Radiolytic Synthesis of —OH Group Functionalized Fullerene Structures and Their Biosensor Application

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ABSTRACT: In this study, radiolytic functionalization of fullerene in methanol/1,2-dichlorobenzene mixtures and its applications with respect to biosensor support materials were studied. To obtain supports for biosensors for electron transfer, fullerene was functionalized by γ-irradiation in a methanol/1,2-dichlorobenzene mixture solution. The hydroxyl group-modified fullerene, F-fullerene, was characterized by Fourier-transform infrared, Raman spectroscopy, MALDI-TOF mass spectroscopy, and elemental analysis. As a result, the main hydroxyl group was successfully introduced on the surface of fullerene. F-fullerene was found to disperse well in water by ultrasonication. The results indicated that F-fullerene is a good candidate for use in biological systems as a biosensor support material. A biosensor based on F-fullerene was prepared by hand-casting the mixture of tyrosinase, F-fullerene, and 2% chitosan solution on an ITO electrode. Furthermore, the prepared biosensor was optimized pH and temperature. The prepared biosensor was then evaluated for its ability to analyze phenolic compounds contained in commercial red wines. The total phenolic concentration was determined to be in the range of 397–895 mg/L. From these results, the electron transfer ability of F-fullerene was improved on an enzyme biosensor. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 1785–1791, 2011

Key words: fullerene electrode; radiolytic functionalization; tyrosinase; phenolics; red wines

INTRODUCTION

Fullerenes are three-dimensional cage-like molecules shaped as closed polyhedra with pentagonal and hexagonal faces. They are essentially composed of a large number of carbon atoms, from 42 up about 1000. The most common fullerene, the C_{60} and the C_{70},were discovered in 1985.[1]

Because of their unique photophysical properties, fullerenes have potential application in many fields, such as electronics and biomedicine.[2,3] Fullerene and its derivatives exhibit satisfactory enzyme-inhibiting,[4] radical quenching,[5] and DNA-cleavage abilities.[6] Their application in biomedicine is limited because fullerenes are highly hydrophobic and insoluble in water. Therefore, investigations into improving the solubility of fullerenes in aqueous solutions are very important. On the other hand, the hydroxyl group, —OH, is well known as being one of the functional groups that has affinity for biomolecules.[7–9] The —OH group also has hydrophilic properties and, consequently, it can easily immobilize an enzyme onto the hydrophilic site via physical methods. However, literature searches did not reveal any publication regarding the introduction of —OH groups onto fullerene surfaces by γ-irradiation techniques.

In a previous article,[10] the functionalization of single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs) using γ-irradiation in an organic solvent with Triton X-100 as a dispersing agent was discussed. Carboxylic acid and sulfonic acid were successfully introduced onto the surface of carbon nanotubes using this technique. The introduction mechanism of that functional group during γ-irradiation was also detailed in the aforementioned article.

Wines, particularly red wines, contain numerous biologically active compounds; the most important of which are polyphenols. The nutritional importance of polyphenols is attributed to their antioxidant properties. In particular, flavonoids and their related phenolic compounds, which are naturally found in red wines, are receiving interest from researchers.[11] Red wines have been reported to be cardioprotective, and they play a possible role in reducing thrombotic and anthropogenic processes. Polyphenols also contribute substantially to the quality of wines as they affect the color, flavor, stability, and aging behavior.[12] However, little has been

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reported with respect to the total amount of phenolic compounds in red wine determined by electrochemical methods.

In earlier articles,[13,14] vinyl polymer-grafted MWNTs via radiation-induced graft polymerization (RIGP) of various vinyl monomers were synthesized in aqueous solutions to immobilize enzymes. The prepared enzyme electrode evaluated the sensing efficiency for phenols in a phosphate buffer solution. However, we could not synthesize the vinyl polymer-grafted fullerene since fullerene does not disperse in aqueous solutions.

We have previously published research regarding the functionalization of MWNTs through γ-irradiation in aqueous and nonaqueous media.[10] In this article, we introduced a carbonyl group via a trapped radical on the surface of the MWNTs in an aqueous solution by γ-irradiation; a sulfonic acid group is also introduced from sulfur radicals induced from sodium thiosulfate or carbon disulfide by γ-irradiation. On the other hand, we also have reported the functionalization of MWNTs by RIGP using vinyl monomers with various functional groups in aqueous solutions.[15] We selected vinyl monomers for the hydrophobic properties of the vinyl group and the hydrophilic properties of the functional group on the vinyl monomers. The vinyl moiety of the monomers faced toward the surface of the MWNTs because of its hydrophobic properties, while the functional group faced the aqueous solution due to its hydrophilic properties. The radical polymerization of these vinyl monomers was performed on the surface of the MWNTs during γ-irradiation. As a result, the functional group was introduced onto the surface of carbon nanotubes in an aqueous solution. However, we cannot functionalize fullerene in an aqueous solution using the aforementioned methods because the fullerene possesses highly hydrophobic properties and does not disperse in aqueous solutions.

In this study, the hydroxyl group was introduced onto the surface of fullerene by radiation-induced synthesis in a methanol/1,2-dichlorobenzene mixture solution. The obtained functionalized-fullerene, F-fullerene, was characterized by Fourier-transform infrared (FTIR), Raman spectroscopy, MALDI-TOF mass spectroscopy, elemental analysis, and cyclic voltammetry to confirm the success or failure of the synthesis. Furthermore, the enzyme electrode was prepared on the ITO electrode via hand-casting of the chitosan solution based on F-fullerene and tyrosinase. The prepared enzyme electrode was evaluated for its sensing efficiency for phenols in a phosphate buffer solution. Optimal conditions for pH and temperature were evaluated for various phenolic compounds. Total phenolic compounds in three commercial red wines were examined using the prepared enzyme electrode.

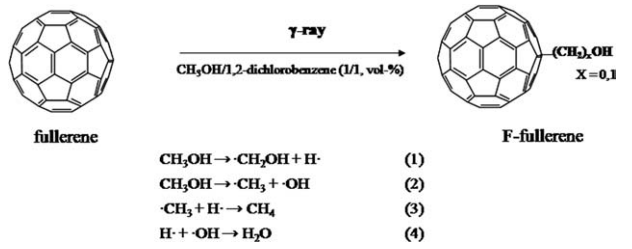

Figure 1 Suggestion mechanism for hydroxyl group introduction under γ-irradiation in the presence of in methanol/1,2-dichlorobenzene mixture solution.

EXPERIMENTAL

Reagents

Tyrosinase from mushroom (EC 1.14.18.1), phenol, *p*-chresol, catechol, chitosan, and fullerene were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ITO electrode as working electrode (working area 0.7 × 1.1 cm^2, 10 Ω cm^{-1} resistance) was purchased from Dasom RMS (Busan, Korea). The red wines used to obtain phenolics were Chateau Manidry, Chateau Mani-sweet, and Chateau Mani-nouveau (Daejeon, Korea). Solutions for the experiments were prepared with water purified in a Milli-Q plus water purification system (Millipore, USA, the final resistance of water was 18.2 MΩ cm^{-1}) and degassed before each measurement.

Synthesis of the functionalized (F)-fullerene by γ-irradiation

Figure 1 shows the preparation procedure of F-fullerene by γ-irradiation. In detail, the fullerene (0.1 g) was dissolved in a methanol/1,2-dichlorobenzene mixture solution (1/1 vol %, 50 mL). The optimized ratio of methanol to 1,2-dichlorobenzene was found to be a very important factor. Nitrogen gas was bubbled through the solution for 30 min to remove oxygen, and the solution was irradiated with a Co-60 source under atmospheric pressure and ambient temperature. A total irradiation dose of 30 kGy (a dose rate = 1.0 × 10^4 Gy/h, exposure time = 3 h) was used. After γ-irradiation, the F-fullerene was precipitated in the methanol/1,2-dichlorobenzene mixture solution (1/1 vol %). Finally, F-fullerene was obtained by centrifuge (3000 rpm for 6 h) and dried at 50°C in a vacuum oven.

Fabrication of the biosensor based on F-fullerene

Figure 2 shows the fabrication procedure of the biosensor based on F-fullerene for measuring phenols. The mixture solution contained F-fullerene (3.2 mg) for electron transfer, chitosan (2.0% sol. × 1.6 mL) as a binder, and tyrosinase (25,000 unit, 0.08 mL). The mixture solution (9.0 μL) was coated on the surface

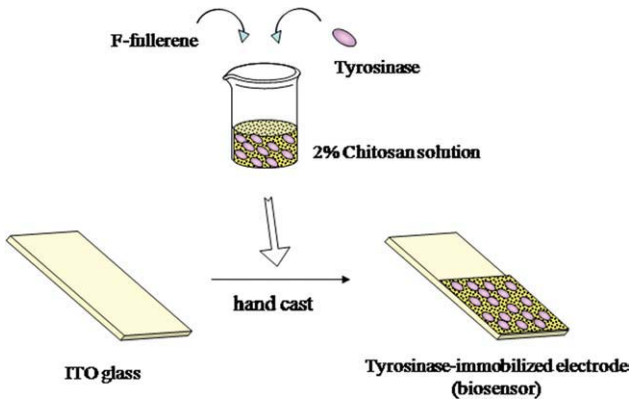

Figure 2 Fabrication of the biosensor based on F-fullerene for measuring phenols. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of a precleaned ITO electrode by a hand-cast method. The prepared biosensor was kept at 4°C until use.

Instrumentation

FTIR absorption spectra were recorded on a FTIR spectrum 1000 spectrometer (PerkinElmer, USA). For the preparation of pellets, KBr powder was carefully dried under vacuum. It was then mixed with the sample solid and made into a transparent pellet (13 mm in diameter and 0.5-mm thick) by using a hydraulic press. The IR measurements were carried out under a nitrogen atmosphere by purging a slow stream of dry nitrogen gas in the sample chamber. All spectra were carefully corrected for background absorption. The NIR Fourier transform (FT) Raman spectra were recorded with a Bruker FT-106 Raman module equipped with a Ge detector cooled by liquid nitrogen and connected to a Bruker FTIR 66 interferometer. For exciting the Raman spectra, a continuous wave diode-pumped Nd:YAG Laser with a radiation wavelength of 1064 nm (9398.4 cm^{-1}) was used. In all cases, the laser power was 300 mW and the spectral resolution was 2 cm^{-1}. Elemental analysis of samples was performed with an automatic elemental analyzer (Thermo Fisher Scientific, Flash EA 1108 series, USA). The samples were analyzed using a MALDI-TOF mass spectrometer (Applied Biosystems, Voyager DE-STR, USA). The surface morphology of the samples was determined using scanning electron microscopy (SEM, S-3000N, Hitachi Science System, Japan). Cyclic voltammetric experiments were performed with a Potentiostat/Gavanostat Model 283 (Ametek PAR, USA). All experiments were carried out with a conventional three-electrode system. The working electrode was ITO electrode coated with the F-fullerene, the counter electrode was a platinum wire, and the reference electrode was Ag/AgCl (saturated KCl).

RESULTS AND DISCUSSION

Synthesis and characterization of F-fullerene

Figure 3 shows the FTIR spectra of the fullerene and F-fullerene prepared by γ-irradiation in the mixture solution of methanol and 1,2-dichlorobenzene (1/1 vol %). In the F-fullerene spectra, the broad peak at 3500 cm^{-1} was assigned to the hydroxyl group (—OH) and the peak at 3000 cm^{-1} was assigned to C—H peaks. A small amount of carbonyl peak due to 1730 cm^{-1} was observed before was not appeared before γ-irradiation and assigned to the carbonyl group and it appeared after γ-irradiation. In an earlier article,[10] it was suggested the carbonyl group be introduced on the surface of carbon nanotubes due to trapped radicals (activated site). The strong stretching attributed to the >C—O peak was observed at 1230 cm^{-1}. As a result, the hydroxyl group (—OH) was introduced onto the surface of fullerene via γ-irradiation in a mixture solution of methanol/1,2-dichlorobenzene.

Raman spectra of the base fullerene powder and F-fullerene powder are shown in Figure 4. F-fullerene has spectroscopic features very similar to those of fullerene powder, indicating that F-fullerene retains the original structure retain of the base fullerene. Seven peaks of fullerene powder are observed at 271, 493, 712, 1405, 1458, and 1590 cm^{-1}. The peak positions at 1458 and 1590 cm^{-1} were shifted to 1462 and 1570 cm^{-1}, respectively, after γ-irradiation. Specifically, the peak intensity of the peak at 1450 cm^{-1}, which is referred to as a D band, for fullerene disappeared and the peak intensity of the peak at 1590 cm^{-1} due to a G band in fullerene also decreased.[16] These results may be attributed to formation of disordered sp^2 carbon structure of F-fullerene after γ-irradiation process.

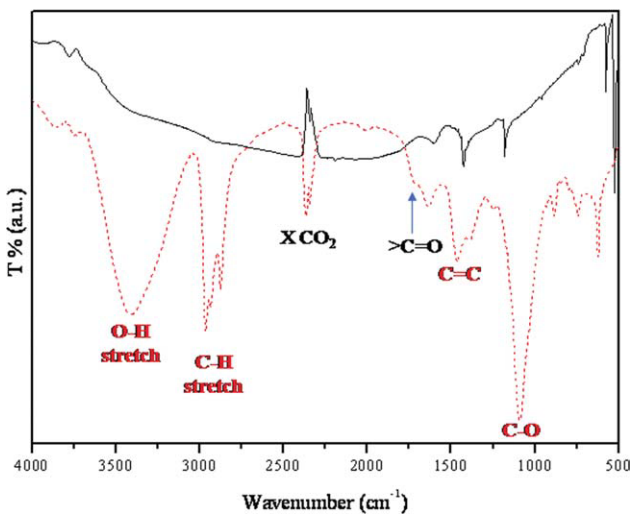

Figure 3 FTIR spectra of fullerene (solid line) and F-fullerene (dashed line) prepared by γ-irradiation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

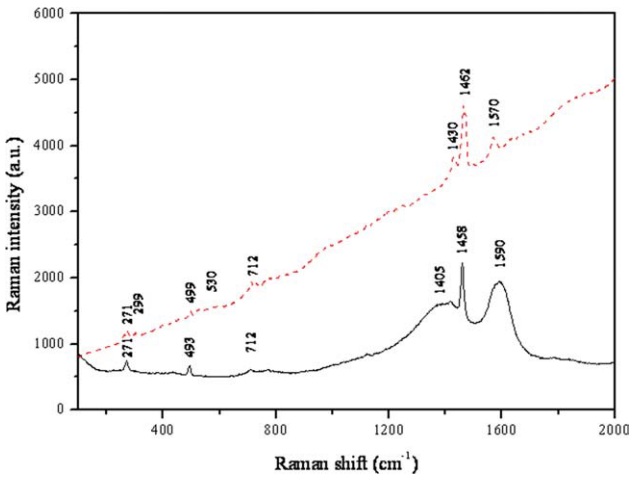

Figure 4 Raman spectra of fullerene (solid line) and F-fullerene (dashed line). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5 shows the MALDI-TOF mass spectra of the fullerene and F-fullerene. Three types of functionalized fullerene were observed. The hydroxyl (—OH), methyl hydroxyl group (—CH_2OH), and carboxylic acid group (—C(=O)OH) were introduced on the surface of fullerene. The obtained F-fullerene was well dispersed in water.

Preparation of the biosensor based on F-fullerene and its optimization

To improve the sensitivity of the phenol biosensor, we added F-fullerene made by radiolytic functionalization to a chitosan and *tyrosinase* mixture solution as mentioned in the Experimental section. Subsequently, we applied the freshly prepared solution to the surface of the phenol biosensor by a hand-casting method, as shown Figure 2. The SEM images of naked ITO, chitosan/ITO, tyrosinase/chitosan/ITO, and tyrosinase/F-fullerene/chitosan/ITO electrode are presented in Figure 6(a–d), respectively. As can be seen in Figure 6(a), ITO surface is almost flat, while the film of chitosan as binder was appeared as an amorphous flat form looks like typical polymer form as shown in Figure 6(b). In contrast, the composite film with tyrosinase and chitosan exhibited an irregular polymer form [Fig. 6(c)]. On the F-fullerene electrode surface, the morphology of the composite film is changed and looks like an irregular mountain pattern [Fig. 6(d)]. The biosensor based on F-fullerene was successfully fabricated.

Selectivity was a very important factor in the biosensor, so three compounds (phenol, *p*-cresol, and catechol) were detected in a 0.1 *M* phosphate buffer solution (pH = 7.0) as shown in Figure 7. In the *p*-cresol and catechol solution, there are weak redox peaks for the biosensor in this experiment. Serra et al.[17] reported that different sensitivity is observed for phenolic compounds with different positions in the utilized enzymatic reaction. The *meta*-position phenol (*m*-cresol) appeared to have a higher sensitivity response than that of ortho- and *para*-position phenols in the utilized enzymatic reaction because of their high affinity for tyrosinase. As shown in Figure 7, the response peak of phenol was observed to be larger than those of *p*-cresol and catechol because of its enzymatic reaction. Tsai et al.[18] reported that *p*-catechol appeared to have higher response

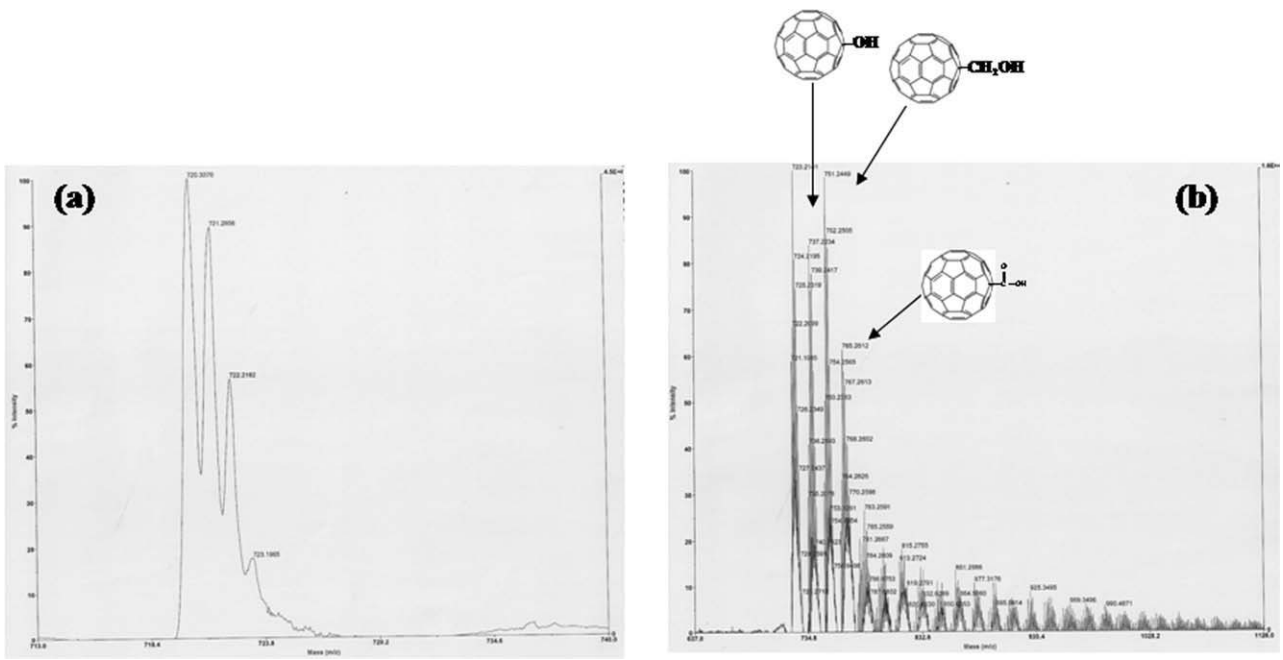

Figure 5 MALDI-TOF mass spectra of fullerene (a) and F-fullerene (b).

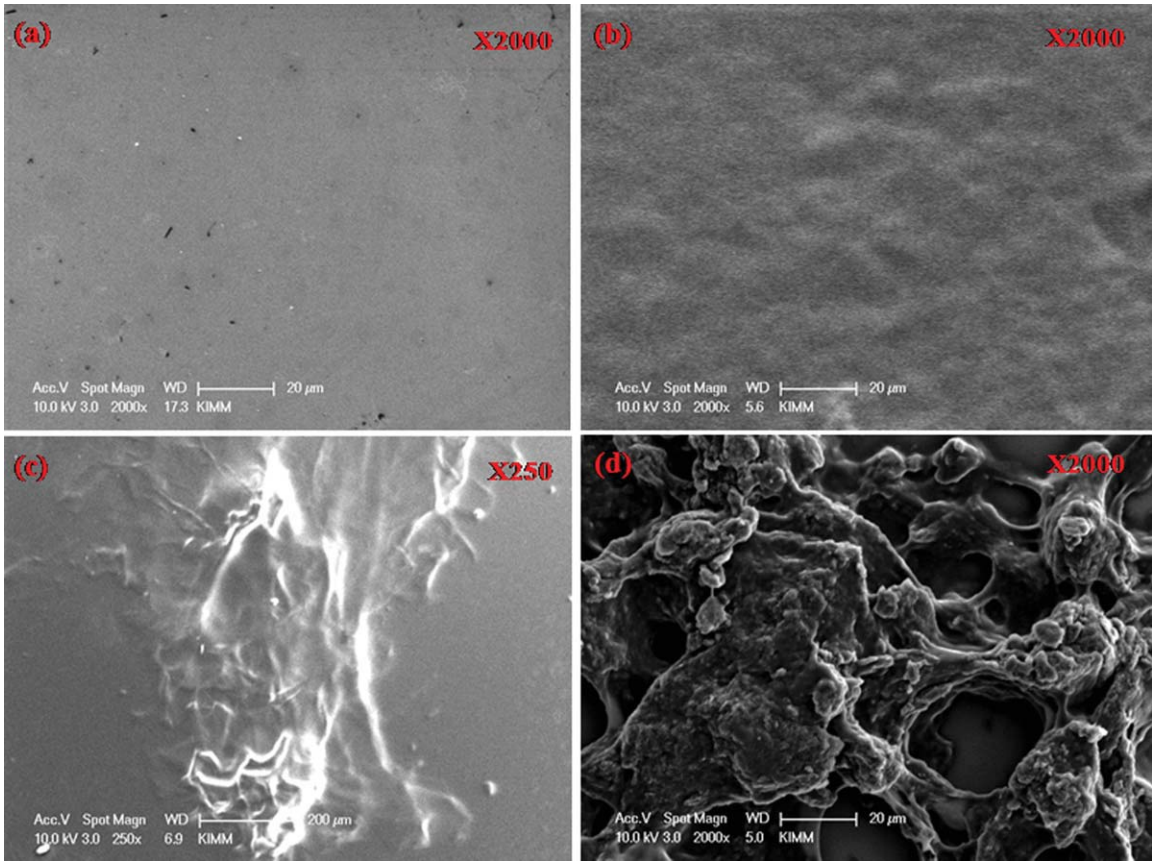

Figure 6 SEM images of naked ITO (a), chitosan/ITO (b), tyrosinase/chitosan/ITO (c), and tyrosinase/F-fullerene/chitosan/ITO electrode (d). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sensitivity than dopamine and epinephrine. However, a low response sensitivity of catechol for the prepared biosensor based on F-fullerene was observed.

The effect of pH on the amperometric response of phenol was also studied [Fig. 8(a)]. The pH value was observed to change when 0.5 m*M* phenol was put into a 0.1 *M* phosphate buffer solution (0.1*M*).

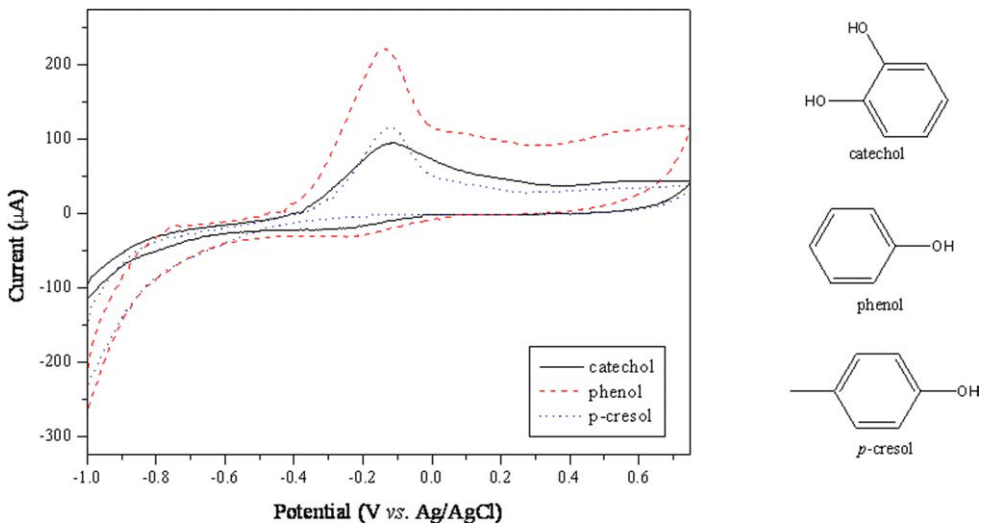

Figure 7 Cyclic voltammograms of the tyrosinase-immobilized biosensor in 0.1 *M* phosphate buffer solution (pH = 7.0) containing 0.5 m*M* catechol (solid line), 0.5 m*M* phenol (dashed line), and 0.5 m*M* *p*-cresol (dotted line) with a scan rate 100 mV/s. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

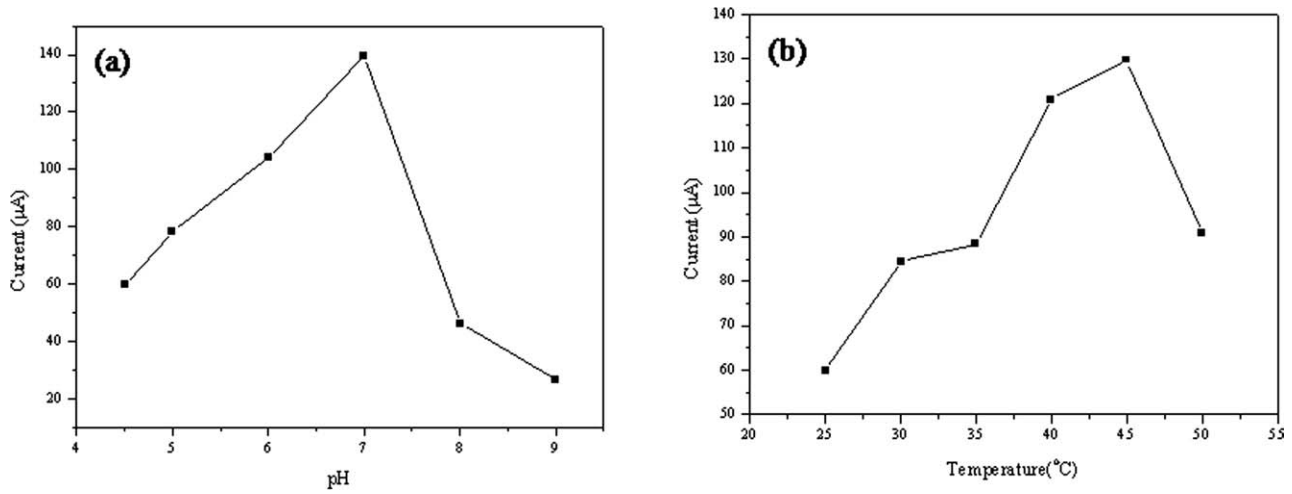

Figure 8 The effect of pH (a) and temperature (b) on the response of the tyrosinase-immobilized biosensor in 0.1 *M* phosphate buffer containing 0.5 m*M* phenol with a scan rate 100 mV/s.

The response sensitivity of the phenol biosensor in pH 4.5–9.0 showed that the more acidified the phenol biosensor became, the lower the peak current became. The activity of the enzymes slowed as the oxidization peak current decreased. The value of the oxidization peak current increased until pH 7.0. As shown in this figure, the current value of the oxidization peak reaches its maximum height at pH 7.0. As the pH became higher, the peak current continued to decrease. As shown in Figure 8(a), the sensing efficiency increased as the pH value increased from 4.0 to 7.0, and then it rapidly decreased above pH 7.0. It was concluded that the biosensor is sensitive to pH due to the enzyme which the phenol biosensor contains.

As shown in Figure 8(b), the phenol biosensor was tested at various temperatures ranging from 25 to 50°C. While oxidization peak slowly increased from room temperature to 35°C, it sharply increased from 35 to 45°C. However, the potential value decreased above 45°C. The maximum sensitivity of the biosensor was attained at 45°C. It appears that the biosensor is affected by temperature, since it contains enzyme, which sensitively reacts to the temperature.

The electrochemical biosensing of phenol was performed under optimized experimental conditions. In Figure 9(a), the CV data show how the potential value varies according to the phenol concentration degree. Figure 8(b) demonstrated the relationship of

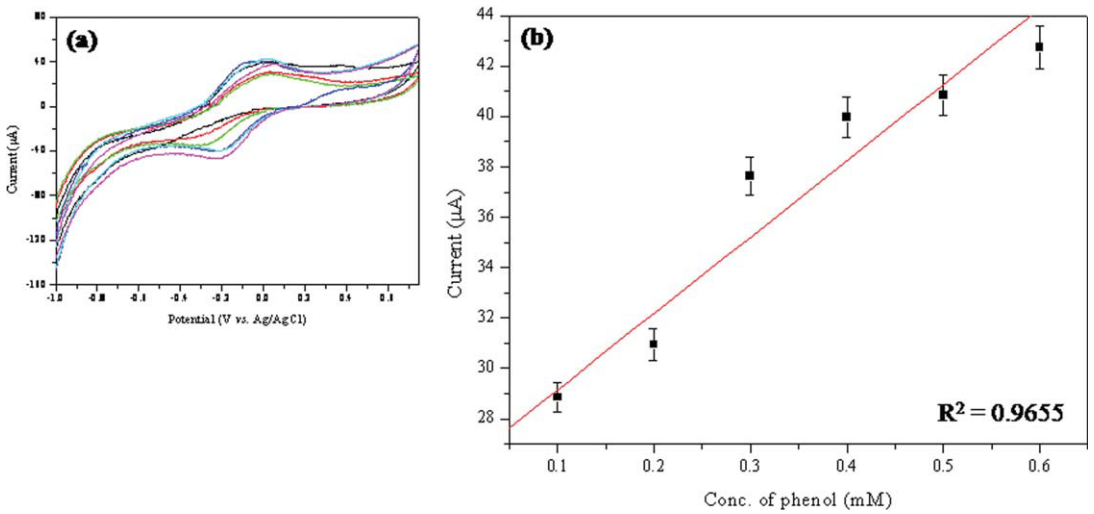

Figure 9 Cyclic voltammograms of the tyrosinase-immobilized biosensor in 0.1*M* phosphate buffer solution (pH = 7.0) containing 0.1–0.6 m*M* phenol with a scan rate 100 mV/s (a) and calibration plot of the concentration of phenol with concentration between 0.1 and 0.6 m*M* (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I
Total Phenolic Amounts in Commercial Red Wines Determined by the Tyrosinase-Immobilized Biosensor Based on F-Fullerene-C_{60}[a]

	Current density (A)	Phenolics[b] (mg/L)	Phenolics[c] (mg/L)
Chateau Mani-dry (Korea)	9.7×10^{-5}	137.9	369.6
Chateau Mani-sweet (Korea)	1.2×10^{-4}	138.9	556.2
Chateau Mani-nouveau (Korea)	2.1×10^{-4}	182.7	895.2

[a] The amounts of total phenolics were calculated from the calibration curve as shown in Figure 10.
[b] The sensing was performed in 2.0 mL commercial red wine.
[c] The sensing was performed in 100 mL commercial red wine with 0.1 *M* phosphate buffer solution.

the concentration of phenol to the amount of current flowing over the phenol biosensor. In the data, the current increase is the concentration of phenol increases. This means phenol is detected in the phenol biosensor. The sensing range of the biosensor was in the range of 0.1–0.6 m*M* for phenol in the phosphate buffer solution.

Total amounts of the phenolic compounds in commercial red wines

Three red wines, Chateau Mani-dry, Chateau Mani-sweet, and Chateau Mani-nouveau that were made in Korea were used in the analysis of amounts of phenolic compounds. The biosensor detected all of the phenol in the three commercial red wines, as shown in Table I. The current density of each of the three wines is as follows: Chateau Mani-dry is 9.7×10^{-5} A, Chateau Mani-sweet is 1.2×10^{-4} A, and Chateau Mani-nouveau is 2.1×10^{-4} A. The total concentration of phenolics was 369.6.5, 556.2, and 895.2 mg/L, respectively. As a result, the biosensor was affected by the alcoholic solution. The data also show that Chateau Mani-nouveau has a higher total concentration of phenolics than do the other red wines. The conclusion is that the high amounts of phenolic compounds in Chateau Mani-nouveau are responsible for the bitter taste of the wine.

CONCLUSIONS

Radiation functionalization of fullerene using γ-irradiation in a mixture of MeOH/1,2-dichlorobenzene was analyzed using FTIR, Raman spectroscopy, MALDI-TOF mass spectroscopy, and elemental analysis and was used as a sensor material. From the data presented, the following conclusions are made:

1. The hydroxyl group (—OH) was easily and simply introduced on the surface of fullerene during γ-irradiation in the mixture solution of MeOH/1,2-dichlorobenzene (1/1 vol %).
2. The obtained F-fullerene was well dispersed in an aqueous solution by ultrasonication, and the F-fullerene was used as sensor materials.
3. The sensing range of the biosensor was 0.1–0.6 m*M* for phenol in a phosphate buffer solution.
4. The prepared biosensor was applied to the determination of phenolics in commercial red wines.

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