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Biofabrication and Characterization of Multispecies Electroactive Biofilms in Stratified Paper-based Scaffolds

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Abstract: Bioelectrochemical technologies have attracted significant scientific interest because the effective bacterial electron exchange with external electrodes can provide a sustainable solution that joins environmental remediation and energy recovery. Multispecies electroactive bacterial biofilms are catalysts that will drive the operation of bioelectrochemical devices. Unfortunately, there is a lack of understanding of key mechanisms determining their electron-generating capabilities and syntrophic relations within microbial communities in biofilms. This is because there are no universally standardized models for simple, rapid, reliable, and cost-effective fabrication and characterization of electroactive multispecies biofilms. The heterogeneous and long-term nature of biofilm formation has hampered the development of those models. This work develops novel biofabrication and analysis platforms by creating innovative, paper-based 3-D systems that accurately recapitulate the structure, function, and physiology of living multispecies biofilms. Multiple layers of paper containing bacterial cells were stacked to simulate different layered 3-D biofilm models with defined cellular compositions and

microenvironments. Overall bacterial electrogenic capabilities through the biofilm structures were characterized by thoroughly monitoring collective electron flows through different external resistors. Changes in the type of species and order of stacking created biofilm modeling which allowed for the study of their electrogenic performance via variation in electron flow rate output. Furthermore, multi-laminate structures allowed for straightforward de-stacking and layer-by-layer separation for analyses of pH distribution and cellular viability. Our multi-laminate structures provide a new strategy for (i) controlling the biofilm geometry of 3-D bacterial cultures, (ii) monitoring the microbial electoral properties, and (iii) constructing an artificial biofilm layer by layer.

1. Introduction

Extracellular electron transfer (EET) in electroactive bacteria is an extraordinary and transformative process through which self-sustaining and self-maintaining intracellular reactions can be linked to external environments having discontinuous and dependent features [1, 2]. In particular, the study of microbial bidirectional electron exchange with external abiotic electrodes in oxygen-limited environments has evolved into a new, separate field of study, named "electromicrobiology." The exchange enables sustainable electrochemical synthesis, effective pollutants treatment and remediation, and renewable energy generation in bioelectrochemical systems. Those rapidly developing systems include microbial electrosynthesis (MES), microbial electrolysis cells (MECs), microbial fuel cells (MFCs), and microbial desalination cells (MDCs) [3, 4]. Moreover, as bioelectronics brings unprecedented changes to conventional semiconductor electronics, electroactive bacteria have received increasing attention as an effective interface to bridge the signal gap between biological systems and abiotic electronics [5, 6]. The two most

critical requirements to translate these promising EET-based techniques from laboratory curiosities to practical applications are "performance" that is at least comparable to non-renewable and abiotic existing techniques and "longevous operation" that can fully and cost-effectively exploit microbiological self-sustainability. The most practical and feasible solution can be found by learning from and then emulating natural microbial forms, behaviors, and processes [7]. Bacteria in natural environments co-exist in densely packed, spatially structured, multicellular communities defined as biofilms [8]. Individual microbial species in biofilms enable division of labor, exchange of nutrients and metabolites between species, and facilitate bacterial survival in and resistance to a hostile environment through collective group responses [9, 10]. The natural bacterial multicellular biofilms find the most optimized condition to maximize their viability and survival by controlling their population and spatial organization [9, 11]. Many previous studies demonstrated that co-cultured bacterial systems generated longer and better bioelectrochemical performance in a more self-sustaining manner than monocultures [12-14]. Furthermore, the bacterial cells embedded in a biofilm enabled a considerably higher EET rate than planktonic cells because of substantially more compact cell densities and more available EET pathways in the biofilm [15]. Because of the heterogeneous and long-term nature of biofilm formation, however, there are no universally used standard biofilm models, and no studies elucidate adequately multispecies biofilms. Although conceptual co-culture models with some selected communities have been successfully demonstrated, the models are limited to conventional 2-D culture platforms that do not reflect the heterogeneous and complicated 3-D natural environments of multispecies biofilms. When electroactive biofilms use anaerobic electrode respiration to perform their EET process, the electron transfer efficiency directly relies on oxygen and electrochemical gradients

formed throughout 3-D biofilms [16, 17]. Depending on microenvironmental changes in

the gradients, bacterial metabolic activities vary, further changing the local cell viability

and pH, and eventually affecting the performance and longevity of the bacterial systems.

Therefore, an in-depth understanding of the key mechanisms underlying the EET process

within the biofilm thickness, and of the fundamental factors responsible for determining

efficient electron exchange with external electrodes will be critical. Unfortunately, none

of the conventional techniques quantify these critical parameters inside biofilms nor

control 3-D biofilm formation and thickness. Last but not least, constructing microbial

communities having microscale spatial microbial structures remains elusive [12].

Conventional lab-based culture models do not form reproducible and controllable

multispecies communities with a spatially structured network. What is needed is the ability to reliably biofabricate 3-D multispecies electroactive biofilm model systems with a spatial arrangement of community members and to practically characterize key parameters inside local biofilm and determine critical factors in limiting bacterial EET efficiency. The model system must mimic *in vivo* environment in biofilms with the ability to modulate the concentration gradients of the metabolites.

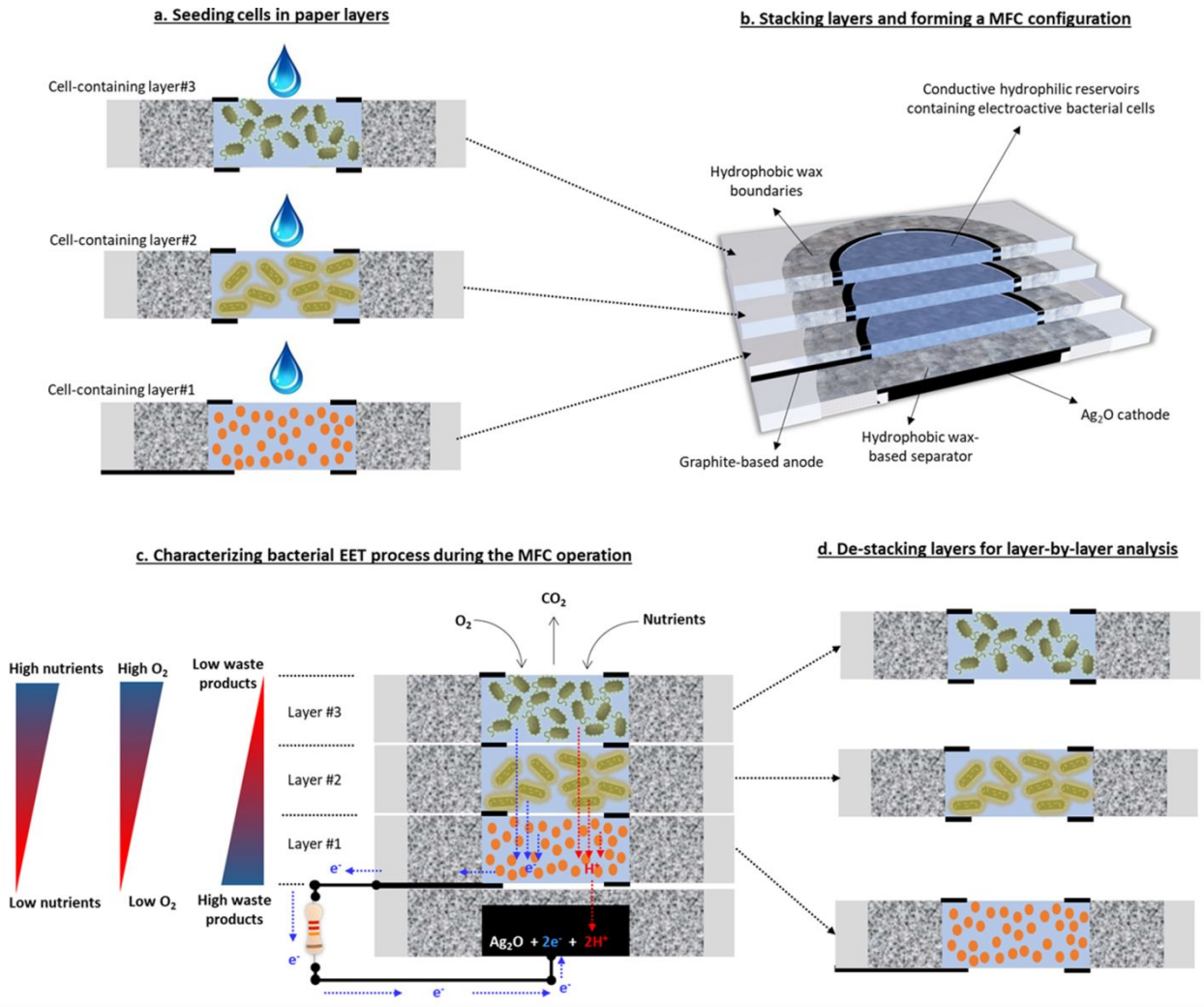

Figure 1. Schematic diagram of our paper-based biofabrication and charaerization platform for 3-D multispecies electroactive biofilm models. After inidividual paper layers are inoculated with electroactive bacteria as scaffolds (a), they are stacked to form a layered 3-D multispecies biofilm model (b). Mass transport of nutrients and oxygen into the 3-D system are regulated to explore the bacterial EET process and current generation within contolled biofilms (c). De-stacking the multi-layered biofilm constrcut allow layer-by-layer analysis for pH distribution and cellular viability (d).

In this work, we create innovative, paper-based biofabrication and characterization platforms for 3-D multispecies electroactive biofilm engineering (Figure 1). Previously, paper-based cell culture platforms have been successfully demonstrated for various human cells [18-20]. However, no studies have explored the biofabrication of stratified biofilm for better electrochemical performance and to control biofilm functions to optimize the efficiency of electron transfer. Stacking individual layers of cell-containing paper offers a modular platform that can mimic 3-D complex biofilm structures with defined cellular compositions and microenvironments. Multiple layers of paper allow a single species to form a biofilm with thicknesses determined by the number of layers or a biofilm where individual species that are confined in separate layers can exchange metabolites. Individual paper layers can be well patterned by wax printing to define hydrophilic and hydrophobic regions. The printing is followed by a conductive coating of the non-conductive hydrophilic areas where bacterial cells are inoculated. Their metabolically produced electrons can be harvested. By vertically adding another paper layer integrating a wax-patterned region as a separator and an Ag_2O electrode as a cathode from the bottom of the bacteria-containing stack, a microbial fuel cell (MFC) can be constructed (Figure 1). The MFC effectively evaluates the metabolically produced electrons through the bacterial EET process by allowing a collective measurement of the electron flows as current. The electrical current values will vary according to different stack configurations and the number of bacterial layers. The microfibers of paper closely resemble those in extracellular polymeric substances of the biofilms, providing mechanical integrity and structural robustness to scaffolds the bacteria use to form biofilms. The porous structure of paper provides natural gradients of oxygen, nutrients, and bacterial waste products as a result of diffusional limitations throughout stratified biofilm constructs, consequently

leading to different metabolic activities of bacteria within local biofilms and thus better simulating 3-D in-vivo environments. The biocompatibility and the strong capillary force of paper improve cell adhesion in liquid cultures and stimulate rapid biofilm formation. Finally, multi-laminate biofilm structures allow for straightforward de-stacking and layer-by-layer separation at the end of an MFC operation and provide spatially resolved microbial colonies for further analysis such as pH distribution and cellular viability. This work will revolutionize knowledge about and applications of electromicrobiology by developing novel biofabrication and analysis platforms with a controllable 3-D biofilm model. Those models will help users mimic different biofilm interactions to further study them via de-stacking the 3D paper system. This will support the fundamental study and characterization of microbial EET interactions with electrodes and contribute to an understanding of their EET activities within biofilm thickness and syntrophic relations within microbial communities. Optimizing the spatial arrangement of community members in biofilms and maximizing the metabolic interactions between biofilm layers will be the next-generation strategy for engineering electromicrobiology [12, 13]. The application value of this simulation tool will motivate more innovation in multiple fields of bio-energy conversion, remediation of organic pollutants and toxic metals, and biomedicine.

2. Results and discussion

2.1 Seeding bacterial cells in individual paper layers and stacking the layers for 3-D thick biofilm formation. Bacterial EET-based electrochemical techniques will be able to be realizable when their performance is sufficient for actual applications. Given that the performance depends on the collective EET capabilities of individual cells, it is desirable

to design and construct densely packed aggregates of cells [15]. While bacteria can exist in the form of planktonic cells or biofilms, the cells in biofilm play a more critical role in the EET process and improve electrochemical performance [21]. The contribution of planktonic cells represents less than 20% of the total bioelectrochemical performance in a system [22]. However, electro-microbiological studies have traditionally been limited because of the lack of models of natural biofilms. Previous static or dynamic biofilm formation methods with laboratory-scale bioreactors require a relatively long time, skilled staff, and a considerable amount of reagents, leading to non-reproducible and non-controllable outputs [23]. Recent microfluidic approaches are limited to small populations that cannot mimic a thick *in vivo* biofilm environment [24, 25]. Even the latest advances in 3-D printing require complicated and time-consuming processes for bioink preparation, and it is controversial whether the 3-D printed biofilm resembles the actual *in vivo* biofilm environments [26, 27]. Above all, reported work with electrogenic bacterial cells for electroactive biofilm formation is unavailable or quite limited.

Here we propose a 3-D, paper-based biofilm model as a new technique for simple, rapid, and cost-effective electroactive biofilm fabrication and characterization. A hydrophilic paper can serve as a scaffold for biofilm formation and multiple layers of paper containing bacterial cells can be readily stacked to control the biofilm's thickness (Figures 2 and 3). First, hydrophobic and hydrophilic regions of the individual papers were well defined by printing and thermally impregnating hydrophobic wax (Figures 2a and 2b). Then, we coated non-conducting hydrophilic regions with a conducting material, poly(3,4-ethylened ioxythiophene):polystyrene sulfonate (PEDOT:PSS). The coating enabled the regions to have open pores and hydrophilic features for subsequent liquid-based bacterial sample introduction [28]. Because of the excellent biocompatibility,

bacterial affinity, and electrocatalytic capability of the PEDOT:PSS, bacterial cell adhesion can be significantly improved in the engineered paper (Figure 2c). Circular graphite electrodes were then screen-printed at the periphery of the conductive regions on both sides of the paper to act as the current collector and the conductive interface when being stacked (Figures S1, 2, and 3). When bacterial cells with an optical density at 600 nm (OD600) of 1.0 were introduced into the engineered hydrophilic region, the capillary force pulled the cells through the paper layers, resulting in fast accumulation and acclimation of the cells. To ensure reducing the unintended biases caused by the user fabrication process, we made it where every step of the fabrication process is micro-fabricable and every step can be automated.

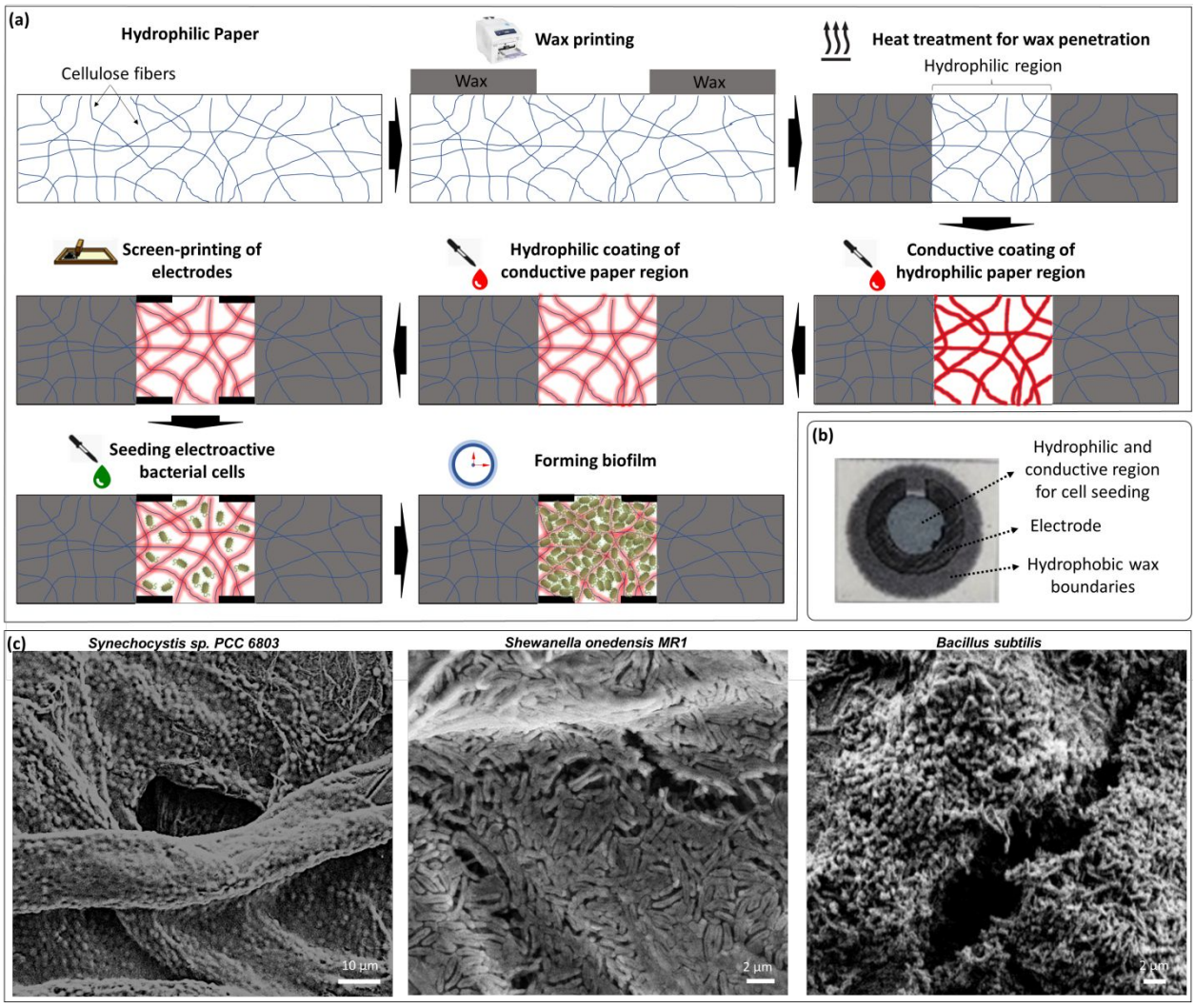

Figure 2. Seeding cells in engineered paper layers. The part of paper is engineered to be conductive and hydrophilic for bacterial cell inoculation and their biofilm formation. To effectively harvest microbial electrons metabolically produced, the non-conductive paper fibers are coated with a conductive PEDOT:PSS polymer. Hydrophilic coating is necessary to improve the capilary force of the treated paper region for rapid wicking of aquatic cell culture and cell adhesion. (a) Process flow for paper engineering and biofilm formation, (b) a picture of the engineered paper, and (c) scanning electron microscope (SEM) images of three individual bacterial biofilms formed in the engineered papers.

Rapid biofilm formation can be attributed to (i) paper's porosity throughout its large surface area and 3-D nature-like bacterial habitat, (ii) the biocompatibility and bio-affinity of the coated PEDOT:PSS to promote cell adhesion, and (iii) paper's hydrophilicity to strongly absorb the aquatic bacterial culture. The paper-based biofilm model can standardize simple and rapid biofilm formation and EET evaluation by allowing easy

seeding of bacterial cells with a micropipette. The procedure has potential high-throughput capabilities and allows easy assembly of a thick biofilm by simply stacking the layers. Individual layers were tightly attached by using a spray adhesive and paper clips, resulting in precise control of the thickness of the 3-D biofilm (Figure S1). The spray was only on the unused sides of the paper to hold the structure still to maintain consistency and did not affect any parameters of the experiments conducted. A mask was used to completely prevent any adhesive chemical from getting in contact with the anode, cathode, or separator. Overall bacterial electrogenic EET capabilities through the biofilm thickness were characterized by thoroughly monitoring the collective electron flows through different external resistors (Figure 3). Comprehensive polarization curves and output power densities as a function of current density were well obtained for different bacterial species and their different biofilm thickness (Figure S2). *Synechocystis sp. PCC6803*, *Shewanella oneidensis MR1*, and *Bacillus subtilis* were inoculated in BG-11, Luria Broth (LB), and Soy Broth (SB), respectively. Each medium without bacterial cells generated negligible electrical output compared with the cell-containing culture, indicating that all current/power productions originated from bacterial metabolism (Figure S3). Although this system-level analysis does not include cellular or genetic level assessment, it is sufficient to obtain a comprehensive understanding of the electrogenic dynamics of microbial biofilms.

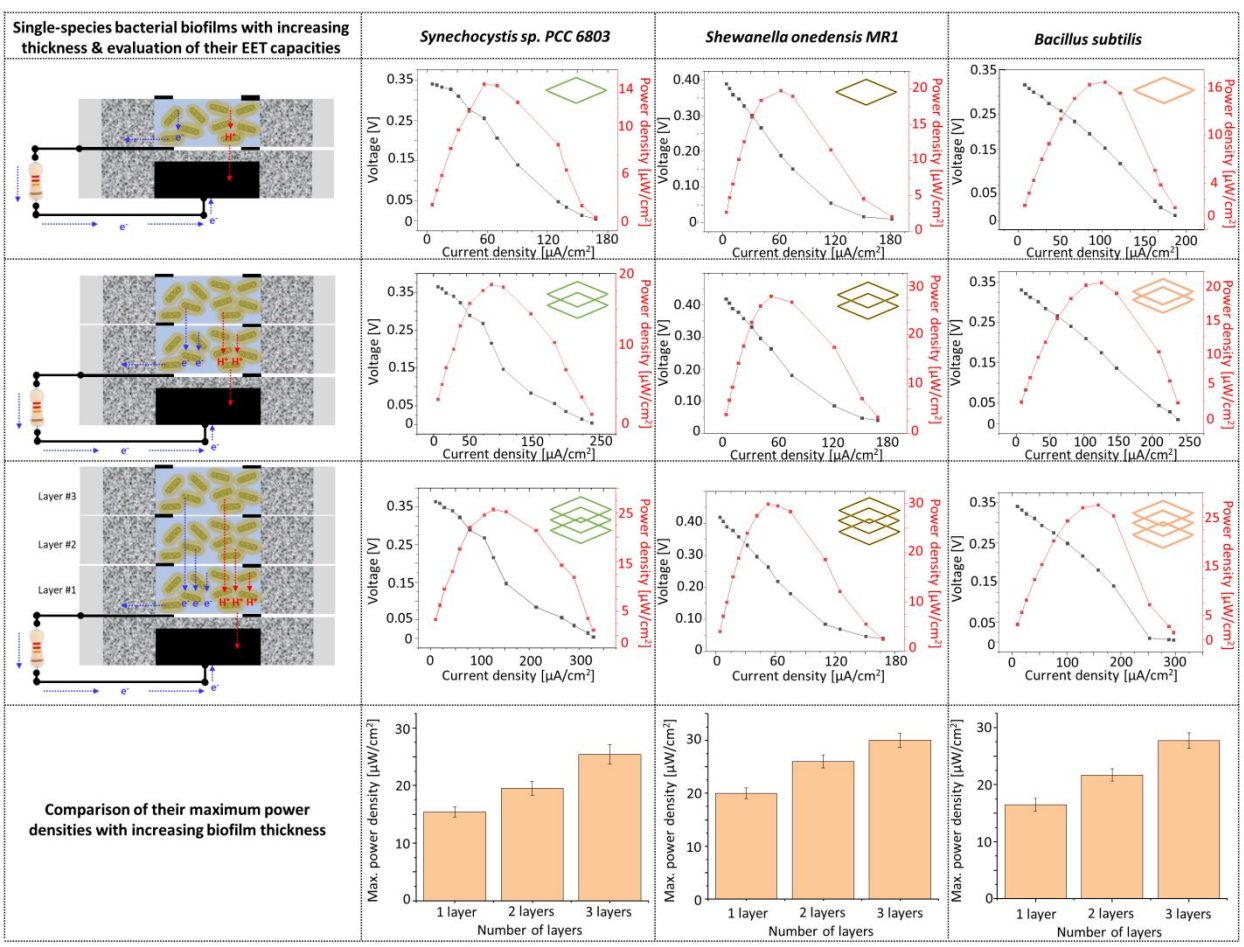

Figure 3. Evaulation of single-species bacterial EET capacities with increasing biofilm thickness. The schematic illustration of multi-laminate structures with patterned papers as a scaffold shows bacterial biofilm formation with different thickness. Three single-species, *Synechocystis sp. PCC6803*, *Shewanella oneidensis MR1*, and *Bacillus subtilis*, are tested. Polarization curves and output powers measured as a function of current are demonstrated. Finally, their maximum power densities with increasing biofilm thickness are compaired.

Obviously, as the thickness of the biofilm increases, all three species have increased electrical performance. For a single layer, the best performance was clearly shown from *S. onedensis* (*S. onedensis*: 20 μW/cm^2, *Synechocystis sp.*: 15 μW/cm^2, and *B. subtilis*: 17 μW/cm^2). Even the other two generated comparable electrical outputs with increasing biofilm thickness (*S. onedensis*: 30 μW/cm^2, *Synechocystis sp.*: 26 μW/cm^2, and *B. subtilis*: 28 μW/cm^2 for three layers). This is very surprising given that *Synechocystis sp.* and *B. subtilis* have weak electrogenic capabilities because of their low

EET efficiencies compared with *S. onedensis* having various EET mechanisms for much greater electrogenic and electrochemical activities [3]. Thicker biofilms improve the EET efficiencies of bacterial cells because of their increased numbers and the many conductive conduits for electrons to move directly from the cells to the conductive fibers. Moreover, when stacking the layers of the platform, the condition of the layer is impacted by conductive conduits of the hosted species and the other EET mechanisms of the other species surrounding it. Although further studies of chemical components of biofilms and their conductivity are required, this result proves their better EET capabilities in biofilms, providing one of the fundamental factors that can maximize bacterial electrochemical performance.

Because the multi-layered paper system is patterned by hydrophobic wax boundaries and its bottom cathodic layer is sealed, the system can only receive oxygen and nutrients through the top window. (Figure 1 and Figure 3) [18, 19]. As nutrients and oxygen diffuse into the 3-D biofilms from the top, the model provides decreasing gradients of nutrients and oxygen while the simultaneous microbial metabolism increases gradients of waste products (e.g. protons, CO_2, etc.). Our paper model can revolutionarily recapitulate the *in vivo* biofilm environment having physiological stratification where each area is differently developed along those gradients. Electrogenic bacterial cells on the top layer (#3) can rely more on oxygen for their respiration, decreasing the electrode respiration and the current generation while the cells on the bottom layer (#1) rather respire with the electrode and generate more electrons than the top layer. However, the lack of nutrients on the bottom layer can negatively affect cell metabolism and viability. When the cells oxidize the nutrients and produce electrons and protons in the multi-layered system, the electrons move to the current collector on the top of the bottom layer and flow through the external circuit to reach the cathode at the bottom of the bottom layer (Figure 3). In

the meantime, the protons diffuse to the bottom layer and travel to the cathode through the wax-based separator. The Ag_2O is eventually reduced by the protons and electrons at the cathode to maintain the charge neutrality of the MFC system. Because of the mass transfer limitation of the protons, however, the protons can pile on the bottom layer decreasing pH and cell viability. Understanding the microscale development of pH inside biofilms and its effect on cell viability can allow for better-characterized correlations between bacterial EET activities in biofilm thickness. MEMS microneedles were proposed as an alternative method for quantifying pH inside biofilms, but their invasive technique uses ferricyanide, which kills the biofilm and thus does not allow for time series measurements [29, 30]. The recently-developed pulsed-field gradient Nuclear Magnetic Resonance (NMR) technique suffers from inherently low sensitivity because of its low energies [30]. It requires careful optimization to reduce measurement times and detect low concentrations. NMR systems are bulky, heavy, and expensive, further limiting their capability and accessibility. Moreover, none of the techniques control biofilm thickness and provide an effective analysis of cell viability across the biofilm thickness. De-stacking the multi-layered biofilm construct can offer the most effective layer-by-layer analysis for pH distribution and cellular viability at different biofilm locations. To determine the effect of the pH directly on the cell viability after the MFC operation, we first avoided other potential factors to affect the cell viability such as the nutrient and oxygen gradient. Before stacking the individual layers, they were replenished with a fresh medium to eliminate a nutrient gradient. Furthermore, to avoid a significant oxygen gradient, the biofilm construct was operated right after stacking. After 5 hours of operation, we peeled apart the stacked layers and measured the pH and the cell viability of each layer (Figure 4). The pH level was evaluated by using pH test strips and compared with the initial culture pH (Figure 4a). For all bacterial species, a clear drop in pH was observed as each layer got closer to the separator. Because other nutrient and gas gradients throughout the biofilm

were intentionally minimized, the increased acidification reduces cellular fitness and proliferation.

To better understand the change in bacteria viability caused by the decreased pH levels, the

bacterial cells were harvested from the individual layers, specifically containing *S. oneidensis,* and the dead/live cells were identified with fluorescent dyes with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) (Figure 4b). As the layers come closer to the separator and have lower pH, the fluorescent images indicate that the number of dead cells gradually outnumbers the live cells. The experiment demonstrates that there can be a steep decrease in local pH throughout biofilms during microbial metabolism, and the cell viability can be significantly reduced by the unequal pH distribution. This finding establishes fundamental knowledge to determine critical factors in improving bacterial EET activities within a thick biofilm.

(a)	*Synechocystis sp. PCC 6803*	*Shewanella onedensis MR1*	*Bacillus subtilis*
Culture in media	7.6	8	7.5
Paper layer #3 (Top)	6.3	6.5	6.3
Paper layer #2 (Middle)	5.3	6.2	6
Paper layer #1 (Bottom)	4.8	6	5.8

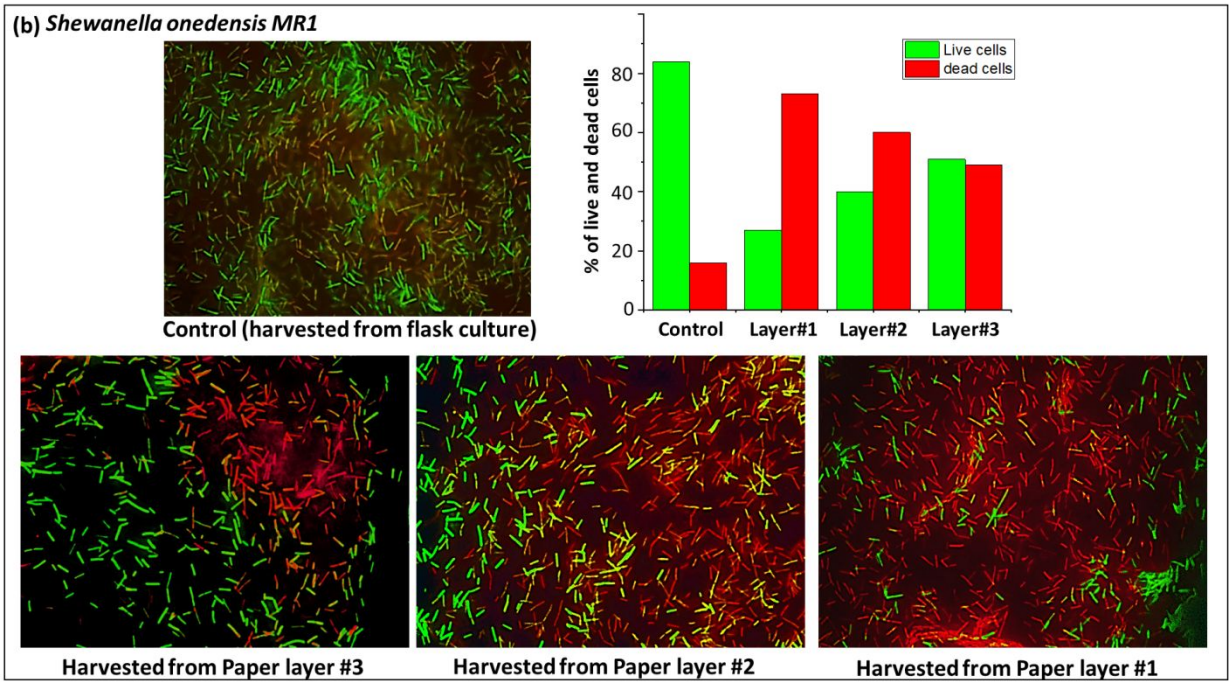

Figure 4. Layer-by-layer analysis after de-stacking three-layered biofilm constructs. (a) pH measurements of individual paper layers cultured with *Synechocystis* sp., *Shewanella* sp., and *Bacillus* sp, respectively, compared with the values of species cultured in media. (b) Cell viability tests of individual three layers with *Shewanella* sp. Control is about the one harvested from flast media culture.

2.2 Forming multilayered microbial consortium. In the natural environment, bacteria live in mixed communities having a complex heterogeneous environment with diverse metabolic exchanges and co-dependencies [9-11]. Recent studies in multispecies microbial consortia demonstrated their potential as an innovative approach enabling self-sustainable, eco-friendly, and cost-effective biomanufacturing, biopowering, and bioprocessing [12, 13, 31, 32]. On the other hand, many chronic infections require a better understanding of such communities because they are related to polymicrobial biofilms which are extremely difficult to be correctly diagnosed and effectively cured [33]. Therefore, the design and construction of artificial bacterial communities have opened a new avenue in synthetic biology not only to develop new biofabrication technologies for desired tasks but also to prevent and eradicate polymicrobial biofilms having detrimental issues. However, multispecies biofilms are extremely difficult to model. Conventional techniques are limited to uncontrollable and time-consuming mixed populations without engineered *in vivo* stratification. Even the latest microfluidic approaches suffered from very limited experimental setups with only a few selected strains. In that sense, our 3-D multi-laminate paper stack can provide a new strategy for simple, rapid, and reliable layer-by-layer polymicrobial biofilm formation in a controllable manner. Our paper-based platform can potentially generate many different experimental setups to better understand the individual and synergistic roles of key microbial populations. To quickly demonstrate the potential and practicability of our technique for 3-D multispecies electroactive biofilm engineering and compare its performance, we selected four two-species biofilms and two three-species biofilms. In our previous study [12], we comprehensively explored synergistic co-operations by using other biofabrication approaches having time-

consuming and complicated processes (Figure 5). Individual biofilm formation in paper layers and multi-layered polymicrobial biofilm stacking followed the same procedure described above but with different bacterial species. Our modular layer-by-layer platform can design nature-like microscale spatial structures with different species while maximizing their interaction and avoiding competition without physical contact between species. While all individual bacterial biofilm layers showed a very similar range of current generation as a result of collective EET activities in very conductive biofilms (Figure 3), different combinations of bacterial species and their location generated significantly different electrical performances (Figure 5). Under the illumination in a fluorescent lamp-controlled chamber, the electrical performances of the two-species biofilms were lower when *Synechocystis* sp. were located on the bottom than on the top (Figure 5a). When the bacteria were in the bottom layer, light energy could not be fully delivered to *Synechocystis* sp. through another species located on the top, and the EET activities of *Synechocystis* sp. could not be added to the collective performance. Given that *S. oneidensis* have a stronger electrogenic capability than *B. subtilis*, it is very interesting that the combination with *S. oneidensis* (Figure 5a-1)) has a weaker electrical performance than that with *B. subtilis* (Figure 5a-3)). Although the detailed chemical and metabolic communications must be explored, it can be concluded that synergetic cooperation can generate better and unexpected outputs. The three-species biofilm model with *S. oneidensis* on the bottom produced the best performance among all multispecies biofilms which is in good agreement with our previous study [12]. In this consortium, *B. subtilis* located in the middle plays a critical role in improving the collective EET activities. *B. subtilis* oxidize the organic compounds produced from *Synechocystis* sp. located above and produce riboflavin as a product that can effectively mediate the EET

process of *S. oneidensis* on the bottom (Figure 5b-1)). When *B. subtilis* was located on the bottom, the electrical performance dramatically decreased because the organic compounds were not effectively delivered to the bottom (Figure 5b-1)). All the experiments were conducted in triplicate, and data were represented as mean ± standard deviation (Figure S4). Although we have not provided additional data in this work, de-stacking the three-layered biofilm construct will enable layer-by-layer analysis for further microbial and genetic analyses. Our innovative modularity of the layer-by-layer construction of the multi-species biofilm system can allow for emulating and elucidating those key parameters and interactions between different biofilm layers, which have never been explored in any previous studies.

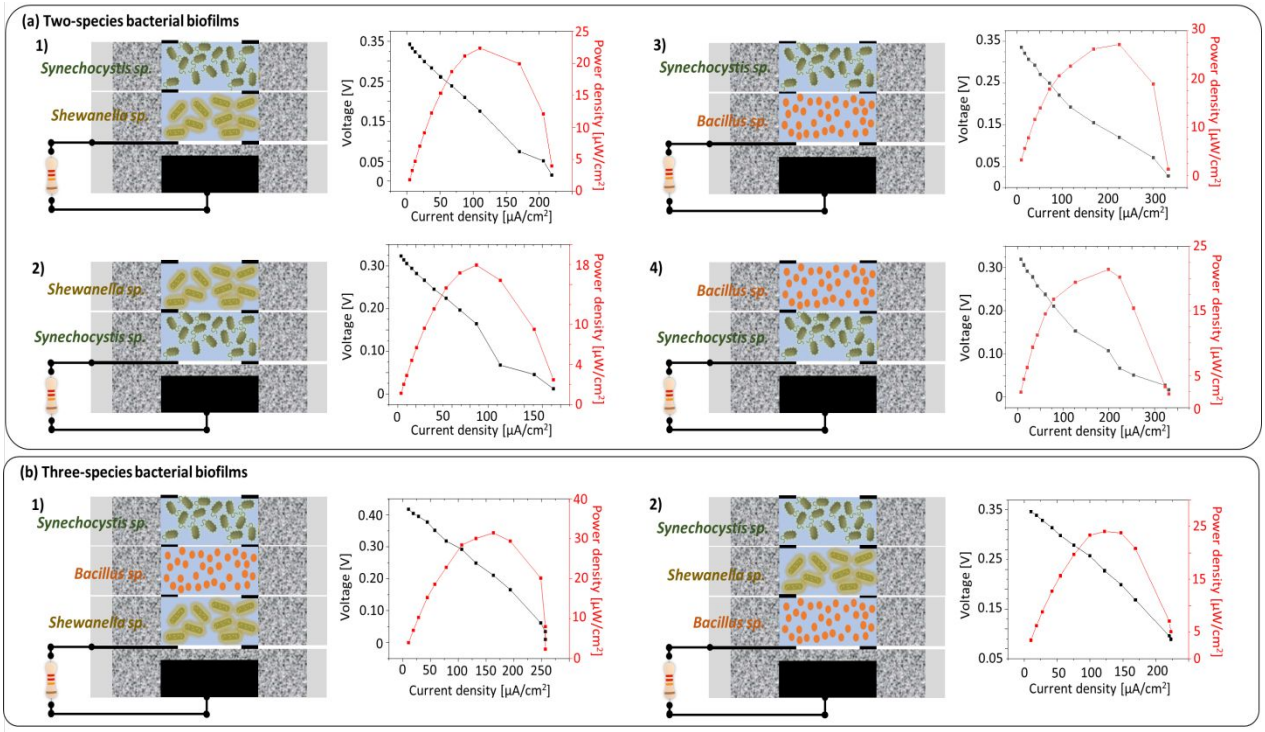

Figure 5. Evaulation of multiple-species bacterial EET capacities. (a) Schematic illustration of various two-species biofilms and their polarization/power curves (1. *Synechocystis* sp. (top) and *Shewanella* sp. (bottom); 2. *Shewanella* sp. (top) and *Synechocystis* sp. (bottom); 3. *Synechocystis* sp. (top) and *Bacillus* sp. (bottom); 4. *Bacillus* sp. (top) and *Synechocystis* sp. (bottom)). (b) Schematic illustration of two three-species biofilms and polarization/power curves (1. *Synechocystis* sp. (top), *Bacillus* sp. (middle), and *Shewanella* sp. (bottom); 2. *Synechocystis* sp. (top), *Shewanella* sp. (middle), and *Bacillus* sp. (bottom)).

3. Conclusion

In this work, we provided a new technique for biofabrication and characterization of a 3-D multi-species electroactive biofilm by using 3-D multi-laminate structures of papers as a scaffold. Multiple layers of paper containing individual bacterial cells were stacked to form a layered 3-D biofilm model that allowed us to control the thickness of the overall biofilm construct and the composition of each layer. Diffusions of the gas, nutrients, ions, and metabolites through the stack and the shapes of their gradients resemble the *in vivo* microenvironment of 3-D natural biofilms. By measuring the current generated from a

different number of layer stacks that integrated a MFC configuration, the overall EET activities with different biofilm thicknesses were quantitatively characterized. De-stacking the multi-layered biofilm construct allowed straightforward layer-by-layer analysis for pH distribution and cellular viability. Multi-cultured stacks composed of paper layers formed a multifunctional polymicrobial biofilm and increased the EET efficiency through microbial syntrophic relationship. Overall electrogenic properties of different combinations of bacterial cell types were readily investigated. The modularity of the layer-by-layer construction of the culture system probed the complex interactions between biofilm layers in the 3-D biofilm stack. Our paper-based platforms will revolutionize characterizing and optimizing multi-species biofilm communities through the metabolic engineering of organisms that can be incorporated into a practical bioelectrochemical system.

Materials and methods

Bacterial inoculum All three bacterial species, *Synechocystis* sp. PCC 6803, *Shewanella oneidensis* MR-1, and *Bacillus subtilis* were acquired from the American Type Culture Collection (ATCC). *Synechocystis* sp. were grown at 30°C in a BG-11 medium in light /dark cycles (12 hours/12 hours) for about two weeks. *S. oneidensis* were grown in an SB medium with shaking for 24 hours at 30°C. *B. subtilis* were cultured overnight in an LB medium at 37°C with shaking. Bacterial growth was monitored by using a spectrophotometer with a wavelength of 600nm (OD_{600}) and all bacterial cultures were used for tests when their OD_{600} reached 1.0.

Fabrication of layers of paper Each layer of paper was prepared by directly patterning wax onto Whatman Grade 1 filter paper sheets using a commercial wax printer (ColorQube 8570). The wax-printed paper was placed in a 150°C oven for 60 seconds to allow the impregnation of the wax through the entire thickness of the paper and to define hydrophilic regions in the center with hydrophobic peripheral boundaries. The individual papers were precisely cut by a laser cutter (Universal Laser System VLS 3.5) for further biofilm formation and multi-laminate stacking. Conductive regions were prepared by introducing a 20 μL mixture of 1 wt% PEDOT:PSS and 5 wt% dimethyl sulfoxide (DMSO) into the hydrophilic areas. 20 μL of 2 wt% 3-glycidoxypropy-trimethoxysilane was added to the treated regions to improve their hydrophilicity and thus uniformly distribute the liquid bacterial samples [28]. Circular graphite electrodes were then screen-printed at the periphery of the conductive regions on both sides of the paper to act as the current collector and the conductive interface when the individual papers are stacked. An additional paper layer was prepared to integrate a wax-based separator and a cathode. The penetration depth of the wax was carefully controlled so that the hydrophilic region was secured from the back side of the paper sheet for the cathode. The cathode was prepared by introducing 500 mg of silver (I) oxide (Ag_2O) in 10 mL PEDOT:PSS solution with 500 μL of DMSO. That layer was attached to the bacteria-containing layers to form an MFC configuration. When the cells oxidize nutrients generating electrons and protons, the electrons electrically move to the cathode through an external resistor and the protons ionically flow to the cathode through the wax-based separator. Those collective electrons are measured as a measure to determine the bacterial EET efficiencies.

pH measurements and fluorescence imaging To monitor the pH and the cell viability of the individual paper layers, the layers of the stack were peeled apart. The pH level of each layer was evaluated by pressing pH test strips on the layer and compared to the initial culture pH. For the cell viability tests, the individual layers were submerged in phosphate-buffered saline for the cells to be harvested through gentle sonication. The harvested cells were double-stained with cFDA and PI, causing the live cells to fluoresce bright green and dead cells to fluoresce red.

SEM imaging The SEM imaging protocol involved bacterial fixation in glutaraldehyde 2.5% in 0.1M PBS for 10 hours and dehydration steps in ascending ethanol series (35%, 50%, 75%, 95%, and 100%). The samples were further placed in a desiccator and left to dry 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).

Electrical measurement setup The voltage drops across various external resistors (No resistor, 470k, 250k, 162k, 100k, 71k, 47.5k, 32k, 22k, 15k, 10k, 2k, 1.5k, 0.45k, and 0.35k) were monitored with a data acquisition system (National Instruments, USB-6212). The power outputs and polarization curves were acquired through calculation with the voltage values at those resistors. Power and current densities were normalized to the anode surface area.

Author contributions

Anwar Elhadad: Investigation; Methodology; Formal analysis; Data curation; Validation; Software

Seokheun Choi: Conceptualization; Supervision; Project administration; Funding acquisition; Writing – original draft; Writing – review, editing, and finalization.

Conflicts of interest

There are no conflicts to declare.

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