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IMMOBILIZED XANTHINE OXIDASE WITH BIO-COMPOSITE USING GAS BIOSENSOR

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ABSTRACT

The paper describes the development of biosensor for gas detection using bio-composite-xanthine oxidase. The xanthine oxidase enzyme is immobilized by bio-composite including gelatine and chitosan. The high stability of immobilization enzymes onto a solid substrate can make them ideal alternatives as recognition elements for sensors. Xanthine oxidase was constructed in a very simple way using ammonium sulfate precipitation and affinity chromatography. Xanthine oxidase was immobilized with bio-composite on glass and quartz crystal substrates. The structural properties of bio-composite films have been investigated via ultraviolet visible spectrophotometer and quartz crystal microbalance systems. In order to investigate the sensing properties of bio-composite films, five vapors such as chloroform, acetone, toluene, ethyl acetate and carbon tetrachloride were selected and quartz crystal microbalance system were employed to characterize the sensing properties. Results were remarkable that the bio-composite gas sensor showed selective and reproducible responses against organic vapors. The gas sensor can be used for application in the monitoring and screening of diabetes through the detection of low concentrations of breath acetone vapor.

KEYWORDS:
Gas biosensor, bio-composite, xanthine oxidase, gas vapors

INTRODUCTION

Biosensors have been used to detect the toxic materials, various pollutants, hazardous materials and hazardous gases. In the recent past, biosensors are widely used in military, medicine, industrial process control, environmental monitoring, food industries, veterinary medicine, pharmacy and microbiology. Biosensor technology has improved with in vitro studies based on enzymes.

Enzyme-based biosensors are mostly preferred with prominent properties such as specificity, rapid reaction in mild conditions, and low cost [1, 2]. One of the most important features of effective use of enzyme is enzyme immobilization [3, 4]. Advantages of immobilized enzyme according to free enzymes have reusability and storage stability [5, 6]. Biosensors can be used for a long time with these properties of immobilized enzymes [7, 8].

Xanthine oxidase (XO) is a member of the molybdenum hydroxylase family of proteins contains binding sites for molybdopterin, iron and flavin cofactors XO catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid [9]. High amount of uric acid generation is associated with various pathological functions such as gout, inflammation, ischemia-reperfusion damage, cardiovascular diseases, renal diseases and even cancer [10-13]. Therefore, XO has been considered as a promising target for treating hyperuricemia, gout and other associated pathological conditions [14, 15]. XO primarily produces ROS such as superoxide and hydrogen peroxide by preferentially using molecular oxygen as an electron acceptor [16, 17]. XO is an enzyme which is purified from milk in abundance and this makes it cheap [17]. XO was highly used in biosensors systems [18-21].

This work is aimed at determining the detection of low concentrations of five vapors such as chloroform, acetone, toluene, ethyl acetate and carbon tetrachloride for possible application in the monitoring and screening of organic vapors. Diabetes is a common disease in the world due to genetic and environmental factors. Diabetes causes permanent damage to eyes, blood vessels, kidneys, nerves and heart [22].

The most prominent feature of diabetic patients is the presence of acetone in the breath. Therefore, it is important to develop systems that can easily detect acetone vapor in the breath. Deposition process of bio-composite films was monitored using UV-visible and quartz crystal microbalance (QCM) techniques.

MATERIALS AND METHODS

Sepharose 4B, l-tyrosine, xanthine, 4-aminobenzamide dihydrochloride, cysteine, APTES, HCl hydrogen peroxide, sulfuric acid and ammonium sulfate, toluene, carbon tetrachloride, ethyl acetate, chloroform and acetone were purchased from

Sigma-Aldrich (St Louis, MO, USA). Tris-base, glycine and NaCl were purchased from Merck (Darmstadt, Germany). All other chemicals were of the highest quality available. Spectrophotometric measurements were measured with PerkinElmer Lambda Spectrophotometer.

Purification of Xanthine Oxidase from Bovine Milk. XO was purified from bovine milk with ammonium sulfate precipitation and affinity chromatography methods. Bovine milk was centrifuged at 15000 rpm for 60 minute before precipitation with 38 % ammonium sulfate to remove the bulk of contaminants. The supernatant was brought to 50 % saturation with solid ammonium sulfate. The precipitate formed was collected by centrifugation at 15000 rpm for 60 minute and dissolved 0.1 mol/L Tris-base (pH 9.0) buffer. The pooled precipitate obtained from bovine milk by using ammonium sulfate precipitation was subjected to affinity chromatography [17]. Buffer fractions of 2.0 mL were collected and their absorbance measured at 280 nm.

Bio-composite preparation. Chitosan (1 g) and gelatin (1 g) were prepared separately by dissolving in acetic acid and then were mixed together in 1:1 proportion. This mixture was stirred for 3 hours at room temperature for drying. The bio-composite was stored at 4 °C with 0.1 mol/L phosphate buffer (pH: 7.0) before use [23].

Preparation of Biosensor. A piranha solution was made by adding 10 ml hydrogen peroxide to 30 ml concentrated sulphuric acid. The glass substrate and quartz crystal were immersed in the piranha solution for 30 s. After cleaning, the glass was rinsed thoroughly with deionized water. The piranha solution removes any organic compounds remaining on the glass substrate and quartz crystal leaving a clean surface on the gold electrode for further modification.

The glass substrate and quartz crystal were loaded into a spin-coater and 100 µl of bio-composite was pipetted onto the glass surface by the Laurell WS-650MZ-23NPP spin coater. After spin coating bio-composite, the glass substrate and quartz crystal were placed in an incubator at 30 °C for 24 h with calcium chloride to act as a desiccant. After incubation, the glass substrate and quartz crystal were loaded into a spin-coater and 100 µl of xanthine oxidase was pipetted onto the surface at room temperature.

Quartz crystal microbalance (QCM) system. Quartz crystal microbalance system is capable to detect very small mass changes via measuring the frequency of crystal. In an attempt to determine the reducibility of bio-composite films the frequency change and loaded mass is investigated. This crystal consists of a thinly cut wafer of raw quartz which is sandwiched between two gold electrodes and resonate at an extremely well defined frequency. That is called resonant frequency which is extremely sensitive to small mass changes. The relation between the resonance frequency and the deposited mass is described by the following equation [24]:

$$\Delta f = \frac{-2f_o^2}{\rho_q^{1/2}\,\mu_q^{1/2}\,A}\Delta m$$

Where f_o is the resonance frequency of quartz crystal (Hz), Δm is the mass change (g), A is the piezoelectrically active area (cm^2), ρ_q is the density of quartz (2.648 g cm^{-3}), μ_q is the shear modulus of quartz (2.947×10^{11} g cm^{-1} s^{-2}).

Owing to that mass sensitive system quartz crystal can be used as a substrate for thin film measurements. In this study 10 MHz quartz crystal were used with a commercial system obtained from Open QCM Company. All measurements were performed at room temperature and the frequency shift of coated crystal was recorded considering the f_0 resonance frequency of un-coated crystal.

Optical Measurements. UV-Vis spectra of biosensor were recorded using a PerkinElmer Lambda Spectrophotometer with absorbance scan mode in a region from 200 nm to 900 nm. After the deposition of each layer onto glass substrate, UV-VIS measurements were performed and compared with each other.

Vapor Sensing Measurements. The investigation of the kinetic response of bio-composite films involved recording the dynamic response upon exposure to the analyte vapor. Chloroform, toluene, ethyl acetate, acetone and carbon tetrachloride were chosen as analytes in order to test the film against different type of vapors. The dynamic response of the thin films upon exposure to analyte vapor was recorded using QCM system. Figure 1 shows the schematic diagram of QCM system which has main three parts quartz crystal, gas cell and electronic circuit. QCM measurements were monitored by using computer controlled system which was commercially purchased from Open QCM Company. 10 MHz quartz crystal was used for the control of deposition process and dynamic vapour sensing measurements. The dynamic response of bio- composite films on exposure of analyte vapors was carried out by measuring the resonance frequency change versus time. As shown in Figure 1 gas in and out holes allow introducing vapors for exposure or dry air for recovery of thin film. Bio-composite film was exposed to analyte vapors for 90 seconds and followed 90 seconds dry air was sent into gas cell. Two exposure and three recovery cycles were performed in order to observe the reproducibility of bio-composite sensor.

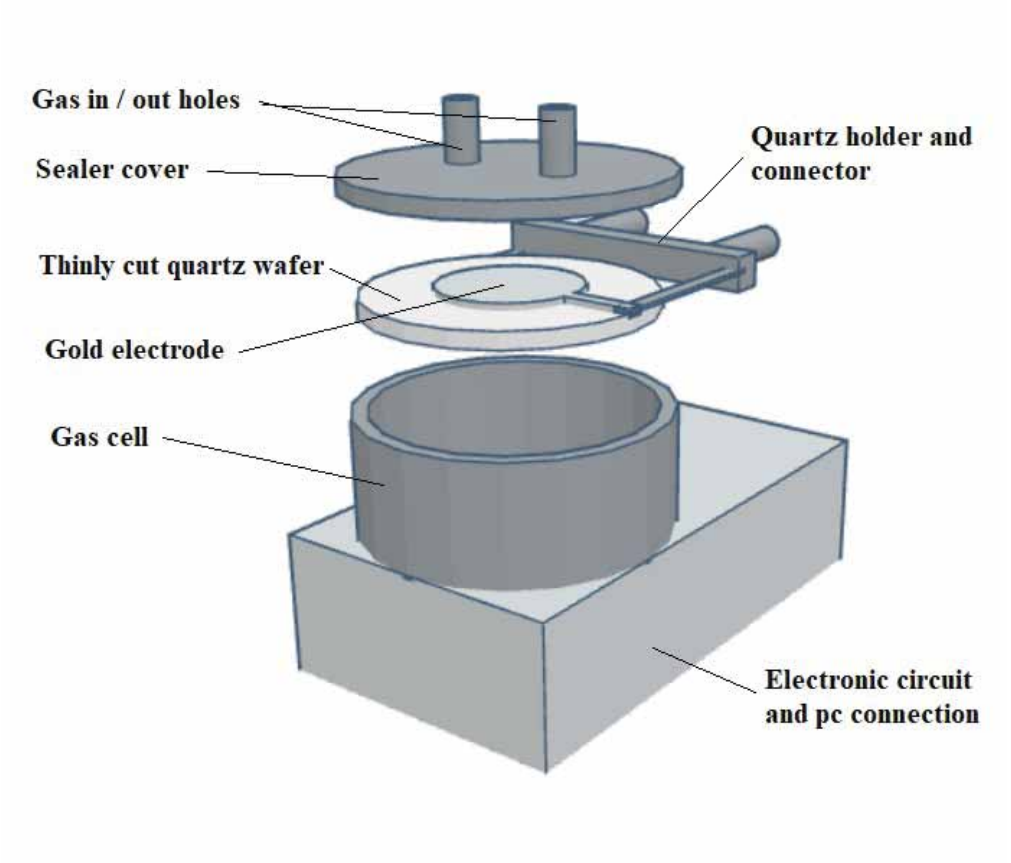


FIGURE 1
Figure of QCM system

RESULTS AND DISCUSSION

Optical Characterization. UV-VIS absorption spectroscopy is often used to characterize the optical properties. In the present study it was used for the check the reproducibility of bio-sensor thin films. The optical property, absorption (α) spectra were obtained at ambient temperature in the wavelength ranges of 300-900 nm by a PerkinElmer Lambda Spectrophotometer with bio-composite. Figure 2 shows the UV-VIS absorption spectra of bio-composite and its spin coated thin film. It has recognizable spectra which consist of several peaks and these peaks correspond to specific transitions. These compounds have σ-σ *, n-σ *, π-π * and n-π * transitions as σ electrons and n electrons are also found in compounds containing π electrons. Since the bio-composite contains of an aromatic group in chitosan-gelatin, it absorbs about n-π * electron transition at a wavelength of about 328 nm. This value is consistent with the literature [25]. The main focus of recording UV-VIS spectra is to control the thin film reproducibility. The increase in absorption of bio-composite after XO layer deposition is due to the increase of thickness of the sample which has bio-composite layer sample. It indicates that bio-composite and bio-composite with XO layer was successfully deposited onto the glass substrate (Figure 2). As seen on the figure thin film has weak spectra because of the low material concentration according to bio-composite. Nevertheless it exhibits same peaks with bio-composite but the only the difference is peaks are low density and overlapped because of the low material concentration of the film.

Gas Sensor Results. Figure 3 shows the dynamic response for the bio-composite film sample against various organic vapors (chloroform, toluene, ethyl acetate, acetone and carbon tetrachloride) at room temperature. The bio-composite film responses to these organic vapors are almost reversible except carbon tetrachloride vapour. The response and recovery times are a few seconds when vapor and dry air are injected to the gas cell. Although carbon tetrachloride vapor produced fast response and recovery has as like the other vapors the recovery was not completely.

As seen on the graph, there is not any significant loss in sensitivity which exhibits the thin film coatings were stable and have been used many times. The bio-composite film sample is found to be reasonably selective and significantly sensitive to chloroform vapor than other organic vapors. Other vapors exhibit quite similar response levels to each other. Figure 4 shows the maximum response values for each vapor.

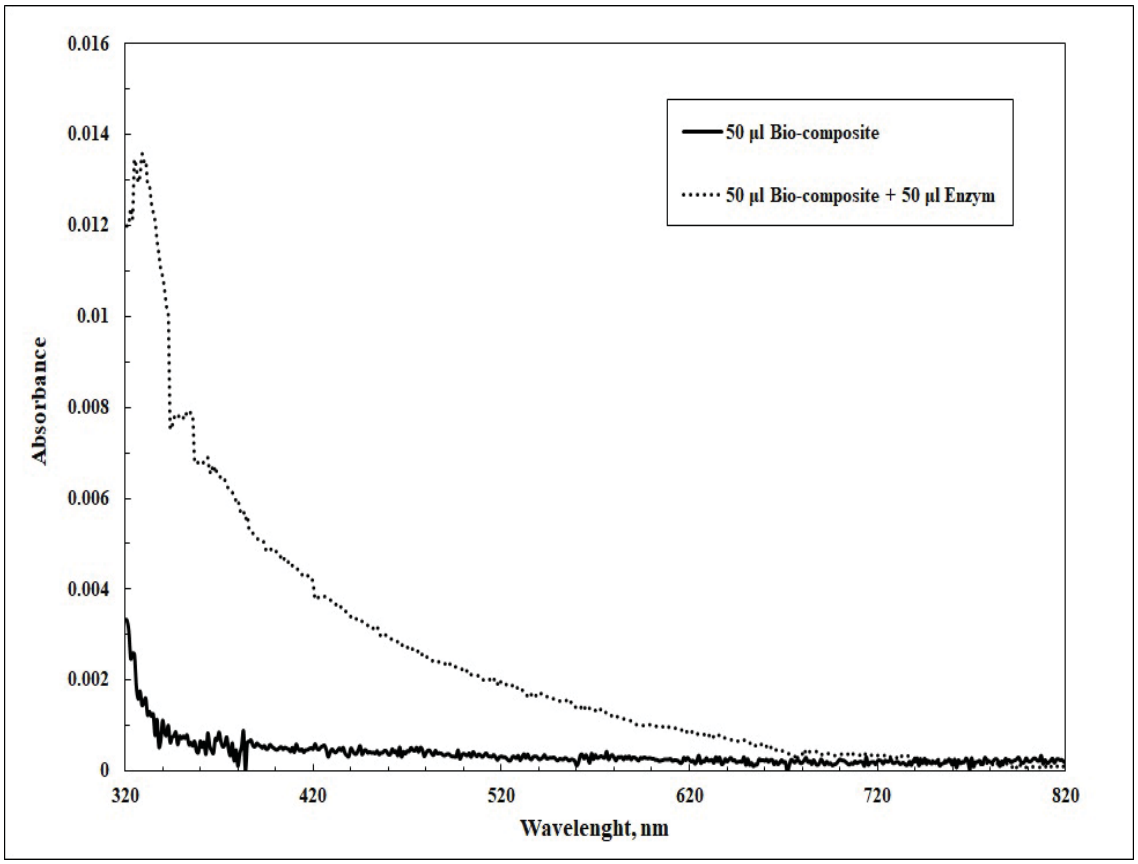
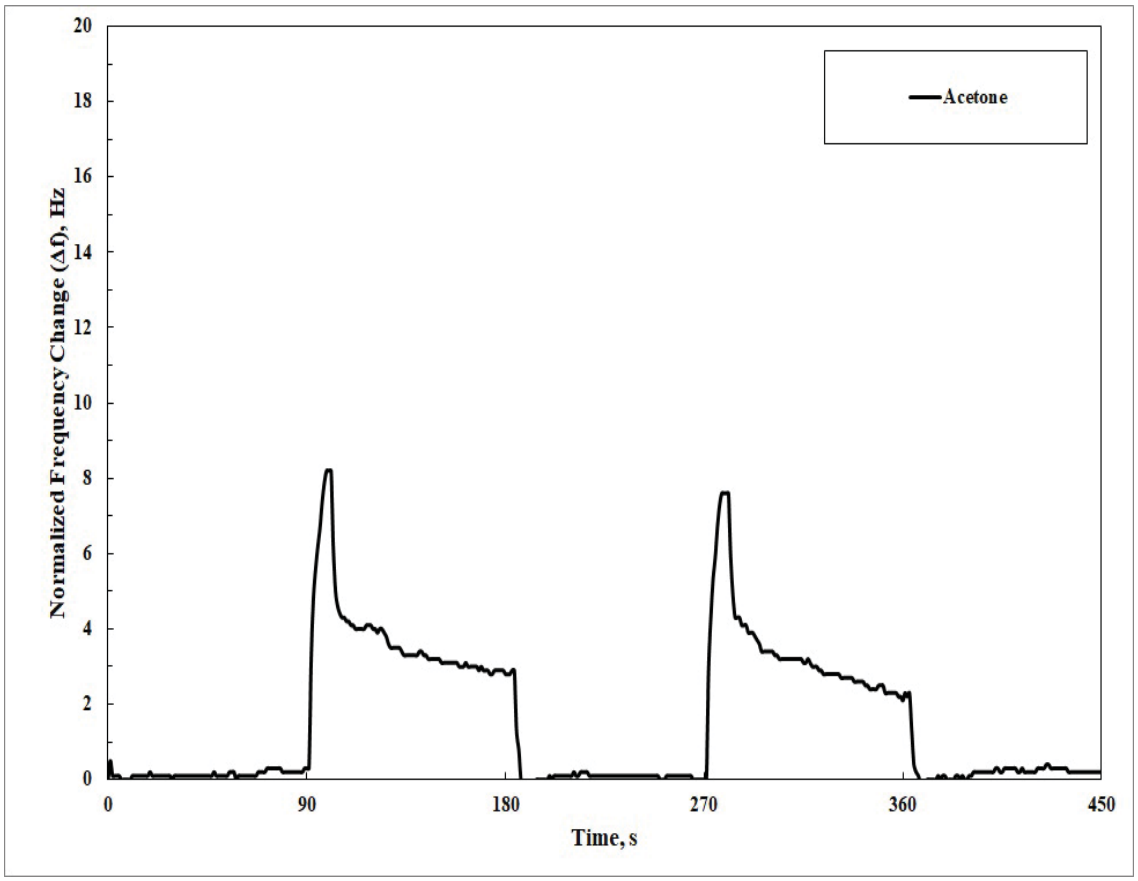
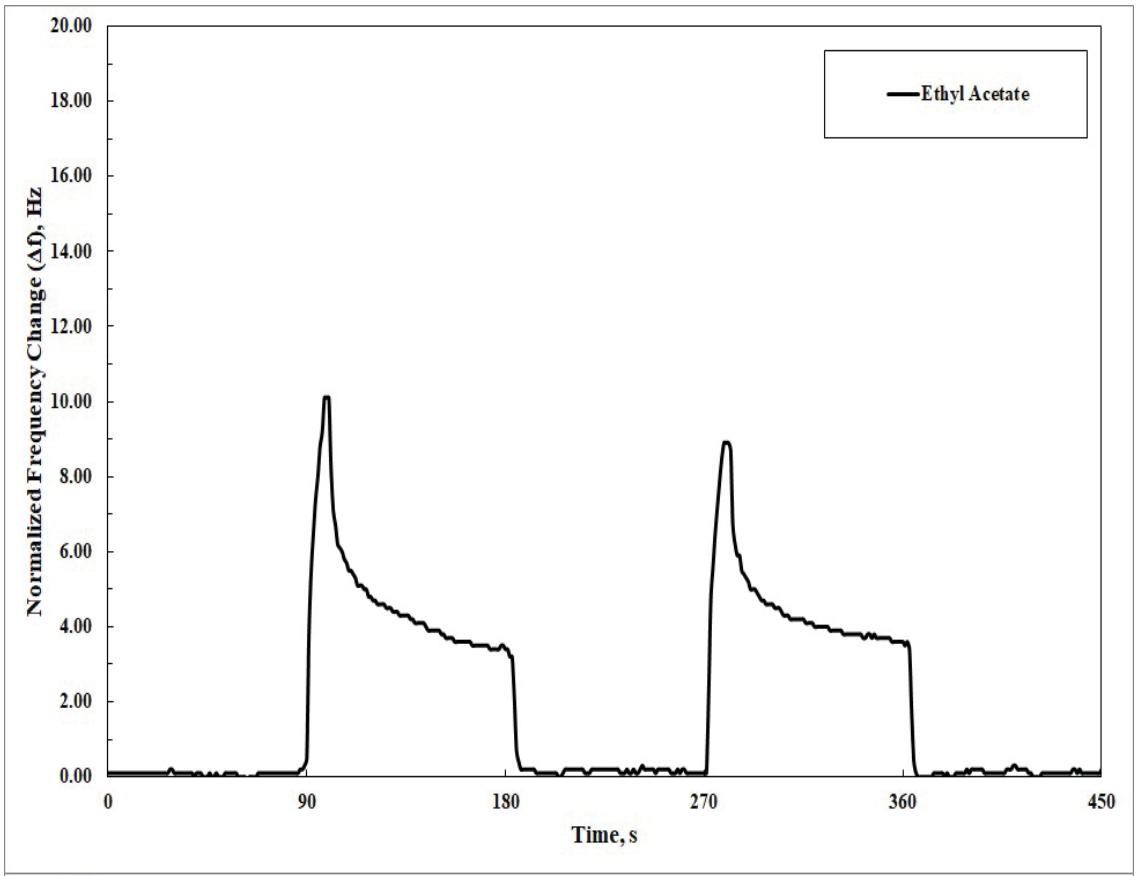
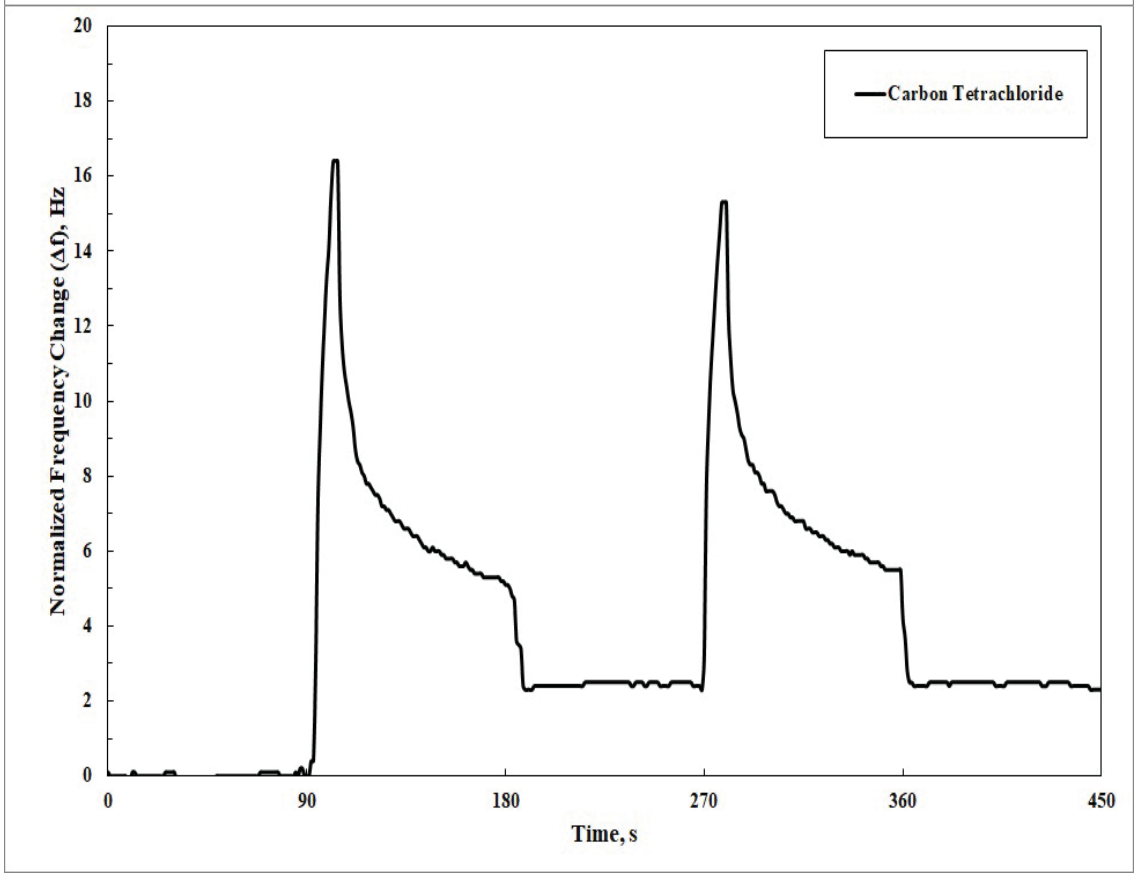


FIGURE 2
The UV-VIS absorption spectra of bio-composite before and after enzyme




Ethyl Acetate
Normalized Frequency Change (Δf), Hz
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
90
180
270
360
450
Time, s


Carbon Tetrachloride
Normalized Frequency Change (Δf), Hz
20
18
16
14
12
10
8
6
4
2
0
0
90
180
270
360
450
Time, s

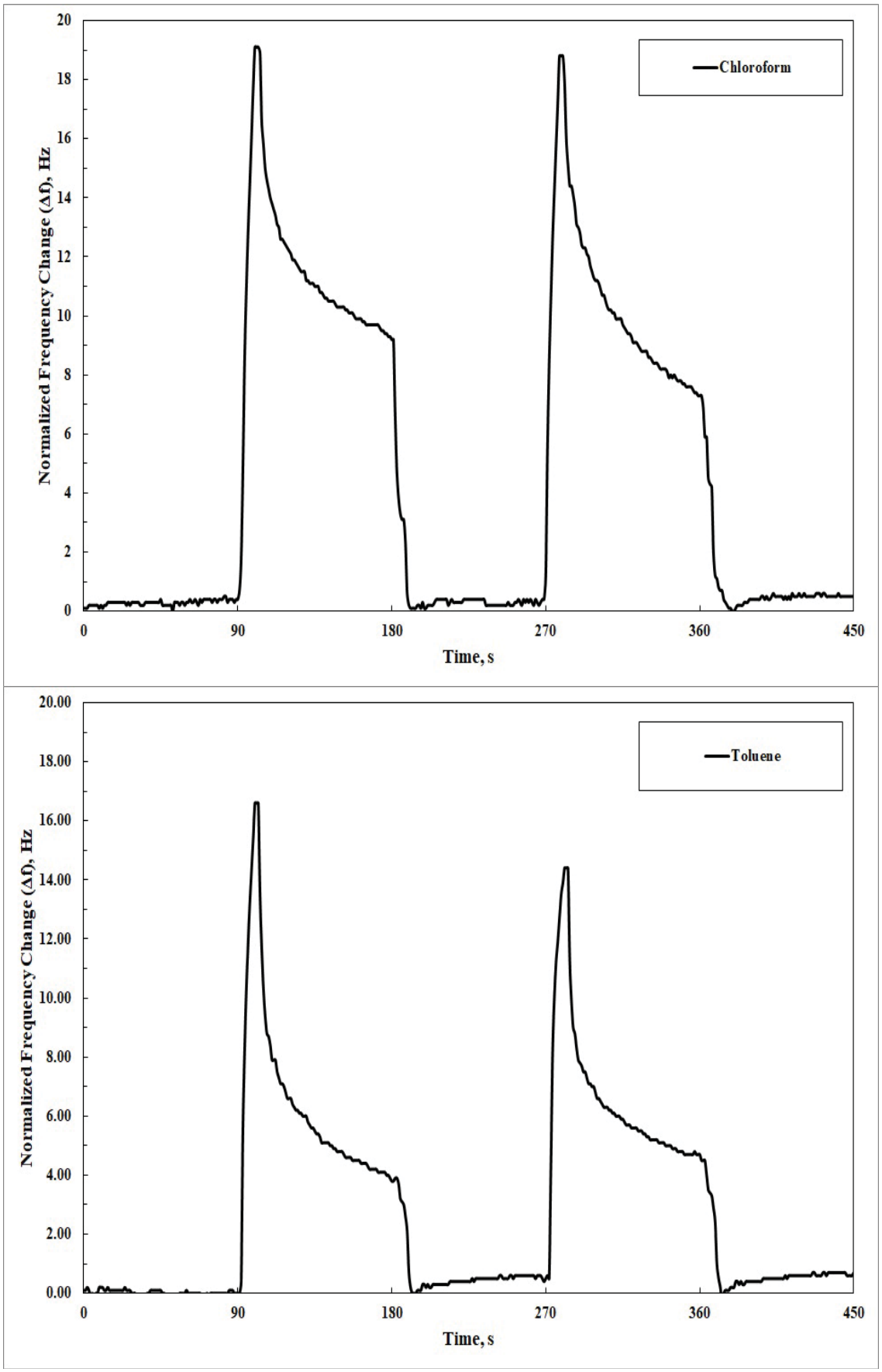


FIGURE 3
The bio-composite film sample against various organic vapors

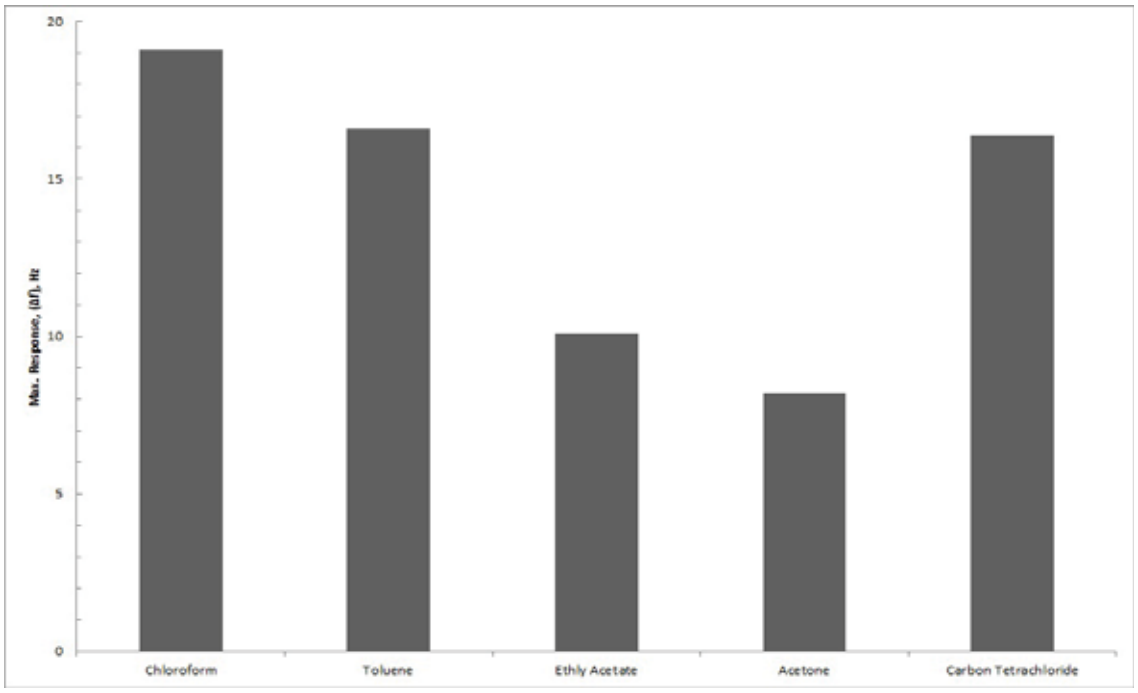


FIGURE 4
The maximum response values for organic vapors

TABLE 1
Vapor properties and response results of bio-composite film

	The concentration of the analyte vapors (ppm)	Vapor pressure (20 °C) [Sigma Aldrich]	Response per concentration (Hz/ppm)	Molecular weight g/mol
Toluene	30.09	22 mmHg	0.13	92.14
Carbon tetrachloride	33.22	91 mmHg	0.15	153.82
Ethyl acetate	32.82	73 mmHg	0.10	88.11
Chloroform	39.98	160 mmHg	0.23	119.38
Acetone	43.66	184 mmHg	0.06	58.08

Table 1 gives the vapor properties of analytes. Vapor pressure and the concentration of vapor are crucial for sensor studies. Intermolecular interactions of volatile compounds are relatively weak and contribute more to vapor pressure. Thus the vapor pressure and the intermolecular interactions are inversely proportional.

In all studied vapors, chloroform is one of the compounds with the highest molecular weight and the highest vapor pressure. Therefore it is one of the gases with the most volume per unit and causes the greatest response with the enzyme.

Since carbon tetrachloride has the highest molecular weight and vapor pressure is less than chloroform, it is seen in the graph that it interacts with the enzyme less than chloroform. Besides the magnitude of the response, the sensor is not fully recovered after the vapor has been removed. The reason for this was determined to be irreversible inhibition between carbon tetrachloride and enzyme. In this sense; an irreversible inhibition can be effected from one or more functional groups of enzymes. It is known that this inhibition occurs when the inhibitor covalently attaches to the enzyme or forms a strong complex which is difficult to separate [26].

Although the molecular weight of ethyl acetate is less than the other compounds, intermolecular interaction is higher due to the low vapor pressure. Therefore, the amount of analyte molecule passing into the vapor phase is fewer. Thus the number of ethyl acetate molecules interacting with the enzyme is lower and due to the low molecular weight, a lower value and less response were observed in the graph.

Acetone has the highest vapor pressure among the studied compounds and therefore it has the greatest amount of analyte per unit area. Wherefore the low molecular weight, it caused a lowest response than other analyte vapors.

Toluene has high molecular weight and lower vapor pressure than the other studied compounds which indicates that the interaction between toluene molecules and the sensor is high. Thus toluene was produced the third highest response besides it showed fully recovers rather than carbon tetrachloride.

CONCLUSIONS

The aim of this study was to investigate the sensor response of bio-composite thin film upon exposure to several organic vapors. Bio-composite film consists of two layers, the first layer is gelatin and chitosan mixture, the second layer is xanthine oxidase. Xanthine oxidase was chosen for the active layer of the vapor sensor which interacts with the analyte vapors and gives response depending on the amount of the interaction. Thin films of bio-composite with enzyme layer were successfully produced and controlled with the UV-vis. Bio-composite films were exposed to chloroform, toluene, ethyl acetate, acetone and carbon tetrachloride vapors. In order to perform a comparative study, dynamic measurements were carried out using QCM measurement systems. The dynamic measurement technique gives an opportunity to control the sensitivity of the sensor system against each analyte vapor. The results obtained show that bio-composite sensor demonstrate significant responses to all vapors and responses are fast, reproducible and reversible to all vapors except carbon tetrachloride. A larger response to chloroform occurred than other organic vapors. Finally, bio-composite film with xanthine oxidase enzyme can be used as a sensor material and can be used in the development of room temperature organic vapor sensor devices. In addition, by using the method of determining acetone vapor from the breath, patients can be diagnosed with an easy and painless method and the patient's disease process can be monitored.

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REFERENCES

[1] Dey, D.S., Raj, C.R. (2010) Development of an amperometric cholesterol biosensor based on graphene-Pt nanoparticle hybrid material. The Journal of Physical Chemistry C. 114 (49), 21427-21433.

[2] Komathi, S., Muthuchamy, N., Lee, K.P., Gopalan, A.I. (2016) Fabrication of a novel dual mode cholesterol biosensor using titanium dioxide nanowire bridged 3D graphene nanostacks. Biosensors and Bioelectronics. 84, 64-71.

[3] Shen, J. Liu, C.C. (2007) Development of a screen-printed cholesterol biosensor: Comparing the performance of gold and platinum as the working electrode material and fabrication using a self-assembly approach. Sensors and Actuators B: Chemical. 120 (2), 417-425.

[4] Teke, M., Sezgintürk, M.K., Dinçkaya, E., Telefoncu, A. (2008) Two biosensors for phenolic compounds based on mushroom (Agaricus bisporus) homogenate:comparison in terms of some important parameters of the biosensors. Preparative Biochemistry and Biotechnology. 38 (1), 51-60.

[5] Shen, L., Cheng, K.C.K., Schroeder, M., Yang, P., Marsh, E.N.G., Lahann, J., Chen, Z. (2016) Immobilization of enzyme on a polymer surface. Surface Science. 648, 53-59.

[6] Verma, N., Kumar, N., Upadhyay, L.S.B., Sahu, R., Dutt, A. (2017) Fabrication and characterization of cysteine functionalized zinc oxide nanoparticles for enzyme immobilization. Analytical Letters. 50 (11), 1839-1850.

[7] Bayramoglu, G., Akbulut, A., Arica, M.Y. (2013) Immobilization of tyrosinase on modified diatom biosilica: enzymatic removal of phenolic compounds from aqueous solution. Journal of Hazardous Materials. 244-245, 528-536.

[8] Carvalho, F., Paradiso, P., Saramago, B., Ferraria, A.M., Rego, A.M.G., Fernandes, P. (2016) An integrated approach for the detailed characterization of an immobilized enzyme. Journal of Molecular Catalysis B: Enzymatic. 125, 64-74.

[9] Serrano, J.L., Figueiredo, J., Almeida, P., Silvestre, S. (2020) From xanthine oxidase inhibition to in vivo hypouricemic effect: An integrated overview of in vitro and in vivo studies with focus on natural molecules and analogues. Hindawi Evidence-Based Complementary and Alternative Medicine. 17, Article ID: 9531725.

[10] Pathey, S., Venkatesan, P., Jeyaraja, K., Chandrasekar, M., Pandiyan, V. (2015) Xanthine oxidase as a biochemical marker of dilated cardiomyopathy in dogs. Indian Journal of Animal Research. 49 (2), 187-190.

[11] Gliozzi, M., Malara, N., Muscoli, S., Mollace, V. (2016) The treatment of hyperuricemia. International Journal of Cardiology. 213, 23-27.

[12] Meneshian, A., Bulkley, G.B. (2002) The physiology of endothelial xanthine oxidase: from urate catabolism to reperfusion injury to inflammatory signal transduction. Microcirculation. 9 (3), 161-175.

[13] Burmaoglu, S., Ozcan, S., Balcioglu, S., Gencel, M., Noma, S.A.A., Essiz, S., Ates, B., Algul, O. (2019) Synthesis, biological evaluation and molecular docking studies of bischalcone derivatives as xanthine oxidase inhibitors and anticancer agents. Bioorgic Chemistry. 91, 103149-103157.

[14] Gao, J., Zhang, Z., Zhang, B., Mao, Q., Dai, X., Zou, Q., Lei, Y., Feng, Y., Wang, S. (2020) Novel 3-[4-alkoxy-3-(1H-tetrazol-1-yl) phenyl]-1,2,4-oxadiazol-5(4H)-ones as promising xanthine oxidase inhibitors: Design, synthesis and biological evaluation. Bioorganic Chemistry. 95, 103564-103576.
[15] Luna, G., Dolzhenko, A.V., Mancera, R.L. (2019) Inhibitors of xanthine oxidase: scaffold diversity and structure-based drug design. ChemMedChem. 14 (7), 714-743.
[16] Tang, H., Zhao, D. (2019) Investigation of the interaction between salvianolic acid C and xanthine oxidase: Insights from experimental studies merging with molecular docking methods. Bioorganic Chemistry. 88, 102981-102991.
[17] Beyaztaş, S., Arslan, O. (2015) Purification of xanthine oxidase from bovine milk by affinity chromatography with a novel gel. Journal of Enzyme Inhibition and Medicinal Chemistry. 30 (3), 442-447.
[18] Uzunoglu, T., Evyapan, M., Beyaztas, S., Yildirim, G., Kusku, T. (2020) Immobilization xanthine oxidase on interdigitated electrode for biosensor investigation. Fresen. Environ. Bull. 29 (8), 7122-7129.
[19] Becker, M.M., Ribeiro, E.B., Marques, P.R.B.O., Marty, J.L., Nunes, G.S., Catanante, G. (2019) Development of a highly sensitive xanthine oxidase-based biosensor for the determination of antioxidant capacity in Amazonian fruit samples. Talanta. 204, 626-632.
[20] Sharma, N.K., Monika, Kaushal, A., Thakur, S., Thakur, N., Sheetal, Kumar, D., Bhalla, T.C. (2021) Nanohybrid electrochemical enzyme sensor for xanthine determination in fish samples. 3 Biotech. 11 (5), 212-218.
[21] Boluda, A., Casado, C.M., Alonso, B., Armada, M.P.G. (2021) Efficient oxidase biosensors based on bioelectrocatalytic surfaces of electrodeposited ferrocenyl polyclosiloxanes-pt nanoparticles. Chemosensors. 9 (4), 81-85.
[22] Fahad, U., Dennis,J.O., Mkawi, E.M., Al-Hadeethi, Y., Meriaudeau, F., Ferrell, T.L., Aldaghri, O., Sulieman, A. (2020) Investigation of acetone vapour sensing properties of a ternary composite of doped polyaniline, reduced graphene oxide and chitosan using surface plasmon resonance biosensor. Polymers. 12 (11), 2750-2765.
[23] Agarwal, P., Dubey, S., Singh, M., Singh, R.P. (2016) Aspergillus niger PA2 tyrosinase covalently ımmobilized on a novel eco-friendly biocomposite of chitosan-gelatin and its evaluation for l-dopa production. Frontiers in Microbiology. 7, 2088-2098.
[24] Sauerbrey, G. (1959) The use of quartz oscillators for weighting thin layers and for microweighting. Zeitschrift für Physik. 155 (2), 206-222.
[25] Uzunoglu, S.B., Beycic, T., Uzunoglu, T., Evyapan, M., Kusku, T., Arslan, O. (2020). Inhibition effect of some chemical on lactoperoxidase and covalently immobilized on a bio-composite for biosensor. Fresen. Environ. Bull. 29 (11), 9924-9933.
[26] Berg, J.M., Tymoczko, J.L., Stryer, L. (2002). Biochemistry, 5th edition. New York: WH Freeman. 209-216.

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