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Bioelectricity production from sweat-activated germination of bacterial endospores

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ABSTRACT

A microbial fuel cell is created that uses a bacterium's natural ability to revive from dormancy to provide on-demand power for next-generation wearable applications. In adverse conditions, *Bacillus subtilis* responds by becoming endospores that serve as a dormant biocatalyst embedded in a skin-mountable paper-based microbial fuel cell. When activated by nutrient-rich human sweat, the germinating bacteria produce enough electricity to operate small devices, such as the calculator that we operated to test our methodology. The spore germination is artificially accelerated by nutritious germinants, which are pre-loaded on the skin-contacting bottom layer of the device, absorb the released sweat, and deliver a mixture of the dissolved germinants and sweat to the spores. When the skin-mountable device is applied to the arm of a sweating volunteer, it can generate a maximum power density of 16.6 μW/cm^2 through bacterial respiratory activity. A potential risk of bacteria leakage from the device is minimized by packaging with a small pore size paper so that bacterial spores and germinated cells cannot pass through. When three serially connected devices are integrated into a single on-chip platform and energized by sweat, a significantly enhanced power density of 56.6 μW/cm^2 is generated, powering an electrical calculator. After three weeks of dormant storage, the device exhibits no significant decrease in electrical output when activated by sweat. After use, the device is easily incinerated without risking bacterial infection. This work demonstrates the promising potential of the spore-forming microbial fuel cell as a disposable and long storage life power source for next-generation wearable applications.

1. Introduction

As material science and sensing technologies advance, single-use disposable sensors have experienced tremendous growth in popularity, and their market is expected to reach $8.5 billion by 2025 (Dincer et al., 2019; Killard, 2017; ReportLinker, 2021). The disposable sensor is a device platform designed for one-time use or short-term measurement cost-effectively and efficiently, enabling simple, rapid, and easy-to-use analytic tests for anyone, anywhere, and anytime. In particular, the field of disposable sensors holds great potential in clinical diagnostics, the food industry, and environmental monitoring as they provide point-of-care or point-of-need testing without degradation, recalibration, and contamination issues (Dincer et al., 2019; Killard, 2017). Furthermore, their disposability avoids contributing to the dramatic increase in electronic waste (Killard, 2017). In recent years, disposable sensors have been integrated into wearables to monitor the fatigue and fitness levels of athletes for short-term training and competitive sports or to collect sweat non-invasively for one-time or short-term continuous measurement of health-related biomarkers (Lee et al., 2018; Tu et al., 2020; Vinu Mohan et al., 2020). The wearable sensors establish intimate interfaces with the human body, directly monitoring human activities and multiple biochemical indicators and allowing self-management without bulky external equipment or trained personnel (Barlya et al., 2018; Parlak et al., 2020). After a single on-body use, the sensors can be readily disposed of without potential spreading of contamination.

To ensure that the wearable sensors can work independently and self-sustainably for the expected short operating lifetime and then be economically and eco-friendly disposed of, it is critical to develop

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efficient and disposable power supplies. Microwatt-level energy harvesting technologies are attractive for meeting short-term operation or single-use measurement compared with widely used longer-operating batteries and energy storage devices that are wasteful for short-term low-power applications and environmentally problematic for disposal (Fan et al., 2020; Hasan et al., 2021). Among many energy harvesting technologies for wearables, biofuel cells offer an attractive energy solution as they can directly harvest bioelectricity from human sweat (Bandodkar et al., 2020; Wu et al., 2020). Sweat is readily and constantly available in sufficient quantities and can be obtained non-invasively or on-demand through local chemical stimulation. Although many enzymatic biofuel cells have been successfully demonstrated as power sources for short-term wearable applications (Jeerapan et al., 2020; Yu et al., 2020), the promise of this energy technology has long been limited by the low operational and storage stability of the enzymes (Xiao et al., 2019). Several innovative approaches have been proposed to address these issues such as improving immobilization, engineering enzymes, and adding mediators (Huang et al., 2020; Xiao et al., 2019), but these methods are time-consuming and labor-intensive, running counter to the goal of inexpensive and disposable applications.

Instead of redox enzymes, microorganisms can be used as a more stable and cost-effective biocatalyst for the biofuel cell without requiring complicated bioreagent preparation and immobilization processes (Osman et al., 2010). Microorganisms contain complete enzyme pathways and regenerate biocatalytic enzymes as part of their natural metabolism (Osman et al., 2010), providing superior self-sustaining features with long-term operational stability. Many microbial fuel cells have been developed as disposable power sources (Gao et al., 2019; Fraiwan et al., 2016; Nguyen and Taguchi, 2020; Veerubhotla et al., 2019), and their wearable platforms have recently been demonstrated that use many non-human or human skin-inhabiting bacteria (Gao et al., 2020; Mohammadifar et al., 2020; Pang et al., 2018; Ryu et al., 2021). Because human skin is a great habitat for millions of bacteria and organic food for bacteria is readily available in sweat, the direct use of the bacteria as the biocatalyst for power generation is conceivable for wearable applications. Furthermore, using non-toxic bacteria or skin-inhabiting bacteria directly extracted from the host can reduce potential health concerns related to bacterial cytotoxicity (Mohammadifar et al., 2020). The best strategy to adapt microbial fuel cells for wearable applications is to directly use the bacteria living on human skin, continuously generating power through their metabolism in sweat. However, this approach is not feasible in reality because electricity-producing bacteria must be selected from the skin microbiome and a sufficient number of the cells be cultivated through time-consuming and complicated multistep culturing processes, requiring tens of hours for an initial power generation, and hampering on-site analytic testing. Recently, flexible and wearable microbial fuel cells were pre-inoculated with a sufficient number of well-cultivated electricity-producing bacteria and freeze-dried to be stored until used (Mohammadifar et al., 2020). The freeze-dried bacteria were easily activated for power generation through rehydration with sweat. Despite excitement about this innovation, the freeze-dried cells had a critical limitation in long-term storage. The power density dramatically decreased with storage time, requiring special procedures for increasing their stability during storage (Mohammadifar and Choi, 2017). Maintaining the optimized conditions for storage is very challenging especially in resource-limited environments. Furthermore, the freeze-drying technique requires well-controlled multiple processes or very expensive bulky equipment.

In this work, by using spore-forming bacteria *Bacillus Subtilis* as the biocatalyst, we established an innovative and practical strategy to revolutionize the microbial fuel cell for on-demand wearable power generation having a long battery shelf-life. *B. subtilis* tolerates a variety of prolonged external stresses such as nutrient depletion, freezing, hot temperature, desiccation, and high pressure, by transitioning into a dormant state as endospores, ensuring their long-term preservation without degradation or denaturation (Christie and Setlow, 2020; Khanna et al., 2020). The endospores pre-loaded in the microbial fuel cell demonstrated long-term shelf-life stability and stabilization and the on-demand power generation was readily activated when the spores returned to life in the process of germination with nutrient-rich sweat (Fig. 1). Three skin-mountable microbial fuel cells connected in series produced a maximum power density of 56.6 μW/cm^2 from human sweat, which was more than enough electrical energy to power an electrical calculator. Although the electrogenicity of the Gram-positive *Bacillus* species was demonstrated in previous reports (Chen et al., 2019; Nimje et al., 2009; Zhao et al., 2020), no other studies have proposed using endospores to create a long-term shelf life for the microbial fuel cells and investigated their electricity-producing capabilities through spore germination in sweat for wearable power generation. The microbial fuel cell was constructed with multiple layers of paper and enclosed in a small-pore-sized paper wrapper, generating an entirely paper-based battery platform and facilitating device incineration. Although *Bacillus Subtilis* belongs to a group generally recognized as safe (GRAS) (Zhang et al., 2020), the packaging kept the endospores within the device preventing them from posing health concerns. To promote the spore germination and reduce the start-up time for the power generation, a mixture of nutrient germinants was pre-loaded on the bottom packaging layer through which the sweat moved along with the germinants to the spores (Fig. 1). Instant power was produced by the sweat, which grew significantly for an hour. The power performance sustained even after 3-week shelf storage in our uncontrolled lab environment, indicating the spores' long-term viability without specific storage and handling conditions.

2. Results and discussion

2.1 Changes in the electrogenic activity of *B. subtilis* in spore germination. The electricity-producing bacteria can transfer electrons through the cell membrane to their extracellular environment (Lovley, 2012). This unique ability is widely harnessed in microbial fuel cells for direct power production from the broad diversity of organic substrates such as wastewater, soiled water, and body fluids, making the technique a promising wearable power source on the human body (Mohammadifar et al., 2020). While most microbial fuel cell technologies have advanced with Gram-negative bacteria such as *Geobacter sulfurreducens* and *Shewanella oneidensis* (Zhao et al., 2020), even Gram-positive bacteria with their thick non-conductive cell walls were showed to extracellularly transport electrons to an electrode in a microbial fuel cell (Pankratova et al., 2019; Tahernia et al., 2020). Many Gram-positive skin bacteria were successfully shown to perform direct electricity generation, which created opportunities for the design of wearable microbial fuel cell technologies (Mohammadifar et al., 2020). However, the direct use of the human bacteria from collection, separation, cultivation, preparation to long-term storage until used remained a major challenge for use in practical applications. In this work, we provided an innovative method that uses *B. subtilis* endospores as dormant biocatalysts in the disposable wearable microbial fuel cell for simple preparation of the bacterial sample and their long-term storage. *B. subtilis* is a well-known Gram--positive electricity-producing bacterium, widely found in soil and the gastrointestinal tract of humans (Zhang et al., 2020). Because of their favorable culturing characteristics, easy genetic manipulation, highly efficient protein secretion, and non-pathogenicity, *B. subtilis* has been widely used for industrial bio-manufacturing applications (Su et al., 2020). The bacteria can form endospores to endure adverse environmental conditions such as heat, freezing, high pressure, UV radiation, and nutrient depletion (Riley et al., 2021), enabling long-term storage and survival even in various manufacturing processes. Their endospores are extremely resistant and metabolically inert forms and a 500-year storage experiment that started in 2014 shows no significant decrease in spore viability yet (Ulrich et al., 2018). Recently, the Voigt group at MIT demonstrated that *B. subtilis* endospores endured harsh 3-D printing

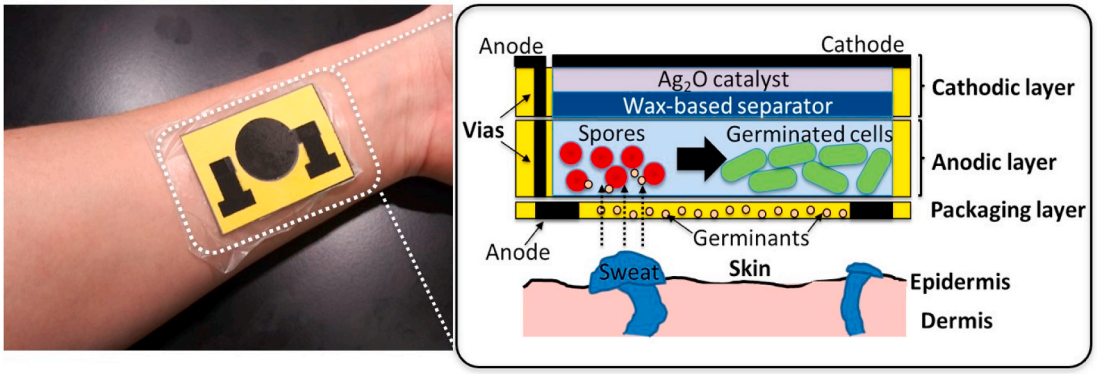

Fig. 1. Conceptual image of the proposed work on a spore-forming microbial fuel cell as a disposable and wearable power source.

conditions and successfully germinated even after various extreme stresses with low pH, high osmolarity, ethanol, UV light, and radiation (Gonzalez, et al., 2020). Because *B. subtilis* can change its structure from a metabolically dormant spore to a vegetative reproductive state through germination when nutrients become available, the bacteria can be excellent biocatalysts for wearable on-demand power generation in the presence of nutrient-rich sweat. The endospores allow long battery shelf life by maintaining their dormant, non-reproductive, and environmentally resistant structure. Although the processes of sporulation and germination in *B. subtilis* have been extensively studied, its electrogenic characteristics during spore germination have never been explored.

The flexible paper-based microbial fuel cell was first developed to characterize the electrogenic capability of *B. subtilis* in its process of germination. The preliminary in vitro studies monitored bioelectricity production of *B. subtilis* during its stages of germination from endospores upon the introduction of Luria-broth (LB) growth medium. The paper-based microbial fuel cell consisted of three functional paper layers; a cathodic layer, an anodic layer, and a packaging layer (Fig. 1 & Fig. S1). The cathodic layer contained a solid-state Ag_2O catalyst for the reduction and a wax-penetrated separator to contact the anode layer when assembled. The anode paper layer was engineered with poly(3,4-ethylenedioxythiophene): polystyrene sulfonate (PEDOT: PSS) to promote conductivity and enhance the rate of electron transfer from the bacteria (Fig. S1) (Gao and Choi, 2018). While the cathodic and anodic papers were Whatman Grade 1 filter paper having particle retention of 11 μm, Whatman Grade 410 filter paper with particle retention of 1 μm was carefully selected as the packaging layer to prevent the bacterial cells and spores from moving out of the device and posing potential health and environmental risks (Fig. 2). In the meantime, the packaging layer is gas- and liquid-permeable allowing nutrient-rich samples such as LB and sweat to be collected and delivered to the anodic layer by capillary action (Fig. S2). To make sure that the packaging layer effectively preserves and protects the bacterial cells and spores, two different

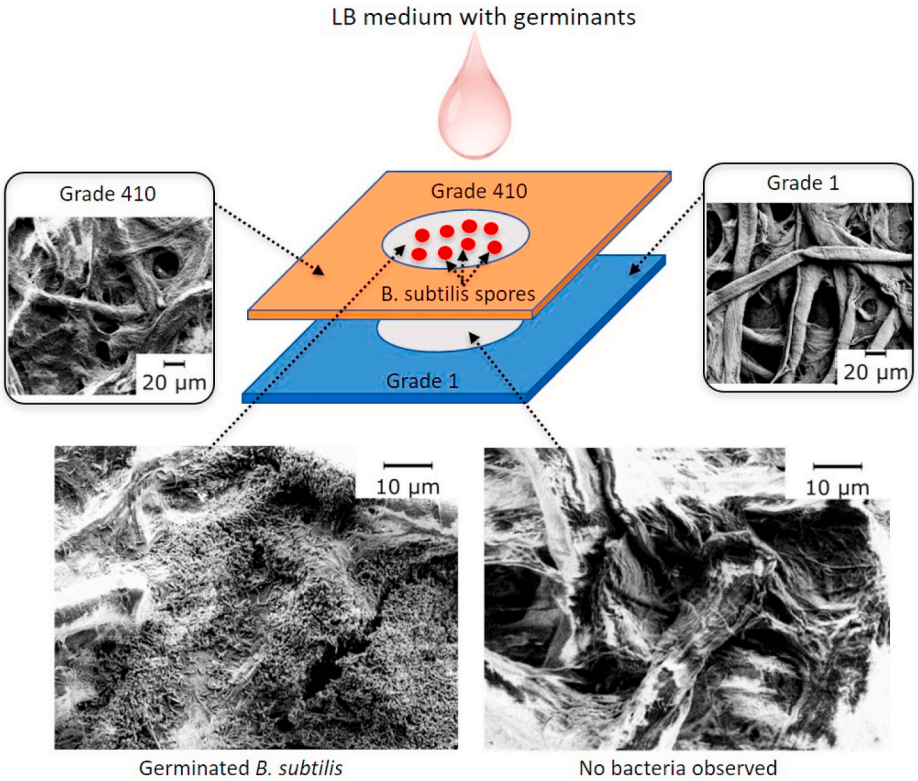

Fig. 2. SEM images of Whatman Grade 1 and 410 filter papers before and after the bacterial filtration test. The pore size of the Grade 1 filter paper is much larger than that of Grade 410. The spores and the germinated cells are not able to pass through 1 μm pore size paper (Grade 410).

paper layers were prepared and attached. The top Whatman Grade 410 filter paper was pre-loaded with endospores and the bottom Whatman Grade 1 paper had none (Fig. 2). When the LB medium with special germinants was introduced from above, the bottom paper worked as an absorbent pad drawing in the medium from the top. As shown in scanning electron microscope (SEM) images (Fig. 2), the LB with germinants triggered the germination in the bacterial spores of the top layer but none of the bacterial cells were observed on the bottom layer, indicating that the spores and the germinated cells cannot pass through the Whatman Grade 410 filter paper. The *B. subtilis* spore has a mean length of 1.33 μm with an aspect ratio of 1.86 while the size of the geminated cell is much larger than the endospore.

Bacterial endospores can rapidly germinate in response to nutrients, returning to a vegetative state for power generation. *B. subtilis* possess three germinant receptors, GerA, GerB, and GerK, to detect nutrients and trigger spore germination (Sayer et al., 2019). Normally, the use of additional germinants that directly interact with the germinant receptors can significantly reduce the concentration of sweat necessary to trigger germination (Sayer et al., 2019; Stewart et al., 2012), improving germination speed and start-up time for power generation. In particular, L-Valine is recognized by GerA and a mixture of L-Asparagine, D-glucose, D-fructose, and K+ ions (AGFK) can interact with GerB and GerK, allowing the germination within minutes of their exposure (Sayer et al., 2019; Stewart et al., 2012). Here, a mixture of L-Valine and AGFK was pre-loaded and dried on the packaging layer where the released in vivo sweat or the introduced in vitro LB sample was first absorbed. The sweat or the LB penetrated through the packaging layer by capillary action and delivered the pre-loaded germinants to the endospores on the upper anodic layer, allowing rapid germination and power production.

After the LB medium was introduced into the device, the polarization curve and power output of the microbial fuel cell were measured at 15 min, 30 min, and 1 h, based on the current at a given external resistance (Fig. 3a). Identical experiments were conducted on three different devices to demonstrate the reproducibility, and the results are shown by error bars in the data. The medium without the spores and the spore sample without germination were used as the negative controls. The microbial fuel cell started to generate the electrical current in only 15 min, which is distinctively higher than the control without bacterial spores or the sample only with the spores, indicating that the output originates from the extracellular electron transfer of the germinated cells. As the endospores took the germination process with the time, the open circuit voltage (OCV), the power density and the current density increased significantly and saturated in 1 h when all spores germinated, producing 0.47 V of OCV, 13 μW/cm^2 of power density, and 86 μA/cm^2 of current density (Fig. 3a). The fluorescent images with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) show that the germination with green cFDA dye starts to gradually outnumber the spores with red PI dye in 15 min and is completed in an hour (Fig. 3b). The electrical outputs and images demonstrate that the spore is metabolically dormant without electrogenic capability. The electricity production can be initiated when the vegetative cells are formed by germination followed by a more extended outgrowth phase. The OCV value increased with the germination proceed (Fig. 3a). Given that the OCV is a function of the potential of the individual electrodes and the anode potential is set by the respiratory enzymes of the bacteria, the OCV can be a critical indicator to germination progress while the vegetative cells increase the electrochemical metabolic activities. When we used freeze-dried *S. oneidensis* as the biocatalysts and the power generation was activated through rehydration with the LB introduction (Mohamaadifar and Choi, 2017), their OCV values did not change even though their power and current increased with time. This indicates that freeze-dried cells already contain respiratory enzymes to contribute to the OCV even though they are not active yet before the full rehydration (Fig. S3). Therefore, the OCV cannot be used to determine the number of rehydrated cells.

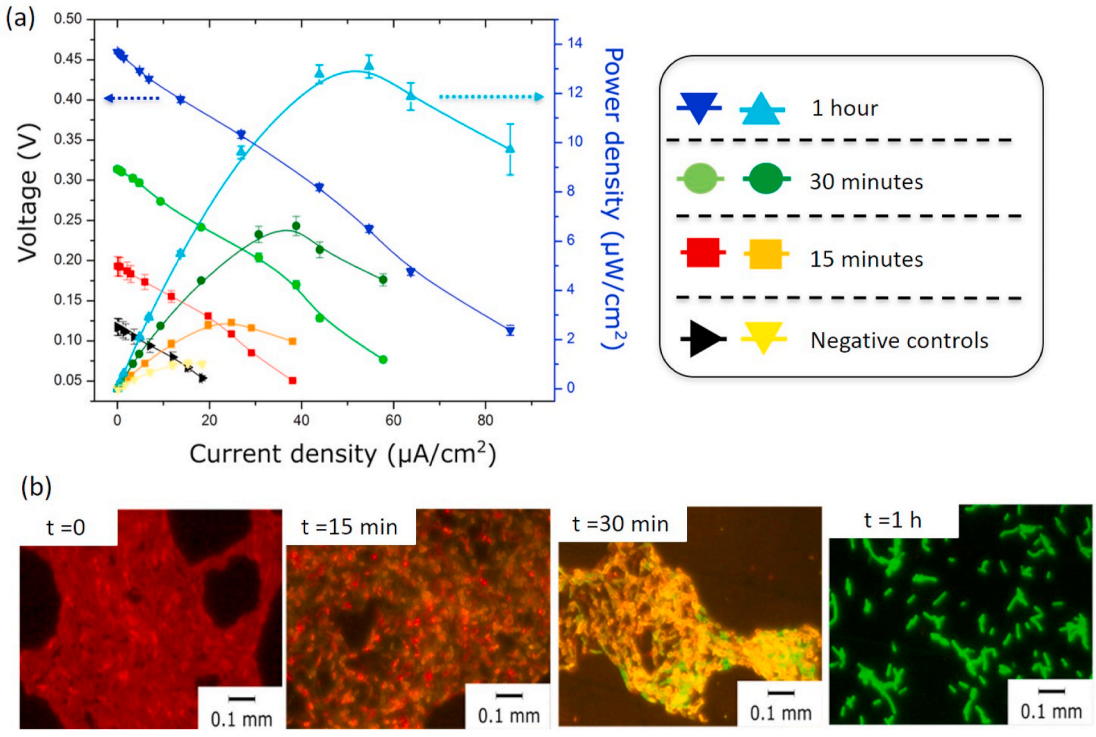

Fig. 3. (a) Polarization curve and power output of the microbial fuel cell with *B. Subtilis* endospores 15 min, 30 min and 1 h after the LB medium is introduced. The negative controls have no bacterial spores or contain spores only without germination. (b) Fluorescence images showing spore germination. Red shows the spores while green represents the germinated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.1. Device shelf-life and serial connection

The bacterial ability to form endospores allows them to survive external stress and storage in an uncontrolled environment. Although their longevity under potential environmental extremes has not been explored comprehensively, many short-term storage experiments show potentially stable spore viability for many years (Gonzalez et al., 2020; Pandey et al., 2013; Sanchez-Salas et al., 2011; Ulrich et al., 2018). In this task, three-week-long storage experiments were carried out to study the stability of the bacterial electrogenicity every week for three weeks. 40 μL of the harvested spore sample was inoculated in the anodic region of the device and then air-dried for 2 h before being sealed with the packaging paper. Nine device samples were prepared and stored in our uncontrolled lab environment (having about 22.8 °C and 45% of relative humidity) where three were used once a week for each of the three weeks. After every week's storage, the devices were activated with the LB medium to germinate the dried endospores. After the three weeks, their electrical outputs exhibited no significant decrease showing only ±5% variation (Fig. 4a). Although further long-term scale storage experiments under various extreme stress are required for more practical applications, this shelf-life is outstanding compared with the freeze-dried technique, which loses 22% of its power reduction within three weeks in the same storage environment (Mohammadifar and Choi, 2017).

The typical sustainable output voltage and power generated from a single microbial fuel cell (~0.3 V and ~ a few μW) may limit its ability to power useful wearable applications in practice. Most conventional wearable devices require voltage and power on the order of >1.0 V and > tens of μW for energy-consuming functions. The electric outputs can be boosted by integrating an additional power management system including a DC-DC converter and an energy storage device, but it may increase complexity and cost, making the battery system unsuited for disposable applications. Furthermore, those necessary electronic components contain toxic wastes and require destructive manufacturing processes, generating negative effects on the environment and public health (Mohammadifar et al., 2018). Therefore, to produce sufficient voltage and power for practical applications, it is indispensable to connect multiple device units. Single device units can be stacked in series to produce higher power and voltage outputs. Fig. 4b shows how cell performance improves with a serial connection of the units. The OCV of the two-cell and three-cell serial connections was ~0.8 V and ~1.25 V, respectively, which is equal to the sum of the single unit's OCV (~0.4 V). The maximum power density of the two- and three-cell stacks was 32.3 μW/cm^2 and 56.6 μW/cm^2, respectively. The connection of multiple microbial fuel cells can provide a realistic solution to achieve a sufficient energy performance for wearable applications.

2.2. Skin-mountable microbial fuel cells and in vivo power generation

Once the body produced sweat after a workout, the flexible paper-based microbial fuel cell was tightly attached to the skin with good conformity by using a skin-adhesive tape. Three healthy human subjects were involved in the in vivo power generation with their sweat. While instant power generation was observed, the full performance analysis was performed by measuring the polarization curve and power output after an hour when the germination was considered complete. As shown in Fig. 5a, the electrical output exhibited great variation among individual subjects mainly because of differences in human sweat composition depending on their dietary habits and water contents. During the testing, sweat evaporation was minimized by the multi-layered device structure. The maximum power density was obtained from subject #3, generating 16.6 μW/cm^2 while subjects #1 & #2 produced 9.31 μW/cm^2 and 7.41 μW/cm^2, respectively. Subject #3 showed the highest OCV value (0.44 V) presumably because his sweat sample contained the most

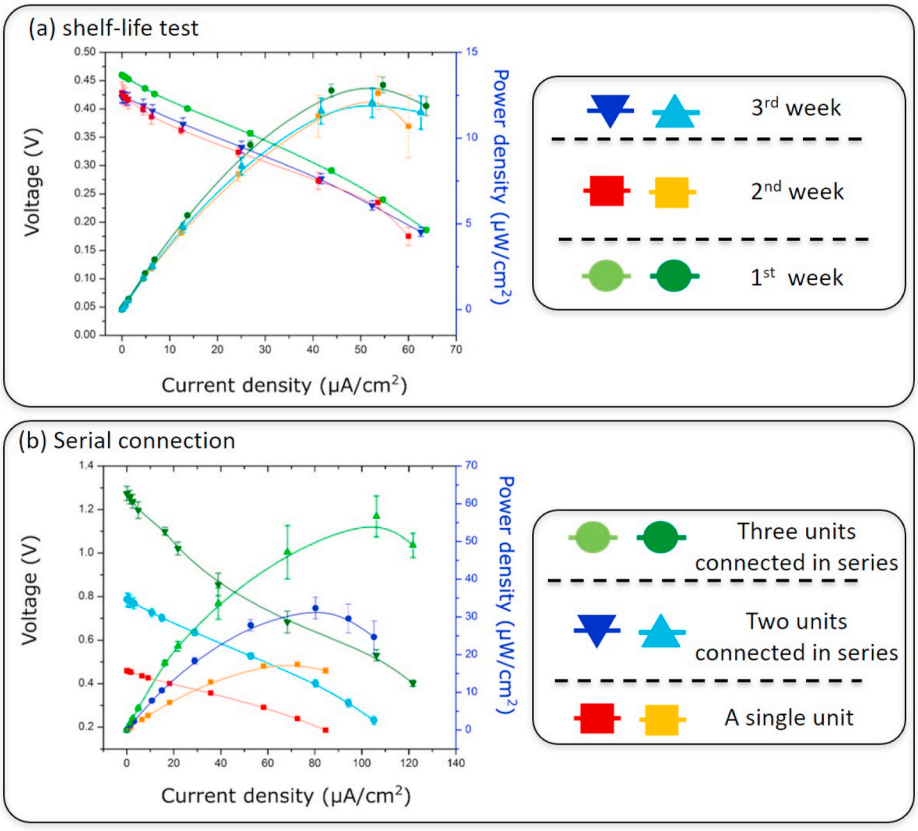

Fig. 4. (a) Polarization curve and power output of the microbial fuel cell with *B. Subtilis* in germination after 1 week, 2 weeks, and 3 weeks of storage. (b) Polarization curve and power output of the microbial fuel cell when connected in series.

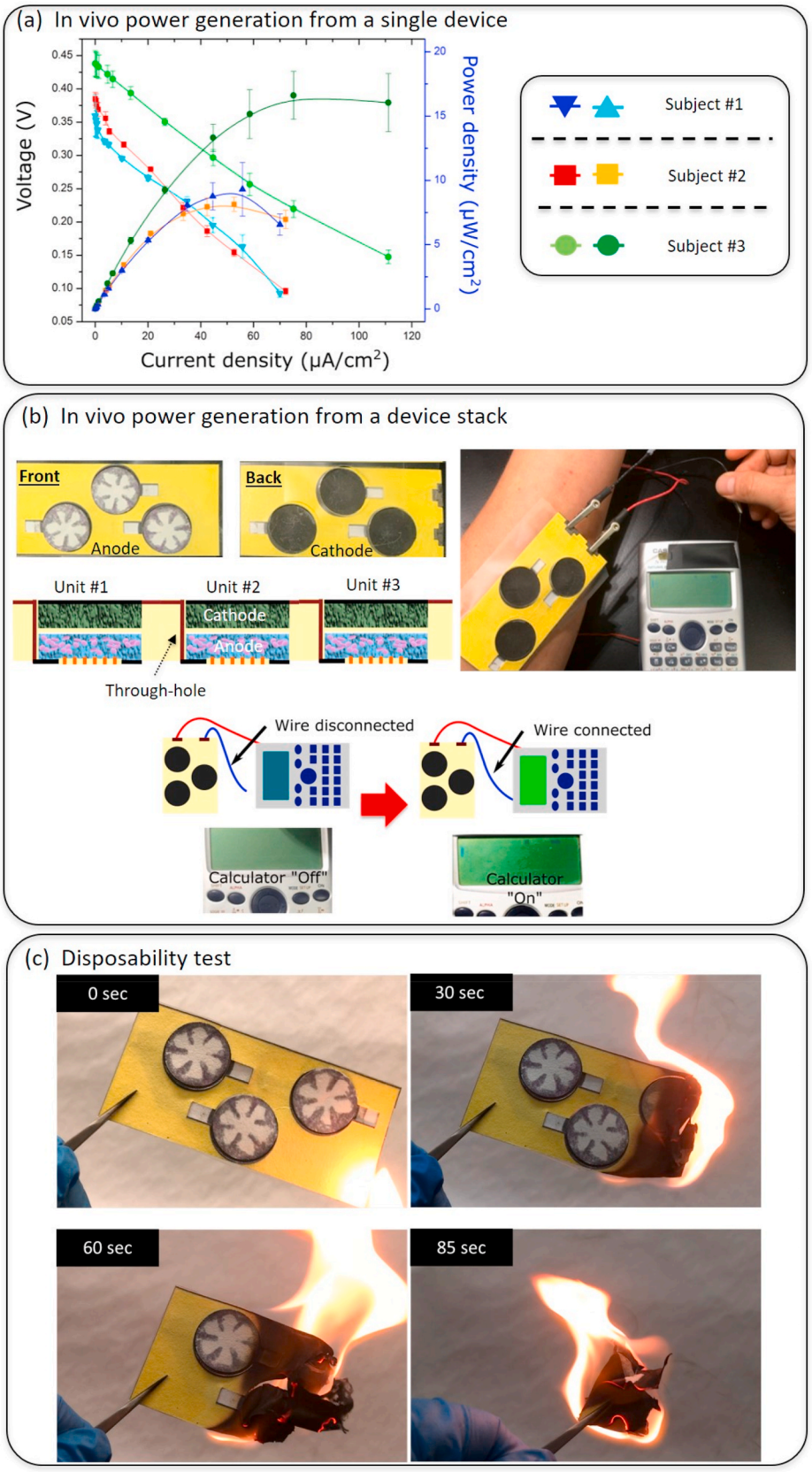

Fig. 5. In vivo power generation directely from human sweat. (a) Polarization curve and power output of the single microbial fuel cell applied to three human subjects. (b) A stack with three microbial fuel cells connected in series powers the electrical calculator. (c) Disposal of the paper device by incineration. The whole process took about 85 s.

chemical energy (Fig. 5a).

For more practical power generation, three microbial fuel cells were designed to be integrated into a single on-body device platform. Individual unit cells were connected in series by using through-holes (Fig. 5b). The entire paper-based system was batch-fabricated by screen-printing or spraying materials and patterning hydrophilic and hydrophobic regions on paper with a commercial wax printer. The skin-mountable stack was attached to the skin of subject #3 when perspiration was observed. The on-body device was connected to an electrical calculator an hour after the sweat was introduced, successfully powering the calculator. After the use, the devices required only 85 s to be completely disposed of by incineration (Fig. 5c). This demonstrates the promising potential of the spore-forming microbial fuel cell as a disposable power source for next-generation wearable applications.

2.3. Future direction

Although the use of the bacterial endospores as biocatalysts for biological fuel cells provided an innovative method to improve device practicability and shelf-life, the device requires an hour to provide the maximum performance capacity with the full germination of the spores, hampering on-site analytic testing when perspiration takes place during exercise. For short-term possible solutions, we may increase the number of spores to be inoculated so that the initial power generation at the early stage of germination can be improved. Or, the device can be first pre-inoculated with a sufficient number of vegetative cells allowing a thick biofilm tightly adhered on the anode, followed by the sporulation process inside the device. In this way, a start-up time for bacterial accumulation and acclimation for bacterial respiration with the anode and their electricity generation can be considerably reduced. Another possible solution is that some other geminants to accelerate the germination can be explored.

Furthermore, the effects of sporulation techniques and storage conditions on the stability and electrogenicity of the spores for a long-term period must be examined for commercial and wider applications. Also, various external stresses must be applied during their storage to provide a practical strategy for the microbial fuel cells. Finally, the mechanical stability and performance of the device will be required.

3. Conclusion

In this work, we, for the first time in research regarding microbial fuel cells, used *B. subtilis* endospores as a dormant biocatalyst, which became the metabolically active form of the bacterium, the vegetative cell, when their environment became nutrient-rich. This paper-based, disposable, wearable, spore-infused microbial fuel cell produced harvestable electricity when human sweat prompted the spores to germinate. Because the endospores can remain dormant for extended periods and develop a remarkable resistance to adverse environmental conditions, the shelf-life of the microbial fuel cell can be extended without requiring special procedures. Even after three weeks of storage, the electrical output of the device exhibited no significant decrease. With additional nutrient germinants (i.e. L-Valine and AGFK), spore germination was rapidly promoted. During in vitro experiments with the LB medium, the germinant-loaded microbial fuel cell produced 0.47 V of OCV, 13 $\mu W/cm^2$ of power density, and 86 $\mu A/cm^2$ of current density. For in vivo experiments with the skin-worn microbial fuel cell, the human sweat generated a maximum power density of 16.6 $\mu W/cm^2$. Bacterial spores and germinated cells were preserved and protected by packaging the device with a filter paper having a pore size of less than 1 μm. The skin-mounted device stack containing three units connected in series had a high OCV of 1.25 V and a maximum power density of 56.6 $\mu W/cm^2$, which produce more than enough electrical energy to power an electrical calculator. After use, the paper-based device was simply disposed of safely by incineration without posing a potential risk from bacteria. Although challenges to reduce start-up time need to be addressed and further stability studies for a longer-term period are required, this proof-of-concept spore-forming microbial fuel cell can provide a realistic and practical solution for a novel sweat-activated power source that can achieve the long shelf-life vision of self-sustaining wearable applications.

4. Experimental procedure

4.1. Bacterial inoculum and sporulation

B. subtilis strain 168 was originally obtained from the American Type Culture Collection (ATCC) and its overnight precultures were grown in 20 mL of LB medium with gentle shaking overnight at 37 °C. Sporulation in *B. subtilis* was induced by nutrient exhaustion (Pandey et al., 2013). The sporulation progress was monitored with a traditional optical microscope. When 90% of the bacteria cells formed endospores, the culture was pelleted by centrifugation at 4000 rpm for 4 min three times. The harvested spores were resuspended with distilled water and stored at 4 °C. All remaining vegetative cells or germinating spores were inactivated by thermal treatment at 70 °C for 30 min.

4.2. Germinant preparation and fluorescence visualization during spore gemination

The LB medium including L-Valine (10 mM) and AGFK (10 mM L-Asparagine, 33.6 mM D-Glucose, 33.6 mM D-Fructose, 60 mM KCl) was prepared as the germinant which was pre-loaded in the patterned hydrophilic region of the packaging layer and then dried in air. The spore germination was first confirmed on a glass slide with the germinant by a fluorescence microscope. A combination of two dyes with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) allowed visualization of the spore germination. The PI's red fluorescent dye showed the dormant spores while the cFDA stained the metabolically active germinated cells.

4.3. Bacterial fixation and SEM imaging procedures

The paper samples were treated with 4% glutaraldehyde solution in 0.1 M phosphate buffer saline (PBS) overnight at 4 °C for bacterial fixation. The fixing reagent was rinsed three times with 0.1 M PBS. To dehydrate the inoculated bacterial cells, the device went through a series of baths made up of 35%, 50%, 75%, 95%, and 100% ethanol. Finally, it was immersed in hexamethyldisilazane (HMDS), a highly volatile organic compound, for 10 min to minimize the distortion. The samples were further placed in a desiccator and dried overnight. The samples were sputter-coated with carbon (208HR Turbo Sputter Coater, Cressington Scientific Instruments, UK) and examined with a field emission SEM (Supra 55 VP, Carl Zeiss AG, German).

4.4. Fabrication of skin-mountable microbial fuel cells and their stack

The skin-mountable microbial fuel cell consisted of the engineered conductive paper anodic layer, the cathodic paper layer with the wax-based separator, and the packaging paper layer having a smaller pore size than the bacterial spores (Fig. 1, Fig. S1, Fig. S2, & Fig. S4). Whatman Grade 1 filter paper having particle retention of 11 μm was selected as the paper substrate for the anodic and the cathodic layers. The relatively large pores of the paper allowed incorporation of a large number of bacterial spores and the cathodic catalysts to be well-prepared. Wax boundary patterns were printed on both sides of the paper by a Xerox Phaser printer (ColorQube 8570) and heated at 140 °C for 50 s to allow the impregnation of the hydrophobic wax into the paper. The well-patterned wax on the cathode was used as the separator. The conductive anodic layer was prepared by introducing a 20 μL mixture of 1 wt% PEDOT:PSS and 5 wt% dimethyl sulfoxide (DMSO) into the paper followed by air-drying for 8 h. After that, 20 μL of 2 wt%

3-glycidoxypropy-trimethoxysilane was added to the region and air-dried for 2 h to improve its hydrophilicity and thus uniformly distribute the liquid bacterial samples. A graphite ink was screen-printed on top of the peripheral of the engineered anodic layer leaving the window in the middle for the spore inoculation and the sweat introduction. The cathode was prepared by introducing 300 mg of silver (I) oxide (Ag_2O) in 10 mL PEDOT:PSS ink. The Ag_2O catalyst has been widely used for the cathode because it remains in a solid state in a water environment and shows stable activity (Xie et al., 2013). The cathode was prepared by screening-printing the graphite ink on top of the catalyst. The anodic and the cathodic layers were carefully aligned and attached by using adhesives (Super 77, 3 M Company, USA). The harvested spores were loaded in the engineered anodic region and then Whatman Grade 410 filter paper pre-loaded with the nutrient germinants was placed as a packaging layer. Because the pore size of the packaging layer is smaller than the bacterial spores, no leakage of any bacterial-related materials is expected. A skin-adhesive double-side tape (PC2723U, Scapa Healthcare) allowed tight and conformal attachment of the flexible paper-based microbial fuel cell to the skin. Three microbial fuel cells were integrated into a single on-chip platform and connected in series to increase their electrical output. The individual anodes facing the skin were extended toward the top layer by using through-hole technology so that the devices can be readily connected through the top layer without any additional wires.

In our microbial fuel cell, the endospores germinate and oxidize the organic fuel in sweat and produce electrons and protons. The electrons move to the cathode through an external circuit while the protons diffuse from the anode to the cathode through a wax separator to maintain electroneutrality. At the cathode, Ag_2O is reduced to Ag by the electrons and the protons that traveled from the anode.

4.5. Freeze-drying procedures

40 μL of *S. oneidensis* MR1 with 10% sucrose cryoprotectant was pipetted onto the anodic reservoirs for lyophilization. The devices with bacterial cells were then frozen at −80 °C and placed in a freeze-dryer (FreeZone Plus 2.5 L Cascade Benchtop Freeze Dry System) to undergo the lyophilization process for 12 h at 25 × 10^{-6} bar pressure. After the lyophilization was complete, the devices were stored at 4 °C.

4.6. Bioelectricity measurement

The potential difference between the anode and the cathode was measured by a data acquisition system every 12 s. The data acquisition system (USB-6212 from National Instrument) was coupled with a customized LabView interface. Current and power generation were calculated from the measured voltage outputs with the connected external resistors. Current and power densities were normalized to the anodic area.

CRediT authorship contribution statement

Jihyun Ryu: Investigation, Methodology, Data curation, Writing – original draft. **Seokheun Choi:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113293.

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