A Molecularly Imprinted Polymer-modified Potentiometric Sensor for the Detection of Glutathione

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Potentiometric glutathione (GSH) sensors were fabricated using a molecularly imprinted polymer prepared from GSH, methacrylic acid (MAA), and ethylene glycol dimethacrylate as the template molecule, functional monomer, and cross-linker, respectively. Five GSH sensors were prepared with different ratios of GSH to MAA. Their potential responses were measured in a GSH aqueous solution using Ag/AgCl as the reference electrode. A GSH sensor prepared with a GSH:MAA ratio of 2:32 had the best responsivity, while the sensor synthesized from a non-imprinted polymer prepared without GSH (NIP sensor) showed a potential response value of almost zero after the addition of GSH. The ratio of the potential responses of the GSH sensor to the NIP sensor was 8.21. Additionally, the GSH sensor had good linearity over a GSH concentration range of $1 \times 10^{-5}$ to $2 \times 10^{-4}$ mol L$^{-1}$. The GSH sensor provided good quantification and high specificity for GSH and is expected to be applicable for easy and direct determinations of GSH.

Keywords Molecularly imprinted polymer, potentiometric sensor, glutathione, quantification, specificity, methacrylic acid, functional monomer ratio, non-imprinted polymer, polymerization initiator, plasma polymerized membrane

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Introduction

Chemosensors are devices that transduce the existence and concentrations of chemical substances and biological events into measurable signals. The equipment is simple because it consists of two parts: a sensing element that recognizes the analyte and a transducer that converts the signals. Additionally, chemosensors do not require a sample pretreatment or a skilled operator. Biosensors, the group of chemosensors that use an enzyme,1 antigen–antibody,2 or microorganism3 as the sensing element, have been developed for their advantages of high sensitivity and specificity. However, they employ biological molecules, have a limited shelf life and stability, are expensive, and are not robust under harsh conditions.

To overcome the drawbacks of biosensors, we focused on using a molecularly imprinted polymer (MIP) as the sensing element for a chemosensor. A MIP is a highly cross-linked polymer capable of selectively recognizing and binding target molecules with high affinity. MIPs are low cost and easy-to-prepare synthetic polymers and are typically manufactured by forming template molecule-functional monomer complexes, which are polymerized in the presence of a cross-linker prior to removal of the template molecule. Therefore, a MIP provides binding sites that are complementary in size, shape, and functionality to the template molecule and can be tailored for a wide range of molecules with various affinities and selectivities. Furthermore, MIPs are stable in aqueous solutions ranging from strongly acidic to basic and organic solvents, can be used repeatedly, and have long shelf lives. As a result of these characteristics, a MIP can be used as an alternative to a biological sensing element, and many examples exist where this approach has been successfully achieved, including the development of MIP-templated herbicides,4 peptides,5 proteins,6 and cells.7 Furthermore, MIPs have been used as the packing in liquid chromatography8 and solid-phase extraction columns.9–12 Additionally, some MIP-based surface plasmon resonance,13 voltammetric,14,15 and potentiometric sensors16,17 have been developed.

Glutathione (GSH), which consists of glycine (Gly), cysteine (Cys) and glutamic acid (Glu), is the most abundant thiol in mammalian cells (Fig. 1). GSH plays an important role as an antioxidant in physiological processes.18 For example, GSH is involved in protection against peroxide and reactive oxygen species and detoxification.19 GSH protect cells from peroxide and reactive oxygen by being oxidized from a reduced form to

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Fig. 1 Structure of glutathione.
oxidized glutathione disulfide. Therefore, the balance of the concentration of oxidized glutathione disulfide and GSH is considered to be an indicator of the oxidative stress in vivo.\textsuperscript{20} Furthermore, the concentration of GSH is associated with diseases and cancers, and cancer cells maintain GSH at high levels.\textsuperscript{21-23} Hence, the determination of GSH is significant. Various GSH determination methods have been developed, including GSH determination kits that are used to measure the absorbance, fluorescence, or luminescence of the product obtained from the oxidation-reduction of GSH. Dai et al. quantified GSH by using fluorescence enhancement or quenching of a directly bound fluorescence probe.\textsuperscript{24-26} Another laboratory has proposed a unique electrochemical sensing strategy.\textsuperscript{27} Taken together, these methods allow for the determination of GSH with high sensitivity and selectivity. However, they require expensive and large equipment to determine the GSH concentration. More importantly, they cannot directly determine the presence of GSH itself.

In this study, we aimed to develop a MIP-modified potentiometric sensor for direct and easy determination of GSH. A potentiometric sensor measures the change of the surface potential by binding GSH with a MIP; therefore, the GSH concentration can be directly monitored in real time. Furthermore, this type of sensor is not only easy to miniaturize, but it is also not expensive because its construction is simple. For the development of a MIP-modified potentiometric sensor for GSH with good responsivity and selectivity, first, the conditions for the fabrication of the MIP on the GSH sensor were examined, including the polymerization temperature and the ratios of the template molecule and functional monomer. Methacrylic acid (MAA) having a carboxyl group was selected as a functional monomer, because GSH also has two carboxylic groups in the chemical structure, and it is well-known that the molecules having the carboxylic groups easily interact via the hydrogen bond with each other. Next, the quantification and selectivity of the GSH sensor prepared using the most suitable preparation condition were investigated.

**Experimental**

**Materials**

Graphite rods were purchased from Strem Chemicals Inc. (Newburyport, MA). Toluene and L-glutamic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Glycine was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ethylbenzene was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Ethylene glycol dimethacrylate (EDMA) and poly(vinyl alcohol) (PVA) 1000 (partially hydrolyzed PVA) were purchased from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan) and used after the hydroquinone monomer was extracted with a 5% NaOH aqueous solution. Other reagents were purchased from Wako Pure Chemical Industries, Ltd.

**GSH sensor synthesis**

The GSH sensor was prepared following a previously reported procedure.\textsuperscript{28} A transducer graphite rod (diameter 3 mm, length 50 mm) was polished with sandpaper prior to being sonicated five times for 5 min in distilled water. A plasma polymerized thin film (PPTF) using ethylbenzene was deposited onto the surface of the polished graphite rod via a plasma deposition system (BP-1, Samco Inc. Kyoto, Japan). The first swelling step involved immersing the PPTF-coated graphite rod in a suspension of sodium dodecyl sulfate (0.55 mmol) as a surfactant, radical initiator (1.37 mmol), dibutyl phthalate (14.3 mmol) as a plasticizer, and distilled water (40 mL). The mixture was maintained for 24 h at room temperature. The second swelling step involved immersing the PPTF-coated graphite rod in a PVA solution containing GSH (2 mmol), appropriate amounts of MAA as a functional monomer, EDMA (25 mmol) as a cross-linker, and toluene (47 mmol). After 24 h of stirring at room temperature, the mixture was degassed using helium gas and polymerized by subjecting the system to a heat treatment stage for 12 h to form the MIP in PPTF. Thereafter, the PPTF-coated graphite rod was immersed in distilled water to remove GSH from the MIP. Finally, the MIP-containing graphite rod was employed as a GSH sensor electrode and stored in distilled water. The NIP sensor was prepared using the same procedure in the absence of GSH.

**Sensor measurements**

The GSH sensor (or NIP sensor) and an Ag/AgCl reference electrode were immersed in distilled water (100 mL) and the potential response of the sensor was measured at room temperature against the reference electrode by a potentiometer (pH meter F-52, Horiba Inc., Kyoto, Japan). When the potential response was stable, the recording was started. One minute after starting the measurement, 1 mL of each chemical substance stock solution of various concentrations was injected into the distilled water. The assembly of the potential cell was as follows: Ag/AgCl|3.33 mol L\textsuperscript{-1} KCl aqueous solution|chemical substance stock solution|MIP membrane|graphite. The potential response value at the start of recording was set to 0 mV, and the potential change was defined as the difference between the potential response values before and after adding of the stock solution.

**Results and Discussion**

**Selection of the radical initiator**

Radical initiator initiates polymerization by the decomposition to generate radicals. If the decomposition of the initiator is too fast compared to the propagation reaction rate, it decreases the efficiency of polymerization initiation. These decomposition rates of the initiators depend on each radical initiator and are affected by the temperature. To investigate the suitable polymerization temperature for fabricating the MIP in the PPTF on the graphite rod, two radical initiators, 2,2′-azobis(2,4-dimethylvaleronitrile) (V-65) and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) with polymerization temperatures of 70 – 75°C and 50 – 55°C, respectively, were chosen. Using the appropriate polymerization temperatures, two GSH sensors were prepared at a GSH:MAA:EDMA ratio of 2:16:25. The potential responses of the two GSH sensors were measured by the addition of 1 mL of a 10 mmol L\textsuperscript{-1} GSH aqueous solution, as shown in Fig. 2. Although the two GSH sensors responded to the GSH binding to each MIP after the addition of GSH, the GSH sensor initiated using V-65 showed a larger potential change to GSH than that prepared using V-70. The polymerization reaction initiated by V-65 may proceed easier without inhibiting the intermolecular interaction between GSH and MAA than that initiated by V-70 because V-65 formed an immobilized MIP on the graphite rod that was visually tighter than V-70. Therefore, it was likely that the formation of the GSH templates was insufficient in the MIP prepared by the polymerization using V-70. This result showed that the GSH sensor, and this polymerization condition was adopted for the subsequent studies.
MAA content optimization

The interaction between the functional groups of the template molecule and the functional monomer is one of the important factors governing the molecular recognition of a MIP. Therefore, the ratio of the amounts of the template molecule and functional monomer in the MIP preparation affects the molecular recognition performance of the sensor. In this study, GSH sensors were prepared by varying the amounts of MAA with 2 mmol GSH, and the responsivities of each GSH sensor to GSH were examined. The potential response curves are shown in Fig. 3(a), when 1 mL of 10 mmol L⁻¹ GSH aqueous solution were added, *i.e.*, final GSH concentration was $1 \times 10^{-4}$ mol L⁻¹. As shown in Fig. 3(a), every GSH sensor showed the potential change immediately after adding GSH, but the behavior of the potential response of each GSH sensor was different. In GSH sensors with a GSH:MAA ratio of 2:8, 2:16 and 2:32, their potential response was constant until 25 min. In particular, the GSH sensor with a GSH:MAA ratio of 2:32 showed the most stable and largest potential response value among all GSH sensors. In contrast, for the sensors with GSH:MAA ratios of 2:4 and 2:64, GSH was recognized after its addition but, thereafter, the potential response value gradually decreased. To examine whether the MIP of the GSH sensor can recognize GSH in a sample solution, the NIP sensor, which was prepared at a GSH:MAA ratio of 0:32 and did not have the GSH templated on the polymer, was measured; the result is also shown in Fig. 3(a). The behavior of the potential response of the NIP sensor after the addition of the GSH aqueous solution was similar to the response of the GSH sensors with GSH:MAA ratios of 2:4 and 2:64, *i.e.*, the potential response value increased, but thereafter gradually decreased. This result may be due to the fact that once GSH non-specifically binds to the polymer surface (the carboxyl group of MAA) of the NIP sensor, it gradually becomes free in the sample solution apart from the surface. The same phenomenon may occur in the GSH sensors with GSH:MAA ratios of 2:4 and 2:64. In the sensor with a GSH:MAA ratio of 2:4, the molecular recognition sites were not formed properly because the MAA amount was too low, while in the sensor with a GSH:MAA ratio of 2:64, an excessive amount of MAA resulted in the binding sites being buried inside the polymer. Therefore, when MAA is present at ratios of GSH:MAA of 2:4 and 2:64, it does not serve as an effective functional monomer. Li *et al.* have also reported similar results.²⁹ As the potential responses of the GSH sensors with GSH:MAA ratios of 2:8, 2:16 and 2:32 were stable, it is possible that in these three GSH sensors, GSH immediately, specifically, and strongly binds to the templates of the MIP surface. Furthermore, to compare the responsivity and repeatability of these GSH sensors, the potential change of each sensor at 25 min was read, as shown in Fig. 3(b). The GSH sensor with a GSH:MAA ratio of 2:32 showed the highest responsivity to GSH with good repeatability. From these results, although the amount of MAA was a little higher compared with the other reported determination methods using a MIP,⁸,₃₀ we concluded that a GSH:MAA ratio of 2:32 was suitable for preparing a GSH sensor with the best responsivity and repeatability among the other GSH:MAA ratios tested.

### Quantification of the GSH sensor

The quantification of the GSH sensor with a GSH:MAA ratio of 2:32 was determined over a GSH concentration range of $1 \times 10^{-6}$ to $5 \times 10^{-4}$ mol L⁻¹; the results are shown in Fig. 4. As shown in Fig. 4(a), the potential response value immediately increased after the addition of the GSH aqueous solution and became larger as the GSH concentration increased, except when the GSH concentration was $5 \times 10^{-4}$ mol L⁻¹. The calibration curve of the GSH concentration and the
external cells, respectively. Therefore, these results demonstrated
that the GSH sensor specifically recognized GSH and was
useful to quantify GSH. On the other hand, the error bar was
limited to measure analytes in distilled water.

Selectivity
To evaluate the selectivity of the GSH sensor, the potential response of the GSH sensor was measured for the three amino
acid building blocks of GSH, i.e., Gly, Cys, and Glu, and their
values at 25 min were compared with that of GSH. The final
centration of each substance was 1 × 10⁻⁴ mol L⁻¹. The result is shown in Table 1. The GSH sensor had very small
response to Gly and Cys, but showed the same potential value to
Glu compared with that for GSH. To examine whether the potential
to GSH and Glu was due to specific binding with the MIP surface of the GSH sensor, the potential response of the NIP sensor to these substances was also measured, and is
given in Table 1. For GSH, the potential change value of the
NIP sensor was significantly reduced compared with that of the
GSH sensor. In contrast, the NIP sensor showed different
to Glu, and tended to be slightly
For GSH, the ratio of the potential responses of the
GSH sensor to the NIP sensor shows the index of the specificity
for the GSH sensor to each substance. Therefore, the ratio of
the GSH sensor/NIP sensor was calculated and is also shown in
Table 1. For GSH, the ratio of the GSH sensor/NIP sensor was
significantly larger than those for the other substances. This
result clearly shows that the GSH sensor preferentially
recognizes the GSH that is specifically binding with the MIP on
the GSH sensor. Although the GSH sensor showed a large
potential response for Glu, the ratio of the GSH sensor response
/NIP sensor response for Glu was small. This means that the
large potential response of the GSH sensor for Glu was due to
Glu non-specifically binding to the MIP surface on the GSH
sensor. The isoelectric point (pI) values of GSH, Gly, Cys, and
Glu are 5.9, 6.0, 5.1, and 3.2, respectively. In distilled water
(at pH 5.5), GSH, Gly, and Cys, are in a zwitterion state and
have almost a net zero charge, whereas Glu is in a net negatively
charged state. Despite the fact that GSH and Glu are different
in their molecular states, the fact that the GSH sensor gave Glu
the same potential change value as GSH indicates that the two
carboxyl groups of GSH and Glu play an important role. MAA,
which is the functional monomer in the MIP, also has a carboxyl
group in its chemical structure. Therefore, the carboxylic
GSH and Glu may interact with that of MAA via a hydrogen
bond. However, the chemical state of the carboxyl groups in
GSH is different from that for Glu; i.e., one carboxyl group of
GSH is in a neutral state and one is in a dehydrogenated state,
while both carboxyl groups in Glu are in dehydrogenated states.

potential change at 25 min are illustrated in Fig. 4(b). Over a
GSH concentration range from 1 × 10⁻³ to 2 × 10⁻⁴ mol L⁻¹, a
linearity of r = 0.88 was obtained. This result indicated that our
GSH sensor reflects the amount of GSH binding to the template
on the MIP surface as the potential change. Over this linear
range, the GSH sensor may be insufficient in terms of its
sensitivity and dynamic range compared with other methods.
However, the GSH sensor is sufficiently sensitive to allow for a
quantitative determination of the GSH because its concentration
is on the order of mmol L⁻¹ and μmol L⁻¹ in the internal⁴⁺ and
external⁴⁻ cells, respectively. Therefore, these results demonstrated
that the GSH sensor specifically recognized GSH and was
useful to quantify GSH. On the other hand, the error bar was
significantly large. This may be mainly due to the pH change in
the sample solution. If the pH value changes, the molecular
states of GSH are changed, and the amount of GSH binding to
the GSH sensor is also changed. As the distilled water was used
as a solvent in this study, the pH value of the sample solution
after the addition of GSH aqueous solution may change at not
only each GSH concentration, but also the same one. In general,
some suitable buffer, e.g., phosphate buffer or Good’s buffer,
should be used to maintain the pH value of the sample solution.
However, in our preliminary study, this GSH sensor was affected
by inorganic ions in the buffer solution, and showed a reduction
of the potential response. Therefore, the GSH sensor is currently
limited to measure analytes in distilled water.

Selectivity
To evaluate the selectivity of the GSH sensor, the potential response of the GSH sensor was measured for the three amino
acid building blocks of GSH, i.e., Gly, Cys, and Glu, and their
values at 25 min were compared with that of GSH. The final
centration of each substance was 1 × 10⁻⁴ mol L⁻¹. The result is shown in Table 1. The GSH sensor had very small
response to Gly and Cys, but showed the same potential value to
Glu compared with that for GSH. To examine whether the potential
to GSH and Glu was due to specific binding with the MIP surface of the GSH sensor, the potential response of the NIP sensor to these substances was also measured, and is
given in Table 1. For GSH, the potential change value of the
NIP sensor was significantly reduced compared with that of the
GSH sensor. In contrast, the NIP sensor showed different
potential response behaviors to other substances; i.e., the
potential response of the NIP sensor negatively increased for
Cys, showed no change for Gly, and tended to be slightly
reduced for Glu. The ratio of the potential responses of the
GSH sensor to the NIP sensor shows the index of the specificity
for the GSH sensor to each substance. Therefore, the ratio of
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Glu are 5.9, 6.0, 5.1, and 3.2, respectively. In distilled water
(at pH 5.5), GSH, Gly, and Cys, are in a zwitterion state and
have almost a net zero charge, whereas Glu is in a net negatively
charged state. Despite the fact that GSH and Glu are different
in their molecular states, the fact that the GSH sensor gave Glu
the same potential change value as GSH indicates that the two
carboxyl groups of GSH and Glu play an important role. MAA,
which is the functional monomer in the MIP, also has a carboxyl
group in its chemical structure. Therefore, the carboxylic
GSH and Glu may interact with that of MAA via a hydrogen
bond. However, the chemical state of the carboxyl groups in
GSH is different from that for Glu; i.e., one carboxyl group of
GSH is in a neutral state and one is in a dehydrogenated state,
The MIP on the GSH sensor specifically recognizes the neutral state and dehydrogenated states of the two carboxyl groups of GSH via a hydrogen bond. The dehydrogenated state of the carboxyl group may contribute to the non-specific binding of these substances on the MIP surface because the potential responses were observed in the GSH sensor for Cys and Gly and the NIP sensor for GSH. Therefore, Glu, which has two dehydrogenated states, non-specifically bound to the MIP surface of the GSH sensor much more than the other substances, which are in a dehydrogenated state, and gave a large potential response in the GSH sensor. Unfortunately, in this study, the GSH sensor showed poor selectivity for compounds having two carboxyl groups, and the challenge remains to develop a method to enhance the selectivity of the sensor by reducing the non-specific binding of substances with dehydrogenated carboxyl groups to the MIP surface. To suppress the non-specific binding of chemical substances to the MIP, the carboxyl group of MAA localized on the MIP surface should be converted to a suitable functional group, e.g., methyl and trifluoromethyl groups. These MIP surface-modified GSH sensor are currently undergoing testing.

Conclusions

Potentiometric GSH sensors based on the MIP prepared from GSH, MAA, and EDMa as a template molecule, functional monomer, and cross-linker, respectively, were fabricated. By comparing the potential change of the GSH sensors after the addition of a GSH aqueous solution to a sample solution, the GSH sensor prepared with a GSH:MAA ratio of 2:32 showed the best responsiveness and repeatability among the various GSH sensors prepared with different ratios of GSH to MAA. Additionally, the GSH sensor prepared with a GSH:MAA ratio of 2:32 had good linearity with a r = 0.88 over a GSH concentration range of 1 × 10^-4 to 2 × 10^-4 mol L^-1 and good specificity for GSH. This study demonstrated that this GSH sensor should be applicable for the easy and direct determination of GSH. Future work should include an investigation of methods to further enhance the selectivity of the sensor so that it can achieve a potential response value that can differentiate GSH from Glu. To suppress the non-specific binding of chemical substances to the MIP, the carboxyl group of MAA localized on the MIP surface should be converted to a suitable functional group, e.g., methyl and trifluoromethyl groups. These MIP surface-modified GSH sensor are currently undergoing testing.

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