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Preparation, characterization, and antioxidant properties of gallic acid-*grafted*-chitosans

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ABSTRACT

Gallic acid-*grafted*-chitosans (GA-*g*-chitosans) with four different grafting ratios were prepared by a free radical-induced grafting reaction in order to improve antioxidant and water-solubility. To verify the synthesis of GA-*g*-chitosans, ^{1}H NMR and thin layer chromatography were employed, and the results revealed that GA was grafted onto the chitosan. The antioxidant properties of the GA-*g*-chitosans were evaluated using several *in vitro* models. GA-*g*-chitosan (I), which has the highest GA content, showed 92.26% scavenging activity against 2,2-diphenyl-1-picrylhydrazyl and 93.15% hydrogen peroxide scavenging activity at 50 μg/mL. GA-*g*-chitosan (I) was also showed higher reducing power compared to others. All GA-*g*-chitosans showed improved antioxidant capacities compared to plain chitosan treated in the same conditions without gallic acid grafting. Furthermore, the GA-*g*-chitosans also exhibited good cytocompatibility and effectively inhibited the formation of intracellular reactive oxygen species (ROS) in time- and dose-dependent manner in RAW264.7 macrophages.

1. Introduction

The uncontrolled generation of reactive oxygen species (ROS) and free radicals is involved in the pathogenesis of several human diseases including cancer, aging, and atherosclerosis (Moskovitz, Yim, & Chock, 2002). Antioxidants reduce or retard free radical and ROS generation and prevent the oxidation of biomacromolecules including DNA, membrane lipids, and proteins. Several synthetic antioxidants are extensively used; however, these antioxidants must be used under strict regulations due to their potential risk *in vivo*. Therefore, much attention has been given to the use of naturally occurring antioxidants to inhibit oxidative damage.

Chitosan has been chemically and enzymatically modified to impart new physical and chemical properties, and numerous research works regarding antioxidant activity have been carried out (Guo et al., 2005; Muzzarelli & Muzzarelli, 2005; Sousa, Guebitz, & Kokol, 2009; Xing et al., 2005; Zhong, Zhong, Xing, Li, & Mo, 2010).

Polyphenols are usually referred to as a diverse group of naturally occurring compounds containing multiple phenolic functionalities (Tuchmantel, Kozikowski, & Romanczyk, 1999). Naturally occurring polyphenols are known to have numerous biological activities; in particular, they have recently received attention as antioxidants. It has been suggested that ingesting polyphenols may be beneficial to human health (Flight & Clifton, 2006). Gallic acid (GA) is a natural phenolic antioxidant extractable from plants, especially green tea (Lu, Nie, Belton, Tang, & Zhao, 2006), and is widely used in foods, drugs, and cosmetics. It is possible to reduce the intra- and intermolecular hydrogen bond networks of chitosan by means of the bulky group of the benzene ring of GA, and to increase the antioxidant ability of chitosan by conjugation of GA onto chitosan.

The objective of this study was to synthesize GA-*grafted*-chitosans and to evaluate their antioxidant properties in different antioxidant assays, in order to verify the possibility for applications in functional foods and/or the pharmaceutical industry.

2. Materials and methods

2.1. Materials

The chitosan was kindly donated by Kitto Life Co. (Seoul, Korea). The average molecular weight and degree of deacetylation were 310 kDa and 90%, respectively. Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteau's phenol reagent, hydrogen peroxide, 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), peroxidase, and potassium ferricyanide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

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2.2. Preparation of gallic acid-grafted-chitosans (GA-g-chitosans)

The synthesis of GA-g-chitosans was performed according to the method of Curcio et al. (2009) with slight modifications. Briefly, the chitosan (0.5 g) was dissolved in 50 mL of 2% acetic acid (v/v) and then 1 mL of 1.0 M H_2O_2 containing 0.054 g of ascorbic acid was added. After 30 min, gallic acid was added to the mixture at different amounts with the following molar ratios of repeating unit of chitosan to gallic acid: 1:1, 1:0.5, 1:0.25, and 1:0.1. These GA-g-chitosans were designated as GA-g-chitosan (I), GA-g-chitosan (II), GA-g-chitosan (III), and GA-g-chitosan (IV), respectively. Finally, the mixture was allowed to rest at 25 °C for 24 h under atmospheric air, and then dialyzed with distilled water for 48 h in order to remove unreacted gallic acid.

2.3. Analytical determinations

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 70 °C for samples dissolved in 2% CD_3COOD/D_2O (v/v) using a JEOL JNM ECP-400 NMR spectrometer under a static magnetic field of 400 MHz.

To verify whether gallic acid was grafted onto chitosan, TLC analysis was performed. Gallic acid, plain chitosan, and GA-g-chitosan separations were performed on a silica gel plate by development with chloroform–ethyl acetate–acetic acid (50:50:1). The separated TLC plate was detected using 30% H_2SO_4 followed by heating at 100 °C for 10 min.

Gallic acid contents were determined by the method of Singleton, Orthofer, and Lamuela-Raventos (1999). Briefly, 40 μL of GA-g-chitosans (1 mg/mL) was mixed with 200 μL of Folin-Ciocalteau reagent and 1160 μL of distilled water for 3 min, followed by 600 μL 20% sodium carbonate (Na_2CO_3). The mixture was shaken for 2 h at room temperature and then a 200 μL aliquot of the mixture was added to each well of a 96-well microplate. Absorbance was measured at 720 nm using a microplate reader (SpectraMax® M2/M2^e, CA, USA). Gallic acid was used as a standard.

The DPPH scavenging activity of the GA-g-chitosans was measured according to the method of Blois (1958), with slight modifications. Briefly, the DPPH solution (1.5×10^{-4} M, 100 μL) was mixed with and without GA-g-chitosans (100 μL), after which the mixture was incubated at room temperature for 30 min. The absorbance of the mixture was then determined at 517 nm using a microplate reader.

The hydrogen peroxide scavenging activity of the GA-g-chitosans was determined according to the method described by Müller (1985). Briefly, 100 μL of 0.1 M phosphate buffer (pH 5.0) and GA-g-chitosans were mixed in a 96-well microplate. Next, 20 μL of hydrogen peroxide was added and the mixture was incubated at 37 °C for 5 min. Following incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, which was subsequently incubated at 37 °C for 10 min. The absorbance was then measured at 405 nm using a microplate reader.

The reducing power of the GA-g-chitosans was determined using the method described by Oyaizu (1986). Briefly, the GA-g-chitosans were mixed with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of potassium ferricyanide (1%, w/v). The mixture was then incubated at 50 °C for 20 min. Next, 0.5 mL of TCA (10%, w/v) was added to the mixture, which was then centrifuged at $1036 \times g$ for 10 min. Finally, a 0.5 mL aliquot of the upper layer of the solution was mixed with 0.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1%, w/v), after which the absorbance was measured at 700 nm. A higher absorbance was regarded as greater reducing power.

2.4. Cell culture

RAW264.7 macrophage cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were incubated at 37 °C in a humidified atmosphere (5% CO_2).

2.5. Cytotoxicity assay

The cytotoxicity of the GA-g-chitosans was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were grown in 96-well plates at a density of 1×10^4 cells/well. After 24 h, the cells were treated with control medium or medium supplemented with different concentrations of GA-g-chitosans. After incubation for 24 h, MTT solution (1 mg/mL) was added and incubated for another 4 h. Finally, 100 μL of DMSO was added to solubilize the formed formazan crystals. The amount of formazan crystals was determined by measuring the absorbance at 540 nm using an ELISA reader (SpectraMax® M2/M2^e, CA, USA).

2.6. Determination of intracellular ROS formation using DCFH-DA labeling

Intracellular ROS formation was assessed according to a previously described method, employing the oxidation sensitive dye DCFH-DA as the substrate (Engelmann, Volk, Leyhausen, & Geurtsen, 2005). The RAW264.7 macrophage cells growing in black microtiter 96-well plates were labeled with 20 μM DCFH-DA in HBSS for 20 min in the dark. The cells were then treated with different concentrations of GA-g-chitosans and incubated for 1 h. After washing the cells three times with PBS, 500 μM H_2O_2 (in HBSS) was added. The formation of 2′,7′-dichlorofluorescein (DCF), due to the oxidation of DCFH in the presence of various ROS, was read after every 30 min at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (SpectraMax® M2/M2^e, CA, USA). Following a maximum rate of fluorescence increase, each well was normalized to cell numbers using the MTT cell viability assay. The dose-dependent and time-dependent effects of the treatment groups were plotted and compared with the fluorescence intensity of a control group in which samples were not treated.

3. Results and discussion

3.1. Preparation and characterization of GA-g-chitosans

The synthesized GA-g-chitosans were characterized by ^{1}H NMR spectroscopy. Fig. 1 shows the ^{1}H NMR spectrum of GA-g-chitosan (I). As shown in Fig. 1, plain chitosan peaks occurred at $\delta = 2.5$ (H-Ac), 5.3 (H-1), 3.6 (H-2), and 4.0–4.5 (H-3 to H-6 of pyranose ring), and the GA-g-chitosan showed a new peak at 7.6 ppm belonging to phenyl protons as compared to the chitosan. This confirms the conjugation of GA onto chitosan.

As shown in Fig. 2, gallic acid alone was detected on the chromatogram but it was not detected in the plain chitosan and GA-g-chitosan. This further verifies that gallic acid chemically grafted onto the chitosan and this was not a mixture condition.

A possible mechanism of GA insertion onto chitosan is related to the formation of macromolecular chitosan radicals by hydroxyl radicals that are generated by interactions between redox pair components. The major attack target of hydroxyl radicals in chitosan is NH_2 at C-2, and OH groups at the C-6 position, and the minor attack target is OH groups at the C-3 position due to steric hindrance. Xie, Xu, & Liu (2001) also suggested that the scavenging mechanism of

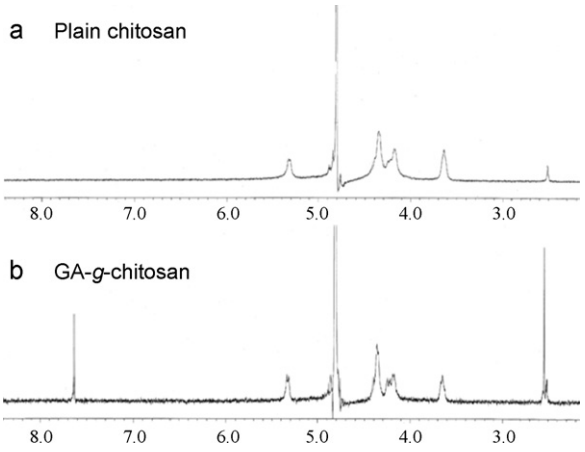

Fig. 1. ^{1}H NMR spectrum of gallic acid-*grafted*-chitosan (I).

chitosan against free radicals may be related to the active hydrogen of NH_2 at C-2 and the OH groups at C-6 positions. Therefore, GA can interact with macromolecular chitosan radicals generated by redox pair systems, particularly NH_2 at C-2 and OH groups at the C-6 position.

3.2. Determination of GA contents in GA-g-chitosans

GA contents in the GA-*g*-chitosans increased with increasing molar ratio. The GA contents of GA-*g*-chitosan (I), GA-*g*-chitosan (II), GA-*g*-chitosan (III), and GA-*g*-chitosan (IV) were 118.92, 82.91, 67.62, and 53.87 mg GA/g GA-*g*-chitosan, respectively (Fig. 3). The grafting ratio was then calculated from the GA content in the GA-*g*-chitosans and the utilized amount of GA in the preparation of the GA-*g*-chitosans. Therefore, the grafting ratios of GA onto chitosan were 11.89%, 16.98%, 27.05%, and 53.88% with regard to GA-*g*-chitosan (I), GA-*g*-chitosan (II), GA-*g*-chitosan (III), and GA-*g*-chitosan (IV), respectively. According to these results, the optimal condition for the synthesis of GA-*g*-chitosan was 1:0.1 as a molar ratio of repeating unit of chitosan to GA.

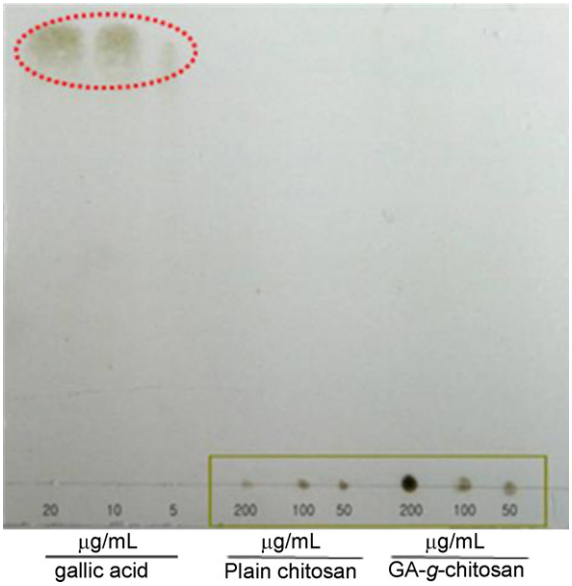

Fig. 2. TLC chromatogram of gallic acid, plain chitosan, and gallic acid-*grafted*-chitosan (I).

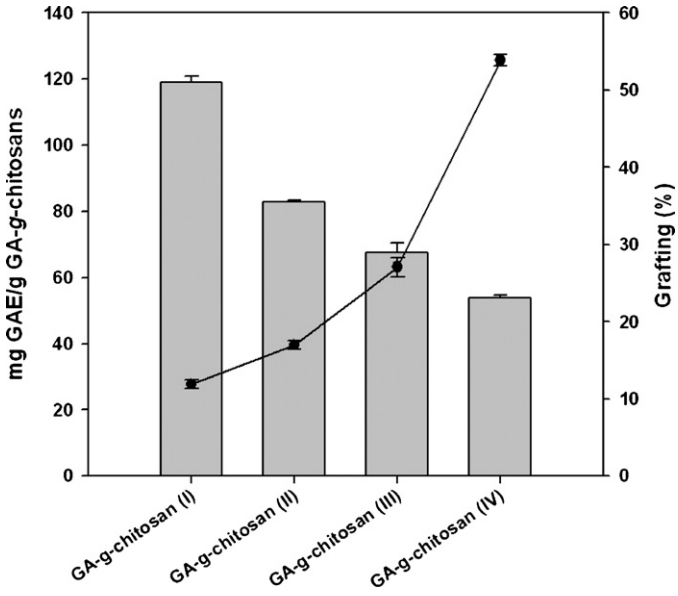

Fig. 3. GA content and grafting percentage of gallic acid-*grafted*-chitosan.

3.3. DPPH scavenging capacity

As shown in Fig. 4, the GA-*g*-chitosans strongly quenched DPPH radicals in a dose-dependent manner. DPPH scavenging activity was in the order of GA-*g*-chitosan (I) > GA-*g*-chitosan (II) > GA-*g*-chitosan (III) > GA-*g*-chitosan (IV) > plain chitosan. This activity corresponds to the GA content of the GA-*g*-chitosan, in which a high GA content resulted in high DPPH scavenging activity. We also evaluated GA alone, and the concentration of GA alone was similar to that in GA-*g*-chitosan (I). Plain GA showed 49.03% DPPH scavenging capacity at 5 μg/mL, and this activity was lower than that of GA-*g*-chitosan (I). These results indicate that the grafting of GA onto chitosan improved the antioxidant capacity of plain chitosan.

It has been documented that chitosan has antioxidant activity in various *in vitro* systems, and the hydrogen-donating ability of

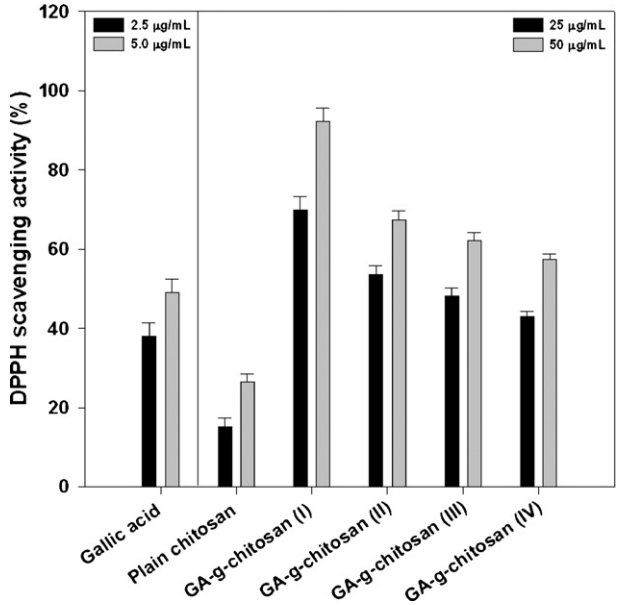

Fig. 4. DPPH radical scavenging capacities of gallic acid, plain chitosan, and gallic acid-*grafted*-chitosans. All statistical analyses were performed with three independent experiments and data are represented as means ± S.E.

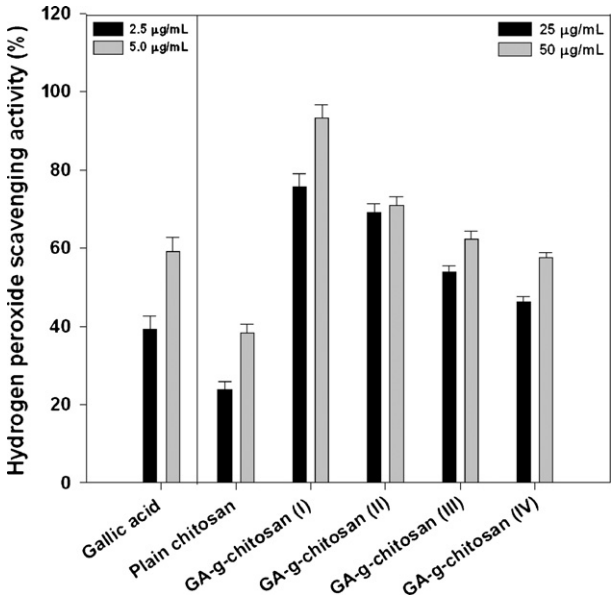

Fig. 5. Hydrogen peroxide scavenging capacities of gallic acid, plain chitosan, and gallic acid-*grafted*-chitosans. All statistical analyses were performed with three independent experiments and data are represented as means ± S.E.

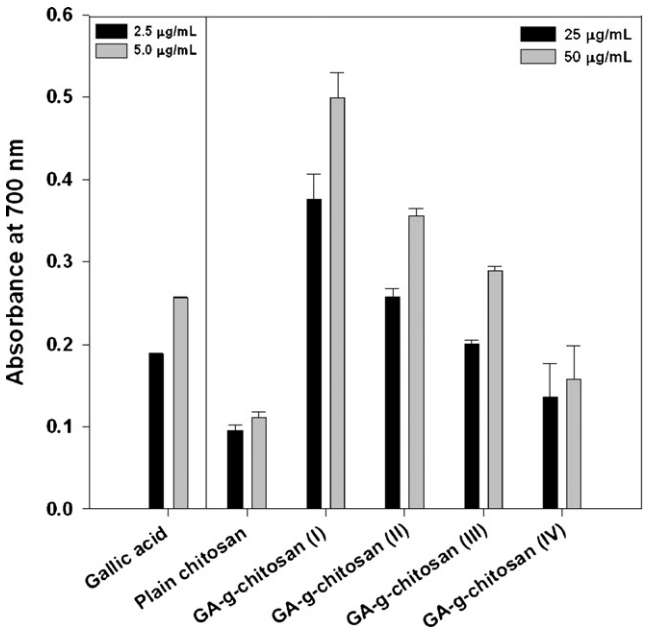

Fig. 6. Reducing power of gallic acid, plain chitosan, and gallic acid-*grafted*-chitosans. All statistical analyses were performed with three independent experiments and data are represented as means ± S.E.

chitosan is a major antioxidant mechanism (Curcio et al., 2009). In addition, GA is well known for its antioxidant ability by its hydrogen-donating capacity. Therefore, we attempted to improve not only the water-solubility of chitosan by interfering with intra- and intermolecular hydrogen bonds, but also its antioxidant activity by grafting of GA onto chitosan. According to the above results, plain chitosan showed weak DPPH radical scavenging capacity whereas GA-*g*-chitosans exhibited improved DPPH radical scavenging activities, and the GA-*g*-chitosans dissolved well in water up to 5 mg/mL. These results indicate that GA grafting onto chitosan by redox pair systems increased the antioxidant capacity and water-solubility of plain chitosan. Two kinds of experiments using different methods have been reported for the grafting of GA onto chitosan (Curcio et al., 2009; Pasanphan, Buettner, & Chirachanchai, 2010). The DPPH radical scavenging capacities of the reported materials were 92% at 1000 μg/mL (Curcio et al., 2009) and 87% at 230 μg/mL (Pasanphan et al., 2010). In the present study, GA-*g*-chitosan (I) had a 92.26% scavenging activity at 50 μg/mL, and GA-*g*-chitosan (IV), which had the lowest DPPH scavenging activity, showed a scavenging activity of around 57.51% at 50 μg/mL. These comparisons indicate that the GA-*g*-chitosans prepared in this study had superior antioxidant activities compared to others in the literature. Furthermore, the previous two studies did not carry out comparison experiments with respect to antioxidant activity between the GA and the GA-*g*-chitosans. Our findings demonstrate that the antioxidant capacity of plain chitosan was improved by the grafting of GA onto plain chitosan.

3.4. Hydrogen peroxide scavenging capacity

Hydrogen peroxide, a reactive non radical, is very important because it can penetrate biological membranes, thereby it is easily converted into more reactive species such as singlet oxygen and hydroxyl radicals, which can then initiate lipid peroxidation or induce toxic effects in cells. Therefore, the measurement of hydrogen peroxide scavenging activity is known as a useful method for determining the ability of antioxidants to decrease levels of prooxidants such as hydrogen peroxide (Czochra & Widensk, 2002), and the scavenging of hydrogen peroxide by antioxidants may be attributed to their electron donating ability. As shown in Fig. 5, the GA-*g*-chitosans scavenged hydrogen peroxide as concentration increased, and GA-*g*-chitosan (I) showed a 93.15% hydrogen peroxide scavenging capacity at 50 μg/mL, whereas plain chitosan showed weak hydrogen peroxide scavenging capacity. GA alone also scavenged 59.20% hydrogen peroxide at 5 μg/mL, which was similar GA content to that in GA-*g*-chitosan (I). The results also indicate that the activity of plain chitosan was improved through the grafting.

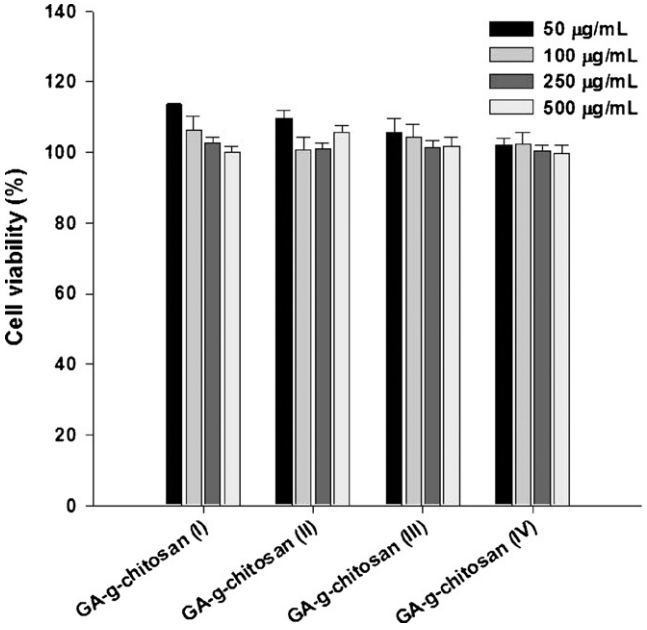

Fig. 7. Cytotoxic effects of GA-*g*-chitosans on RAW264.7 macrophages. Cells were grown at a density of 1×10^4 cells/well and different concentrations of gallic acid-*grafted*-chitosans were treated. After 24 h of incubation, MTT solution was treated to each well and incubated for another 4 h. The formation of formazan crystals was solubilized using DMSO and absorbance was measured at 540 nm. All statistical analyses were performed with three independent experiments and data are represented as means ± S.E.

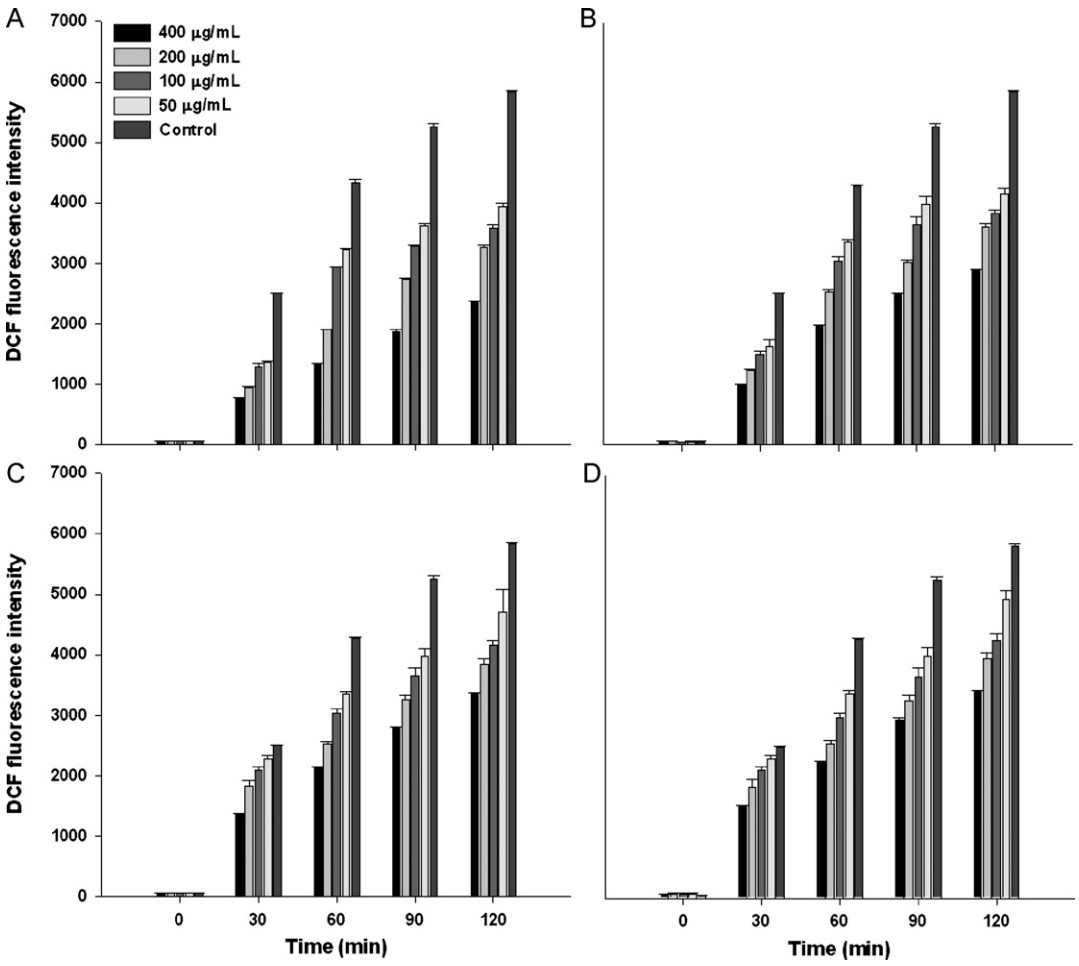

Fig. 8. Effect of gallic acid-*grafted*-chitosans on the inhibition of intracellular generation of ROS in RAW264.7 macrophages. Intracellular ROS formation was assessed using the oxidation sensitive dye, DCFH-DA. Formation of DCF due to DCFH oxidation was read at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

3.5. Reducing power

Fig. 6 depicts the reducing power of the GA-*g*-chitosans, which were found to have good ability for reducing ferric iron (III) to ferrous iron (II). In addition, the reducing power of the GA-*g*-chitosans increased as concentration increased, and the reducing power of the GA-*g*-chitosans was higher than that of the plain chitosan. GA also showed good reducing power but this activity was lower than that of GA-*g*-chitosan (I).

3.6. Cytotoxicity and intracellular ROS determination

As depicted in Fig. 7, the GA-*g*-chitosans did not exert any significant ($p < 0.01$) toxic effects on the RAW264.7 mouse macrophage cells under the tested concentrations after 24 h of treatment. Therefore, non-toxic concentrations of GA-*g*-chitosans were used for the cell-based antioxidant assays with regard to intracellular ROS formation.

Intracellular ROS were determined using the fluorescence sensitive dye, DCFH-DA. During labeling, non-fluorescent DCFH-DA dye, which freely penetrates into cells, was hydrolyzed by intracellular esterases to DCFH and trapped inside the cells. DCFH further oxidized to DCF by ROS, which emitted fluorescence. Intracellular ROS formation in the presence of GA-*g*-chitosans is shown in Fig. 8. The fluorescence emitted by DCF following ROS-mediated oxidation of DCFH followed a time course increment up to 120 min. Pre-treatment with GA-*g*-chitosans decreased DCF fluorescence dose- and time-dependently. Considerable ROS scavenging effects were observed by the GA-*g*-chitosans even after 30 min, at all tested concentrations. In particular, intracellular ROS formation was significantly inhibited by GA-*g*-chitosan (I) as compared to GA-*g*-chitosans (II), (III), and (IV). These activities are in agreement with antioxidant abilities in the non-cellular systems.

4. Conclusion

In the present study, four different grafting ratios of the GA-*g*-chitosans were prepared by a free radical-induced grafting reaction and the grafting of GA onto chitosan was confirmed via ^{1}H NMR and TLC. The GA-*g*-chitosans exhibited obviously antioxidant activity against DPPH radical scavenging, hydrogen peroxide scavenging, and reducing power and the activity was superior to plain chitosan. In addition, the antioxidant activities were augmented with increasing GA content in GA-*g*-chitosans. The GA-*g*-chitosans exhibited good cytocompatibility against RAW264.7 mouse macrophages. Furthermore, the GA-*g*-chitosans effectively inhibited the formation of intracellular ROS in a time- and dose-dependent manner in RAW264.7 mouse macrophages. These results suggest that the optimized grafting ratio for GA-*g*-chitosans might provide beneficial effects in the treatment of diseases related to oxidative damage.

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