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Electronic Supplementary Information

Towards the fabrication of bio-hybrid silk fibroin materials: entrapment and preservation of chloroplast organelles in silk fibroin films

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Supplemental Materials and Methods

Silk processing: Production of silk fibroin solution has been previously described. The purification of silk fibroin from *Bombyx mori* cocoons initially involves the removal of sericin, by boiling the cocoons in 0.02 M aqueous solution of sodium carbonate for 60 minutes. The remaining fibroin bundle was washed in deionized water and dried overnight, and then dissolved in 9.3M aqueous lithium bromide at 60°C for three hours. Dialysis of the solution against deionized water (dialysis cassettes Slide-a-Lyzer, Pierce, MWCO 3.5K) enables the production of 6% w/v silk fibroin solution. The whole process results in the partial - but predictable - denaturation of the silk fibroin protein, to which corresponds a decrease in the protein molecular weight. Silk fibroin heavy chain has a molecular weight of circa 390 kDa. Within the context of this study, silk fibroin materials possessed molecular weight in the 268-41 kDa range, with a distribution peak at 50 kDa.

Extraction of chloroplast from spinach leaves: Baby spinach leaves (*Spinacia oleracea*) were freshly harvested, washed, and dried before chopping. Leaves were homogenized in a cooled (4 °C) chloroplast isolation buffer (CIB) (330 mM sorbitol, 50 mM HEPES, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) containing 0.1% bovine serum albumin (BSA). The homogenate was filtered through cheese cloth (spacing ~100 μm) to remove large debris before centrifugation at 200 x g for 3 minutes to remove remaining cell debris. The pellet was discarded and the supernatant was centrifuged for 7 minutes at 1000 x g. The pellet was suspended in CIB to the desired concentration. Chlorophyll was

extracted by mixing the chloroplast suspension in 80% acetone for 15 minutes and centrifuging at 3000 x g for 5 minutes to remove broken organelle debris.

Film preparation and storage: Silk and PVA films were prepared using solutions of 5% (w/w) reconstituted silk fibroin and 5% (w/w) polyvinyl alcohol (PVA) (M_w 30,000-70,000, 99+% hydrolyzed from Aldrich). The chloroplast and chlorophyll solutions were diluted to 0.4 mg/ml as determined by the mass of chlorophyll before mixing with silk or PVA solutions. Solution of silk or PVA were mixed with ratios 10:1.5 and were cast as films with a surface area of 9.6 cm^2. Films were prepared in quadruplicate and left at different conditions (4 °C no illumination, 25 °C no illumination, 25 °C with 1.1 mW white light illumination, 60 °C no illumination, and 90 °C no illumination).

Spectroscopic measurements: The absorbance and fluorescence of each film was measured using a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). Absorbance of the films was measured at 440 nm and 680 nm. Fluorescence detection was taken on the films with an excitation wavelength at 432 nm and measured emission wavelength of 665 nm. All samples were baseline corrected to the absorbance and fluorescence of silk and PVA films.

Fourier transform infrared spectroscopy (FTIR): FTIR analysis of chloroplast samples were performed in a JASCO FTIR 6200 spectrometer (JASCO, Tokyo, Japan) in

attenuated total reflectance (ATR). For each sample, 64 scans were coded with a resolution of 1 cm^{-1}, with a wavenumber range from 400-4000 cm^{-1}. Fourier self-deconvolution of the infrared spectra covering the amide I region (1595-1705 cm^{-1}) was performed by Opus 5.0 software.

SEM characterization: After initial purification, the chloroplasts were attached to a cellulose substrate, embedded in a silk film, or embedded in a PVA film. To reduce the solubility of the PVA, it was submerged in a 3% boric acid solution which does not alter the embedded chloroplasts (1–3). Before imaging, each sample was fixed in a solution of 2.5% aqueous glutaraldehyde, stained with 0.1% osmium tetroxide, washed 3 times with PBS, dehydrated in a series of ethanol rinses at concentrations of 50%, 70% 80%, 90%, 100%, and 100% for 15 minutes, and dried above the critical point of carbon dioxide (Tousimis 931 GL Critical Point Dryer, Rockville, MD, USA) before being sputter coated with 5 nm of gold. The effectiveness of the chloroplast isolation and entrapment was verified by scanning electron microscopy (SEM, Zeiss EVO MA 10, Carl Zeiss SMT, UK) collected at 3 keV.

TEM characterization: For the TEM sampling after initial purification, the chloroplasts were stained with 0.1% osmium tetroxide for 30 minutes and washed 3 times with PBS. Chloroplasts were mixed with water, silk fibroin solution, or PVA solution before casting onto TEM grids. The effectiveness of chloroplast isolation in silk and PVA films was

verified using transmission electron microscopy (TEM, Zeiss Libra 200 aberration corrected TEM, Carl Zeiss SMT, UK) at 80 keV.

Photochemical activity measurement: The photochemical activity of the silk and PVA films was monitored in a Clark cell vessel (Oxy-lab, Hansatech, UK). The rate of oxygen evolution was measured in the presence of an artificial electron acceptor buffer (1 mM potassium ferricyanide ($K3[Fe(CN)_6]$), 5 mM ammonium chloride, and 10 mM sodium bicarbonate in phosphate buffered saline, pH=7.4). Oxygen measurements were taken in 4 ml of electron acceptor buffer with samples containing 0.2 ml chloroplast suspension with a concentration of 2 mg/ml (as measured by the amount of chlorophyll) illuminated by a red lamp centered at 650 nm. Films were kept at room temperature, and oxygen measurements were taken at days 1, 3, 5, 7, and 10.

Figure S1

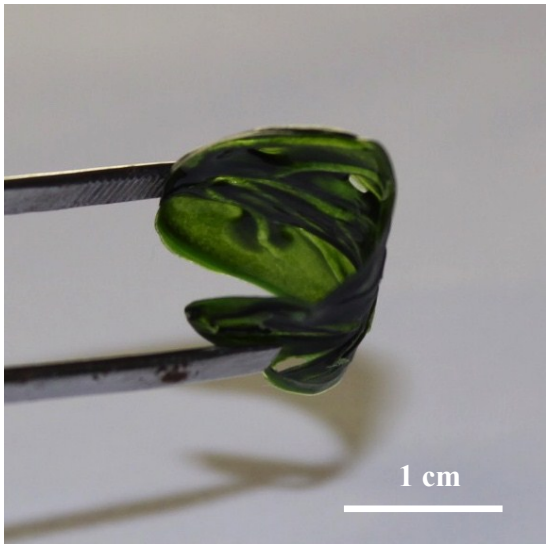

Fig. S1: Image of bendable biohybrid silk-chloroplast films.

Figure S2

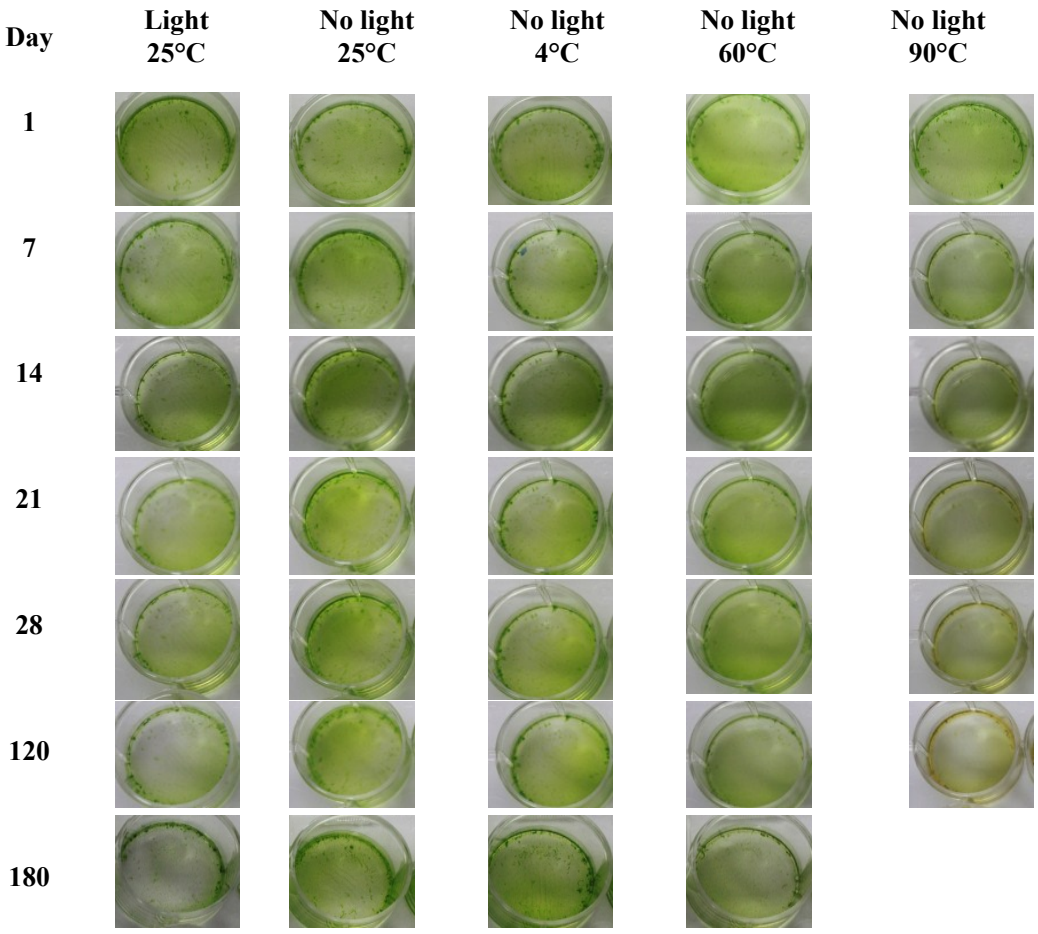

Fig. S2. Images of chloroplasts entrapped in silk films at different lighting conditions (with or without) at different temperatures (4°C, 25°C, 60°C, and 90°C) up to 180 days.

Figure S3

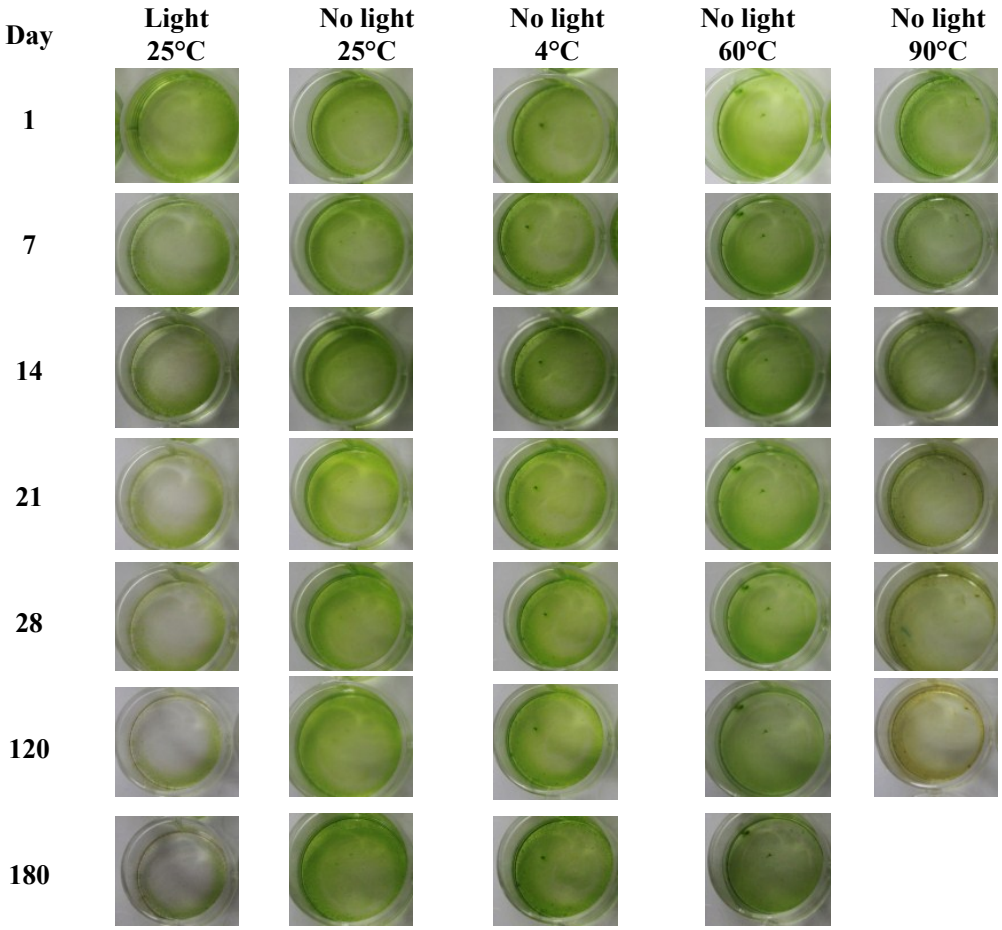

Fig. S3. Images of chloroplasts entrapped in PVA films at different lighting conditions (with or without) at different temperatures (4°C, 25°C, 60°C, and 90°C) up to 180 days.

Figure S4

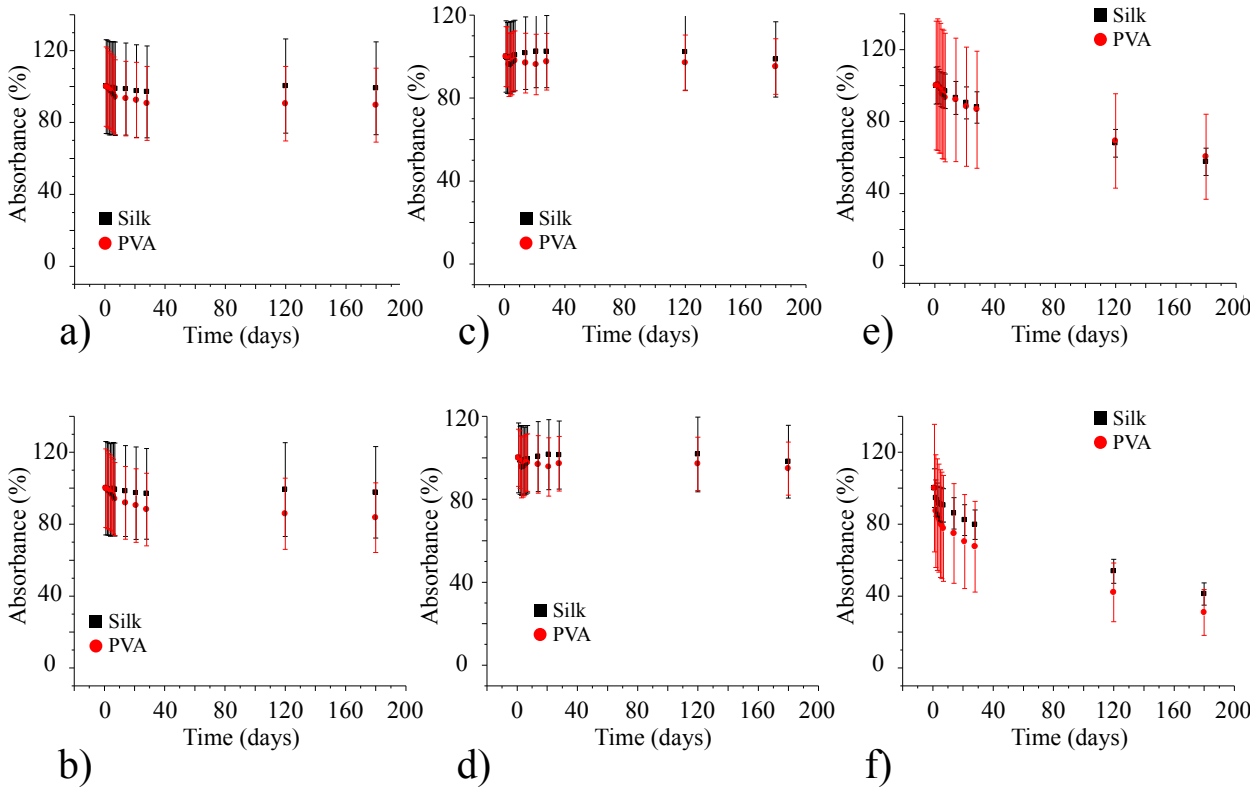

Fig. S4: Absorbance measurements of chloroplasts entrapped in silk films and PVA films exposed to different variables at 440 nm and 680 nm. a-b) Preserved activity compared to the initial condition for films stored in the dark at 25°C with absorbance measured at (a) 440 nm and (b) 680 nm. c-d) Preserved activity compared to the initial condition for films stored in the dark at 4°C with absorbance measured at (c) 440 nm and (d) 680 nm. e-f) Preserved activity compared to the initial condition for films stored in the dark at 60°C with absorbance measured at (e) 440 nm and (f) 680 nm.

Figure S5

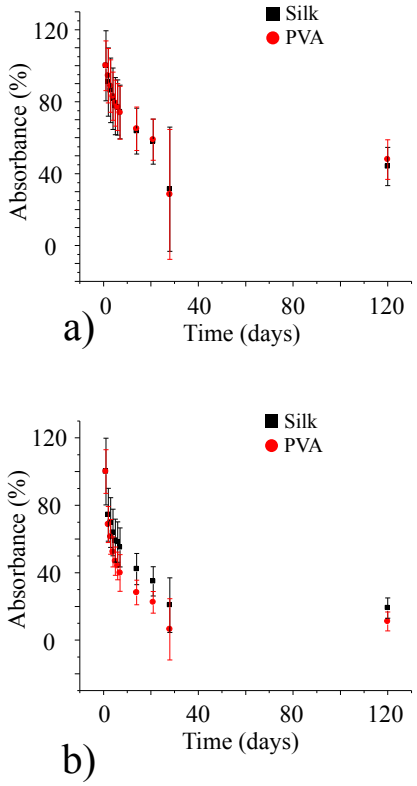

Fig. S5: Absorbance measurements of chloroplasts entrapped in silk films and PVA films exposed to different variables at 440 nm and 680 nm. a-b) Preserved activity compared to the initial condition for films stored in the dark at 90°C with absorbance measured at (a) 440 nm and (b) 680 nm.

Figure S6

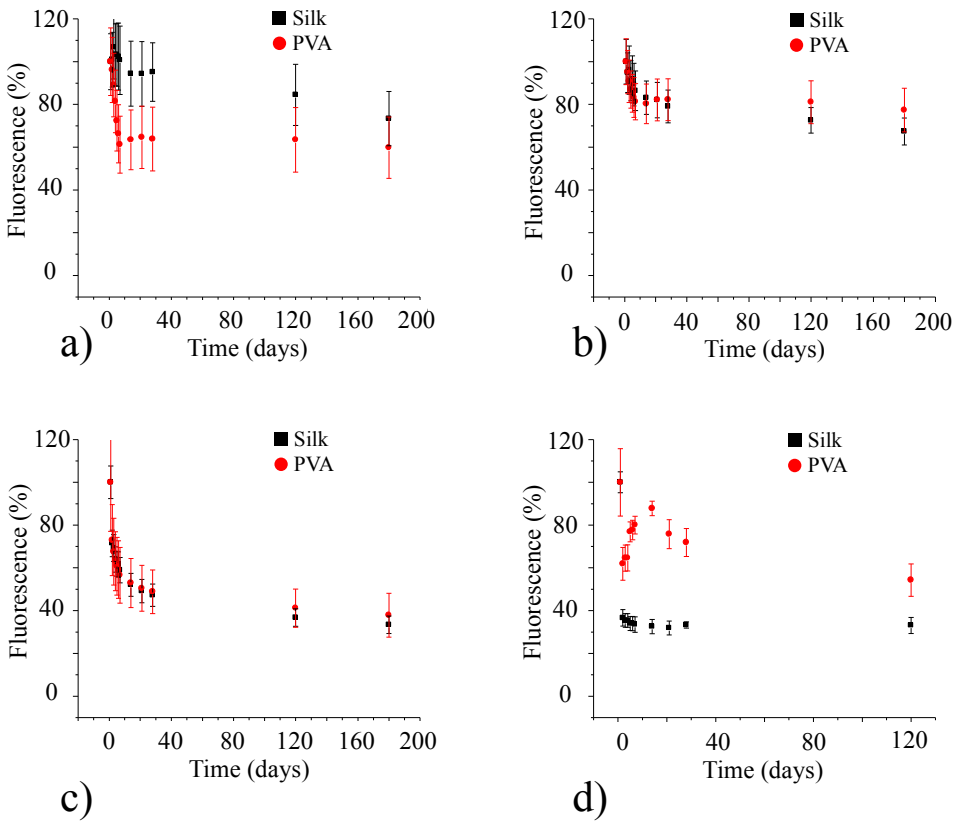

Fig. S6: Fluorescence measurements of chloroplasts entrapped in silk films and PVA films exposed to different variables at ex=432 nm, em=685 nm. Preserved activity compared to the initial condition for films stored in the dark at (a) 25°C, (b) 4°C, (c) 60°C, and (d) 90°C.

Figure S7

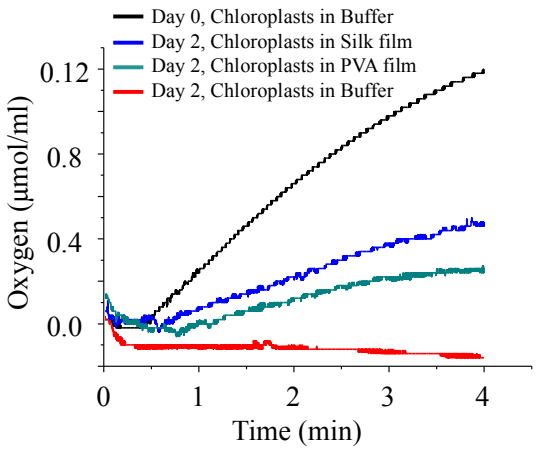

Fig. S7: Oxygen production over time of chloroplasts in free suspension, silk films, and PVA films at different time periods**.**