An Amperometric Sensor Based on Gold Electrode Modified by Soluble Molecularly Imprinted Catalyst for Fructosyl Valine

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ABSTRACT

An amperometric sensor based on a soluble molecularly imprinted catalyst (MIC) has been developed for the detection of fructosyl amine compounds. A soluble MIC containing water-soluble functional monomers, an imidazole catalyst, and small amounts of a hydrophilic cross-linker is developed and used as a fructosyl amine oxidase mimic and for amperometric sensor construction. Fructosyl valine (Fru-val), a model compound of glycated hemoglobin, HbA1c, is used as the template. The MIC specifically oxidizes Fru-val in the presence of 1-methoxyphenazine methosulfate (electron acceptor) and reacts with the glycated peptide, fructosyl-valine-histidine sequence at the N-terminal of the β-globin in HbA1c. The biosensor was fabricated by immobilizing the soluble MIC on Au electrodes via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-mediated amidation coupling. Using the soluble MIC-based sensor, 0.05 to 0.6 mM Fru-val could be determined at 40°C and neutral pH. The steady-state current increase for this sensor was 33 nA in the presence of 0.05 mM Fru-val. The sensor showed 1.4 times higher sensitivity to Fru-val than to Fru-ε-lys, the competitor in HbA1c detection.

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1. Introduction

Molecular imprinting technology (MIT) is a versatile tool for the preparation of synthetic materials that are capable of specific recognition and binding to target molecules. By MIT, specific binding sites for target molecules can be easily tailored in a polymer network. One of the most challenging applications of MIT is the development of enzyme mimics, which are also known as artificial enzymes. An enzyme mimic that shows high substrate specificity can be prepared by combining a molecularly imprinted polymer that specifically binds to a target with a catalyst. While several artificial enzymes have been developed by MIT and used as catalysts and sensor elements, most of them are highly cross-linked rigid polymers with low flexibility, as a result, their activity is much lower than that of natural enzymes. Developing flexible and conformationally adaptable MIT-based artificial enzymes is a challenging task but is considered the possible solution to the aforementioned problem. Several research groups have been involved in the preparation of molecularly imprinted microgels and nanogels, which can be homogeneously dissolved in aqueous solvents. Resmini et al. reported the first synthesis of molecularly imprinted soluble acrylamide-based microgels having arginine and tyrosine, which act as special functional monomers, in their side chains and studied the catalytic activity of these microgels for the hydrolysis of carbonates. Recently, Diaz-Diaz et al. reported a molecularly imprinted microgel based on 2,4,6-trichlorophenol and its application in electrochemical sensing.

Glycated hemoglobin (HbA1c) has been identified as the most important indicator of glycemia control. Since the amount of glycated protein in serum depends on the glucose concentration in the serum and the lifetime of the protein, the relative proportions of glycated and non-glycated proteins is a good and reliable indicator of glycemic control in diabetic patients. We reported an MIT-based artificial dehydrogenase for fructosyl valine (Fru-val), a model compound of HbA1C. We first demonstrated that 1-vinylimidazole acts as a catalyst for the oxidation of Fru-val and then prepared a molecularly imprinted catalyst (MIC) by imprinting the co-polymer of 1-vinylimidazole and 4-vinylphenylboronate using Fru-val as the template. The MIC showed high affinity to the template molecule, Fru-val; further, the rate of oxidation of Fru-val was higher when using this MIC than when using a non-imprinted catalyst in the presence of an electron acceptor, indicating that the MIC acts as an artificial enzymatic catalyst. We constructed an amperometric sensor based on a carbon-paste electrode modified with the aforementioned MIC and used it for measured Fru-val detection. We also optimized the operation conditions for the MIC-based sensor in order to increase its reactivity and specificity to Fru-val. However, the synthesized MIC was a rigid bulk polymer with low flexibility, and most of the active imidazole catalyst sites were located in the inside of the polymer, and not on the surface. As a result, the Fru-val oxidation activity of the MIC was unsatisfactory for detecting the product formed from the proteolytic digestion of HbA1C in blood.

In this study, the Fru-val oxidation activity of MIC is shown to improve when the flexibility of the polymer chains is increased. Furthermore, the ability of the soluble-MIC-based amperometric sensor to detect Fru-val with higher sensitivity than that of a bulk MIC-based sensor is demonstrated. To improve the catalytic activity of the MIC, an acrylamide-based soluble MIC having water-soluble functional monomers is employed; very low concentrations of cross linkers have been used in order to ensure high flexibility of the MIC. Since a polyacrylamide gel with a low degree of cross-linking would be soft with a macroporous structure, the synthesized polymer would be conformationally flexible, and show high aqueous solubility. N-methacryloyl-L-histidine, which is soluble in aqueous solvents, is used as the catalyst for the MIC preparation. The high activity of the soluble MIC for fructosyl amine oxidation is assessed by using a homogeneous assay system. Then, the performance of an amperometric sensor based on the soluble MIC is evaluated.
2. Experimental

2.1 Chemicals

Fructosyl amine compounds were synthesized as per a previously reported method.27,28 1-Methoxyphenazine methosulfate (m-PMS) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Acrylamide, 4-vinylphenylboronate, 3,3′-dithiodipropionic acid, N,N′-methylene-bis-acrylamide, N,N,N′,N′-tetrakis(ethyl)-hydroxysuccinimide (TEMED), and ammonium persulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-Hydroxysuccinimide (NHS) was purchased from Wako Pure Chemicals (Osaka, Japan). Phannazine methosulfate (PMS), 1-ethyl-3-(3-dimethylaminopropyl)-NHS was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acrylamide, 4-vinylphenylboronate, 3,3′-dithiodipropionic acid, N,N′-methylene-bis-acrylamide, N,N′,N′-tetrakis(ethyl)-hydroxysuccinimide (TEMED), and ammonium persulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

We first investigate the feasibility of using the MIC as the catalyst for the oxidation of fructosyl amine compounds. In the presence of the electron acceptor PMS and the imidazole-based catalyst, the fructosyl amine compound is oxidized; subsequent electron transfer to DCIP results in the decolorization of DCIP [Fig. 2(a)]. Three fructosyl amine compounds are used as substrates. Fru-val has been used as a model compound of HbA1C. The μ-terminal region of the β-globin in HbA1C is typically characterized by the fructosyl-valine-histidine (Fru-val-his) sequence. Fructosyl-s-lysine (Fru-s-lysine) was used as a model substrate. The electrochemical oxidation of fructosyl amine compounds was monitored in a 9:1 mixture of 100 mM PBS and 100 mM DCIP, pH 7.0, at room temperature under continuous stirring. The oxidation of the MIC and NIC was carried out for 5 min and washed with sterilized water. This process was repeated two times. Then, the wires were immersed for 30 min in 3,3′-dithiodipropionic acid (2 mM, 1 mM) at room temperature with continuous stirring and washed with sterilized water. The Au wires were immersed in 2 mL of a solution containing 100 mg NADH and 100 mg EDC for 20 min at room temperature under continuous stirring and washed with HEPES buffer (2 mL, 1 mM, pH 8.5). These Au wires, in which succinimide groups were introduced, were immersed for 2 d at room temperature in 200 µL of a 9:1 mixture of PPB (10 mM, pH 7.0) and DMSO containing 2.0 mg of the soluble polymer under continuous stirring. For blocking the free succinimide groups, the Au wires were immersed in Tris-HCl buffer (2 mL, 1 M, pH 8.0) for 1 h at room temperature under continuous stirring.

2.6 Operation of amperometric sensor

Amperometric measurements were carried out using a potentialstat HA-151 (Hokutomo Denko, Tokyo, Japan) with a three-electrode system. The working electrode, reference electrode (Ag/AgCl electrode, Bioanalytical Systems), and platinum counter electrode (diameter: 0.5 mm; Tanaka Noble Metal, Tokyo, Japan) were inserted into the cell via holes on the Teflon cover. All measurements were carried out at 4°C in 10 mL of a 9:1 mixture of PPB (10 mM, pH 7.0) and DMSO containing 1 mM m-PMS (electron acceptor), under stirring at 250 rpm. The applied anodic potential for the oxidation of m-PMS was +150 mV versus a Ag/AgCl (3.0 M NaCl) electrode.

3. Results and Discussion

3.1 Polymer preparation

Figure 1 shows a schematic of the preparation of soluble microgel MICS using MA histidine as the catalyst. In this reaction, MA histidine, 4-vinylphenylboronate, and acrylamide acted as functional monomers. Histidine derivatives and imidazole compounds such as 1-vinylimidazole11,12 and 4(5)-vinylimidazole,35–36 have frequently been used as histidine residue mimics and model catalytic centers for the development of artificial enzymes, because the histidine residue acts as a nucleophile catalytic center and/or a general base catalyst for enzyme catalysis. In this study, MA histidine was used as the catalyst because it had superior solubility in aqueous solutions than did 1-vinylimidazole, the previously used catalyst for the rigid MIC synthesis. Since the boronate group forms covalent bonds with the cis diol of the target molecule, 4-vinylphenylboronate was used to increase the affinity of the MIC to fructosyl amine compounds, and acrylamide was added to make the polymer chain hydrophilic. Because of the use of low concentrations of the cross-linker N,N′-methylene-bis-acrylamide, the polymer remained dissolved in the solvent even after the polymerization reaction. The polymer solution was dialyzed to remove the template and unpolymerized monomer and then lyophilized. The synthesized MIC and NIC were soluble in a 9:1 mixture of PPB (10 mM, pH 7.5) and DMSO and afforded a homogeneous soluble microgel.
lys) is the model compound of glycated albumin. For highly specific fructosyl amine detection, the sensor must be capable of discriminating Fru-val from Fru-X-lys. The existing biochemical methods for HbA1c measurement require proteolytic digestion of HbA1c, so that the resulting low-molecular-weight fructosyl amine compounds can be measured by the enzyme fructosyl amine oxidase.\textsuperscript{37-39} In this process, however, the proteolysis products of glycated albumin containing Fru-X-lys are also generated. Therefore, an acceptable catalytic component of an HbA1c diagnostic kit should be selective to Fru-val in the presence of Fru-X-lys. Figure 3 shows the effect of the polymer concentration on the rates of oxidation of Fru-val and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Preparation of acrylamide-based soluble MIC using MA histidine as a catalyst.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Colorimetric methods (a) and amperometric methods (b) for the detection of fructosyl amine compound using soluble MIC.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Fru-val (a) and Fru-X-lys (b) oxidation catalyzed by MIC (filled marks) and NIC (open marks).}
\end{figure}
Fru-ε-lys. Fructosyl amine compounds are oxidized spontaneously in the presence of 1 mM PMS in the buffer. The spontaneous-oxidation rates of Fru-val and Fru-ε-lys are 0.63 ± 0.13 and 0.98 ± 0.04 nmol/min, respectively. The rate of Fru-val oxidation increases with the amount of MIC in the reaction solution. On the other hand, the rate of Fru-ε-lys oxidation does not increase when the amount of the polymer is increased. Because of the spontaneous oxidation of Fru-ε-lys in the buffer, the role of the MIC in the oxidation is insignificant. The NIC oxidizes Fru-val and Fru-ε-lys to the same extent as that in the spontaneous reaction. These results indicate that because of molecular imprinting, the MIC recognizes and specifically oxidizes Fru-val, thus showing high selectivity toward Fru-val. On the basis of the result, it is proposed that molecular imprinting of Fru-val using 4-phenylvinyl boronate and MA histidine as the functional monomers may result in the formation of a cavity that recognizes the cis diol on the basis of covalent bond formation between the cis diol and the boronate, and the imidazole units act as cleavage catalyst sites. Table 1 shows the relative rates of oxidation of Fru-val-his induced by the MIC, NIC, and spontaneous reaction. The Fru-val-imprinted MIC shows higher oxidation activity than does NIC. Although the rate of MIC-induced oxidation to fructosyl dipeptide (V_{MIC}) was lower than the spontaneous oxidation rate (V_{buffer}), increasing the amount of polymer in assay solution might enable to increase V_{MIC} to fructosyl dipeptide. The MIC can be utilized for fructosyl peptide detection.

3.3. Electrochemical detection

One of the advantages of a soluble MIC is that a homogeneous solution of the polymer can be obtained, so that the MIC can be treated as a natural enzyme. When an amino group is incorporated in the polymer chain of the soluble MIC, the catalyst can be immobilized on various material surfaces by the standard immobilization methods used for natural proteins. In this study, an amperometric sensor was developed for fructosyl amine compounds by using the MIC prepared in the presence of Fru-val. The MIC was immobilized on a Au electrode by EDC-mediated amidation coupling. Control experiments were carried out on a bare Au electrode. Measurements were based on the re-oxidation of the reduced m-PMS, which was formed during the oxidation of the fructosyl amine compounds, as shown in Fig. 2(b). The measurements were carried out in a 9:1 mixture of PPB (10 mM, pH 7.0) and DMSO at 40°C. After the injection of Fru-val or Fru-ε-lys, the steady-state current increased immediately. Calibration curves were obtained by plotting the steady-state current versus the concentration of the injected sample. Figure 4 shows the calibration curves for Fru-val and Fru-ε-lys compounds when using the MIC- and NIC-based electrodes. The MIP-based Au electrode showed 1.4-fold higher current response to Fru-val than to Fru-ε-lys. The NIP-based Au electrode and bare Au electrode showed almost the same response to Fru-val and Fru-ε-lys. These results indicated that the template Fru-val orient itself in the optimal position with respect to the functional monomers. The selectivity of the polymer catalyst, therefore, could be improved by molecular imprinting. In our previous study, the selectivity of the bulk MIP-based sensor to Fru-val against Fru-ε-lys increased from 1.9 to 5.7 when the ion strength of the assay buffer solution was increased from 10 to 100 mM. The hydrophobic interaction between the valine residue of Fru-val and the polymer became stronger with the ionic strength of the buffer solution. Therefore, the selectivity of the soluble MIC-based sensor is expected to increase when using a high-ionic-strength buffer. The increase in the steady-state current for the soluble MIC-based sensor in the presence of 0.4 mM Fru-val is more than 180 nA, while that for the rigid bulk MIC-based sensor that we previously constructed is 60 nA in 10 mM PPB (pH 7.5). Thus, in the present study, we have successfully improved the catalytic activity of MIC by increasing the flexibility of the polymer chains. Using the soluble-MIC-based sensor, 0.05 to 0.6 mM Fru-val can be detected. Considering that the physiological HbA1c level in blood is 4 to 15% of the total hemoglobin level (8–20 g dL⁻¹), the corresponding fructosyl valine concentration is estimated to range from 0.05 to 0.5 mM. Thus, our soluble-MIC-based sensor fulfills the required detection range for HbA1c measurement.

4. Conclusion

We developed an acrylamide-based soluble MIC using MA histidine as the catalyst. The MIC could be converted into a homogeneous soluble microgel and used as an enzyme mimic catalyst for fructosyl amine oxidation. We also fabricated an amperometric biosensor for Fru-val determination by immobilization of the soluble MIC onto an Au electrode via EDC-mediated amidation coupling. The amperometric MIC-based sensor showed higher sensitivity to Fru-val than to Fru-ε-lys as well as high flexibility and activity for Fru-val oxidation.

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References