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Immobilization of nitrate reductase onto epoxy affixed silver nanoparticles for determination of soil nitrates

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ABSTRACT

Epoxy glued silver nanoparticles were used as immobilization support for nitrate reductase (NR). The resulting epoxy/AgNPs/NR conjugates were characterized at successive stages of fabrication by scanning electron microscopy and fourier transform infrared spectroscopy. The immobilized enzyme system exhibited reasonably high conjugation yield (37.6 ± 0.01 μg/cm^2), with 93.54 ± 0.88% retention of specific activity. Most favorable working conditions of pH, temperature and substrate concentration were ascertained to optimize the performance of epoxy/AgNPs/NR conjugates for soil nitrate quantification. The analytical results for soil nitrate determination were consistent, reliable and reproducible. Minimum detection limit of the method was 0.05 mM with linearity from 0.1 to 11.0 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 0.556% and 1.63% respectively. The method showed good correlation ($R^2 = 0.998$) with the popular Griess reaction method. Epoxy/AgNPs bound NR had a half-life of 18 days at 4 °C and retained 50% activity after 15 reuses.

1. Introduction

Nitrate, leached from soil, is a common surface and ground water contaminant which causes eutrophication of water bodies and health complications in humans and animals [1]. Further, high nitrate content in soil contribute to air pollution and climate change through emission of nitrogen oxides from nitrification and denitrification. Direct determination of nitrate in soil is required for improving nitrogen application management and reducing environmental pollution. The commonly employed methods for nitrate determination are spectrophotometric [2,3], ion exchange chromatography [4,5], high performance liquid chromatography [6], gas chromtography–mass spectrometry [7], capillary electrophoresis [8] and electrochemical [9,10] methods. However, most of these methods have disadvantages like employment of large volumes of toxic reagents, low reproducibility, time-consuming procedures and complicated systems [11]. Fabrication of nitrate biosensors employing nitrate reductase (NR) is an attractive alternative due to its simplicity and selectivity. However, limited stability and activity of NR over the electrode surface is restricting the application of nitrate biosensors for routine purposes [12–14]. Poor performance of immobilized NR in these biosensesors has partly been attributed to the partial hydrophobic character of the immobilization matrix and partly to the complex multisubunit structure of NR. Hence, research focused on the use of new immobilization supports that could stabilize the three dimensional structure of NR is highly desirable.

Recently, a growing interest has been shown in using surface functionalized AgNPs for immobilizing enzymes due to their biocompatibility, high surface area to volume ratio and minimal diffusional limitations [15]. So far, AgNPs have been reported to stabilize and preserve the activity of immobilized β-D-glucanase, [16] papain [17], horseradish peroxidase [18], diastase [19], urease [20], glucose oxidase [21], β-galactosidase [22] and alkaline phosphatase [23]. Additionally, nanoparticles may also be attached to some insoluble support such as epoxy for easy separation of nanobioconjugates from the reaction medium [24–26].

Hence, in the present work epoxy affixed and NHS terminated AgNPs were used as immobilization support for NR. Kinetic parameters, operational, thermal and storage stabilities of immobilized NR were studied and compared with that of free enzyme. A simple, sensitive and inexpensive colorimetric assay for determination of soil nitrate using immobilized NR has also been designed.

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2. Experimental

2.1. Reagents

11-mercaptoundecanoic acid (MUA), nitrate reductase (NR; NAD(P)H) from *Aspergillus niger*, N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and β-nicotinamide adenine dinucleotide (β-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 were obtained from Himedia, Mumbai, India. Ethylenediaminetetraacetic acid (EDTA) from Thomas Baker, Mumbai, India and silver nitrate ($AgNO_3$) from Loba Chemie 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. Deionized water was used as solvent in all the experiments.

2.2. Synthesis, surface modification and characterization of AgNPs

The citrate stabilized AgNPs were freshly prepared through reduction of silver nitrate by sodium citrate [27] as per the following reaction:

$$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \rightarrow 2Ag^{\circ} + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2$$

To synthesize AgNPs, 50.0 ml aqueous solution of silver nitrate (2.0 mM) was stirred on a magnetic stirrer for 15 min at 80 °C. Then, 50.0 ml aqueous solution of tri sodium citrate (20.0 mM) was added quickly into the constantly stirring solution. Change in the color of the solution from light yellow to golden yellow indicated the formation of silver nanoparticles.

MUA–modified NPs were prepared by exchange of mercaptocarboxylic acid with citrate group in the presence of non-ionic surfactant Tween-20 [28]. 5.0 ml of colloidal AgNPs (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 the 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 gently shaken for 5 h for complete chemisorption of alkane thiol on the AgNPs surface. MUA-modified AgNPs were further terminated with NHS based on EDC/NHS coupling reaction [29]. These NHS terminated NPs were dispersed under ultrasonication (MisonixQ125, U.S.A.) at 20 °C for 10 min at 70% amplitude.

Size of citrate stabilized AgNPs was confirmed by transmission electron microscopy (TEM-JEOL 2100F). Modification of AgNPs was confirmed by UV–vis spectra (Thermo Scientific, Evolution 201) and fourier transform infrared spectroscopy (FT-IR, Alpha, Bruker, Germany).

2.3. Fabrication and characterization of epoxy/AgNPs/NR strip

To prepare the epoxy support, epichlorhydrin and bisphenol A (epoxy resin and hardener) were mixed in a ratio of 85:15 and spread evenly on the surface of polyethylene sheet (size 5 cm × 5 cm) at 26 °C [30]. After 30 min, the resin reached a state, when it was neither very soft nor very hard. Now, 2.0 ml of the NHS terminated AgNPs were sonicated at 20 °C for 10 min at 70% amplitude and gently spread evenly over the polymerized epoxy support. At this stage, the preparation was 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/AgNPs support. The resultant epoxy/AgNPs/NR conjugates were kept covered for 48 h at 4 °C for complete immobilization of enzyme over the support [29]. In order to remove the unbound enzyme, the immobilized enzyme preparation was 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. [30] using bovine serum albumin as standard protein. The enzyme bound epoxy/AgNPs support was stored in 25.0 mM potassium phosphate buffer pH 7.3 at 4 °C when not in use.

Surface morphology and bonded interactions of the bare epoxy, epoxy/NR, epoxy/AgNPs and epoxy/AgNPs/NR conjugates were studied using scanning electron microscopy (SEM, Jeol JVSM 6510, Japan) and fourier transform infrared spectroscopy (FT-IR, Alpha, Bruker, Germany) respectively.

2.4. Nitrate reductase activity assay

The assay of the free and epoxy/AgNPs bound NR was based on spectrophotometric stop rate determination method [31]. The reaction mixture having 24.0 mM potassium phosphate buffer (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 at 540 nm was read. The assay for immobilized NR was performed in the same way except that free enzyme was replaced by epoxy/AgNPs/NR strip measuring 5 cm × 5 cm. One unit of NR activity is defined as the amount of enzyme required to reduce 1.0 μmole of nitrate into nitrite per min in β-NADH system at 30 °C and pH 7.3.

2.5. Optimization of NR kinetics

All experiments related to optimization of NR kinetics in both free and bound form 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.

In order to judge the suitability of immobilization support, data for epoxy/AgNPs bound NR was compared to the data acquired for free NR. To determine optimum pH, 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 6.0–8.0, and 10.0 mM borate buffer for 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 (E_a) was calculated from Arrhenius plot by plotting inverse of temperature (in degree Kelvin) vs. log of enzyme activity. In order to study the effect of substrate (KNO_3) concentration, it was varied from 0.01 to 13.0 mM under optimum conditions of pH and temperature. Kinetic parameters K_m and V_{max} were calculated from Lineweaver–Burk plot. V_{max} values were used to assess the turnover number (k_{cat}).

2.6. Stability studies

To ascertain thermal stability, free and immobilized NR were exposed to temperatures ranging from 25 °C to 60 °C at an interval of 5 °C for 30 min and then the residual activity was measured under optimum conditions of pH, temperature and substrate concentration. Shelf life of free as well as epoxy/AgNPs bound NR, stored in potassium phosphate buffer (25.0 mM, pH 7.3) at 4 °C, was determined by measuring their activity on alternate days up to 50 days. For reusability studies, immobilized NR was repeatedly assayed at 25 °C in batch mode till the point its activity was significantly