionalised by enzymatic grafting of catechin. The sample solutions were prepared then lyophilised. The KBr powder was used for background scanning. Same amount of KBr was mixed with lyophilised each sample for further analysis. The spectra were collected at a resolution of 8 cm^{-1} , with 60 scans, over the range of $800\text{--}4000\text{ cm}^{-1}$ at room temperature. A background scan with no sample and no pressure was acquired before collecting the spectra of the samples.

2.6.3. Swelling and degradation behaviours

The swelling and degradation behaviours of chitosan films were studied in SIF (simulated intestine fluid without enzyme) and SGF (simulated gastric fluid without enzyme) which are commonly used for chitosan film characterization [27,28]. Films were cut $1.5\text{ cm} \times 1.5\text{ cm}$ pieces, then vacuum dried overnight before weighting (W_0). Samples were submerged in 50 mL of medium and incubated in a shaking water bath at 37°C . Samples were withdrawn from the medium at certain time intervals (0, 0.5, 1, 2, 3, 5, 8 and 24 h) and weighted (W_t) after removing the water from the surface of films with filter paper. The swelling rate was calculated following Eq. (2). The measurements were carried out in triplicate and the average values were determined

$$\text{Swelling rate (\%)} = \frac{W_t - W_0}{W_0} \times 100 \quad (2)$$

Finally, after 24 h of incubation in medium, films were taken and again vacuum dried overnight to measure the erosion rate. The erosion rate of chitosan films was obtained by measuring the weight of films before and after incubation and presented as weight loss (%) following the Eq. (3). All measurements were carried out in triplicate and their average values were determined.

$$\text{Erosion rate (\%)} = (W_0 - W_t) \times 100 \quad (3)$$

2.6.4. Tensile strength measurements

The mechanical properties of chitosan films were determined by tensile tests. A bench top tensile testing machine (ESM Mark-10), equipped with a 100N ($\pm 0.5\%$) load cell was used, with a crosshead speed set at 10 mm/min . Three samples were tested per chitosan type, and each sample was cut into strips of $0.5\text{ cm} \times 3\text{ cm}$. These tests were performed at room temperature. Tensile strength, strain at break and Young's modulus values were recorded. Measurements were done in triplicate and average values and standard deviation are presented.

2.6.5. Antioxidant activity measurement

The antioxidant properties of chitosan films were evaluated according to a modified ABTS radical cation decolourization assay method [10,29]. ABTS^{•+} free radical in solution was obtained from the reaction with potassium persulphate overnight in the dark at room temperature and then diluted with distilled water until the absorbance (Abs) reached 0.7 ± 0.02 at 734 nm. Film samples of 10 mg were immersed in 3 mL of diluted ABTS^{•+} free radical solution at room temperature and taken after 0, 30, 60 and 120 min. The absorbance was measured at 734 nm and the scavenging ability of chitosan films was evaluated following Eq. (4). In Eq. (4), the Abs_{control} is the absorbance values of diluted ABTS^{•+} free radical solution without samples and Abs_{sample} is the absorbance values of and diluted ABTS^{•+} free radical solution with chitosan films. The measurements were carried out in triplicate and the average values were determined.

$$\text{Antioxidant activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (4)$$

Table 1

DDA values obtained from ^1H NMR spectra and molecular weight determined from Mark–Houwink–Sakurada (MHS) equation of chitosan from Sigma–Aldrich and prepared by deacetylation of chitin provided by Idebio were presented.

	DDA (%)	MW (kDa)
Chitosan from Sigma–Aldrich	69	1370
chitosan obtained by deacetylation	94.7	670

2.6.6. 'In vitro' leaching behaviour

To study the leaching behaviour of chitosan and modified chitosan films, methylene blue (MB) and coomassie brilliant blue (CB) dyes were employed as model molecules. MB and CB dyes are broadly used for biology, biochemistry and medicine applications. The MB dye is a basic dye which reacts with negatively charged compounds [30,31] and sulfonic acid groups in CB dye react with positively charged amino groups by ionic interactions [32]. They have different acidity/basicity properties in solution, MB becomes positively charged and CB negatively charged. 0.025 g of dyes were mixed to 50 mL of chitosan solutions (0.5 g L^{-1} dye conc.) and stirred for 3 h. Chitosan solutions mixed with dyes were then employed to the preparation of chitosan films. The chitosan films containing MB and CB dyes were further tested to verify their leaching profiles in different buffer medium. 0.03 g was taken from each film of chitosan and modified chitosan containing dyes and then suspended in test tubes containing 50 mL of buffer medium. The incubation was performed using water bath at 37°C for 24 h under mild agitation. A 2 mL of the medium was withdrawn after certain time intervals and replaced with an equal volume of fresh medium. The release of molecules was quantitatively monitored by UV–vis spectroscopy in the range of 190–800 nm. The buffer medium for leaching profiles of dyes from chitosan films were SIF and SGF. The maximum absorbance of dyes of MB was detected at 670 nm in both SIF and SGF solution. The maximum absorbance of CB was obtained at 595 nm and 615 nm in SIF and SGF buffer solutions, respectively. The measurements were carried out in triplicate and the average values were determined.

2.7. Statistic analysis

Data was statistically analysed through *t*-test in excel and *p*-value < 0.05 was considered as statistically significant. Data are presented as mean $\pm$ standard deviation (SD) with samples number (*n*) □ 3.

3. Results and discussion

Chitosan is the N-deacetylated derivative of chitin and can be obtained by deacetylation of chitin. In this study, we prepared chitosan with high degree of deacetylation (DDA) and the properties for film formation were compared with commercial chitosan. DDA of two types of chitosan were identified by NMR spectroscopy analysis and the molecular weight (MW) was calculated by the Mark–Houwink–Sakurada (MHS) equation. Both values of DDA and MW were presented in Table 1. The MW and DDA of chitosan are affecting their physiochemical and biological properties such as crystallinity, hydrophilicity, degradation and cell response so on [6,33]. Chitosan obtained by deacetylation (CS95) has higher rate of DDA and lower value of MW, 95% and 670 kDa, respectively, comparing to commercial chitosan (CS69) with 69% and 1370 kDa, respectively.

The effects of the DDA and MW of CS, catechin (CA) presence, and laccase (LAC) catalysis on the viscosity were studied and the results obtained are presented in Table 2. Since the MW and DDA of CS are decisive factors on viscosity, the CS69 showed a higher viscosity than CS95. An increase in molecular weight and decrease

Table 2
Viscosity of chitosan with DDA 69% (CS69) and DDA 95% (CS95) before and after incubation with CA, and with CA and LAC at pH 5. The numbers in the graph present the value of viscosity changes (%).

Samples	CS69	CS69 + CA	CS69 + CA + Lac	CS95	CS95 + CA	CS95 + CA + Lac
Viscosity (cps)	863	806	744	469	461	482
Increase of viscosity (%)	Control	–7	–14	Control	–2	3

Bold values show the degree of viscosity change after incubation with CA, and with CA + LAC.

Fig. 1. Estimated schematic presentation of enzymatic oxidation of catechin by laccase and non-enzymatic grafting with chitosan.

in DDA cause low solubility and high viscosity due to entanglement of chains and low numbers of free amino groups [34]. The DDA of chitosan is directly related to the viscosity which plays an important role in the preparation of medical/pharmaceutical applications [6,7,35]. Compared to CS95, the viscosity of CS69 was influenced more by enzymatic CA grafting, showing higher rate of reduction (–14%). The addition of CA and enzymatic catalysis decreased the viscosity in all samples except in the case of the incubation of CS95 with CA + LAC (3% increase). This could be explained by an increase of electrophoretic mobility by catechin conjugating into the structure of chitosan molecules [10]. The phenolic compounds form *o*-quinones which are highly reactive electrophilic compounds formed by a radical reaction and this radical reaction can occur naturally but can be accelerated by enzymatic catalysis. The reactive *o*-quinones formed are further covalently attached to nucleophilic amine groups in chitosan through Schiff-base and/or Michael-type addition reaction [36,37]. The expected mechanism is presented in Fig. 1. In the case of CS95 incubated with CA + LAC, the high rate of CA oligomers or polymers was substituted to the site of the hydrophilic and free amine groups in CS. The phenolic polymers with high molecular weight generated by enzymatic oxidation are less soluble than phenolic monomers or oligomers [38] and might provoke a slight increase in the viscosity of CS95 solu-

tion since it has a higher number of free amino groups available to react with CA polymers than CS69.

In this study, laccase-mediated reactions of CA and CS were spectrophotometrically studied using a UV–vis spectrophotometer. The spectra were collected before and after laccase addition to each solution of CS69 and CS95 in the presence of catechin. The obtained results are presented in Fig. 2. After incubation with laccase, the mixed solutions of CS and CA turned dark brown producing an increase of band absorption in the visible area, which indicates a polymerisation or/and crosslinking [13,37,39]. A significant peak at 280 nm was detected in both CS69 + CA and CS95 + CA solutions and their intensity increased by addition of laccase. Resulting bands emerged at 380 and 450 nm and their intensity increased constantly with reaction time which is typically observed in the coupling reaction between reactive *o*-quinones and amino groups in a Michael addition reaction [37]. The rate increase of the peak intensity in the visible region with time was higher in CS95 than in CS69 solution. The difference of DDA and the number of free amino groups in CS might affect the reaction rate. The higher DDA means a higher amount of available amino groups to react with *o*-quinone in oxidised CA. Most chemical and biological reactions of CS relate to its cationic character, therefore, it should be expected that CS95, which has a higher DDA, would interact more favourably with CA polymers. At the same time, the higher molecular weight

Fig. 2. UV-vis spectroscopy analysis of the laccase catalysis reaction of CS69 and catechin (A), CS95 and catechin (B). Spectra were collected for 6 h at room temperature. The spectra was also collected in the CS69 and CS95 solutions without laccase (C).

of CS65 might supplied higher structure hindrance to react with CA polymers than CS95. In a previous research, the high affinity of CA polymers obtained from laccase catalysis for cationic materials resulted in stronger colour absorbance onto cationised fibres than non-cationized fibres [10]. As controls, CS69 + CA and CS95 + CA solutions without laccase were also studied (Fig. 2C) and resulted in no peak appearance. The spectra were collected for 6 h but there were no notable changes in peak shape or intensity in both UV and visible area.

The thermo behaviour of native CS and modified CS films by enzymatic grafting was studied using DSC. The common peak, close to 88–90 °C, attributed by evaporation of absorbed water not detected due to pre heating cycle performing (~200 °C). The thermograms showed an exothermic peak of CS films at around 280–290 °C (Fig. 3). These peaks are accounted for by the decomposition of the amide group of chitosan [40,41] and the peak appeared at higher temperature on CS 95 (291.4 ± 0.2) comparing to CS 69 (281.7 ± 0.4). This might be due to their different DDA level. The higher DDA and higher crystallinity of chitosan bring an increase

of stability to molecules and it might result in an increase of degradation temperature [41]. In Fig. 3, the temperature of degradation slightly decreases when the blending CS with CA and an enzymatic grafting of CA to CS occurred. Comparing to CS95, the CS65 films were less affected by CA presence and enzymatic grafting to CA. The thermograms of CS films can be differed from those of physical mixture and chemical bonds compositions [42].

The catechin grafting onto CS95 by laccase catalysis was evidenced by FT-IR spectra. Fig. 4 shows the CS, CS+CA and CS functionalized by enzymatic grafting of CA. The spectrum of chitosan without any modification presents a strong absorption in the region 3200–3500 cm^{-1} and attributed to NH-stretching [14]. The two specific peaks at 1670 cm^{-1} and 1580 cm^{-1} corresponding to amide I and amide II bands and a peak at 1380 cm^{-1} attributed to NH-bending of the glucosamine unit [11,13]. The characteristic peaks of CS saccharide structure were found at 1150 cm^{-1} (anti-symmetrical stretching of C–O–C bridge) and at 1080 cm^{-1} (skeletal vibration involving C–O stretching) [11,13,14]. The spectrum of CS functionalized by enzymatic grafting of CA shows the

Fig. 3. Thermal behaviours of chitosan films were analysed with DSC. The CS films were heated from 30 °C to 400 °C with a heating rate of 3 °C/min. The exothermal peaks of films of CS native (CS69, CS95), CS mixed with catechin (CS69 + CA, CS95 + CA) and CS modified by enzymatic grafting to catechin (CS69 + CA + LAC, CS95 + CA + LAC) were observed near 280–290 °C.

Fig. 4. FT-IR spectra of CS95, CS95 and catechin, and CS95 functionalized by enzymatic grafting of catechin. The spectra were showed from 1000 to 4000 cm^{-1} .

Table 3

Data of weight, thickness, equilibrium water uptake (EWu, %) and weight loss (%) of CS films in SIF solution after 24 h at 37 °C is presented as mean $\pm$ SD (n = 3).

Samples	Initial weight (mg)	Thickness (μm)	EWu (%)	Weight loss (%)
CS69	18.7 $\pm$ 1.7	47.5 $\pm$ 0.7 [*]	203.2 $\pm$ 23.4	−0.4 $\pm$ 0.4
CS69 + CA	13.7 $\pm$ 1.7 [*]	36.5 $\pm$ 0.5 [*]	201.5 $\pm$ 18.3	−3.0 $\pm$ 0.8
CS69 + CA + Lac	16.7 $\pm$ 1.3	38.2 $\pm$ 1.0 [*]	160.5 $\pm$ 8.9 [*]	−0.8 $\pm$ 1.1
CS95	10.3 $\pm$ 0.4[*]	31.5 $\pm$ 2.8	90.7 $\pm$ 19.6	0.0 $\pm$ 0.6
CS95 + CA	13.3 $\pm$ 2.0 [*]	36.0 $\pm$ 1.2	92.7 $\pm$ 2.5	−3.0 $\pm$ 1.0
CS95 + CA + Lac	15.0 $\pm$ 2.8 [*]	38.8 $\pm$ 2.0 [*]	94.2 $\pm$ 21.2	−2.5 $\pm$ 1.2

Bold values present the most different sample among all film samples (CS 95).

^{*} Significantly different was $p < 0.05$ compared with non modified chitosan films.

bands that evidence the grafting of CS and CA, catalysed by the enzyme. The two adsorption bands are corresponding the phenolic oligomers or polymers and the first peak at 1023 cm^{-1} is evidenc-

ing the extended polymerisation by showing the attribution to the C–O stretching vibration and the second peak at about 1053 cm^{-1} contributing to the phenolic acids' C–C linkages [11]. The specific

Fig. 5. Swelling behaviour data of CS films (A) in SIF and (B) SGF solutions at 37 °C under gentle agitation, collected for 24 h. SIF: Simulated Intestinal Fluid, SGF: Simulated Gastric Fluid.

Table 4

Tensile strength (UTS) and elongation of CS, CS + CA, and CS + CA + LAC films.

Samples	CS69	CS69 + CA	CS69 + CA + Lac	CS95	CS95 + CA	CS95 + CA + Lac
UTS (MPa)	57.0 ± 0.9	33.2 ± 1.6	33.2 ± 1.6	41.3 ± 2.0	33.4 ± 1.4	36.6 ± 2.4
Elongation (%)	37.2 ± 1.2	29.4 ± 6.2	20.9 ± 3.2	35.5 ± 1.2	39.4 ± 6.4	30.1 ± 7.4

Bold values present the referring values of control CSfilms for modified films.

peak proving the Schiff-base formation between free amine groups and phenolic functional groups occurring near at 1650 cm^{-1} ($\text{C}=\text{N}$ stretching mode of imines) was not clearly detected from our samples and it might be due to the reaction condition differences. The polymer products obtained by enzyme mediated processes are varied depending on the reaction conditions and Božič and his colleagues detected the products variety depending on pH condition and this peak was detected only at pH 5.5 and pH 6.5 conditions

[11,13]. However, the enzymatic synthesis was carried out at pH 5 in our study and showed a new peak appearance at 1610 cm^{-1} after reaction with catechin and laccase which might be occurred by Schiff-base formation between chitosan and polycatechin [36]. The NH stretching at $3300\text{--}3500\text{ cm}^{-1}$ become broader due to an increase of aromatic and non-aromatic stretching hydroxyl groups of phenols which result from a Michael addition reaction [11,13,14].

Fig. 6. Radical scavenging capacity of CS69, CS69 + CA, CS69 + CA + LAC, CS95, CS95 + CA and CS95 + CA + LAC films. Antioxidant capacity of films is presented as mean $\pm$ SD, $n = 4$; significantly different ($p < 0.05$) compared with non modified chitosan films.

In wound dressing materials, the efficiency of fluid absorbance is an important factor to be considered to provide and maintain a moisturizing environment over the wound bed [43]. Moreover, swelling and erosion properties are important mechanisms to be considered as critical factors for drug release from hydrophilic matrices [42]. To study CS film behaviour in gastrointestinal environments, CS films were incubated in SIF and SGF separately at 37 °C for 24 h. The DDA, molecular weight, solvent pH, types of acids, and thickness of films considerably affect the water permeability of the CS films [44,45]. The swelling capacity and erosion rate (weight loss) of each CS film in SIF for 24 h are presented in Table 3. The results showed no correspondence to the previous work of Motta de Moura and his colleagues that the lower molecular weight and higher DDA of CS have higher hydrophilicity and water vapour permeability [45]. Films prepared with CS69 showed a higher swelling rate than films prepared with CS95. CS69 and CS69 + CA films absorbed the highest volume of water (EWu near 200%). In the case the coupling reaction occurred between poly (CA)s and CS69 by enzymatic oxidation, the swelling rate decreased. Polymers produced by laccase mediation are highly insoluble [38] which might have affected on the level of water uptake. The films prepared with CS95 showed swelling rate values around 90 ~ 94% which is less than half of swelling rate of CS65 films. This can be explained by the difference of the thickness of the films. All samples tested had a same size ($1.5 \times 1.5 \text{ cm}^2$) but had different initial weight and thickness (Table 3). CS95 had noticeably less weight and thickness, therefore, promoting less space for water retention capacity.

In the erosion test, the weight loss was measured before and after incubation for 24 h, there was no significant weight loss detected, showing less than 3% of weight loss (Table 3) in all CS films. This might be due to pH dependence of CS (pK_a 6.3) and the semi-crystalline nature of CS which is derived mainly from inter and intra molecular hydrogen bonds [46]. Thus CS is hardly soluble in SIF (pH 6.8) resulting in no notable weight change after incubation for 24 h. On the contrary, CS films in SGF were rapidly dissolved and it was difficult to withdraw the films from the solution to be weighed [46,47].

Continue with the swelling and erosion test, water permeability of CS films at certain time intervals is presented in Fig. 5. The films prepared with CS69 and CS95 show a slight difference in swelling behaviour over time. As chitosan is a pH sensitive compound, swelling behaviours of chitosan films at different buffer solutions were expected. Generally, the swelling rate of chitosan films was much higher in SGF (pH 1.2) than in SIF (pH 6.8) [47]. All CS films absorbed water rapidly in the first period in SIF (Fig. 5(A)). The swelling rate of CS69 and CS69 + CA was higher than others and similar between them from two hours till 24 h of incubation time. The films prepared with CS95 showed three stages of swelling processes, they first increased, then decreased and finally became constant.

Weighing of CS films in SGF was very limited due to their high solubility and fast degradation in acidic solution (pH 1.2) (Fig. 5(B)). All CS films were decomposed and precipitated at the bottom of the test tube as a gel during the first stage of incubation (30 min) except CS69 + CA + LAC and CS95 + CA + LAC films. The only sample that could be weighed without problems after 24 h of incubation was CS69 + CA + LAC. The swelling rate of CS95 + CA + LAC in SGF reached near 1400% in 30 min, however, after 1 h, the film decomposed and precipitated as other films.

Table 4 shows the ultimate tensile strength (UTS), and the elongation (%) of CS films. CS69 film presented higher values of UTS, and elongation than CS95 film. In the literature, the DDA and MW were found to influence on the crystallinity and it can consequently affect on the mechanical properties of the chitosan films and the high UTS was obtained for high DDA CS than low DDA CS due to more active ionic sites in their molecules [45]. However, in our study, CS65 presented higher UTS than CS95 and it can be explained with the difference of thickness between CS65 and CS95. In Table 3, the CS65 was 1.5 times thicker than CS95, 47.5 and 31.5 μm , respectively and it might influence on the mechanical strength of films. The decrease of UTS was found in the CS films modified by enzymatic grafting with CA. The tensile strength of CS films was also affected by preparation conditions. The tensile strength of CS films decreases when they prepared with counter ions and the concentration of counter ion and hydroxyl groups are decisive factors for tensile strength of films [48]. It might be the reason of UTS decrease

Fig. 7. Cumulative release profiles of MB (Methylene Blue dye) loaded CS films (A) in SGF and (B) in medium SIF. SIF: Simulated Intestinal Fluid, SGF: Simulated Gastric Fluid.

in CS films prepared with CA (CS + CA) which contains hydroxyl groups in the molecules. After occurring the enzymatic grafting of CA to CS, the slightly increased than CS + CA films but still lower than native CS films. The elongation (E) was not very affected by DDA and molecular weight of chitosan showing similar values [45] but affected by enzymatic grafting. Both CS65 and CS95 presented a decrease of elongation after modification and it might be same reason with UTS decrease.

ABTS•+ decolourisation assay method was applied to evaluate the antioxidant activities of CS films obtained by LAC mediated modification with CA. CS films prepared with CA in the presence and in the absence of LAC presented relatively strong scavenging activity against ABTS cation radicals (Fig. 6). The flavonoids are well known antioxidant agents with strong inhibition of auto-oxidation and scavenging of free radicals and have been broadly applied to the surface modification of materials to transfer the

antioxidant activity [10,11,13,31,37]. The initial value of scavenging activity of CS95 + CA film was the highest but CS69 + CA film sample reached 100% of reduction of cation radical in shortest period of incubation time (in 15 min). The laccase mediated polymerisation was identified by both an electrostatic interactions and additional ester-bond formation between the hydroxyl groups on C-6 CS and carboxyl group on flavonoid [11]. When phenolic acids in flavonoids formed oligomers or polymers by enzymatic oxidation, their antioxidant activity was reduced resulting similarly to monomers. This might be due to the low solubility and steric hindrance of the poly(CA)s [11,38]. The increase of antioxidant activity was observed in the films prepared with CA over time. Božič and his colleagues reported that this possibly occurred by the reproduction of reactive hydroxyl groups in the polymerised products, the high radical stability caused by greater electron delocalization and the structural characteristics of the oligomeric and polymeric products

Fig. 8. Cumulative release profiles of CB (Coomassie Blue dye) loaded CS films (A) in SGF and (B) in medium SIF. SIF: Simulated Intestinal Fluid, SGF: Simulated Gastric Fluid.

[11]. Although much lower than other samples the native CS films also showed some antioxidant activity. Chitosan has been studied as natural antioxidant and their scavenging activity was examined as radical scavengers against 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH•), hydroxyl radical ($\cdot\text{OH}$), and superoxide radical ($\cdot\text{O}_2^-$) [49,50]. There are several suggested mechanism explaining the chitosan scavenging the radicals and one of them is the elimination of free radicals by the action of nitrogen on the C-2 position. The scavenging activity of chitosan derivatives against hydroxyl radicals may be derived from some or all the following: i) the hydroxyl groups in the polysaccharide unit can react with hydroxyl radicals by the typical H-abstraction reaction ii) OH can react with the residual free amino groups NH_2 to form stable macromolecules radicals; iii) the NH_2 groups can form ammonium groups NH_3^+ by absorbing H^+ from the solution, then they react with OH through addition reactions [37,50].

Natural biopolymers are becoming of greater interest for the controlled release of drugs. Chitosan is one of the most used natural polymers for medical and pharmaceutical applications for external and internal use, due to its properties like non-toxicity, antimicrobial activity and biocompatibility.

In this study, we have employed two different model compounds, MB and CB, having different ionic charges. The chemical structure of drugs is very important to determine their release rates since their solubility and molecular weight are decisive parameters influencing their delivery by diffusion as well as the dissolution of the delivery matrix [42]. The release action of molecules from polymeric matrices may occur due to the mechanisms of Fick's diffusion, polymer matrix swelling, polymer matrix erosion and degradation [50,51]. The effect of the system environment on the release rate was monitored at different buffer media like SGF and SIF, and firstly described for MB molecules in CS films. During the washing pro-

cess of the dried films, a high rate of colour loss occurred in MB loaded films, especially in native CS films. The colour loss might be related to the solubility of MB and the electrostatic interaction between MB and CS. MB is highly water soluble at room temperature, compared to CB, which is only slightly soluble in water therefore warming is needed to increase its solubility. With respect to the electrostatic charges, MB is positively charged in water and might have no charge attraction to bind to CS, which is either neutral or positively charged depending on pH. From the obtained results shown in Fig. 7, the CA and LAC addition to the film is an important factor influencing on loading efficiency and release rate. The dye absorbance onto the films with CA or CA + LAC was much higher than unmodified CS69 and CS95 films, showing a stronger colouration. It was also observed that the MB loading delayed the degradation time in SGF medium showing an increase of release rate till 5 h. The CS95 + CA film presented the highest release rate from the initial time, increasing to a constant value. Three other samples, CS69 + CA, CS69 + CA + LAC and CS95 + CA + LAC, showed very similar release behaviours all throughout the same period of time. On the other hand, MB was poorly released in SIF medium (pH 6.8) and continuously released without showing a constant value. This could be explained by the pH sensibility of CS which is hardly soluble at pH 6.8. Even at pH 6.8, 24% of amino groups of CS were still protonated [42] which explains the poor release of MB occurred in SIF medium. In both SGF and SIF media, the MB loaded native CS69 and CS95 films showed the lowest release rates.

The controlled release profiles of CB dye loaded CS films were also tested in SGF and SIF media and results obtained are presented in Fig. 8.

CB loaded CS films presented a stronger coloration but a lower release rate than MB loaded films. In this case, the strong electrostatic interaction between positively charged CS and negatively charged CB molecules increased the dye absorption onto films and prolonged the release time. In the reaction solution, the opposite charges of CB and CS form a strong ionic bond and thus decrease the CB release rate. Besides the charge interaction, the lower solubility and higher molecular weight of CB dye as compared to MB dye might influence on their release behaviours. In contrast to MB in CS films, CB was released faster from native CS films than CA grafted films, especially from CS95.

In SIF medium, the release rate of the dye was very low and it was difficult to differentiate the release profiles of the different CS films. The strong electrostatic interaction of CB with CS films together with the low solubility of chitosan in this medium might be responsible for this behaviour.

4. Conclusions

In the present study, laccase was used to catalyse catechin oxidation to produce reactive o-quinones and their subsequent reaction with free amino groups in chitosan through Schiff-base and Michael addition reactions. These reactions were proved by UV-vis and FT-IR spectroscopy which showed the characteristic peaks of the new functional groups. This functionalization was performed on chitosan with DDA of 69% and 95%. The swelling, erosion, thermal and mechanical properties of functionalized CS films were studied. Antioxidant activity was highly improved when enzymatic grafting of CA occurred. The controlled release study of model compounds MB and CB, showed that the DDA of CS, the type of loaded molecule, the release medium, and the enzymatic grafting of CA were important parameters that affected the release profiles of CS films.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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