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Novel Urea and Highly Sensitive L-Cysteine Sensors Fabricated by Combining Urease Adsorbed Gold Nets with Ammonia Electrodes

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Urease adsorbs to metal surfaces such as gold, silver or copper in partly inhibited states. The activities of urease-adsorbed metals are highly dependent on the type of metal, and it has been found that urease-adsorbed gold has a much higher activity than urease-adsorbed silver or copper. Therefore, a novel urea electrode could be fabricated by combining a urease-adsorbed gold net and an ammonia electrode. The sensor response of urea was strongly influenced by the electrode potential applied to the gold net during urease adsorption, and was also significantly influenced by the concentration of L-cysteine in the urease solution used for adsorption. The activity of adsorbed urease increased as the concentration of L-cysteine decreased, and the calibration curve for L-cysteine showed a linear relationship down to a concentration as low as 10^{-12} M. In this study, we also measured the activities of urease adsorbed to other metal nets, and the activity of urease adsorbed to a silver net was much lower than that adsorbed to a gold net. The low activity of urease adsorbed to a silver net was dramatically recovered by the addition of dithiothreithol to the urea solution, however, the urease adsorbed to a copper net was still inactive even after dithiothreithol addition and was not recovered for 10 h of observation. The reason why the activity of urease adsorbed to silver and copper nets is low is that the conformation of urease is destroyed because the adsorptive forces of these metals are stronger than those of gold.

1. Introduction

Self-assemblies of many thiol compounds such as alkanethiols,[1,2] L-cysteine[3] and glutathione[4] are of major interest in the field of electroanalytical chemistry. In general, many thiol and disulfide groups exist in proteins such as enzymes or antibodies. Some of

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these groups are located in the center of the proteins, resulting in a lack of communication with the electrode surface, but the remainder of thiol and disulfide groups may be located at or near the surface of proteins, and they should be in contact with the gold or silver electrode surface under appropriate conditions. In fact, it is known that many enzymes adsorb to metal surfaces strongly, and the participation of thiol and disulfide groups in adsorption must be taking place. If an enzyme can be attached to a gold surface by only thiol adsorptions, the self-assembly of native enzymes might be realized in the future; however, other processes responsible for chemisorption and physisorption of enzymes to the metal surface have to be eliminated in advance.

Urease catalyses the hydrolysis of urea[5–7] to ammonia and carbon dioxide. This enzyme consists of six identical subunits,[8] and each subunit has five sulfydryl groups which can adsorb to a metal surface. Although it has been considered that the sulfydryl groups in the enzyme are located in the inner body of the enzyme, some of these can contact the electrode surface, because the electrochemical reduction and oxidation of sulfydryl or disulfide groups in the enzyme have been realized at mercury[9] and carbon electrodes.[10,11]

Urease directly adsorbs to a gold surface in an active state,[12] but the sites of protein adsorption to the metal surface are difficult to specify, because there is no control over the degree of denaturation,[13,14] and self-assembly of directly adsorbed enzymes should not occur. Urease also adsorbs to mercury not only through sulfydryl groups but also disulfide bonds, and reversible changes in activity based on the redox reactions of SS bonds have been reported.[9]

In this work, the activity of urease-adsorbed metals has been measured and a novel urea electrode using an urease-adsorbed gold net has been fabricated. A variety of urea sensors using urease have been reported using a combination of ammonia or carbon dioxide electrodes and immobilized urease,[15–18] but for most of these sensors, the immobilization of urease is rather tedious and time consuming. On the other hand, the adsorption of urease requires only the immersion of the gold net into a urease solution for a very short time. Moreover, we found that the adsorption of urease is interrupted by L-cysteine even if the L-cysteine concentration is extremely low. The adsorption rate of L-cysteine is expected to be much higher than that of the sulfydryl groups on the urease molecule, because the sulfydryl group of L-cysteine can contact the gold surface easily without steric hindrance.

Urease has many SH groups which have a very strong affinity to surfaces of metals such as gold, silver and copper. The activity of urease should be changed when it adsorbs to a metal because the conformation of the enzyme must be altered by the adsorption. The degree of destruction of urease conformation caused by adsorption should considerably depend on the identity of the metal.

2. Experimental

Two kinds of urease from jack beans were used: crude (undialyzed) and purified (dialyzed) enzymes. Urease was adsorbed by immersing metal nets (gold: 100 mesh; silver: 40 mesh; copper: 40 mesh) into jack bean urease solution (pH 7.0). For every experiment, 0.1 M phosphate buffer solutions were used. To study the effect of electrode potential on the adsorbed urease activity, the adsorption of urease was carried out by electrolyzing the urease solution at a constant potential. The constant potential electrolytic

experiment was carried out using a potentiostat (NPOT-1), and a platinum wire and saturated calomel electrodes were used as counter and reference electrodes, respectively. The adsorption of urease was carried out at pH 7.0, and the measurement of the activity of the urease-adsorbed nets was carried out at pH 8.0.

The urea sensor was fabricated by combining the enzyme-adsorbed net and an ammonia electrode, and the enzyme-adsorbed net was immobilized onto the ammonia membrane with a dialysis membrane. A cross section of the urea electrode is shown in Fig. 1. The ammonia produced from urea penetrates the metal net to reach an ammonia gas permeable membrane and the electrode potential changes. The electrode potential of the urea electrode was measured with a potentiometer (TOA Ion meter IM-5S). To compare the types of metals, gold, silver and copper nets were used to adsorb urease. The influence of dithiothreithol on the activity of adsorbed urease was also investigated. After the background potential of the urea electrode reached the steady-state value, urea was added and the change in the electrode potential was measured.

The amount of urease adsorbed to gold nets was changed by dissolving an aliquot of L-cysteine in the enzyme solution. The activity of adsorbed urease should be highly dependent on the concentration of L-cysteine, because the adsorption of L-cysteine competes with that of urease.

3. Results and Discussion

3.1 *Undialyzed urease*

First, the adsorption of crude urease to a gold net was carried out, because if treatment of urease is not needed in advance, the fabrication of a urea sensor becomes very simple. The activity of the enzyme immobilized on the gold net was achieved by only adsorption and was significantly increased by the application of a positive electrode potential during adsorption, mainly because the thiol compound (stabilizer) contained in the crude enzyme

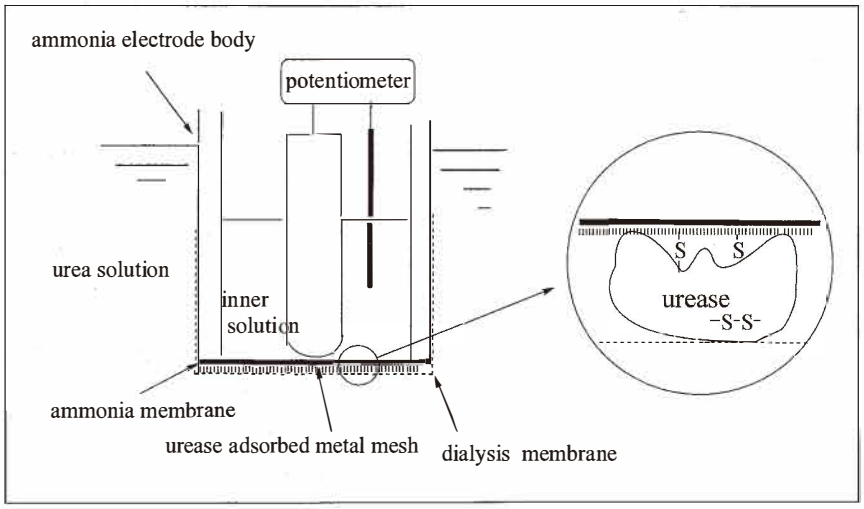

Fig. 1. Cross section of ammonia electrode equipped with urease-absorbed gold net.

preparation is eliminated by oxidation. Thiol compounds have a strong affinity to gold surfaces and may interfere with the adsorption of urease. Then, the urease-adsorbed gold net prepared by one minute of electrolysis at + 1.0 V vs SCE was used to fabricate the urea sensor. After adsorption, the gold net was washed with a buffer solution. The response curve obtained by successive additions of urea is shown in Fig. 2. This result indicates that the adsorbed urea exhibited excellent hydroxylase activity for urea, and its response time was approximately 15 min. This good response means that the affinity between urease and the gold surface is so weak that the conformation of urease is not significantly destroyed by adsorption.

Figure 3 shows the calibration curve of urea obtained at pH 8.0 using a urease-adsorbed gold net equipped with an ammonia electrode. The curve shows a relatively broad dynamic range from 5×10^{-5} to 1×10^{-2} M with a detection limit of 1×10^{-5} M. The response characteristics of the urea sensor should be highly dependent on the characteristics of the gold net, such as mesh size, the diameter of the gold fiber, and the response time.

The urease-adsorbed gold net retained its activity for at least 14 days when stored in a refrigerator, and a number of measurements of urea could be performed using the same urease-immobilized gold net. From this fact, it was confirmed that the urease-adsorbed gold net provided a very convenient immobilized enzyme for sensor fabrication.

3.2 *Dialyzed enzyme*

Crude urease, as purchased, contains large amounts of impurities such as thiol compounds and glycerin. The small molecules were eliminated by dialysis for one 24 hour period to purify the urease. The adsorptions of dialyzed urease were carried out at the electrode potentials of the gold net. Figure 4 shows the effect of the electrode potential during adsorption of urease on the activity of the adsorbed urease. This result indicates that the activity gradually increases as the potential increases positively, but decreases abruptly over + 0.70 V vs SCE. This result indicates that the adsorption rate is highly dependent on

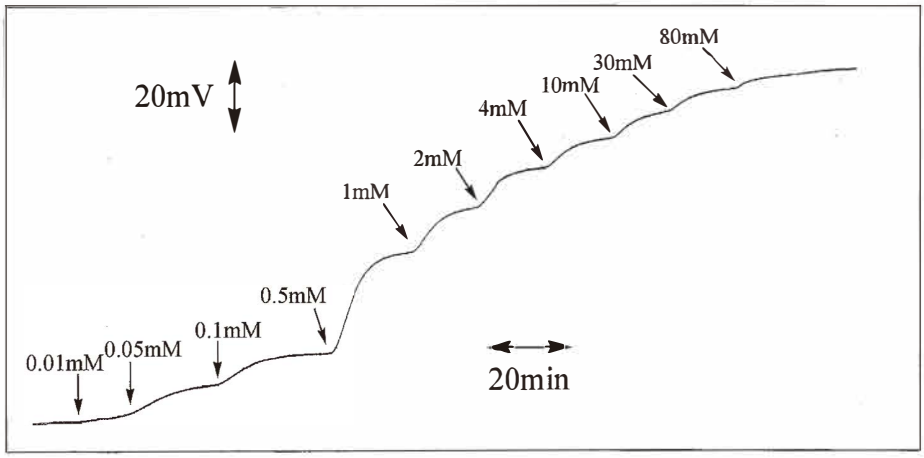

Fig. 2. Response curve obtained from the ammonia electrode with crude urease (1 mg/ml) adsorbed to a gold net. Direct electrolysis of the urease solution using a gold net was carried out at +1.0 V vs SCE for 5 min. This response curve was measured 2 days after adsorption.

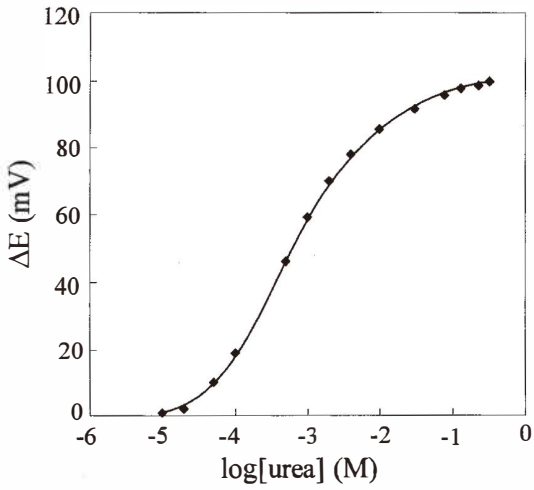

Fig. 3. Calibration curve for urea obtained with the urea electrode with a 1 mg/ml urease-adsorbed gold net in a pH 7.0 phosphate buffer.

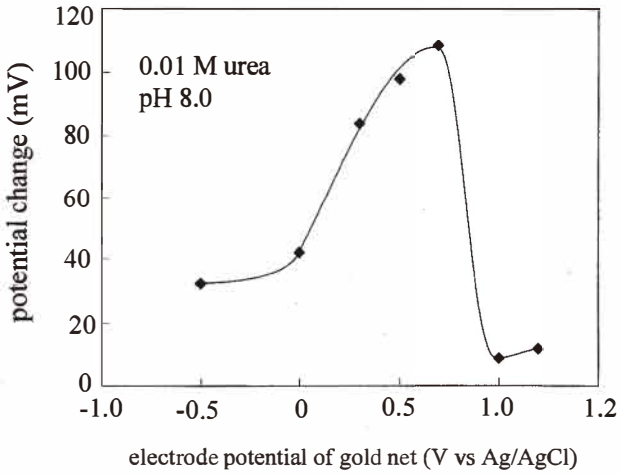

Fig. 4. Effect of electrode potential on the activity of the 6 mg/ml dialyzed urease-adsorbed gold net at pH 8.0 in phosphate buffer. Potential changes were measured 60 min after addition of 1×10^{-2} M urea.

the electrode potential. Urease has a negative charge at pH 7.0 and the migration rate of urease should be increased as the potential becomes more positive. Adsorption must be accelerated by the electric potential, and the activity increases as the potential increases. However, at very high potential, the denaturation of the enzyme occurs, and the activity of the adsorbed urease abruptly decreases. L-cysteine undergoes six-electron transfer to form cysteinic acid, and oxidation of the sulfydryl group to cysteinic acid would take place at

such high positive potentials.[19] This result was different from that obtained using the crude enzyme solution, and this discrepancy suggests that the electrical denaturation is prevented by thiols or other impurities contained in the crude enzyme solution. Different from the crude enzyme, the dialyzed enzyme was strongly adsorbed and was denatured at high positive potentials.

3.3 *L-cysteine detection*

From these results, it can be recognized that the concentration of thiol molecules in the enzyme solution for adsorption should affect the activity of enzyme adsorbed on the gold net. The influence of L-cysteine concentration on the adsorbed dialyzed urease activity was studied. The adsorption of urease was carried out by immersing the gold net for 18 h at an open circuit potential. For such a long period for adsorption, L-cysteine should be accumulated on the surface of gold net and replace the physical adsorption sites of urease; consequently, the amount of adsorbed urease may decrease.

The relationship between L-cysteine concentration in the urease solution for adsorption and the urea electrode response is shown in Fig. 5. The potential changes were measured when three hours had passed after urease was added. This result indicates that the adsorbed enzyme activity increased as L-cysteine concentration decreased, mainly because the adsorption of L-cysteine can apparently decrease the amount of adsorbed enzyme. Surprisingly, the potential change was observed at extremely low concentrations (lower than 10^{-12} M). This fact indicates that L-cysteine is accumulated to such an extent that the adsorption of urease is prevented, even if L-cysteine concentration is extremely low.

The relationship between concentration of L-cysteine and the potential change may provide a novel, extremely sensitive analytical method for the detection of thiol compounds. The principle of this new method is based on the inhibition of thiol on the adsorption of urease. Recently, highly sensitive measurements of cholinesterase activity using accumulation of adsorbed thiocholine on gold and silver electrode surfaces have

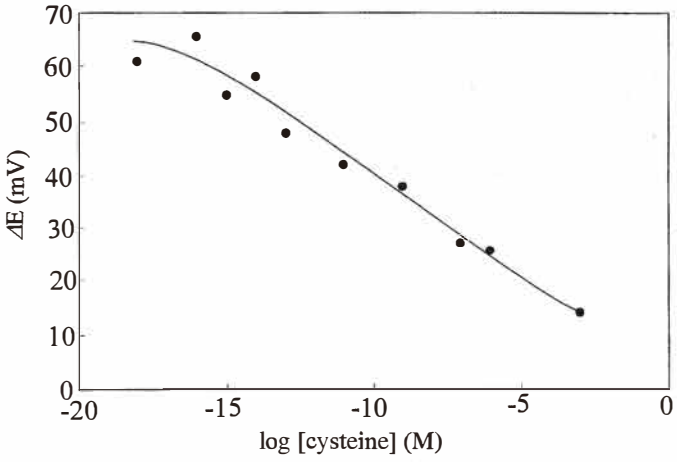

Fig. 5. Effect of the concentration of urea dissolved in the urease solution (6 mg/ml) used for adsorption on the response to urea.

been studied, and amplification of the signal has been achieved.[20,21] Figure 6 illustrates the principle of this method.

3.3 *Comparison of adsorbed urease activities on other metal nets*

The response curves of 1×10^{-2} M urea obtained using gold, silver and copper nets are shown in Fig. 7. All adsorption times were 20 h at room temperature. This result indicates that the activity of urease adsorbed to silver is significantly lower than that adsorbed to gold; however, the activity increased remarkably when dithiothreithol was added to the sample solution. Dithiothreithol has two sulfydryl groups which have very strong affinities for the gold surface. On the other hand, the activity of urease completely disappeared when the copper net was used, and its activity was not recovered by the addition of dithiothreithol. The reason why the adsorbed urease activities were diminished is considered to be based primarily on denaturation of urease caused by breaking disulfide bonds, because the adsorption strengths of thiol and disulfide bonds to silver and copper are very large. In fact, the inactivation of urease by the adsorption of its disulfide bonds to mercury has been reported.[9] It can be suggested that the recovery of adsorbed urease to silver was caused by the desorption of disulfide bonds of urease when dithiothreithol was adsorbed.

If disulfide bonds of urease adsorb to silver, the electroreduction current of the adsorbed disulfide bond should be observed. We found that the reduction current of urease (–1.0 V vs SCE) appeared at a silver disk electrode, but no reduction current of urease was observed at a gold disk electrode. This fact supports the hypothesis that the adsorption of the disulfide bonds of urease takes place at a silver electrode.

An illustration of the influence on urease adsorption and desorption of dithiothreithol at a silver surface is shown in Fig. 8. The urease-adsorbed gold net can be used as an effective

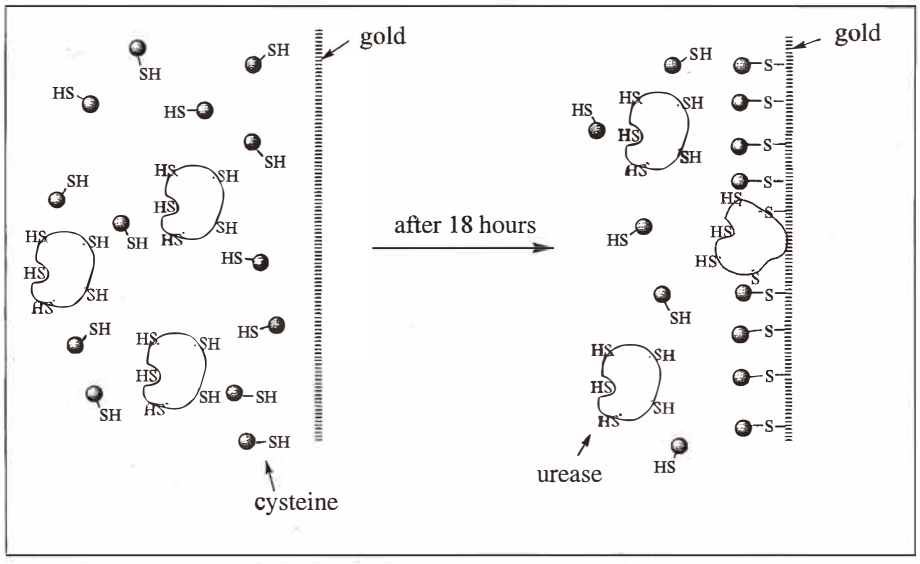

Fig. 6. Illustration of how L-cysteine adsorption changes urease activity.

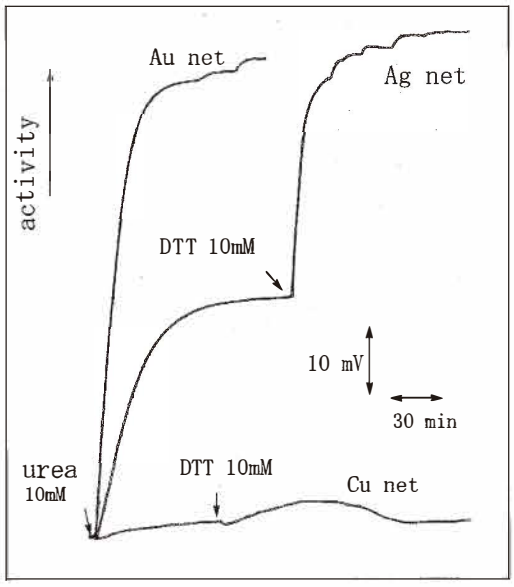

Fig. 7. Comparison of urea responses to gold, silver and copper nets and the effects of dithiothreithol addition on the responses. Metal nets were immersed into dialyzed urease solution (6 mg/ml) for 20 h.

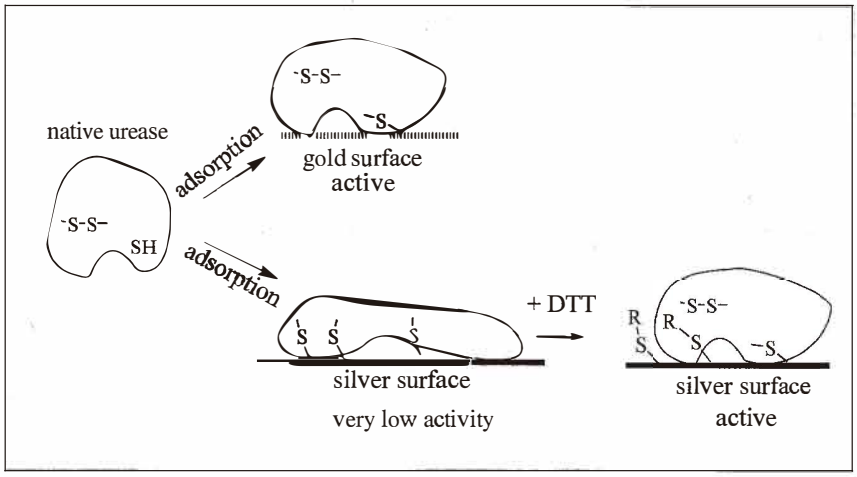

Fig. 8. Illustration of adsorbed urease and the recovery of the activity of adsorbed urease by dithiothreithol addition.

immobilized enzyme in a biosensor. The activity of the adsorbed enzyme depends significantly on the type of metal net and the thiol concentration in the urease solution used for adsorption. The relationship between the concentration of L-cysteine and the activity of adsorbed urease revealed the possibility of the detecting extremely low concentrations of thiol molecules.

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