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Immobilization of Stable Thylakoid Vesicles in Conductive Nanofibers by Electrospinning

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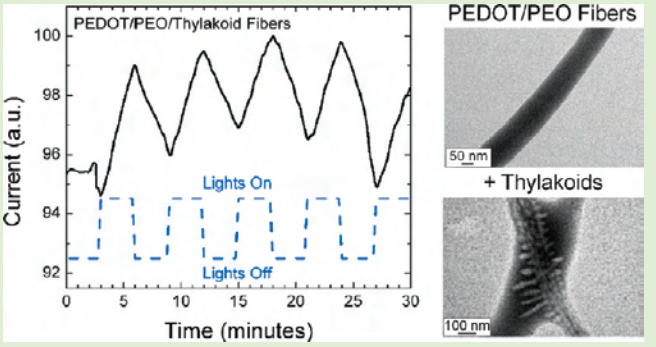
ABSTRACT: Electrospun fibers consisting of poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate) (PEDOT/PSS) and poly(ethylene oxide) (PEO) have been used to successfully encapsulate and stabilize thylakoid membrane vesicles isolated from spinach. Light-driven electronic properties were measured. Fibers with immobilized thylakoids show higher electrical conductivity compared with fibers without thylakoids under white light conditions. This is attributed to the electron-generating photosynthetic reactions from the thylakoids. Electron and optical microscopy show the presence of thylakoid vesicles within the fibers using lipid-specific stains. After electrospinning into fibers, the thylakoid vesicles still exhibit an ability to produce a light-driven electron gradient, indicating that activity is preserved during the electrospinning process. These electrospun fibers provide an excellent example of incorporating photosynthetic function into an artificial system.



1. INTRODUCTION

Photosynthetic biological materials recently have been receiving much attention[1] for potential use in optoelectronic, organic photovoltaic (OPV), and sensor applications given their superior photoresponsive properties and inherent biodegradability/renewability. The structural hierarchy of available photosynthetic materials starts with the chromophore (such as chlorophyll molecules, derivatives of chlorophyll, and other accessory pigments) and moves up to the pigment-containing membrane-spanning proteins (such as photosystems I and II in plants and reaction center in bacteria). These proteins are part of a double membrane envelope called the thylakoid membrane (or simply thylakoid), which houses the light reactions of photosynthesis that are ultimately responsible for the synthesis of ATP in photosynthetic species (inset, Figure 1).[2] Biological photosynthetic chromophores have been previously used in the fabrication of OPVs[3] and dye-sensitized solar cells,[4−7] whereas photo systems and reaction centers have shown promise in OPVs/photoelectronic[8−15] and sensors applications.[16,17] The inclusion of larger structural units such as thylakoids in a material's matrix is of particular interest because the encapsulation of intact and functioning thylakoids would be a step toward a photosynthetic "living material".[18] Previously, thylakoids embedded in a silica matrix were shown to have improved activity when compared with a free thylakoid suspension,[19] whereas thylakoids immobilized in a cross-linked albumin-glutaraldehyde have exhibited the ability to detect herbicides.[20]

Electrospinning[21,22] is an established method for creating continuous fibers ranging from tens of nanometers to micrometers in diameter. This is accomplished by applying a high voltage between a droplet of solution at the end of a spinneret and a collecting substrate. Under the proper conditions (solution conductivity, viscosity, etc.), the applied electric field causes a liquid jet to eject from the droplet toward the collecting substrate. During this process, the jet quickly elongates, reduces in diameter, and loses solvent, resulting in a nonwoven mesh of fibers with high surface-to-volume ratio. Previously it has been demonstrated that biological macrostructures such as living cells,[23] bacteria,[24−27] viruses,[28] and enzymes[29,30] can be incorporated into electrospun fibers while still retaining their biological function.

In this work, electrospinning is used to create nanofibers from a solution of thylakoids, poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate) (PEDOT/PSS, herein referred to as PEDOT), and poly(ethylene oxide) (PEO). By immobilizing thylakoids into electrically conductive PEDOT/PEO nanofibers, changes in electronic properties with response to light can be measured. These nanofibers exhibit light-induced changes in electronic properties attributed to the photosystems inside the thylakoids. Transmission electron micrographs show that

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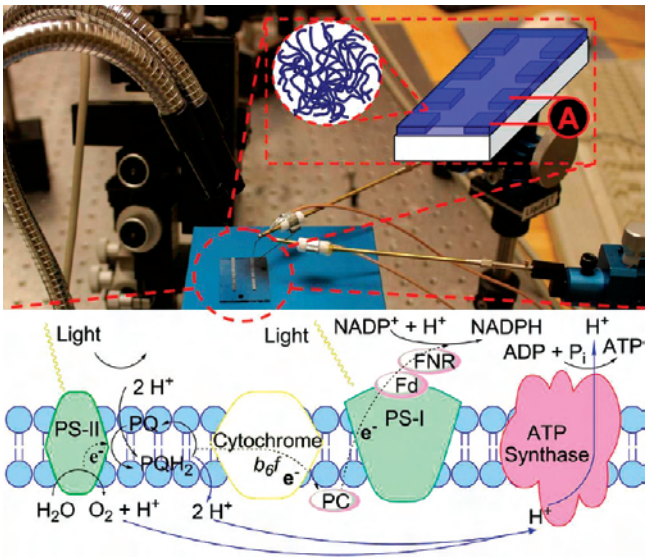

Figure 1. Photograph of the electrical testing experiment, with diagrams of the micropatterned substrates and the light reactions of the photosynthesis within the thylakoids. Spacing between the electrodes is 100 μm. For reference: photosystem I (PS-I), photosystem II (PS-II), plastoquinone (PQ), reduced plastoquinone (PQH_2), plastocyanin (PC), ferredoxin (Fd), ferredoxin-NADP-reductase (FNR), adenosine diphosphate (ADP), adenosine triphosphate (ATP), and phosphate (P_i). Proton movement is shown in blue solid lines, and electron movement is shown in black dashed lines.[2].

thylakoid vesicles are present within the fibers, whereas lipid-specific fluorescence staining also indicates the presence of a stable lipophilic environment within the nanofibers. The viability of the thylakoids during the fiber formation process was tested by measuring light-induced pH change prior to and after electrospinning. To the authors' knowledge, this study represents the first attempt to encapsulate and immobilize active thylakoids into electrospun fibers, which could potentially be used in optoelectronic applications.

2. EXPERIMENTAL SECTION

2.1. Thylakoid Isolation. The thylakoid isolation was adapted from Izawa and Good.[31] In brief, spinach leaves were washed and deveined, then homogenized in a Waring blender three times for 10 s each in a cold aqueous solution of 2 mM Na_2EDTA, 40 mM K_2HPO_4, 10 mM KH_2PO_4, and 0.35 mM NaCl. The homogenate was filtered through four layers of cheesecloth and centrifuged at 3000*g* for 5 min. All centrifugation steps were performed at 4 °C (Sorvall RC6 Plus). The resulting pellet was then suspended in a cold solution of 0.2 M sucrose, 50 mM tricine, 3 mM KCl, and 3 mM $MgCl_2$ and centrifuged at 750*g* for 1 s. The supernatant was then filtered and centrifuged at 3000*g* for 5 min. The pellet was resuspended in the same tricine buffer solution and centrifuged again at 3000*g* for an additional 5 min. The supernatant was decanted, and pellets were concentrated in the tricine buffer solution at a chlorophyll (chl) concentration of ~25 mg/mL, as determined using the method of Arnon.[32]

2.2. Solution Preparation. In a typical procedure, 10 mL of aqueous PEDOT solution (HC Starck, under the trade name Clevios P) was evaporated until it reached a solid form under vacuum at 35 °C, resulting in ~100 mg of solid material. This solid was then redispersed in 2.25 mL of deionized H_2O by vortex mixing and magnetic stirring for at least 1 h, followed by the addition of 2.5 mL of ethanol. After the addition of 0.045 g of PEO (Acros, MW = 900 000 g/mol) to the dispersion, it was vigorously mixed by magnetic stirring for at least 8 h. PEO is added to the solution to assist in electrospun fiber formation. The motivation for concentrating the initial PEDOT solution was to increase the ratio of PEDOT/PEO, which increases the overall electrical conductivity. Where appropriate, 1 mL of the isolated thylakoid solution was added and allowed to stir in the polymer blend for 15 min prior to electrospinning. The thylakoid solution was increased to 1.513 mL for osmium tetroxide (OsO_4)-stained samples used in TEM analysis. The stained thylakoid solution consisted of 0.5 mL of as-isolated thylakoid solution, 0.5 mL of 4% paraformaldehyde (Acros) in 23 mM NaH_2PO_4/77 mM Na_2HPO_4 buffer, 13 μL of 8% glutaric dialdehyde (Acros), and 0.5 mL of 4 wt % OsO_4 (Acros). The solution was incubated for 30 min at 4 °C after the addition of the fixation agents to the thylakoids. This was followed by an additional 30 min incubation period at 4 °C after OsO_4 was added to the solution. For fluorescence testing, a stock solution of Nile red dye (Acros) was made in acetone at 1 mg/mL and added to electrospinning solutions at a final dye concentration of 50 μg/mL. All solutions for light-induced pH measurements had an addition of $K_3[Fe(CN)_6]$ to a concentration of 0.3 mM for electron transfer purposes. Isolated thylakoid solutions and electrospinning solutions were used as-is (with the exception of the $K_3[Fe(CN)_6]$) while electrospun fibers were redissolved in the isolation buffer medium to a final volume of 10 mL.

2.3. Electrospinning. The electrospinning apparatus consisted of a high-voltage power supply (Gamma High Voltage), a syringe pump (Stoelting), a spinneret, and a 5 × 5 cm^2 aluminum collecting plate. Electrospinning solutions were fed into an 18 gauge blunt needle at a rate between 0.1 and 0.3 mL/h. A positive voltage of 25 kV was applied between the spinneret and an aluminum collecting ground electrode separated by a distance of 20 cm. For electrical testing, fibers were collected on glass substrates with aluminum micropatterned electrodes separated by 100 μm. The glass substrate was placed on top of the aluminum collector. All electrospinning experiments were performed at room temperature.

2.3. Light-Dependent Electrical Measurements. Fibers were electrospun directly onto precleaned glass slides with micropatterned Al electrodes with an electrode spacing of 100 μm. The same amount of material (200 μL) was used for each experiment to better control the amount of electrospun fibers on the device substrates. Because of the random landing of fibers during the electrospinning process, the location and distribution of the deposited fibers on the Al-patterned substrates inevitably changed to some degree from experiment to experiment. Given the difficulty of measuring the amount of fibers between the electrodes for each experiment, all electrical measurements were normalized to a value of 100, corresponding to the maximum value recorded. Electrical current levels were typically on the order of several hundred microamperes. All light-dependent electrical measurements were performed using a Hewlett-Packard 4140B ammeter/DC voltage source with LabView data acquisition software using probe manipulators (Cascade Microtech). Silver paste was used to enhance the connection between the Al electrodes and probes. A Schott ACE 1 halogen light source was used for all white light experiments at a power density of ~13 mW/cm^2. When red (λ_p = 625 nm), green (520 nm), or purple (470 nm) filters were used, the power density was ~24, ~7, or ~14 mW/cm^2, respectively. All electrical characterization was done under ambient conditions and repeated multiple times to ensure good reproducibility.

2.4. Characterization. Fiber morphology and thylakoid membrane inclusion were studied using a Phillips CM20 transmission electron microscope operating at 80 kV. Fluorescence imaging was done on a Nikon Eclipse Ti−U fluorescence inverted microscope using a Texas Red filter cube and analyzed using the Image J program.[33] Light-induced pH measurements were done using a MeasureNet pH meter and data acquisition software (MeasureNet Technology, Ltd.). We used 10 μL of 0.1 N HCl to calibrate the change in proton concentration for each solution tested.

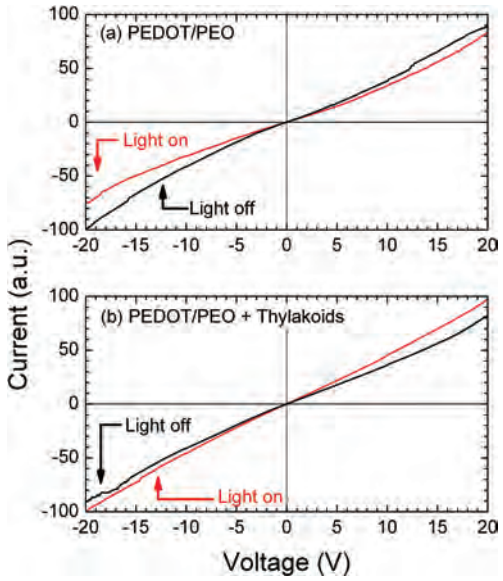

Figure 2. I−V sweeps of (a) PEDOT/PEO fibers and (b) PEDOT/PEO + thylakoid fibers under white light illumination.

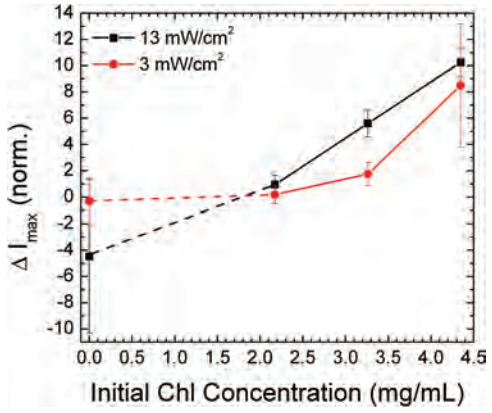

Figure 3. Maximum photocurrent versus the initial chl concentration in solution for 13 (squares, black line) and 3 mW/cm^2 (circles, red line).

3. RESULTS AND DISCUSSION

PEDOT/PEO fibers with and without the inclusion of thylakoids were electrospun onto micropatterned Al-electrodes with an electrode gap distance of 100 μm (inset, Figure 1). Light-induced changes in electrical properties were then measured using the microprobe station shown in Figure 1. The light-dependent I−V sweeps are shown in Figure 2 for PEDOT/PEO fibers without (Figure 2a) and with (Figure 2b) the inclusions of thylakoids. For fibers without thylakoids, there is a drop in electrical conductivity under white light illumination (compared with dark conditions) at higher voltages. This reduction in electrical conductivity is likely due to the degradation of PEDOT under light and the ambient testing conditions.[34,35] When thylakoids are added to the electrospun fibers, this trend is reversed, with slightly higher electrical conductivities reached under white light illumination.

The concentration of thylakoids in the electrospinning solution was varied to examine the effect of thylakoids on the overall photocurrent (Figure 3). The normalized differences in photocurrent measured at the maximum voltage increase monotonically with thylakoid concentration. The minimum thylakoid concentration

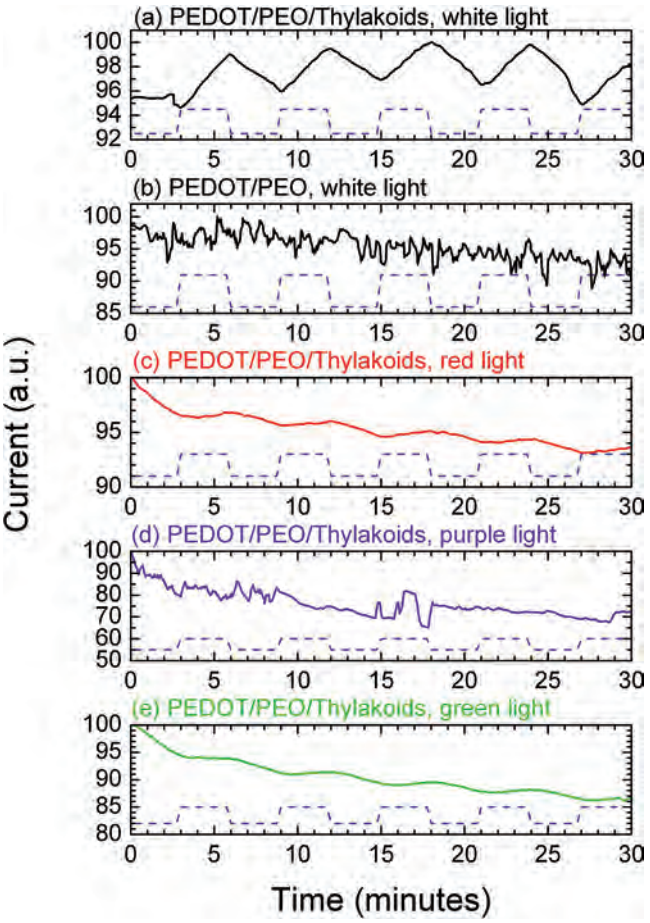

Figure 4. Current versus time measurements with response to light for (a) PEDOT/PEO/thylakoid fibers under white light, (b) PEDOT/PEO fibers under white light, (c) PEDOT/PEO/thylakoid fibers under red light, (d) PEDOT/PEO/thylakoid fibers under purple light, and (e) PEDOT/PEO/thylakoid fibers under green light. The dashed line corresponds to light being on or off.

needed to produce an increase in photocurrent was found to be ~2 mg/mL (in chl). The effect of power density on the photocurrent was also examined (Figure 3). Unsurprisingly, it was found that a larger power density produced a higher photocurrent response for each thylakoid concentration tested. At power densities lower than 1 mW/cm^2, the change in photocurrent was negligible. Therefore, to produce a measurable photocurrent, a power density >1 mW/cm^2 must be used on fibers electrospun from a solution containing a chl concentration of at least 2 mg/mL. For reference, the changes in PEDOT/PEO fiber for both power densities are also shown in Figure 3.

The electrical current response to a train of light pulses was evaluated for PEDOT/PEO fibers with and without thylakoids for several wavelength regimes (Figure 4). The voltage was held at +5 V, and light was pulsed on and off at 3 min intervals (Figure 4, blue dashed lines). Under white light exposure (Figure 4a), the PEDOT/PEO fibers with immobilized thylakoids display a gradual increase (decrease) in current during (after) exposure. The current response is roughly constant with sequential light pulses. Fibers without thylakoids (Figure 4b) show no marked changes in current coincident with light exposure and exhibit only a slight overall decrease throughout the duration of the experiment.

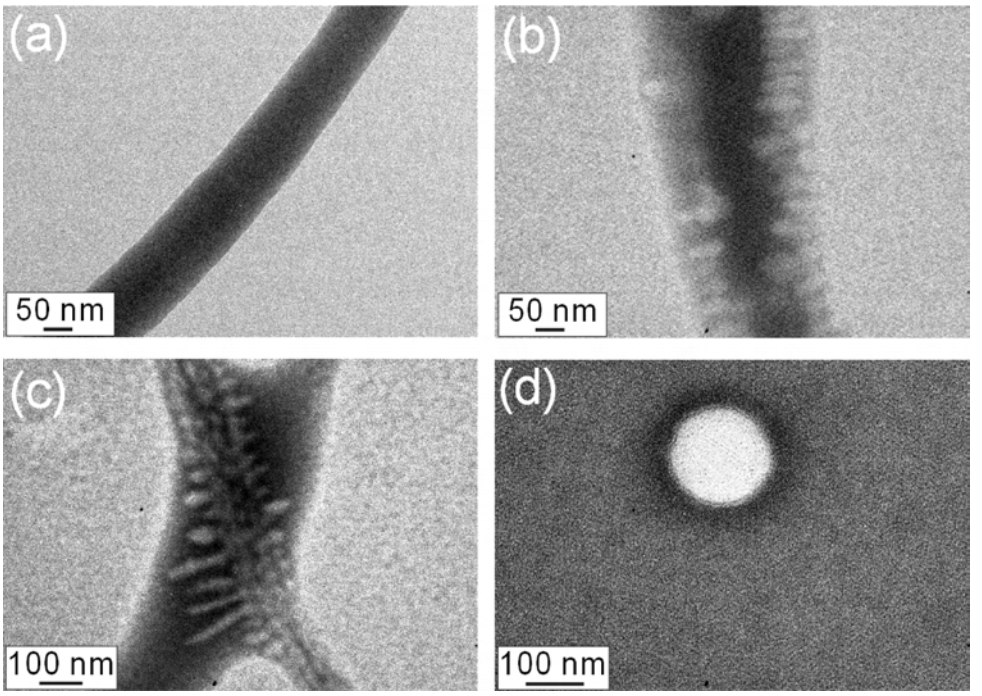

Figure 5. TEM micrographs of (a) PEDOT/PEO fibers, (b) PEDOT/PEO/thylakoids fibers, (c) PEDOT/PEO/thylakoid fibers + OsO_4 and fixing agents, and (d) an as isolated thylakoid vesicle.

The wavelength dependence of the observed increases in photocurrent for the PEDOT/PEO/thylakoid fibers is shown in Figure 4c−e. The wavelength regimes selected correspond to the main absorption regions for both chlorophyll *a* and chlorophyll *b* (red and purple), as well as a green wavelength region where the two chromophores do not absorb light.[36] As can be seen in Figure 4c−e, light of all selected wavelength regions does not produce the same electrical response as does white light. Relatively small current increases were observed at the moment of illumination. A general decrease in current was observed as the experiment progressed.

The wavelength sensitivity can be attributed to the interplay between the distinct photosynthetic pigments during the energy transfer processes of photosynthesis and their corresponding absorption of light in the visible spectrum. For an electron to be given off in photosynthesis, light is first absorbed by various pigments in the antenna complexes within the photosystem. These pigments, each with their own absorption characteristics, transfer the excitation energy via fluorescence resonance energy transfer (FRET) to a pair of chlorophyll molecules, which then in turn produces a free electron. Absence of photon absorption by any pigment in the photosystem would prevent the energy transfer process. This would then severely reduce the efficiency of generating free electrons and thus prevent an increase in electrical current in the electrospun fibers during illumination. This is analogous to the Emerson enhancement effect.[37] The photocurrent response (on the order of minutes) observed for white light illumination is much slower than the electron transfer processes seen in photosystem proteins (~200 ps)[2]. This indicates that the photoresponse is not rate-limited by the photosystems, but likely by the electron transfer from thylakoid to the fiber material. Nonetheless, these findings clearly show that photocurrent is observed only in the presence of thylakoids in the fibrous material and during exposure to a white light source.

The PEDOT/PEO fiber morphology and immobilization of thylakoids into PEDOT/PEO fibers and was studied using TEM (Figure 5). The single PEDOT/PEO fiber shown in Figure 5a has a diameter of ~100 nm. With thylakoids added to the electrospinning solution, the resulting fiber diameter is approximately the same (Figure 5b), although a clear variation in contrast is present along the fiber. The ovular shapes are the immobilized thylakoids, as they are less dense then the polymeric fibers and thus exhibit a lighter shading in the TEM micrograph. It is interesting to point out that the thylakoids tend to arrange themselves perpendicular to the fiber axis. Many other reports[38−41] of high aspect ratio particles in electrospun fibers display an alignment along the fiber axis. To confirm that these vesicles are indeed thylakoids, we added the lipid stain OsO_4 and fixation agents to the thylakoid solution prior to electrospinning, as mentioned in the Experimental Section (Figure 5c). The periphery of the vesicles is clearly darker in contrast with the addition of OsO_4, indicating that a lipid environment is present, which is likely the structurally intact thylakoids. The density of the thylakoids in certain regions of the fibers also becomes higher because of the addition of cross-linking moieties in the stained thylakoid solution. The inclusion of the OsO_4 stained thylakoids has a drastic effect on electrospun fiber morphology because the fibers become more beaded in nature and electrospraying becomes more prevalent. A single isolated thylakoid from the starting solution is shown in Figure 5d for reference.

To investigate further whether stable thylakoids were present in the electrospun PEDOT/PEO fibers, the lipid-specific dye Nile red[42] was added to the electrospinning solutions. Inverted fluorescence microscopy images of fibers with and without thylakoids are shown in Figure 6b,d, respectively. Corresponding bright field images are shown in Figure 6a,c. Comparing the images, it is clear that the fluorescence is much stronger in fibers where thylakoids were included in the electrospinning solution. Using gray scale analysis, the fluorescence of the

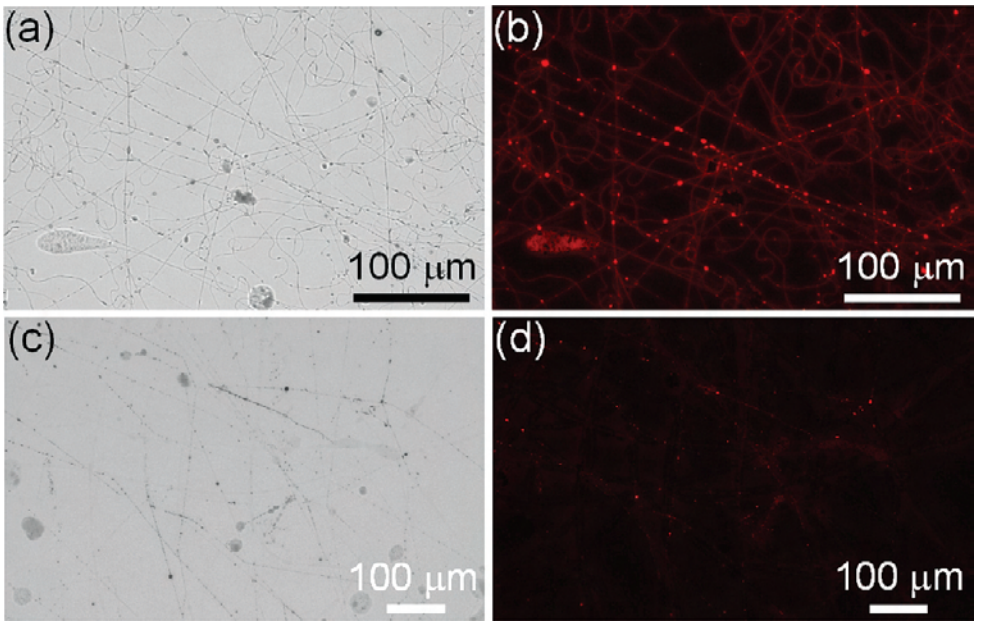

Figure 6. Bright field microscopy images of (a) Nile-red-stained PEDOT/PEO/thylakoid fibers and (c) Nile-red-stained PEDOT/PEO fibers and fluorescence microscopy images of (b) Nile-red-stained PEDOT/PEO/thylakoid fibers and (d) Nile-red-stained PEDOT/PEO fibers taken with a Texas Red filter at 2 s exposure time.

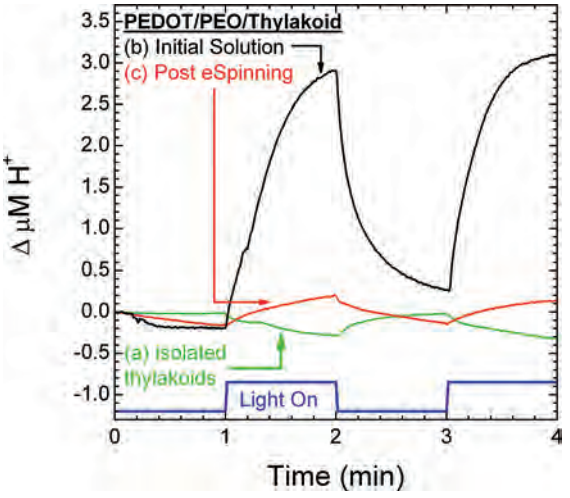

Figure 7. pH versus time measurements with responses to light for (a) isolated thylakoid solution, (b) PEDOT/PEO/thylakoid electrospinning solution, and (c) redispersed electrospun fibers in tricine buffer solution. Solutions were under white light illumination. The dashed line corresponds to light being on or off.

PEDOT/PEO/thylakoid fibers is determined to be ~800 times higher than fibers without thylakoids for the same concentration of Nile red, indicating the presence of an ordered lipid environment within the fibers. Fiber diameters are larger here than in previous experiments because of the acetone present in the electrospinning solution. (See the Experimental Section). The addition of acetone (solvent for Nile red) increases the overall volatility of the electrospinning solution, which has shown to increase fiber diameter in certain polymeric solutions.[43−46] Acetone is also a poor solvent for both PEDOT and PEO, resulting in overall morphology that includes a higher density of beaded fibers and electrosprayed globules.[47,48]

We performed white-light-driven proton gradient measurements to probe the viability of the thylakoids through the electrospinning process. Thylakoids were tested after isolation (Figure 7a, green line), after being added to the PEDOT/PEO solution (Figure 7b black line), and redissolved following electrospinning, all with the addition of 0.3 mM $K_3[Fe(CN)_6]$ and with a pH of 2 to 2.25. An increase or decrease in the concentration of protons can yield information on the orientation of the thylakoids vesicles, namely, whether the conformation is right-side-out or inside-out.[49] The isolated thylakoid solution shows a decrease in proton concentration with light (Figure 7a), indicating an uptake of protons from solution due to a majority right-side-out configuration. The time constant of the proton response is on the order of minutes, similar to other reports.[49,50] When thylakoid solution is mixed with PEDOT/PEO solution, a reversed and much larger change in proton concentration response to light is observed (Figure 7b). This increase in protons in solution is consistent with inside-out thylakoids.[50] The overall magnitude of the change in proton concentration between the as-isolated thylakoids and the PEDOT/PEO/thylakoid solution suggests that the PEDOT, PEO, or both contribute to a configuration change of thylakoids from a mixture of inside-out and right-side-out vesicles to mainly inside-out vesicles. After the PEDOT/PEO/thylakoid solution was electrospun into fibers, it was redissolved in the tricine buffer. The thylakoids in the redissolved solution were tested for their ability to establish light-induced proton gradients. As seen in Figure 7c, the thylakoids still exhibit the ability to generate a proton gradient and show the same orientation as the electrospinning solution. The lower magnitude of the overall change in proton gradient is likely due to the lower percentage of thylakoids in the solution tested (~300 μL of solution electrospun redissolved in 10 mL of tricine buffer), although a loss of thylakoid activity cannot be ruled out. The use of stabilizing agents, such as trehalose,[51−54] could potentially improve cell

viability, although it is uncertain how electrical properties may be affected. Nonetheless, the ability to establish light-induced proton gradients illustrates that thylakoids are still active after the electrospinning process.

4. CONCLUSIONS

Thylakoid vesicles were successfully electrospun into nanofibers of PEDOT/PEO and show viability postelectrospinning. Because of the relatively high electrical conductivity of the polymer nanofiber, light-induced electronic properties can be measured. The increase in electrical current of the PEDOT/PEO/thylakoid nanofibers due to exposure to light is likely caused by the still functioning light-driven reactions of photosynthesis in the stable thylakoids. This photocurrent was found to increase monotonically with thylakoid concentration. The presence of thylakoid environments within the fibers was verified by TEM and fluorescence microscopy with the lipid-specific stains OsO4 and Nile red, respectively. By measuring the light driven changes in pH in various thylakoid solutions, it was determined that thylakoids still have the ability to produce proton gradients after the electrospinning process. This coupled to the fibers' ability to produce increases in electrical current upon white light illumination shows that the thylakoids survive the electrospinning process and still perform basic biological functions. The fluorescence and TEM experiments show the presence of stable lipid environments. These results represent a significant step toward incorporating photosynthetic biological function into an artificial system, which may be used in optoelectronic applications.

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