BIOCHIP JOURNAL, Vol. 1, No. 3, 188-192, September 2007

Thin Film Fabrication of Electroactive Protein with Heme Group

Sang-Uk Kim[1], **Young Jun Kim**[1],
Jeong-Woo Choi[1,2] **& Byung-Keun Oh**[1,2]

[1]Interdisciplinary Program of Integrated Biotechnology, Sogang University, 1 Shinsoo-Dong, Mapo-Gu, Seoul 121-742, Korea
[2]Department of Chemical & Biomolecular Engineering, Sogang University, 1 Shinsoo-Dong, Mapo-Gu, Seoul 121-742, Korea
Correspondence and requests for materials should be addressed to B.-K. Oh (bkoh@sogang.ac.kr)

Accepted 14 August 2007

Abstract

Horse heart myoglobin is a member of the metalloproteins, and harbors iron-containing porphyrins in its active site. Myoglobin evidences redox properties, and can transfer electrons from one site to other sites. These molecules can be used in bioelectronic applications, when fabricated as a self-assembled bio-film. We utilized 11-mercaptoundecanoic acid (11-MUA) as a chemical linker in order to immobilize myoglobin on gold (Au) substrates. The immobilization of the protein and the surface morphology of the thin film were confirmed via surface plasmon resonance (SPR) and atomic force microscopy (AFM), respectively. The results indicated that the proposed immobilization technique should prove useful for the fabrication of high-quality protein film, and this technique could be applied to the fabrication of nano-scale bioelectronics.

Keywords: *Horse heart* Myoglobin, Self assembly (SA), Metalloprotein, Surface plasmon resonance (SPR), Atomic force microscopy (AFM), Cyclic voltammetry (CV), Bioelectronics

Introduction

In a biological electron transfer system, photoelectronic conversion occurs, followed by long-range electron transfer. It takes place unidirectionally in a very efficient manner, via the activity of biomolecules[1-3]. Recently, a variety of artificial bioelectronic devices have been fabricated by mimicking the electron transport function of the biological electron transfer system. The control of the arrangements of these molecules in a solid state is crucial to efforts to construct high-efficiency bioelectronic devices[2-6]. Thus, the organism employed in this work has an optimized signal translation system[7].

According to the results of biochemical studies, metalloproteins evidence redox properties, such as cytochrome *c*, azurin, ferredoxin, ferritin, *etc*. It has also been determined that myoglobin can function as an electron donor or electron acceptor. However, in general, the protein is unstable and fragile in its solid state. Therefore, efficient film fabrication techniques are clearly required for protein to be useful as a bioelectronic device component. If the arrangement of the biomolecule in the solid state can be adequately controlled, the development of a high performance nano-scale bioelectronic device should be an achievable goal.

In the past decade, the self-assembly (SA) technique has been pioneered, and appears to be a useful method for the fixation of a thin layer onto a metal substrate for a vareity of applications, including biosensors, field-effect transistors (FET), *et cetera*[8-10]. The most general self-assembly system involves the use of alkanethiols as chemical linkers. In long hydrocarbon chain structures, one side is reacted with a solid substrate, and the other side is reacted with a target protein. It has been determined that sulfur compounds coordinate quite strongly with a variety of metal surfaces, including Au, Ag, Cu, Pt, *etc*. Among these, Au has been most frequently used in the self-assembly monolayer (SAM) formation of alkanethiols, since gold is not readily oxidized[11-14].

In this study, we demonstrate a technique for thin film fabrication using the electroactive protein, myoglobin, with a chemical linker, in order to make an effective nano-bioelectronic device. No reports have been previously filed regarding the immobilization of myoglobin using a chemical linker and the characterization of its electrochemical properties. Figure 1 provides a schematic diagram of this system, which is an electrostatic adsorption system that exploits the charges between the target materials and the chemical linker materials. Initially, we fabricated an 11-mercaptoundecanoic acid (11-MUA)-modified gold electrode. 11-MUA harbors a thiol group (-SH) and a carboxyl group (-COOH) at both ends. Thus, 11-MUA can alter the surface charge of the gold substrate to a negative charge under aqueous conditions. Myoglobin harbors several amine groups in its amino acid sequence. These opposite charges allow for the interaction between the target materials and the charge-mod-

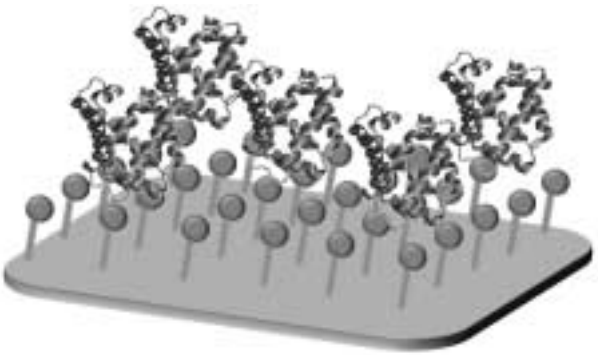

Figure 1. Schematic description of myoglobin immobilization.

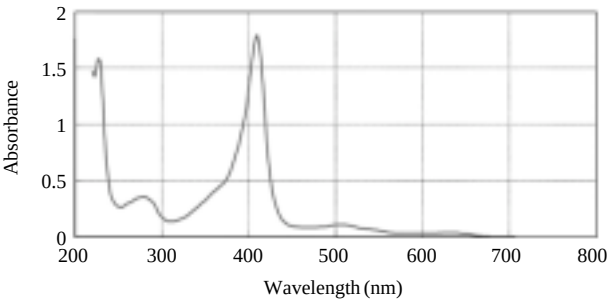

Figure 2. UV-visible spectroscopy of myoglobin.

ified gold electrode. The immobilization of myoglobin on the basis of electrostatic adsorption could be verified by surface plasmon resonance (SPR) and atomic force microscopy (AFM). Surface plasmon resonance (SPR) was utilized for confirming optical thickness of myoglobin layer, and atomic force microscopy was utilized for analyzing surface morphology. The electrochemical redox properties of the immobilized myoglobin on the solid surface were determined via cyclic voltammetry (CV).

Results and Discussion

Initially, the existence of ferric ion in the myoglobin utilized in this study was assessed via UV-visible spectroscopy. In the myoglobin solution, three specific absorption peaks are shown (Figure 2). The myoglobin evidences an absorption spectrum with a maximum at 410-411 nm band, induced by ferric ions.

The immobilization of myoglobin on the Au surface was evaluated via surface plasmon resonance (SPR), as is shown in Figure 3. The SPR angle was shifted from 43.00 degrees to 43.17 degrees, and from 43.17 degrees to 43.74 degrees by the adsorption of

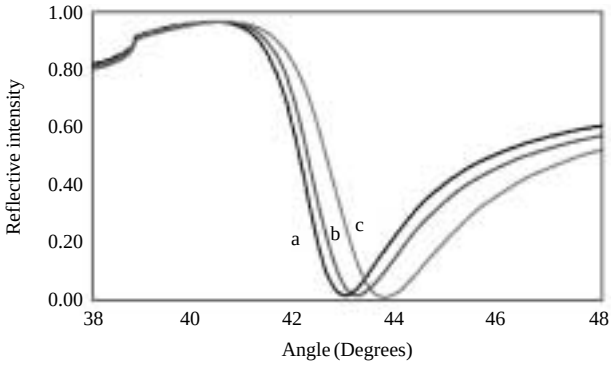

Figure 3. SPR spectroscopy of bare Au and 11-MUA, and myoglobin on the 11-MUA modified Au surface (a: bare Au, b: 11-MUA, c: Myoglobin).

11-MUA on the Au surface and the binding of myoglobin to the 11-MUA-modified Au surface via the charge interaction between the myoglobin and the carboxylic terminal group of 11-MUA, respectively. In principle, a surface plasmon is a bound electromagnetic wave which propagates at the metal-dielectric interface. An external laser field drives the free electron gas of metal in a distinct mode. The spatial charge distribution creates an electric field that is localized at the metal-dielectric interface. Thus, the plasmon resonance is exceedingly sensitive to the interfacial architecture. An adsorption process results in a shift in the plasmon resonance and allows for the monitoring of the mass coverage at the surface with a high degree of accuracy. Therefore, the shift of the SPR angle confirmed that a thin layer of 11-MUA was formed on the Au surface, and myoglobin was bound to the 11-MUA-modified Au surface via a charge interaction occurring between the myoglobin and the carboxylic terminal group of 11-MUA.

Figure 4 shows the atomic force microscope (AFM) image of the electrostatically adsorbed myoglobin layer, as compared to that of bare gold. In general, AFM analysis can be utilized as a complement to the SPR technique. The benefit of combining SPR and AFM imaging is that it allows for the inter-relationships between surface topologies and the interactions between biomaterials to be analyzed efficiently. It was observed that the aggregated protein clusters are formed with a globular shape in a solid-like state, while retaining a random cloud-like structure, as in a bulk solution. Also, the sizes of those clusters differed from those observed on bare Au. From the results shown above, it could be concluded that myoglobin was fabricated on the Au substrate functionalized with 11-MUA.

In general, the original structure of the biomolecule

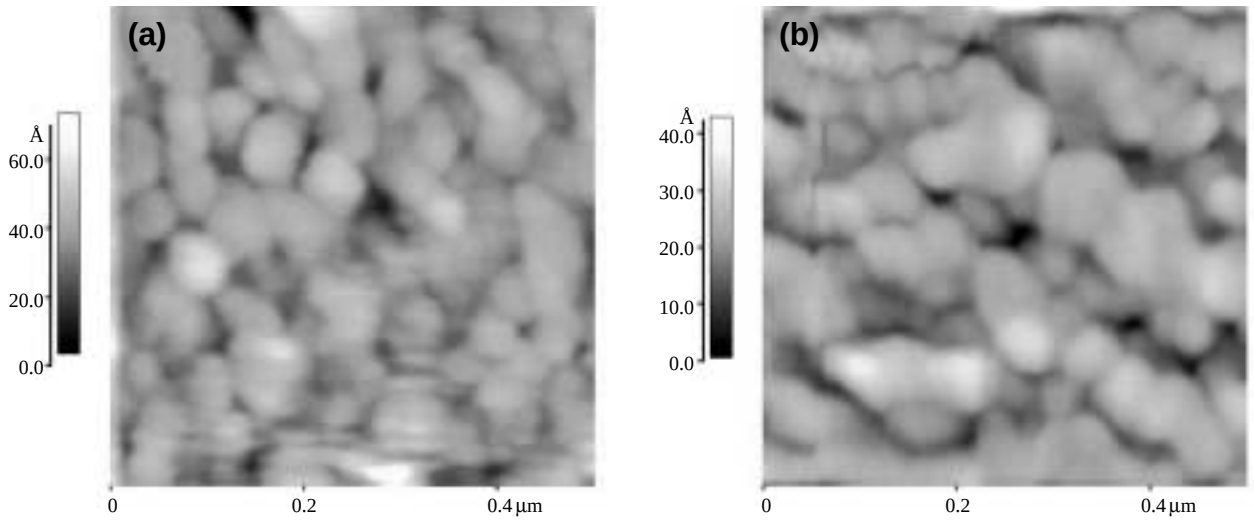

Figure 4. AFM topography analysis (a: Au, b: Myoglobin layer on 11-MUA modified Au surface).

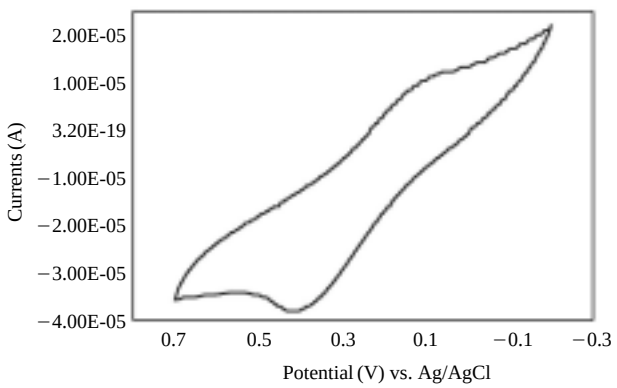

Figure. 5. Cyclic voltammetry of myoglobin immobilized on Au electrode.

in solid-state should be maintained in order to acquire the redox signal of the electroactive protein in a bioelectronic device. However, in cases in which the redox protein layer was fabricated on the metal electrode, the protein structure was readily denatured. The supporting layer between the protein and metal substrate not only increased the immobilization capability, but also assisted in the maintenance of the protein structure on the metal substrate. In this study, an alkyl chain was utilized as the supporting layer, to adsorb myoglobin on the Au electrode. The CV measurement of the adsorbed myoglobin layer was evaluated in a range of 700 mV to −100 mV (Scan rate= 50 mV/s). Figure 5 shows the redox characteristics of the myoglobin adsorbed onto the 11-MUA modified Au electrode. As is shown in Figure 5, the immobilized myoglobin on the Au electrode was oxidized and reduced at 400 mV and 100 mV, respectively. From these results, we could assume that the 11-MUA modified gold electrode created excellent conditions for the maintenance of the redox properties of the electroactive molecules.

Conclusions

In this study, we demonstrated a thin film fabrication technique with the electroactive protein, myoglobin, using a chemical linker to construct an effective nano-bioelectronic device. An 11-MUA thin layer was fabricated on a gold electrode via the self-assembly method. The myoglobin was immobilized on an 11-MUA-modified Au electrode via electrostatic interaction between the myoglobin and carboxyl groups of 11-MUA. The immobilization of the myoglobin based on electrostatic adsorption was verified via SPR and AFM. The electrochemical properties of the myoglobin immobilized on the solid surface were assessed via CV. In conclusion, the proposed immobilization technique could be utilized in the construction of high quality protein films, and could be applied to the fabrication of nano-scale bioelectronics.

Materials and Methods

Materials

Horse heart myoglobin was purchased from Sigma -Aldrich (USA). 2-(N-morpholino) ethanesulfonic acid (MES, Sigma-Aldrich, USA) and phosphate buffer saline Tween 20 (PBS-T Sigma-Aldrich, USA) were

utilized as solvents for the preparation and washing of the myoglobin sample and washing buffer solution. The water used in all experiments was distilled and deionized by Millipore [(Milli-Q) water (DDW; $>18\,M\Omega$)].

Thin Film Fabrication

For the fabrication of the gold (Au) substrate, a cover glass composed of BK7 (18 mm × 18 mm, Superior, Germany) was used as a solid support. Chromium (Cr) was initially sputtered onto the glass substrate as an adhesion material to a thickness of 20Å, followed by gold (Au) sputtering to a thickness of 430Å. The sputtered Au substrate was then cleaned with pirana solution composed of 30 vol% H_2O_2 (Sigma-Aldrich, USA) and 70 vol% H_2SO_4 (Duksan Chemical Co. Ltd, Korea) at 70˚C for 5 min, and then the cleaned Au substrate was immersed in pure ethanol solution for 1 hr. The Au substrate was rinsed with acetone and deionized water. To prepare the myoglobin sample, 0.100 mg/mL of myoglobin was dissolved in 10 mM MES (pH 6.25). 0.100 mg/mL of myoglobin solution was dropped onto the substrate for 6 hrs. After 6 hrs, the substrates were gently washed in deionized water, taking great care to wash the substrate sufficiently. The residual solution on the Au surface was then removed with an N_2 gun.

Surface Plasmon Resonance (SPR)

SPR depends on a bound electromagnetic wave propagating at a metal-dielectric interface. The attenuated total reflection (ATR) configuration by Kretchmann, which is well known as the design for the SPR instrumental method, relies on the phenomenon of total internal reflection. The external laser field drives the free electrons of metal in a distinct mode. The spatial change distribution creates an electric field localized at the metal-dielectric interface. Bi-molecular interactions were monitored via surface plasmon resonance spectroscopy (Multiskop™, Optrel GmbH, Germany) using a He-Ne laser light source with a wavelength of 632.8 nm. The p-polarized light beam generated by the polarizer was utilized as a reference and the intensity of the reflected beam was with a photo multiplier tube (PMT) sensor. A glass prism (BK 7, n=1.5168) with a 90˚ angle was used as a Kretchmann ATP coupler. The plane face of the 90˚ glass prism was coupled to the cover glass via index matching oil. The resolution of the angle reading of the goniometer was 0.01 degree. All samples were monitored at a constant temperature of 20˚C. The angle of incidence was verified from 38 degrees to 50 degrees.

Topography Analysis using Atomic Force Microscopy (AFM)

The topographies of the thin films were evaluated via AFM (Autoprobe CP, Park Scientific Instruments, USA). AFM was conducted in contact mode in air, at room temperature. The images were acquired at a 0.3 Hz scan rate with a silicon cantilever (Ultralever 06B, PSI, USA), at a scan size of 500 nm.

Elctrochemistry

In order to determine the redox potential of myoglobin, we constructed a working electrode via gold deposition, with a 0.5 cm × 0.5 cm working electrode. The working electrode was composed of the myoglobin/11-MUA/Au substrate, an Ag/AgCl electrode as the reference, and a platinum electrode as the counter. The reduction-oxidation spectra of myoglobin were acquired with a cyclic voltammetry (CV) measurement system (CHI 660A potentiostat (USA)) in HEPES buffer solution.

Acknowledgements

This research was supported by the Nano/Bio Science & Technology Program (M10536090001-05N3609-00110) of the Ministry of Science and Technology (MOST), and by the Core Environmental Technology Development Project for Next Generation funded by the Ministry of Environment of Korea, and by Sogang University (20061019).

References

1. Chi, Q., Zang, J. & Nielsen, J.U. Molecular monolayers and interfacial electron transfer of *Pseudomonas aeruginosa* azurin on Au (111). *Journal of American Chemical Society* **122**, 4047-4055 (2000).
2. Fristrup, P. *et al*. Voltammetry of native and recombinant *Pseudomonas aeruginosa* azurin on polycrystalline Au- and single-crystal Au (111)-surfaces modified by decanethiol monolayers. *Journal of Electro-Analytical Chemistry* **511**, 128-133 (2001).
3. Pozdnyakova, I. & Stahshede, P.W. Biological relevance of metal binding before protein folding. *Journal of American Chemical Society* **123**, 10135-10136 (2001).
4. Choi, J.-W. & Fujihira, M. Molecular-scale biophotodiode consisting of a green fluorescent protein/cytochrome c self-assembled heterolayer. *Applied Physics Letters* **84**, 2187-2189 (2004).
5. Choi, J.-W. *et al*. Nanoscale fabrication of biomolecular layer and its application to biodevices. *Biotechnology and Bioprocess Engineering* **9**(2), 76-85 (2004).

6. Choi, J.-W., Nam, Y.S., & Lee, W.H. Nanoscale fabrication of biomolecular layer and its application to biodevices. *Applied Physics Letters* **79**(10), 1570-1572 (2001).
7. Bott, A.W. Redox properties of electron-transfer Metalloproteins. *Current Separations* **18**, 47-54 (1999).
8. Nuzzo, R.G. & Allara, D.L. Adsorption of bifunctional organic disulfides on gold surfaces. *Journal of American Chemical Society* **105**, 4481 (1983).
9. Porter, M.D., Bright, T.B., Allara, D.L. & Chidsey, C.E.D. Active site generation of a protonically unstable suicide substrate from a stable precursor: Glucose oxidase and dibromonitromethane. *Journal of American Chemical Society* **39**(38), 11808-11817 (2000).
10. Strong, L. & Whitesides, G.M. Structures of self-assembled monolayer films of organosulfur compounds adsorbed on gold single crystals: electron diffraction studies. *Langmuir* **4**(3), 546-558 (1988).
11. Lee, W., Lee, D.B., Oh, B.-K., Lee, W.H. & Choi, J.-W. Nanoscale fabrication of protein A on self-assembled monolayer and its application to surface plasmon resonance immunosensor. *Enzyme and Microbial Technology* **35**(6-7), 678-682 (2004).
12. Li, H., Luk, Y.-Y. & Mrksich, M. Catalytic asymmetric dihydroxylation by gold colloids functionalized with self-assembled monolayers. *Langmuir* **15**(15), 4957-4959 (1999).
13. Dubrovsky, T.B., Hou, Z., Stroeve, P. & Abbott, N.L. Self-assembled monolayers formed on electroless gold deposited on silica gel: A potential stationary phase for biological assays. *Analytical Chemistry* **71**(2), 327-332 (1999).
14. Sigal, G.B., Bamdad, C., Barberis, A., Strominger, J. & Whitesides, G.M. A self-assembled monolayer for the binding and study of histidine-tagged proteins by surface plasmon resonance. *Analytical Chemistry* **68**(3), 490-497 (1996).