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REVIEW ARTICLE

Common Causes of Glucose Oxidase Instability in *In Vivo* Biosensing: A Brief Review

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Abstract

Clinical management of diabetes must overcome the challenge of *in vivo* glucose sensors exhibiting lifetimes of only a few days. Limited sensor life originates from compromised enzyme stability of the sensing enzyme. Sensing enzymes degrade in the presence of low molecular weight materials (LMWM) and hydrogen peroxide *in vivo*. Sensing enzymes could be made to withstand these degradative effects by (1) stabilizing the microenvironment surrounding the sensing enzyme or (2) improving the structural stability of the sensing enzyme genetically. We review the degradative effect of LMWM and hydrogen peroxide on the sensing enzyme glucose oxidase (GOx). In addition, we examine advances in stabilizing GOx against degradation using hybrid silica gels and genetic engineering of GOx. We conclude molecularly engineered GOx combined with silica-based encapsulation provides an avenue for designing long-term *in vivo* sensor systems.

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Introduction

Efficient and accurate glucose-sensing devices are a critical component in current diabetes treatment protocols. These devices are often based on the molecular recognition and catalysis of glucose by the enzyme glucose oxidase (GOx). The development of long-term implantable glucose sensors for more efficient diabetes management faces several immediate limitations, most notably sensor lifetime. A long-term implantable sensor based on GOx will require the enzyme to retain a functional level of catalytic activity for months. While GOx maintains this level of activity *in vitro*, its stability *in vivo* is of the order of 10–14 days, necessitating novel immobilization and enzyme modification strategies to extend the functional lifetime of these oxygen-sensitive sensors.

Glucose sensor instability depends on many environmental and internal factors. Gough and coauthors[1–8] published several papers elaborating on the origins of these environmental and internal factors. Environmental factors occur *in vivo* due to lack of biocompatibility and include membrane biofouling, electrode passivation, and fibrous encapsulation. Internal factors result from both external penetrants [low molecular weight materials (LMWM)] and internal sensor

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Abbreviations: (CBD) chitin-binding domain, (ELP) elastin-like polypeptide, (GAX) glutaraldehyde cross-linking, (GOx) glucose oxidase, (H_2O_2) hydrogen peroxide, (LMWM) low molecular weight materials, (OPH) organophosphorus hydrolase, (PBSA) protein-based stabilizing agent, (PEG) polyethylene glycol

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issues. For an excellent review on the effects of environmental factors on sensor performance, refer to Wisniewski and coauthors.[9] The internal factors include lead detachment, electrical short, membrane delamination, membrane degradation, and sensing-enzyme degradation. Gough and coauthors[1–8] state that GOx degradation stems from either spontaneous inactivation or peroxide mediated inactivation. They suggest that spontaneous inactivation occurs throughout the immobilized enzyme phase but inactivation occurs by an unknown mechanism. Gough and coauthors[3] suggest that these mechanisms could include "a temperature-dependent protein conformational or reversible FAD binding." Conformational changes due to temperature changes directly relate to GOx stability. We suggest that LMWM degradation and epoxy formation within the immobilized enzyme layer encompasses the spontaneous inactivation observed by Gough and coauthors.[1–8] The work of Gough and coauthors[1–8] motivated synthesis of this review.

However, sensing-enzyme degradation, a significant internal factor connected to enzyme stability, has not received thorough review. Enzyme degradation severely limits the functional life of GOx *in vivo* and remains a significant challenge in continuous glucose monitoring. Suspected causes of GOx degradation include hydrogen peroxide (H_2O_2) generated at the electrode[10–15] and intrinsic LMWM from blood and interstitial fluid.[16] However, eliminating these factors is not enough to ensure optimum GOx performance; in addition, we must engineer a more stable environment for GOx and make the GOx enzyme itself more intrinsically stable.

Several groups have attempted to improve stability by engineering various membrane types [silica sol-gel,[17–19] glutaraldehyde cross-linking (GAX),[20,21] carbon nanotubes[22,23]] and by manipulating the molecular structure of GOx itself (addition of oligomers and point mutations to improve functionality of GOx). However, each of these strategies fails to create a stable enough environment for GOx to function effectively over the target lifetime of a continuous glucose sensor. A stable environment (**Figure 1**) requires stoichiometrically controlled diffusion of reactants (oxygen, glucose) and products (H_2O_2) to and from the electrode and the bulk surface, hydrophilicity within the matrix, a localized pH near the isoelectric point of GOx (isoelectric point 4.2), and a mechanically strong and thin matrix layer on the electrode

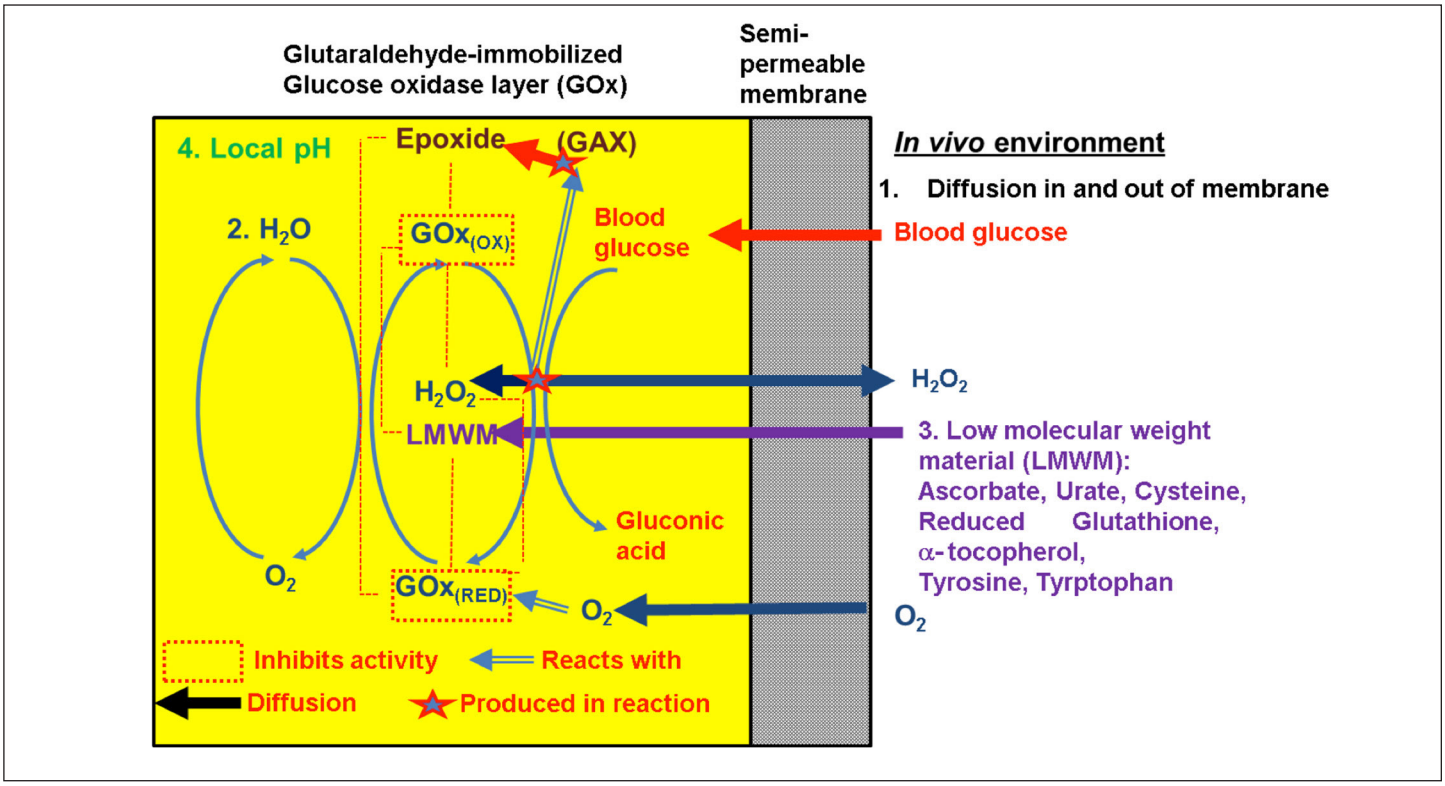

Figure 1. The factors to create an optimum environment for entrapped GOx stability: (1) ample diffusion of key reactants and products in and out of the matrix/GOx/platinum surface, (2) adequate supply of reactants and structural molecules to promote proper folding (potential barriers toward GOx stability), (3) potential degradation from LMWM and H_2O_2, and (4) loss of localized pH near the isoelectric point of GOx.

surface. This review will discuss why these previous stabilization strategies have failed and explore new approaches to achieve a stable GOx environment for the development of a functional, implantable continuous glucose monitor.

Previous Internal Strategy: Glutaraldehyde Cross-Linking of Glucose Oxidase

Enzyme-based glucose sensors provide excellent specificity for a given analyte, yet often suffer from problems with long-term stability and biocompatibility. Glutaraldehyde plays a critical role in the design of biosensors by cross-linking enzymes at amine groups on electrode surfaces. Nonetheless, the effectiveness of GAX-based biosensors is still unclear. In fact, some studies suggest that GAX impairs proper enzyme conformation by cross-linking vital surface residues, resulting in decreased enzymatic activity and limited functionality.[24] The following subsection details how GAX alters GOx structure in the absence and presence of H_2O_2 and LMWM.

Hydrogen Peroxide Degradation of Glucose Oxidase

Glucose oxidase stability decreases over time, in part, due to H_2O_2 oxidation of active site methionine residues to methionine sulfoxide.[10–15] Oxidative damage is a critical issue in maintaining enzyme stability. Methionine sulfoxide formation affects the active site coordination of substrate recognition, catalysis, and specificity. Both soluble and immobilized GOx are susceptible to H_2O_2-mediated oxidative damage.[11,12] Kleppe[12] also suggests that H_2O_2 inactivates the redox states of GOx at different rates. Semiquinone (one electron reduced) and the two-electron-reduced GOx display no activity or severely reduced activity to glucose, respectively. Researchers have shown that H_2O_2 inactivates the reduced GOx 100 times more than the oxidized GOx.

Studies also demonstrate that H_2O_2 deactivates oxidized GOx in the absence of glucose slower than reduced GOx in the presence of glucose.[14,25] Malikkides and Weiland[14] postulated that the predominant mechanism of GOx inactivation by H_2O_2 involves the attack of peroxide on the glucose–GOx complex. They suggest that glucose changes the oxidized GOx conformational structure by expanding the active site, allowing for attack by peroxide. Bao and coauthors[25] determined that immobilized GOx was competitively inhibited by H_2O_2. The reduced form of GOx competitively binds H_2O_2 and oxygen with similar specificity. This affinity for H_2O_2 results in an inactive complex of reduced GOx and glucose, i.e., the semiquinone state discussed previously. Ultimately, glucose sensor stability suffers due to elimination of key active site residues by H_2O_2 or enhanced susceptibility to oxidative attack of H_2O_2 in the presence glucose. If H_2O_2 concentrations reach critical levels, the glucose sensor will experience a decrease in sensor accuracy, substrate sensitivity, and half-life. However, Von Woedtke and coauthors[26] report that *in vivo* glucose sensor operation fails to produce H_2O_2 levels sufficient to effect GOx. Von Woedtke and coauthors[26] concede that local H_2O_2 concentration depends on the diffusion of glucose, oxygen, and H_2O_2 on the electrode surface and the configuration of the active electrode surfaces. It is plausible that H_2O_2 exposure, in addition to potential influence of LMWM, may lead to premature failure of *in vivo* glucose sensors.

In designing glucose sensors, the central challenge is maintaining consistent GOx conformation, which requires controlling the redox state of GOx during immobilization to the surface of the electrode and during *in vivo* operation. The differences in the oxidative states of GOx between different glucose sensors also makes it difficult to evaluate glucose sensor performance; lower stability may arise from a combination of design flaws and a higher relative concentration of reduced GOx. However, it is difficult to identify a single cause of GOx instability since, currently, there is no reliable method of measuring the percentage of oxidized/reduced GOx immobilized on the surface of an electrode.

Low Molecular Weight Materials Degradation of Glucose Oxidase

Kerner[16] first discovered that LMWM of <10 kDa (e.g., ascorbate, urate, cysteine, reduced glutathione and alpha-tocopherol, tyrosine, and tryptophan) could lead to the rapid degradation of GOx and a dramatically lowered sensitivity. Pinpointing the cause of LMWM degradation of GOx is difficult due to numerous unknown events that occur in the surrounding tissue and within the sensor itself that can lead to gradual failure. For example, enzyme often accumulates at the surface of the sensor, resulting in a substantial concentration of GOx at the membrane–bulk interface.[16,26,28–31] This masks the effect of oxidative degradation and prohibits deconvolution of the impact of LMWM

from the effect of H_2O_2.[16,26,28–31] As a result, the specific mechanisms of LMWM degradation of GOx continue to elude the scientific community.

Degradation of Glutaraldehyde Cross-Linked Glucose Oxidase

Glutaraldehyde cross-linking severely limits enzyme leaching[24,32] in sensor applications; however, it can lead to heavily altered tertiary and secondary structures and compromised stability and activity of the enzyme. Researchers have attempted to improve GOx stability by combining GAX with alternative immobilization strategies. For example, Gouda and coauthors[33] showed that the use of lysozyme as a protein-based stabilizing agent (PBSA) resulted in a significant increase in the stability of GOx compared with soluble GOx and immobilized GOx with other PBSAs (PBSA and gelatin). Likewise, Betancor and coauthors[34] demonstrated, without the use of PBSAs, that, if GOx was adsorbed onto a cationic support combined with subsequent treatment with glutaraldehyde, it was 100 times more stable than soluble wild-type GOx.

A number of studies suggest that GAX can provide either a destabilizing or stabilizing environment for GOx; however, the parameters controlling GOx stabilization are largely unknown. López-Gallego and coauthors[24] suggested that a key factor involves the use of supports that are preactivated[35–39] versus nonactivated supports.[40,41] Preactivation allows only primary amino groups of the enzyme to react with the aldehyde groups introduced by modification of the amino groups of the support.[42,43] With nonactivated supports, primary amino groups as well as other amino groups on the enzyme's surface react with the support layer, leading to greater conformational changes and lower stability.[24]

The deleterious effects of GAX on GOx also derive from side reactions of H_2O_2 with glutaraldehyde to produce epoxides. Peracchia[44] demonstrated the production of epoxides from H_2O_2 and glutaraldehyde using nuclear magnetic resonance spectroscopy. Epoxy groups result from a reaction between H_2O_2 and double bonds of α-β-unsaturated aldehydes. Unsaturated aldehydes of glutaraldehyde, a product of aldol condensation, are ubiquitous in commercial glutaraldehyde and are very active in protein cross-linking.[45–47] These epoxy products interact with GOx as well as with glutaraldehyde during the cross-linking step. For example, the addition of epoxy groups may enhance the activity of glutaraldehyde and result in modification of more than the primary amino group in the presence of preactivated supports. Epoxides may also react with GOx and bind not only the primary amino group, but the imino, hydroxyl, and mercapto groups as well.[48] This process is similar to a technique used by Mateo and coauthors[49,50] for covalent attachments to epoxy supports. Enzyme attachment to epoxy supports produce intense multipoint covalent attachment, which enhances the stability of the attached enzyme in controlled environments.[34] Though the potential of multipoint covalent attachment is possible, baseline glutaraldehyde reactivity with H_2O_2 may not produce the controlled conditions observed in Mateo's procedure due to a lack of preactivation of the support. Glutaraldehyde cross-linking shows great promise as a technique to extend GOx stability but requires additional studies to address these side reactions with intermediate species produced in the oxidation of glucose.

Proposed Strategies for Improving Glucose Oxidase Stability

Silica Sol-Gel Encapsulation

Innovations in sol-gel chemistry have led to development of silica hybrids in which organic and inorganic species are mixed at the molecular level.[51] These hybrids range from brittle glasses to flexible gel-like materials. Several groups have encapsulated enzymes, antibodies, and other proteins within silica composites to address stability issues in the design of biosensors, biocatalysts, and bioreactors.[52–57] To date, most studies on sol-gel entrapped biomolecules use TMOS (tetramethyl orthosilicate) and TEOS (tetraethyl orthosilicate) as silica precursors.[58,59] Most TMOS- or TEOS-based silica gels lead to significant changes in enzyme conformation, making them unsuitable for practical applications. However, scientists have developed new sol-gel methods for functional stabilization of biomolecules entrapped in silica gels using different precursors, additives, and aging methods.

For most sensing applications, silica gels in the form of thin films must be uniform. Drying of such films may lead to severe cracking due to differences in drying rates for different pore sizes within the silica gel. Smaller pores remain

wet while larger pores dry quickly, creating large internal pressure gradients. These gradients cause fractures during drying or when dry sensors encounter an aqueous environment. Strategies to overcome fracture formation include the use of chemical additives, such as formamide and Triton-X, in the sol-gel precursors.[60] Cationic surfactants, such as trimethylalkylammonium chlorides, form electrostatic bonds with deprotonated silanol groups during gelation of the silica gel and prevent fractures after immersion in aqueous solutions. Minimizing these fractures limits leaching of the enzyme from the silica matrix.

Numerous industrial and medical applications use ormosils (e.g., methyltriethoxysilane, propyltrimethoxysilane, dimethyldimethoxysilane) to preserve the native activity of biomolecules. Ormosils are organically modified silanes that incorporate various functional groups (amino, glycidoxy, epoxy, hydroxyl) in alkoxide monomers resulting in modified sol-gels.[61,62] The wettability of composite silica gels with ormosils can be controlled by altering the ratio of hydrophilic to hydrophobic monomers,[62] thus controlling fracture formation. Wang and coauthors[63] reported that the incorporation of copolymers into silica gels enhanced the activity of entrapped GOx for amperometric detection of glucose. Ormosils offer a promising option for enzyme stabilization by preventing surface fractures and stabilizing the enzyme within the silica gel.

In addition, adding polymers such as polydimethylsiloxane, polyamides, polyacrylates, and polyethylene glycol (PEG) may allow control over the inorganic condensation–polymerization process. For example, including polyethers in the sol-gel process allows control over the pore size distribution.[64] Adding PEG improves resistance to cracking due to greater hydration of the films during aging, which lowers the hydration stress upon immersion in aqueous solutions.[65] The addition of PEG also reduces the surface area the gel without changing the pore size.[64] However, PEG may also compromise mobility and alter the conformation of enzymes entrapped in silica gel.[66]

Several groups have begun to include additives, such as sorbitol and N-methylglycine (osmolytes), in the sol-gel process. Osmolytes can increase the thermal stability and preserve the activity of entrapped enzymes.[67] Increased pore size allows also greater diffusion of water to the surface of encapsulated enzymes and allows greater substrate accessibility.[68] Enzymes maintain their intrinsic stability largely in an aqueous microenvironment with suitable diffusion to and from the active site of the enzyme.[69]

While silica gels have potential benefits, sol-gel formation also produces harmful organic solvent byproducts that can destabilize the encapsulated enzymes. For example, methanol and ethanol, common organic solvent byproducts, denature entrapped enzymes. This leads to decreased catalytic activity (k_{cat}), decreased substrate specificity (K_M), and increased inhibition (K_I) with increased organic solvent concentration. To minimize organic intermediate formation, scientists are using new biocompatible silane precursors (including glycerated silanes[68] and sodium silicate[70,71]) and developing aqueous processing methods to evaporate out the organic solvent or to evaporate in the silica precursor, avoiding organic synthesis all together (chemical vapor deposition).[72] Besanger and coauthors[58] reported that a new precursor, diglyceryl silane, extends the functional life of entrapped enzymes by liberating glycerol from diglyceryl silane. The glycerol stabilizes the enzyme through molecular crowding and impedes enzyme denaturation by restricting enzyme movement. We reported that glucose sensors fabricated using chemical vapor deposition with GOx dispersed in silica gels condensed at pH 2, 7, and 12. Glucose sensors fabricated at a condensation pH of 12 exhibited the fastest response time, the most extended linear range, greatest specificity, and longest half-life.[20] Increased pore size led to increased diffusion of molecular waters and substrates, ensuring extended stability and activity of the entrapped enzymes.

Studies show that macromolecular crowding and increased hydration increase the stability of encapsulated enzymes.[73] Zhou and Dill[74] report that macromolecular crowding improves stability by introducing folding forces not available for proteins in solution. These forces include ionic and hydrogen bonds as well as Van der Waals and hydrophobic forces. Dipole–dipole forces from molecular waters create a cage structure that stabilizes entrapped enzymes. Zhou and Dill[74] predict that these cages increase the stability of an enzyme's native state by as much as 15 kcal/mol. As reviewed here, polymer dopants, ormosils, proper aging techniques, new silane precursors, and new sol-gel processing techniques are promising approaches for new sensing platforms in long-term *in vivo* applications.

Glucose Oxidase Molecular Cloning

Despite these promising techniques for improving enzyme stability *in vitro*, immobilized enzymes demonstrate nominal increases in stability during *in vivo* applications, resulting in little improvement in sensor lifetimes. However, molecularly engineered enzymes that include protein tags and/or point mutations have shown increased intrinsic molecular stability compared to wild-type homologs. Zhu and coauthors[75] adapted directed evolution protocols to improve the catalytic performance, thermal resistance, and pH stability of GOx; this approach resulted in a 4 °C jump in melting temperature, increased functionality at pH range 8–11, and 1.9-fold improvement in k_{cat}. Chen and coauthors[76] modified GOx to include a poly-lysine tag on the C-terminus to anchor ferrocenecarboxylic acid mediators to the enzyme, improve enzyme stability, and increase sensitivity of glucose biosensors. This modified GOx maintained a 90% sensor response after 70 days of storage at 4 °C. Finally, Holland and coauthors[77] mutated 15 residues in *Aspergillus niger* GOx to mirror residues found in the more catalytically active *Penicillium amag* GOx. The mutant GOx displayed a 4.5-fold improvement in k_{cat} and marginal improvement in thermal stability at 50 °C (increase in half-life from 28 to 40 h).[77]

Another synthetic approach is to engineer recombinant versions of the enzyme at the genetic level to include components or chimeras that lead to stabilization. Engineered protein tags that may improve stability include the chitin binding domain from *Pyrococcus furiousus*[78] and elastin-like polypeptides (ELPs).[79] We attached the chitin-binding domain (CBD) to the N-terminus of a xylose isomerase from *Thermotoga neapolitana* and immobilized the fusion enzyme to chitin beads. Addition of the CBD led to increased isomerization of glucose to fructose, increased melting temperature, and increased half-life of the enzyme (5-fold versus soluble fusion protein and 10-fold versus the soluble wild-type). The xylose isomerase remains folded for a longer period due to the imparted rigidity of the CBD tag anchored to chitin. Similar modifications of GOx may yield enhancements in its stabilities.

The ELP tags can also enhance stability while providing a facile method for purification of enzymes. Shimazu and coauthors[79] attached a [78-VPGVG] repeat ELP to the C-terminus of organophosphorus hydrolase (OPH). Incorporation of an ELP fusion sequence markedly improved OPH stability, maintaining 100% activity over 3 weeks.[79] The long-term stability of aggregated OPH–ELP in storage far exceeded the stability of soluble OPH–ELP at various temperatures or the stability achieved by the addition of a stabilizing agent (PBSA). Elastin-like polypeptide aggregation drives the fusing enzyme partner out of solution due to dehydration at the surface of the ELP tag while maintaining enzyme structure and activity. In comparison with previously discussed strategies, modification of GOx's molecular structure is a versatile and perhaps, ultimately, most promising method to improve GOx stability without affecting sensor performance. Engineered GOx may display enhanced *in vitro* stability that might counterbalance the damaging effects of H_2O_2 and LMWM *in vivo* while at the same time incorporate molecular components that allow enhanced protein immobilization to result in improved functional lifetimes for the enzyme and thus implanted sensor devices.

Conclusions

This review discusses GOx degradation in the presence of H_2O_2, LMWM, and GAX and describes several techniques to overcome these effects. These techniques include silica sol-gel encapsulation (to minimize H_2O_2 and GAX effects) and molecular cloning (to minimize H_2O_2 and LMWM effects). Previous studies have demonstrated that (1) silica encapsulation carries less risk of disrupting GOx functionality than techniques utilizing chemical modification of the GOx surface, (2) polymer dopants and ormosils improve stability by increasing the enzyme's access to molecular waters and substrate, (3) aging of silica prevents enzyme leaching and preserves sensor stability, and (4) molecular engineering of GOx is a versatile and promising method to improve GOx stability without affecting sensor capabilities. Further studies are necessary in combining these approaches to eliminate the effects of H_2O_2, LMWM, and GAX. In particular, molecularly engineered GOx combined with silica-based encapsulation stands out as a promising approach for designing long-term *in vivo* sensor systems.

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