— Review —

Design and Fabrication of a Molecular Interface on an Electrode with Functional Protein Molecules for Bioelectronic Properties

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Electrochemical reactions can combine with efficient biochemical reactions, as in enzymatic catalytic reactions. In the case of man-made bio-catalytic (enzymatic) electrochemical reaction systems, there are two interfaces on an electrode; a solution phase-enzyme layer interface and an enzyme layer-electrode interface. These interfaces act as a chemical reaction interface and a physicochemical reaction interface respectively. In order to prepare efficient biomolecular electrochemical devices, the molecular interfaces have to be designed as optimized physical and chemical structure. The author has studied the functional and physical structures of macromolecular interfaces in bioelectronics properties and others. The present review describes the fabrication of molecular interfaces on an electrode, using functional protein molecules in order to obtain specific bioelectronic properties.

Key Words : Bioelectronics, Biosensor, Biofuel Cell, Molecular Interface

1 Introduction

Protein molecules, such as enzymes, have efficient and important functions, *e.g.* molecular recognition, organization and catalytic activity. These functions have been the principal object of molecular design. However, such studies have resulted in the development of many types of artificial molecules. But these protein-mimic artificial molecules are not very efficient in practical purposes. In contrast, Natural functional proteins have been employed in molecular devices for practical purposes, such as immobilized catalysts, biosensors, molecular chips, and immune plates. Some novel molecular devices are under development, *i.e.* bio-batteries and bio-fuel cells. Especially in the case of bioelectronic devices, such as biosensors, protein molecules are immobilized on an electrode surface and provide both catalytic reactions and electrochemical reactions as hypothetically illustrated in Fig. 1. The enzyme layer immobilized on a solid (electrode) surface acts as a “molecular interface”. Actually, the molecular interface consists of two functional interfaces; solution layer/protein layer interface and a protein layer/solid phase (electrode) interface. As illustrated in Fig. 1, at the upper interface, the reaction occurs

between the solution layer and the protein layer. The lower interface, on the other hand, performs electrochemical reaction(s), also acting as a transducer. This is the theory of “three phases two interfaces” which has been proposed by the author.¹⁾ There are three different phases (water phase, molecular phase and electrode (solid phase)) and two functional interfaces, as illustrated in Fig. 1. In order to achieve efficient molecular interface functionality, the interfacial structures in the “three phases two interfaces” must be carefully designed and their fabrication must be orderly and accurate.

The author has studied the physical and functional structures of molecular interfaces consisting of functional proteins and other molecules. Bio-molecules, especially enzymes, are macro-molecules with sizes ranging from a few nm to less than about 50 nm. In the case of such macro-molecules, preparation of a molecular interface on an electrode poses many difficulties, *i.e.* steric hindrance, nonspecific adsorption, multiple chemical reactive sites on the protein molecule, etc. Due to these difficulties, most of the current methodologies of molecular immobilization on solid substrates (electrodes) are incapable of producing appropriate molecular interface structures. (In

Fig. 1 Conceptual illustration of interfacial structures in the “three phases two interfaces”; it is consisting from a molecular phase, solid phase, solution phase and two functional interfaces in between them.²⁾

cases of molecular interfaces with low-molecular-weight molecules, some useful methodologies have been developed and are already well known. However, in the past few years, some epoch-making methodologies and techniques have been reported, regarding both the fabrication of macro-molecular interfaces and the investigation of *in situ* nano-structures. The significant development of genetic engineering and protein engineering is also making possible the establishment of novel approaches to the development of biomolecular (bio-catalytic and related molecules, such as enzyme) interfaces. In the present review, the most recent methodologies for the fabrication of functional nano-structured molecular interfaces which have specific bioelectronic properties are discussed.

2 Pioneering Biomolecular Electrochemistry

Cytochrome C is well known as both a metal protein and a redox protein. It has been well studied electrochemically. In order to perform efficient electrochemical reactions with cytochrome C, the electrode surface must be modified with pyridine thiol, which acts as an adsorption layer for cytochrome C, ensuring its correct molecular orientation on the electrode.³⁾ A previous study has yielded a theory of the electron transfer and structure of this absorption layer and the absorbed cytochrome C. This type of research has advanced with the development of scanning probe microscope (SPM) technology. These researches suggest the importance of the orderliness of the macro-molecular interface for efficient functionalities.

Catalytic proteins (*i.e.*, enzymes) have also been studied from the electrochemical viewpoint, in view of applying them to biosensors, among other devices. The first biosensor reported was an enzyme electrode which can measure glucose, based on the coupling of glucose oxidase with an electrochemical reaction.⁴⁾ Hydrogen peroxide detection was also successfully performed in a pioneering work in the research field of bioelectronics.⁵⁾ In the case of electron transfer between a redox enzyme and an electrode, direct electron transfer is not easy; it must be mediated by a separate electron mediator which is dissolved in the solution. By enclosing the redox enzyme molecule inside a conducting polymer, smooth electron transfer between the redox enzymes and the electrode can be achieved. In this case, the conducting polymer was polymerized electrochemically in the enzyme-containing solution. Then, the polymerization on the electrode proceeded simultaneously with the enclosure of the enzyme molecules into the polymer.⁶⁾ The other things of enzyme electrodes for biosensor, some other types of enzyme electrode had been studied, *e.g.* conducting organic salt electrode,⁷⁾ and cross-linked redox gel-glucose oxidase on electrode.⁸⁾

As suggested by these pioneering research achievements, the careful design of physical structure and/or the functional structure is required to create molecular-solid (electrode) interfaces which can perform efficient electrocatalytic reactions, including enzymatic catalytic reactions and electrochemical reactions (electron transfer).

3 Conventional Molecular Immobilization Methods

The conventional methods of immobilizing molecules on a solid substrate can be classified into three types; chemical cross-linkage (between the molecules and the electrode substrate), bioaffinity assembly and self-assembly on an electrode.

Chemical cross-linkage is the most popular method of immobilizing protein molecules on any type of solid substrate. Immobilized enzymes have been employed as catalysts in many industrial production processes. Taking advantage of the electrochemical properties of the immobilized molecules, biosensors and other bio-molecular electronic devices have been constructed using protein-immobilized electrodes.

The silane coupling reaction is probably the most popular means of immobilizing proteins on inorganic substrates. All silane coupling reactions require an OH base on a solid substrate. Glasses and glass-based transparent electrodes can acquire an OH base (or an OH base can be introduced) on their surface if properly treated, especially with NaOH solution.⁹⁾ On other solid substrates, an OH base can be installed through a plasma irradiation.¹⁰⁾ A number of silane coupling reagents are commercially available, as such as alkoxy silane,^{11, 12)} trichlorosilane¹³⁻¹⁶⁾ and dimethyldichlorosilane¹⁷⁾ respectively. The reaction efficiency depends to a high degree on the reaction conditions, including temperature, reagent purity, type of solvent and anaerobic environment, but the reaction is controllable at the substrate/silane coupling reagent interface. For other interfaces, the protein molecules react with the free end of silane coupling reagent. In the most popular system, the end bears an active base, *e.g.* maleimide,¹⁸⁾ succinimide or aldehyde. However, protein molecules have more than one amino acid residue of the same type on the surface which can bind to these active bases. This means that the structure of the immobilized protein layer cannot be controlled through this immobilization method. The silane coupling reaction has been employed to immobilize molecules on inorganic substrates. It is a very powerful method when a low-molecular-weight organic substance is immobilized. However, in the case of protein (high-molecular-weight-macro-molecule) immobilization, an ordered molecular layer cannot be prepared on the electrode surface merely by applying the silane coupling method.

Bio-affinity assembly is based on the typical behavior of biomolecules. Some functional protein molecules have accurate and specific affinity to some part of the target molecule. Using these characteristics, a few types of functional protein layer have been prepared on a solid substrate. The most well-known bioaffinity molecule is the avidin-biotin complex. Avidin is a protein which has a strong and specific affinity to biotin. Biotin is also known as vitamin H or vitamin B7. If biotin is immobilized on a solid surface, avidin can bind to the immobilized biotin through the strong bioaffinity.¹⁹⁾ However, such an affinity reaction between proteins and small immobilized molecules is hardly controllable. Affinity-bound molecules tend to localize on solid surface.²⁰⁾

The specific bioaffinity between the FC part of IgG (antibody protein) and protein A (which can bind to the FC part of IgG specifically) is also often used to immobilize antibodies on an electrode or QCM crystal.²¹⁾ Avidin-biotin affinity binding has also been employed for molecular immobilization and molecular recognition.²²⁾ However, in order to achieve ordered molecular immobilization on an electrode or QCM crystal, the first layer (in the cases discussed, either protein A or biotin) must be ordered. If the first layer is not accurately arranged, the following layers are not able to form an orderly structure. In most cases of molecular immobilization based on bioaffinity, the first layer is not sufficiently well ordered.

Observations have suggested that the preparation of a molecular (protein) - solid juncture is crucial to the construction of an ordered protein layer on a solid surface.

Mercaptide bond-based^{23, 24)} molecular self-assembly is probably the most utilized method of modifying molecules on an Au electrode surface in electrochemistry research. Many pioneering studies have relied on an Au electrode modified through a mercaptide bond between SH base-tagged molecules and the epitaxial Au electrode surface.

As described in this section, most conventional molecular immobilization techniques cannot be applied to achieve the orderly immobilization of macro-molecules on a solid surface, such as an electrode.

4 Fabrication of an Ordered Nano-structured Macro Molecular Interface on an Electrode and Its Electrochemical Properties

As described in the previous section, a protein molecular layer is prepared by immobilizing protein molecules on an electrode surface. In this case, the formation of a molecule-solid juncture is key to accurately prepare a bottom-up structure. In cases of protein immobilization (preparation of a protein layer on an electrode), chemical modification cannot ensure the proper arrangement of the protein molecules. Since protein molecules have multiple active bases on their molecular surface, site-specific chemical reactions cannot be successfully performed. The fabrication of a macro-molecular (enzyme, antibody, etc.) layer on a solid surface is quite difficult to achieve, especially in an ordered and accurate fashion. However, an ordered molecular layer is indispensable for good molecular interfacial functionality. Some of studies on molecular interfaces have shown clearly the importance of carefully designing the molecular layer on the solid surface in order to induce efficient interface functionality.

Buckmann *et al.* have immobilized apo-form glucose oxidase on an Au electrode by means of a coenzyme-modified thiol compound which was immobilized on the Au electrode in advance through a mercaptide bond.²⁵⁾ This method can be used for site-specific bonding at both interfaces (electrode/thiol compound and thiol compound/enzyme), but cannot be used for other types of proteins (*i.e.*, it is applicable only to apo-enzymes or holo-enzymes). However, the accurate design and fabrication of a molecular layer on an electrode surface is crucial to

ensure the efficient functionality of the immobilized functional proteins (enzymes, antibodies, receptors, etc.) and other functional macro-molecules. In the case of an immobilized enzyme, its apparent catalytic activity is drastically altered according to its molecular orientation on the electrode surface.²⁶⁾ Antibodies also require a suitable molecular orientation on the solid substrate in order to provide good affinity activity. Nock *et al.* have prepared various types (size and orientation on the solid surface) of specific antibodies and compared their affinities on a solid substrate.²⁷⁾ In their works also, the affinity of the antibodies was found to depend on their molecular orientation on the solid surface. The redox properties are also drastically dependent on the manner of molecular immobilization. Anderson *et al.* have investigated the redox properties of immobilized azurin, a simple copper protein having two different molecular orientations, on two types of mercaptide-modified electrodes. The two types of azurin-modified electrodes showed drastically different electrochemical behaviors.²⁸⁾ One showed a reversible redox behavior, while the other showed an irreversible electrochemical behavior. Similar observations have been made for myoglobin (metal protein)-modified electrodes.²⁹⁾ Haruyama *et al.* have designed and synthesized four different types of α -helical peptides that have the same length but different sequences and chemical conditions on their surfaces. α -helical peptides had thiol at one end which could bind to an Au electrode, while the other end was modified with flavin for use in electrochemical investigations. The four electrodes modified with different types of flavin-peptide-SH molecules (all having the same length) showed reversible flavin redox reaction electrochemically. However, the electron transfer coefficients were very different (Fig. 2). The fact needs some of more investigation in order to judge what is happened. However, the results strongly suggest that the chemical environment around the redox molecules and the electrode can effect the ratio of electron transfer.³⁰⁾ As shown in Fig. 2, the efficient electron transfer was performed from redox molecule to an electrode through protein (peptidic) interface. In the current data, the electron transfer observed as if the hydrophobic interfacial structure gives interfacial environment to be efficient electron transfer. A very similar observation was reported with some types of ferrocene-modified SH ended molecules on an Au electrode. In this case, the electron transfer co-efficients also changed depending on the peptide structure.³¹⁾

Various types of molecular interface design using molecules in dynamic motion have been reported. pH-sensitive polymers have been employed to prepare a dynamic motion layer on an electrode surface. In such a system, the enzyme molecule is not immobilized directly on the electrode but positioned in its vicinity, on a pH-sensitive mediator polymer co-immobilized on the electrode. The immobilized pH-sensitive mediator layer can promote catalytic reactions and electron transfers on the electrode, because the pH-sensitive layer fluctuates in response to pH in the immediate environment and controls the reactions by catalase on the electrode.³²⁾ In

Fig. 2 Designed molecular interface for the investigation of electron transfer which is depend on peptidic monolayer structures.³⁰⁾

another study, ferrocenyl alkanethiol and thio cyclodextrin were allowed to co-self-assemble on an Au electrode. The ferrocene part clathrated into the cyclodextrin, which had been immobilized on the electrode surface in advance. The clathration/declathration reactions were modulated by the electrochemical redox reaction of ferrocen.³³⁾ Dynamic-motion molecular interfaces seems to have certain advantages. However, most of the molecular motion is too slow to ensure prompt electrochemical reactions.

All the research achievements described above are fraught by the same obstacle to the design/fabrication of a molecular layer. Immobilized macro-molecules (heterogeneously conditioned macro-molecules) require the accurate design and fabrication of the molecule-solid (electrode) juncture in order to have and maintain efficient interfacial molecular functionality on the electrode. However, it is very difficult to modulate both the density of the immobilized molecules and the molecular orientation through conventional molecular immobilization methods, especially in the cases of macro-molecules. In order to clarify the late limitation of macro-molecular interfaces consisting of functional proteins and to fabricate efficient macro-molecular electronic devices, efficient methodologies for the fabrication of designed macro-molecular layers on a variety of solid materials are very much required. This is also an indispensable factor of the reproducibility of each type of molecular interface.

Epoch-making novel approaches preparation of macro-molecular interfaces have been reported in recent years.

5 Novel Methodologies for the Preparation of a Macro-molecular Layer on an Electrode

As hypothetically illustrated in Fig. 1, in order to implement efficient functionality in both a “molecule/liquid interface” and a “molecule/solid interface”, certain structural aspects have to be considered: (1) the distance between the molecules and the solid surface, (2) the macro-fluctuation of the immobilized molecules, (3) accurate layer structure (distance, orientation, etc), (4) the chemical environment at the junctures. In order to control these structural aspects, epoch-making discoveries

are required in macro-molecular electronics.

Electronic molecular adsorption has been investigated; it is a variation of electrophoresis and electrostatic adsorption.^{34, 35)} In contrast, Haruyama has developed the ECTag method, by which electrochemical molecular immobilization (not adsorption) on any conductive material can be achieved in an orderly fashion and reversibly.¹⁾ The ECTag method is based on the electrochemical deposition of a coordinated metal ion in peptidic ligand.³⁶⁾ The peptidic ligand (at least 3 amino acids in length) can be introduced at any site on the enzyme molecule by genetic techniques and can be tagged onto synthesized organic compounds (*e.g.*, oligo-peptides) or inorganic materials (*e.g.*, nanoparticles).²⁾ Using the ECTag method, a well-ordered molecule-solid juncture can be constructed electrochemically (Fig. 3). The ECTag method can use most divalent metal ions and can perform immobilization on most conductive materials, *e.g.*, metals, carbon, semiconductors, etc. In the author most current achievement, the immobilized peptide (an oligo-polymer consisting of 12 amino acids) has an affinity sequence which specifically binds to the receptor protein if the peptide is immobilized by the ECTag method on a solid substrate. But the same peptide does not show any affinity for the same receptor protein when the peptide is immobilized on the solid substrate by either physical unspecific adsorption or chemical covalent modification. This indicates that the affinity between the peptide and the protein is easily affected by molecular steric hindrance, especially if it is immobilized on a solid surface (heterogeneous condition). But if it is immobilized by the ECTag method, a molecule-solid juncture is formed. The peptide is immobilized and stands perpendicularly on the solid surface. This vertical orientation of the immobilized molecules is a key factor of their functionality and can be regulated by the ECTag method.

Another epoch-making method is based on molecular self-organization and self-adsorption. The author has focused on the remarkable characteristics of proteins “hydrophobin” (originally cloned from the cell wall of fungi), because they have the outstanding ability to self-organize (*i.e.*, they can form an ordered self-organized monolayer on an air/liquid interface) and adsorb (they

Fig. 3 Schematic illustration of the ECTag method for electrochemical molecular immobilization.^{1, 36)}

Fig. 4 Schematic illustration of the HFB drop-stamp method for the preparation of a self-organized molecular layer on a solid surface.³⁷⁾

play a role in the adsorption of fungi).³⁸⁻⁴⁰⁾ Haruyama *et al.*, have employed hydrophobins (they are divided into different subclasses) as carriers in order to achieve the ordered self-organization and adsorption of the objective molecules on a solid surface without nonspecific adsorption. In order to prepare such an ordered protein layer on a solid substrate, the drop-stamp method is used, as illustrated in Fig. 4.⁴¹⁾ Since the hydrophobin protein forms a horizontal self-organized monolayer on the air/liquid interface, the objective functional protein-hydrophobin fusion molecule (obtained by a genetic fusion technique) can also form an ordered monolayer on the surface of the solution droplet, as well as on solid material surface by stamping the droplet on the solid

substrate,³⁷⁾ as illustrated in Fig. 4. Using this method, the immobilized density (number of molecules per unit area), molecular orientation and other aspects can be controlled with great accuracy on the solid material surface. The ordered fusion protein layer with the hydrophobin carrier can be prepared on a variety of solid material surfaces, *e.g.*, silanated glass, HOPG, gold, mica, silicon, ITO, FTO, GaAS etc. The concept and techniques behind the construction of an accurate, designed protein layer have been developed as a result of attempts to induce interfacial molecular functionality electrochemically. Various design of electrode modified with an ordered protein layer demonstrate remarkable electrochemical properties, as has already been reported.

6 Design and Synthesis of Artificial Enzymes for Bioelectronic Devices

Interfacial molecular functions can also be induced by designing and synthesizing artificial enzymes. Artificial enzymes are man-made organic catalysts in which the reaction rate is dramatically increased by lowering the reaction activation energy.⁴²⁾ They act in the same manner as natural enzymes. Practical applications for the coupling of artificial enzymes with electrochemical reactions are very few. However, there are some pioneering studies on molecular interfaces consisting of artificial enzymes. Sode *et al.*, have designed and synthesized a catalyst which mimic dehydrogenase and cleaves fructosyl valine on HbA1c. The artificial enzyme was successfully coupled with the electrochemical measurement of HbA1c using m-PMS as the electron mediator.⁴³⁾

Haruyama *et al.*, have developed an artificial enzyme based on a self-assembled polymer, and have applied it to the construction of an amperometric sensor. This enzyme does not mimic natural counterpart, functionally speaking; it has a new type of molecular specificity. The artificial enzyme can be prepared using two types of polymer and a metal ion through self-coordinative assembly. In our molecular design strategy, the Cu^{2+} -complex cavity acts as the catalytic active site which runs the catalytic hydrolysis reaction at the phosphoric acid hydride bond part of the substrate. The active-center

Fig. 5 Artificial enzyme based amperometric biosensor for one-stop-biosurveillance.⁴⁴⁾ The sensor reproducibility is sufficiently high (coefficient of variation $\leq 3\%$) to perform biosensing without references. It is storable for the prescribed term in dry conditions. After 3 months of storage, the sensor response still shows good reproducibility ($\text{CV} \leq 3\%$).

cavities are accumulated in the membrane to ensure suitable catalytic activity in every unit section of the membrane matrix covering the electrode surface. The membrane is designed and synthesized through coordinative self-assembly among a metal coordinative cation polymer, Cu (II) ions and a counter anion polymer. The artificial enzyme is easily prepared in the form of a thin membrane on the electrode surface. The artificial enzyme membrane recognizes and hydrolyses the phosphoric acid hydride structure, which is a structure common to all biological phosphoric substances. The hydrolysis reaction was successfully coupled with amperometric electrochemical detection (Fig. 5).⁴⁴⁾ The sensor performs biosurveillance based on the detection of biological phosphate from viruses, fungi, pollen and other microorganisms.⁴⁵⁾

7 *In Situ* Nano-structural Investigation of the Molecular Interface

In order to design and develop the functionality of molecular interfaces constructed using proteins, it is necessary to utilize scanning probe microscopy (SPM) technology, especially atomic force microscopy (AFM), to observe and to investigate the *in situ* physical structure of the molecular layer on the solid surface. Many pioneering studies have carried out structural investigations on molecule-modified electrodes, mostly with low-molecular-weight organic and inorganic functional molecules. In the case of protein interfaces, the structures have to be investigated in liquid conditions because maintain their functional and structural integrity only in the appropriate liquid conditions. Moreover, proteins can be bulky, flexible or soft macro-molecules. It is difficult to investigate them in liquid conditions by means of conventional contact-mode AFM. In contact mode AFM, the horizontal friction between the cantilever and the object sample is too large to obtain clear image of the sample. Therefore, tapping-mode AFM (AC-AFM) has been developed for the purpose of soft-material imaging.

However, tapping-mode AFM is ineffective when a soft object is imaged with a soft (lower restoring force) cantilever. In this case, the tip is easily adsorbed on the object molecule, which lowers the accuracy of the Z axis modulation of the cantilever. If a hard (higher restoring force) cantilever is used, cantilever stays separate from the object molecule; however, the modulation of the tip along the Z axis becomes more difficult mechanically.

In recent years, new AFM trials have been developed to enable the high resolution imaging of soft objects in solution. Frequency-modulated AFM (FM-AFM) can modulate the Z axis movement of the cantilever with high accuracy when a soft and elastic object is being imaged.⁴⁶⁾ FM-AFM yields very clear images in solution and related surface information based on its outstanding resolution along the Z axis. Taking advantage of this high resolution, FM-AFM can be used to image a hydrated layer on a mica surface⁴⁷⁾ in solution as well as on a lipid bilayer⁴⁸⁻⁵⁰⁾ in solution. It is important to understand the relationship between the functional structure and the physical structure of the molecular interface.

The recent development of the scanning probe electrode expands the range of phenomena which can be investigated. In a recent report, the combination of a Kelvin probe force microscope (KFM) with a scanning probe near-field optical microscope (SNOM) is described. The novel SNOM-KFM system employs near-field photo excitation through a fiber probe during KFM measurement. Using SNOM-KFM, it may be possible to investigate the functionality of energy transfer and its dependency on the interfacial structure.⁵¹⁾ These new SPM technologies can be employed as potential investigation tool to clarify the *in situ* physical and functional structures of biomolecular interfaces.

8 Conclusion

Molecules display various functionalities in homogeneous conditions such as in solution. Especially the functions of protein molecules (*i.e.*, molecular recognition,

enzymatic catalysis, energy transduction) are important for the creation of biomolecular electronic devices. Immobilized molecules should provide not only molecular functions but also interfacial functions in heterogeneous conditions (in the form of immobilized molecules on a solid surface). Molecular interfaces based on immobilized molecules have been employed in the construction of various molecular devices, including electrochemical molecular devices. It is not easy to immobilize a molecule (especially a functional protein molecule) on a solid surface while maintaining its whole range of functionality to obtain a functional molecular interface. The structure of the immobilized layer and steric hindrance on a solid surface are the peculiar difficulties associated with this type of construction. In order to ensure efficient functionality of the molecular interface, the optimization of the molecular layer structure on the solid surface (electrode) is crucial.

In this review, the main lines of the current research on the electrochemical properties of biomolecular interfaces, including related research, have been described. Molecular interfaces are a topic of electrochemistry, but they belong, in fact, to an interdisciplinary research field. There are many pioneering studies of molecule-modified electrodes. These current endeavors to which I am referring here represent just some of the work being done in this field. However, I would like to emphasize that the seeds of the next developments in this field are based on wide interdisciplinary concept, and approaches to the realization of efficient biomolecular interface and its application to electrochemical molecular devices must take this concept into consideration.

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