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Glycosylation site-targeted PEGylation of glucose oxidase retains native enzymatic activity

Dustin W. Ritter[a], Jason R. Roberts[a], Michael J. McShane[a,b,*]

[a] Department of Biomedical Engineering, Texas A&M University, College Station, TX 77843, USA
[b] Materials Science and Engineering Program, Texas A&M University, College Station, TX 77843, USA

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ABSTRACT

Targeted PEGylation of glucose oxidase at its glycosylation sites was investigated to determine the effect on enzymatic activity, as well as the bioconjugate's potential in an optical biosensing assay. Methoxy-poly(ethylene glycol)-hydrazide (4.5 kDa) was covalently coupled to periodate-oxidized glycosylation sites of glucose oxidase from *Aspergillus niger*. The bioconjugate was characterized using gel electrophoresis, liquid chromatography, mass spectrometry, and dynamic light scattering. Gel electrophoresis data showed that the PEGylation protocol resulted in a drastic increase (ca. 100 kDa) in the apparent molecular mass of the protein subunit, with complete conversion to the bioconjugate; liquid chromatography data corroborated this large increase in molecular size. Mass spectrometry data proved that the extent of PEGylation was six poly(ethylene glycol) chains per glucose oxidase dimer. Dynamic light scattering data indicated the absence of higher-order oligomers in the PEGylated GOx sample. To assess stability, enzymatic activity assays were performed in triplicate at multiple time points over the course of 29 days in the absence of glucose, as well as before and after exposure to 5% w/v glucose for 24 h. At a confidence level of 95%, the bioconjugate's performance was statistically equivalent to native glucose oxidase in terms of activity retention over the 29 day time period, as well as following the 24 h glucose exposure. Finally, the bioconjugate was entrapped within a poly(2-hydroxyethyl methacrylate) hydrogel containing an oxygen-sensitive phosphor, and the construct was shown to respond approximately linearly with a 220 ± 73% signal change (n = 4, 95% confidence interval) over the physiologically-relevant glucose range (i.e., 0–400 mg/dL); to our knowledge, this represents the first demonstration of PEGylated glucose oxidase incorporated into an optical biosensing assay.

1. Introduction

Protein PEGylation—the covalent attachment of poly(ethylene glycol) (PEG) to a protein or peptide—has been widely employed for therapeutic purposes since its introduction in 1977 by Abuchowski et al. [1]. A number of reviews outline the benefits that PEGylation can impart upon therapeutic proteins, such as enhanced circulation half-life in vivo and decreased immunogenicity [2–5]. Until recently, modification of the ε-amino group of superficial lysine residues with an amine-reactive PEG has been most commonly employed due to the large number of these reactive groups (lysine residues comprise 10% of a typical protein [6]); however, conjugates prepared using this technique are typically heterogeneous and often require purification to isolate the preferred isoform [4,5,7]. Furthermore, in enzymes, activity loss is an issue when random multi-site PEGylation is applied, which has been partially attributed to lower substrate binding affinity due to steric hinderance of the binding site and disruption of the protein tertiary structure [8–11]. To overcome this issue, Zalipsky and co-workers designed a PEG-hydrazide (PEG-Hz) derivative [12] that could be used to target oligosaccharides on glycoproteins, allowing for PEGylation without affecting the primary structure of the enzyme [2,13].

Glucose oxidase (GOx) is a dimeric enzyme that is used widely in the food industry to produce gluconic acid, act as a food preservative, and determine the glucose content in foodstuffs [14]. Many of the properties that make GOx an ideal choice for glucose sensors in the food industry also make it the most suitable choice for incorporation into glucose biosensors for biomedical applications [15]. Our lab has developed optical glucose biosensors based on GOx [16–18], and recent work has focused on extending the operating lifetime of these biosensors [19]. Loss of enzymatic activity of GOx can result in undesirable changes in sensor response characteristics (requiring recalibration and decreasing operational

Abbreviations: PEG, poly(ethylene glycol); PEG-Hz, poly(ethylene glycol)-hydrazide; GOx, glucose oxidase; pHEMA, poly(2-hydroxyethyl methacrylate).

* Corresponding author. Tel.: +1 979 845 7941; fax: +1 979 845 4450.
E-mail addresses: dritter918@bme.tamu.edu (D.W. Ritter), jasonrr@tamu.edu (J.R. Roberts), mcshane@bme.tamu.edu (M.J. McShane).

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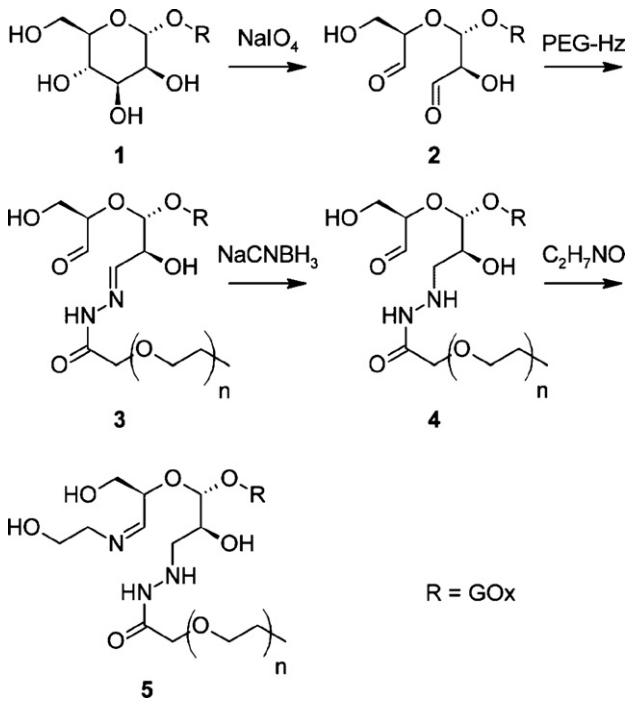

Scheme 1. Glycosylation site-targeted PEGylation of GOx.

lifetime); therefore, we hypothesized that PEGylation of GOx would provide stability against denaturation or hydrolytic cleavage. Moreover, GOx is highly glycosylated, with a total carbohydrate content of 18.8 ± 0.6% of its molecular mass [20], so PEG-Hz is an attractive option to avoid blocking the binding site or affecting the protein conformation. Therefore, in this work, we PEGylated GOx using PEG-Hz to target glycosylation sites, compared the native and PEGylated GOx enzymatic activities, and demonstrated the bioconjugate's ability to function as a glucose sensor. To our knowledge, this is the first reported study to assess the enzymatic activity of GOx modified with PEG-Hz and to incorporate PEGylated GOx into a hydrogel and demonstrate its function as an optical biosensor.

2. Materials and methods

2.1. Materials

GOx from *Aspergillus niger* (type VII, 168.8 U/mg solid, 80% protein by biuret) and peroxidase from *Amoracia rusticana* (type II, 188 U/mg solid) were obtained from Sigma. Methoxy-poly(ethylene glycol)-hydrazide (PEG-Hz, 4.5 kDa by gel permeation chromatography) was obtained from Laysan Bio.

2.2. Preparation of PEGylated GOx

A modification of Zalipsky's PEGylation protocol was used (Scheme 1) [21]. GOx (6 mg, **1**) was dissolved in 1.8 mL of 10 mM sodium phosphate containing 154 mM sodium chloride (pH 7.2). Separately, 8.6 mg of sodium periodate was dissolved in 200 μL of deionized water and protected from light. The sodium periodate solution was immediately added to the GOx solution, and the sample was slowly agitated. The mixture was reacted in the dark for 1 h at room temperature to yield **2**. It is important to note that proteins exposed to oxidants such as periodate have been reported to form higher-order oligomers in certain cases due to intermolecular crosslinking; however, Nakamura et al. exposed GOx from *A. niger* to a five-fold higher concentration of periodate for 5 h and found that the size and shape of the periodate-oxidized GOx was essentially the same as the native GOx [22]. The reaction was quenched by the addition of 2.5 μL of glycerol, corresponding to a 20-fold molar excess of glycerol to sodium periodate. The oxidized GOx was purified using a desalting column equilibrated with 100 mM sodium phosphate containing 154 mM sodium chloride (pH 6.0). PEG-Hz (33.8 mg) was added to the oxidized GOx solution, yielding a 200-fold molar excess of PEG-Hz to GOx. The extremely low pK_a of the hydrazide reactive group (pK_a = 3), paired with its smaller size and large molar excess as compared to the GOx, makes attachment of PEG more favorable than intermolecular crosslinking between oxidized sugars and superficial amines on GOx. The reaction solution was reacted in the dark for 2 h at room temperature under gentle agitation to yield **3**. In a fume hood, 20 μL of 5 M sodium cyanoborohydride in 1 N sodium hydroxide was added. Caution: sodium cyanoborohydride is extremely toxic; as such, all operations should be performed with care in a fume hood. The sodium cyanoborohydride is reacted with the PEGylated GOx for 30 min at room temperature under gentle agitation to yield **4**. Unreacted aldehyde sites were blocked by addition of 100 μL of 1 M ethanolamine (pH 9.6) and reaction for 30 min at room temperature under gentle agitation to yield **5**. The PEGylated GOx was purified from low-molecular-weight contaminants using a desalting column equilibrated with 10 mM sodium phosphate containing 154 mM sodium chloride (pH 7.2).

2.3. Gel electrophoresis

Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed to test for an increase in the hydrodynamic size of GOx as a result of the PEGylation process. Protein samples were combined with sample buffer (containing 54 mg/mL DL-dithiothreitol), vigorously agitated, and loaded onto a 10-well, 10% precast polyacrylamide gel; all samples were duplicated symmetrically on the gel (i.e., the first sample was loaded onto lane 1 and lane 10, etc.). Following electrophoresis, the gel was rinsed three times with deionized water and cut between lanes 5 and 6 so half of the lanes could be stained for protein and the other half for PEG. To stain for protein, one half of the gel was placed in 30 mL of the Coomassie staining solution for 1 h, followed by rinsing with deionized water overnight. To stain for PEG, the other half of the gel was placed in 30 mL of perchloric acid for 15 min, and then 10 mL of 5% w/v barium chloride and 4 mL of 0.1 N iodine were added. The gel was stained for 10 min, followed by extensive rinsing with deionized water [23]. Both stained halves of the gel were imaged separately using a gel imaging system (Bio-Rad model 170-8270).

2.4. Liquid chromatography

Gel-filtration chromatography was performed to separate the PEGylated GOx from unattached PEG-Hz (i.e., purify the conjugate), but also served as an independent secondary characterization tool to confirm the gel electrophoresis data. The samples were injected into a liquid chromatography system (GE Healthcare Life Sciences model ÄKTAexplorer 10) equipped with a gel-filtration column (GE Healthcare Life Sciences model HiLoad Superdex 200 PG) equilibrated with 10 mM sodium phosphate containing 154 mM sodium chloride (pH 7.2). Absorbance at 280 nm was monitored and 2 mL fractions were collected.

2.5. Mass spectrometry

Mass spectra were acquired with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Shimadzu model Axima-CFR) operating in linear mode to determine the extent of PEGylation (i.e., the number of PEG chains attached per GOx). Protein samples (3 mg/ml) in 10 mM sodium phosphate containing 154 mM sodium chloride (pH 7.2) were concentrated and desalted (Millipore model $ZipTip_{C4}$ Pipette Tips); the eluate was spotted directly onto a steel sample plate, where it was combined with an equal volume of saturated sinapinic acid solution and air-dried.

2.6. Dynamic light scattering

A photon correlation spectrometer (Malvern model Zetasizer Nano ZS) was used to acquire size distributions of the GOx and PEGylated GOx samples. This was necessary to determine the change in size after modification, as well as to gauge the extent of oligomerization during oxidation or subsequent PEGylation. Disposable 3.5 mL cuvettes were filled with protein samples (0.6 mg/mL) in 10 mM sodium phosphate containing 154 mM sodium chloride (pH 7.2). In all cases, the derived count rate exceeded 300 kcps.

2.7. Enzymatic activity experiments

Enzymatic assays of PEGylated GOx and native GOx were performed to determine activity [24], and in all cases, enzymatic activity measurements were performed in triplicate at pH 5.1 and 35 °C. All enzyme concentrations were determined using a UV/Vis spectrophotometer (PerkinElmer model LAMBDA 45), with a molar extinction coefficient (λ = 280 nm) of $2.672 \times 10^5\ M^{-1}\ cm^{-1}$ and a molecular mass of 160 kDa for the GOx dimer (based on the manufacturer's datasheet). To observe the effect PEGylation has on the spontaneous denaturation of GOx, enzymatic activity was assayed over the course of 29 days stored at 37 °C (elevated temperature to accelerate deactivation) in the absence of glucose. To compare the rates of deactivation for PEGylated GOx and native GOx under operating conditions, 0.25 mL of each protein solution (0.25 mg/mL) was injected into a dialysis cassette (10 kDa molecular-weight cutoff, 0.5 mL capacity) and placed into 1 L of 10 mM sodium phosphate containing 154 mM sodium chloride and 5% w/v glucose (pH 7.2). Both solutions were stirred and air-equilibrated by bubbling air through a gas diffuser. After 24 h at room temperature, the dialysis cassettes were transferred into 1 L of 10 mM sodium phosphate containing 154 mM sodium chloride (pH 7.2). The dialysate was tested for the presence of glucose using a biochemical analyzer (YSI Life Sciences model 2700 SELECT Biochemistry Analyzer) and exchanged for