Additive Manufacturing of Living Electrodes

Yang Gao, Jeonghwan Kim, Jihyun Ryu, and Seokheun Choi, *Senior Member, IEEE*

***Abstract*— The combination of electrochemically active microorganisms with miniaturized electronics has immense potential for augmenting a wide range of practical bioelectronic applications such as biosensing, bioenergy harvesting, and bio-electrosynthesis. However, a major challenge to the controllable and seamless integration of biological systems and external electronics is the fundamental mismatch in physicochemical properties and weak signal transmission between the biotic-abiotic interfaces. Here, we demonstrated an innovative method to fabricate the conductive-polymer-bacteria living electrode with a rapid and controllable 3-D printing platform and simple electrochemical polymerization techniques. The monomer precursor 3,4-ethylenedioxythiophene (EDOT) was printed into a 3-electrode electrochemical cell containing the exoelectrogen *Shewanella oneidensis* wild type MR-1. The monomer was in-situ electrochemically polymerized to the conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT), entrapping and connecting the electrochemically active bacteria to the electrode within 35 min. Using cyclic voltammetry (CV), the living electrode showed enhanced bio-electrochemical activities, providing transformative development for bioenergy harvesters, biosensors and biomedical devices. [2020-0082]**

***Index Terms*— Living electrode, exoelectrogens, conductive biocomposite, additive manufacturing.**

I. Introduction

ACTIVE, selective, and seamless biotic-abiotic interfacing is critical for the field of bioelectronics. Chemical, biological, mechanical and electrical information measured from biological systems across the biotic-abiotic interfaces will have far-reaching implications in important applications such as diagnostics, neuroscience, energy generation, bio-electrosynthesis, and environmental monitoring. Recent advances in micro-/nano-technologies and material science/engineering provided opportunities for realizing seamless interfaces between the biological systems and external electronics [1], [2]. These strategies include using mechanically compliant micro-/nano-electrodes to reduce mechanical mismatch at the biotic-abiotic interfaces [3], applying bioactive conducting polymer coatings to control the phenotype of the biological systems [4], and modifying electrode architectures to reduce a high interface impedance between the interfaces [5]. However, all existing research efforts to improve the biotic-abiotic interaction suffer from its fundamental mismatch in physicochemical properties and signal transmission. Living electrodes that exploit biological electron conduits and bridge biological and inorganic electrical circuits can make an effective coupling at the biotic-abiotic interface. Electrogenic bacteria are known for their extraordinary capability to electrochemically interact with external redox-active materials, which could promote seamless contact between biological systems and external electronics. Conventionally, such living electrodes are obtained by natural biofilm formation, increasing the surface-area-to-volume ratio of the electrode, or modifying the electrode surface free energy to favor bacterial attachment. More recently, the conductive polymer poly(3,4-ethylenedioxythiophene) (PEDOT) gained considerable research attention in the field of bioelectronics due to its biocompatibility, good electric and ionic conductivity, and chemical stability [6]. Encouraging results were reported from conductive tissue scaffolding [7], neural probing [8], and electroactive living materials [9]. Nevertheless, there still exists an unaddressed need to develop a simple, rapid, and controllable method to combine microorganisms with electronics.

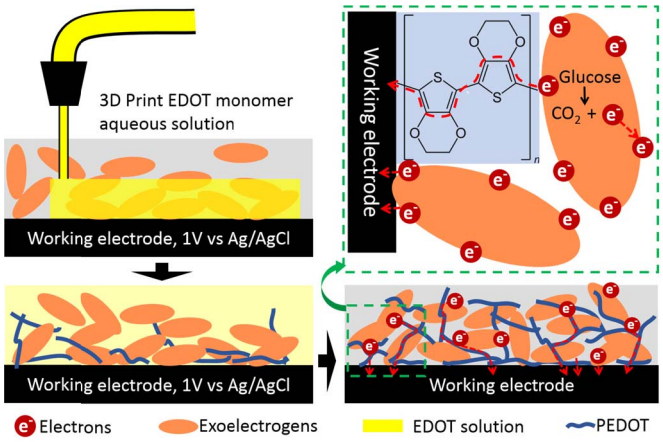

Fig. 1. Conceptual illustration of the living electrode fabrication process. The monomer precursor EDOT was 3-D printed into the electrochemical cell chamber containing exoelectrogens. The electropolymerized PEDOT trapped the exoelectrogens to the working electrode surface, forming the living electrode.

In this work, we constructed a living electrode by 3-D printing the polymer precursor 3,4-ethylenedioxythiophene (EDOT) in electrogenic bacteria-containing liquid and forming an electrochemically active biomaterial onto an abiotic graphite electrode through the in-situ electropolymerization of the monomer to the redox-active conducting PEDOT (Fig. 1 and 2). Within just 35 minutes, we successfully created a living bacterial electrode that effectively improved

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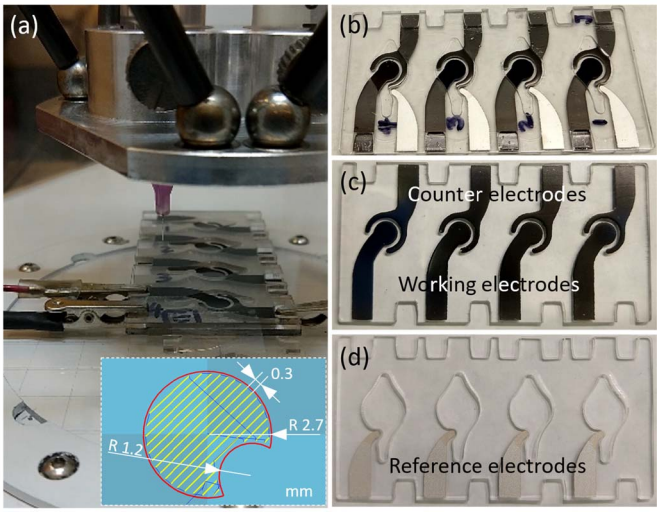

Fig. 2. Photo images of (a) 3-D printing monomer EDOT solution into (b) the 3-electrode electrochemical cell. (c, d) The two electrode sheets before cell assembly. (Insert: The printing patterns are marked in yellow).

the electrochemical interaction at the biotic-abiotic interface by 113% (Fig. 3). Electropolymerized PEDOT was exploited as conductive bridges to harvest the extracellularly transferred electrons from *S. oneidensis* respiratory, constructing a seamless contact between the biological processes and the external abiotic systems. A comprehensive understanding of the microbe-electrode exchange for extracellular electron transfer could provide new insight into the integration of biological and electronic systems. This work can provide a new method to design an ideal biotic-abiotic interface for the next generation of bioelectronics.

II. Materials and Experimental Set-Up

A. Electrochemical Cell Fabrication

The electrochemical three-electrode micro-chamber was fabricated by micromachining two polymethyl methacrylate (PMMA) layers and screen-printing the working electrode (WE), counter electrode (CE) onto one layer, and the reference electrode (RE) onto the other layer (Fig. 4a). After drying the electrode inks (Graphite Ink and Silver / Silver Chloride Ink, Ercon Inc.), the graphite WE and CE electrodes were further wet polished by P1200 sandpaper and ethanol with two overlaying crosshatch patterns, one horizontal-vertical and the other in diagonal directions. Debris from the polishing were cleaned by sonification in a deionized water bath for 15 mins. Before assembly, both the WE and CE layer, and the Ag/AgCl RE layer were treated with oxygen plasma for 45 s to free the surface from contaminants. To combine the two PMMA layers, the thermoplastic Parafilm®was laser-cut into a sealing gasket, sandwiched between the two PMMA layers, and heated at 82 °C for 15 mins (Fig. 4b). Metal clamps and glass sheets were used to apply even compression force over the assembly during the heating.

B. Bacteria Culture

Freshly prepared M9 medium with glucose as the carbon source was used for the living electrode fabrication and electrochemical characterization while the LB medium was

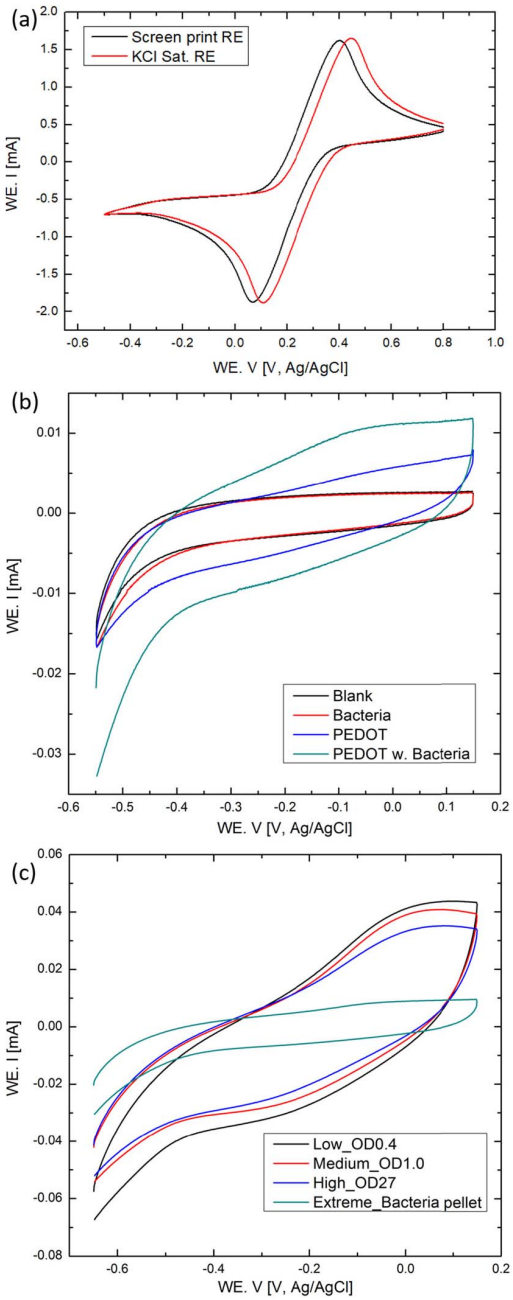

Fig. 3. Cyclic voltammetry curves of (a) the abiotic electrochemical cell with $K_3[Fe(CN)_6]$ in KCl solution, (b) the as-fabricated living electrodes with negative controls and (c) different initial bacteria loading concentrations.

used for the bacteria culture. Cryostock *S. oneidensis* MR-1 was grown in LB medium overnight at 37 °C with constant shaking. Then the bacteria culture was centrifuged at 4000 rpm for 4 mins to obtain the cell pellet. The pellet was washed in M9 medium 3 times by resuspending in fresh medium and subsequent centrifugation. Afterward, the pellet was adjusted with M9 medium to the desired density by measuring the OD_{600} reading from a spectrometer. The prepared bacteria culture was stored in 4 °C no longer than 3 hours before use.

C. Living Electrode Fabrication

The monomer precursor EDOT was dissolved in the M9 medium at a concentration of 10 mM by sonification in an

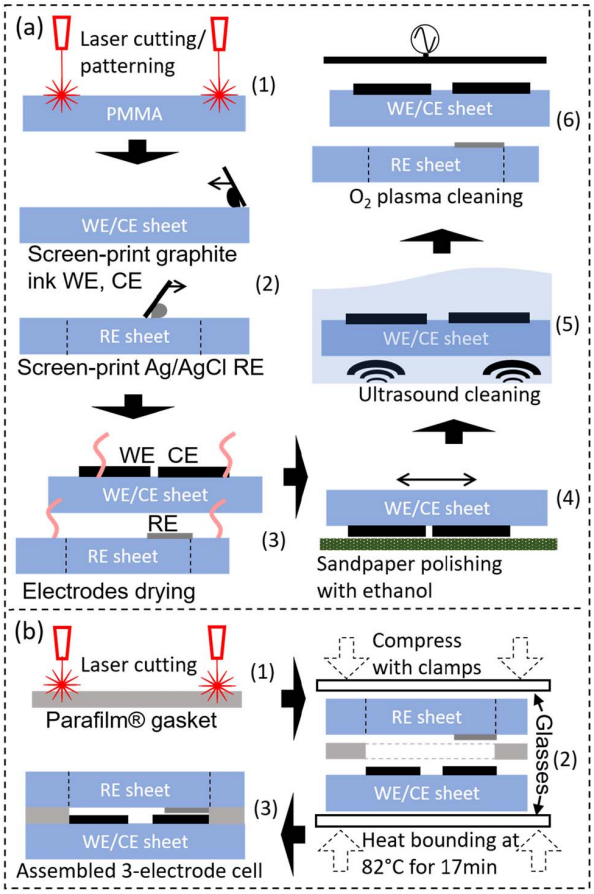

Fig. 4. Schematics illustration of the 3-electrode electrochemical cell fabrication process. (a) Fabrication process of the two PMMA sheets of the 3-electrode electrochemical cell, and (b) assembly of the two PMMA sheets into the complete electrochemical device. (WE: working electrode, CE: counter electrode, and RE: reference electrode).

ice bath for 3 h. Then the EDOT solution was filled into the cartridge of the 3-D bioprinter (Pensées Inc.). The printing pressure was set to 5 MPa and a 30-gauge flat nozzle was used for the printing job. The printing pattern was created by Cura software with a layer height of 0.5 mm, a wall thickness of 0.25mm (Fig. 2a insert, red), a printing velocity of 30 mm/s, and a motion path (Fig. 2a insert, yellow) spacing of 0.3 mm.

The 3-electrode electrochemical cell was positioned and taped onto the printing platform with the electrodes connected to a potentiostat (Squidstat Plus, Admiral Instruments). Bacteria culture in M9 medium was pipetted into the electrochemical cell chamber. Then the WE was biased at 1 V versus the RE in chronoamperometry mode right before the printing. Three printing iterates was used with a holding time of 10 mins after each print for the electropolymerization of the monomer to PEDOT. The total printing and polymerization process took 35 min. Following the last print, the electrochemical cell chamber was triple washed and replenished with the fresh M9 medium to remove unreacted EDOT monomer. Parafilm®was used to cover the device chamber to avoid water evaporation and contamination after the living electrode fabrication.

D. Electrochemical Measurement

For the 3-electrode electrochemical cell validation, 10 mM potassium ferricyanide ($K_3[Fe(CN)_6]$) in 1 M of KCl was used as the electrolyte. CV with the scan rate at 50 mV/s was performed on the electrochemical cell. In the reference voltage control test, a commercial Ag/AgCl glass RE in saturated KCl was positioned over the center of the WE with the ceramic tip fully immersed in the electrolyte. The electrochemical characterization of the living electrode was conducted with CV measurement at a scan rate of 100 mV/s. Three scans were performed ensuing the electrode sample fabrication and the third CV scan from different samples was used for comparison.

E. Fluorescence Microscopy Imaging

After the electrochemical measurement, the PEDOT-bacteria samples were stained with 6-Carboxyfluorescein diacetate (CDFA) fluorescent dye and kept in the dark at room temperature for 30 min. Then the PEDOT-bacteria composite was scraped from the WE surface with a sterile inoculation loop and transferred to microscope slides with cover glass sealed with silicone grease. The images were acquired by Nikon TS100 inverted phase-contrast microscope with an excitation light wavelength of 460 nm.

F. Scanning Electron Microscopy (SEM) Sample Preparation

Samples for SEM were fixed with 4% glutaraldehyde solution in 0.1 M phosphate buffer saline (PBS) for 2 h at 4 °C. To avoid contamination from the Parafilm®wax gasket and screen-printed Ag/AgCl electrode, the microchambers were disassembled with the residual wax cleaned before dehydration. Dehydration solution was applied onto the WE by pipet and removed by carefully soaking with filter paper. The samples were dehydrated through ethanol series and HMDS from 35%, 50%, 75%, 95%, 100% ethanol to HMDS with 10 mins immersion interval. The 95% and 100% ethanol steps were performed twice. The dehydrated samples were placed in a fume hood and dried overnight before the SEM examination.

III. Results and Discussion

Before the living electrode fabrication, the electrochemical performance of the 3-electrode cells was first determined by CV scans carried out in $K_3[Fe(CN)_6]$ and KCl solution with aseptic devices. A commercial Ag/AgCl reference electrode in saturated KCl was used as a control to validate the reference voltage of the build-in screen-printed electrode. As shown in Fig 3a, the corresponding oxidation peaks occurred at 403 mV and 448 mV for the screen-printed and the commercial saturated KCl RE, respectively. Whereas the reduction peaks appeared at 70 mV and 109 mV, respectively. On average, a voltage shift of -42 mV was observed from the screen printed RE comparing to the commercial electrode.

After determining the basic electrochemical performance of the 3-electrode electrochemical cells, exoelectrogens was utilized for the in-situ living electrode electropolymerization. The micro-chambers were filled with *S. oneidensis* MR-1 culture at an OD_{600} reading of 1.5, the monomer EDOT was introduced onto the graphite WE by 3-D printing and electropolymerized with the chronoamperometry method at a constant potential of 1 V versus Ag/AgCl RE. Bacterial cells placed on the working electrode were entrapped through this in-situ polymerization process, forming an electrochemically

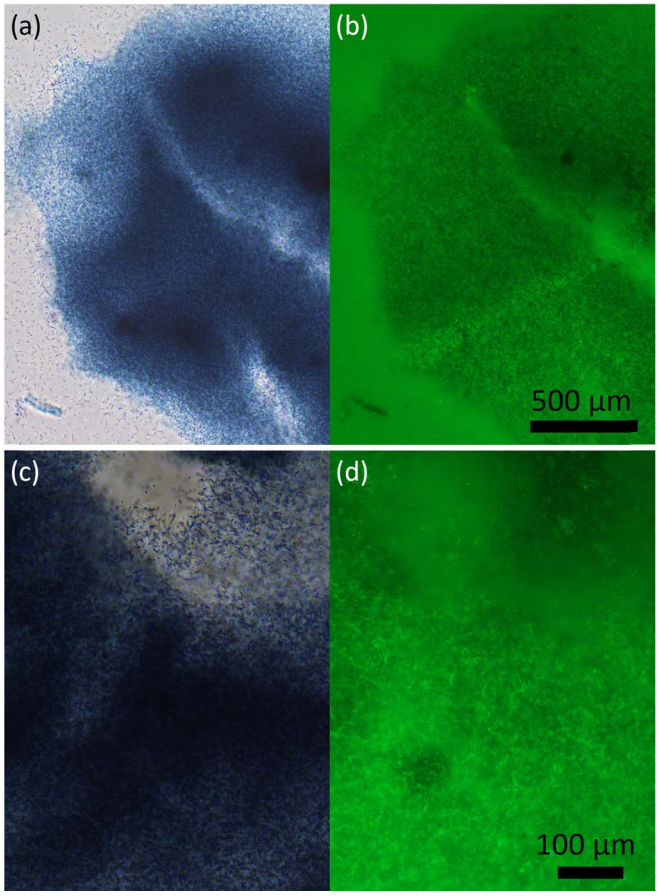

Fig. 5. Fluorescent microscope images of the living electrode fabricated with initial bacteria concentration of 1.0 (OD_{600}). Samples were transferred to microscope slides from the living electrode after the electrochemical measurements. Fluorescent images taken with normal/excitation light conditions. (a & b) and (c & d) are the same spot, respectively.

active PEDOT-bacteria living electrode. For comparison, samples with aseptic M9 medium (denoted as Blank), bacteria in M9 (denoted as Bacteria) and 10 mM EDOT in M9 (denoted as PEODT) also underwent the same chronoamperometry process as the living electrodes (denoted as PEDOT w. Bacteria). As shown in Fig. 3b, CV measurement showed higher electrochemical activities than that of the PEDOT only and bacteria only samples. A characteristic reduction peak around -54 mV was observed from the PEODT living electrode, which was related to the direct electron transfer (DET) meditated by the outer membrane-bound cytochromes in *Shewanella* sp. [10]–[12].

As the number of electrogenic bacteria entrapped to the PEDOT, the living electrode directly affected the measurable bio-electrochemical signal. Different initial bacteria loadings were tested for the living electrode fabrication. *S. oneidensis* MR-1 cultures with OD_{600} readings of 0.4, 1.0, 27 were used as low, medium, and high bacteria loading conditions while the bacteria pellet was used directly after centrifugation without any dilution as the extreme case. To minimize the local dilution of the bacteria concentration over the WE surface caused by the monomer solution jet from the nozzle, only one printing iterant was performed with a holding time of 30 min for this sequence of living electrode experiments. As illustrated

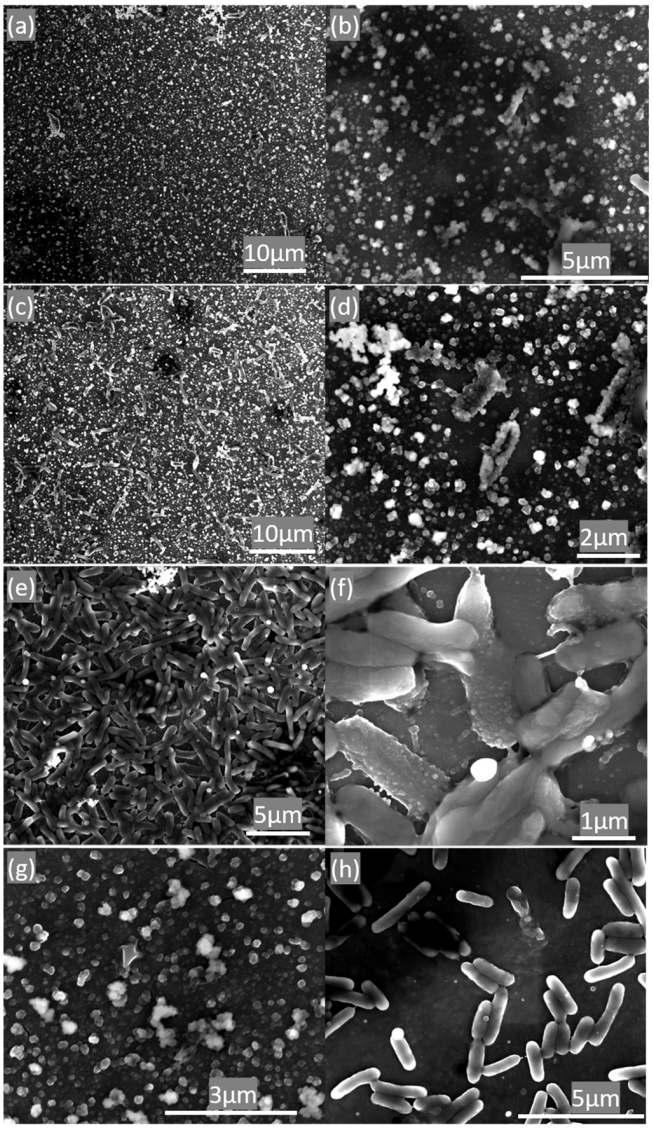

Fig. 6. Scanning electron microscopy images of the living-electrode fabricated with initial bacteria concentration from OD_{600} readings of (a, b) 0.5, (c, d) 1.0, and (e, f) the centrifugation pellet of bacteria. Controls of (g) aseptic PEDOT only and (h) bacteria only samples went through the same fabrication process.

in Fig. 3c, the resultant CV curves for these initial bacteria loading conditions showed decreased pseudo-capacitance from the polymerized PEDOT with increased initial bacteria loading. This phenomenon could be attributed to two factors that (i) the exoelectrogen *S. oneidensis* MR-1 is redox-active and competing with the monomer EDOT to donate electrons to the oxidative WE, (ii) the bacteria cell would block the active site of the WE for PEDOT electropolymerization. After the CV measurement, samples were examined with fluorescent microscopy to determine the bacterial entrapment in the PEDOT. As shown in Fig. 5, a large portion of live bacteria was observed in the dark blue PEDOT with small numbers of planktonic cells in the liquid environment. From the scanning electron microscopy (SEM) images, shown in Fig. 6a-6f, with increased bacteria initial loading, less PEDOT and more bacteria were observed on the WE. The bacteria pellet sample showed little resemblance to the PEDOT

only sample (Fig. 6g) yet more similarities to the bacteria only sample (Fig. 6h). Thus, we conclude that the amount of polymerized PEDOT was inversely proportional to the bacteria concentration in the reaction chamber, and a suitable initial bacteria loading should be found on the application bases. For example, for bioelectricity harvesting, higher bacteria loading is desirable to maximize the biocatalyst activity, whereas, for biosensing, higher signal-to-noise ratio with and an optimal bacteria loading would be preferable [13], [14].

IV. Conclusion

In this study, we demonstrated a controllable, rapid, and simple method to fabricate an electroactive living electrode by combining exoelectrogens onto the electrodes by the conductive polymer PEDOT. The in-situ electropolymerized PEDOT entrapped bacteria on the WE surface, enhancing the bioelectrochemical signals. With the help of 3-D printing, the fabrication time reduced from hours to only 35 min, and the exposure of the bacteria to the cytotoxic monomer precursor EDOT was reduced. The living electrode produced higher current in CV measurement comparing to the control. The presented method to integrate biological systems and conventional man-made systems will be critical for advancing practical applications in bioelectronics such as in vitro biosensors, electronic medical implants, neuroprosthesis, and biofuel cells.

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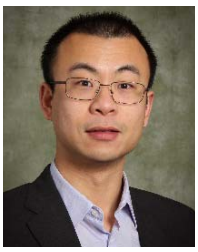

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