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Chitosan-based tyrosinase optical phenol biosensor employing hybrid nafion/sol–gel silicate for MBTH immobilization

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Abstract

The development of an optical biosensor based on immobilization of 3-methyl-2-benzothiazolinone hydrazone (MBTH) in hybrid nafion/sol–gel silicate film and tyrosinase in chitosan film for the detection of phenolic compounds has been described. Tyrosinase was immobilized in chitosan film deposited on the hybrid nafion/sol–gel silicate film containing MBTH. The enzymatic oxidation product of phenolic compounds were stabilized through formation of adduct with MBTH to produce a maroon color adduct. The color intensity of adduct was found to increase proportionally with the increase of the substrate concentrations after 5 min exposure. The linearity of the biosensor towards phenol, catechol and *m*-cresol were in the respective concentration range of 0.5–7.0, 0.5–10.0 and 1.0–13.0 mg/L with detection limit of 0.18, 0.23 and 0.43 mg/L, respectively. The biosensor shows a good stability for at least 3 months.

Keywords: Optical biosensor; Tyrosinase; Hybrid nafion/sol–gel silicate; Chitosan; Phenols

1. Introduction

Phenols are important contaminants in ground and surface water [1]. They have been widely used in wood preservatives, textiles, herbicides and pesticides [2]. Spectrophotometry and chromatography are the commonly used analytical methods for measuring phenols [3,4]. However, these methods are complicated, time consuming and require expensive instrumentation. They are also not suitable for field monitoring. Therefore, there is an interest in developing a simple, sensitive, accurate but portable device for the determination of phenols.

Many efforts have been made for simple and effective determination of phenols. Biosensors based on tyrosinase have proved to be sensitive and convenient tools for this purpose [5]. In such devices, tyrosinase catalyses two reactions: first, ortho-hydroxylation of phenols to catechols and then further oxidation of catechols to ortho-quinones with the presence of molecular oxygen [6]. In the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH), ortho-quinone react to form a maroon color adducts, which can be monitored spectrophotometrically [7–9].

Recently, sol–gel chemistry offers many new and interesting applications in the fields of chemical sensors and biosensors [10–12]. An organic reagent dyes are mixed as dopants with the starting solution to develop optochemical sensors for pH [13], ammonia [14] and iron [15]. Even biologically active species such as enzymes can be immobilized in sol–gel glasses [16,17]. The sol–gel process, allows the preparation of porous glasses at low temperature, high purity of the starting materials for the production of ceramic materials by hydrolysis and polycondensation of alkoxides [11,12]. A prominent feature of silica sol–gel based material such as chemical inertness, physical rigidity, negligible swelling in aqueous and organic solutions, tunable porosity, high photochemical and thermal stability and optical transparency [12,18] have led to intensive research in the area of an optical and electrochemical biosensors. Numerous advantages of the sol–gel matrix application in sensor have been reported, however, the cracking of sol–gel matrix with time is still a problem. Many research have been carried out to prevent the cracking problem such as forming hybrids of sol–gel with poly(vinylalcohol)-poly(vinylpyridine) [19], natural poly-

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mer chitosan [20], hydroxyethyl carboxymethyl cellulose [21] and polysaccharide [22].

In this work, we have studied the fabrication of an optical biosensor with two layers of films containing of immobilized MBTH in hybrid nafion/sol–gel derived silicate and tyrosinase enzyme in chitosan for the detection of phenolic compounds. This type of hybrid material can overcome the brittleness and shrinkage of the pure sol–gel derived silicate [18]. Another advantage of this material is providing a moderate hydrophobic environment for dye immobilization and permits permeation of the analyte/enzymatic product into the material structure where the recognition can occur. However, it requires a careful control of pore size and ratio of nafion to sol–gel silicate to facilitate the accessibility of the analyte/enzymatic product without leaching of the dye.

Barroso-Fernandez et al. [23], Khramov and Collinson [24] and Xu et al. [25] reported the use of hybrid nafion/sol–gel silicate to immobilize dyes (methyl viologen and tris(2,2′-bipyridyl)ruthenium(II)) for the development of chemical sensors and biosensors. The concept of dye immobilization in their work was based on the ion exchange of the cationic groups of the dyes (MV^{2+} and $Ru(bpy)_3{}^{2+}$) with the sulfonate sites of nafion. To our knowledge, there are no reports on MBTH immobilization in hybrid nafion/sol–gel silicate matrix for optical biosensor development has been reported to-date. This work reports the immobilization of hydrophilic MBTH reagent in the moderate hydrophobic nafion/sol–gel silicate film. The idea is to utilize the moderate hydrophobic environment and a great porosity of the hybrid material to retain the hydrophilic MBTH reagent and to prevent leaching from the biosensor film. Optical based biosensors have offered many advantages [8,9,12] and this include the ability of the sensors to give a quick indication on the presence of analyte of interest based on the color changes. The chitosan film consisting immobilized tyrosinase was used for enzymatic reaction with phenol to produce quinone before react with MBTH to form quinone-MBTH adduct.

2. Experimental

2.1. Reagents

Tyrosinase from mushroom (EC 1.14.18.1), phenol, catechol, *m*-cresol, *o*-cresol, *p*-cresol, hydroquinone and 4-chlorophenol were purchased from Sigma. Nafion (5% solution in a mixture of alcohol) was supplied by Aldrich. Hydrochloric acid (37%) and acetic acid glacial were obtained from R&M Chemicals and Ajax Chemicals whereas MBTH was purchased from Merk. Tetraethyl orthosilicate (TEOS) was acquired from Fluka. Chitosan was supplied by Chito-Chem (M) Sdn. Bhd. All chemicals were used without any further purification.

2.2. Preparation of stock solution

A homogeneous stock sol–gel solution was prepared by vigorously mixing 1000 μL of TEOS, 200 μL of distilled water and 10 μL of 0.1 M HCl in a glass vial. The sol–gel solution was stirred for 24 h. The sol–gel silicate solution was mixed homogeneously with nafion solution at volume ratio of 1 to 1 (v/v) and kept at room temperature (25 °C) for overnight. A small portion of 100 mM MBTH solution was mixed to hybrid nafion/sol–gel silicate mixture at volume ratio of 0.3–1 (v/v) and the mixture was stirred until homogeneous solution was obtained.

Chitosan solution (2%, w/v) was prepared by dissolving 2 g of chitosan powder in 100 mL of acetic acid (1%, v/v). The viscous chitosan solution was stirred overnight at room temperature. A homogeneous stock solution of tyrosinase/chitosan mixture was prepared by mixing chitosan solution and tyrosinase solution (150 mg/mL) at volume ratio of 15.7–1.0 (v/v) [7] in an eppendorf tube. The mixture was stirred gently for 15 min. This stock solution was freshly prepared before the fabrication of the optical film. The formulation was changed accordingly when certain experimental parameters were investigated.

2.3. Construction of biosensor

Initially, 10 μL of hybrid nafion/sol–gel silicate-MBTH mixture was deposited onto a glass slide (25 mm × 9 mm) and coated in an area of 9 mm × 10 mm. It was spun at 3000 rpm for 5 s. The glass slide containing hybrid nafion/sol–gel silicate-MBTH film was kept in air-tight bottle at 4 °C for overnight for drying purposes. A volume of 10 μL of the stock tyrosinase/chitosan mixture was then pipetted onto a dried hybrid nafion/sol–gel silicate-MBTH film on a glass slide and coated gently over an area of 9 mm × 10 mm. Then it was spun at 2000 rpm for 3 s and dried at 4 °C for overnight.

2.4. Procedure for the study of MBTH leaching from hybrid nafion/sol–gel silicate film and pure sol–gel silicate film

The biosensor was washed with 50 mM phosphate buffer solution pH 6.0 or immersed in 50 mM phosphate buffer solution pH 6.0 for 0, 5, 15, 25 and 40 min prior to evaluation of sensor performance with 9.4 mg/L phenol. The sensor response was monitored at 490 nm and was recorded at an interval of 1 min for duration of 5 min. All measurements were carried out in triplicates.

2.5. Evaluation of the biosensor response

All absorption measurements were conducted by using an ultraviolet-visible spectrophotometer (model Varian-Cary win UV 50). The glass slide coated with two layered of films was immersed in 3 mL of 50 mM phosphate buffer solution of pH 6.0 for 5 min and then washed with distilled water. It was then exposed to 3 mL of 50 mM phosphate buffer solution pH 6.0 containing phenol. The absorption spectra were recorded between 300 and 800 nm at an interval of 1 min for 5 min. The response of the biosensor was studied at a fixed wavelength of 490 nm determined from the spectra.

2.6. Validation of the biosensor response

The response of the constructed biosensor at various concentration of phenol standard was validated with a standard

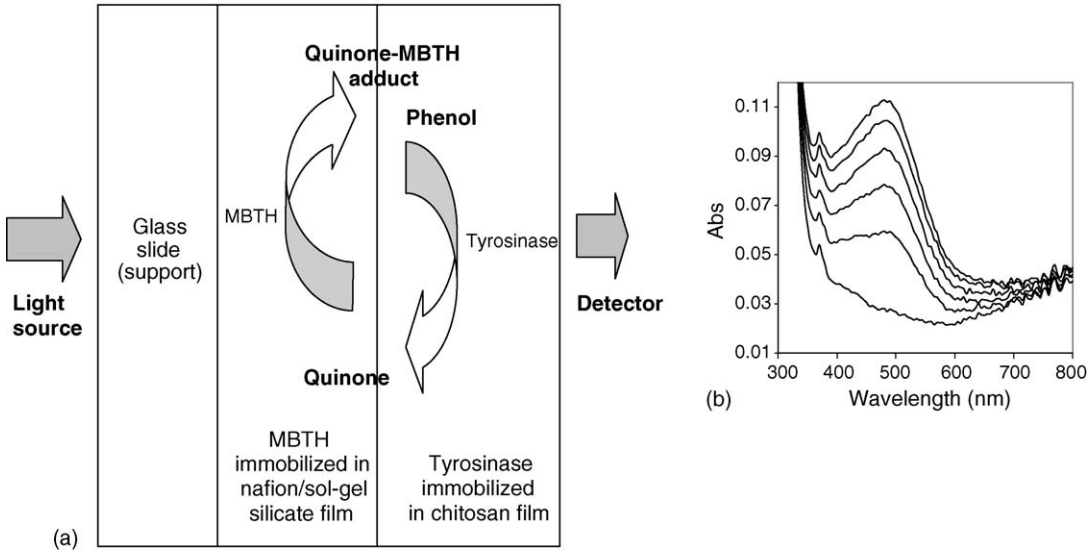

Fig. 1. Reaction mechanism for hydroxylation of phenol by tyrosinase in the presence of MBTH on the surface of the biosensor film (a). Absorption spectra of the biosensor in the presence of 9.4 mg/L phenol (b).

colorimetric method (APHA), which based on the reaction of phenolic compounds with 4-aminoantipyrine at pH 7.9 in the presence of potassium ferricyanide to form a red color antipyrine dye. The absorbance of the dye was measured at 500 nm [26].

3. Results and discussions

The mechanism of MBTH-quinone adducts formation for the diphenolase activity of tyrosinase has been previously reported [27]. The oxidation of a phenolic substrate by tyrosinase (polyphenol oxidase) produces a dihydroxybenzene (catechol), which is then further oxidized to *o*-quinone. As shown in Fig. 1(a), *o*-quinone produced by enzymatic reaction of phenol with immobilized tyrosinase in chitosan film, reacts with immobilized MBTH in the hybrid nafion/sol–gel silicate film to produce a maroon color adduct, which can be monitored spectrophotometrically as depicted in Fig. 1(b).

3.1. The effect of the nafion/sol–gel silicate ratio on biosensor response

The optimum parameters for tyrosinase immobilization in chitosan film were previously reported [7]. These parameters were used for subsequent studies.

The sol–gel silicate polymer is formed by hydrolysis of an alkoxide precursor with simultaneous condensation to yield a polymeric oxo-bridged SiO_2 network [12]. In this work, TEOS was chosen as a precursor of sol–gel silicate for immobilization of MBTH reagent. The properties of the sol–gel silicate matrix such as surface area, pore size and its distribution can be affected by various factors. Among them, the pH and the ratio of Si to water for sol–gel silicate preparation are the most important parameters.

Nafion has a hydrophobic fluorocarbon backbone but hydrophilic cation-exchange site and thus exhibits moderate hydrophobicity [18], which can assists in retaining the MBTH in the film and reduces leaching. Therefore, in this study nafion was mixed with sol–gel silicate to form an organic–inorganic hybrid material. This hybrid material also solved the cracking problem of pure sol–gel silicate film [12,18,20]. Fig. 2 illustrates the effect of various ratios of nafion/sol–gel silicate on the biosensor response. In this work, the biosensor was immersed in 50 mM phosphate buffer (pH 6.0) for 20 min prior to evaluation of biosensor response with 9.4 mg/L phenol. The lowest response of the biosensor was observed when pure sol–gel silicate matrix was used. This behaviour could be due to the hydrophilic nature of TEOS [23] that could not retain much of the hydrophilic MBTH reagent in the immobilization matrix. The hybrid material with ratio of 1:1 showed the optimum biosensor response. Further increase in nafion content decreased the sensor response. This may attributed to the decreased of porosity of sol–gel silicate film when the content of nafion increased in the hybrid

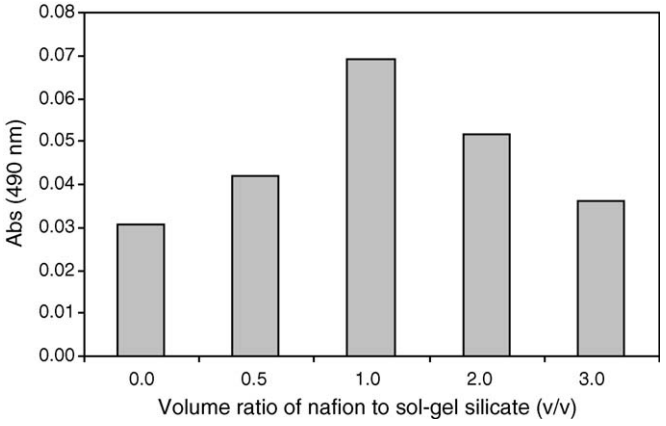

Fig. 2. The effect of nafion/sol–gel silicate ratio on the biosensor response. Phenol concentration used was 9.4 mg/L in 50 mM phosphate buffer pH 6.0 at room temperature.

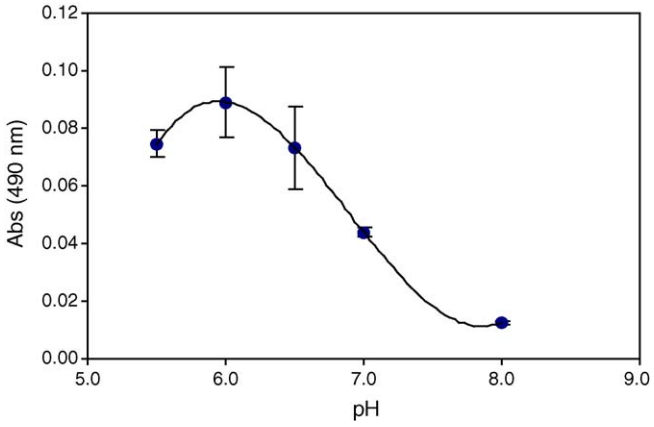

Fig. 3. The effect of pH on the response of the biosensor when phenol concentration used was 9.4 mg/L in 50 mM phosphate buffer solution ($n = 3$).

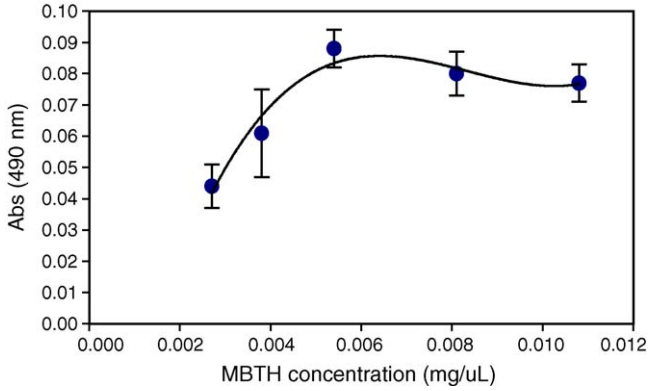

Fig. 4. The effect of MBTH concentration on the biosensor response when the phenol concentration used was fixed at 9.4 mg/L in 50 mM phosphate buffer solution (pH 6.0) ($n = 3$).

materials as well as the hydrophobicity of the matrix. Thus, it affect the amount of MBTH trapped in the hybrid nafion/sol–gel silicate network since decreased in porosity of the hybrid material resulting in low amount of MBTH trapped in the hybrid film. Similar finding was also reported by Miao and Tan [20] where the decreased of porosity of hybrid silica sol–gel/chitosan resulting low amount of horseradish peroxidase enzyme trapped in the matrix thus low response of the sensor was observed. The decrease in the sensor response could also be due to the increased hydrophobicity of the matrix, thus increased the diffusion barrier of the product formed into the MBTH layer. Consequently, a poor response of the biosensor was obtained. In this study, the thickness of the film was estimated based on the weight of the microscope glass slide before and after the formation of nafion/sol–gel silicate and tyrosinase-chitosan layers. By using this method, the thickness of the biosensor film at nafion:sol–gel silicate ratio of 1:1 was estimated to be in the range of 4–5 μm.

The pH of the working buffer was also investigated using 50 mM phosphate buffering system covering the pH ranging from 5.5 to 8.0 (Fig. 3). The optimum response of the biosensor was obtained at pH 6. This finding is in agreement with the work reported for immobilized tyrosinase on the surface of the modified core-shell magnetic nanoparticles [28]. They observed that the optimum response of the immobilized tyrosinase was achieved in the pH range of 6–7 in the presence of 25 μM phenol in 50 mM phosphate buffer solution. Therefore, pH 6.0 was chosen in subsequent experiments.

The effect of a different amount of MBTH on the MBTH-quinone adducts formation was investigated by measuring the absorbance at fixed wavelength (490 nm). It was found that the absorbance of MBTH-quinone adduct was optimum at initial MBTH concentration of 0.0054 mg/μL (Fig. 4). Thus a concentration of 0.0054 mg/μL of MBTH reagent was employed for all subsequent studies.

Fig. 5 shows the leaching effect on the sensor response when the sensor was immersed in the buffer solution at various times, prior to insertion of the sensor in the phenol solution. Time = 0 min represent the response of the sensor when it was quickly immersed and removed from the buffer solution before insertion of the sensor in phenol solution for 5 min to study its response. The result reveals that from 0 to 5 min immersion time, the leaching of MBTH was about 40% and 80% for hybrid nafion/sol–gel silicate film and pure sol–gel silicate film, respectively. The leaching is probably caused by MBTH, which was adsorbed on the surface of the biosensor film. After 5 min the biosensor shows a constant response, which indicated no further leaching of MBTH from the film was observed. Therefore, the biosensor was immersed in the phosphate buffer solution for 5 min to remove excess MBTH from the biosensor film prior to the detection of phenol.

3.2. Analytical performance of the optical biosensor

Fig. 6 shows the calibration curves for phenol, catechol and *m*-cresol over the concentration range of 0.5–13.0 mg/L. In this work, the wavelengths chosen for the determination of phenol, catechol and *m*-cresol were 490, 490 and 430 nm, respectively (Fig. 7). Table 1 presents the response characteristics of the biosensor including sensitivity, detection limit, linearity, correlation coefficient and the apparent Michaelis–Menten constant (K_m) for the different phenolic compounds. Seven phenolic compounds (phenol, catechol, *m*-cresol, *o*-cresol, *p*-cresol, hydro-

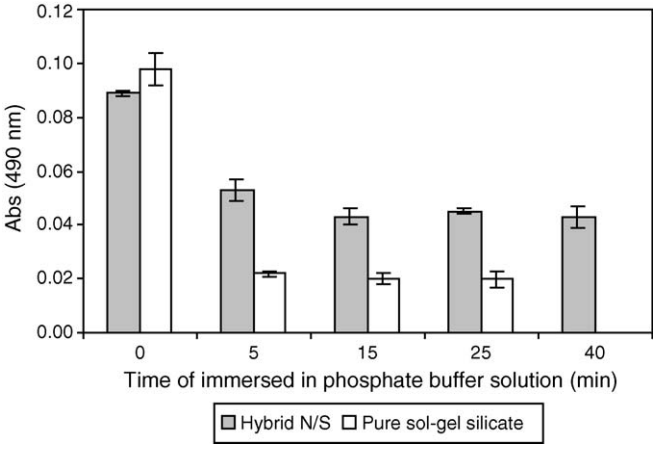

Fig. 5. The leaching study of the biosensor when it was immersed in 50 mM phosphate buffer solution pH 6.0 for 0, 5, 15, 25 and 40 min prior to response measurement. Phenol concentration used was 9.4 mg/L ($n = 3$).

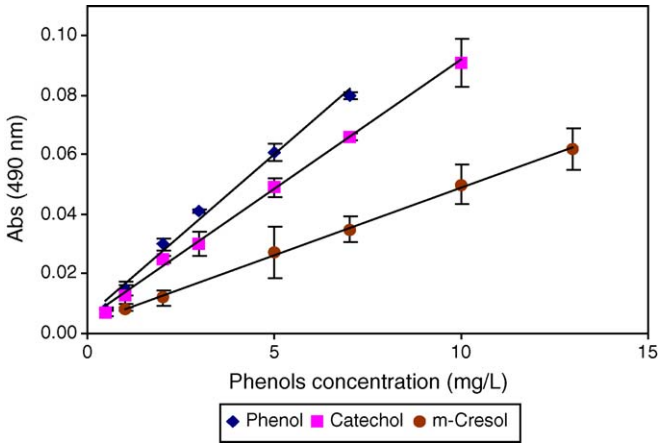

Fig. 6. The linear dynamic range of the biosensor towards phenol, catechol and *m*-cresol ($n = 3$).

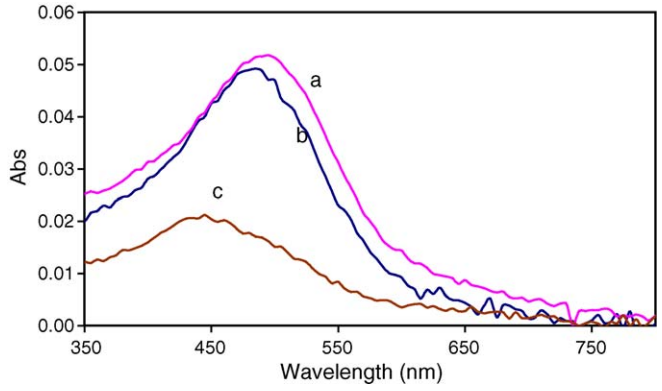

Fig. 7. The absorption spectra of a two-layer film with immobilized MBTH in nafion/sol–gel silicate film and tyrosinase in chitosan in the presence of 5 mg/L of phenol (a), catechol (b) and *m*-cresol (c) after 5 min reaction time.

quinone and 4-chlorophenol) were monitored by the biosensor. It indicates that the sensitivity of the biosensor followed the order of phenol > catechol > *m*-cresol, which is in the same order with the phenol biosensor as reported by Campuzano et al. [29]. Other phenolic compounds such as *o*-cresol, *p*-cresol, hydroquinone and 4-chlorophenol did not give any response. The restricted activity of the biosensor to certain phenolic compounds could be caused by the hydrophobic characteristics of the matrix [30,31] and/or steric restrictions in the transition state of the phenolic compound with tyrosinase [32]. In addition, the nafion/silicate films contain negatively charged sulfonate groups, thus preventing the negatively charged product formed/analyte from partitioning into the biosensor film. The apparent Michaelis–Menten constant (K_m) was estimated using the Lineweaver–Burk plots and the values were calculated to be 15.63, 15.38 and 8.62 mg/L for phenol, catechol and *m*-cresol, respectively. K_m is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity [33]. A lower K_m indicates that the enzyme requires only a small amount of substrate to become saturated concentrations and also shows greater affinity of the enzymes towards substrate. Hence, the maximum velocity is reached at relatively low substrate. In this work, it reveals that the higher affinity of the biosensor was towards *m*-cresol compared to catechol and phenol. Therefore, the lower concentration of *m*-cresol needed to achieve maximum reaction velocity.

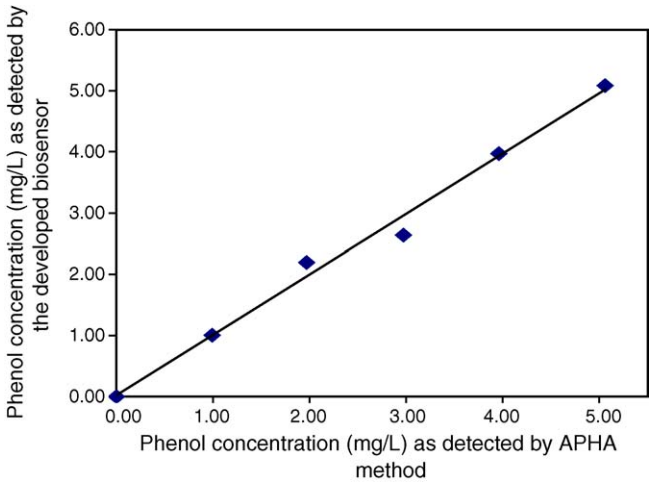

Fig. 8. A comparison of the developed optical biosensor with the APHA method in the detection of phenol.

The reproducibility in fabrication of the biosensor was investigated at a phenol concentration of 9.4 and 5.0 mg/L. The relative standard deviation of the fabricated biosensor was 8.9% ($n = 8$) and 5.6% ($n = 12$), respectively. The stability of the biosensor was tested using 5.0 mg/L phenol and the biosensor displayed good storage stability for at least 3 months when stored dry at 4 °C (Fig. 8). They retained almost 90–98% of their initial activity after 3 months of storage at 4 °C.

The linearity of the biosensor towards phenol, catechol and *m*-cresol were in the concentration range of 0.5–7.0, 0.5–10.0 and 1.0–13.0 mg/L with detection limit of 0.18, 0.23 and 0.43 mg/L.

Table 1
Determination of different phenolic compounds by the biosensor

Phenolic compounds	Sensitivity (Abs/mg/L)	Detection limit (mg/L)	Linearity range (mg/L)	Correlation coefficient (*r*)	K_m (mg/L)
Phenol	0.011	0.18	0.5–7.0	0.992	15.63
Catechol	0.009	0.23	0.5–10.0	0.997	15.38
m-Cresol	0.005	0.43	1.0–13.0	0.999	8.62
o-Cresol	nd	–	nd	–	nd
p-Cresol	nd	–	nd	–	nd
Hydroquinone	nd	–	nd	–	nd
4-Chlorophenol	nd	–	nd	–	nd

nd: Not detected.

The detection limit was calculated based on the method as previously reported by Day and Underwood [34] and Navas Diaz et al. [35].

3.3. Validation of the biosensor performance

Fig. 8 shows the performance of the biosensor compared with an APHA method for phenol [26] from the concentration of phenol from 0.0 to 5.0 mg/L. A good correlation was found between these two methods ($r = 0.9909$, slope = 0.987). Similar finding also reported by the work for self-assembled monolayer-based tyrosinase biosensors [29] where a good correlation was obtained ($r = 0.9999$, slope < 1).

4. Conclusion

An optical biosensor based on the use of two layer films for the immobilization of MBTH-hybrid nafion/sol–gel silicate film and tyrosinase-chitosan film for the determination of phenolic compounds was successfully designed. The response of the biosensor was found to be linear with substrate concentrations after 5 min exposure. The biosensor demonstrated good sensitivity, reproducibility and stability. This work demonstrates that the use of a hybrid nafion/sol–gel material is suitable for immobilizing the hydrophilic dye MBTH.

Acknowledgements

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