Multienzyme-Immobilized Modified Polypropylene Membrane for an Amperometric Creatinine Biosensor

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ABSTRACT: Creatinine has become an important clinical analyte that is used for the determination of renal and muscular dysfunction. It is essential to determine its concentration in the serum of patients suffering from renal insufficiency. Therefore, an amperometric creatinine biosensor fabricated from a covered platinum/silver electrode with a thin layer of an immobilized multienzyme membrane was studied. Poly(acrylic acid) was introduced onto an argon-plasma-treated porous polypropylene membrane surface by graft copolymerization. Subsequently, three different enzymes (sarcosine oxidase, creatinase, and creatininase) were immobilized onto this novel grafted membrane simultaneously via a carbodiimine agent to form a thin layer. The sensor performance was evaluated with a biochemistry analyzer. Moreover, attenuated total reflection/Fourier transform infrared, electron spectroscopy for chemical analysis, and scanning electron microscopy were used to confirm the progression of these reactions. The developed sensor showed a linear detection range of 3.2–320 μM for creatinine in a pH 7.4 buffered solution with 0.1M phosphate. The immobilized multienzyme membrane could be used for at least 3 weeks. The results obtained in our study will hopefully lead to the successful application of modified polypropylene for the development of a creatinine sensor. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 92: 3126–3134, 2004

Key words: poly(propylene) (PP); enzymes; membranes

INTRODUCTION

Creatinine is the final product of creatine metabolism in mammals and is a clinically important index of the renal glomerular filtration rate.[1] The reference range for serum/plasma creatinine is 35–140 μM,[2] but during kidney dysfunction or muscle disorder, the creatinine concentration in serum/plasma may rise to values higher than 1000 μM.[3] Therefore, the assessment of creatinine levels in human blood becomes very important clinically.

For creatinine detection, the most commonly used biosensor principle was proposed by Tsuchida and Yoda.[4] A three-enzyme sequence consisting of creatininase (CRN), creatinase (CR), and sarcosine oxidase (SOX) catalyzes the conversion of creatinine via creatine and sarcosine to glycine, formaldehyde, and hydrogen peroxide, as depicted in the following reaction sequence:

$$\text{Creatinine} + H_2O \xleftrightarrow{\text{CRN}} \text{Creatine} \quad (1)$$

$$\text{Creatine} + H_2O \xleftrightarrow{\text{CR}} \text{Sarcosine} + \text{Urea} \quad (2)$$

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{\text{SOX}} \text{Formaldehyde} + \text{Glycine} + H_2O_2 \quad (3)$$

With this enzyme sequence, both, the amperometric detection of enzymatically consumed oxygen or enzymatically generated hydrogen peroxide gives direct access to the original creatinine concentration. As creatine may also be present in physiological samples, its influence on the sensor signal has to be taken into account.

The differentiation of recent creatinine biosensors with the aforementioned principle is mainly based on various coimmobilization methods. The three enzymes have, for example, been crosslinked with glutaraldehyde,[5] adsorbed on carbon in the presence of poly(ethylene glycol),[6] adsorbed on platinum black and covered by a gelatin layer,[7] and entrapped in a polypyrrole layer by electropolymerization.[8]

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The combination of plasma treatment and plasma deposition polymerization provides a unique and powerful method for the surface chemical modification of polymeric materials without alterations to their bulk properties.[9] These techniques offer the possibility of improving the performance of existing biomaterials and medical devices and developing new biomaterials.[10]

Polymer surface modification by graft polymerization has also become possible with free radicals or peroxides, which are generated by plasma treatment. Surface modification with plasma techniques has received only slight attention so far in comparison with other plasma polymerization and plasma techniques. Ikada et al.[11] recently demonstrated the possibility of surface modification through graft polymerization under mild conditions for a series of polymers. In our previous work, the method was thoroughly implemented for plastic polymers such as polyethylene and poly(4-methylpentene) (TPX) to develop a biosensor.[12,13] Mild conditions could be achieved after the oxidation of surfaces with a chemical reagent, glow discharge, corona discharge, UV, or an ozone exposure method.

The purpose of this study was to prepare an amperometric creatinine biosensor from a covered platinum/silver electrode with a thin layer of an immobilized multienzyme membrane. The graft polymerization of acrylic acid onto the polypropylene (PP) membrane surface was achieved by plasma-induced graft polymerization. Subsequently, three enzymes were immobilized onto the modified surface together. The immobilized process for the amine group of an enzyme and a carboxylic group of the modified surface was carried out. The surface characterizations of various surfaces, such as attenuated total reflection/Fourier transform infrared (ATR–FTIR), electron spectroscopy for chemical analysis (ESCA), and scanning electron microscopy (SEM), and the optimum preparation conditions, including the compositions of the three enzymes in the enzyme solutions, were also accomplished. Finally, the linear range of the calibration curve and the stability of the multienzyme membrane with respect to the performances of the amperometric creatinine biosensor were studied for a more thorough understanding of the applications of the modified surfaces.

EXPERIMENTAL

Materials

A PP membrane (1 mil thick, 38% porosity, 0.02-μm effective pore size) was obtained from Celanese Fibers Co. (Narrows, VA). The acrylic acid monomer (Merck, Whitehouse Station, NJ) was redistilled *in vacuo* before use. CRN (E.C. 3.5.2.10., 79 U mg^{-1}) from *Pseudomonas sp.*, CR (E.C. 3.5.3.3., 14 U mg^{-1}) from *Actinobacillus sp.*, SOX (E.C. 1.5.3.1., 50 U mg^{-1}) from *Bacillus sp.*, creatinine hydrochloride, anhydrous creatine, and sarcosine (SigmaUltra) were purchased from Sigma (New York). A phosphate buffer solution (PBS) was prepared through the mixing of 0.1*M* K_2HPO_4 and 0.1*M* KH_2PO_4. All the other solutions were prepared with analytical-grade reagent chemicals and deionized water.

Plasma treatment

A glow-discharge reactor (a model PD-2 plasma deposition system) with a bell-jar reactor cell, manufactured by Samco Corp. (Kyoto, Japan), was used. The processing for the plasma treatment has been described in detail in our previous studies.[14] The PP membranes were used and activated at 200 mTorr and at a predetermined discharge power of Ar plasma for a predetermined time. The peroxide group was introduced by the exposure of samples in oxygen gas.[15] A schematic diagram of the glow-discharge treatment can be found elsewhere.[16] The peroxide group was then measured via 1,1-diphenyl-2-picrylhydrazyl (DPPH) in agreement with earlier reports.[17]

Graft polymerization

The detailed process of graft polymerization was described in a recent article of ours.[15] The membrane exposed to oxygen after argon-plasma treatment was immersed in a glass ampule in a monomer solution prepared at a given acrylic acid concentration. The ampule was then sealed by fire after being degassed three times and were maintained at 80°C for a predetermined reaction time. Next, the grafted PP membrane was taken out from the ampule and washed with 95% ethanol for 24 h to remove the poly(acrylic acid) (PAA) homopolymer. The amount of the carboxylic group in the PAA grafted onto the PP membrane was determined as follows: each PAA grafted onto the PP membrane was reacted for 6 h, at 75°C, with 10 mL of 0.01*M* NaOH. Finally, 5 mL of the supernatant was back-titrated with 0.01*M* HCl with a Mettler DL 21 autotitrator (Trace Analytical Co., Michigan).[18]

Multienzyme immobilization

The immobilizing experiments were performed in an aqueous, heterogeneous mixture with insoluble PP grafted with PAA and a soluble enzyme solution and carbodiimide agent. Single-enzyme (SOX) and multienzyme (SOX/CR and SOX/CR/CRN) solutions of predetermined concentrations and compositions were prepared in 1 mL of 0.1*M* PBS, at various pH regions, containing 200 mg mL^{-1} 1-cyclohexy-3-(2-morphoilnoethyl) carbodiimide metho-*p*-toluene sulfonate

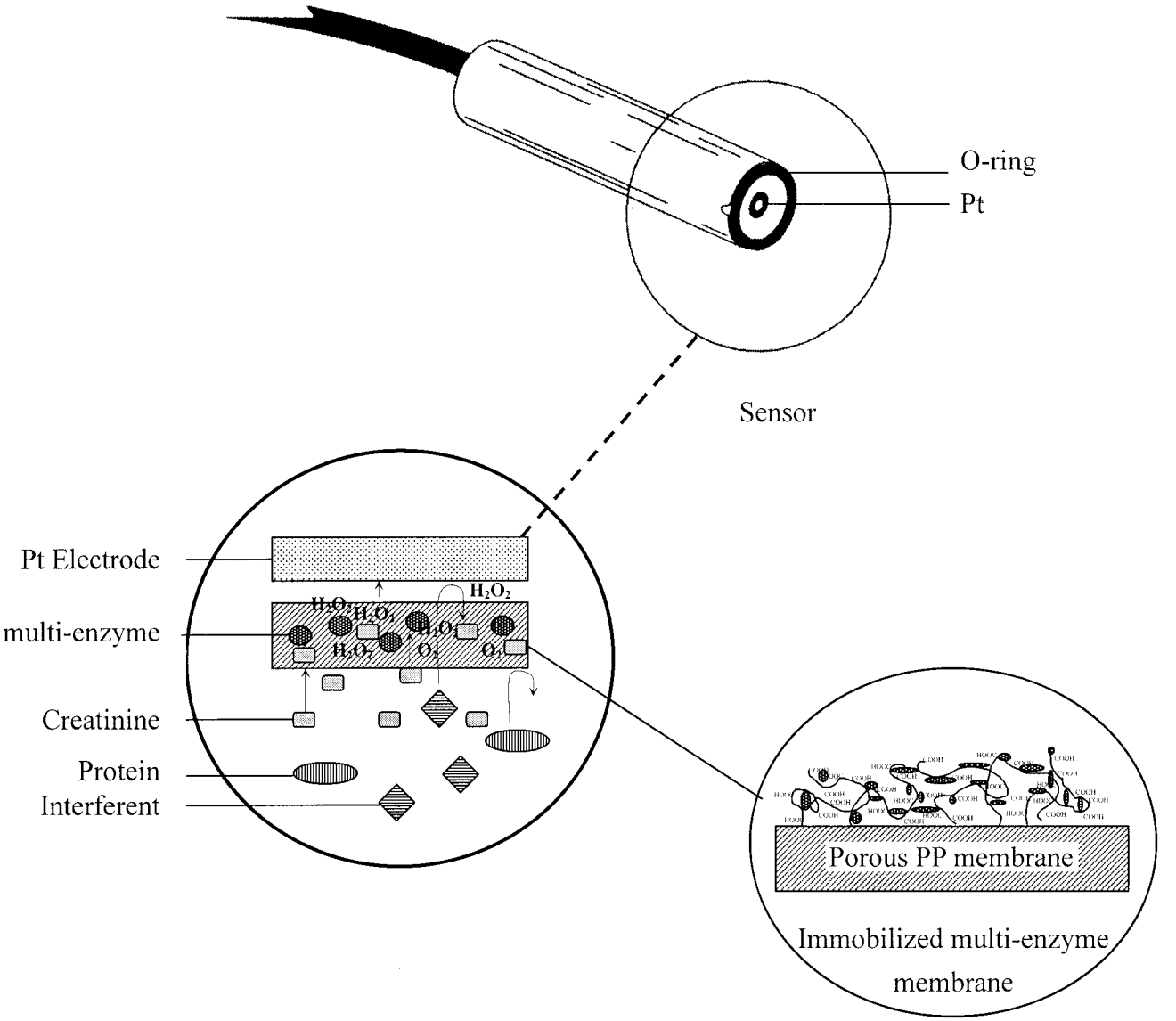

Figure 1 Schematic diagram of the creatinine biosensor.

(CMC).[19] The immobilizing reaction was performed at 4°C in a mixture of water and ice for 24 h. The PP immobilized with the enzyme was removed and washed with PBS three times. These single-enzyme and multienzyme membranes (PAA–SOX, PAA–SOX/CR, and PAA–SOX/CR/CRN) were then stored at 4°C.

Surface property analysis

A Bomem DA 3.002 ATR–FTIR spectrometer (Zurich, Switzerland) was used to confirm the polymer surfaces. A PerkinElmer PHI 1600 ESCA spectrometer (Massachusetts) was used to measure the various PP membranes at a pass energy of 1253.6 eV with an Mg Kα X-ray source. ESCA data were processed with a double-pass PHI 15-225 GAR CMA (ULVAC-PHI, Inc., Kanagawa, Japan). A Hitachi 570 scanning electron microscope (Tokyo, Japan) was used to analyze the polymer surface morphology.

Preparation of the creatinine sensors

A hydrogen peroxide electrode (YSI, Ohio) was used to obtain amperometric creatinine sensors. For this purpose, a thin layer of an immobilized multienzyme membrane (PAA–SOX/CR/CRN) was cut into desired pieces and retained on the Pt sensing electrode (0.1-mm diameter) with an O-ring (Fig. 1). When not in use, all these biosensors were stored at 4°C.

Amperometric measurements

Amperometric measurements were performed with a YSI 2700 Select biochemistry analyzer. Standard solu-

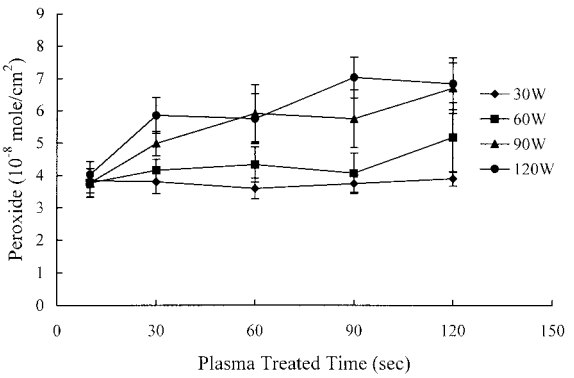

Figure 2 Changes in the peroxide concentration (mol/cm^2) with different plasma-treatment times (s) and powers (W; n = 9).

tions with various concentrations of sarcosine, creatine, and creatinine were prepared with pH 7.4 PBS. The sensor was situated in a buffer-filled sample chamber into which a sample was injected. All experiments were carried out in magnetically stirred PBS (pH 7.4) solutions. The temperature probe in the sample chamber monitored the fluid temperature very near the enzyme sensor. The sample results were temperature-corrected for the difference in the temperature between the sample and the calibration. After the desired potential was applied, the background current was allowed to stabilize. The appropriate amount of the concentrated substrate solution was added to the cell for a preselected concentration, and when it come into contact with the immobilized oxidase enzyme, it was rapidly oxidized, producing hydrogen peroxide. The faradaic current increased as a function of time and eventually reached a constant value. The increased current was taken as the sensor response for any particular substrate concentration.

RESULTS AND DISCUSSION

Peroxide destruction

The surface concentration of generated peroxide measured via DPPH is plotted in Figure 2 as a function of the argon-plasma-treatment time and discharge power. The maximum value (7.03×10^{-8} mol cm^{-2}) of the peroxide group was found at 90 s and 120 W, which could be the most active point for plasma-induced graft polymerization. At 120 W, the value of the peroxide group decreased with an increase in the argon-plasma-treatment time beyond 90 s; this could be as a result of etching, which occurred on the treated surfaces of PP membranes.

Graft polymerization

The graft polymerization of PAA onto the PP membrane was performed at different polymerization intervals of 24 h to find a suitable reaction time of polymerization, as shown in Figure 3. The amount of PAA, plotted as a function of the reaction time, leveled off beyond 20 h. Consequently, the amount of PAA was maintained at 178.2 μg cm^{-2} throughout the subsequent experiments. The influences of various amounts of PAA-grafted PP surfaces on the amount of the immobilized enzyme are also described in previous reports.[12,13] The amount of the immobilized enzyme increased with an increase in the amount of PAA-grafted PP. This indicated that the reactive site of the modified PP membrane with the enzyme depended on the amount of the carboxyl group on the PAA-grafted PP surface.

Multienzyme immobilization

Effect of the composition of the immobilized multienzymes

Creatinine is converted into electroactive H_2O_2 by three consecutive enzymatic reactions with three different enzymes. The ratio and amount of these three enzymes in the enzyme solution have a big effect on the performance of the creatinine sensors. Several groups have prepared sensors with enzyme solutions with different compositions of the three enzymes. These compositions vary significantly from system to system, depending on the method of preparation and the sensor structure. Aizawa et al.[20] used a concentration of 5 mg mL^{-1} for SOX, CR, and CRN in an electrolyte solution for the preparation of polypyrrole-matrix-based sensors. Yamato et al.[8] reported the influence of the SOX (from *Arthrobacter sp.*) concentration in a solution on the preparation of sensors with fixed concentrations of CR and CRN (5 mg mL^{-1} each) and observed that the maximum creatinine response of the SOX concentration was 5 mg mL^{-1}. In this study, the immobilization of a multienzyme onto the PAA-grafted PP surface by the carbodiimide method

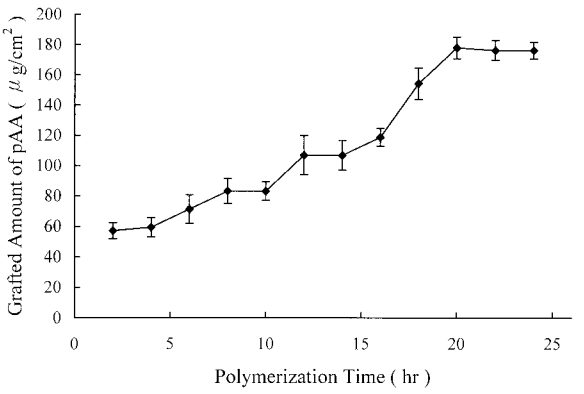

Figure 3 Effect of the polymerization time (h) on the amount of PAA-grafted PP membranes (n = 3).

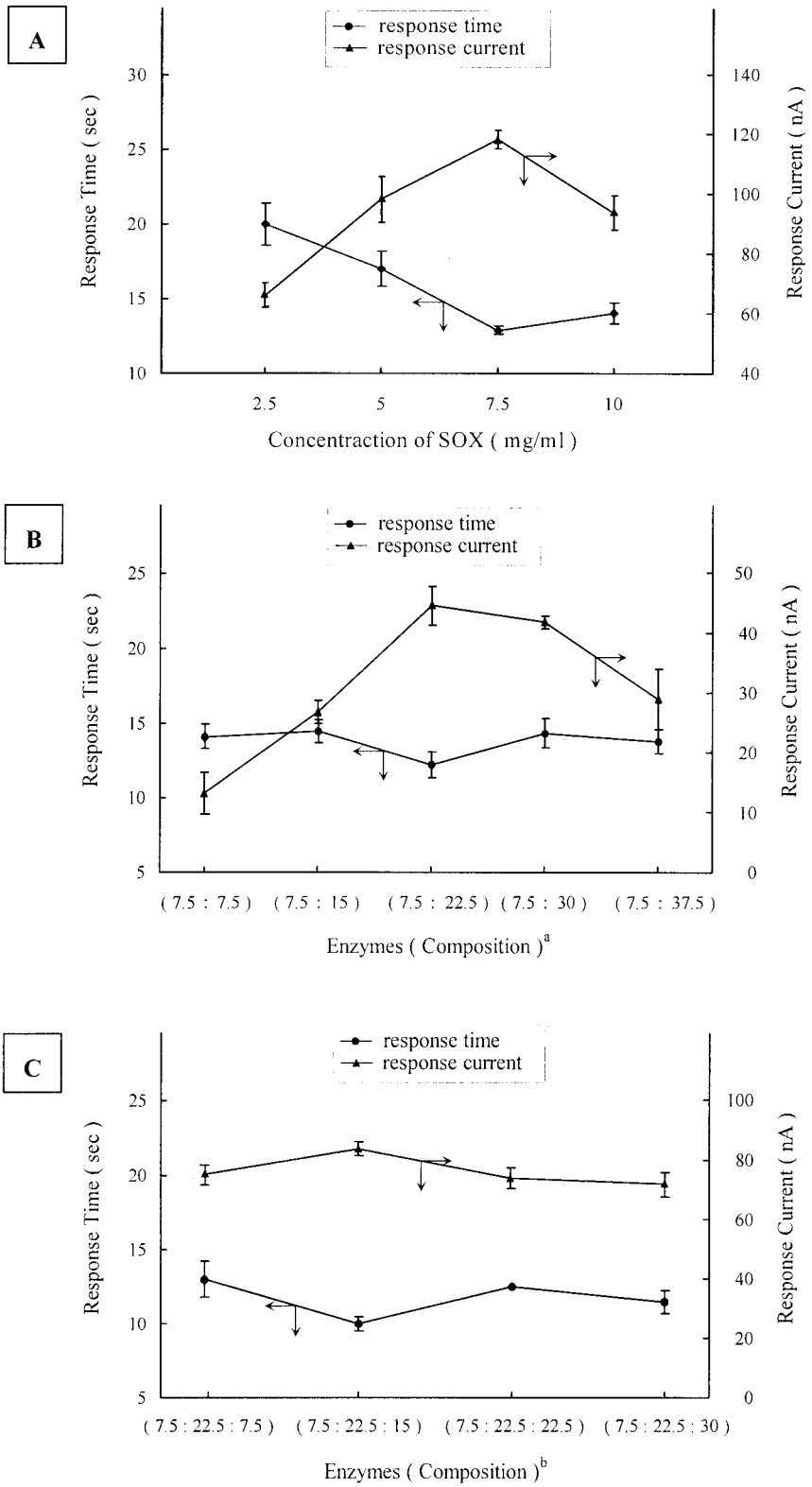

Figure 4 Effect of (A) the SOX concentration, (B) the SOX/CR composition, and (C) the SOX/CR/CRN composition on the response time and current of hydrogen peroxide electrodes covered with immobilized SOX, SOX/CR, and SOX/CR/CRN membranes, respectively (n = 4). The samples contained 50 μL of 300 μM (A) sarcosine, (B) creatine, or (C) creatinine. Superscript a indicates milligrams of SOX and CR per milliliter of the buffer solution; superscript b indicates milligrams of SOX, CR, and CRN per milliliter of the buffer solution

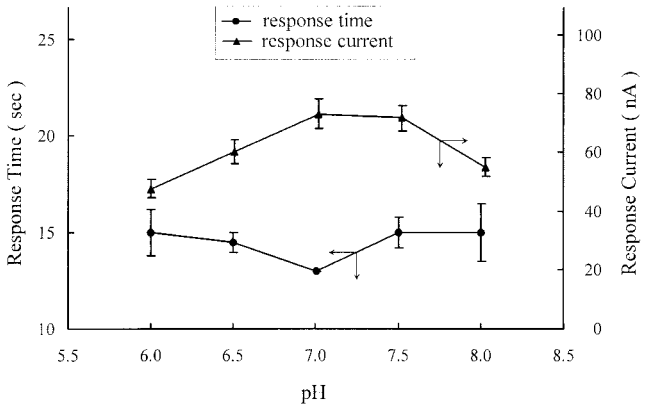

Figure 5 Effect of pH on the response time and current of a hydrogen peroxide electrode covered with an immobilized SOX/CR/CRN membranes (n = 4). The sample contained 50 μL of 300 μM creatinine.

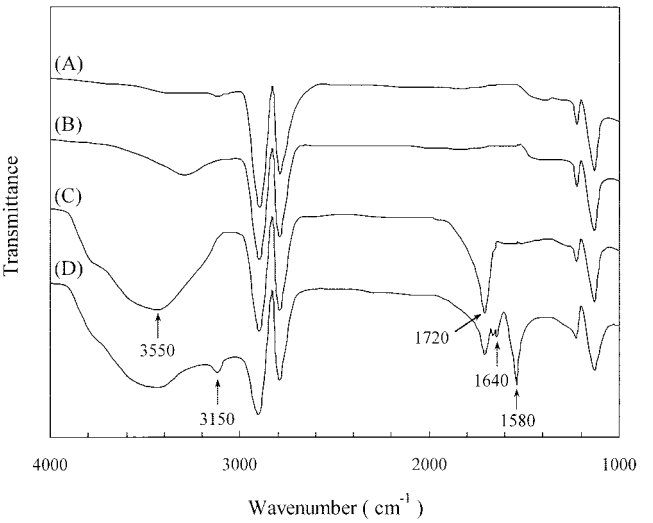

Figure 6 ATR–FTIR spectra of (A) original PP, (B) plasma-treated PP, (C) PAA-grafted PP, and (D) PAA–SOX/CR/CRN.

was performed with different compositions of these enzymes (SOX, CR, and CRN) in the enzyme solution to find a suitable condition. The responses to the time and current for a hydrogen peroxide electrode, covered with PAA–SOX, PAA–SOX/CR, and PAA–SOX/CR/CRN have been plotted as functions of the concentration and composition of single-enzyme (SOX) and multienzyme (SOX/CR, SOX/CR/CRN) solutions with a fixed pH of 7.0 in Figure 4(A–C), respectively. The hydrogen peroxide electrode covered with PAA–SOX was measured for sarcosine. The minimal response time and the maximal response current were observed at a concentration of 7.5 mg mL^{-1} SOX [Fig. 4(A)]. The electrode covered with PAA–SOX/CR was prepared with a fixed concentration of SOX (7.5 mg mL^{-1}), and the optimum creatine response was observed at a CR concentration of 22.5 mg mL^{-1} [Fig. 4(B)]. Finally, for the creatinine sensor prepared with fixed concentrations of SOX and CR (7.5 and 22.5 mg mL^{-1}, respectively), the minimal response time and the maximal current were observed at a CRN concentration of 15.0 mg mL^{-1} [Fig. 4(C)].

Effect of pH

The effect of pH of the enzyme solution on the response of the amperometric biosensor for creatinine was examined in the pH range of 6–8 in multienzyme solutions with fixed concentrations of SOX, CR, and CRN (7.5, 22.5, and 15.0 mg mL^{-1}, respectively). The minimal response time and the maximal response current were observed at pH 7.0, as shown in Figure 5. This optimum pH differed from the pH optimum of CRN (pH 8.0), CR (pH 7.5), and SOX (pH 8.3). The pH shift for the three immobilized enzymes could be explained as follows. As a carrier is negatively charged, a high concentration of positively charged ions (H^+) will accumulate at the boundary between this carrier and the surrounding solution, and as a result, the pH at the carrier surface will become lower than that of the bulk solution. Immobilized enzymes will, therefore, function at pHs lower than that of the bulk solution.[21] Consequently, enzyme solutions with a pH of 7.0 and SOX, CR, and CRN concentrations of 7.5, 22.5, and 15.0 mg mL^{-1}, respectively, were used for further experiments.

Surface property analysis

ATR–FTIR

Figure 6 shows the ATR–FTIR spectra for an original PP membrane as a control, an argon-plasma-treated PP membrane, a 178.2 μg cm^{-2} PAA-grafted PP membrane, and a multienzyme (7.5, 22.5, and 15.0 mg mL^{-1} SOX, CR, and CRN, respectively) membrane. A strong C═O adsorption band appeared at 1715 cm^{-1},

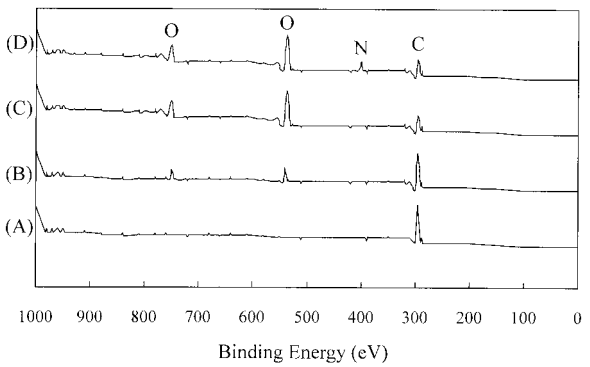

Figure 7 ESCA survey scan of (A) original PP, (B) plasma-treated PP, (C) PAA-grafted PP, and (D) PAA–SOX/CR/CRN.

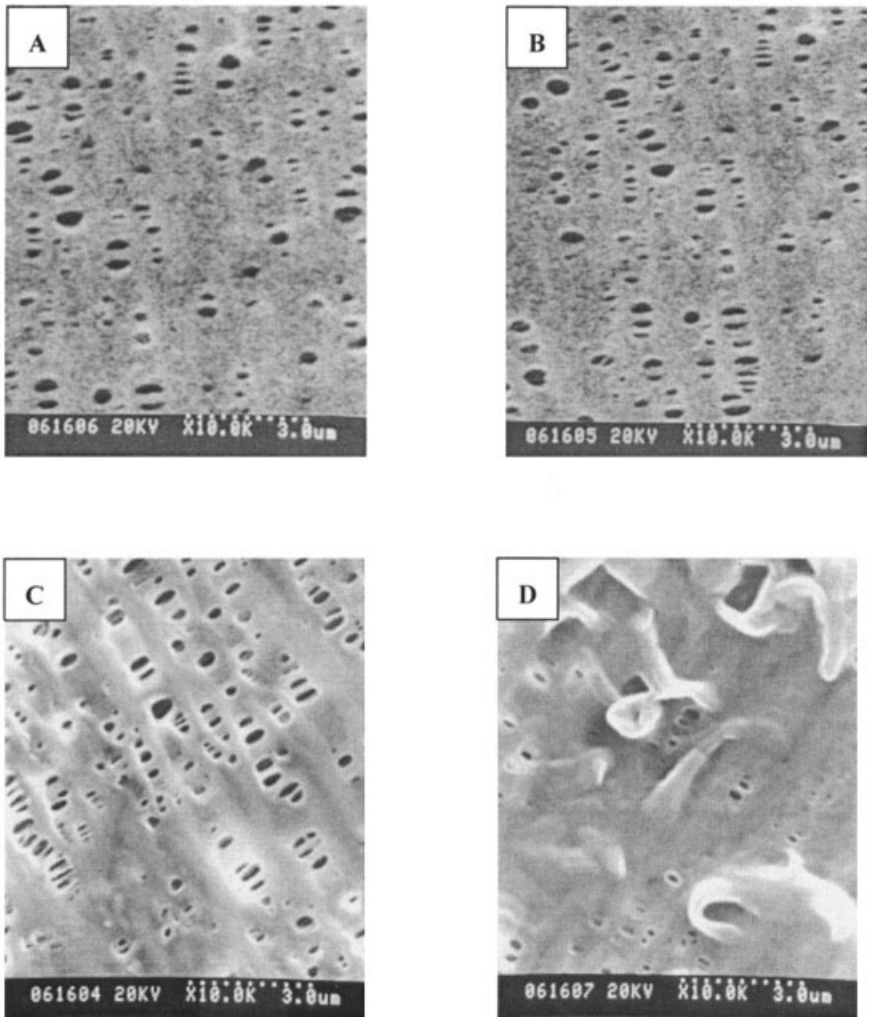

Figure 8 SEM photographs of (A) original PP, (B) plasma-treated PP, (C) PAA-grafted PP, and (D) PAA–SOX/CR/CRN

and a broad COOH adsorption also appeared at 3200–3500 cm^{-1} [Fig. 6(C)]. A successfully induced graft copolymerization of the PP membrane grafted with acrylic acid was achieved by glow discharge. Figure 6(D) confirms the multienzyme (SOX/CR/CRN) immobilized onto the COOH group of PAA-grafted PP by the CMC coupling agent. In this figure, 3340 cm^{-1} is the adsorption peak of hydrogen bonding for the enzyme, and 3150 cm^{-1} is the single bond of N—H. In addition, 1640 and 1680 cm^{-1} were associated with a carbonyl group (C═O). The C═O produced according to the amide group (O═C—NH) was at 1680 cm^{-1} when the amine (—NH_2) of the enzyme reacted with the carboxyl group (COOH) of PAA grafted onto the PP membrane, whereas 1640 cm^{-1} was the amide group of the enzyme.

ESCA

The ESCA spectra for an original PP membrane, an argon-plasma-treated PP membrane, a PAA-grafted PP membrane, and a multienzyme-immobilized modified PP membrane were obtained in each of the modified stages. An initial survey scan and low-resolution spectra were performed to establish which composition elements were available on the various surfaces (Fig. 7). From the survey scan, only the carbon peak (300 eV) present for the original PP membrane was demonstrated [Fig. 7(A)]. After the original PP membrane was treated with argon plasma and exposed to oxygen, oxygen peaks (530 and 750 eV) were observed [Fig. 7(B)]. Furthermore, the carboxylic acid groups of PAA revealed similar results in Figure 7(C). A nitrogen peak (401 eV) was only found on the modified surface immobilized with an enzyme [Fig. 7(D)]. From the special analysis, we concluded that the enzyme was successfully introduced onto the modified surfaces.

SEM

After various kinds of membrane surfaces were coated with gold by ionic sputtering (IB-2, Eiko,

Hitachi, Tokyo, Japan), the morphologies were observed with a scanning electron microscope. The morphologies of the original PP membrane, PP treated with argon plasma, PAA-grafted PP, and multienzyme-immobilized PP were observed with a scanning electronic microscope (Fig. 8). The original PP and argon-plasma-treated PP membrane morphologies [Fig. 8(A,B)] were smoother than those of PAA-grafted PP [Fig. 8(C)] and enzyme-immobilized PP membranes [Fig. 8(D)]. A morphology comparison was made between PAA-grafted PP and enzyme-immobilized PP. The morphology of enzyme-immobilized PP was rougher than that of PAA-grafted PP. In addition, the microporous structure, which provided a high surface for enzyme immobilization, mostly disappeared in Figure 8(D), whereas such a phenomenon could not be observed in Figure 8(C).

Amperometric measurements

Linear calibration curve

The relationship between the creatinine concentration and the response current was examined with a YSI 2700 S biochemistry analyzer. The results are shown in Figure 9. A linear relationship was obtained in the range of 3.2–320 μM creatinine. The calibration curve could be shown by the equation $y = 0.2515x + 3.5535$, where y is the response current (nA) and x is the creatinine concentration (μM). The correlation coefficient was 0.9993.

Stability of the creatinine sensor

The stability of the creatinine sensor was examined as shown in Figure 10. The slope of the calibration curve fluctuated from day to day, but the linear range was always maintained. More than 250 experiments were performed with the same multienzyme membrane. The decline of the stability is also shown in the figure, indicating that the immobilized enzymes were very stable and could be used for at least 3 weeks.

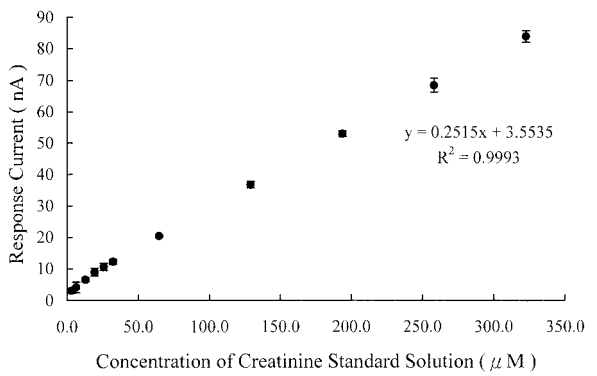

Figure 9 Calibration curve of a hydrogen peroxide electrode covered with an immobilized SOX/CR/CRN membrane ($n = 3$)

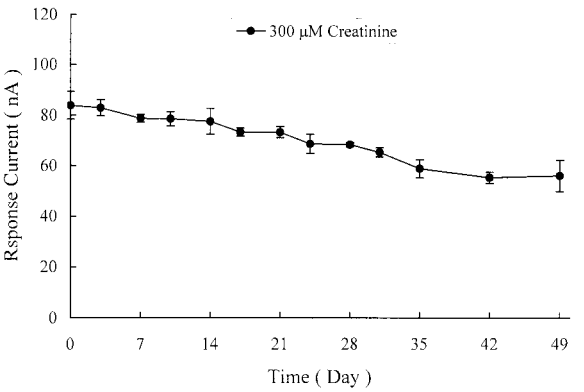

Figure 10 Stability of a hydrogen peroxide electrode covered with an immobilized SOX/CR/CRN membrane ($n = 3$). The sample included 50 μL of 300 μM creatinine.

CONCLUSIONS

An amperometric creatinine biosensor covered by a platinum/silver electrode with a thin layer of an immobilized multienzyme membrane was constructed. First, PAA had to be introduced onto the PP membrane surface by plasma-induced graft polymerization. Subsequently, three different enzymes (SOX, CR, and CRN) were immobilized onto the modified surface simultaneously via a carbodiimine agent. ATR–FTIR, ESCA, and SEM surface analysis were performed to confirm the possibility of the modification process. Moreover, a linear calibration curve was obtained for creatinine in the range of 3.2–320 μM. The immobilized multienzyme membrane could be used for at least 3 weeks. On the basis of these results, modified PP membranes immobilized with multienzymes have potential for the development of a creatinine sensor.

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