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Radiolytic Introduction of Multiple Functional Groups to Multiwalled Carbon Nanotubes and Their Application as Biosensor Supports

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ABSTRACT: Tyrosinase was immobilized on multiwalled carbon nanotube (MWNT) supports that were functionalized with multiple groups. It was then used for the detection of phenolic compounds. The radiation-induced graft polymerization of 1-[(4-ethenylphenyl)methyl]-3-buthyl-imidazolium chloride and vinyl ferrocene introduced functional groups derived from both species onto the nanotubes' surfaces: imidazolium salts that contained sites for the enzyme's immobilization via ionic bonding and ferrocene compounds that acted as electron transfer mediators via redox reactions. Using these additives at a 1 : 4 molar ratio resulted in an electrode with optimized current. The multifunctionalized nanotube supports were characterized by X-ray photoelectron spectroscopy, transmission electron microscopy, and thermogravimetric analysis. The prepared tyrosinase-immobilized biosensor showed a sensing range of 1.0×10^{-4} *M* to 7.0×10^{-4} *M* and was used for the detection of phenolic compounds in red wines. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: nanotubes; graphene and fullerenes; grafting; radical polymerization

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INTRODUCTION

Amperometric enzymatic biosensors are potentially useful in chemical and biomedical analyses, pollution monitoring, biotechnology, and food and agricultural processing.[1–3] They are suitable for biochemical analysis because of their good selectivity, sensitivity, rapid responses, compactness, and reproducible results.[4,5] However, redox enzymes' electron transfer efficiencies are poor in the absence of mediator because of their deeply embedded active sites. Therefore, biosensors' sensitivities can be significantly improved by the addition of mediators to their matrices.

Ferrocene and its derivatives have been used as electron transfer mediators due to their relatively low molecular mass, reversibility, regeneration at low potential, and generation of stable redox forms.[6–9] The immobilization of electron transfer mediators on electrodes' surfaces is well studied because low-molecular weight, soluble mediators can easily diffuse away from an electrode's surface into the electrolyte during a biosensor's continued use, significantly decreasing the electron signal and the sensor's performance and lifetime. The covalent immobilization of ferrocene derivatives onto electrode supports can reduce this problem.

Radiation-induced graft polymerization (RIGP) can introduce specific properties onto the surfaces of functional polymers' matrices, these include thermal stability, mechanical strength, electronic properties, and crystallinity. Enzymatic biosensors have been prepared by the RIGP of variously functionalized vinyl monomers onto MWNTs at room temperature.[10–14] The vinyl monomers' functional groups can be used as physical interaction sites due to their hydrophilic properties that are compatible with those of the enzyme. This allows the functional groups to interact easily on the surface of the electrode. MWNTs have been used as supporting materials because of their high chemical stability, high surface area, and unique electronic properties.[15]

Tyrosinase has been immobilized on MWNT supports via 1-butylimidazole bromide immobilization sites through the RIGP of glycidyl methacrylate with epoxy groups.[16,17] The resulting biosensors could be used to detect phenolic compounds in red wine. Sensors based on ionically modified MWNTs have been prepared in aqueous solutions at room temperature using vinyl monomers such as 3-(butyl imidazol)-2-(hydroxyl)propyl methyl methacrylate and 1-[(4-ethenylphenyl)methyl]-3-buthyl-imidazolium chloride (EMBI) with ionic properties. Tyrosinase immobilized via imidazolium sites has been used to detect total phenolic compounds, mainly caffeine, in coffee. Overall, biosensors based on MWNTs functionalized with EMBI are promising for the detection of target organic molecules in electrolytes. However, there are no reports of biosensors based on supports

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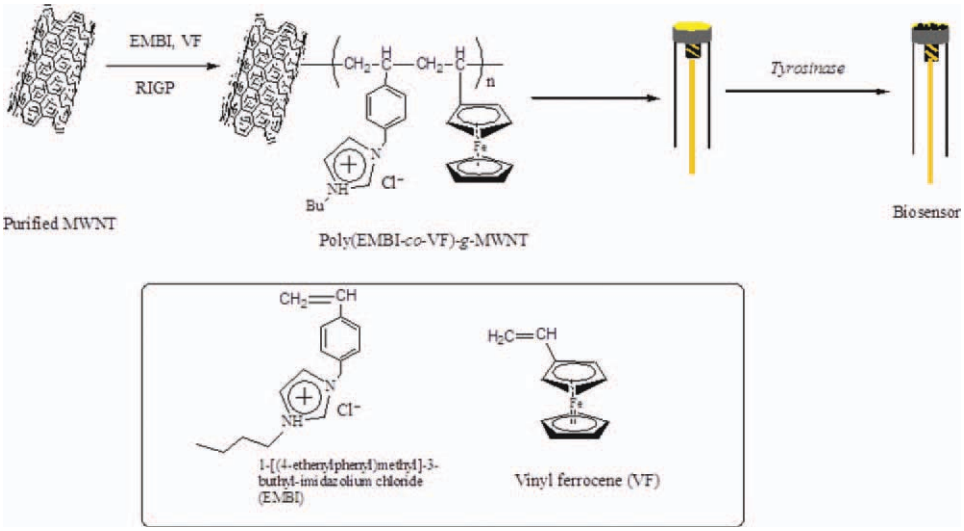

Figure 1. Preparation procedure of tyrosinase-immobilized biosensor based on MWNT supports with multifunctional group for detection of phenolic compounds. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with multiple functional groups, e.g., both imidazolium salts that can immobilize enzymes via ionic bonding and ferrocene compounds that can mediate electron transfer via redox reactions.

This work reports the fabrication by RIGP of biosensors through the immobilization of tyrosinase on MWNT supports that contained various amounts of both imidazolium salts, for enzyme immobilization, and ferrocene groups, for electron mediation. The resulting MWNT supports were analyzed by X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM), and thermogravimetric analysis (TGA). Electrodes were prepared by hand casting the MWNT supports onto the surfaces of glass carbon (GC) electrodes and their currents were measured by cyclic voltammery. A tyrosinase-immobilized biosensor was fabricated by immobilizing tyrosinase onto the surface of the most suitable electrode in 0.1 *M* phosphate buffer solution (1.0 mL, pH = 7.0). It was then tested in the detection of phenolic compounds and its sensing range for phenol was evaluated in the phosphate buffer solution. Its optimal operating conditions—pH, temperature, and phenol detection range—were evaluated and the sensor was used to measure total phenolic concentrations in red wines.

EXPERIMENT DETAILS

Reagents

Tyrosinase from mushrooms (EC 1.14.18.1), phenol, *p*-chresol, catechol, and vinyl ferrocene (VF) were from Aldrich–Sigma Chemical MWNTs (CM-95) were from Hanwha Nanotech (Korea). Solutions were prepared with water from a Milli-Q puls water purification system (Millipore, final resistance, 18.2 $M\Omega$ cm^{-1}) that was degassed prior to each measurement. Other chemicals were of reagent grade.

Synthesis of 1-[(4-Ethenylphenyl)methyl]-3-Buthyl-Imidazolium Chloride as Electron Transfer Mediator

The 1-[(4-ethenylphenyl)methyl]-3-buthyl-imidazolium chloride was synthesized by an elsewhere reported method.[17] In detail, butyl imidazole (5.45 mL, 0.04 mol) was dissolved in acetonitrile (30 mL) in a 100-mL round bottom flask under magnetic stirring. The 4-vinylbenzyl chloride (6.94 mL, 0.04 mol) was added and the mixture was stirred overnight at 50°C. When the reaction completed, the mixture was added to Et_2O, and then frozen for several hours. Decanting the solvent left the product that was then dried in a vacuum oven at room temperature. Yield = 66.4%, LC-Mass = 301.2.

Tyrosinase-Immobilized Biosensor Preparation based on MWNT Supports with Multiple Functional Groups

Tyrosinase-immobilized biosensors were fabricated using MWNT supports with multifunctional groups (Figure 1). The MWNTs were first purified to remove any catalyst and noncrystallized carbon impurities by treatment with phosphoric acid solution for 6 h at 60°C. They were then used to support the RIGP of the binary vinyl monomers, EMPI and VF. MWNTs (0.2 g) and various compositions of the binary vinyl monomers (Table I) were mixed in methanol/water (95/5, vol %) mixture (350 mL). Nitrogen was bubbled through the solution for 30 min to remove oxygen. The solution was then irradiated by a 30 kGy dose of γ-rays from a ^{60}Co source at 1.0×10^4 Gyh^{-1} under atmospheric pressure and ambient temperature to induce free radicals. The prepared MWNT supports were washed using chloroform, acetone, and methanol, sequentially, to remove homopolymer and then dried in a vacuum oven at 50°C and 3.0 mg was then dissolved in dimethylformaide (DMF, 1.0 mL) to prepare the coating solution. MWNT electrodes were fabricated by hand casting 6.0 μL coating solution onto GC electrodes (0.2×0.2 cm^2), which were dried in a vacuum oven at 50°C for 24 h. The tyrosinase was strongly immobilized on the positively charged imidazolium salts of the most suitable MWNT electrode by immersing the electrode in 0.1 *M* phosphate buffer (1.0 mL, pH = 7.0) with tyrosinase at 37°C for 20 h. The resulting biosensor was stored in phosphate buffer solution (PBS, pH = 7.0).

Table I. Properties of the MWNT Supports with Multifunctional Group Prepared by RIGP[a]

No.	Feed		Graft yield (%)[b]	Fe content (%)[c]	N content (%)[c]	CV current (A)
	EMPI (mol %)	VF (mol %)				
1	100	0	26.8	-	1.90	7.57×10^{-5}
2	80	20	24.3	0.20	1.20	9.10×10^{-5}
3	60	40	21.5	0.36	1.20	1.21×10^{-4}
4	50	50	23.0	0.38	1.12	2.68×10^{-4}
5	40	60	21.9	0.39	0.91	3.15×10^{-4}
6	20	80	24.5	0.55	0.85	3.48×10^{-4}
7	0	100	22.3	0.70	-	7.078×10^{-5}

[a]Reaction condition: MWNT 0.2 g, solvent 350 mL.
[b]Determined by TGA.
[c]Determined by XPS.

Instrumentation

Cyclic voltammograms were measured using a potentiostat/galvanostat (model 283. Ametek PAR, USA) in a conventional three-electrode system comprising a working electrode of the glass carbon (GC) GC MWNTs, a platinum wire counter electrode, and a Ag/AgCl (sat'd KCl) reference electrode. Samples' surface morphologies were determined by HR-TEM (JEOL, JEM-2010, USA). X-ray photoelectron spectra were measured using on a MultiLab ESCA2000 (Thermo Fisher Scientific). Thermogravimetric analysis (TGA) was conducted on a Scinco TGA S-1000 (Seoul, Korea) under an N_2 flow from 25 to 700°C at a heating rate of 20°C min^{-1}.

RESULTS AND DISCUSSION

Preparation and Characterization of MWNT Supports with Multiple Functional Groups for Use as Biosensors

Various vinyl monomers such as acrylic acid, methacrylic acid, glycidyl methacrylate, maleic anhydride, and vinylphenyl boronic acid have previously been grafted onto MWNTs by aqueous RIGP at room temperature.[15] Vinyl monomers were selected for this work because they possess hydrophobic sites to complement the hydrophilic functional groups that were to be attached to them. The vinyl groups interacted with the nanotubes' surfaces through hydrophobic–hydrophobic interactions, and the functional groups attached to the vinyl monomers interacted with the aqueous solution through their hydrophilic properties. Radical polymerization of the vinyl monomers was induced on the nanotubes' surfaces by γ irradiation, which introduced various functional groups to the nanotubes' surfaces while maintaining their tubular morphology. Tyrosinase has been immobilized on biosensors incorporating MWNT supports with anion-exchange,[11] hydroxy,[12] and carboxylic acid[13] groups and have been used for the detection of phenolic compounds. In these previous cases the enzyme was physically adsorbed onto the nanotubes by RIGP. Biosensors prepared by physical adsorption are of limited use as the adsorbed enzyme can dissociate into the electrolyte during sensing, greatly reducing sensing efficiency. To overcome this, the enzyme should be strongly

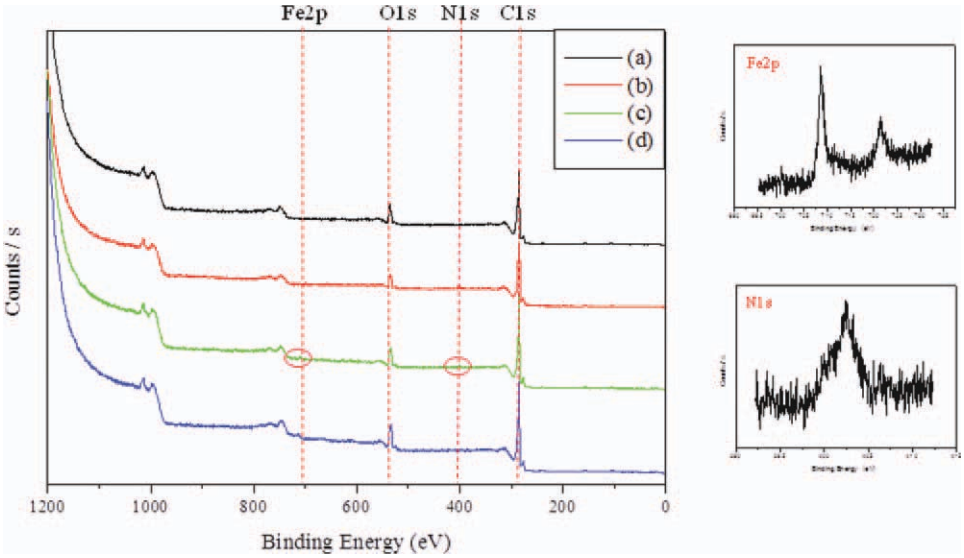

Figure 2. XPS survey scan spectra of the MWNT (a), No. 1 (b), No. 6 (c), and No. 7 (d) prepared by RIGP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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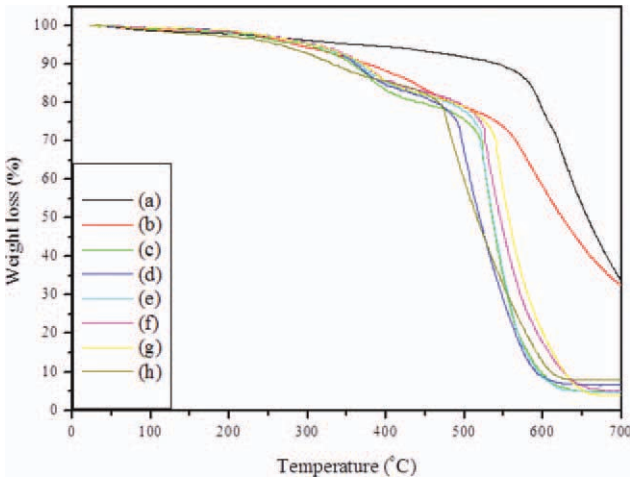

Figure 3. TGA curves of the purified MWNT (a), No. 1 (b), No. 4 (c), No. 6 (d), No. 5 (e), No. 3 (f), No. 2 (g), and No. 7 (h) prepared by RIGP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

immobilized on the nanotubes' surfaces by ionic bonding. Therefore, EMBI was chosen here to provide ionic bonding, and hence strong enzyme immobilization, and vinyl ferrocene was selected as an electron transfer mediator, to increase the efficiency of sensing phenolic compounds via redox reactions.

Various compositions of EMPI and VF underwent RIGP onto the MWNTs in MeOH at room temperature (Table I). Grafting yields were found to be 21–27% by TGA. Fe content increased and N content decreased with the feed's increasing VF content. The maximum CV current was displayed by the electrode with a EMPI : VF molar ratio of 1 : 4 (sample 6, Table I). The results show that MWNT supports with multiple functional groups—ionic and enhanced electron properties—were successfully synthesized by a one-step radiation-induced copolymerization in MeOH at room temperature.

Comparison of the XPS spectra of the pure nanotubes and the MWNT supports with multiple functional groups prepared by RIGP shows that grafting the monomers resulted in significantly different spectra (Figure 2). Peaks characteristic of Fe2p at 713 eV and N1s at 402 eV appeared after grafting, indicating the successful functionalization of the nanotubes by RIGP.

The TGA curve of the purified and functionalized MWNTs showed initial mass losses at 50–250°C that were attributable to moisture loss. This mass loss was increased in the vinyl grafted samples due to their increased hydrophilicity (Figure 3). A second, greater, mass loss at 250–600°C was shown by the vinyl-grafted samples. It was

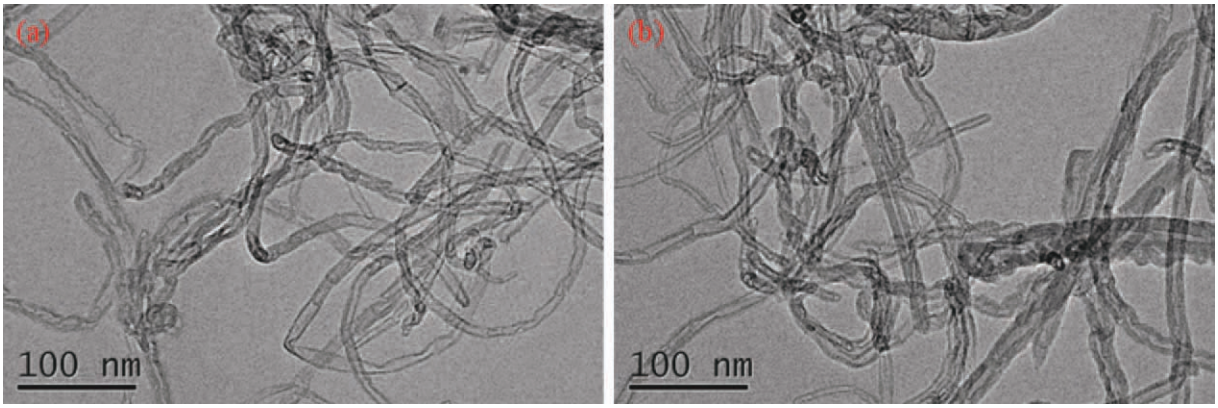

Figure 4. TEM images of the purified MWNT (a) and No. 6 (b) prepared by RIGP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

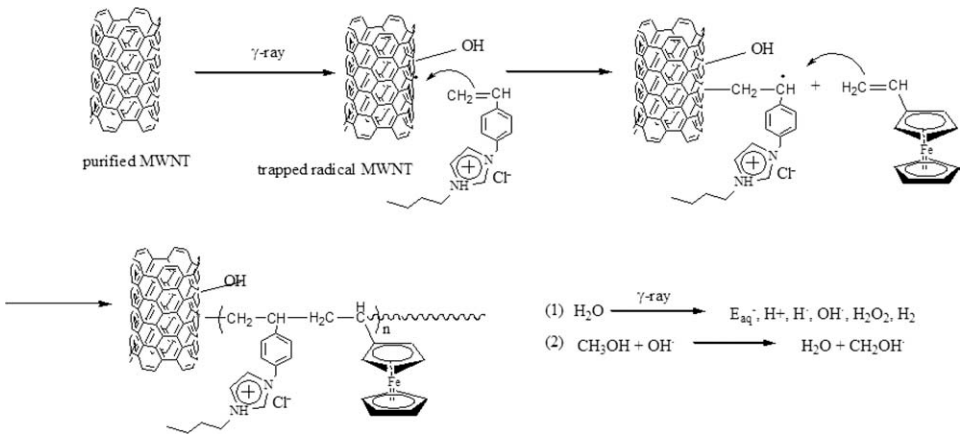

Figure 5. Possible grafting mechanisms of the vinyl monomers onto the surface of MWNT in H_2O/MeOH mixture solution during g-irradiation.

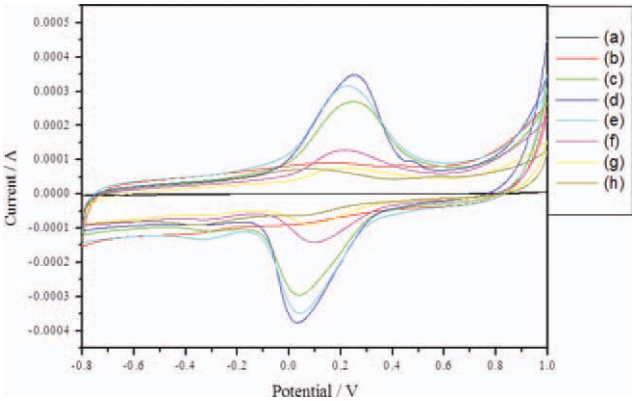

Figure 6. Cyclic voltammograms of GCE (a), No. 1 (b), No. 4 (c), No. 6 (d), No. 5 (e), No. 3 (f), No. 2 (g), and No. 7 (h) supporting electrode in 0.1 *M* PBS (pH = 7.0); scan rate: 50 mVs^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

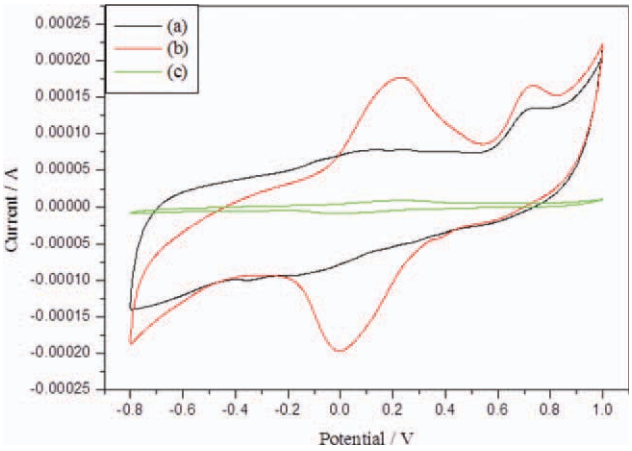

Figure 7. Cyclic voltammograms of tyrosinase-modified biosensor prepared by No. 1 (a), No. 6 (b) and No. 7 (c) in 6.0 mL phosphate buffer solution (pH = 7.0) containing 0.1 m*M* phenol; scan rate: 50 mVs^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

attributed to mass loss through the grafted vinyl polymer. Vinyl graft yields were calculated to be about 21–27% after RIGP.

Samples' TEM images show that a fine coating on the nanotubes' surfaces emerged after the RIGP of the comonomers (Figure 4). The increased diameter of the MWNT supports after RIGP indicates the successful attachment of the multiple functional groups.

We can obtain two active species such as free radical and the solvated electron ($e_{aq}-$) in water/methanol mixture solution when γ-ray irradiated in aqueous solutions, as shown in the following equation.

$$H_2O \rightarrow e_{aq}^-, H^+, H\bullet, OH\bullet, H_2O_2, H_2 \quad (1)$$

$$CH_3OH + OH\bullet \rightarrow H_2O + \bullet CH_2OH \quad (2)$$

The hydroxyl radical (OH●) or hydroxymethyl radical (●CH_2OH) can be immobilized onto MWNT surface as shown in Figure 5. As results the formed radical trapped MWNT can be initiated for grafting polymerization of vinyl monomers on the surface of MWNT.

Application of the Multifunctionalized MWNTs as Biosensor Supports

MWNT supports are generally coated onto the surfaces of GC electrodes using polymer binder. However, the functionalized MWNT supports prepared by RIGP could be coated onto the surfaces of GC electrodes using a 2 : 1 v/v mixture of DMF and water without polymer binder. Cyclic voltammetry was performed in PBS at pH = 7.0 after hand-casting the MWNT supports onto the GC electrodes' surfaces (Figure 6). Current increased as the binary monomer mixture's Fe content increased (Table I). This is considered that electron transfer is increased because of synergetic effects between ionic group of EMPI and ferrocene group as mediator of VF.

Tyrosinase was immobilized on biosensors based on MWNT supports with only EMPI, both EMPI and VF, and only VF. The biosensors were used to assess phenol in 0.1 *M* phosphate buffer solution (pH = 7.0) at a scan rate of 50 mVs^{-1} (Figure 7).

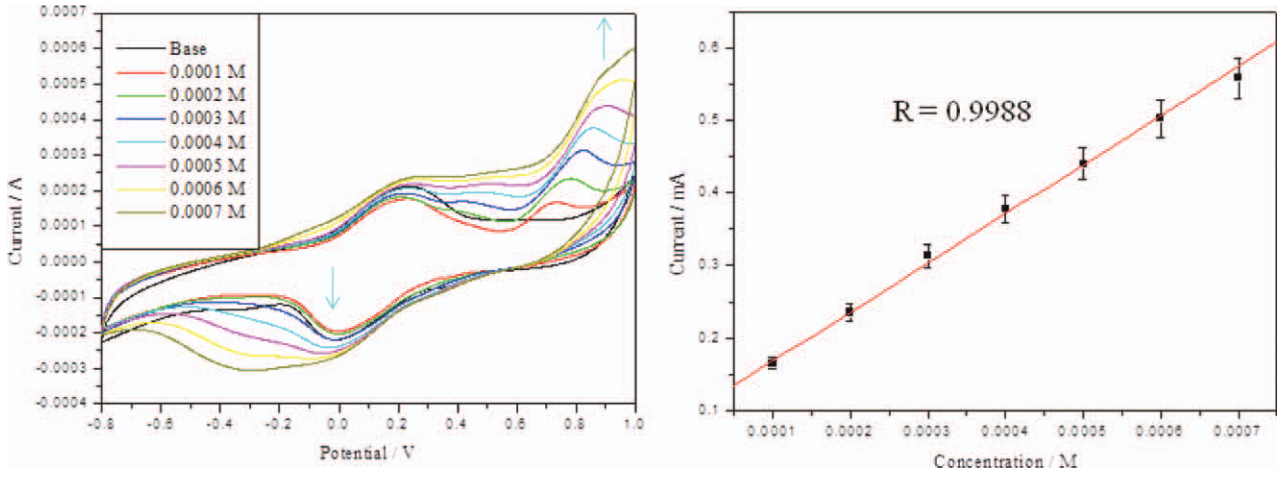

Figure 8. Cyclic voltammograms of tyrosinase-modified biosensor prepared by No. 6 in 6.0 mL phosphate buffer solution (pH = 7.0) containing 0.1–0.7 m*M* phenol; scan rate: 50 mVs^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Total Phenolic Amounts in Commercial Red Wines Determined by the Tyrosinase-Immobilized Biosensor Based on No. 6 Supports[a]

	Current density	Phenolics[b]
BLUE NUN (France)	2.7×10^{-4} A	937.5 mg L^{-1}
Carlo Rossi (USA)	1.9×10^{-4} A	925.6 mg L^{-1}

[a]The amounts of total phenolics were calculated from the calibration curve as shown in Figure 7.
[b]The sensing was performed in 60 μL commercial red wine with 0.1*M* phosphate buffer solution.

Tyrosinase immobilized on nanotubes prepared with a mixture of EMPI and VF showed the best response, demonstrating the importance of both functionalizing species.

On the other hand, the biosensors based on MWNT for determination of phenolic compounds have been studied many researchers.[18–23] However, there are no reports that the preparation of MWNTs supporting with multifunctional group using radiation-induced graft polymerization until now.

The biosensor's sensing range, an important property, was determined by cyclic voltammetry of various concentrations of phenol in 50 m*M* phosphate buffer at pH = 7.0 (Figure 8). The sensor's detection range for phenol was found to be 1.0×10^{-4} *M* to 7.0×10^{-4} *M* at oxidation peaks.

The biosensor was used to assess the total phenolic contents of red wines in a phosphate buffer at room temperature. Concentrations ranged between 926 and 940 mg L^{-1}. The biosensor demonstrated that multifunctionalized MWNT supports could be used as the basis for determining phenol concentrations in red wines.

CONCLUSION

Tyrosinase was immobilized on MWNT supports that were multifunctionalized by radiation-induced graft polymerization. The resulting biosensor's sensing range for phenol was 1.0×10^{-4} *M* to 7.0×10^{-4} *M*. These results show that MWNT supports prepared by RIGP can be used in enzyme-immobilized biosensors for the detection of phenolic compounds in red wine.

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