Indian Journal of Experimental Biology
Vol. 59, December 2021, pp. 916-920

Low temperature co-fired ceramic (LTCC)-based biosensor for detection of vanadium using immobilized *Arachis hypogaea* alkaline phosphatase on multi walled carbon nanotubes ethyl cellulose sponge matrix

Vijaya D. Giramkar[1]*, Girish J. Phatak[1] &
Sushma G. Sabharwal[2]

[1]Centre for Materials for Electronics Technology, Panchwati, Off Pashan road, Pune-411 008, Maharashtra, India
[2]Biochemistry Division, Department of Chemistry, Savitribai Phule Pune University, Pune-411 007, Maharashra, India

Received 31 May 2021; *revised 03 August 2021*

Studies on enzyme based thermometric biosensor are limited. Here, we report on fabrication of an alkaline phosphate based thermometric biosensor. We designed alkaline phosphatase inhibition based biosensor for detection of vanadium using immobilized alkaline phosphatase on multi walled carbon nano tubes (MWCTs) ethyl cellulose sponge matrix. We isolated protein from plant source, partially purified and fabricated a miniature ceramic viz. LTCC (low temperature co-fired ceramics technology) based biosensor for detection of vanadium. This biosensor consists of a microreaction chamber with buried heaters. Alkaline phosphatase has been isolated from the seeds of '*Arachis hypoghaea*' was studied for its biochemical properties viz. optimum pH and temperature. The partially purified enzyme was immobilized using carboxyl-functionalised carbon nanotubes (CNTs) by cross linking with epichlorohydrin (ECH) along with a matrix of ethyl cellulose. The developed LTCC based biosensor on testing indicated its linear response to vanadium concentration up to 9 mM with a relatively high sensitivity of about 147 nA/mM. Thus, we have demonstrated a LTCC based biosensor using immobilized alkaline phosphatase for detection of vanadium.

Keywords: Alkaline phosphatase, Electronic packaging, Heavy metal toxicity, MWCNTs, Thermometric sensor

Biosensors are widely used in various fields with major applications in the fields of medicine and health, industry, pollution monitoring and control along with chemical warfare. They have significance as appropriate detectors of heavy and toxic metal ions. They demonstrate promising for environmental monitoring, since the system is simple, rapid and selective. Several techniques based on spectroscopy, ion-selective electrodes, polarography and voltammetry have been described in the past, tyrosinase, L-lactate dehydrogenase and nitrate reductase have been used for detection of heavy metals[1].

Heavy metal ions are observed as one of the majority toxic substances affecting the environment[2]. Although some of the heavy metals are essential trace elements, majority are toxic to all forms of life at high concentrations due to formation of complex compounds within the cell. Even in minute concentration, heavy metals are hazardous to the environment as they are non-biodegradable[3]. Monitoring of heavy metal levels in the environment are essential for effective pollution control. Heavy metals which inhibit cell activity in concentrations of less than 1.0 mg/L are considered highly toxic and include Ag, Be, Hg, Sn, Co, Ni, Pb, and Cr. If the metal inhibition is between 1-100 mg/L concentration, it is said to be semi-toxic and includes As, Se, Al, Cd, Cr, Fe, Mo as well as Zn. Metals like Ca, Mg, Sr, Li which show inhibition at concentrations above 1800 mg/L show low metal toxicity[3]. Vanadium detection at very low concentrations is significant as at even low concentration, vanadium ions influence both the ecosystem and human health[3]. In most cases, the toxic metals like vanadium when comes in contact with proteins through some reactive groups they change their configuration and leads to inactivation of enzymes[4]. Protein based biosensors which monitor enzyme inhibition or activation offer fast response times[5]. Many enzyme catalyzed reactions are exothermic, generating heat (Table 1) which may be used as a basis for measuring the rate of reaction and, hence, the analyte concentration[6].

Since vanadium is a strong inhibitor of *Arachis hypogaea* alkaline phosphatase, here, we have made an attempt to fabricate a thermometric biosensor based on immobilized *Arachis hypogaea* alkaline phosphatase. Alkaline phosphatase immobilized on multiwall carbon nano tube-ethyl cellulose composite sponge matrix was used for fabrication of the biosensor and tested for detection of vanadium ions (V^{3+}).

*Correspondence:
Phone: +91 9766606748 (Mob.)
E-Mail: vijaya@cmet.gov.in

Table 1 — Heat output (molar enthalpies) of enzyme catalysed reactions

Reactant	Enzyme	Heat output -DH (kJ x mol^{-1})
Cholesterol	Cholesterol oxidase	53
Esters	Chymotrypsin	4 - 16
Glucose	Glucose oxidase	80
Hydrogen peroxide	Catalase	100
Penicillin G	Penicillinase	67
Peptides	Trypsin	10 - 30
Starch	Amylase	8
Sucrose	Invertase	20
Urea	Urease	61
Uric acid	Uricase	49

Materials and Method

'Arachis hypoghaea' alkaline phosphatase was isolated and purified as described in our earlier paper[7]. The pH and temperature optima and stability of the enzyme was determined. Alkaline phosphatase was immobilized on commercially available carboxyl functionalized multi walled carbon nanotubes MWCNTs by cross-linking with various cross linkers viz. epichlorohydrin (ECH), citric acid, glutaraldehyde and 1-ethyl -3-(3-dimethyl amino propyl) carbodiimide (EDC) as coupling agents. Also, the immobilized enzyme activity studies were also carried out using ECH-ethyl cellulose matrix and MWCNTs. For this, ethyl cellulose was dissolved in minimum required ethanol and then continuously mixed with ECH with constant stirring followed by MWCNTs. The optimum pH, pH stability, optimum temperature, temperature stability and effect of metal ions also studied for both types of immobilized enzyme. The effect of concentration of enzyme and composite matrix was also studied.

To study the change in effect of metal ions on alkaline phosphatase after immobilization, the immobilized enzyme was pre-incubated with different metal ions Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, Fe^{3+}, $Mn^{2+,}$ Ba^{2+}, Mo^{5+}, Ni^{2+}, Al^{3+}, Na^{+}, Cu^{2+}, Co^{2+}, Be^{2+} and V^{5+} (5 mM each) solutions for 1 h. The residual enzyme activity was then determined as described for the native enzyme using *p*-nitrophenyl phosphate as substrate. Corresponding controls without metal ions were also run simultaneously. For the assessment of reusability, the stored immobilized alkaline phosphatase was reused 8-10 cycles and the residual activity was measured to calculate the immobilized activity[8].

Fabrication of LTCC based thermometric biosensor

Alkaline phosphatase inhibition based biosensor for detection of vanadium was fabricated in our laboratory using above immobilized alkaline phosphatase on multi walled carbon nano tubes-ethyl cellulose sponge matrix. The low temperature co-fired ceramics (LTCC) based thermometric biosensor was fabricated as follows.

Fabrication of multilayer LTCC package

The LTCC processes have for many years been used in fabrication of electronic circuits[9]. LTCC device or package consisted of dielectric tapes, connecting vias, external and internal conductors and passive components (resistors, capacitors, inductors)[10]. The LTCC has a range of applications. Chemical reactor is one of them. The reactions often need to carry out into effect strictly controlled thermal conditions. The LTCC material combines: good electrical properties with simplicity in machining and high chemical resistance with relatively low costs of production. The substrate is relevant to build chemical or bio-chemical reactors. Furthermore, the device could be used once. It resolves contamination obscurity[11]. Currently, LTCC micro systems market is growing fast. New LTCC micro devices are becoming more and more refined. They have cooling and heating systems, sensors (gas[12], flow[13], temperature, pressure [14], proximity[15]) and actuators (micro valve, micro pump[16,17]). A LTCC micro reactor device was constructed using a 10 layered LTCC package of DuPont 951 PX tape (thickness 2.54 mm before firing) with a central open well (micro reactor cavity). The microreactor cavity was formed by punching a circular well with 5 mm diameter in the top three layers of the LTCC package.

Detection of vanadium using the sensor

The above thermometric biosensor was used for detection of vanadium based on inhibition of alkaline phosphatase by vanadium. The reactants were added in the central microreactor cavity with a final reaction volume of 150 µL. To begin with, the temperature of the reactor was maintained at 60°C (the optimum temperature of the enzyme) by adjusting voltage and current. For detection of vanadium ions, the immobilized enzyme - MWCNT-ethyl cellulose sponge matrix (50 µL, 10 mg/mL), and 50 µL of buffer (20 mM, pH 8.0) was pre-incubated with 50 µL of vanadium (of different concentrations). Temperature in the reactor well was continuously monitored. Finally after 10 min, the substrate and *p*-nitrophenyl phosphate (2 mM, 50 µL) was added and change in temperature due to addition of the substrate was determined. Measurements were done using different

concentrations of vanadium. Corresponding controls in absence of vanadium were run simultaneously. Sensor performance was also evaluated using other metals (2 mM) in order to establish the selectivity of the sensor in detection of vanadium.

Since, the effects of pH, operating temperature optimized earlier, the sensitivity, accuracy of the alkaline phosphatase assay were investigated accordingly. The assay performed satisfactorily at the pH 8.0. An operating temperature of 60°C was chosen as enzyme showed higher activity at 60°C as optimized earlier[18].

Results and Discussion

The sensitivity and accuracy of the sensor response y were investigated. The enzyme assay was carried out at its optimum pH (pH 8.0) and temperature (60°C)[18]. Fig. 1 shows the response of sensor as a function of concentration of V^{3+}. Temperature difference with respect to control (without enzyme) was checked with external thermocouple dipped in the bioreactor. Bioreactor was already set to the optimum temperature of 60°C by applying 1 mV voltage to screen printed heaters at the bottom of the microreactor. Temperature difference was measured after addition of incubated immobilized enzyme and vanadium solution. Response of the sensor was found to be linear in the range of 0.5 to 10 mM. Sensor was found to be saturated above 8 mM concentration of V^{3+} as complete inhibition of immobilized *Arachis hypogaea* alkaline phosphatase was observed at this concentration.

Immobilized phosphatase based optical biosensor was found to show detection up to 1×10^{-3} M concentration of V^{3+}. Normalized temperature difference shows a good R^2 = 0.9681 plotted per concentration of vanadium as seen in Fig. 2.

As the biosensor sensing material here is immobilized enzyme, the most outstanding advantages of immobilized enzyme is that it could be used repeatedly. To evaluate the reusability of immobilized enzyme, the same concentration of vanadium ion solutions incubated with the same enzyme for different times, and the enzymolysis rates were summarized. As displayed in Fig. 3, incubation time for immobilized enzyme matrix and vanadium solutions was optimized for 1 h. The immobilized enzyme on multi walled carbon nano tubes still could maintain 80% sensor response, after 8-10 runs repeatedly. This may due to thickening of surface layer at this loading of enzyme concentration which acts as a barrier to diffusion[19].

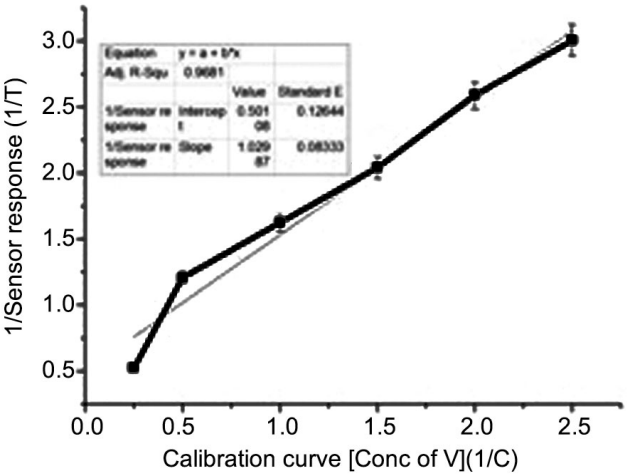

Fig. 2 — Calibration curve of 1/T as a function of concentration of V3+

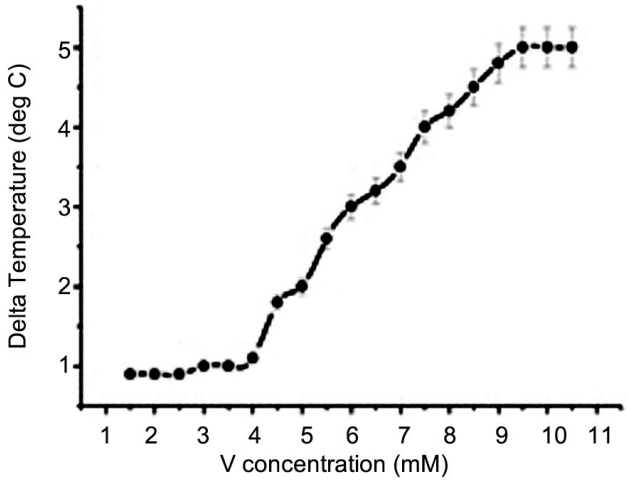

Fig. 1 — Change in temperature difference (Delta temperature) as a function of concentration of vanadium

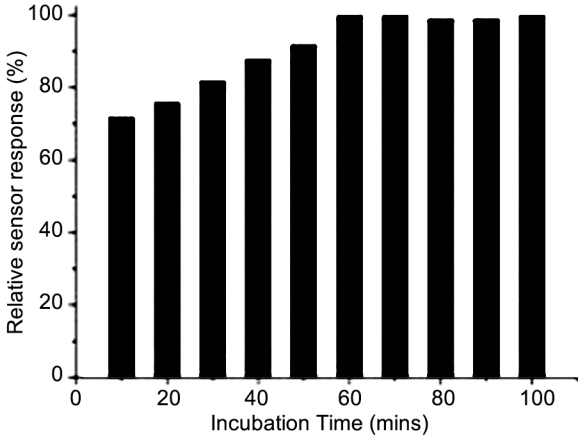

Fig. 3 — Optimization of incubation time of vanadium with immobilized enzyme composite matrix

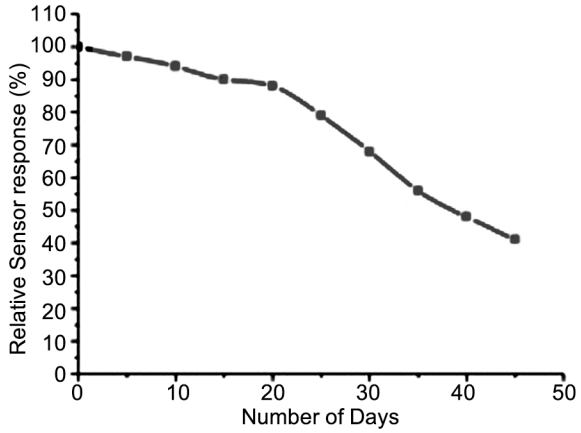

Fig. 4 — Storage stability of immobilized *Arachis hypogaea* alkaline phosphatase and carbon nanotube and ethyl cellulose composite matrix

The immobilized *Arachis hypogaea* alkaline phosphatase was stored in refrigerator at 4°C. For consistency of the biosensor, determination of vanadium was done after every five days. The biosensor was stable up to 25 days of storage at 4°C without any appreciable loss in activity. After 25 days a gradual decline in sensor performance was observed. About 50% sensor performance was observed even after 35 days (Fig. 4).

Conclusion

In the present investigation, isolation, purification and biochemical characterization of alkaline phosphatase from the *Arachis hypogaea* seeds has been carried out. An attempt has been made to evaluate the potential application of the enzyme. *Arachis hypogaea* alkaline phosphatase was found to be active in the pH range 7.0 to 10.6 with maximum activity at pH 8.0. The optimum temperature of *Arachis hypogaea* alkaline phosphatase was found to be 60°C. The alkaline phosphatase from *Arachis hypogaea* showed high catalytic activity towards *p*-nitrophenyl phosphate.The effect of metal ions on alkaline phosphatase activity revealed that vanadium ions were a potent inhibitor of the enzyme. Using immobilized *Arachis hypogaea* alkaline phosphatase a simple and portable thermometric biosensor for the detection of vanadium using LTCC (Low Temperature co-fired ceramic) based multilayer ceramics based on inhibition of the enzyme. A multi layered low temperature co-fired technology based portable thermometric biosensor was developed and used for detection of vanadium ions (V^{3+}). The ceramic materials used in LTCC designs are characteristically very temperature stable. Therefore, the necessity to compensate for variations in temperature is greatly reduced. The performance of the developed sensor was successfully evaluated using vanadium standard samples.

Conflict of Interest

Authors declare no competing interests.

References

1 Zhylyak GA, Dzyadevich SV, Korpan YI, Soldatkin AP & Elskaya AV, Application of urease conductometric biosensor for heavy-metal ion determination. *Sens Actuators B Chem*, 24 (1995) 145.

2 Bontidean I, Ahlqvist J, Mulchandani A, Chen W, Bae W, Mehra RK, Mortari A & Csoregi E, Novel synthetic phytochelatin-based capacitive biosensor for heavy metal ion detection. *Biosens Bioelectron*, 18 (2003) 547.

3 Peavy HS, Rowe DR & Tchobanoglous G, *Environmental Engineering*, (McGraw Hill Inc., Singapore), 1988.

4 Kabata-Pendias A & Dudka S, Trace metal contents of *Taraxacum officinale* (dandelion) as a convenient environmental indicator. *Environ Geochem Health*, 13 (1991) 108.

5 Oehme I & Wolfbeis S, Optical sensors for determination of heavy metal ions. *Mikrochim Acta*, 126 (1997) 177.

6 Verma N & Singh M, Biosensors for heavy metals. *BioMetals,* 18 (2005) 121.

7 *Zhang D*, Hegab HE, Dale Snow YLL & Palmer J, Im*mobilizat*ion of cellulase *on a silica ge*l substrate modified using a 3-APTES self-assembled monolayer. *Springerplus*, 5 (2016) 48.

8 https://www.lenntech.com/periodic/elements/v.h*tml*

9 Smetana W, Balluch B, Stangl G, Gaubitzer E, Edetsberger M & Köhler G, A *multi-sensor* biological monitoring module built up in LTCC-technology. *Microelectron Eng*, 84 (2007) 1240.

10 Golonka LJ, New application of LTCC technology. *Proceeding of the 28 th International Spring Seminar on Electronics Technology, Wiener Neustadt*, 2005.

11 Pawel Bembnowicz, Golonka LJ, LTCC Microreactor with Thermal Stabilization. *IEEE* (2008) 276.

12 Teterycz H, Kita J, Bauer R, Golonka LJ, Licznerski BW, Nitsch K & Wiśniewski K, New design of an SnO2 gas sensor on low temperature co-firing ceramics. *Sens. Actuators B*, 47 (1998) 100.

13 Gongora-Rubio M, Sola·-Laguna LM, Moffett PJ & Santiago-Avile JJ, The utilisation of low temperature cofired ceramic (LTCC-ML) technology for mesoscale EMS, a simple thermistor based flow sensor. *Sens. Actuators A*, 73 (1999) 215.

14 Golonka LJ, Dziedzic A, Roguszczak H, Tankiewicz S & Terech D, Novel technological and constructional solutions of pressure sensors made in LTCC technology. *Proceeding of SPIE on Optoelectronic and Electronic Sensors IV*, 4516 (2000) 10–14.

15 Gongora-Rubio MR, Espinoza-Vallejos P, Sola-Laguna L & Santiago-Aviles JJ, Overview of low temperature cofired ceramics tape technology for meso-system technology (MsST). *Sens. Actuators A*, 89 (2001) 222–241.

16 Wilcox DL, Multilayer ceramic microsystems technology. *Proceeding of the Thirteenth European Microelectronics and Packaging Conference, Strasbourg*, 2001, 115–117.
17 Martin Chaplin, Calorimetric biosensors. In: *Enzyme Technology*, 2014.
18 Vijaya D. Giramkar, Dr. Girish J. Phatak & Prof. Sushma G. Sabharwal, Biochemical Studies and Characterization of Immobilized Alkaline Phosphatases on Carboxyl-Functionalised Carbon Nanotubes. *Int J Sci Res Sci Technol*, 5-1 (2018) 2395.
19 Basu AK, Chattopadhyay P, Roychudhuri U & Chakraborty R, Development of Biosensor based on L-Glutamate oxidase for determination of monosodium glutamate in food. *Indian J Exp Biol*, 44 (2006) 392.