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Immobilization of barley oxalate oxidase onto gold–nanoparticle-porous $CaCO_3$ microsphere hybrid for amperometric determination of oxalate in biological materials

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ABSTRACT

Objectives: The use of Au nanoparticles (AuNPs)–$CaCO_3$ porous microsphere hybrid as a matrix for enzyme immobilization to increase the performance of an amperometric biosensor.

Design and methods: A method is described for construction of an amperometric oxalate biosensor by immobilizing a barley root oxalate oxidase (OxOx) onto AuNPs–$CaCO_3$ porous microsphere hybrid encapsulated in silica sol and deposited on Au electrode.

Results: The biosensor showed optimum response within 4 s at pH 5.0 and 30 °C, when polarized at +0.4 V vs. Ag/agCl. The biosensor possesses high sensitivity and measures oxalate concentrations as low as 1.0 μmol/L. The working linear range was from 1.0 to 1000 μmol/L of oxalate. The biosensor measured urinary and plasma oxalate of normal persons and stone formers.

Conclusion: The use of AuNPs–$CaCO_3$ porous microsphere hybrid as a support for immobilization of OxOx has resulted into an improved amperometric oxalate biosensor.

Introduction

The measurement of oxalate in urine and plasma is of great importance in the diagnosis and medical management of primary hyperoxaluria, calcium oxalate urolithiasis, malabsorption, steatorrhoea, illeal disease and ethylene glycol poisoning [1]. The normal level of oxalic acid is 0.8–2.50 μmol/L in plasma [2] and 20–30 mg/24 h in urine [3]. Due to endogenous synthesis under certain pathological conditions or increased absorption of oxalate from the diet, the oxalate accumulates in the body leading to hyperoxaluria and finally manifestation of calcium oxalate urinary stones. This results into increased excretion of urinary oxalate in stone formers [4]. Among the various methods available for oxalic acid analysis such as titrimetric [5], colorimetric [6], enzymic colorimetric [7], isotachophoresis [8], isotope dilution and mass spectrometry [9], atomic absorption spectro-photometric [10], continuous flow method [11], HPLC [12], capillary electrophoresis [13], ion chromatography and chemiluminescence [14], enzyme electrode/biosensing methods [15] are comparatively more simple, sensitive, rapid and specific and hence considered better for routine analysis.

Gold nanoparticles (AuNPs) are an ideal material for biosensors, especially for those with electrochemical detection, because the gold surface area is suitable for binding of biomolecules and the metal facilitates direct and fast electron transfer [16]. If AuNPs are assembled on porous $CaCO_3$ via electrostatic interaction, the resulting AuNPs–$CaCO_3$ composite would couple the advantages of both the nanoparticles and porous material such as satisfying biocompatibility, strong adsorption, good stability and fast electron transfer [17].

The present report describes the construction and application of an amperometric oxalate biosensor based on immobilization of barley oxalate oxidase (OxOx) onto AuNPs–$CaCO_3$ porous microsphere hybrid encapsulated in silica sol.

Materials and methods

Chemicals and reagents

4-Aminophenazone, oxalic acid, DEAE-Sephacel and Sephadex G-100 were from Sigma-Aldrich, St. Louis, USA. $CaCl_2$ $2H_2O$, Na_2CO_3, $HAuCl_4$ $4H_2O$ and tetraethyl orthosilicate (TEOS) were from Sisco Research Laboratory, Mumbai, India. The seeds of barley (BH 393, purity—98%) and gold wire (1.5 cm × 0.05 cm) (23 carat) were purchased from local market, Rohtak, India. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used throughout the experiments.

Apparatus

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a Potentiostat/

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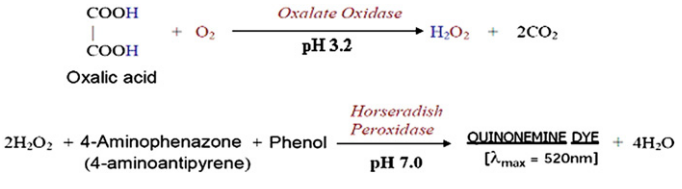
Galvanostat (Autolab, Eco Chemie, The Netherland. Model: AUT83785) with a three electrode system composed of a Pt wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode and AuNPs–$CaCO_3$ porous hybrid modified Au wire as a working electrode. Scanning electron microscopy (SEM) measurements were carried out at Department of Chemistry, M. D. University, Rohtak. Transmission Electron Microscopy (TEM) for AuNPs–$CaCO_3$ solution was performed at Punjab University, Chandigarh.

Extraction and purification of OxOx

All steps of extraction and purification of OxOx were carried out at 4 °C. OxOx was extracted from the roots of 15 days old barley seedlings as described by Chiriboga [18] with modification. The roots (150 g) of 15 day old barley seedling plants grown in soil in an earthen pot were homogenized (1500×*g*) with two volumes of DW in a warring blender for 2 min. The extract was stirred for 6 h with an overhead stirrer and the insoluble material was removed by filtration through gauze, followed by centrifugation at 15000×*g* for 30 min. The supernatant was collected and treated as crude enzyme. All enzyme preparations were tested for OxOx activity and protein by Lowry method. The crude enzyme was purified by combination of conventional purification techniques of enzymes such as 0–80% $(NH_4)_2SO_4$, gel filtration on Sephadex G-100 and ion exchange chromatography on DEAE-Sephacel using linear gradient of KCl (0.1 M to 0.6 M) at 4–10 °C. The purified enzyme showed a single band in polyacrylamide gel electrophoresis using $AgNO_3$ as protein stain and had a specific activity of 22 U/mg protein, which represented 16% yield of the activity of initial crude enzyme.

Assay of barley OxOx

The assay of OxOx was carried out in dark as described by [19] with modification. In a 15 mL test tube wrapped with black paper. The reaction mixture containing 1.8 mL 0.05 M sodium citrate buffer pH 3.2, 0.1 mL enzyme (1.5 mg protein/mL) was preincubated at 30 °C for 10 min. The reaction was started by adding 0.1 mL of oxalate solution (0.5 M). After incubation at 40 °C for 15 min, 1 mL color reagent (50 mg 4-aminophenazone, 100 mg phenol in 1 mg horse radish peroxidase per 0.4 M sodium phosphate buffer pH 7.0) was added and kept at the same temperature in water bath for 20 min to develop the color. The blank was also run in the same manner except that the enzyme solution was replaced with reaction buffer. The color intensity of the reaction mixture was read at 520 nm against the blank in Spectronic-20. The content of H_2O_2 generated in the reaction was extrapolated from standard curve of H_2O_2. The following chemical reactions occurred during the assay of oxalate oxidase:



One unit of oxalate oxidase is defined as amount of enzyme required to generate 1 μmol H_2O_2/min/mL under standard assay conditions.

Preparation of AuNPs and $CaCO_3$ microspheres

AuNPs were prepared as described [20] with slight modification. Twenty mL of $HAuCl_4$ solution (1 mg/5 mL) was taken in a 50-mL flask kept on a stirring hot plate. A magnetic stir bar was added and the solution was boiled. To this boiling solution, 2 mL of 1% trisodium citrate dehydrate was added dropwise. An AuNPs suspension gradually formed, as the citrate reduced the gold. Change of color of the solution from blackish to wine red confirmed formation of gold nanoparticles. The colloidal gold solution was then stored in dark bottles at 4 °C after cooling.

The porous $CaCO_3$ microspheres were prepared rapidly by pouring a 10 mL 0.33 M Na_2CO_3 solution into an equal volume of a 0.33 M $CaCl_2$ solution at room temperature (RT) (30 °C) on a magnetic stirrer. The precipitate was removed by centrifugation, washed with DW and dried at RT.

Preparation of AuNP–$CaCO_3$ hybrid material

The porous $CaCO_3$ microsphere (0.5 g) were dispersed into 50 mL of Au colloid solution (pH 7) and sonicated for 20 min. After centrifugation, the light purple colored AuNP/$CaCO_3$ composites were collected, while the colorless supernatant was discarded. The AuNP–$CaCO_3$ composites were further washed with DW for three times and dried at RT [21]. The diameter of the prepared AuNPs–$CaCO_3$ hybrid was measured by TEM.

Preparation of OxOx–AuNP–$CaCO_3$ bioconjugates

The AuNP–$CaCO_3$ hybrid material (10 mg) was dispersed into a 1 mL purified OxOx containing 22 U (1 mg/mL, pH 7.0) and incubated for 1 h at 4 °C for enzyme adsorption. The bioconjugates were then centrifuged and washed with DW three times. The concentrations of the gold colloid and OxOx solutions before and after the assembly process were measured by UV–vis spectra.

Preparation of silica sol

Silica sol was prepared according to the reference [21] by mixing 600 μL of ethanol, 50 μL of TEOS, 10 μL of 5 mM NaOH and 60 μL of DW in a small test tube and sonicating it for 20 min at RT. The sol so formed was stored at 4 °C.

Fabrication of the OxOx–AuNP–$CaCO_3$ modified Au electrode

An Au wire was polished successively with 1.0 μm alumina powder and rinsed thoroughly with DW between each polishing step. The OxOx–AuNP–$CaCO_3$ bioconjugates (0.5 g) were resuspended in 0.5 mL of DW and 10 μL of this suspension was smeared on the surface of the pretreated Au electrode and then left it at RT to dry. Silica sol (10 μL) was then added on the surface of Au electrode to encapsulate the bioconjugates. The electrode was then dried at RT and stored at 4 °C for 24 h.

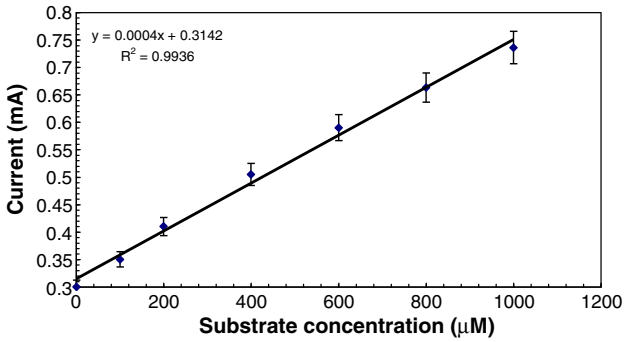


Fig. 1. Standard graph between oxalic acid concentration and current response of oxalate biosensor based on OxOx–AuNPs–$CaCO_3$/Au electrode.

Response measurement of OxOx–AuNP–$CaCO_3$ modified Au electrode and its optimization

Cyclic voltammogram of OxOx–AuNP–$CaCO_3$/Au electrode was recorded in the potential range +0.1 to +0.9 V vs Ag/AgCl as reference and Pt as auxiliary electrode in a 0.05 M sodium succinate buffer (pH 4.5) containing 0.5 M oxalate. The maximum response was observed at +0.4 V and hence the subsequent studies were carried out at this voltage. To test the functioning of the biosensor, three electrodes immersed into 15 mL 0.05 M sodium succinate buffer pH 4.5 in a 50 mL beaker. The reaction was started by adding 1.0 mL oxalate solution (0.01 M) to reach a final concentration of 10 mmol/L and the current (mA) generated was recorded at +0.4 V. Cyclic voltammetric studies were recorded in 0.05 M sodium succinate buffer pH 4.5 containing oxalic acid concentration varying from 1.0 to 1000 μmol/L at 0.0 to +0.6 V with a scan rate of 50 mV s^{-1}.

Determination of oxalate in urine and plasma

Twenty four h urine samples from apparently healthy individuals and kidney stone formers of different age groups and sex from hospital of Pt. BDS Post Graduate Institute of Medical Science, Rohtak were collected in 2 L plastic bottles containing 15 mL concentrated HCl and its pH was adjusted to pH 5 by adding HCl or NaOH and stored at 4 °C until use. To avoid possible ascorbate interference, 200 mg of activated charcoal was added to 2.0 mL of neutralized urine sealed with Parafilm and vortex mixed for 5 min in a 15 mL test tube. The urine was filtered through Whatman No. 1 filter paper and filtrate was used for oxalate assay. Fresh blood samples of these apparently healthy individuals and urinary stone formers were withdrawn intravenously with sterilized needle and syringe and transferred into plastic vial containing a pinch of heparin and centrifuged in cold at 1300×*g* for 10 min. The supernatant/plasma was collected and stored at 4 °C

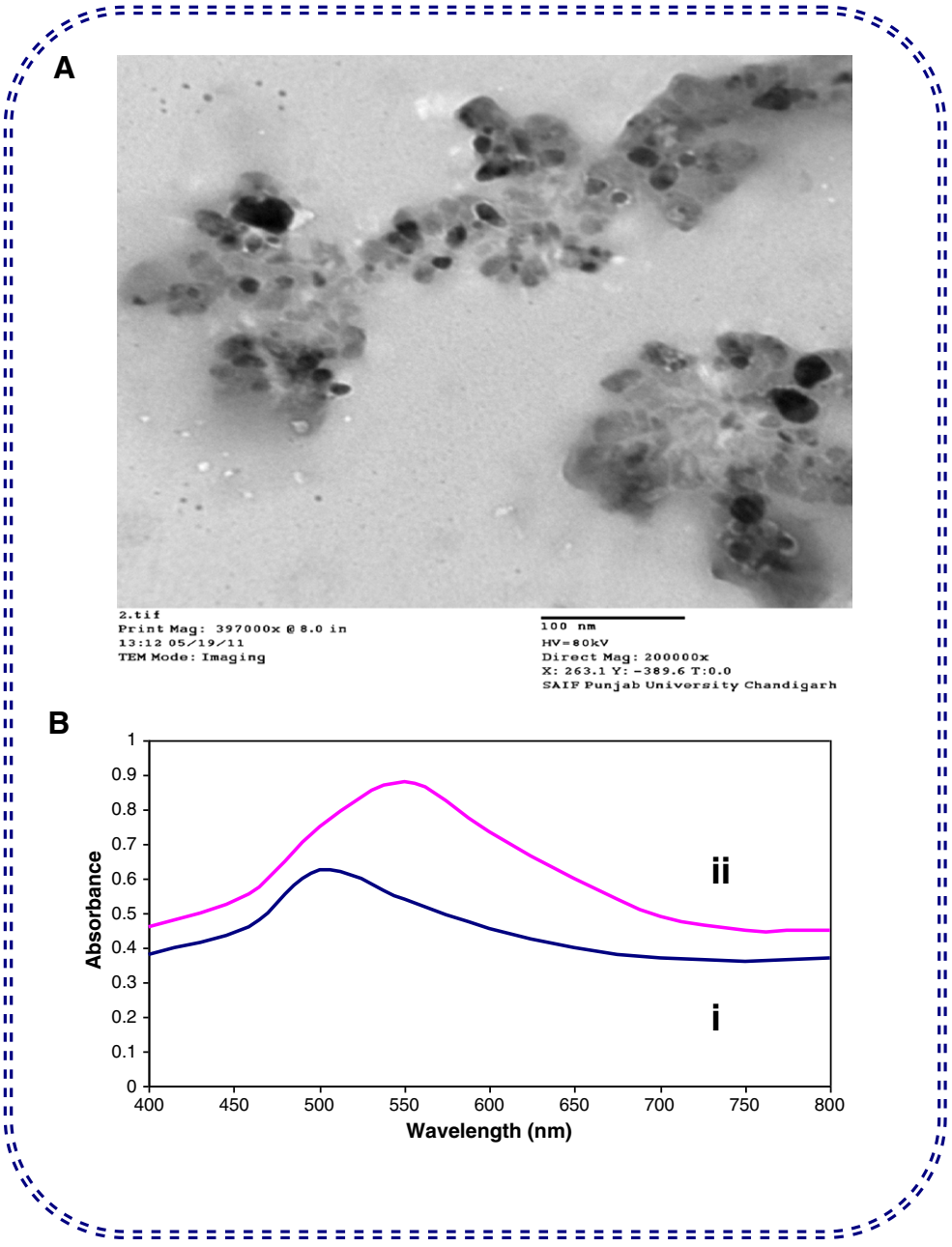


Fig. 2. (A) Transmission electron microscopic image of AuNP–$CaCO_3$. (B) UV–vis absorption spectra for AuNP (i) and AuNP–$CaCO_3$ (ii).

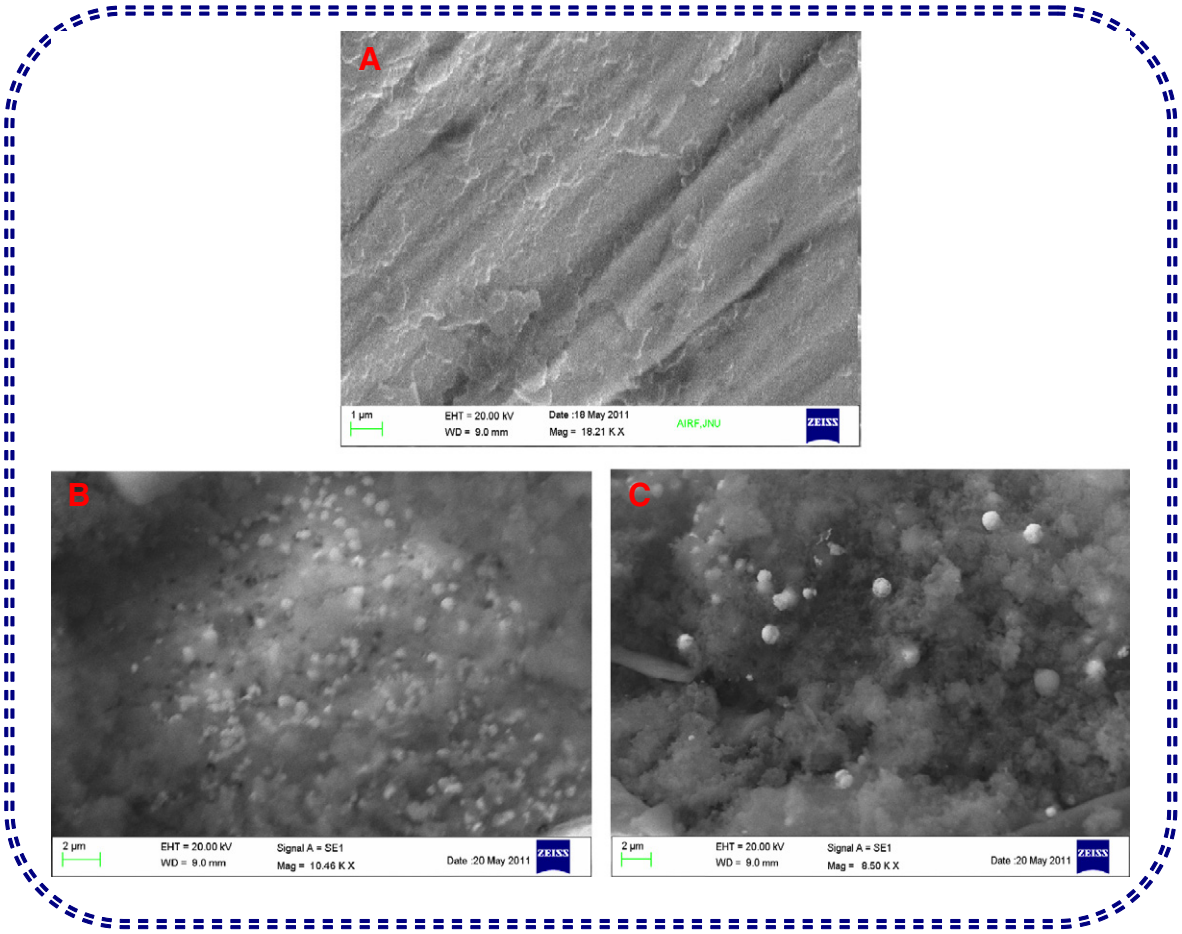
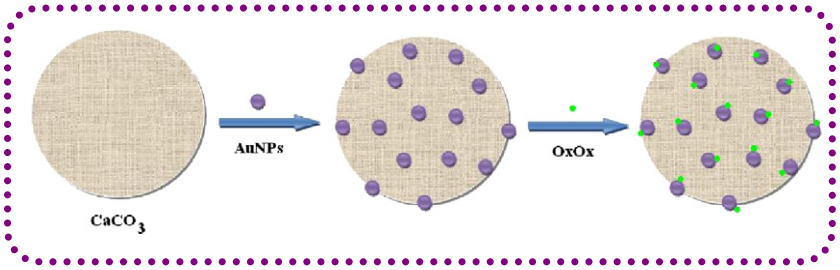
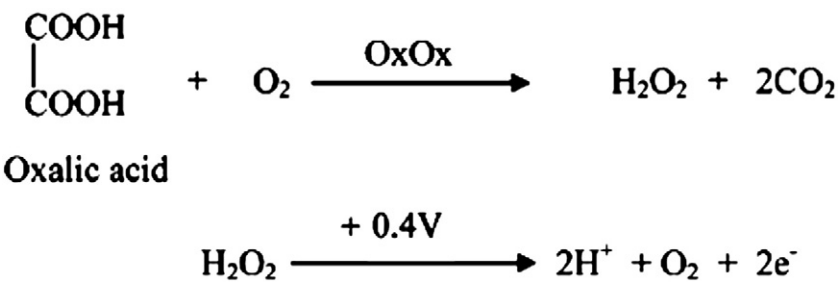


Fig. 3. Scanning electron microscopy of (A) bare Au electrode, (B) AuNP–$CaCO_3$/Au electrode and (C) OxOx–AuNP–$CaCO_3$ modified Au electrode.



Scheme 1A. Formation of OxOx–AuNPs–$CaCO_3$ bioconjugates.



Scheme 1B. The electrochemical reactions involved in response measurement of oxalate biosensor based on OxOx–AuNPs/$CaCO_3$/Au electrode.

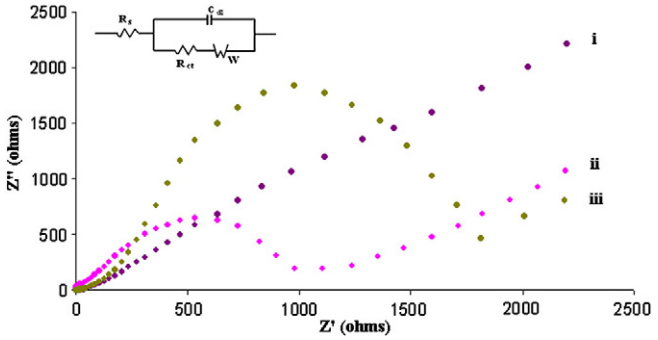


Fig. 4. Electrochemical impedance Nyquist plot of bare AuE (i), AuNP–$CaCO_3$/AuE (ii) and OxOx–AuNP–$CaCO_3$/AuE (iii) at 0.05 M sodium succinate buffer (pH 5.0) containing 0.5 mM $[Fe(CN)_6]^{3-}$ + 0.5 mM $[Fe(CN)_6]^{4-}$ and 0.5 M oxalic acid. Frequency range: 0.01 Hz to 10 KHz. Inset shows the equivalent circuit for 'mixed kinetic and diffusion control'.

until use. To avoid possible ascorbate interference, 10 μL of 5 mM $NaNO_2$ was added to 0.1 mL plasma and vortexed out vigorously. To determine oxalate in urine/plasma, three electrode systems were immersed into 15.0 mL 0.05 M sodium succinate buffer pH 5.0 in a 50 mL beaker. The reaction was started by adding 1.0 mL pretreated urine/plasma and the current (mA) generated was recorded at +0.4 V Vs. Ag/AgCl under its optimal working conditions. The concentration of oxalate in urine/plasma was interpolated from standard curve of oxalate between oxalate concentrations vs. current (mA) (Fig. 1).

Results and discussion

TEM study of AuNPs–$CaCO_3$ porous hybrid

AuNP–$CaCO_3$ porous hybrid synthesized in this study was essentially very fine and monodisperse with a mean diameter of ca. 40 nm, as seen from their TEM image (Fig. 2A). Fig. 2B shows the UV–vis spectra of the prepared colloidal AuNPs solution (i) and the gold solution after being sonicated with $CaCO_3$ for 20 min and centrifuged (ii). AuNPs alone showed an absorption peak at 500 nm, which was increased and shifted to 540 nm after their assembly with $CaCO_3$ microspheres, indicating capture of AuNPs. The shift in the absorbance (blue to red) indicated aggregation of the AuNPs on the $CaCO_3$ microspheres surface [17].

SEM study of OxOx–AuNPs–$CaCO_3$/Au electrode

SEM images of bare Au electrode (Fig. 3A), AuNPs–$CaCO_3$ hybrid material onto Au electrode (Fig. 3B) and OxOx–AuNPs–$CaCO_3$/Au electrode are shown in Fig. 3C. The surface of bare Au electrode was smooth (Fig. 3A). The AuNPs–$CaCO_3$ microspheres were rough and consisted of a great number of carbonate nanoparticles. These nanoparticles were combined to form a specific morphology (Fig. 3B), which played important role in the immobilization of enzyme onto AuNPs assembly. The assembly of the OxOx–AuNPs–$CaCO_3$ resulted in rough surface texture decorated with small spherical enzyme granules on AuNPs–$CaCO_3$ hybrid material (Fig. 3C).

Characteristics of OxOx–AuNPs–$CaCO_3$/Au electrode

Porous $CaCO_3$ microspheres were used as template for enzyme immobilization. The presence of nanometer-sized pores and channels in $CaCO_3$ particles offered a great opportunity for capturing biomacromolecules such as proteins via physical adsorption/pore diffusion, thus enabling very high substrate loading. With the isoelectric point at pH 8.5 $CaCO_3$ microspheres were positively charged at pH 7.0, whereas AuNPs were negatively charged at the same pH. The electrostatic interaction caused the assembly of AuNPs on the surface of the oppositely charged $CaCO_3$ microspheres; the unique structure of $CaCO_3$ microspheres greatly facilitated this assembly process (Scheme 1A). The electrochemical reactions occurring during biosensor response measurements are summarized in Scheme 1B.

Electrochemical impedance spectroscopic (EIS) study

Fig. 4. shows electrochemical impedance spectra (EIS) of (i) Au electrode (ii) AuNPs–$CaCO_3$/Au electrode and (iii) OxOx–AuNPs–$CaCO_3$/Au electrode. The charge transfer process in OxOx–AuNPs–$CaCO_3$/Au bioelectrode has been studied by monitoring charge transfer resistance (Rct) at the electrode and electrolyte interface. The value of the electron transfer resistance (semicircle diameter) (Rct) depends on the dielectric and insulating features at the electrode/electrolyte interface. The Rct values for the bare Au, AuNPs–$CaCO_3$/Au and OxOx–AuNPs–$CaCO_3$/Au electrodes have been obtained as 50, 1000 and 1870 Ω, respectively. The increased Rct value of OxOx–AuNPs–$CaCO_3$/Au electrode was due to the immobilization of enzymes onto AuNPs–$CaCO_3$ modified Au surface. This increase in Rct is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz) and cause hindrance to the electron transfer.

Optimization of biosensor

The optimal pH of biosensor/immobilized OxOx was 5.0, which is higher than that of free enzyme (pH 3.5). This shift in optimum pH could be related to the microenvironment of the immobilized enzyme. The optimum temperature of biosensor/immobilized OxOx was 30 °C, which is lower than that of free enzyme (37 °C). The present biosensor showed a fast response time i.e. it showed maximum response within 4 s, which is lower than earlier reported oxalate biosensors [22–25]. Fig. 5 shows the cyclic voltammogram of OxOx–AuNP–$CaCO_3$/Au electrode response at different oxalic acid

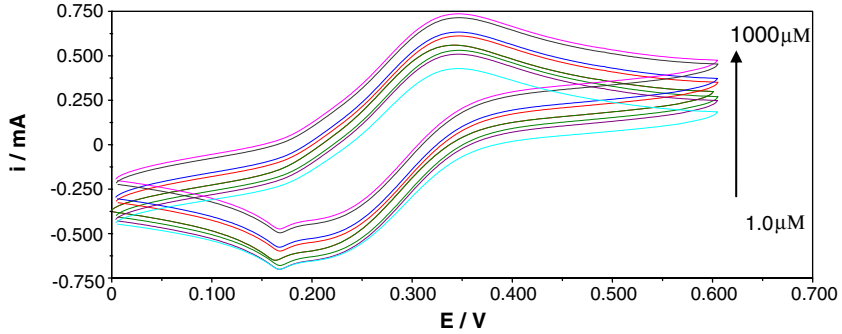


Fig. 5. Cyclic voltammogram of OxOx–AuNP–$CaCO_3$/Au electrode obtained at 0.0 to +0.6 V with scan rate 50 mV/s in the presence of different concentration of oxalic acid (1–1000 μM).

concentrations (1.0–1000 μmol/L). There was a linear increase in oxidation current with increase in oxalic acid concentration in the range 1.0–1000 μmol/L, after which it was constant (Fig. 1). K_m for oxalate as calculated from Lineweaver-Burk plot was 46.6 μmol/L.

Evaluation of biosensor

The minimum detection limit of the present biosensor was 1.0 μmol/L, which is lower than earlier reported biosensors (Supplementary Table 1). The mean analytic recoveries of added oxalate-at-50.0 and 100.0 mmol/L (final concentration in urine) as determined by present biosensor were 94.7% and 95.1% respectively. To test the reproducibility and reliability of the present oxalate biosensor, oxalic acid content in six urine samples was determined on single day (within batch) five times and again after storage at −20 °C for one week (between batch). The results showed that determinations were consistent and within and between coefficients of variation (CVs) were 4.7% and 5.8% respectively (Supplementary Table 2). These results indicated the reproducibility and consistency of the present method. The oxalic acid values of our method were in good agreement with those by standard enzymic colorimetric method of Sigma with a good regression coefficient ($r = 0.984$). These results show the high accuracy of the method.

Practically no interference was observed during measurements of oxalate by the present biosensor response in presence of glucose, cholesterol, urea, pyruvate, bilirubin, $CuSO_4$, KCl, FAD, NaCl, $ZnSO_4$, NADH, $CaCl_2$, EDTA, NEM, riboflavin, $MnCl_2$, FMN and sodium azide. However ascorbic acid cause 30% interference, while the oxalate sensor reported earlier showed maximum decrease in activity by ascorbic acid [26] and phthalate [27] (Supplementary Table 3).

Application

Oxalate value in 24 h urine from apparently healthy male and female in three age groups i.e. children (1–20 yr), young (21–45 yr) and old persons (>45 yr) ($n = 20$ in each group) and urinary stone formers in same groups were measured by the present enzyme electrode. The results are given in Supplementary Table 4, which show that the urinary oxalate value was in the range, 12.40 to 28.38 mg/L and 27.96 to 43.96 mg/L with a mean of 20.17 and 36.23 mg/L in healthy and stone formers respectively. The oxalate value in serum of apparently healthy individuals and stone formers were in the range of 1.3 to 3.1 μmol/L and 6.7 to 10.6 μmol/L with a mean of 2.2 and 8.7 μmol/L in healthy and stone formers respectively (Supplementary Table 5). The urinary and plasma oxalate values in stone formers were significantly higher ($p<0.05$) in stone formers compared to apparently healthy persons, similar to earlier reports [19–22].

Stability and reusability

The enzyme electrode lost only 50% of its initial activity after its 200 uses over a period of 6 months, when stored dry at 4 °C. A comparison of analytical properties of present oxalate biosensor with earlier amperometric oxalate biosensors is summarized in Supplementary Table 1.

Conclusion

The use of nanohybrid of AuNP–$CaCO_3$ porous microsphere in preparation of an amperometric oxalate biosensor has resulted into its improved analytical performance in terms of faster response (4 s), minimum detection limit (1.0 μmol/L) and higher storage stability (6 months) and no interference compared to earlier oxalate biosensors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.clinbiochem.2011.12.004.

References

[1] Hogkinson A. Oxalic acid in biology and medicine. New York: Academic press; 1977. 104-158.
[2] Pundir CS, Kuchhal NK, Thakur M. Satypal. Determination of plasma oxalate with chloride ion insensitive oxalate oxidase. Indian J Biochem Biophys 1998;35:120–2.
[3] Pundir CS, Thakur M, Satypal. Determination of urinary oxalate with Cl^- and NO_3^- insensitive oxalate oxidase purified from sorghum leaves. Clin Chem 1998;44:1364–5.
[4] Earnest DL, Johnson G, Williams HE, Admirand WH. Hyperoxaluria in patients with ileal resection: an abnormality in dietary oxalate absorption. Gastroenterol 1974;66:1114–22.
[5] Archer HE, Dormer AE, Scowen EF, Watts RWE. Studies on the urinary excretion of oxalate by normal subjects. Clin Sci 1957;16:405–11.
[6] Hodgkinson A, Williams A. An improved colorimetric procedure for urine oxalate. Clin Chim Acta 1972;36:127–32.
[7] Crider QE, Curran DE. Simplified method for enzymatic urine oxalate assay. Clin Biochem 1984;17:351–5.
[8] Schmidt K, Hagmaier V, Bruchelt G, Rutishauser G. Analytical isotachophoresis: a rapid and sensitive method for determination of urinary oxalate. Urol Res 1979;8:177–80.
[9] France NC, Holland PT, Meghie TK, Wallace MR. Measurement of urinary oxalate by capillary gas chromatography and its validation by isotope dilution mass spectrometry. J Chromatogr 1988;433:1–7.
[10] Levya JAM, Artiga MPH, Mendez MMA, Perez JJQ. Atomic absorption and UV–vis absorption spectrophotometric determination of oxalate in urine by ligand exchange extraction. Clin Chim Acta 1990;195:47–56.
[11] Goldsach K, Ginman RFA, Wright JM. Direct determination of urinary oxalate by a continuous flow method. Med Lab Sci 1990;47:73–9.
[12] Fry Ian DR, Starkey BJ. The determination of oxalate in urine and plasma by high performance liquid chromatography. Ann Clin Biochem 1991;28:581–7.
[13] Nelson BC, Uden PC, Rockwell GF, Gorsky KM, Aguilera ZB. Determination of oxalate in parenteral nutrition solutions by capillary electrophoresis. J Chromatogr A 1997;771:285–99.
[14] Balion CM, Thibert RJ. Determination of oxalate by luminol chemiluminescence. Clin Chem 1994;40:1096–7.
[15] Pundir CS, Sharma M. Oxalate biosensor: a review. J Sci Indus Res 2010;69:489–94.
[16] Andreescu S, Luck LA. Studies of the binding and signaling of surface-immobilized periplasmic glucose receptors on gold nanoparticles: a glucose biosensor application. Anal Biochem 2008;375:282–90.
[17] Cai WY, Xu Q, Zhao XN, Zhu JJ, Chen HY. Porous gold–nanoparticle–$CaCO_3$ hybrid material: preparation, characterization, and application for horseradish peroxidase assembly and direct electrochemistry. Chem Mater 2006;18:279–84.
[18] Chiraboga J. Purification and properties of oxalic acid oxidase. Arch Biochem Biophys 1965;116:516–23.
[19] Bias R, Potezny N, Edward JB, Rofe AM, Conyer RAJ. Oxalate determination by immobilized oxalate oxidase in a continuous flow system. Anal Chem 1980;52:508–51.
[20] McFarland AD, Haynes CL, Mirkin CA, Duyne RPV, Godwin HA. Color my nano world. J Chem Educ 2004;81:544A-544B.
[21] Wang QL, Lu GX, Yang BJ. Direct electrochemistry and electrocatalysis of hemoglobin immobilized on carbon paste electrode by silica sol–gel film. Biosens Bioelectron 2004;19:1269–75.
[22] Yadav S, Devi R, Kumari S, Yadav S, Pundir CS. An amperometric oxalate biosensor based on sorghum oxalate oxidase bound carboxylated multiwalled carbon nanotubes–polyaniline composite film. J Biotech 2011;151:212–7.
[23] Pundir CS, Chauhan N, Rajneesh, Verma M, Ravi. A novel amperometric biosensor for oxalate determination using multi-walled carbon nanotube-gold nanoparticle composite. Sens Actuat B 2011;155:796–803.
[24] Mishra R, Kumar H, Pundir CS. An amperometric oxalate biosensor based on sorghum leaf oxalate oxidase immobilization on carbon paste. Anal Lett 2009;42:1–11.
[25] Chaudhary R, Pundir CS. An electrochemical oxalate biosensor based on CA membrane bound sorghum oxalate oxidase. Sens Transducers 2010;113:127–39.
[26] Milardovic S, Kerekovi I, Nodilo M. A novel biamperometric biosensor for urinary oxalate determination using flow-injection analysis. Talanta 2008;77:222–8.
[27] Amini AS, Vallon JJ. Comparison of performances and analytical application of two immobilized oxalate oxidase sensors. Anal Chim Acta 1994;299:75–9.