Functionalization of chitosan by a free radical reaction: Characterization, antioxidant and antibacterial potential

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Abstract

Chitosan was functionalized with epigallocatechin gallate (EGCG) by a free radical-induced grafting procedure, which was carried out by a redox pair (ascorbic acid/hydrogen peroxide) as the radical initiator. The successful preparation of EGCG-grafted-chitosan was verified by spectroscopic (UV, FTIR and XPS) and thermal (DSC and TGA) analyses. The degree of grafting of phenolic compounds onto the chitosan was determined by the Folin-Ciocalteu procedure. Additionally, the biological activities (antioxidant and antibacterial) of pure EGCG, blank chitosan and EGCG-grafted-chitosan were evaluated. The spectroscopic and thermal results indicate chitosan functionalization with EGCG; the EGCG content was 25.8 mg/g of EGCG-grafted-chitosan. The antibacterial activity of the EGCG-grafted-chitosan was increased compared to pure EGCG or blank chitosan against S. aureus and Pseudomonas sp. (p<0.05). Additionally, EGCG grafted-chitosan showed higher antioxidant activity than blank chitosan. These results indicate that EGCG grafted-chitosan might be useful in active food packaging.

Keywords:
Grafting
Modified chitosan
Epigallocatechin gallate
Antibacterial
Antioxidant

1. Introduction

The antioxidant/antimicrobial molecules covalently grafted onto polymers improve their stability and functional properties as well as enlarge the field of the potential applications of polymers (Akagawa, 2008; Lee, Woo, Ahn, & Je, 2014; Zhu & Zhang, 2016).
Different synthetic strategies such as free radical grafting by the hydrogen peroxide/ascorbic acid pair redox have been developed for this purpose. This strategy, rather than another grafting reaction, allows the chemical functionalization of polymers with high reaction yields without the generation of toxic compounds (Spizziri et al., 2010; Yuan, Qiu, Su, Cao, & Jaong, 2016). These modified polymers could offer numerous advantages in active packaging technology such as decreasing the concentration of antimicrobial/antioxidant (Božič, Štrnar, & Kokol, 2013), homogeneous distribution of the active compound onto the polymeric matrix, and the increased thermal stability of the antimicrobial/antioxidant (Curcio et al., 2009). This increased thermal stability is the most important because extrusion is one of the main processes used in the preparation of packaging technology. Previous studies have reported degradation of active compounds at temperatures higher than 100 °C (Beigommamadi et al., 2016).

Chitosan is a biopolymer that presents reactive functional groups susceptible to chemical modification and has been shown to be a functional polymer to covalently graft antioxidant/antimicrobial activity onto its backbone (Choi, Nam, & Nah, 2016; Wang et al., 2016). Several forms of bioactivity such as antifungal, antimicrobial, and antioxidant activity of chitosan have been reported (Xie, Hu, Wang, & Zeng, 2014). Therefore, a synergistic effect of the antimicrobial or antioxidant compound grafted onto chitosan could exist. Aljawish, Chevalot, Jasiewski, Paris et al. (2014) grafted ferulic acid (FA) onto chitosan by laccase-catalyzed oxidation and reported an increase in antioxidant activity (20%) of the modified chitosan compared to blank chitosan. However, the FA grafted onto chitosan showed lower scavenging activity than pure FA.

Many active compounds such as citral (Jin, Wang, & Bai, 2009), eugenol, carvacrol (Chen, Shi, Neoh, & Kang, 2009), gallic acid (Hu et al., 2015), fluoroquinolone (Cirillo et al., 2014), hydroxycinnamic acid (Lee et al., 2014; Liu et al., 2015), catechins (Curcio et al., 2009; Mi, 2013; Zhu and Zhang, 2014), and oxazolidine (Parisi et al., 2014) have been covalently grafted onto polymers. However, the covalently grafted polymers do not inhibit the growth of gram-negative bacteria, particularly Pseudomonas sp., which is one of the bacteria mainly responsible for the deterioration of refrigerated products containing meat and fish and is also the group of bacteria least sensitive to the action of essential oils and bioactive components of plant origin (Tajkarimi, Ibrahim, & Cliver, 2010). However, epigallocatechin gallate (EGCG), a flavonoid and the principal active compound of green tea (Camellia sinensis), has received considerable attention and is considered a potential alternative to synthetic additives. EGCG is categorized as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration and can inhibit the growth of Pseudomonas sp. (also Stenotrophomonas maltophilia) (Gordon & Wareham, 2010), Escherichia coli (Nakayama et al., 2013), Pseudomonas aeruginosa (Zhang et al., 2014), Streptococcus mutans (Hu et al., 2013), Staphylococcus aureus (Novy, Rondevaldova, Koulimska, & Kosoksa, 2013), Vibrio cholera (Friedman, 2007), Campylobacter jejuni (Yanagawa, Yamamoto, Haru, & Shimamura, 2003) and Clostridium perfringens (Lee, Kim, Kim, & Kim, 2009).

Additionally, antioxidant activity of EGCG has been reported in vitro assays (Cvetkovic et al., 2015; Fernando & Soysa, 2015; Hu & Kitts, 2001; Potapovich & Kostyuk, 2003; Zorilla, Liang, Remondetto, & Subirade, 2011) and food systems such as pork muscle (Zhong & Shahidi, 2012), mackerel (Scomber scombrus) (Banerjee, 2006), and minced beef (Tang et al., 2006). Studies have reported that EGCG shows a higher antioxidant activity than natural compounds such as carvacrol, eugenol, and thymol as well as synthetic antioxidants such as THBQ, BHT, BHA and α-tocopherol, which are commonly employed by the food industry (Wanasundara & Shahidi, 1996).

No previous studies have attempted to evaluate the antioxidant and antibacterial activity that may be achieved simultaneously when EGCG is grafted onto a chitosan backbone. In the present work, EGCG was covalently grafted onto a chitosan backbone and characterized. Additionally, antioxidant and antimicrobial activity against two typical foodborne bacteria Staphylococcus aureus (gram-positive) and Pseudomonas sp. (gram-negative) were evaluated.

2. Materials and methods

2.1. Materials

Epigallocatechin gallate (EGCG) (94 wt%) was purchased from Teavigo®, DSM Nutritional Products (Kaiseraugst, Switzerland). Chitosan from crab shells (average MW = 121 kDa, 80% deacetylation) (Martín—Camacho et al., 2010), hydrogen peroxide (H2O2), ascorbic acid, acetic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylethen-2-carboxylic acid (Trolox), Folin Ciocălteu reagent and sodium carbonate were purchased from Sigma—Aldrich (Sigma Chemical Co., St. Louis, MO).

2.2. Synthesis of EGCG grafted-chitosan

EGCG grafted-chitosan was prepared according to the method of Curcio et al. (2009), with a slight modification. Chitosan (0.5 g) was dissolved in 50 mL of acetic acid water solution (0.4% v/v). Then, 1 mL of 1.0 M hydrogen peroxide containing 0.054 g of ascorbic acid was added. After 30 min, 0.5 g of EGCG was introduced into the reaction. The grafting reaction was maintained at 25 °C for 24 h under atmospheric conditions. Finally, the reaction was purified into Amicon ultra centrifugal filter tubes (MWCO 100,000 Da) with washes of acetic acid water solution (0.4% v/v) by centrifugation for 35 min at 3500 rpm and 25 °C (Sorvall Legend XTR Centrifuge, Eppendorf, Hamburg, Germany). EGCG grafted-chitosan was verified to be free of unreacted EGCG and any other compounds by spectrophotometric analysis after the purification step. EGCG grafted-chitosan solution was lyophilized. Blank chitosan as a control was prepared under the same conditions but in the absence of EGCG.

2.3. Structural characterization of EGCG grafted-chitosan

To verify that the EGCG was covalently grafted onto chitosan, the EGCG, EGCG grafted-chitosan and blank chitosan as a control were characterized using UV–vis, FTIR, XPS, DSC and TGA techniques.

2.3.1. UV–vis analysis

To prove the existence of a chemical bond between EGCG and chitosan, pure EGCG (0.3 mg/mL), blank chitosan (10 mg/mL) and EGCG grafted-chitosan (10 mg/mL) solutions were analyzed using a UV–vis Spectrophotometer (Varian Co. Ltd, California, USA). Acetic acid water solution (0.4% v/v) was the blank solution.

2.3.2. FTIR analysis

The EGCG, blank chitosan and EGCG grafted-chitosan were dispersed in KBr, pelletized and analyzed using a Perkin-Elmer Spectrum 2000 spectrometer (Perkin-Elmer Co., Norwalk, CT). The absorbance measurements were carried out within the 4000–400 cm−1 range, with 16 scans and a resolution of 4 cm−1.

2.3.3. X-ray photoelectron spectroscopy analysis

Surface composition of blank chitosan and EGCG grafted-chitosan was determined by X-ray photoelectron spectroscopy (XPS), using a photoelectron spectrometer PHI 5100 Perking-Elmer with an Al Kα X-ray source (1486.6 eV photons) at a constant dwell
time of 50 ms and a pass energy of 17.90 eV. The anode voltage was 15 kV, and the anode current was 10 MA (150 W). The spectra were fitted assuming a Gaussian–Lorentzian distribution for each peak, with a linear background to determine the binding energy of the various element core levels. Peak fit results were imported into a graphic software package (Origin, OriginLab Corp.) to display the modeled data.

2.3.4. Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) analysis was carried out using a differential scanning calorimeter (Perkin-Elmer 8500). Six mg of dried pure EGCG, blank chitosan or EGCG grafted-chitosan were placed inside a hermetic aluminum pan, and the pan was then sealed tightly by a hermetic aluminum lid. The thermal analyses were performed from 25 to 350 °C under a dry nitrogen atmosphere with a flow rate of 25 mL/min and a heating rate of 5 °C/min.

2.3.5. Thermogravimetric analysis

Analysis was determined according to Woranuch and Yoksan (2013) using a Pyris 1 TGA (Perkin-Elmer Ltd., USA).

2.4. Evaluation of grafting efficiency by the Folin–Ciocalteu procedure

Total EGCG content (EGCG grafted onto chitosan) was determined according to Curcio et al. (2009). The EGCG content of the chitosan was calculated by the following equation (Eq. (1)) obtained from the calibration curves of EGCG.

\[ y = 0.0036x - 0.0251 \]  

where \( x \) and \( y \) are the absorption value at 760 nm and the concentration of grafted EGCG (mg/mL), respectively.

2.5. Determination of antibacterial activity of EGCG grafted-chitosan in vitro

2.5.1. Preparation of stock solutions

Stock solutions were prepared according to the procedure of Aljawish, Chevalot, Jasniowski, Revol-Junelues et al. (2014). The pure EGCG, blank chitosan and EGCG grafted-chitosan were dissolved in acetic acid water solutions (0.4% v/v, 10 mg/mL), and then the solutions were each diluted to obtain 0.9, 1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000, 4000 and 8000 μg/mL.

2.5.2. Bacteria and culture conditions

Representative gram-positive and gram-negative bacteria (Staphylococcus aureus, ATCC 25923 and Pseudomonas sp., ATCC 13867) were obtained from the American Type Culture Collection (ATCC) and used to evaluate the antibacterial activity of blank chitosan and EGCG grafted-chitosan. These strains were propagated in liquid Mueller–Hinton broth (MHB) (21 g/L of water) at pH 7.2 and were diluted with MHB to obtain a final cell concentration of 10⁶ CFU/mL.

2.5.3. Bacterial growth inhibition (%)

Bacterial growth inhibition by pure EGCG, blank chitosan and EGCG grafted-chitosan was evaluated by spectrophotometric analysis, according to Aljawish, Chevalot, Jasniowski, Revol-Junelues et al. (2014) with a slight modification. A 96-well microplate was filled with 210 μL of bacterial strains at a cell concentration of 10⁶ CFU/mL in double Mueller–Hinton broth (2X). Then, 90 μL of each sample (pure EGCG, blank chitosan or EGCG grafted-chitosan) solution (pH 5.2) was added to the wells, yielding a pH of 6.2. Two controls were used, the first with 90 μL of sterile distilled water, and the second with 90 μL of acetic acid adjusted by 0.1 M NaOH (both controls present a final pH of 6.2). The samples were loaded in triplicate. Then, the microplates were shaken for 15 min. Bacterial growth before incubation (T₀) was determined by optical density at 630 nm according to the procedure of Sanchez-Maldonado, Schieber, and Ganzle (2011) on a microplate reader (Bio-Rad Microplate Reader 550). The blank sample consisted of sterile MHB. Then, microplates were incubated and shaken at 37 °C for 24 h. After incubation, the optical density of each well was determined (Tᵢ). Bacterial growth inhibition of chitosan and EGCG-grafted chitosan were reported as inhibition percentage (]% by the following equation (Eq. (2)), according to Cueva et al. (2010):

\[ \text{Inhibition (\%)} = 1 - \frac{(T_{\text{sample}} - T_{\text{blank}})}{(T_{\text{growth}} - T_{\text{blank}})} \times 100 \]  

where \( T_{\text{sample}} \) and \( T_{\text{blank}} \) are the optical densities at 630 nm of the strain growth in the presence of pure EGCG, blank chitosan or EGCG-grafted chitosan before (T₀) and after (Tᵢ) incubation, respectively; \( T_{\text{blank}} \) corresponded to the MHB medium with pure EGCG, blank chitosan or EGCG-grafted chitosan before and after incubation, respectively; and \( T_{\text{growth}} \) correspond to the strain growth in the presence of MHB medium (positive control) before and after incubation, respectively.

2.5.4. Minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of pure EGCG, blank chitosan and EGCG-grafted chitosan were tested according to the procedure of Chandrasekar and Venkatesalu (2004). Using the same 96-well microplates prepared for bacterial growth inhibition, differences in absorbance between (Tᵢ) and after (Tᵢ) incubation, respectively: Tᵢblank and Tᵢsample lower than 0.02 implied no bacterial growth. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of pure EGCG, blank chitosan or EGCG-grafted chitosan at which bacterial growth was not detected (Becerril, Gómez-Lus, Goni, López, & Nerín, 2007).

2.5.5. Minimum bactericidal concentrations

The minimum bactericidal concentration (MBC) of pure EGCG was tested according to the procedure of Chen et al. (2009). TriPLICATE tests were performed with each strain with pure EGCG, blank chitosan and EGCG-grafted chitosan.

2.6. Determination of antioxidant activity of EGCG grafted-chitosan in vitro

2.6.1. DPPH radical scavenging assay

A 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH)-scavenging assay was performed according to the procedure of Xie et al. (2014), with a slight modification. Pure EGCG, blank chitosan or EGCG grafted-chitosan were homogeneously dispersed in an acetic acid water solution (0.4% v/v) at a series of concentrations (250, 500, 1000, 2000, 3000, 4000, and 5000 μg/mL). A 50 μL sample was added to each well of the 96-well microplate and mixed with 200 μL of methanolic DPPH solution (0.4 mM). The reactions were carried out in the dark at room temperature for 60 min. Absorbance was measured at 517 nm using a microplate reader (Bio-Rad Microplate Reader 550). The scavenging activities of pure EGCG, blank chitosan and EGCG grafted-chitosan were measured as the decrease in absorbance of the DPPH, and the scavenging activities were expressed as the percent inhibition of DPPH radicals calculated according to the following equation (Eq. (3)).

\[ \text{Inhibition (\%)} = \left( \frac{1 - \frac{Abs_1 - Abs_2}{Abs_0}} \right) \times 100 \]  

where \( Abs_0 \) is the absorbance of the control (water), \( Abs_1 \) is the absorbance of the sample and \( Abs_2 \) is the absorbance of the sample only (methanol instead of DPPH solution).
2.6.2. ABTS radical scavenging assay

Preformed radicals of ABTS were generated by the oxidation of ABTS (7.0 mM) with potassium persulfate (4.95 mM K2S2O8) for 12 h in the dark at room temperature. After generation, ABTS was diluted with distilled water to an appropriate concentration (absorbance of 0.70 ± 0.02 at 734 nm), affording the working solution. The absorbance of a mixture of a 20 μL of sample (250–5000 μg/mL) and a 200 μL of working solution was measured at 734 nm after reaction for 1 h. The scavenging activity of pure EGCG, blank chitosan and EGCG grafted-chitosan was measured as the decrease in absorbance of the ABTS and was expressed as percent inhibition of ABTS radicals calculated according to the following equation (Eq. (4))

\[
\text{Inhibition(\%)} = \left( 1 - \frac{\text{Abs}_0 - \text{Abs}_2}{\text{Abs}_0} \right) \times 100
\]  

where \( \text{Abs}_0 \) is the absorbance of the control (water instead of sample), \( \text{Abs}_1 \) is the absorbance of the sample, and \( \text{Abs}_2 \) is the absorbance of the sample only (water instead of ABTS).

2.7. Statistical analysis

Data from thermal analysis (DSC and TGA) were expressed as the mean ± standard deviation (SD) and subjected to analysis of variance (ANOVA). Statistical analyses of antibacterial and antioxidant activities were performed by a two-way analysis of variance on JMP 10 (SAS Institute Inc, Cary, NC, USA) for Windows. In both cases, the differences between means were evaluated by Tukey’s test at the significance level of 5%.

3. Results and discussion

3.1. Structural characterization of EGCG grafted-chitosan

3.1.1. UV-vis analysis

Fig. 1 compares the UV spectra of pure EGCG, blank chitosan and EGCG grafted-chitosan in acetic acid water solution (0.4% v/v). The analysis was recorded using acetic acid water solution (0.4% v/v) as a blank to remove the interference of the solvent. Pure EGCG showed an absorption peak at 254 nm. Blank chitosan showed an absorption peak at 217 nm, and the UV-vis absorption peak of EGCG grafted-chitosan was shifted toward a longer wavelength (229 nm) compared with pure EGCG. This behavior indicates a reduction in energy for the \( \pi \rightarrow \pi^* \) electron transition during light absorption (Liu, Lu, Kan, Tang, & Jin, 2013) due to the extension of the conjugation between the reaction groups of chitosan and the EGCG aromatic ring (Aljawish et al., 2012). Additionally, in EGCG grafted-chitosan, the absorption peak of pure EGCG (254 nm) does not appear.

3.1.2. FT-IR analysis

Fig. 2 shows pure EGCG, blank chitosan and EGCG grafted-chitosan FTIR spectra. The pure EGCG spectrum showed bands at 1606 and 1149 cm\(^{-1}\), which are related to C=O (aromatic ring) and C=O bonds (pyranose heterocyclic chain). Additionally, the EGCG structure possesses eight –OH groups, which are shown in a band at 3500–3200 cm\(^{-1}\) due to the vibration of this functional group (Zhong, Ma, & Shahidi, 2012). In the blank chitosan spectrum, a band at 1600 cm\(^{-1}\) represents the N–H bending of the primary amine. C–N stretching (amide III), N–H bending (amide II) and C=O (amide I) stretching of the residual N-acetyl groups were represented by bands at 1320, 1420 and 1650 cm\(^{-1}\), respectively (Fig. 2). Additionally, a band at 3600–3200 cm\(^{-1}\) represents the vibration of the –OH groups (Hu et al., 2015; Ryu, Hong, & Lee, 2015).

The EGCG grafted-chitosan spectrum presented differences compared to blank chitosan. Bands within the 3200–3550 cm\(^{-1}\) range appeared broader as a consequence of the high amount of free and H-bonded –OH groups (Božić, Gorgievka, & Kokol, 2012). Additionally, bands became broader than blank chitosan at 1550 and 1400 cm\(^{-1}\), although a decreased band intensity of N–H of the primary amine at 1550 cm\(^{-1}\) as well as an increased band intensity of N–H bending (amide II) and C=O (amide I) was observed. According to Muzzarelli, Litarru, Muzzarelli, and Tosi (2003), this behavior could be due to the presence of an aromatic ring (C=C stretching), in this case due to EGCG. Additionally, this spectrum showed two new bands at 1066 cm\(^{-1}\) and 2132 cm\(^{-1}\) which are typical of ether and C=N bands. These bonds could be indicative of the graft that could occur at the –OH and –NH2 groups of chitosan. Curcio et al. (2009) indicated that alpha-methylene (CH\(_2\)) is a possible insertion of flavonoids onto chitosan. This binding can be observed in this study because the EGCG grafted-chitosan spectrum shows a decreased band intensity at 2926–2878 cm\(^{-1}\).

3.1.3. XPS analysis

The N 1s core-level spectra of blank chitosan and EGCG grafted-chitosan are shown in Fig. 3. The N 1s core-level spectrum of blank chitosan shows three peaks at 399.3, 400.3 and 401.2 eV, which are characteristic of N–H, –O=C–N and positively charged nitrogen (N\(^+\)). In the EGCG grafted-chitosan N 1s core-level spectrum, a new peak appears at 398.3 eV, which according to Chen et al. (2009), indicates –N=C=N– binding. This result could indicate that the EGCG is linked with the –NH2 groups of chitosan.

3.1.4. DSC analysis

The DSC thermograms of pure EGCG, blank chitosan and EGCG grafted-chitosan are shown in Fig. 4a. Calorimetric analysis of pure EGCG shows an endothermic peak at 123.8 °C ± 2.33. This transition indicates the conversion of catechins from green tea into the corresponding epimers (Chen, Zhu, Tsang, & Huang, 2001; Li, Taylor, Ferruzzi, & Mauer, 2012). The second endotherm peak at 225 °C ± 3.67 corresponds to the melting temperature of pure EGCG (product data sheet, DSM Nutritional Products). The third transition at 230.9 °C ± 0.99 could be due to decarboxylation and polymerization reactions of EGCG (Brennan, Brennan, Derbyshire, & Tiwari, 2011). The blank chitosan thermogram exhibited 2 transitions, endothermic and exothermic peaks at 101.2 and 309.6 °C, respectively, which have been assigned to evaporation of bound water from the polymer chains and a crosslinking reaction between the degradation products of chitosan (Woranuch & Yoksan, 2013). The EGCG grafted-chitosan thermogram shows the same 2 transitions as blank chitosan as well as endothermic and exothermic peaks at 109.4 and 291.6 °C, respectively. A second transition appears at a temperature lower than the second transition in blank chitosan (p < 0.05), possibly indicating that EGCG grafted-chitosan shows a lower decomposition temperature, which could be due to a reduction in crystallinity because of the presence of EGCG. Jin et al. (2009) describe a decrease in the crystallinity of chitosan modified by citral compared to blank chitosan, this was attributed to the deformation of the strong hydrogen bond in the chitosan backbone with the substitution of citral group on the N atoms of chitosan. Additionally, the EGCG grafted-chitosan thermogram shows the disappearance of the melting enthalpy of EGCG is observed, which indicates the binding of chitosan with EGCG (Lee et al., 2014).

3.1.5. TGA analysis

TGA was used to study the thermal stability of the samples. Fig. 4b shows the mass loss of pure EGCG, blank chitosan and EGCG grafted-chitosan as a function of temperature. The thermogram of pure EGCG presents a two-step mass loss, which is attributed to the loss of water (T\(_{\text{max}}\) at 65 °C) and degradation (T\(_{\text{max}}\) at 254 °C) (Moreno-Vásquez et al., 2016). Blank chitosan also exhibited a two-step mass loss. These stages are attributed to loss of water (T\(_{\text{max}}\)
Fig. 1. UV–vis spectra: pure EGCG, blank chitosan, and EGCG grafted-chitosan.

Fig. 2. FT-IR spectra: pure EGCG, blank chitosan, and EGCG grafted-chitosan.
42 °C and thermal degradation of the polymer ($T_{\text{max}}$ 302 °C), which is a complex process and includes dehydration of the saccharide rings, depolymerization and decomposition of the acetylated and deacetylated units of the polymer (Kumar, Deepak, Kumari, & Dutta, 2016; Liu, Wen, Lu, Kan, & Jin, 2014).

The EGCG grafted-chitosan thermogram showed a three-step mass loss. The first stage ($T_{\text{max}}$ 34 °C) is due to the loss of adsorbed and bound water (Zhu & Zhang, 2014). The second stage ($T_{\text{max}}$ 172 °C) is a preliminary decomposition of modified polymers (Jiao et al., 2011), which could be attributed to the degradation of chitosan depolymerization products. According to previous studies, polymers such as starch undergo free radical attack (ascorbic acid/H₂O₂, 0.025 M) and can be depolymerized (Farahnaky, Gray, Mitchell, & Hill, 2003). The third stage ($T_{\text{max}}$ 294 °C) suggests the thermal decomposition of material that is lower than blank chitosan ($p < 0.05$), possibility due to a decreased crystallinity in chitosan because of the presence of EGCG in the polymer chains. However, this decreased temperature is lower than previously reported. Woranuch and Yoksan (2013) describe a decrease of 30 °C in the temperature degradation of chitosan modified by grafted ferulic acid compared to blank chitosan, which could indicate the possible effect of the antioxidant on EGCG, which inhibits the thermal degradation of chitosan. This observation is in agreement with De Dicastillo, del Mar Castro-López, López-Vilarino, and González-Rodríguez (2013), who reported an improvement in thermal stability of polypropylene films by the immobilization of green tea extract (the main active compound of which is EGCG). This information is important because one of the potential applications of polymers modified by active compounds is active packaging technology (Woranuch, Yoksan, & Akashi, 2015).

3.2. Evaluation of grafting efficiency by the Folin–Ciocalteu procedure

Control experiments were also performed with blank chitosan, and no EGCG was detected. The results indicated 28.7 mg EGCG/g of dry chitosan, and this concentration is higher than that observed in previous studies (Curcio et al., 2009; Hu et al., 2015; Lee et al., 2014; Liu et al., 2013; Puoci et al., 2008; Spizzirri et al., 2010). The difference could be due to the modification of the reaction. According to previous studies, many factors such as pH and temperature as well as polymer molecular weight and active compound concentration influence grafting efficiency (Božič et al., 2012; Lee et al., 2014). In this study, we employed low molecular-weight chitosan, which is easier to dissolve than high molecular-weight chitosan due to a decreased number of hydrogen bonds. Therefore, the interaction between active compounds and polymers could increase (Aytök, Morimura, & Kida, 2011). Additionally, the reaction pH is important. At a pH of 4.5 (or lower), the amine groups of chitosan, being fully protonated, are not nucleophilic and therefore cannot react with electrophiles (such as EGCG), which could produce a lower active compound concentration grafted onto chitosan (Božič et al., 2012). In this study, the pH of the reaction was 5.2.

3.3. Determination of antibacterial activity of EGCG grafted-chitosan in vitro

3.3.1. Bacterial growth inhibition (%)

According to spectrophotometric analysis, EGCG grafted-chitosan presented a greater growth inhibition than pure EGCG or blank chitosan against S. aureus and Pseudomonas sp. ($p < 0.05$) (Fig. 5). Additionally, the inhibition of growth was concentration dependent ($p < 0.05$).

These results differ from those reported for gallic and caffeic acids grafted onto chitosan films, which showed less antibacterial activity than control films (blank chitosan) (Božič et al., 2012). This lack of activity might be because the available free amino groups (responsible for the antibacterial activity of chitosan) were reduced by modification. However, in this study, the reduction of available free —NH₂ could have been offset by —OH groups that are available in EGCG (responsible for the antibacterial activity of EGCG). The possible mechanism of antibacterial action by EGCG grafted-chitosan could be due to the chitosan attack mainly on the outer surface of the bacteria where the positively charged —NH₃⁺ group interacts with negatively charged bacterial cell membranes, leading to the leakage of proteins and constituents of the bacteria to cause cell agglutination (Shahidi, Arachchi, & Jeon, 1999). Mechanisms of action for catechins are the formation of cell aggregates. Nakayama et al. (2013) reported that the interaction between —OH groups form catechins and proteins or enzymes from the cell wall, and this interaction interferes with the synthesis of the cell wall and membrane. Based on the above, EGCG grafted-chitosan could inhibit bacterial growth through an interaction between unreacted —NH₂ and —OH groups and the cell membranes. Additionally, EGCG grafted-chitosan presented a greater growth inhibition against S. aureus than Pseudomonas sp. due to differences in cellular walls composition (Nakayama et al., 2013).
Fig. 4. DSC thermograms (endo up) (a) and TGA and derivative thermogravimetric (DTG) thermograms (b) of pure EGCG, blank chitosan, and EGCG grafted-chitosan.

Table 1
Minimum inhibitory concentration MIC (µg/mL) and minimum bactericidal concentration MBC (µg/mL) of pure EGCG, blank chitosan and EGCG grafted-chitosan against the tested bacteria.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Pure EGCG</th>
<th>Blank Chitosan</th>
<th>EGCG grafted-chitosan</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>S. aureus</td>
<td>62.5 ± 0.21a</td>
<td>62.5 ± 0.24a</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp.</td>
<td>500 ± 0.01b</td>
<td>125 ± 0.81b</td>
</tr>
<tr>
<td>MBC</td>
<td>S. aureus</td>
<td>125 ± 0.00a</td>
<td>125 ± 0.00a</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp.</td>
<td>1000 ± 0.01a</td>
<td>250 ± 0.51b</td>
</tr>
</tbody>
</table>

Note: The data are represented as mean values ± standard deviation (n = 6). Different letters within the same row indicate statistically significant differences (p < 0.05).

3.3.2. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The MIC and MBC values of pure EGCG, blank chitosan and EGCG grafted-chitosan against S. aureus and Pseudomonas sp. are summarized in Table 1.

According to the MIC and MCB values, EGCG grafted-chitosan exhibited higher antibacterial activity than pure EGCG or blank chitosan (p < 0.05). MICs and MBCs obtained in this study are lower than previously reported for polymers modified with active natural compounds (in a concentration range of 6–40 µg/mL) against...
S. aureus and Pseudomonas sp. strains (Chen et al., 2009; Lee et al., 2014; Sousa, Guebitz, & Kokol, 2009).

3.4. Determination of the antioxidant activity of EGCG grafted-chitosan in vitro

3.4.1. DPPH radical scavenging assay

Fig. 6a shows the inhibition percentages of the DPPH radical by pure EGCG, blank chitosan and EGCG grafted-chitosan. In this test, an antioxidant molecule reduces the DPPH radical to diphenylpicrylhydrazine (a yellow-colored compound), and the extent of discoloration will depend on the hydrogen-donating ability of the antioxidant molecule. The results indicate that pure EGCG shows $100 \pm 0.53\%$ of inhibition of DPPH radicals at 250 $\mu$g/mL. However, blank chitosan did not show inhibition. This behavior has been reported previously. The absence of activity may be partly due to the inhibition of radical scavenging because of inter- and intra-molecular hydrogen links, especially for high-molecular-weight chitosan (Aljawish, Chevalot, Jasniewski, Paris et al., 2014).

EGCG grafted-chitosan activity was dependent on concentration ($p < 0.05$) and shows an $80 \pm 0.38\%$ of inhibition of DPPH radicals at 5000 $\mu$g/mL. These results indicate that inhibition of DPPH radicals could be attributed to the binding of EGCG and chitosan backbone. The inhibition of DPPH radicals obtained in the present study is
higher than reported previously by other reports such as Puoci et al. (2008), who evaluated the effect of ferulic acid grafted onto methacrylic acid on the inhibition of DPPH radicals. However, a 30 and 80% inhibition of DPPH radicals by 8000 μg/ml blank chitosan and ferulic acid grafted onto methacrylate was reported.

### 3.4.2. ABTS radical scavenging assay

Fig. 6b shows the inhibition of ABTS radical cation decolorization by blank chitosan and EGCG grafted-chitosan. In this test, an antioxidant molecule reduces the ABTS radical to 2,2’-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (a green-colored compound), and the extent of discoloration will depend on the
hydrogen-donating ability. In the present study, the ability of EGGC grafted-chitosan was compared to pure EGGC and blank chitosan. According to the results, pure EGGC shows 100±0% inhibition of ABTS radicals at 250 µg/mL. Blank chitosan did not show inhibition, which are agree with DPPH assay. However, EGGC grafted-chitosan activity was dependent on concentration (p < 0.05), showing 100% inhibition of ABTS radicals at 4000 µg/mL, which could be due to the introduction of the H-atom donating group from EGGC being grafted onto the chitosan backbone. EGGC grafted-chitosan presents a higher inhibition of ABTS radicals than previous studies, which reported an antioxidant activity of the modified polymer in the 500–6000 µg/mL concentration range (Aljawish, Chevalot, Jasniowski, Paris et al., 2014; Aljawish, Chevalot, Jasniowski, Revol-Junelles et al., 2014; Božič et al., 2013).

4. Conclusions
Chitosan modified by EGGC was successfully synthesized using a water-soluble radical initiator system (ascorbic acid/hydrogen peroxide), with some modifications. The covalent bond was confirmed by spectroscopy (UV–vis and FTIR) and thermal analysis (DSC and TGA). Additionally, thermal analysis showed the thermal stability of modified chitosan at temperatures higher than 150 °C. These results clearly showed that the bond between chitosan and EGGC was an effective method to improve chitosan and EGGC biological properties (antibacterial and antioxidant activities). Thus, our results showed that the modification of the reaction provided an efficient method for the modification of chitosan which resulted in an increase in the concentration of linked EGGC. These results indicate that EGGC grafted-chitosan might show potential in active food packaging.

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