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Stability and Stabilization of Enzyme Biosensors: The Key to Successful Application and Commercialization

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Abstract

Fifty-five years have passed and more than 100,000 articles have been published since the first report of an electrochemical enzyme biosensor. However, very few biosensors have reached practical application and commercialization. The bulk of the research effort has been on increasing sensitivity and selectivity. In contrast, the number of publications dealing with stability or stabilization of enzyme biosensors is very small. Here, we critically review enzyme stabilization strategies as well as the progress that has been done in the past 20 years with respect to enzyme biosensor stabilization. Glucose oxidase, lactate oxidase, alcohol oxidase, and xanthine oxidase are the focus of this review because of their potential applications in food. The inconsistency in reporting biosensor stability was identified as a critical hurdle to research progress in this area. Fundamental questions that remain unanswered are outlined.

INTRODUCTION

Biosensors are devices that "incorporate a biologically active element in intimate contact with an appropriate transduction element for the purpose of detecting (reversibly and selectively) the concentration or activity of chemical species in any type of sample" (Arnold & Meyerhoff 1988, p. 149). Biological elements include enzymes, antibodies, nucleic acids, organelles, and microorganisms. There are electrochemical, optical, acoustic, and calorimetric transduction elements. Almost 100,000 articles have been written on biosensors since 1985. Since then, the number of publications has increased following a quadratic function with respect to time, as indicated in the trend line in **Figure 1**. The first biosensor developed in the 1960s was an enzyme biosensor (Clark & Lyons 1962). Approximately 24% of all biosensors reported used electrochemical transducers and approximately 24% of the biosensor reports, i.e., almost 23,000 articles, documented the use of enzymes as biological elements. Some of the advantages of using enzymes in biosensors are their high catalytic activity, which results in effective signal amplification, their high substrate selectivity, and their ability to operate at moderate potentials (Borgmann et al. 2012). A detailed description of the principles of electrochemical biosensors can be found elsewhere (Reyes-De-Corcuera 2015). Hundreds of papers on amperometric enzyme biosensors have been published. Glucose biosensors are the most studied because of their applications in glucose monitoring for diabetes management. Detection of contaminants, monitoring of raw materials, and verification of product contents and freshness are the areas of potential biosensor applications in the food industry (Anand et al. 2013). Applications of enzyme biosensors in foods and food processing have been frequently proposed since the 1980s and include detection of glucose, lactate, and alcohol in beverages (Goriushkina et al. 2009) as well as of lactose (Campuzano et al. 2004), putrescine (Boka et al. 2012), and xanthine (Dolmaci et al. 2012). In contrast, relative to the total number of publications on biosensors, only 3.4% of the articles published since 1985 (3,312 articles) refer to food, as indicated in **Figure 1**. Although biosensors are valuable analytical tools, their use within the food industry remains limited. Few biosensors have been commercialized specifically for use within the food industry (approximately 1% of food analysis methods, including all types of biosensors) (**Figure 2*a***), 12% of the methods of pathogen detection in foods (**Figure 2*b***) and 7% of the methods used for antibiotic detection in foods (**Figure 2*c***). Comprehensive reviews

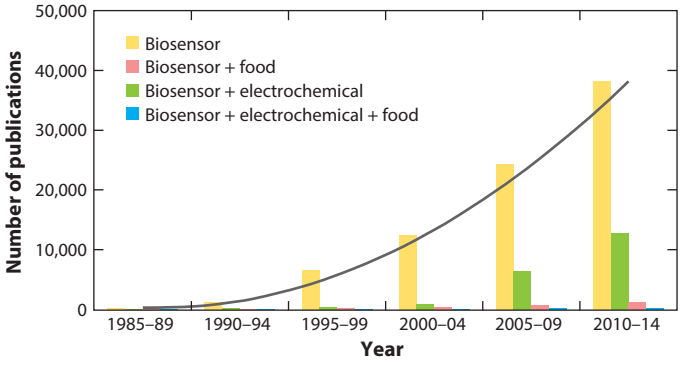

Figure 1

Number of published articles on biosensors between 1985 and 2014 as reported by ISI Web of Science accessed on May 26, 2016 using the keywords "biosensor," "biosensor + food," "biosensor + electrochemical," and "biosensor + electrochemical + food."

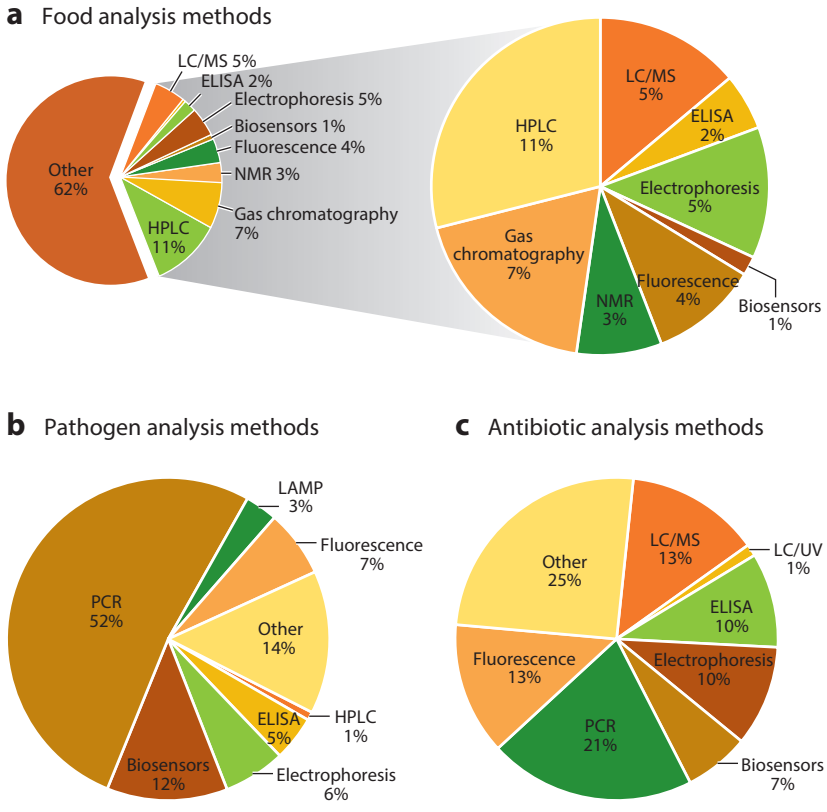

Figure 2

Analytical methods used for the analysis of (*a*) foods, (*b*) pathogens, and (*c*) antibiotics. These charts are based on the number of articles published between 2007 and 2017 as indicated by searches in the Web of Science accessed on September 25, 2017, using "only articles" for the type of document. Keywords were "food analysis" under the Food Science and Technology category; "pathogen detection" + "food"; and "antibiotic detection" + "food" for panels *a*, *b*, and *c*, respectively. Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LAMP, loop-mediated isothermal amplification; LC, liquid chromatography; MS, mass spectroscopy; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; UV, ultraviolet.

of enzyme biosensors for food have been published (Monošík et al. 2012c, Palleschi & Cubadda 2001); however, like most of the literature, they barely document the operational life of the reviewed biosensors. Of almost 23,000 articles reporting enzyme biosensors, only 13.6% report on the stability of the biosensor and only 1% focus on enzyme stabilization as suggested by the use of the keywords "stability" or "stabilization" in addition to "enzyme biosensors." In most cases, operational life is reported as number of assays (Akyilmaz & Yorganci 2007, Yilmaz et al. 2012) or after storage under refrigerated conditions (~4°C) (Chen et al. 2012, Villalonga et al. 2011, Yilmaz et al. 2012, Zhang et al. 2011). Very few publications have provided kinetic information on the rate of loss of biosensor sensitivity, which is directly related to the immobilized enzyme activity (Shkotova et al. 2006). The issue of sensor stability, in particular enzyme stability, is not new and was thoroughly discussed almost two decades ago (Gibson 1999). Gibson defined stability and rightfully differentiated shelf stability from operational stability. The review also outlined the

mechanisms of enzyme inactivation and the methods used to stabilize enzymes as the foundation of a "rational stabilisation design" (Gibson 1999) that would allow the production of practical commercial biosensors. Indeed, most commercial biosensors are single shot; i.e., instruments that couple disposable enzyme-sensing elements to a transducer. These disposable sensing elements have a long shelf life but are not suitable for continuous monitoring of the analyte of interest. Gibson's group at Leeds University created Applied Enzyme Technology Ltd. as perhaps the first spin-off company devoted to the stabilization of enzymes and partnered with Gwent Electronic Materials Ltd. One of the best-known enzyme biosensor companies is Yellow Springs Instruments (YSI), now a Xylem Inc. brand. YSI developed several enzyme membranes that can be used for multiple analyses. The stability of these membranes, depending on the enzyme, lasts from days to a few weeks. Several other enzyme biosensor companies have been created and many have disappeared. Despite the fact that in the research community the effort toward enzyme biosensor stabilization is small relative to other interests such as nanostructuring for third generation biosensors (i.e., biosensors with direct electron transfer between the enzyme and the electrode), there is already an important body of literature on the topic that deserves to be reviewed. Here, we first give an overview of the main strategies for enzyme stabilization. We then review biosensor publications between 1997 and 2017 that report the stability and/or the stabilization of electrochemical enzyme biosensors for the quantification of glucose, ethanol, lactate, and xanthine. More specifically, we focus on oxidase-based biosensors. There are several other enzymes used for biosensor design, but glucose oxidase, lactate oxidase (LOX), alcohol oxidase (AOX), and xanthine oxidase (XOX) are among the most researched and most relevant to food quality, for example, during wine or beer fermentation (Gamella et al. 2010, Goriushkina et al. 2009, Mazzei et al. 2007, Parra et al. 2006) or in other products such as fruit juices, pastes, and brines (Dimakis et al. 2002, Monošík et al. 2012c). Detailed review of these biosensors allows covering the most relevant stabilization strategies without excessive redundancy. Glucose oxidase (GOX) is the most successful enzyme for use in biosensors because of its widespread biomedical application, stability at human physiological pH and temperature, and relatively low cost (Monošík et al. 2012b). GOX is frequently used for the primary evaluation of new biosensor designs because it is easy to obtain and inexpensive, and its performance can be easily compared to the reports in the literature. Because of the disproportionately large number of GOX-based biosensors relative to the other biosensors, we reviewed in detail only the past five years. Some overlap with other reviews and references to publications before 1997 are necessary for context and to provide a broader picture, but they are kept to a minimum. Here, we attempt to elucidate the current opportunities and gaps of knowledge that would explain the disparate growth of scientific research on biosensors relative to a rather small rate of commercialization of amperometric enzyme biosensors.

ENZYME STABILIZATION

The major strategies for enzyme stabilization that have been explored for decades, not only for biosensors but also for industrial biocatalysis, are the addition of stabilizing substances such as sugars, sugar alcohols (e.g., lactitol), polyols, and polyelectrolytes [e.g., diethylaminoethyl (DEAE)-Dextran]; chemical modification of enzyme side groups (e.g., alkylation of lysine residues, iodination of tyrosine residues, and hydrophobization of carboxylates with hydrophobic amines using carbodiimide bioconjugation); and enzyme immobilization (Colacino & Crichton 1997; Fagain 1995; Gupta 1991; Klibanov 1983; Longo & Combes 1999; Martinek et al. 1977a,b; Martinek & Torchilin 1988; Torchilin et al. 1978). Genetic modification, mining for hyperthermophilic microorganisms (Daniel 1996), and, more recently, application of high hydrostatic pressure (HHP) (Eisenmenger & Reyes-De-Corcuera 2009) are becoming more widely researched.

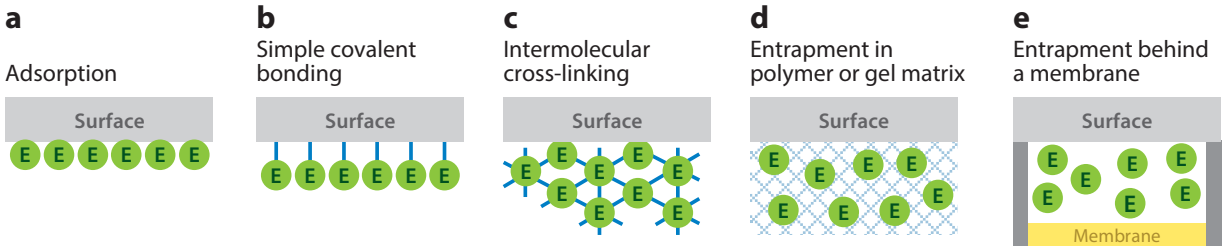

Figure 3

Schematic representation of different methods of enzyme immobilization.

ENZYME IMMOBILIZATION

The effects of immobilization on activity, stability, and even selectivity of some enzymes have been documented (Mateo et al. 2007). The most common methods of enzyme immobilization are physical adsorption, covalent tethering, cross-linking, and entrapment (see **Figure 3**). Physical adsorption (**Figure 3*a***) results from hydrophobic, hydrophilic, or ionic interactions between the protein and a solid surface. There is evidence that physical adsorption of enzymes may stabilize their activity (Zoungrana et al. 1997). However, this type of immobilization often leads to enzyme leakage, especially with changes in pH and temperature. Therefore, adsorption is frequently used to build layer-by-layer architectures in combination with other immobilization techniques such as cross-linking and/or entrapment behind a membrane. To the best of our knowledge, immobilization by adsorption alone has not resulted in practical biosensors. Enzymes have been entrapped behind or between selective membranes (**Figure 3*e***) for the fabrication of commercial biosensors. Sol-gels have been used to immobilize enzymes (**Figure 3*d***). These techniques provide an environment similar to that of the enzyme in solution. However, the properties of the sol, namely volume and pore size, change as condensation reactions continue. Once the sol is dry and stable, the pore size is so small that the enzyme reaction is limited by diffusion (Dave et al. 1994). To overcome these limitations, thin films have been tried (Wang et al. 1999). To prevent cracking and swelling of the hydrogel, organic-inorganic hybrid materials are being investigated (Guo & Dong 1997, Li et al. 1998). Several studies on enzyme entrapment in sol-gels for the development of biosensors focus on increasing sensitivity, measuring range, and decreasing response time (Liu et al. 1998, Zhu et al. 2000). Entrapment can also be achieved by chemical, electrochemical, or photo-polymerization in a monomer-enzyme solution (**Figure 3*d***). Amperometric biosensors entrapping GOX in electrochemically generated polymers have been developed (Reyes-De-Corcuera et al. 2005).

Covalent immobilization of enzymes on gold nanoparticles (AuNPs) was done for the development of amperometric enzyme biosensors for detection of glucose (Zhang et al. 2005). In our laboratory, GOX has been entrapped in nanofilms electrochemically deposited on porous platinum, producing large increases in rates of catalysis and resulting in highly sensitive enzyme biosensors (Reyes-De-Corcuera et al. 2005). The nanostructure served mainly to increase sensitivity. Increased stability of glucose was reported for GOX covalently attached to citrate-stabilized AuNPs by derivatizing the nanoparticle (NP) with carboxyl groups and coupling the enzyme-amino side groups using the carbodiimide bioconjugation method. Attachment to NPs has also been done with glutaraldehyde (GA). Enzymes have been covalently tethered to, e.g., polysaccharides, vinyl polymers, polyamides (nylon), polyalkylenes, and polyester (**Figure 3*b***). Frequently, covalent attachment of enzymes to solid supports has been done by activation of enzyme carboxylic side chains

with a carbodiimide followed by condensation with support surface amino groups. Alternatively, activation of a carboxyl-derivatized solid surface with a carbodiimide followed by condensation with amino groups of the enzyme side chains has been used to covalently attach enzymes to biosensor transducers (Divya et al. 1998, Schuhmann 1991, Wolowacz et al. 1992). Covalent tethering eliminates enzyme leakage and covalently immobilized enzymes sometimes show greater resistance to pH and temperature variations (Taylor 1996). Intermolecular cross-linking (**Figure 3*c***) has been widely used in multipoint covalent immobilization (Divya et al. 1998). Intermolecular cross-linking can result in activity loss. Because of the random mechanism and fast reaction rate of the cross-linking reaction, it is difficult to obtain reproducible sensors. However, indirect (e.g., through a dried gelatin layer) mild cross-linking with GA in combination with other immobilization techniques for biosensor development has resulted in devices with increased stability (Khan & Wernet 1997). Intramolecular cross-linking for enzyme stabilization has been demonstrated using bifunctional reagents such as GA and other dialdehydes (Fernández-Lafuente et al. 1993, 1995). Covalent bonds result from the reaction between aldehydes of the cross-linker and amines of the protein. Multipoint covalent attachment has been reported using soluble polysaccharides (Lenders & Crichton 1984) or solid agarose gel supports (Guisán 1988). Multiple covalent bonds result in reinforced quaternary and tertiary structures providing stabilization of up to 30,000-fold for a thermophilic esterase (Fernández-Lafuente et al. 1995).

ENZYME STABILIZATION AT HIGH HYDROSTATIC PRESSURE

In attempts to inactivate enzymes that are deleterious to food quality using nonthermal technologies, it was found that HHP activates and stabilizes some important endogenous food enzymes such as pectinesterase (Crelier et al. 2001), polyphenol oxidase (Anese et al. 1995), and α-chymotrypsin (Rariy et al. 1995). HHP affects weak molecular interactions, such as hydrophobic, ionic, and hydrogen bonds, in proteins. In general, the change in the structure of proteins follows the Le Châtelier principle (Hendrickx et al. 1998). The effects of HHP below 1,000–1,500 MPa on enzyme activity and stability are attributed to changes in noncovalent molecular interactions (Balny 2004). HHP produces a shift in equilibrium and may have positive, negative, or undetectable effects on enzyme catalysis (Northrop 2002). However, from a practical point of view, changes in equilibrium and kinetics last only if the enzyme does not lose its activity. A three-stage denaturation was observed for carboxypeptidase in which the carbohydrate moiety contributed to pressure stability (Dumoulin et al. 1999). We extensively reviewed the stabilization effects of HHP on 26 enzymes (Eisenmenger & Reyes-De-Corcuera 2009). The effects of HHP on the stability of most enzymes have not been characterized. The relationship between protein structure and stability under HHP remains unclear. To the best of our knowledge, of all the enzymes that have been stabilized under HHP, there is no report of retained stability upon depressurization.

GLUCOSE OXIDASE–BASED BIOSENSORS

The main application of glucose biosensors in the food industry is to determine sugar content in beverages. More than 5,000 papers have been published on GOX biosensors in the past two decades. Although this review covers only the past five years of research publications for GOX biosensors, it is pertinent to cite two 1997 studies that report that highly stable GOX can be used as a reference for stability improvement. Glucose was immobilized onto controlled-pore glass beads and stabilized by forming an enzyme-polyelectrolyte complex cross-linked with GA, which caged the enzyme and helped to give it a more rigid conformation. Adding trehalose and sorbose

to the GOX buffer solution helped GOX remain fully active for 15 min at 80°C, suggesting that this method of immobilization could be used in biosensor fabrication (Appleton et al. 1997). In the same year, GOX's stability was increased by entrapping the enzyme in a carbon paste previously used in the fabrication of reagentless renewable biosensors. The thermal stability of the sensors was measured by subjecting the biosensor to elevated temperatures of 60–80°C. Thermal stability (60°C) was compared for GOX in solution, GOX entrapped in poly-*o*-phenylenediamine (PPD), and GOX immobilized in the carbon paste. The enzyme entrapped in the carbon paste was by far the most stable and retained 95% of its original activity after 10 days of stress. After four months, the biosensor retained 85% of its original electrical current response at 60°C. After 60 days of storage in an oven at 80°C, the GOX–carbon paste electrode retained 65% of its electrical current response. Biosensors were removed from the oven and their electrical current response was tested at room temperature at selected time intervals (Wang et al. 1997).

From 2012 to 2017, twenty-five publications were found after searching the Web of Science using the keywords "glucose oxidase," "biosensor," and "stabilization." Colak et al. (2012) developed a glucose biosensor using a polypyrrole (PPy)-poly(vinyl sulfonate) composite film combined with cross-linking via GA at different concentrations for the immobilization of GOX. An electrode prepared with 30 μL of a 2.5% GA solution yielded the maximum electrical current response. The biosensor had no decrease in sensitivity after 27 assays in one day and retained 63% of its original activity after 93 days of storage in a phosphate buffer (pH 7.5) at 4°C. Dong et al. (2013) developed a GOX biosensor using $CuGeO_3$ nanowires modified onto a glassy carbon electrode. To test the operational stability, the biosensors were stored dry at 4°C and measurements were taken every three days for a month; there was little loss of activity. There have been many biosensors fabricated using carbon nanomaterials in recent years. Han et al. (2014) developed a glucose biosensor using a carbon nanosphere/sodium alginate (CNS/SA) composite matrix for GOX immobilization. This material combined the chemical stability of the carbon nanosphere with the biocompatibility and stabilizing effect of sodium alginate. Absorbance spectra of CNS/SA/GOX films at 280–345 nm deposited on quartz slides suggested that GOX maintained its native conformation in the composite material. The electrode was stored at 4°C and used intermittently with no obvious decrease in electrical current response for 28 days. After 55 days, the biosensor had retained 83% of its initial response. Miao et al. (2015) developed a glucose biosensor by electrodepositing AuNPs-polyvinylpyrrolidone-polyaniline (AuNP-PVP-PANI) nanocomposites onto a glassy carbon electrode. GOX was immobilized on the AuNP-PVP-PANI nanocomposites using Nafion as the preventive layer. Nafion has been reported to have good biocompatibility and mechanical and thermal stability, and, most importantly, it serves as a protective layer to aide in biosensor stabilization. Incorporating AuNPs into the polyaniline matrix resulted in large surface areas and enhanced electrocatalytic activity. Under dry conditions, the biosensor was stored at 4°C when not in use. After two weeks, the biosensor retained 89.9% of its initial activity. Shamsazar et al. (2016) fabricated a GOX biosensor using a modified carbon paste electrode with zinc oxide (ZnO) NPs. When GOX was adsorbed on the modified electrode surface, it performed a rapid electron transmission via the electrode, reducing GOX. Operational stability was measured intermittently for four weeks and when not in use the electrode was stored at 4°C. After the four-week period, it retained 95% of its initial electrical current response. ZnO has been employed in other ways as well for the fabrication of amperometric biosensors. Zhou et al. (2016) fabricated a glucose biosensor by immobilizing GOX via physical adsorption on ZnO nanotubes. Operational stability was measured intermittently over a 20-day period. When stored at 4°C, the biosensor retained 90.7% of its initial activity after 10 days; after 20 days, it retained 80.8% of its initial activity, which does not represent an enhancement relative to previous reports or commercially available sensors (Zhou

et al. 2016). Carbon nanotubes are commonly employed in the fabrication of biosensors. Shrestha et al. (2016) prepared a bio-nanohybrid fold of a PPy-Nafion-functionalized multiwalled carbon nanotube nanocomposite on a glassy carbon electrode. A solution of GOX in phosphate buffer and chitosan in aqueous acetic acid was then cast on the surface of the modified glass carbon electrode. The energy-dispersive spectroscopy (EDS) data confirmed that immobilizing with a cationic redox polymer such as chitosan enhances the enzyme immobilization capacity, which helps to improve the physical and chemical stability of the fabricated biosensor. The biosensor was stored in a phosphate buffer (pH 7.4) at 4°C when not in use. The performance was measured every 3 days for 45 days. Within the first 21 days, the biosensor retained almost all its initial activity, and after 45 days the electrode retained approximately 93% of its original response. A 3D nitrogen-doped carbon nanotube supported by a carbon-foam hybrid was fabricated as GOX's carrier matrix. The uniform pore distribution and high surface area of the nanostructured N-doped carbon clarified foam provide a large quantity of active sites for GOX immobilization, which improves sensitivity. After three weeks of storage at 4°C, the electrode retained 91.2% of its original sensitivity (Fan et al. 2017).

AuNPs may improve biosensor stability because of their excellent biocompatibility and large specific-surface area. German et al. (2017) fabricated a glucose biosensor using a graphite rod (GR) modified with AuNPs and GOX. PPy was applied to the surface of the GOX/AuNPs/GR electrode as a conduction polymer to improve the stability of the biosensor. The electrodes were stored at 4°C in a closed vessel hanging over a 0.05 mol/L sodium acetate buffer (pH 6.0) to maintain constant humidity. Operational stability was measured over a 34-day period, at the end of which the GOX/AuNPs/GR-modified electrode decreased to 7.91% of its initial signal and the PPy(21H)/GOX/AuNPs/GR-modified electrodes decreased to 28.4% (German et al. 2017). Although the modified electrodes had poor stability, the authors claimed that the results showed that PPy may play a role in improved stability. Sanaeifar et al. (2017) also reported that polymers aided in the stabilization of glucose biosensors. They developed a biosensor based on a Fe_3O_4 NP–polyvinyl alcohol composite. Operational stability was measured intermittently for a month, and the electrode was stored at 4°C in a phosphate buffer (pH 7) when not in use. After a month, the biosensor retained approximately 81% of its initial response. Similarly, a glucose biosensor fabricated using a composite of AuNPs, iron oxide, and bovine serum albumin (BSA) composite NPs retained 81% of its initial activity after 30 days of storage in the same phosphate buffer at 4°C, with intermittent testing every 3 days for 30 days (He et al. 2016). This biosensor had greater stability than the one fabricated by German et al. (2017), probably because of the incorporation of a BSA shell. The BSA shell was shown to help minimize conformational changes in the structure of GOX. The greatest operational stability for GOX-based biosensors in the recent literature is an amperometric glucose biosensor based on a fabricated MXene nanocomposite. Glucose biosensors were fabricated using a nanocomposite of gold and MXenes. MXenes are two-dimensional metal carbides with hydrophilic surfaces and metallic conductivity. This biosensor takes advantage of the synergistic relationship between MXene sheets and AuNPs. The Au nanostructures help GOX retain its biological activity when used in electrochemical biosensors. Operational stability was measured every day for two months, and GOX retained 93% of its initial activity when stored at 4°C in a phosphate buffer (pH 7) (Rakhi et al. 2016). However, this biosensor was not as stable as those studied by Colak et al. (2012) and Wang et al. (1997). **Table 1** summarizes the stability performance of recently reported GOX biosensors. Modification with phenyl groups by derivatization of 28 amino side groups with benzoate slightly destabilized GOX. In contrast, derivatization of 20 carboxyl side groups with aniline increases the temperature of denaturation by 8°C (Halalipour et al. 2017b). In the same study, the pseudo-first-order rate constant of inactivation of GOX was 50 times smaller at 240 MPa and 74.5°C than at atmospheric pressure and 74.5°C.

Table 1 Stability performance of selected glucose oxidase (GOX) biosensors (1997 and 2012–2017)

Analyte/Source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
GOX/*Aspergillus niger*	GOX immobilized onto controlled-pore glass	To increase thermal stability an enzyme-polyelectrolyte complex was formed that cages the enzyme and helps give the enzyme a more rigid conformation. Adding trehalose and sorbose to the GOX buffer solution helped to decrease activity loss	Retained 100% of the sensor response for 15 min at 80°C when trehalose and sorbose were added; biosensors	NS	Appleton et al. 1997
GOX/*A. niger*	GOX immobilized in the carbon paste. Immobilizing enzymes in carbon paste has been shown to aid in the fabrication of reagentless renewable biosensors	NS	Retained 85% of its original response after 4 months at 60°C. At 80°C, GOX entrapped in carbon paste retained 65% of its activity after 60 days	NS	Wang et al. 1997
GOX/*A. niger*	GOX immobilized via cross-linking with glutaraldehyde on an electrochemically polymerized polypyrrole-poly(vinyl sulfonate) film on a platinum electrode surface	NS	Biosensor used for 27 assays in one day	Additional activity assays performed for 93 days to determine storage stability. On the 93rd day, biosensor retained 63% of its original response	Colak et al. 2012
GOX/NS	GOX adsorbed on $CuGeO_3$ nanowire-modified glassy carbon electrode	NS	NS	Biosensor was stored dry at 4°C in a refrigerator when not in use. Measurements were taken every 3 days for a month; the biosensor retained almost all of its response after one month	Dong et al. 2013

(*Continued*)

Table 1 (*Continued*)

Analyte/Source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
GOX/NS	GOX immobilized on the surface of a graphene and Cu-nanomodified electrode	NS	NS	The biosensor lost approximately 4.2% and 8.6% of its original response after 10 days and 20 days, respectively	Huang et al. 2013
GOX/*A. niger* type X-S	GOX immobilized at gold nanoparticle (AuNP)-decorated graphene-carbon nanotubes	NS	NS	The biosensor stored at 4°C when not in use. Retained 94.37% of its initial response electrical current even after a 2-month storage period	Devasenathipathy et al. 2015
GOX/*A. niger* type II	GOX AuNP–polyvinylpyrrolidone–polyaniline was immobilized on the nanocomposites using Nafion as the preventing layer	Nafion is a protective layer that helps retain the stability of biosensors	NS	Under dry conditions, biosensor stored at 4°C when not in use. After 2 weeks, biosensor retained 89.9% of its initial response	Miao et al. 2015
GOX/*A. niger*	GOX immobilized on a carbon nanosphere/sodium alginate composite matrix. This material combines the benefits of sodium alginate and the carbon nanosphere	A carbon nanosphere is a carbon nanomaterial that has good chemical stability. Alginate biocompatibility and film forming ability favored stability	NS	Biosensor stored at 4°C for 28 days and measured intermittently with no obvious decrease in electrical current response. After 55 days, biosensor retained 83% of its initial response	Han et al. 2014
GOX/*A. niger* type II	In the proper concentration of the Co(II)-based metallo-supramolecular polymer (~0.7 mM), effective immobilization carried out by coimmobilizing GOX with the polymer	NS	NS	Measurements carried out once a week for 4 weeks. After 4 weeks stored at 4°C, biosensor sensitivity retained 98.7% of its initial response	Hsu et al. 2016

(*Continued*)

Table 1 (*Continued*)

Analyte/Source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
GOX/*A. niger*	GOX immobilized on Nafion-solubilized Au/MXene nanocomposite over a glassy carbon electrode	An MXene hydrophilic surface gives the sensor greater stability in aqueous environments	NS	Stored at 4°C in phosphate buffer when not in use. Operational stability measured every day for 2 months; retained 93% of its initial response	Rakhi et al. 2016
GOX/*A. niger* type X-S	GOX immobilized with chitosan	The energy-dispersive spectroscopy data confirmed that immobilizing with a cationic redox polymer such as chitosan enhances the enzyme immobilization capacity, which helps improve the physical and chemical stability of the fabricated biosensor	NS	Stored in a phosphate buffer at 4°C when not in use. Stability measured every 3 days for 45 days. After 45 days, electrode retained 93% of its original response	Shrestha et al. 2016
GOX/*A. niger*	Regulated etching temperature is used to alter the surface morphology of the zinc oxide (ZnO) nanotubes for immobilization of GOX to enhance the sensor	ZnO nanotubes have been shown to enhance biosensor performance because of their large surface area and hollow structure	NS	Stability measured intermittently over a 20-day period, stored at 4°C. Biosensor retained 90.7% of its initial response after 10 days. After 20 days, it retained 80.8% of its initial response	Zhou et al. 2016
GOX/NS	GOX adsorbed on a carbon paste electrode surface modified with zinc oxide nanoparticles	ZnO nanoparticles are believed to play an important role in the stabilization process because of their increased surface to volume ratio	NS	Biosensor stored at 4°C when not in use; stability measured intermittently for 4 weeks. After 4-week period, retained 95% of its initial electrical current response	Shamsazar et al. 2016

(*Continued*)

Table 1 (*Continued*)

Analyte/Source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
GOX/NS	A bovine serum albumin (BSA) shell is shown to help minimize conformational changes in the structure of GOX, making it a promising substrate for enzyme immobilization in biosensor design	Integrating AuNPs and Fe_3O_4 with the BSA improved electron transfer and biocompatibility	NS	Biosensor stored in phosphate buffer at 4°C when not in use; stability measured every 3 days for 30 days. After 30 days, biosensor retained 81% of its initial response	He et al. 2016
GOX/*A. niger*	NS	Uniform pore distribution and high surface area of the N-doped carbon nanotube@carbon foam provides large quantity of active sites for GOX immobilization, which improves sensing	NS	Stability measured after 3 weeks of storage at 4°C; biosensor retained 91.2% of its original response	Fan et al. 2017
GOX/NS	NS	3D graphene was reported to be effective biosensor support because of significant sensitivity and a low detection limit	NS	Stored dry; activity measured weekly for a month. After one month, the biosensor retained 76.9% of its original response	Mansouri et al. 2017
GOX/*A. niger* type VII-S	GOX immobilized on the nanocomposite by physical adsorption and drop cast on a tin electrode	Polymers are often used to help stabilize enzymes in biosensor design	NS	Biosensor stored at 4°C in phosphate buffer when not in use; stability measured intermittently for a month. After a month, biosensor retained approximately 81% of its initial response	Sanaeifar et al. 2017
GOX/*A. niger* type TX-SG7141–50 KU	NS	GOX encapsulated in the liposome, which may have improved stability	NS	After 1-week dry storage at room temperature, biosensor retained 75% of its original response	Yu et al. 2017

(*Continued*)

Table 1 (*Continued*)

Analyte/Source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
GOX/NS	Alginate hydrogel was used as the immobilization matrix for GOX and CuO nanoparticles	Combination of the alginate hydrogel with covalently cross-linked GOX and copper oxide may have provided a good catalytic environment to aid in stabilization	NS	Stored at 4°C when not in use. After 15 days, electrode retained 78% of its original response	Buk et al. 2017
GOX/NS	TiO_2 hollow nanofibers (HNF-TiO_2) were prepared by the sol-gel method to use for GOX immobilization	TiO_2 hollow nanofibers and GOX have strong interactions that help to enhance stability	In O_2-free conditions, biosensor retained 97.5% of its response after 2 weeks. In O_2-saturated conditions, biosensor retained 95.7% of its response after 2 weeks	Stored at 4°C for 2 weeks	Guo et al. 2017
GOX/*A. niger* type X-S	Inclusion of the outer layer, which helped to retain GOX	NS	NS	Stored for 2 months without significant sensitivity loss (6.3% relative standard deviation)	Ayenimo & Adeloju 2017
GOX/*A. niger*	Reduced graphene oxide offers an ideal surface for the fabrication of large levels of AuNPs for enzyme immobilization	NS	NS	Stored at 4°C when not in use. After 2 weeks, biosensor retained 90% of its original response	Mazar et al. 2017

Abbreviation: NS, not stated.

LACTATE OXIDASE–BASED BIOSENSORS

The main application for lactate biosensors in the food industry is for monitoring malolactic fermentations. The number of publications (a little over 240) using the keywords "lactate oxidase" and "biosensor" in 1997–2007 was almost the same as in 2008–2017. When the keyword "stability" was added to "lactate oxidase" and "biosensor," the number of publications in 1997–2007 was 64 and increased to 82 in 2008–2017. In contrast, when adding the keyword "stabilization," the total number of publications decreased from 9 to 3 in those two decades. The storage stability of LOX increased with the addition of DEAE-dextran/lactitol. Ink-printed biosensors were fabricated using LOX with or without DEAE-dextran/lactitol and Gafquat, a cationic form of a copolymer of vinylpyrrolidone and dimethylaminoethylmethacrylate quarternized with diethyl sulfate. At the beginning of a 172-day experiment at 25°C, the mean electrical current response was greater than the initial electrical current response and did not decrease below 90% of the initial electrical current for the entire duration of the experiment (Hart et al. 1996). Khan & Wernet (1997) developed a three-layer LOX biosensor using several approaches for each layer. The first layer was a polyanion-doped PPy electrodeposited on stainless steel. A platinum black layer was then deposited by the heat-press method, and a LOX layer was cast by adding a LOX solution and allowing it to dry. Finally, a gelatin layer was cast and cross-linked with GA. This study by Khan & Wernet (1997) is one of the very few articles that documents the operational life and shelf life of a biosensor. Unfortunately, Khan & Wernet (1997) did not document electrode-to-electrode reproducibility. Even though the time course data of inactivation were presented, there was no attempt to characterize its kinetics. Khan & Wernet (1997) pointed out that over the previous two decades very little effort had been put into producing stable biosensors, hence their enthusiasm for the subject. Their stabilized biosensor retained 50% of its activity after almost 60 days of continuous electrode polarization at 37°C compared to fewer than 15 days for the control biosensor. The stabilization effect was attributed to indirect and mild cross-linking with GA on the dried enzyme and gelatin layers. In the same year, Wang et al. (1997) reported stabilization of several enzyme biosensors using carbon paste as an immobilization matrix, attributing the stabilization to the hydrophobic nature of the paste. The residual response of LOX electrodes exposed to 60°C for 1 and 5 days was 40% and 15%, respectively, but the loss of the electrochemical response of a control electrode fabricated using a different immobilization method or the loss of residual activity of LOX in solution incubated under the same conditions was not reported. Also, Wang et al. (1997) did not report the stability of their LOX biosensors at room or physiological temperature, making it difficult to compare their study to other LOX electrode studies in the literature. Immobilization of poly(1-vinylimidazole)-LOX complexes in tetramethyl orthosilicate gel decreased the LOX activity but increased its half-life 150-fold. Such stabilization was attributed to the charge balancing of the polyanionic enzyme with a polycation and to entrapment in the gel (Chen et al. 1998). Even though this study and others by Chen's group dealt with dispersed enzymes rather than biosensors, it inspired the work of Cox et al. (2003), who did a similar study and claimed its potential use in biosensors. However, they did not couple the sol-gel to a transducer and hence did not fabricate a biosensor. Serra et al. (1999) fabricated graphite-Teflon-LOX-POD biosensors with operational stability of up to 55 days of intermittent use (21 tests) but required surface regeneration (polishing) approximately every 7–10 days, and the electrode was kept at 4°C when not in use. Storage stability under refrigeration lasted six months. This study, unlike most, documented sensor-to-sensor reproducibility, providing strong evidence that the procedure is reproducible. Unfortunately, Serra et al. (1999) did not test their electrodes under continuous operation. It is unclear whether these biosensors were stored in solution or dry when not in use. Because the enzyme is adsorbed onto the immobilization matrix only, one can speculate that storage in solution or continuous usage

may lead to enzyme leaching and decreased operational life. Immobilization of LOX stabilized with 1% DEAE-dextran and 5% lactitol by UV-induced polymerization of metacrylates resulted in lactate biosensors that showed almost no decrease in response for 96 h, presumably at room temperature with a standard deviation of less than 6% in subsequent measurements (Schumacher et al. 1999). This study, however, did not report the loss of activity or the detachment of the immobilization matrix beyond 200 h (~8 days). Adsorption of a LOX-DEAE-dextran mixture in porous carbon electrodes resulted in electrodes with a half-life of at least 240 h. A control using a polarized electrode with only LOX adsorbed into the electrode was not reported. Therefore, it was impossible to elucidate the extent of stabilization of the adsorbed enzyme and whether the observed stability was due to stabilization of the enzyme by the polyelectrolyte or by prevention of the enzyme leaching into the bulk of the solution (Gavalas & Chaniotakis 2000). The same group expanded their research to other enzymes and used polyethyleneimine as an alternative polyelectrolyte to DEAE-dextran, but no improvement over DEAE was reported. Mutants of LOX using random mutagenesis and selection for thermostability produced biosensors with a half-life of more than 40 min after incubating at 65°C compared to fewer than 10 min for the wild type (Minagawa et al. 1998). Random and site-directed mutagenesis stabilized LOX from *Aerococcus viridans* with 17% and 12% residual activity, respectively, after heating for 10 min at 70°C. However, random and site-directed mutations decreased the enzyme activity by 59% and 94%, respectively (Kaneko et al. 2005). None of the mutants were used to fabricate biosensors, but this approach was furthered by the same group using directed evolution to produce a mutant with six amino acid replacements that retained 71% of its initial activity after 20-min incubation at 70°C. The half-life of the mutant was 36 times longer than that of the wild type (Minagawa et al. 2007). Covalent immobilization of LOX, malate dehydrogenase, and horseradish peroxidase (HRP) on a nylon 6.6 membrane functionalized with carboxyl groups and via the addition of a polyazetidine prepolymer produced biosensors that allowed 200–300 measurements showing loss of 30% activity. Continuous use was not determined because samples required dilution (Mazzei et al. 2007). Goriushkina et al. (2009) immobilized LOX in electrochemically generated poly(3,4-ethylenedioxythiophene) (PEDT). The stability of these biosensors lasted only four days, which contrasts with the extended operational life of GOX and AOX biosensors immobilized by cross-linking with GA that were reported in the same study. The rationale for selecting GA was that PEDT was not selective to interfering species in wine. Yet Goriushkina et al. (2009) contradicted themselves, stating that PEDT has good selectivity when selecting it for LOX biosensors. Gamella et al. (2010) co-immobilized LOX and HRP along with mediator tetrathiafuvalene on a 3-mercaptopropionic acid self-assembled monolayer and reported 91% retention of the stability after five days, but no information was provided regarding longer usage. The incorporation of the mediator in the immobilization matrix obviated the need for its addition ex situ. These authors contrasted the operational stability of their sensor to nine other reports published between 1998 and 2008. However, the stability in other studies was reported in terms of storage stability or number of tests, not under continuous exposure to the substrate of interest and constant electrode polarization, making an accurate comparison impossible. Monošík et al. (2012a) proposed a lactate sensor based on sandwiching LOX and HRP between chitosan layers cast on multiwall carbon nanotubes. The shelf or operational life of such biosensors did not improve compared with previous reports. Similarly, Shimomura et al. (2012) proposed a different immobilization matrix based on deposition of LOX previously absorbed in mesoporous silica dispersed in a polymer of denatured polyvinyl alcohol. The storage stability of the sensor was longer than previously reported. However, it was shorter than that of Monošík et al. (2012a), which was published at about the same time. Shimomura et al. (2012) did not report biosensor operational life. Finally, a

LOX-HRP lactate biosensor with 40-day operational life was proposed by Gimenez-Gomez et al. (2016). The stability of the conducting polymer is due to its operation at rather low overpotentials (0.075 mV versus Ag/AgCl), which prevents the overoxidation and degradation of PPy. However, the mediator ferrocyanide must be added; thus, this biosensor can only be used ex situ. The authors compared the stability of their PPy-based biosensor to six other reports that used other immobilization matrices and/or mediators to highlight the better stability (42 days of discontinuous measurements) of their sensor. However, biosensors reported by Hart et al. (1996) or Serra et al. (1999) appear to have a longer operational life. Gimenez-Gomez et al. (2016) did not explain the nature of the stabilization effect on the enzyme and only reported the change in sensitivity of one single electrode. Therefore, it is not possible to assess the reproducibility of the operational stability. In summary, over the past 20 years, the documentation of the stabilization of LOX biosensors has not improved much, and the stability of recently reported sensors does not appear to have improved either. **Table 2** summarizes the stability performance of LOX biosensors reported recently.

ALCOHOL OXIDASE–BASED BIOSENSORS

The main application of AOX biosensors is in the beverage industry because alcohol quantification is needed for quality control of the fermentation process. Fewer publications resulting from searches using the keywords "alcohol oxidase" and "biosensor" were found in 1997–2007 (135) than in 2008–2017 (193). The number of studies using the keyword "stability" in addition to "biosensor" and "alcohol oxidase" was higher (67) in 2008–2017 than in 1997–2007 (52). However, the number of publications using the keyword "stabilization" in addition to "biosensor" and "alcohol oxidase" was 6 in 1997–2007 and 5 in 2008–2017. Vijayakumara et al. (1996) developed an amperometric alcohol biosensor consisting of AOX and HRP in a carbon paste matrix. The two electrode formulations with the best operational and storage stability were (*a*) AOX from *Candida boidinii* + HRP + polyethylenimine (CB+HRP+PEI) and (*b*) AOX from *Pichia pastoris* + HRP wired with osmium containing the three-dimensional redox hydrogel poly[1-vinyl imidazole osmium (4,4′-dimethyl-bipyridine)]$2Cl^{+/2+}$ (PP+HRP-Os). The first electrode (CB+HRP+PEI) had an operational stability of 13% loss in response over 8 h at a sample throughput of 30 injections per hour. However, the second electrode (PP+HRP-Os) had an operational stability of 10% loss in response when continuously operated at room temperature for 9 h at a rate of 30 injections per hour. The storage stability of both electrodes depended on storage conditions; if stored dry, an 80% drop in sensitivity was observed, but if kept on a phosphate buffer, the drop was 35%. After 6 days, 45% and 85% of the original response was retained by electrodes CB+HRP+PEI and PP+HRP-Os, respectively. The improved stability on the PP+HRP-Os electrode was attributed to the effect of the redox polymer–cross-linker network. Wang et al. (1997) also improved the thermostability of AOX among other enzymes by immobilizing the enzymes within carbon paste electrodes. The AOX-containing carbon paste electrode resulted in retention of 70% and 30% of the initial response after 1 and 5 days, respectively, at 60°C. Thermostability was attributed to the conformational rigidity of the biocatalyst within the highly hydrophobic pasting liquid. Disposable biosensors using screen-printing technology with AOX immobilized by poly(carbamoyl)sulfonate (PCS) hydrogel were developed. In this study, performance and stability of AOX from *C. boidinii*, *Pichia pastoris*, or *Hansenula* sp. were studied. The electrodes fabricated with AOX from *Hansenula* sp. had the longest storage life (Patel et al. 2001). Smutok et al. (2006) used a thermotolerant methylotrophic yeast *Hansenula polymorpha* to develop a biosensor that retained 50% of the initial electrical current response after 14 days of storage at 4°C. The authors attributed the stability

Table 2 Stability performance of selected lactate oxidase (LOX) biosensors (1997–2017)

Enzyme/source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
LOX/*Pediococcus* sp.	Adsorption, gelatin casting, and cross-linking with glutaraldehyde	Attributed to mild cross-linking with glutaraldehyde	Retained more than 50% response relative to initial response for 55 days of continuous polarization at 37°C	Retained sensitivity after 7 months of storage at room temperature and for at least 18 months when stored at −18°C	Khan & Wernet 1997
LOX/*Pediococcus* sp.	Adsorption/entrapment by mixing with carbon/rhodium/mineral oil paste	Attributed to the hydrophobic properties of the mineral oil that would increase enzyme rigidity	NS	Retained 40% response relative to initial response after 1 day of storage at 60°C	Wang et al. 1997
LOX/NS/HRP	Adsorption/entrapment by mixing with enzymes/graphite and Teflon	NS	Retained sensitivity for up to 55 days of intermittent use (21 tests) but required surface regeneration (polishing) every 7–10 d	Reported as stable after 6 months of storage at 4°C	Serra et al. 1999
LOX/*Pediococcus* sp. HRP	Entrapment in UV-induced polymerization of metacrylates	Multiple effects hypothesized but no attribution	Sensor response did not decrease for 96 h after measurements every 2 h in murine hybridoma cells. No indication of how electrodes were stored between measurements was provided	Retained 100% of response after 200 h of storage at 4°C	Schumacher et al. 1999
LOX/*Pediococcus* sp.	Adsorption of LOX/diethylaminoethyl (DEAE)-dextran polyelectrolyte into porous carbon electrodes	Attributed to DEAE-dextran	Retained 56% of initial response after 240 h of continuous polarization in a flow injection system, presumably at room temperature	Retained 100% enzyme activity after 5 months, lyophilized	Gavalas & Chaniotakis 2000
LOX/*Pediococcus* sp. Lactate dehydrogenase HRP	Covalent co-immobilization of LOX and HRP onto functionalized Nylon 6.6 membranes using polyazetidine prepolymer	NS	Retained 70% of initial response after 200–300 determinations with electrodes kept at room temperature in buffer	NS	Mazzei et al. 2007

(*Continued*)

Table 2 (*Continued*)

Enzyme/source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
LOX/*Pediococcus* sp.	Entrapment by electrochemical polymerization in poly(3,4-ethylenedioxythiophene)	NS	Retained 30% of initial response after 4 days using continuous measurement	Retained 70% of sensor response after 3 h of continuous work, and no additional drop in the succeeding 5 h. 30% sensor response was reported after 4 days. Intermittent use with dry storage of the sensor at 4°C between measurements	Goriushkina et al. 2009
LOX/*Pediococcus* sp.	Self-assembled monolayer coimmobilized with HRP along mediator tetrathiafuvalene on 3-mercaptopropionic acid (MPA), entrapment behind a dialysis membrane	Attributed to MPA, but no details about the stabilization mechanism were provided	NS	Retained 91% of the sensor response after 5 days	Gamella et al. 2010
LOX/NS	Casting and drying chitosan and LOX and HRP layers to produce a sandwiched catalytic layer on multiwalled carbon nanotubes	NS	Retained 100% of the initial sensor response after 60 discrete measurements or 10 h when sensors were stored at room temperature for up to 12 h	Retained 90% of the sensor response after 15 months; sensors stored dry	Monošík et al. 2012
LOX/NS	Immobilization by deposition of LOX previously absorbed in mesoporous silica dispersed in a polymer of denatured polyvinyl alcohol	Attributed to the absorption and interaction of the enzyme in the mesoporous silica	NS	Retained 100% of the sensor response for more than 9 months of dry storage at 4°C	Shimomura et al. 2012
LOX/HRP	Entrapment in polypyrrole by electropolymerization at 850 mV versus Ag/AgCl	NS	NS	Retained sensitivity after 42 days of intermittent operation. Biosensor stored at 4°C and used approximately every other day	Gimenez-Gomez et al. 2016

Abbreviations: HRP, horseradish peroxidase; NS, not stated.

of the biosensor to the generic "sandwich" set up using anodic and cathodic electrodeposition paints.

GA cross-linking with BSA is a versatile immobilization technique. Carelli et al. (2006) used overoxidized PPy (PPy_{ox}) and PPD as anti-interferent perm-selective films in a gold electrode and GA cross-linking to immobilize AOX. Authors concluded that the use of a gold instead of platinum electrode prevented fouling problems and the use of the PPy_{ox} and PPD films improved response rate and prevented interference from other metabolites. Electrodes had no sensitivity loss after 3 h and lost 5% after 6 h of continuous operation in a flow injection system. Similarly, Barsan & Brett (2008) used poly(neutral red) (PNR) as a redox mediator electropolymerized on carbon film electrodes combined with GA cross-linking. The use of PNR increased sensitivity by a factor of five. In addition, the replacement of O_2 by PNR overcame the disadvantage of electrochemical biosensors having difficulty controlling the O_2 partial pressure. Kamanin et al. (2015) immobilized multiple enzymes, including AOX, using GA cross-linking and modified screen-printed electrodes to develop a biosensor array for monitoring of fermentation. The long-term stability of the AOX biosensor was three days with more than 25% response relative to newly prepared sensors. Wen et al. (2007) developed an alcohol biosensor consisting of an AOX/chitosan-immobilized eggshell membrane. The biosensor retained 86.6% of its original electrical current response after three months of storage in phosphate buffer (pH 7.4) at 4°C, with testing in 0.5-mM ethanol every three days. The operational life was determined over the course of 36 determinations within 10 h. No loss of sensitivity was detected within 8 h and 20 measurements. The stabilization was attributed to the biocompatibility of chitosan and the eggshell membrane.

AuNPs have also been used to develop an amperometric alcohol biosensor. The AuNPs-AOX assembly was stabilized on a glassy carbon electrode (GCE) by a chitosan-Nafion mixture. Chinnadayala et al. (2015) explained that the improved stability is due to the amplified activity and high loading of AOX obtained by its conjugation with activator AuNPs and the favorable conformation to AOX provided by the PANI film involved in the process. These electrodes have an operational life of 5 h with less than 7% loss of sensitivity and a storage life of approximately 93% response retention after five weeks of storage at 4°C in phosphate buffer (pH 7.5). In the supplementary material of their article, Chinnadayyala et al. (2015) also compared their biosensor to others, including some studies that we cite here, but they did not cite Wen et al. (2007), who reported longer storage times. Ling & Heng (2010) developed a potentiometric AOX-based formaldehyde biosensor fabricated by immobilizing AOX on acryloxysuccinimide-modified acrylic microspheres. The long-term stability of 48 days at 4°C with a decrease in sensitivity of 20% was attributed to the immobilization matrix. Fang et al. (2016) developed a bienzyme electrochemical biosensor to detect methyl salicylate, a volatile organic compound released by pathogen-infected plants. AOX and HRP were co-immobilized on a carbon nanotube matrix. The biosensor kept 79% of initial electrical current after 10 days of storage at 4°C. More recently, Farina et al. (2017) developed an ethanol amperometric biosensor integrated with a wireless telemetry system to monitor fermentation processes. AOX was immobilized by electropolymerization in a PPD membrane. The biosensor showed good agreement ($r^2 = 0.994$ to 0.997) with the validation method [near-infrared spectroscopy (NIR)], but operational and storage life were not evaluated. Similarly, Mohan et al. (2017) developed a minimally invasive microneedle sensor array for continuous monitoring of alcohol that could be attached to the fingertip. In this case, AOX was immobilized onto a chitosan and Nafion layer that was then attached to a pyramidal microneedle structure. The reproducibility of that sensor was determined as the RSD of 10 successive measurements over 100 min (2.85%), but no evaluation of operational or storage life was performed. From this review, Wen et al.'s (2007) AOX biosensor appeared to be the most stable among those reported in the past 20 years.

However, continuous operation was reported for only 10 h. **Table 3** summarizes the stability performance of recently reported AOX biosensors.

XANTHINE OXIDASE–BASED BIOSENSORS

The main application for xanthine biosensors in the food industry is for monitoring fish freshness. Upon the death of a fish, adenosine triphosphate (ATP) degrades to eventually produce xanthine, which can then be detected by an XOX biosensor (Ghosh et al. 1998). The issue of the stability of XOX is not new. The loss of activity has been attributed to the presence of copper, or hydrogen peroxide, as well as thermal denaturation. The addition of sodium salicylate inhibited inactivation by metals (Bergel & Bray 1959). One of the first reports of an XOX electrode reported an operational stability of 10 days with measurements on days 1, 2, 4, 7, 10, and 11 (Ianniello et al. 1982). A search using the keywords "xanthine oxidase biosensor" and "stabilization" returned five articles, and a search using the keywords "xanthine oxidase biosensor" and "stability" returned 45 papers. The emphasis on stability and stabilization decreased in the past decade. In their study of screen-printed electrodes, Schumacher et al. (1999) also fabricated an XOX biosensor, but the operational life was not clearly assessed. Nakatani et al. (2005) immobilized XOX via covalent cross-linking with GA and BSA cast on a Nafion-coated platinum electrode. The operational life was 10 days without loss of sensitivity. A biosensor with 93% response retention after 21 days resulted from supramolecular immobilization of carboxymethylcellulose-β-cyclodextrin-modified XOX adsorbed onto one adamantyl-derivatized gold electrode. The stabilization effect was attributed to the surface modification of the protein with cyclodextrin (Villalonga et al. 2007). Zhang et al. (2014) reported that the inactivation of XOX from *Arthrobacter* M3 is due to the exposure of hydrophobic residues and that the stabilizing effects of trehalose and betaine are due to preferential exclusion and prevention of aggregation, respectively. Behera et al. (2016) immobilized XOX and graphene-platinum NPs on glassy carbon electrodes by drop-casting in Nafion solutions. The stability of the transducer platform toward hydrogen peroxide was studied. However, the stability of the immobilized XOX was not determined. Hence, the authors' conclusion regarding the practicality of their electrodes was not fully supported by experimental data. Mesostructured electrodes prepared by electrodeposition or electrografting of mesoporous silica nanospheres onto epoxy-functionalized glassy carbon that then adsorbed XOX and cross-linked with 3-glycidyloxypropyl trimethoxysilane retained 80% response after 21 days of storage at 4°C (Saadaoui et al. 2016). Narang et al. (2017) reported a 40% loss of sensor response after 60 days for XOX adsorbed onto a matrix of titanium oxide NPs adsorbed onto multiwalled carbon nanotubes attached to gold electrodes. Electrochemical impedance spectroscopy measurements suggested that the loss of electrode sensitivity was due to enzyme activity loss. Fish-freshness test paper is a paper onto which XOX and 2.6-dicholophenolindophenol are immobilized to form a colorimetric test that correlates xanthine concentration to fish freshness. In an effort to extend the storage life of fish freshness paper, XOX was freeze-dried using combinations of several stabilizing agents, including sucrose, trehalose, BSA, and dextran. A mixture of sucrose, dextran, and BSA was the most effective in stabilizing the enzyme, as indicated by storage tests at 25°C to 60°C (Srirangsan et al. 2010). GOX was modified by attaching phenyl groups to 12–17 amino side groups with benzoate or to 8–14 carboxyl side groups with aniline; in both cases, carbodiimide-mediated coupling was used. Unlike GOX, such modification did not affect the stability of XOX (Halalipour et al. 2017a). In the same study, HHP-stabilized XOX decreased the pseudo–first order of inactivation rate constant by a factor of 9.5. **Table 4** summarizes the stability performance of recently reported XOX biosensors.

Table 3 Stability performance of selected alcohol oxidase (AOX) biosensors (1996–2017)

Enzyme/source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
AOX/*Candida boidinii*, *Pichia pastoris*, or *Hansenula polymorpha* + horseradish peroxidase (HRP)	Carbon paste matrix on carbon paste in a syringe with a silver thread	Stability attributed to beneficial effects of the redox polymer-cross-linker network of the hydrogel	Lost 10%–13% response at a throughput of 30 injections/h	Retained 45%–85% of initial response after 6 days	Vijayakumara et al. 1996
AOX/*Hansenula* sp.	Carbon paste matrix	Encapsulating AOX within the hydrophobic carbon paste enhances thermal stability and storage life of biosensor	NS	Retained 30% of initial response after 5 days at 60°C	Wang et al. 1997
AOX	Poly(carbamoyl)sulfonate hydrogel	Dialysis step performed to remove any additives such as salts and sugars from the AOX because the additives may influence stability	Retained 59%–95% of initial response after 12 measurements over 12 h	Retained 40–50% of initial response after 18 days under humid atmosphere at 4°C	Patel et al. 2001
AOX/*Pichia pastoris* + HRP	Graphite–70% Teflon pellets	Stability may be attributed to the daily polishing of the electrodes; without it, electrode responses not obtained	NS	Retained 100% of initial response after 3 months of dry storage at 0°C	Guzmán-Vázquez de Prada et al. 2003
AOX/*C. boidinii* Carboxyl esterase/ porcine liver	Graphite epoxy composite electrode	Attributed to the gelatin membrane integrated with graphite epoxy composite electrode used for the immobilization of AOX	No significant decrease of initial activity after 40 measurements over 16 h	NS	Kirgoz et al. 2006
AOX/*H. polymorpha* + HRP	Electrodeposition paints on graphite rods or glassy-carbon electrodes	Stability attributed to the "sandwich" architecture of the bienzyme sensor, using anodic and cathodic electrodeposition paints to include different enzymes according to specific enzyme properties	NS	Retained 50% of initial response after 14 days at 4°C	Smutok et al. 2006

(*Continued*)

Table 3 (*Continued*)

Enzyme/source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
AOX/*P. pastoris*	Electrodeposition of overoxidized polypyrrole (PPy_{ox}) or poly-*o*-phenylenediamine (PPD) on gold	Using a bilayered membrane consisting of an enzyme entrapping gel and electropolymerized nonconducting (PPy_{ox}) film aids in stability	Retained 100% response after 90 injections carried out in 3 h; 5% sensitivity loss after 6 hours of continual online monitoring	NS	Carelli et al. 2006
AOX/*H. polymorpha*	Glutaraldehyde cross-linking in presence of bovine serum albumen on poly(neutral red)-mediated carbon film	NS	Retained 57.6% sensitivity after use 2–3 times per week for 3 weeks	Retained 88% of initial response after 6 weeks at 4°C	Barsan & Brett 2008
AOX/*P. pastoris*	Graphite-pasted, screen-printed electrode coated with Prussian blue	NS	Retained 100% of initial response within 2% relative standard deviation after 15 successive measurements	Retained at least 25% of initial response after 3 days of storage	Kamanin et al. 2015
AOX/*P. pastoris*	Polyaniline-AOX-gold nanoparticles immobilized in chitosan deposited on glassy carbon electrodes	NS	Retained 93.7% of initial response after 25 measurements	Retained 93% of initial response after 5 weeks of storage at 4°C	Chinnadayyala et al. 2015
AOX/*P. pastoris*	Carbon nanotube matrix	NS	NS	Retained 79% of initial electrical current after 10 days of storage at 4°C	Fang et al. 2016
AOX/*P. pastoris*	Electropolymerization of PPD membrane	NS	NS	NS	Farina et al. 2017
AOX/*P. pastoris*	Chitosan and Nafion layer on a pyramidal microneedle structure	NS	Retained 100% of initial response within 2.85% relative standard deviation of 10 successive measurements during 100 min	NS	Mohan et al. 2017

Abbreviation: NS, not stated.

Table 4 Stability performance of selected xanthine oxidase (XOX) biosensors (1992–2017)

Enzyme/source	Method of immobilization	Method of stabilization	Operational life	Storage life	Reference
XOX/Cow milk	Co-immobilization of xanthine oxidase, uricase, nucleoside phosphorylase, and catalase mixed in a gelatin solution and cast on the tip of a Clark-type O_2 electrode	Consumption of H_2O_2 by catalase	Retained 100% of initial response after 8 days in buffered solutions at 25°C	NS	Wollenberger et al. 1992
	Screen printed with UV-polymerizable paste and addition of stabilizers	Addition of stabilizers, in particular, polylysine	Unclear report of stability. Measurements taken every 1.5 min for 2.5 h	NS	Schumacher et al. 1999
	Cross-linking with glutaraldehyde and bovine serum albumin on Nafion-coated platinum	Cross-linking	Retained 100% of initial response after 80 measurements in 3.98×10^{-5}-M hypoxanthine solution during a period of 10 days. Storage conditions not stated	NS	Nakatani et al. 2005
	Adsorption of carboxymethylcellulose/cyclodextrin-modified (CMC-CD) XOX on one-adamantil-derivatized gold electrodes	CMC-CD surface modification of the enzyme	NS	Retained 93% of initial response after 3 weeks of intermittent determinations during storage at 4°C in phosphate buffer pH 7	Villalonga et al. 2007
	Adsorption onto mesoporous surface formed from silica nanoparticles bound to glassy carbon	Attributed to the interaction of the enzyme with silica nanoparticles	NS	Retained 80% of initial response after 21 days with discrete measurement after storage at 4°C	Saadaoui et al. 2016
	Adsorption onto a matrix of titanium oxide particles adsorbed onto multiwalled carbon nanotubes	NS	NS	Retained 60% of initial response after 60 days of dry storage at 4°C	Narang et al. 2017

Abbreviation: NS, not stated.

CONCLUSIONS

Although there have been some improvements over the past two decades in the stability of enzyme biosensors, such improvements are small and unclear. We found no evidence that new architectures have improved the stability of enzyme biosensors to an extent that would induce a large increase in commercialization. Strategies for stabilization of enzyme biosensors in the past two decades built upon and refined previous approaches such as the use of stabilizers (e.g, polymers of sugars, polyols, polyelectrolytes, and cyclodextrins) and cross-linking as well as combining novel nanostructured materials with cross-linking and electrochemically generated polymers. Of the methods described in this review, inter- or intramolecular cross-linking combined with stabilizing agents and immobilization on carbon paste are the ones that have the most stability retention. Unfortunately, the degree of stabilization relative to free enzymes in solution is not reported in most cases. Most reports attribute any stabilization to the interaction of the enzyme and the immobilization matrix without shedding light onto the actual mechanism by which the enzyme is stabilized. The very large increase in stability induced by HHP on GOX and XOX, although not applicable directly to biosensor development, indicates that not only surface interactions induce stability and suggests that greater stabilization is achievable. Adequate comparison of the stability of enzyme biosensors among the published literature is practically impossible because most studies differ in the experimental conditions under which stability is tested and in the way it is reported. It is critical to establish standardized methods of reporting stability. We propose to test the stability of enzyme biosensors by continuous operation (continuous polarization) at standard ambient temperature (i.e., 25°C) or at 60°C for accelerated tests. Tests should be carried out in solutions with analyte concentrations of $K_M/2$ because these concentrations fall within the linear range of the sensor. The pH optimum of the enzyme in solution and a standard ionic strength of 0.1 M should be used. As a measure of stability, the half-life of the sensor is a minimal piece of information, but kinetic characterization via determining the rate constant of inactivation when possible is desirable. Finally, the most robust means of testing the improvement of the stability of a sensor is to test it side-by-side against the sensor that displays the greatest stability previously reported. The proportion of GOX, LOX, AOX, and XOX biosensor articles that report stability as a keyword has decreased by 4.5% to only 27% in the past decade. Those articles reporting stabilization as a keyword decreased to 1% and 2% for LOX and AOX, respectively, and increased to 3% and 2% for XOX and GOX, respectively. The emphasis on developing nanostructured materials has increased the number of reports on direct electron transfer because on such materials certain enzymes can be immobilized with their active site in closer proximity to the electrode surface. However, such efforts have perhaps diverted the attention from biosensor stabilization, even though stabilization strategies most likely lie at the nanoscale.

Several fundamental questions remain. What is the maximal level of stabilization that these enzymes can achieve? How can that be determined based on their crystalline structure? What are the dominant mechanisms that drive the stability of any given protein? There is no doubt that additional efforts, including mathematical modeling and empirical research, are needed to answer these questions. However, we will not answer them efficiently unless the scientific community agrees on standardizing the reporting of enzyme stability.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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