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폴리(글리시딜 메타크릴레이트)가 그래프트된 다중벽 탄소나노튜브에 다양한 아민 그룹의 도입과 바이오센서 지지체로서의 응용

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Introduction of Various Amine Groups onto Poly(glycidyl methacrylate)-*g*-MWNTs and their Application as Biosensor Supports

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초록: 다양한 아민 그룹으로 개질된 다중벽 탄소나노튜브(이하 MWNT) 지지체를 기반으로 하여 페놀 화합물을 검출하기 위한 티로시나아제가 고정된 바이오센서를 개발하였다. 방사선 중합법을 이용하여 MWNT에 글리시딜메타크릴레이트를 중합한 후 중합 사슬의 아미노화 반응을 통해 MWNT에 다양한 아민 그룹을 도입시켰다. 이렇게 제조된 물질의 물리적, 화학적 특성은 SEM, XPS 그리고 TGA에 의해 평가되었다. 그리고 제조된 물질을 기반으로 제작된 티로시나아제가 고정화된 바이오센서의 전기화학적 특성도 평가하였다. 본 효소 바이오센서는 0.1-0.9 mM의 페놀을 검출할 수 있다. 결합효과, pH, 온도 그리고 다양한 페놀화합물에 대한 반응과 같은 여러 가지 변수에 대하여도 최적화하였고 상용 레드와인에서의 페놀화합물 검출도 연구하였다.

Abstract: A *tyrosinase*-immobilized biosensor was developed based on various amine-modified multi-walled carbon nanotube (MWNT) supports for the detection of phenolic compounds. MWNTs with various amine groups were prepared by radiation-induced graft polymerization of glycidyl methacrylate (GMA) onto MWNT supports and the subsequent amination of poly(GMA) graft chains. The physical and chemical properties of the poly(GMA)-grafted MWNT supports and the aminated MWNT supports were investigated by SEM, XPS, and TGA. Furthermore, the electrochemical properties of the prepared tyrosinase-modified biosensor based on MWNT supports with amine groups were also investigated. The response of the enzymatic biosensor was in the range of 0.1-0.9 mM for the concentration of phenol in a phosphate buffer solution. Various parameters influencing biosensor performance have been optimized: binder effects, pH, temperature, and the response to various phenolic compounds. The biosensor was tested on phenolic compounds contained in two different commercial red wines.

Keywords: tyrosinase-immobilized biosensor, MWNT supports with various amine groups, radiation-induced graft polymerization, detection of phenolic compounds.

Introduction

In recent years, radiation-induced graft polymerization (RIGP) has become an important research subject, because it has been known as a good method for the modification of the physical and chemical properties of polymeric materials including carbon materials. To obtain various kinds of functional polymers, monomers containing easily modifiable functional groups have been used. Glycidyl methacrylate (GMA) is a monomer that is easily modified into various functional groups. As GMA is polymerized, the epoxy groups in poly(GMA) can be modified to alcohol,[1] amine,[2] phosphonic acid,[3] sulfonic acid,[4] etc.[5] In our previous works,[6,7] we grafted the GMA onto various polymeric supports in order to introduce an amine group. This amine group can be used as a chilating site for the removal of heavy metal ions such as Pb^{2+} and Pd^{2+} and for the adsorption of proteins such as urokinase for use as

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biomaterials. We obtained that the amine group-modified polymer materials have good adsorption properties for protein.

Recently, direct electrochemistry and catalytic activity of many biomolecules have been obtained at electrodes modified with nanomaterials, such as carbon nanotubes (CNTs).[8-11] CNTs have been one of the most actively studied electrode materials in the past few years due to their unique electronic and mechanical properties.[12,13]

On the other hand, the MWNTs were functionalized with various monomers by RIGP in an aqueous solution at room temperature using γ-irradiation.[14] The boronic acid-functionalized MWNTs were used as a supporting material for detecting glucose that has no enzymes. We also introduced a carboxylic acid group onto the surface of the MWNT by RIGP of acrylic acid and methacrylic acid to increase the affinity of the enzymes. When we used the carboxylic acid-functionalized MWNT, the sensitivity of the biosensor could be enhanced due to an increase in affinity between the MWNT surface and the enzymes.[15] The hydroxyl group, –OH, is well known as being one of the functional groups that has affinity to biomolecules.[16-18] The –OH group also has hydrophilic properties and consequently it can easily immobilize the enzyme onto the hydrophilic site by physical methods. Therefore, we also performed the introduction of –OH groups onto CNT surfaces by grafting techniques in order to prepare an enzymatic biosensor.[19] The amine group is also well known as a functional group with an affinity to biomolecules. However, little study has been done regarding the introduction of amine groups onto the surfaces of carbon nanotubes and their use for biosensor supports.

Wines, particularly red wines, contain numerous biologically active compounds, the most important of which are poly-phenols. The nutritional importance of poly-phenols is attributed to their antioxidant properties. In particular, flavonoids and their related phenolic compounds, which are naturally found in red wines, are gaining interest.[20,21] However, there have been few reports on the total amount of phenolic compounds in red wine as determined by electrochemical methods.

In this study, a *tyrosinase*-immobilized biosensor based on amine group-modified MWNT supports was designed to detect or sense the phenolic compounds in red wines. The biosensor was fabricated on a glassy carbon electrode via hand casting of the amine group-functionalized MWNT supports. The biosensor was evaluated for its efficiency in sensing phenols in a phosphate buffer solution. Various parameters influencing the response of the biosensor were tested and optimized: binder effects, pH, temperature, and different phenolic compounds. The total concentration of phenolic compounds in commercial red wines was also measured.

Experimental

Chemicals. *Tyrosinase* from mushrooms (EC 1.14.18.1, Aldrich), glycidyl methacrylate [CH_2=C(CH_3)COOCH_2-CHOCH_2, GMA; Aldrich], trimethylamine [$N(CH_3)_3$, TMA, Showa, Osaka, Japan], triethylamine [$N(CH_2CH_3)_3$, TEA; Showa], ethylenediamine [$NH_2(CH_2)_2NH_2$, EDA; Duksan], diethylenetriamine [$NH_2(CH_2)_2NH(CH_2)_2NH_2$, DETA; Aldrich], triethylenediamine [$C_6H_{12}N_2$, TEDA; Aldrich], hexamethyldiamine [$NH_2(CH_2)_6NH_2$, HMDA; Aldrich] and all the other chemicals of reagent grade were used without further purification. MWNTs (CM-95) were supplied by Hanwha Nanotech (Korea). Phosphate buffer solution (PBS) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 and then adjusting the pH. Solutions for the experiments were prepared with water purified in a Milli-Q plus water purification system (Millipore, the final resistance of water was 18.2 MΩcm^{-1}) and were degassed prior to each measurement.

Fabrication of the *Tyrosinase*-immobilized Biosensor Based on Various Amine Group-modified MWNT Supports. The MWNTs were purified in order to remove the catalyst and non-crystallized carbon impurities. Briefly, the MWNTs were treated with phosphoric acid solution in order to remove the metallic catalysts. The purified MWNTs were used as the supporting materials for grafting with GMA. The MWNTs (0.2 g) and GMA (0.2 g) were mixed in an aqueous solution (100 mL). Nitrogen gas was bubbled through the solution for 30 min to remove oxygen gas, and the solution was irradiated by γ-rays from 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) was used. The obtained samples were separated by centrifugation at 2000 rpm and then dried in a vacuum oven at 50 ºC for 8 hrs.

The poly(GMA)-*g*-MWNT (0.1 g) and TEDA (0.1 g) were dissolved in DMSO solution (50 mL). The reaction was performed at 60 ºC for 8 hrs, which was sufficient to complete the conversion. The amine group-functionalized MWNTs were washed sequentially with acetone and deionized water and the sample was then dried under reduced pressure. The introduction of other amine groups onto poly(GMA)-*g*-MWNTs was done by a similar method to that described above.

In order to prepare the MWNT-modified electrode, slurry

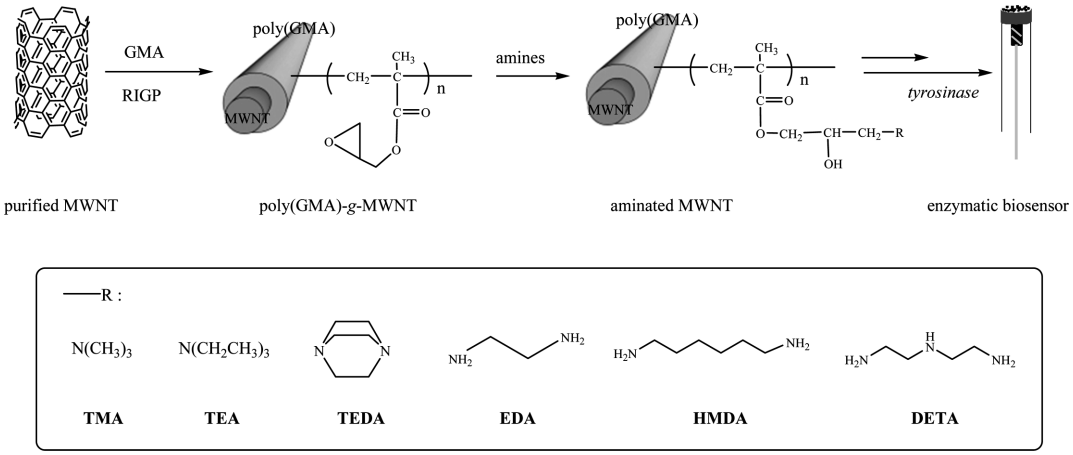

Figure 1. Schematic fabrication of electrochemical enzymatic biosensor based on the aminated MWNT supports.

inks were prepared using a DMF/water (vol/vol, 1/1) mixture solution (40 μL) and amine group-functionalized MWNT supports (1.0 mg) by stirring for 5 hrs. The mixed solution (10 μL) was coated on the surface of a pre-cleaned GC electrode (0.2 × 0.2 cm) by the hand-casting method and then dried in a vacuum oven at 50 °C for 24 hrs. Finally, we prepared the *tyrosinase*-immobilized biosensor by the immobilization of 10 μL of tyrosinase solution (2.0 mgmL^{-1}) onto the amine group-functionalized MWNT-modified electrode. The prepared *tyrosinase*-immobilized biosensor was dried for 1 hr at room temperature and was kept at 4 °C until use.

Instrumentation. The X-ray photoelectron spectra (XPS) of the samples were obtained using MultiLab ESCA2000 (Thermo Fisher Scientific, USA). Surface morphologies were determined by HR-TEM (JEOL, JEM-2010, USA). The thermal gravimetric analysis (TGA) was conducted on a Scinco TGA S-1000 (Seoul, Korea) under N_2 flow from 25 to 800 °C at a heating rate of 20 °C/min. Cyclic voltammetry and chronoamperometry were carried out using a potentiostat Versa STAT3 (Princeton Applied Research). Electrochemical experiments were carried out using a conventional three-electrode system. Working electrodes made of glassy carbon electrodes (SPI supplies, V25 Grade GC) were prepared from 2.0 mm diameter rods encased in epoxy resin. Platinum wire was used as the counter electrode. All potentials are reported versus the reference electrode Ag/AgCl (sat'd KCl).

Results and Discussion

Characterization of the Aminated MWNT Supports Prepared by RIGP. An amine group can be introduced onto the epoxy group of the grafted poly(GMA) on the surface of the MWNT via an SN_2 reaction. The amination was performed in the DMSO by refluxing for 6 hrs in the presence of the poly(GMA)-*g*-MWNT supports. Various amines were used (see Figure 1, structure of the used amines). As a result, the amine group could be introduced onto the epoxy group of the poly(GMA)-*g*-MWNT. However, tertiary amines such as TMA and TEA cannot be introduced onto the epoxy group of the poly(GMA)-*g*-MWNT supports, except for TEDA (see Table 1).

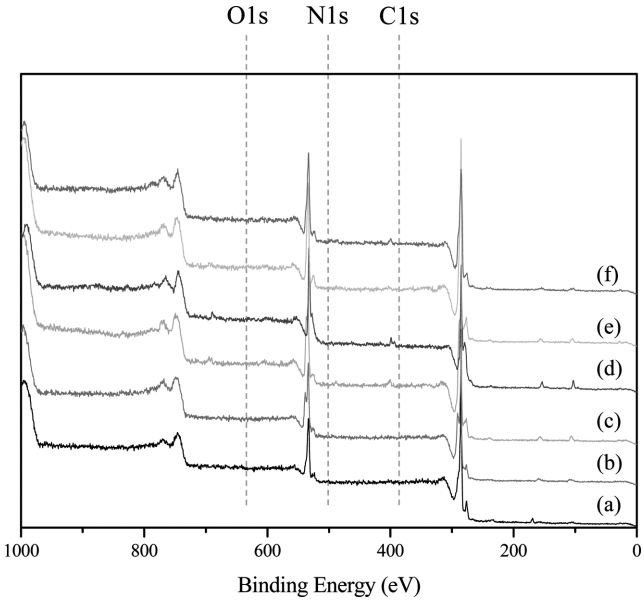

Figure 2. XPS survey scan spectra of the purified MWNT (a); poly(GMA)-*g*-MWNT (b); TEDA- (c); EDA- (d); HMDA- (e); DETA-modified MWNT (f).

Figure 2 shows XPS survey scan spectra of (a) the puri-

fied MWNT, (b) poly(GMA)-*g*-MWNTs, and the aminated MWNT supports with (c) TEDA-, (d) EDA-, (e) HMDA-, and (f) DETA-. The characteristic N1s peak at 400 eV appeared after the amination of poly(GMA)-*g*-MWNT supports. This indicated that the amine group was successfully introduced onto the epoxy group of the poly(GMA)-*g*-MWNT supports via the SN_2 reaction.

Figures 3 and 4 exhibit the high-resolution XPS spectra of the C1s and N1s of (a) the purified MWNTs, (b) poly(GMA)-*g*-MWNTs, and the aminated MWNT supports with (c) TEDA-, (d) EDA-, (e) HMDA-, and (f) DETA-. In Figure 3(a), a small peak of the carbonyl group (>C=O) appeared at 286~287 eV on the purified MWNTs spectrum. This arises because oxidation of the MWNTs occurred during strong acid treatment for removing the metallic catalysts. Grafting the monomers significantly affected the XPS data (see, Figure 3(b)). Grafting with GMA resulted in an additional peak at 288.7~ 289.5 eV due to carbonyl groups in the polymer chains. These data support the successful functionalization of the MWNTs by RIGP. After amination, the characteristic peak of C-N appeared at 290 eV as shown in Figure 3, and an N1s peak also appeared at around 400 eV as shown in Figure 4. A small amount of TEDA was introduced onto the epoxy group of the poly(GMA)-*g*-MWNT supports because of their cationic property (Figure 4(c)). As a result, the aminated MWNT supports for the biosensor were successfully prepared by amination after radiation-induced graft polymerization of GMA with an epoxy group onto the MWNT.

Table 1 summarizes the element composition (%) of the purified MWNTs, poly(GMA)-*g*-MWNTs, and the aminated MWNTs with TMA, TEA, TEDA, EDA, HMDA and DETA, as determined by XPS analysis. Tertiary amines such as TMA and TEA were not introduced onto the epoxy group on the poly(GMA)-*g*-MWNT supports, except for TEDA via the SN_2

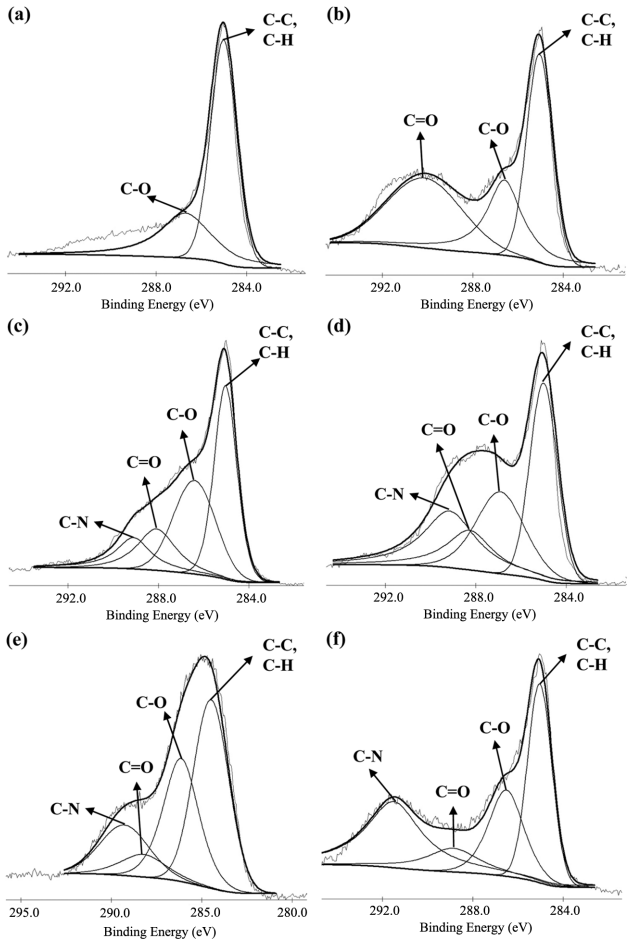

Figure 3. High-resolution XPS spectra of the C1s of the purified MWNT (a); poly(GMA)-*g*-MWNT (b); TEDA- (c); EDA- (d); HMDA- (e); DETA-modified MWNT (f).

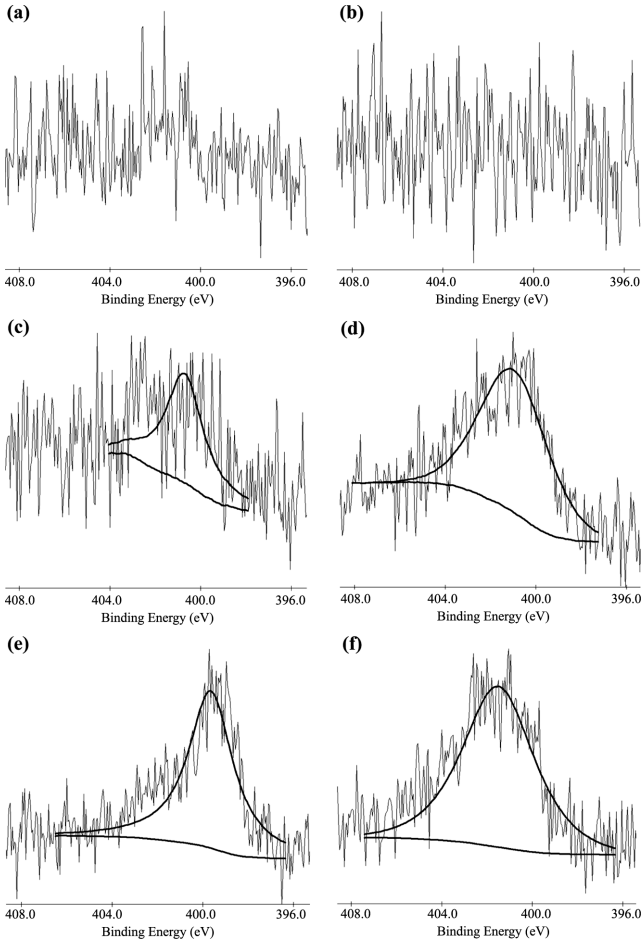

Figure 4. High-resolution XPS spectra of the N1s of the purified MWNT (a); poly(GMA)-*g*-MWNT (b); TEDA- (c); EDA- (d); HMDA- (e); DETA-modified MWNT (f).

Table 1. XPS Surface Compositions of the Purified MWNT, Poly(GMA)-*g*-MWNT, TMA-, TEA-, TEDA-, EDA-, HMDA- and DETA-modified MWNT

Structure			Element surface compositions determined by XPS (at%)		
			C1s	O1s	N1s
Purified MWNT			83.3	16.7	-
Poly(GMA)-*g*-MWNT			78.5	21.5	-
TMA-modified MWNT	$-N(CH_3)_3$	Tertiary	82.3	17.7	-
TEA-modified MWNT	$-N(CH_2CH_3)_3$	Tertiary	81.2	18.8	-
TEDA-modified MWNT	$-NC_6H_{12}N$	Tertiary	81.5	17.7	0.79
EDA-modified MWNT	$-NH_2(CH_2)_2NH_2$	Primary	74.5	23.2	2.27
HMDA-modified MWNT	$-NH_2(CH_2)_6NH_2$	Primary	76.2	21.6	2.11
DETA-modified MWNT	$-NH_2(CH_2)_2NH(CH_2)_2NH_2$	Primary	75.5	20.9	3.57

reaction. However, primary amines were successfully introduced onto the epoxy group on the poly(GMA)-*g*-MWNT supports prepared by RIGP. The N content decreased in the following order: DETA-modified MWNT > EDA-modified MWNT > HMDA-modified MWNT > TEDA-modified MWNT. These aminated MWNTs could be applied as biosensor supports in order to immobilize biomolecules such as enzymes, DNA, etc.

Figure 5 shows TEM images of the purified MWNTs, poly(GMA)-*g*-MWNTs, and the aminated MWNTs with TEDA. A fine coating on the surfaces of the MWNTs, which slightly increased their diameter, is observable in the case of the poly(GMA)-*g*-MWNTs in Figure 5(b). The increased diameter of the MWNTs indicates the successful attachment of epoxy groups by the radiation-induced graft polymerization of GMA. Amines can be covalently introduced onto these MWNT supports with epoxy groups by an SN_2 reaction. In Figure 5(c), the diameter of the MWNT is slightly increased compared to that of the purified MWNT. However, the successful introduction of an amine group onto the epoxy group of the poly(GMA)-*g*-MWNT could not be confirmed perfectly from the TEM image data.

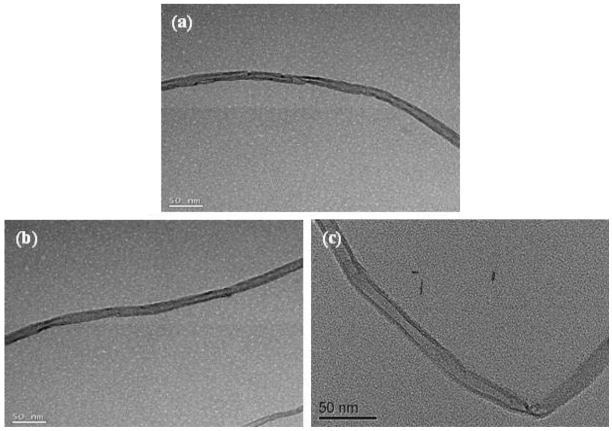

Figure 5. TEM images of the purified MWNT (a); poly(GMA)-*g*-MWNT (b); TEDA-modified MWNT (c).

In order to confirm the introduction of an amine onto the epoxy group of the poly(GMA)-*g*-MWNT, thermal analysis was performed. Figure 6 shows TGA curves of (a) the purified MWNTs, (b) poly(GMA)-*g*-MWNTs, and the aminated MWNT supports with (c) TEDA, (d) EDA, (e) HMDA, and (f) DETA. In Figure 6(a), the first weight loss from 50 to 250 °C for the purified MWNT was attributable to moisture loss because of the change in the hydrophilic properties due to the modification of the carboxylic acid onto the MWNT surface. A sec-

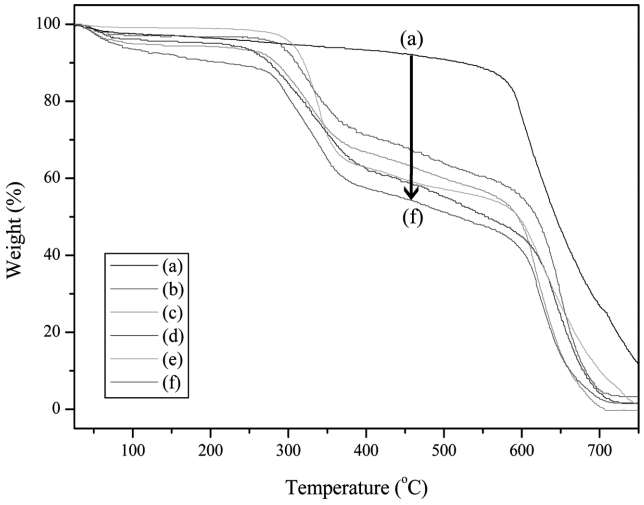

Figure 6. TGA curves of the purified MWNT (a); poly(GMA)-*g*-MWNT (b); TEDA- (c); EDA- (d); HMDA- (e); DETA-modified MWNT (f).

ond weight loss appeared at 600 ºC due to the MWNT backbone. In the spectrum of the poly(GMA)-*g*-MWNT in Figure 6(b), the second weight loss at 250~600 ºC was due to weight loss through the grafted poly(GMA). These results show that the graft yields were ca. 20.0% after RIGP of GMA monomers. After amination, as shown in Figure 6(c), (d), (e), and (f), the weight loss pattern was similar to that of poly(GMA)-*g*-MWNTs. However, the weight loss amounts were larger than those of poly(GMA)-*g*-MWNT, except in the case of the TEDA-modified MWNTs. From these results, we can conclude that the amines were successfully introduced onto the epoxy group of the poly(GMA)-*g*-MWNT by the SN_2 reaction. These aminated MWNTs can be used as biosensor supports in order to immobilize biomolecules such as enzymes, proteins, etc.

Application of the Aminated MWNT as Biosensor Supports. In order to coat MWNT supports onto GC electrodes, a polymer binder is generally used. However, the aminated MWNT supports prepared here could be coated onto the surfaces of GC electrodes using a DMF/water mixture without a polymer binder because a polymer was already present on the surfaces of the MWNTs. After hand-casting the MWNTs onto the GC electrodes' surfaces, the *tyrosinase* was immobilized on the coated MWNT supported electrode, and then CV data were recorded in PBS at pH 7.0 (Figure 7). There was no response to phenol for the electrodes modified with the purified MWNT or poly(GMA)-*g*-MWNT supports, as shown in Figure 7(a) and (b). On the other hand, a significant response to phenol was observed for the aminated MWNT electrodes, except for the electrode modified with TEDA. This result indicates that the amine group on the electrode was an important factor in the immobilization of enzymes. In previous papers,[15,19,22-26] *tyrosinase* immobilized-biosensors based on MWNT supports have also been prepared through the physical adsorption of *tyrosinase* onto electrodes supporting MWNTs with hydrophilic functional groups. In this case, a polymer binder, usually Nafion, was used to coat the MWNT supports with hydrophilic groups. When a polymeric binder was used, the CV response could affect both the polymeric binder and the MWNT supports with hydrophilic groups.

In order to investigate only MWNT supports with amine groups, we prepared an electrode with MWNT supports using Nafion as a polymeric binder, after which we recorded its reponse to phenol in PBS (pH=7.0). Figure 8 shows the cyclic voltammograms of the *tyrosinase*-immobilized biosensor based on DETA-modified MWNT electrodes (a) without Nafion as a binder, and (b) with Nafion as a binder in 0.1 M phosphate buffer solution (pH = 7.0) with a scan rate of 100 mV/s. A significant response was recorded for the *tyrosinase*-immobilized biosensor based on an MWNT electrode

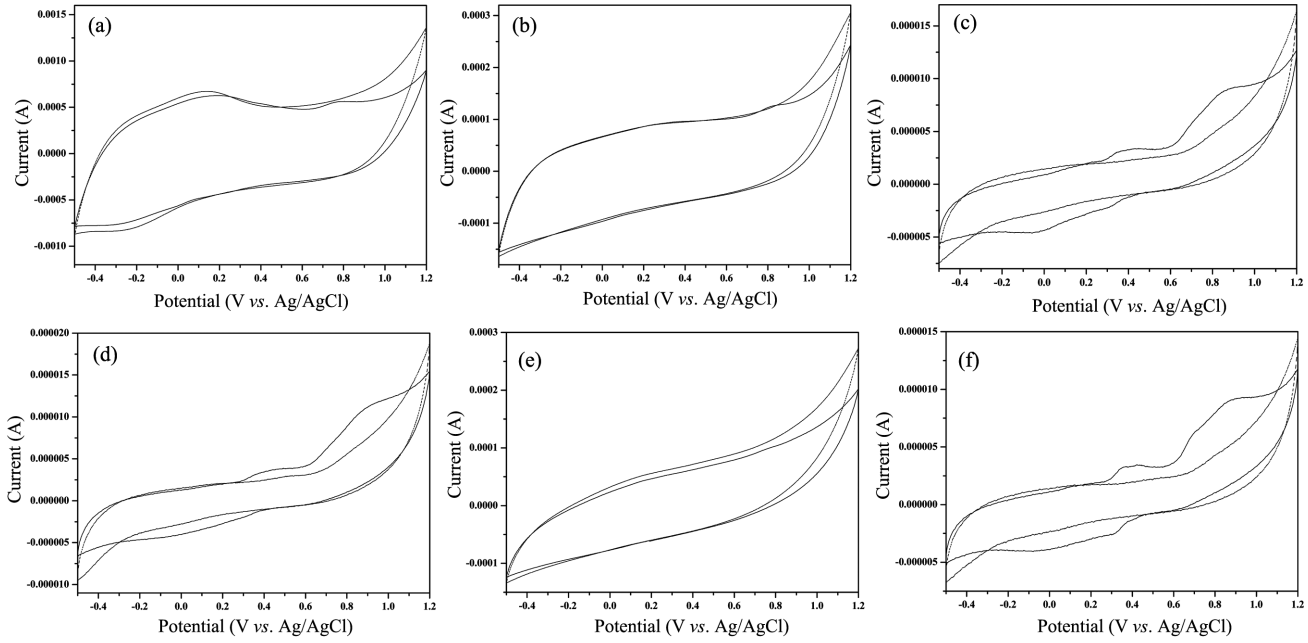

Figure 7. Cyclic voltammograms of the *tyrosinase*-immobilized biosensor based on the purified MWNT (a); poly(GMA)-*g*-MWNT (b); EDA- (c); DETA- (d); TEDA- (e); HMDA-modified MWNT (f) electrode in the absence (--), and presence (—) of 1.0 mM phenol in 0.1 M phosphate buffer solution (pH = 7.0) at a scan rate of 100 mV/s.

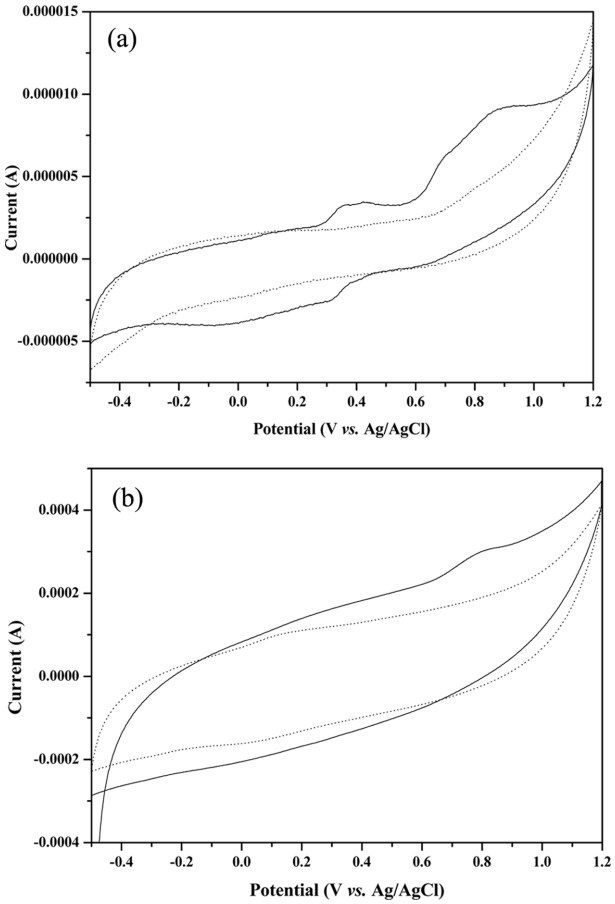

Figure 8. Cyclic voltammograms of the *tyrosinase*-immobilized biosensor based on DETA-modified MWNT electrode without Nafion as binder (a), with Nafion as binder (b) in the absence (--), and presence (—) of 1.0 mM phenol in 0.1 M phosphate buffer solution (pH = 7.0) at a scan rate of 100 mV/s.

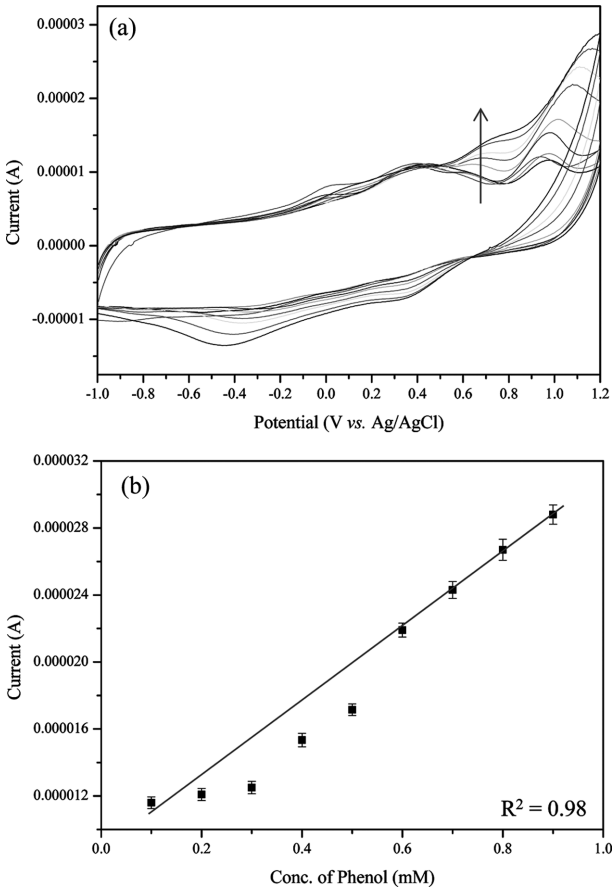

Figure 9. Cyclic voltammograms of the *tyrosinase*-immobilized biosensor based on DETA-modified MWNT electrode in 0.1 M phosphate buffer solution (pH = 7.0) containing 0.1~0.9 mM phenol at a scan rate 100 mV/s (a); calibration plot of the concentration of phenol with concentration between 0.1~0.9 mM (b).

without Nafion as a polymeric binder, as shown in Figure 8. From these results, we can conclude that the aminated MWNTs were very effective supports for increasing the CV response in the enzymatic biosensor.

Sensing range is a very important charcteristic of a biosensor. Cyclic voltammograms of phenols on the biosensor were recorded in 100 mM phosphate buffer at pH 7.0 as a function of phenol concentration (Figure 9). The detection response range for phenol was found to be 0.1~0.9 mM.

The total amounts of phenolics in red wine samples that were detected in a phosphate buffer using the *tyrosinase*-immobilized biosensor at room temperature were in the range of 899~937 mg/L as shown in Table 2. The prepared bioseonser based on the aminated MWNT supports can be used for the determination of phenol concentrations in real red wines.

Table 2. Total Phenolic Amounts in Commercial Red Wines Determined by the *Tyrosinase*-immobilized Biosensor Based on DETA-modified MWNT Electrode[a]

	Current density (A)	Phenolics[b] (mg/L)
BLUE NUN (France)	2.7×10^{-5}	937.1
Carlo Rossi (USA)	1.9×10^{-5}	898.6

[a]The amounts of total phenolics were calculated from the calibration curve as shown Figure 9.
[b]The sensing was performed in 60 μL commercial red wine 0.1 M phosphate buffer solution.

Conclusions

MWNTs with various amine groups were prepared by radiation-induced graft polymerization of glycidyl methacrylate (GMA) onto MWNT supports and the subsequent amination

of poly(GMA) graft chains. Then, a *tyrosinase*-immobilized biosensor was fabricated based on the various amine-modified multiwall carbon nanotube (MWNT) supports for the detection of phenolic compounds. Its sensing range for phenols was 0.1~0.9 mM. It was used to determine phenolic compounds in commercial red wines in a phosphate buffer solution, finding 899~937 mg/L of phenolics in various red wines. These results were calculated from a calibration curve of phenols compiled for the sensor. These results show that the aminated MWNT supports can be used in enzyme-immobilized biosensors as good electron transfer materials and as supports for enzyme immobilization.

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