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Control of heme peptide activity by using phase transition polymers modified with inhibitors

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Abstract

Catalytic activity of a heme peptide (HP) modified-electrode for H_2O_2 reduction was controlled by use of poly(*N*-isopropylacrylamide) modified with an inhibitory moiety, imidazole group. The polymers inhibited the catalytic activity below their lower critical solution temperature (LCST) where the polymers were dissolved and did not inhibit the activity above the LCST where the polymers were precipitated. A polymer with a longer side chain connecting with the imidazole group was more inhibitory than a polymer with a shorter side chain at temperatures below the LCST. Formation constants of dissolved HP-imidazole complexes were evaluated by spectroscopic means, and it was found that the polymers were more inhibitory than the corresponding monomers.

Keywords: Activity regulation; Heme peptide; Thermoresponsive polymer; Inhibition

1. Introduction

Activity regulation of biocatalysts by external signal or stimuli such as temperature [1–4], light [5–11], pH [12–14], electric field [15] and magnetic field [16] is one of the promising biotechnologies. It is potentially applicable to devices switching or regulating biochemical reactions and chemical sensors with controllable dynamic ranges [17–20].

In most of the studies concerning the activity regulation of biocatalysts, diffusion of the substrate to the active site of the biocatalyst was controlled by changing, for instance, permeability of a membrane covering the biocatalyst [1–3,8,9,15,17–19]. On the other hand, we have envisaged developing biocatalytic electrochemical devices (e.g., electrochemical biocatalysts and biosensors) that do not regulate the diffusion of the substrate but the intrinsic activity of the biocatalyst. Although several research groups have reported control of intrinsic biocatalytic activities [4–7,10], only our group has applied such a system to electrochemical devices [20] to the best of our knowledge. In this study, the biocatalytic activity of a heme peptide (HP; Fig. 1a) immobilized on the electrode surface was controlled by the use of a thermoresponsive phase transition polymer modified with an inhibitor.

HP is a model compound of the active site of peroxidase, and its catalytic activity is similar to that of peroxidase; it catalyzes electron transfer from an electron donor to H_2O_2. Since HP also transfers electrons from electrodes to H_2O_2, it has been used for electrochemical biosensors detecting H_2O_2 [21] and inhibitors for HP (e.g., imidazole [22] and urocanic acid [23]).

The inhibitors for HP are detected by an HP-modified electrode on the basis of inhibition of the H_2O_2 reduction current. The dynamic range of the inhibitor sensing depends on the rate-determining step of the H_2O_2 reduction. The highest sensitivity is obtained under the kinetically controlled conditions [22,24] because a drop of the biocatalytic activity is directly reflected by a drop of the H_2O_2 reduction current. On the other hand, under the diffusion-controlled conditions, the reduction current is not decreased by an inhibitor as far as the current is solely limited by the diffusion of H_2O_2. Therefore, the dynamic range shifts to

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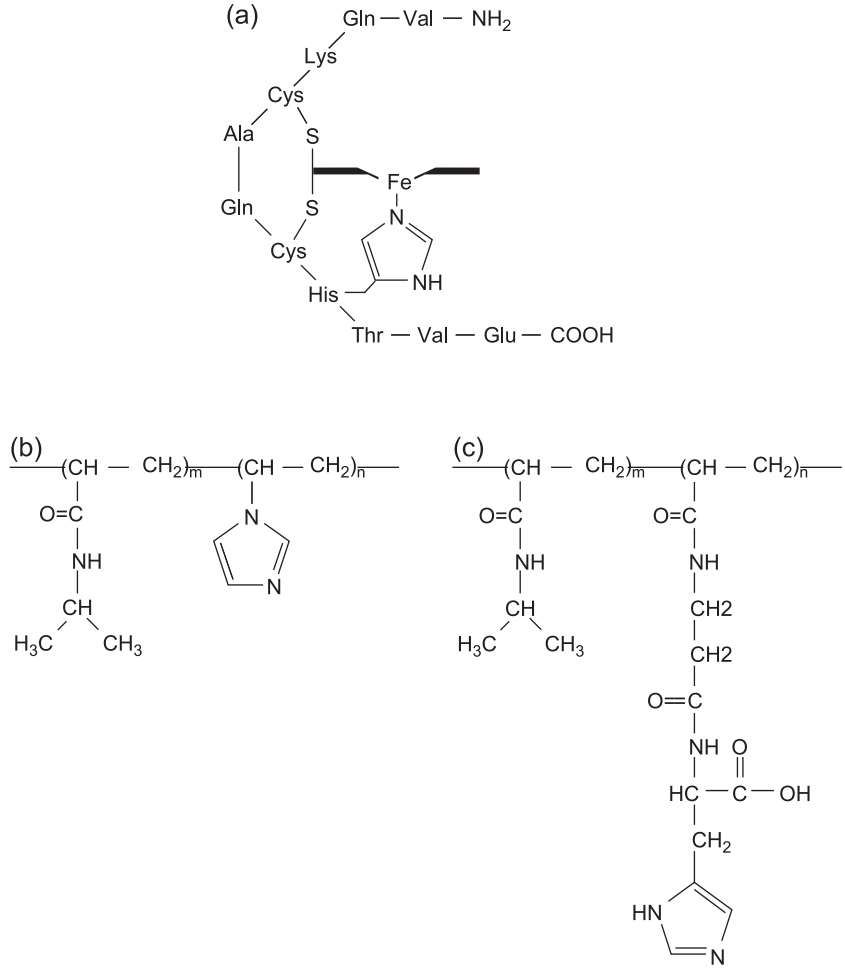

Fig. 1. Structures of (a) heme peptide, (b) poly(NIPA-VI) and (c) poly(NIPA-Car).

higher concentration ranges [24]. Namely, if the rate-determining step can be also controlled reversibly by regulating the catalytic activity of HP for the reduction of H_2O_2, the dynamic range for the inhibitor sensing can be controlled.

For the control of the HP activity, we used poly(*N*-isopropylacrylamide) (poly(NIPA)) modified with imidazole derivatives. Poly(NIPA) dissolves in water at temperatures below the lower critical solution temperature (LCST) and precipitates at temperatures above LCST [25–28]. On the other hand, it is known that imidazole derivatives coordinate to the active site of HP, heme. In our preliminary work [20], we synthesized poly(*N*-isopropylacrylamide-*co*-vinylimidazole) (poly(NIPA-VI); Fig. 1b) and used it to regulate the activity of HP immobilized on the electrode surface. The activity was inhibited by the dissolved poly(NIPA-VI) at temperatures below LCST and was not inhibited by the precipitated poly(NIPA-VI) at temperatures above LCST. However, even at low temperatures, the inhibition ratio (%inhibition) was as low as 20%. In this study, we attempted to enhance the inhibition ratio by using L-carnosine (Car) instead of VI. Since the synthesized copolymer (poly(NIPA-Car); Fig. 1c) has the longer side chain connected with the imidazole group than does poly(NIPA-VI), we expect lower steric hindrance and a stronger inhibitory effect. Moreover, a complex formation constant, K (M^{-1}), was examined on the basis of spectroscopic measurements. Effects of NIPA on the complex formation were also studied.

2. Experimental

2.1. Synthesis of the polymers

Poly(NIPA-VI) was synthesized under nitrogen from an aqueous solution containing 665 mM NIPA, 35.0 mM VI, 3.50 mM ammonium peroxodisulfate as an initiator and 20.0 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine as an accelerator. In synthesis of poly(NIPA-Car), VI was replaced with 105 mM Car and 35.0 mM *N*-acryloxysuccinimide (NASI; Acros Organic). The weight-average molecular weights (M_w) of poly(NIPA-VI) and poly(NIPA-Car) estimated by means of gel permeation chromatography (GPC) were 64,000 and 56,000, respectively.

The GPC system consisted of a HPLC pump PU 714, a degassing device DG 660B, a column oven CO 705 and a refractive index detector RI 704 (GL Science). An autosampler MIDAS (Spark, Holland) was used for the injection of polymer solutions. A GPC column PROTEIN 2004 (packed with porous silica gel, 20 mm i.d.×300 mm) and a guard column PROTEIN KW-LG (Shodex) were used at 20 °C. The flow rate of a 1/15-M pH 7.4 phosphate buffer (Iatron) solution as an eluent was 5.0 mL min^{-1}. Polyethylene oxide as molecular weight standards (Polymer Laboratories) and a software EZ Chrom Elite (Scientific Software) were used to determine M_w of the synthesized polymers. Polymers of $2000<M_w<50{,}000$ were collected by the use of dialytic membranes, unless otherwise noted. Poly(NIPA-Car) with lower molecular weight was synthesized in the presence of 0.30 mM mercaptoacetic acid, which terminates the polymerization as a polymer endgroup. M_w of the obtained poly(NIPA-Car) was 13,000. Polymer concentration was estimated on the basis of the polymer weight after evaporation of the dialyzed polymer solutions. The proportion of monomer units in a copolymer was assumed to be equal to the concentration ratio of the starting materials ([NIPA]:[inhibitor]=95:5). Quantitative analysis of the proportion was difficult due to much lower concentration of the imidazole group in comparison with that of NIPA.

2.2. Electrochemical measurements

The working electrode was prepared as follows. An indium–tin oxide (ITO)-coated glass plate (area ~0.25 cm^2) treated with a 1.0 M sodium hydroxide aqueous solution was immersed in a 2.5% acetic acid aqueous solution containing 2.5% 3-aminopropyltriethoxysilane and then treated with a 2.5% glutaraldehyde aqueous solution for 12 h. After each treatment, the electrode was thoroughly rinsed with distilled water. The electrode was then treated with 1.0 mM HP (Sigma) aqueous solution and stored at 4 °C. HP should have been immobilized onto the electrode surface via the amino group of lysine or valine of HP.

The HP activity was measured by means of amperometry. The measurement was performed in a batch system. The temperature of a 1/15-M pH 7.4 phosphate buffer solution as the electrolyte was controlled with a thermostat. Reference and counter electrodes were a Ag/AgCl/KCl (sat.) and platinum wire, respectively. The electrode potential was regulated with a potentiostat LC-4C (BAS). After the working electrode was polarized at +150 mV, H_2O_2 solution was added in the electrolyte (final concentration, 10 μM), followed by the addition of a polymer. Inhibition ratios of the HP activity were evaluated from decreases in the H_2O_2 reduction current.

2.3. Spectroscopic measurements

In order to evaluate the formation constant K of HP with inhibitors (including the polymers), absorption spectra of 2.0 μM HP were collected in a stirred pH 7.4 phosphate buffer containing various concentrations of inhibitors with a spectrophotometer MCPD-3000 and MC2530 (Otsuka Electronics). The light-path length was 1 cm. For comparison, monomer solutions before polymerization were also examined.

3. Results and discussion

3.1. LCSTs of the polymers

Poly(NIPA) dissolved in water at a temperature below LCST precipitates immediately when the temperature is raised across LCST ([25–28]. In order to estimate the LCSTs of poly(NIPA-VI) and poly(NIPA-Car) of $2000<M_w<50{,}000$, the transmittance changes of the solutions were monitored at 500 nm during temperature scan from 25 to 42 °C. As a result, the transmittances of poly(NIPA-VI) and poly(NIPA-Car) rapidly declined at around 36–38 °C (Fig. 2), reflecting polymer precipitation.

3.2. Effect of the polymers on the response of the HP-modified electrode

The HP-modified electrode was polarized at +150 mV (vs. Ag/AgCl) in pH 7.4 phosphate buffer, and H_2O_2 was added to the solution after the current was stabilized. A cathodic current observed in the presence of 10 μM H_2O_2 was approximately 2.0 nA cm^{-2}. The current is due to the electron transfer from the ITO electrode to HP [21]. This reaction mechanism is as follows:

$$\text{ferric HP} + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

$$\text{compound I} + H^+ + e^- \rightarrow \text{compound II} \quad (2)$$

$$\text{compound II} + H^+ + e^- \rightarrow \text{ferric HP} + H_2O \quad (3)$$

when an inhibitor such as cyanide or imidazole exists in the reaction system at a sufficiently high concentration, the

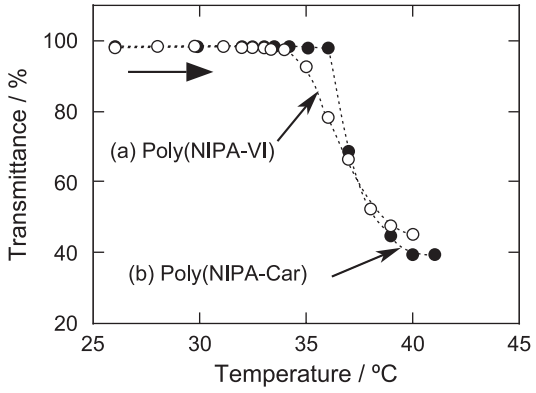

Fig. 2. Transmittance changes (500 nm) of (a) poly(NIPA-VI) and (b) poly(NIPA-Car) solutions during temperature scan (4 °C min^{-1}). The concentration of monomer unit was approximately 5 mM.

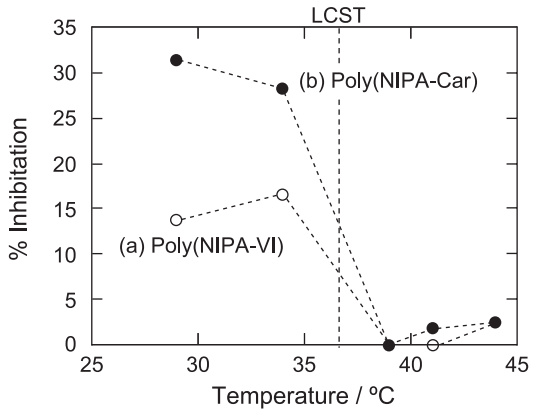

Fig. 3. Temperature dependencies of the inhibition effects of (a) poly(NIPA-VI) and (b) poly(NIPA-Car) on amperometric responses of the HP-modified electrode to 10 μM H_2O_2 at +150 mV vs. Ag/AgCl. Estimated concentrations of the imidazole groups were 3.0 μM (see Experimental).

reaction described above does not occur because the inhibitor coordinates to the sixth coordination site of ferric HP, which is the reaction site for H_2O_2 [22]. Therefore, the catalytic current decreases in the presence of an inhibitor. The %inhibition value was defined as follows:

$$\%Inhibition = (1 - i/i_0) \times 100 \quad (4)$$

where i_0 and i represent the current responses to H_2O_2 before and after the addition of the inhibitor, respectively.

Fig. 3 shows the %inhibition values of the HP-modified electrode in the presence of poly(NIPA-VI) or poly(NIPA-Car) at various temperatures. Concentration of imidazole groups was estimated to be 3.0 μM (see Experimental). The activity was inhibited at temperatures below LCST and was not inhibited at temperatures above LCST.

Since poly(NIPA) (M_w~37,000, LCST~31 °C) did not inhibited the HP-modified electrode at 29 and 41 °C, we can ascribe the inhibition observed for poly(NIPA-Car) and poly(NIPA-VI) to their imidazole groups. In addition, although VI and Car monomers inhibited the catalytic activity of HP, the inhibition was almost independent of the temperature in the range examined (29–41 °C). Therefore, the critical changes in the %inhibition values for the poly(NIPA-VI) and poly(NIPA-Car) at around their LCSTs should be explained in terms of the dissolution–precipitation processes of the polymers. Imidazole groups of the dissolved polymer can coordinate to HP, while those of the precipitated polymer cannot even approach HP. It has been known that Soret band of HP is red-shifted due to the coordination of imidazole derivatives to its heme [22]. Since Soret band of HP was red-shifted in the presence of the dissolved polymer, the inhibition should be ascribed to the coordination of the polymer to HP. This is mentioned in further detail in Section 3.5. Although we have reported the behavior of poly(NIPA-VI) in our preliminary paper [20], here, we found that poly(NIPA-Car) also exhibited similar behavior.

3.3. Effect of side chain length on the inhibition

Dependencies of the %inhibition values of the HP-modified electrode on the concentrations of the polymers were investigated at 29 and 41 °C (Fig. 4). In the concentration range examined, poly(NIPA-Car) exhibited stronger inhibition effect than did poly(NIPA-VI) at 29 °C, where the polymers were dissolved. In the case of poly(NIPA-VI), the isopropyl groups may interfere with the coordination of the imidazole group to HP, due to steric hindrance (Fig. 1b). On the other hand, since the imidazole group of poly(NIPA-Car) has a longer side chain (Fig. 1c), it may not readily suffer from the steric hindrance. Greater degree of freedom of Car, owing to its longer side chain, should also be advantageous for the coordination to HP.

For comparison, we also examined the inhibition effect of Car monomer (Fig. 4). However, the effect of the monomer was weaker than that of the polymer. This result suggests that we underestimate the amounts of imidazole groups in the polymer (i.e., imidazole groups are concentrated in comparison with NIPA during the polymerization and dialysis) or that coordination ability of the imidazole groups is enhanced by the polymerization. This will be discussed further below. On the other hand, the inhibition

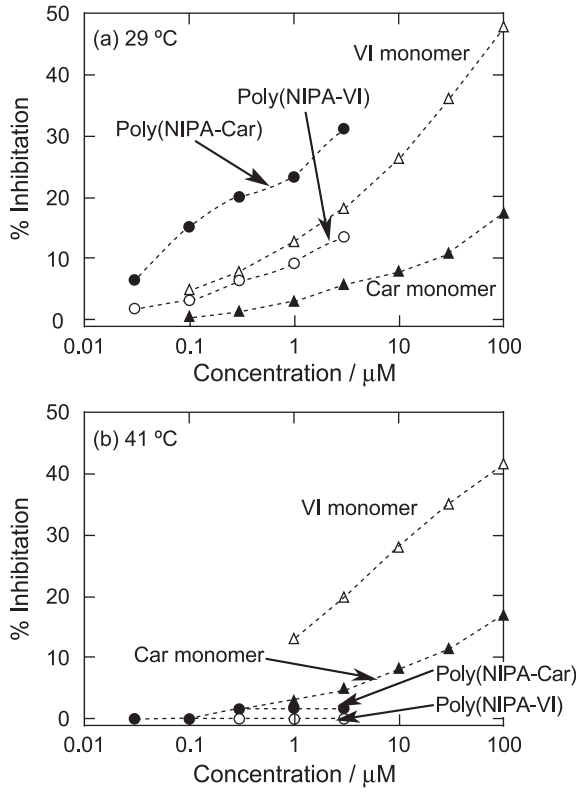

Fig. 4. Concentration dependencies of the inhibition effects of poly(NIPA-VI), poly(NIPA-Car), VI monomer and Car monomer on amperometric responses of the HP-modified electrode to 10 μM H_2O_2 at (a) 29 and (b) 41 °C. The concentrations are those for imidazole groups (see Experimental). Potential of +150 mV vs. Ag/AgCl was applied to the electrode in pH 7.4 phosphate buffer.

effect of VI monomer was almost the same as that of poly(NIPA-VI) (Fig. 4). The apparently smaller enhancement of the coordination ability by polymerization of VI in comparison with Car should be explained again in terms of the greater steric hindrance due to the shorter side chain.

3.4. Effect of main chain length on the inhibition

Next, we examined influence of main chain length on the inhibition for the HP-modified electrode. We anticipate that a polymer with a longer main chain exhibits greater steric hindrance as well as higher adsorptivity on the electrode surface. In this study, poly(NIPA-Car) (M_w~13,000 or 56,000) was used. LCSTs of the shorter and larger polymers were 37 and 35 °C, respectively. This difference is reasonable because smaller molecules are more soluble in general. However, no significant difference in the inhibition effect was observed at 29 °C, where both of the polymers were soluble.

3.5. Formation constants of the HP-inhibitor complexes

From the results of electrochemical measurements, we found the possibility that the inhibition effect of Car is enhanced by copolymerization with NIPA. In order to confirm this, formation constants of dissolved HP-inhibitor complexes were examined in pH 7.4 phosphate buffer by means of visible spectroscopy.

It is known that the Soret band of heme, the active center of HP, is red-shifted when an inhibitor is coordinated to the heme. Formation constant K can be estimated from the following equation:

$$\log[(A - A_E)(A_{EI} - A)] = \log K + \log[I]$$

where A is the absorbance at 399.0 nm of a mixed solution at the inhibitor concentration of $[I]$. A_E and A_{EI} are the absorbance for free HP ($[I]$=0) and that for fully complexed HP (sufficiently high $[I]$), respectively. This equation is suitable for analysis in the wide range of the inhibitor concentration.

At first, it was examined whether poly(NIPA) coordinates to HP or not. Although the concentration of poly (NIPA) was sufficiently increased, the peak of the Soret band was not red-shifted; poly(NIPA) does not coordinate to HP. NIPA (1.3 M) monomer also showed no evidence of the coordination.

Next, poly(NIPA-Car) and Car monomer were examined. Poly(NIPA-Car) was used without dialysis to not overestimate the amount of imidazole groups (i.e., Car side chain and Car monomer) in the solution. For comparison, a solution containing the monomers (NIPA, NASI and Car, mole ratio=95:5:10) before polymerization as well as a Car monomer solution (without NIPA and NASI) was also examined.

As to all the three solutions examined [(a) the poly (NIPA-Car) solution, (b) the mixed monomer solution and (c) the Car monomer solution], $\log[(A-A_E)/(A_{EI}-A)]$ vs. $\log[I]$ plots showed good linearity with a slope of 1.0. Therefore, HP and Car may form a 1:1 complex. On the basis of the plots and Eq. (5), K values were estimated to be (a) 9000±1000, (b) 50,000±10,000 and (c) 6000±1000 (mean±standard error, n=3), respectively. Thus, we can conclude that NIPA monomer does not coordinate to HP by itself, but it promotes the coordination of the Car to HP. There is a possibility that the NIPA monomer unit in poly(NIPA-Car) also has the same effect. Although hydrophobic interaction and/or hydrogen bonding between NIPA (and/or NASI) and HP or Car may be responsible for the promotion; detailed mechanism has not yet been revealed. However, these results imply that inhibition effects could be enhanced further by modifying the polymer with appropriate substituents. As described above, both the inhibitory effect and the coordination effect of Car are enhanced by copolymerization with NIPA. However, the increment of the %inhibition value is much larger than that of the K value. This difference might be explained in terms of the concentration of the polymer in the vicinity of the electrode surface due to the interaction between the immobilized HP and the polymer, resulting in the apparent increase in the coordination and inhibition effects in comparison with the homogeneous systems, in which K values were evaluated.

In the meantime, similar results were obtained when Car and NASI were replaced with VI, although the difference in K value between poly(NIPA-VI) (5000±1000) and VI monomer (4000±1000) was much smaller. Much greater steric hindrance described above might have canceled the enhancement in coordination ability.

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References

[1] L.C. Dong, A.S. Hoffman, Thermally reversible hydrogels: III. Immobilization of enzymes for feedback reaction control, J. Control. Release 4 (1986) 223–227.
[2] T. Shiroya, M. Yasui, K. Fujimoto, H. Kawaguchi, Control of enzymatic activity using thermosensitive polymers, Colloids Surf., B Biointerfaces 4 (1995) 275–285.
[3] T. Shiroya, M. Yasui, K. Fujimoto, H. Kawaguchi, Activity of enzyme immobilized on microspheres with thermosensitive hairs, Colloids Surf., B Biointerfaces 8 (1997) 311–319.
[4] P.S. Stayton, T. Shimoboji, C. Long, A. Chilkoti, J.M. Harris, A.S. Hoffman, Control of protein–ligand recognition using a stimuli-responsive polymer, Nature 378 (1995) 472–474.

[5] J. Bieth, S.M. Vratsanos, N. Wassermann, B.F. Erlanger, Photoregulation of biological activity by photochromic reagents: II. Inhibition of acetylcholinesterase, Biochemistry 64 (1969) 1103–1106.
[6] P.R. Westmark, J.P. Kelly, B.D. Smith, Photoregulation of enzyme activity. Photochromic, transition-state-analogue inhibitors of cysteine and serine proteases, J. Am. Chem. Soc. 115 (1993) 3416–3419.
[7] C. Amato, A. Fissi, L. Vaccari, E. Balestreri, O. Pieroni, R. Felicioli, Modulation of a proteolytic enzyme activity by means of a photocromic inhibitor, J. Photochem. Photobiol., B Biol. 28 (1995) 71–75.
[8] I. Willner, S. Rubin, Control of the structure and functions of biomaterials by light, Angew. Chem., Int. Ed. Engl. 35 (1996) 367–385.
[9] I. Willner, B. Willner, Photoswitchable biomaterials as grounds for optobioelectronic devices, Bioelectrochem. Bioenerg. 42 (1997) 43–57.
[10] T. Shimoboji, E. Larenas, T. Fowler, S. Kulkarni, A.S. Hoffman, P.S. Stayton, Photoresponsive polymer–enzyme switches, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 16592–16596.
[11] K. Komori, K. Yatagai, T. Tatsuma, Activity regulation of tyrosinase by using photoisomerizable inhibitors, J. Biotechnol. 108 (2004) 11–16.
[12] K. Ishihara, M. Kobayashi, N. Ishimaru, I. Shinohara, Glucose induced permeation control of insulin through a complex membrane consisting of immobilized glucose oxidase and a poly(amine), Polym. J. 16 (1984) 625–631.
[13] E. Kokufuta, T. Sodeyama, T. Katano, Initiation–cessation control of an enzyme reaction using pH-sensitive poly(styrene) microcapsules with a surface-coating of poly(iminoethylene), J. Chem. Soc., Chem. Commun. 9 (1986) 641–642.
[14] E. Kokufuta, Y.Q. Zhang, T. Tanaka, Biochemo-mechanical function of urease-loaded gels, J. Biomater. Sci., Polym. Ed. 6 (1994) 35–40.
[15] M. Kubo, I. Karube, S. Suzuki, Electric field control of enzyme membrane activity, Biochem. Biophys. Res. Commun. 69 (1976) 731–736.
[16] R. Hirsch, E. Katz, I. Willner, Magneto-switchable bioelectrocatalysis, J. Am. Chem. Soc. 122 (2000) 12053–12054.
[17] T. Tatsuma, Y. Watanabe, N. Oyama, Controllable electrocatalytic activity of a hemin-containing phase transition gel, Electrochem. Solid-State Lett. 1 (1998) 136–138.
[18] T. Tatsuma, H. Mori, A. Fujishima, Selective control of imidazole derivatives of interference-based biosensors by use of a phase transition gel, Chem. Commun. (1999) 2395–2396.
[19] T. Tatsuma, Y. Fujimoto, N. Oyama, Controllable electrocatalytic activity of heme peptide in a phase transition gel, Electrochem. Solid-State Lett. 3 (2000) 283–285.
[20] T. Tatsuma, H. Mori, H. Takahashi, A. Fujishima, Activity control of a heme peptide modified electrode by an inhibitor bound to a thermoresponsive polymer, Electrochem. Solid-State Lett. 4 (2001) E5–E7.
[21] T. Tatsuma, T. Watanabe, Peroxidase model electrodes: heme peptide modified electrodes as reagentless sensors for hydrogen peroxide, Anal. Chem. 63 (1991) 1580–1585.
[22] T. Tatsuma, T. Watanabe, Peroxidase model electrodes: sensing of imidazole derivatives with heme peptide-modified electrodes, Anal. Chem. 64 (1992) 143–147.
[23] T. Tatsuma, K. Okamura, K. Komori, A. Fujishima, Interference-based electrochemical biosensor for the measurement of the concentration and isomer ratio of urocanic acid, Anal. Chem. 74 (2002) 5154–5156.
[24] T. Tatsuma, N. Oyama, H_2O_2-generating peroxidase electrodes as reagentless cyanide sensors, Anal. Chem. 68 (1996) 1612–1615.
[25] H. Heskins, J.E. Gullet, Solution properties of poly(*N*-isopropylacrylamide), J. Macromol. Sci., Chem., A 2 (1968) 1441–1455.
[26] L.D. Taylor, L.D. Cerankowski, Preparation of films exhibiting a balanced temperature-dependence to permeation by aqueous-solutions—study of consolute behavior, J. Polym. Sci., Polym. Chem. 13 (1975) 2551–2570.
[27] H.G. Schild, Poly(*N*-isopropylacrylamide): experiment, theory and application, Prog. Polym. Sci. 17 (1992) 163–249.
[28] H. Feil, Y.H. Bae, J. Feijen, S.W. Kim, Effect of comonomer hydrophilicity and ionization on the lower critical solution temperature of *N*-isopropylacrylamide copolymers, Macromolecules 26 (1993) 2496–2500.