



Short communication

Strip-based amperometric detection of myeloperoxidase

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ABSTRACT

The development of a screen-printed strip-based amperometric biosensor for the determination of myeloperoxidase (MPO) levels is reported. The biosensor utilizes 3,3',5,5'-tetramethylbenzidine (TMB) as a redox mediator to enable high-sensitivity quantification of physiological levels of MPO. A multivariate parameter optimization was performed. Under the optimal conditions, physiological levels of MPO between 3 and 18 U/L were detected in both acetate buffer (pH 4.5) and human serum using flexible screen-printed electrodes (SPE). The potential interference generated by common serum-based electroactive compounds and a similar peroxidase enzyme was also investigated. The proposed detection methodology offers a simpler, more rapid, and cost-effective alternative to conventional MPO immunoassays, thereby leading to further development in point-of-care testing of acute cardiac events.

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1. Introduction

Immediate and reliable diagnostic information regarding unpredicted cardiovascular events is crucial for providing timely, life-saving therapeutic intervention. However, identifying patients with cardiac injury remains a primary challenge. Major emphasis has thus been given to point-of-care testing that provides a rapid means for early diagnosis (Brogan and Bock, 1998). Although advanced diagnostic tools such as the electrocardiogram offer a wealth of information regarding the cardiac pathophysiology (Zimetbaum and Josephson, 2003), this technique is not amenable to point-of-care testing. Bioassays, however, show the promise for improved point-of-care evaluation and decision making for the diagnosis of acute cardiac events (Hamm et al., 1997). Despite extensive development in the field of bioassays, current assay tests performed in the hospital are still costly and time-consuming. A high-fidelity, low-cost point-of-care assay offering rapid results could enable a faster and better-suited treatment, thereby leading to decreased mortality rates from life-threatening cardiovascular events.

Cardiovascular disorders manifest a wide range of symptoms and characteristics, hence providing a challenging domain for

diagnostics. Particular attention has been given to the development of blood tests for detecting injury to the heart muscle at the early stages among people with chest discomfort and for the diagnosis of acute coronary syndromes (ACS) (Wu et al., 1999). Myeloperoxidase (MPO), a heme peroxidase has been given much attention as a prognostic indicator of suspected ACS (Morrow, 2007). MPO catalyzes the conversion of hydrogen peroxide and chloride ion to hypochlorous acid. As a pro-inflammatory enzyme biomarker, MPO becomes elevated upon neutrophil activation resulting from inflammation caused by eroded or ruptured arterial lesions (Brennan et al., 2003). Increased serum levels of MPO (11.9 U/L vs. 6.6 U/L under normal cardiovascular conditions) have been shown to precede cardiac events such as acute myocardial infarction (AMI) (Brennan et al., 2003) even in the absence of established indicators of myocardial necrosis (Baldus et al., 2003). Moreover, increased serum MPO has been shown to predict an elevated risk for subsequent cardiovascular events among patients with ACS and as a risk indicator for long-term mortality following AMI (Mocatta et al., 2007).

Numerous MPO immunoassays have been reported (Suzuki et al., 1983; Graff et al., 1998; Babu et al., 2009; Lin et al., 2010) and are commercially available. These assays require long incubation times and entail complex, labor-intensive preparation and analysis. A strip-based sensor, analogous to those used for blood glucose monitoring (Wang, 2008), would allow for the immediate diagnosis of life-threatening acute cardiac events and facilitate timely thera-

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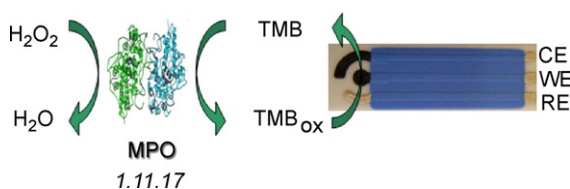


Fig. 1. Schematic representation of the enzymatic detection of MPO using screen-printed electrode (SPE).

peutic intervention, which will ultimately lead to increased survival rates.

The present communication reports the development of a strip-based assay for the amperometric detection of physiologically relevant levels of MPO. The assay employs 3,3',5,5'-tetramethylbenzidine (TMB) as a redox mediator for the detection of MPO in the presence of a peroxide substrate (Fig. 1). The results demonstrate the feasibility of employing simple electrochemical techniques for the direct and highly-sensitive detection of MPO.

2. Materials and methods

2.1. Chemicals and reagents

Myeloperoxidase from human leukocytes (E.C. 1.11.1.7; 250–300 U/mg) was purchased from Alexis Biochemicals (San Diego, CA). Ascorbate oxidase from *Cucurbita* sp. (E.C. 1.10.3.3), glutathione peroxidase from bovine erythrocytes (E.C. 1.11.1.9), other reagents and serum from human male (Type-AB) were purchased from Sigma–Aldrich and were used as supplied. Ultra pure deionized water (18.2 MΩ cm) was used in all experiments.

Acetate buffer solutions (0.1 M) with pH between 3.0 and 6.0 were prepared in deionized water. In order to comply with the 50 μL electrode reservoir, 39.7 μL acetate buffer (0.1 M), 3.0 μL H₂O₂ (19.6 mM), and 6.3 μL TMB (4.8 mM) along with 1.0 μL MPO solution (diluted to yield physiological levels) were mixed, incubated in a vial, and later dispensed in the electrode reservoir. This protocol was followed throughout the optimization experiments. Calibration curves were performed in acetate buffer and serum. The calibration performed in buffer followed the same protocol as used for the optimization. In the case of serum samples, 0–18 U/L MPO in 40.7 μL of serum was used to perform the calibration.

2.2. Instrumentation and electrode fabrication

A CH Instruments model 630C potentiostat was used for all electrochemical measurements. A Thermo Scientific NESLAB RTE-7 temperature-controlled circulating bath was employed for the temperature characterization. The fabrication of the flexible SPE is described in the literature (Chuang et al., 2010). The screen-printed three-electrode strip (Fig. 1) consisted of a circular carbon working electrode (area: 3 mm²) inscribed in carbon hemispherical counter (area: 10 mm²) and Ag/AgCl reference electrodes (area: 2 mm²).

3. Results and discussion

As a basis for the experimental realization of highly sensitive detection of MPO, several parameters including the applied potential, pH, concentration of TMB, incubation time, and temperature were investigated and optimized. The optimized parameters were utilized in calibration measurements employing physiological levels of MPO. Common interferences in serum were also investigated in the context of MPO detection fidelity. The results are as follows.

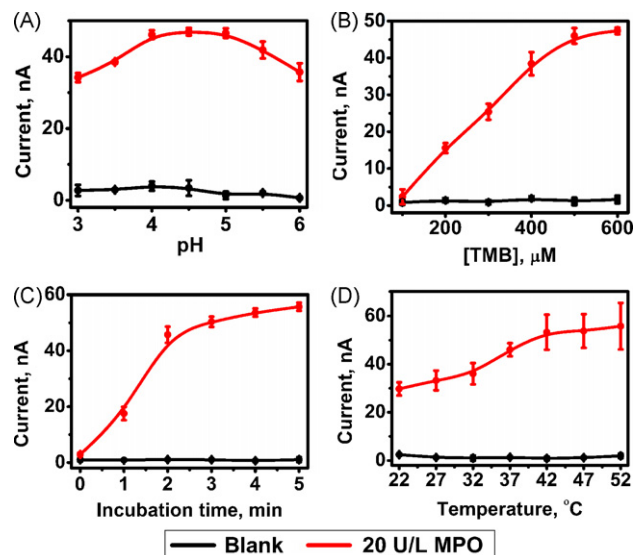


Fig. 2. Effect of (A) pH, (B) concentration of TMB, (C) incubation time, and (D) temperature on the detection of MPO (20 U/L) at SPE. All experiments were conducted in 0.1 M acetate buffer with an applied potential of 0 mV (vs. Ag/AgCl) and 1.2 mM of H₂O₂.

3.1. Effect of applied potential

In order to determine the precise TMB reduction potential, the applied potential was scanned from –200 to +200 mV in 50 mV increments in the acetate buffer containing 0.6 mM TMB and 1.2 mM H₂O₂. At an applied potential of 0 mV, a highly sensitive current response for the reduction of the oxidized TMB product was observed. Therefore, this reduction potential was selected to enable low-noise detection.

3.2. Parameter optimization

With the reduction potential established at 0 mV, chronoamperograms were recorded for acetate buffer solutions with pH values of ranging from 3.0 to 6.0 in 0.5 increments. For each buffer pH, current readings were extracted at the 10th sec following the application of 0 mV for both blank (0 U/L MPO) and 20 U/L MPO concentrations, as shown in Fig. 2A. The current response exhibited a negative parabolic profile with a maximum at pH 4.5. The decrease in the current response profile at pH values greater than 5 can be attributed to the instability of the TMB oxidation product near neutral pH values (Suzuki et al., 1983). Accordingly, a pH 4.5 acetate buffer was selected for further measurements.

Subsequent to pH studies, the concentration of TMB was varied between 100 and 600 μM in 100 μM increments with a fixed concentration of H₂O₂ (1.2 mM). Chronoamperograms were recorded and a sampling time of 10 s was established. An increase in the magnitude of the current response was obtained for TMB between 100 and 500 μM, as elucidated in Fig. 2B. The onset of saturation in the current response was observed at 600 μM. Due to the diminishing returns afforded by increasing the level of TMB beyond 600 μM, ensuing experiments were performed with this concentration of TMB.

To determine the reaction duration that resulted in the optimal current response, the reagents (including 20 U/L MPO) were mixed and stored in a vial for 0–5 min (in 1 min increments). Once the desired incubation time transpired, the sample was dispensed on the electrode surface and chronoamperometry was performed. As illustrated in Fig. 2C, an increase in the reduction current was observed for reaction durations from 0 to 2 min. However, once the

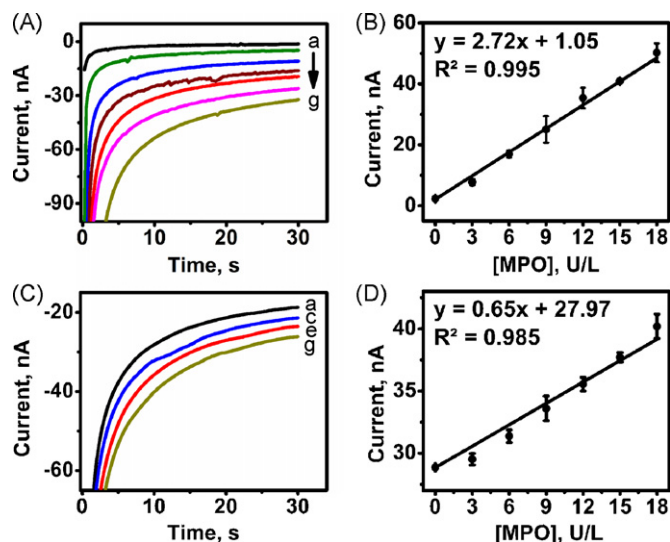


Fig. 3. Chronoamperograms recorded for the detection of MPO in (A) 0.1 M acetate buffer (pH 4.5) and (C) human serum at SPE; (B) and (D) show the calibration curves for MPO detection in buffer and serum, respectively; a → g: 0 to 18 U/L MPO (3 U/L increments). For clarity, only the 0, 6, 12 and 18 U/L MPO signals are displayed in (C). Experiments were conducted at 37 °C with 600 μ M of TMB, 1.2 mM of H_2O_2 , 2 min incubation time, and an applied potential of 0 mV (vs. Ag/AgCl).

2 min reaction duration was attained, the current response saturated and further escalating the incubation time had little effect on the current response. Thus, an incubation time of 2 min was chosen for subsequent investigations.

Employing a temperature-controlled recirculating water bath, the reaction temperature was increased from 22 °C to 52 °C in 5 °C increments. All reagents were incubated in this bath following preparation and allowed to reach the desired temperature. Once all the compounds to be analyzed were mixed, the solution was placed in a vial and incubated in the bath for two min prior to being dispensed on the electrode surface. Chronoamperograms were subsequently recorded and the results of these measurements (current sampled at $t = 10$ s) are shown in Fig. 2D. The measured current response increased from 22 °C to 37 °C. However, the current response saturated at temperatures of 42 °C and greater.

As indicated from Fig. 2A–D, the measured background (blank) current was independent of TMB concentration, incubation time and temperature, although it displayed a slight pH dependence, as expected from the optimal assay conditions of TMB (Suzuki et al., 1983).

3.3. Calibration in buffer solution

With the above mentioned optimization complete, all parameters were established at their respective optimal values and a calibration was performed between 0 and 18 U/L MPO to emulate circulating concentrations of the enzyme in human serum. Acetate buffer solutions (pH 4.5) containing varying concentrations of MPO were mixed with TMB and H_2O_2 , and allowed to incubate at 37 °C for 2 min prior to dispensing on the electrode surface. A chronoamperogram was recorded for 30 s for the blank buffer solution and for each MPO concentration. The corresponding current–time signals are shown in Fig. 3A. The current was sampled at $t = 10$ s, and a linear regression shows a high degree of correlation among increasing levels of MPO with a correlation coefficient of 0.995 ($n = 3$). Extrapolating from the regression line intercept, the noise level was 1.05 nA and the regression line slope indicated a sensitivity of 2.72 nA (U/L) $^{-1}$ (Fig. 3B). The detection limit of MPO was determined to be 0.4 U/L when considering a three standard deviation ($3\text{-}\sigma$) separation from the blank buffer solution.

3.4. Interference investigation

An interference study was conducted whereby the common serum-based compounds ascorbic acid (AA) and uric acid (UA) were employed to study their effect on the detection of MPO. The presence of 0.4 mM UA resulted in a slight deviation in the measured current of 20 U/L MPO in buffer while 0.1 mM AA yielded more than 100% deviation in the MPO signal. As such, AA served to directly reduce the TMB oxidation product generated by the presence of MPO. The study indicates that, even when adhering to the optimal conditions discussed earlier, the performance of the biosensor was noticeably altered by the presence of physiological concentrations of AA. This interference can be rectified through the inclusion of the enzyme ascorbate oxidase, AOx (Anzai et al., 1998). Consequently, further investigation into the remedial properties of AOx in the assay is warranted.

In order to mitigate the deleterious effect of AA, acetate buffer solutions (pH 4.5) were spiked with elevated levels of the circulating compounds under investigation: MPO (20 U/L) and AA (0.1 mM). The level of AOx in the buffer sample was optimized until the contribution of AA to the reduction current was negated. 650 U/L was identified as the most effective AOx level and this quantity was employed for further investigations. As in the calibration experiment, the reagents were incubated at 37 °C for 2 min prior to dispensing on the electrode surface and chronoamperograms were recorded for 30 s. The current–time signals are shown in Fig. S1(A) (see Supplemental information) for the assay with 20 U/L MPO (baseline) and the assay with 20 U/L MPO, 0.1 mM AA, and 650 U/L AOx (current sampled at 10th sec). As indicated from Fig. S1(B) (see Supplemental information), the response deviated by less than 15% from the baseline (MPO alone) when 0.1 mM AA and 650 U/L AOx were included in the assay. A low standard deviation of 3.4% ($n = 3$) from the mean value was obtained in these measurements. As such, sensitive detection of relevant levels of MPO in buffer using TMB was achieved even in the presence of a potent reducing agent such as AA.

In addition to a close examination of serum-based electroactive interferents such as AA and UA, the effect of other peroxidases that exist in the blood (Passardi et al., 2007) was also explored. Given that peroxidases catalyze the reduction of H_2O_2 in the presence of a reducing substrate, many of these enzymes exhibit similar catalytic functionalities as MPO and therefore may be able to displace this compound in enzymatic detection schemes such as the one proposed. When multiple peroxidases are present (such as in serum), H_2O_2 will be consumed by each of these enzymes. However, by employing a sufficient concentration of H_2O_2 (1.2 mM) as in the aforementioned assay protocol, the interference effect of other peroxidases can be mitigated. In the present investigation, as an example, a human serum sample was spiked with a physiological level of glutathione peroxidase (GPx), a common antioxidant enzyme found in the blood at relatively high levels (676 U/L) (Guemouri et al., 1991). The measured current from the chronoamperometric experiments resembled baseline readings, thereby indicating that the presence of GPx could not be detected when employing the conditions used for MPO detection. Subsequently, a serum sample was spiked with both GPx and MPO (at physiological levels) in order to verify that TMB demonstrated specificity towards MPO alone. The measured current in this experiment was in close agreement with MPO signal obtained earlier and did not show an “additive” effect, thus confirming that the presence of GPx did not interfere with the detection of MPO. Reports have also indicated that Ellman’s Reagent can be utilized to react with the thiol groups of the glutathione substrate, thereby inhibiting the catalytic oxidation of TMB (Sedlak and Lindsay, 1968).

Although the results imply that amperometry could lead to straightforward detection of MPO, the presence of other electroac-

tive compounds and high levels of peroxidases (categorized under the E.C. 1.11.1.7 family) in serum could potentially interfere with the amperometric detection of this enzyme. Accordingly, the electrochemical detection of MPO should only be considered when it is evaluated in conjunction with other well-established prognostic indicators of acute myocardial conditions.

3.5. Detection of MPO in human serum

In order to demonstrate the practical application of the new method, a calibration study was also performed in human serum. Serum samples were spiked with physiological levels of MPO (ranging from 0 to 18 U/L), and the corresponding chronoamperograms were recorded. The corresponding current signals and the calibration curves are shown in Fig. 3C and D, respectively. The current was sampled at 10th sec following the initialization of the measurement. As with the buffer experiments, a favorable response is observed for these MPO increments, leading to a linear response with a correlation coefficient of 0.985 ($n=3$). The noise level and sensitivity of the experiment were 27.97 nA and 0.65 nA, respectively. In this case, a limit of detection of 0.6 U/L was achieved (using the 3- σ methodology).

4. Conclusions

An electrochemical biocatalytic scheme for detecting MPO has been presented. A multivariate parameter optimization for this electrochemical strip-based assay was performed. Such optimization ensured effective amperometric detection of physiological levels of MPO at SPE in buffer and human serum using TMB as a redox mediator. It is anticipated that an inexpensive and easy-to-operate strip-based sensor, such as the one presented here, will yield a more rapid diagnosis than current methods provide, thereby leading to improved management of acute coronary syndromes. However, prior to its use as a diagnostic tool, extensive studies must be performed to examine and address all the potential interferences in relevant clinical serum samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.07.031.

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