Preparation and Characterization of Eugenol-Grafted Chitosan Hydrogels and Their Antioxidant Activities

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ABSTRACT: The hydrogels composed of chitosan and eugenol were prepared to enhance and sustain antioxidant activities. The vinyl groups of eugenol monomer were directly grafted on the amino groups of chitosan, using ceric ammonium nitrate. The graft of eugenol onto chitosan was confirmed by using Fourier-transform infrared and proton nuclear magnetic resonance spectroscopies. Results from the swelling behavior, thermal stability, and wide-angle X-ray diffraction revealed that the equilibrium water content decreased with increase of graft yields, because of the hydrophobicity of eugenol, although the introduction of eugenol as a side chain disturbed the ordered arrangement of chitosan's crystalline structure. The eugenol-grafted chitosan hydrogels showed lower pH sensitivity in comparison with chitosan alone, because the amino groups, which were pH sensitive, of chitosan were grafted with eugenol. The scavenging activity of the tested hydrogels increased with graft yield of eugenol, because phenolic groups in the eugenol could play a major role as potent free-radical terminators, in the results of improved antioxidant activity in eugenol-grafted chitosan hydrogel in comparison with chitosan alone. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 99: 3500–3506, 2006

Key words: antioxidant; chitosan; graft copolymers; hydrogel

INTRODUCTION

Chitosan, a deacetylated form of chitin, having a subunit of β-(1,4)-2-amido-2-deoxy-D-glucopyranose, is the second, most abundant, naturally occurring biopolymer and is found in the exoskeleton of crustaceans, in fungal cell walls, and in other biological material.[1] Because of its biocompatibility, biodegradability, antibacterial properties, and remarkable affinity to proteins, it has been found to increase applications in areas, such as hematology, immunology, wound healing, drug delivery, and cosmetics.[2–5] The amino group, which is rare in polysaccharides, of chitosan can be used as a reactive site and also to chemically alter its properties under mild reaction conditions.[6–10]

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. The main role of antioxidants is to help the body to protect itself against damage, caused by reactive oxygen species and degenerative diseases.[11] To simultaneously impart the antioxidant activity in the chitosan, eugenol molecules were grafted on the amino groups of chitosan, because eugenol and its related compounds could play an important role in antioxidation. They owe their activity to trap the chain-carrying peroxy radicals by donation of the phenolic hydrogen atom reaction.[12] In biological applications, eugenol was widely used in dentistry, oral environment, and as flavoring agent in cosmetic and food products, because eugenol is generally recognized as safe materials by the Food and Drug Administration.[12] Natural antioxidants constitute a broad range of compounds, including phenolic compounds, nitrogen compounds, and carotenoids.

Thus, the goals of the present study are to prepare eugenol-grafted chitosan (EuCs) hydrogels and to investigate their antioxidative potency. Furthermore, the difference of swelling behavior is also discussed by comparing chitosan alone with the EuCs hydrogels of various compositions.

EXPERIMENTAL

Materials

Chitosan (degree of deacetylation = 85%) was purchased from Sigma Chemicals (St. Louis, MO) and was used after dissolving in 2 wt % acetic acid, and filtered using a glass filter to remove any insoluble residues. Then, it was precipitated by adding 0.1*N* sodium hydroxide solution. The precipitates were washed with

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deionized water, ethanol, and ether, and dried at 60°C under vacuum. Eugenol (2-methoxy-4-(2-propenyl)-phenol) and ceric ammonium nitrate (CAN) were purchased from Sigma Chemicals (St. Louis). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and formaldehyde were purchased from Aldrich Chemical Co. (Milwaukee, WI). Water was first treated with a reverse-osmosis system (Sambo Glove, Ansan, Korea) and further purified with a Milli-Q Plus system (Water, Millipore, Billerica, MA). Other chemicals were reagent grade and used without any further purification.

Synthesis of EuCs

Chitosan (4 g) and a predetermined amount of eugenol ($4 \times 10^{-3}M$) were added into a 500-mL reactor, with 200 mL of 2 wt % acetic acid, and stirred for 4 h under nitrogen atmosphere, with heating at 40°C. The CAN, dissolved in 100 mL of 1*N* HNO_3, was slowly added into the reactor to initiate the graft polymerization. Reaction products were precipitated in 300 mL of acetone, filtered, and dried at 50°C for 6 h. After the graft, any unreacted eugenol residues, with chitosan amino groups, were removed by using Soxhlet's extractor, with methanol for 2 days, and dried at 30°C. The graft yield based on the weight change was calculated using the following equation:

$$\text{Graft yield (wt \%)} = (W_{\text{gm}}/W_c) \times 100 \qquad (1)$$

where W_{gm} and W_c are the weights of grafted monomer and chitosan, respectively.

Preparation of EuCs hydrogels

Chitosan and EuCs were dissolved in 2% acetic acid, with 1.5 wt % concentration, at room temperature. The solutions were poured into a petri dish and dried at room temperature in a vacuum oven. To crosslink the chitosan and EuCs, the formed films were immersed in 0.25% formaldehyde solution for 5 min, and washed three times with deionized water to remove excess formaldehyde. The crosslinked films were immersed in 0.1*N* NaOH solution for 3 h, to extract the complexed acetic acid molecules from amino groups of chitosan. The neutralized films were washed three times with deionized water, and then dried at 50°C in a vacuum oven.

Characterizations

Fourier-transform infrared (FTIR, Nicolet model Magna IR 550, Madison, WI) spectroscopy was used to confirm the synthesis of the EuCs. In addition, structural analyses of eugenol, chitosan, and EuCs were performed by a 500 MHz proton nuclear magnetic resonance (^{1}H NMR, Bruker AMX-500, Karlsruhe, Germany), using $CDCl_3$, D_2O/DCl, and D_2O solutions, respectively. Thermal stability of chitosan and EuCs hydrogels was measured using thermogravimetric analysis (TGA, Perkin–Elmer TGA-7, Shelton, CT). Decomposition profiles of TGA were recorded at a heating rate of 10°C/min in nitrogen, between 30 and 650°C. Wide-angle X-ray diffraction (WAXD) patterns were recorded by reflection method, using nickel-filtered Cu Kα radiation and Rigaku Denki X-ray diffractometer (Tokyo, Japan), which was operated at 50 kV and 180 mA, in the 2θ scanning mode between 5 and 40°.

Swelling behaviors

A swelling study was conducted on the EuCs hydrogels to observe the swelling behaviors in the medium. To measure the swelling behaviors, preweighed dry samples were immersed in deionized water. After wiping off the excess water on the samples' surface, the weight of the swollen sample was measured at various time intervals. The swelling ratio was calculated using the following formulae:

$$\text{Swelling ratio} = (W_s - W_d)/W_d \qquad (2)$$

where W_s is the weight of the hydrogel in the swollen state at a particular temperature and W_d is the dry weight of the hydrogel after drying the gels in a vacuum oven for 2 days. The weight of the hydrogels at various pH conditions was measured in buffer solutions of pH ranging between 3 and 9.

Free radical scavenging of hydrogels

DPPH (16 mg) was dissolved in a mixing solution composed of deionized water (100 mL) and ethanol (100 mL). The film samples (1.5 × 1.5 cm2) were immersed in DPPH (8 mL). The reaction mixtures were continuously shaken in the dark, until the absorbance of the solution was measured. The absorbance of the resulting solutions was determined using quantitative ultraviolet (UV) spectrophotometry (Shimadzu Model UV-2101 PC, Kyoto, Japan), at $\lambda = 515$ nm, against blank in which DPPH was absent. The percentage of inhibition was calculated as follows:

$$\text{Inhibition ratio (\%)} = [(A_b - A_s)/A_b] \times 100 \qquad (3)$$

where A_b and A_s are the absorbance of blank without the sample and the measured absorbance with the sample at a determined time, respectively.

RESULTS AND DISCUSSION

Preparation of EuCs hydrogel

Figure 1 shows the molecular scheme for preparing the EuCs hydrogel. To covalently graft the eugenol on

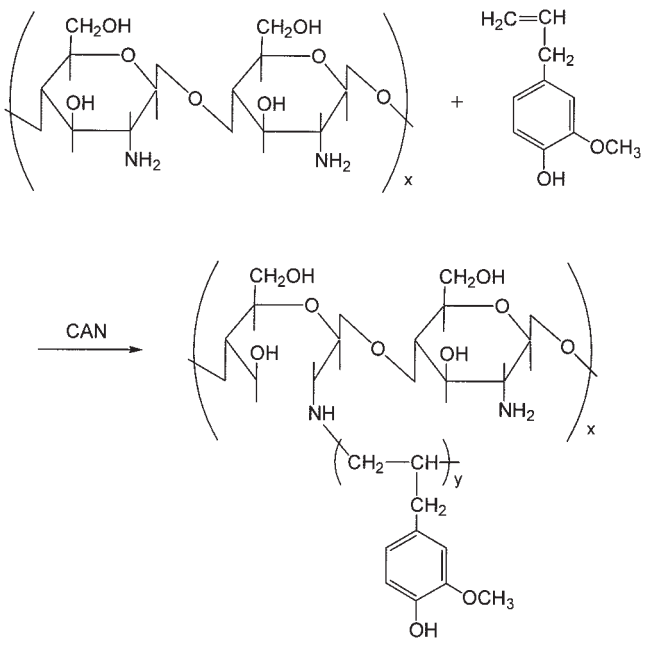

Figure 1 Molecular scheme for the preparation of EuCs hydrogel.

the amino groups of chitosan, CAN was used as an initiator. The grafting mechanism using Ce(IV) as an initiator was assumed to proceed via a redox mechanism in three steps: (1) the solvation of water to chitosan, (2) the formation of the complex between solvated chitosan and Ce(IV), and (3) grafting initiation by radicals from the complex.

Table I shows the graft yield of the EuCs hydrogels. The graft yield increased with the concentration of CAN. However, the graft yield was not improved above the concentration of $6 \times 10^{-3}M$. It is because when the concentration of initiator CAN was above the critical concentration, the polymerization was terminated by Ce(IV), reacting with the primary radicals of chitosan or propagational radicals of eugenol.

Preparation and characterization of chitosan and EuCs hydrogels

FTIR spectroscopic measurement was carried out to confirm the formation of graft, based on the changes in chemical structure of the EuCs. Figure 2 shows the FTIR spectra for (a) chitosan, (b) EuCs copolymer, and (c) eugenol. The FTIR spectrum of chitosan, with 85% deacetylation degree, indicated that peaks appearing at 1653 and 1598 cm^{-1} could be assigned to a carbonyl stretching vibration (amide I) and N—H bending vibration (amide II) of a primary amino group, respectively. In addition, Figure 2(c), obtained from eugenol monomer, shows characteristic peaks at 3443, 1610, and 1434 cm^{-1}, which can be attributed to the characteristic peaks of —OH attached in the benzene and C═C and CH_2═in the vinyl group, respectively. In the EuCs copolymer [Fig. 2(b)], the formation of a covalent linkage between the amine group of chitosan and eugenol was confirmed by the peak shift of amide II in chitosan to 1553 cm^{-1} and the disappearance of the free amino group of chitosan at 1598 cm^{-1}, when compared with chitosan itself. In addition, the characteristic peaks at 1610 cm^{-1} (C═C), 1434 cm^{-1} (CH_2═), and C—H vinyl out-of-plane bending vibrations, observed in the spectrum of the monomer [see Fig. 2(c)], disappeared in EuCs copolymer [see Fig. 2(b)]. From the results, we could confirm the graft of the eugenol molecules on the chitosan.

Figure 3 shows the ^1H NMR spectroscopy to identify the graft of eugenol on the chitosan. The spectrum of eugenol monomer exhibited peaks at 5.7–6.0 ppm because of CH_2═CH—, and at about 6.8 ppm because of the hydrogen atoms attached in the benzene. The copolymer showed new signals at 1.9 and 2.2 ppm and disappearance of peaks at 5.7–6.0 ppm, because the double bonds of vinyl groups in the eugenol were changed into single bonds (—CH_2—CH_2—) after grafting. However, the EuCs peaks at 6.8 ppm, caused by protons in the benzene of copolymer, were not detected, although the benzene groups existed in the copolymer. The absence of benzene peaks in the EuCs copolymer is due to the usage of hydrophilic solvent for dissolving the copolymer, because the benzene groups, which are hydrophobic, of the copolymer might not be soluble in the D_2O solution.

Thermal stability and crystallinity of EuCs hydrogels

Thermal stabilities of chitosan alone and EuCs hydrogels were measured using TGA analysis. Figure 4 shows the weight loss curves recorded at a heating rate of 10°C/min in nitrogen, between 30 and 650°C. The EuCs shows a faster thermal decomposition in comparison with that of chitosan alone.

Figure 5 shows the crystal structure and crystallinity of chitosan and the EuCs copolymer, using the WAXD measurement. The chitosan gives patterns at around $2\theta = 10$ and 20°, which were assigned to be a

TABLE I
Graft Yield of EuCs Hydrogels

Sample code	Concentration of CAN (10^{-3} mol)	Graft yield[a] (wt %)
EuCs-1	2	10.1 ± 0.9
EuCs-2	4	14.1 ± 1.2
EuCs-3	6	21.7 ± 1.0
EuCs-4	8	18.7 ± 1.3

[a] Chitosan, 4 g; reaction time, 4 h; concentration of eugenol, $4\times10^{-3}M$; reaction temperature, 40°C.

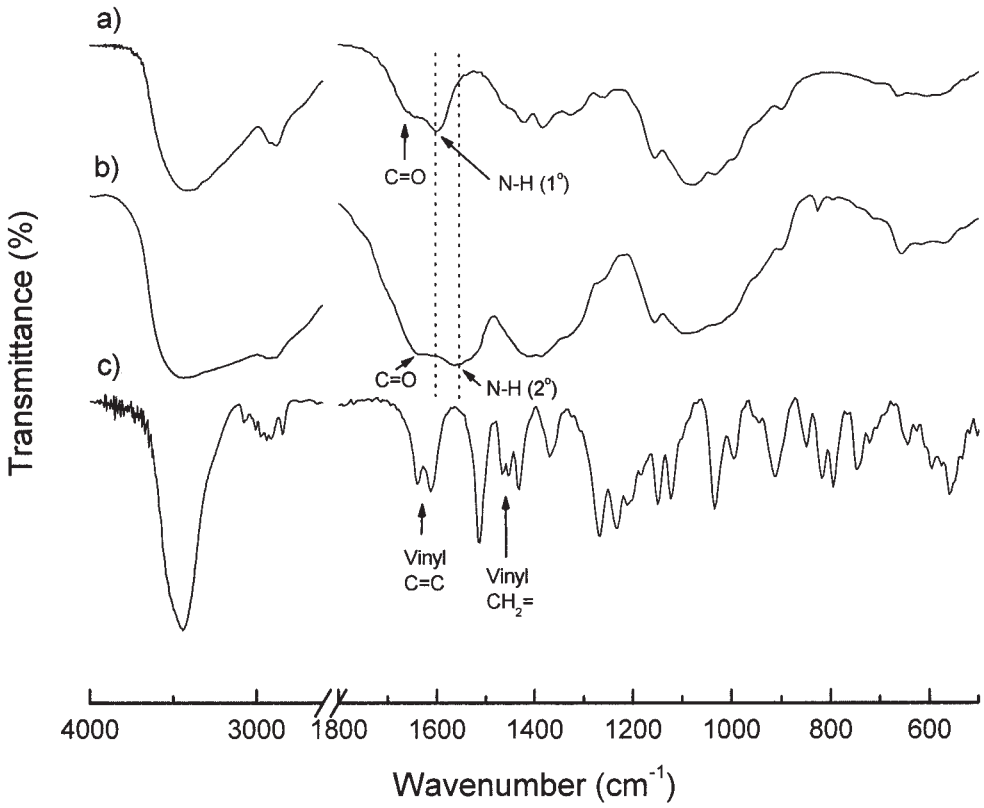

Figure 2 FTIR spectra of (a) chitosan, (b) EuCs-2, and (c) eugenol.

mixture of (001) and (100) of (101) and (002), respectively. The diffraction intensity of EuCs hydrogel was reduced in comparison with those of chitosan alone.

Thus, the results from TGA and WAXD revealed that the introduction of the bulky side chain inside hydrogel decreased the thermal stability caused by the breakdown of the crystalline region of chitosan.

Swelling behaviors

Figure 6 shows the swelling behaviors of chitosan and EuCs hydrogels in deionized water. Their swelling ratios reached an equilibrium swelling state within 20 min. The equilibrium–swelling ratios of EuCs hydrogels were smaller than that of chitosan, which is 2.21, and decreased with the increase of graft yield.

Introducing the bulky group in the main chain of chitosan improved the solubility of chitosan, because of the breakdown of crystalline region.[13] The side chain attached on the chitosan may disturb the ordered arrangement of the molecule during the hydrogel formation, resulting in an increase of swelling ratio in the grafted hydrogel. However, the graft of eugenol in the chitosan main chain had a reverse tendency in the swelling ratio, although the breakdown of the crystalline structure was confirmed from the results of

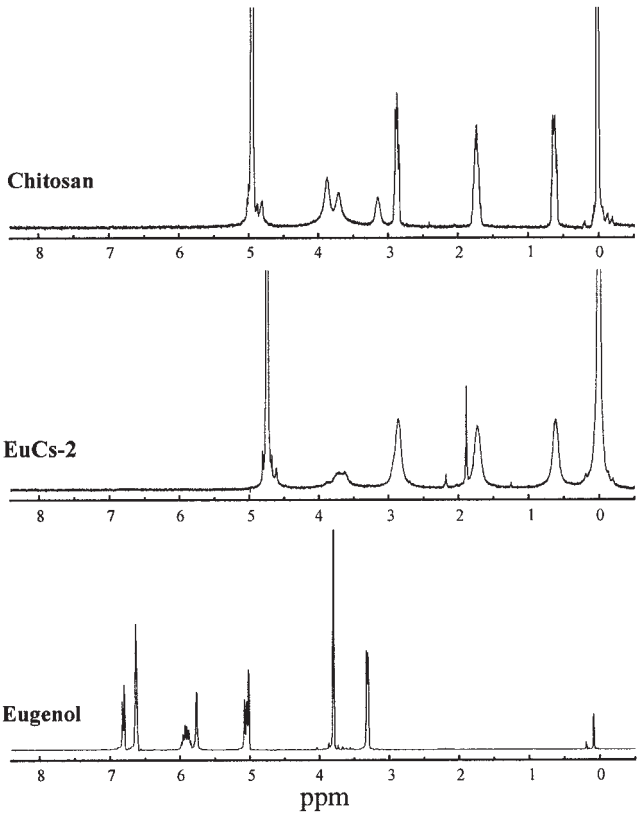

Figure 3 1H NMR spectra of chitosan, EuCs-2, and eugenol.

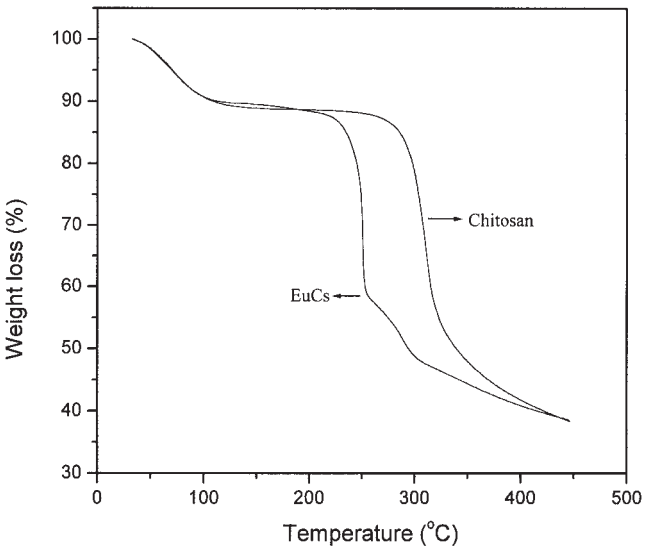

Figure 4 TGA of chitosan and EuCs-2 hydrogels.

TGA and WAXD (see Figs. 4 and 5). The decrease of swelling ratio after graft is due to the hydrophobicity of the attached eugenol molecules, indicating that the grafted eugenol disturbed the permeation and retention of water.

pH-dependent swelling behavior

Figure 7 shows pH-sensitive characteristics of hydrogels, which were investigated by swelling test under various pH ranges between 3 and 9. The pH sensitivity is mainly affected by chitosan amino groups, which is a weak base with an intrinsic pK_a of about 6.5; namely, the chitosan hydrogels swelled at low pH, because of the ionic repulsion of the protonated amine groups, and collapsed at high pH because of the influence of unprotonated amine groups. As the pH value of the buffer solution increases, ionized NH_3^+ groups become NH_2 groups, and the resulting neutralization of ionic groups causes the hydrogels to be precipitated. As shown in Figure 7, the swelling ratio of chitosan hydrogels continuously decreased, with increasing pH values. On the other hand, the swelling ratios of EuCs hydrogels were not affected by the pH of the media because chitosan amino groups, which were pH sensitive, were grafted with eugenol.

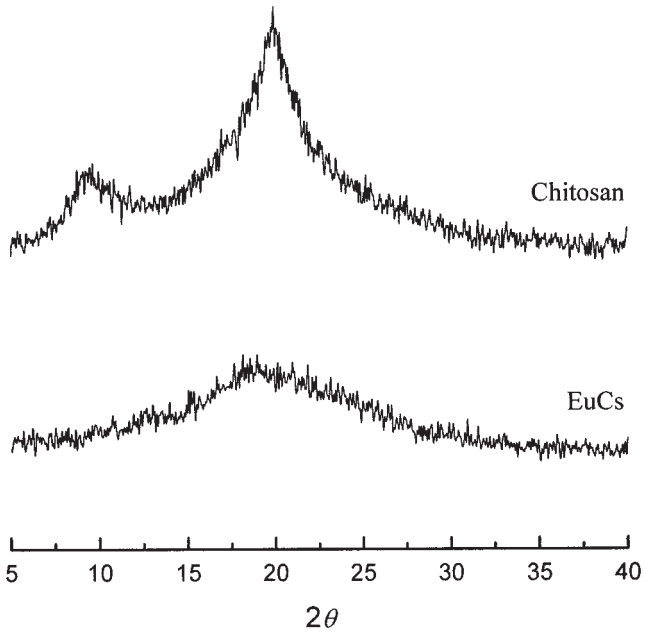

Figure 5 WAXD patterns of chitosan and EuCs-2 hydrogels.

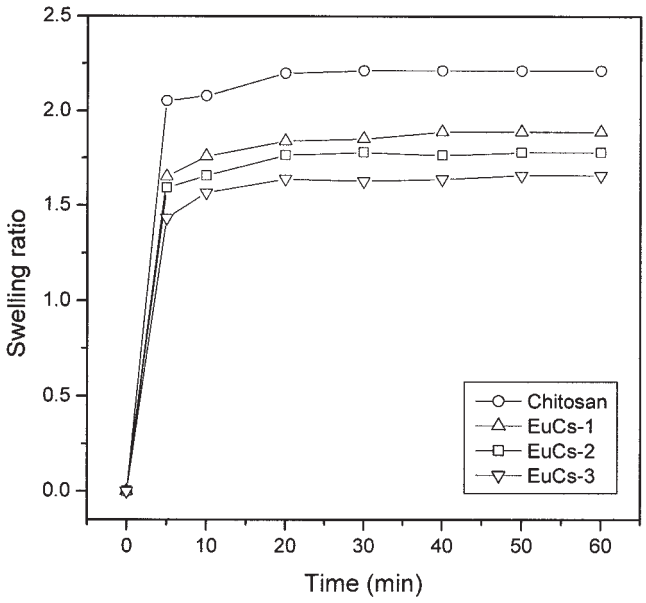

Figure 6 Swelling kinetics of chitosan and EuCs hydrogels in deinoized water (pH = 5.4) at 25°C.

Free radical scavenging activity of hydrogels

Figure 8 shows the free-radical scavenging activity of the EuCs hydrogels. The model of scavenging stable DPPH free radicals can be used to evaluate the antioxidative activities in a relatively short time compared to other methods.[11] The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. As shown in Figure 8, the scavenging activity order of the test hydrogels was EuCs-3 > EuCs-2 > EuCs-1 > chitosan alone, because the phenolic groups in eugenol are potent free-radical terminators.[12] Thus, the scavenging activity increased with the graft yield of eugenol.

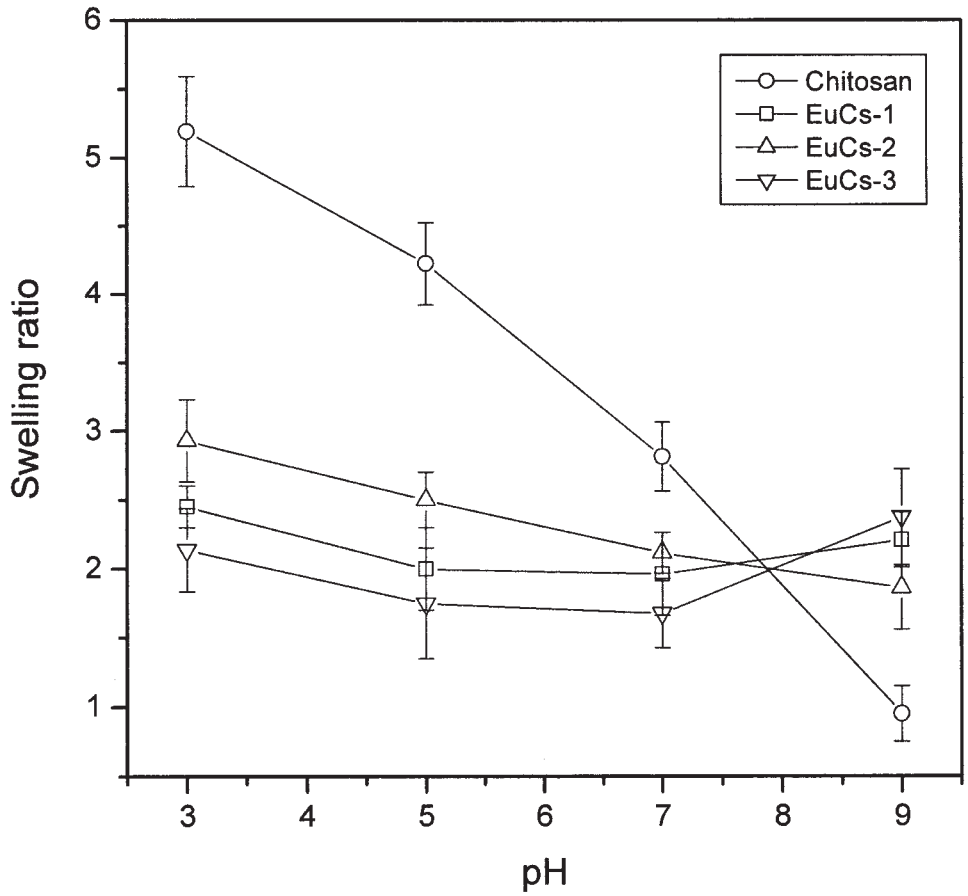

Figure 7 pH-Dependent swelling behaviors of chitosan and EuCs hydrogels at 25°C.

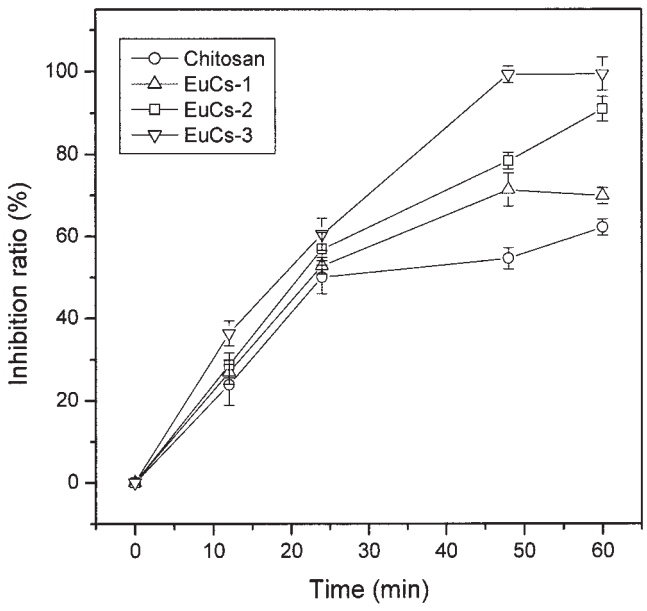

Figure 8 Free-radical scavenging activity of chitosan and EuCs hydrogels.

CONCLUSIONS

The graft of eugenol onto the chitosan was confirmed by using FTIR and ^{1}H NMR spectroscopies. The graft yield increased with the concentration of CAN. The results from TGA and WAXD revealed that the introduction of the bulky side chain inside hydrogel collapsed the crystalline region of chitosan, resulting in a decrease of thermal stability. In the swelling kinetics, the swelling ratios reached an equilibrium swelling state within 20 min. The equilibrium–swelling ratios of EuCs hydrogels were smaller than that of chitosan and decreased with increase of graft yield because of the hydrophobicity of the attached eugenol molecules. At various pHs, the swelling ratio of chitosan hydrogels continuously decreased with increasing pH values, whereas the swelling ratios of EuCs hydrogels were not affected by the pH of the media. The scavenging activity of the tested hydrogels increased with graft yield of eugenol because of the phenolic antioxidants

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