

## Fabrication of Hemoglobin/Silver Nanoparticle Heterolayer for Electrochemical Signal-enhanced Bioelectronic Application

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**Abstract** – A hemoglobin/silver nanoparticle heterolayer was fabricated for bioelectronic device with electrochemical signal-enhancement effect. As a device element, a hemoglobin, the metalloprotein, contained the heme group that showed the redox property was introduced for charge storage element. For electron transfer facilitation, a silver nanoparticle was introduced for electrochemical signal facilitation, the hemoglobin was immobilized onto Au substrate using chemical linker 6-mercaptopentanoic acid (6-MHA). Then, the silver nanoparticle was immobilized onto fabricated hemoglobin/6-MHA heterolayers by layer-by-layer (LbL) method. The surface morphology and surface roughness of fabricated heterolayer were investigated by atomic force microscopy (AFM). The redox property of hemoglobin/silver nanoparticle heterolayer was investigated by a cyclic voltammetry (CV) experiment for obtaining an oxidation potential and reduction potential. Moreover, for the assessing charge storage function, a chronoamperometry (CA) experiment was conducted to hemoglobin/silver nanoparticle-modified heterolayer electrode using oxidation and reduction potentials, respectively. Based on the results, the fabricated hemoglobin/silver nanoparticle heterolayer showed that an increased charge storage effect compared to hemoglobin monolayer-modified electrode.

Key words: Hemoglobin, Silver nanoparticle, Electrochemical bioelectronic device, Cyclic voltammetry, Atomic force microscopy

### 1. Introduction

Nanobioelectronics is a cornerstone of the silicon industry for developing new concept electronic components such as transistors, logic gates and microprocessors [1,2]. Among them, the information storage device has been developed using biomolecules. Several groups have attempted to fabricate several types of information storage devices [3-5]. Especially, the electrochemical bioelectronic devices were broadly proposed to develop field-effect transistors (FET), biosensors because of various functionalities, ease-of-fabrication and future applications [6,7].

Several groups have developed various bioelectronics devices; the Winfree group developed DNA-based programmable computing with applications [8,9]. The Yang group developed a virus-nanoparticle-based digital memory device [10]. The Smolke group suggested the RNA-based information processing system. [11]. The Willner group also suggested enzyme-based information processing [12]. The Cho group developed the metalloprotein-nanoparticle based resistive charge storage system that shows excellent electrical resistive property for future memory devices [13].

Since 2000, Choi's group has focused on a biomolecule-based apparatus which mimics the natural characteristics of a biological organism in inorganic devices. In an initial stage, they suggested protein-based photodiode and visual information controller [14-16]. After that, they developed a metalloprotein-based electron charge system using redox property of metalloproteins [17]. Also, the metalloprotein can be modified using DNA recombinant technique for introducing additional functional group such as thiol group. Several types of information storage devices have been developed, for example, a multi-level biomemory, a multi-functional biomemory and a DNA-based WRER-type biomemory [18-20]. Recently, they developed a bioprocessing device consisting of protein-DNA-nanoparticles and RNA-nanoparticle-based resistive memory device [21]. Like this, to fabricate the various bioelectronics devices, fabricating the protein-nanoparticle heterolayer is an essential technique.

Hemoglobin is a well-known metalloprotein. It has the four electroactive ferrous ions ( $\text{Fe}^{2+}$ ) in a heme group; the heme groups are oxidized to ferric ions ( $\text{Fe}^{3+}$ ) that provide the redox property. The hemoglobin has been widely investigated in the life sciences, medicine and nanobiotechnology [22,23]. In particular, the electron transfer property of hemoglobin is very interesting for biosensor and bioelectronic device applications. If the electron transfer facilitation can be enhanced by additional extra nanomaterials, this can be very effective for constructing the charge storage device. The silver nanoparticle is suitable to facilitate electron transfer because of biocompatibility, cheap cost and ease of preparation. In this study, we fabricated the

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‡This article is dedicated to Prof. Choon Han on the occasion of his retirement from Kwangwoon University.

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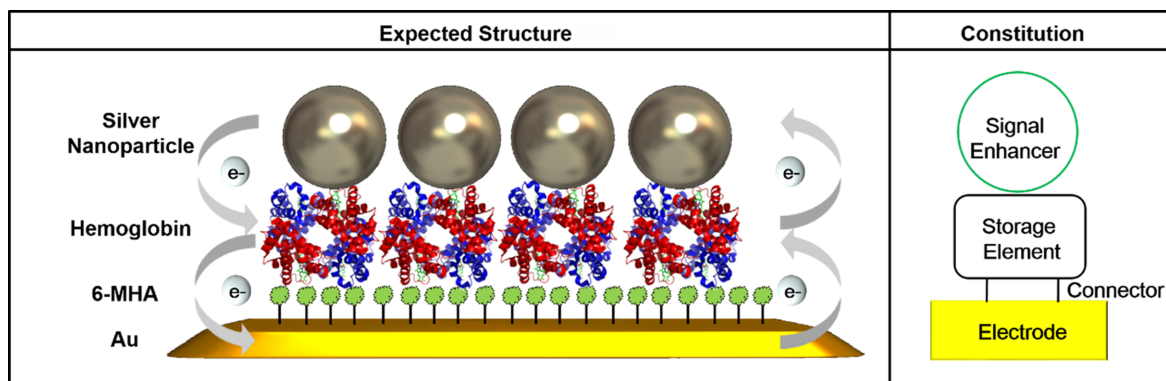


Fig. 1. Schematic diagram of Hemoglobin/Silver Nanoparticle Heterolayer.

hemoglobin/silver nanoparticle heterolayer for confirming the electrochemical signal-enhanced charge storage property. The hemoglobin was self-assembled onto the Au substrate through additional linker 6-Mercaptohexanoic acid (6-MHA). Then, the silver nanoparticle was immobilized onto hemoglobin-modified substrate using layer-by-layer (LbL) technique. The prepared hemoglobin/silver nanoparticle heterolayer was investigated by atomic force microscopy (AFM). The surface roughness analysis was carried out to confirm the surface properties. Furthermore, the redox properties of hemoglobin and silver nanoparticle/hemoglobin were monitored by cyclic voltammetry (CV). The charge storage property of hemoglobin and silver nanoparticle/hemoglobin was assessed by chronoamperometry (CA). Fig. 1 shows the schematic diagram of fabricated hemoglobin/silver nanoparticle immobilized on the Au substrate. The following sections describe the preparation of hemoglobin/silver nanoparticle heterolayer and its characteristics.

## 2. Experimental Details

### 2-1. Materials

To fabricate the bioelectronic device electrode, Au substrate [Au (43 nm)/Cr (2 nm)/SiO<sub>2</sub> (200 nm) Si (p-type) wafers] was manufactured as the working electrode (G-MEK, South Korea). For the electrochemical experiments and charge storage test, Pt wire and the Ag/AgCl reference electrode were purchased from BAS (USA) for 3 electrode electrochemical system. The hemoglobin was extracted from *horse heart*, 6-Mercaptohexanoic acid (6-MHA) and obtained from Sigma-Aldrich (USA). Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) was obtained from Thermo-Fisher Scientific (USA). A 10  $\mu$ M of hemoglobin solution was diluted in 10 mM PBS buffer at pH 7.4. The silver nanoparticle (20 nm) was obtained from BBI (UK).

### 2-2. Fabrication of hemoglobin/silver nanoparticle heterolayer

For the electrode preparation, Au substrates were washed by piranha solution (H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>SO<sub>4</sub>, 3:7 composition) at 70 °C for 3 min to remove the residues on the surface of substrates. Then, the sub-

strates were rinsed with ethanol, DI water, respectively, and dried by N<sub>2</sub> gas. The prepared 1 mM of the 6-MHA solution (20  $\mu$ l) was dropped onto the Au surface for connection between hemoglobin and Au substrate for 12 hrs at 4 °C. The head group (thiol group) of 6-MHA was bound to Au substrate through the covalent bonding. Continuously, the prepared 10  $\mu$ M of the hemoglobin solution (20  $\mu$ l) was dropped onto the 6-MHA-modified Au surface through interaction between tail group (carboxylic acid) and amine group of hemoglobin by EDC/NHS reaction for 6 hrs. The modified substrates were cleaned with deionized water and dried under N<sub>2</sub> gas stream. Then, 1 mM solution of the Sulfo-SMCC solution was immersed in prepared substrate for 6 hrs. Next, 0.2 mg/ml of silver nanoparticle solution (20  $\mu$ l) was added onto the substrate for 12 hrs. Finally, the modified substrates were washed with DI water and dried under N<sub>2</sub> gas flow. The fabrication process was carried out in a humidity chamber at 25 °C [24].

### 2-3. Surface morphology investigation of hemoglobin/silver nanoparticle heterolayer using AFM

To confirm the immobilization of hemoglobin/silver nanoparticle heterolayer, the surface morphologies of hemoglobin monolayer, hemoglobin/silver nanoparticle heterolayer were investigated using AFM. The AFM experiment was conducted using a Nanoscope IV / Multimode (Bruker, USA). The AFM tip was purchased from Bruker (USA). The tip was composed of phosphorous (n-type doped Si). Spring constants of 30~80 N/m were used. Before scanning the sample, the auto set point and gain values were adjusted to optimize the force between the tip and the surface [18].

### 2-4. Electrochemical analysis of hemoglobin-silver nanoparticle heterolayer

Electrochemical experiments and charge storage validation test of hemoglobin/silver nanoparticle heterolayer were confirmed using a CHI660A electrochemical workstation (CH Instruments, USA). The electrochemical analysis was performed in 10 mM PBS buffer solution (pH = 7.4) at room temperature [16]. All electrochemical experiments were repeated with five samples.

### 3. Results and Discussion

#### 3-1. Investigation of hemoglobin-silver nanoparticle heterolayer formation

To confirm the fabrication of hemoglobin-silver nanoparticle heterolayer on the Au substrate, AFM was operated for the surface morphology investigation. Fig. 2 shows the AFM results of hemoglobin-silver nanoparticle heterolayer and hemoglobin monolayer. Fig. 2(a) shows the surface morphology of hemoglobin monolayer which exhibits the immobilized hemoglobin with around 15 nm diameter and small aggregated circular shapes. Compared to the surface morphology of hemoglobin monolayer, AFM result of fabricated hemoglobin/silver nanoparticle heterolayer is shown in Fig. 2(b). There existed a hemoglobin-silver nanoparticle heterolayer with around 30 nm size diameter and some irregular aggregated shapes which were bigger than the result of hemoglobin monolayer on average. This result was induced by the introduction of silver nanoparticles on hemoglobin monolayer. Also, Fig. 2(c) exhibits the roughness analysis of fabricated biofilms. Roughness average (Ra), RMS roughness (Rq) and maximum height (Rmax) values of hemoglobin monolayer were  $0.488 \pm 0.175$  nm,  $1.687 \pm 0.439$  nm and  $2.436 \pm 1.285$  nm, respectively. In the case of hemoglobin/silver nanoparticle heterolayer, those values were  $1.476 \pm 0.302$  nm,  $4.874 \pm 1.238$  nm and  $3.974 \pm 0.671$  nm, respectively. By comparison of Ra, Rq and Rmax values of hemoglobin monolayer and hemoglobin/silver nanoparticle heterolayer, hemoglobin/silver nanoparticle heterolayer showed the increased roughness due to the immobilization of silver nanoparticle on the hemoglobin monolayer. Based on the AFM results, the fabrication of hemoglobin/silver nanoparticle heterolayer self-assembled on the Au substrate via 6-MHA was confirmed well.

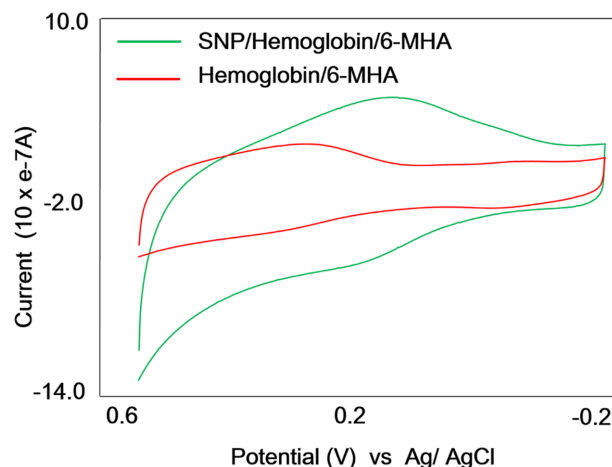


Fig. 3. Cyclic voltammogram of hemoglobin (Red line) and hemoglobin/silver nanoparticle (Green line) self-assembled on 6-MHA layer-modified Au substrate, respectively.

#### 3-2. Electrochemical investigation of hemoglobin-silver nanoparticle heterolayer

The electrochemical property of fabricated hemoglobin-silver nanoparticle heterolayer was investigated by CV. Fig. 3 shows the cyclic voltammogram of hemoglobin monolayer and hemoglobin/silver nanoparticle heterolayer. The cyclic voltammogram showed the reduction and the oxidation potential peak of the fabricated films. The reduction and the oxidation potential values of hemoglobin monolayer were  $320 \pm 18$  mV and  $375 \pm 31$  mV, respectively. Also, those values for hemoglobin-silver nanoparticle heterolayer were  $173 \pm 27$  mV and  $251 \pm 32$  mV, respectively. The cyclic voltammogram showed that hemoglobin/silver nanoparticle heterolayer exhibited the electrochemical signal amplified currents compared to the results of hemoglobin monolayer. The amplification of the electrochemical signal was induced by immobi-

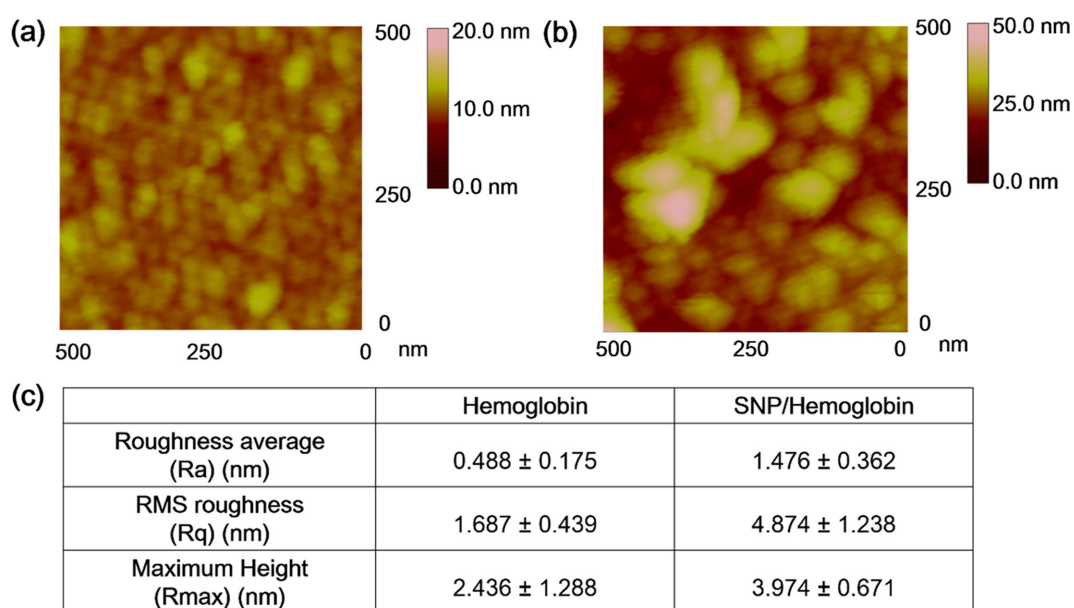
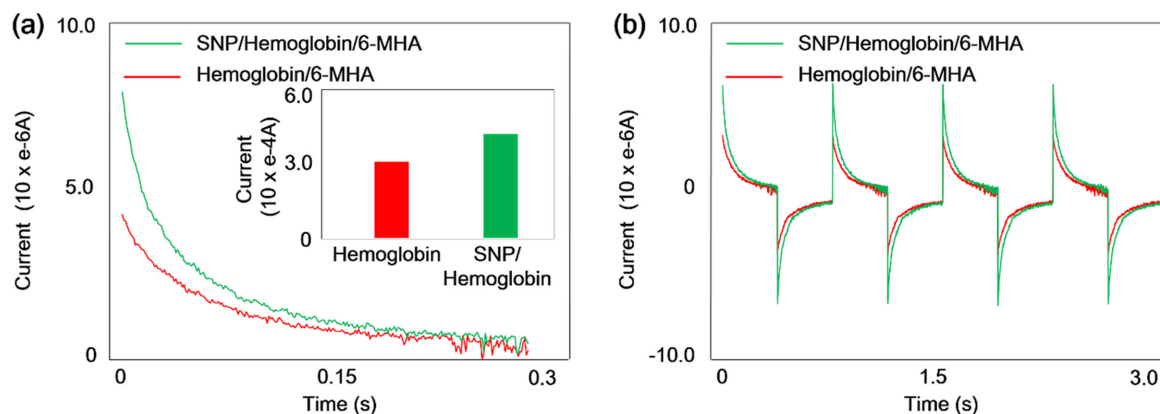


Fig. 2. AFM images of (a) Hemoglobin self-assembled on 6-MHA layer, (b) Silver nanoparticle/Hemoglobin self-assembled on 6-MHA layer, (c) Surface roughness analysis of each biofilm.



**Fig. 4. (a) Confirmation of charge storage properties. The current response curve corresponding to the applied oxidation potentials (Red line: hemoglobin, Green line: silver nanoparticle/hemoglobin), inset figure shows the charged current value of each electrode. (b) The current response corresponding to the applied potential series for a total duration for 4 cycles.**

lization of silver nanoparticle. The silver nanoparticle facilitated the electron transfer rate of the fabricated heterolayer biofilm due to the increment of the electron coupling effect between hemoglobin and the Au substrate. Presumably, the hemoglobin/silver nanoparticle heterolayer showed the amplified electrochemical signals compared to the hemoglobin monolayer prepared without silver nanoparticle.

### 3-3. Charge storage investigation of hemoglobin-silver nanoparticle heterolayer for biomemory application

To investigate the charge storage function of hemoglobin/silver nanoparticle heterolayer on the Au substrate, CA was done for biomemory application. For validating charge storage function, the reduction potential value and the oxidation potential value acquired from CV were used as the parameters to operate CA technique. The reduction potential of 320 mV and the oxidation potential of 375 mV obtained from CV were applied for hemoglobin monolayer. Like this, the reduction potential of 173 mV and the oxidation potential of 251 mV were applied to hemoglobin/silver nanoparticle heterolayer-modified electrode. Fig. 4 shows the charge storage properties of the fabricated biofilms. In Fig. 4(a), CA graphs of hemoglobin-silver nanoparticle heterolayer and hemoglobin monolayer from 0 sec to 0.3 sec are shown and charged current values of them are shown in the inserted Fig 4(a). To estimate the charged current value for charge storage function, the formula of ' $Q = \int i \cdot dt$ ' was used. Based on the formula, the area under the acquired graph was calculated to obtain the charged current value of each biofilm. During 0.3 sec, CA graph of hemoglobin/silver nanoparticle heterolayer showed the amplified electrochemical signal compared to the result of hemoglobin monolayer. Therefore, the calculated charge current value of hemoglobin/silver nanoparticle heterolayer  $4.34 \times 10^{-4}$  A was higher than the value of hemoglobin monolayer  $3.18 \times 10^{-4}$  A. That indicates the silver nanoparticle facilitates the charge transfer between hemoglobin and Au substrate around 136% more. Also, the hemoglobin/silver nanoparticle heterolayer showed a repetitive current response by applied potentials for a total duration for 4 cycles with amplified electrochemical signal compared

to the hemoglobin monolayer fabricated without silver nanoparticle. From CA results, the charge storage property of hemoglobin/silver nanoparticle heterolayer for bioelectronic device application was verified well.

## 4. Conclusion

A hemoglobin/silver nanoparticle heterolayer was fabricated with the electrochemical signal-enhancement effect for bioelectronic device application. For this purpose, the hemoglobin was immobilized through 6-MHA chemical linker on the Au substrate. After that, the silver nanoparticle was immobilized onto hemoglobin-modified electrode. The heterolayer fabrication was confirmed by AFM and surface analysis well. AFM results showed that each hemoglobin, hemoglobin/silver nanoparticle heterolayer was well organized. Moreover, the electrochemical property of hemoglobin/silver nanoparticle heterolayer was well investigated by CV. CA experiment showed the increased charge storage values of hemoglobin/silver nanoparticle heterolayer compared to hemoglobin monolayer around 136%. Based on the electrochemical experiments, the hemoglobin/silver nanoparticle heterolayer can be used for bioelectronic device applications such as biomemory, bioprocessing devices and biosensor applications in the near future.

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