Laccase-mediated Functionalization of Chitosan with 4-hexyloxyphenol Enhances Antioxidant and Hydrophobic Properties of Copolymer

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ABSTRACT

An effective method to functionalize chitosan with 4-hexyloxyphenol (HP) under a homogeneous reaction condition was developed using laccase as the catalyst. The resulting copolymer was characterized for chemical structure, grafted-HP content, surface morphology, thermal stability, antioxidant capacity, hydrophobic properties and tensile strength. Solid-state ^{13}C-NMR spectrum confirmed the incorporation of HP onto chitosan. X-ray diffraction (XRD) showed a decrease in the degree of crystallinity for laccase/HP treated compared to pure chitosan. The grafted-HP content in laccase/HP-treated chitosan first increased and then declined with increase of the initial HP/chitosan ratio. A heterogeneous surface with spherical particles on the laccase/HP treated chitosan was observed by environmental scanning electron microscopy (ESEM) and scanning probe microscopy (SPM). The laccase/HP treatment of chitosan improved the thermal stability of copolymer. More significantly, the HP functionalized chitosan showed greatly improved $ABTS^{\cdot+}$ and DPPH radicals scavenging capacity, compared with pure chitosan. The hydrophobicity property of the HP functionalized chitosan also significantly increased although its tensile strength decreased. This new type of composite with double functionalities (i.e., antioxidant and hydrophobic) could potentially be used as food packaging materials or coating agents.

Keywords: Chitosan; Laccase; 4-hexyloxyphenol; Antioxidant; Hydrophobic

1. Introduction

As a natural resource, chitosan derived from the deacetylation of the chitin has several unique properties such as biocompatibility, antimicrobial, film-forming capacity, and low toxicity. Chitosan and its derivatives have been widely studied in a variety of applications such as food packing or preservation (Li et al., 2011; Wu et al., 2016), metal ions adsorption (Ayoub et al., 2013), adhesives (Mati-Baouche et al., 2014; Patel, 2015), dye removal, and pharmaceutical applications (Zou et al., 2016). Particularly due to its good film-forming capacity, chitosan can be used to fulfill the needs of various packaging in the form of transparent films or coatings to improve the quality of fresh food and extend the food's shelf life (Arancibia et al., 2014). The antioxidant capacity of native chitosan may be due to the capacity of residual free amino groups of chitosan to react with free radicals forming stable macromolecular radicals and ammonium groups (Yen, Yang, & Mau, 2008). However, chitosan lacks a hydrogen atom that can be easily donated in order to serve as a good antioxidant (Schreiber et al., 2013). The low antioxidant activity of chitosan is a major problem affecting food quality and biological applications (Nunes et al., 2013). The application of chitosan has also been restricted due to its hygroscopicity, which has been attributed to the reactive amine groups especially in humid environments or in an acid media (Dutta et al., 2002). Phenolic compounds, classified as primary antioxidants, readily scavenge free rediclas by donating a hydrogen atom or an electron (Schreiber et al., 2013). Therefore, introducing a hydrophobic phenolic compound onto chitosan would provide a new chitosan derivate with antioxidative and hydrophobic properties.

Chemical functionalization of chitosan can improve the antioxidant capacity (Hu et al., 2016; Schreiber et al., 2013; Xie et al., 2014) and hydrophobic property (Höhne et

al., 2007; Pradal et al., 2011) of chitosan composites by the incorporation of small functional groups onto chitosan. However, chemical functionalization typically has several drawbacks, including the requirement of reactive reagents and harsh experiment conditions, as well as the use of crosslinking agents may lead to toxic side effects (Lin et al., 2005). Chemo-enzymatic modifications provide an alternative method for functionalization of chitosan that could avoid the safety and environmental concerns mentioned above (Aljawish et al., 2015). Research efforts have been conducted regarding the improvement of the antioxidant activity of chitosan-based polymers through enzymatic grafting of phenolic compounds (Aljawish et al., 2016; Hu & Luo, 2016). This type of enzymatic modification of chitosan is usually carried out using polyphenol oxidases such as tyrosinase, peroxidase and laccase (Aljawish et al., 2015). Tyrosinase has been used to graft flavonoids or 4-hexyloxyphenol (HP) onto chitosan, conferring new capacities to chitosan such as antioxidative, antibacterial (Sousa et al., 2009) and hydrophobic properties (Chen et al., 2000). Tyrosinase-mediated chitosan derivatives can also be used as functional coatings for food packaging materials (Liu et al., 2014). Peroxidase has also been used to graft phenolic compounds onto chitosan to enhance its antioxidative (Zavaleta-Avejar et al., 2014), antibacterial (Sakai et al., 2014), and hydrophobic properties (Vachoud et al., 2001). Laccases are blue multi-copper oxidases and have shown encouraging potential as biocatalysts in organic synthesis mainly because the catalytic reaction only requires oxygen molecular and releases water as the only by-product (Kudanga et al., 2017). The laccase activated phenolics are potential cross-linkers of chitosan as a novel approach to synthesizing chitosan hydrogels (Huber et al., 2017). There have been several investigations on laccase-catalyzed functionalization of chitosan with phenolic compounds to enhance the

antioxidative activity (Aljawish et al., 2012; Božič et al., 2013), antibacterial activity (Aljawish et al., 2014b; Božič et al., 2012), and barrier properties (Aljawish et al., 2016).

In the previous studies of chitosan-phenolic-enzyme systems, the hydrophobic functionalization of chitosan was performed using peroxidase (Vachoud et al., 2001) or tyrosinase (Chen et al., 2000; Liu et al., 2014) as the catalyst. In terms of functionalization of chitosan with phenolic compound by laccase, most of studies focused on improving the antioxidant properties of chitosan derivate. Furthermore, most of the phenolic compounds being used in these studies contained carboxyl group or at least two phenolic hydroxyl groups, and was soluble in water. However, to the best of our knowledge, few publications have reported on the functionalization of chitosan with hydrophobic monophenols by laccase targeted at improving the antioxidant and hydrophobic properties of chitosan at the same time.

The objective of this work is to functionalize chitosan with 4-hexyloxyphenol (HP) under homogeneous conditions for enhancing the antioxidant and hydrophobic properties of chitosan through the laccase-mediated coupling reactions. The incorporation of HP into chitosan was supported by solid-state ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopy studies. The surface morphology of the chitosan and its derivative were further characterized by environmental scanning electron microscope (ESEM) and scanning probe microscope (SPM). The crystallographic structure and the thermal behavior of chitosan derivatives were analyzed by X-ray diffraction (XRD) and thermogravimetric analysis (TGA), respectively. Finally, the chitosan and its derivative films were characterized in terms of antioxidant, hydrophobic, and mechanical properties.

2. Materials and methods

106 *2.1. Chemicals*

107 Chitosan with a degree of de-acetylation of 85% was obtained from Sinopharm
108 Chemical Reagent Co. Ltd. 4-Hexyloxyphenol (HP), 2,2′-azino-bis-(3-
109 ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH)
110 were all purchased from Sigma-Aldrich. All other reagents were of the analytical grade
111 and used as received.

112 *2.2. Laccase*

113 Laccase (NS51003) from *Ascomycete myceliophthora thermophila* was provided
114 by Novozymes (Tianjin, China) with a specific activity of 1000 U/ml. 1 U was defined
115 as the amount of enzyme that oxidizes 1 μmol of ABTS per minute in a sodium acetate
116 buffer (pH 4.5) at 25 ºC.

117 *2.3. Enzymatic functionalization of chitosan*

118 Enzymatic functionalization of chitosan was performed according to a modified
119 homogeneous grafting method (Božič et al., 2012). First, the chitosan solution (0.3%
120 w/v, dissolved in 0.1 M acetate buffer solution, pH 4.5) and the HP solution (10% w/v,
121 dissolved in methanol) were prepared accordingly. The chitosan solution was heated to
122 45 ºC. The HP solution was then added into the chitosan solution under a constant
123 stirring rate. The ratio of the HP to chitosan was 1:2 (w/w). Afterwards, laccase (200
124 U/g HP) was slowly dropped into the chitosan-HP mixture solution. The laccase dosage
125 used in this paper was optimal for improving the antioxidant properties of chitosan
126 derivates. The laccase/chitosan-HP mixture was allowed to react for 15 h at 45 ºC. The
127 resultant solution was centrifuged at 5000 rpm for 5 min to remove the solid residue. 1
128 M NaOH solution was added to the supernatant to precipitate the solid product. After
129 centrifugation, the precipitation was extracted with methanol for 12 h to remove any

unreacted HP and then washed extensively with distilled water to remove the methanol. The resulting samples were lyophilized and stored at room temperature. The pure chitosan and the HP-chitosan solutions were prepared following the same procedures except the steps involving laccase were skipped.

2.4. Preparation of chitosan films

The chitosan solution was prepared by dissolving 0.10 g chitosan particles in 10.00 ml of acetic acid solution (2%, v/v) according to the method described by Aljawish et al (Aljawish et al., 2014c). Chitosan films were formed by adding 5.00 ml of the above solution to a 7 cm-diameter Petri dish, and the solution was then oven-dried overnight at 45 ºC. The dried films were neutralized by immersion in 1 M NaOH solution for 3 h, thoroughly washed with methanol and water, and then dried overnight at 40 ºC. The untreated chitosan and HP-treated chitosan were used as controls. All of the film samples were placed in sealed polyethylene bags and stored at room temperature.

2.5. Chemical evidence for chitosan functionalization

The structural characteristics of chitosan and its derivatives were analyzed by solid-state ^{13}C-NMR to confirm the grafting of 4-hexyloxyphenol onto chitosan by laccase. Solid-state ^{13}C-NMR experiments were performed on an AVIII 400 MHz WB spectrometer (Bruker Inc., Germany) operating at a ^{13}C frequency of 100.62 MHz equipped with a double resonance H/X CP-MAS 4 mm probe. The crystallinity of chitosan was recorded by a Brooke D8-ADVANCE X-ray diffractometer (AXS Company, Germany) over a 2θ range from 3° to 50°.

2.6. Determination of 4-hexyloxyphenol content onto chitosan derivative

The content of HP in chitosan composite was indirectly determined by measuring the content of the remaining HP in the reaction medium according to a modified Folin-

Ciocalteu assay method (Liu et al., 2013b). Briefly, ~10 mg of chitosan derivative was dissolved in 50 ml of aqueous acetic acid (0.5%, v/v). ~1 ml of sample solution was mixed with 1 ml of Folin-Ciocalteu reagent (10 times dilution) and allowed to react at 30 ^{o}C for 5 min in the dark. Then, ~5 ml of saturated Na_2CO_3 solution was added and mildly shaken periodically for 1 h before measuring the UV absorbance at 760 nm. HP was used to calculate the standard calibrations curves. The content of HP in grafted chitosan was expressed as mg of HP per g of laccase/HP-chitosan (mg HP/g). The grafting percentage of HP was then calculated from the HP content in the laccase/HP-chitosan and the utilized amount of HP in the preparation of laccase/HP-chitosan. Each point represents the average of three independent experiments.

2.7. Surface morphology characteristics

The surface morphology of the chitosan films was scanned using a QUANTA 200 Environmental scanning electron microscope (ESEM, FEI, USA). Three-dimensional surface topography was examined using a scanning probe microscope (SPM) model Mutimode 8 Nanoscope V system (Bruker Company, USA).

2.8. Thermogravimetric analysis

The thermal properties of the chitosan derivatives were determined using a Mettler-Toledo (Schwerzenbach, Switzerland) TGA 1 STAR System thermogravimetric analyzer, where the sample (5-7 mg) was heated from 30 ºC to 800 ºC under nitrogen atmosphere at a heating rate of 10 ºC/min and a nitrogen flow rate of 50 mL/min.

2.9. Evaluation of antioxidant activity

Antioxidant efficiency of the chitosan films was evaluated by the $ABTS^{\cdot+}$ and DPPH· methods. The $ABTS^{\cdot+}$ radical scavenging capacity of the control and HP-chitosan samples were determined according to the method described by Aljawish et al.

178 (Aljawish et al., 2014a), with a slight modification. An $ABTS^{\cdot+}$ solution was prepared
179 by mixing 7 mM ABTS in H_2O and 2.45 mM potassium per-sulfate at the proportion of
180 1:1 (v:v) at room temperature in the dark for 12 h. Before usage, 1 mL of the $ABTS^{\cdot+}$
181 solution was diluted into 80 mL of ethanol to reach an absorbance of 0.700 ± 0.025 at
182 734 nm. 6 mg of the film was placed in 4 ml of $ABTS^{\cdot+}$ solution and left in water bath
183 (30 ºC) in the dark for 20 min. The UV-absorbance of $ABTS^{\cdot+}$ was measured at 734 nm.
184 All measurements were performed in triplicate. The scavenging capability was
185 calculated using the following Eq. (1).

186 $$\text{Inhibition or scavenging activity (\%)} = (1 - A_{sample} / A_{control}) \times 100 \quad (1)$$

187 where $A_{control}$ is the absorbance of initial concentration of $ABTS^{\cdot+}$ and A_{sample} is the
188 absorbance of the remaining concentration of $ABTS^{\cdot+}$ in the presence of the sample.
189 The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed according to the
190 method of Woranuch et al. (Woranuch & Yoksan, 2013) with minor modification.
191 Briefly, 0.60 g of chitosan film was added into an ethanol solution of the stable
192 $DPPH\cdot$(100 μM) in brown bottle and the solution was then incubated in water bath (30
193 ºC) in the dark for 20 min. The optical absorbance of the reaction liquid was measured
194 at 517 nm. All measurements were performed in triplicate. The radical scavenging
195 activity was defined as a decrease in the absorbance of $DPPH\cdot$, and was calculated using
196 Eq. (1), where $A_{control}$ is the absorbance of initial concentration of $DPPH\cdot$ and A_{sample} is
197 the absorbance of the remaining concentration of $DPPH\cdot$ in the presence of the sample.
198 *2.10. Water contact angle and swelling property*
199 In order to characterize the hydrophobicity of each film sample, the contact angle
200 was measured according to the method described by Moradi et al. (Moradi et al., 2012)
201 at ambient humidity and temperature by using an OCA20 static contact angle analyzer

(Dataphysics Co, Germany). Briefly, deionized water was deposited on each film sample mounted on the side of glass slides with double-side tape. After the drop was steady, the water contact angles were measured by a contact angle goniometry.

Swelling behavior was evaluated according to the method described by Jung et al. (Jung et al., 2006). Firstly, the pre-weighted dry samples were immersed in deionized water. After wiping off the excess water on the samples' surface with filter paper, the weight of the swollen sample was measured at 10 min intervals. The swelling ratio was calculated using the following Eq. (2), where W_s and W_d are the weight of the swollen and the initial sample, respectively.

$$Swelling\ ratio = (W_s - W_d)/W_d \quad (2)$$

2.11. Tensile properties

Tensile test was performed at room temperature using a universal testing machine at a crosshead speed of 1 mm/min according to the Chinese National Standard GB/T13022-91. For each data of tensile properties, five samples were tested.

3. Results and discussion

3.1. Chemical structure of chitosan and chitosan derivatives

Chitosan and its derivatives were characterized by solid-state ^{13}C-NMR. As shown in Fig. 1, for the chitosan sample, the signals centered at 57, 60, 75, 83 and 105 ppm were assigned to C-2, C-6, C-3 and C-5, C-4 and C-1 carbons, respectively (Aljawish et al., 2012; Liu et al., 2013a). Carbonyl group C-7 and methyl group C-8 of acetylglucosamine showed peaks around 174 and 24 ppm, respectively. The HP-treated chitosan without laccase (HP-chitosan) spectrum exhibited an almost identical spectrum compared to the pure chitosan because HP was extensively washed out by methanol during the work-up, indicating there were no chemical bonds formed between chitosan

and HP. Compared with the chitosan and the HP-chitosan samples, new signals were observed in the spectrum of the laccase/HP-chitosan sample (Fig. 1). These new signals in the laccase/HP-CTS sample were attributed to the CH_3 (C-10', δ=14 ppm,) and unshielded CH_2 groups (C-6'~9' and 5', δ=22~35 and 68 ppm) on the hexyl group of HP (Jin et al., 2009). Additionally, only the laccase/HP-chitosan sample showed the oxygen substituted aromatic carbon (C-4', C-1') at bands 153/157 ppm and protonated aromatic carbon on the benzene ring of HP (C-3', C-2') at bands 115/125 ppm. These additional peaks clearly confirmed that the HP was grafted onto the chitosan.

Enzyme-mediated chitosan functionalization is generally based on the activation of a substrate into a reactive intermediate. It is well known that the phenolic compound can be oxidized to phenoxy radical by laccase (Polak & Jarosz-Wilkolazka, 2012). As proposed by Angerer et al. (Angerer et al., 2005), the phenoxyl radicals from oxidized HP could be delocalized across the aromatic ring on HP and further polymerize into the corresponding dimer or oligomer which might undergo additional oxidation into highly reactive quinonoid structures. These activated intermediates react spontaneously with initial substrate to produce new free radicals or react non-enzymatically by covalent bonds with the free amino groups of chitosan at pH < 6.3 (Aljawish et al., 2015). The oligomer-forming reactions with other quinones led to complex mixture of monomers or oligomers grafted onto chitosan.

Fig. 1

3.2. Crystallographic structures of chitosan and chitosan derivatives

As shown in Fig. 2, the X-ray diffraction pattern of chitosan and chitosan derivatives exhibited a characteristic peak at $2\theta = 20°$, corresponding to its crystal form II. Compared with chitosan and HP-chitosan, the laccase/HP-chitosan showed a

250 decreased intensity of reflection at $2\theta = 20°$. The diffraction pattern of original chitosan
251 showed hydrated polymorphism with a 020 reflection appearing at around $2\theta = 10°$, a
252 characteristic of "tendon" form. For the laccase/HP-chitosan, the peak of 020 reflection
253 at $2\theta = 9.71°$ became weaker, indicating the decrease in the acetamido group and the
254 loss of ordered structure (Kumar et al., 2004). The XRD results revealed that the
255 introduction of HP onto chitosan caused a decrease in crystallinity and loose structure of
256 HP-grafted copolymer, suggesting the decrease of inter- and intra-molecular hydrogen
257 bonds of the original chitosan after grafting.
258 **Fig. 2**

259 *3.3. HP content in laccase/HP-chitosan*

260 Total phenolic group determined by Folin-Ciocalteu assay method can evaluate the
261 content of grafted phenolic compound on the chitosan (Hu et al., 2015; Liu et al.,
262 2013b). As shown in Fig. 3, the content of HP incorporated onto chitosan product
263 tended to increase with increasing initial ratio of HP to chitosan (up to 1:2). The
264 maximum HP incorporation was 41.6 mg/g product. Further augmentation of the initial
265 HP/chitosan ratio resulted in decreased HP incorporation. There was a high probability
266 that the self-polymerization of oxidized HP became a dominant reaction due to an excessive
267 dosage of HP even though the self-polymerization reaction could be simultaneous with the graft
268 reaction at the low or high concentration of HP. More polymerization products could produce
269 the steric hindrance that was adverse to grafting HP into chitosan, which resulted in the
270 decrease of phenolic content on chitosan. In addition, the grafting percentage of HP

271 decreased significantly with the increase of HP dosage (Fig.3). Therefore, the
272 HP/chitosan ratio should be optimized in light of the application property of chitosan
273 derivative.
274 **Fig. 3**

275 *3.4. Surface morphology of chitosan films*

276 The surface morphology and topography of chitosan, HP-chitosan and laccase/HP-
277 chitosan films were studied by ESEM and SPM. As shown in the ESEM micrographs
278 (Fig. 4), the pure chitosan and HP-chitosan films presented relatively smooth and
279 continuous surfaces. However, the laccase/HP-chitosan film exhibited a much more
280 roughened and heterogeneous surface. The 3D images from SPM clearly demonstrated
281 the differences in surface morphology of these samples. The pure chitosan film
282 appeared to have a surface with some grooves. The HP-chitosan film showed an almost
283 same surface as the chitosan film. The laccase/HP-chitosan film, by contrast, clearly
284 appeared as a matrix with globular units of various sizes and the surface coarseness
285 significantly increased. It could be attributed to the laccase oxidized HP onto chitosan.
286 **Fig. 4**

287 *3.5. Thermal properties of chitosan particles*

288 Thermal properties of chitosan and its derivative were determined by
289 thermogravimetric analysis (TGA) and are shown in Fig. 5. The pure chitosan and HP-
290 chitosan samples showed two distinct stages (Fig.5a). The first weight loss of 5%
291 occurred below 100 ºC which was attributed to the loss of water in the materials (Ou et
292 al., 2009). Compared to the pure chitosan, the HP-chitosan and laccase/HP-chitosan

293 showed a much slower decrease of weight at this stage. It indicated that less water was
294 released in HP-chitosan and laccase/HP-chitosan possibly due to the presence of HP,
295 which can be also observed in the derivative thermo gravimetric analysis (DTG) curves
296 (Fig. 5b).

297 The second and third stages of weight loss in the range of 235–500 ºC was ascribed
298 to the thermal degradation of the chitosan components including dehydration of the
299 sugar rings, depolymerization, and decomposition of the acetylated and deacetylated
300 units of the polymer (Wang et al., 2007). It was also shown that all the chitosan films
301 started to degrade at the temperature of 233 ºC (Fig. 5a) and the maximum
302 decomposition rate appeared at 287 ºC (Fig. 5b). It was noteworthy that the laccase/HP-
303 chitosan showed a lower peak height in the DTG curves compared to the other samples,
304 indicating a better thermal stability after the laccase HP treatment (Ou et al., 2009).

305 However, the laccase/HP-chitosan sample started to degrade at 336 ºC with another
306 rapid weight loss appeared at 424 ºC (Fig. 5b). The weight loss in the range of 400–600
307 ºC is mainly due to the cleavage of pyranose ring and decomposition of carbonized
308 residue (Ou et al., 2009). In this paper, the thermal stability of grafted chitosan was
309 decreased above 336 ºC. In general, the introduction of a functional group can obstruct
310 chitosan chain packing, resulting in the decrease of thermal stability (Pasanphan &
311 Chirachanchai, 2008). Therefore, the graft of HP led to the loose structure of chitosan
312 and subsequently decreased the thermal stability above 336 ºC. The weight loss should
313 be caused by the cleavage of sugar ring and decomposition of grafted HP.

Fig. 5

3.6. Evaluation of antioxidant activities of chitosan films

The ABTS·$^{+}$ radical scavenging activities of all chitosan samples (1.5 mg/ml) at different times were shown in Fig. 6a. The pure chitosan sample presented a very low inhibitory activity towards ABTS·$^{+}$ radicals and its scavenging activity changed slowly with the increase of time, which may be partly due to the inter- and intra-molecular hydrogen links especially for the high molecular weight of chitosan (Aljawish et al., 2014b). Compared with the chitosan sample, the HP-chitosan showed a slightly higher but similar stable radical inhibitory activity. However, the scavenging activity of laccase/HP-chitosan was significantly higher than the others at any time and it increased rapidly as a function of time. The scavenging activity of ABTS·$^{+}$ radical of the chitosan increased from 21.97% to 48.92% within 20 min after laccase/HP treatment. As presented in Fig. 6b, the antioxidant efficiencies of pure chitosan and HP-chitosan increased slightly with the increase of chitosan concentration, however, the scavenging activity of the laccase/HP-chitosan increased rapidly. At a chitosan concentration of 2 mg/ml, the scavenging activity of laccase/HP-chitosan was increased by 3.02 times compared to that of pure chitosan.

Fig. 6

Fig. 7 shows the DPPH· scavenging activities of all chitosan samples at different times (Fig. 7a) and different concentrations of chitosan (Fig. 7b), respectively. The variation trends of DPPH· scavenging activities are similar to those of ABTS·$^{+}$ scavenging activities. Compared with the HP-chitosan and the pure chitosan, the laccase/HP-chitosan sample showed the highest antioxidant activity at different times and concentrations. In conclusion, the laccase/HP-chitosan film exhibited much stronger

inhibitory activity towards the ABTS$^{\cdot+}$ and DPPH$^{\cdot}$ as time extended and chitosan concentration increased. This improved antioxidant capacity of functioned chitosan would be extremely helpful in terms of opening a wide range of applications such as food packaging, biomedical, and cosmetics industries (Woranuch & Yoksan, 2013).

Fig. 7

3.7. Hydrophobicity of chitosan films

As presented in Fig. 8, chitosan and HP-chitosan had almost same contact angles around 45°-50°, while the contact angle of laccase/HP-chitosan increased to 70º. The hydrophobicity of chitosan derivatives was significantly enhanced, which could be possibly attributed to the introduction of HP with hydrophobic methylene units and benzene ring. Furthermore, the contact angle of laccase/HP-chitosan was quite stable as time extended to 30 s.

Fig. 8

As shown in Table 1, the swelling ratio of the laccase/HP-chitosan was decreased by 14.6% compared with the pure chitosan film, which had a very similar swelling ratio to HP-chitosan. The result was consistent with the increase of hydrophobicity properties. In the research work from Chen et al.(Chen et al., 2000), the contact angle of chitosan modified by tyrosinase with HP was increased by around 20% compared with the pure chitosan. In this work, the contact angle of chitosan derivate increased by around 42% after the laccase/HP treatment. A higher hydrophobicity property for the HP-chitosan material may have potential beneficial implications for packing materials.

Table 1

3.8. Tensile strength of chitosan films

As shown in Table 1, compared with the pure chitosan, the HP-chitosan showed comparable tensile strength and elongation at break. For the laccase/HP-chitosan film, the tensile strength and elongation at break decreased by 18.5% and 26.4%, respectively.

The remarkable strength decrease of the laccase/HP-chitosan might be attributed to the destruction of inter- and intra-molecular hydrogen bonds of chitosan by the introduction of HP (Woranuch & Yoksan, 2013), which are consistent with the result from XRD. Additionally, this loose structure but decreased swelling property for laccase/HP-chitosan indicated that the chitosan itself was functionalized with the hydrophobic group originated from HP.

4. Conclusions

The chitosan was functionalized under acidic conditions (pH 4.5) with 4-hexyloxyphenol (HP) by laccase catalysis. The maximum of grafted-HP content on chitosan was 41.6 mg/g laccase/HP-chitosan at the initial ratio of HP to chitosan of 1:2 (w/w). This new composite material exhibited a heterogeneous surface and better thermal stability. It is worth noting that the HP functionalized chitosan showed greatly improved antioxidant characteristics and increased hydrophobic properties while its tensile strength decreased. This new type of composite with double functionalities (i.e., antioxidant and hydrophobic) could potentially be used in some important biotechnological domains such as food packing or coating agent.

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Figure Captions

Fig. 1 Solid state ^{13}C-NMR spectrum of chitosan, HP-chitosan, and laccase/HP-chitosan

Fig. 2 XRD spectra of chitosan, HP-chitosan and laccase/HP-chitosan

Fig. 3 Contents and grafting percentages of 4-hexyloxyphenol (HP) in laccase/HP-chitosan with different initial ratios of HP to chitosan

Fig. 4 ESEM micrographs and SPM images (3D) of chitosan (a), HP-chitosan (b) and laccase/HP-chitosan (c)

Fig. 5 TGA (a) and DTG (b) curves for chitosan, HP-chitosan and laccase/HP-chitosan

Fig. 6 $ABTS^{\cdot+}$ radical scavenging activity of chitosan, HP-chitosan and laccase/HP-chitosan as function of time (a) and chitosan concentration (b)

Fig. 7 DPPH· scavenging activity of chitosan, HP-chitosan and laccase/HP-chitosan as a function of time (a) and chitosan concentration (b)

Fig. 8 Contact angle over the time of chitosan, HP-chitosan and laccase/HP-chitosan

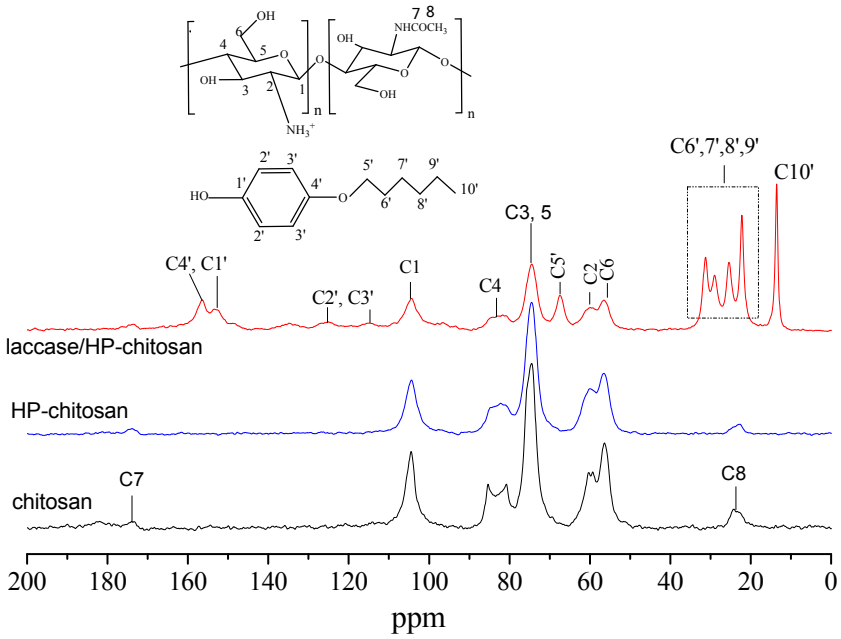
C6',7',8',9'
C10'
C3, 5
C4', C1'
C1
C4
C5'
C2
C6
C2', C3'
laccase/HP-chitosan
HP-chitosan
chitosan
C7
C8
200
180
160
140
120
100
80
60
40
20
0
ppm

Fig. 1

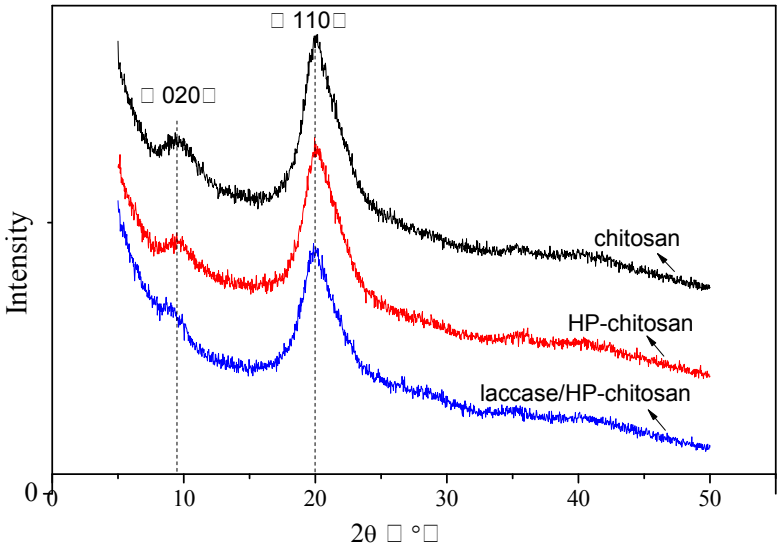
□ 110□
□ 020□
chitosan
HP-chitosan
laccase/HP-chitosan
Intensity
0
0
10
20
30
40
50
2θ □ °□

Fig. 2

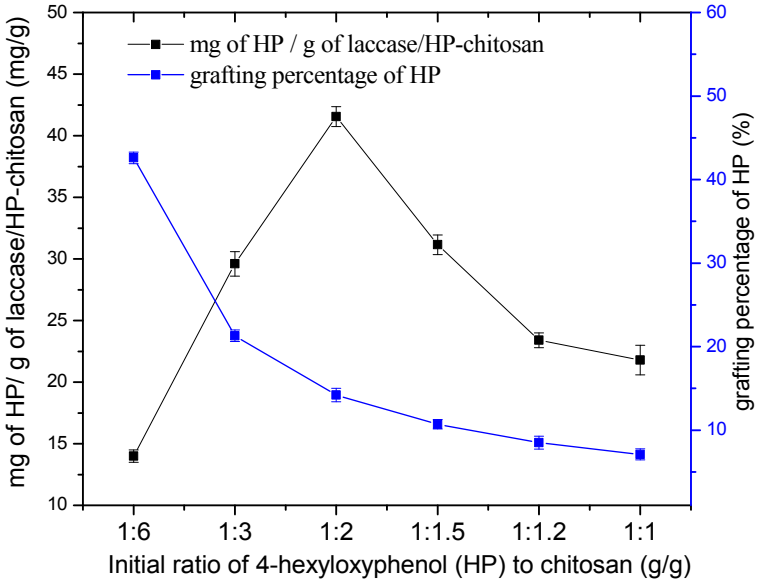
mg of HP / g of laccase/HP-chitosan
grafting percentage of HP
mg of HP/ g of laccase/HP-chitosan (mg/g)
grafting percentage of HP (%)
Initial ratio of 4-hexyloxyphenol (HP) to chitosan (g/g)
1:6
1:3
1:2
1:1.5
1:1.2
1:1

Fig. 3

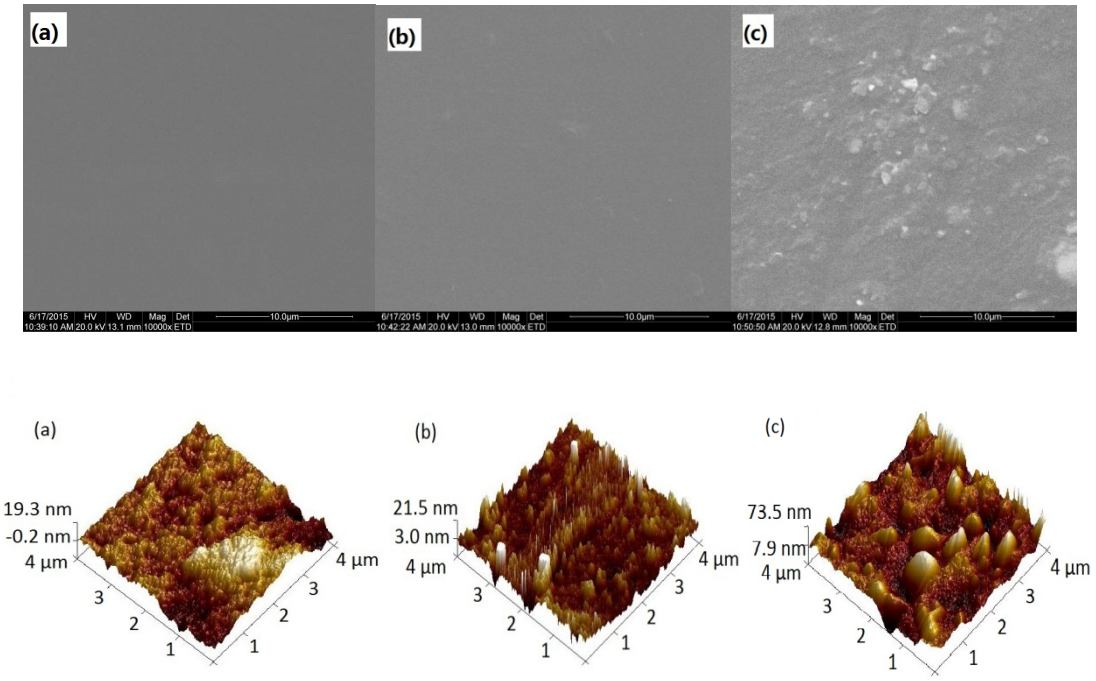

Fig. 4

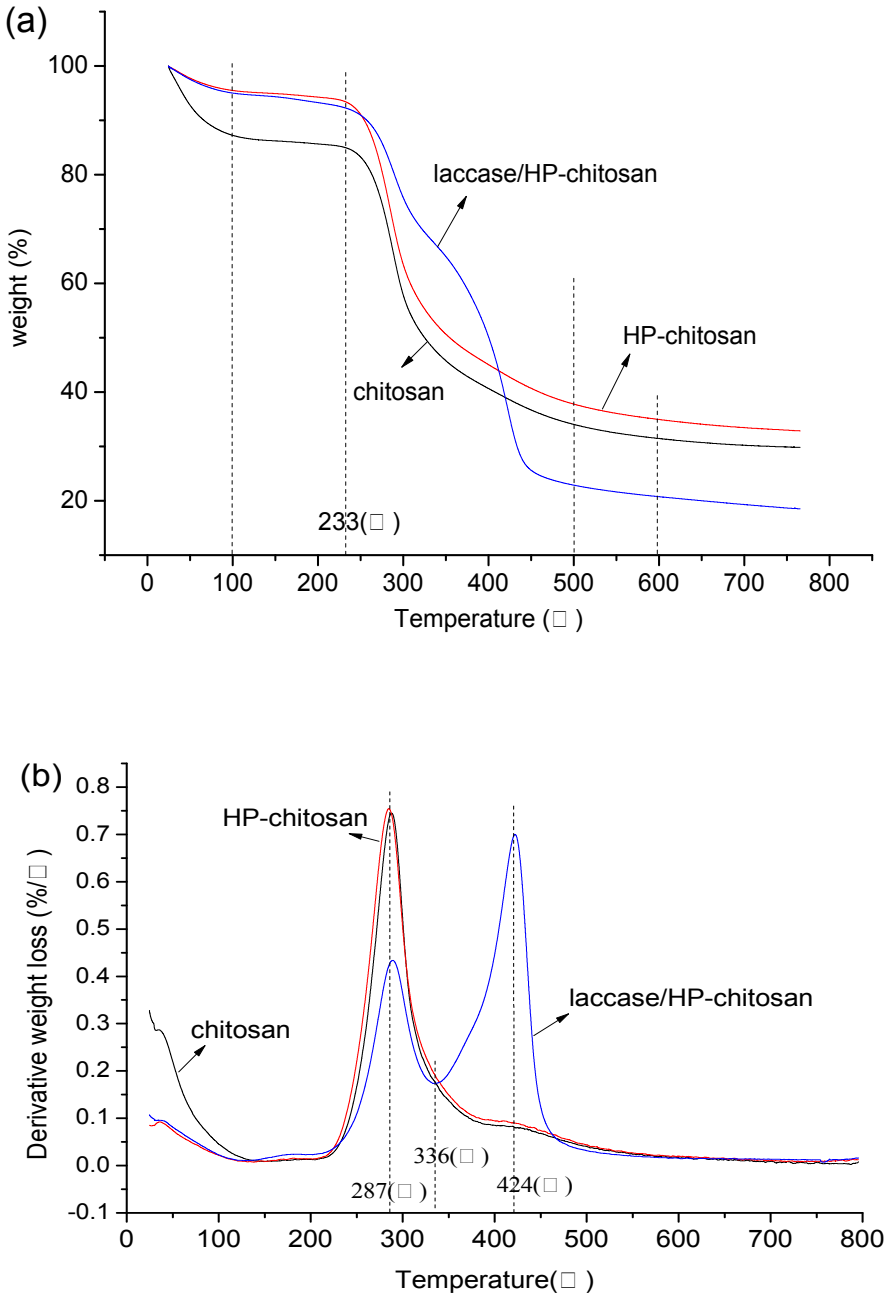
(a)
100
80
60
40
20
weight (%)
laccase/HP-chitosan
HP-chitosan
chitosan
233(□)
0
100
200
300
400
500
600
700
800
Temperature (□)
(b)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
Derivative weight loss (%/□)
HP-chitosan
chitosan
laccase/HP-chitosan
336(□)
287(□)
424(□)
0
100
200
300
400
500
600
700
800
Temperature(□)

Fig. 5

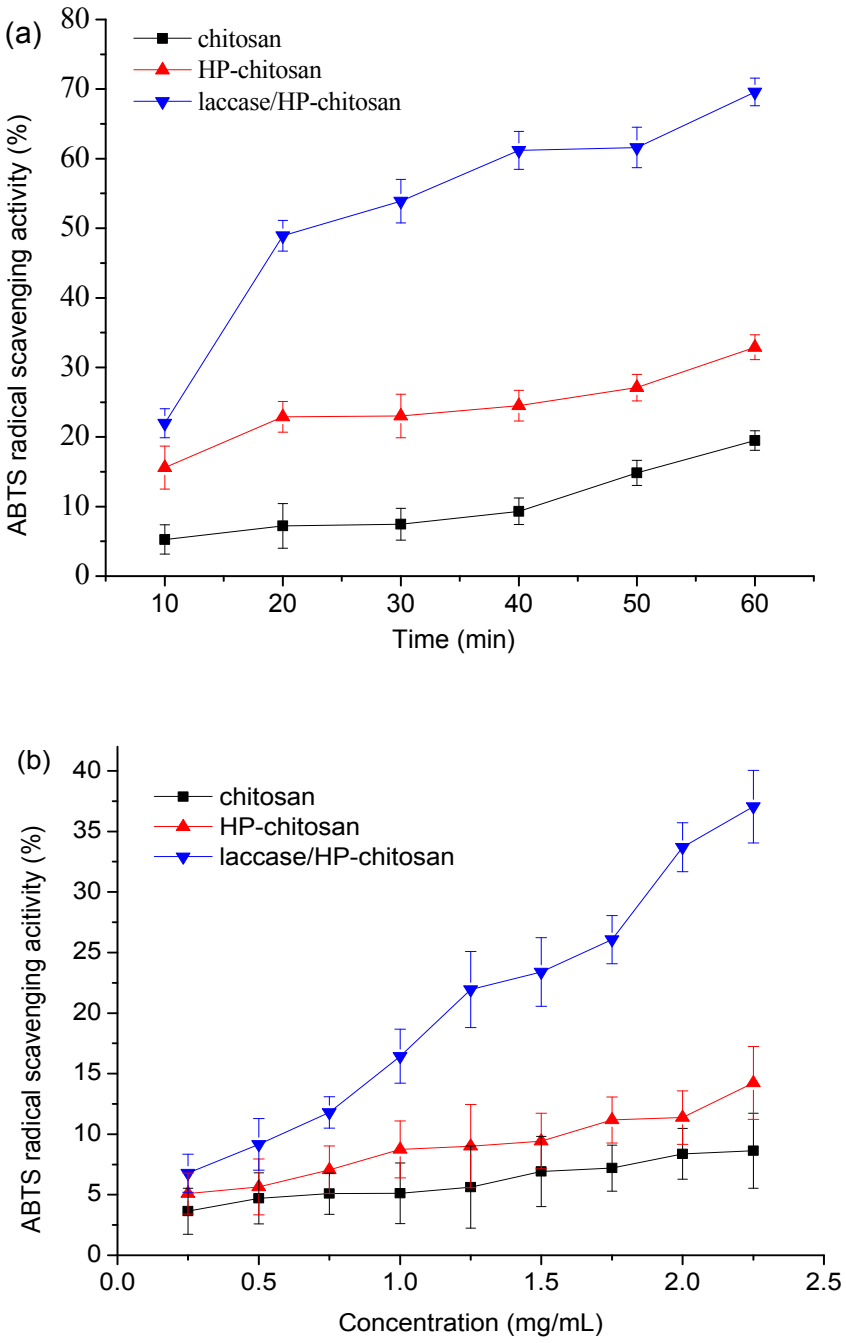
(a)
chitosan
HP-chitosan
laccase/HP-chitosan
ABTS radical scavenging activity (%)
Time (min)
0
10
20
30
40
50
60
70
80
10
20
30
40
50
60
(b)
chitosan
HP-chitosan
laccase/HP-chitosan
ABTS radical scavenging acitivity (%)
Concentration (mg/mL)
0
5
10
15
20
25
30
35
40
0.0
0.5
1.0
1.5
2.0
2.5

Fig. 6

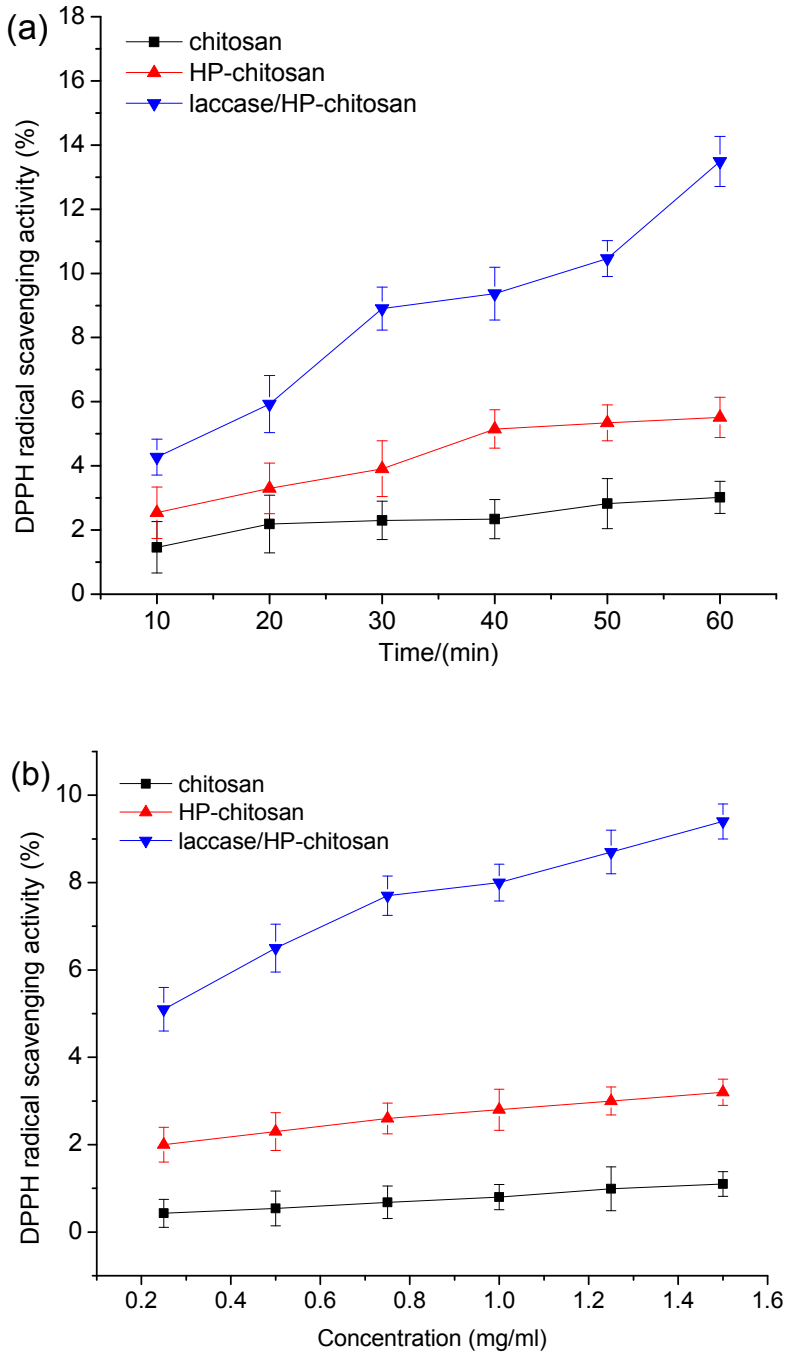
(a)
chitosan
HP-chitosan
laccase/HP-chitosan
DPPH radical scavenging activity (%)
Time/(min)
0
2
4
6
8
10
12
14
16
18
10
20
30
40
50
60
(b)
chitosan
HP-chitosan
laccase/HP-chitosan
DPPH radical scavenging activity (%)
Concentration (mg/ml)
0
2
4
6
8
10
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

Fig. 7

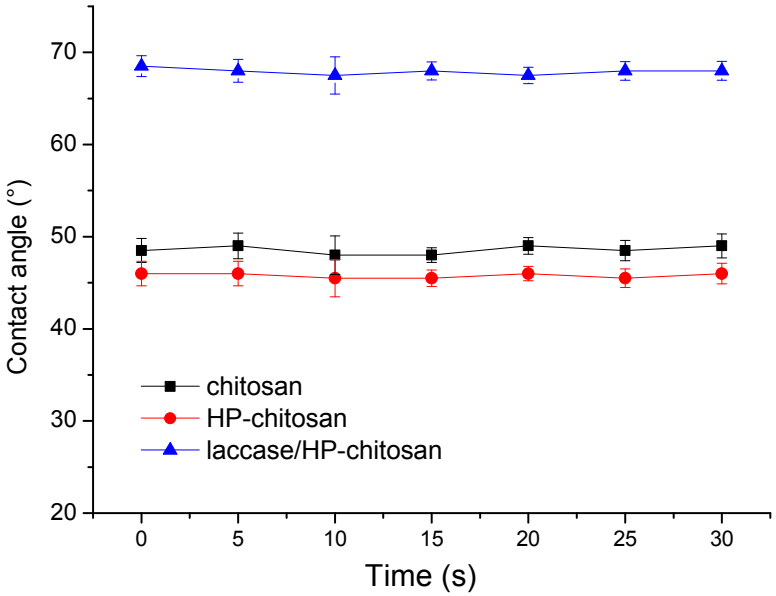
Contact angle (°)
70
60
50
40
30
20
0
5
10
15
20
25
30
Time (s)
chitosan
HP-chitosan
laccase/HP-chitosan

Fig. 8

Table 1 Swelling ratio and tensile properties of chitosan, HP-chitosan and laccase/HP-chitosan

Samples	chitosan	HP-chitosan	laccase/HP-chitosan
Swelling ratio (%)	37.0 ±1.9	38.0 ±1.8	31.6 ± 1.9
Tensile strength (Mpa)	39.00 ± 1.15	37.76 ±1.26	31.78 ±1.04
Elongation at break (%)	11.95 ± 0.96	11.98 ±1.10	8.80 ± 0.82

Each value is expressed as mean ± standard deviation (n = 3).