Increased Thermal Stability of a Glucose Oxidase Biosensor under High Hydrostatic Pressure

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Title: Increased Thermal Stability of a Glucose Oxidase Biosensor under High Hydrostatic Pressure

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Highlights:

- Immobilization and high pressure synergistically stabilized glucose oxidase.
- Thermal inactivation of immobilized glucose oxidase followed pseudo-second order.
- The stabilizing effect of high pressure was not retained upon depressurization.

Abstract

We report the effects of high hydrostatic pressure (HHP), immobilization in electrochemically generated poly-*o*-phenylenediamine nano-films, and reticulation with glutaraldehyde on the thermal stability of glucose oxidase (GOx). The pseudo-first-order rate constant of inactivation of immobilized GOx inactivated at 70 °C and atmospheric pressure was

20.6 times smaller than that of GOx in solution under the same conditions. Immobilized GOx inactivated at 70 °C and 180 MPa was 87.6 times more stable than GOx in solution inactivated at 70 °C and atmospheric pressure. However, applying high pressure during electropolymerization or cross-linking with glutaraldehyde only had minor influences on GOx thermal stability. The stabilizing effect of HHP was not retained upon depressurization.

Keywords: glucose oxidase; high hydrostatic pressure; biosensor; electropolymerization; cross-linking; enzyme stabilization

1. Introduction

Glucose oxidase (GOx) (EC 1.1.3.4) catalyzes the oxidation of β-D-glucose by oxygen to D-glucono-1,5-lactone and hydrogen peroxide. It is a flavin-dependent homodimer of approximately 160 kDa. Glucose oxidase has been widely used in industries including clinical, food, chemical, and biotechnology [1]. Glucose biosensors that use immobilized GOx have been extensively researched and used in the clinical industry for the last four decades [2]. Among all glucose biosensors, electrochemical glucose biosensors are the most frequently researched and are further classified as potentiometric, amperometric, or impedimetric/conductometric biosensors based on their transduction signal [2]. In first-generation amperometric glucose biosensors like the ones presented in this research, glucose oxidase is immobilized onto the surface of an electrode. The electrode potential is typically set to 700 mV *vs.* Ag|AgCl. As glucose diffuses into the immobilized GOx layer, it is oxidized into gluconic acid and hydrogen peroxide. Then, at the surface of the metal electrode, hydrogen peroxide is oxidized exchanging two electrons and producing an electrical current that is a function of the concentration of glucose in the sample. Glucose biosensors are calibrated by correlating the current response to solutions of known concentration of glucose.

Practical enzyme biosensors overcome the long sample analysis time and the complexity of conventional methods such as chromatographic or electrophoretic separations followed by spectrophotometric or electrochemical detection of individual analytes. Thus, biosensors provide a low-cost, fast, sensitive, and specific response to analytes in complex mixtures [3]. The global market of all types of biosensors was estimated at approximately 12 billion dollars in 2015 [4]. In the food industry, biosensors can be used to detect food components, pathogens, and food contaminants [5, 6]. Potentially, some biosensors can be integrated into food packaging [7].

However, the major market for enzyme biosensors is in medical care. The most successfully commercialized biosensors are GOx biosensors used to detect glucose for individuals with diabetes [4]. The main limitation that greatly hampers the development of practical enzyme biosensors, including GOx biosensors, is their short operational life which is due to the poor stability of enzymes [8].

High hydrostatic pressure (HHP) has been normally used to kill microbes and inactivate enzymes in food processing. However, at moderate levels, high pressure stabilizes and activates some enzymes [9]. The protective effect of HHP enables enzymes to remain active at temperatures above those that, at atmospheric pressure, would denature them. The increase in temperature also results in a faster reaction rate [10]. This stabilization effect has mainly been observed at temperatures above 50 ˚C and pressures below 400 MPa [11]. However, the stabilizing effect of HHP is lost upon depressurization, limiting the application of this approach to the availability of HHP reactors. We have recently reported the stabilization of glucose oxidase [12], alcohol oxidase [13], xanthine oxidase [14], and pyruvate oxidase [15] by HHP.

Stabilization of enzymes by immobilization has been well documented and reviewed [16-18]. A simple method of enzyme immobilization is the physical entrapment in electrochemically generated polymers. These polymers can be grown from aqueous solutions that contain both the enzyme and the monomer. When a sufficiently oxidizing potential is applied to the electrode, the monomer loses electrons and polymerizes. These polymers grow on the surface of the electrode and typically adhere well to the electrode surface [19]. Non-conducting polymers like poly(*o*-phenylenediamine) (PoPD), polyphenol, or poly(dichlorophenolindophenol) produce very thin, compact films that result in biosensors with fast response time, improved selectivity, and anti-fouling properties [20]. High storage stability of glucose biosensors with GOx immobilized in

PoPD films has been reported [21-26]. Among other immobilization methods, inter- and intra-molecular cross-linking have also been commonly used to stabilize proteins. Glutaraldehyde (GA) is one of the most commonly used cross-linkers [27]. Cross-linking with GA has increased the thermostability of enzymes [28]. Glutaraldehyde reacts mainly with the primary amino side groups of proteins and increases the stability of enzymes by restricting the conformation changes, i.e. protein unfolding. Three approaches to immobilize GOx by cross-linking with GA are commonly used: 1) Cross-linking GOx before the enzyme is immobilized onto supports with other techniques [29-31]; 2) Cross-linking as the method to bind GOx onto the support [32]; 3) Cross-linking after the enzyme has been immobilized [33]. In order to better cross-link with GA, glucose oxidase is often mixed with bovine serum albumin (BSA) before the addition of GA [34-37]. However, though the thermostability of the enzymes can be improved by the newly created microenvironment and the increased rigidity, the harsh conditions of immobilization sometimes inactivate the enzymes [38]. The effectiveness of the methods of immobilization to increase the stability of an enzyme depends also on the enzyme itself and has to be evaluated on a case-by-case basis [39].

To the best of our knowledge, high pressure has not been applied during enzyme immobilization or inactivation of immobilized enzymes to improve the stability of enzyme biosensors against thermal treatment. We hypothesized that combining HHP with electropolymerization of *o*-phenylenediamine (oPD) or cross-linking improves the stability of GOx-based biosensors.

Therefore, the objectives of this study were 1) to assess the effect of HHP during electropolymerization on the thermal stability of GOx biosensors, 2) to combine HHP and cross-

linking with GA to enhance the stability of GOx biosensors, and 3) to assess the effect of HHP during thermal inactivation on the stability of immobilized GOx.

2. Material and methods

2.1. Materials

Glucose oxidase from *Aspergillus Niger* (Product Number G7141), D-(+)-glucose, bovine serum albumin, and chloroplatinic acid hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium phosphate monobasic, sodium acetate trihydrate, glutaraldehyde (25%), potassium chloride, and sulfuric acid were purchased from Fisher Scientific (Hampton, NH, USA). Lead (II) acetate trihydrate and *o*-phenylenediamine were purchased from Acros Organics (Geel, Belgium). All chemicals were reagent grade and solutions were made with ultrafiltered water, resistivity 18.2 MΩ cm^{-1}.

2.2. Equipment

Reference 600 potentiostats from Gamry Instruments (Warminster, PA, USA) were used for cyclic voltammetry, chronoamperometry, and chronocoulometry experiments. Framework or Echem computer programs from Gamry were used to operate the potentiostat or analyze data, respectively.

The high pressure system used in this research consisted of a piston pump (model MP5), a jacketed high pressure reactor (model U111), and a controller unit from Unipress Equipment (Warsaw, Poland). The system was previously described in the report by Halalipour, et al. [12], with the difference that the cap of the high pressure reactor had electrical feed-through connectors rated up to 700 MPa to carry out the electropolymerization under high pressure, shown in Fig. 1A.

2.3. Methods

2.3.1. Fabrication of electrochemical cell

An electrochemical cell was designed and fabricated specifically for the high pressure reactor in this research as shown in Fig. 1A. The cell was made of a silicone tubing (3 cm long, 0.95 cm outside diameter, 0.64 cm inside diameter) enclosed with one polytetrafluoroethylene cap (0.5 cm thick, 0.8 cm diameter) that holds the working and counter electrodes (platinum wire, 1.6 cm long, 0.8 mm diameter) (Fig. 1B) and one reference electrode. Pellets of Ag|AgCl mounted on silver wires were purchased from Warner Instruments (Hamden, CT, USA). The Ag|AgCl pellet was inserted in a heat shrinking tube filled with 3 M KCl with a porous glass frit heat-sealed at one end. Then, the silver wire was sealed at the other end with epoxy glue to form the bottom cap of the cell.

2.3.2. Fabrication of GOx biosensor

The method of fabricating a first generation glucose oxidase biosensor in this research was modified from Reyes-De-Corcuera, et al. [25].

2.3.2.1. Platinum electrodes preparation

The cap with platinum electrodes (Fig. 1B) was polished and cleaned before each use. The electrodes were sequentially polished with abrasive paper P320 grit, a nylon disk with 0.3 µm alumina slurry, and a microcloth disk with 0.05 µm alumina slurry. When reused, only the microcloth disk with 0.05 µm alumina slurry was needed. The electrodes were immersed in an ultrasound bath (output frequency 40 kHz) for 15 min in deionized water and immersed in concentrated sulfuric acid overnight. The electrodes were finally electrochemically cleaned in 0.1 M H_2SO_4 by cyclic voltammetry from -0.189 V to 1.461 V *vs.* Ag|AgCl in 3.5 M KCl at 500 mV s^{-1} for 80 cycles. The electrodes were rinsed with deionized water between each step.

To increase the surface area, platinum electrodes were platinized by chronocoulometry in 2 mM H_2PtCl_6, 1 mM $Pb(C_2H_3O_2)$, 0.1 M KCl deaerated solution at -0.089 V *vs.* Ag|AgCl in 3.5 M KCl to reach the total charge density 2.0 mC cm^{-2}. The electrodes were then ultrasonicated in 0.1 M phosphate buffer (pH 7) for 10 min to remove any loosely bound platinum deposit. Cyclic voltammetry in 0.5 M H_2SO_4 was carried out at 500 mV s^{-1} from -0.238 V to 1.211 V *vs.* Ag|AgCl in 3.5 M KCl for 10 cycles after the ultrasonic treatment to determine the electrochemical surface area of the electrodes as shown in Fig. 1C.

2.3.2.2. Enzyme immobilization and electrode characterization

Glucose oxidase was immobilized on the working electrode by entrapment in poly-*o*-phenylenediamine (PoPD) films electrochemically generated from the deaerated solution containing 10 mg mL^{-1} glucose oxidase, 5 mM oPD, and 0.2 M acetate acid buffer (pH 5.2). Cyclic voltammetry was carried out at 100 mV s^{-1} from 0 to 0.65 V *vs.* Ag|AgCl in 3.0 M KCl for 100 cycles. Platinized electrodes were immersed in the solution for 5 min before starting the electropolymerization to allow solution diffusion into the black platinum porous surface of the electrode.

The amperometric response of the electrodes was measured at 0.711 V *vs.* Ag|AgCl in 3.5 M KCl in stirred 0 to 20 mM glucose solutions. The sensitivity of the biosensors was determined as the slope of the calibration curves (current density *vs.* glucose concentration).

2.3.3. Effect of HHP during immobilization on the stability of GOx biosensors

The electropolymerization was carried out at 0.1 to 420 MPa. Immobilization treatments were carried out in triplicate. Then, each electrode was heated at 70 °C in 0.1 M phosphate buffer (pH 7) in a water bath for an accumulated time of 20, 40, 60, 120, and 180 min and were placed in 0.1 M phosphate buffer (pH 7) on ice for 5 min before measuring the amperometric response

to 20 mM glucose solution at 25 °C. The stability of each biosensor was characterized in terms of the percent residual amperometric response *vs.* heat treatment time relative to the response at time 0 (fresh electrodes).

2.3.4. Effect of cross-linking on the stability of GOx biosensors

Before electropolymerization, GOx was either cross-linked with 0.5% (w/v) GA in 0.2 M acetate acid buffer (pH 5.2) at room temperature for 6 or 12 h at 0.1 or 180 MPa, or cross-linked with the mixture of 0.5% (w/v) GA and BSA (BSA: GOx = 1:1) at room temperature for 1 h at 0.1 or 180 MPa. Cross-linking agents were separated by pumping through a 5 mL HiTrap™ desalting column from GE Healthcare (Pittsburgh, PA, USA) to stop the reaction. The concentration of GOx was adjusted with 0.2 M acetate acid buffer (pH 5.2) to 10 mg mL^{-1} by measuring the absorbance at 280 nm relative to fresh enzyme. The cross-linked GOx was immobilized at 0.1 or 180 MPa and the resulting biosensors were characterized as described in sections 2.3.2 and 2.3.3.

Cross-linking was also carried out after electropolymerization. Glucose biosensors fabricated at 0.1 or 180 MPa were immersed in 2.5 or 5% (w/v) glutaraldehyde solutions at room temperature for 10 or 30 min at 0.1 MPa. The stability of the biosensors against thermal treatments was done as described in sections 2.3.2 and 2.3.3. All electrode fabrication conditions were done in triplicate.

Pressure 180 MPa was chosen because it was optimal for GOx stability [12].

2.3.5. Effect of HHP during thermal treatment on the stability of immobilized GOx

Enzyme immobilization by electropolymerization was carried out at 0.1 or 180 MPa. Electrodes fabricated at atmospheric pressure were heated at 70 °C in 0.1 M phosphate buffer (pH 7) in the high pressure reactor for an accumulated time of 0, 20, 40, 60, 120 and 180 min at

0.1, 180, or 360 MPa. Electrodes fabricated at 180 MPa were heated at 180 MPa. The electrodes were cooled to 10 °C before measuring the amperometric response at room temperature. All treatments were done in triplicate.

2.4. Data analysis

Pseudo-first-order and pseudo-second-order models were used to fit the residual current density response of the electrodes as a function of time. For the pseudo-first-order model, the rate constant of inactivation (k_{inact}) was calculated from the linear regression of the natural logarithm of relative current density response to 20 mM glucose solution *vs.* treatment time. For the pseudo-second-order model, the rate constant of inactivation (k'_{inact}) was calculated from the linear regression of the reciprocal of the relative current density response *vs.* treatment time. Mean values ± 95% confidence intervals are reported. Statistical analysis was conducted by Minitab 16 software (State College, PA, USA) and JMP Pro 14 software (Cary, NC, USA). MATLAB software was used for non-linear model fitting. Tukey's method and Dunnett's method for multiple comparisons were used.

3. Results and discussion

3.1. Electrode inactivation model fitting

During thermal treatment, the current response of GOx biosensors decreased with treatment time. This can be attributed to the thermal inactivation of GOx, the leaching of GOx out of the oPD polymer matrix when heated, and/or the degradation of oPD polymer [40]. The decrease in the biosensor current response was most likely due to enzyme inactivation because there were only minor changes of current response to phosphate buffer in the absence of glucose throughout the entire duration of the thermal treatment. Therefore, it was unlikely that the degradation of polymer had a pronounced effect on the decrease in current response. Biosensor

current response was calculated as the difference of the current response to 20 mM glucose solution in 0.1 M phosphate buffer pH 7.0 and a glucose-free buffer solution.

The rate constant of inactivation was calculated using pseudo-first-order or pseudo-second-order models. The means of coefficient of determination (R^2) were used to partially assess model fit for all the experimental conditions of this study (Tables 1-4). The means and standard deviations of R^2 for all the experimental conditions of the pseudo-first-order and pseudo-second-order models were 0.84 ± 0.05 and 0.96 ± 0.03, respectively. Non-linear fitting of relative current density response *vs.* treatment time for the pseudo first and second order models confirm that the pseudo-second-order model fitted better the inactivation of the enzyme biosensor as shown in Fig. S1. As the level of inactivation decreased, the fit to the pseudo-first-order model improved but remained inferior to the pseudo-second-order model (Fig. S2). Although MATLAB and other statistical software calculate R^2 for non-linear regressions as a partial indicator of fit, it is not valid to compare it to that of a linear regression [41]. Figures S1 and S2 show that the fitting of the non-linear regression is not better than the linear fitting of the pseudo-second-order linearized data. Therefore, the pseudo-second-order model fitted better than the pseudo-first-order model and was used to compare results within this report. This is in agreement with Dumont and Fortier [40], stating the second-order model was adequate for the inactivation at 55 to 65 °C of immobilized GOx by electropolymerization into the films of polyaniline, polyindole, polypyrrole, PoPD, or poly(aniline/*p*-phenylediamine). Similarly, Ulbrich, et al. [42] found that the thermal inactivation of immobilized α-amylase, α-chymotrypsin, and trypsin followed biphasic kinetics rather than first-order kinetics of enzymes in solution. They suggested that the biphasic kinetics resulted from two different pathways of inactivation of the same enzyme caused by immobilization. However, many other studies have

fitted a first-order model to the inactivation of GOx: in solution at 70 °C [43], encapsulated in liposomes inactivated at 45 to 55 °C [44], and immobilized by absorption and cross-linking inactivated at room temperature [45]. Therefore, to compare results with other reports specifically concerning soluble GOx, pseudo-first-order model is also reported and discussed. Moreover, our data does not offer a mechanistic explanation for the pseudo-second order, though deviation from the most often reported pseudo-first-order indicates that the immobilization matrix contributes non-linearly to the kinetics of inactivation.

3.2. Effect of high pressure during immobilization on the stability of GOx biosensors

Table 1 shows the sensitivity, apparent Michaelis-Menten constant (K_m^{app}), k_{inact} and k'_{inact} for GOx biosensors fabricated at 0.1 to 420 MPa. For GOx biosensors fabricated at different pressures k'_{inact} ranged from $(1.9 \pm 2.0) \times 10^{-4}$ min^{-1} to $(3.3 \pm 1.0) \times 10^{-4}$ min^{-1}. Immobilization pressure didn't have a significant influence ($p > 0.05$) on the thermostability of biosensors based on k'_{inact} when treating the biosensors at 70 °C and 0.1 MPa. However, in our previous study, pressure had a stabilizing effect on the soluble GOx against thermal treatment and was at most ~20 times slower when inactivated at HHP and 69.1 °C [12]. This contradicted our hypothesis that the conformation changes of an enzyme under high pressure would be retained after depressurization when the enzyme was entrapped at HHP and would result in a more stable biosensor. Immobilization at HHP didn't have a significant influence ($p > 0.05$) on either the sensitivity or the K_m^{app}. Therefore, the enzyme was not inactivated at the pressures applied. For all the electrodes tested, the current response began to level off at glucose solution concentrations above 20 mM. This suggested that the entrapment of the enzyme was consistent for all electrodes, and pressure didn't affect the permeability of the polymer. The sensitivity of the biosensors, $(3.0 – 4.5) \times 10^{-3}$ $\mu A\ mm^{-2}\ mM^{-1}$, was in accordance with the other report using

electropolymerization of oPD to immobilize GOx which was in the range of $(2.0 – 7.0) \times 10^{-3}$ $\mu A\ mm^{-2}\ mM^{-1}$ [23]. The K_m^{app}, (21.2 ± 22.9 – 35.2 ± 13.2) mM, at any immobilization pressure at pH 7.0 was higher than 17.5 mM at pH 5.1 reported by Halalipour, et al. [12] and was not significantly different from 33 mM at pH 5.6 reported by Swoboda and Massey [46], both using soluble GOx from *A. niger*. The fact that the K_m^{app} of the immobilized enzyme is not different from the K_m^{app} of the enzyme in solution indicates that the biosensor response is under kinetic and not under diffusion control. This is explained by the small thickness of PoPD film (~20 nm) [25, 47]. Therefore, it is adequate to use the current density as the indirect measurement of enzyme activity and as an indicator to determine the enzyme inactivation.

3.3. Effect of cross-linking on the stability, sensitivity and K_m^{app} of GOx biosensors

3.3.1 Cross-linking before immobilization

Table 2 shows the sensitivity, K_m^{app}, k_{inact}, and k'_{inact} of GOx biosensors using GOx that was cross-linked with 0.5% (w/v) GA at 0.1 or 180 MPa before immobilization. The results when BSA was added or not were analyzed separately. In the absence of BSA, there was no statistically significant interaction between immobilization pressure, cross-linking pressure, and cross-linking reaction time ($p > 0.05$). In contrast, the simple main effect of pressure during immobilization on k'_{inact} was significant ($p < 0.01$) using the general linear model (GLM). The k'_{inact} of GOx biosensors immobilized at 180 MPa was up to two times greater than for GOx immobilized at 0.1 MPa, but it was not affected significantly by the reaction time (6 or 12 h) with GA (without BSA) or by the pressure applied during cross-linking. Adding BSA during cross-linking with GA did not affect the stability of the biosensors as indicated by the lack of significant difference ($p > 0.05$) on the k'_{inact}. Compared to the data in Table 1, biosensors cross-linked without BSA immobilized at 180 MPa and biosensors cross-linked with BSA immobilized

at either 0.1 or 180 MPa had k'_{inact} significantly higher than biosensors fabricated at 0.1 MPa without any cross-linking based on Dunnett's method. Therefore, cross-linking without BSA at 180 MPa or cross-linking with BSA at either pressure before immobilization decreased the thermostability. The lowest k'_{inact} when cross-linked with GA was $(5.1 \pm 1.8) \times 10^{-4}$ min^{-1} and the thermostability was 2.4 times less than the untreated GOx biosensors with k'_{inact} $(2.1 \pm 0.7) \times 10^{-4}$ min^{-1}. This was in agreement with the report by Busto, et al. [48], showing the humic-carboxymethyl cellulase cross-linked with GA became less stable. But the reason why thermostability decreased is unclear. Therefore, under the conditions of this study, cross-linking with GA or with GA and BSA did not add to the stability of GOx biosensors. Furthermore, immobilization at HHP by entrapment with cross-linked GOx resulted in less stable biosensors which contradicted our hypothesis. This suggests that either the cross-linking destabilized the enzyme or affected the growth of the film, resulting in a less compact polymer that restricts the enzyme less and prevents to a lesser extent the unfolding of the protein.

As for the sensitivity, though the immobilization pressure and the cross-linking pressure didn't have significant influence ($p > 0.05$) on the sensitivity when using BSA during cross-linking, two-way ANOVA and GLM indicated an interaction of immobilization pressure and cross-linking reaction time, an interaction between immobilization pressure and cross-linking pressure, and the main effect of cross-linking pressure had significant influence ($p < 0.05$) on the sensitivity of biosensors without using BSA during cross-linking. Without using BSA, the sensitivity was significantly higher when cross-linked at 0.1 MPa than when cross-linked at 180 MPa. Compared to the data in Table 1, the sensitivity of biosensors with cross-linking was higher than the biosensors without cross-linking. The lowest sensitivity when using cross-linked GOx was $(5.6 \pm 0.9) \times 10^{-3}$ $\mu A\ mm^{-2}\ mM^{-1}$ and the highest was $(9.6 \pm 0.9) \times 10^{-3}$ $\mu A\ mm^{-2}\ mM^{-}$

^{1}, which was 1.3 and 2.2 times greater than that of the control biosensor $(4.4 \pm 0.9) \times 10^{-3}$ μA mm^{-2} mM^{-1}. This was in contrast to the findings in literature where the activity of enzymes was decreased after cross-linking or increasing the concentration of GA [48, 49]. One possible explanation is that greater concentrations of GOx were entrapped when immobilizing the cross-linked conjugate than the free enzyme. However, we do not have a quantitative measurement of the amount of GOx immobilized.

Similar to sensitivity, the immobilization pressure and cross-linking pressure didn't have significant influence ($p < 0.05$) on the K_m^{app} when using BSA, but the interaction between cross-linking pressure and cross-linking reaction time and the main effect of immobilization pressure had significant influence ($p < 0.05$) without using BSA. When the cross-linked GOx was immobilized at 180 MPa without using BSA, the K_m^{app} was higher than the biosensors immobilized at 0.1 MPa. Compared to the biosensors immobilized at 0.1 MPa without cross-linking in Table 1 with Dunnett's method, there was no significant difference in K_m^{app} between the control biosensors and the cross-linked GOx without using BSA and immobilized at 0.1 MPa, or between the control and the cross-linked GOx using BSA. But the cross-linked GOx without using BSA and immobilized at 180 MPa had a K_m^{app} that was significantly higher than the untreated biosensors. The highest K_m^{app} was 50.0 ± 10.4 mM which was 1.8 times higher than the untreated biosensors with K_m^{app} 28.4 ± 5.7 mM. This suggests that rearrangement of the conformation of the cross-linked enzyme conjugate entrapped in PoPD under high pressure hindered the access of the substrate to the active sites or produced a more compact polymer with reduced substrate permeability, which may also be the reason for the lower sensitivity mentioned above.

3.3.2. Cross-linking after immobilization

The sensitivity, k_{inact}, and k'_{inact} for the biosensors cross-linked with 2.5 or 5% (w/v) GA after the GOx immobilized at 0.1 or 180 MPa are shown in Table 3. There was no significant difference ($p > 0.05$) among the rate constant of inactivation by different immobilization pressure, GA concentration, or cross-linking reaction time. However, all the k'_{inact} (0.5 ± 0.4 – 1.0 ± 0.4) $\times 10^{-4}$ min^{-1} were significantly lower ($p < 0.01$) than the biosensors without cross-linking immobilized at 0.1 MPa with the k'_{inact} (2.1 ± 0.7) $\times 10^{-4}$ min^{-1}. The cross-linking after immobilization made the biosensors approximately two times more stable than the untreated biosensors. Therefore, glutaraldehyde was able to diffuse into the polymer structure and probably resulted in intermolecular and/or intramolecular cross-linking with GOx which further restricted the unfolding of proteins under thermal treatment. The increase in stability can also be due to the prevention of enzyme leaching out from the polymer. Bhushan, et al. [50] and Kumar, et al. [51] used GA to cross-link with xylanase entrapped in alginate beads which reduced the impact of enzyme leaching by forming aggregates, thus improved the stability. Kowal and Parsons [52] also reported that GA cross-linking decreased the leakage of lactoperoxidase immobilized on Sepharose; and the higher concentration of GA resulted in less leakage and lower enzyme activity. However, Betancor, et al. [33] reported that the different concentration of GA didn't make any significant difference on the thermostability of enzymes which was in accordance with our results. This suggested that the different cross-linking conditions used in this research resulted in similar degree of cross-linking. However, the degree of cross-linking was not verified. In our preliminary experiments, excessive cross-linking from high concentration of GA (> 5% w/v) and long reacting time (overnight) with or without BSA resulted in non-functional electrodes.

The immobilization pressure significantly influenced ($p < 0.01$) biosensor sensitivity. Compared to Table 1, cross-linking GOx after entrapment in PoPD films electropolymerized at 0.1 MPa had minor effect on the sensitivity. In contrast, cross-linking GOx after entrapment in PoPD films electropolymerized at 180 MPa decreased the sensitivity up to approximately four times, suggesting cross-linking with the enzyme whose conformation was changed by HHP hindered the access of the substrate to the enzyme. Unlike cross-linking before immobilization, the amount of GOx entrapped in PoPD could not have changed. The inter- or intra- cross-linking with GOx entrapped in PoPD didn't affect the activity of the enzymes if crosslinked at 0.1 MPa.

3.4. Effect of HHP during thermal inactivation on the stability of immobilized GOx

Calculated k_{inact} for the first 60 min and k_{inact} and k'_{inact} for the entire 180-min thermal treatment are shown in Table 4. The values of k_{inact} calculated based on the first 60 min and the entire 180 min are different. At longer treatment times, the pseudo-first-order model deviates from linearity as reflected by a lower R^2 and the better fit of the pseudo-second-order model. To compare with our previous report using soluble GOx, the pseudo-first-order model for the first 60 min of heating was used which corresponded approximately to the longest heating time by Halalipour, et al. [12]. Inactivation pressure had significant influence ($p < 0.01$) on the thermostability while immobilization pressure (0.1 or 180 MPa) had no significant ($p > 0.05$) effect. Tukey's method didn't find any significant difference between 180 MPa and 360 MPa treatments but both of them were significantly different from 0.1 MPa. The immobilized GOx inactivated at 180 MPa and 70 °C was 4.3 times more stable than when inactivated at 0.1 MPa and 70 °C (0 – 60 min).

Fig. 2 shows the pseudo-first-order plot of thermal inactivation with linear regression lines (0 – 180 min). There was an evident leveling-off in the trend when residual activity

decreased below ~15%, suggesting the pseudo-second-order model was a better fit. Fig. 2 also includes the calculated inactivation of soluble GOx inactivated at 0.1 or 180 MPa based on the kinetic parameters reported by Halalipour, et al. [12]. The calculated k_{inact} of GOx in solution was 536×10^{-3} min^{-1} at 70 °C and 0.1 MPa, and 36×10^{-3} min^{-1} at 70 °C and 180 MPa. Therefore, immobilization alone with k_{inact} $(26.0 \pm 4.1) \times 10^{-3}$ min^{-1} at 70 °C and 0.1 MPa (0 – 60 min) increased the thermostability of GOx by 20.6 times, which was similar to the effect of applying 180 MPa during inactivation for soluble GOx at 70 °C with the increase by 14.9 times. At 180 MPa and 70 °C, k_{inact} $(6.1 \pm 3.6) \times 10^{-3}$ min^{-1} of immobilized GOx (0 – 60 min) was 87.6 times smaller than the soluble GOx inactivated at 0.1 MPa and 70 °C. This suggested that there was a synergistic effect between immobilization and high pressure on the thermostability, since the combined stabilizing effect was bigger than the sum of the individual effects.

The stabilizing effect of immobilization on enzymes have been widely reported. As a method of immobilization, electropolymerization has been widely used to develop biosensors due to the easily controlled one-step process [19, 20], and was reported to be able to increase the thermal stability of the immobilized GOx [40, 53]. *o*-phenylenediamine is one of the non-conducting monomers used during electropolymerization, and a number of reports had adopted it to produce GOx biosensors and showed high storage stability with the response retained from 41% to 88% after 30 days storage at 4 °C [21-26]. Electropolymerization only physically entraps the enzymes without any chemical bond formation between the enzyme and the polymer. The increase in thermal stability by electropolymerization can be attributed to the spatial restriction of the enzyme inside the polymer which reduces enzyme unfolding at high temperatures [53].

The stabilizing effect of HHP on enzymes has been reported, but the mechanism of stabilization is unclear [54-57]. Pressures higher than 400-500 MPa can denature proteins while

low pressures result in reversible changes. Pressures above 200 MPa can change tertiary structures while pressures lower than 150-300 MPa affect quaternary structures [58]. High pressure decreases the volume of proteins following the Le Chatelier principle and compresses non-rigid internal voids through the displacement of helices [11, 59-61]. At moderate pressures, the protein is stabilized by increased Van der Waals forces resulting from compressed cavities [62]. At high pressures, the volume decrease is mainly from the penetration of water molecules into the cavities which causes the inactivation of proteins by weakening electrostatic and hydrophobic interactions and disturbing the tertiary structures [63-65]. An antagonistic effect of pressure and temperature on the inactivation of enzymes is often observed, and the native state of enzymes is favored at high temperatures and moderately high pressures [9, 65]. Pressures typically below 300 MPa can counteract the unfolding of proteins caused by high temperature to increase the thermal stability of enzymes. High temperature can cause the disruption of highly ordered structure of electrostricted water, resulting in the essential water loss when the thermal inactivation starts, which will lead to structural rearrangement and protein denaturation. High pressure, on the other hand, can strengthen the hydration of charged and non-polar groups, compress the protein hydration shell, and counteract the process from high temperature [9, 11, 65, 66]. High temperature weakens the intermolecular oxygen-hydrogen interactions, resulting in a softened hydrogen bond network. On the contrary, HHP strengthens the intermolecular oxygen-hydrogen interactions by shortening the distance between hydrogen bonds and thereby stabilizing them [9, 10]. High pressure can also strengthen the ion pairs which further stabilizes the tertiary and quaternary structures of enzymes [10].

The mechanism of the synergistic stabilizing effect of high pressure and immobilization against thermal treatment observed in this study is unclear. It is reasonable to hypothesize that

high pressure compresses surfaces, cavities, and hydrophobic regions while immobilization restricts protein unfolding and prevents enzyme aggregation. Also, compaction of the PoPD under HHP may contribute to the synergism. Nevertheless, this confirms that the stabilization mechanisms of the two treatments are different and complex. Structural analysis by fourier-transform infrared spectroscopy, fluorescence, or nuclear magnetic resonance spectroscopy are needed to elucidate the origin of the observed synergism. However, these studies are beyond the scope of this report.

4. Conclusions

Among the different methods tested, only applying HHP during thermal inactivation of immobilized GOx had a large increase in the thermostability. Cross-linking with GA at atmospheric pressure or at HHP had too small of an effect to be of practical significance. To the best of our knowledge, this was the first time reporting the synergistic effect of HHP and enzyme immobilization on improving the thermostability of enzymes. These combined stabilizing effects provided a new method to improve the biocatalysis of enzymes at high temperatures, which has the potential to be applied to other enzymes and result in economic saving in industry. However, the application of HHP to biosensor stability is not practical since its stabilization effect disappears after depressurization.

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Figure captions

Fig. 1. (A) Electrochemical cell connected to the cap of the high pressure reactor; (B) polytetrafluoroethylene cap with platinized platinum electrodes; (C) cyclic voltammograms of (— • • —) polished, (—) platinized, and (- - -) platinized and ultrasonicated electrodes.

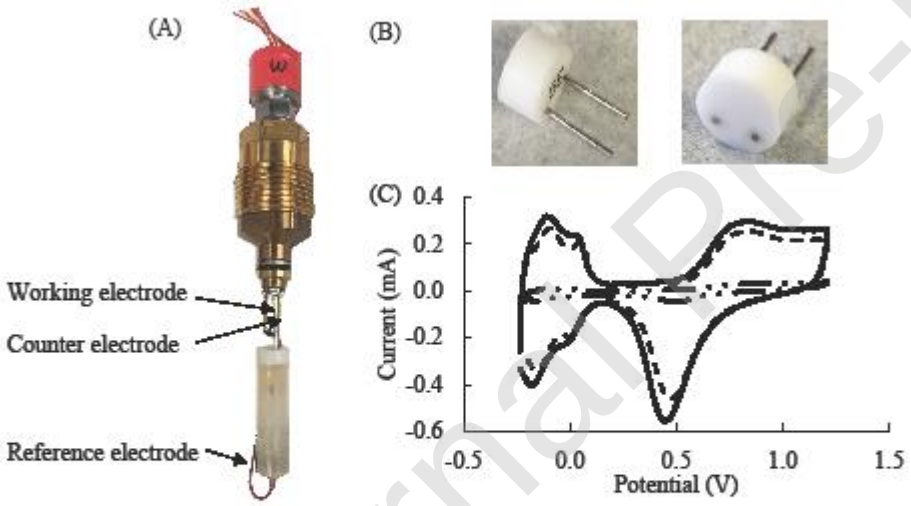

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Fig. 2. Inactivation of GOx immobilized in PoPD at 0.1 MPa and inactivated at 70 °C and 0.1 MPa (■), 180 MPa (◆), or 360 MPa (×), or immobilized at 180 MPa and inactivated at 70 °C and 180 MPa (▲). Inactivation of GOx in solution at 70 °C and 0.1 MPa (— • • —) or 180 MPa (— • —) was calculated based on the data reported by Halalipour, et al. [12]. Error bars represent ± standard deviations. Each treatment was done at least in triplicate.

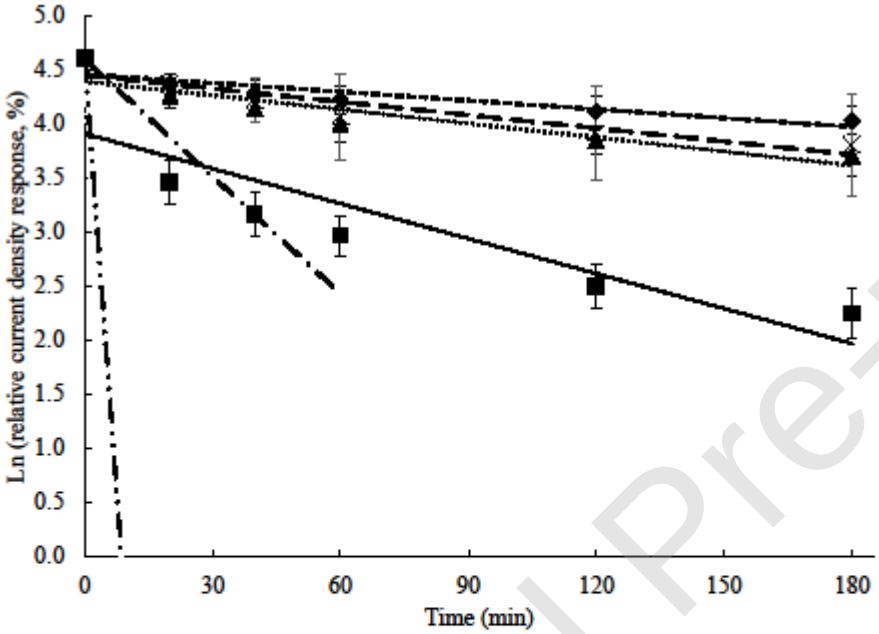

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Table 1. Sensitivity, apparent Michaelis-Menten constant, and pseudo-first-order and pseudo-second-order rate constant of inactivation of GOx biosensors immobilized at pressures from 0.1 to 420 MPa. The biosensors were inactivated at 70 °C and 0.1 MPa. The values are reported as mean ± 95% confidence interval.

Pressure (MPa)	Sensitivity ($\mu A\ mm^{-2}\ mM^{-1}$) × 10^3	K_m^{app} (mM)	k_{inact} (min^{-1}) × 10^3	R^2	k'_{inact} (min^{-1}) × 10^4	R^2
0.1	4.4 ± 0.9	28.4 ± 5.7	6.6 ± 1.0	0.84	2.1 ± 0.7	0.95
180	3.0 ± 1.4	38.6 ± 8.7	7.7 ± 1.4	0.85	3.3 ± 1.0	0.97
240	3.8 ± 2.2	35.2 ± 13.2	5.7 ± 1.7	0.84	3.1 ± 1.2	0.94
300	4.5 ± 2.2	34.6 ± 13.2	5.6 ± 1.7	0.86	2.7 ± 1.2	0.98
420	4.4 ± 3.7	21.2 ± 22.9	7.6 ± 2.9	0.92	1.9 ± 2.0	0.98

No significant differences ($p < 0.05$) were found in means of sensitivity, K_m^{app}, k_{inact}, or k'_{inact}.

Table 2. Sensitivity, apparent Michaelis-Menten constant, and pseudo-first-order and pseudo-second order rate constant of inactivation of GOx biosensors. Glucose oxidase was cross-linked with 0.5% (w/v) glutaraldehyde at 0.1 or 180 MPa with or without the addition of BSA before electropolymerization at 0.1 or 180 MPa. The biosensors were inactivated at 70 °C and 0.1 MPa. The values are reported as mean ± 95% confidence interval.

Immobilization pressure (MPa)	Cross-linking pressure (MPa)	Cross-linking reaction time (h)	BSA added (Y/N)	Sensitivity ($\mu A\ mm^{-2}\ mM^{-1}$) × 10^3	K_m^{app} (mM)	k_{inact} (min^{-1}) × 10^3	R^2	k'_{inact} (min^{-1}) × 10^4	R^2
0.1	0.1	6	N	9.3^{abc} ± 0.9	21.2^{f} ± 10.4	9.0 ± 1.6	0.84	3.0 ± 1.4	0.97
0.1	180	6	N	7.5^{bcde} ± 0.9	31.7^{fgh} ± 10.4	8.1 ± 1.6	0.84	2.4 ± 1.4	0.98
0.1	0.1	12	N	8.6^{abcd} ± 0.9	24.2^{fg} ± 10.4	7.5 ± 1.6	0.84	2.1 ± 1.4	0.97
0.1	180	12	N	6.8^{de} ± 0.9	21.9^{f} ± 10.4	7.7 ± 1.6	0.80	2.3 ± 1.4	0.93
180	0.1	6	N	9.6^{ab} ± 0.9	37.8^{fgh} ± 10.4	9.9 ± 1.6	0.86	3.9 ± 1.4	0.99
180	180	6	N	5.6^{e} ± 0.9	46.6^{gh} ± 10.4	9.4 ± 1.6	0.81	3.6 ± 1.4	0.98
180	0.1	12	N	9.6^{a} ± 0.9	50.0^{h} ± 10.4	9.1 ± 1.6	0.84	3.2 ± 1.4	0.98
180	180	12	N	7.3^{cde} ± 0.9	35.4^{fgh} ± 10.4	10.2 ± 1.6	0.70	4.8 ± 1.4	0.91
0.1	0.1	1	Y	8.5^{i} ± 3.6	18.0^{j} ± 17.8	7.6 ± 1.8	0.81	2.3 ± 1.8	0.97

0.1	180	1	Y	$7.5^{i} \pm 3.6$	$27.7^{j} \pm 17.8$	9.6 ± 1.8	0.75	3.7 ± 1.8	0.95
180	0.1	1	Y	$7.6^{i} \pm 3.6$	$40.1^{j} \pm 17.8$	9.1 ± 1.8	0.82	3.5 ± 1.8	0.99
180	180	1	Y	$7.2^{i} \pm 3.6$	$28.2^{j} \pm 17.8$	10.7 ± 1.8	0.77	5.1 ± 1.8	0.96

No significant differences ($p < 0.05$) were found in means of k_{inact} or k'_{inact} in the absence of BSA or not.

[a-e]Differences in means of sensitivity with different letter without adding BSA were significant ($p < 0.05$).

[f-h]Differences in means of K_m^{app} with different letter without adding BSA were significant ($p < 0.05$).

[ij]No significant differences ($p < 0.05$) were found in means of sensitivity or K_m^{app} when the BSA was added.

Table 3. Sensitivity, pseudo-first-order and pseudo-second-order rate constant of inactivation of GOx biosensors inactivated at 70 °C and 0.1 MPa. Glucose oxidase was immobilized at 0.1 or 180 MPa and then cross-linked with 2.5 or 5% (w/v) glutaraldehyde at 0.1 MPa for 10 or 30 min. The values are reported as mean ± 95% confidence interval.

Immobilization pressure (MPa)	GA concentration (%)	Cross-linking reaction time (min)	Sensitivity ($\mu A\ mm^{-2}\ mM^{-1}$) × 10^3	k_{inact} (min^{-1}) × 10^3	R^2	k'_{inact} (min^{-1}) × 10^4	R^2
0.1	2.5	10	4.2^{a} ± 1.2	3.3 ± 1.5	0.95	0.5 ± 0.4	0.98
0.1	5	10	3.0^{ab} ± 1.2	4.6 ± 1.5	0.91	0.9 ± 0.4	0.97
180	2.5	10	1.4^{b} ± 1.2	5.0 ± 1.5	0.91	0.9 ± 0.4	0.95
180	5	10	1.1^{b} ± 1.2	3.7 ± 1.5	0.86	0.7 ± 0.4	0.91
0.1	2.5	30	4.1^{a} ± 1.2	4.8 ± 1.5	0.92	1.0 ± 0.4	0.98
180	2.5	30	1.6^{ab} ± 1.2	3.9 ± 1.5	0.82	0.7 ± 0.4	0.90

180	5	30	$2.0^{ab} \pm 1.2$	4.9 ± 1.5	0.87	1.0 ± 0.4	0.96

No significant differences ($p < 0.05$) were found in means of k_{inact} or k'_{inact}.

[a-b]Differences in means of sensitivity with different letter were significant ($p < 0.05$).

Table 4. Pseudo-first-order and pseudo-second-order rate constant of inactivation of GOx biosensors immobilized at 0.1 or 180 MPa. The biosensors were inactivated at 70 °C and 0.1 to 360 MPa. The values are reported as mean ± 95% confidence interval.

Immobilization pressure (MPa)	Inactivation pressure (MPa)	k_{inact} (min^{-1}) $\times 10^3$ (0 – 60 min)	R^2	k_{inact} (min^{-1}) $\times 10^3$ (0 – 180 min)	R^2	k'_{inact} (min^{-1}) $\times 10^4$ (0 – 180 min)	R^2
0.1	10	$26.0^a \pm 4.1$	0.84	$11.0^a \pm 1.4$	0.77	$5.2^a \pm 0.8$	0.98
0.1	180	$6.1^b \pm 3.6$	0.89	$2.6^b \pm 1.3$	0.83	$0.4^b \pm 0.7$	0.89
0.1	360	$8.8^b \pm 4.7$	0.91	$4.0^b \pm 1.6$	0.86	$0.8^b \pm 0.9$	0.93
180	180	$9.6^b \pm 4.7$	0.92	$4.3^b \pm 1.6$	0.84	$0.8^b \pm 0.9$	0.92

[a-b]Differences in means of k_{inact} or k'_{inact} with different letter within each column were significant ($p < 0.05$).