

An amperometric polyphenol biosensor based on laccase immobilized on epoxy resin membrane

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A polyphenol biosensor employing epoxy resin membrane bound *Ganoderma* sp. laccase has been developed. The biosensor showed optimum response within 30 s, when operated at 0.4 V in 0.1 M acetate buffer, pH 6.0 and 35 °C. Detection limit of the biosensor was 3.0×10^{-7} M. Analytical recovery of added guaiacol was 96.66%. Within batch and between batch coefficients of variation were <1.35% and <2.97% respectively. The biosensor was employed for amperometric determination of polyphenols in fruit juices and alcoholic beverages. The enzyme electrode was used 200 times over a period of 10 months, when stored at 4 °C.

Introduction

Polyphenolic compounds are byproducts of large-scale production of man-made organics such as drugs, dyes, antioxidants, paper pulp, plastic and pesticides, which have ecologically harmful effects.¹ Most phenols exhibit different toxicities and their determination is very important when evaluating the total toxicity of an environmental sample. Their role as antioxidants is significant in terms of prevention of cardiovascular diseases and there are claims that some may prevent cancer, these are free radical scavengers, neutralizing oxygen reactive species and chelating metal ions. Most analytic methods used in qualitative and quantitative determination of polyphenols in juices, beverages, red wine *etc* include chromatography methods HPLC, GC-LC,² ELISA (enzyme linked immune-sorbent assay)³ and spectrophotometry.⁴ However, these methods are expensive, time-consuming, require costly reagents, and several operations and separation steps. Furthermore, these methods lead to the emergence of large amount of effluents, which are not environment friendly.⁵ A number of biosensors have previously been developed using the catalytic activity of redox enzymes for phenol determination, such as tyrosinase, peroxidase, laccase *etc*.⁶ Laccase does not require H₂O₂ as a co-substrate or any other cofactors for its catalytic action making the construction of a laccase-based biosensor easier. Laccase has the capability of catalyzing 4-electron reduction of oxygen to water with concomitant oxidation of a broad range of substrates such as phenols, anilines, benzenethiols and phenothiazines⁷ which make it useful for the amperometric determination of phenolic compounds.⁸ However, most applications require laccase in its immobilized form, which is significant for its performance. The

development of an enzyme-based biosensor with excellent performance requires advances in the support matrix and methods for its immobilization.⁹ The immobilization of laccases on various carriers, including activated glass beads, Sepharose, gelatin, polyurethane, platinum and organic gels has been reviewed.¹⁰ Important recent supports used for immobilization include woven nylon supports,¹¹ Fe₃O₄-SiO₂ nanoparticles,¹² granocel,¹³ and silver nanoparticles.¹⁴ The present report describes the construction of an electrochemical biosensor based on 'Araldite' epoxy resin membrane bound laccase purified from *Ganoderma* sp. *Rckk02*. The Araldite membrane is composed of epoxy resins with epoxy groups on both sides of a single epoxy resin, which forms a highly cross-linked network that is long lived and water permeable. The membrane has certain advantages such as high affinity for the enzyme, low cost, easy synthesis, high temperature stability, chemical resistance and porous nature and thus is an ideal material for forming an enzyme electrode.¹⁵

Materials and methods

Materials

Sephadex G-100 and DEAE-Sephacel from Sigma Aldrich, Guaiacol from SRL, Mumbai were used. The source of 'Araldite' epoxy resins and polyamine crosslinker, manufactured by Huntsman Advanced Materials Pvt. Ltd., Mumbai was purchased from the local market. All other chemicals used were of AR grade. Fruit juices and alcoholic beverages of various commercial brands were also obtained from the local market.

Purification of laccase

The cell free extract of *Ganoderma* sp. *Rckk02* grown in malt extract broth (MEB)^{16,17} was used as crude laccase. The crude laccase was purified at 4 °C using 0–80% ammonium sulfate

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precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephacel using a linear gradient of KCl (0.1M to 0.6M). The activity and protein content of various enzyme preparations were assayed as follows.

Assay of laccase

The laccase assay was based on oxidative polymerization of guaiacol.¹⁶ Guaiacol (0.4 μ moles) and acetate buffer (300 μ moles) in a total volume of 4.0 ml (pH 5.0) was used as the substrate. Changes in absorbance of 0.01 $\text{min}^{-1} \text{ml}^{-1}$ at 470 nm was defined as one unit of the enzyme activity. Protein content in various enzyme preparations was determined by the Lowry method using bovine serum albumin as the standard protein.

Preparation of laccase–epoxy resin biocomposite membrane

The epoxy resin and hardener of Araldite were mixed on a plastic (polythene) sheet (size $4 \times 4 \text{ cm}^2$) in a 85 : 15 ratio at room temperature for 5 min. Two ml of purified enzyme was added to this mixture and spread equally to enable polymerization and crosslinking for 48 h. The Araldite membrane with entrapped laccase was stripped from the plastic sheet and washed with 20 mM acetate buffer pH 5.0.¹⁵ Araldite membrane with and without enzyme was subjected to scanning electron microscopy (SEM) to confirm immobilization, this procedure was carried out at the AIIMS, New Delhi.

Construction of an amperometric polyphenol biosensor and the response measurement

An amperometric biosensor for measurement of polyphenols was constructed using a Pt electrode mounted with Araldite membrane containing immobilized laccase, as the working electrode, Ag/AgCl as the reference electrode and a Cu wire as the auxiliary electrode. Araldite membrane was wrapped and fixed firmly onto the Pt wire with the help of a parafilm. The working, reference and auxiliary electrodes were connected through a three-terminal electrometer/high resistance meter (Keithley Japan Model 6517A/E). To avoid any possible interference of copper ions generated from Cu wire (auxiliary electrode), a control was also run in which the working electrode had no immobilized enzyme and negligible interference was found.^{15,18,19} To test the activity of the biosensor, all three electrodes were dipped into 4 ml reaction mixture [1.0 ml of 0.4 mM guaiacol and 3.0 ml of 0.1 M acetate buffer pH 5.0]. The electrodes were polarized at different potentials (at 0.1–0.6 V) and the current was measured through an electrometer. The maximum current was generated at 0.4 V, hence in subsequent amperometric experiments the current (mA) was read at 0.4 V. The current was also measured at varying concentration of guaiacol in acetate buffer (0.1 M, pH 5.0). The principle illustrating how this biosensor works is described as follows. Guaiacol is oxidized into its corresponding o-quinone by Araldite membrane bound laccase. It is then regenerated through electrochemical reduction of o-quinone, thus it forms a bioelectrocatalytic amplification cycle generating electrons, which are passed to a Pt electrode from the solution through the pores of the Araldite membrane. Fig. 1 shows a schematic diagram of laccase immobilization onto an epoxy resin membrane.

Study of analytical and kinetic properties of immobilized laccase

Various analytical and kinetic properties of Araldite membrane bound enzyme such as optimum pH, incubation temperature, response time and effect of substrate (guaiacol) concentration were studied amperometrically to determine the optimum working conditions of the biosensor.

Amperometric determination of polyphenols in fruit juices and alcoholic beverages

The polyphenols levels in fruit juices and alcoholic beverages were determined by the laccase biosensor as described for its optimal working conditions except that guaiacol was replaced by these samples. The current (mA) was recorded and the amount of polyphenols was extrapolated from the standard curve between guaiacol concentrations and current (mA) prepared under optimal working conditions.

Results and discussion

Purification of laccase

Laccase was purified from a cell free extract of *Ganoderma* sp. by 84.12 fold with 76.44% yield (Table 1). The purified enzyme showed a single band in PAGE stained with coomassie blue.

Immobilization of laccase onto the Araldite membrane

Laccase purified from *Ganoderma* sp. was immobilized onto an Araldite membrane with 93% retention of initial activity of free enzyme. The –OH groups of epoxy (based on dihydroxydiphenylpropane and epichlorohydrin) containing polymers reacted with the bifunctional polyamine and –NH₂ groups of the enzyme to form a C–N linked enzyme – epoxy amine resin composites¹⁵ (Fig. 1).

Scanning electron microscopic (SEM) study of the epoxy resin membrane

The Scanning Electron Microscopic images of surface of the Araldite membrane with and without enzyme is shown in Fig. 2A and 2B. High resolution scanning electron micrographs of Araldite membrane with immobilized enzyme revealed clusters along some beaded structures that were not observed in the membrane without the immobilized enzyme present. This might be due to both the chemical coupling as well as adsorption of the enzyme. A change in surface morphology of the membrane after the immobilization process is evidence of enzyme immobilization. The formation of clusters instead of regular globular beaded structures may be due to high concentrations of enzyme on the surface of the Araldite membrane.

Construction of the polyphenol biosensor based on epoxy resin membrane bound laccase

A method is described for construction of an amperometric polyphenol biosensor based on epoxy resin membrane bound laccase. Earlier same membrane based biosensors have been reported from this laboratory for uric acid¹⁵ and ascorbate¹⁸

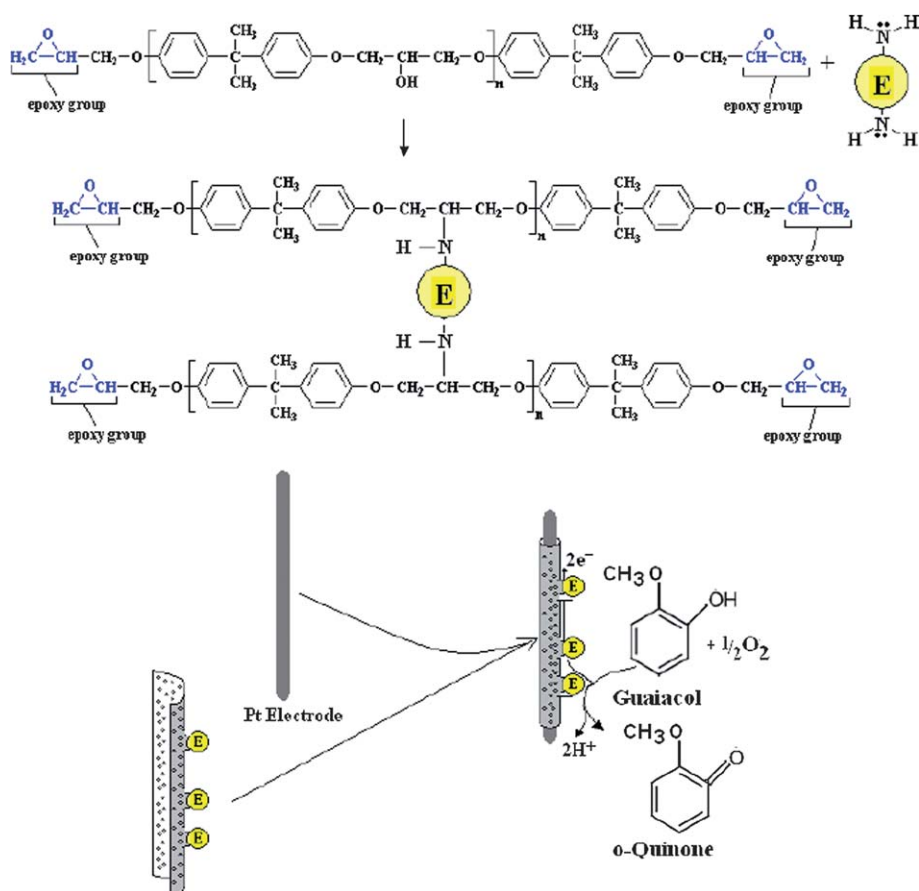


Fig. 1 Scheme of the chemical reactions involved in the immobilization of laccase onto epoxy resin membrane and the construction and response measurement of the working electrode.

Table 1 Purification of laccase from *Ganoderma lucidum*

Purification step	Total vol (ml)	Protein (mg ml ⁻¹)	Activity ^a (units/ml min ⁻¹)	Specific activity	Purification fold	Yield (%)
Crude enzyme	150	1.85	11.7	6.324	1	100
Ammonium sulfate precipitation 0–80%	7.5	2.12	44.8	21.132	3.3415	19.145
Sephadex G-200	26	0.148	37.2	251.35	39.745	55.111
DEAE Sephacel	26	0.097	51.6	531.96	84.1176	76.44

^a One unit of laccase is defined as the change in absorbance of 0.01 min⁻¹ ml⁻¹ at 470 nm.

Optimum pH

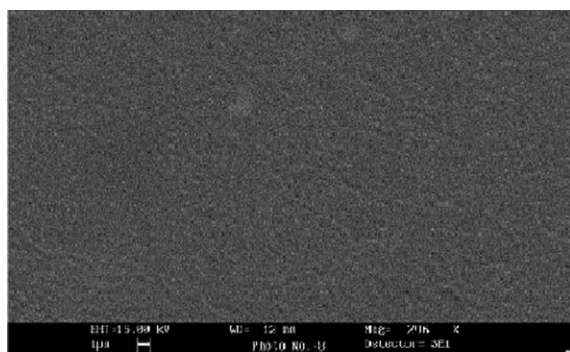
The effect of pH of reaction buffer on the biosensor response was studied in the range, pH 4.0 to 6.5 for 2 μM guaiacol solution using 10 mM acetate buffer and pH 7.0 to 8.0 using 10 mM sodium phosphate buffer. The biosensor response increased as the pH was increased up to pH 6.0 and thereafter it declined (Fig. 3). The optimum pH of the immobilized laccase enzyme in this study (pH 6.0) was similar to that of free enzyme (pH 6.0) but higher than the immobilized enzyme on different supports, e.g. chitosan membrane modified with tripolyphosphate (pH 4.0)²⁰ and hydrotalcites (pH 4.0),²¹ polyethersulfone membrane (pH 4.5)⁵ and lower than that of the immobilized enzyme on carbon paste modified graphite electrode (pH 7.0)²² and comparable to that of carbon nanotube–chitosan composite (pH 6.0)²³ and nitrocellulose membrane (pH 6.5).²⁴

Optimum incubation temperature

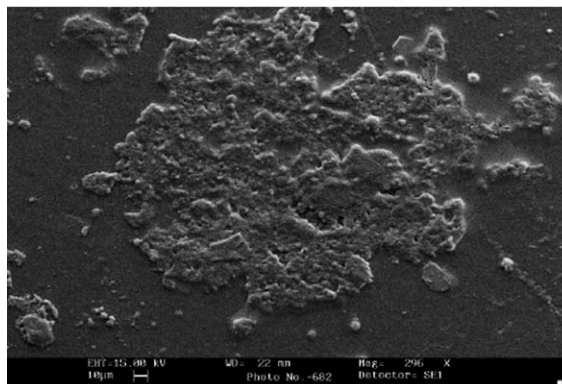
The optimum temperature for the biosensor was investigated by varying the incubation temperature in a temperature controlled water bath and was found to be 35 °C (Fig. 4) which is lower than that of the immobilized enzyme on hydrotalcites²¹ and nitrocellulose membrane²⁴ but higher than that of immobilized enzyme on carbon paste modified graphite electrode.²²

Effect of substrate concentration on the biosensor

To study the effect of substrate concentration on biosensor response, the concentration of guaiacol was varied from 10 μM to 80 μM . A hyperbolic relationship was found between this guaiacol concentration range *versus* current (mA) (Fig. 5). K_m for guaiacol, as calculated from a Lineweaver–Burk plot (inset



(A)



(B)

Fig. 2 SEM of Araldite membrane (A) without enzyme at 2500x magnification and (B) with immobilized enzyme at 296x magnification.

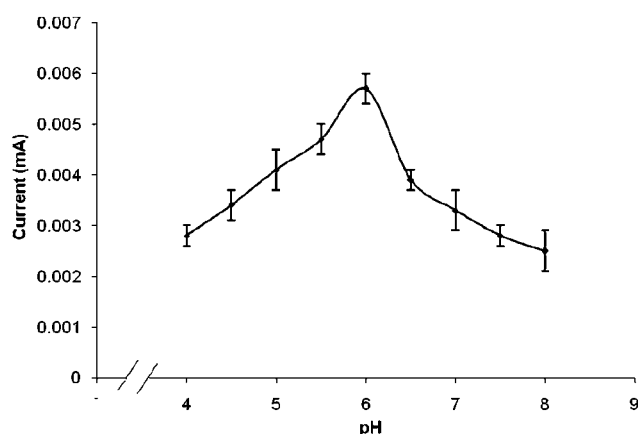


Fig. 3 Effect of pH on response of polyphenol biosensor based Araldite membrane bound laccase.

Fig. 5) at $38.46 \mu\text{M}$, which was lower than that of a carbon paste modified graphite electrode (3.87 mM)²² and carbon nanotube–chitosan composite (3.22 mM).²³ I_{max} was $0.31 \mu\text{A ml}^{-1} \text{ min}^{-1}$. The following listed criteria were studied to evaluate the performance of the biosensor

Linearity

There was a linear relationship between current (mA) and guaiacol concentration ranging from 5×10^{-7} to $5 \times 10^{-5} \text{ M}$

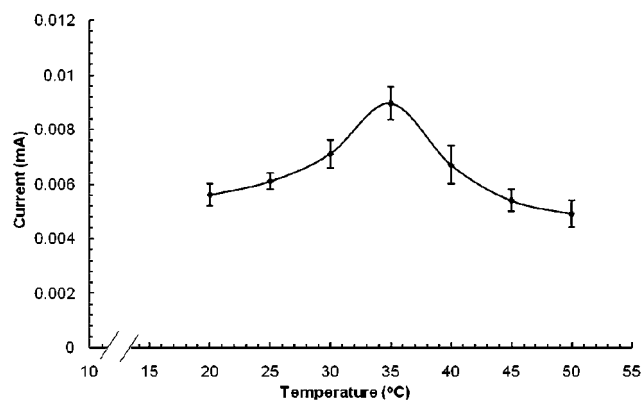


Fig. 4 Effect of temperature on the response of polyphenol biosensor based Araldite membrane bound laccase.

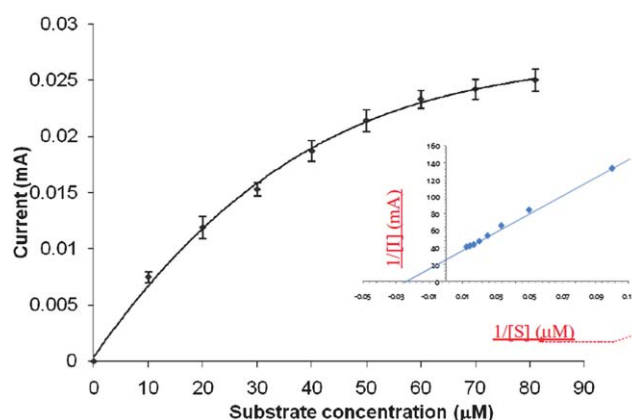


Fig. 5 Effect of substrate concentration on the response of polyphenol biosensor based on Araldite membrane bound laccase. Inset: Lineweaver–Burk plot.

(Fig. 5), which was found to be better than an earlier biosensor based on polyethersulfone membrane (1×10^{-5} to $8 \times 10^{-5} \text{ M}$)⁵ and a carbon paste modified graphite electrode (1.97×10^{-4} to $3.24 \times 10^{-3} \text{ M}$)²² and carbon nanotube–chitosan composite (1.2×10^{-6} to $3 \times 10^{-5} \text{ M}$)²³ but comparable to that based on chitosan membrane modified with tripolyphosphate (5.99×10^{-7} to $3.92 \times 10^{-6} \text{ M}$).²⁰

Detection limit

The detection limit of the biosensor was $3 \times 10^{-7} \text{ M}$, which is lower and better than polyethersulfone membrane [$1 \times 10^{-5} \text{ M}$],⁵ carbon paste modified graphite electrode ($1.97 \times 10^{-4} \text{ M}$)²² and carbon nanotube–chitosan composite ($6.6 \times 10^{-7} \text{ M}$).²³ But it was higher than that based on chitosan membrane modified with tripolyphosphate ($6.23 \times 10^{-8} \text{ M}$).²⁰

Recovery

To study the analytical recovery, exogenous guaiacol of known concentration was added to tea leaf extract ($3.0 \mu\text{M}$ and $6.0 \mu\text{M}$). The concentration of polyphenol was measured in fruit juice before and after addition of exogenous guaiacol. The % recovery of added guaiacol in fruit juice was calculated. The % recovery

Table 2 Within and between assay coefficients of variation (CV) for the determination of polyphenols in fruit juice samples as measured by a polyphenol biosensor based on Araldite membrane bound laccase

<i>n</i>	Guaiacol (μM)	CV (%)
Within assay (5)	1.87 ± 0.04	1.35
Between assay (5)	1.98 ± 0.05	2.97

measured by the present sensor was 90.00 % and 96.66 %, respectively.

Precision

To check the reproducibility and reliability of the present method, the polyphenols in the five fruit juice samples were determined six times on a single day (within batch) and six times again in the same samples after storage at -20°C for one week (between batches). The results showed that the determinations were consistent, and within and between coefficient of variation (CVs) were $<1.35\%$ and $<2.97\%$, respectively (Table 2).

Reusability and storage

The enzyme electrode was reused 200 times over a period of 10 months when stored at 4°C , 60% retention of original response was obtained when stored at 4°C (Fig. 6). This stability of the Araldite membrane bound enzyme was better than that obtained with earlier sensors.^{5,19,20,22}

Accuracy

To determine the accuracy of the method, the level of polyphenol in 15 fruit juice samples was determined by standard spectrophotometric method (x) and by the present method (y). The polyphenol values obtained by both methods were in good agreement with each other with correlation coefficient ($r = 0.98$) (Fig. 7). Evaluation studies showed that the method was consistently reliable with high recovery and in agreement with the standard method.

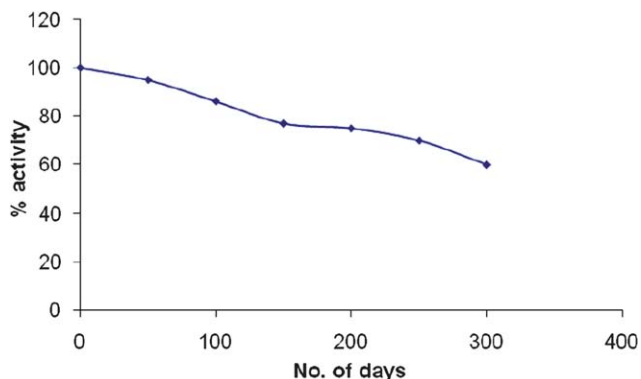


Fig. 6 Storage stability of laccase electrode in reaction buffer at $4\text{--}8^\circ\text{C}$.

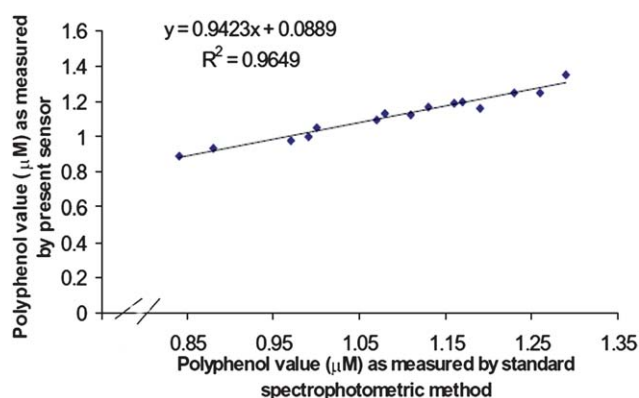


Fig. 7 Correlation between polyphenol values determined by standard spectrophotometric methods and the present method.

Table 3 Polyphenol levels in different brands of tea and alcoholic beverages as measured by polyphenol biosensor based on Araldite membrane bound laccase

Biological samples	Polyphenol level (μM) Mean $\pm$ SD ($n = 4$)
1. Fruit juices	
a) Mixed	0.88 ± 0.03
b) Frooti	0.81 ± 0.03
c) Litchi	1.23 ± 0.15
d) Appy	1.92 ± 0.19
e) Grape	1.27 ± 0.10
2. Alcoholic Beverages	
a) Vodka	2.9 ± 0.21
b) Royal challenge	2.5 ± 0.28
c) Beer	3.0 ± 0.45
d) Whisky	2.2 ± 0.18
e) Local	1.9 ± 0.24

Determination of polyphenols in fruit juices and alcoholic beverages

Polyphenols levels as measured by the present sensor ranged from $0.81\text{--}1.92\ \mu\text{M}$ in fruit juices and $1.9\text{--}3.0\ \mu\text{M}$ in alcoholic beverages (Table 3).

Conclusion

An amperometric polyphenol biosensor was constructed by immobilizing laccase from *Ganoderma* sp. on an epoxy resin membrane. The advantage of the biosensor is its lower detection limit and longer life time compared to earlier designed biosensors.

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