Talanta 79 (2009) 1135–1141

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Carbon felt-based biocatalytic enzymatic flow-through detectors: Chemical modification of tyrosinase onto amino-functionalized carbon felt using various coupling reagents

Yue Wang[a,b], Yasushi Hasebe[a,c,*]

[a] Department of Materials Science and Technology, Graduate School of Engineering, Saitama Institute of Technology, 1690 Fusaiji, Fukaya, Saitama 369-0293, Japan
[b] School of Chemical Engineering, University of Science and Technology Liaoning, 185 Qianshan Road, Hi-tech zone, Anshan, Liaoning 114501, PR China
[c] Department of Life Science and Green Chemistry, Saitama Institute of Technology, 1690 Fusaiji, Fukaya, Saitama 369-0293, Japan

ARTICLE INFO

Article history:
Available online 21 February 2009

Keywords:
Flow-biosensor
Carbon felt
Tyrosinase
Chemical modification
Coupling reagents

ABSTRACT

Tyrosinase (TYR) was covalently immobilized onto amino-functionalized carbon felt (CF) surface via eight different coupling reagents. Prior to the TYR-immobilization, primary amino group was introduced to the CF surface by the treatment with 3-aminopropyltriethoxysilane (APTES). The APTES modification of the CF surface was confirmed by XPS and SEM measurements. The terminal amino groups on the CF surface were cross-linked with protein lysine group (or cysteine group) using various coupling reagents. The resulting TYR-immobilized CF (TYR-CF) was utilized as a working electrode unit of a biocatalytic enzymatic flow-through detector. Catechol and 4-chlorophenol (4-CP) were used as model analytes for the evaluation of catecholase activity and phenolase activity, respectively, and flow injection peaks based on the electro-reduction of the enzymatically produced *o*-quinone species were monitored at −0.05 V vs. Ag/AgCl. Among eight coupling reagents, glutaraldehyde (GA) exhibited the best results on the sensitivity, the operational stability and the storage stability. The detection limits of catechol and 4-CP obtained by the GA-coupling method were found to be 6.0×10^{-9} M and 1.5×10^{-8} M, respectively with the sample through-put of 36 samples/h. No serious degradation of the peak current was observed over 30 consecutive samples injections on the GA-coupling method, while gradual decrease in the peak currents was observed on other seven coupling reagents. The GA-coupling method showed the best results on the storage stability, and 85% of original activity for catechol oxidation remained after 25 days storage.

1. Introduction

Various immobilization approaches of enzyme such as physical adsorption, covalent binding, encapsulation, entrapment and cross-linking have been established. Method of enzyme immobilization is made by weighing different factors. A number of practical aspects should be considered before embarking on experimental work to ensure that the final immobilized enzyme preparation is fit for the planned purpose or application and will operate at optimum effectiveness [1,2]. Among various immobilization methods, covalent binding often exhibits the highest stabilization of enzyme activities because the active conformation of the immobilized enzyme is stabilized [3]. Covalent binding has the advantage that the enzyme is generally strongly immobilized on the surface and therefore unlikely to detach from the surface during the repeated use. Another advantage is the variety of functional groups available for covalent immobilization which allow the active site of the binding process to be avoided [4].

Biomolecule-functionalized support surfaces are of fundamental importance for analyte recognition in biosensor technology. One key factor for the fabrication of biosensors is usually integration of the biorecognition molecule onto the surface of the transducer. To develop a sensitive, stable and reusable support surface, numerous bi-functional coupling reagents were applied with different degrees of success. In these cases, surface-bound functional groups such as terminal amino group ($-NH_2$) is useful for covalent coupling of protein to the surface. Despite the long history of enzyme immobilization, there are, only a few examples of systematic investigations of the influence of coupling reagents on the properties of the immobilized enzymes [5,6], while most studies investigate the influence of the support [7,8].

Carbon felt (CF) is a microelectrode ensemble of micro carbon fiber (ca. 7 μm diameter), and possesses a random three-dimensional structure with high porosity (>90%). The CF has high

* Corresponding author at: Department of Materials Science and Technology, Graduate School of Engineering, Saitama Institute of Technology, 1690 Fusaiji, Fukaya, Saitama 369-0293, Japan.
E-mail address: hasebe@sit.ac.jp (Y. Hasebe).

0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.02.028

surface area (estimated to be 0.3–30 m^2/g) and shows a high conductivity and excellent electrolytic efficiency. In addition, porous structure of the CF causes very low diffusion barrier against the solution flow. On the basis of these characteristics, we reported recently that the CF is useful as a working electrode unit of electrochemical flow-through detector [9,10]. Thus, the establishment of novel immobilization strategy of biomolecules onto the CF surface is attractive research topic to develop CF-based flow biosensing systems. Although a few surface modification strategy of CF has been reported [11,12], the covalent modification protocol of biomolecule onto the CF surface for biosensor application has not been established so far, at least from our best knowledge.

It is well known that three functional groups (i.e., phenolic, carboxylic and quinine) are present on carbon surface, and R.W. Murray et al. have reported that graphite and glassy carbon surface readily react with organosilanes via surface phenolic (–OH) group [13]. In this study, we introduced terminal amino group (–NH_2) onto the CF surface by the reaction with 3-aminopropyltriethoxysilane (APTES) via phenolic OH group at graphite edge on the CF surface without any thermal or chemical pre-oxidation. Then tyrosinase (TYR) was covalently coupled to the amino-functionalized CF surface by eight different coupling reagents. TYR is bi-nuclear copper–protein that catalyzes two distinct oxidation reactions toward mono- and di-phenol compounds [14]. Up to now, various TYR-immobilized carbon electrodes have been developed for the electrochemical determination of phenol and catechol compounds using graphite [15–17] carbon paste [18–21], reticulated vitreous carbon (RVC) [22] and glassy carbon [23–25]. However, CF-based TYR biosensor has not been reported so far. In addition, in regard to the immobilization strategy, chemical modification of TYR to amino-functionalized carbon materials has not been achieved, although carbodiimide coupling to graphite [16,21], covalent linking to polymer [23,24] and physical adsorption and entrapping in hybrid materials [14–16,18,22,25] have been reported. Principal aim of the present study is to investigate the effect of different coupling reagents on the activity of the immobilized TYR on the APTES-functionalized CF surface. The resulting TYR-immobilized CF (TYR-CF) was used as a working electrode unit of biocatalytic enzymatic flow-through detector. The activities of the immobilized TYR toward catechol and 4-chlorophenol (4-CP) were evaluated by measuring the flow injection peak current based on the electroreduction of enzymatically liberated *o*-quinone species. The sensor performance of the TYR-CF-based flow detector was strongly influenced by the kinds of coupling reagents. Among eight coupling reagents examined, glutaraldehyde (GA) showed the best results on the sensitivity, the operational stability and the storage stability. This would be a first report on the chemical modification of redox enzyme onto the amino-functionalized CF using various coupling reagents for the construction of the CF-based biocatalytic enzymatic flow-through detector.

2. Experimental

2.1. Reagents

Tyrosinase (TYR, polyphenol oxidase, EC 1.14.18.1, 5370 unit/mg from mushroom) was purchased from Sigma Co., and used as received. Catechol, glutaraldehyde, ascorbic acid (AA), cyanuric chloride (CC), dimethyl sulfoxide (DMSO), N,N-dimethylacetamide dehydrated (DMA), methanol and ethanol were obtained from Wako Pure Chemicals. 4-Chlorophenol, 1,4-benzoquinone (BQ) were obtained from Tokyo Kasei Kogyo. 3-Aminopropyltriethoxysilane, terephthaloyl chloride (TCL), dimethyl suberimidate dihydrochloride (DMS), N-[ε-maleimidocaproyloxy] succinimide ester (EMCS), and disuccinimidyl suberate (DSS) were obtained from Aldrich Co. All of the other chemicals were of the highest grade available, and used without further purifications. Doubly distilled water was used for the preparation of buffer solution, sample standard solution and enzyme solution.

2.2. Preparation of the NH_2-functionalized CF

The CF sheet [from Nihon Carbon Co. which has three-dimensional random ensemble structure of carbon fiber (ca. 7 μm diameter)] was cut into 10 mm × 3 mm × 3 mm in size (weight, ca. 12 mg), and washed with doubly distilled water under ultrasonication (As One) for 10 min, and was dried in vacuum for 1 h. Then the CF was dipped into a 25% (w/v) solution of 3-aminopropyltriethoxysilane in toluene. After 1 h incubation at room temperature, the CF was washed with toluene under ultrasonication for 2 min and dried in vacuum for 1 h.

2.3. Enzyme immobilization procedures

2.3.1. GA activation

The APTES-modified CF was immersed in 20% (w/v) aqueous GA solution (2 mL) and incubated at room temperature for 15 min. The activated CF surfaces were then washed thoroughly with pure water and the CF was placed in enzyme solution in pure water.

2.3.2. AA activation

The APTES-modified CF was placed in a saturated solution of AA in DMA (1.5 mL, 400 mg/mL) and incubated at room temperature for 15 min. The CF was thoroughly rinsed with pure water and added to an enzyme solution in pure water immediately.

2.3.3. BQ activation

The APTES-modified CF was placed in a BQ/DMSO solution (1.5 mL, 200 mg/mL). After incubation for 15 min at room temperature, the CF was rinsed thoroughly with pure water until the solution was no longer yellowish, and then added to an enzyme solution in pure water.

2.3.4. Activation by means of DMS, EMCS and DSS

The APTES-modified CFs were immersed into a 1 mM DMS, EMCS and DSS solutions (5 mL) in DMSO-methanol (1:9 in volume ratio) for 15 min at room temperature. Each CF was rinsed thoroughly with methanol and pure water, respectively, and then added to an enzyme solution in 0.1 M phosphate buffer (pH 7.0).

2.3.5. Activation by means of TCL and CC

The APTES-modified CF was added to a solution of 50 mg of TCL and/or CC dissolved in 5 mL of DMA, and was incubated at room temperature for 15 min. The activated surfaces were then thoroughly rinsed with ethanol until they became clear, quickly washed with 0.1 M phosphate buffer (pH 7.0), and then immediately placed into an enzyme solution in the same phosphate buffer, which had previously been cooled to 4 °C.

2.3.6. Enzyme immobilization

In the case of GA-, AA-, and BQ-activated CFs, the activated NH_2-functionalized CFs were immersed in 2 mL of TYR aqueous solutions (0.5 mg of TYR per 2 mL of pure water). In the case of DMS-, EMCS-, DSS-, TCL- and CC-activated CFs, the activated NH_2-functionalized CFs were immersed in 2 mL of TYR buffer solutions (0.5 mg of tyrosinase per 2 mL of 0.1 M phosphate buffer, pH 7.0). After the incubation at 4 °C for 16 h, the weakly adsorbed enzyme was removed by rinsing with pure water and 0.1 M phosphate buffer (pH 7.0). To evaluate the approximate amount of immobilized enzyme at the CF surface, the absorbance at λ = 280 nm of TYR solutions before and after the immobilization treatment were measured

with spectrophotometer (BIO-RAD, SmartSpec Plus spectrophotometer) using disposable UV Cuvettes (sample volume, 50 μL), and the amount of immobilized enzyme was calculated according to similar method of the literature [26]. The surface coverage of TYR at the CF was estimated using the value of effective surface area of CF (1 m^2/g) [9].

2.4. X-ray photoelectron spectroscopy

The XPS data of bare-CF and the APTES-modified CF were acquired with a Physical Electronics Industries Model 5500 multitechnique surface analysis system equipped with a hemispherical analyzer, a monochromator, and a multichannel detector. Monochromatic Al K_α radiation (1486.6 eV) at 300 W was used for excitation. The photoelectrons were collected at 10° from the surface parallel direction to maximize surface detection sensitivity. The binding energies were referenced to the C (1s) emission band at 284.3 V. The base pressure of the ion-pumped ultrahigh vacuum (UHV) chamber was less than 1×10^{-9} Torr during analysis.

2.5. Scanning electron microscopy

The scanning electron microscope (SEM) analysis of bare-CF and the APTES-modified CF were performed with a JEOL JSM-5500LV microscope operating at 8.0 kV.

2.6. Flow injection analysis

Flow injection analysis (FIA) system is composed of a double-plunger pump (DMX 2000T, SNK) with a six way injection valve (SVM-6M2, SNK, 200 μL injection loop) and CF-based electrochemical flow-through detector [9,10]. All FIA experiments were carried out at room temperature. Air-saturated 0.1 M phosphate buffer (pH 7.0) was used as a carrier. Before the measurements, carrier solution was flowed at flow rate of 3.0 mL/min for 1000 s under the applied potential of −0.05 V vs. Ag/AgCl to remove weakly adsorbed TYR from the CF surface and to reduce the background current. After the background current had been reached to steady-state value, 200 μL of standard solutions of catechol and 4-CP were injected, and the cathodic peak currents based on the electroreduction of *o*-quinone species produced by TYR reaction were detected at −0.05 V vs. Ag/AgCl. Although more cathodic potential is effective for the electroreduction of *o*-quinones, induces electrochemical reduction of dissolved oxygen at the same time. Thus we selected this potential to avoid increase in the background current and decrease in the oxygen concentration at the vicinity of the electrode surface [19,29]. The operational stability and the storage stability were checked by measuring the flow injection peak currents by 30 consecutive injections of 10 μM samples at every five days during 25 days. The TYR-immobilized CF was stored in 0.1 M phosphate buffer (pH 7.0) at 4 °C in refrigerator when not in use.

2.7. Electrochemical impedance spectra (EIS) measurement

To obtain the information of the interfacial property of the TYR-immobilized surface, we measured electrochemical impedance spectra (EIS) of the TYR-immobilized CF with electrochemical analyzer (ALS 6122A) using $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ as probe at room temperature. Deoxygenized 0.1 M phosphate buffer (pH 7.0, 15 mL) containing 1 mM 1:1 $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ was used as an electrolyte. Prior to the measurement, nitrogen gas was bubbling into the electrolyte for 15 min to remove the dissolved oxygen in electrolyte solution. The TYR-immobilized CF (size: 10 mm × 3 mm × 3 mm) with Au lead wire (0.5 mm diameter, 50 mm length) was used as working electrode. Pt wire counter electrode (1 mm diameter, 50 mm length) and Ag/AgCl reference electrode (BAS, RE-1B) were used. The EIS were obtained by sweeping an applied potential of 0.2 V (formal potential of the $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ redox system in the present medium) from 0.01 Hz to 10^4 Hz, superimposed on dc offset equivalent to an open-circuit potential.

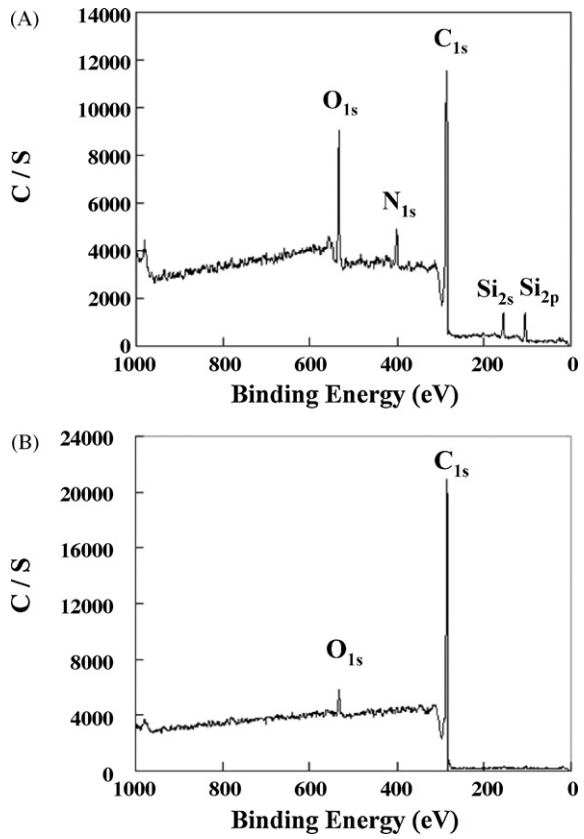

Fig. 1. XPS spectra of (A) the APTES-modified and (B) bare-CF.

3. Results and discussion

3.1. XPS and SEM measurements of the APTES-treated CF

Organosilan modification is useful for the introduction of various functional groups onto the support materials. APTES has widely been used for the introduction of primary amino group (–NH_2) at the surface of glass [27] and silicon [28] via silanol group. Similarly, the organosilane modification of carbon electrodes (glassy carbon and graphite rod) has successfully been conducted via OH group at graphite edge of the electrode surface [13]. Fig. 1 shows the XPS spectra of the CF with and without APTES treatment. A significant amounts of attached N (1s), Si (2s) and Si (2p) were observed after APTES treatment (Fig. 1A), although N atom and Si atom were not detected on the bare-CF (Fig. 1B). The binding energy of N 1s and Si 2p bands of the APTES-treated CF (Table 1) is essentially similar to those of the APTES-modified glassy carbon electrode previously reported by Eliott and Murray [13]. Thus it is clear that APTES was modified onto the CF surface under the

Table 1
Binding energies of each XPS band of bare-CF and APTES-treated CF.

	O 1s	N 1s	Si 2p
Bare-CF	532.3 ± 0.5	ND	ND
APTES-CF	533.5 ± 0.5	400.4 ± 0.5	103.7 ± 0.5

ND: not detected.

Table 2
XPS band area ratios of bare-CF and APTES-treated CF.

	C 1s (%)	O 1s (%)	N 1s (%)	Si 2p (%)
Bare-CF	96.4	3.6	ND	ND
APTES-CF	74.8	14.4	6.1	4.6

ND: not detected.

present modification conditions. However, surface density of OH group on the CF surface required for chemical modification of organosilane would not be so high as compared with glass and silicon surfaces. Therefore, judging from the relatively high content of O, N, and Si of the APTES-modified CF (shown in Table 2), it can be supposed the formation of three-dimensional siloxane polymer at the CF surface based on the multiple bridges of each APTES molecules. This assumption was supported by the SEM observation (Fig. 2). Differing from the bare-CF surface (Fig. 2B), micrometer-size island-like structure and/or film-like structure were observed on the APTES-treated CF surface (Fig. 2A). In any event, large amounts of terminal amino group introduced to the CF surface would be useful for the covalent immobilization of the enzyme via various coupling reagents at least for the purpose of developing enzyme-immobilized CF-based flow-biosensor.

3.2. Sensitivity of the TYR-CF-based biocatalytic enzymatic flow-through detector

TYR was covalently immobilized via eight different coupling reagents, shown in Fig. 3, onto the NH_2-functionalized CF surfaces using the various reaction possibilities of NH_2 groups. In all cases, we used in great excess of coupling reagents to avoid cross-linking of themselves. This is necessary because each reagent, if applied in low concentration, may lead to a different extent of cross-linking resulting in a different extent of swelling, which can affect the immobilization of enzyme [29].

Table 3 shows the comparison of the peak currents of 10 μM catechol and 4-CP obtained for each immobilization procedure. Among eight coupling reagents, the GA-coupling method exhibited the highest sensitivity for both catechol and 4-CP determination. Especially, the sensitivity of 4-CP by the GA-coupling method was much higher than other seven coupling reagents. In all cases, the sensitivity of catechol was higher than that of 4-CP. This would be attributed to the difference between the catecholase activity and the phenolase activity of TYR [14]. Differences of the sensitivity and the selectivity observed by various coupling reagents may be originated from the following factors: (1) Differences in the reactivity of each coupling reagent, affecting the enzyme loading. (2) Differences in spacer effect and molecular geometry effect of each coupling reagent, probably due to the differences in the length and the flexibility of the molecular structure. In fact, it has been reported

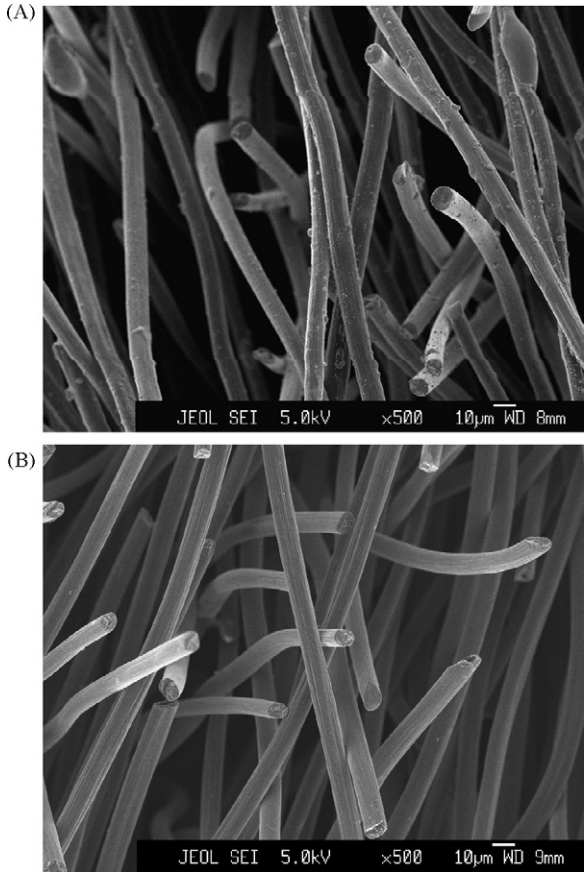

Fig. 2. SEM images of (A) the APTES-treated and (B) bare-CF.

that the ratio of the catecholase activity to the phenolase activity is changed depending on the microenvironment of the immobilized TYR [20].

To discuss about the surface structure and microenvironment of TYR-immobilized CF surface, we estimated the immobilization amount of TYR by measuring absorbance at 280 nm of TYR solution before and after the TYR-immobilization. The estimated apparent surface coverage of TYR (Γ_{TYR}^{app}) was summarized in Table 3. Furthermore, we measured electrochemical impedance spectra and estimated the electron-transfer resistance (R_{CT}) values at the electrode surface by Nyquist plot. EIS is powerful tool to study interfacial properties of surface-modified electrode, and the electron-transfer resistance (R_{CT}) is an important parameter [30]. In this case, the increased R_{CT} values would correspond to larger TYR-immobilization because of the macromolecular structure of TYR hinder the electron transfer. The estimated R_{CT} values at each elec-

Table 3
Comparisons of the peak currents to 10 μM catechol and 10 μM 4-CP, the amounts of immobilized TYR and the surface resistance of TYR-CFs prepared by various coupling reagents.

No.	Coupling reagent	Peak current (μA) for 10 μM substrates (1st day)		Γ_{TYR} (μg/cm^2) [a]	Surface resistance Z' (Ohm)[b]
		Catechol	4-Chlorophenol (4-CP)		
1	Glutaraldehyde (GA)	50.4	20.7	0.233 ± 0.009	25.5
2	Cyanuric chloride (CC)	21.5	4.3	0.320 ± 0.013	20.0
3	Ascorbic acid (AA)	31.2	0.6	–	34.9
4	1,4-Benzoquinone (BQ)	10.1	2.7	–	69.6
5	Terephthaloyl chloride (TCL)	16.7	2.6	0.411 ± 0.017	41.2
6	Dimethyl suberimidate dihydrochloride (DMS)	18.2	1.5	0.259 ± 0.010	20.1
7	N-[ε-Maleimidocaproyloxy] succinimide ester (EMCS)	21.8	1.2	–	21.6
8	Disuccinimidyl suberate (DSS)	20.0	2.5	0.183 ± 0.007	16.3

[a] Surface coverage of immobilized TYR (Γ_{TYR}) was estimated by using the value of effective CF surface area of 1 m^2/g [9].
[b] The surface resistance (the electron-transfer resistance) of the TYR-immobilized CF was estimated from the Niquist diameter of EIS using $Fe(CN)_6^{4-/3-}$ as redox probe.

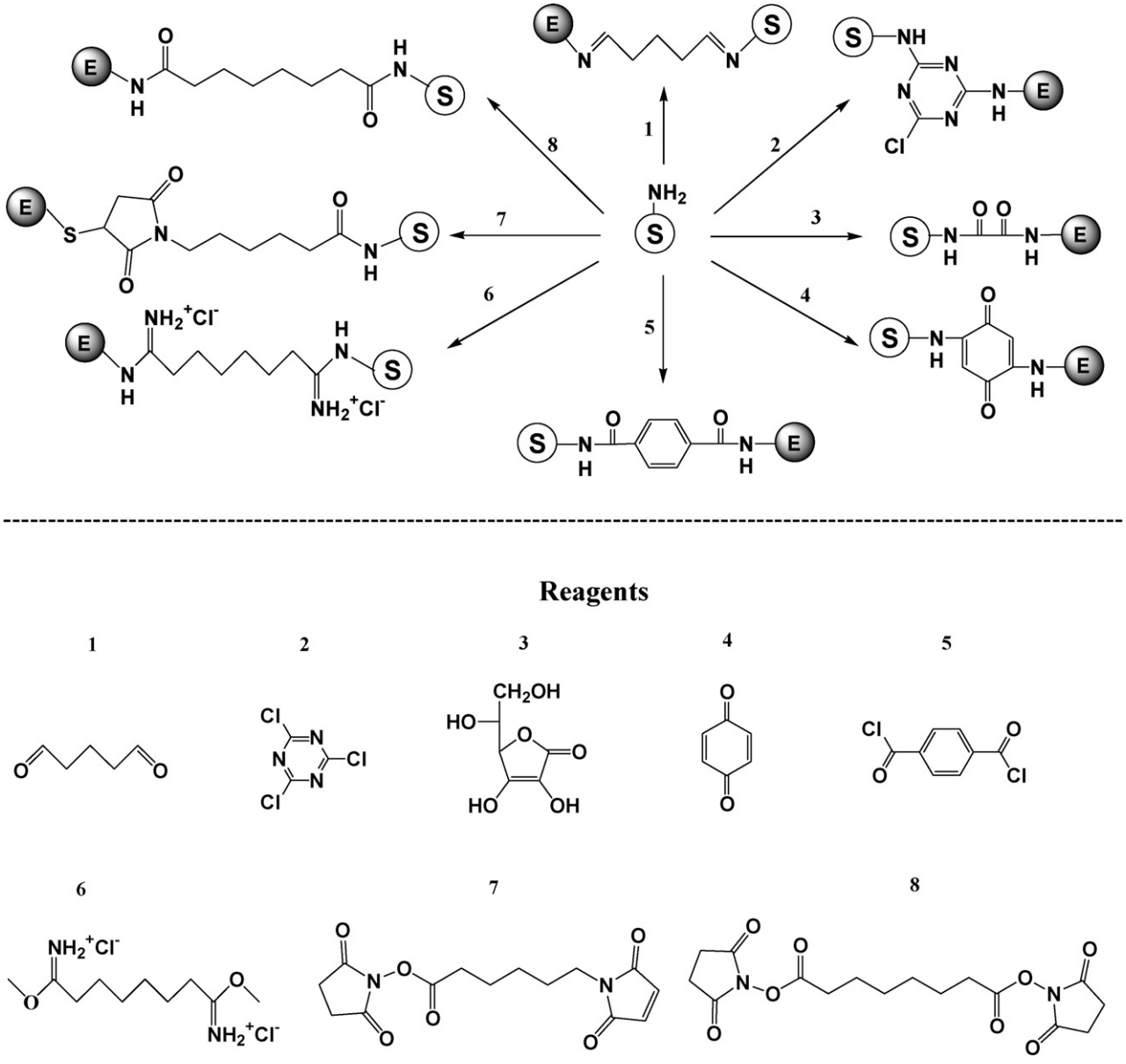

Fig. 3. Structural formulas: coupling reagents and coupling structures between NH_2 support (S) and enzyme protein (E).

trode surface were also summarized in Table 3. Judging from these data, the amount of immobilized TYR does not necessarily depend on the catechlase and phenolase activities. Therefore, it is reasonable to assume that the spacer effect and molecular geometry effect of each coupling reagent preferentially influence the microenvironment of immobilized TYR at the APTES-treated CF surfaces, and results in the difference of catalytic activity of the immobilized TYR.

Fig. 4 shows the calibration curve of the TYR-CF prepared by the GA-coupling method for catechol and 4-CP. The peak currents of catechol increased linearly up to 3 μM (correlation coefficient, 0.9998; slope, 4.97 μA/μM), and the detection limit was found to be 6.0×10^{-9} M (S/N = 3; noise level is ca. 0.1 μA order). On the other hand, the peak current of 4-CP increased linearly up to 10 μM (correlation coefficient, 0.9992; slope, 2.05 μA/μM), and the detection limit was found to be 1.5×10^{-8} M This sensitivity is much higher than previously reported other TYR-based flow biosensors using graphite-epoxy resin matrix [17], poly-L-lysine-coated porous stainless steel [31] and cation-exchanger film-coated graphite electrode [20], probably due to the large effective surface area of the CF. The apparent Michaelis–Menten constant $K_{m,}^{app}$ was evaluated by fitting the approximated line of the electrochemical Lineweavor-Burk plots. Evaluated $K_{m,}^{app}$ were 22.2 μM for catechol and 42.7 μM for 4-CP, respectively. The observed $K_{m,}^{app}$ values were much lower than those of free enzyme in solution (280 μM for catechol, 700 μM for phenol) [32]. The observed $K_{m,}^{app}$ values are lower than the values found for other TYR-based biosensors [100 μM and 210 μM for phenol [33], 130 μM [30] and 240 μM [22] for catechol. This small K_m values reflects the high sensitivity of this system, and this feature would be one of the important characteristics of this TYR-immobilized CF-based biosensor system. Similar small K_m value was observed for TYR-adsorbed colloidal gold modified carbon paste electrode [22]. The relatively smaller K_m values could be explained by an electroenzymatic recycling phenomenon and a preconcentration effect of the matrix on the enzyme substrate leading to a local increase in substrate concentration [22].

The fabrication reproducibility of the present APTES-GA method was examined for three CFs made independently by same protocol using 10 μM catechol and 4-CP. The relative standard deviation

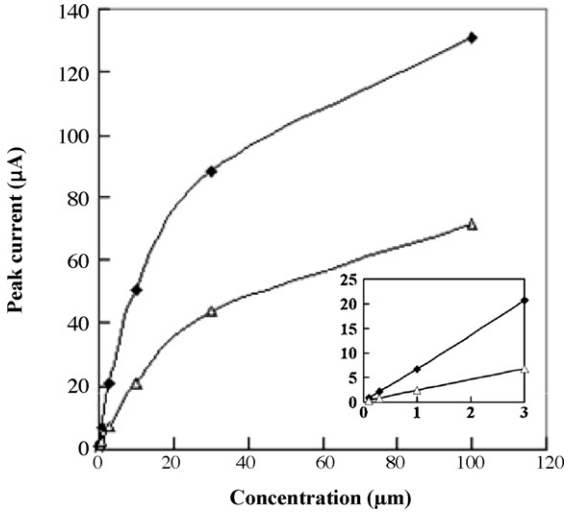

Fig. 4. Calibration curves of catechol (♦) and 4-CP (△) by the TYR-CF prepared by the GA-coupling method. Inset graph is enlargement of lower concentration region. Each plot is an average of three measurements.

($n = 3$) was evaluated to be 8.0% and 6.3%, for catechol and 4-CP, respectively, suggesting that the fabrication reproducibility is not so bad.

3.3. Operational stability of the TYR-CF-based biocatalytic enzymatic flow-through detector

Operational stability is one of the important factors for the practical use of enzymes in biosensors or as biocatalysts [34]. To investigate the operational stability, 30 consecutive injection peaks were measured at carrier flow rate of 3.0 mL/min at pH 7.0 (Fig. 4). In the case of the GA-coupling method, after slight increase in the peak current for first five injections, no serious peak degradation was observed over 30 consecutive injections. Some geometry effect of GA would keep the conformation of the active TYR and may prevent the gradual denaturing of TYR at the CF surface. On the contrary, seven coupling reagents except for GA showed apparent decrease in the peak current during the consecutive injections of catechol (Fig. 5A) and 4-CP (Fig. 5B). It is known that quinone compounds are highly unstable and easily polymerized and inactivates the TYR [35,36]. The polymerized product causes the electrode fouling of the TYR-based enzyme electrode, leading to the serious degradation of the response [15,17].

Fig. 6 shows typical consecutive flow injection peaks of catechol obtained by TYR-CF prepared by the GA-coupling method after the storage of 15 days in 0.1 M phosphate buffer (pH 7.0). The operational stability was much improved as compared with that of first day as shown in Fig. 5A. The relative standard deviation (RSD) was 0.87% for 30 successive assays (average, 53.1 μA; range, 51.6–54.1 μA), this is superior to TYR-incorporated graphite-epoxy resin matrix (RSD = 1.4%, $n = 40$) [17] and TYR-entrapped carbon paste (RSD = 2.5%, $n = 30$) [19]. The sample through-put of this system under the present conditions was 36 samples/h.

3.4. Storage stability of the TYR-CF-based biocatalytic enzymatic flow-through detector

Storage stability is an important factor for the applicability of immobilized enzymes, because native enzymes usually quickly lose their activity [37]. Fig. 7 shows the relative remaining activities for the determination of catechol and 4-CP over the 25 days storage period. Each point is the average value of peak current for 30 repetitive injections of each sample measured at every 5

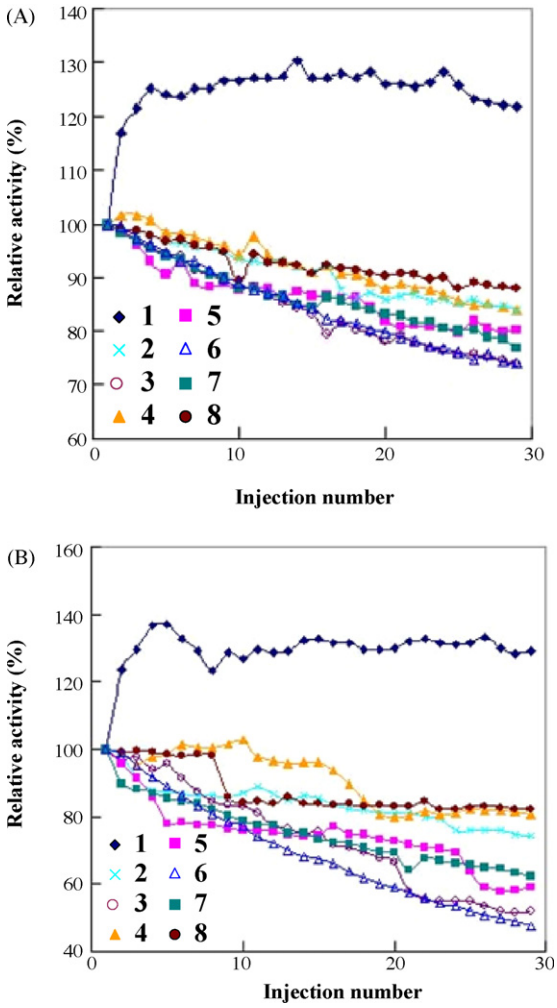

Fig. 5. Comparison of the operational stability of the TYR-CF prepared by various coupling regents over 30 consecutive injections of 10 μM (A) catechol and (B) 4-CP. 0.1 M phosphate buffer (pH 7.0) was used as a carrier at flow rate of 3.0 mL/min. Applied potential was −0.05 V vs. Ag/AgCl.

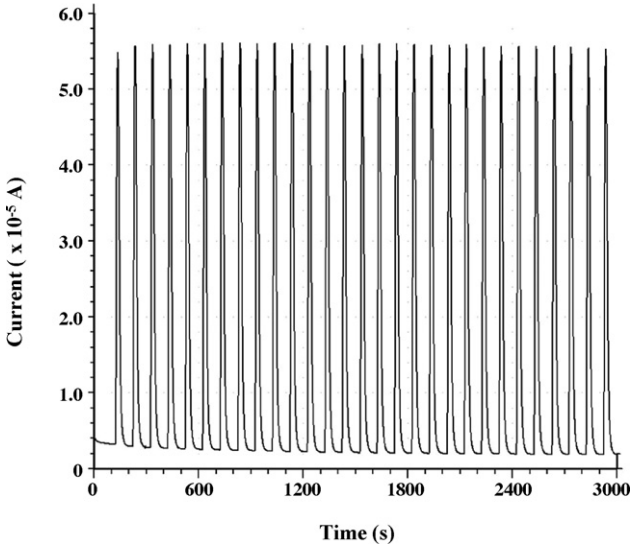

Fig. 6. Typical peak responses of 30 consecutive injections of 10 μM catechol obtained with the TYR-CF prepared by the GA-coupling method, after 15 days storage in 0.1 M phosphate buffer (pH 7.0) at 4 °C. The carrier flow rate was 3.0 mL/min.

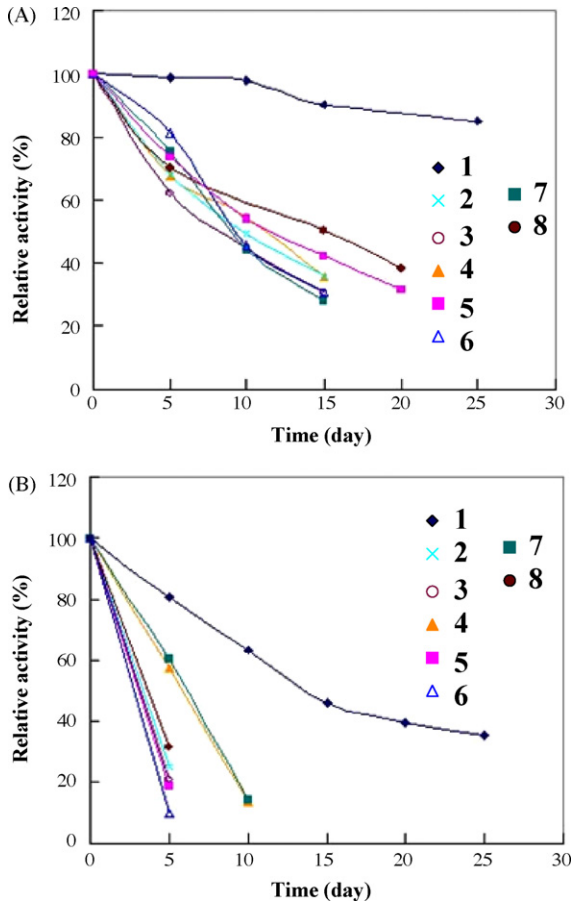

Fig. 7. Comparison of storage stability of the TYR-CFs prepared by various coupling reagents for the determination of catechol (A) and 4-CP (B). Each point was average value from 30-conscutive measurements of 10 μM analytes.

days. When not in use, the TYR-CF was stored in 0.1 M phosphate buffer (pH 7.0) at 4 °C. It is clear that the best storage stability was achieved when the TYR was immobilized by the GA-coupling method. As shown in Fig. 7A, the peak currents of catechol obtained by the GA-coupling method were almost unchanged during initial 10 days, and the 85% of original activity was remaining after 25 days storage. In contrast, as can be seen in Fig. 7B, the storage stability for 4-CP measurement was significantly inferior to that of catechol measurement even by the GA-coupling method, and the peak current decreased to 35% of initial day after 25 days storage. These results imply that the inactivation mechanism of the phenolase activity and the catechol activity of the immobilized TYR by this method is different. The storage stability for 4-CP determination is unfortunately inferior to the TYR-immobilized onto the colloidal gold modified carbon paste electrode (after 20 days, 72% of initial response to phenol is retained) [22] and the TYR-immobilized on the graphite-epoxy resin matrix (after 20 days storage in dry state, 90% original activity for phenol detection is remained) [17], and the TYR-modified reticulated vitreous carbon (during 20 days, no significant loss of the amperometric response to phenol) [21]. Therefore, the development of novel strategy to maintain the phenolase activity of immobilized TYR for long period would be future important research topics. It has been reported that the reduction of Schiff base double bonds (C=N) to single bond (C—N) using suitable reducing agents such as sodium cyanoborohydride ($NaCNBH_3$) enhances the stability of immobilized enzymes [26]. Therefore, such attempts would be made in near future to improve the long-term storage stability of the present system.

4. Conclusions

Eight kinds of coupling reagents were used to immobilize TYR onto the APTES-modified CF surface. The GA-coupling method showed the best results on the sensitivity, the operational stability and the storage stability. The detection limits of catechol and 4-CP obtained by the TYR-CF prepared by the GA-coupling method were 6.0×10^{-9} M and 1.5×10^{-8} M, respectively. The TYR-CF prepared by GA-coupling method exhibited excellent operational stability over repetitive 30 injections, and maintained 85% original catecholase activity after 25 days storage.

Since TYR has broad selectivity toward various mono- and diphenol compounds, by combining with the separation column, this TYR-CF-based biocatalytic enzymatic flow-through detector would be useful for the continuous monitoring of highly toxic phenol compounds and clinically important catecholamines. Furthermore, this enzyme immobilization strategy onto the CF would be useful for not only biosensors but also enzyme-based biofuel cells.

Acknowledgements

This research was financially supported in part by the Open Research Center Project of the Ministry of Education, Science and Culture of Japan. We would like to express our grateful acknowledgement to Prof. Y. Okabe and Prof. M. Shimojo of Saitama Institute of Technology, for supporting the XPS and SEM measurements.

References

[1] M.J. Núñez, J.M. Lema, Enzyme Microb. Technol. 9 (1987) 642.
[2] J. Woodward (Ed.), Immobilized Cells and Enzymes A Practical Approach, IRL, Oxford, UK, 1985.
[3] M. Arica, H.J. Yakup, Chem. Technol. Biotechnol. 58 (1993) 287.
[4] A.F. Collings, F. Caruso, Rep. Prog. Phys. 60 (1997) 1397.
[5] A. Subramanian, S.J. Kennel, P.I. Oden, K.B. Jacobson, J.D. Woodward, J. Mitchel, Enzyme Microb. Technol. 24 (1999) 26.
[6] T.T. Ngo, J. Ivy, H.M. Lenhoff, Biotechnol. Lett. 2 (1980) 429.
[7] J. Emneus, L. Gorton, Anal. Chim. Acta 276 (1993) 303.
[8] O. Valentova, M. Marek, F. Svec, J. Stamberg, Biotechnol. Bioeng. 23 (1981) 2093.
[9] Y. Hasebe, T. Shirai, T. Nagashima, T. Gu, S. Uchiyama, Bunseki Kagaku (in Japanese) 57 (2006) 1197.
[10] Y. Hasebe, R. Imai, M. Hirono, S. Uchiyama, Anal. Sci. 23 (2007) 71.
[11] E. Coulon, J. Pinson, Langmuir 17 (2001) 7102.
[12] E. Coulon, J. Pinson, J.-D. Bourzat, A. Commercon, J.-P. Pulicani, J. Org. Chem. 67 (2002) 8513.
[13] C.M. Eliott, R.W. Murray, Anal. Chem. 48 (1976) 1247.
[14] S.-Y. Seo, V.K. Sharma, N. Sharma, J. Agric. Food. Chem. 51 (2003) 2837.
[15] F. Ortega, J.L. Cuevas, J.I. Centenera, E. Domínguez, J. Pharm. Biomed. Anal. 10 (1992) 789.
[16] F. Ortega, E. Dominguez, G.J. Petterson, L. Gorton, J. Biotechnol. 31 (1993) 289.
[17] J. Wang, L. Fang, D. Lopez, The Analyst 119 (1994) 455.
[18] G.M. Varga, E. Burestedt, C.J. Svensson, J. Emnéus, L. Gorton, T. Ruzgas, M. Lutz, K.K. Unger, Electroanalysis 8 (1996) 1121.
[19] J. Wang, F. Lu, S.A. Kane, Y.-K. Choi, M.R. Smyth, K. Rogers, Electroanalysis 9 (1997) 1102.
[20] C. Nistor, J. Emnéus, L. Gorton, A. Ciucu, Anal. Chim. Acta 387 (1999) 309.
[21] N. Peña, A.J. Reviejo, J.M. Pingarrón, Talanta 55 (2001) 179.
[22] S. Liu, J. Yu, H. Ju, J. Electroanal. Chem. 540 (2003) 61.
[23] F. Daigle, D. Leech, Anal. Chem. 69 (1997) 4108.
[24] O. Adeyouju, E.I. Iwuoha, M.R. Smyth, D. Leech, Analyst 121 (1996) 1885.
[25] Q. Zhao, L. Guan, Z. Gu, Q. Zhuang, Electroanalysis 17 (2005) 85.
[26] Y. Li, X. Xu, C. Deng, D. Pengyuan, X. Zhang, J. Proteom. Res. 6 (2007) 3849.
[27] S.-R. Kim, N.L. Abbott, Langmuir 18 (2002) 5269.
[28] J. Yakovleva, R. Davidson, A. Lobanova, M. Bengtsson, S. Ermin, T. Laurell, J. Emnéus, Anal. Chem. 74 (2002) 2994.
[29] J.C. Tiller, R. Rieseler, P. Berlin, D. Klemm, Biomacromolecules 3 (2002) 1021.
[30] E. Han, D. Shan, H. Xue, S. Cosnier, Biomacromolecules 8 (2007) 971.
[31] M.W. Ducey Jr., M.E. Meyerhoff, Electroanalysis 10 (1998) 157.
[32] J.L. Smith, R.C. Krueger, J. Biol. Chem. 237 (1962) 112.
[33] B. Wang, S. Dong, J. Electroanal. Chem. 487 (2000) 45.
[34] J.C. Tiller, D. Klemm, P. Berlin, Des. Monomers Polym. 4 (2001) 315.
[35] N.H. Horowiz, M. Fling, G. Horn, in: A.H. Tabor, C.W. Tabor (Eds.), Methods in Enzymology, vol. XVII, Academic Press, New York, 1970, p. 615.
[36] D.A. Robb, in: R. Lontie (Ed.), Copper Proteins and Copper Enzymes, vol. II, CRC Press, Boca Raton, FL, 1984, p. 207.
[37] K. Buchholz, J. Klein, in: K.V. Mosbach (Ed.), Methods Enzymology, vol. 135, Academic Press, New York, 1987, p. 3.