Electrochemical Properties of Honeycomb-like Structured HFBI

Self-Organized Membranes on HOPG Electrodes

Ryota Yamasaki^{1,2,3}, Yoshiyuki Takatsuji^{1,2,3}, Michael Lienemann⁴, Hitoshi Asakawa⁵,

Takeshi Fukuma⁵, Markus Linder^{4,6}, and Tetsuya Haruyama^{1,2,3*}

*1 Department of Biological Functions Engineering, Kyushu Institute of Technology,
Kitakyushu Science and Research Park, Fukuoka, 808-0196, Japan*

*2 Research center for Eco-fitting Technology, Kyushu Institute of Technology, Kitakyushu
Science and Research Park, Fukuoka, 808-0196, Japan*

*3 Advanced Catalytic Transformation program for Carbon utilization (ACT-C), Japan
Science and Technology Agency (JST), Tokyo, 102-0076, Japan*

4 VTT Biotechnology, Espoo, Finland

5 Kanazawa University, Ishikawa, 920-1192, Japan

6 Aalto University, School of Chemical Technology, Kemistintie 2, FI-00076, Espoo, Finland

*All correspondences should be addressed;

Tetsuya Haruyama, PhD Professor (Kyushu Institute of Technology)

Email: haruyama@life.kyutech.ac.jp

Acknowledgement

This research was partly supported financially by Japan Science and Technology Agency (JST), advanced catalytic transformation program for carbon utilization (ACT-C)

This research was partly supported financially by Japan Science and Technology Agency (JST), Japan-Finland Joint Research Program

ABSTRACT: HFBI (derived from *Trichoderma* sp.) is a unique structural protein, which forms a self-organized monolayer at both air/water interface and water/solid interfaces in accurate two-dimensional ordered structures. We have taken advantage of the unique functionality of HFBI as a molecular carrier for preparation of ordered molecular phase on solid substrate surfaces. The HFBI molecular carrier can easily form ordered structures; however, the dense molecular layers form an electrochemical barrier between the electrode and solution phase. In this study, the electrochemical properties of HFBI self-organized membrane-covered electrodes were investigated. Wild-type HFBI has balanced positive and negative charges on its surface. Highly oriented pyrolytic graphite (HOPG) electrodes coated with HFBI molecules were investigated electrochemically. To improve the electrochemical properties of this HFBI-coated electrode, the two types of HFBI variants, with oppositely charged surfaces, were prepared genetically. All three types of HFBI-coated HOPG electrode perform electron transfer between the electrode and solution phase through the dense HFBI molecular layer. This is because the HFBI self-organized membrane has a honeycomb-like structure, with penetrating holes. In the cases of HFBI variants, the oppositely charged HFBI membrane phases shown opposite electrochemical behaviors in electrochemical impedance spectroscopy. HFBI is a molecule with a unique structure, and can easily

form honeycomb-like structures on solid material surfaces such as electrodes. The molecular membrane phase can be used for electrochemical molecular interfaces.

1. Introduction

The functionalization of an electrode surface requires precise interface design at the nanoscale [1-3]. A precisely designed molecular layer on an electrode surface can provide various properties such as molecular and electrochemical functions. Proteins are divided into two types: structural proteins and functional proteins. Some structural proteins can form precise structures at air/water or water/solid interfaces by self-organization. These proteins can be used as molecular carriers for the formation of precise molecular structures on electrode surfaces. Most functional molecules, including proteins, have maximum activity under homogeneous conditions, e.g., in solution. However, under heterogeneous conditions, such as at an electrode/solution interface, the partly immobilized molecules cannot perform the required function, because the immobilized functional molecules oriented unevenly or their functions are deactivated. The use of a structural protein as a molecular carrier can immobilize functional molecules while maintaining high activity [4-6]. Extensive research has been performed on the use of electrodes modified with organic molecules [2,7,8].

Hydrophobins are small proteins produced by filamentous fungi [9-15]. The most characteristic feature of hydrophobin is that they self-organize at air/water or water/solid interfaces [16-18]. Their ability to self-organize at an air/water or

water/solid interface is based on their ability to form a protective coating on a cell wall on the fungal surface when growing into the air [19-20]. Hydrophobins are divided into two classes, on the basis of the hydropathic pattern of their amino acid sequences and their solubility properties [10,22]. We have investigated class II hydrophobin, HFBI, which is a superior interfacial self-organized protein. HFBI is used for high through-put nanostructure control or formulation at molecular interfaces. This protein originates from the cell walls of fungi and is a small amphiphilic protein [10]. The molecular mass of HFBI is about 7.5 kDa. A characteristic property of HFBI is adsorption to air/water interfaces, at which they form self-organized membranes. We found that HFBI can form nanostructured self-organized membranes at water/solid interfaces [23]. We also found that HFBI can be used as carrier to form nanostructured self-organized membranes at solid interfaces, because HFBI can be tagged with a protein of interest, using genetic engineering [23,24]. The ability of HFBI to modify surface properties by interfacial self-assembly and its high surface activity make it suitable for several potential applications [4]. Furthermore, HFBI has a number of charged amino acid on its surface. The HFBI surface charges can affect molecules in solution. We therefore prepared two types of point-mutated HFBI. These have predictable amino acid sequences on the surface, which were designed to provide different electrochemical behaviors.

In this study, highly oriented pyrolytic graphite (HOPG) electrodes coated with HFBI self-organized membranes were investigated electrochemically to clarify whether HFBI can be used as a molecular carrier to prepare molecular interfaces on electrodes. In the experiments, HOPG electrodes coated with wild-type HFBI [balanced charged (neutral) surface] and HFBI variant (negatively or positively charged surfaces) were investigated using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

2. Experimental

2.1. Reagents

Sodium phosphate, acetic acid, potassium hexacyanoferrate(III) $[\text{K}_3\text{Fe}(\text{CN})_6]$, and potassium hexacyanoferrate(II) trihydrate $[\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}]$ were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Hexammineruthenium(III) chlorides (99%; $[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$) were also purchased from Wako Pure Chemical Industries, Ltd. HOPG (10×10×1mm, Grade SPI-1) was purchased from Structure Probe, Inc. (PA, USA). Hydrophobin from *Trichoderma reesei* (HFBI wild type) was isolated and purified as previously reported [25]. Two types of point-mutated HFBI [R45Q/K50Q (HFBI-nega) and D40Q/D43N (HFBI-posi) variants] were prepared [26]. Details of these proteins are shown *Table 1*. HFBI wild type originally has three positive charged amino acids (D30, D40 and D43) and three negative charged amino acids (K32, R45 and K50). Four of them (D40, D43, R45 and K50) are located on the hydrophilic side surface of HFBI wild type (the surface to liquid phase when it is self-organized at the water/solid interface). The charge mutants of these amino acids may give rise to a drastic difference in the electrical behavior. $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and $[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$ with concentrations of 25mM were prepared. A 1mM acetate buffer (pH5.0) consisting of acetic acid and sodium acetate was used to dissolve proteins. A 0.1M phosphate buffer

(pH 7.0) consisting of Na_2PO_4 and NaH_2PO_4 was used as the supporting electrolyte. All the chemical reagents were dissolved in deionized, distilled water. All experiments were performed at room temperature, approximately 25 °C.

2.2. Preparation of self-organized HFBI protein

HFBI was prepared in two-phase system using a series of non-ionic surfactants with different characteristics. Most of the HFBI was associated with the fungal mycelium, and therefore preparative purification of HFBI was performed using mycelium pellet as the starting material [25]. The HFBI self-organize at air/water or water/solid interfaces to give an amphiphilic membrane.

2.3. Electrode modification with HFBI protein

The surface of an HOPG electrode (10 mm × 10 mm) was cleaved with Scotch tape. The electrode was used in an electrochemical device. An HFBI solution of concentration 15 μM was prepared and sonicated at 28 kHz for 1 min to disperse the protein in solution. The cleaved HOPG electrode was modified with this HFBI protein, using the drop-stamp method; details of this method are shown in Figure 1. The electrode was rinsed in deionized, distilled water. Other HFBI-modified electrodes were

prepared using the same method. The surface roughness of the HFBI/HOPG electrodes was observed using frequency modulation atomic force microscopy (FM-AFM).

2.4. AFM measurements

AFM is a measurement technique with subnanometer spatial resolution in the vertical and lateral directions [27]. The surface structures of the electrodes modified with HFBI self-organized membranes were examined using FM-AFM. In this FM technique, the cantilever serves as the frequency-determining element of a constant amplitude oscillator [28]. Therefore it can image organic materials with true molecular resolution, on both insulators and conductive surfaces [29]. Three types of electrode were prepared. Wild-type HFBI, and HFBI-nega and HFBI-posi variants were used to modify cleaved HOPG substrates using the Drop-stamp method. All the drop-state times were 15 min. These modified substrates were imaged in a liquid, and the measurements were performed in buffer (100mM phosphate buffer, pH 7.0); 100 μ L of the buffer was loaded on the substrate. In the liquid measurements, an NCH probe (42mN/m, Nanoworld) was used for imaging.

2.5. Electrochemical measurements

Electrochemical measurements were performed using an electrochemical polarizer (Hokuto Denko Co., Japan), with platinum wire as the counter electrode, Ag/AgCl wire as reference electrode, and HOPG as the working electrode. The electrochemical investigations were performed using a three-electrode system. The electrolyte was freshly prepared 0.1 M phosphate buffer (pH 7.0). All potentials were reported with respect to Ag/AgCl. CV and EIS were performed on the HOPG electrodes covered with HFBI or mutated HFBI in a 0.1 M phosphate buffer solution containing two types of redox molecule, i.e., $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and $[\text{Ru}(\text{NH}_3)_6]^{2+/3+}$. All the cyclic voltammograms of the electrodes were determined from the second sweep. Measurements were carried out at room temperature (25 °C). Impedance spectra in the range 10^5 – 10^{-1} Hz or 10^5 – 10^{-2} were recorded with a 10mV root-mean-square amplitude potential perturbation.

3. Results and discussion

3.1. AFM imaging of the protein layer

Cleaved HOPG substrates were coated with self-organized membranes with wild-type HFBI (neutral charged surface) and mutated HBIs (HFBI-posi and HFBI-nega) (Figure 2a–c). The HFBI molecular layer structures were investigated using AFM in a liquid (Figure d–f). Figure 2d shows an image of the self-organized membrane of wild-type HFBI. The images in Figure 2e and f are of the HFBI-posi and HFBI-nega membranes, respectively. As can be seen from the AFM images in Figure 2d–f, similar densely packed monolayer structures were formed. These membranes also formed honeycomb-like structures. The diagrams in Figure 2g–i show the hypothetical structures of these self-organized membranes. The honeycomb-like structure with through-holes may help electron transfer between the HOPG electrode and solution phase through HFBI self-organized membrane. These images show that the three types of HFBI self-organized membrane, with neutral, positive or negative charges, all have the same typical structure.

3.2. Electrochemical measurement of HFBI/HOPG

Figure 3 shows the cyclic voltammograms of HOPG or HFBI/HOPG electrodes in 0.1M phosphate buffer (pH 7) solution containing 25mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The voltammogram of the bare HOPG electrode shows a reversible redox current response, as shown in Figure 3, left, line a. Line b shows a suppressed redox current response for the electrode coated with wild-type HFBI. Oxidation and reduction current peaks can both be seen. Despite the presence of HFBI self-organized membrane on HOPG, this electrode maintained its basic function as an electrode. The AFM images (Figure 2d–f) show that the HFBI self-organized membranes have regular porosity. A redox molecule such as $[\text{Fe}(\text{CN})_6]^{3-/4-}$ can therefore participate in a redox reaction on the conductive surface through the pores of the HFBI membrane, i.e., the performance of an electrode modified with an HFBI self-organized membrane was not greatly inferior to that of a non-modified electrode. Figure 3, right, shows Nyquist plots recorded under the same conditions as the CV measurement. The charge-transfer resistance of an HFBI-coated HOPG electrode is higher than that of the bare HOPG electrode. The two HFBI variants were therefore used to improve the electrochemical properties of the HFBI-coated HOPG further (Figure 4).

3.3. EIS properties of HFBI/HOPG electrode

Three types of HFBI-coated HOPG electrodes were investigated using EIS, in two different solutions, containing either a negatively charge redox molecule, $[\text{Fe}(\text{CN})_6]^{3-/4-}$, or positively charged redox molecule, $[\text{Ru}(\text{CN})_6]^{2+/3+}$. These electrochemical data are shown in Nyquist plot (“ Z_{im} ” and “ Z_{re} ”) (Figure 4). These equivalent circuits include the ohmic resistance of the electrolyte solution, R_s , the Warburg impedance, Z_w , resulting from the diffusion of ions from the bulk electrolyte to the electrode, the Nernst diffusion impedance within the HFBI membrane pores, including factor for the HFBI charge, R_p , the double-layer capacitance, C_{dl} , and the interfacial charge-transfer resistance, R_{ct} (Scheme 1).

(Scheme 1)

The charge-transfer resistance corresponds to the diameter of the semicircle in the Nyquist plot. The double-layer capacitance and the interfacial charge-transfer resistance components in the equivalent circuit depend on the dielectric and insulating features at the electrode/electrolyte interface or protein/electrolyte interface, and are controlled by electrode surface modification [30].

In the case of impedance measurements in $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution, charge-transfer resistance is quite small, because HOPG is a conductive substrate (Figure 4A-a). The HFBI-posi (D40Q/D43N)-coated HOPG electrode shows small charge-transfer resistance compared with the other protein-coated electrodes (Figure 4A-b). In contrast, the HFBI-nega (R45Q/K50Q)-coated HOPG electrode shows a large charge-transfer resistance (Figure 4A-d). The neutral HFBI (wild-type)-coated HOPG electrode shows a medium charge-transfer resistance (Figure 4A-c).

In contrast, the following results were obtained for impedance measurements in the positively charged $[\text{Ru}(\text{CN})_6]^{2+/3+}$ solution. The HFBI-nega (R45Q/K50Q)-coated HOPG electrode shows a small charge-transfer resistance (Figure 4B-d). The HFBI-posi (D40Q/D43N)-coated HOPG electrode shows large charge-transfer resistance (Figure 4B-b). The neutral HFBI-coated electrode shows medium charge-transfer resistance (Figure 4B-c). *The present EIS analysis data show good reproducibility, with deviations of less than $\pm 7\%$ (n=5). All the measured data are shown in *Supporting Information*.*

These results show that the surface charge on the HFBI molecular layer controls the charge-transfer resistance, depending on the charge on the redox species. The use of two types of HFBI self-organized membrane showed that electrostatic interactions occurred. If the electrode surface has a positive charge and the redox molecule has a negative

charge, they are attracted to each other; conversely, If the electrode surface has a negative charge, they repulse each other. When using positive charge redox molecules, the opposite effects are observed [31-34]. These results show that an HFBI self-organized membrane layer modulates electrostatic interactions.

4. Conclusion

Electrodes coated with HFBI self-organized membranes maintain the same electrode function as bare electrode. This shows that HFBI can be used to design and fabricate specific molecular interfaces for electrochemical applications. As found in our current study, sterically disposed enzyme on a solid substrate using HFBI molecular carrier shows prominent catalytic activity, which is comparable to those of enzymes under homogeneous (non-immobilized) conditions.⁴ The results of the present study prove that the HFBI molecular layer does not affect the electrochemical reaction. HFBI molecular carrier methodology will enable the development of efficient molecular interfaces for electrochemical coupled reaction.

Reference

1. Y. Yourdshahyan, A. Rappe, Structure and energetics of alkanethiol adsorption on the Au(111) surface, *Journal of Chemical Physics*, 117 (2002) 825–833.
2. I. Yamashita, Fabrication of a two-dimensional array of nano-particles using ferritin molecule, *Thin Solid Films*, 393 (2001) 12–18
3. B. Kim, M. Bruening, pH-dependent growth and morphology of multilayer dendrimer/poly(acrylic acid) films, *Langmuir*, 19 (2003) 94–99.
4. Y. Takatsuji, R. Yamasaki, A. Iwanaga, M. Lienemann, M. Linder, T. Haruyama, Solid-support immobilization of a "swing" fusion protein for enhanced glucose oxidase catalytic activity, *Colloids and Surfaces B-Biointerfaces*, 112 (2013) 186–191.
5. T. Baby, S. Aravind, T. Arockiadoss, R. Rakhi, S. Ramaprabhu, Metal decorated graphene nanosheets as immobilization matrix for amperometric glucose biosensor, *Sensors and Actuators B-Chemical*, 145 (2010) 71–77.
6. . Noorbakhsh, A. Salimi, E. Sharifi, Fabrication of glucose biosensor based on encapsulation of glucose-oxidase on sol-gel composite at the surface of glassy carbon electrode modified with carbon nanotubes and Celestine blue, *Electroanalysis*, 20 (2008) 1788–1797.
7. J. Love, L. Estroff, J. Kriebel, R. Nuzzo, G. Whitesides, Self-assembled monolayers of thiolates on metals as a form of nanotechnology, *Chemical Reviews*, 105 (2005) 1103–1169.
8. G. Hahner, R. Hofer, I. Klingenfuss, Order and orientation in self-assembled long chain alkanephosphate monolayers adsorbed on metal oxide surfaces, *Langmuir*, 17 (2001) 7047–7052.
9. H. Hektor, K. Scholtmeijer, Hydrophobins: proteins with potential, *Current Opinion in Biotechnology*, 16 (2005) 434–439.
10. M. Linder, G. Szilvay, T. Nakari-Setälä, M. Penttilä, Hydrophobins: the protein-amphiphiles of filamentous fungi, *FEMS Microbiology Reviews*, 29

(2005) 877–896.

11. J. Wessels, Developmental regulation of fungal cell-wall formation, *Annual Review of Phytopathology*, 32 (1994) 413–437.
12. M. Sunde, A. Kwan, M. Templeton, R. Beever, J. Mackay, Structural analysis of hydrophobins, *Micron*, 39 (2008) 773–784.
13. M. De Vocht, I. Reviakine, W. Ulrich, W. Bergsma-Schutter, H. Wosten, H. Vogel, A. Brisson, J. Wessels, G. Robillard, Self-assembly of the hydrophobin SC3 proceeds via two structural intermediates, *Protein Science*, 11 (2002) 1199–1205.
14. J. Bayry, V. Aimanianda, J. Guijarro, M. Sunde, J. Latge, Hydrophobins-unique fungal proteins, *Plos Pathogens*, 8 (2012).
15. L. Gruner, K. Ostermann, G. Rodel, Layer thickness of hydrophobin films leads to oscillation in wettability, *Langmuir*, 28 (2012) 6942–6949.
16. H. Wosten, F. Schuren, J. Wessels, Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces, *EMBO J.* 13 (1994) 5848–5854.
17. H. Wosten, T. Ruardy, H. Vandermei, H. Busscher, J. Wessels, Interfacial self-assembly of a schizophyllum-commune hydrophobin into an insoluble amphipathic protein membrane depends on surface hydrophobicity, *Colloids and Surfaces B-Biointerfaces*, 5 (1995) 189–195.
18. H. Wosten, M. de Vocht, Hydrophobins, the fungal coat unravelled, *Biochimica Et Biophysica Acta-Reviews on Biomembranes*, 1469 (2000) 79–86.
19. M. Linder, Hydrophobins: Proteins that self assemble at interfaces, *Current Opinion in Colloid & Interface Science*, 14 (2009) 356–363.
20. V. Aimanianda, J. Bayry, S. Bozza, O. Kniemeyer, K. Perruccio, S. Elluru, C. Clavaud, S. Paris, A. Brakhage, S. Kaveri, L. Romani, J. Latge, Surface hydrophobin prevents immune recognition of airborne fungal spores, *Nature*, 460 (2009) 1117–U1179.
21. H. Valo, P. Laaksonen, L. Peltonen, M. Linder, J. Hirvonen, T. Laaksonen, Multifunctional hydrophobin: Toward functional coatings for drug nanoparticles, *Acs Nano*, 4 (2010) 1750–1758.
22. S. Ikeno, G. Szilvay, M. Linder, H. Aritomi, M. Ajimi, T. Haruyama, Protein dot stamp using hydrophobin as immobilization carrier, *Sensors and Materials*, 16 (2004) 413–420.
23. A. Iwanaga, H. Asakawa, T. Fukuma, M. Nakamichi, S. Shigematsu, M. Linder, T. Haruyama, Ordered nano-structure of a stamped self-organized protein layer on a HOPG surface using a HFB carrier, *Colloids and Surfaces B-Biointerfaces*,

84 (2011) 395–399.

24. H. Asakawa, S. Tahara, M. Nakamichi, K. Takehara, S. Ikeno, M. Linder, T. Haruyama, The amphiphilic protein HFBII as a genetically taggable molecular carrier for the formation of a self-organized functional protein layer on a solid surface, *Langmuir*, 25 (2009) 8841–8844.
25. M. Linder, K. Selber, T. Nakari-Setälä, M. Qiao, M. Kula, M. Penttilä, The hydrophobins HFBI and HFBII from *Trichoderma reesei* showing efficient interactions with nonionic surfactants in aqueous two-phase systems, *Biomacromolecules*, 2 (2001) 511–517.
26. M. Lienemann, J. Gandier, J. Joensuu, A. Iwanaga, Y. Takatsuji, T. Haruyama, E. Master, M. Tenkanen, M. Linder, Structure-function relationships in hydrophobins: Probing the role of charged side chains, *Applied and Environmental Microbiology*, 79 (2013) 5533–5538.
27. T. Fukuma, Water distribution at solid/liquid interfaces visualized by frequency modulation atomic force microscopy, *Science and Technology of Advanced Materials*, 11 (2010).
28. J. Sader, S. Jarvis, Accurate formulas for interaction force and energy in frequency modulation force spectroscopy, *Applied Physics Letters*, 84 (2004) 1801–1803.
29. T. Albrecht, P. Grutter, D. Horne, D. Rugar, Frequency-modulation detection using high-Q cantilevers for enhanced force microscope sensitivity, *Journal of Applied Physics*, 69 (1991) 668–673.
30. J. Roy-Mayhew, D. Bozym, C. Punckt, I. Aksay, Functionalized graphene as a catalytic counter electrode in dye-sensitized solar cells, *Acs Nano*, 4 (2010) 6203–6211.
31. L. Liu, D. Deng, Y. Xing, S. Li, B. Yuan, J. Chen, N. Xia, Activity analysis of the carbodiimide-mediated amine coupling reaction on self-assembled monolayers by cyclic voltammetry, *Electrochimica Acta*, 89 (2013) 616–622.
32. V. Pardo-Yissar, E. Katz, O. Lioubashevski, I. Willner, Layered polyelectrolyte films on Au electrodes: Characterization of electron-transfer features at the charged polymer interface and application for selective redox reactions, *Langmuir*, 17 (2001) 1110–1118.
33. N. Wei, J. Chen, J. Zhang, K. Wang, X. Xu, J. Lin, G. Li, X. Lin, Y. Chen, An electrochemical biosensor for detection of PML/RARA fusion gene using capture probe covalently immobilized onto poly-calcon carboxylic acid modified glassy carbon electrode, *Talanta*, 78 (2009) 1227–1234.

34. W. Marmisolle, D. Capdevila, E. de la Llave, F. Williams, D. Murgida, Self-assembled monolayers of NH₂-terminated thiolates: Order, pK(a), and specific adsorption, *Langmuir*, 29 (2013) 5351–5359

Figure 1. Illustration of drop-stamp method. A solution containing HFBI protein was kept in a droplet; 15 min later, an HFBI self-organized membrane was formed at the air/water interface. The hydrophobic substrate was contacted from bottom side. After wash, a precise, self-organized membrane was formed on substrate.

Figure 2. Wild-type HFBI and HFBI variants: (a) HFBI without point mutation, (b) HFBI variant, negative amino acid was changed to neutral amino acid (D40Q/D43N); (c) HFBI variant, positive amino acid was changed to neutral amino acid (R45Q/K50Q); and (d–f) AFM images of HFBI wild-type or variant HFBI self-organized membranes. These HFBI self-organized membranes have a periodic and highly dense packed structures. (g–i) Hypothetical self-organized membrane structures.

Figure 3. (Left) Cyclic voltammograms and (right) Nyquist plots recorded in the presence of 25mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at (a) HOPG electrode and (b) HOPG electrode

modified with wild-type HFBI. Magnified plot shows between 0 and 600 Ω . Scan speed, 50mV s⁻¹.

Figure 4. Nyquist plots for electrochemical impedance spectroscopy in the presence of 25mM [Fe(CN)₆]^{3-/4-} (A) or [Ru(NH)₆]^{2+/3+} (B) at an HOPG electrode (a), and HOPG electrodes modified with HFBI-posi (D40Q/D43N) (b), wild-type (c), and HFBI-nega (R45Q/K50Q) (d). The measurements were performed at 10mV in the frequency range from 10 kHz to 10 mHz (A) or 10 kHz to 100 mHz (B).

Table 1

Molecular Weight and pI Values of Wild-Type HFBI and Mutated HFBI

HFBI variants	MW (Da)	pI
HFBI wild type	7532	5.74
HFBI-posi (D40Q/D43N)	7544	8.16
HFBI-nega (R45Q/K50Q)	7504	3.93

Figure 1

Figure 2

Figure 3

Figure 4

Graphical abstract

The unique protein molecule HFBI forms a precision honeycomb-like structure through self-organization at the air/water interface and water/solid interface such as an electrode. The self-organized HFBI membrane does not prevent electron transfer even when coated on an electrode surface. Thus, HFBI can be used for molecular interface fabrication in precision structures.