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Full paper

A fully disposable 64-well papertronic sensing array for screening electroactive microorganisms

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ABSTRACT

A papertronic 64-well sensing array for rapid and sensitive screening of electroactive microorganisms was developed. This device offers a simple and facile approach to accurately characterize and simultaneously monitor bacterial electrogenicity without complicated fluidic feeding systems and electrical wires. After use, the entirely paper-based device platform can be disposed of safely by incineration without posing a risk of bacterial infection. Paper-based printed circuit boards integrated into the array effectively measured bioelectricity generated from spatially distinct 64 microbial fuel cells (MFCs) without cumbersome electrical connections. The flexible array performed stably through repeated folding cycles. Within 30 min, we successfully determined electrogenicity of 15 bacteria including two known wild-type electroactive microorganisms and thirteen isogenic mutants of *P. aeruginosa*. The paper-based MFC array could help transform electromicrobial technology from bench-top settings to practical applications that would demand proof of high performance.

1. Introduction

Microorganisms are extremely diverse and can thrive in highly diverse environments [1]. They comprise the major source of biomass on earth, functionally maintain ecosystems, and can impact human health [1,2]. Recently, select microorganisms have been used in various biotechnological applications, as a bioreceptor to detect a wide range of chemical and biological substances, as biofactory's to produce value-added chemicals and biofuels, and as microrobots for drug delivery [3–5]. In particular, microorganisms that can divert electrons to conductive materials through their metabolic activity (called *electroactive microorganisms*) have received much attention as a new generation of sustainable and green energy technology [6,7]. Electroactive microorganisms can directly interact with external electrodes via extracellular electron transfer (EET), thereby producing an electrical current. Their EET mechanisms are based on extracellular conductive nanowires (or type IV pili), outer-membrane or periplasmic extensions, or redox-active mediators [8]. The microbial electrogenic capability allowed the development of innovative bioelectrochemical technologies, including microbial fuel cells (MFCs), microbial electrolysis cells (MECs), microbial desalination cells (MDCs) and microbial electrosynthesis (MES), for energy recovery, wastewater treatment and new material synthesis [9]. However, these technologies remain laboratory novelties mainly because a very limited number of electroactive microorganisms have been discovered coupled with the extremely challenging process of screening and characterizing microbial electrogenic capabilities [10]. Bioelectrochemical technologies can be dramatically revolutionized from the pilot-scale levels to practical use in real-world applications if more electroactive microorganisms were identified from the natural environment, genetic manipulation and most recently through synthetic biology. The current urgent and immediate need is an easy-to-use and rapid parallel analysis platform for high-throughput screening of microbial electrochemical activities. Still, very few research groups have attempted to develop high-throughput approaches for such measurements. Previous studies include an electrochemiluminescence-based assay [11], electrochromic approaches [12], colorimetric analysis [13,14], and dielectrophoresis [15]. However, these techniques have often been indirect, insensitive, or reagent-intensive. Furthermore, each technology uses only one microbial EET mechanism at a time, which results in difficulty in characterizing potential electroactive microorganisms having unknown EET mechanism(s). In contrast, MFC-based electricity measurements are the most direct, precise, and reliable approach to examine and characterize all types of electroactive microorganisms [16–18]. MFCs are typically composed of anodic and cathodic compartments externally connected through a load and separated by a proton exchange membrane (PEM) such that harvested

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electrons (e^-) from the anode travel to the cathode through the external circuit while the produced protons (H^+) move to the cathode through the PEM, maintaining the electroneutrality of the MFC. By measuring the voltage drop through the external load, the electrical current can be directly and sensitively measured to characterize microbial electrogenic capability regardless of their EET mechanisms. Despite the promise of this technology, it often requires complex operation, measurement, or maintenance because of hundreds of electrical wires for parallel electrical characterization of the sensing units and complicated handling of fluidic feeding system with hundreds of tubes and external multichannel pumps, which make it time-consuming, expensive, and low-throughput. Furthermore, the screening array should be carefully decontaminated, discarded or incinerated because of a high potential risk of microbial infection after its use, which requires considerable time-consuming disposal procedures and expensive facilities. Unfortunately, the conventional screening techniques cannot easily and safely be disposed, and their developers seem to have overlooked potential biohazards for the environment and human health.

In this work, we demonstrate a universally papertronic 64-well MFC array for rapid and sensitive characterization of electroactive microorganisms without complicated fluidic feeding systems and electrical wires (Fig. 1), followed by safe disposal by incineration. Fast and strong capillary-driven flow of the aqueous samples on paper allowed the system to operate without tubes and external pumps. Paper-based printed circuit boards integrated into the array effectively and simultaneously measured electrical currents from spatially distinct 64 MFC units without cumbersome electrical connections. The flexible MFC array demonstrated a stable performance through repeated folding cycles. Full integration of highly-sensitive MFC-based sensors on paper was achieved by improving the bacterial EET efficiency with the electrode in an electrochemically engineered paper reservoir and reducing cathodic overpotential by using a solid-state Ag_2O cathode on paper. Within 30 min, we successfully determined electrogenicity of 16 samples (each sample loaded on four MFC units) including two known wild-type electroactive microorganisms (*Pseudomonas aeruginosa* PA01 and *Shewanella oneidensis* MR1), thirteen isogenic mutants of *P. aeruginosa* (*wapR, pilQ, PA0962, pilY1, moaA1, gor, lasl rhll, norCB, pmbA, mvfR, rplY, pilT nirS,* and *acnC*), and Luria-Bertani (LB) broth media as a negative control. Simple batch-fabrication methods of printing, brushing, and spraying that can simultaneously construct 64 individual sensing units in an array are easily directed toward the development of much higher-throughput arrays (e.g., 96 or 384 wells).

2. Experimental

2.1. Materials

Conductive graphite ink (#E34561000G) was purchased from Fisher Scientific Company, LLC. Whatman™ Grade 3MM chromatography paper and Ag_2O (AA11407-14) were obtained from VWR International, LLC. Poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PEDOT:PSS) (Clevios PH1000) was purchased from Heraeus. Tryptone, yeast extract, Sodium chloride (NaCl), dimethyl sulfoxide (DMSO), glutaraldehyde solution, phosphate buffer saline (PBS) and 3-glycidoxypropy-trimethoxysilane (GLYMO) were purchased from Sigma-Aldrich.

2.2. Preparation of paper-based sensing array

The entirely paper-based sensing array is illustrated in Fig. 1a and contains spatially distinct 64 MFC units on 7 cm × 7 cm multi-laminate paper substrates (Fig. S1). All the detailed dimensions of the wells, the lines, and the devices are in Supporting information (Fig. S1 & Fig. S2). The array was assembled by sandwiching six functional layers of paper: (i) cathodic cover, (ii) cathodic wiring, (iii) cathodic MFC, (iv) anodic MFC, (v) anodic writing, and (vi) anodic cover (Fig. 1b). The middle two MFC layers formed 3-D MFC units consisting of a cathode, PEM, anodic reservoir, and anode (Fig. 1c). Bacteria inoculated into the reservoir produce electrons and protons for a fuel cell operation in which the electrical current is collected through anodic and cathodic wiring layers and measured through the external load (Fig. 1c and d). All fluidic boundaries and electrical insulators were defined with hydrophobic wax on the paper layers simply by using a Xerox ColorQube wax printer (Fig. 2). The identical wax patterns in full alignment were

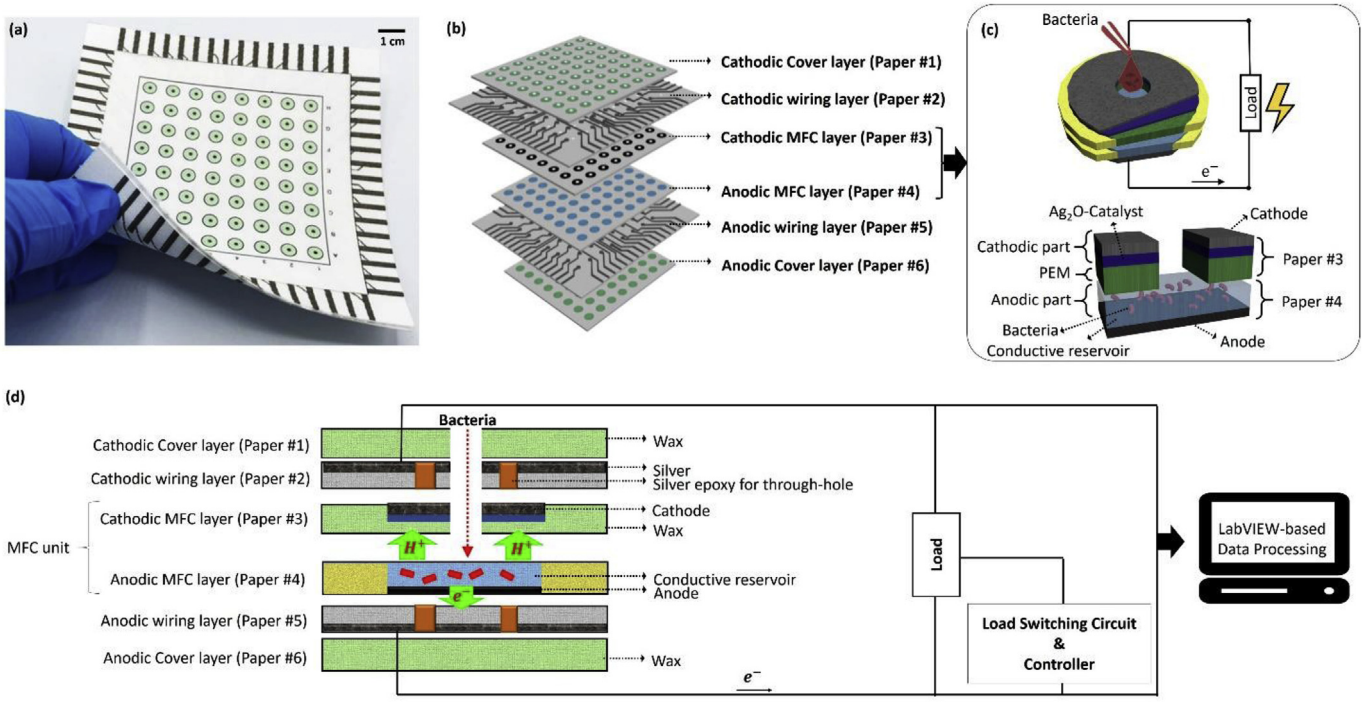

Fig. 1. (a) Photo image of the assembled MFC array, schematic diagrams of (b) its individual layers) and (c) an individual MFC unit. (d) Schematic diagram of a cross section of the system unit showing the individual layers.

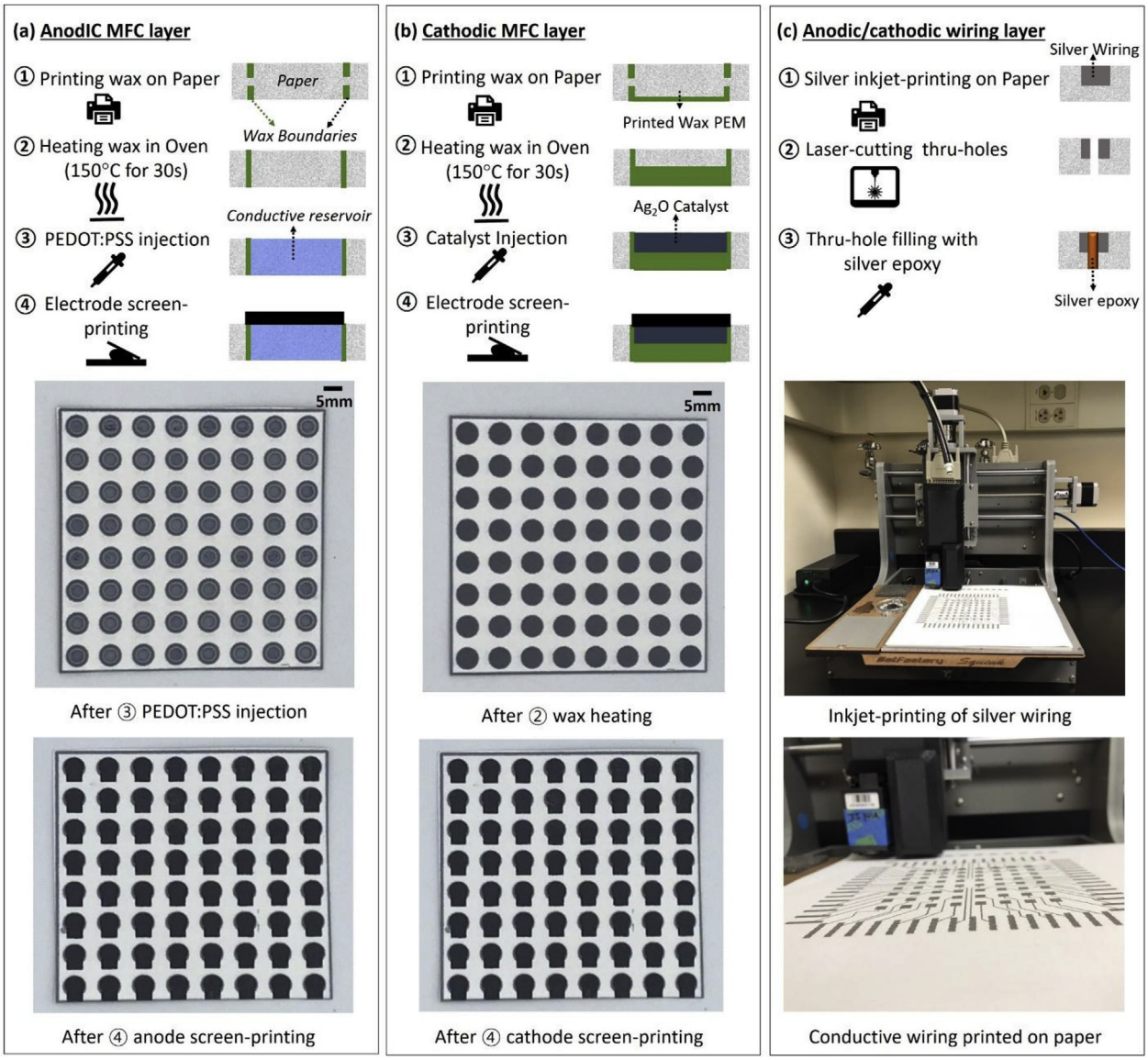

Fig. 2. Schematic illustrations and photoimates of the process used to create the papertronic MFC array integrating (a) anodic MFC layer, (b) cathodic MFC layer and (c) electrode wiring layers.

printed on both sides of the paper. This double-sided printing decreased the line features down to ~1 mm as the wax penetrates through the paper from both directions. The wax patterns were subsequently melted into the paper by heating in an oven at 150 °C for 30 s. The conductive anodic reservoirs were formed by adding a mixture of 1 wt% of PEDOT:PSS and 5 wt% of DMSO into the defined regions (Fig. 2a). A 2 wt% of GLYMO solution was added to the reservoirs to increase the hydrophilicity of the paper. On the cathodic MFC layer, the wax was also printed as the PEM, separating the cathode from the anode while allowing for proton transfer (Fig. 2b). The solid-state cathode was prepared by mixing 500 mg of Ag_2O in the 10mL of PEDOT:PSS solution. Finally, the graphite ink was screen-printed on top of the anodic reservoirs and cathodic wells through the paper-based masks with alignment cuts. Cathodic and anodic wiring layers were prepared on paper by ink-jet printing silver (BotFactory Squink Desktop PCB Printer) (Fig. 2c). We designed all the circuit wires by using the free version of PCB design software (Eagle) (Fig. S2). The printer was capable of printing conductive lines with a minimum trace with 0.25 mm. The through-holes were subsequently prepared by precisely laser-cutting the wiring layers (Universal Laser System VLS 3.5) and filling silver epoxy (Ted Pella, Inc.) (Fig. 2c). All functional paper layers were carefully assembled through alignment cuts and sandwiched using an adhesive spray (3 M Super77SprayAdhesive).

2.3. Electrical measurement setup

Alligator clips were used to hold wiring connections for testing. The potentials between the anodes and the cathodes were measured with a data acquisition system (National Instruments, USB-6212), and recorded every 30 s via a customized LabView interface. The open circuit voltage (OCV) was measured for the first 30 min. Then, external resistors (1 MΩ, 0.5 MΩ, 248 kΩ, 68 kΩ, 47 kΩ, 22 kΩ, 10 kΩ, 5 kΩ, 3.3 kΩ, 2.2 kΩ, 1 kΩ, 0.5 kΩ, 330 Ω, and 220 Ω) were subsequently connected between the anodes and cathodes to measure the current flow through the resistor by Ohm's law (I = V/R).

2.4. Bacterial inoculum

To demonstrate the papertronic sensing array for characterization of electroactive microorganisms, *Pseudomonas aeruginosa* PAO1 was selected as a model organism. The genome of this organism has been sequenced and it is highly genetically tractable. For decades,

researchers have produced mutant and controlled overexpression/repression constructs through developments in genetic engineering. Our hypothesis is that the genetic engineering of bacterial metabolic pathways greatly affects the electrogenic capability of *P. aeruginosa* mutants, and the papertronic sensing array provides a rapid, sensitive, and high-throughput platform to evaluate the electrogenicity of such strains. In this work, wild-type *P. aeruginosa* PAO1, wild-type *Shewanella oneidensis* MR1, and thirteen genetically engineered *P. aeruginosa* mutants (*wapR, pilQ, PA0962, pilY1, moaA1, gor, lasl rhll, norCB, pmbA, mvfR, rplY, pilT nirS,* and *acnC*) were tested while Luria-Broth (LB) medium was used as the negative control. The mutants are genetically modified by using classical allelic replacement or transposon (Tn) mutagenesis techniques. All bacteria were grown in LB media (1 w/v% tryptone, 0.5 w/v% yeast extract and 0.5 w/v% NaCl) with the cell titers controlled by monitoring the optical density at 600 nm.

2.5. Bacterial fixation and SEM imaging

Scanning electron microscopy (SEM) samples were washed gently with 0.1 M PBS and fixed using 4% glutaraldehyde solution overnight at 4 °C. Then, they were washed with PBS and dehydrated by 5-min serial transfers through 50, 70, 80, 90, 95, and 100% ethanol. The samples were then placed in hexamethyldisilizane (HMDS) for 10 min followed by air drying overnight. The fixed samples were examined using a FESEM (Field Emission SEM) (Supra 55 VP, Zeiss).

3. Results and discussion

3.1. Sample introduction

All 16 samples were added into the 64 wells of the array by repeatedly using an 8-channel pipette. Each sample was loaded on four units in the array to demonstrate its reproducibility, which was shown with error bars in the electrical outputs. Bacterial samples were cultivated until their optical densities at 600 nm (OD_{600}) reached 2.5, which was carefully chosen to saturate the ~10 μL volume of the anodic reservoir. SEM images show that a sample with an OD_{600} of 2.5 of forms densely packed bacterial aggregates, completely covering all paper reservoirs. Using suspensions with an OD_{600} of 0.5 and 1.5, the anodes were occupied by only sparse bacterial aggregates (Fig. 3). Bacterial current generation is proportional to the number of viable bacteria in the reservoir cells. Thus, having completely saturated cells removes a possible confounding factor that could produce unreliable comparisons of the electrogenicity of the mutants.

3.2. High-throughput characterization

Previously, we created a paper-based MFC as a realistic and accessible power solution that can enable a self-powered diagnostic test in resource-limited settings [19–22]. Paper's unique fluidic feature with capillary action allow fast bacterial cell attachment to the anode and thus rapid power generation. To improve electrical performance for practical applications, the anodic paper layer was electrochemically engineered with PEDOT:PSS and a solid-state Ag_2O cathode was developed on paper. The PEDOT:PSS-treated reservoirs enhanced bacterial electrocatalytic activity and improved bacterial adhesion while the solid-state cathode reduced cathodic over-potential, ultimately increasing the total power performance of the device [20]. In this work, multiple MFC devices were miniaturized and arranged in an array as a much more rapid, sensitive, and high-throughput, screening tool for microbial electricity generation studies compared to even our latest 48-well MFC array [23]. Although our 48-well MFC array was capable of simultaneous evaluation of some selected bacteria cells, it was limited to poor sensitivity and low practicability. The device could not measure the collective electricity generation harvested from all the bacterial cells placed throughout a non-conducting paper while it required

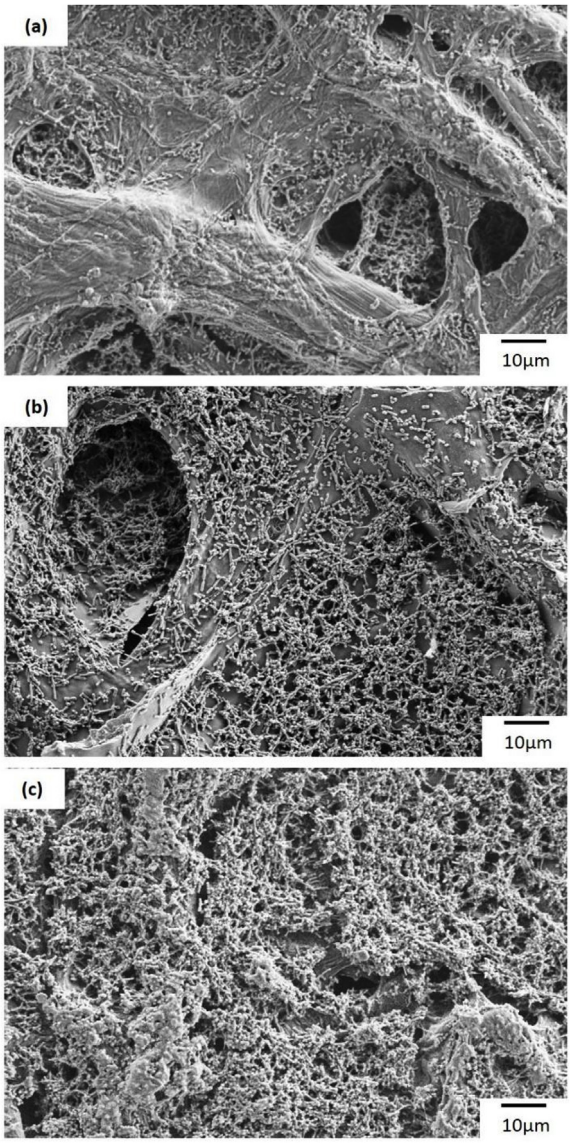

Fig. 3. SEM images of anodic reservoirs with difference concentration of *P. aeruginosa* PAO1; (a) 0.5, (b) 1.5, and (c) 2.5 of OD_{600}.

complicated device fabrication and operation due to the aqueous cathodic acceptors. To improve electrical performance and operation for practical applications, the anodic paper layer was electrochemically engineered with PEDOT:PSS and a solid-state Ag_2O cathode was developed on paper. Prior to this, we first tested the reproducibility and reliability of the 64-well array by measuring the OCV and power output from individual MFCs. The OCVs and the maximum power outputs of the 64 units in the array vary by 4% with *P. aeruginosa* PAO1. This low percent deviation is attributed to the miniatured anodic volume (~10 μL), which improved performance. After injecting all 16 bacterial samples into the 64 MFCs, we monitored bacterial accumulation and acclimation by monitoring the OCV values while the bacteria adhered to the anodic surface. Their OCVs gradually increased and reached saturation values about 30 min after injection of the bacterial strains (data not shown). Next, the polarization curves and power outputs were measured and calculated based on the voltage values every 30 s at given external resistances (Fig. 4a and b) [24]. All of the experiments were rapidly performed at 25 °C to minimize environmental changes and the measurement time and intervals with different resistors were carefully selected. All OCVs and maximum power densities obtained from the 64 sensing units were summarized with standard deviations in Fig. 4c and

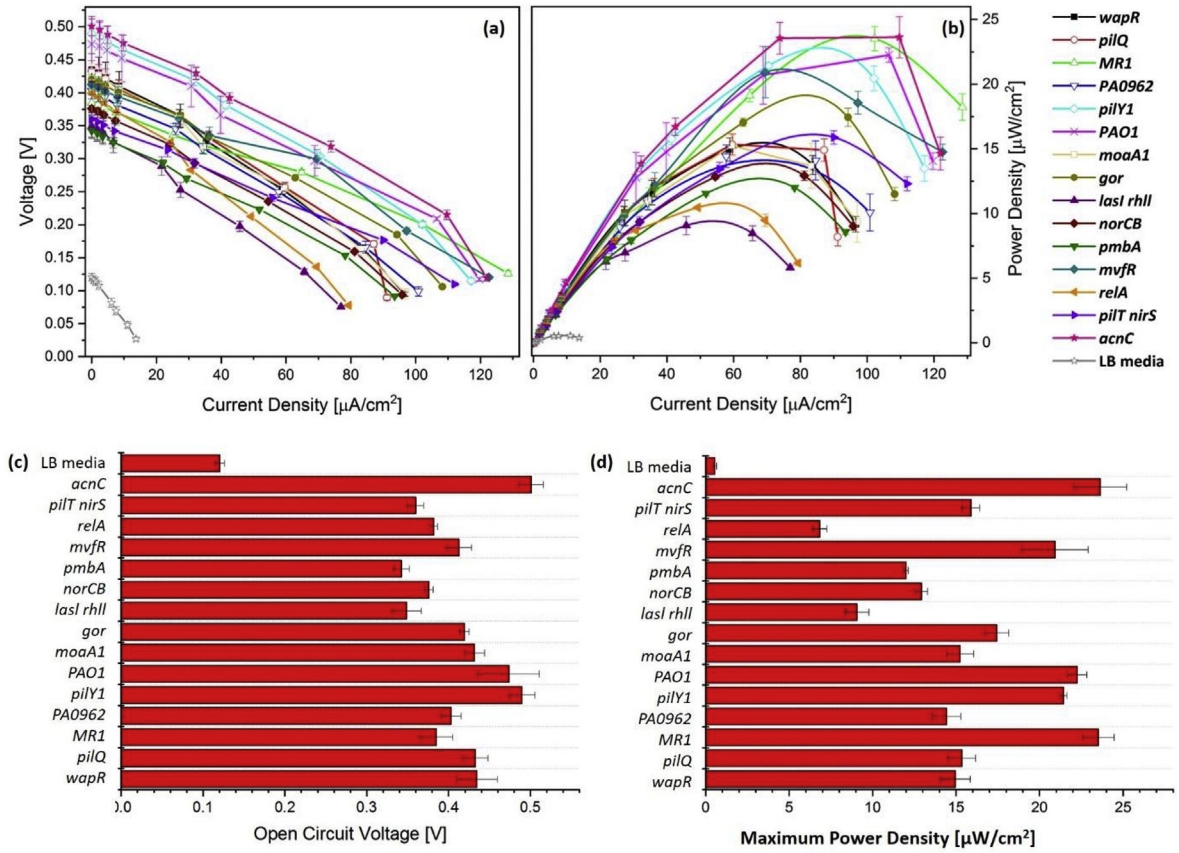

Fig. 4. (a) polarization curves and (b) power outputs of 16 samples, from which (c) open circuit voltages and (d) maximum power densities are obtained (MR1: *S. oneidensis* MR1 and PAO1: *P. aeruginosa* PAO1).

d. The negative control produced a negligible output compared to the bacterial samples, indicating that all power generations were a result of microbial metabolism. Our MFC achieved rapid and highly sensitive (~120 μA/cm^2) electricity generation even from the well-known exoelectrogen, *Shewanella* species, which is about 100 times stronger than the previous MFC arrays and paper-based MFCs [16–23]. The *acnC* mutant of the weak electrogen *P. aeruginosa* PAO1, showed the highest OCV and power density among all 16 samples, which is comparable to that of the strong electrogen *S. oneidensis* MR1. This is very significant because it demonstrates that strategic genetic modifications can improve bacterial electrogenicity. The *acnC* mutant minimized the production of one of three aconitases that are part of the tricarboxylic acid (TCA) cycle, which was indirectly involved in electrochemical activity of the microorganism. The OCV values of the bacterial samples ranged from 0.32 V to 0.5 V, indicating that the genetically modified mutants provided the different thermodynamic differences in oxidizing organic matter. The relA mutant, that lacks GTP pyrophosphokinase, produced the lowest power density. Although further studies are needed to fully understand the effects of those gene deletions on bacterial electrogenic capabilities, this work has enormous positive potential for the field of bioenergy. Comprehensive data acquisition for electrochemical activities of the 64 bacterial samples was rapidly performed without any external pumps, tubes, and electrical wires.

3.3. Flexibility

Flexibility is an important property of material when considered as a supporting substrate for biosensing [25,26]. This is due to the fact that the material is typically lightweight, foldable, portable and disposable. Furthermore, many additional functions such as self-healing and versatile multimodal sensors, can be integrated into flexible materials Also, flexible devices can operate by being directly attached to curved and soft surfaces to collect real-time information. Among all potential flexible substrate materials, we were particularly attracted by paper because of its versatility, low-cost, porosity and eco-friendliness [27]. Fig. 5 shows the folding reliability of our papertronic sensing array. Three devices were assessed with the bending angle of 90°. It exhibits no noticeable change in resistance of the graphite electrodes, PEDOT:PSS reservoirs, and silver lines formed on the paper through 25 folds. Even with bacteria in the device, we expected that the device

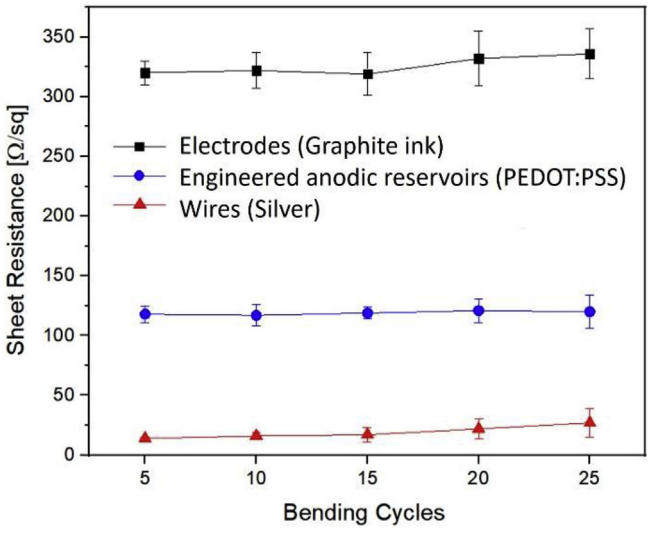

Fig. 5. Sheet resistance changes of (i) electrodes, (ii) anodic reservoirs and (iii) silver wires under repeated bending cycles.

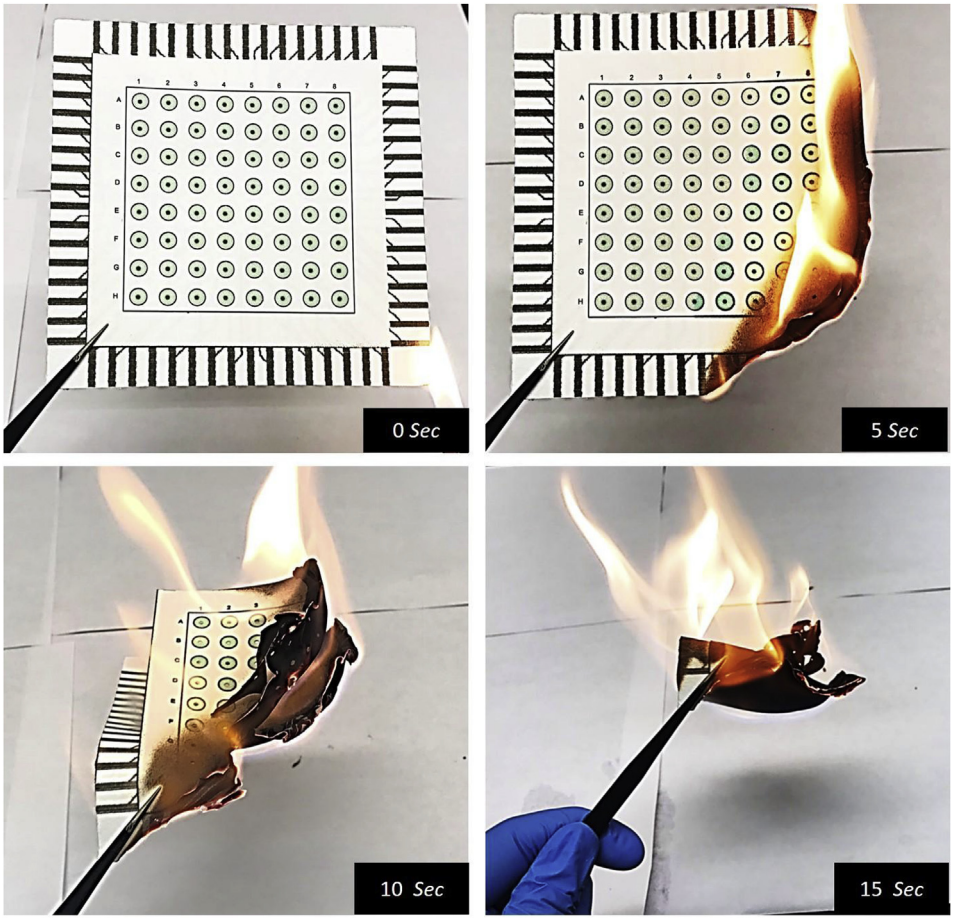

Fig. 6. Physical disposal of the device using incineration. The device showed the decomposition in the short burning time.

performance will not be damaged because of reproducible bacterial biofilm formation and cellular maintenance make the device resilient on the flexible paper. Various exciting devices can be used for high-throughput sensing in healthcare, food safety, biomedical research, and environmental monitoring.

3.4. Disposability

This device, potentially harboring 64 different bacterial species or mutants of a single organism, can pose a high risk of bacterial infection if not safely and properly disposed of after use [28]. Conventional plastic-based non-disposable devices for high-throughput screening require extra caution and complicated procedures for their complete disposal. Our entirely paper-based device platform can be revolutionarily decomposed by incineration. Fig. 6 shows the simple disposal process using incineration, where the bacterial sensing array on all paper substrates were completely burned in 15 s, demonstrating simple and rapid disposal. Given that most electronic wastes are deposited in landfills that pose environmental concerns [29], the physical destruction of our device using incineration is another useful benefit. As there are no harmful plastics or a significant amount of metal coating on our paper substrates, our device is attractive for disposable "green" electronics.

4. Conclusion

In this work, we demonstrated a flexible and disposable biosensing array on paper for a rapid, sensitive, and high-throughput characterization of microbial electrochemical activities. Electrogenic capability of two wild-type electroactive microorganisms and thirteen genetically modified mutants was examined and characterized on the papertronic 64-well sensing array. The capillary force of paper enabled passive fluidic control for storing and moving bacterial aqueous samples without using external tubing and pumps. Furthermore, all conductive lines for the 64 devices in the array were integrated onto the paper layer, leading to simple and simultaneous electrical measurement without complicated electrical wires. The *acnC* mutant of *P. aeruginosa* generated the highest power density, which is higher than those of the wild-type control strain, and even the strongly electrogenic *S. oneidensis*. Finally, our high-throughput MFC paper array will provide fast, reliable, and accurate information and a quantitative assessment of more electrochemically active microbes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nanoen.2019.104026.

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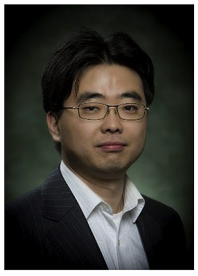

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