Supporting Information

Covalent Interlocking of Glucose Oxidase and Peroxidase in the Voids of Paper: Enzyme-Polymer “Spider Webs”

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1. Experimental

1.1 Materials
Glucose oxidase from *Asperigillus niger* (GOx, EC 1.1.3.4) was purchased from Sigma-Aldrich. Peroxidase from horseradish (HRP, EC 1.11.1.7) was purchased from Calzyme. 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) was purchased from TCI America. Poly(acrylic acid) (PAA, Mw = 8,000) was purchased from Sigma-Aldrich. Filter paper (Whatman Grade 1, typical 180 μm thickness, 87 g m⁻² weight, and with 11 μm pore size) was purchased from Sigma-Aldrich. Household kitchen wax paper was purchased from the local grocery store. Agarose (high gel temperature) was purchased from USBiological Life Sciences. Sodium phosphate, D-(+)-glucose, potassium iodide, brilliant blue G250 and R250, sodium dodecyl sulfate (SDS), urea, acetic acid, isopropanol, ethanol, phosphoric acid, sodium bicarbonate, dimethyl sulfoxide (DMSO), Fluoresceinamine Isomer I (FA), 5(6)carboxy-x-rhodamine N-succinimyd ester (ROX) were all purchased from Sigma-Aldrich.

1.2 Preparation of Waxed Paper
Circles (8 mm diameter) were cut into wax paper using a precision cutter. The cut wax paper was placed on top of a single sheet of Whatman filter paper, and a heat press was applied (280 °C, 20 seconds). The wax-coated filter paper wells had an inner diameter of ~ 3.5 mm. The wells were verified for uniform coating by wetting with distilled water and then observing if the water diffused into the waxed areas.

1.3 Synthesis of GOx-HRP-PAA/Cellulose
The conjugation-activated polymer solution was prepared by mixing poly(acrylic acid) (PAA, 10 mM), phosphate buffer (20 mM, pH 7.0), EDC (100 mM), and stirred for 10 minutes. The GOx (16 μM) and HRP (3.2 uM) solution was pre-mixed and added to the activated polymer. The resulting GOx/HRP/PAA/EDC mixture was then immediately drop cast on each filter paper well (2.5 μL per well) and left to air dry for about 1 hour. The resulting GOx-HRP-PAA/cellulose was washed three times in 25 mL distilled water to remove any unreacted PAA and EDC-urea, and was subsequently dried again for 1 hour.
2. Characterization

2.1 Enzymatic Colorimetric Activity
Colorimetric enzymatic activity was measured by the addition of potassium iodide (0.3 M, 2 μL) to the filter paper wells. Upon the addition of glucose (2-40 mM), GOx forms hydrogen peroxide and gluconic acid. The HRP, with hydrogen peroxide, oxidizes potassium iodide to elemental iodine (yellow to brown color). The yellow to brown color intensity was digitally scanned with a photo scanner (Canon, color setting, 600 dpi resolution, no color adjustment). The resulting image was converted to gray scale and analyzed for intensity using Image-J histogram software.

2.2 Agarose Gel Electrophoresis
Agarose gel electrophoresis was used for evaluating the presence of unreacted enzyme upon conjugation in solution-phase. The gels were prepared by microwaving a solution of agarose (0.5% w/v) in Tris-acetate buffer (40 mM, pH 7.0) on high for 1 minute. The agarose solution was cured at room temperature for 30 minutes. Meanwhile, samples were prepared by mixing 5 - 6 μM enzyme with loading buffer (50% v/v glycerol and 0.1% w/v bromophenol blue). Once loaded, the agarose gel was run at 100 V for 30 minutes in a horizontal gel electrophoresis apparatus (Gibco Model 200, Life Technologies Inc.) The resulting agarose gel was stained with brilliant blue R250 (0.1% w/v) for 4 hours, and then destained with acetic acid (10% v/v) overnight.

2.3 SDS-PAGE
For SDS-PAGE, the gels were prepared using a 7% polyacrylamide separating gel with a 5% stacking gel. The samples were prepared by adding loading buffer (10 μL, 2% w/v SDS, 10% v/v β-mercaptoethanol) to sample solutions and incubated in a hot bath (~60 °C) for 15 minutes. Samples were loaded so that each well contained about 4 μg of enzyme per well. The SDS gel was run at 200 V in a Bio-Rad Mini-Protean Electrophoresis apparatus until the dye front nearly reached the bottom of the gel. The gel was stained with acetic acid (10% v/v), isopropanol (10% v/v), and brilliant blue R250 (0.1% w/v) overnight. The gel was subsequently placed in a second stain bath containing acetic acid (10% v/v) and brilliant blue R250 (0.1% w/v) for 4 hours, and then destained in acetic acid (10% v/v) overnight.

2.4 Circular Dichroism
Retention of molar ellipticity of the GOx-HRP-PAA conjugate in solution was found by measuring the circular dichroism (CD) spectra with a JASCO model J715 spectropolarimeter. All spectra are an average of 10 accumulations, measured with a 1 nm data pitch, and scan speed of 50 nm/minute. Cuvette path lengths and sample concentrations were 0.05 cm and ~ 3 μM (UV-CD), 1 cm and ~ 15 μM (Soret-CD). All ellipticities were normalized against path length and exact sample concentration.

2.5 Scanning Electron Microscopy/Energy Dispersive Spectroscopy
Field-emission scanning electron microscopy (FE-SEM) and energy-dispersive spectroscopy (EDS) were performed on the Strata 400 STEM DualBeam system.
equipped with Focused Ion Beam (FIB) technology with an accelerating voltage of 10 kV for imaging and 20 kV for EDS analysis. Sample dispersed at an appropriate concentration was sputter-coated with gold using Edward Evaporator/sputter coater prior to SEM/EDS examination. With EDS, the elemental composition of the conjugate on paper can be visualized, and atomic percentages of the elements can be identified. The identified elements were quantified by eZAF Smart Quant Results method.

2.6 Mercury Intrusion Porosimetry
The porous structure of the conjugate within the filter paper (26 mg GOx total enzyme loaded) was determined by mercury porosimetry. The measurements were conducted automatically on an AutoPore IV 9500 V1.09 (Micromeritics, Georgia, USA). Samples were sealed in a penetrometer, weighed, and subjected to mercury intrusion. GOx-HRP-PAA/cellulose (loaded with 26 mg GOx) had a total pore volume of 0.65 mL/g, a total pore area of 14 m2/g, a median pore diameter of 3.2 \( \mu \text{m} \), and porosity of 21%. The corresponding control of bare cellulose had a total pore volume of 1.4 mL/g, a total pore area of 7.3 m2/g, a median pore diameter of 12 \( \mu \text{m} \), and porosity of 48%.

2.7 Synthesis of FA-labeled PAA and ROX-labeled GOx
EDC (1 M) was added to a solution of PAA (9 mM) and stirred for 10 minutes. FA (10 mM) was slowly added to the PAA solution with stirring, and reacted for 4 hours in the dark at room temperature. The solution was then centrifuged for 30 minutes (10,000 rpm) to remove any unreacted solid FA. The FA-PAA was then purified by acetone precipitation. After adding equal volume of 100% acetone, the FA-PAA precipitated and the solution was centrifuged for 10 minutes (10,000 rpm). The supernatant was removed, and the PAA was resuspended in distilled water. Acetone precipitation was repeated 3 times.

ROX in DMSO (10mg/mL, 100 \( \mu \text{L} \)) was slowly added to a solution containing GOx (20 mg/mL) in bicarbonate buffer (0.2 M, pH 8.3), followed by gently stirring for 4 hours in the dark at room temperature. The solution was then dialyzed against phosphate buffer (pH 7.0, 20 mM) for 4 hours to remove the unreacted fluorescent dye.

2.8 Laser Confocal Fluorescence Microscopy
Fluorescence images were collected with a Nikon A1R Confocal Microscope equipped with a 10x dry objective lens. Filter paper samples were placed directly on the microscope stage. The FA dye was excited by a 488 nm argon laser (power level 2.4) and the fluorescence emission was monitored at 525 nm, with a PMTHighVoltage of 125. ROX dye was excited by a 561 nm argon laser (power level 2.0) and monitored at 595 nm, with a PMTHighVoltage of 120. The instrument pinhole was 21.7 \( \mu \text{m} \).

2.9 Loading of Enzyme
Enzyme loading was examined by increasing the concentration of GOx (16, 30, 60, 90, 180 \( \mu \text{M} \)) and by maximizing the concentration of EDC (from 100 mM to 1.11 M). This much EDC is sufficient to activate all the carboxyl groups on the PAA and maximize the amount of enzyme crosslinked. To further enhance enzyme loading, a sample of GOx/HRP/PAA/EDC containing 180 \( \mu \text{M} \) GOx (26 mg GOx total) was loaded onto the
paper four subsequent times, drying for 30 minutes in between each application. Samples were synthesized under the same conditions as stated previously. The GOx-HRP-PAA/cellulose samples were washed three times in 25 mL of distilled water for one hour.

2.10 Analysis of Wash by Bradford Assay
Bradford reagent was prepared by dissolving brilliant blue G-250 (100 mg, Sigma) in ethanol (95% w/v, 50 mL). To this solution, phosphoric acid was added (85% w/v, 100 mL) and the resulting solution was then diluted to a final volume of 1 L with distilled water. Final concentrations of the reagent were 0.01% w/v brilliant blue G-250, 4.7% w/v ethanol, 8.5% w/v phosphoric acid. The reagent was filtered through Whatman 1 filter paper before each use until the color was light brown. Enzyme standards containing either GOx-HRP-PAA or GOx were prepared with 1, 3, 5, 7, 8.5, and 10 μg enzyme in 0.1 mL. Respective control of only PAA was made with identical PAA mass present in GOx-HRP-PAA standards. To these standards, 1 mL of Bradford reagent was added and mixed. Absorbance of the solution at 595 nm after 10 minutes was measured against a blank of phosphate buffer (20 mM, pH 7.0) and reagent.

2.11 Liquid-Phase Activity Assay – Denaturation Study
To confirm the denaturation of the GOx and HRP in the presence of 5 M urea, a solution-phase activity assay was done using liquid samples of GOx-HRP-PAA and respective controls of GOx/HRP and GOx/HRP/PAA. A FlexStation3 plate reader (from Molecular Devices) was used to measure the enzymatic activity. A solution of GOx (0.5 μM), HRP (0.1 μM) phosphate buffer (20 mM, pH 5.5) guaiacol (10 mM) and urea (5 M) were incubated in a 96-well plate (200 μL per well) for 30 minutes at room temperature. Glucose (70 μL, 2 mM) was deposited into each well after about 20 seconds. Increase in absorbance (470 nm) was monitored for 200 seconds. Respective controls in the absence of urea were also measured for comparison.
3. Results

3.1 Grey Scale Intensity

Figure S1. Calibration of grey scale intensity with respect to increasing glucose concentration. GOx/HRP was deposited onto the filter paper (16 μM GOx, and 3.2 μM HRP). The samples were assayed for grey scale color intensity by adding potassium iodide (0.3 M, 2 μL), and increasing concentrations of glucose. Samples were prepared in triplicate, and images were scanned after 30 minutes of developing color.
3.2 Agarose Gel Electrophoresis and SDS-PAGE
Covalent attachment and formation of the GOx-HRP-PAA conjugate, and verification of the increase in molecular weight was confirmed agarose gel electrophoresis and by SDS-PAGE.

Figure S2. (A) Agarose gel of the GOx-HRP-PAA conjugate in solution (lane 3), corresponding controls of GOx/HRP (lane 1) and GOx/HRP/PAA physical mixture (lane 2). Samples (5 - 6 μM enzyme) were spotted in the center of the gel with loading buffer, and the agarose gel was run in Tris acetate buffer (40 mM, pH 7.0) for 30 minutes. Each well is highlighted in a black rectangle. (B) SDS-PAGE of the GOx-HRP-PAA conjugate (lane 4) shows the increased molecular weight compared to the corresponding controls of GOx/HRP (lane 2) and GOx/HRP/PAA physical mixtures (lane 3). Standard molecular weight markers are shown (lanes 1, 5). Samples were loaded so that each well contained about 4 μg of enzyme per well. The GOx-HRP-PAA showed increased electrophoretic mobility towards the positive electrode and a higher molecular weight compared to GOx/HRP and GOx/HRP/PAA controls, indicating no unconjugated enzyme remained after the EDC reaction was complete.
3.3 Circular Dichroism

Figure S3. (A) UV and (B) Soret Circular Dichroism (CD) of GOx-HRP-PAA conjugates and corresponding GOx/HRP and GOx/HRP/PAA physical mixture controls. All spectra are an average of 10 accumulations, and enzyme concentrations were ~ 3 μM for UV and 16 μM for Soret. Ellipticities were normalized against each sample’s concentration and cuvette path length (0.05 cm). These spectra strongly suggest that enzyme structure is largely retained upon crosslinking with PAA.
3.4 Scanning Electron Microscopy
The formation of the conjugate within the filter paper was visualized using SEM.

Figure S4. SEM images of (A) bare cellulose and (B) GOx-HRP-PAA/cellulose made with higher loading of GOx (26 mg GOx, 1 M EDC). Controls of (C) GOx/HRP/PAA/cellulose and (D) GOx/HRP/cellulose physical mixtures are also shown. All samples loaded with enzyme were washed with distilled water for 30 minutes to remove any unbound materials before imaging.
3.5 Energy Dispersive Spectroscopy

Figure S5. Energy dispersive spectroscopy of GOx-HRP-PAA/cellulose (loaded initially with 26 mg GOx). The SEM image of the area assayed by EDS is highlighted in a white rectangle (inset). A control of bare cellulose was also done to identify the nitrogen to carbon ratio in the enzyme-polymer conjugate embedded in the paper. Samples were washed and prepared according to the procedure listed in ESI section 2.6. Nitrogen to carbon ratio was calculated by dividing the N wt.% by the C wt.%. EDS indicated that GOx-HRP-PAA/cellulose had a nitrogen content of 14±2% and N/C ratio of 0.33±0.07 while bare cellulose had no nitrogen.
3.6 Agarose Gel Electrophoresis of FA-labeled PAA and ROX-labeled GOx

Figure S6. Agarose gel electrophoresis of the fluorescence labeled conjugate ROX-GOx-FA-PAA in solution (lane 8), and corresponding controls of unlabeled GOx (lane 1) ROX dye (lane 2), labeled ROX-GOX (lane 3), PAA (lane 4), FA dye (lane 5), labeled FA-PAA (lane 6), and physical mixture of ROx-GOx/FA-PAA (lane 7). Samples were spotted at the negative side of the gel with loading buffer, and the gel was run in Tris acetate buffer (40 mM pH 7.0) for 40 minutes. Each well is highlighted in a white rectangle. The agarose gel was first imaged under a black light, then stained for PAA with Carolina Blu indicator, and then stained for protein with brilliant blue. The gel was destained with acetic acid for 3 hours before imaging. Both PAA and GOx were successfully labeled, and conjugation of ROX-GOx to FA-PAA occurred as expected.
3.7 Multi-Channel Confocal Fluorescence Microscopy

Figure S7. Multi-channel fluorescence microscopy images of filter paper taken at 10x magnification. GOx was labeled with ROX dye (channel 1, red) and PAA was labeled with FA dye (channel 2, green), then merged together (channel 4, overlay of red green/gray). (A) Conjugate ROX-GOx-FA-PAA/cellulose was compared to the corresponding controls of the (B) physical mixture ROx-GOx-FA-PAA/cellulose, and (C) ROX-GOx/cellulose. Additional controls where only one component was labeled are also shown. (D) The conjugate GOx-FA-PAA/cellulose and (E) for physical mixture GOx/FA-PAA/cellulose. (G) The conjugate ROX-GOx-PAA/cellulose and (H) for the physical mixture ROX-GOx/PAA/cellulose. Control of bare cellulose is shown in (F). Microscope images clearly show that both the enzyme and the polymer are confined to the cellulose fibers.
3.8 Loading of Enzyme

Figure S8. Colorimetric assay of GOx-HRP-PAA/cellulose 30 minutes after the addition of potassium iodide (0.3 M) and glucose (40 mM) with respect to increasing PAA concentration (1, 5, 10, and 20 mM) used during synthesis. Two concentrations of GOx were examined, (A) 16 μM and (B) 30 μM. EDC (100 mM) and HRP (3.2 μM) concentrations were maintained constant. (C) Colorimetric intensities were scanned using ImageJ and are plotted with respect to increasing concentration of PAA used during the synthesis of GOx-HRP-PAA/cellulose. The concentration of COOH groups from PAA is in large excess compared to the number of lysine residues in the system; therefore, varying [PAA] did not affect the enzymatic activity.
Figure S9. (A) Agarose gel electrophoresis of the GOx-HRP-PAA solutions synthesized with increasing enzyme concentration (16, 30, 60, 90 μM). Samples (5 - 6 μM enzyme) were spotted in the center of the gel, and the agarose gel was run in Tris acetate buffer (40 mM, pH 7.0) for 30 minutes. Each sample well is highlighted in a black rectangle. It was found that in all GOx-HRP-PAA samples synthesized, there was no unreacted enzyme left over in solution. Agarose gel electrophoresis showed no unreacted enzyme in solution, even at high GOx concentrations.
Figure S10. Calibration graph of enzyme standards (1, 3, 5, 7, 8.5, and 10 μg enzyme in 0.1 mL) by the net absorbance at 595 nm of after adding Bradford reagent. Enzyme standards of GOx (blue), GOx-HRP-PAA conjugate (red), and control of PAA without enzyme (green) are shown. Net absorbance was calculated by subtracting the absorbance of buffer and reagent from the mixture of enzyme and reagent.
Figure S11. (A) Percent loading of GOx (grams enzyme) per gram of paper. Increasing [GOx] was loaded onto cellulose and washed. The wash was analyzed for GOx concentration by Bradford assay. Percent loading was then calculated by dividing the mass of enzyme retained in the paper by the total mass of the cellulose, multiplied by 100. (B) The percent enzyme retained within the paper was calculated by dividing the mass of enzyme retained by the original mass of enzyme added, multiplied by 100. In all experiments, total filter paper weight was ~270 mg. All samples were synthesized and analyzed in triplicate. Error bars are provided but some are smaller than the points.

Table S1. Summary of % Loadings and % Retentions of GOx-HRP-PAA in cellulose.

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<th>Loading</th>
<th>% Loading</th>
<th>% Retention</th>
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<td>(g enzyme retained / g paper *100) by Bradford</td>
<td>(g enzyme retained / g enzyme added *100) by Bradford</td>
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3.9 Enzyme Denaturation

Solution phase activity in the presence of denaturing agents was done to confirm denaturation of the enzymes.

Figure S12. (A) Solution phase kinetic traces of GOx-HRP-PAA (solid red line) and respective controls of GOx/HRP (solid black line) and GOx/HRP/PAA (solid green line) physical mixtures. Glucose oxidase (0.5 μM) and HRP (0.1 μM) were mixed with phosphate buffer (10 mM, pH 5.5), and guaiacol (10 mM). A solution of glucose (2 mM) was added to the solution and absorbance at 470 nm was monitored. Activity was also monitored in the presence of urea (5 M), where the urea was added to the GOx solution 30 minutes before assaying. (B) Relative initial activity was calculated by measuring the slope of the first 20 seconds of the kinetic trace and normalizing it against GOx/HRP in the absence of urea. All samples retained ≤5% activity in the presence of urea.
3.10 Effect of Molecular weight on Enzyme Retention

Figure S13. Effect on enzyme retention when GOx (12 mg) is conjugated in the filter paper to two different molecular weights of PAA (8 k or 450 k). Samples were washed three times with distilled water, and enzyme content was quantified by the Bradford assay. The percent enzyme retained within the paper was calculated by dividing the mass of enzyme retained by the original mass of enzyme added, multiplied by 100. In all experiments, total filter paper weight was ~270 mg. All samples were synthesized and analyzed in triplicate. Error bars are provided but some are smaller than the points. Higher molecular weight PAA (450 k) increased efficiency of enzyme loading (82-98%) when compared to that obtained with lower molecular weight PAA (50-80%).