Electrochemical reconstitution of biomolecules for applications as electrocatalysts for the bionanofuel cell

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ABSTRACT

Platinum-cored ferritins were synthesized as electrocatalysts by electrochemical biomineralization of immobilized apoferritin with platinum. The platinum cored ferritin was fabricated by exposing the immobilized apoferritin to platinum ions at a reduction potential. On the platinum-cored ferritin, oxygen is reduced to water with four protons and four electrons generated from the anode. The ferritin acts as a nano-scale template, a biocompatible cage, and a separator between the nanoparticles. This results in a smaller catalyst loading of the electrodes for fuel cells or other electrochemical devices. In addition, the catalytic activity of the ferritin-stabilized platinum nanoparticles is enhanced by the large surface area and particle size phenomena. The work presented herein details the immobilization of ferritin with various surface modifications, the electrochemical biomineralization of ferritin with different inorganic cores, and the fabrication of self-assembled 2-D arrays with thiolated ferritin.

Keywords: Biofuel cell, ferritin, immobilization, site-specific reconstitution, electrochemical biomineralization

1. INTRODUCTION

In the most widely used methanol-O_2 fuel cell, methanol is oxidized to CO_2 at the anode under a reduction potential and oxygen is reduced to water at the cathode under an oxidation potential.[1,2] The anode and cathode compartments of most fuel cells are separated by an ion exchange membrane, such as Nafion, because the electro-oxidation products coming off the anode poison the metallic platinum electrocatalysts of the cathode. The performance of other biofuel cells[3-9] is limited by a small cell potential and a low power density due to their slow reaction rate. These existing biofuel cells barely operate under physiological conditions or only in living organisms that are sustained at a neutral pH level, because the electron transfer mediators function poorly with a very low potential. The known biofuel cells employ a complicated immobilization method using a long chain cross linker between the electroactive biomolecules and the electron transfer mediator. Thus, the power density and cell potential are very poor due to the slow electron transport through the long chains. Therefore, the immobilization of biomolecules on a substrate by a short chain linker is one of the key issues for the enhancement of biofuel cell performance.

The immobilization of biomolecules on electrode surfaces is of great importance and interest in research areas for biosensors[10-11] and bioelectronics[12,13] applications. The ferritins used in this work are natural iron storage proteins that present a high degree of structural similarity across a wide range of biological species.[14] The ferritin molecule is composed of 24 organic subunits that build a segmented hollow protein shell with an outer diameter of 12.5 nm and an inner diameter of 7.5 nm. The mineral core of naturally existing ferritins is composed of an antiferromagnetic iron oxide (ferrihydrite) within its hollow and spherical protein interior. The assembled structure of ferritin is remarkably stable and robust, and able to withstand biological extremes of high temperature (up to 70 °C) and wide pH variations (2.0 ~ 10.0).[15] Ferritin proteins have hydrophobic and hydrophilic molecular channels through the protein shell, which enables the removal of the inorganic phase *in vitro* by reductive dissolution. The reconstitution of ferrihydrite cores into a ferritin protein cage proceeds through remineralization of apoferritin by Fe^{2+} oxidation usually with O_2 as the oxidant. A synthesis of ferrimagnetic ferritin (γ-Fe_2O_3) was prepared by a chemical remineralization procedure[16,17] using H_2O_2 as the oxidant in 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid (AMPSO) buffer (pH 8.5) at 65 °C under N_2 gas. Biomineralization of some other

metals into the ferritin cavity has been reported. Cobalt[18] can be accumulated as a cobalt oxyhydroxide (CoOOH) state within the core with the aid of H_2O_2. Manganese[19-21] can also be reconstituted in the cavity as manganese oxyhydroxide (MnOOH) by natural oxidation. Nickel[22] can be encaged as nickel hydroxide ($Ni(OH)_2$) using a hydroxylation process of nickel ion solutions that contain dissolved CO_2 while precisely controlling the pH. Wong et al. demonstrated the magnetic transition from superparamagnetic to ferromagnetic CoPt cored ferritin.[23,24] Tetrachloroplatinated anions can be moved into the ferritin core and converted to Pt nanoparticles by chemical reduction. The electrochemical behavior of physically-adsorbed ferritin molecules on indium-tin oxide (ITO) glass was studied via cyclic voltammetry.[25,26] Zapien et al. showed that ferritins in a solution were well adsorbed onto an ITO surface at a controlled electrode potential and could be electrochemically induced to release the iron core without the need for a reducing agent.

The metal-cored biomolecules, acting as both the electrocatalysts and the electron transfer mediators, are properly modified via biomineralization techniques and are immobilized with short cross linkers. Enzymes or whole cells can be used as a biocatalyst for biofuel cells through two different routes, such as glucose oxidation or oxygen reduction. In this work, we focused on a platinum-cored ferritin used as the cathode for a ferritin-based biofuel cell. The electrode surface was modified to ensure a strong and stable adhesion of ferritin molecules and to reconstitute the core material with another metal. An electrochemically-controlled site-specific biomineralization was demonstrated through the immobilization of biomolecules on a substrate to produce biologically-derived nanoparticle arrays. We used the electrochemical biomineralization technique for the direct reconstitution of immobilized ferritins on a gold electrode.

2. EXPERIMENTAL

2.1 Immobilization of ferritins

Biologically derived nanoparticles can be fabricated from the electrochemically-controlled biomineralization of immobilized ferritin. This process is very important to precisely position ferritin molecules on conducting substrates (Au, Pt, Ag, ITO, etc.). The immobilization of ferritins is achieved by modifying the substrate with thiol-derivatized molecules such as dithiobis-N-succinimidyl propionate (DTSP) and alkanethiols, whereupon the ferritin molecules react with the modified substrate. This method has two approaches using different substrate modifiers, but results in the same reaction product. One uses a DTSP reaction and another uses a 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) coupling reaction. The DTSP is used as a protein cross-linking reagent through the acylation of free primary or secondary amino groups.[27] The DTSP is also adsorbed onto gold surfaces through the disulfide group, so that the terminal succinimidyl groups are available to react with amino-containing biomolecules.[28] The immobilization procedure begins with a cleaned polycrystalline gold electrode immersed in a 5 mM DTSP solution in dioxane (ACS grade) for 1 hr at room temperature. Then the DTSP-modified electrode is thoroughly rinsed with acetone and finally with the 25 mM of N-[2-hydroxyethyly] piperazine-N'-[2-ethanesulfonic acid] (HEPES, $C_8H_{18}N_2O_4S$, 99.5 %) buffer (pH 7.3). After preparation of the DTSP-modified electrode, it is immediately submerged in 50 mM HEPES/NaCl buffer solutions (pH 7.3) containing 2 mg/ml ferritin (Type I from horse spleen, 77 mg/ml) molecules. The electrode is immersed into the ferritin solution for 18 hr at 4 ^oC and then rinsed with 25 mM HEPES buffer.

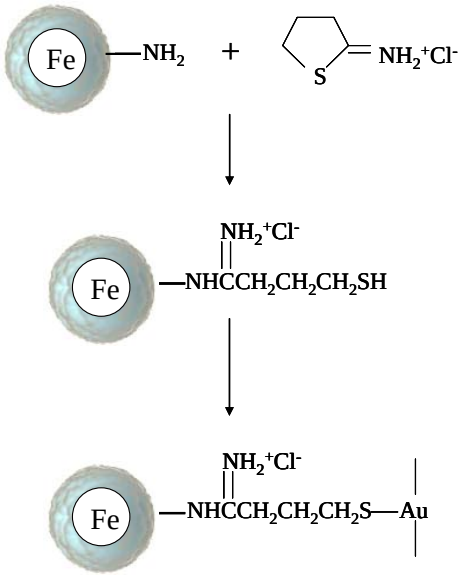


Figure 1. Schematic diagram of ferritin thiolation.

2.2 Thiolation of ferritins

The procedure for ferritin thiolation is based upon a method described by Traut et al.[29,30] With proper modifications the free thiol groups are introduced to the gold surface for immobilizing the protein molecule. First, a buffer solution was prepared (triethanolamine hydrochloride buffer) consisting of triethanolamine hydrochloride (50 mM, $C_6H_{15}NO_3 \cdot HCl$, 99.5 %), KCl (50 mM, 99.999 %), and $MgCl_2$ (1 mM, 98 %). Second, a stock solution was prepared immediately before use, consisting of 2-iminothiolane (0.5 M) triethanolamine hydrochloride (1.0 M), and triethanolamine (1.0 M, 98 %), with a pH of 8.0. The reaction

mixture was prepared as follows: 77.73 µl of ferritin (77 mg/ml), 200 µl of 2-mercaptoethanol (98 %), and 24 µl of the stock solution were placed in a vial and diluted to 20 ml with triethanolamine hydrochloride buffer. The reaction was then incubated for 20 min at 0 ^{o}C. After completion, the reaction was quenched by reducing the pH to 4, and the excess reagent was removed by dialysis against 100 volumes of triethanolamine hydrochloride buffer at 4 ^{o}C for 4hr. Then the mixture was diluted by a factor of 100 in phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$, pH 7.8). Inserting an Au substrate into the solution for 18 hr completed the adsorption of the thiolated ferritin.

2.3 Electrochemical measurements and spectroscopic analysis

Electrochemical biomineralization of immobilized ferritins was carried out in a 0.05 M phosphate buffer (pH 7.5) solution either with or without a chelating agent of EDTA at room temperature. Cyclic voltammograms (CVs) were recorded using a BAS model 100B/W potentiostat. Each solution was purged with nitrogen for 10 min before the acquisition of the CV measurements. A BAS stationary voltammetry Au electrode was prepared as working electrode, according to the above immobilization methods. The Ag/AgCl (in 3 M NaCl) electrode was used as reference electrode. The counter electrode was a spiral platinum wire.

Field emission-scanning electron microscopy (FE-SEM) and energy dispersive spectroscopy (EDS) analyses were carried out on a Hitachi S-5200 on the immobilized ferritin layers and the electrochemically reconstituted Pt-cored ferritin. The immobilized ferritins layer was thoroughly rinsed with 25 mM HEPES buffer and doubly distilled, deionized water, dried in a nitrogen atmosphere, and then subjected to the microscopic analysis.

3. RESULTS AND DISCUSSION

3.1 Design of bionanofuel cells

The ferritin-based biofuel cell is to convert the chemical energy in molecules such as glucose, alcohols, and oxygen into electrical energy. The oxidation of glucose at an electroactive enzyme of flavine adenine dinucleotide (FAD, chemical composition: $C_{27}H_{33}N_9O_{15}P_2$) in the glucose oxidase (GOx) protein occurs at anode of the biofuel cell (see Fig. 2). In the reduction half reaction, glucose is oxidized to gluconolactone and two protons and two electrons are transported to GOx under physiological conditions. Through the sequential immobilization processes, ferritin and GOx are immobilized on the electrode surface. This provides for an electron cascade at the anode in the direction of glucose → GOx → ferritin → current collector. Meanwhile, the cathode process includes the oxygen reduction to water on the platinum-cored ferritin (see Fig. 2). Electrons generated from anode are transported from the current collector to oxygen via platinum-cored ferritin. Figure 2 shows a schematic diagram of whole ferritin-based biofuel cell system[31,32] proposed here with the electron transfer direction and redox cycles of the biomolecules. The overall reaction is represented by

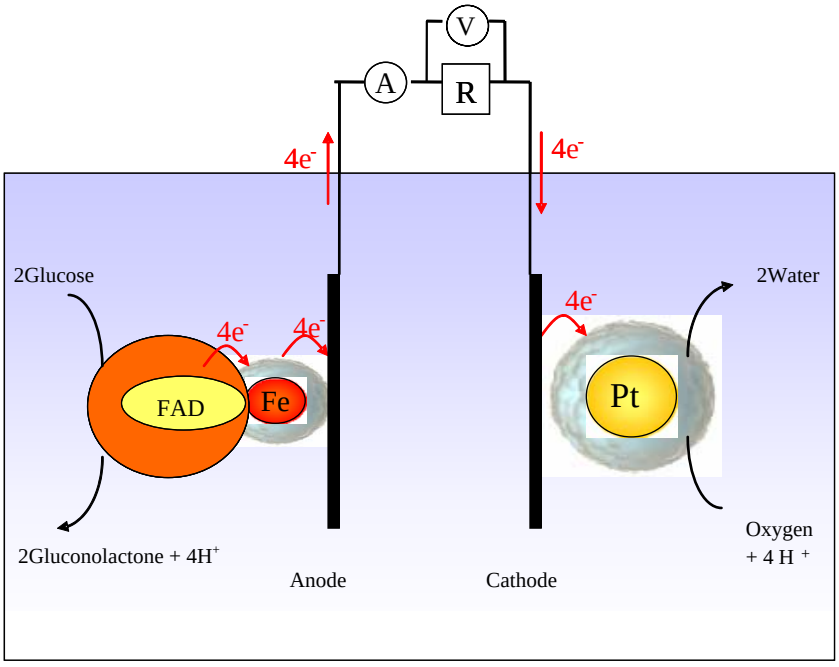


Figure 2. Schematic diagram of ferritin-based fuel cell. The two electrodes, immobilized with a cross linker, reside in the same solution under physiological conditions.

$$\beta - D - glu\cos e \rightarrow \delta - gluconolac\,tone + 2H^+ + 2e^-$$

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

$$2\beta - D - glu\cos e + O_2 \rightarrow 2\delta - gluconolac\,tone + 2H_2O.$$

The repeated generation of Fe(III)/Fe(II) and GOx(ox)/GOx(red) redox couples is used as an electrocatalyst for the electro-oxidation of glucose to gluconolactone at the anode while platinum-cored ferritin is used as an electrocatalyst for the reduction of oxygen to water at the cathode under physiological conditions. The iron-cored ferritin is used as an electron transfer mediator through the Fe(III) (FeOOH)/Fe(II) ($Fe(OH)_2$) redox couple having an equilibrium potential of – 0.31 V (vs. NHE). Oxygen is reduced to water with four protons and four electrons generated from the anode, thus having an equilibrium potential of 1.23 V (vs. NHE). So, the ferritin-based biofuel cell theoretically has a cell potential of 1.54 V (vs. NHE) at pH 7.0.

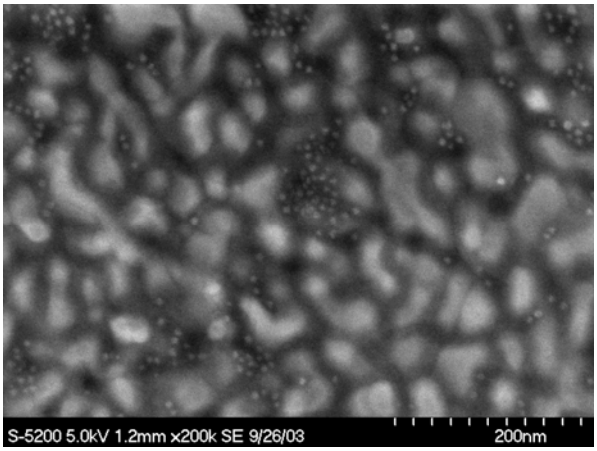


Figure 3. FE-SEM image of immobilized ferritin on a DTSP-modified Au electrode.

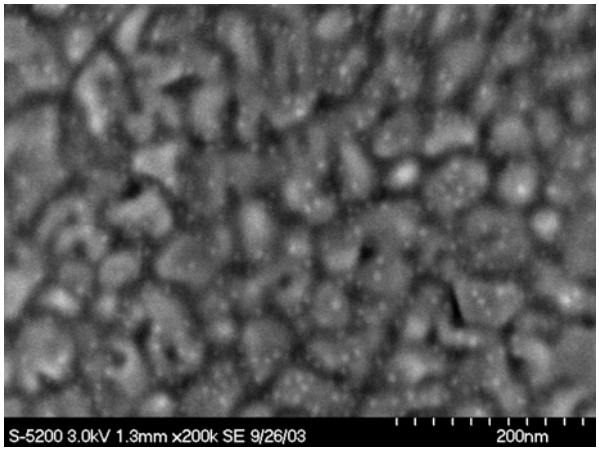


Figure 4. FE-SEM image of physically adsorbed ferritin on a Au electrode.

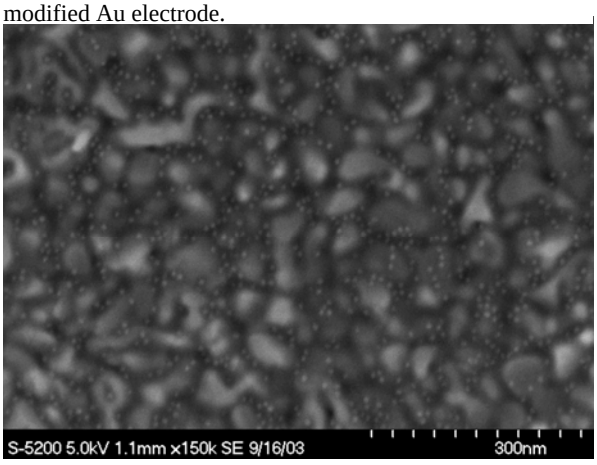


Figure 5. FE-SEM image of immobilized ferritins on a DTSP and MPA-modified Au. The mixed SAM is adsorbed on Au for 1 hr at room temperature. The DTSP and MPA-modified Au is then immediately inserted into the ferritin solution for 18 hr at 4 ^oC.

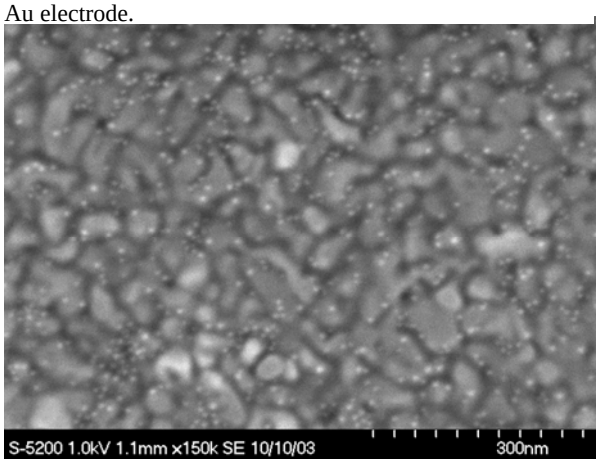


Figure 6. FE-SEM image of immobilized ferritins on a DTSP and MHA-modified Au. The mixed SAM is adsorbed on Au for 1 hr at room temperature. The DTSP and MHA-modified Au is then immediately inserted into the ferritin solution for 18 hr at 4 ^oC.

3.2 Surface characterization of immobilized ferritins and fabrication of 2-D arrays

Immobilization of the electron transfer mediator between an electrocatalyst and the current collector is one of the key issues of ferritin-based biofuel cell. Figure 3 shows a FE-SEM image of ferritins immobilized on a DTSP modified Au electrode. Immobilized ferritin molecules are well separated from each other on the Au electrode. Single molecules of ferritin were isolated with a sphere-like protein shell of 12 nm diameter. The electrode surface coverage was about 20 % showing preferential deposition at the high surface energy grain boundaries. The

physically adsorbed ferritins are well adsorbed across the entire gold electrode with coverage of more than 60 % as shown in Fig. 4. A mixed self-assembled monolayer (SAM) of DTSP and 3-mercaptopropanoic acid (MPA, 95 %) or 6-mercaptohexanoic acid (MHA, 97 %) allowed for the preparation of an isolated ferritin layer across the entire Au surface as shown in Figs. 5 and 6, respectively. The electrode was kept in the 1 mM DTSP solution containing either 10 mM MPA or (10 mM MHA) in dioxane for 1 hr at room temperature. Immobilization of ferritin was achieved by immersion of the mixed SAM-modified electrode in the ferritin solution for 18 hr at 4 ^{o}C temperatures. MPA has the same chain length compared to DTSP after reaction with ferritin while MHA has a similar chain length to the anchored DTSP with a succinimidyl group. The ferritin molecules immobilized on the DTSP- and MHA-modified electrodes have the highest population after the dipping for 2 hr in ferritin solution at room temperature (Fig. 7). The reaction rate between the ferritin and DTSP increases with the rise of reaction temperature. Another immobilization method using a Au surface modification is to use an EDC coupling reaction between the carboxyl terminal groups of a pretreated mercaptopropanol SAM and the amino groups of the ferritin shell. All of these reactions are carried out in 4-morpholinoethanesulfonic acid (MES) buffer solutions between pH 4.5 to 5.0 reacting with N-hydroxysuccinimide (NHS) molecules. The same results can be obtained from immobilized ferritin prepared by the DTSP modification with three carbon chains.

Fabrication of 2-D arrays of ferritins is achieved by the modification of the ferritin shell with thiol terminal groups. Adsorption of the thiolated ferritins was performed by inserting a Au substrate into the solution for 18 hr. Figure 8 shows the FE-SEM image of thiolated ferritins on a Au surface. The thiolated ferritin SAM shows a highly packed and well-dispersed monolayer of ferritin on across the entire Au electrode.

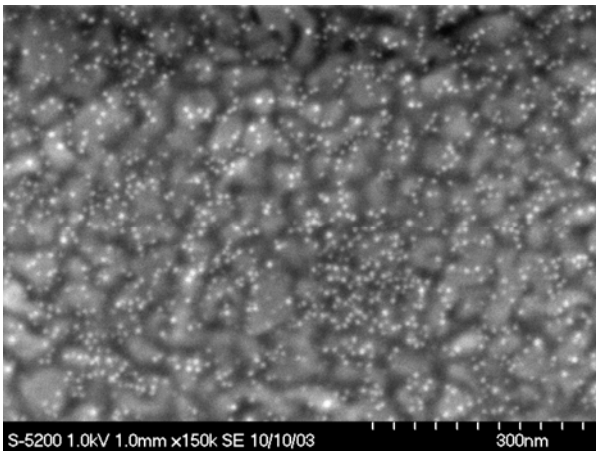


Figure 7. FE-SEM image of immobilized ferritins on a DTSP and MHA-modified Au. The mixed SAM is adsorbed on Au for 18 hr at room temperature. The DTSP and MHA-modified Au is then immediately inserted into the ferritin solution for 2 hr at room temperature.

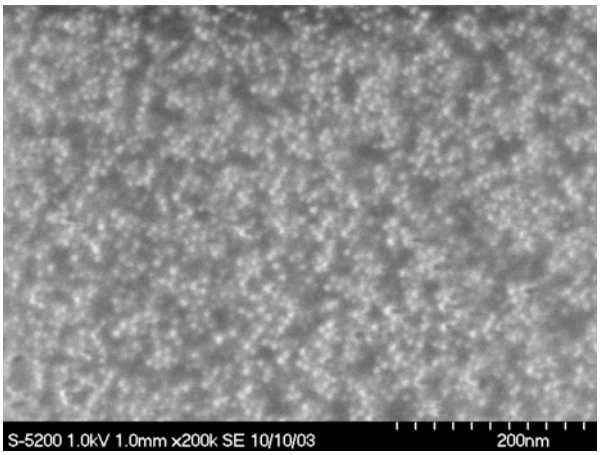


Figure 8. FE-SEM image of thiolated ferritin on a Au

3.3 Electrochemical biomineralization of ferritins with different inorganic cores

Figure 9 shows a schematic diagram of an electrochemical reconstitution procedure using immobilized ferritin electrodes. Immobilization of ferritins on a Au electrode is achieved by the methods based on DTSP and alkanethiols. The Fe-core inside ferritin is removed during a potential sweep at a given potential that is higher than the Fe(III) reduction potential in the presence of chelating agents such as bipyridine (bipy, 99 %) and ethylenediaminetetraacetic acid (EDTA, 99 %) bisodium salt. This electrochemical reconstitution produces the analogous product of immobilized apoferritins on DTSP-modified Au electrodes. The electrochemical biomineralization of ferritins is accomplished by a potential sweep or a constant potential step with a more negative potential than the metal ion reduction potential in metal ion solutions. Using this method, we can control the number of metal atoms deposited inside the ferritin by controlling the total charge passed and the valance of the target metal is determined by the electrolyte type and pH and the engaged potentials. In addition, this procedure allows for the electrochemical biomineralization with various metals and an easy reconstitution without maintaining the stringent conditions of an anaerobic environment with a precise control of pH and temperature. Electrochemical biomineralization of the immobilized ferritins was carried out in a 0.05 M phosphate buffer (pH 7.5) solution containing a chelating agent of EDTA at room temperature. The ferritin-immobilized Au electrode was prepared

following the ferritin immobilization procedure. A cleaned Au electrode was inserted into the DTSP and MPA solutions for 1 hr and then washed with acetone and HEPES buffer in succession. The mixed SAM-modified Au electrode was immersed into the ferritin solution for 18 hr at 4 ^{o}C to produce the ferritin immobilized Au electrode.

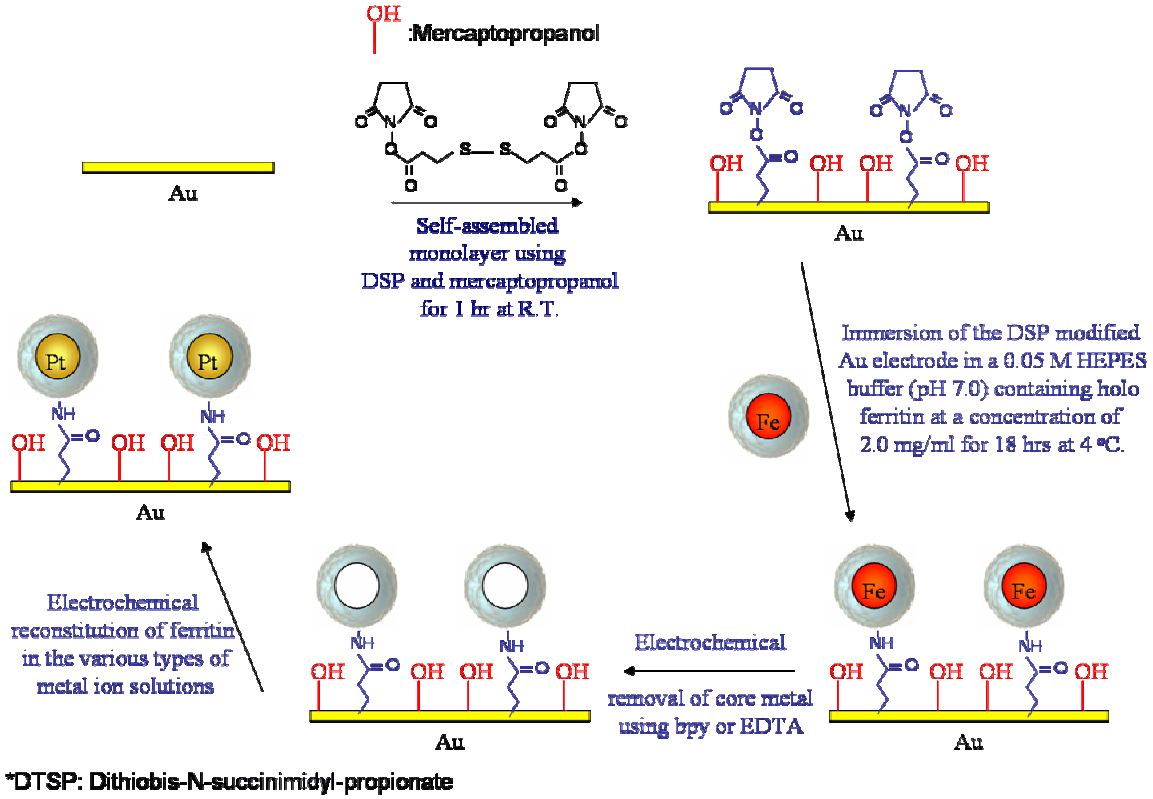


Figure 9. Schematic diagram on the electrochemical reconstitution of ferritin on immobilized ferritin

The electrochemical behavior of this electrode is shown in Fig. 10a. The electron transfer between the ferritin core metals and substrate occurred easily on the immobilized ferritin electrode. The reduction of Fe(III) occurred at – 0.65 V then gradually the current decreased depending on the number of cycles, but the oxidation of Fe(II) was not observed in the reverse potential scan. The results from the CVs are consistent with the fact that although Fe(II) is soluble in phosphate buffer, Fe(III) is not. This difference in solubility is particularly apparent when Fe(II) solutions are exposed to air. The rapid oxidation of Fe(II) to Fe(III) occurs at pH 7 by forming a white precipitate of $FePO_4$ within a few seconds, since the phosphate anions have a strong affinity for iron oxyhydroxide.[33] The ratio of phosphate to iron for mammalian ferritin is 10 % with phosphate possibly adsorbed on the surface of the iron oxyhydroxide. Thus, the reduced Fe(II) ions are released to the solution as free Fe(II) ions, followed by a reaction with phosphate ions to form the $FePO_4$ precipitate. Therefore, the oxidation current of Fe(II) is not reversible for the reverse scan. A new cathodic peak grew at – 0.3 V as the cycling repeated. This faradaic reaction is likely caused by the reduction of accumulated and/or diffused $FePO_4$ precipitate onto the substrate. The immobilized ferritin on a DTSP and MPA-modified Au electrode was inserted into a 0.05 M phosphate buffer containing 0.01 M EDTA and then electrochemically cycled (see Fig. 10b). In the first cycle, the reduction peak of Fe(III) to Fe(II) appeared at -0.65 V, the same result in the absence of EDTA. In the second cycle, the reduction process of Fe(III) disappeared because the released Fe(II) ions were released outside the ferritin shell and combined with EDTA, and the faradaic current did not appear at – 0.3 V. During this process, the state of immobilized ferritins was changed from holo to apoferritin. After thoroughly washing with 25 mM HEPES buffer, the immobilized ferritin, without the metal core, was immersed into a solution with the volume ratio of 3 to 1 of HEPES buffer (0.05 M) and $(NH_4)_2PtCl_4$ (0.05 M) for 30 min. Platinum anions likely entered the ferritin through the channels of the protein shell. The apoferritin

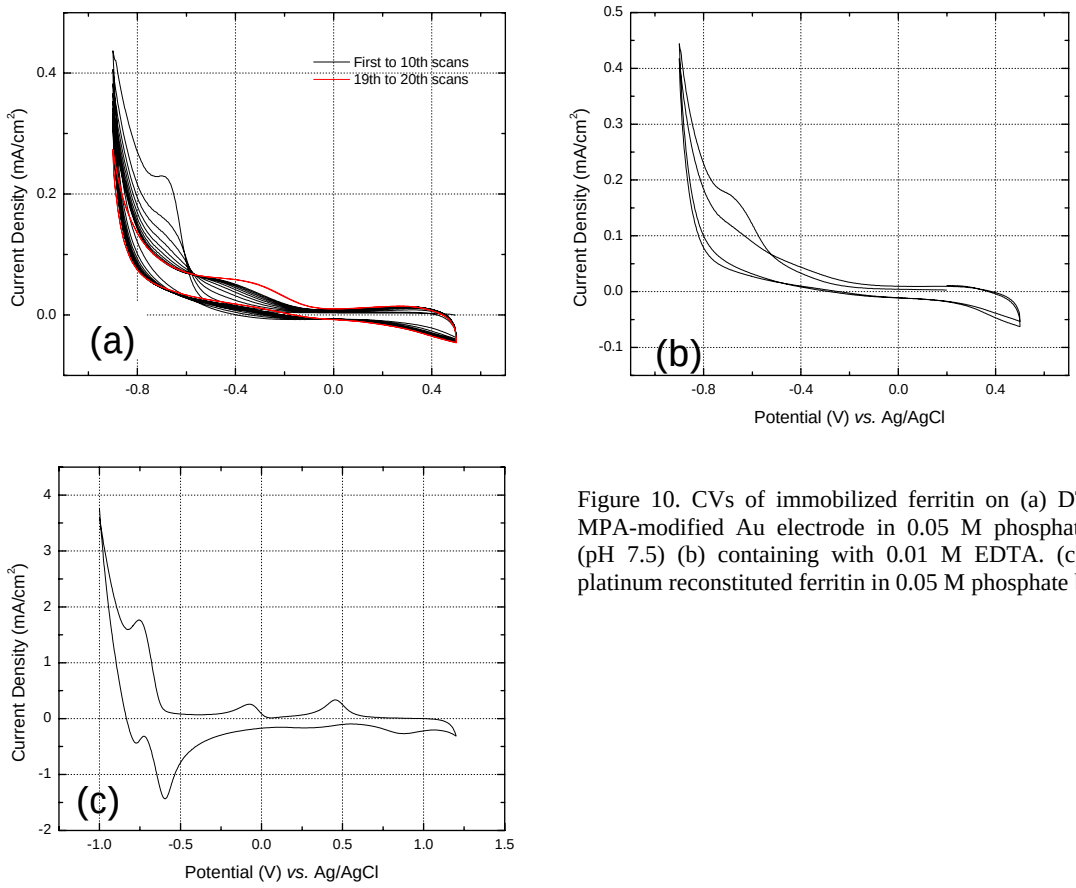
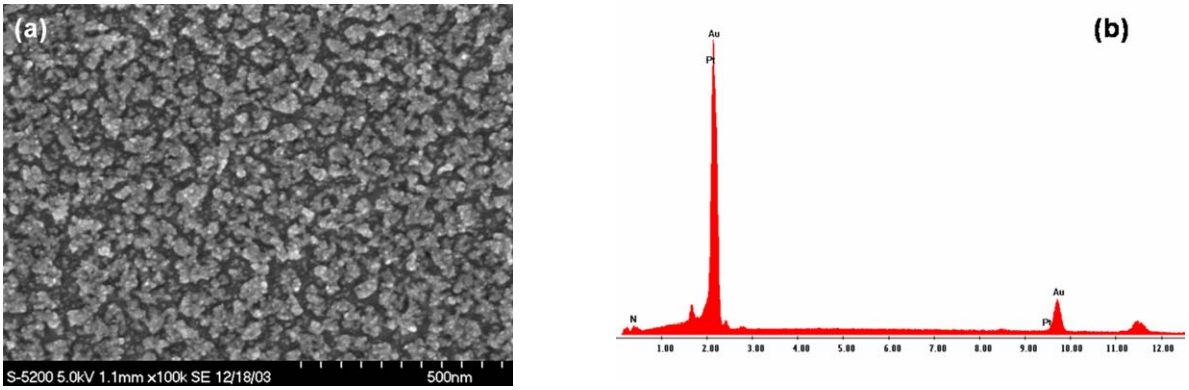


Figure 10. CVs of immobilized ferritin on (a) DTSP and MPA-modified Au electrode in 0.05 M phosphate buffer (pH 7.5) (b) containing with 0.01 M EDTA. (c) CV of platinum reconstituted ferritin in 0.05 M phosphate buffer.

containing platinum ion was washed with 0.025 M HEPES buffer several times and inserted into 0.05 M phosphate buffer again. Platinum ion is reduced through the potential sweep from 0.2 to – 1.0 V. The CV of platinum reconstituted ferritin in 0.05 M phosphate buffer (pH 7.5) is shown in Fig. 10c. New reduction and oxidation peaks were induced from Pt metal in the phosphate buffer. Two reduction peaks and the corresponding oxidation peaks at a more negative potential than – 0.5 V might be attributed to phosphate ion reduction and oxidation. The reduction of platinum oxide and gold oxide occurred at – 0.1 and 0.4 V, respectively.



(c)

Element	Wt. %	At. %
C K	3.09	29.52
N K	1.83	14.99
Pt M	10.35	6.09
Au M	84.74	49.41
Total	100	100

Figure 11. FE-SEM image and (b) EDS spectrum of Pt-cored ferritin on Au electrode surface and (c) the data table from EDS analysis of C K, N K, Pt M, and Au M energy. EDS spectrum obtained at 20 keV from apoferritin immobilized on the Au electrode after addition of $PtCl_4^{2-}$ for 30 min and then reduction with $NaBH_4$ for 10 min.

We also identified the platinum presence on the Au electrode surfaces by FE-SEM with EDS (see Fig. 11). Platinum exists on the electrode surface with about 6 at. % from analysis of Pt and Au M edge energy. In the natural state, the ferritin has negatively charged the surface. Therefore, the platinum anion is repelled from ferritin surface due to electrostatic repulsion. Nevertheless, the ferritin molecule might have different path ways and binding sites for anions in the ferritin molecule itself.

4. SUMMARY

The ferritin-based biofuel cell reported herein can be operated under physiological conditions due to the biocompatibility of the electrodes. Glucose oxidase and metal-cored ferritin are activated under a neutral pH and room temperature while conventional fuel cells operate under excessive pH levels and a high temperature due to the activation of electrocatalysts. Also, the fuel of the ferritin-based biofuel cell is glucose and oxygen. These are abundant biomass products in bio-organisms and living plants unlike existing energy sources such as fossil fuels and hydrogen. The Fe(III)/Fe(II) redox couple was used as an electron transfer mediator at the anode and the platinum-cored ferritin was used as an electrocatalyst for the reduction of oxygen to water at the cathode. The ferritin-based biofuel cell will have a high cell potential and output power due to the fast electron transfer through the metal-cored biomolecules and a fast reaction rate through the surface reaction of the nanoparticles. The platinum-cored ferritin used as the cathode consists of encapsulated nanoparticles having large surface areas. In this work, we demonstrated the modification of the electrode surface using immobilization and electrochemical biomineralization methods with various metal ion solutions without the concern of complicated chemical treatments such as extraction, dialysis, and column treatments for removing the unbind metal ions. This method may open up numerous possibilities for practical applications of immobilized ferritins such as high-density data storage devices, bioelectronic devices, and biosensors.

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