CHAPTER V

CHITOSAN FUNCTIONALIZATION WITH GALLIC ACID: A NOVEL POTENTIAL BIOPOLYMER-BASED ANTIOXIDANT BASED ON EPR STUDIES

5.1 Abstract

A novel biopolymer-based antioxidant, chitosan conjugated with gallic acid (chitosan-GA), is proposed. Electron paramagnetic resonance (EPR) declares a wide range of antioxidant activity of chitosan-GA as evidenced from the reactions with oxidizing free radicals, i.e. 1,1-diphenyl-2-picryl-hydrazyl (DPPH), horseradish peroxidase (HRP)-H_2O_2, carbon-centered alkyl radicals, and hydroxyl radicals. The EPR spectrum of the radical formed on chitosan-GA is relevant to the semiquinone radical of gallic acid. The stoichiometry and effective concentration (EC_{50}) between DPPH free radical and chitosan-GA show that the radical scavenging capacity is maintained even after thermal treatment at 100°C for an hour. Although the substitution degree of GA on chitosan is about 15%, antioxidant capacity to stabilize carbon-centered radical and hydroxyl radical is comparable to that of GA.

Keywords: Chitosan, gallic acid, antioxidant, electron paramagnetic resonance (EPR).

5.2 Introduction

Chitin-chitosan is the second most naturally abundant copolysaccharide next to cellulose obtained from shells of crustaceans, cuticles of insects, cell-walls of fungi and yeasts. The pyranose rings of β–(1-4)-2-acetamido-2-deoxy-β-D-glucose and β–(1-4)-2-amino-2-deoxy-β-D-glucose linked with glycoside linkage offer chitosan the specific properties, for example; biodegradability,[1] biocompatibility,[2] bioactivity,[3] non-toxicity,[4] antimicrobial activities,[5,6] and ion absorption ability.[7] Based on those specific properties, chitin-chitosan has received much attention as a biomaterial for value-added products, especially food[8] and drugs[9] including cosmetics.[10]

Development of chitosan and its derivatives are covered in more areas, especially in biomedical one. When we consider the biological molecules such as lipids, proteins, enzymes, DNA, and RNA, one may recognize how the free radicals involve the aging, atherosclerosis, carcinogenesis, including food deterioration. [11-15] Thus, developing either synthetic or natural antioxidants is an appropriate to reduce or retard free radical generation. Recently, chitosan has been exploited for an alternative natural antioxidant as well as its antibacterial[16] and antimutagenetic[17] properties. On the basis of the free radical theory, chitosan should form the most stable macroradicals via the hydroxyl and amino groups,[18] however, Alexandrova et al.[19] reported that the antioxidant activity of chitosan was equal to zero. Li et al.[20] used the very high EC_{50} (1.12×10^{6} $\mu g \cdot mL^{-1}$) of chitosan from EPR studies to point out that inter- and intramolecular hydrogen bonds obstructs the radical scavenging. On this viewpoint, the development of chitosan for antioxidant activity has to overcome; (i) the poor solubility and chemical inertness based on the strong inter- and intra-molecular hydrogen bond network, and (ii) the poor H-atom donating ability to serve as a good chain breaking antioxidant.

For (i), Sun et al.[21] showed that by introducing the long hydroxyl group or carboxylic group onto chitosan, the EC_{50} was decreased to be as low as about 3×10^{2} $\mu g \cdot mL^{-1}$. It is important to note that the radical scavenging, in most cases, is in water-based system, especially when we consider chitosan as marine polysaccharide drugs

with antioxidant activities. Therefore, currently, water soluble chitosans and their antioxidant activities have been reported. For example, Xing et al.[22,23] reported about a series of chitosan sulfate derivatives with radical scavenging ability (EC_{50}) for superoxide anion and hydroxyl radical to be around 0.01-0.03 and 1.3-3.3 $mg \cdot mL^{-1}$, respectively, which is 10^2-10^5 times less than that of chitosan. The fact that chitosan shows a strong metal ion chelating ability, chitosan is a potential natural product antioxidant when we consider the deactivation of the metal ions in radical generation, especially Fe^{2+} or Fe^{3+}.[24] Based on this viewpoint, Xing et al.[25] and Guo et al.[26] investigated the ferrous ion-chelating effect for chitosan sulfate to find that the deoxyribose oxidation was effectively inhibited.

In the case of (ii), it is important to mention that the H-atom donating ability to serve as a good chain breaking antioxidant of chitosan is possible if we functionalize chitosan with some specific groups. To our knowledge, this strategy has not been reported yet. The functional molecules either the synthetic ones, such as butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) or the natural ones, such as green tea, rosemary, and tannin, are good candidates.

Gallic acid (3,4,5-trihydroxybenzoic acid, Figure 1(a)), a natural phenolic antioxidant from grape seeds, is attractive since the low O-H bond dissociation enthalpy (BDE)[27] serves as one- or two-electron reductant. Gallic acid transfers hydrogen atom[28] and yields a delocalized aroxy radical[29] to form a stable semiquinone free radical[30] in a wide pH range.[31]

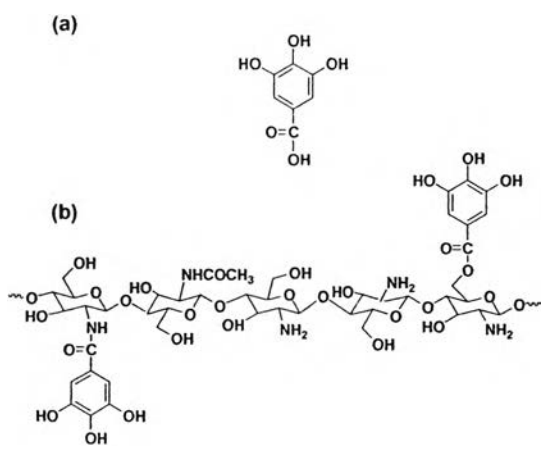


Figure 1. Chemical structure of GA and chitosan-GA.

Taking gallic acid in our choice, currently, we succeeded in introducing gallic acid onto chitosan. The derivative obtained shows the swelling in water implying the loosely bounded inter- and intramolecular hydrogen bonding of chitosan due to the bulky group of gallic acid.[32] The present work, thus, focuses on how chitosan conjugated with gallic acid show its radical scavenging potential to be a novel type of bio-based antioxidant by demonstrating a series of EPR studies.

5.3 Results and discussion

5.3.1 Conjugation of chitosan with GA and structural characterization

As reported in previous,[32] the conjugation of chitosan with GA possibly occurs between the carboxylic acid group of GA and the amino group (C-2 of pyranose ring of chitosan), to obtain an amide linkage, or the hydroxyl group (C-3 and C-6 of pyranose ring of chitosan), to obtain an ester linkage. As compared to the FT-IR spectrum of chitosan, the compound obtained shows the new significant peaks at 1640 and 1730 cm^{-1} confirming both amide and ester linkages (Figure 2 (A)).

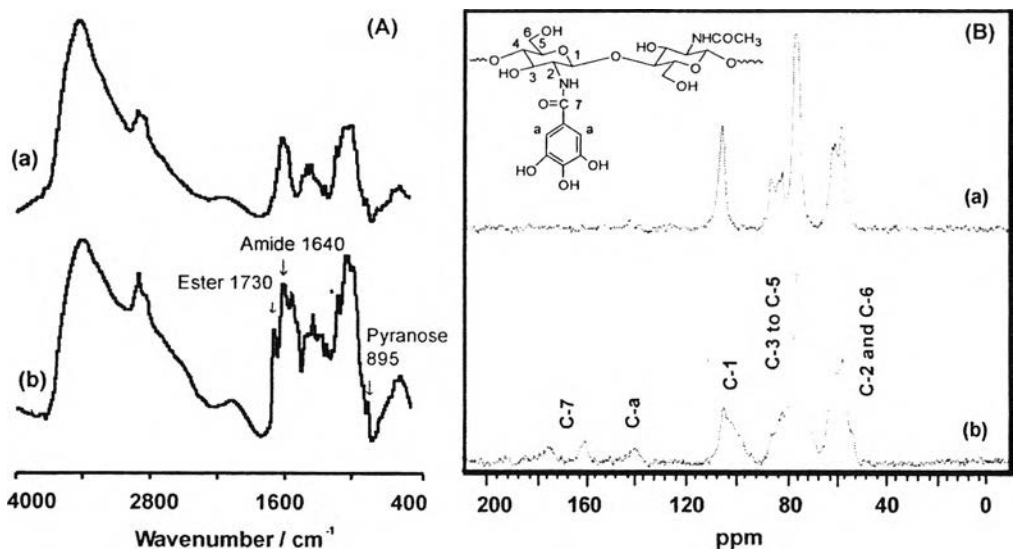


Figure 2. (A) FT-IR spectra and (B) ^{13}C-NMR spectra of (a) chitosan and (b) chitosan-GA.

The ^{1}H-NMR spectrum of GA:chitosan showed a new peak at 7.6 ppm belonging to the protons of phenoxyl GA. In addition, ^{13}C CP/MAS NMR confirms the aromatic ring of galloyl group on chitosan at 140 ppm (C=C, aromatic) and at 160.2 ppm (C=O, ester) (Figure 2(B)).

When %DS was evaluated by elemental analysis using the C/N ratio, it was found to be ~15.6% (for 3:1 GA:chitosan molar ratio). Based on this substitution degree, we calculated the average molecular weight per monosaccharide unit of chitosan-GA to be 187.5 g/mole and used this value for preparing chitosan-GA solutions in molar.

It is important to develop the water solubility together with antioxidant activity, here, we clarified the solubility of the compound by dispersing it in water and observed the transmittance at 500 nm. Chitosan-GA obtained from the 3:1 GA:chitosan molar ratio showed almost clear solution as clarified from the transmittance above 80%.

5.3.2 Semiquinone radical formation of chitosan-GA

For the radical scavenging processes of phenolic antioxidants (ArOH), H-atom donation is the dominant mechanism. This reaction occurs by two separate, but related pathways to form ArO$^{\bullet}$. Eq. (1) is the simple single step H-atom-transfer whereas Eq. (2) represents the stepwise electron-transfer/proton-transfer.

$$ROO^{\bullet} + ArOH \longrightarrow ROH + ArO^{\bullet} \quad (1)$$

$$ROO^{\bullet} + ArOH \longrightarrow ROO^{-} + ArOH^{\bullet+} \longrightarrow ROOH + ArO^{\bullet} \quad (2)$$

The ArO$^{\bullet}$ radical is relatively stable and thus ArOH can serve as a good chain-breaking antioxidant.[33,47] Semiquinone radicals (GA-semiquinone radicals) are less reactive than phenoxyl radicals.[34,35]

In order to preliminarily determine if the galloyl group of chitosan-GA is capable in forming the GA-semiquinone radical, chitosan-GA and GA were incubated in alkaline aqueous solution (NaOH, pH 13). The EPR spectrum of the GA-semiquinone free radical derived from chitosan-GA (Figure 3(A)(a)) shows the same g-value and gives the same three-line EPR spectrum ($a_1{}^{H}{}_1 = a_1{}^{H}{}_2 = 1.07$ G) with

1:2:1 intensity ratio as the free radical derived from GA (Figure 3(A)(b), which is similar to the report by Oniki *et al.* (a^{H} (2) = 1.1 G).[36]

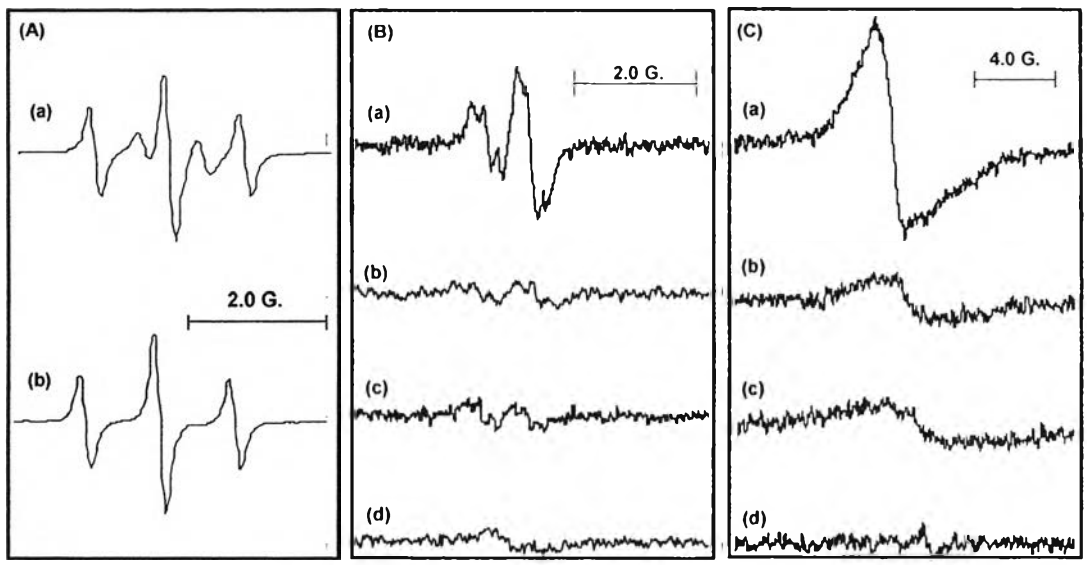


Figure 3. (A) EPR spectra generated in alkaline solution. (a) chitosan-GA; and (b) GA in alkaline solution (NaOH, pH 13). (B) EPR spectra of GA radicals formed by the HRP-H_2O_2 system: (a) GA (4 mM), HRP (0.8 μg/mL), and H_2O_2 (0.4 mM) in PBS solution (pH 7.4) (g = 2.0054, a^{H}_{1} = 0.96 G and a^{H}_{2} = 0.26 (2) G); (b) as in (a), but without HRP and H_2O_2 (g = 2.0054; (c) as in (a), but without HRP; and (d) as in (a), but without GA (g = 2.0052). (C) EPR spectra of chitosan-GA radicals formed by the HRP-H_2O_2 system: (a) chitosan-GA (30 mM), HRP (0.8 μg/mL), and H_2O_2 (0.4 mM) in PBS solution (pH 7.4) (g = 2.0055); (b) as in (a), but without HRP and H_2O_2 (g = 2.0055); (c) as in (a), but without chitosan-GA, and (d) as in (a), but without chitosan-GA (g = 2.0052).

The spectrum of the radicals derived from chitosan-GA shows additional unidentified peaks consistent with hyperfine splitting of a single spin one-half species, a_2^{H} = 0.86 G. Although further studies are needed, we suspect that it might come from the functional groups of chitosan, i.e. the hydroxyl and amino groups.

To probe the radical formation of chitosan-GA at neutral pH, we used HRP-H_2O_2 oxidizing system. A series of GA in various conditions were studied to understand the EPR spectrum of chitosan-GA under HRP-H_2O_2 system. For example, when GA is subjected to the HRP-H_2O_2 system at pH 7.4 a second unidentified free radical with the same *g*-value (2.0054) as in the alkaline pH was observed (Figure 3(B)(a)). This spectrum shows a doublet of triplets with hyperfine splitting from three protons (a^H_1 = 0.96 G and a^H_2 = 0.26 (2) G).

As shown in Figure 3(B) (b) and (c), only low level background EPR spectra are observed when there was neither HRP-H_2O_2 (b), nor HRP (c). This confirms that the hyperfine splitting is from the GA not from any other components. Moreover, the weak signals might be due to the auto-oxidation of GA in atmospheric oxygen at pH>7, which has been reported by Friedman *et al.*[37] When the system contains only HRP-H_2O_2 (without GA), no signal is observed (Figure 3(B)(d)).

Chitosan-GA with HRP-H_2O_2 shows the EPR spectrum (Figure 3(C)(a) and Figure 3(C)(a)) with the same *g*-value as that of the GA (Figure 3(B)(a)). This implies that the chitosan-GA generates GA-semiquinone radical. Figure 3(C)(b) and (c)) declares that the EPR signal in Figure 3(C)(a) is from chitosan-GA not from the HRP-H_2O_2.

Comparing Figure 3(C)(a) to Figure 3(B)(a), it is clear that chitosan-GA gives spectra with a broad linewidth and no resolvable hyperfine splittings. This may be due to the fact that the galloyl group is tethered to the chitosan polymer chain; as a result, the isotropic motion of the generated GA radical is restricted.

5.3.3 Semiquinone radicals formation of chitosan-GA by H-atom transfer to carbon-centered radicals

Lipid peroxidation in a biological system is a good example, which the function of antioxidants on carbon-centered radical is needed.[47] In order to investigate the reactivity of chitosan-GA, the free radicals produced by the azo initiator AAPH were used as a model. AAPH is a hydrophilic compound that produces carbon-centered radicals *via* thermal decomposition.[38] Although the temperature control in decomposing AAPH in the standard (TE) cavity could be done during the EPR measurement, the fact that the signal-to-noise ratio of this

cavity was not as good as in the transverse mode (TM_{110}) cavity with the flat cell one, we, then, used the UV irradiation to increase the AAPH radical formation rate. The radical formation follows the Eqs. (3)- (6).

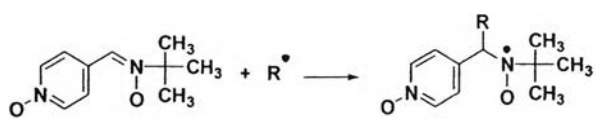
$$\text{R-N=N-R} \xrightarrow{\text{UV-light}} 2R^{\bullet} + N_2 \quad (3)$$

$$2R^{\bullet} + 2O_2 \longrightarrow 2ROO^{\bullet} \quad (4)$$

$$R^{\bullet} + ArOH \longrightarrow RH + ArO^{\bullet} \quad (5)$$

$$ROO^{\bullet} + ArOH \longrightarrow ROOH + ArO^{\bullet} \quad (6)$$

POBN is known as an excellent spin trap for carbon-centered radicals, producing persistent spin adducts (Eq. (7)).[47] Using EPR spin trapping with POBN, the thermal decomposition of AAPH at room temperature yields an EPR spectrum with hyperfine splitting constants (a^N = 15.6 G and a^H = 2.6 G). This hyperfine splitting constant is consistent with that of the POBN spin adduct of carbon-centered radicals, POBN/R$^{\bullet}$.[38,39]



POBN POBN/R$^{\bullet}$ (7)

If UV does increase the rate of radical production from AAPH, the formation of POBN spin adducts will be increased. When the samples were exposed to UV, the EPR intensity of POBN/R$^{\bullet}$ was increased up to 10 μM within 200 s. Here, the concentration of POBN/R$^{\bullet}$ was quantified based on the known concentration of 3-carboxy-proxyl (3cp) standard free radical.

When GA was incubated with AAPH and exposed to UV in the EPR cavity, the EPR spectrum (a^H_1 = 0.98 G and a^H_2 (2) = 0.26 G) obtained (Figure 4(A)(a)) is similar to that of the GA radical in the HRP-H_2O_2 system (Figure 3(A)(a)). The result implies that GA undergoes hydrogen-atom transfer to repair the

carbon-centered radicals generated by AAPH, leading to a stable semiquinone free radical similar to the case of the HRP-H_2O_2 system. Only a small doublet spectrum of GA-semiquinone radicals is observed when there was neither UV exposure (Figure 4(A)(b)) nor AAPH addition (Figure 4(A)(c)). This confirms that the EPR spectrum in Figure 4(A)(a) is generated from GA. The GA-semiquinone radicals are formed according to Eqs. (5)-(6).

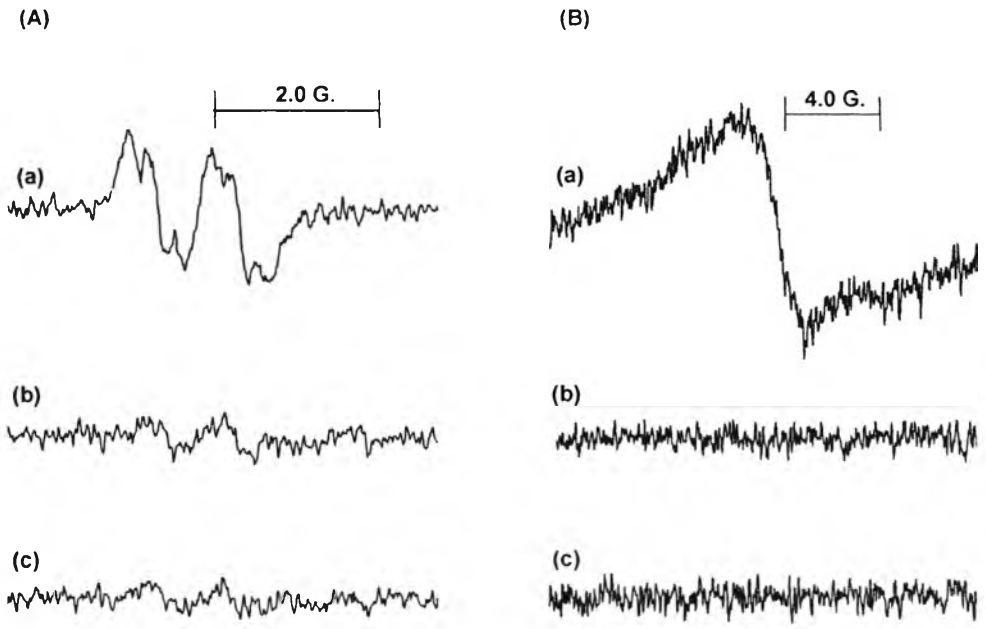


Figure 4. EPR spectra generated using AAPH. (A): (a) GA (4 mM) and AAPH (15 mM) in PBS solution (pH 7.4) with UV exposure at room temperature (g = 2.0054, a^{H}_{1} = 0.98 G, a^{H}_{2} = 0.26 (2) G); (b) as in (a), but without UV exposure (g = 2.0054); and (c) as in (a), but without AAPH (g = 2.0054). (B): (b) chitosan-GA (24 mM) and AAPH (15 mM) in PBS solution (pH 7.4) with UV exposure at room temperature (g = 2.0055); (b) as in (a), but without UV exposure; and (c) as in (a), but without AAPH.

Chitosan-GA in AAPH gives a broad EPR spectrum (Figures 4B (a)) with the same g-values (g = 2.0055) as that of chitosan-GA in HRP-H_2O_2 system (Figure 3B (a)). This confirms that chitosan-GA is a hydrogen atom donor to repair carbon-centered. If any of the components omitted, the spectra with only "baseline"

are observed (Figure 4(B)(b) and (c)). This confirms that: (i) chitosan-GA exhibits antioxidant activity to repair carbon-centered radicals; and (ii) the exposure of chitosan-GA to UV hardly induces radical formation.

5.3.4 DPPH radical scavenging capacity

1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH$^{\bullet}$) is a relatively stable free, thus, it is useful and practical for the evaluation of antioxidative potential by EPR technique.[28,40] The free radical scavenging activity was investigated from the activity of antioxidant (ArOH) to reduce DPPH$^{\bullet}$. The antioxidant capacity was evaluated by plotting the concentrations of antioxidant against the percent of free radical scavenging capacity. Figure 5(A) shows comparative studies on DPPH$^{\bullet}$ scavenging capacity of chitosan-GA and that of chitosan acetic acid solution. In the case of chitosan, no DPPH$^{\bullet}$ reduction is observed even though its concentration was as high as 0.23 mg·mL^{-1} (1200 μM). The scavenging capacity of chitosan-GA for DPPH$^{\bullet}$ increases as its concentration increases and is up to 87.3% when the concentration reached 0.23 mg·mL^{-1} (1200 μM). The EC_{50} value which expresses the

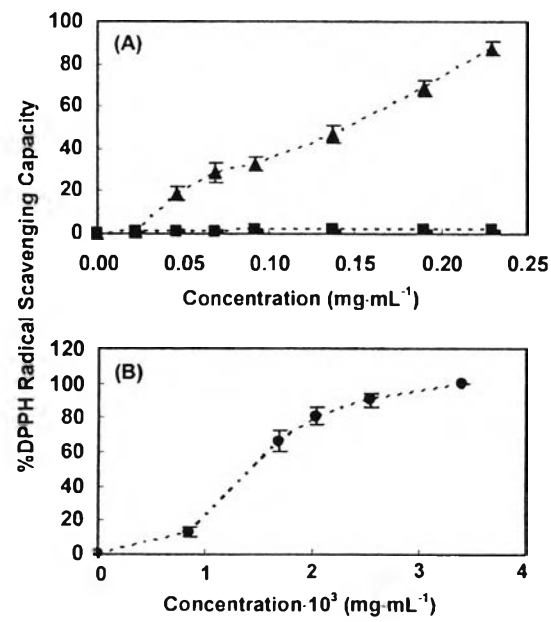


Figure 5. Scavenging capacity of (A) chitosan-GA (▲), chitosan (■) and (B) GA (●) on DPPH radical. Results are mean ±SD (n=3).

antioxidant concentration to reduce the radicals by 50% is a good indicator to quantify the antioxidant capacity. As shown in Figure 5(A), the EC_{50} in scavenging the DPPH$^{\bullet}$ of chitosan-GA is 0.14 mg·mL^{-1} (740 μM). Here, it should be noted that in the cases of sulfated chitosans reported by Xing et al.[25], TSCTS (the sulfated chitosan of $C_{3,6}$ sulfonation) showed a good radical scavenging ability at 0.025 mg·mL^{-1} (EC_{50}), whereas the other sulfated chitosan derivatives exhibited DPPH$^{\bullet}$ scavenging ability less than 20% at the concentration as high as 0.40 mg·mL^{-1}. Taking this into our consideration, the introduction of H-atom donating group, i.e. gallic acid, onto chitosan is a good strategy to develop chitosan antioxidant capacity.

It is important to verify that the hydroxyl groups of GA on chitosan were not oxidized to quinones during the reaction since this is also another possible factor to lower the DPPH$^{\bullet}$ radical scavenging ability of chitosan-GA. If quinones were present, the introduction of Zn^0 would reduce these quinones to hydroquinones. This would increase the apparent stoichiometry for the reaction of chitosan-GA with DPPH$^{\bullet}$. When the experiment was carried out by treating chitosan-GA with Zn^0, the stoichiometry of the reaction of chitosan-GA with DPPH$^{\bullet}$ was found to be the same, i.e. 0.11± 0.03. This confirms that GA (OH groups) on chitosan-GA was not oxidized to quinone during synthesis and storage. It can be concluded that the scavenging ability of chitosan-GA observed was not related to any prior oxidation of the galloyl group.

5.3.5 Carbon-centered radical scavenging capacity

In free radical-mediated oxidations, especially lipid peroxidation, an oxidant abstracts a hydrogen atom from a carbon-hydrogen bond resulting in the formation of carbon-centered radicals. These carbon-centered radicals will react rapidly with oxygen to form peroxyl radicals that propagate the chain reactions of lipid peroxidation.[41] To examine the efficiency of GA and chitosan-GA in repairing carbon-centered radicals, the EPR signal intensities of POBN/R$^{\bullet}$ with various concentrations of GA and chitosan-GA were compared. It is important to note that the higher the antioxidant potential of ArOH (Eq. (5)), the smaller the EPR signal of POBN/R$^{\bullet}$ (Eq. (7)). The EPR signal intensity in the system with chitosan-GA or GA (Figure 6(A)(a) or (b)) is smaller than that observed without chitosan-GA or GA

(Figure 6(A)(c)). This confirms the carbon-centered radical scavenging capacity of chitosan-GA.

In order to confirm that UV enhances only the rate of radical generation from AAPH, and not from GA or chitosan-GA, a sample without AAPH was examined. As no spin adduct formation is observed when samples (GA or chitosan-GA) were exposed to UV light (Figure 6(A)(d) and (e)), it can be concluded that the carbon-centered radicals were from AAPH.

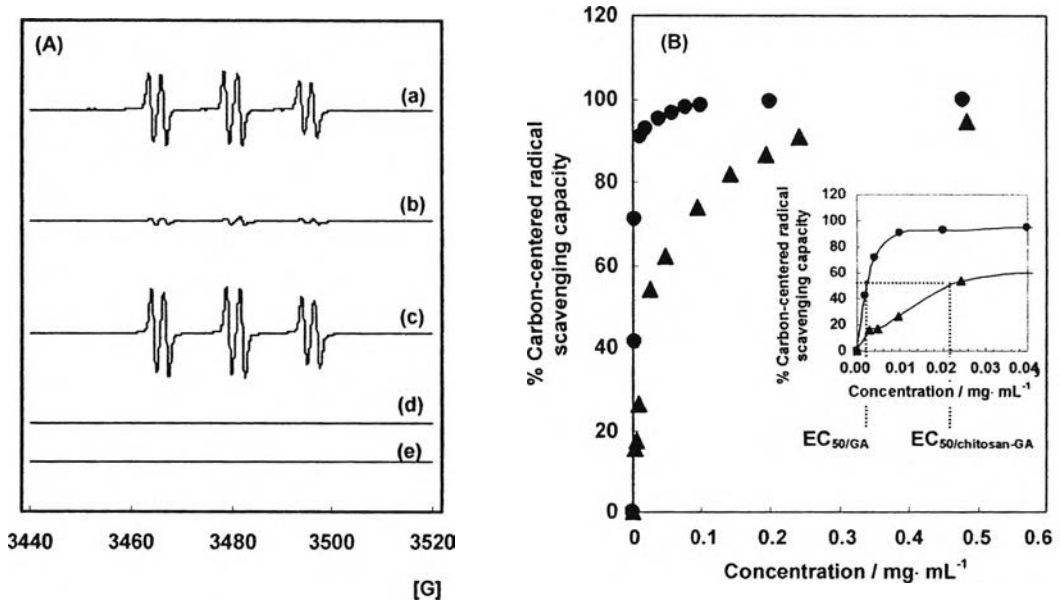


Figure 6. Chitosan-GA scavenges carbon-centered radicals. (A): EPR spectra of POBN/R$^{\bullet}$ spin adduct from: (a) the mixture of chitosan-GA (0.01 mM), POBN (25 μM), AAPH (15 mM), and PBS buffer pH 7.4 with UV exposure; (b) as in (a), but with GA (0.01 mM) instead of chitosan-GA; (c) as in (a), but without chitosan-GA and GA; (d) as in (a), but without AAPH; and (e) as in (b), but without AAPH. (B): carbon-centered radicals scavenging capacity of GA (●) and chitosan-GA (▲). Results are mean ±SD (n=3).

Figure 6(B) shows that the scavenging capacity of GA and chitosan-GA in stabilizing the carbon-centered radical is 100% when the concentration reaches 0.19 mg·mL^{-1} (1000 μM) and 0.45 mg·mL^{-1} (2400 μM), respectively. The EC_{50} values for GA and chitosan-GA are about 0.0019 mg·mL^{-1} (10 μM) and 0.021

$mg{\cdot}mL^{-1}$ (110 μM), respectively. In term of GA moiety, as 110μM of chitosan-GA contains 1.65×10^{-8} moles (110 μM×15%DS) of GA, the reducing capacity of chitosan-GA can be mentioned to be 1.65 times less than that of GA (10μM of GA contains 10^{-8} moles of GA). This value implies an effectiveness of radical scavenging capacity of chitosan-GA when we consider the substitution of galloyl group is about 15%. We suspect the role of chitosan in stabilizing carbon-centered radical, which will be further studies.

Based on the previous reports, chitosan sulfate showed antioxidant activity in carbon-centered radical scavenging capacity by 60% when the concentration is about 0.125 $mg{\cdot}mL^{-1}$, whereas in our case it was only 0.038 $mg{\cdot}mL^{-1}$ (200 μM) in stabilizing the same amount of $R^{\bullet}$.[42] This means that the conjugation of antioxidant molecule, i.e. GA, on chitosan assists the antioxidant activity to chitosan.

5.3.6 Hydroxyl radical quenching assay via the Fenton reaction

In the past, chitosan-Fe complexation was reported to enhance the antioxidation by preventing hydroxyl radical.[43] Here, the hydroxyl radical scavenging capacity of chitosan-GA was further studied via Fenton reaction (Eq. (8)).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\bullet} \qquad (8)$$

It is important to note that the reactivity of $HO^{\bullet}$ and its broad linewidth preclude the direct detection by EPR. Thus, the EPR spin trapping agent, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was used to detect $HO^{\bullet}$. DMPO forms a long-lived spin adduct with $HO^{\bullet}$ (DMPO/$HO^{\bullet}$; $a^N = a^H = 14.9$ G), allowing determination of the relative amount of $HO^{\bullet}$ in the system, Eq. (9).

DMPO DMPO/HO• (9)

A decrease in EPR signal intensity for DMPO/HO$^{\bullet}$ with an increase in GA or chitosan-GA concentration, confirms the quenching capacity of GA and chitosan-GA for HO$^{\bullet}$ (Figure 7). Figure 7 also provides some important information

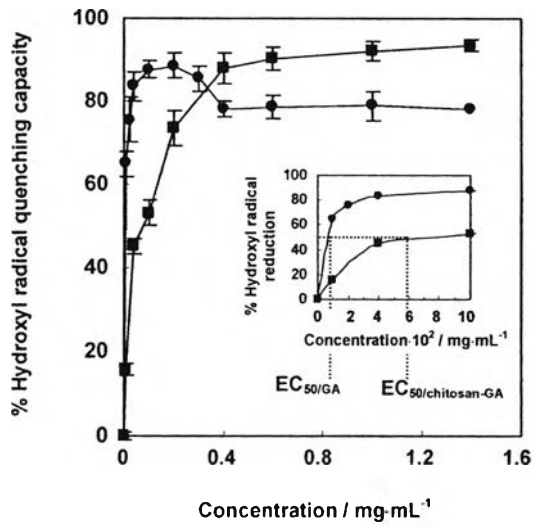


Figure 7. Hydroxyl radical quenching capacity of GA (•) and chitosan-GA (■). Results are mean ±SD (n=3).

about the function of chitosan related to the Fenton reaction. That is, the percentage of the HO$^{\bullet}$ quenching capacity is as high as 90% at a GA concentration of 0.19 mg·mL^{-1} (1000 μM), and starts to decrease when the GA concentration is higher than this. A separate experiment was carried out while increasing the H_2O_2 concentration. A similar trend was found where the HO$^{\bullet}$ quenching capacity decreased after a level of GA concentration. This might be due to the pro-oxidant chemistry of GA in the Fenton reaction. As GA is a strong reducing agent, it may reduce Fe^{3+} to Fe^{2+} (Eq. 10), as a result, increasing of the HO$^{\bullet}$ formation (Eq. (8)).

$$GA + Fe^{3+} \longrightarrow Fe^{2+} + GA^{\bullet} \qquad (10)$$

For chitosan-GA, the HO$^{\bullet}$ quenching capacity increases as the concentration of chitosan-GA increases. The HO$^{\bullet}$ quenching capacity is close to 90% at a chitosan-GA concentration above 0.38 mg·mL^{-1} (2000 μM). As it is known that

chitosan chelates metal ions[24,44] especially transition metals, we suspect that chitosan may form a complex with Fe^{2+}or Fe^{3+}, resulting in the retardation of HO$^{\bullet}$ formation *via* the Fenton reaction. Andres et al.[43] reported that the chelating of Fe^{2+} participated in Fenton reaction leaded to the decrease of hydroxyl radical generation. Here, we suspect that the amino group of chitosan effectively functions as a Fe^{2+} chelating group by forming chitosan-Fe^{2+} complex.[45] Moseley et al.[46] showed that the more substitution of SO_4^{2-} groups at amino groups the less HO$^{\bullet}$ scavenging ability.

The EC_{50} values of chitosan-GA and GA for decreasing [DMPO/HO$^{\bullet}$] are 0.066 mg·mL^{-1} (350 μM) and 0.009 mg·mL^{-1} (50 μM), respectively (Figure 7). In comparison, it is important to note that the EC_{50} in term of GA moiety of chitosan-GA (350 μM of chitosan-GA contains 5.25×10^{-8} moles of GA) is almost equal to that of GA (50 μM of GA contains 5×10^{-8} moles of GA). In other words, the antioxidant activity of chitosan-GA is comparable to that of GA even though the degree of substitution of galloyl group on chitosan is only 15%.

Guo et al.[26] reported that the antioxidant capacity to reduce HO$^{\bullet}$ by 25% required 2.5 mg·mL^{-1} of chitosan Schiff bases, whereas, in our case, it required only 0.057 mg·mL^{-1} (300 μM). At the same time, the EC_{50} of sulfated chitosan reported by Xing et al.[22] was 3.269 mg·mL^{-1}; however, our chitosan-GA was only 0.067 mg·mL^{-1}.

5.3.7 Thermal stability

The antioxidant potential, after being subjected to 100°C for 30 or 60 min, was examined by using the reduction of DPPH$^{\bullet}$. It was found that the stoichiometry ratios (DPPH$^{\bullet}$:GA) and EC_{50} of both GA (i.e., 9.2±1.3 and 8.6±1.24, respectively) and chitosan-GA (0.10±0.01 and 769±46 μM, respectively), before and after incubation at 100°C, are almost the same. The time of thermal treatment also has little effect on the antioxidation potential. This suggests the possibility of using chitosan-GA as a natural antioxidant polymer product to be used in high temperature.

5.4 Conclusions

The present work demonstrated that by simply functionalizing chitosan with H-atom donating group, i.e. gallic acid, we can achieve a novel type of chitosan antioxidant. Although the degree of substitution of gallic acid onto the chitosan was about 15%, the comparative studies of chitosan-GA and GA confirmed that the galloyl group on chitosan could effectively transfer H-atom and could subsequently form stable semiquinone radicals resulting in a wide range of antioxidation activity of chitosan-GA with oxidizing free radicals, such as carbon-centered and hydroxyl radicals. As compared to other chitosan derivatives, such as sulfated chitosan (EC_{50} = 0.1 mg·mL^{-1} for $R^{\bullet}$[42] and 3.269 mg·mL^{-1} for $HO^{\bullet}$[22]), chitosan-GA (EC_{50} = 0.021 mg·mL^{-1} for $R^{\bullet}$ and 0.066 mg·mL^{-1} for $HO^{\bullet}$) showed the lower EC_{50} approximately 5 times (for $R^{\bullet}$) and 50 times (for $HO^{\bullet}$). Based on comparative EC_{50} of chitosan-GA/GA, chitosan-GA exhibited the radical scavenging ability in the order of $HO^{\bullet}$ (0.066/0.063 mg·mL^{-1} = 1.05 times) > $R^{\bullet}$ (0.021/0.0127 mg·mL^{-1} = 1.65 times) > $DPPH^{\bullet}$ (0.14/0.01 mg·mL^{-1} = 14 times). The equivalent EC_{50} of chitosan-GA and GA for the hydroxyl radical quenching system suggested the synergistic function of chitosan to retard the pro-oxidation of GA in the Fenton reaction. The equivalent DPPH radical scavenging ability of chitosan-GA, before and after thermal treatment, confirmed the stability of galloyl group in chitosan-GA. As both chitosan and GA are natural products, chitosan-GA is expected to provide novel practical antioxidation when safety reasons are a main concern, for example food preservation, biomedical products, etc.

5.5 Experimental

5.5.1 Chemicals

Chitosan with percent degree of deacetylation (%DD) of 92 (M_v = 9.5 ×10^5 Dalton) was provided from Seafresh Chitosan (Lab) Company Limited, Thailand. Gallic acid, horseradish peroxidase (HRP, Type I), α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (POBN), and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy

(Tempol) were purchased from Sigma, USA. 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was purchased from Aldrich, USA. Zinc metal (dust), hydrogen peroxide solution (30%), sodium hydroxide solution (1 M), ferrous ammonium sulfate ($Fe(NH_4)_2 \cdot 6H_2O$), and methanol were bought from Fisher Scientific Co., Fair Lawn, NJ, USA. 2,2 -Azo*bis* (2-amidinopropane)•HCl (AAPH) was from Polysciences, Inc., USA. 1-Ethyl-3-(3 -dimethylaminopropyl) carbodiimide (EDC) and 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was from TCI, Tokyo, Japan. *N*-hydroxysuccinimide (NHS) was from Wako, Osaka, Japan. Ethanol and acetic acid (AR grade) were from Lab Scan, Co., Ltd, Thailand. Phosphate buffer saline (PBS) pH 7.4 was prepared from KH_2PO_4 (210 mg/L), Na_2HPO_4 (407 mg/L), and NaCl (9000 mg/L). All chemicals were used without further purification.

5.5.2 Instruments

Fourier transform infrared spectroscopy (FTIR) analysis was carried out by using a Thermo Nicolet Nexus 670 with 32 scans, 2 cm^{-1} resolution. Elemental analysis was carried out by using a YANAKO CHN CORDER MT-3, MT-5 with a combustion temperature at 950°C under air atmosphere with O_2 as a combustion gas (flow rate 20 mL/min) and He as a carrier (flow rate 200 mL/min). ^{13}C Cross-polarization magic angle spinning nuclear magnetic resonance (^{13}C CP/MAS-NMR) spectra were taken from a BRUKER DPX-300 at 23 ± 1°C. Proton nuclear magnetic resonance (^1H-NMR) spectra were recorded by a Bruker UXNMR/XWIN-NMR Avance DPX400 at 70°C using CD_3COOD/D_2O as a solvent. All EPR spectra were obtained from a Bruker EMX using version 3.2 software. A transverse mode (TM_{110}) cavity and flat cell were used for aqueous solution samples, and a high sensitivity (HS) cavity was used in the kinetic studies. The *g*-value was calibrated with the field by using the solid DPPH standard. Typical EPR spectrometer settings were: receiver gain, 2.5×10^5; microwave power, 10 mW; modulation frequency, 100 kHz; frequency, 9.780 GHz; modulation amplitude, 0.10 G; time constant, 40.96 ms; number of scans, 20; and scan rate, 10 G/ 21 s. UV radiation was generated by a UV PhotoMax, Model 60100 (Oriel Corp., Stratford,

USA) with a 75 W Xe bulb. The current density and applied voltage were 70 amp and 35 volt, respectively.

5.5.3 Preparation of chitosan conjugated with gallic acid (chitosan-GA)

The conjugation of chitosan with gallic acid in details was reported previously.[32] Here, the optimum condition was used. Gallic acid (GA, 3 moles equivalent to chitosan, 0.510 g) was reacted with reprecipitated chitosan (1 mole, 0.163 g) in the presence of 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC, 3 moles equivalent to GA, 1.725 g) and *N*-hydroxysuccinimide (NHS, 3 moles equivalent to GA, 1.036 g) in ethanol (50 mL). The heterogeneous mixture was left to react at ambient for 24 h. The crude product was filtered and washed thoroughly with ethanol five times to obtain chitosan-GA (Figure 1).

$(C_{27}H_{23}O_{16}N)_{0.92}(C_{21}H_{21}O_{12}N)_{0.08}$: Calcd. C 54.04, H 3.84, N 1.62, O 40.50; Found for GA:chitosan = 3:1 (DS ~ 15.62%) C 36.93, H 6.70, N 7.07, O 49.30

IR (KBr): 1640 (amide I), 1580 (amide II), 1730 (C=O ester), 3464 (OH), and 895 (pyranose ring).

^{13}C NMR: δ = 160-180 (C=O ester), 140 (aromatic), 104.6 (C-1 of pyranose ring) 75.5-82.5 (C-3 to C-5 of pyranose ring), 57.3-61.1 (C-2 and C-6 of pyranose ring).

^{1}H NMR (2% CD_3COOD in D_2O): δ = 7.6 (2H, s, H-a), 2.5 (3H, s, H-Ac), 5.3 (1H, d, H-1), 3.0 (1H, d, H-2), 3.9-4.5 (5H, m, H-3 to H-6 of pyranose ring).

5.5.4 Formation of semiquinone radicals by air-oxidation in alkaline Condition

As GA is oxidized easily at high pH, NaOH (1 M, 300 μL) was added to chitosan-GA (30 mM, 200 μL) or GA (2.5 mM, 200 μL) at room temperature to generate GA-semiquinone radicals. The solution was placed into a quartz flat cell and EPR spectra were recorded immediately using a TM_{110} cavity.

5.5.5 Formation of semiquinone radicals by horseradish peroxidase-H_2O_2

To an aqueous solution of chitosan-GA (40 mM, 375 μL), 10 μL of phosphate buffer saline (PBS, pH 7.4) solution containing H_2O_2 (20 mM) was added.

The mixture was well stirred before adding the buffer solution of HRP (0.04 μg·mL^{-1}, 10 μL). More amount of PBS buffer was added until 500 μL. The mixture was rapidly transferred to a quartz flat cell and EPR spectra were recorded by using a TM_{110} cavity. Similarly, GA (20 mM, 50 μL) in PBS was prepared.

5.5.6 Formation of semiquinone radicals by H-atom transfer to carbon centered radicals

To an aqueous solution of chitosan-GA (40 mM, 300 μL), 25 μL of PBS buffer (pH 7.4) containing AAPH (300 mM) was added. More amount of PBS buffer was added until 500 μL. The mixture was transferred to a quartz flat cell and placed to a TM_{110} cavity followed by UV exposure. EPR spectra were recorded during UV exposure at room temperature. Similarly, GA (20 mM, 50 μL) in PBS was prepared.

5.5.7 DPPH radical scavenging assay

Chitosan-GA (250 μL) solutions with the concentrations of 0.09, 0.18, 0.27, 0.37, 0.55, 0.76, and 0.92 mg·mL^{-1} (480-4850 μM) were mixed with methanolic DPPH$^{\bullet}$ solution (200 μM, 750 μL). Each mixture was reacted at room temperature under the subdued light and left for 30 min before collecting EPR spectrum. Relative percent of DPPH$^{\bullet}$ scavenging capacity was calculated from $[(h_0-h_c)/h_0] \times 100$, where h_c and h_0 are the peak heights belonging to the middle peak of DPPH$^{\bullet}$ spectrum of with and without antioxidants, respectively. Similarly, chitosan solution (in 2%v/v acetic acid) with the same concentrations as those of chitosan-GA solutions was prepared and studied. In the case of GA, the solutions were prepared at the concentrations of 3.4×10^{-3}, 6.8×10^{-3}, 8.16×10^{-3}, 10.2×10^{-3}, 13.6×10^{-3} mg·mL^{-1} (20-80 μM).

5.5.8 Stoichiometric reaction of GA, chitosan-GA, and the Zn-reduced chitosan-GA with DPPH$^{\bullet}$

The reduced chitosan-GA was prepared by treating chitosan-GA aqueous solution (4840 μM) with zinc powder (7.84 mg, 6 moles equivalent to

chitosan-GA). The reduced chitosan-GA solutions (250 μL) at 970, 1450, 1940, and 2900 μM were mixed with methanolic DPPH solution (200 μM, 750 μL) and left for 30 min under subdued light at room temperature before transferring to a quartz flat cell to collect EPR spectra using a TM_{110} cavity. Similarly, GA aqueous solutions (250 μL) at 20, 40, 60, and 80 μM and chitosan solutions (250 μL) at 970, 1450, 1940, and 2900 μM, were prepared.

5.5.9 Carbon-centered radical scavenging assay

Carbon-centered radicals were generated from AAPH *via* UV radiation. POBN was used as a spin trapping agent.[47] A series of chitosan-GA aqueous solutions (0.76 $mg{\cdot}mL^{-1}$ (4mM), for 2, 3, 6, 15, 30, 60, 90, 120, 150, and 300 μL) were mixed with PBS buffer (pH 7.4) solution containing AAPH (300 mM, 25 μL) and POBN (500 mM, 25 μL). More amount of PBS buffer was added until 500 μL. Each mixture was transferred to a quartz flat cell. The EPR spectrum of POBN/$R^{\bullet}$ spin adducts was measured while being exposed to UV radiation directly in the TM_{110} cavity at room temperature. The relative percent of carbon-centered radical scavenging capacity was calculated from $[(h_0-h_c)/h_0] \times 100$, where h_c and h_0 are the peak heights of the low field line of the POBN/$R^{\bullet}$ spectrum (a^N = 15.6 G and a^H = 2.6 G) with and without antioxidant, respectively. Similarly, the studies on carbon-centered radical scavenging capacity of GA were carried out by using 0.85 $mg{\cdot}mL^{-1}$ (5 mM) of GA for 1, 2, 5, 10, 20, 30, 40, 50, 100, and 240 μL.

5.5.10 Hydroxyl radical quenching assay via the Fenton reaction

Ferrous ammonium sulfate was dissolved in distilled water and the pH was adjusted to 2.5 by HCl (1 M) to prepare Fe^{2+} stock solution (100 μM).[48] Chitosan-GA aqueous solutions (1.89 $mg{\cdot}mL^{-1}$ (10 mM), for 2.5, 5, 10, 25, 50, 75, 100, 150, 250, 250, or 350 μL) were mixed with PBS buffer solution (pH 7.4) containing H_2O_2 (250 μM, 20 μL) and DMPO (1 M, 5 μL). The Fe^{2+} stock solution (10 μL) was added into the mixtures and more amount of PBS buffer was added until 500 μL. The mixtures were transferred to a quartz flat cell and incubated for 3 min before collecting EPR spectra. The relative percent of hydroxyl radical-quenching

capacity was calculated from $[(h_0-h_c)/h_0] \times 100$, where h_c and h_0 are peak heights of the second low-field line of the DMPO/HO$^{\bullet}$ spin adduct ($a^N = a^H = 14.9$ G) with and without antioxidants, respectively. Studies on hydroxyl radical quenching capacity of GA were carried out with the same procedures.

5.5.11 Thermal stability

Chitosan-GA aqueous solutions (4840 μM) were incubated at 100°C under aerobic conditions for 30 and 60 min. Chitosan-GA were diluted to 0.09, 0.18, 0.27, 0.37, 0.55, 0.76, and 0.92 mg·mL^{-1} (480- 4850 μM). An aliquot (250 μL) of each chitosan-GA concentration was mixed with DPPH methanolic solution (200 μM, 750 μL). The reactions were carried out at room temperature under subdued light and left for 30 min before collecting EPR spectra. In the case of GA, the similar procedures were carried out but using 100 μM of GA incubated at 100°C and diluted to 1.73×10^{-3}, 4×10^{-3}, 6.8×10^{-3}, 8.16×10^{-3}, 10.2×10^{-3}, 13.6×10^{-3} mg·mL^{-1} (10-80 μM).

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