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Modification of PEGylated enzyme with glutaraldehyde can enhance stability while avoiding intermolecular crosslinking†

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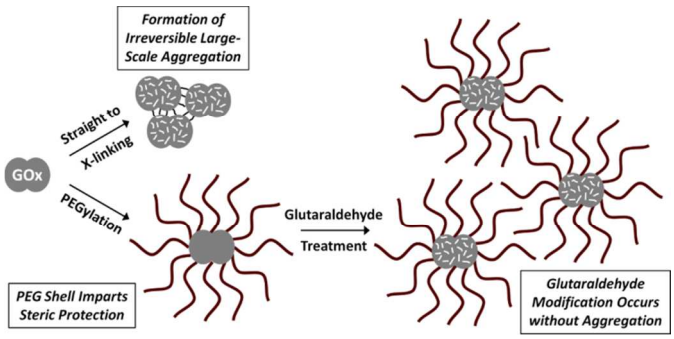
We demonstrate an enzyme stabilization approach whereby a model enzyme is PEGylated, followed by controlled chemical modification with glutaraldehyde. Using this stabilization strategy, size increases and aggregation due to intermolecular crosslinking are avoided. Immediately following synthesis, the PEGylated enzyme with and without glutaraldehyde modification possessed specific activities of 372.9 ± 20.68 U/mg and 373.9 ± 15.14 U/mg, respectively (*vs.* 317.7 ± 19.31 U/mg for the native enzyme). The glutaraldehyde-modified PEGylated enzyme retains 73% original activity after 4 weeks at 37 °C (*vs.* 8.2% retention for control).

Enzymes are employed across a broad range of applications, including biosensors, tissue engineering, drug delivery, and bioprocess engineering. However, the utility of these biomolecules is often limited by their relatively short lifespan, a result of natural processes such as denaturation and degradation. These processes are accelerated when enzymes are exposed to harsh environmental conditions or industrial processing.[1, 2] As an example, glucose oxidase (GOx) is widely used in enzymatic glucose sensors due to its high affinity and selectivity for glucose,[3] and our lab has developed GOx-based implantable optical glucose biosensors.[4, 5] However, significant enzymatic activity is lost during sensor fabrication (due to solvent exposure, local heating, *etc.*) and upon implantation (exposure to physiological conditions), which ultimately necessitates premature sensor replacement.

Many enzyme stabilization approaches have been demonstrated, with a vast number of recent reviews dedicated to the subject.[2, 6-13] A common class of enzyme stabilization strategies involves single- or multi-point covalent, electrostatic, or physical attachment of an enzyme to a rigid support (*e.g.*, an activated support or ion exchange resin).[14] Often immobilization on a support is very effective in stabilizing enzymatic activity, especially for multimeric enzymes where attachment to a support prevents subunit dissociation.[6, 8-10, 12] However, the support matrix can present a significant transport barrier, and the enzyme is no longer in the solution phase. Carrier-free techniques, such as crosslinked enzyme crystals (CLECs) and crosslinked enzyme aggregates (CLEAs), are also effective at stabilizing the enzyme's tertiary and quaternary structure (*i.e.*, preventing denaturation and subunit dissociation) and do not "dilute" the activity.[15-17] However, the resulting particles are large and polydisperse (*ca.* 5-50 μm), and transport barriers are expected. Recently, Jiang and co-workers proposed enzyme-based inverse opals as a related alternative to address transport issues.[18] While each of these approaches has merit, native properties (*e.g.*, native size and residence in solution phase) are sacrificed for increased stability.

In this work, we demonstrate a novel approach to enzyme stabilization, which offers marked advantages over previously reported techniques. Our straightforward approach involves the attachment of poly(ethylene glycol) (PEG) chains (*i.e.*, PEGylation) to an enzyme to provide steric protection, followed by chemical modification in an effort to stabilize the tertiary and quaternary structures (Scheme 1). We developed this technique based on the hypothesis that deposition of an inert polymeric shell around the enzyme would prevent intermolecular crosslinking, which results in irreversible, large-scale aggregate formation.[1] This can lead to a decrease in the apparent enzyme activity—a result of substrate inaccessibility to its binding site—and precludes the use of the stabilized enzyme in applications which require the enzyme to be in solution phase and/or near-native size. Our intention is to stabilize GOx in a way that permits subsequent homogenous distribution and entrapment within an optical biosensing hydrogel; however, this approach could be applied to a myriad of other proteins for different applications, which might include tissue engineering and therapeutic biologicals. We demonstrate that our technique results in dramatic increases in long-term stability for GOx, while avoiding undesirable aggregate formation.



Scheme 1 PEGylation of the enzyme and subsequent chemical modification allows for enzyme stabilization. Covalently bound PEG chains sterically protect against intermolecular crosslinking.

GOx is a homodimeric flavoenzyme with a high degree of glycosylation (*ca.* 18–20%).[19] Glycosylation sites of GOx were targeted for the attachment of PEG chains.[20, 21] This PEGylation route was chosen to preserve the enzyme's reactive primary amines, which are key to permit effective modification by an amine-reactive dialdehyde such as glutaraldehyde (GA).[1] Our previously reported protocol was employed,[21] wherein sugar residues on GOx's surface are oxidized and reacted with 5 kDa methoxy-PEG-hydrazide. After sodium cyanoborohydride reduction, the PEGylated GOx (PEG-GOx) is purified using GFC. Dynamic light scattering (DLS) reveals that PEG-GOx has a hydrodynamic diameter of 16.98 ± 2.68 nm, as compared to native GOx, which has a hydrodynamic diameter of 10.76 ± 0.95 nm (Fig. 1A).

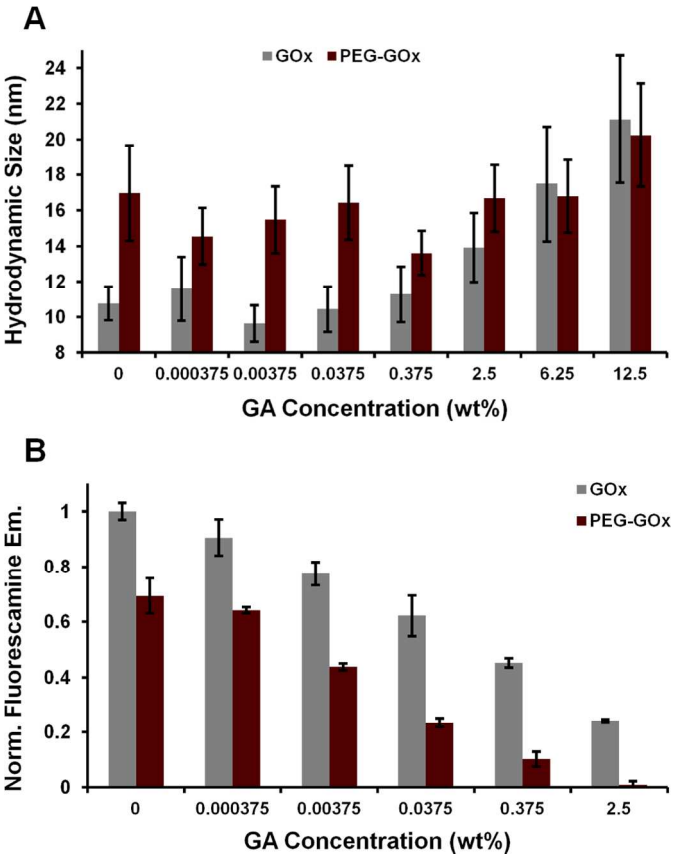


Fig. 1 Hydrodynamic size (A) and free primary amine content (B) of native GOx and PEG-GOx exposed to various GA concentrations. Error bars represent 95% CI.

Subsequently, PEG-GOx was modified with GA at various concentrations ranging from 3.75e-4 wt% to 12.5 wt%. DLS was used to determine the hydrodynamic size of the modified PEG-GOx (n = 5), which provides insight into the extent of intermolecular crosslinking at various GA concentrations. As we hypothesized, there was no statistically significant change in the size of the initially PEGylated GOx across the range of GA concentrations (Fig. 1A). In a control experiment, GOx exposed to GA concentrations of 2.5 wt% or higher was determined to be larger than native GOx, indicating formation of multi-enzyme aggregates (Fig. 1A). Upon visual inspection, the GOx samples exposed to the two highest GA concentrations (*i.e.*, 6.25 wt% and 12.5 wt%) contained easily identifiable large-scale aggregates. These GA concentrations were omitted for the remainder of the study to prevent possible misrepresentation of enzymatic activity due to inaccurate estimates of protein concentrations or significant apparent activity reduction due to blockage of substrate diffusion to active sites.

A fluorescamine assay was employed to test for free primary amines (*e.g.*, ε-amino group of superficial Lys residues)[22] as an indication of the extent of GA modification of PEG-GOx and native GOx (n = 4). The data clearly show that with increasing GA concentration, the amine content of both PEG-GOx and native GOx is decremented (indicated by a decrease in fluorescamine emission)—attributable to modification of the enzyme by GA (Fig. 1B). Further, the decrease in amine content of the PEG-GOx with increasing GA concentration appears to be similar to that of native GOx; thus, the presence of PEG does not appear to interfere with the reaction between GA and PEG-GOx. Finally, because the fluorescamine emission from PEG-GOx is initially and persistently lower than that of native GOx, this might suggest that PEG-GOx contains fewer primary amines than native GOx. However, we believe this is more likely due to the steric hindrance of PEG: a portion of the amines on PEG-GOx are inaccessible by fluorescamine, which is more bulky and has a molecular mass nearly three times that of GA.

To further characterize the modified and unmodified enzymes, DLS was employed to investigate the effect of heating on size distribution (n = 3). The hydrodynamic diameter of each form of enzyme was monitored while incrementally increasing the temperature of the sample from 25 °C to 90 °C, and the percent change from the starting size was plotted as a function of temperature. Fig. 2 shows that the size distribution of native GOx begins to drastically shift toward larger size at 60 °C (*ca.* 150% change at 75 °C), which is indicative of thermal denaturation and subsequent aggregation of the enzyme at these temperatures. This is consistent with reports from other groups that place the melting temperature for GOx between approximately 56 °C and 58 °C.[23-25] Moreover, GOx is reported to form primarily trimers and tetramers upon thermal denaturation, which is supported by the magnitude of the size increase as well.[25] For the GA-modified GOx, the size distribution still shifts toward larger size at elevated temperature; however, the onset is delayed until about 80 °C is reached and the size change is attenuated by a factor of two. Reduced aggregation from the GA-modified GOx could indicate reduced thermal denaturation, possibly due to increased rigidity resulting from GA modification. In the case of both unmodified and GA-modified PEG-GOx, no significant increase in size was observed across the range of exposure temperatures. This could signal an increase in thermal stability, but we believe it is more likely that the presence of PEG merely prevents thermally denatured enzyme from aggregating.

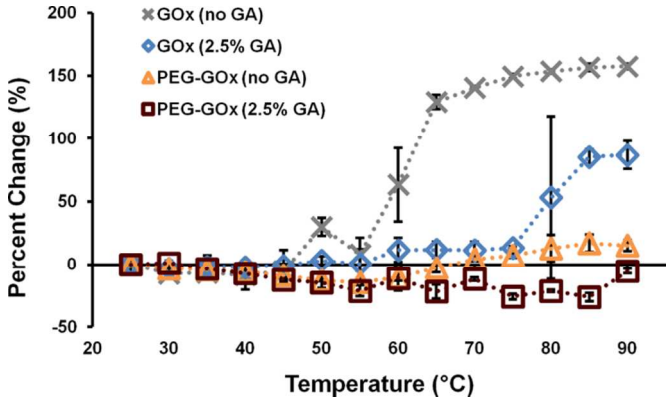


Fig. 2 Effect of heating on the size of GA-modified and unmodified PEG-GOx and native GOx. Error bars represent 95% CI.

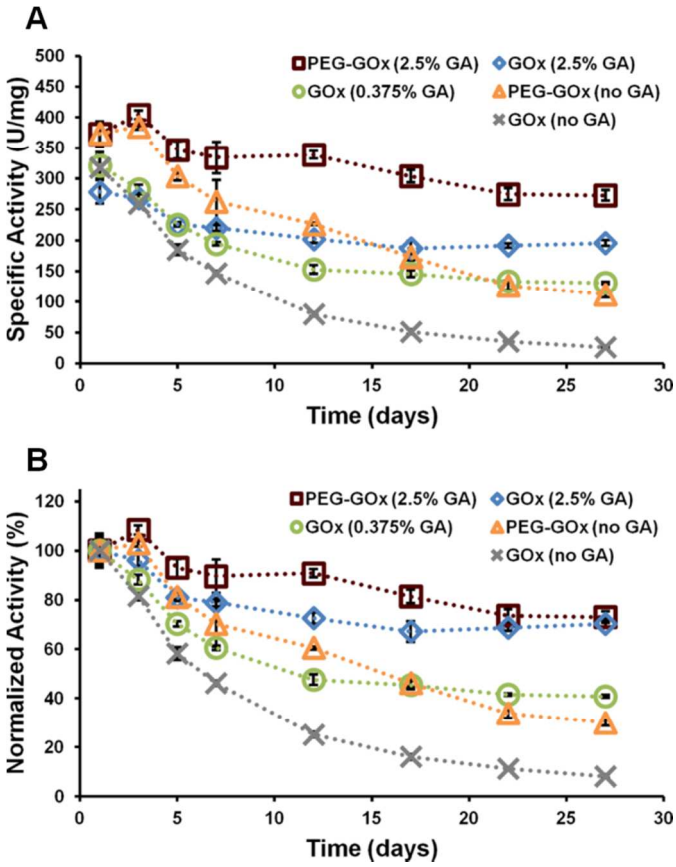


Fig. 3 Specific activity (A) and normalized activity (B) of GA-modified PEG-GOx and native GOx, as well as unmodified controls, over four weeks at 37 °C. Error bars represent 95% CI.

To investigate the effect of GA modification on the long-term retention of enzymatic activity, samples of PEG-GOx and native GOx—either unmodified or modified with GA concentrations ranging from 3.75e-4 wt% to 2.5 wt%—were stored in phosphate buffered saline (PBS) for four weeks at 37 °C. Enzymatic activity was assayed at various points in time using a standard colorimetric assay.[26] The initial time point reveals that GOx modified with 2.5 wt% GA has only 87.6% the specific activity of unmodified GOx, whereas there is no statistically significant difference between the specific activities of PEG-GOx modified with 2.5 wt% GA and its unmodified counterpart (Fig. 3A). Also, no statistically significant difference is observed between the specific activities of GOx modified with 0.375 wt% GA and unmodified GOx after 1 day. The data show that after four weeks, PEG-GOx modified with 2.5 wt% GA retains more than twice its initial specific activity compared to unmodified PEG-GOx (273 U/mg *vs.* 112 U/mg or 73.1% *vs.* 30.0% retention, respectively). Similarly, Fig. 3B shows that GOx modified with 2.5 wt% GA retains 70.3% specific activity after four weeks, which is greater than GOx modified with 0.375 wt% GA (40.8% retention) and greater still than unmodified GOx (8.19% retention). Further, all PEG-GOx samples exposed to GA concentrations less than 2.5 wt%, including unmodified PEG-GOx, displayed similar stability to GOx modified with 0.375 wt% GA (Fig. S2, ESI†). Likewise, GOx exposed to GA concentrations less than 0.375 wt% behaved similarly to unmodified GOx (Fig. S3, ESI†).

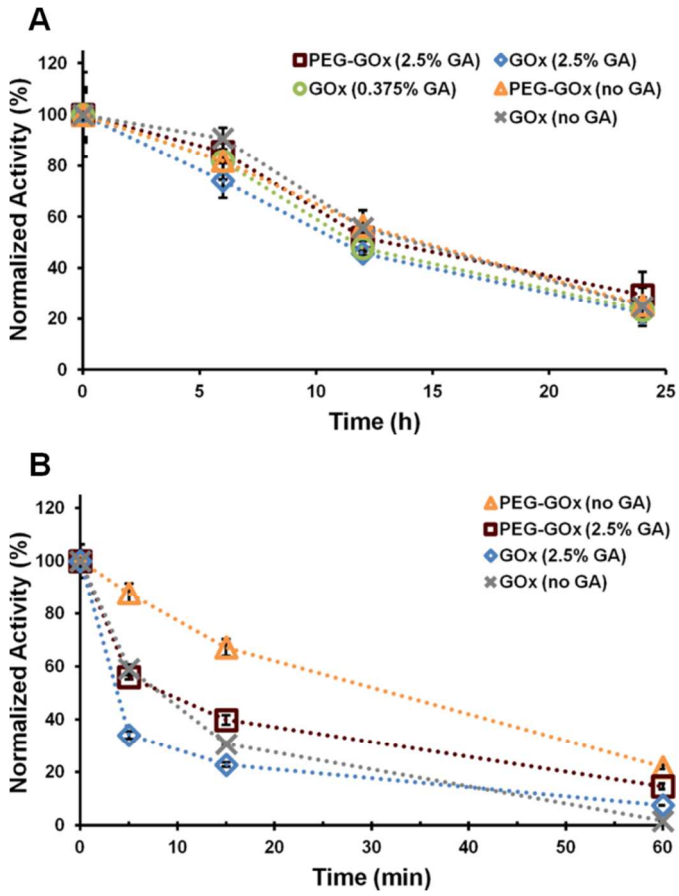


Fig. 4 Normalized activity of GA-modified and unmodified PEG-GOx and native GOx with exposure to glucose (A) and heat (B). Error bars represent 95% CI.

It is well-known that exposure of GOx to glucose hastens its activity loss *via* production of and modification by hydrogen peroxide,[27] and we have previously reported this effect with respect to PEG-GOx.[21] Betancor and co-workers have shown that immobilization of GOx can mitigate deactivation by hydrogen peroxide to a large extent, presumably due to increased rigidity.[28] We tested for this effect on GA-modified and unmodified PEG-GOx and native GOx by continuously exposing samples to PBS containing glucose at room temperature *via* a previously commercially-available dynamic dialysis system in a microwell plate format (Fig. S4, ESI†). Enzymatic activity was assayed initially and after 6 h, 12 h, and

24 h. As shown in Fig. 4A, there were no major differences among the various samples in their response to glucose exposure; after 24 h, all samples lost approximately 75% of their initial specific activity. However, at the 6 h time point, native GOx exposed to 2.5 wt% GA had lost more activity than native GOx, and after 12 h, both of the GA-modified GOx samples (*i.e.*, 0.375 wt% GA and 2.5 wt% GA) had lost more activity than native GOx. At all tested time points, unmodified and GA-modified PEG-GOx were not significantly different from unmodified native GOx ($\alpha = 0.05$).

For many enzymes, exposure to extreme temperature results in thermally-induced denaturation accompanied by enzymatic activity loss. For GOx specifically, thermal denaturation involves an irreversible transition to a compact denatured state, which is accompanied by dissociation of the FAD co-factor but not dissociation of the dimer into its subunits.[25] To determine if PEGylation and GA modification of the enzyme provided stability at elevated temperatures, enzymatic activity was assayed following exposure to 60 °C for up to 1 h. Interestingly, while PEGylation proved beneficial at all time points, GA modification of both native GOx and PEG-GOx appeared to have a largely deleterious effect (Fig. 4B). Seymour and Klinman have shown that PEGylation of GOx increases its melting temperature, which is in good agreement with our results.[24] The most likely explanation for the reduced thermostability observed from the GA-modified samples is an inability for these forms of the enzyme to undergo thiol-disulfide exchange. GOx, which contains two disulfide bonds and two thiol groups per homodimer, relies heavily upon thiol-disulfide exchange for stability at higher temperatures according to Ye and Combes.[29] They have shown that blocking of the free thiols of GOx with *N*-ethylmaleimide does not affect the initial activity, but drastically reduces the enzyme's stability at 60 °C. Therefore, it is possible that GA modification prevents thiol-disulfide exchange, either through increased rigidity or creation of a microenvironment in which thiol-disulfide exchange is unfavorable.

In summary, we have demonstrated the utility of PEGylation to function as a steric stabilizer during chemical modification with GA. PEG chains do not appear to inhibit GA modification, but seem to prevent intermolecular crosslinking, which leads to irreversible aggregation. Likewise, the PEGylated enzyme appears to resist aggregation during and following thermal denaturation. The utility of enzymes modified in this manner is dependent upon the intended use. At physiological temperature and in the absence of glucose, PEG-GOx modified with 2.5 wt% GA retains nearly an order of magnitude more of its initial activity compared to native GOx. Neither PEGylation nor GA modification imparts any significant protective effect upon exposure to glucose. Finally, PEGylation appears to help preserve enzymatic activity of GOx exposed to extreme temperature, especially in the short-term, but modification with GA actually has a detrimental effect.

Future work could focus on further exploring the exact mechanism of stabilization/destabilization, evaluating higher GA concentrations for PEG-GOx, and employing a detachable PEG chain that could prevent aggregation during chemical modification, but allow for subsequent removal and return of the enzyme to its native size. Furthermore, it would be interesting to apply this technique to other glycoenzymes (or a modified version of the approach for non-glycosylated proteins), especially in cases where subunit dissociation of multimeric proteins is expected.

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Notes and references

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We demonstrate a novel and effective enzyme stabilization approach whereby an enzyme is initially PEGylated, followed by controlled glutaraldehyde modification.