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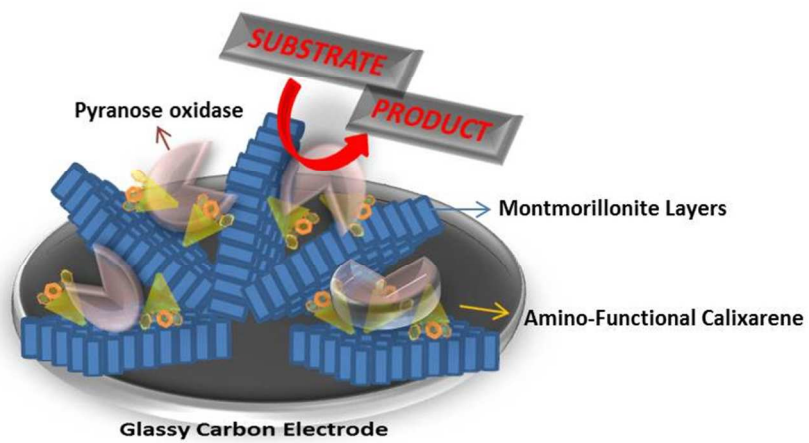


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Calixarene Modified Montmorillonite: A Novel Design for Biosensing Applications

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Here we report the synthesis, characterization and application of calixarene (Calix) modified montmorillonite (Mt) as a platform for the bio-applications such as biomolecule immobilization and biosensing technologies. This modification enhanced the biomolecule immobilization capability of Mt. Initially, amino-functional calixarenes (Calix-NH₂) were synthesized and used as a modifier. X-ray diffraction, Fourier transform infrared spectroscopy, zeta potential and thermal gravimetric analysis were performed to verify the modification of clay minerals. For the biosensor construction, Calix-NH₂ modified Mt (Calix-NH₂/Mt), bovine serum albumin (BSA), glutaraldehyde (GA) and pyranose oxidase were immobilized on the surface of glassy carbon electrode which is then called as Calix-NH₂/Mt/PyOx biosensor. After optimization of the enzyme amount and pH, analytical characteristics were investigated in detail.

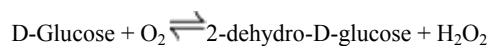
Introduction

Immobilization of the enzymes in a suitable matrix is crucial for fabrication of biosensor systems and other enzyme-based diagnostic techniques. In the literature, various techniques and immobilization materials have been attempted to fabricate enzyme biosensors.¹⁻⁵ Clays are one of the advantageous materials to design different immobilization matrices for biomolecule immobilization due to their high stability, good adsorptive capacity, large specific surface area, stick out adhesiveness and low costs.⁶ Laponite, montmorillonite (Mt), nontronite and layered doublehydroxides (LDHs) are well known Smectite clay minerals that already were exploited in numerous biosensor studies.⁷⁻¹¹ Mt is a naturally occurring cationic phyllosilicate and composed of silica tetrahedral sheets layered between alumina octahedral sheets. To date it has been utilized in different biotechnological and biomedical applications.¹²⁻¹⁴ For instance, Joshi et al. used Mt as drug carrier for timolol maleate and demonstrated the intercalation of timolol maleate into the interlayer of Mt at different pH.¹⁵ Folic acid modified Mt was used as a cell specific adhesion material for HeLa cells.¹⁶ Demir et al were successfully applied trimethylamine modified Mt to design microbial biosensor systems.¹⁷ Besides, glucose oxidase and laccase were also, immobilized on dimethylamine and histidine modified Mt matrices for glucose and phenol sensing, respectively.^{9, 18}

Calixarenes which are cyclic oligomers, are synthesized traditionally via oligomerization of phenol and formaldehyde.¹⁹ Due to their excellent ability to form host-guest complexes and their multi center

bonding with guest molecules, calixarenes can selectively bind different inorganic and organic compounds.²⁰⁻²² Chen et al. reported the synthesis of p-tert-butylthiacalix[4]arene tetra-amine and demonstrated the use of calixarenes as enzyme immobilization material.²³ Recently, 5,11,17,23-tetra-tert-butyl-25,27-bis(3-thiol-1-oxypropane)-26,28-dihydroxycalix[4]arene (SH-Calix) was synthesized and used to modify gold electrode via formation of self-assembled monolayers. Then, glucose oxidase was immobilized on the SH-Calix-modified surface.²⁴

This work composed of three parts as follows; (i) the synthesis and characterization of 5,17-diamino-25,27-bis[N-(2-aminoethyl)]calix[4]azacrown(Calix-NH₂), (ii) modification of Mt with Calix-NH₂ salt which is then, used as a platform for the enzyme immobilization, (iii) biosensor (Calix-NH₂/Mt/PyOx) applications. Pyranose oxidase (PyOx) was selected as a model enzyme. Glutaraldehyde (GA) and bovine serum albumin (BSA) were used to test the specificity of the sensor. Amine groups of Calix-NH₂ played an important role in the stable immobilization of the enzyme by crosslinking with GA. Calix-NH₂, BSA, GA and PyOx were mixed and immobilized onto Glassy carbon electrode (GCE). The enzymatic reaction of PyOx is given below,²⁵



The consumption of the oxygen due to the enzymatic activity was related to the substrate concentration. The response signals were followed at -0.7 V vs Ag/AgCl. After optimization studies, Calix-

NH₂/Mt/PyOx biosensor was calibrated for glucose and applied for the glucose analysis in a real sample.

Materials and Methods

Materials. Pyranose oxidase (EC 1.1.3.10, from *Coriolus* sp., ≥ 2.7 Units/mg), GA solution (25%, v/v), bovine serum albumin (BSA) glucose and all other chemicals were ordered from Sigma Aldrich. Mt was supplied by Southern Clay Products, Inc. (CEC = 92 meq/100 g). Thin layer chromatography (TLC) analyses were carried out on DC Alufolien Kieselgel 60 F₂₅₄ (Merck). The solvents were dried by storing them over molecular sieves (Aldrich; 4 Å, 8-12 mesh). All reactions, unless otherwise noted, were conducted under nitrogen atmosphere. All starting materials and reagents used were of standard analytical grade from Merck or Aldrich and used without further purification. All aqueous solutions were prepared with deionized water that was passed through a Millipore Milli-Q Plus water purification system.

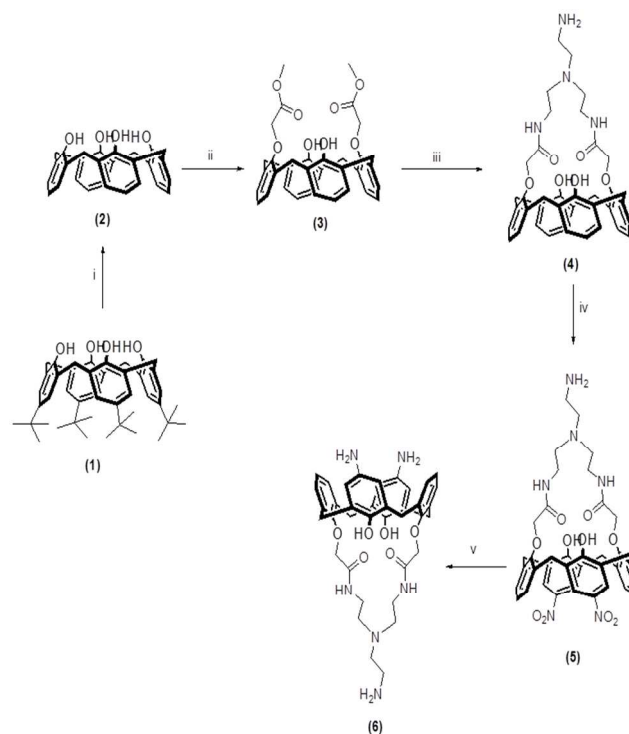
Synthesis of Calix-NH₂. *p*-*tert*-Butylcalix[4]arene (1), calix[4]arene (2), 1,3-O-dimethyl ester calix[4]arene derivative 3 and the compound 4 N-(2-aminoethyl)calix[4]azacrown were synthesized according to literature procedures.²⁶⁻²⁹ The compounds 5 5,17-dinitro-25,27-bis[N-(2-aminoethyl)]calix[4]azacrown and 6 5,17-diamino-25,27-bis[N-(2-aminoethyl)]calix[4]azacrown are herein reported for the first time.

Synthesis of compound 4. Yield; 70.5%, m.p.; 250-252 °C. FTIR (ATR): 1678 cm⁻¹ (C=O). ¹H NMR (400 MHz CDCl₃): δ 2.13-2.33 (m, 2H, -NH₂), 2.53 (t, 2H, $J=5.6$ Hz, -CH₂-N), 2.74-2.81 (m, 6H, -CH₂-), 3.46 (d, 4H, $J=13.6$ Hz, Ar-CH₂-Ar), 3.53-3.56 (m, 4H, -CH₂-), 4.21 (d, 4H, $J=13.6$ Hz, Ar-CH₂-Ar), 4.55 (s, 4H, O-CH₂-), 6.73-6.79 (m, 4H, ArH), 6.85 (d, 4H, $J=7.6$ Hz, ArH), 7.12 (d, 4H, $J=7.6$ Hz, ArH), 7.19 (s, 2H, -OH), 8.32 (t, 2H, $J=5.6$ Hz, -NH). Anal. Calcd. For C₃₈H₄₂N₄O₆: C, 70.13; H, 6.51; N, 8.61. Found (%); C, 70.05; H, 6.63; N, 8.55.

Synthesis of compound 5. HNO₃ (65%, 20 mL) was added to the solution of 4 (0.7 g, 1.076 mmol) in 20 mL of CH₂Cl₂. The mixture was stirred at room temperature for 1 h. Then, 50 mL water was poured into the mixture, and then, filtered. The solid was washed with water to neutralize and then, dried. The crude was recrystallized from acetone. Yield; 56.5%, m.p.; 208-210 °C. FTIR (ATR): 1660 cm⁻¹ (C=O). ¹H NMR (400 MHz DMSO): δ 2.60-2.70 (m, 5H, -CH₂- and -NH₃⁺), 3.15-3.47 (m, 6H, -CH₂-over shielded by the solvent), 3.69-3.73 (m, 8H, -CH₂- and Ar-CH₂-Ar), 4.19-4.30 (m, 4H, Ar-CH₂-Ar), 4.58 (brs, 4H, O-CH₂-), 6.89 (t, 2H, $J=7.6$ Hz, ArH), 7.21 (d, 4H, $J=7.6$ Hz, ArH), 7.63 (brs, 2H, -OH), 8.18-8.24 (m, 6H, ArH and -NH). Anal. Calcd. For C₃₈H₄₁N₆O₁₀: C, 61.53; H, 5.57; N, 11.33. Found (%); C, 61.44; H, 5.81; N, 11.38.

Synthesis of compound 6. Raney Ni (1.0 g) was added to the solution of 5 (0.35 g, 0.473 mmol) and hydrazine monohydrate (2.8 mL) in MeOH. The reaction mixture was refluxed for 10 h. The reaction temperature was then cooled to room temperature,

and then, filtered and evaporated to dryness. The crude was recrystallized from acetone. Yield: %95, m.p.; 269-272 °C. FTIR (ATR): 1660 cm⁻¹ (C=O). ¹H NMR (400 MHz DMSO): δ 2.69 (t, 3H, $J=4.4$ Hz, -NH₃⁺), 2.92-2.95 (m, 2H, -CH₂-), 3.16-3.45 (m, 10H, -CH₂-over shielded by the solvent), 3.63-3.74 (m, 4H, Ar-CH₂-Ar), 3.98-4.04 (m, 8H, Ar-CH₂-Ar and Ar-NH₂), 4.52-4.59 (m, 4H, O-CH₂-), 6.89 (t, 2H, $J=7.6$ Hz, ArH), 7.21 (d, 4H, $J=7.6$ Hz, ArH), 7.57-7.74 (m, 2H, -OH), 7.99-8.23 (m, 6H, ArH and -NH). ¹³C NMR (100 MHz DMSO): δ 31.07 (Ar-CH₂-Ar), 37.26 (NH-CH₂), 38.98 (NH₂-CH₂), 53.04 (N-CH₂), 54.81 (N-CH₂), 74.20 (O-CH₂), 124.96 (Ar-C), 126.02 (Ar-C), 129.46 (Ar-C), 130.10 (Ar-C), 133.56 (Ar-C), 144.60 (NH₂-Ar-C), 152.99 (ArO-C), 153.60 (ArO-C), 169.26 (C=O). Anal. Calcd. For C₃₈H₄₅N₆O₆: C, 66.94; H, 6.65; N, 12.33. Found (%); C, 67.01; H, 6.73; N, 12.28.



Scheme 1. The synthetic route for compound 6. Reaction conditions: (i) Phenol, AlCl₃; (ii) K₂CO₃, CH₃CN, methylbromoacetate; (iii) Tris (2-aminoethyl)amine, MeOH/toluene; (iv) HNO₃, CH₂Cl₂; (v) Raney-Ni, hydrazine, MeOH.

Preparation of Calix-NH₂/Mt. The Mt was organically modified by a cation-exchange reaction between Mt and Calix-NH₂. The Calix-NH₂, as an intercalating agent, was firstly protonated with 1.0 M HCl aqueous solutions to adjust the pH to 2.0–3.0. The required amount (two time equivalent of the CEC) of the Calix-NH₂ salt was dissolved in deionized water at approximately 50 °C. 1.0 g of Mt was preliminarily dispersed in deionized water by using a mechanical stirrer. The Calix-NH₂ solution was added to the dispersion of the clay mineral particles, and the mixture was exchanged for 24 h at room temperature using vigorous stirring. The resulted organo-clay mineral (Calix-NH₂/Mt) was collected by filtration and repeatedly washed with hot deionized water until no chloride ions were detected

upon adding 0.1 M aqueous AgNO₃. It was dried in a vacuum oven at 50 °C for 2 days to a constant weight.

Characterization of Calix-NH₂/Mt. For the characterization, initially, modified and pristine samples were examined by X-ray diffraction (XRD) Spectrometers (Philips Expert Pro; Cu-K α radiation, $\lambda=1.54056 \text{ \AA}$) to determine the interlayer spaces of Mt and Calix-NH₂/Mt. The samples were scanned under the diffraction angle 2θ in the range of 4.0–70.0 with a scanning rate of 2.0 min⁻¹. The interlayer distance was determined by the diffraction peak, using the Bragg equation ($n\lambda=2d\sin\theta$). Fourier transform infrared (FTIR) spectra of clay minerals were recorded using a Perkin Elmer Pyris 1 FTIR Spectrometer with KBr pellet. Thermal degradation was studied with a thermogravimetric analyzer (Perkin Elmer Pyris 1 TGA/DTA) by heating the samples from ambient temperature to 1000 °C at 10 °C min⁻¹ under a 10 bar dry air atmosphere. The electrokinetic properties of clay minerals were determined by measuring the zeta-potential of particles with a Zeta-Meter 3.0+ (with a Zeiss DR microscope, GT-2 type quartz cell, molybdenum-cylinder anode, and platinum-rod cathode electrode). For this purpose, the clay mineral samples were stirred overnight in the deionized water to obtain well dispersed particles. The zeta potential of the Mt dispersions was estimated from the measured electrophoretic mobility by employing the Smoluchowski equation.³⁰ The value of the zeta potential assigned to the dispersions was the average of the data obtained from at least 10 experiments. All of the samples were dried in a vacuum oven before the analyses at 50 °C for 2 days.

Scanning Electron Microscopy (SEM) images were registered by using SEM (JEOL5600-LU). The images were taken via using an acceleration voltage of 20 kV

Calix-NH₂/Mt biosensor. Glassy carbon electrodes (GCE) were polished with 0.5 mm alumina slurry. To remove alumina residues on the electrode, ultrasonic cleaning for about 2–3 min in 1:1 ethanol distilled water was carried out. Afterwards, 1.0 mg PyOx (in 2.5 μL , sodium phosphate buffer pH 7.0, which equals to 2.7 Units), 5.0 μL of Calix-NH₂/Mt solution (1.0 mg/mL in sodium phosphate buffer pH 7.0), 2.5 μL of BSA (1.0 mg/mL in sodium phosphate buffer, pH: 7.0) and 2.5 μL of glutaraldehyde (5.0%, in sodium phosphate buffer, pH 7.0) were mixed. Finally, 12.5 μL of this mixture was dropped on the electrode surface and allowed to dry at room temperature for 1 h. The same procedure was used to prepare the Mt modified PyOx biosensors in which only pristine Mt was used instead of Calix-NH₂ modified clay mineral.

Electrochemical measurements. Amperometric measurements performed using a Palm Sens Potentiostat (Palm Instruments, Houten, Netherlands). All experiments were carried out in a reaction cell in working buffer (10 mL sodium phosphate buffer, 50 mM, pH 6.0) at room temperature using of three electrode configuration. GCE as a working electrode, Ag/AgCl as a reference electrode (3.0 M KCl, Metrohm, Switzerland) and Pt as a counter electrode (Metrohm, Switzerland) were used. Initially, the biosensors were equilibrated in the working buffer in the reaction cell and then, glucose was added. The response signals which were proportional to

the substrate concentration were followed at $-0.7 \text{ V vs Ag/AgCl}$. The consumption of molecular oxygen in the enzymatic reaction was monitored at given potential. PyOx oxidizes glucose to 2-dehydro-D-glucose and the signals were correlated indirectly to the substrate concentrations consumed during the enzymatic reaction.^{9,17,18,24}

After each measurement, the electrodes were washed with distilled water and the working buffer was refreshed.

Sample application. Calix-NH₂/Mt/PyOx biosensor was used to analyze the glucose concentration in coke and fizzy samples. The sample was also analyzed with Trinder reagent which is a commercial enzyme assay kit based on spectrophotometric analysis. This assay includes oxidation of glucose to D-gluconate catalyzed by glucose oxidase with the formation of hydrogen peroxide. In the presence of peroxidase, a mixture of phenol and 4-aminoantipyrine is oxidized by hydrogen peroxide to form a red quinoneimine dye proportional to the glucose concentration in the sample.³¹ The glucose concentration in the real sample was measured by direct addition of samples into working buffer, instead of glucose. The amount of glucose in the samples was calculated from corresponding calibration curves obtained with the standard glucose solutions.

Results

Synthesis of a calixarene derivative substituted at the both rims with primary amine groups. *p*-tert-Butylcalix[4]arene1 was chosen as the starting material, and it was transformed to the dealkylated derivative **2** and then the diester derivative **3** according to known procedures (see Scheme 1).^{26–28} Treatment of the diester derivative **3** with tris(2-aminoethyl)amine in the presence of MeOH/toluene led to produce compound **4** according to the literature procedure.²⁹ Then, a mixture of compound **4** with 65% HNO₃ in DCM was reacted at room temperature for 1 h yielded the *p*-nitro substituted derivative **5**. Upon reduction of nitro groups with Raney-Ni, the target compound **6**, which was functionalized at the both rims of Calix with the primary amine groups, was synthesized in 95% yield (see Scheme 1).

The characterization of the synthesized Calix derivatives was performed by FTIR, ¹H and ¹³C NMR spectroscopy and given as supplementary data (Fig.S1- Fig. S5). The FTIR spectra of **4–6** signify that their characteristic peaks(C=O) appear at 1678 cm⁻¹ for **4** and 1660 cm⁻¹ for both **5** and **6** (Fig. S1). In the ¹H NMR spectrum of **4**, the additional peaks of Tris (2-aminoethyl) aiming at δ 2.13–2.33 (m, 2H, -NH₂), 2.53 (t, 2H, $J= 5.6 \text{ Hz}$, -CH₂-N), 2.74–2.81 (m, 6H, -CH₂-), 3.53–3.56 (m, 4H, -CH₂-) and 8.32 ppm (t, 2H, $J= 5.6 \text{ Hz}$, -NH) placed on the spectrum. Thus, these peaks confirm the structure of **4** (Fig. S2). ¹H NMR spectrums of **5** which are nitro derivative of **4** clearly verifies the structure of **5** by disappearing of two protons of aromatic at *para* positions on the spectrum (Fig. S3). Obtained compound **6** by reduction of nitro groups has four protons at 3.98 ppm belonging Ar-NH₂ groups in the ¹H-NMR spectrum (Fig. S4). In addition, in the ¹³C NMR spectra of **6**, it can be seen the peak belonging to the C=O groups appears at 169.26 ppm, (Fig. S5).

Characterization of Calix-NH₂/Mt. Each layer of natural Mt consists of octahedrally coordinated cations (typically Mg, Al and Fe) sandwiched by tetrahedrally coordinated cations (typically Si and Al). Isomorphous substitution of Si⁴⁺ by Al³⁺ leads to a net negative surface charge that is compensated by an interlayer of exchangeable, hydrated cations (Ca²⁺, Mg²⁺, Cu²⁺, Na⁺, H⁺). The surface of Mt is hydrophilic and this makes difficult the interaction of the clay mineral with the enzyme substrate. Therefore, it is necessary to make the surface of Mt less hydrophilic to compatible with a chosen enzyme. Various methods have been proposed for making clay minerals more hydrophobic.³² In our case, the methodology is based on the replacement of the exchangeable hydrated cations with organophilic cations. The clay minerals were characterized by FTIR, XRD, TGA/DTG and zeta potential measurements. The FTIR spectra of pristine Mt and Calix-NH₂/Mt are presented in Fig. 1. In the FTIR spectrum of Mt (Fig. 1 A), the band at 3630 cm⁻¹ is ascribed the -OH bond corresponding to the characteristic stretching for Al-OH bond in the octahedral layer of Mt. The band at around 1630 cm⁻¹ is related with H-OH stretching vibration. An intense peak at around 1000 cm⁻¹ is the characteristic absorption band for Mt corresponding to Si-O stretching. After modification with Calix-NH₂ (Fig. 1B) characteristic absorption bands for both Mt and modifier were observed. In addition to Mt band, characteristic peaks (C=O) of Calix-NH₂ were shifted towards to 1730 cm⁻¹. Also, vibration peaks at 2980 cm⁻¹ and 2850 cm⁻¹ for -CH stretching and at 1360 cm⁻¹ for -CH₂ bending are observed, supporting the intercalation of Calix-NH₂ salt between the silicate layers.

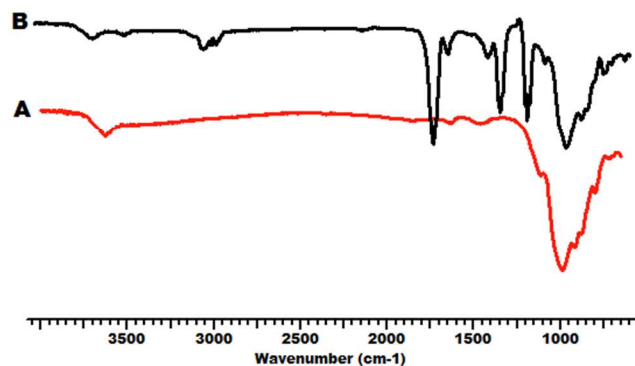


Fig.1 FTIR spectra of Mt (A) and Calix-NH₂/Mt (B).

The X-ray diffraction (XRD) technique is frequently used to detect the basal spacing between the interlayer of the clay mineral particles. XRD pattern of pristine Mt and Calix-NH₂/Mt is shown in Fig. 2. Pristine Mt yields characteristic diffraction peaks at $2\theta = 7.74$. This diffraction peak corresponds to the basal spacing d_{001} (11.4 Å). From the XRD pattern of Calix-NH₂/Mt (Fig. 2), it can be seen a diffraction peak appeared at the lower angles of the diffractogram indicated the intercalation of Calix-NH₂ molecules in the interlayer spaces of Mt. The ions of the clay mineral were exchanged with ammonium cations of calixarene to prepare the organoclays,

and the exchange resulted in an increase in the basal spacing. The basal spacing of Calix-NH₂/Mt is increased to 13.06 Å ($2\theta = 6.76$). The interlayer space height, Δd , has been calculated as 1.66 Å by subtracting the average layer thickness of Mt (11.4 Å) from that of Calix-NH₂/Mt. It is attributed that the cation exchange process achieved between the interlayer cations of Mt and calixarene salt intercalated into the Mt layers.

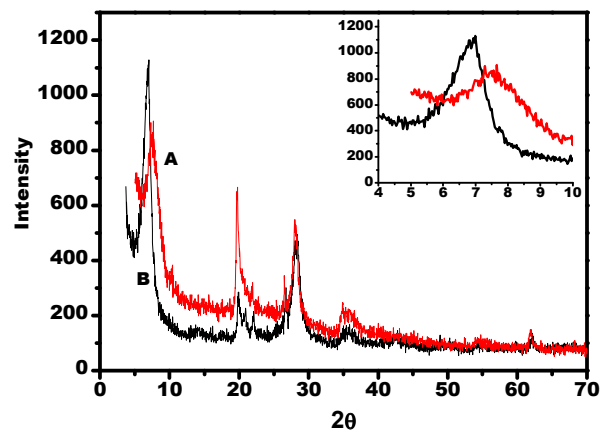


Fig.2 XRD patterns of Mt (A) and Calix-NH₂/Mt (B).

The thermogravimetric (TG) and derivative thermogravimetric (DTG) curves for pristine Mt and Calix-NH₂/Mt are shown in Fig. 3 and Fig. 4, respectively. The TGA thermogram of pristine Mt reveals two major temperature regions of mass loss, one below 200 °C and another above 500 °C. The first step observed from room temperature is attributed to the dehydration of physically adsorbed water and dehydration of water molecules adsorbed around metal cations on exchangeable sites in Mt. The last step observed in the temperature region of 500-700 °C is attributed to the dehydroxylation of Mt. After the exchange with the Calix-NH₂ salt, thermal decomposition of the Calix-NH₂/Mt takes place in three regions. In the first region, below 150 °C, takes place the evolution of adsorbed water. The second region occurs between 400-500 °C due to the decomposition of incorporated organic molecules, and the last one over the temperature range of 600–700 °C which is ascribed to the dehydroxylation of the structural OH units of the organoclay mineral. From Fig. 4, it is observed that the Calix-NH₂/Mt presents lower thermal stability than the pristine Mt.

Also, the TGA results were used to examine the amount of organic component. The organic content of the Calix-NH₂/Mt was calculated from the residue of the Calix-NH₂/Mt after being burnt for over 30 min at 800 °C, using the following equations:³³

$$Mt (\%) = \frac{Mt_{\text{residue at } 800^{\circ}\text{C}}}{0.987} \quad (1)$$

$$Org (\%) = 100\% - Mt\% \quad (2)$$

The factor of 0.987 in the first equation corrects for loss of physically adsorbed water of the pristine Mt, being in this case 1.3% mass loss as taken from TGA thermogram. From these equations, the amount of organic component is calculated as 16.67%.

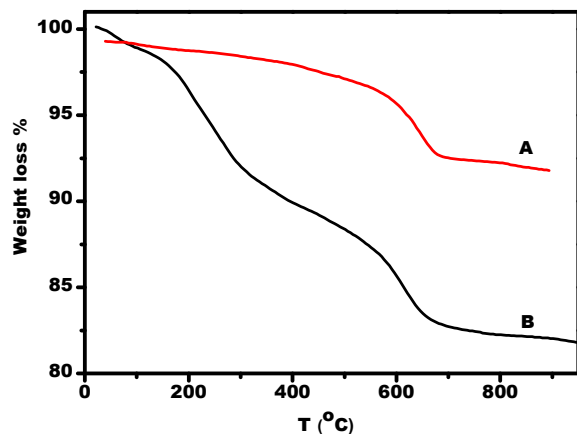


Fig.3 TG thermograms of Mt (A) and Calix-NH₂/Mt (B).

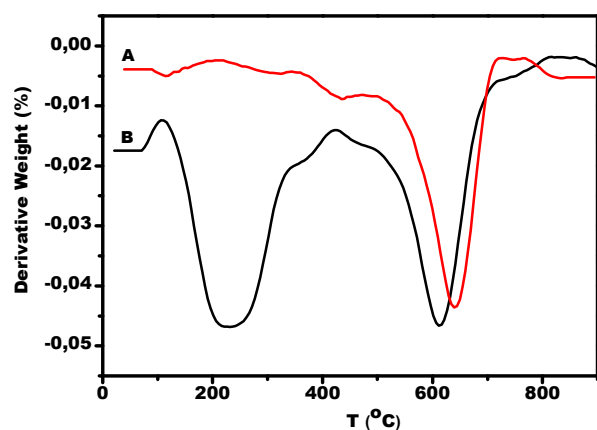


Fig.4 DTG thermograms of Mt (A) and Calix-NH₂/Mt (B).

The zeta potential is an indicator of the surface charge properties of a colloid or a particle in solution and varies depending on the surface potential and the thickness of the electric double layer. It is usually characterized by the measurement of the electrophoretic mobility of the colloidal particles in dispersion, which is a key technique for the determination of the surface properties of the particles.³⁴ The zeta potential can be used for studying and predicting colloidal stability and particle surface charge, effectively. It is also an important parameter for a number of applications including characterization of biomedical polymers, electrokinetic transport of particles or blood cells, sensors and biosensors, membrane efficiency and microfluidics.³⁵⁻³⁸ In this study, the

zeta potentials of the samples were measured as -42.0 ± 2.24 and -29.70 ± 0.63 mV for Mt and Calix-NH₂/Mt, respectively. After each modification step, the zeta potentials shifted to less-negative values as a result of adsorption of positively charged calixarene salt at the surface or interlayer of the mineral.

Optimization studies. Effect of pH and the modifier on the biosensor response were investigated for Calix-NH₂/Mt/PyOx biosensor. The effect of pH on the response of the biosensor was studied between pH 5.5 and 7.0 in sodium acetate and sodium phosphate buffer (50 mM) by using glucose (0.25 mM). The lower biosensor response obtained with lower or higher pHs than 6.0. Therefore, pH 6.0 is selected as the optimum for Calix-NH₂/Mt/PyOx biosensor (Fig. 5). Previously, the optimum pH was obtained at 10.5 for the PyOx biosensor in which the enzyme was immobilized in the Osmium redox polymer.²⁵ In another work PyOx immobilized in carbon nanotube modified carbon paste electrode and optimum pH was reported as 7.5.³⁹ It is known that the optimum pH of free PyOx is 7.0.⁴⁰ In our work, optimum working pH of PyOx was shifted to an acidic pH due to the presence of positive groups in the structure of Calix-NH₂ intercalated Mt. It is clear that optimum pH is varied depending on the matrices used for the biomolecule immobilization.

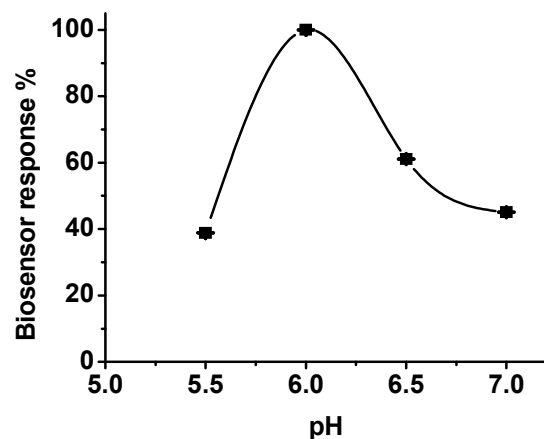


Fig. 5 Effect of pH on the response of Calix-NH₂/Mt/PyOx

Furthermore, the amount of immobilized enzyme affects biosensor sensitivity directly.^{41,42} To observe this effect, three different Calix-NH₂/Mt biosensors prepared with 0.5 mg, 1.0 and 2.0 mg of PyOx and the amounts of other components were kept constant. Fig. 6 demonstrates that lower enzyme amounts (0.5 mg) cause lower responses due to inadequate enzyme activity. When enzyme amounts were increased to 1.0 mg the highest signal responses were obtained. Furthermore, if 2.0 mg of enzyme was immobilized on the electrode, bioactive layer becomes thicker and this causes decreasing of signals.

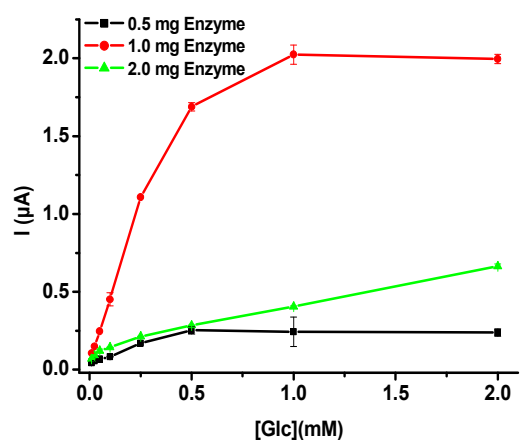


Fig. 6 Effect of enzyme amount on the biosensor response (in sodium phosphate buffer, 50 mM, pH 6.0, at -0.7 V; error bars show standard deviation (S.D) of three measurements).

Both types of clay mineral samples, pristine and Calix-NH₂ modified Mt were applied to examine the effect of Calix-NH₂ modification on the biosensor response. The amperometric responses of the Mt/PyOx and Calix-NH₂/Mt/PyOx biosensors were linear in the range 0.01–0.5 mM glucose by the equations of $y = 2.33x + 0.03$ ($R^2 = 0.99$) and $y = 3.92x + 0.07$ ($R^2 = 0.99$) respectively. Fig. 7 shows that Calix-NH₂/Mt/PyOx biosensor has higher current values as well as higher sensitivity (that can be seen from the slope of the linear graph) in compared to Mt/PyOx.

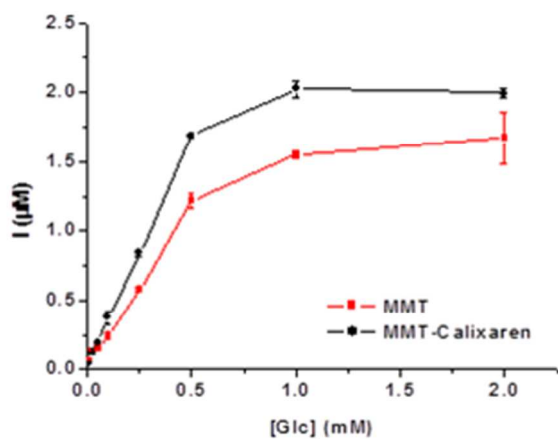


Fig. 7 Effect of the presence of Calix-NH₂ as a modifier in the matrix structure on the biosensor response (in sodium phosphate buffer, 50 mM, pH 6.0, at -0.7 V; error bars show S.D of three measurements).

Analytical Characterization. Calix-NH₂/Mt/PyOx biosensor was calibrated under optimum conditions by the addition of glucose standards between 0.01–0.5 mM. All the response signals were

measured three times with a 20 s response time. The calibration curve of Calix-NH₂/Mt/PyOx biosensor was defined by the equation of $y = 3.16x + 0.02$ ($R^2 = 0.997$) and demonstrated in Fig.8. Data were given as mean \pm S.D. The limit of detection (LOD) was also calculated as 0.50 μ M using $S/N=3$. Various clay mineral based glucose biosensors using laponite,^{43,44} alginate/layered double hydroxides,⁴⁵ dimethyl and trimethyl modified Mt were reported previously.^{17,18} When Calix-NH₂/Mt/PyOx is compared with the earlier works, a lower LOD has been observed.¹⁸ The comparison of various PyOx biosensors reported in literature was also given in the supplementary data as Table S1.

Moreover, repeatability and operational stability of the biosensor were investigated and 10 succeeding measurements were performed using 0.1 mM glucose. The S.D and variation coefficient (c.v) were calculated as 0.1 ± 0.001 mM and 1.06%, respectively. The reproducibility of the biosensor was also evaluated using 4 different enzyme electrodes prepared at different times, using 0.1 mM glucose. The relative standard deviation (RSD) was found as 0.05%. For the operational stability of Calix-NH₂/Mt/PyOx biosensor, 80 measurements during 72 h were carried out and after these measurements only 30% decrease was observed. Besides, SEM images, obtained for Mt (Fig. 9 A) and Calix-NH₂/Mt (Fig. 9 B), proved the decoration of Mt with Calix-NH₂. The changes in the surface morphologies depending on the modifier types were shown in Fig. 9 C and Figure 9 D, respectively. According to these images, it can be said that the surfaces of Mt/PyOx biosensors were different from Calix-NH₂/Mt/PyOx biosensors. In the presence of glutaraldehyde as a cross-linker, covalent bonds between the free amine groups of Calix-NH₂, PyOx and BSA were formed and the surface morphology of Calix-NH₂/Mt/PyOx biosensors was differentiated from Mt/PyOx biosensors.

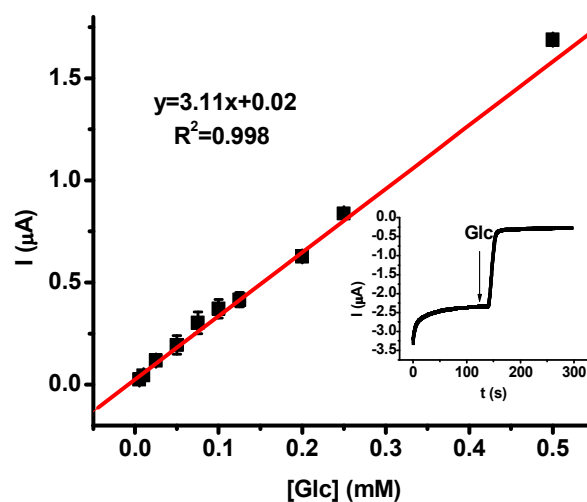


Fig. 8 Calibration curve for glucose (in sodium phosphate buffer, 50 mM, pH 6, -0.7 V; error bars show S.D of three measurements. Inset: time dependent current response with the addition of 0.5 mM glucose).

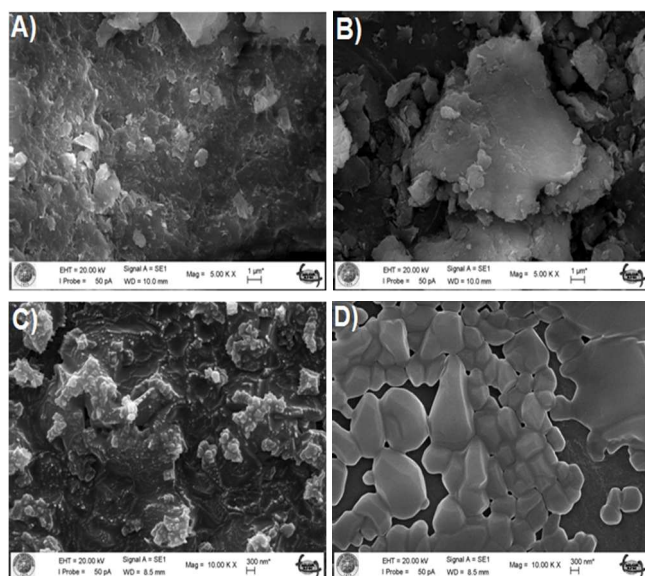


Fig. 9 SEM images of Mt (A), Calix-NH₂/Mt (B) with 5000 X magnification, Mt/PyOx (C), and Calix-NH₂/Mt/PyOx (D) with 10000 X magnification. Scale bars show 1.0 μm for A and B and 300 nm for C and D.

Interferences. Ascorbic acid, uric acid and 3-acetamino phenol (0.1 mM of each compound) were used to evaluate the inference effect on the Calix-NH₂/Mt/PyOx biosensor. It is known that at higher potentials, most common metabolites such as uric acid and ascorbic acid get oxidized and interfere with the electrochemical signal.⁴⁶ Therefore, effect of interferences to biosensor signals was evaluated by using the working potentials of -0.7 V. The differences in current levels were compared with that of standard glucose solutions and were calculated as a relative response value. Except ascorbic acid, no change was observed in the current response to glucose in the presence of interfering compounds.

Sample application. Under optimized conditions the glucose concentrations of coke and fizzy were detected using Calix-NH₂/Mt/PyOx biosensor. The concentration of glucose in the samples was calculated using the calibration graph. The results were compared with calculated glucose concentrations which were analyzed by reference method (Trinder Reagent) to confirm the accuracy of designed biosensor. The calculated glucose amounts of both samples are shown in Table 1. According to data the use of the Calix-NH₂/Mt/PyOx biosensor provided very similar results for the kit data (with the recovery of 99.2%).

Table 1. Glucose content of some beverages obtained by the biosensor and the reference method.

Sample	Calix-NH ₂ /Mt/PyOx (mM \pm SD)	Trinder Reactive (mM)	Recovery (%)
Coke	5.00 \pm 0.0	5.04 \pm 0.1	99.2
Fizzy	5.79 \pm 0.47	6.0 \pm 0.15	96.5

Conclusions

In conclusion, a promising material which is a good candidate for several biotechnological applications was synthesized, characterized and applied as an enzyme immobilization and biosensor platform. PyOx was chosen as a model enzyme to fabricate Mt based biosensors. The Calix-NH₂/Mt/PyOx sensor has shown good linearity with low detection limit. At the final step, the biosensor was applied for glucose analysis in real samples and the obtained data for the clay biosensor were in good agreement with reference method. This system could apply different bio-analysis systems for the biotechnological applications.

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