Electronic Supplementary Information

Modification of PEGylated Enzyme with Glutaraldehyde can Enhance Stability while Avoiding Intermolecular Crosslinking

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Synthesis of PEGylated GOx (PEG-GOx)

Materials

Glucose oxidase from *Aspergillus niger* (GOx, 338 U/mg solid, 82% protein) was obtained from BBI Enzymes, and methoxy-poly(ethylene glycol)-hydrazide (PEG, 4.5 kDa by gel permeation chromatography) was obtained from Laysan Bio. All other reagents were obtained from Sigma.

Synthesis of PEG-GOx

The procedure used in this work to synthesize PEG-GOx, which is depicted schematically in Fig. S1, is slightly modified from our previous work.[1] GOx (6.6 mg, **1**) was dissolved in 1.8 mL of 100 mM sodium phosphate containing 154 mM NaCl (pH 7.2). Separately, $NaIO_4$ (8.6 mg) was dissolved in 200 μL of deionized water (protected from light). Immediately following dissolution of the $NaIO_4$, it was added to the GOx solution and slowly agitated in the dark for 1 h at room temperature to yield **2**. Glycerol (20:1 molar ratio of glycerol to $NaIO_4$) was added to quench the reaction. The oxidized GOx was immediately purified using a desalting column equilibrated with 100 mM sodium phosphate containing 154 mM NaCl (pH 7.2). PEG (37.18 mg) was then added to the oxidized GOx solution (200:1 molar ratio of PEG to GOx). These conditions favor attachment of PEG rather than intermolecular crosslinking between oxidized sugars and superficial amines on GOx because of the hydrazide group's low pK_a (*ca.* 3), along with the smaller size and large molar excess of PEG as compared to GOx. The solution containing PEG and GOx was reacted in the dark for 2 h at room temperature under gentle agitation to yield **3**. In a fume hood, 20 μL of $NaBH_3CN$ (5 M, in 1 N NaOH) was added to the mixture. The $NaBH_3CN$ was reacted with the PEG-GOx for 30 min at room temperature under gentle agitation to yield **4**. PEG-GOx was then purified from low-molecular-weight contaminants using a desalting column equilibrated with 10 mM sodium phosphate containing 154 mM NaCl (pH 7.2). Gel-filtration chromatography was performed to further purify PEG-GOx (*i.e.*, remove unattached PEG) and analyze products. Sample was injected into a liquid chromatography system (GE Healthcare Life Sciences, ÄKTAexplorer 10) equipped with a gel-filtration column (GE Healthcare Life Sciences, HiLoad Superdex 200 PG) equilibrated with 10 mM sodium phosphate containing 150 mM NaCl (pH 7.2). Absorbance at 220 nm, 280 nm, and 450 nm was monitored, 2 mL fractions were collected, and protein-containing fractions were pooled and concentrated to 6 mg/mL (relative to GOx). Liquid chromatography data, as well as gel electrophoresis and mass spectrometry data, are provided in Ref. 1.

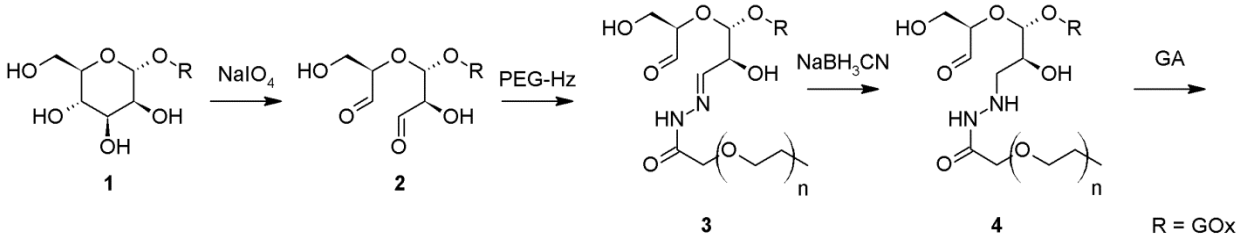

Fig. S1 Synthesis of PEG-GOx. Glycosylation sites on GOx are oxidized by $NaIO_4$ to yield reactive aldehydes. The hydrazide group of the PEG is covalently attached at the glycosylation sites, and the resulting hydrazone linkages are reduced with $NaBH_3CN$ for stability.

Chemical Modification of PEG-GOx and Native GOx

To initiate chemical modification, equal volumes of enzyme solution (PEG-GOx or native GOx, 6 mg/mL in 10 mM sodium phosphate containing 150 mM NaCl, pH 7.2) and glutaraldehyde solution (GA, ranging from 7.5e-4 wt% to 25 wt%) were combined and reacted for 1 h at room temperature. The final GA concentrations in the reaction solution ranged from 3.75e-4 wt% to 12.5 wt%, corresponding to a molar excess of GA (relative to GOx monomer) ranging from 1 to 3.33e4, respectively. Excess GA was removed using a 30 kDa centrifugal filter, and the modified enzyme was transferred back into 10 mM sodium phosphate containing 154 mM NaCl (pH 7.2).

Particle Sizing

A photon correlation spectrometer (Malvern model Zetasizer Nano ZS) was used to acquire size distributions of the modified and unmodified PEG-GOx and native GOx samples (n = 5). This was necessary to determine the change in size after GA

modification, as well as the extent of oligomerization during oxidation, subsequent PEGylation, and GA exposure. Disposable 3.5 mL cuvettes were filled with enzyme (1 mL, 0.6 mg/mL) in 10 mM sodium phosphate containing 154 mM NaCl (pH 7.2).

Primary Amine Content

A fluorescamine assay was used to determine the presence of free primary amines on GA-modified and unmodified PEG-GOx and native GOx samples as an indication of the extent of modification ($n = 4$). In a 96-well solid black flat bottom polystyrene microplate, enzyme (200 µL, 25 µg/mL) in 10 mM sodium phosphate containing 154 mM NaCl (pH 7.2) was mixed with 1 mM fluorescamine (50 µL, 0.3 mg/mL) in acetone for 60 s. Fluorescence was excited from samples at 405 nm and fluorescamine emission was collected at 485 nm using a multimode microplate reader (Tecan model Infinite M200 PRO series).

Thermal Denaturation Study

A photon correlation spectrometer (Malvern model Zetasizer Nano ZS) was used to determine the effect of heating on the size of the modified and unmodified PEG-GOx and native GOx samples ($n = 3$). Standard 3.5 mL glass cuvettes were filled with enzyme (1 mL, *ca.* 0.735 mg/mL) in 10 mM sodium phosphate containing 154 mM NaCl (pH 7.2). The temperature of the cuvette holder was incrementally increased from 25 °C to 90 °C in steps of 5 °C, and the enzyme solution was allowed to equilibrate to each temperature for 2 min prior to collection of sizing data.

Enzymatic Activity Assays

Enzymatic assays of GA-modified and unmodified PEG-GOx and native GOx samples were performed to determine activity following exposure to various challenges. In all cases, enzymatic activity measurements were performed in triplicate at pH 5.1 and 35°C using a UV/Vis spectrophotometer (Agilent model Cary 300) equipped with a water-thermostatted multicell holder to maintain the appropriate temperature. Absorbance data were collected at *ca*. 30 Hz for *ca*. 2.5 min. Data were fit *via* linear regression using the maximum number of points to obtain a norm of residuals < 0.025. This procedure was adopted to minimize the effects of nonlinearities present at later time points in absorbance data collected for samples of high catalytic activity.

Long-Term Storage Stability

To observe the effects of PEGylation and GA modification on the spontaneous denaturation of GOx, enzymatic activity was assayed over a period of four weeks with the enzyme samples stored at 37°C in the absence of glucose. To limit artifacts from sample contamination, replicate samples were prepared for each timepoint and each sample to prevent the need for samples to be re-opened multiple times; that is, samples were only opened on the day they were assayed for enzymatic activity. Figs. S2 and S3 show complete specific activity data for all GA-modified and unmodified PEG-GOx and native GOx samples.

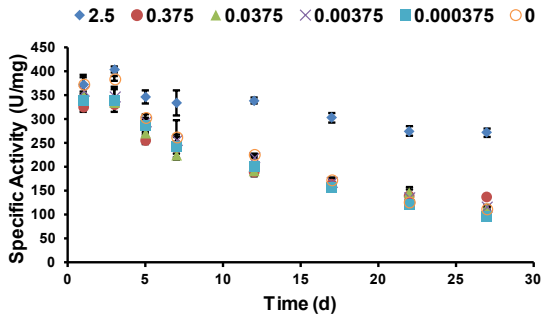

Fig. S2 Specific activity of GA-modified PEG-GOx, as well as unmodified PEG-GOx control, over four weeks at 37°C ($n = 3$; error bars represent 95% CI).

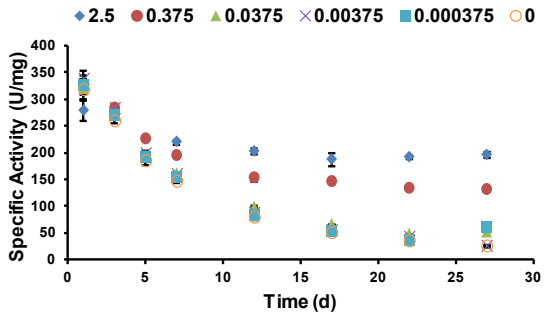

Fig. S3 Specific activity of GA-modified native GOx, as well as unmodified native GOx control, over four weeks at 37°C (n = 3; error bars represent 95% CI).

Glucose Stability

For the glucose exposure experiments, a dynamic dialysis system in a microplate format (10-well MicroDialyzer) was obtained from Spectrum Labs (Note: this product has since been discontinued). The MicroDialyzer has ten wells, each of which can be filled with up to 500 µL of sample that is in direct contact with a 12-14 kDa regenerated cellulose dialysis membrane (Spectrum Labs model Spectra/Por 2). Beneath the dialysis membrane, a stirred dialysate solution is pumped through the dialysis chamber. For our purposes, enzyme solutions (250 µL, *ca*. 0.25 mg/mL) were added to wells (n = 3), and PBS containing glucose (100 mg/dL) was pumped through the dialysis chamber at 4 mL/min using a peristaltic pump. Fig. S4 (*left*) shows the assembled MicroDialyzer on top of a magnetic stir plate in the foreground with the peristaltic pump shown in the background. Fig. S4 (*right*) illustrates the flow and diffusion path for glucose to enter the enzyme-containing wells while product (*i.e.*, H_2O_2 and gluconic acid) is also removed from the system. After the appropriate glucose exposure time (6 h, 12 h, or 24 h), glucose-containing PBS was removed from the dialysis chamber, and 1 L of fresh PBS (no glucose) was pumped through the system at 4 mL/min (*ca.* 4 h) to remove excess glucose and product from the sample wells. Because the samples may change volume due to osmotic and hydrostatic forces—and because there is a possibility for the membrane to be compromised during testing—the samples were transferred to a standard microplate and checked for concentration using a multimode microplate reader (Tecan model Infinite M200 PRO series). Finally, the samples were assayed for enzymatic activity.

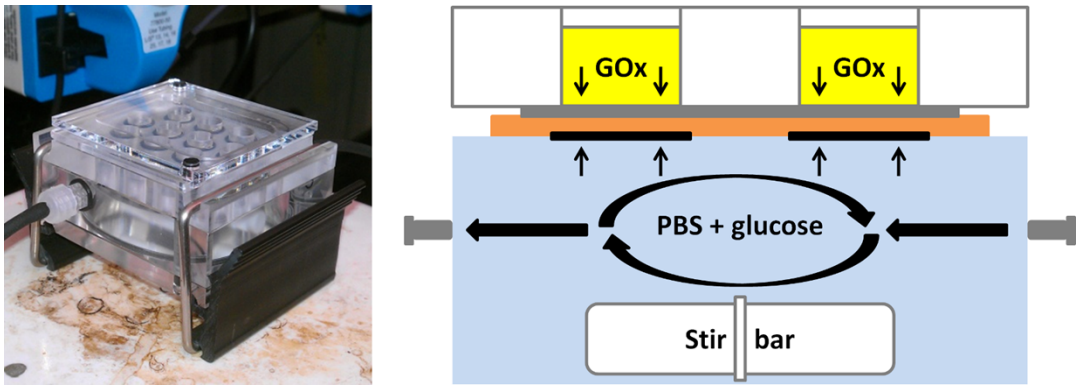

Fig. S4 Apparatus used for glucose exposure experiments. The photograph on the left depicts the MicroDialyzer during operation, while the schematic on the right illustrates how the MicroDialyzer is utilized for these experiments.

Elevated Temperature Stability

To determine if PEGylation and GA modification provides stabilization of the enzyme at elevated temperatures (*i.e.*, where thermally-induced denaturation dominates), enzyme samples (0.73 mg/mL) were immersed into a 60°C water bath for up to 1 h. Separate replicate samples (n = 3) were prepared for each time point (5 min, 15 min, and 1 h). Upon removal from the water bath, samples were immediately cooled to 4°C, then tested for enzymatic activity.

References

1 D. Ritter, J. Roberts and M. McShane, *Enzyme Microb. Technol.*, 2013, **52**, 279.