

Enzyme–Electropolymer-Based Amperometric Biosensors: An Innovative Platform for Time–Temperature Integrators

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A novel exogenous time–temperature integrator (TTI) based on an amperometric glucose oxidase biosensor is presented. The TTI consists of the enzyme entrapped within an electrochemically generated poly(*o*-phenylenediamine) (PoPD) thin film deposited on the interior wall of a platinum (Pt) or a platinized stainless steel (Pt–SS) capsule. After thermal treatment, the TTI is mounted in a continuous flow system and connected to a potentiostat for amperometric detection of residual enzyme activity. A measurement is completed within 10 min. Isothermal treatments were carried out between 70 and 79.7 °C. Thermal inactivation of the immobilized enzyme followed apparent first-order kinetics with *z* values of 6.2 ± 0.6 and 6.6 ± 0.8 °C for Pt and Pt–SS capsules, respectively. These *z* values suggest that the proposed TTIs have the potential to assess pasteurization processes that target microorganism such as *Listeria monocytogenes* and *Escherichia coli* O157:H7.

KEYWORDS: Time–temperature integrator; glucose oxidase; amperometric biosensor; pasteurization

INTRODUCTION

In view of the need for assessing the efficacy of thermal processing of food in operations where traditional instrumentation like thermocouples or resistance temperature detectors cannot be implemented, several research groups have been developing different strategies to evaluate the time–temperature history of foods (1, 2). One strategy is the use of so-called time–temperature integrators (TTIs). Exogenous and endogenous TTIs from different origins (microbiological, enzymatic, chemical, and physical) have been compared (3). Enzymatic TTIs offer the advantage of being relatively easy to assay, they can be inexpensive, and the dependency of the rate of heat inactivation of some enzymes is similar to that of several pathogens of interest to the food industry. A detailed review of intrinsic endogenous TTIs with potential for use in the heat treatment of milk has been published (4), and six of these potential TTIs have been recently investigated (5). However, oftentimes, intrinsic endogenous TTIs have the disadvantage of the intrinsic variability of the concentration of the TTI component and its thermal stability. For example, triose phosphate isomerase has been proposed to verify roast beef processing (6), but its activity and thermal stability varied with muscle type. Other components

present in the food in variable concentrations can also affect the thermal stability of the TTI. In contrast, exogenous TTIs can operate under controlled composition. The stability of some enzymes used as TTIs can be adjusted by changing their pH (7, 8) or adding stabilizing agents (9). Amylase (10–14), horseradish peroxidase (15–18), and r-phycoerythrin (19) have been used as exogenous TTIs for food pasteurization. Some drawbacks of exogenous TTIs include the following: (i) if the TTI volume is relatively large (i.e. gel cubes), temperature gradients may develop throughout its volume and their application may be limited to processes where the heating transient is negligible with respect to processing time; (ii) when the substance used as the indicator needs to be extracted from a capsule, the substance might adhere to the surface of the capsule, making its recovery difficult for rapid assay; and (iii) once the substance used as indicator is retrieved, analysis often requires long preparation, which sometimes includes grinding, centrifugation, incubation, and finally the assay time itself (13). Most of the recent research in TTIs has focused on the use of α -amylases. The group of Hendrickx has focused on TTIs for food sterilization (3, 14, 20) and has recently developed TTIs based on *Bacillus licheniformis* α -amylase, sucrose, and salts immobilized on glass beads and equilibrated at low moisture content to increase the stability of the enzyme (21). This research provided not only a thermostable TTI for sterilization processes but a rapid spectrophotometric reading of the TTI response that obviates the need for an expensive differential scanning calorimeter used in previous reports. A disadvantage of that TTI is that, as mentioned above, it still requires collecting and

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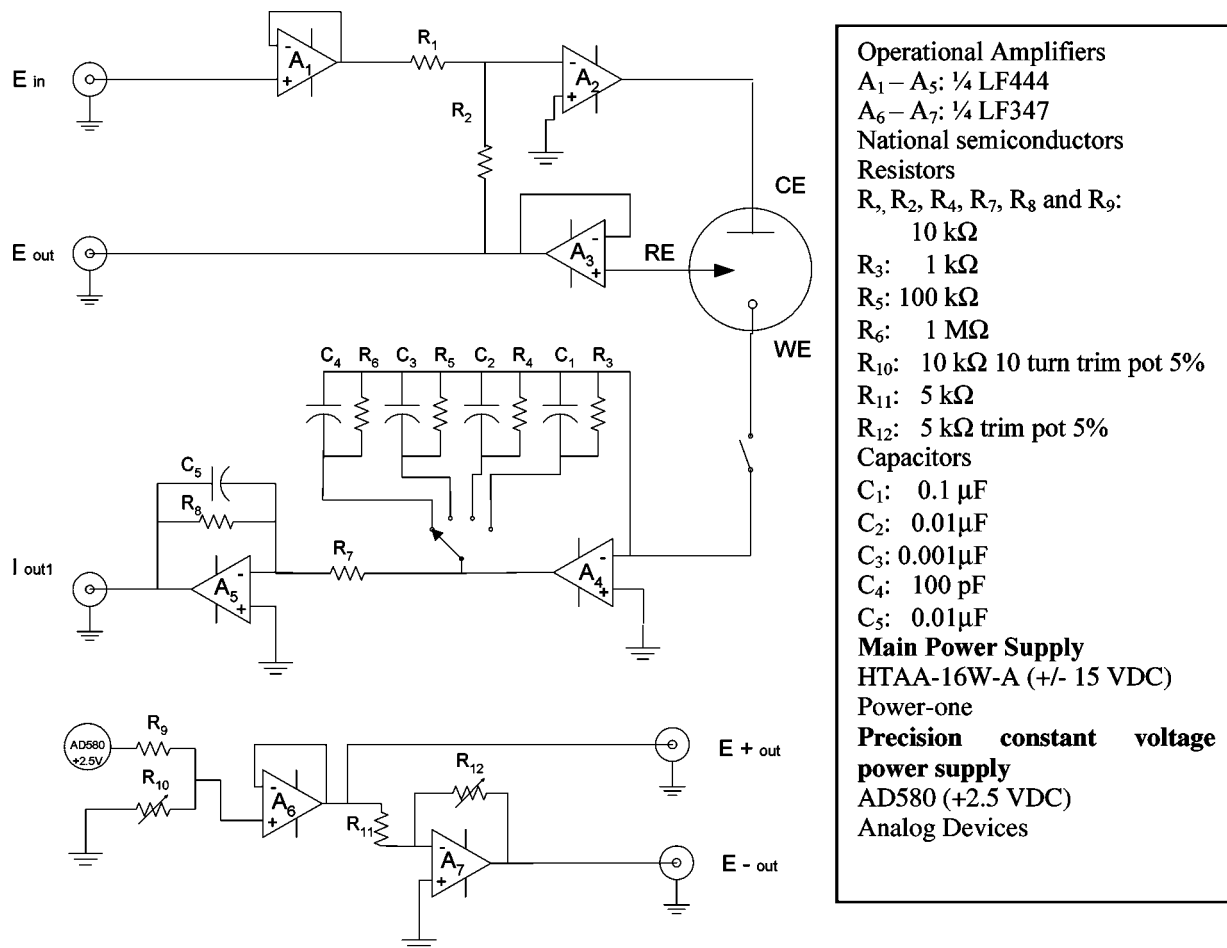


Figure 1. Schematic of the potentiostat used for the research.

precisely weighing and dissolving the content of the capsule prior to the spectrophotometric reading.

Amperometric enzyme-based biosensors have been studied since the early 1960s (22). However, it was not until 1986 that the first enzyme–electropolymer biosensors were reported (23, 24). These sensors were based on glucose oxidase (GOD) immobilized in conducting polypyrrole. Since then, hundreds of research papers dealing with amperometric enzyme-based biosensors using enzymes immobilized in electrochemically generated conducting and nonconducting polymers have been published and reviewed (25–29). The main advantage of this type of sensors is that such polymers can be grown to a controlled thickness from aqueous buffered solutions in which most enzymes are soluble and stable. Resulting sensors are reproducible and some films proved to have selective permeability to the substrate of interest (mainly glucose) (30). Most of these amperometric sensors have been grown on noble metals or glassy carbon electrodes. Inert substrates need to be used because electrochemical polymerization takes place at potentials (typically 700 mV vs Ag/AgCl) where other metals oxidize. We recently studied the effects of Pt platinization conditions on the sensitivity of biosensors based on GOD entrapped in electrochemically generated poly(*o*-phenylenediamine) (PoPD) (31). More information on biosensors can be found in books by Turner (32) and Kress-Rogers (33).

The objective of this research was to design, fabricate, and characterize inexpensive rapid-response TTIs based on amperometric enzyme–electropolymer biosensor technology. GOD immobilized in an electrochemically generated PoPD film

deposited on the interior wall of Pt or Pt–SS capsules is presented as a potential TTI for food pasteurization.

MATERIALS AND METHODS

Glucose oxidase (EC 1.1.3.4 type X-S from *Aspergillus niger*), *o*-phenylenediamine free base, chloroplatinic acid hexahydrate, hydrogen peroxide, and potassium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Lead acetate trihydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Platinum wire (0.4 mm diameter) was purchased from Fisher Scientific (Fair Lawn, NJ). Pt tube (1.57 mm o.d., 1.32 mm i.d.) was purchased from Alfa Aesar (Ward Hill, MA). Stainless steel type 316 tubing (3.17 mm o.d., 1.59 mm i.d.) was purchased from Microgroup Inc. (Medway, MA). All other reagents and solvents were purchased from Sigma-Aldrich or Baker Analyzed and were reagent grade. Solutions were prepared in deionized ultrafiltered ($\Omega > 18 \text{ M}\Omega \text{ cm}$) water.

Platinization, electropolymerization, and cyclic voltammetry experiments were performed with a potentiostat/galvanostat, EG&G 263A (Perkin-Elmer Instruments, Oak Ridge, TN), interfaced to a personal computer, through a GPIB board (National Instruments, Austin, TX). Amperometric measurement of residual enzyme activity of the TTIs was carried out with a low-cost, “in-house-built” potentiostat interfaced to a personal computer using an analog to digital board (PCI 6032E) (National Instruments, Austin, TX). A schematic of the potentiostat with a list of its components is shown in Figure 1. All potentials are reported with respect to Ag/AgCl, 3.0 M KCl reference electrode model EE008 from Cypress Systems Inc. (Lawrence, KS) A Pt wire was used as the counter electrode. A syringe pump model 210 from Kd Scientific (New Hope, PA) was used for the injection of buffer and glucose solutions. The syringe pump was interfaced to the PC through a serial RS-232 port. The cost of all the potentiostat components was under

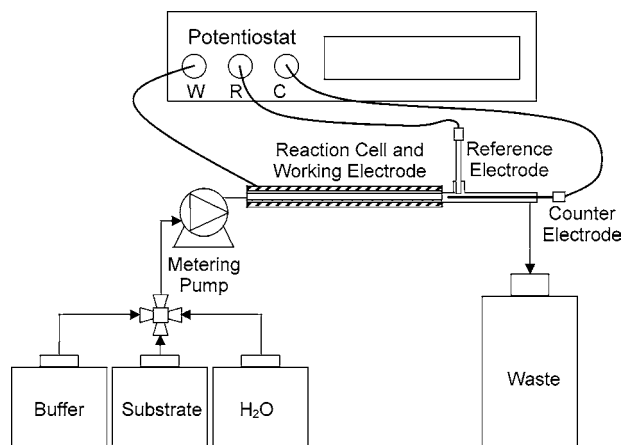


Figure 2. Schematic representation of the TTI flow system.

\$350 US. The estimated total cost of the equipment required for the measurement of the residual enzyme activity of the TTIs including a personal computer and the data acquisition board was under \$5000 US. Data acquisition and control of the potentiostats was done using computer programs written using LabVIEW 6.0 Professional Development System (National Instruments, Austin, TX).

Electrode Cleaning. Pt tubes were cut to make 8-mm-long capsules. Each capsule was polished with 5- μm alumina slurry (Buehler, Lake Bluff, IL) using miniature brushes purchased from Tanis Inc. (Waukesha, WI), rinsed with deionized water, and immersed in an ultrasound bath for 5 min to remove any adherent alumina particle. Tubes were rinsed with deionized water and left immersed in water until used. For reuse, prior to polishing with alumina, Pt tubes were immersed in concentrated H_2SO_4 at 21 °C for at least 1 h and rinsed with deionized water to remove the polymer–enzyme film. SS capsules were made by cutting 15-mm-long tubes. The interior wall of the tubes was washed with soap and thoroughly rinsed with deionized water, polished with 5 μm alumina slurry, rinsed again with deionized water, and immersed immediately in 5 M H_2SO_4 solution at 60 °C. The capsules were thoroughly rinsed with deionized water and immediately platinized.

Electrode Platinization. Potentiostatic platinization of the SS capsules was carried out at -100 mV vs Ag/AgCl in 4 mM H_2PtCl_6 , 1 mM $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ (used as crystal growth promoter), 0.1 M KCl oxygen-free solution for 60 min at room temperature (21 °C). Oxygen was removed from KCl solutions by vigorously sparging with nitrogen for 15 min. A Pt wire inserted in the capsule was used as the counter electrode. To prevent the counter electrode from touching the working electrode, thin glass beads were fused on the wire. Platinized capsules were rinsed with deionized ultrafiltered water. The presence of the Pt layer was verified by cyclic voltammetry in 0.5 M H_2SO_4 between -0.2 and 1.18 V vs Ag/AgCl. Cathodic currents are represented as positive and anodic currents as negative.

Enzyme Immobilization. Potentiostatic electropolymerization was carried out in 5 mM *o*-phenylenediamine, 0.2 M acetate buffer (pH 5.2), and 2.5–10 g L^{-1} GOD oxygen-free solutions at 600 mV vs Ag/AgCl for 30 min at 21 °C. Pt–SS capsules were filled with monomer–enzyme solution. Prior to applying the polymerization potential, monomer and enzyme were allowed to diffuse for 5 min into the porous Pt deposit. Three electrodes were prepared for each level of GOD concentration. The effect of glucose oxidase concentration was studied to determine the range of glucose oxidase concentration that would allow the TTI to operate under a kinetically controlled regime. Because at enzyme concentrations higher than 5 g L^{-1} the increase in TTIs response was small and presumably diffusion started to affect the response, 5 g L^{-1} was chosen for the inactivation experiments.

Adjustment of Operating Conditions. Freshly prepared TTIs were tested in a continuous flow system depicted in **Figure 2**. Background current was measured with buffer solutions in the absence of glucose and subtracted from the current produced in the presence of glucose each time the activity of a TTI was determined.

Electrodes prepared using a solution containing 2.5 g L^{-1} glucose oxidase were used to determine the apparent kinetics of the immobilized

electrode and determine the glucose concentration necessary to reach V_{max} . Operation at V_{max} ensures a large and reproducible amperometric response that is independent of substrate concentration and proportional to the concentration of active enzyme. Finally, substrate flow rate was adjusted to further increase the amperometric response.

Thermal Inactivation Studies. A thermocouple type T was tightly inserted into the TTI at one end, capped at the other, and immersed into a temperature controlled water bath at 84.8 °C for 20 s, followed by immersion in iced water to assess the heating transient phase of the TTI. After fabrication, each TTI was calibrated. The background-subtracted current response was defined as 100% activity. TTIs were capped and immersed in a water bath at 70.0, 72.4, 74.8, 77.2, or 79.7 °C for five different periods of time adjusted to obtain a decrease of at least 50% of the initial activity for the longest incubation time at each temperature. TTIs were immersed in iced water immediately after incubation until tested for activity. Each temperature and each incubation time was carried out in triplicate using a different capsule for each treatment. The experiment was blocked by incubation temperature treatment so that, for a given temperature, incubation times were carried out in random order before the next temperature level was used. The order in which each temperature treatment was applied was also random. Pseudo-first-order inactivation kinetics was assumed and linear regression was used for the calculation of the kinetic constant of inactivation. Arrhenius-type behavior was assumed and linear regression was used for the calculation of the apparent activation energy.

RESULTS AND DISCUSSION

Electrode Platinization. SS 316 was selected for construction of the TTI because this material is approved by the FDA for use in contact with foods. Electrochemical polymerization on SS for corrosion protection has been carried out from aniline (34–38), pyrrole (39, 40), and using multilayers of polyaniline and polypyrrole (41). However, in most cases, polymers are generated in organic solvents unsuitable for enzyme immobilization. We attempted polymerization from 5 and 50 mM *o*-phenylenediamine in 0.2 M acetate buffer (pH 5.2). However, because of the high potential (500–700 mV vs Ag/AgCl) SS oxidation occurred at a faster rate than the polymerization and no polymer adherent deposit was obtained. As an alternative, we coated the interior wall of the SS capsule by electrodepositing a black Pt layer. Platinization of Pt electrodes is a common practice to increase the surface area of amperometric biosensors (42–44), and we have recently improved this method (31). Platinization of SS has been reported to result in fragile Pt black layers with poor adhesion due to the presence of the metal oxide passive layer that forms on SS in contact with air or oxygen-containing solutions (45). Polishing and activation of SS with hot sulfuric acid prior to electroplating has been recommended (46). We used that activation procedure and obtained Pt black deposits that adhered well. Pt black deposits on the interior wall of the TTI were characterized by cyclic voltammetry in 0.5 M H_2SO_4 . Bare SS tubes showed reproducible voltammograms after the first cycle that is characterized by a passivation peak at around 0.1 mV vs Ag/AgCl, as shown in **Figure 3A**. The presence of a porous Pt deposit can be detected by an approximately 10-fold increase in current, shown in **Figure 3B**, compared to a bare SS tube, shown in **Figure 3A**, and by the appearance of an even more pronounced increase in current response in the range of -0.2 to approximately 0.1 mV, which corresponds to hydrogen adsorption and desorption peaks. These peaks are not well resolved as in a pristine polycrystalline Pt surface (**Figure 3C**), probably because the porous deposit did not completely cover the SS surface area. Some SS components may have dissolved and may have been incorporated into the black Pt layer.

Enzyme Immobilization. Electrochemical polymerization of *o*-phenylenediamine on Pt or Pt–SS was carried out success-

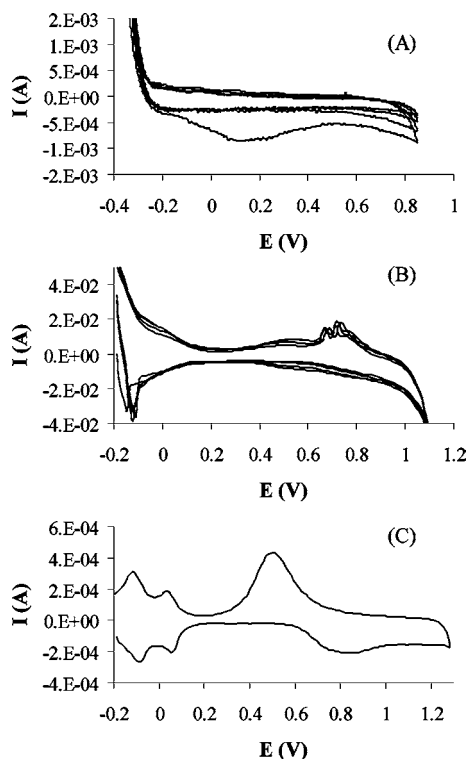


Figure 3. Cyclic voltammograms in 0.5 M H_2SO_4 at 100 mV s^{-1} of (A) SS 316, (B) Pt–SS, and (C) platinized Pt.

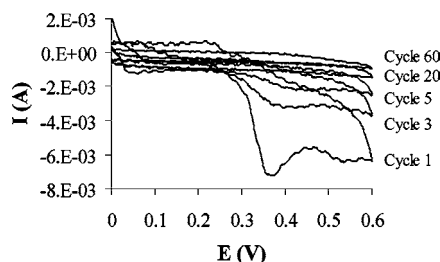


Figure 4. Cyclic voltammograms of Pt–SS tube in 5 mM *o*-phenylenediamine, 5 g L^{-1} GOD in 0.2 M acetate buffer (pH 5.2).

Scheme 1. GOD-Catalyzed Oxidation of Glucose



fully, as evidenced by the shape of the voltammograms and the decrease in current response upon potential cycling depicted in **Figure 4**. As the nonconducting polymer grows, less monomer can reach the metal surface, where it oxidizes, resulting in a current signal decrease. Similar results have been reported for PoPD films grown on polished Pt disk electrodes (47).

Adjustment of Operating Conditions. Glucose oxidase catalyzes the oxidation of glucose according to **Scheme 1**. The mechanism of this reaction has been proven to follow a two-substrate ping-pong mechanism, and the reaction is product inhibited (48). If the O_2 supply is sufficient and glucose, gluconolactone, and H_2O_2 concentrations are below inhibition levels, the kinetics of the reaction can still be described using a simple one-substrate Michaelis–Menten with apparent parameters. In the reaction–diffusion system, glucose diffuses into the enzyme immobilization matrix and glucose oxidase catalyzes the production of H_2O_2 and gluconolactone following **Scheme 1**. H_2O_2 diffuses toward the surface of the electrode, where it is oxidized, and toward the bulk of the solution, driven by concentration gradients as shown on the schematic representation

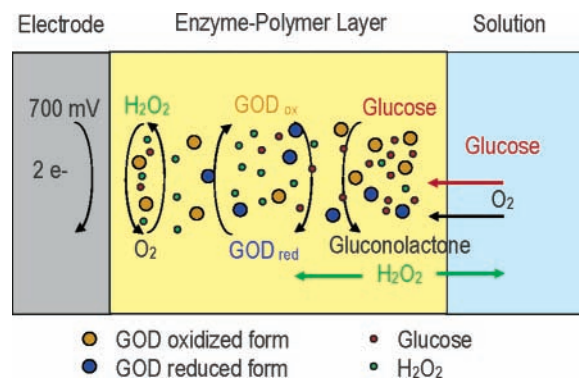


Figure 5. Schematic representation of the amperometric detection of glucose at a GOD–PoPD biosensor.

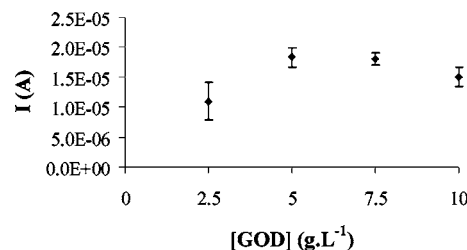


Figure 6. Effect of GOD concentration on SS TTIs current response to 120 mM glucose solution in 0.1 M phosphate buffer (pH 7.0). Error bars represent \pm one standard deviation of three TTIs.

of the reaction diffusion system in **Figure 5**. Because at 700 mV vs Ag/AgCl H_2O_2 oxidation is rapid and because the flow of glucose solution is sufficiently high, both at the metal electrode–polymer and at the polymer–solution interfaces, the concentration of H_2O_2 is practically zero. Furthermore, it has been shown that platinized Pt electrodes with a small concentration of GOD immobilized in thin poly(*o*-phenylenediamine) film operate in a strongly kinetic-controlled regime (31). Therefore, a modified Michaelis–Menten equation can be written as

$$i = \frac{i_{\max} C_{\text{Glu}}}{K_M + C_{\text{Glu}}} \quad (1)$$

In excess of glucose, eq 1 becomes

$$i = i_{\max} \propto k_3 C_{\text{EO}} \quad (2)$$

where k_3 is the rate constant of the release of the product and the free enzyme from Michaelis–Menten mechanism and C_{EO} is the initial concentration of active enzyme. Therefore, the current is a measure of the rate of production of H_2O_2 by the enzymatic reaction. At a given glucose oxidase concentration, the current is a measure of glucose concentration. This is the principle of operation of amperometric GOD biosensors. In contrast to GOD biosensors, because we are interested in measuring the concentration of active enzyme, experiments were run at constant glucose concentration. To maximize TTI response and ensure its operation in the kinetic regime, the effect of glucose oxidase concentration in the polymerization solution on the current response of the TTIs was investigated as shown in **Figure 6**. The current response approximately doubled as the GOD concentration doubled from 2.5 to 5 g L^{-1} , as to be expected. However, at larger GOD concentrations, the current response remained unchanged and slightly decreased at 10 g L^{-1} . This behavior is in agreement with previous reports (49, 50). At low GOD concentrations, diffusion of glucose, O_2 and H_2O_2 throughout the immobilization matrix is rapid and enzyme

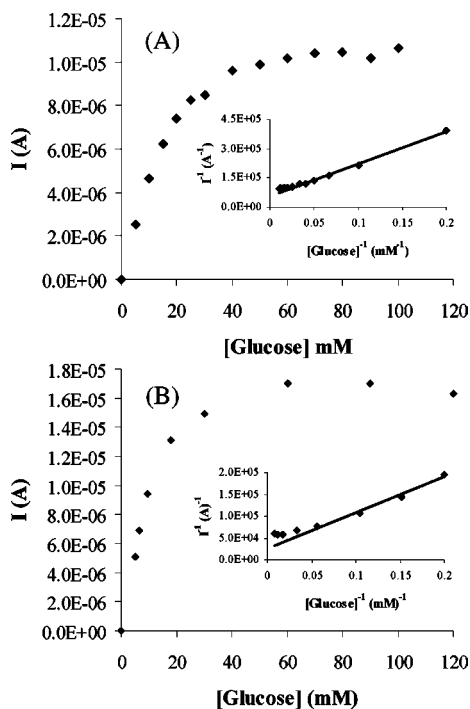


Figure 7. Effect of glucose concentration on TTIs current response. (A) Polymer film was grown from a 5 mM *o*-phenylenediamine, 10 g L⁻¹ GOD in 0.2 M acetate buffer (pH 5.2) on a Pt capsule. (B) Polymer film was grown from a 5 mM *o*-phenylenediamine, 2.5 g L⁻¹ GOD in 0.2 M acetate buffer (pH 5.2) on a Pt-SS capsule. Insets represent the double reciprocal plots used to determine kinetic parameters. A single TTI of each type was used for these experiments.

kinetics limits the overall rate of reaction and is proportional to enzyme concentration, as described by eq 2. Conversely, when GOD reaches a high enough level, glucose is consumed at a faster rate than it diffuses from the bulk of the solution through the polymer film. Diffusion then starts to control the overall rate of reaction. At even greater GOD concentrations, glucose is rapidly oxidized at an outer layer of the polymer film; more H₂O₂ diffuses back to the bulk of the solution than to the surface of the electrode, resulting in a decrease of current response. It has also been reported (51) that at high GOD concentrations electrochemically generated films have reduced permeability, resulting in a decrease in the rate of diffusion of substrates and products and of the current response. Therefore, to operate under kinetic regime, for thermal inactivation studies, all TTIs were fabricated using 5 g L⁻¹ GOD solutions.

To obtain the largest current response, glucose concentration is increased to operate at $i = i_{\max}$. The apparent kinetic parameters of the immobilized GOD-catalyzed oxidation of glucose were determined from eq 1 using the Lineweaver-Burk approach. K_M values were 28 ± 1 and 34 ± 3 mM glucose for Pt and Pt-SS TTIs, respectively. Calculated apparent K_M values were very similar to the values reported for GOD in solution (52), indicating that these TTIs operate under a kinetically controlled regime. Therefore, the amperometric response of both types of TTIs will be proportional to the concentration of active enzyme. Theoretically, i_{\max} is reached at infinite substrate concentration and is equal to 2 times the rate of reaction at substrate concentration equal to K_M . From a practical approach, i_{\max} is reached when glucose concentration is large enough so that the K_M value becomes negligible compared to substrate concentration. **Figure 7** shows the effect of glucose concentration for (A) a Pt TTI and (B) a Pt-SS TTI. For both types of

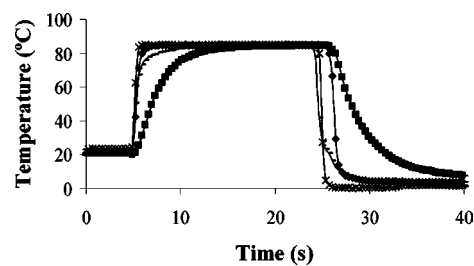


Figure 8. Temperature transient for (x) thermocouple 1, (♦) thermocouple 2, (-) the interior wall of a Pt TTI measured with thermocouple 1, and (■) the interior wall of a SS TTI measured with thermocouple 2. Thermocouples were tightly sealed in contact with the interior wall of the TTIs.

TTI, glucose concentrations of 60 mM or higher produced fairly constant currents with 2.0% and 2.4% relative standard deviation for the Pt TTI and the SS TTI, respectively. A slight decrease in current response was observed at 100 mM for the SS-Pt TTI, in agreement with reported substrate inhibition (48). Glucose concentration of 100 mM was chosen for operation of the system. Because of the inhibition effect, the double reciprocal plot deviates from linearity at low values of the reciprocal of glucose concentration. For that reason, apparent kinetic parameters were determined using only high values of the reciprocal of glucose concentrations in the linear regression, as shown in the insert of **Figure 7**.

TTI Characterization. Heating Transient. Pure metals and metal alloys have high thermal conductivity. SS has thermal conductivity about 5 times smaller than that of Pt but 10 times greater than that of glass and about 30 times greater than that of water. Therefore, metal-capsule-based TTIs with short thermal transient response accurately reflect the temperature history of the product that surrounds them. In addition, electrochemically generated PoPD forms very thin films, on the order of 10-nm-thick (53). This means that the immobilized enzyme reaches the temperature of the interior wall of the TTI very rapidly and without temperature gradients in the enzyme immobilization matrix. Thus, on heating, the immobilized enzyme is uniformly inactivated and the only transient of concern is caused by the thickness of the SS tube. The temperature transient for the heating of Pt and Pt-SS capsules is shown in **Figure 8**. For the thick (0.79 mm) SS tubes, approximately 10 s is required for the interior wall to reach the final temperature after immersion in an 85 °C water bath. For Pt tubes (0.12 mm thick), the temperature transient was 2 s.

Thermal Inactivation. The thermal inactivation at 60 °C of GOD in solution and immobilized in polypyrrole has been reported to follow pseudo-first-order kinetics with k values of 2.4×10^{-4} and 6.7×10^{-5} s⁻¹ respectively (54, 55). To verify whether the pseudo-first-order inactivation approach is adequate, we compared orders 0, 0.5, 1, and 2 for the inactivation of a Pt TTI at 74.8 °C. Correlation coefficients (R^2) were 0.89, 0.97, 0.99, and 0.94, respectively. In this case, a single TTI was used for five heating-cooling cycles to eliminate TTI-to-TTI variability. These results suggest that first-order kinetics best represent the inactivation of the TTI. The kinetics of inactivation of enzymes in solution and the kinetics of inactivation of microorganism have been described with first-order models. Therefore, it is reasonable to assume pseudo-first-order inactivation. The rate constant (k) was calculated from the slope of the linear regression of $\ln(\%$ residual activity) vs time assuming pseudo-first-order kinetics. **Figure 9** shows the thermal inactivation of Pt TTIs at different temperatures. The activation energy for the thermal inactivation of the immobilized GOD was

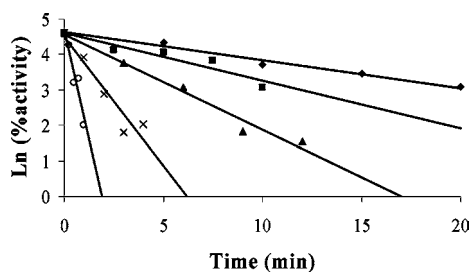


Figure 9. Thermal inactivation of GOD Pt TTIs at (◆) 70 °C, (■) 72.4 °C, (▲) 74.8 °C, (○) 77.2 °C, and (×) 79.7 °C.

Table 1. Thermal Inactivation Kinetic Parameters for GOD Immobilized in an Electrochemically Generated PoPD Film Deposited on Pt Capsules

T (°C)	k^d (s ⁻¹) × 10 ³	r^{2a}	D^d (min)	E_a^d (kJ mol ⁻¹)	r^{2b}	z^d (°C)	r^{2c}
70.0	1.31 ± 0.10	0.98	29.3 ± 2.7	395 ± 54	0.97	6.2 ± 0.6	0.98
72.4	2.25 ± 0.45	0.89	17.0 ± 3.0				
74.8	4.48 ± 0.41	0.97	8.6 ± 0.8				
77.2	11.75 ± 2.22	0.91	3.3 ± 0.5				
79.7	44.86 ± 7.44	0.91	0.9 ± 0.2				

^a Correlation coefficients of $\ln(A/A_0)$ vs time that apply to all k and D values. One replicate for each incubation time was used for the determination of the residual enzyme activity. ^b Correlation coefficient of $\ln(k)$ vs T^{-1} using the values of k obtained from linear regression of $\ln(A/A_0)$ vs time. ^c Correlation coefficient of $\log(D)$ vs T . ^d Standard error was used as the measure of uncertainty in the determination of these values.

Table 2. Thermal Inactivation Kinetic Parameters for GOD Immobilized in an Electrochemically Generated PoPD Film Deposited on Pt–SS

T (°C)	k^d (s ⁻¹) × 10 ⁻³	r^{2a}	D^d (min)	E_a^d (kJ mol ⁻¹)	r^{2b}	z^d (°C)	r^{2c}
70.0	0.43 ± 0.11	0.55	89.0 ± 11.1	335.5 ± 45.7	0.96	6.6 ± 0.84	0.97
72.4	0.70 ± 0.07	0.87	54.5 ± 4.6				
77.2	2.97 ± 0.37	0.80	12.9 ± 1.3				
79.7	12.0 ± 1.82	0.79	3.2 ± 0.4				

^a Correlation coefficients of $\ln(A/A_0)$ vs time that apply to all k and D values. Three replicates for each incubation time were used for the determination of the residual enzyme activity. All replicates were used for linear regression analysis. ^b Correlation coefficient of $\ln(k)$ vs T^{-1} using the values of k obtained from linear regression of $\ln(A/A_0)$ vs time. ^c Correlation coefficient of $\log(D)$ vs T . ^d Standard error was used as measure of the uncertainty in the determination of these values.

calculated from the slope of the linear regression of $\ln(k)$ vs the reciprocal of the absolute temperature, assuming that the effect of temperature on the rate constant can be described using an Arrhenius-type equation. The decimal reduction time (D value) and the temperature increase that produces an increase in the rate of enzyme inactivation by a factor of 10 (z value) were also calculated. Thermal inactivation kinetic parameters of Pt-based and Pt–SS-based TTIs are shown in **Tables 1** and **2**, respectively. The z value for Pt and Pt–SS TTIs was 6.2 and 6.6 °C, respectively, and they were not significantly different. While the z value was essentially the same for both TTIs, the D values for Pt–SS TTIs were greater than those for Pt-TTIs. This suggests that immobilization on Pt–SS has a stabilization effect on the enzyme, but in the range of temperatures investigated here, the dependency of the rate of inactivation on temperature remains unchanged. The decrease in the rate of inactivation may be partially due to the greater thickness of SS capsules compared to Pt capsules. Other interaction of metals from the SS alloy might have affected the stability, too.

A disadvantage of the Pt–SS capsules is that their fabrication requires several additional steps compared to Pt capsules. Several batches of Pt–SS TTIs were not functional. Despite the use of three replicates for each incubation time, variability in Pt–SS TTIs response was large, as shown by small correlation coefficients and large standard errors calculated for k and D values (see **Table 2**). Platinization was a particularly difficult step, because activation of SS in hot H₂SO₄ was not always reproducible. The time required for H₂ evolution in H₂SO₄ to start varied and the rate of H₂ evolution varied among capsules. Conversely, Pt TTIs required simple cleaning and immobilization procedures and gave reproducible results and very few nonfunctional TTIs. We conclude that while using a disposable approach to the fabrication of the proposed TTIs might be economically attractive (the cost of a Pt–SS based TTI is approximately \$3.00 US), the more expensive (\$30.00 US) Pt-based TTI can be recycled and offers a more robust approach. Beyond our main objective of proposing amperometric biosensors as an innovative platform for TTIs, our results suggest that the proposed GOD-based TTIs are suitable for the assessment of microbial inactivation during pasteurization. For example, it has been reported that *Listeria monocytogenes* strain Scott A in dairy products has a z values of 6.08–6.67 °C for pasteurization at 52–68 °C. Strain 1151 has z values of 5.32–6.71 in the same temperature range (56). *Escherichia coli* O157:H7 has z values of 6.50, 6.89, 6.51, 6.0, and 6.79 °C in turkey, lamb, pork, beef, and chicken, respectively (57, 58). Among oxidases, glucose oxidase is a very stable enzyme (59). To the best of our knowledge, this is the first report of GOD as a potential TTI for food pasteurization. For processes where target microorganisms have lower z values, there are a number of oxidases, such as amino acid oxidase and alcohol oxidase, that have lower thermal stability and have the potential to be readily used as TTIs using the proposed method of transduction, i.e., amperometric detection of H₂O₂. Furthermore, there are other enzymes that produce electroactive compounds that can be detected using the proposed technology by simply changing the substrate and the electrode potential. In summary, the proposed TTI technology has the following advantages: (i) TTIs are simple to prepare, (ii) TTIs' response is simple and rapid to read, (iii) inexpensive electronic instrumentation is used, and (iv) a variety of target microorganisms can be easily achieved by changing the enzymes and the substrates. The proposed TTIs and the associated electronic components can be miniaturized to produce a low-cost portable instrument. On the basis of the proposed technology, TTIs with higher z values based on more stable or stabilized enzymes can potentially assess the adequacy of sterilization in the canning industry.

ACKNOWLEDGMENT

The authors are grateful to Alma L. Peña for her assistance in carrying out some the experiments of this research.

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Received for review May 13, 2005. Revised manuscript received September 6, 2005. Accepted September 9, 2005. J.I.R.-D.-C. was supported by a grant from Fulbright-CONACyT (National Council for Science and Technology, Mexico). This project was also supported by a grant from the Washington State University IMPACT Center and by the Washington State University Agricultural Research Center.

JF051103+