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A novel biosensor for the detection and monitoring of β-d-galactosidase of faecal origin in water

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Abstract

A voltammetric sensor prepared by the immobilization of metallophthalocyanine complexes onto a glassy carbon electrode has been developed for the detection of β-d-galactosidase (B-GAL) of faecal origin in water. Electrooxidation of chlorophenol red, a breakdown product of the chromogenic substrate chlorophenol red β-d-galactopyranoside, was used as a measure of β-d-galactosidase activity. At metallophthalocyanine modified electrodes, in particular copper(II) phthalocyanine, a decrease in electrode fouling was observed. The sensor was sensitive to fluctuations in pH, not significantly affected by temperature variations and could detect one colony forming unit/100 mL in 15 min. Loss of 40% sensitivity was observed over a period of 30 days. A strong correlation between sensor sensitivity and colony forming units was observed. The sensor is capable of detecting viable but nonculturable bacteria, overcoming this drawback of the use of culture media for detection of coliforms.

1. Introduction

It is required by public health and environmental protection units globally that drinking water is safe from microbial contamination. The coliform group of bacteria has been used extensively as an indicator of water quality [1], [2] and [3]. Most coliforms are present in large numbers amongst the intestinal flora of humans and other warm-blooded animals, thus readily found in faecal matter [1]. As a result, coliforms detected in water intended for drinking and recreational purposes are used as an index of the potential presence of enteropathogens [1].

The rapid, sensitive and reliable screening for bacterial contamination in drinking water is fundamental to the detection and thus prevention of infection. The routine detection methods for microorganisms are based on a count of colony forming units (CFU) and require selective culture, biochemical and serological characterizations. Although bacterial detection by this method is sensitive and selective, time-spans ranging from days to weeks are required to confirm results. These methods are also costly and time-consuming [4].

β-d-galactosidase (B-GAL), an enzyme produced by coliforms, catalyses the breakdown of lactose into galactose and glucose, and has been used for enumerating this group of bacteria [5], [6] and [7].

Chromogenic and fluorogenic substrates produce colour and fluorescence upon breakdown by specific enzymes. Bacterial diagnosis based on enzymes has attracted extensive research over the years [8]. Several chromogenic substrates are available for such purposes [9]. The chromogene chlorophenol red β-d-galactopyranoside (CPRG) (yellow in colour) can be broken down by the enzyme β-d-galactosidase into chlorophenol red (CPR) (red in colour) [8] and [10] (Fig. 1).

Fig. 1. Conversion of CPRG (yellow) to CPR (red) by the enzyme β-d-galactosidase (B-GAL).

Electrochemical approaches have been investigated for the detection and monitoring of microbial contamination [11] and [12] and have been successfully employed in the detection and or monitoring of some phenolic compounds in water [13], [14], [15] and [16]. Most phenols are readily oxidized at accessible potentials [13]. No literature was found on the electrochemical detection of CPR as a measure of coliform activity.

In general, electrooxidation of phenolic compounds results in the formation of radical intermediates that dimerise or polymerise at electrode surfaces. These oxidation products are problematic as they lead to the passivation of the electrode [17] and [18]. Increased sensitivity in the detection of phenols, as well as a lowering of the fouling of the electrode can be obtained through the use of catalysts such as metallophthalocyanine (MPc) complexes [13].

Although research in the development of sensors for the direct or indirect microbial detection has attracted interest over the years, there is no practical sensor to satisfy market requirements, such as short analysis time, cost-effectiveness, high sensitivity and real time online monitoring [4].

In this work, we examine the electrochemical detection and or monitoring of CPR as a sensitive measure of B-GAL activity from bacterial coliform contamination of water by the use of a range of MPc complexes.

2. Materials and methods

2.1. Materials

Sodium phosphate buffer (0.1 M, pH 7.2) prepared with a Milli-Q system (18 MΩ cm) (Millipore, Milford, CT, USA) purified water was used for all electrochemical analysis. Phthalocyanine metal complexes of cobalt(II) (CoPc), copper(II) (CuPc), zinc(II) (ZnPc), nickel(II) (NiPc), tetrasulphonated metal complexes (MTSPc) of copper(II) (CuTSPc), cobalt(II) (CoTSPc), chlorophenol red β-d-galactopyranoside and chlorophenol red were purchased from Sigma-Aldrich (Deisenhofen, Germany). All the reagents were of analytical grade.

2.2. Cyclic voltammetry and construction of MPc-modified glassy carbon working electrode (GCE)

Cyclic voltammetry experiments were performed using a BAS Epsilon EC-200-XP (Bio-Analytical Systems, Inc., USA). All measurements were carried out in a glass cell with a conventional three-electrode configuration at a scan rate of 100 mV s^{-1}. A glassy carbon working electrode with a surface diameter of 3 mm was used together with a platinum wire counter electrode and a silver/silver chloride (3 mol dm^{-3} KCl) reference electrode. Prior to modifying the GCE with metallophthalocyanine (MPc) complexes, the GCE was polished with alumina on a Buehler felt pad, rinsed with ethanol, followed by water, dilute nitric acid and a further rinse in distilled water.

Solutions of phthalocyanine complexes (1 mM) were prepared in dimethylformamide (DMF) (HPLC grade). The tetrasulphonated MPcs were dissolved in distilled water. Ten microliters of each sample was dropped onto the surface of the GCE and allowed to air dry at room temperature for about 30 min. Cyclic voltammograms of standard CPRG, CPR and the phenolic compounds tested were collected in the presence and absence of the MPc catalysts examined. Triplicate results were obtained in each case unless otherwise stated.

2.3. Effect of temperature and pH on MPc-modified GCE

The effect of temperature on the analysis of CPR was investigated by recording CVs at different temperatures between 5 and 60 °C. The temperature of the reaction cell was maintained at the appropriate level after attaining the required temperature for about 15 min to stabilize, after which a CV was recorded. The pH dependency of the sensor was also evaluated within the pH range 4.0–10.0, using sodium citrate buffer (pH 3.0–6.0, 0.1 M), sodium phosphate buffer (pH 7.0–8.0, 0.1 M) and carbonate bicarbonate buffer (pH 9.0–11.0, 0.1 M).

2.4. Stability of the MPc-modified GCE

Single daily readings were taken with the unmodified and MPc-modified glassy carbon electrodes for 30 days. The electrodes were then rinsed with water and stored at 4 °C. The electrodes were allowed to reach room temperature for about 15 min before the CV was recorded.

In order to determine the effect of electrode fouling on the sensor response, consecutive CV scans of CPR were recorded at the CuPc modified GCE as well as at the unmodified GCE.

2.5. Correlation of CFU/100 ml and electrochemical detection based on the least time required for detection

Polluted water samples were collected from and around Grahamstown, Eastern Cape, South Africa, for this study. One hundred milliliters water samples were filtered through a membrane (Whatmann, 0.45 μm pore size) and placed on CM 1047 (Oxoid media) and incubated at 37 °C for 18–24 h after which the number of CFUs were counted. Aliquots of the corresponding water samples (4 ml of water sample, 5 ml of phosphate buffer and 1 ml of the CPRG) were mixed in the glass cell for electrochemical analysis. Readings were taken every min for the first 15 min after which 5 min time intervals were allowed between subsequent readings.

2.6. Effect of environmental water samples on the sensitivity of the sensor in detecting CPR

In order to determine the effect of compounds that may be in the environmental water sample on the sensor response for CPR detection, different volumes (0–50% of the total reaction volume) were analysed for electrochemical detection. The volume differences were made up with the buffer to maintain a constant final volume.

2.7. Selectivity and sensitivity of sensor to some phenolic compounds

The selectivity and sensitivity of the sensor in detecting phenolic compounds that may be present in the environment were evaluated. Equimolar concentrations of chlorophenol red, 4-chloro-3-methyl phenol; 2,4-dichlorophenol; phenol; 2-nitrophenol; 3-nitrophenol; 2,4-dinitrophenol; 2,4,6-trinitrophenol and 2-chlorophenol were studied.

2.8. Correlation between CFU/100 ml and sensor detection of CPR

A total number of 35 water samples were collected from various sources in the Eastern Cape, South Africa for analysis. CFUs and electrochemical analysis were determined as described in Section 2.5.

3. Results and discussion

3.1. Detection of CPR at MPc modified electrodes

An oxidation wave at 0.72 V was observed for standard CPR as well as CPR generated as a result of the enzymatic breakdown of CPRG by β-d-galactosidase. The oxidation wave attributed to CPR was observed with no interference from CPRG and the enzyme at a GCE as shown in Fig. 2. Increases in current response for detection of CPR were observed at MPc modified electrodes compared to a bare GCE. The sensor response was dependent on the metal as well as the substituents on the phthalocyanine ring with increases in detection of CPR, relative to the bare GCE, ranging from 21% for NiPc to 147% for CuTSPc, in the following order: CuTSPc > ZnPc > CoTSPc > CuPc > NiPc.

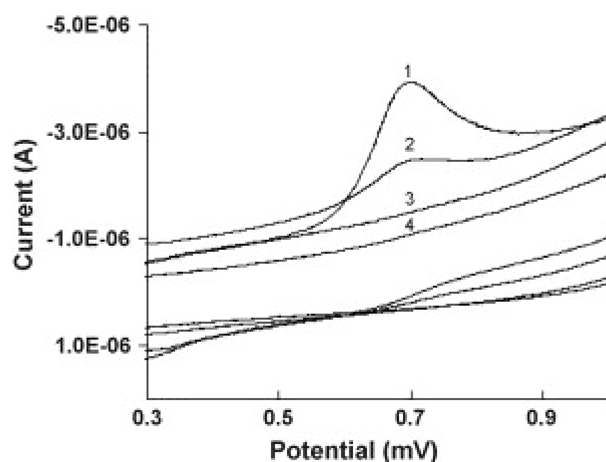

Fig. 2. Comparative cyclic voltammograms (CVs) of (1) standard CPR, (2) CPRG and B-GAL in buffer (peak due to partial breakdown of CPRG to CPR), (3) standard CPRG, and (4) sodium phosphate buffer (0.1 M, pH 7.2).

3.2. Effect of temperature and pH on MPc-modified GCE

Electrodes modified with MPcs were generally sensitive to pH changes for detection of CPR. A reduction in sensitivity from acidic pH to alkaline pH was observed. CuTSPc was however more stable with minimum change of sensitivity (about 17%) as compared with ZnPc, where the relative sensitivity changed from about 70% at pH 10 to 125% at pH 4.0 (Fig. 3).

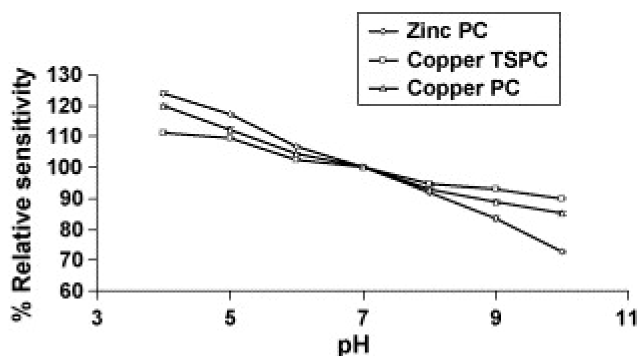

Fig. 3. Effect of pH variation on MPc-modified GCE for detection of 0.004% CPR. A value of 100% was assigned to analyses conducted at pH 7.0. Data points represent mean ± S.D.

The selected MPcs were variable in their responses to temperature fluctuations. ZnPc was the most susceptible to temperature changes while CuTSPc was the most stable (Fig. 4). A 7% change in sensitivity (for ZnPc) between 15 and 60 °C is not significant, thus the MPc sensors were not subject to sharp changes in sensitivity with temperature variation (Fig. 4).

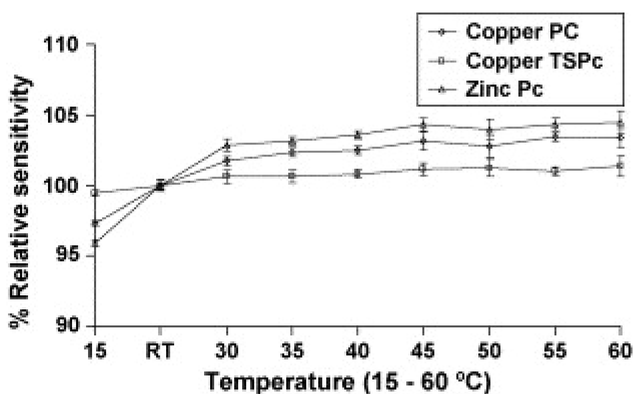

Fig. 4. Effect of temperature on MPc-modified GCE for detection of 0.004% CPR. A value of 100% was assigned to analyses conducted at room temperature (RT: 20 ± 2°C). Data points represent mean ± S.D.

3.3. Stability of MPc-modified GCE

As observed from Fig. 5, the CuPc-modified electrode for CPR detection was the more stable over the 30-day study period retaining 40% of its sensitivity over the period. There was minimal change in sensitivity over the first 5 days of scanning. The least stable was unmodified GCE, with 80% loss of sensitivity in 7 days.

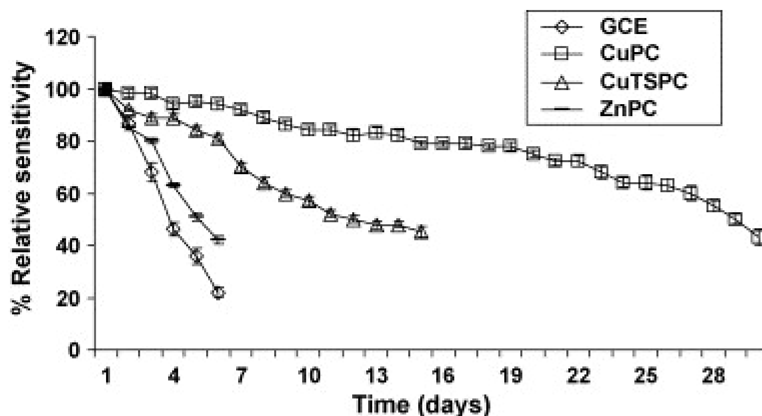

Fig. 5. Stability of MPc-modified and unmodified GCE over a 30-day period for detection of 0.04% CPR in 0.1 M sodium phosphate buffer pH 7.2. Data points represent mean ± S.D.

Unlike with unmodified GCE where only a maximum of five scans are possible consecutively, it was possible to perform 80 consecutive scans upon immobilizing CuPc onto the glassy carbon electrode for detection of CPR. The first 10 scans maintained 100% sensitivity, after which there was a subsequent reduction in sensitivity until the 50th scan when results became inconsistent (Fig. 6). Beyond the 50th scan, it was observed that the peak currents fluctuated.

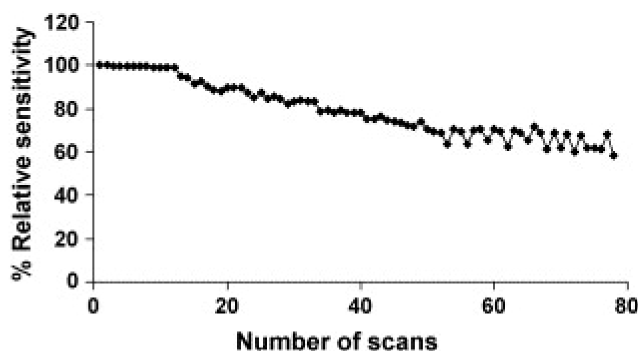

Fig. 6. Consecutive multi-scans with CuPc-modified GCE for detection of 0.004% CPR in sodium phosphate buffer pH 7.2. Data points represent mean ± S.D.

It has been suggested that the decrease in fouling observed in the presence of the MPc catalyst is due to the minimization of the adsorption of the oxidation products onto the electrode as result of steric hindrance caused by the MPc [13]. This may be the case in our studies given the size of the CPR compared to phenols examined in that study. However, the absence of fouling within the first ten successive scans suggests that other factors may increase the resistance to fouling. It is possible that intramolecular rearrangement may occur following hydrogen abstraction without the formation of complex polymeric structures believed to foul the electrode. However, further analysis by spectroscopic methods of the oxidation products formed is required for accurate characterization of these products.

3.4. Correlation between CFU/100 ml and electrochemical detection based on the least time required for detection

While at the unmodified GCE, 1 CFU/100 ml was detected after 8 h (based on detection of CPR generated), only 12 min was required for the detection of the same at a CuPc-modified electrode (Table 1). Cobalt, zinc and the tetrasulphonated phthalocyanine of cobalt required 2:00, 2:15, and 1:45 h, respectively, to detect the same numbers of CFUs. Water samples containing 40 CFU/100 ml were instantly detectable with the CuPc-modified GCE.

Table 1.

Correlation between CFU/100 ml and electrochemical detection based on the least time required for detection

CFU (per 100 ml)	Peak current (nA)	Plain GCE	MPc-modified GCE			
			Cu	Co	Zn	CoTSPc
1	24.52	8 h	15 min	2 h	2: 15 h	1:45 min
5	34.52	5:30 h	7 min	1:15 h	1: 45 min	2:30 min
10	225.17	1:20 h	6 min	50 min	1 h	1:15 h
20	594.85	35 min	2:50 min	30 min	40 min	45 min
40	854.81	16:40 min	Instant	13 min	21:50 min	30 min

3.5. Effect of environmental water samples on the sensitivity of the electrochemical detection of CPR

Fig. 7 shows the effect of environmental water samples on the sensitivity of CPR detection electrochemically at a CuPc modified GCE. There was no significant change in sensitivity with volumes up to 50% of the total assay volume. This was aimed at determining the influence of the environmental water samples on the peak currents determined.

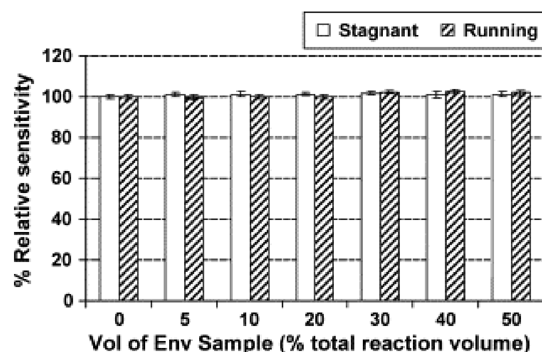

Fig. 7. Effect of environmental water samples on the sensitivity of the electrochemical detection of CPR using CuPc in sodium phosphate buffer pH 7.2. Data points represent mean ± S.D.

3.6. Selectivity and sensitivity of sensor towards some commonly found phenolic compounds in polluted waters

Out of the selected phenolic compounds studied, 4-chloro-3-methyl phenol and 2,4-dichlorophenol were detected by the CuPc-modified sensor with higher sensitivity than chlorophenol red (Table 2). However, these phenolic compounds appear at different potentials, thus making it possible to differentiate between them. Nickel Pc was the most selective of the studied Pcs, in that oxidation waves were only recorded for CPR at the concentrations examined. The modification of electrode surfaces with NiPc has been reported as a way to design selective and sensitive sensors for the electrochemical detection of certain biological analytes such as nitric oxide [19], [20] and [21].

Table 2.

Selectivity and sensitivity of biosensor towards other phenolic compounds

Phenolic compounds	Voltage (mV)	Plain (μA)	CuPc (μA)	ZnPc (μA)	NiPc (μA)	CoTSPc (μA)
Chlorophenol red	692	1.32	4.03	2.10	1.61	2.01
4-Chloro-3-methyl phenol	608	8.90	13.03	1.01	–	9.33
2,4-Dichlorophenol	614	6.2	9.02	–	–	8.56
Phenol	–	–	–	–	–	–
2-Nitrophenol	–	–	–	–	–	–
3-Nitrophenol	–	–	–	–	–	–
2,4-Dinitrophenol	–	–	–	–	–	–
2,4,6-Trinitrophenol	–	–	–	–	–	–
2-Chlorophenol	–	–	–	–	–	–

3.7. Correlation between CFU/100 ml and electrochemical detection of CPR

Generally, a direct correlation between CFUs and current generated in the sensor was observed (Fig. 8). Anodic waves of CPR indicated the presence of coliform enzyme, B-GAL, which hydrolyses the substrate CPRG to CPR. Current was detected in some samples notably 32, 46 and 47 which did not show any colony forming units on the media. It is possible that the current generated in such instances was due to the phenomenon of viable but nonculturable bacteria (VBNC), the major setback in the use of media in detecting microorganisms.

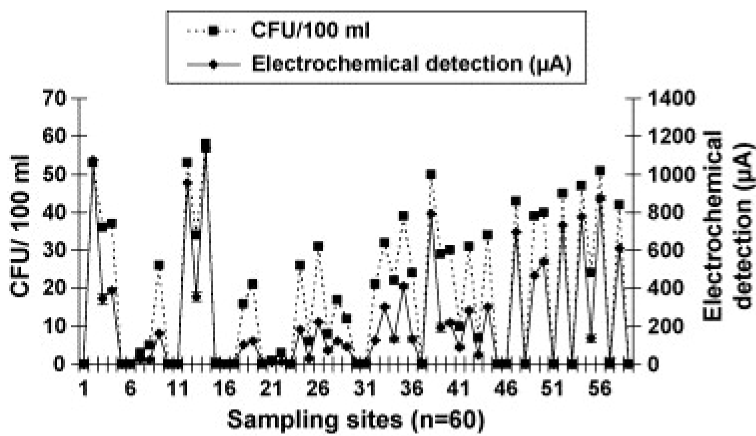

Fig. 8. Graph showing the correlation between CFU/100 ml and electrochemical detection of CPR with CuPc. Data points represent mean ± S.D.

To our knowledge, this is the first report on electrochemical detection of CPR in detecting and monitoring microbial contamination of water. Pérez et al. [22] detected 100 CFU/100 ml in less than 10 h electrochemically. Paitan et al. [23] demonstrated the electrochemical detection of enteric bacteria in general and of *E. coli* specifically. Amperometric detection of 1000 CFU/ml within 60–75 min was achieved. Pre-incubating the samples for 5–6 h further increased the sensitivity to as low as 1 CFU/100 ml within 6–8 h [24]. Mittelmann et al. [25] succeeded in amperometrically determining *E. coli* concentration of 100 CFU/100 ml within a working day. These results match up well with our system that detects 1 CFU/100 ml within 6 h (unmodified GCE). However, our system with the CuPc modified GCE is the most sensitive of all (1 CFU/100 ml in 15 min).

As observed in the correlation of CFU and peak current generation studies, some water samples gave peak currents indicating the presence of CPR hence presence of coliforms but no CFUs on the media. This may be due to the presence of VBNC bacterial cells in the said water sample. These

VBNC cells have always been a problem in the use of the traditional culture method of monitoring coliform contamination of water [1] and [26].

4. Conclusion

In conclusion, electrooxidative detection of CPR as a measure of coliform B-GAL activity was possible. The sensitivity and resistance to fouling of the sensor was highly improved upon modification of the GCE with phthalocyanine metal complexes especially that of copper. This sensor provides a sensitive and robust method in the detection of coliform contamination of water, thus solving the major problem of the lengthy times required for confirming the potability or otherwise in the microbiological water quality industry. It also counters the false negative results obtained due to the presence of VBNC cells in the water environment.

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