

Research article



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A novel immobilization matrix for the biosensing of phenol: self assembled monolayers of calixarenes

Phenolün biyosensörle belirlenmesi için yeni bir immobilizasyon matriksi: kaliksarenlerin kendiliğinden oluşan tek tabakaları

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Abstract

Aim: The development of calixarene based phenol biosensor.

Methods: This study describes the application of a calixarene derivative, 5,17-diamino-25,27-bis(3-thiol-1-oxypropane)-26,28-dihydroxycalix[4]arene (HS-Calix-NH₂) which has both amino and thiol functionalities, in the practical surface modifications for biomolecule binding. The structure of HS-Calix-NH₂ allows easy interaction with Au surface and one-step biomolecule immobilization. Self-assembled monolayers (SAMs) of p-amino-functionalized mercaptoalkylcalixarene (HS-Calix-NH₂) were formed onto the Au electrode. Then, Laccase (Lac)

enzyme was immobilized onto the modified surface by crosslinking with glutaraldehyde (GA). Resulted electrode (HS-Calix-NH₂/Lac) was used for the electrochemical analysis of phenolic compounds at – 50 mV.

Results: The linearity was observed in the range of 0.1–100 µM and 1.0–100 µM for catechol and phenol, respectively. The potential use of the biosensor was investigated for phenol analysis in artificial samples which simulate the industrial waste water, which is highly acidic and composed of concentrated salt, without needing any sample pre-treatment step.

Conclusion: The prepared Lac biosensor has a potential for rapid, selective and easy detection of phenolic contaminations in samples.

Keywords: Functionalized calixarene; Laccase; Phenol detection; Amperometric biosensor; Immobilization of enzymes.

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Özet

Amaç: Kaliksaren temelli fenol biyosensörlerinin geliştirilmesi.

Metod: Bu çalışma amino ve tiyol fonksiyonel gruplara sahip, biyomolekül bağlanmasına olanak sağlayan kaliksaren türevinin (5,17-diamino-25,27-bis(3-tiyol-1-oksiopropan)-26,28-dihidroksikaliks[4]aren (HS-Calix-NH₂) uygulamasını tasvir etmektedir. HS-Calix-NH₂ nin yapısı altın yüzey ile kolay etkilesime ve tek basamaklı biyomolekül immobilizasyonuna izin vermektedir. İlk olarak HS-Calix-NH₂ kullanılarak altın elektrod yüzeyinde kendiliğinden oluşan tek tabaka meydana getirildi. Daha sonra lakkaz enzimi kaliksaren modifiye altın yüzeye glutaraldehyit ile

capraz bağlanarak immobilize edildi. Elde edilen (HS-Calix-NH₂/Lac) elektrod fenolik bileşiklerin -50 mV da elektrokimyasal tayininde kullanıldı.

Bulgular: Katesol ve fenol için lineer aralık sirasi ile 0.1–100 µM ve 1.0–100 µM olarak tayin edildi. HS-Calix-NH₂/Lac biyosensörü kullanılarak yapay endüstriyel atık suyunu simule eden asidik örneklerde fenol tayini hic bir ön uygulama basamagina gerek duyulmadan gerçekleştirildi.

Sonuc: Hazırlanmış olan lakkaz biyosensörü fenol kontaminasyonlarının hizli, secimli ve kolay tayininde kullanilma potansiyeline sahiptir.

Anahtar Kelimeler: Fonksiyonelleştirilmiş kaliksaren; Lakkaz; Fenol tayini; Biyosensör.

Introduction

Calixarenes are cyclic oligomers synthesized by oligomerization of *p*-substitute phenol and formaldehyde. These materials have the flexibility to adjust the cavity dimension and excellent ability to form host–guest complexes [1–3]. Due to their versatility, calixarenes can be considered one of the important member of macrocyclic family, and these are applied intensely in many fields such as molecular recognition, catalysis, nanotechnology, sensing and self-assembly as cyclodextrins and crown ethers [4, 5]. Previously, Oh et al. reported a sensitive protein chip coated with the biofunctional calix crown derivatives that allow efficient immobilization of capture proteins on solid surfaces [6]. Moreover, Jung et al. used the self-assembled monolayer (SAM) of calix[4]crown-5-3 for immobilization of capture antibody monolayer for the electrochemical immunosensing of glucose oxidase labeled C-reactive protein antigen [7]. Furthermore, Snejdarkova et al. presented the usage of 25,26,27,28-tetrakis(11-sulfanylundecyloxy)calix[4]arene on the Au surface for the electrochemical and acoustic dopamine analysis [8]. A sensitive and selective electrochemical method for norepinephrine was developed by Zhang et al. using glassy carbon electrode (GCE) modified with calix[4]arene crown-4 ether film [9]. Additionally, Demirkol et al. synthesized 5,11,17,23-tetra-tert-butyl-25,27-bis(3-thiol-1-oxypropane)-26,28 dihydroxycalix[4]arene (SH-Calix) and used it to modify Au electrode via formation of SAM. In the next step, glucose oxidase was immobilized to the modified surface via 1,1'-carbonyldiimidazole chemistry that forms cross linkages between OH groups of calixarene and amino groups of enzyme [10]. Recently, 5,17-diamino-25,27-bis[N-(2-aminoethyl)]calix[4]azacrown

modified montmorillonite was used to immobilize pyranose oxidase on GCE for glucose biosensing [11].

The formation of SAMs using calixarenes was tested first time in our previous study calixarenes containing hydroxyl (to prepare glucose oxidase biosensor) [10] and thiol and in this study calixarenes containing amine and thiol as a functional group for the covalent immobilization of enzymes (to prepare laccase biosensor). Here, we describe the application of a functional calixarene derivative; 5,17-diamino-25,27-bis(3-thiol-1-oxypropane)-26,28-dihydroxycalix[4]arene (HS-Calix-NH₂) for the practical, one-step immobilization of biomolecules onto the Au surfaces that is very promising in biosensor applications. In order to construct phenolic biosensor, Lac was covalently immobilized onto the surface of calixarene modified gold surfaces, namely HS-Calix-NH₂ which was successfully synthesized, characterized and applied in dye-sensitized solar cells in our previous work [12]. Formation of SAMs on the electrode was performed via bonds between gold and thiol groups of calixarenes, thus free amino groups of HS-Calix-NH₂ remained on exterior side of the surface. These amino functionalities of HS-Calix-NH₂ played a key role for the stable immobilization of the enzyme by crosslinking with glutaraldehyde (GA). GA is a proper bifunctional crosslinker which binds biomolecules to the immobilization support through their amino groups, with suitable reaction time, pH value and temperature [13]. Biosensor preparation was performed by mixing of Laccase (Lac) enzyme, bovine serum albumin (BSA) and GA and then, coated onto the HS-Calix-NH₂-modified Au electrode. During the measurements, Lac is oxidized by molecular oxygen [14] and then, reduced again by the help of phenolic substrates acting as a donor for electrons to regenerate the enzyme, the phenolic substrates are converted to the quinone and/or phenoxy radicals. These oxidized species can be reduced at the electroactive surfaces at potentials below than 0.0 V [15]. After optimization studies, analytical characteristics were investigated and HS-Calix-NH₂/Lac biosensor was applied for analysis of the phenol amount in artificial wastewater samples.

Materials and methods

Materials

Laccase (Lac, EC 1.10.3.2, from *Agaricus bisporus*, lyophilized powder, 6.8 U per mg solid), GA solution (25%, v/v), dimethyl sulfoxide (DMSO), BSA were purchased from Sigma Aldrich. All other chemicals were analytical

grade. TLC analyses were performed on DC Alufolien Kieselgel 60 F254 (Merck). Drying of solvents were carried out by storing them over molecular sieves (Aldrich; 4 Å, 8–12 mesh). All reactions, except noted ones, were conducted under nitrogen atmosphere. All starting supplies used for the synthesis of calixarenes were of standard analytical grade from Merck or Aldrich and used without further purification. To prepare all aqueous solutions, deionized water, passed through a Millipore milli-Q Plus water purification system, was used.

p-tert-Butylcalix[4]arene (1), calix[4]arene (2), 25,27-bis-(bromopropoxy)-26,28-dihydroxycalix[4]arene (3), 5,17-dinitro-25,27-bis(3-bromo-1-oxypropene)-26,28-dihydroxycalix[4]arene (4), 5,17-diamino-25,27-bis(3-bromo-1-oxypropene)-26,28-dihydroxycalix[4]arene (5) and 5,17-diamino-25,27-bis(3-thiol-1-oxypropene)-26,28-dihydroxycalix[4]arene (6) were synthesized according to previous procedures [1–3, 12]. The synthesis of HS-Calix-NH₂ and the applications in dye sensitized solar cells were published [12].

Apparatus

Voltammetric and amperometric measurements were carried out by Palm Sens analyzer (Palm Instruments, Houten, Netherlands). The experiments were performed in a reaction cell (10 mL) at room temperature. A three electrode system consisted of Au (1.6 mm diameter, 99.95% purity; BASI, USA) as the working electrode, a Pt (Metrohm, Switzerland) as the counter electrode and a Ag/AgCl (in 3.0 M KCl, Metrohm, Switzerland) electrode as the reference electrode were used. Surface characterization of the biosensor was carried out on a JEOL5600-LU model scanning electron microscope (SEM). SEM images were taken using an acceleration voltage of 20 kV.

Biosensor preparation

Smoothing and polishing of the electrode surface were made using a piece of cloth with various sized alumina powder (Gamma, 0.05; 0.1; 0.3; 1.0 mm), rinsed with pure water and placed in an ultrasonic bath to remove any substances from the surface. Afterwards, the electrode was cleaned and polished as follows: initially, the electrode was immersed into the ethanol/deionized water mixture (1 : 1 v/v), then it was put in an ultrasound bath type sonicator (Elma Schmidbauer GmbH, Singen, Germany) for 5 min. After washing with Millipore water, the cyclic voltammetry (CV, voltage range between 0.5 and 1.5 V with a scan rate of 50 mV/s) was applied in 0.5 M H₂SO₄ until

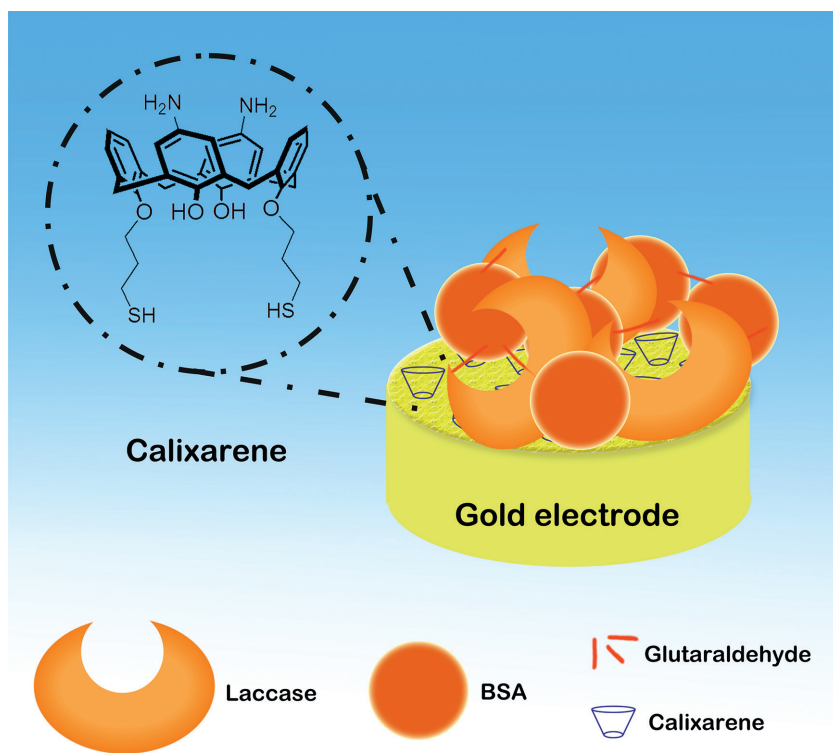
reproducible voltammetric responses were obtained. The electrode was then washed with deionized water. For the surface modification, initially, 0.25 mg HS-Calix-NH₂ was dissolved in 0.025 mL of DMSO and adjust to 0.25 mL with PBS (NaCl 8.0 g·L⁻¹, KCl 0.2 g·L⁻¹, Na₂HPO₄ 1.44 g·L⁻¹, KH₂PO₄ 0.24 g·L⁻¹ and pH 7.4), this solution was dropped on the electrode and allowed to stand 30 min at room temperature. During the incubation time SAMs of *p*-amino-functionalized mercaptoalkylcalixarene (HS-Calix-NH₂) were formed onto the Au electrode. The electrode surface was washed with PBS to remove non-bound molecules. For the enzyme immobilization, 1.0 mg Lac was dissolved in 0.05 M sodium phosphate buffer (0.005 mL, pH 6.0), the mixture of 2.5 μL of 2.5% of GA and 2.5 μL of BSA (1.0 mg·mL⁻¹) solution was prepared in PBS and spread over the surface of HS-Calix-NH₂ modified gold electrodes, dried for 1 h at ambient conditions. The resulted surface was shown in Scheme 1.

Measurements

Electrochemical measurements for the phenolic substrates (phenol and catechol) were carried out at –50 mV in 50 mM sodium phosphate buffer (pH 6.0) [16]. When the phenolic compounds are added into the working buffer, immobilized Lac on the surface of working electrode is oxidized by molecular oxygen. After that it was reduced again by phenolic compounds, acting as electron donors for the enzyme regeneration. Then, the phenolic compounds are oxidized to quinone, phenoxy radicals, or both, and subsequently, these oxidized species can be reduced at the working electrode [17]. After characterization, the performance of HS-Calix-NH₂/Lac biosensor was tested by using artificial waste water sample with highly acidic nature (50 g/L NaCl and 100 g/L phenol in 1.0 M HCl). The sample was diluted with working buffer and added into the cell as a substrate. The phenol calibration curve was used for the calculation of phenol concentration in the sample.

Results and discussion

In this study, an efficient calixarene derivative was used to present the practical surface modifications for biomolecule binding. For this purpose, 5,17-diamino-25,27-bis(3-thiol-1-oxypropene)-26,28-dihydroxycalix[4]arene was successfully synthesized according to the known procedure [12]. Typically, reduction of 5,17-dinitro-25,27-bis(3-bromo-1-oxypropene)-26,28-dihydroxycalix[4]arene



Scheme 1: Schematic representation of HS-Calix-NH₂/Lac biosensors.

with Raney-Ni gave the target compound (5,17-diamino-25,27-bis(3-bromo-1-oxypropane)-26,28-dihydroxycalix[4]arene) in 83% yield. This p-Diamino-substituted derivative was then treated with thiourea in acetonitril under reflux to yield the target 5,17-diamino-25,27-bis(3-thiol-1-oxypropane)-26,28-dihydroxycalix[4]arene in 59.6%.

Biosensing studies

Initially the modified surface was characterized via CV using K₃Fe(CN)₆ as redox probe. The difference in the voltammograms as a result of surface coating was shown in Figure 1. The oxidation and reduction peaks are obtained at about 0.25 and 0.18 V for bare electrode (peak-to-peak separation of 70 mV), 0.27 and 0.15 V for HS-Calix-NH₂ modified surface (peak-to-peak separation of 120 mV). A drop in the peak current when the Au electrode was coated with HS-Calix-NH₂ was observed due to the rather inefficient electron transfer properties. The surface morphologies of HS-Calix-NH₂ and HS-Calix-NH₂/Lac modified surfaces were characterized by SEM and the images were shown in Figure 2. Figure 2 gives information about the surface before and after enzyme immobilization. In Figure 2A, the images of the electrode surface after calixarene covering and Figure 2B shows the big difference after covalent

immobilization of the Lac. It can be seen that the addition of enzyme and the crosslinking enzyme and calixarene on the surface of Au electrode using GA caused the formation of coverage on the Au surface. The membrane structure was formed after immobilization step that includes crosslinking with the bifunctional cross-linker in the presence of Lac on the electrode surfaces (Figure 2B).

The effect of pH on the HS-Calix-NH₂/Lac response was also examined over the range of pH 4.5–7.0 (50 mM sodium acetate and sodium phosphate buffers) by using catechol (250 μM). As shown in Figure 3, lower biosensor response was obtained with lower or higher pHs than 6.0. A further increase or decrease in pH led to decrease in the enzymatic activity. Hence, pH 6.0 is decided as the optimum pH for HS-Calix-NH₂/Lac biosensor, which is similar to that of previously reported Lac biosensors [18]. In another work, the optimum pH was obtained at 5.5 for the Lac biosensor in which the enzyme was immobilized in the histidine modified montmorillonite matrix on a GCE [19]. It can be said that optimum pH for Lac is varied according to the ionic features of immobilization matrices used for biomolecules.

The effect of HS-Calix-NH₂ amount on the biosensor response was investigated using different amount of (0.5, 1.0 and 2.0 mg·mL⁻¹) HS-Calix-NH₂ during Lac immobilization. The results (Figure 4) indicate that using

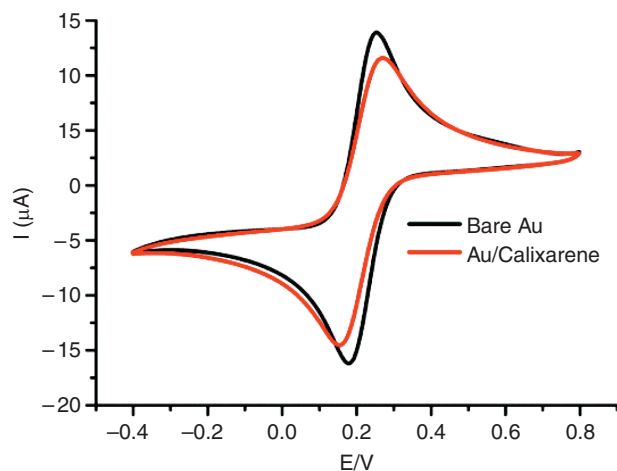


Figure 1: Cyclic voltammogram of bare and HS-Calix-NH₂ modified electrodes in phosphate buffer (pH 6.0; 50 mM) containing 5.0 mM K₃Fe(CN)₆ and 0.1 M KCl (scan rate: 50 mV/s).

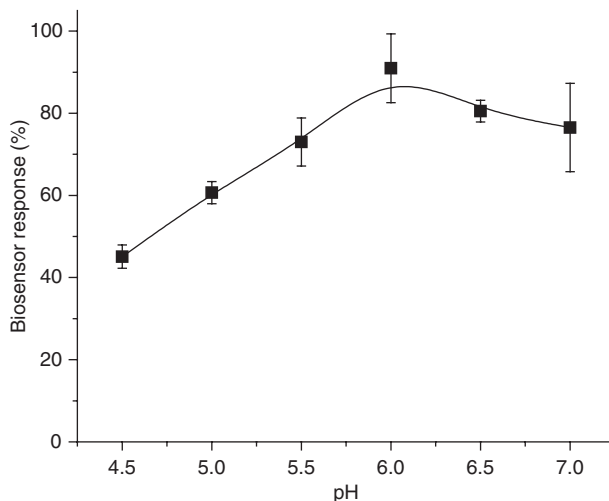


Figure 3: Effect of pH on the response of HS-Calix-NH₂/Lac biosensor (in 50 mM sodium acetate buffers and 50 mM sodium phosphate buffers; 25°C; at -50 mV; in the presence of 250 μM catechol).

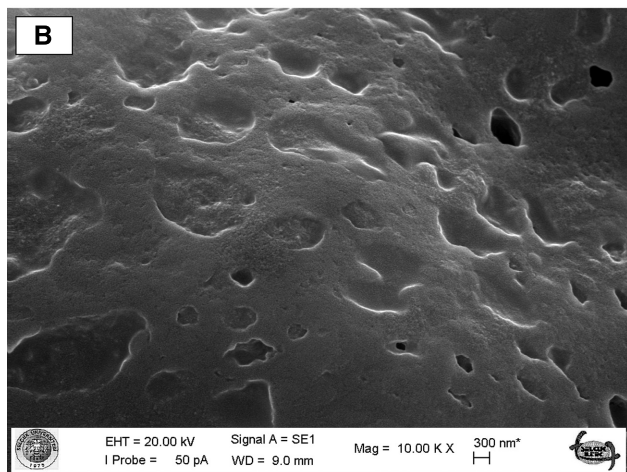
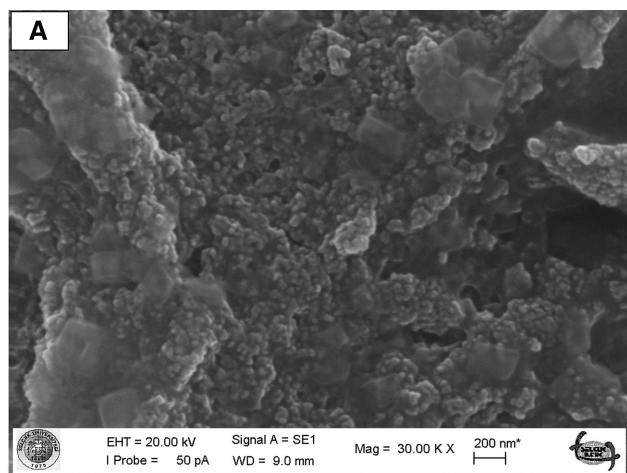


Figure 2: SEM images of HS-Calix-NH₂ (A) and HS-Calix-NH₂/Lac (B) films on the gold electrode surface.

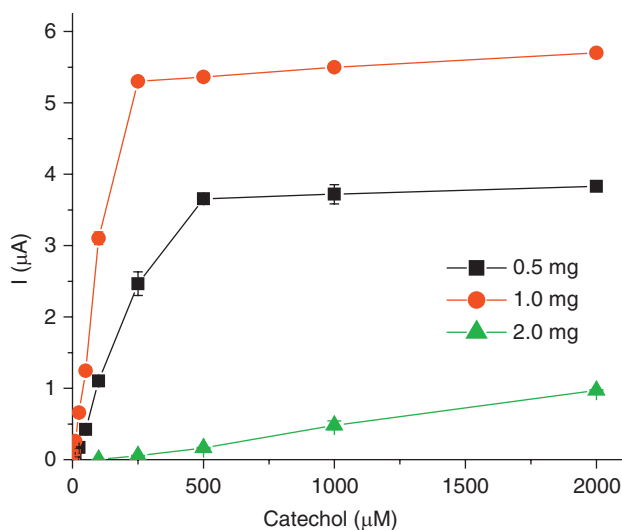


Figure 4: The effect of HS-Calix-NH₂ amount on the biosensor response (in 50 mM sodium phosphate buffer pH 6.0; 25°C; at -50 mV).

1.0 mg/mL HS-Calix-NH₂ showed higher sensor responses (that can be seen from the slope of the linear graph) than 0.5 and 2.0 mg/mL⁻¹. And also the biosensor response showed saturation at 2.5 mM of catechol when the biosensor was prepared using 2 mg of calixarene (data not shown). The lower amount of HS-Calix-NH₂ does not have enough functional group to immobilize enzyme. The decrease on the current with the increased amount of calixarene can be attributed that the limit for the diffusion phenol and of oxygen as a co-substrate of enzymatic reaction. Quinones,

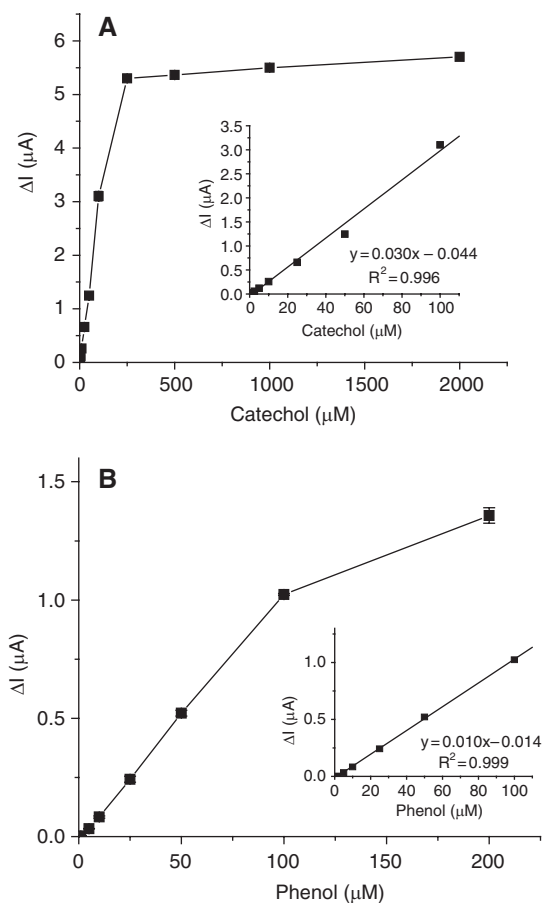


Figure 5: Calibration curve for catechol (in 50 mM sodium phosphate buffer pH 6.0; 25°C; at –50 mV; error bars show S.D. of three measurements.

Inset: Linear range for catechol (A). Calibration curve for phenol (in 50 mM sodium phosphate buffer pH 6.0; 25°C; at –50 mV; error bars show S.D. of three measurements. Inset: linear range for phenol (B).

which are produced in enzymatic reaction, are reduced at the working electrode and the thick layers of calixarenes produce also diffusion barrier for the oxidized species.

As shown in Figure 5, current change was proportional to catechol and phenol concentration in the range

from 0.1 to 100 μM and from 1.0 to 100 μM , respectively. When the concentration of catechol or phenol were more than 100 μM , substrate saturation was observed, showing a typical Michaelis–Menten kinetic mechanism. K_{Mapp} and I_{max} values were calculated (using GraphPad program) as 118 μM and 6.41 μA for catechol and 114 μM and 1.95 μA for phenol, respectively.

For the analytical characterization of HS-Calix-NH₂/Lac electrode, a certain catechol concentration in calibration curve was used for detecting the operational stability, repeatability and limit of detection (LOD). In the repeatability study, optimized HS-Calix-NH₂/Lac biosensor was tested with the addition of 50 μM catechol consecutively. After the measurements ($n=8$), standard deviation and coefficient of variation values were assessed as ± 1.52 and 3.17%, respectively. Furthermore, LOD was estimated via $3S_b/m$ formula where S_b is standard deviation of measurements and m is the slope of standard curve, using eight consecutive measurements at 0.1 μM catechol. It was calculated as 0.06 μM . Concomitantly, to test the operational stability of HS-Calix-NH₂/Lac electrode, 50 μM catechol additions (121 measurements) were accomplished during 42 h and the activity of Lac electrode decreased as 22.7%. The comparison of analytical characteristics of the prepared laccase biosensors were summarized in Table 1. The aim of the preparation of newly designed biosensors is to improve one or more performance characteristics such as linearity for the substrate, operational/storage stability, LOD etc. When compared the HS-Calix-NH₂/Lac to biosensors in the literature, for catechol better linearity and lower LOD were obtained than Tyr biosensor, which was constructed on 3-mercaptopropionic acid (MPA) SAM on a Au disk electrode [20], peroxidase biosensor, which was designed on poly(glycidylmethacrylate-co-vinyl ferrocene) grafted iron oxide nanoparticles [23]. The Lac/NiNPs/cMWCNTs/PANI/AuE electrode lost 15% of its initial activity after its 200 uses during the span of four months when stored at

Table 1: Comparison of the enzyme biosensors for the determination of phenolic compounds.

Support	Enzyme	Substrate	Linearity (M)	LOD (M)	Stability	Ref.
Au	Tyr	Catechol	2×10^{-7} – 1×10^{-4}	1.1×10^{-7}	–	[20]
GE	Lac	Pyrocatechol	2×10^{-6} – 2×10^{-3}	–	–	[21]
GCE	PO	Catechol	2×10^{-9} – 3×10^{-5}	5×10^{-10}	–	[22]
Au	POX	Catechol	5×10^{-4} – 1.7×10^{-2}	2.5×10^{-5}	After 12 measurements 4.0% activity loss	[23]
Au	Lac	Guaiacol	1×10^{-7} – 5×10^{-5}	5×10^{-8}	After 200 measurements 15% activity loss (storage at 4°C for 4 months)	[24]
GCE	PO	Catechol	6×10^{-9} – 2×10^{-5}	4.4×10^{-10}	After 44 measurements no activity loss	[25]
Au	Lac	Catechol	0.1×10^{-6} – 1×10^{-4}	0.06×10^{-6}	After 121 measurements 22.7% activity loss	This work

Au, Gold; GE, Graphite electrode; GCE, Glassy carbon electrode; Tyr, Tyrosinase; Lac, Laccase; PO, Polyphenol Oxidase; POX, Peroxidase.

4°C [24], HS-Calix-NH₂/Lac was used to detect catechol for 121 measurements consecutively.

Additionally, the effect of possible interfering substances such as ethanol, ascorbic acid and uric acid was experimented for the novel proposed biofilm layer to provide higher accuracy in the sample application study. To monitor the effect of interfering substances, initially catechol standard (50 μM) was added into the working buffer (50 mM, pH 6.0, phosphate buffer). Subsequently, ascorbic acid, uric acid and ethanol were added to the working buffer under the same conditions, respectively. Moreover, the same catechol amount was introduced into the reaction cell and it was observed that initial and last catechol signals were similar. On the other hand, there was no dramatic change in the current responses when the 10 μM interfering substances were added into the reaction cell as shown in Figure 6.

In the final step, the developed HS-Calix-NH₂/Lac biosensor system was applied to artificial waste water, which was prepared with 1.0 M HCl solution containing 50 g·L⁻¹ NaCl and 100 g·L⁻¹ phenol [26]. Prior to the sample application, a new calibration curve for phenol was carried out and artificial waste water was diluted as 10 times in working buffer and the final solution was added into the reaction cell to detect the phenol in artificial sample. As a consequence, the corresponding results were compared with known concentration and it was found that there was a 101.5% as a recovery (n=5). The HS-Calix-NH₂/Lac biosensor showed high stability and an extensive linear range for the detection of phenol, and the strategy can be easily broaden to screen other phenolic compounds.

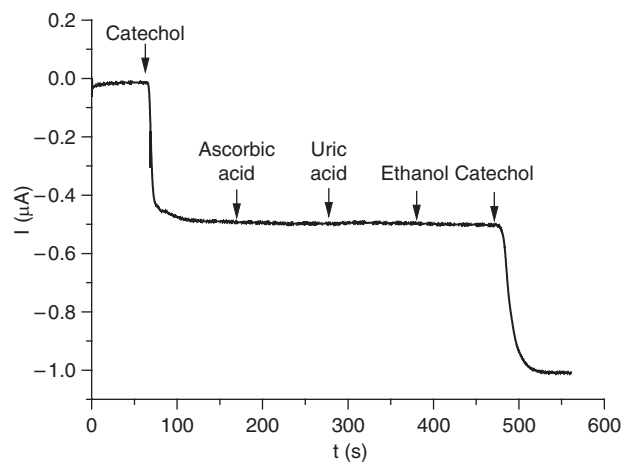


Figure 6: Effect of some chemical compounds added into the cell to be 10 μM on the sensor response measured at -50 mV in pH 6.0 phosphate buffer. Catechol was added to be 10 μM in the cell for comparison.

Conclusions

In conclusion, a functional calixarene derivative ‘HS-Calix-NH₂’ was used to modify the Au electrode via formation of SAMs. Lac was chosen as a model enzyme for the fabrication of phenol biosensors. After optimization of preparation and working conditions, the HS-Calix-NH₂/Lac biosensor was calibrated for catechol in batch systems. The biosensor was applied for phenol analysis in artificial waste water samples. The obtained data for the HS-Calix-NH₂/Lac biosensor was verified the practical application without requiring a sample treatment. And it was shown that the prepared Lac biosensor has a potential for rapid, selective and easy detection of phenolic contaminations in samples.

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Conflict of interest statement: The authors have no conflict of interest.

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