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방사선 그래프트법에 의해 제조된 탄소나노튜브 지지체를 기반으로 한 전기화학 미생물 바이오센서의 제작

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Fabrication of Electrochemical Microbial Biosensor Based on MWNT Supports Prepared by Radiation-Induced Graft Polymerization

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초록: 4급 아민에 의한 이온성 및 3급 아민에 의한 비공유전자쌍의 이중 특성을 갖은 다중벽 탄소나노튜브 지지체를 글리시딜 메타크릴레이트의 방사선 그래프트법을 수행한 후, 아민화 반응을 수행하여 제조하였다. 제조된 이중 다중벽 탄소나노튜브 지지체와 나피온 용액을 혼합 후, 이 코팅용액을 GC 전극 표면에 코팅시킨 후, 여기에 미생물인 *Alkaligenes spp.*를 고정화하여 미생물 바이오센서를 제작하였다. 이 미생물 센서의 페놀에 대한 검출범위는 0.005~7.0 mM이었다. 이 미생물 바이오센서를 이용하여 상용의 적포도주에서 페놀함량을 측정하였다.

Abstract: A multi-walled carbon nanotube (MWNT) support with dual properties, an ionic property via tetra-amine and unpaired electrons via tri-amine, was prepared by radiation-induced graft polymerization of glycidyl methacrylate (GMA) and the subsequent amination of its epoxy group. The electrochemical microbial biosensor (EMB) was then fabricated by immobilization of a microbe *(Alkaligenes spp.)* onto the dual property-modified electrode, which was prepared with the mixture of the MWNT support and a Nafion® solution on a glass carbon (GC) electrode surface by a hand-casting method. The sensing range of the prepared EMB for phenol in a phosphate buffer solution was 0.005~7.0 mM. The total concentration of phenolic compounds in a commercial red wine was also determined using the EMB.

Keywords: radiation-induced graft polymerization, electrochemical microbial biosensor, MWNT supports, amination.

Introduction

Microbial cells have been immobilized on various subtracts to use in bioreactors and for the production of useful compounds, such as amino acids, organic acids, antibiotics, hydrogen, steroids, exopolysaccharides, and enzymes.[1-5] Microbial cells have also been used in constructing biosensors for recognizing elements.[6] Microbial biosensors have the following advantages: (1) The enzyme does not need to be isolated, (2) The microbial cells are more tolerant to inhibition by solutes and sub-optimal pH or temperature conditions, (3) They are more durable than enzyme electrodes since enzymes are more stable in their natural environment in the cells,[7-9] and (4) Microbes are susceptible to genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes.[10]

Recently, nanomaterials have been used as supporting materials for biosensors due to their greatly enhanced electrochemical reactivity and electron transfer rates.[11] Carbon nanotubes (CNTs) are a new type of nanomaterials and they can be considered as the result of folding grapheme layers into carbon cylinders.[12] CNTs are also utilized as an electrode supporting material because of their high electrical conductivity and good physical and chemical properties.[13] However, a major problem of the CNTs to be used as supporting

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materials in biosensor is their insolubility in any solvents due to the lack of functional groups on the surface of the CNTs. Therefore, many researchers have used polymeric materials, such as Nafion®, poly(propionylethylenimine-coethylenimine), tocopheryl polyethylene glycol succinate, chitosan, poly(metaphenylenevinylene), and helical amilose, in the preparation of CNT solutions.[14-17] However, little has been reported on the graft polymerization of the vinyl monomers with various functional groups onto CNT surfaces in order to increase the solubility of CNTs with the desired functional group.

Radiation-induced graft polymerization (RIGP) is a useful technique for the introduction of various functional groups into different polymeric subtracts using specially selected vinyl monomers. There have been several reports about RIGP of polar monomers onto polymer subtracts with hydrophobic properties in order to obtain hydrophilic properties for versatile applications.[18-23] In previous papers,[24,25] RIGP was also performed to introduce functional group onto multi-walled carbon nanotube (MWNT) surfaces using various vinyl monomers in an aqueous solution at room temperature for electrochemical biosensor supports. No papers have reported on the functional group, especially with an amine group with duel properties of a plus (+) charge and unpaired electron. This duel property of MWNT supports can be expected to increase its binding affinity to various biomolecules, such as enzyme, microbe, protein, etc.

On the other hand, 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 related phenolics which are naturally found in red wines have gained increasing interest.[26] Red wines have been reported to be more cardioprotective and they play a possible role in reducing thrombotic and anthrogenic processes. Polyphenols also contribute substantially to the quality of wines and affect their color, flavor, stability, and aging behavior.[27] However, little has been reported regarding the easy determination of the total amount of phenolics in red wine by an electrochemical method.

In this study, we performed radiation-induced graft polymerization of the poly(glycidyl methacrylate), poly(GMA), onto MWNT surfaces in an aqueous solution at room temperature in order to introduce an epoxy group. The epoxy group can be easily converted to alcohols, amines, phosphonic acid, sulfonic acid, etc. Subsequently, we introduced the triethylene diamine (TEDA) onto the epoxy group of the grafted poly(GMA) in order to prepare MWNT supports with an amine group. Finally, we fabricated the electrochemical microbial biosensor (EMB) after immobilization of a microbe (*Alkaligenes spp.*) on the modified electrode which was prepared by hand casting the mixture of the prepared supports and nafion® solution. The prepared EMB was evaluated by its sensing efficiency for phenol in a phosphate buffer solution. The total concentration of phenolic compounds in commercial red wines was determined using the prepared EMB.

Experimental

Chemicals. The microbe (*Alkaligenes spp.*) was obtained from Analytical Science Center Co. (Korea). Glycidyl methacrylate (GMA), triethyldiamine (TEDA), phenol, *p*-chresol, catechol, gallic acid, and Nafion® solution were of analytical reagent grade (Sigma-Aldrich, U.S.A.) and were used without further purification. MWNTs (CM-95) were supplied by Hanwha Nanotech Co., Ltd (Korea). Solutions for the experiments were prepared with water purified in a Milli-Q plus water purification system (Millipore Co. Ltd., the final resistance of water was 18.2 $M\Omega cm^{-1}$) and degassed prior to each measurement. Other chemicals were of reagent grade.

Preparation of the Electrochemical Microbial Biosensor (EMB) Based on MWNT Supports with Duel Properties. Scheme 1 shows the preparation procedure of the MWNT supports with amine groups and the electrochemical microbial biosensor (EMB). In order to use the supporting materials for a biosensor, the MWNTs were firstly purified to remove the catalyst and non-crystallized carbon impurities. MWNTs were treated with phosphoric acid at 50 ℃ for 5 hrs. The purified MWNTs were used as supporting materials for grafting with GMA. The MWNTs (2.0 g) and GMA (2.0 g) were mixed in an aqueous solution (20 mL). Nitrogen gas was bubbled through the solution for 30 min to remove oxygen gas, and the solution was irradiated by γ-ray from a Co-60 source under atmospheric pressure and an ambient temperature. A total irradiation dose of 30 kGy (a dose rate $=1.0\times10^4$ Gy/h) was used. The obtained samples were separated by centrifuge with 2000 rpm, and then dried in a vacuum oven at 50 ℃ for 18 hrs.

The GMA-grafted MWNTs was immersed in 0.5 M TEDA dissolved in toluene. The reaction was performed at 60 ℃ for 8 hrs, which was sufficient to reach the final conversion. The modified MWNTs were washed sequentially with toluene, acetone, methanol, and hot deionized water and the sample was then dried under reduced pressure.

In order to prepare a MWNT-modified electrode, the mixed solution was prepared using 5% Nafion® solution (91 μL) and amine-functionalized MWNT supports (4.0 mg) by stirring for 24 hrs, then the mixed solution (10 μL) was

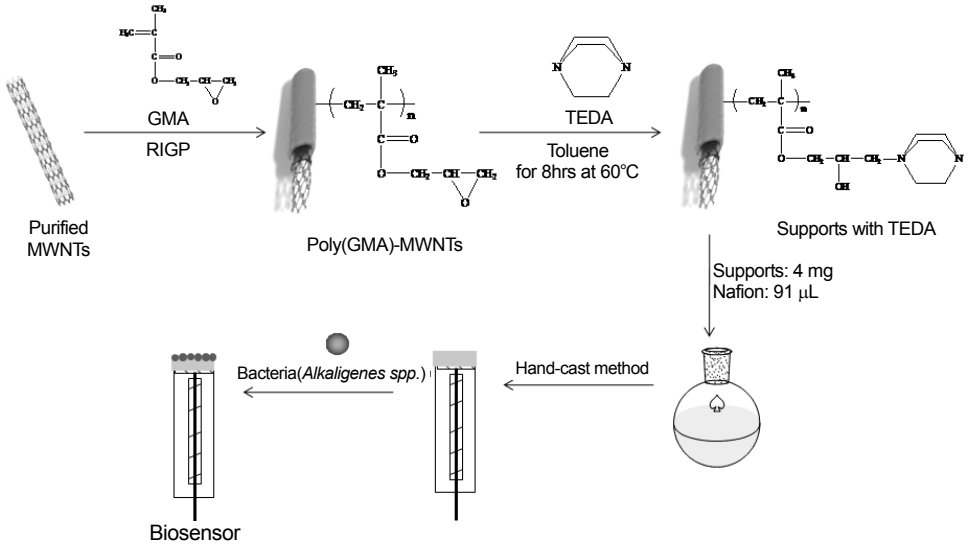
coated on the surface of a pre-cleaned GC electrode (0.02 cm^2) by the hand-cast method. Finally, we prepared an EMB by immobilization of 10 μL of the microbe (*Alkaligenes spp.*, 1.0×10^9 CFU/mL) onto the MWNT-modified electrode.

The prepared EMB was dried for 1 hr at room temperature and was kept at 4 ℃ prior use.

Instrumentation. Cyclic voltammetric experiments were performed with a Potentiostat/Gavanostat model 283 (Ametek PAR, U.S.A.). All experiments were carried out with a conventional three-electrode system. The working electrode was a GC electrode coated with the modified MWNTs, the counter electrode was the platinum wire, and the reference electrode was an Ag/AgCl (sat'd KCl). The X-ray photoelectron spectra of the samples were obtained using MultiLab ESCA2000 (Thermo Fisher Scientific). The surface morphology of the samples was determined by HR-TEM (JEOL, JEM-2010, USA). Thermal gravimetric analysis (TGA) was conducted on a Scinco TGA S-1000 (Seoul, Korea) under N_2 flow from 25 to 700 ℃ at a heating rate of 20 ℃/min.

Results and Discussion

Characterization of MWNT Supports with Amine Groups Prepared by RIGP. Figure 1 shows the XPS survey scan spectra of the purified MWNTs (a), poly(GMA)-*g*-MWNTs prepared by RIGP (b), and MWNTs with TEDA (c). There are significant changes after the introduction of TEDA onto poly(GMA)-*g*-MWNTs in XPS data. The characteristic N 1s peak at 399 eV appears after introduction of TEDA onto the poly(GMA)-*g*-MWNTs. This means that the MWNT support functionalized with TEDA containing a (+) charge via the tetra-amine and the unpaired electron via the tri-



Scheme 1. Preparation procedure of the electrochemical microbial biosensor(EMB) based on MWNT supports with amine groups by RIGP.

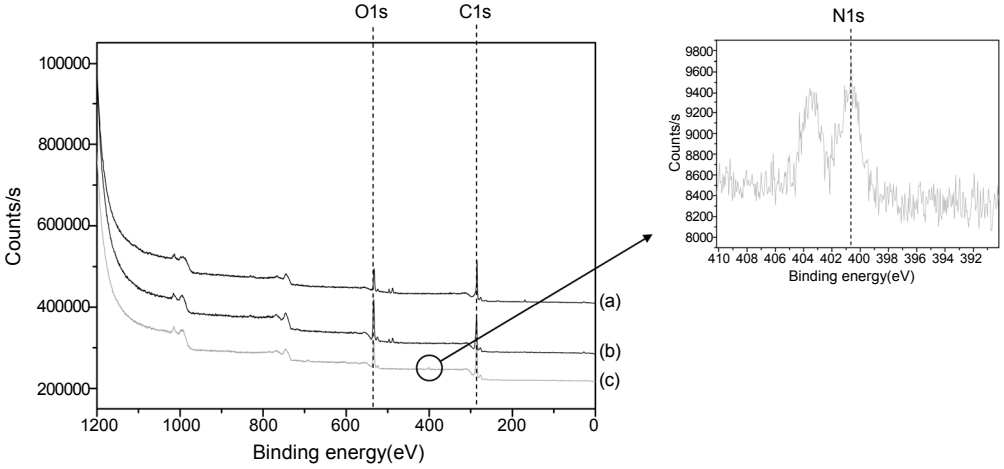


Figure 1. XPS survey scan spectra of the purified MWNTs: (a) poly(GMA)-MWNTs; (b) MWNTs with TEDA; (c) prepared by RIGP.

amine was successfully prepared by the RIGP method. It is expected that this amine functional group will have a strong affinity property for biomolecules, such as enzymes, microbes, antibodies, animal cells, etc. In a previous paper,[28] various vinyl monomers, such as acrylic acid, methacrylic acid, maleic anhydride, and vinylphenyl boronic acid, were grafted onto MWNTs by a one-step reaction of RIGP at room temperature in an aqueous solution. Those vinyl monomers were selected to functionalize MWNTs since they possess both hydrophobic and hydrophilic properties. In this study, we have selected GMA among the vinyl monomers since the epoxy group on GMA can be easily converted to an amine group. We also selected TEDA from the various amine compounds since it reacts with the epoxy group and provides an ionic property and an unpaired electron onto the modified MWNT electrode. The ionic property and unpaired electron can induce strong interaction with the microbe when it is immobilized on the MWNT supports.

We evaluated the morphology of MWNT supports via TEM analysis as shown in Figure 2. The diameter of the purified MWNTs was 40 nm as shown in Figure 2(a) after RIGP, the diameter increased to 60 nm for poly(GMA)-*g*-MWNTs as shown in Figure 2(b). When we introduced TEDA to the epoxy group of the grafted poly(GMA), the diameter increased to 80 nm. The morphology of the poly(GMA)-*g*-MWNTs, as shown in Figure 1(b), exhibited a tubular-type morphology. The likely reason for the tubular-type morphology was as follows: We used the GMA as a vinyl monomer, which is composed of a hydrophilic site of >C=O (carbonyl group) and -C(O)-C- (epoxy group), and a hydrophobic site of a vinyl group. The vinyl group of the GMA comes to the surface of MWNTs because of a hydrophobic-hydrophobic interaction, while the carbonyl and epoxy group of the monomer come to the surface in an aqueous solution because of a hydrophilic-hydrophilic interaction. When irradiated by γ-rays, the radical polymerization of the GMA on the surface of the MWNTs occurs. Hence, we successfully obtained the tubular-type MWNTs as a one-step reaction. After grafting poly(GMA) onto the MWNTs, we performed amination using TEDA because TEDA can induce the ionic property via tetra-amine and the unpaired electron via tri-amine (see the structure in Scheme 1). Thus, we were able to easily immobilize the microbe to the functional group on the MWNTs surface by physical adsorption, and we also used the MWNT as an electron transfer material in order to increase the biosensor sensitivity.

Figure 3 reveals the TGA curves of the purified MWNTs (a), poly(GMA)-*g*-MWNTs (b), and MWNT supports with amine groups (c) prepared by RIGP. The 1st weight loss(%) from 50 to 200 ℃ for the poly(GMA)-*g*-MWNTs and MWNT supports with TEDA appeared on account of moisture because of the hydrophilic properties of the grafted poly(GMA) and amine group. The 2nd weight loss appeared in the range of 250~600 ℃ due to the grafted poly(GMA) weight loss. Results showed that the graft yield was approximately 45% after RIGP of the GMA monomer. From these results, we confirmed the successful preparation of poly(GMA)-*g*-MWNTs. However, there were no remaining MWNTs after introduction of the TEDA as shown in Figure 3(c). It can be considered that the physical and chemical properties of the MWNT supports were very weak during amination in toluene at high temperatures.

Optimization of the Prepared EMB and Determination of Total Amounts of Phenolics in Commercial Red Wines. As mentioned above, MWNTs with the duel property (ionic property and unpaired electron) can be used as supports for microbe immobilization due to their high binding affinity

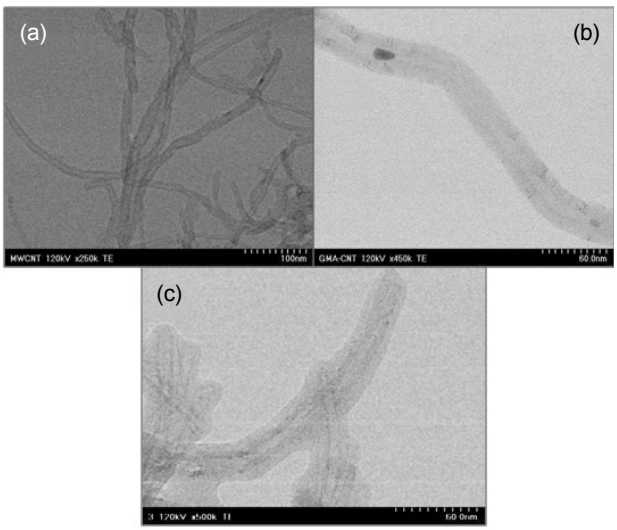


Figure 2. TEM images of the purified MWNTs (a); poly(GMA)-*g*-MWNTs (b); MWNTs with TEDA (c).

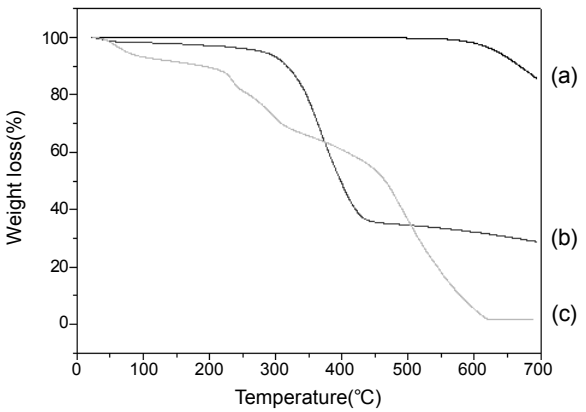


Figure 3. TGA curves of the purified MWNTs (a); poly(GMA)-*g*-MWNTs (b); MWNTs with TEDA (c).

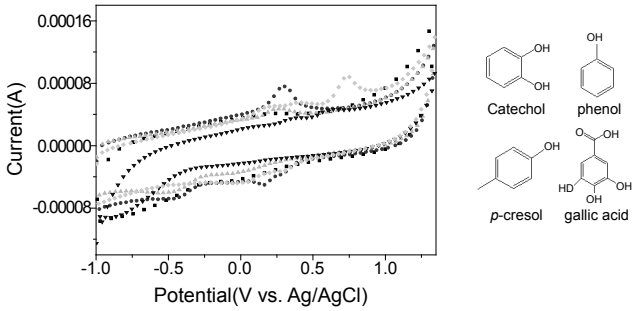


Figure 4. Cyclic voltammograms of the EMB with regards to various phenolic compunds in 0.1 M phosphate buffer solution (■) containing 1 mM catechol (●), 1 mM *p*-cresol (▼), 1 mM gallic acid (▲), and 1 mM phenol (◆) with a scan rate of 100 mV/s.

and improved sensitivity via their ionic property. In previous papers,[7,28] we prepared a *tyrosinase*-immobilized electrode using MWNTs with ion liquid compounds containing ionic properties in order to increase both sensitivity and durability. The prepared enzyme sensor was evaluated based on its efficiency of selectivity, sensitivity, and durability with regards to sensing phenol, *p*-chresol, and catechol in a phosphate solution. As a result, we obtained high selectivity, sensitivity, and durability of the prepared enzyme electrode with regards to sensing phenols when we used MWNTs functionlized with ionic properties. Selectivity, sensitivity, and durability are very important factors for the microbe-immobilized biosensor. Therefore, we firstly evaluated the selectivity of the prepared EMB in sensing various phenolics, such as cartechol, phenol, *p*-cresol, and gallic acid, using cyclic voltammetry as described earlier.[7,28] Figure 4 shows the cyclic voltammograms of various phenolics on the EMB based on the MWNT supports with the dual properties in a 0.1 M phosphate buffer solution (pH=7.0). As shown in Figure 4, the EMB based on MWNT supports with TETA exhibits the redox peaks for both phenol and cartechol compounds. However, there are no redox peaks of other phenolics detected by the prepared EMB.

The sensing efficiency (sensitivity) is also a very important factor in the EMB. We also evaluated the phenol sensing efficiency using an EMS based on MWNT supports. Figure 5 exhibits the cyclic voltammograms of the EMB in a 0.1 M phosphate buffer solution (pH=7.0) containing different phenol concentrations. Figure 5(a) presents cyclic voltammograms of phenol as a function of phenol concentration at a scan rate of 100 mV/s. Followings are the phenol concentrations used: 0, 0.05, 0.10, 0.50, 0.70, 2.00, 4.00, 6.00, 6.50, and 7.00 mM. Figure 5(b) shows the calibration plot of current *vs.* concentration for standard phenol. The prepared biosensor exhibited current responses in the phenol concentration range

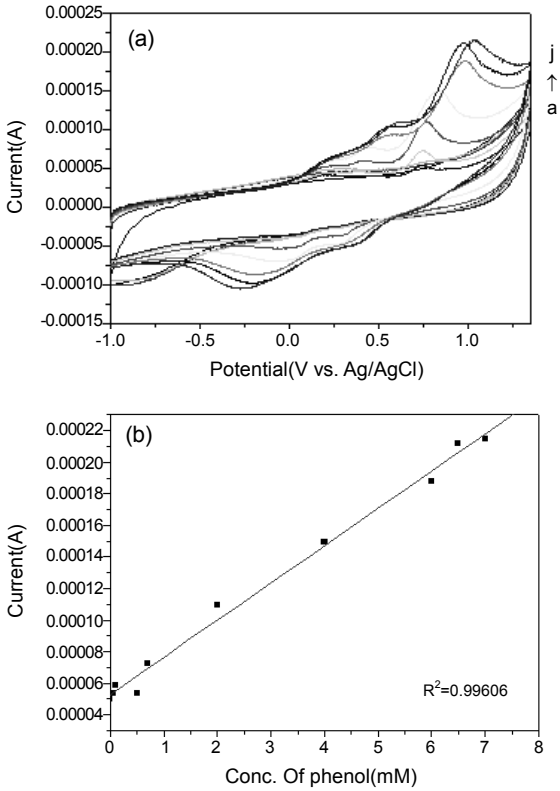


Figure 5. Cyclic voltammograms of the EMB in a 0.1 M phosphate buffer solution (pH=7.0) containing different phenol concentration (a) (from a to j: 0, 0.05, 0.1, 0.5, 0.7, 2, 4, 6, 6.5, 7.0 mM) with a scan rate of 100 mV/s and calibration plot of the concentration of phenol with respect to the current (b).

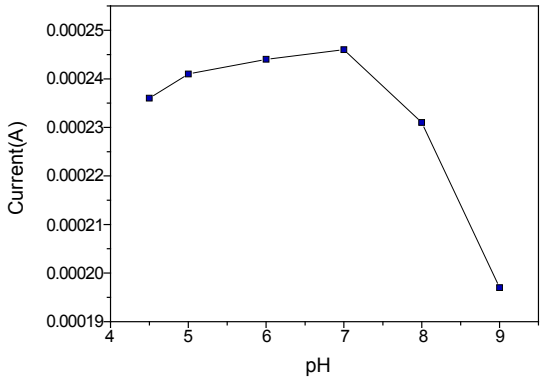
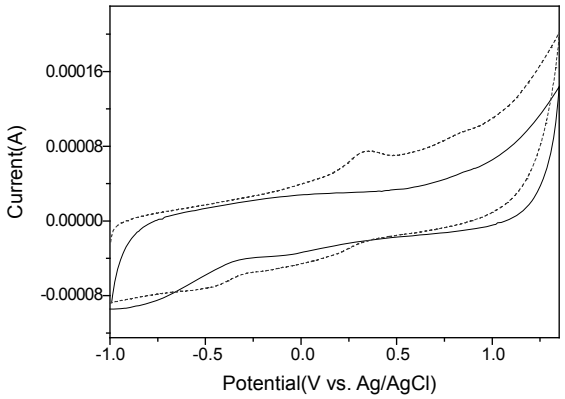


Figure 6. Effect of pH on the response of the EMB in a 0.1 M phosphate buffer solution(pH=4.5, 5, 6, 7, 8, and 9) containing 5 mM phenol at a scan rate of 100 mV/s.

of 0.05 to 7.00 mM.

The electrochemical microbe biosensing of phenols was performed under optimal experimental conditions. Another parameter affecting the sensing efficiency of the EMB based on MWNTs with TEDA is the pH of the supporting electrolyte. Figure 6 reveals the sensing currents of 5.0 mM phenol on the EMB based on MWNTs with TEDA in a 0.1 M



Red wine	Current density	Phenolics
AMOR(Made in Chile)	7.47×10^{-5} A	774.9 mg/L

Figure 7. Cyclic voltammograms of the EMB in a 0.1 M phosphate buffer solution (solid line) containing 100 μL of red wine (AMOR) (dashed line) with a scan rate of 100 mV/s.

phosphate buffer solution as a function of pH. The sensing efficiency was slightly increased from pH=4.0 to pH=7.0, and then it rapidly decreased as the pH levels increased, as shown in Figure 6. This means that at low pH levels, the sensing efficiency of the EMB is not changed due to microbe activity. The anodic peak current dramatically decreased at higher pH. This may be due to the fact that the microbe was unstable and desorption of microbe might occur in alkaline solution.

The commercial red wine, AMOR™ made in Chile, was used in the analysis of phenolic compound amounts in wine. Figure 7 shows cyclic voltammograms of the SMB based on the *Alkaligenes spp.* microbe in a 2.0 mL phosphate buffer solution (pH=7.0) containing 100 μL of red wine. The oxidation peak of red wine in a phosphate buffer using EMB with the microbe appeared at +0.5 V, as shown in Figure 7. As a result, the total amounts of phenolic compounds contained in AMOR™ was 775 mg/L when using the EMB based on the microbe at room temperature. In a previous paper,[28] we also measured the total amounts of phenolic compounds for red wine using a biosensor based on an enzyme in a phosphate buffer solution at room temperature. The total amount of phenolic compounds for a red wine named Shateu Manisweet (made in Korea) was 873 mg/L.

Conclusions

In this study, we fabricated an *Alkaligenes spp.* microbe-immobilized biosensor based on MWNTs with TEDA prepared by radiation-induced graft polymerization. The sensing range of the *Alkaligenes spp.* microbe-immobilized biosensor (EMB) based on the MWNT supports with amine groups for phenol was in the range of 0.005~7.0 mM. The prepared *Alkaligenes spp.* microbe-immobilized biosensor (EMB) was used to determine the level of phenolic compounds in a commercial red wine. As a result, the amount of phenolic compounds in the commercial red wine (AMOR™) was determined to be 775 mg/L which was calculated from a calibration curve of phenol on the prepared biosensor based on MWNT supports with TEDA, as shown in Figure 5(b). From these results, the MWNTs with the amine group containing an ionic property and unpaired electron can be used in microbe-immobilized biosensors as a good electron transfer material and support for microbe immobilization.

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