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Article

Testing HCN1 channel dysregulation in the prefrontal cortex using a novel piezoelectric silk neuromodulator

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SUMMARY

Mental illnesses are prevalent in society, yet patients are widely undiagnosed and mistreated. Within this branch of diseases, schizophrenia is an underrecognized disorder that affects approximately 1% of adult patients that portray behavioral and cognitive impairments. Although no comprehensive characterization of schizophrenia exists, there is a general consensus that patients have electrical dysfunction in the prefrontal cortex. The goal of the first phase of this project was to analyze whether hyperpolarization-activated cyclic nucleotide-gated (HCN) channel expression was increased in a calcium/calmodulin-dependent protein kinase (CAMK2) knockout (KO) mouse model of schizophrenia. Fluorescence analysis of four CAMK2 KO and one wild-type mouse validated that HCN channels were upregulated in the prefrontal cortex of KO mice compared to wild-type, which was demonstrated by high localization in dendrites. Notably, there was a statistical difference between the control and schizophrenic infralimbic regions but not between the control and schizophrenic prelimbic regions, suggesting that electrical dysfunction is localized in the infralimbic prefrontal cortex and future treatments should focus there. In the second phase, we investigated the resynchronization of neuronal firing as a treatment strategy for abnormal electrical firing, which has proven to be effective in other neurological disorders. We designed a novel piezoelectric silk-based implant and optimized electrical output through the addition of conductive materials zinc oxide (ZnO) and aluminum nitride (AlN). Via ultrasound exposure, we determined that the 2 M ZnO-silk composite generated the highest electrical output. With further research and compatibility studies, this implant could rectify electrical misfiring in the infralimbic prefrontal cortex.

INTRODUCTION

Schizophrenia is a common disorder characterized by ailments such as hallucinations, delusions, disordered thinking, and a weakened ability to distinguish reality among a variety of other symptoms that greatly debilitate daily life. This enigmatic disease may arise from genetic, environmental, or neurochemical factors (1).

A rising avenue of research to explain these potential etiologies is electrical dysfunction, particularly with an emphasis on ion channel activity. Ion channels are proteins in the cellular membrane that generate and maintain electrical signals throughout the body. One ion channel family drawing attention is the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel family. HCN family members are the pacemaker channels that regulate rhythmic activity within neurons and cardiac cells (2). They were selected as etiological candidates because previous research suggested that abnormal HCN channel activity plays a role in the pathogenesis of neurological disorders (3). Dysregulated HCN channel activity has been shown to impair cognitive functions by disconnecting the cortical network and disrupting action potentials. Elevated HCN activity also lowers summation, which is the likelihood to fire an action potential (4). Summation is known to be affected in schizophrenics, which highlights the importance of the HCN family. There are four HCN channels that comprise the family. We chose to work with HCN1 knockout mice, which have been shown to have impaired motor learning (5). Furthermore, neural activity has a strong correlation with the expression level of the corresponding HCN channel, so alterations from schizophrenia should affect HCN1 activity (6).

The brain region we investigated is important in schizophrenic research. We chose the prefrontal cortex (PFC) because it regulates planning, social behavior, decision making, motor attention, and working memory which are negatively impacted in schizophrenia (7). Neuronal activity is postulated to regulate the presence of these cognitive impairments (8). Thus, we wanted to characterize electrical dysfunction of schizophrenia in the prefrontal cortex by quantifying HCN1 channel activity. The schizophrenic model we used was a calcium/calmodulin-dependent protein kinase (CAMK2) knockout (KO) model because of its numerous endophenotypes like working memory deficits, impaired neurogenesis, hyperactivity, and reduced long-term potentiation (9). The latter is very important in synaptic plasticity to maintain strong working synapses and increased activity, something injured in schizophrenia (10). In light of these similarities, we believed our KO model would have commonalities with the disease in humans. Within this model, we hypothesized HCN1 ion channel activity would increase

in the CAMK2 KO model.

Interestingly, recent electrophysiological studies reported electrical stimulation of schizophrenic PFCs increased the synchronization of neuronal oscillations and helped alleviate symptoms, especially hallucinations (11). To follow-up on these findings, the second phase of this project proposes a solution to synchronize neuronal firing in schizophrenic individuals. In Parkinson's disease and Tourette syndrome patients, electrical stimulation has proven effective by sending impulses to neutralize tremors and tics (12). Similar therapy could also assist in restoring electrical function in schizophrenic patients. However, neurosurgeries remain risky procedures and implanting electrodes magnifies potential complications. Current models include the use of a 64-electrode array which can cause surgical complications (13). Hence, portable and self-powered systems would facilitate less complicated procedures and decrease the likelihood of adverse reactions. Amongst various models of self-powered systems, we believe piezoelectric nanogenerators are the most suitable candidates for neural stimulation because they eliminate the traditional usage of batteries and reduce the size of the system, which is ideal for surgical procedures.

The self-powered system model we believed most advantageous was a self-generating electrical implant, which would avoid secondary surgeries to recharge batteries, circumvent further damage to the brain, and in principle, not require external energy sources. Such an implant should be made of a minimally invasive thin-film transducer such as ferroelectric, dielectric, or piezoelectric materials. The latter is particularly interesting as these materials can generate electricity more efficiently in the body (14). Piezoelectricity is the conversion of mechanical stress into electrical energy. Piezoelectric materials include non-centrosymmetric crystals that reorient their molecular dipole moments under physical stress (15). Silk holds great potential as a platform for neuromodulation. We chose silk as our biomaterial because it is mechanically resilient and produces sheer piezoelectricity, thus making it an alluring candidate for electrical stimulation (16). This project phase focused on developing a working implant model by maximizing voltage produced. We added various concentrations of two piezoelectric, conductive materials: zinc oxide (ZnO) and aluminum nitride (AlN). We hypothesized that the highest concentration of conductive material, 2 M, would generate the most voltage. Additionally, ZnO was hypothesized to be most efficient because of its hexagonal wurtzite shape which generates a high coupling factor, the degree to which mechanical energy can be converted to electrical energy and vice versa (17).

We determined HCN1 expression was increased in our schizophrenic mouse model and that our 2 M ZnO-silk composite was most effective as a working model for a piezoelectric implant.

RESULTS

HCN1 channel expression was quantified in the PFCs of CAMK2 KO and wild-type mice using immunohistochemistry (IHC). To image HCN1 channels in these samples, we used a Nikon Eclipse 800 microscope and ImageJ software to quantify integrated density and area. Corrected total cell fluorescence (CTCF) minimized non-specific changes and standardized our results. CTCF intensity values were then compared between regions. We used IHC to observe where HCN1 channels localized and to deduce their roles based off their physical locations. We observed that the majority of HCN1 localization is within the dendrites and towards the midline of the brain (**Figures 1-2**). This suggests that HCN1 channels are involved in the integration of input and sending

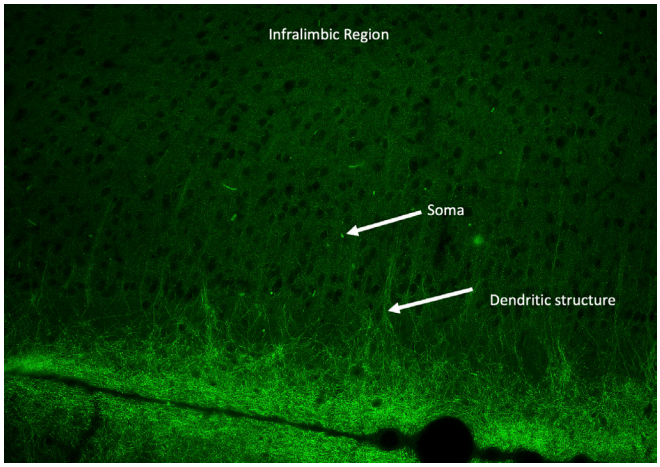

Figure 1. Immunofluorescence stain of HCN1 channels in the schizophrenic infralimbic region using primary antibody anti rabbit HCN1 and secondary antibody anti-rabbit Alexa Fluor 488. The spiny projections are HCN1 channels localized on dendrites.

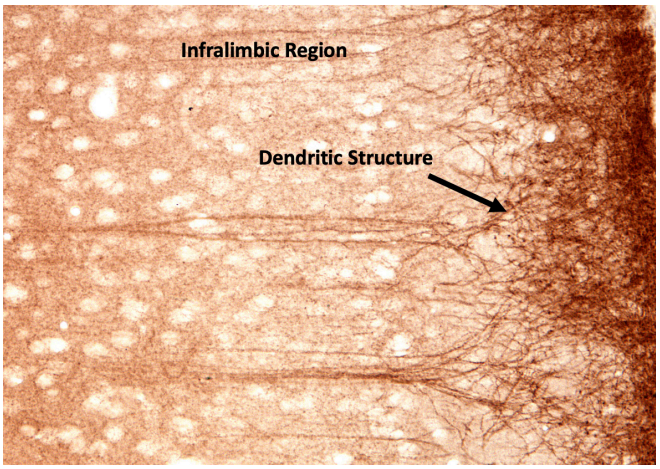

Figure 2. This diaminobenzidine stain of HCN1 channels in the infralimbic region using primary antibody anti rabbit HCN1 and secondary antibody anti-rabbit Alexa Fluor 488 shows dendritic localization.

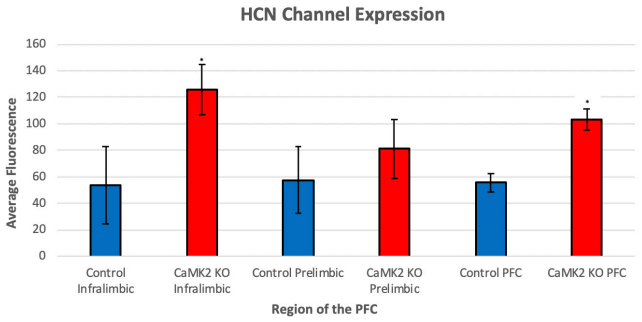

Figure 3. The HCN1 levels measured in the entire PFC, infralimbic, and prelimbic regions in the control and the schizophrenic mouse model.

of messages, so alteration of HCN1 presence might affect these functions. However, further research is needed to test this.

The initial scope of our analysis was the entire PFC of schizophrenic KO and control WT mice. We observed an 85.69% increase in HCN1 channel expression in the PFCs of schizophrenic samples compared to the control samples (**Figure 3**). However, to gain a better understanding of localized electrical dysfunction, we analyzed the subregions of the PFCs—infralimbic and prelimbic regions. We observed the largest increase of HCN1 expression in the infralimbic region of schizophrenic PFCs with a 133.69% increase, whereas in the prelimbic region, we observed a 40.88% increase in HCN1 expression. We ran ANOVAs comparing HCN1 staining in control and schizophrenic mice. The schizophrenic infralimbic regions were statistically upregulated from the control regions with an F-value of 19.05 and a critical value of 4.11. However, the schizophrenic prelimbic regions were not statistically different from the control regions with an F-value of 3.14 and a critical value of 4.09. The HCN1 expression levels within schizophrenic infralimbic were also statistically increased from the schizophrenic prelimbic regions with an F-value of 9.11 and a critical value of 4.11. The results suggest the infralimbic region is the only significantly affected division of the PFC that exhibits altered HCN1 expression levels in the schizophrenic mouse model.

In our second project phase, we quantified the voltage produced from the samples via targeted ultrasound waves and a multimeter. We induced piezoelectric properties into the silk by solvent modification with ethanol which creates beta pleated sheets (secondary protein structure). This process was able to generate an average of 0.15 mV, serving as a proof of concept, and subsequently our control, that this novel method works and also validating the usage of silk as our biomaterial (**Figure 4**). Different concentrations of piezoelectric AlN and ZnO were added to enhance the natural piezoelectric properties of silk, and the voltage readouts of these composite materials were collected (**Figure 4**). Single factor ANOVAs and t-tests were conducted to statistically validate the increases in voltage produced relative to the control samples. The average of AlN samples when compared with the control dataset produced statistically significant differences with an F-value of 2.10 and a critical value of 3.07. All of the *p*-values were greater than 0.05. These results suggest that AlN does not significantly enhance the natural piezoelectric properties of silk.

We observed that samples with 2 M ZnO as an additive produced the highest voltage (0.302 mV), which was greater than twice the control, supporting our hypothesis that increasing concentrations of ZnO can increase the piezoelectricity of silk. The ANOVA for all ZnO concentrations produced an F-value of 7.9356, which was greater than the

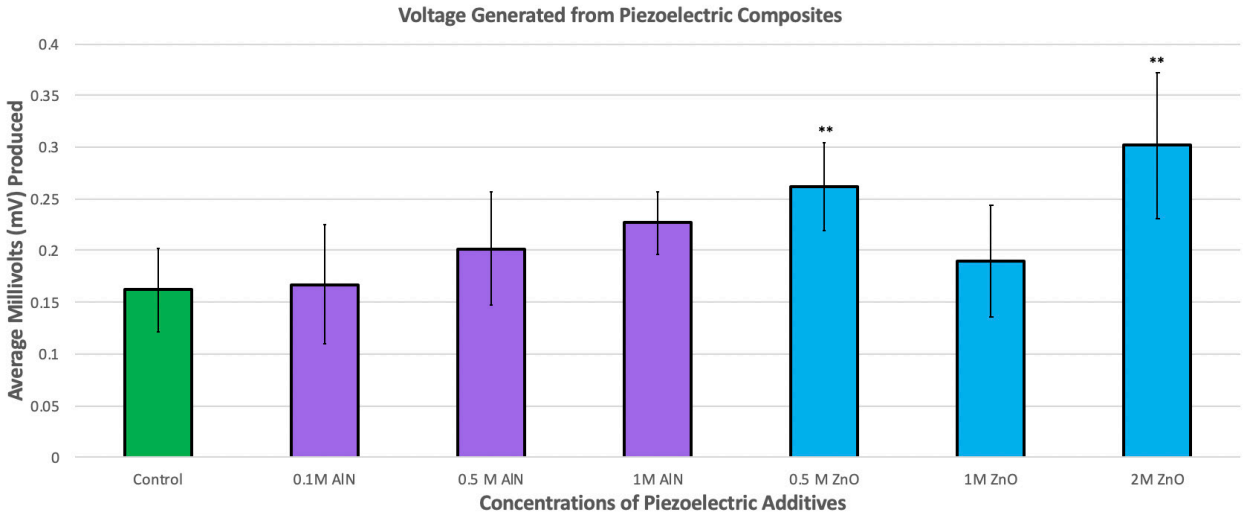

Figure 4. The piezoelectric additives aluminum nitride (purple bars) and zinc oxide (blue bars) generated higher voltages than the control (green bars). The asterisks show statistical increase in comparison to the control sample. Single factor ANOVAs and t-tests were ran with a Tukey-Kramer Post Hoc Test to determine statistical significance.

critical value of 3.0725, suggesting at least one of the added concentrations yielded statistically significant results. Our Tukey-Kramer post-hoc test showed 0.5 M ZnO samples were statistically increased from the control and 1 M ZnO sample groups. Additionally, the 2 M ZnO samples were statistically increased from the control and 1 M ZnO sample groups.

DISCUSSION

Currently, there is limited information concerning the pathology of schizophrenia. This study has identified potential mechanisms behind electrical dysfunction. Ion channels remain important in understanding the development of cognitive deficits in neurological disorders and as targets for resynchronizing the electrical circuit in the brain. The first phase of this project elucidated the trends in HCN1 expression across different regions of the PFC. To characterize electrical dysfunction, we measured HCN1 channel expression with IHC in a schizophrenic mouse model and compared to control mice.

In addition to quantifying the levels of HCN1 expression, IHC experiments also identified the regions where these channels are expressed, allowing us to better understand their roles based on their physical locations. Within the analyzed regions of the PFC, we observed that deeper cortical layers, containing apical dendrite extensions, exhibited the heaviest localization of HCN1. The dendritic presence suggests the importance of HCN1 in sending coordinated messages, receiving inputs, and generating action potentials. In regards to HCN1 expression, there was a 40.88% increase in the prelimbic region of schizophrenic samples compared to the control samples, a 133.69% increase in the infralimbic region of schizophrenic samples compared to the control samples, and an 85.69% overall increase within the entire PFC for schizophrenic samples relative to the control samples (**Figure 3**). The hypothesis that HCN1 channel expression would increase in a CAMK2 KO model was thus supported.

Conducting statistical analysis revealed unexpected trends. The increase in HCN1 presence in the schizophrenic mice compared to the control mice was statistically significant when analyzing the entire PFC and infralimbic regions but not the prelimbic regions. There was also a statistically significant increase from the HCN1 expression levels from the schizophrenic infralimbic to prelimbic region. These two combined results suggest that only the infralimbic region is differentially characterized by HCN1 upregulation. We believe that our studies provide three routes for further research: 1) Target existing treatments to the infralimbic region. 2) We should conduct other studies to determine whether other ion channels have differential expression throughout the PFC. 3) Investigate where the neuronal fibers in the infralimbic region originate from and where they go to in order to determine what additional regions may also be affected by abnormal electrical activity in schizophrenic individuals.

This study validated the usage of Bombyx mori silk as a piezoelectric biomaterial and identified processes to enhance its piezoelectric properties. Its numerous advantages include biocompatibility, low immunogenicity, degradability, and capacity for piezoelectricity generation (18). Bombyx mori silk may also be utilized in other applications such as harnessing energy sources, building transducers, and drug delivery mechanisms. The generation of our piezoelectric implant involved creating novel procedures in response to limitations in budget and availability of equipment (**Figure 5**). For example, rather than increasing the piezoelectric response through costly poling procedures i.e. application of strong electric fields, we induced β-sheet formation via ethanol treatment, a new protocol for silk modification (19). Afterwards, two hexagonal wurtzites (ZnO and AlN) were added into silk-agarose composites to increase piezoelectric response under low frequency mechanical deformations. These wurtzite's non-centrosymmetric crystals have the physical arrangement to make dipole moments and thus generate electricity (20). We used an ultrasound device to deliver sustained mechanical deformations to our piezoelectric device and a digital multimeter to measure voltage produced. Mainstream techniques such as atomic force microscopy and crystallography are expensive and do not produce physiologically relevant data, or at least data which we believe would be more applicable and transferrable in translational studies. Ultrasound therapy, however, is the optimal medium of mechanical deformation in a human, so the data we collected is readily transferrable. To activate an electrical response, 1 MHz of ultrasound was applied for a minute. To the best of our knowledge, no human trials have investigated ultrasound therapy in combination with piezoelectric implants, which makes it difficult to gauge whether our settings are reasonable for use on humans. However, from studies that have used different frequencies of ultrasound to penetrate the skull and blood-brain barrier, we believe our selected frequency still has value (21). Another concern was the amount of voltage being produced. Previous work suggests that 120 to 52,000 volts causes neurological damage, which is significantly larger than the voltage our implant generates so ours is not capable of causing such

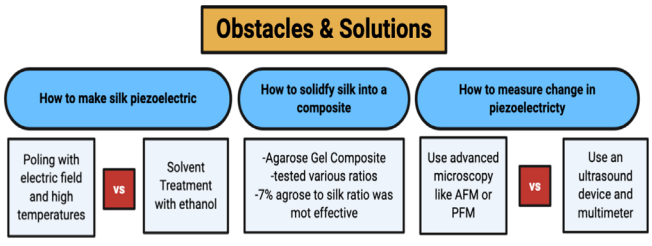

Figure 5. The piezoelectric experimentations necessitated innovative solutions due to a lack of materials and budgets. For example, atomic force microscopy required technical skill and access to an advanced facility. So, we decided to use ultrasound therapy and a multimeter

injury (22).

Of the additive concentrations tested, 0.5 M ZnO and 2 M ZnO were the only concentrations that produced statistically significant results relative to the control untreated silk samples with a 6.6% and 76.3% increase in voltage and *p*-values of 0.0047 and 0.0034, respectively (**Figure 4**). Surprisingly, we observed non-linear data where the intermediate concentration, 1 M ZnO, did not produce higher results than 0.5 M ZnO. Our replicates consistently portrayed similar voltages, so we do not think this is an issue of repetition. More data collection with this concentration of ZnO would be ideal to understand if something was conducted incorrectly. Of the two conductive materials added, ZnO was most effective in generating voltage, which supports our hypothesis that 2M ZnO would generate the most piezoelectricity. ZnO is a suitable model to incorporate because it has been validated to be biocompatible, affordable, and easy to obtain, as it is present in numerous day-to-day products like sunscreen (23).

A limitation in the first project phase could be that our groups of CAMK2 KO and control mice were immunostained at different times. However, we standardized our imaging process so that the pixel intensities could be compared with certainty. Another limitation in this work is that the piezoelectricity project was not tested in either an in vitro or in vivo model, which prevents having a holistic understanding of the effects the implant may have in humans. Conducting cell studies on the piezoelectric platform will provide insight on how neurons will grow in conjunction with this implant. The piezoelectric properties of silk could potentially stimulate cellular proliferation, neurogenesis, and normalization of abnormal electrical fields, which would be beneficial to overall brain health. Another factor to be optimized is degradation of the silk. The effectiveness of an implant depends on its stability over its lifetime. With existing models, the neuro-technical interface of an implant must be recalibrated as it loses efficacy. A benefit of using silk is that the rate of degradation can be altered by modifying its pore size, weight distribution, or crystallinity which can account for calibration prior to implantation.

The end goal of our research is to restore homeostatic plasticity in the neuronal network. The path to a working solution for schizophrenia would require numerous optimization procedures and years of more research. The first phase of this project provides an understanding of the spatial differences of HCN1 channel activity in the PFC. This is a key step towards deconstructing the etiology of the disease. Perhaps an HCN1-inhibiting medication can be incorporated into treatment plans for schizophrenics such as ketamine, isoflurane, and halotane (24). The second phase of this project developed a novel silk-based piezoelectric nanogenerator that can be further enhanced by various procedures. Testing various geometric models of the silk composites, adding other piezoelectric substrates, and evaluating the effects of porosity on current flow are further avenues of research. Overall, optimizing an implant to rectify electrical dysregulation within the brain is another potential solution for schizophrenia.

METHODS

All procedures were conducted using appropriate personal protection (proper shoes, safety goggles, gloves, and a lab coat). The student researcher did not come in contact with vertebrate organisms; tissues were obtained from animals sacrificed for a different experiment at Northwestern University. The four male CAMK2 KO mice used were approximately 2.5 - 3 months old. The tissue was fixed in 4% paraformaldehyde and 14 µm slices were prepared for immunofluorescence while 25 µm slices were prepared for diaminobenzidine (DAB) staining. Sample groups were not immunostained together, but they were stained in the same manner.

For the HCN1 experimentation, we did two types of stains: immunofluorescence and a DAB stain. For both stains, antibodies were prepared as follows: primary antibody of rabbit anti-HCN1 at a dilution of 1:500 and a secondary antibodies of anti-rabbit Alexa Fluor 488 (green) at a dilution of 1:600, and anti-mouse Alexa Fluor 488 (green) at a dilution of 1:60. Alexa Fluor 488 was chosen because it is a standard and validated secondary antibody with low rates of self-quenching. Tissue sections were then distributed into wells and washed 3 times in TBS for 2 minutes. They were then transferred into 3% normal goat serum and 1% BSA in TBS-T (TBS with 0.2% Triton-X) for one hour. They were left overnight in the primary antibody of rabbit anti-HCN1 in TBS-T containing 1% NGS and 1% BSA at 4˚C. The following day, the samples were brought back to room temperature and washed 4 times in TBS for 20 minutes each. They were then incubated in the secondary antibody of Alexa Fluor 488 in TBS-T containing 1% NGS and 1% BSA. A series of 3 washes was conducted in TBS for 20 minutes and the last wash was left overnight in 4 degrees Celsius. After being brought back to room temperature the following day, the tissue sections were washed once in TBS for 20 minutes and twice in PBS for 10 minutes. The samples were mounted, coverslipped, and visualized under a Nikon Eclipse 800 microscope. ImageJ was used to analyze the results. The regions were selected and fluorescent intensity was quantified and compared. We corrected non-specific changes by using a formula for CTCF: Integrated Density - (Area of selected cell X Mean fluorescence of background readings) to minimize non-specific changes.

In the piezoelectricity experimentation, we first acquired silk fibroin by cutting ten grams of Bombyx mori cocoons into small pieces. To induce β-sheet formation to activate piezoelectricity, we placed the silk into 70% ethanol for a duration of 2 hours (25). The fragmented cocoons were then boiled for 30 min in an aqueous solution of 0.02 M sodium bicarbonate (NaHCO3) and rinsed thoroughly to remove sericin. Sericin can adversely impact immune health so it is degummed for biocompatibility reasons (26). The remaining

silk was degummed by dissolving it in a 9.3 M lithium bromide (LiBr) solution for 4 hours at 60°C. The solution was autoclaved for 15 minutes at 121°C and centrifuged twice at 10,000 rpm for 25 minutes to remove silk fibroin aggregates. The LiBr solution was removed using a sieve leaving the degummed and treated silk. Until further usage, the dry silk solution was stored at 4°C.

We then designed the silk composites. Six samples were created for each testing condition (concentration of respective treatment). Agarose gel was created by heating and dissolving 2 grams of agarose powder in 100 mL of water. The final composite had a 70% silk-to-gel ratio, which involved adding 7 mL of dissolved silk to 10 ml of agarose gel. Next, 2 mL of the different concentrations of zinc oxide (ZnO) and aluminum nitride (AlN) were added and mixed to the solution. Cooling should be avoided to ensure that the additives set in the agarose prior to hardening of the gel. To measure voltage, we exposed the composites to 1 MHz of ultrasound for one minute. The probes were placed on top of the gel composites. After this duration, we inserted the testing probes (black and red rods) to measure voltage.

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