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A new immobilization and sensing platform for nitrate quantification

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ABSTRACT

Nitrate reductase (NR) from *Aspergillus niger* was covalently coupled to the epoxy affixed gold nanoparticles (epoxy/AuNPs) with a conjugation yield of 35.40 ± 0.01 μg/cm^2 and 93.90 ± 0.85% retention of specific activity. The bare and NR bound epoxy/AuNPs support was characterized using scanning electron microscopy and Fourier Transform Infrared Spectroscopy. The immobilized enzyme system was optimized with respect to pH, temperature and substrate concentrations and successfully employed for determination of nitrate contents in ground water. The minimum detection limit of the method was 0.05 mM with linearity from 0.1 to 10.0 mM. The % recoveries of added nitrates (0.1 and 0.2 mM) were >95.0% and within-day and between-day coefficients of variations were 1.012% and 3.125% respectively. The method showed good correlation ($R^2=0.998$) with the popular Griess reaction method. Epoxy/AuNPs bound NR showed good thermal and storage stabilities and retained 50% activity after 16 reuses.

1. Introduction

Nitrate is a well-known environmental contaminant largely encountered in ground and stream water [1]. High nitrate levels together with phosphate in water bodies have been implicated in the frequent eutrophication of lakes and coastal waterways [2,3]. Excessive ingestion of nitrate beyond normal human intake is related to the formation of carcinogenic nitrosamines and methemoglobinemia in blood, especially in children [4,5]. Since most countries have imposed limits for nitrate in drinking water of 25–50 mg/L (0.4–0.8 mM), the determination of nitrate is of significant concern [6–8].

Over the last decade, few nitrate biosensors based on the immobilization of nitrate reductase (NR) in a partially hydrophobic polymer film over the electrode surface [9-13] have been developed. However, limited stability and activity of NR over the electrode surface due to partial hydrophobic character of the immobilization matrix is restricting the application of nitrate biosensor for routine purposes [14]. As a result, consistent effort lies in improving the activity and stability of NR by using biocompatible supports.

Recently, a growing interest has been shown in using gold nanoparticles (AuNPs) as carriers to achieve enzyme immobilization in order to increase enzyme activity, stability, reuse capacity and storage stability [15,16]. AuNPs are biocompatible, have high surface area for adequate enzyme loading and impose minimal diffusional limitations needed for optimization of immobilized enzymes. Lysozyme [17], glucose oxidase [18,19], trypsin [20], urease [21], aminopeptidase [16] and alcohol dehydrogenase [22] have been successfully immobilized over gold nanoparticles with enhanced stability and good retention of enzyme activity. However, separation of the suspended nanobioconjugates from the solution is troublesome and tedious, as after every assay, the reaction mixture has to be centrifuged in order to reuse the enzyme. A better approach is to affix the nanoparticles onto some insoluble support. Epoxy, activated supports, as such could be deemed as perfect for this purpose, since they are known to impart stability to the matrix and glue the conducting molecules on its surface [23]. Epoxy activated polyethylene membrane was also successfully employed for retention of calcium carbonate nanoparticles [24]. Such epoxy adhered AuNPs can then be decorated with the biomolecule of interest.

Hence, in the present study NR was immobilized onto the epoxy affixed AuNPs. The kinetic parameters, operational, thermal and storage stability of the immobilized NR were studied and compared with the native enzyme. Use of immobilized NR to develop a simple, sensitive and inexpensive colorimetric assay for determination of nitrate levels in underground water has also been demonstrated.

2. Materials and methods

2.1. Reagents

11-mercaptoundecanoic acid (MUA), nitrate reductase (NR; NAD(P)H) from *Aspergillus niger*, N-ethyl-N′-(3-dimethylaminopropyl)

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carbodiimide hydrochloride (EDC) and β-nicotinamide adenine dinucleotide (ß-NADH) were purchased from Sigma-Aldrich Co. St. Louis (USA). N-(1-naphthyl) ethylenediaminedihydrochloride (NED), tri-sodium citrate, N-hyhroxysuccinimide (NHS), sulfanilamide and Tween 20 from Himedia, Mumbai (India), Ethylenediaminetetraacetic acid (EDTA) from Thomas Baker, Mumbai (India) and tetrachloroauric acid ($HAuCl_4$) from Sisco Research Laboratories, Mumbai (India) were procured. Epoxy resin and bisphenol A available as a popular adhesive under the trade name "Araldite" were purchased from the local market. All other chemicals purchased were of analytical reagent (AR) grade.

2.2. Synthesis, surface modification and characterization of AuNPs

The citrate stabilized AuNPs were freshly prepared through reduction of chloroauric acid by sodium citrate [25]. 50.0 ml of 2.0 mM aqueous solution of chloroauric acid was stirred on a magnetic stirrer for 15 min at 80 °C. Then, 50.0 ml of 0.01 mM aqueous solution of sodium citrate was added quickly into the constantly stirring solution. The solution turned from dark gray to dark purple to bright red.

MUA-modified NPs were prepared by exchange of mercaptocarboxylic acid with citrate group in the presence of non-ionic surfactant Tween-20 [20]. 5.0 ml of colloidal AuNPs (2.0 nM) were gently added to 5.0 ml of phosphate buffer (10.0 mM, pH 6.8 with 0.02 ml Tween-20) and mixture was incubated for 30 min. Thereafter, 5.0 ml of MUA solution (0.5 mM in 1:3/alcohol:H_2O) was added into the mixture and was gently shaken for 5 h for complete chemisorption of alkane thiol on the AuNPs surface. MUA-modified AuNPs were further terminated with NHS based on the EDC/NHS coupling reaction [26]. MUA-NPs were added to 200 mM EDC and 50 mM NHS solution and the reaction mixture was incubated for 10 min. These NHS terminated NPs were dispersed under ultrasonication (Misonix Q125, U.S.A.) at 20 °C for 10 min at 70% amplitude.

Size of citrate stabilized AuNPs was confirmed by Transmission Electron Microscopy (TEM-JEOL 2100F) at Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi. Modification of AuNPs was confirmed by UV–vis spectra and Fourier Transform Infrared Spectroscopy (FTIR, Alpha, Bruker, Germany) at Department of Genetics, Maharshi Dayanand University, Rohtak.

2.3. Fabrication of epoxy/AuNPs/NR and epoxy/NR strip

The epoxy support was prepared by mixing epichlorhydrin and bisphenol A (epoxy resin and hardener) in the ratio of 85:15. This polymerized bubble free emulsion was spread evenly on the surface of polyethylene sheet of size measuring 5×5 cm^2 and left undisturbed for 30 min at 26 °C to allow the resin to set as per the manufacturer's instructions. After 30 min, the resin reached the gel state where it was still wet and no longer a liquid. Now, 2.0 ml of the NHS terminated AuNPs (0.4 nM/ml) was sonicated at 20 °C for 10 min at 70% amplitude and gently spread evenly over the polymerized epoxy support and left covered for 4 h, till epoxy was completely dry and hard. Finally, 0.2 ml of NR (30 units) solution in cold potassium phosphate buffer (25.0 mM, pH 7.3) was slowly poured over the epoxy/AuNPs support. The resultant epoxy/AuNPs/NR conjugates were kept covered for 48 h at 4 °C for the complete immobilization of enzyme over the support [24]. Epoxy/NR strip was also prepared in the similar manner except for the addition of AuNPs. In order to remove the unbound enzyme, the immobilized enzyme preparations were washed with 25.0 mM potassium phosphate buffer (pH 7.3) several times, until no protein was detected in the washing. The protein content of enzyme solution and washings was determined by the method of Lowry et al. [27] using bovine serum albumin as standard protein. The enzyme bound epoxy and epoxy/AuNPs supports were stored in 25.0 mM potassium phosphate buffer pH 7.3 at 4 °C when not in use.

2.4. Characterization of the support

Surface morphology of the bare epoxy, epoxy/AuNPs and epoxy/AuNPs/NR conjugates was studied using scanning electron microscopy (SEM, Jeol JVSM 6510, Japan) at Maharshi Dayanand University, Rohtak. To reveal the bonded interactions infrared spectrum of bare epoxy, epoxy/NR, epoxy/AuNPs and epoxy/AuNPs/NR conjugates were recorded by Fourier Transform Infrared Spectroscopy (FTIR, Alpha, Bruker, Germany) at Maharshi Dayanand University, Rohtak.

2.5. Nitrate reductase activity assay

The assay of the free and immobilized NR was based on a spectrophotometric stop rate determination method [28]. The reaction mixture having 24.0 mM potassium phosphate pH 7.3, 0.05 mM EDTA, 9.5 mM potassium nitrate, 0.10 mM β-NADH and 15.0 units of NR in a total volume of 2.0 ml, was mixed by swirling and incubated at 30 °C for 2 min. The reaction was stopped by adding 1.0 ml of 0.58 mM sulfanilamide solution (prepared in 3.0 M hydrochloric acid solution) and 1.0 ml of 0.77 mM NED solution. After 10 min at 25 °C, the solution was transferred to cuvette and absorbance read at 540 nm. The assay for immobilized NR was performed in the same way except that free enzyme was replaced by epoxy/AuNPs/NR strip measuring 5 cm × 5 cm. One unit of enzyme activity is defined as the amount of enzyme required to reduce 1.0 μmole of nitrate into nitrite per minute in β-NADH system at 30 °C and pH 7.3.

2.6. Optimization of free and immobilized NR

All experiments related to optimization of free and immobilized nitrate reductase were carried out in triplicate, and results were presented as means of three different experiments. Standard error was used to represent the variability of data in all graphs.

To determine optimum pH, free and immobilized NR was assayed using 10.0 mM acetate buffer for pH 5.0 and 5.5, 10.0 mM potassium phosphate buffer in the pH range of 6.0–8.0, and 10.0 mM borate buffer at pH 8.5 and 9.0. The temperature for maximum activity was determined by varying the incubation temperature from 15 to 60 °C with an interval of 5 °C. Energy of activation (*Ea*) was calculated from the Arrhenius plot by plotting inverse of temperature (in degree Kelvin) vs. log of enzyme activity. In order to study the effect of KNO_3 concentration, it was varied from 0.01 mM to 13.0 mM under optimum conditions of pH and temperature and kinetic parameters K_m and V_{max} were calculated by Lineweaver–Burk plot. V_{max} values were used to assess the turnover number (k_{cat}).

2.7. Stability studies

Thermal stability of free and immobilized NR was ascertained by exposing the enzyme for 30 min to various temperatures ranging from 25 °C to 60 °C and then measuring the residual activity under optimum conditions of pH, temperature and substrate concentration. Shelf life of free as well as epoxy/AuNPs bound NR was determined by measuring their activity on alternate days up to 50 days, when stored in potassium phosphate buffer (25.0 mM, pH 7.3) at 4 °C.