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Development of Electrochemical Biosensing Surfaces Based on the Heat-sensitive Structural Transition of Poly (*N*-isopropylacrylamide)

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Abstract

In this study, we describe a controllable biointerface with an external stimulus based on an intelligent polymer-modified electrode. In order to confer heat-sensitivity to the surface, the interface for the immobilization of glucose oxidase (GOX) as a model enzyme was modified using poly (*N*-isopropylacrylamide) (PNIPAAm), which evidences a rapidly reversible hydrophilic/hydrophobic transition of its conformation in response to changes in temperature. As an underlying surface for modification with PNIPAAm and protein adsorption, and for electrochemical sensing, a dendrimer monolayer was self-assembled onto a gold substrate and utilized. For the facile attachment of PNIPAAm to the monolayer, N-hydroxysuccinimide-esterified PNIPAAm (NHS-PNIPAAm) was synthesized. Following modification, GOX was selectively immobilized on the basis of the structural transition of PNIPAAm via the alteration of the environmental temperature across its lower critical solution temperature (LCST). GOX immobilization levels were determined from the bioelectrocatalytic signals via cyclic voltammetry. The dependence of the GOX immobilization levels on the structural transition of PNIPAAm was also assessed. Following optimization, a difference in GOX surface coverage of approximately 4.96×10^{-13} mol · cm^{-2} was achieved between the two operational modes, via merely via alterations in the working temperature. Furthermore, we applied this strategy directly to selective antibody binding and electrochemical immunosensing on the basis of a biocatalyzed precipitation reaction.

Keywords: Electrochemical, PNIPAAm, Heat-responsive, Biosensor

Introduction

Surface chemistry for biomolecular association in a biosensor is a crucial technique in the construction of an interface consisting of biomolecules on a solid substrate[1-3]. To obtain the desired performance, with regard to the sensitivity, selectivity, detection limit, etc. from biosensors, recent efforts for surface modification have relied upon layer formation techniques, focusing on high density[4], orientation[5,6] and activity maintenance[2] of biomolecules on the surface. Additionally, signaling strategies involving efficient communication between elements generated by biorecognition events and electrodes have been previously developed[7-10]. With the recent development of MEMs (microelectromechanical systems), miniaturized biosensor systems, Lab-on-a-Chip or μ-TAS (micro-total analysis system), must now be fabricated with electrode surfaces possessing biocompatible interfaces and acceptably sensitive, quantifiable, and non cross-reactive signaling under artificial microenvironment conditions[11,12]. Considering these factors, biosensor fabrication should focus on multifunctional surface construction, which is associated intimately with the functionality requisite to the desired purposes.

Stimuli-responsive polymers have attracted a fair amount of attention for applications in a variety of biomedical sciences[13,14] and bioengineering[15-18] applications, because their physical properties in solutions changed sensitively in response to slight changes in bioenvironmental conditions, including temperature, pH, ionic strength etc. Poly (*N*-isopropylacrylamide) (PNIPAAm), one of the most frequently utilized thermo-responsive polymers, has the characteristic of hydration/dehydration alterations which respond reversibly and rapidly to specific temperature in a solution[19]. In other words, the PNIPAAm structure is altered into a soluble extended chain of PNIPAAm below the lower critical solution temperature (LCST) and insoluble retracted chain above LCST. PNIPAAm has been demonstrated to mark a LCST of 32°C in pure water and 26°C in aqueous buffer solutions[18,20],

thereby guaranteeing a relatively favorable environment for biomolecules during bioprocessing[21]. Applications utilizing stimuli-responsive polymers, including PNIPAAm, have been made in drug delivery systems[12,22], systems for the modulation of cell attachment/detachment[15,23,24], chromatography apparatus[16,18,20] and biosensors[25-27]. The Hoffman group has published a number of studies for applications involving conjugation between biomolecules and stimuli-responsive polymers[17,28], thereby suggesting the impact of stimuli-sensitivity on biomolecular interactions[17,28,29]. One of their researches showed that the binding of biotin-labeled proteins with streptavidin, of which poly (*N*, *N*-dietyhylacrylamide) (PDEAAm), the heat-sensitive polymer, was conjugated at a distance of 20Å from the binding pocket was inhibited by steric hindrance by the extended form of PDEAAm with temperature changes[30]. With regard to surface modifications with thermo-responsive polymers, cell attachment/detachment control[15,23,24], chromatography[16,18,20] and immunoassay[26] applications were successfully carried out via grafting onto support or bead surfaces of thermo-responsive polymers, in addition to hydrophobic/hydrophilic and structural changes.

In this study, we have attempted to confer heat-sensitivity on an electrode surface via modification with PNIPAAm and the investigation of selective protein binding. As a platform surface for PNIPAAm binding, a fourth generation poly (amidoamine) (PAMAM) dendrimer monolayer was employed, thereby guaranteeing ordered and high-density deposition[4,31,32]. Glucose oxidase was utilized as a model enzyme, and the bioelectrocatalytic signals from the enzyme electrode could be measured quantitatively via voltammetry, as the dendrimer monolayer on the gold electrode allowed for the ready amplification of bioelectrocatalytic signals in electrochemical sensors via its permeable structure, as has been described elsewhere[31-33]. Above and below LCST, the GOX immobilization levels under two structural conditions of surface PNIPAAm were estimated from electrodes with various PNIPAAm modification levels. Then, GOX immobilization was conducted with the PNIPAAm-modified surfaces under two conditions, involving the extended and shrunken forms of PNIPAAm below and above LCST, respectively, and the responsiveness of the surfaces can be estimated from the bioelectrocatalytic signals measured via cyclic voltammetry. In addition, the current strategy was employed in order to effect the selective binding of antibiotin antibody molecules on surfaces, and to quantify the reaction on the basis of a biocatalyzed precipitation.

Results and Discussion

Surface Modification with PNIPAAm

In this study, the platform surface of the enzyme electrode was constructed using the G4 PAMAM dendrimer as a building block for the efficient im-

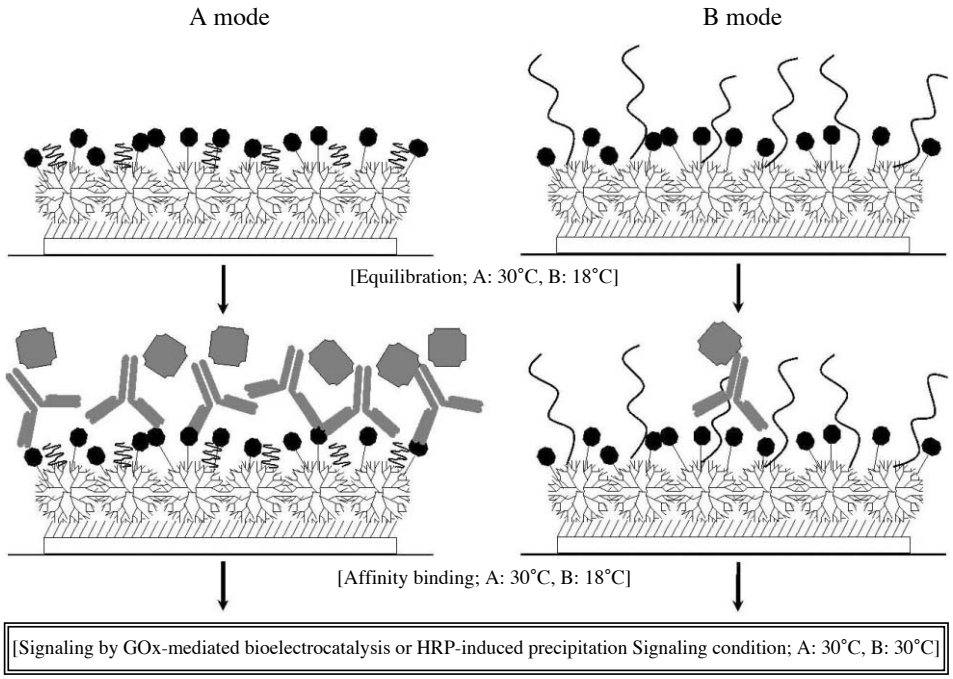

Figure 1. Schematic representation of the processes in selective protein binding and biosensor signaling. The dimensions of the components are not drawn to scale for the sake of simplicity (see experimental section).

mobilization of biomolecules and for further functionalization[4,31-33]. In an effort to achieve covalent grafting at the amine-activated surfaces, PNIPAAm was synthesized and manipulated to harbor an amine-reactive succinimidyl group at one end of the polymer chain, in accordance with the method developed by Pan and Chien[34]. Consequently, NHS-esterified PNIPAAm (NHS-PNIPAAm, MW 4244) was prepared as a heat-sensitive modifier. The chemical structure of the synthesized polymer was identified via ^{1}H-NMR spectroscopy.

Prior to the fabrication of the enzyme electrodes, we verified that abtained polymers were formed covalently on the surface via a coupling reaction between the terminal NHS groups of PNIPAAm and the amine groups of the dendrimer monolayer. This was confirmed by the changes in the FT-IR spectra of the dendrimer/DTSP SAM prior to and after reaction with NHS-PNIPAAm (data not shown). Two discernible spectra that were registered from the experiment revealed the principal adsorption peaks, thereby confirming the modification with NHS-PNIPAAm.

Heat-sensitive Control of the PNIPAAm-modified Electrodes for Selective GOX Immobilization

For selective immobilization to the electrode surfaces, GOX was pretreated via periodate oxidation in an effort to provide amine-reactive moieties at the GOX surface. GOX binding was accomplished via the formation of Schiff bases between the remaining amine groups of the dendrimer on the surface and the surface aldehydes of the periodate-oxidized GOX. It should be noted that the remaining surface amine groups of the dendrimer, which had not been consumed during the PNIPAAm reaction, performed roles in this covalent binding reaction. Therefore, we expected that the GOX immobilization level would be reduced in correlation with increasing quantities of PNIPAAm on the dendrimer monolayers. From this perspective, the PNIPAAm-modified electrodes were prepared with NHS-PNIPAAm concentrations ranging from 20 nM to 2 mM in their modification steps. Also, in an effort to assess the changes in the quantity of GOX immobilization during the structural transition of PNIPAAm on the interface, GOX reaction solutions and the functionalized electrode surfaces were equilibrated above and below LCST, respectively. Then, GOX immobilization was conducted and signaling was carried out via GOX-mediated electrocatalysis.

Figure 2 shows the cyclic voltammograms of the electrodes modified with NHS-PNIPAAm at various concentrations, the signals of which were amplified via GOX-mediated electrocatalysis. For comparison, Figures 2A and 2B evidenced bioelectrocatalytic signals from the electrodes subjected to GOX treatment with shrunken and extended PNIPAAm structures on the interface via phase transition in response to solution temperatures. From the results of the voltammetry experiments, we determined that significant levels of signal differences were developed from 0.02 to 2 mM, but no discernible differences were observed at concentrations of below 0.02 mM. As expected above, the decrement in bioelectrocatalytic signals with increasing PNIPAAm concentrations as shown in both 2A and 2B is attributable to the shortage of surface amine groups from the occupation by PNIPAAm and/or the steric limitation by the modified PNIPAAm for GOX immobilization.

It should be noted that the signal differences from the enzyme electrodes modified with the proper modification of PNIPAAm levels were generated by the temperature control during the GOX immobilization step. The signal differences between Figures 2A and 2B at concentrations of above 0.02 mM are attributed principally to the transition of surface properties having the shrunken form of PNIPAAm above LCST and the extended chain of it below LCST. While the extended polymer layer may prove difficult for the access of GOX to the surface as the result of long chain length and limited polymer mobility, the shrunken form of PNIPAAm may alleviate the steric hindrances for GOX immobilization. This different steric hindrance via the structural transition of polymers may serve different environments for protein binding.

From the results of this test, we determined that selective GOX immobilization could be accomplished on the PNIPAAm-modified electrodes by the proper degree of PNIPAAM modification and temperature control across LCST.

Modulation of Bioelectrocatalytic Signals in Two Modes on the Basis of the Structural Transition of PNIPAAm

On the basis of the differences in the degree of GOX immobilization in response to temperature changes and surface properties, we selected the NHS-PNIPAAm concentration of 0.02 mM as a reaction condition, evidencing a maximum signal difference (Figure 2C). The successive operation processes of the two electrodes which modulate GOX immobilization levels via the transition of PNIPAAm were designated as A mode (high signal) and B mode (low signal). The procedure of the PNIPAAm-modified electrode fabrication was similar to that in the aforementioned test, including the modification step with

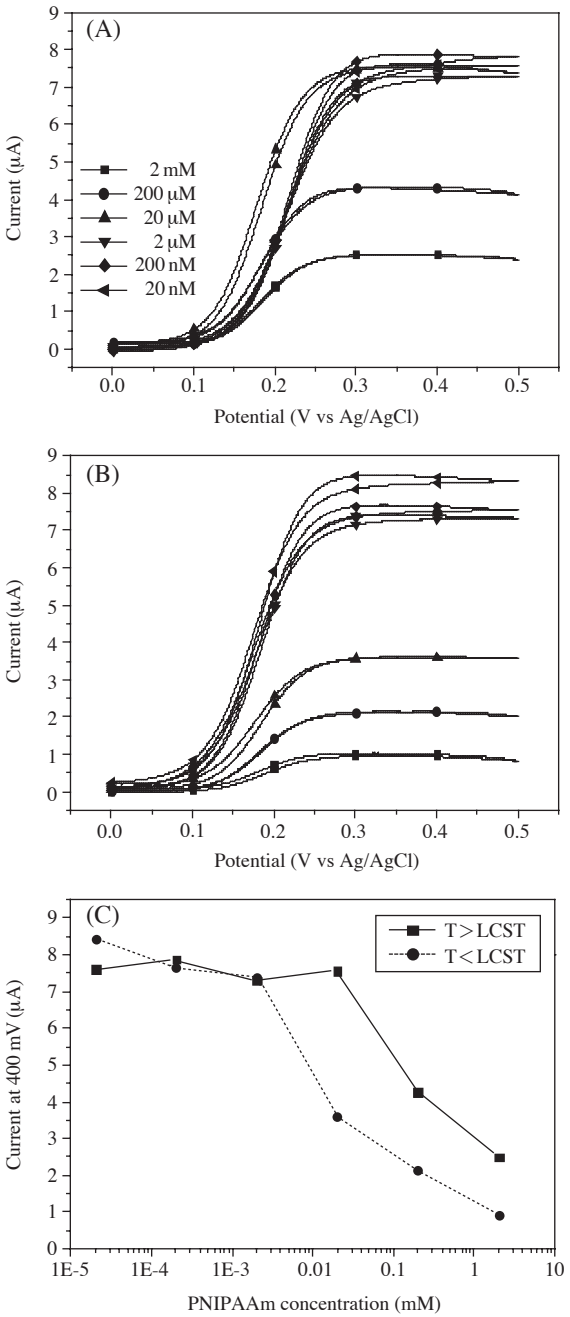

Figure 2. Electrochemical characterization for the covalent binding of oxidized-GOX to the dendrimer monolayers as a function of NHS-PNIPAAm concentration: 2 mM, 200 μM, 20 μM, 2 μM, 200 nM, and 20 nM. The registered cyclic voltammograms of the electrodes to which GOX was immobilized above LCST (A) and below LCST (B) are shown, and the peak currents from the voltammograms were collected (C).

the NHS-PNIPAAm concentration set at 0.02 mM, and the operation process in the A and B modes was composed of three consecutive steps-equilibration, immobilization, and washing (Figure 1).

The cyclic voltammograms evidencing the change in bioelectrocatalytic signals obtained from the two electrodes, namely the A and B modes, are as shown in Figure 3A. The two voltammograms manifested typical catalytic curves for GOX bioelectrocatalysis in electrolytes with a ferrocene electron-transferring mediator. Figure 3B shows the data collected from two modes, A and B, as well as the maximum achievable signal for the electrode surfaces which were not treated with NHS-PNIPAAm. From this result, we observed significant signal differences between modes A and B, thus bolstering the notion that the selective immobilization of protein by polymer transition worked as intended.

Also, to obtain detailed information in this experiment, active GOX concentrations on surfaces were analyzed kinetically from the quantifiable current signals[31,33]. On the basis of the following equation, the difference in GOX surface concentrations by modes A and B can be calculated as follows:

$$\frac{1}{i_p}=\frac{1}{2FS\Gamma_E}\cdot\frac{1}{k_3[Fc]}+\frac{1}{k_2}+\frac{1}{k_{red}[G]},$$

in which F is the Faraday's constant, S is the electrode area, Γ_E is the surface concentration of the enzyme, [Fc] is the mediator concentration, [G] is the glucose concentration in solution, and $k_{red}=k_1k_2/(k_{-1}+k_2)$. On the basis of the above equation and the known rate constant values ($k_2=700\ s^{-1}$, $k_3=1.2\times10^7\ M^{-1}s^{-1}$, $k_{red}=1.1\times10^4\ M^{-1}s^{-1}$), we determined the density of GOX associated with the PNIPAAm-modified electrode surfaces. Table 1 shows the sensor signals for the A and B modes and maximum signal, in addition to the GOX surface concentrations calculated from the current values of the electrodes for suitable substitution. As is shown in the table, the differences in the signal and the GOX surface concentration between the electrodes from modes A and B were determined to be 4.96×10^{-13} mol cm^{-2}. This result indicates that a relatively large difference in GOX binding was achieved successfully. However, the ideal case for the complete adsorption/desorption of GOX was not accomplished because the signals registered in B mode were inconsistent with the background signal, which can be regarded as a signal of complete desorption. It can be assumed that the complete adsorption/desorption process caused by the steric hindrance from the interfacial property should be accompanied by considerations regarding the steric control of the surface and the matching of the molecular components.

In addition, this approach was extended to selective antibody immobilization and immunosensing. Via the

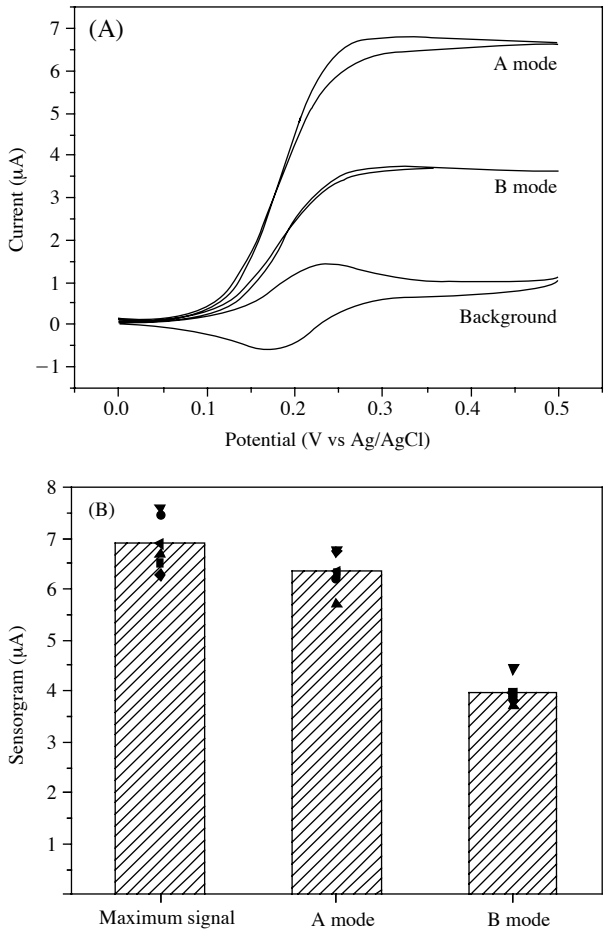

Figure 3. Comparisons of (A) cyclic voltammograms traced and (B) sensor signals for the bioelectrocatalytic signals from the fabricated electrodes in this study. (A) The background cyclic voltammogram was traced in a 0.1 M PBS solution containing 0.1 mM ferrocene methanol without glucose as a substrate for bioelectrocatalysis, and the cyclic voltammograms in modes A and B were traced from the electrodes fabricated in modes A and B. (B) Sensor signals were registered from the anodic currents at +400 mV of cyclic voltammograms in each experiment. Columns represent the averages of signals collected from these. The maximum signal represents the results from the electrodes fabricated without the modification step with NHS-PNIPAAm.

use of peroxidase-mediated precipitation reactions and the subsequent electrochemical tracking of the change in surface resistance, we were able to significantly magnify the signal changes between modes A and B. Figure 4 shows the results from the biotin and PNIPAAm dual-modified interface that was allowed to react with antibiotin-HRP. After biospecific antibody recognition, a precipitation reaction was conducted with 4-chloro-1-naphtol by the labeled peroxidase. As is shown in the figure, the signal changes in modes A and B were substantially increased, supporting the notion that the electrochemical technique combined with precipitation works efficiently. Also from the results of the test, the signaling attributable to nonspecific binding proved insignificant (Figure 4B).

Table 1. Comparison of electrochemical signals and GOX surface coverage for the A and B modes.

	Sensor signal[a] (μA)	Surface coverage of GOX[b] (mol · cm^{-2})
Maximum Signal	6.90	10.2×10^{-13}
A mode	6.35	9.05×10^{-13}
B mode	3.96	4.09×10^{-13}
Diff.[c]	2.39	4.96×10^{-13}

[a]The sensor signal is the average of data collected from anodic current from voltammograms. [b]The surface coverage of GOX is a value which was calculated from sensor signal through the kinetic equation. [c]Diff. value denotes the difference between data from mode A and mode B.

In conclusion, we have described electrode surfaces modulating the access of proteins via the modification of the dendrimer monolayer using the heat-sensitive polymer, PNIPAAm. As the two signaling processes described in this work were focused on electrochemical ones, direct applications for electrocatalyzed enzyme electrodes and immunosensors are possible. Additionally, via this method, the fabrication of protein micro-patterned surfaces and its biosensing applications appear to be very promising.

Materials and Methods

Materials

N-Isopropylacrylamide (NIPAAm), 3-mercaptopropionic acid (MPA), 2, 2′-azobisisobutyronitrile (AIBN), *N*, *N*′-dicyclohexylcarbodiimide (DCC), poly (amidoamine) (PAMAM) generation 4 dendrimer, and ferrocenemethanol were acquired from Aldrich. N-Hydroxysuccinimide (NHS), 3, 3′-dithiopropionic acid bis-N-hydroxysuccinimide ester (DTSP), (+)-biotin N-hydroxysuccinimide ester, glucose oxidase (GOX, from *Aspergillus niger*), monoclonal antibiotin antibody-peroxidase (antibiotin-HRP, clone BN-34), and 4-chloro-1-haphtol were purchased from Sigma. For the buffer solution, a phosphate-buffered saline solution containing 0.1 M phosphate and 0.15 M NaCl (PBS, pH 7.2) was prepared in doubly distilled and deionized water with a specific resistance in excess of 18 MΩ · cm, and utilized throughout the study. All other solvents employed herein were of the highest available quality, and were purchased from regular sources.

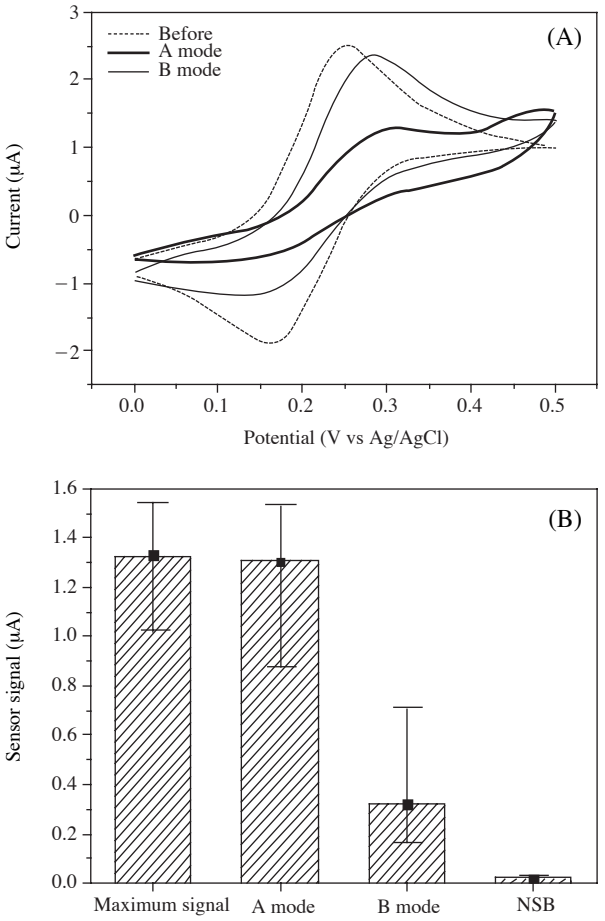

Figure 4. (A) Comparison of cyclic voltammograms of immunosensor electrodes fabricated in A and B mode prior to HRP-mediated precipitation reactions (dashed line) and after (solid line). (B) Comparison of sampled sensor signals at the peak position from voltammograms. NSB stands for non-specific binding.

Synthesis of NHS-PNIPAAm

To achieve coupling to the reactive surface amine groups with the amine-terminated dendrimer SAM, NHS-esterified PNIPAAm (NHS-PNIPAAm) was synthesized. We adopted the synthetic protocol of NHS-PNIPAAm as previously described by Pan and Chien[34]. In order to transfer a carboxyl group to one end of the polymerized chain, MPA and AIBN were used as a chain transfer agent and a radical polymerization initiator in a nitrogen atmosphere. In brief, after 15 g of NIPAAm was completely dissolved in 105 mL of DMF, it was treated with 0.33 g of MPA . To the mixture was then added 0.12 g of AIBN and was stirred for 5 h at 70°C. After the termination of reaction, the resultants were repeatedly purified via precipitation with diethyl ether.

The purified polymer, carboxyl-terminated PNIPAAm, was reacted with DCC and NHS for the amine-reactive activation of carboxyl groups. The obtained PNIPAAm of 5.35 g and DCC of 2 mmol were dissolved in 53.5 mL of dichloromethane, to which was added 2 mmol of NHS. The mixture was then allowed to react by 4 h of stirring in an ice bath. The resulting NHS-PNIPAAm was repeatedly purified via precipitation with diethyl ether, and was collected via vacuum evaporation. The synthetic result was verified via ^{1}H-NMR spectrometry.

Preparation of the Dendrimer-functionalized Electrode Surface

In order to fabricate the enzyme electrodes, freshly evaporated thin-firm gold surfaces were prepared via the resistive evaporation of 200 nm of Au (99.999%) onto titanium-primed (20 nm Ti) Si[100] wafers. Prior to the construction process, the evaporated gold surfaces were cleaned via immersion in piranha solution for 5 min. (*CAUTION: piranha solution reacts violently with most organic materials and must be handled with extreme care*.) After cleaning the electrodes completely with double distilled water, amine-reactive SAM was formed via 2 hours of immersion in a 5 mM DTSP solution in DMSO. As a washing step, the resultant electrodes were rinsed in DMSO and ethanol. After the formation of DSTP SAM, the electrodes were transferred to an 0.5% (w/w) poly (amidoamine) G4 dendrimer solution in ethanol and incubated for 30 min. The amine-terminated dendrimer surfaces were prepared for the modification of NHS-PNIPAAm and the immobilization of GOX, and were utilized throughout the study.

Measurement of the Bioelectrocatalytic Signals for GOX Immobilization Above and Below LCST

For the adsorption of GOX onto the amine-activated surface, GOX was treated via the oxidation of its carbohydrate residues with sodium periodate. 1 mg of GOX was permitted to react for 1 hour with 1.5 mg of sodium periodate in 1 mL of PBS in darkness at 4°C. Oxidized-GOX was purified via an ultrafiltration membrane (Centriprep®, Millipore) with a molecular cut-off of 30,000 and finally concentrated to 1 mg/mL in a PBS solution.

Prior to GOX immobilization, the PNIPAAm-modified surfaces were prepared with various modification levels of PNIPAAm for the evaluation of GOX immobilization. The modification levels of PNIPAAm were controlled via the application of a predetermined concentration of NHS-PNIPAAm for a fixed reaction time. The dendrimer-functionalized el-

ectrodes were immersed for 1 hour in NHS-PNIPAAm solution. After the surface modification of NHS-PNIPAAm, the electrodes were rinsed thoroughly with DMSO and PBS, then stored in a PBS solution for subsequent GOX immobilization.

Considering the LCST of PNIPAAm (26°C in aqueous-buffered solutions), the temperature condition was determined to 30°C as above LCST and 18°C as below LCST. For the precise maintenance of the solution temperatures of 30°C and 18°C in the experiment, two incubators with thermostat equipment (Model LTI-1001SD, EYELA) were utilized. The PNIPAAm-modified electrodes were transferred to champers set to 30°C and 18°C respectively, followed by the equilibration of the electrodes, oxidized-GOX samples, and PBS in each chamber for 15 min. The electrodes were then allowed to react with oxidized-GOX at each temperature for 30 minutes. After GOX immobilization, the electrodes were rinsed in PBS. In order to carry out cyclic voltammetry under the same temperature conditions, the electrodes in the 18°C chamber were transferred into a 30°C chamber, and all electrodes were equilibrated for 15 minutes at 30°C, followed by the voltammetric measurements. For the bioelectrocatalysis of covalently immobilized GOX, the cyclic voltammograms were conducted in the presence of 0.1 mM ferrocenemethanol and 10 mM glucose dissolved in PBS. All registered cyclic voltammograms were collected as background-subtracted cyclic voltammograms.

Modulation of Bioelectrocatalytic Signals from the Electrodes Formed by A and B Modes

In an effort to modulate the bioelectrocatalytic signals from the enzyme electrodes via the structural transition of PNIPAAm, the PNIPAAm-modified electrode surfaces were fabricated by the surface modification with NHS-PNIPAAm at a concentration of 0.02 mM, selected due to the generation of a profound signal difference between the electrodes on which GOX was bound, above and below LCST.

We named the modes A and B for the purposes of the simplicity of a series of the modulating processes of transition of PNIPAAm structures, GOX adsorption, and the detection of the bioelectrocatalytic signals(Figure 1). Modes A and B both began from the preparation of the PNIPAAm-modified electrodes. Mode A consisted of the equilibration step of the storage of GOX samples and PNIPAAm-modified electrodes for 15 minutes in a 30°C chamber, followed by the GOX immobilizing step for 30 min. After GOX immobilization, the washing step was conducted. On the other hand, mode B was similar to mode A in the equilibration and GOX immobilization steps, with the exception that the temperature is controlled at 18°C. However, the washing step of mode B was that after washing with PBS, the electrodes were equilibrated at 30°C in PBS to ensure an equivalent enzymatic activity in subsequent electrochemical measurements. After the equilibration step, cyclic voltammetry was performed.

Electrochemical Immunosensing by Using Similar Principle

As an extension of the above study, we attempted the selective immobilization of the antibody on surface and signaling. Rather than GOX, we employed antibiotin antibody as a model protein, and in particular the peroxidase conjugate antibody (antibiotin-HRP). For the functionalization of the interface, (+)-biotin N-hydroxysuccinimide ester was applied to the PNIPAAm-modified surface. Then, the target protein immobilization and subsequent signaling steps were conducted in a fashion similar to the GOX test as described above. Electrochemical signaling from the immune-reaction was conducted on the basis of HRP-mediated precipitation, by which electrochemical resistance could be correlated with the quantity of biospecifically bound antibodies[7]. Figure 1 shows the schematics for the processes and conditions of the tests.

Apparatus

Cyclic voltammetric measurements were conducted using an electrochemical analyzer model 630B (CH Instruments) connected to a laptop computer. A standard three-electrode configuration with an evaporated gold working electrode, a platinum auxiliary electrode, and an external Ag/AgCl (3 M NaCl, BAS) reference electrode were utilized.

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