JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 5933–5938 5933
DOI:10.1021/jf900778u

Covalent Insertion of Antioxidant Molecules on Chitosan by a Free Radical Grafting Procedure

Manuela Curcio, Francesco Puoci,* Francesca Iemma, Ortensia Ilaria Parisi, Giuseppe Cirillo, Umile Gianfranco Spizzirri, and Nevio Picci

Dipartimento di Scienze Farmaceutiche, Università della Calabria, Edificio Polifunzionale, Arcavacata di Rende (CS) 87036, Italy

In this work, the synthesis of gallic acid−chitosan and catechin−chitosan conjugates was carried out by adopting a free radical-induced grafting procedure. For this purpose, an ascorbic acid/hydrogen peroxide redox pair was employed as radical initiator. The formation of covalent bonds between antioxidant and biopolymer was verified by performing UV, FT-IR, and DSC analyses, whereas the antioxidant properties of chitosan conjugates were compared with that of a blank chitosan, treated in the same conditions but in the absence of antioxidant molecules. The good antioxidant activity shown by functionalized materials proved the efficiency of the reaction method.

KEYWORDS: Grafting; redox initiators; chitosan; antioxidant

INTRODUCTION

Chitosan is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine obtained by alkaline N-deacetylation of chitin. The sugar backbone consists of β-1,4-linked glucosamine (*1*), and it has been known as a bioactive molecule. Several bioactivities such as antitumor activity (*2*), immunoenhancing effects (*3*), wound healing effects (*4*), antifungal and antimicrobial properties (*5*), and antioxidant activity (*6*) of chitosan have been reported.

These characteristics, together with several unique properties such as nontoxicity, biocompatibility, and biodegradability, offer chitosan good potential for biomedical applications, in the food industry as an edible coating for fruits and vegetables (*7*) or packaging film (*8*), and in wastewater purification (*9*).

It is well-known that for some specific polymeric products, especially medical equipment and food packaging, sterilization via radiation is needed with a potential risk of degradation, that is, chain scission and/or cross-linking, resulting in discoloration, cracking of the surface, stiffening, and loss of mechanical properties (*10*).

These serious drawbacks could be controlled by performing chemical modifications of the polymeric backbone.

Specifically for chitosan, to improve the polymer processability, chemical and enzymatic modification reactions were designed. However, chemical modifications are generally not preferred for food applications because of the formation of potential detrimental products (*11*).

In addition, several research works report the applicability of antioxidants as additives for polymers, as they stabilize the polymer from resin extrusion to the molded pieces production. During processing, the antioxidant retards thermal and/or oxidative degradation (*12*). On the other hand, antioxidants with low molecular weight are less effective owing to their poor thermal stability. To overcome this limitation, a useful approach is the covalent linkage of these molecules on a polymeric matrix, enhancing their stability and reducing the effects of migration and blooming. These can cause antioxidants to be easily removed from the host polymer by mechanical rubbing-off, volatilization, or leaching (*13*).

In recent years, several synthetic strategies (*14*, *15*) have been proposed to obtain macromolecular systems, consisting of antioxidant−polymer conjugates, that, combined with the advantages of both components, show a higher stability and a slower degradation rate than molecules with low molecular weight but preserve the unique properties of antioxidant molecules.

In the literature, many studies about the synthesis of chitosan−antioxidant conjugates are reported, but multistep organic syntheses are required (*16*, *17*). This work reports a rapid and ecofriendly procedure for the covalent insertion of antioxidant molecules on chitosan by employing a free radical grafting procedure.

Our synthetic strategy is based on the use of an H_2O_2/ascorbic acid redox pair to functionalize, in a single-step, chitosan with (2*R*)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychroman-4-one [(+)-catechin] and 3,4,5-trihydroxybenzoic acid (gallic acid). The use of this redox system allows the chemical functionalization of chitosan to be performed without the generation of toxic compounds and with high reaction yields.

Gallic acid is a natural phenolic antioxidant extractable from plants, especially green tea (*18*). It is widely used in foods, drugs, and cosmetics to prevent rancidity induced by lipid peroxidation and spoilage.

Catechins are one of the main classes of flavonoids and are present in tea, wine, chocolate, fruits, etc. They are potentially beneficial to human health as they are strong antioxidants, anticarcinogens, antiinflammatory agents, and inhibitors of platelet aggregation in in vivo and in vitro studies (*19*).

*Corresponding author (telephone 0039 0984 493151; fax 0039 0984 493298; e-mail francesco.puoci@unical.it).

 Published on Web 06/12/2009

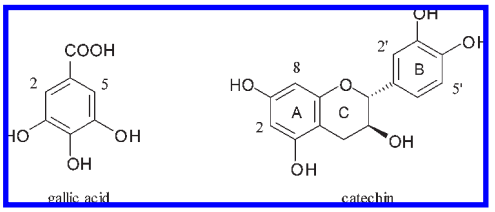

Figure 1. Chemical structures of gallic acid and (+)-catechin.

The conjugates were characterized by DSC, UV, and FT-IR analyses, and then their antioxidant properties were tested by performing different antioxidant assays.

MATERIALS AND METHODS

Materials. Gallic acid, (+)-catechin (**Figure 1**), chitosan from crab shells (MW = 95 kDa, 85% deacetylation), hydrogen peroxide (H_2O_2), ascorbic acid, 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), Folin−Ciocalteu reagent, sodium carbonate, sulfuric acid (96% w/w), trisodium phosphate, ammonium molybdate, β-carotene, linoleic acid, Tween 20, deoxyribose, $FeCl_3$, ethylenediaminetetraacetic acid disodium salt (EDTA), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid, trichloroacetic acid, and hydrochloric acid (37% w/w) were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO).

Ethanol and chloroform were of HPLC-grade and provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

Synthesis of Chitosan Conjugates. The synthesis of both catechin-*grafted*-chitosan and gallic acid-*grafted*-chitosan was performed as follows: in a 25 mL glass tube, chitosan (0.5 g) was dissolved in 10 mL of acetic acid water solution (2% v/v). Then, 1 mL of 1.0 M H_2O_2 containing 0.054 g of ascorbic acid was added. Finally, after 30 min, 0.35 mmol of antioxidant molecule was introduced into the reaction flask, and the mixture was maintained at 25 °C for 24 h under atmospheric air. The obtained polymer solution was introduced into dialysis tubes (MWCO 12000−14000 Da) and dipped into a glass vessel containing distilled water at 20 °C for 48 h with eight changes of water. The copolymer was checked to be free of unreacted antioxidants and any other compounds by HPLC analysis after the purification step.

The resulting solution was frozen and dried with a "freezing−drying apparatus" to afford a vaporous solid. Blank chitosan, which acta as a control, was prepared in the same conditions but in the absence of antioxidant agents.

Instrumentation. The liquid chromatography consisted of a Jasco BIP-I pump and a Jasco UVDEC-100-V detector set at 230 nm. A 250 mm × 4 mm C-18 Hibar column, particle size = 5 μm, pore size = 120 Å (Merck, Darmstadt, Germany), was employed. As reported in the literature (*20*), the mobile phase adopted for the detection of catechin and gallic acid was methanol/water/orthophosphoric acid (20:79.9:0.1), and the flow rate was 1.0 mL/min. The column was operated at 30 °C. The sample injection volume was 20 μL. IR spectra were recorded as films or KBr pellets on a Jasco FT-IR 4200. A freeze-dryer Micro Modulyo, Edwards, was employed.

UV−vis absorption spectra were obtained with a Jasco V-530 UV−vis spectrometer. Calorimetric analyses were performed using a Netzsch DSC200 PC. In a standard procedure about 6.0 mg of sample was placed inside a hermetic aluminum pan, and the pan was then sealed tightly by a hermetic aluminum lid. Thermal analyses were performed from 25 to 400 °C under a dry nitrogen atmosphere with a flow rate of 25 mL min^{-1} and a heating rate of 5 °C min^{-1}.

Determination of Scavenging Effect on DPPH Radicals. To evaluate the free radical scavenging properties of both chitosan−antioxidant conjugates, their reactivity toward a stable free radical, 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), was evaluated (*21*). For this purpose, 20 mg of each polymer was dissolved in 1 mL of distilled water in a volumetric flask (25 mL), and then 4 mL of ethanol and 5 mL of ethanol solution of DPPH (200 μM) were added, obtaining a solution of DPPH with a final concentration of 100 μM. The sample was incubated in a water bath at 25 °C and, after 30 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The same reaction conditions were applied on the blank chitosan to evaluate the interference of polymeric material in the DPPH assay. The scavenging activity of the tested polymeric materials was measured as the decrease in absorbance of the DPPH, and it was expressed as percent inhibition of DPPH radicals calculated according to eq 1

$$\text{inhibition } \% = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without any polymers, and A_1 is the absorbance of polymeric samples. Each measurement was carried out in triplicate, and data are expressed as means (±SEM).

β-Carotene−Linoleic Acid Assay. The antioxidant properties of synthesized functional polymers were evaluated through measurement of percent inhibition of peroxidation in a linoleic acid system by using the β-carotene bleaching test (*22*). Briefly, 1 mL of β-carotene solution (0.2 mg/mL in chloroform) was added to 0.02 mL of linoleic acid and 0.2 mL of Tween 20. The mixture was then evaporated at 40 °C for 10 min in a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 mL of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion. The emulsion (5 mL) was transferred to different test tubes containing 50 mg of antioxidants-*grafted*-chitosan dispersed in 0.2 mL of 70% ethanol, and 0.2 mL of 70% ethanol in 5 mL of the above emulsion was used as a control. The tubes were then gently shaken and placed in a water bath at 45 °C for 60 min. The absorbance of the filtered samples and control was measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at the initial time (t = 0) and successively at 60 min. The same reaction conditions were applied by using blank chitosan.

The antioxidant activity ($A_{ox}A$) was measured in terms of successful bleaching of β-carotene using eq 2

$$A_{ox}A = \left(1 - \frac{A_0 - A_{60}}{A_0^{\circ} - A_{60}^{\circ}}\right) \quad (2)$$

where A_0 and A_0° are the absorbance values measured at the initial incubation time for samples and control, respectively, whereas A_{60} and A_{60}° are the absorbance values measured in the samples and in control, respectively, at t = 60 min. All samples were assayed in triplicate, and data are expressed as means (±SEM).

Evaluation of Disposable Phenolic Groups by Folin−Ciocalteu Procedure. Amount of total phenolic equivalents was determined using Folin−Ciocalteu reagent procedure, according to the literature with some modifications (*23*).

Twenty milligrams of chitosan−antioxidant conjugates was dissolved in distilled water (6 mL) in a volumetric flask. Folin−Ciocalteu reagent (1 mL) was added, and the contents of the flask were mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2%) was added, and then the mixture was allowed to stand for 2 h with intermittent shaking.

The absorbance was measured at 760 nm against a control prepared using the blank polymer under the same reaction conditions. The amount of total phenolic groups in each polymeric materials was expressed as gallic acid and catechin equivalent concentrations, respectively, by using the equations obtained from the calibration curves of each antioxidant. These were recorded by employing five different gallic acid and catechin standard solutions. Half a milliliter of each solution was added to the Folin−Ciocalteu system to raise the final concentrations to 8.0, 16.0, 24.0, 32.0, and 40.0 μM, respectively. After 2 h, the absorbance of the solutions was measured to record the calibration curve, and the correlation coefficient (R^2), slope, and intercept of the regression equation obtained were calculated by using the method of least-squares.

Determination of Total Antioxidant Activity. The total antioxidant activity of polymeric materials was evaluated according to the method reported in the literature (*24*). Briefly, 100 mg of chitosan−antioxidant conjugates was mixed with 2.4 mL of reagent solution (0.6 M sulfuric acid, 28 M sodium phosphate, and 4 M ammonium molybdate) and 0.6 mL of methanol, and then the reaction mixture was incubated at 95 °C for 150 min. After cooling to room temperature, the absorbance of the mixture

was measured at 695 nm against a control prepared using blank polymer in the same reaction. The total antioxidant activity of each polymeric material was expressed as equivalent concentration of the respective antioxidant molecule.

By using five different gallic acid and catechin standard solutions, a calibration curve was recorded. An amount of 0.3 mL of each solution was mixed with 1.2 mL of reagent solution to obtain final concentrations of 8.0, 16.0, 24.0, 32.0, and 40.0 μM, respectively. After 150 min of incubation, the solutions were analyzed by UV−vis spectrophotometry, and the correlation coefficient (R^2), slope, and intercept of the regression equation obtained by using the method of least-squares were calculated.

Determination of Scavenging Effect on Hydroxyl Radical (OH•). The scavenging effect on hydroxyl radical was evaluated according to the literature (*25*). Briefly, 20 mg of chitosan−antioxidant conjugates was dispersed in 0.5 mL of 95% ethanol and incubated with 0.5 mL of deoxyribose (3.75 mM), 0.5 mL of H_2O_2 (1 mM), 0.5 mL of $FeCl_3$(100 mM), 0.5 mL of EDTA (100 mM), and 0.5 mL of ascorbic acid (100 mM) in 2.0 mL of potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C. Then samples were filtered, and to 1 mL of filtrate were added 1 mL of thiobarbituric acid (1% w/v) and 1 mL of trichloroacetic acid (2% w/v); the tubes were heated in a boiling water bath for 15 min. The content was cooled, and the absorbance of the mixture was read at 535 nm against reagent blank without extract.

The antioxidant activity was expressed as a percentage of scavenging activity on hydroxyl radical according to eq 1. All samples were assayed in triplicate, and data are expressed as means (±SEM).

RESULTS AND DISCUSSION

Synthesis of Antioxidant−Chitosan Conjugates. Chitosan was chosen as a polymeric backbone to synthesize two different biomacrolecule-based antioxidants containing the antioxidative groups of catechin and gallic acid, respectively.

The conjugation of the antioxidant moieties on the chitosan chains was performed by free radical-induced grafting reaction. A biocompatible and water-soluble system, an ascorbic acid/hydrogen peroxide pair, was chosen as redox initiator system. The interaction mechanism between the two components of the redox pair involves the oxidation of ascorbic acid by H_2O_2 at room temperature with the formation of ascorbate and hydroxyl radicals, which initiate the reaction (*26*).

Compared to conventional initiator systems (i.e., azo compounds and peroxides), which require relatively high reaction temperature to ensure their rapid decomposition, redox initiators show several advantages. First of all, this kind of system does not generate toxic reaction products; moreover, it is possible to perform the reaction processes at lower temperatures, reducing the risks of antioxidant degradation.

The best reaction conditions involve a first step designed for the chitosan activation toward radical reactions and a second step for the insertion of the antioxidant molecules on the preformed macroradical.

In **Figure 2** a possible mechanism of antioxidant insertion onto chitosan is proposed. The hydroxyl radicals, generated by the interaction between redox pair components, attack H-atoms in α-methylene (CH_2) or hydroxyl groups (OH) of the hydroxymethylene group of the chitosan (step 1) (*27*).

In addition, the reactive amino group in chitosan is important in several of the structural modifications targeted because the deprotonated amino group acts as a powerful nucleophile (pK_a = 6.3), readily reacting with electrophilic reagents (*28*). Even in free radical-initiated copolymerization, NH_2 groups of chitosan are involved in macroradical formation. At those sites, the insertion of the antioxidant molecules can occur (step 2).

On the other hand, in the literature, many research works report on the reactivity of phenolic compounds toward this kind of reaction: monomers with active functional groups (phenolic groups) as side substituents, indeed, were used for the preparation

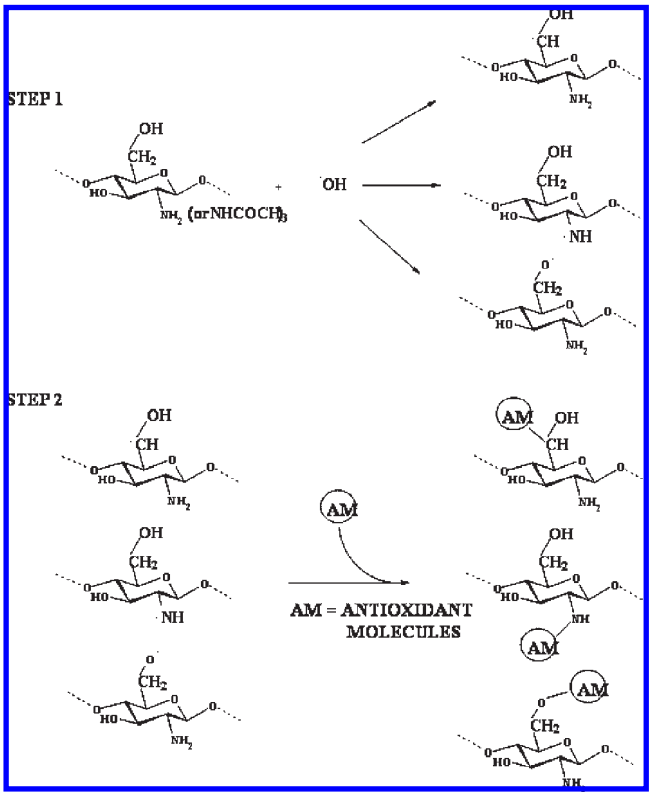

Figure 2. Insertion of antioxidant molecules in chitosan backbone.

of grafted polymeric systems (*29*) using free radical initiators. However, the phenolic group could be directly involved in the polymerization process; it is reported, indeed, that the phenolic radical undergoes dimerization processes by reaction between the hydroxyl radical and aromatic ring in the ortho or para position relative to the hydroxyl group (*30*).

On the basis of these data, it can be reasonably hypothesized that the insertion of antioxidants on the chitosan chains occurs in positions 2 and 5 of the aromatic ring of gallic acid and in positions 2′,5′ (B ring) and 6,8 (A ring) for catechin (**Figure 1**), respectively.

In the reaction feed the amount of antioxidant was 0.7 mmol/g of chitosan for both conjugate systems; this value represents the optimum to obtain a material with the highest efficiency.

Characterization of Antioxidant−Chitosan Conjugates. To verify the covalent insertion of catechin and gallic acid into the chitosan chains, the functionalized materials and the respective control polymers were characterized by Fourier transform IR spectrophotometry, UV, and DSC analyses.

IR spectra of both chitosan−antioxidant conjugates show the appearance of new peaks at 1538 and 1558 cm^{-1}, respectively, awardable to carbon-to-carbon stretching within the aromatic ring of gallic acid and catechin; moreover, in the IR spectrum of gallic acid-*grafted*-chitosan, a new peak at 1771 cm^{-1} ascribable to carbon-to-oxygen stretching within the carbonylic group of gallic acid appeared.

A further confirmation of antioxidant insertion in the biopolymer was obtained by comparing UV spectra of each antioxidant molecule (10 μM) and the respective chitosan conjugates in water (0.6 mg/mL). These were recorded using blank chitosan at the same concentration as baseline to remove the interference of the native polysaccharide.

As depicted in **Figures 3** and **4**, the UV spectra of both conjugates show the presence of absorption peaks in the aromatic region, which can be related to the presence of gallic acid and

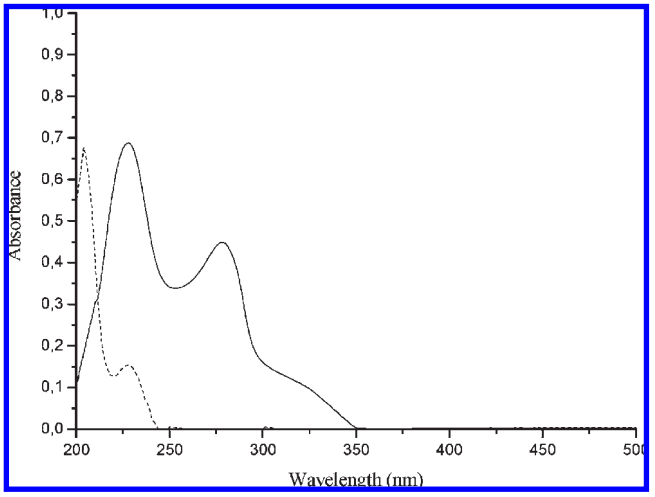

Figure 3. UV spectrum of catechin (- - -) and catechin-*grafted*-chitosan (—).

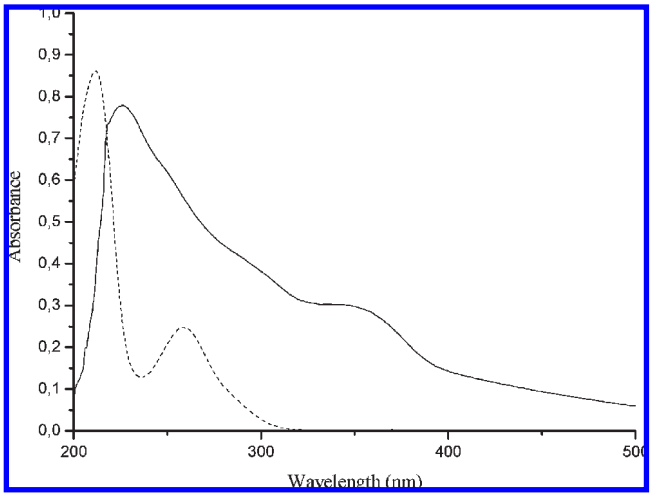

Figure 4. UV spectrum of gallic acid (- - -) and gallic acid-*grafted*-chitosan (—).

catechin in the samples. In addition, the absorption is shifted at higher wavelengths as a consequence of the extension of the conjugation due to the formation of the covalent bonds between chitosan reactive groups and the antioxidant aromatic ring.

Finally, DSC analyses of pure antioxidants, blank chitosan, and each chitosan conjugate were performed (**Figures 5** and **6**).

The calorimetric analysis of pure gallic acid shows a sharp melting endotherm at 266.5 °C, corresponding to the melting point of the antioxidant molecule (**Figure 5c**), whereas for pure catechin a melting endotherm at 155.8 °C was displayed (**Figure 6c**). As far as DSC of blank chitosan is concerned (**Figures 5b** and **6b**), a broad endotherm, located around 39−151 °C, is clearly visible and has been assigned to the glass transition of the polysaccharidic chain; the ΔH_t associated with this transition was −195 J/g. The DSC thermogram of gallic acid-*grafted*-chitosan (**Figure 5a**) displays the disappearance of the melting endotherm of gallic acid and a ΔH_t value (−241 J/g), associated with the polysaccharidic gel transition, higher than that observed in blank chitosan. Similar results were observed in the DSC thermogram of the catechin−chitosan conjugate (**Figure 6a**). Then different thermal behaviors between blank chitosan and these conjugated systems were observed and can be ascribed to the covalent doping of chitosan with antioxidant compounds.

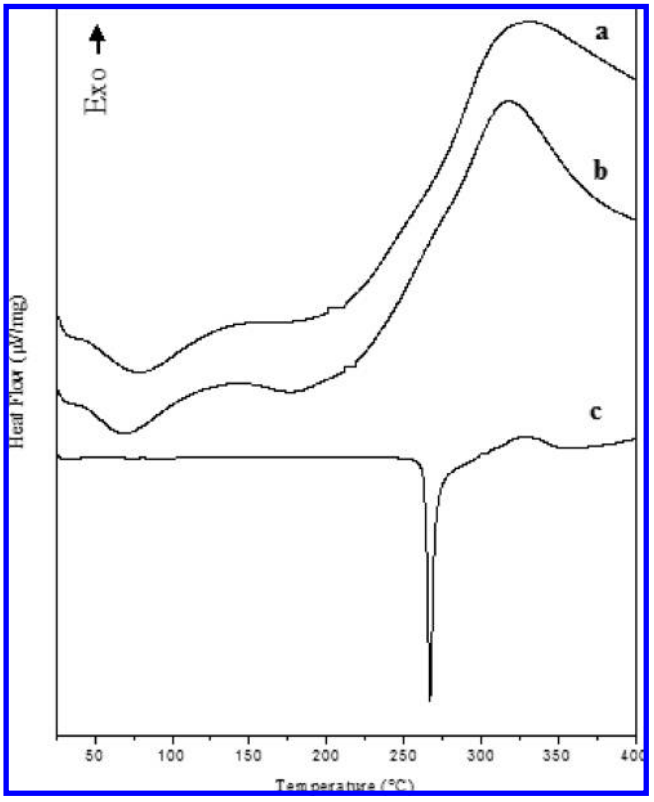

Figure 5. DSC of gallic acid (**c**), blank chitosan (**b**), and gallic acid-*grafted*-chitosan (**a**).

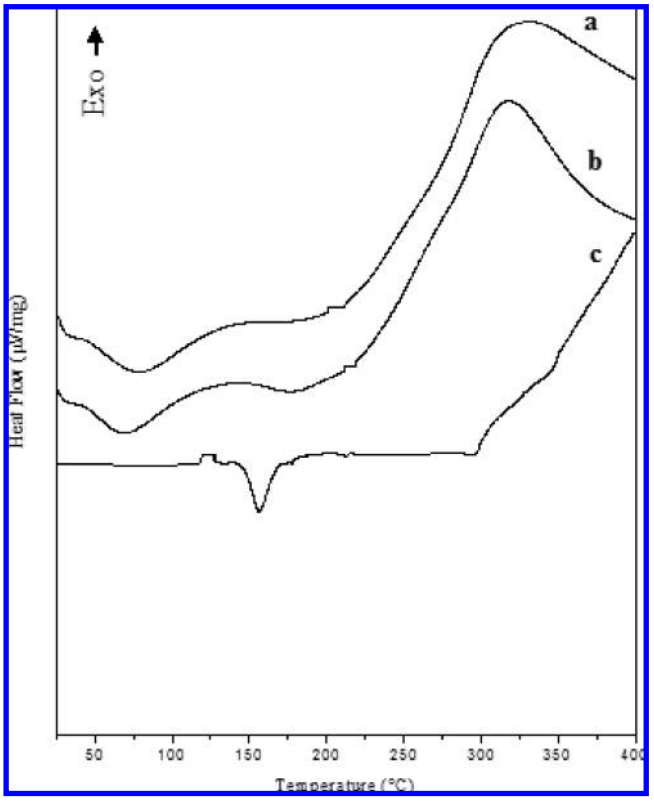

Figure 6. DSC of catechin (**c**), blank chitosan (**b**), and catechin-*grafted*-chitosan (**a**).

Determination of the Scavenging Effect on DPPH Radicals. The DPPH radical is a stable organic free radical with an absorption maximum band around 515−528 nm and, thus, it is

Table 1. Inhibition Percentages of Linoleic Acid Peroxidation, DPPH Radical, and Hydroxyl Radical by Blank Chitosan, Catechin-*grafted*-Chitosan, and Gallic Acid-*grafted*-Chitosan

	inhibition (%)		
sample	linoleic acid peroxidation	DPPH radical	hydroxyl radical
blank chitosan	23 ± 1.2	14 ± 1.1	17 ± 1.4
catechin-*grafted*-chitosan	98 ± 0.8	98 ± 1.1	95 ± 0.9
gallic acid-*grafted*-chitosan	85 ± 0.9	92 ± 1.3	60 ± 1.1

a useful reagent for evaluation of antioxidant activity of compounds.

In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen-donating ability of the antioxidants. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, and polyhydroxy aromatic compounds (e.g., ferulic acid, hydroquinone, pyrogallol, gallic acid) reduce and decolorize 1,1-diphenyl-2-picrylhydrazine by their hydrogen-donating capabilities (*21*).

The polymers' scavenging abilities were evaluated in terms of DPPH reduction using, for each synthesized polymer, gallic acid and catechin as reference compounds, and data are expressed as inhibition (percent).

As reported in **Table 1**, in our operating conditions, both chitosan conjugates can totally inhibit the DPPH radical.

***β*-Carotene−Linoleic Acid Assay.** In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant, which results in a reduction in absorbance of the test solution with reaction time (*22*). This is due to the oxidation of linoleic acid that generates free radicals (lipid hydroperoxides, conjugated dienes, and volatile byproducts) that attack the highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. When this reaction occurs, the β-carotene molecule loses its conjugation and, as a consequence, the characteristic orange color disappears. The presence of antioxidant avoids the destruction of the β-carotene conjugate system, and the orange color is maintained. Also, in this case, good antioxidant activities for both the conjugates were recorded, with inhibition percentages of lipidic peroxidation equal to 98% for the catechin conjugate and 85% for the gallic acid conjugate, respectively (**Table 1**).

Evaluation of Disposable Phenolic Groups by the Folin−Ciocalteu Procedure. Because the antioxidant activity of both the chitosan−antioxidant conjugates is derived from phenolic groups in the polymeric backbone, it is useful to express the antioxidant potential in terms of phenolic content. The Folin−Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of disposable phenolic groups present in the polymer chain. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin−Ciocalteu reactant. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valence (*23*).

For each biopolymer, disposable phenolic groups were expressed as milligram equivalents of the respective functionalizing antioxidant. Particularly, for gallic acid− and catechin−chitosan conjugates these values were 7 and 4 mg/g of dry polymers, respectively. These different values could be due to the presence, in catechin, of a number of free radical reactive sites greater than that existing in the gallic acid molecule.

Determination of Total Antioxidant Activity. The assay is based on the reduction of Mo(VI) to Mo(V) by ferulic acid and subsequent formation of a green phosphate/Mo(V) complex at acid pH (*24*). The total antioxidant activity was measured and compared with that of antioxidants and the control chitosan, which contained no antioxidant component. The high absorbance values indicated that the sample possessed significant antioxidant activity.

Synthesized materials had significant antioxidant activities, and gallic acid and catechin milligram equivalents in the respective functionalized polymers were found to be 3 and 5 mg for 1 g of dry functional polymers, respectively.

Hydroxyl Radical ($OH^{\bullet}$) Scavenging Activity. The deoxyribose test has been considered to be the most suitable means for detecting scavenging properties toward the OH radical.

Hydroxyl radicals exhibit very high reactivity and tend to react with a wide range of molecules found in living cells. They can interact with the purine and pyrimidine bases of DNA. They can also abstract hydrogen atoms from biological molecules (e.g., thiol compounds), leading to the formation of sulfur radicals able to combine with oxygen to generate oxysulfur radicals, a number of which damage biological molecules (*25*). Due to the high reactivity, the radicals have a very short biological half-life. Thus, an effective scavenger must be present at a very high concentration or possess very high reactivity toward these radicals. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is the Fenton reaction, in which a transition metal is involved as a prooxidant in the catalyzed decomposition of superoxide and hydrogen peroxide. These radicals are intermediary products of cellular respiration, phagocytic outburst, and purine metabolism. Hydroxyl radical can be generated in situ by decomposition of hydrogen peroxide by high redox potential EDTA−Fe^{2+} complex, and in the presence of deoxyribose substrate, it forms thiobarbituric acid-reactive substances (TBARS), which can be measured. Antioxidant activity is detected by decreased TBARS formation, which can come about by donation of hydrogen or electron from the antioxidant to the radical or by direct reaction with it. Consequently, the ability of the synthesized polymers to scavenge hydroxyl radical was evaluated by using the Fenton-mediated deoxyribose assay.

Also, this test confirmed the good antioxidant properties of functional materials compared to blank chitosan with the inhibition percentages of hydroxyl radical by gallic acid− and catechin−chitosan conjugates equal to 95 and 60%, respectively, whereas the value for BCH was 17% (**Table 1**).

Grafting Procedure Efficiency. A novel solvent-free synthetic procedure based on the use of water-soluble redox initiators was proposed to covalently bind two antioxidant molecules, catechin and gallic acid, onto chitosan, one of the most widely used natural biopolymers.

The rapidity of the reaction, together with the absence of toxic reaction products, makes this procedure very useful to exalt the biological properties of chitosan.

Furthermore, the high reaction yields, mild reaction conditions, simple setup, and workup procedure are additional merits of our protocol.

The covalent insertion of gallic acid and catechin in the polymeric chain was confirmed by UV and FT-IR analyses, whereas the enhanced thermal stability of the functional materials was demonstrated by DSC thermograms.

Finally, the antioxidant properties of both chitosan−antioxidant conjugates were evaluated by performing five different assays. Particularly, determination of the scavenging activity on DPPH radicals and hydroxyl radical, β-carotene−linoleic acid assay, determination of disposable phenolic groups in polymeric matrices, and determination of total antioxidant capacity were

performed. Good antioxidant properties were recorded in all of the tested conditions, confirming that the antioxidant activity of chitosan was strengthened after its functionalization with the antioxidant molecules.

The obtained results show the applicability of these materials in the food industry as food preservatives.

LITERATURE CITED

(1) Chae, S. Y.; Jang, M.; Nah, J. Influence of molecular weight on oral absorption of water soluble chitosans. *J. Controlled Release* **2004**, *102*, 383–394.

(2) Pae, H. O.; Seo, W. G.; Kim, N. Y.; Oh, G. S.; Kim, G. E.; Kim, Y. H.; Kwak, H. J.; Yun, Y. G.; Jun, C. D.; Chung, H. T. Induction of granulocytic differentiation in acute promyeloocytic leukemia cells (HL 60) by water-soluble chitosan oligomer. *Leukemia Res.* **2001**, *25*, 339–346.

(3) Maeda, M.; Murakami, H.; Ohta, H.; Tajima, M. Stimulation of IgM 15 production in human−human hybridoma HB4C5 cells by chitosan. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 427–431.

(4) Porporatto, C.; Bianco, I. D.; Riera, C. M.; Correa, S. G. Chitosan induces different l-arginine metabolic pathways in resting and inflammatory macrophages. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 266–272.

(5) Hirano, S. Chitin biotechnology applications. *Biotechnol. Annu. Rev.* **1996**, *2*, 237–258.

(6) Yen, M.; Yang, J.; Mau, J. Antioxidant properties of chitosan from crab shells. *Carbohydr. Polym.* **2008**, *74*, 840–844.

(7) Park, H. J. Development of advanced edible coatings for fruits. *Trends Food Sci. Technol.* **1999**, *10*, 254–260.

(8) Caner, C.; Vergano, P. J.; Wiles, J. L. Chitosan film mechanical permeation properties as affected by acid, plasticizer and storage. *J. Food Sci.* **1998**, *63*, 1049–1053.

(9) Knorr, D. Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.* **1991**, *45*, 114–122.

(10) Jahan, M. S.; McKinny, K. S. Radiation-sterilization and subsequent oxidation of medical grade polyethylene: an ESR study. *Nucl. Instrum. Methods Phys. Res., Sect. B* **1999**, *151*, 207–212.

(11) Kanatt, S. R.; Chander, R.; Sharma, A. Chitosan glucose complex—a novel food preservative. *Food Chem.* **2008**, *106*, 521–528.

(12) Pasanphan, W.; Buettner, G. R.; Chirachanchai, S. Chitosan conjugated with deoxycholic acid and gallic acid: a novel biopolymer-based additive antioxidant for polyethylene. *J. Appl. Polym. Sci.* **2008**, *109*, 38–46.

(13) Tseng, T.-W.; Tsai, Y.; Lee, J.-S. Synthesis and characterization of polymeric antioxidants based on oligo(styrene-*co*-chloromethyl styrene). *Polym. Degrad. Stab.* **1997**, *58*, 241–245.

(14) Menichetti, S.; Viglianisi, C.; Liguori, F.; Cogliati, C.; Boragno, L.; Stagnaro, P.; Losio, S.; Sacchi, M. C. Ethylene-based copolymers with tunable content of polymerizable hindered phenols as nonreleasing macromolecular additives. *J. Polym. Sci., Polym. Chem.* **2008**, *46*, 6393–6406.

(15) Puoci, F.; Iemma, F.; Curcio, M.; Parisi, O. I.; Cirillo, G.; Spizzirri, U. G.; Picci, N. Synthesis of methacrylic-ferulic acid copolymer with antioxidant properties by single-step free radical polymerization. *J. Agric. Food Chem.* **2008**, *56*, 10646–10650.

(16) Pasanphan, W.; Chirachanchai, S. Conjugation of gallic acid onto chitosan: an approach for green and water-based antioxidant. *Carbohydr. Polym.* **2008**, *72*, 169–177.

(17) Wu, L.; Embree, H. D.; Balgley, B. M.; Smith, P. J.; Payne, G. F. Utilizing renewable resources to create functional polymers: chitosan-based associative thickener. *Environ. Sci. Technol.* **2002**, *36*, 3446–3454.

(18) Lu, Z.; Nie, G.; Belton, P. S.; Tang, H.; Zhao, B. Structure−activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. *Neurochem. Int.* **2006**, *48*, 263–274.

(19) Mizugaki, M.; Ishizawa, F.; Yamazaki, T.; Hishinuma, T. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat. Res.* **2003**, *523−524*, 9–20.

(20) Wang, H.; Helliwell, K.; You, X. Isocratic elution system for the determination of catechins, caffeine and gallic acid in green tea using HPLC. *Food Chem.* **2000**, *68*, 115–121.

(21) Ardestani, A.; Yazdanparast, R. Antioxidant and free radical scavenging potential of *Achillea santolina* extracts. *Food Chem.* **2007**, *104*, 21–29.

(22) Amin, I.; Zamaliah, M. M.; Chin, W. F. Total antioxidant activity and phenolic content in selected vegetables. *Food Chem.* **2004**, *87*, 581–586.

(23) Pan, Y.; Zhu, J.; Wang, H.; Zhang, X.; Zhang, Y.; He, C.; Ji, X.; Li, H. Antioxidant activity of ethanolic extract of *Cortex fraxini* and use in peanut oil. *Food Chem.* **2007**, *103*, 913–918.

(24) Prieto, P.; Pineda, M.; Aguilar, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal. Biochem.* **1999**, *269*, 337–341.

(25) Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. The deoxyribose method: a simple "test tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **1987**, *165*, 215–219.

(26) Kitagawa, M.; Tokiwa, Y. Polymerization of vinyl sugar ester using ascorbic acid and hydrogen peroxide as a redox reagent. *Carbohydr. Polym.* **2006**, *64*, 218–223.

(27) Mun, G. A.; Nurkeeva, Z. S.; Dergunov, S. A.; Nam, I. K.; Maimakov, T. P.; Shaikhutdinov, E. M.; Lee, S. C.; Park, K. Studies on graft copolymerization of 2-hydroxyethyl acrylate onto chitosan. *React. Funct. Polym.* **2008**, *68*, 389–395.

(28) Chen, T.; Kumar, G.; Harris, M. T.; Smith, P. J.; Payne, G. F. Enzymatic grafting of hexyloxyphenol onto chitosan to alter surface and rheological properties. *Biotechnol. Bioeng.* **2000**, *70*, 564–573.

(29) Nanjundan, S.; Selvamalar, C. S. J.; Jayakumar, R. Synthesis and characterization of poly(3 acetyl-4-hydroxyphenyl acrylate) and its Cu(II) and Ni(II) complexes. *Eur. Polym. J.* **2004**, *40*, 2313–2321.

(30) Uyama, H.; Maruichi, N.; Tonami, H.; Kobayashi, S. Peroxidase-catalyzed oxidative polymerization of bisphenols. *Biomacromolecules* **2002**, *3*, 187–193.

Received January 9, 2009. Revised manuscript received May 11, 2009. Accepted May 21, 2009. This work was financially supported by University funds.