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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.

2.8. Determination of water nitrate using epoxy/AuNPs/NR strip

The detection principle of nitrate by nitrate reductase is based on the redox reaction:

$$\text{Nitrate} + \beta - \text{NADH} \xrightarrow{\text{NitrateReductase}} \text{Nitrite} + \beta - \text{NAD} + H_2O$$

$$\text{Nitrite} + \text{Sulphanilamide} + \text{NED} \rightarrow \text{Nitrite Color Complex}$$

Nitrate present in the water sample was reduced to nitrite using nitrate reductase in the presence of β-NADH as reducing agent. Nitrite thus formed was treated with sulfanilamide to form a diazocompound that couples with NED to give a nitrite color complex. Ground water samples from nearby places were collected in plastic bottles and stored at 4 °C till use. The reaction mixture having 0.1 ml water sample, 1.8 ml potassium phosphate buffer (25.0 mM, pH 7.3 having 0.05 mM EDTA) and 0.1 ml β-NADH (2.0 mM) was poured gently over the epoxy/AuNPs/NR strip 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 incubating the reaction mixture for 10 min at 25 °C, absorbance at 540 nm was read. Nitrate concentration in water was intrapolated from a standard curve between sodium nitrite concentration (ranging from 0.01 to 10.0 mM) and A_{540}. For reusability studies, immobilized NR was repeatedly assayed at 25 °C in a batch mode till the point its activity was significantly lost. After each reaction run, the immobilized enzyme preparation was washed with 10.0 mM potassium phosphate buffer (pH 7.3) to remove any residual activity.

3. Results and discussion

3.1. Characterization of AuNPs

The size characterization of the citrate stabilized AuNPs was done with TEM. As revealed in Fig. 1, very fine AuNPs in the size range 12–24 nm were synthesized which were well dispersed and polyhedral in shape. An optical absorption spectrum of AuNPs at various steps of the modification is shown in Fig. 2. The peak of AuNPs was at 540 nm (curve a). In the presence of MUA, the citrate groups on the AuNPs surface were exchanged with carboxyl groups, resulting in slight shift of peak from 540 to 545 nm (curve b) and the color of nanoparticles changed from deep red to pink. On addition of NHS and EDC onto the MUA modified AuNPs the color of colloidal solution turned purplish blue and the absorption peak broadened, which indicated the aggregation of nanoparticles owing to the loss of surface charges upon formation of active esters. The surface of NHS esters did not carry any charges, and thus AuNPs experience mostly attractive van der Waals forces because repulsive electrostatic forces were eliminated [29]. The difference in the peaks between citrate stabilized, MUA modified and NHS/EDC coupled AuNPs owing to the increase in the refractive index confirmed modification/surface functionalization of AuNPs [30].

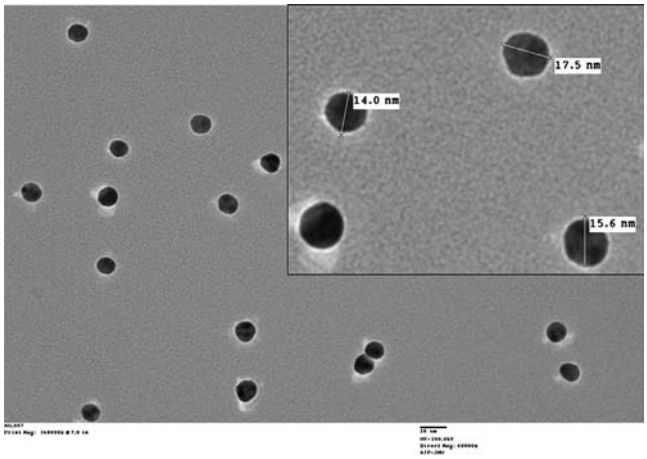


Fig. 1. TEM image of citrate-stabilized AuNPs at a scale bar of 20 nm. In the inset zoomed image shows size labeled AuNPs.

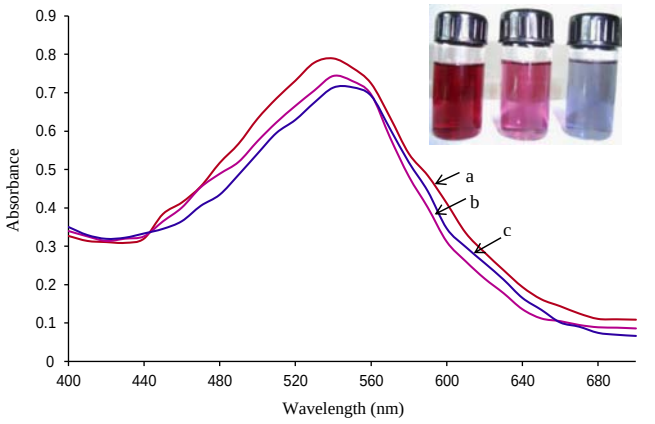


Fig. 2. Optical absorption spectra of citrate-stabilized (a), MUA modified (b) and NHS-terminated AuNPs (c). The solutions of three are also shown in the inset depicting noticeable change in the color of AuNPs at successive stages of modification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

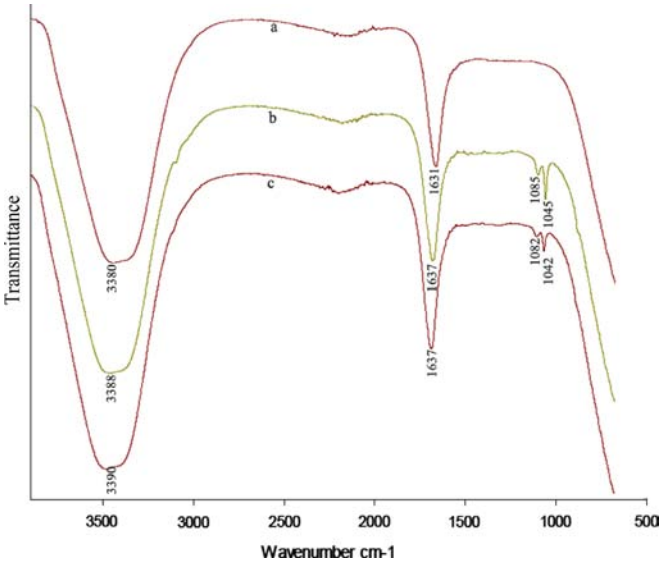
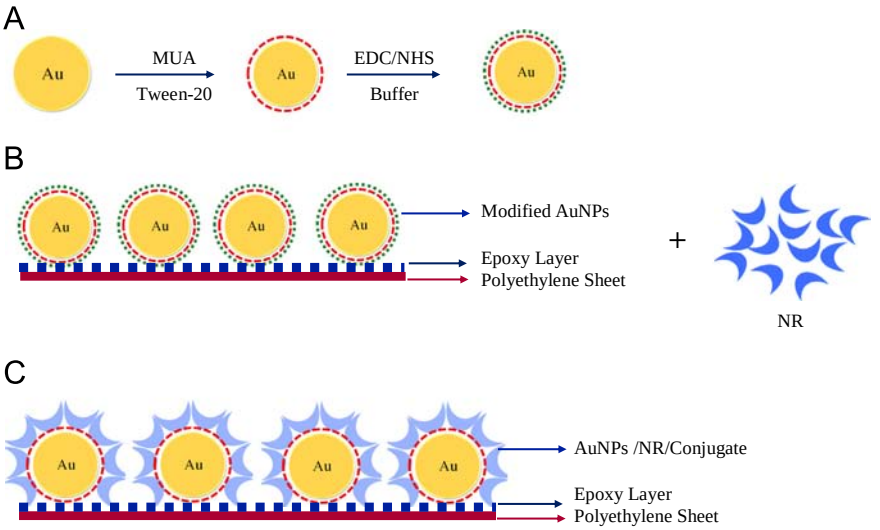


Fig. 3. FTIR spectra of AuNPs at different stages of modification: citrate-stabilized (curve a) MUA modified (curve b) and NHS-terminated AuNPs (curve c).

In FTIR spectra broad absorption band at 3380 cm^{-1}, 3388 cm^{-1} and 3390 cm^{-1} for citrate stabilized AuNPs, MUA modified AuNPs and NHS terminated AuNPs respectively (Fig. 3) can be assigned to O–H stretching of the hydroxyl groups. For citrate-stabilized AuNPs, peak at 1631 cm^{-1} represented the carboxyl group of citrate (curve a). For MUA modified AuNPs, this peak shifted to 1637 cm^{-1} and two weak peaks appeared at 1045 cm^{-1} and 1085 cm^{-1} as the vibrational stretches of $-CH_2$ groups of long alkane chains (curve b) [15]. This indicated the successful modification of AuNPs by MUA groups. For NHS-terminated AuNPs (curve c) there was a slight shift in two



Scheme 1. Immobilization of NR onto epoxy glued AuNPs. Modification of AuNPs (A), attachment of modified AuNPs onto epoxy layer (B) and fabrication of epoxy/AuNPs/NR conjugates (C).

weak peaks to 1042 cm^{-1} and 1082 cm^{-1} with retention of peak at 1637 cm^{-1}.

3.2. Immobilization of NR over epoxy/AuNPs strip

The NHS terminated AuNPs were affixed onto the epoxy layer and NR from *A. niger* was immobilized onto epoxy/AuNPs strip as shown in Scheme 1. Since the enzyme was neither present over the epoxy layer (Fig. 4C) nor did it showed any linkage with epoxy group (Fig. 5C), it may be safely concluded that immobilization of NR occurred only on AuNPs. The side chain amino groups on NR surfaces displaced the terminal NHS groups and formed amide bonds with the carboxyl groups on the AuNPs. NR was immobilized onto epoxy/AuNPs support with a conjugation yield of 35.40 ± 0.01 μg/cm^2 that might be attributed to larger surface area to a volume ration of AuNPs (Table 1). The immobilized enzyme retained 93.90 ± 0.85% of specific activity, which is pretty good considering the complex and multisubunit structure of NR [31]. This might be because biocompatible nature and high surface area of AuNPs renders the immobilized enzyme "quasi-free" [32] and facilitates more efficient electron transfer to and from the redox center of enzyme [33].

3.3. Characterization of epoxy/AuNPs/NR conjugated strip

The surface morphologies of bare and modified epoxy as studied by SEM are presented in Fig. 4. An even polymeric layer of epoxy is shown in Fig. 4A. Addition of AuNPs colloidal solution to partially set epoxy resin resulted in strong physical adherence of AuNPs to the epoxy layer Fig. 4B. The halo around the nanoparticles might correspond to scattering of charges due to the presence of NHS group on the modified AuNPs. The abundance of enzyme all over the surface of AuNPs is well illustrated in Fig. 4C and disappearance of halo indicated the replacement of NHS group by the enzyme. The three figures in Fig. 4 are easily distinguishable supporting the attachment of NHS terminated AuNPs to the epoxy layer and immobilization of NR on epoxy adhered AuNPs.

In order to elucidate the bonding interactions between support and enzyme, FTIR spectra of bare epoxy, epoxy/NR, epoxy/AuNPs and epoxy/AuNPs/NR conjugated strip were recorded. The FTIR spectra for epoxy in Fig. 5 revealed the presence of variable peaks between 2915 and 2847 cm^{-1} for C–H stretching vibrations, characteristic bands for Ar–C=C–H stretching between 1607 and 1509 cm^{-1}, bending $-CH_2$ and $-CH_3$ asymmetrical and symmetrical between 1462 and 1363 cm^{-1} and –C–C–O–C- stretching at 1247, 1183 cm^{-1}. The 1, 4-substitution of aromatic ring and –C–O–C stretching of epoxy ring was seen at 830 cm^{-1}. The aromatic ring vibrations were observed at 719 cm^{-1} [24,34]. The FTIR spectrum of epoxy/NR and epoxy/AuNPs was similar to the bare epoxy (hence peaks were not labeled), ruling out the possibility of any kind of chemical interaction between epoxy and NR or epoxy and AuNPs. However presence of new peak corresponding to the presence of –OH bending at 1638 in spectra of epoxy/NR confirmed the presence of enzyme [35]. FTIR spectra of NR conjugated epoxy/AuNPs showed the emergence of new peaks at 1107 of stretching vibration of –C–N– bond and in plane bending vibration of –C–H– bond of the histidine residue. Peak at 1539 is of secondary amide C=O stretching due to bending of –NH and NH_2 groups present in the enzyme. Presence of a broad peak at 3368 cm^{-1} was attributed to the hydroxyl group of protein [36]. The results confirmed the presence of NR over the epoxy/AuNPs layer. However, retention of characteristic epoxy ring peaks at 1462 and 1509 cm^{-1} and a slight shift in peak from 830 to 831 cm^{-1} confirmed that epoxy groups did not open upon addition of NR. Failure of NR to react with epoxy might be due to unavailability of free epoxy groups on the resin. As the resin hardens it undergoes self-polymerization reaction involving its own epoxy groups and Bisphenol A.

3.4. Kinetic characterization of free and immobilized NR

Although conjugation of NR to epoxy/AuNPs shifted the pH optima from 7.5 to 7.0, the immobilized enzyme retained pretty good activity at pH 7.5 also (Fig. 6). This implies that the epoxy/AuNPs/ NR hybrid can be safely used between pH 7.0 and 7.5 for nitrate determination. The pH optimum is similar to that reported in the literature for NR immobilization onto polypyrrole/carbon nanotubes [37] but lower in comparison to naffion/methyl viologen [12] entrapped NR. Change in optimum pH after enzyme immobilization is a very common phenomenon.

Temperature dependent activity profile of free and immobilized NR showed an optimum temperature of 25 °C for both the enzymes (Fig. 7). The % retention of activity at each temperature was almost the same for both the enzyme preparations from 5 to 25 °C, but further increase in temperature denatured the free enzyme faster than the immobilized form. At 45 °C, the free

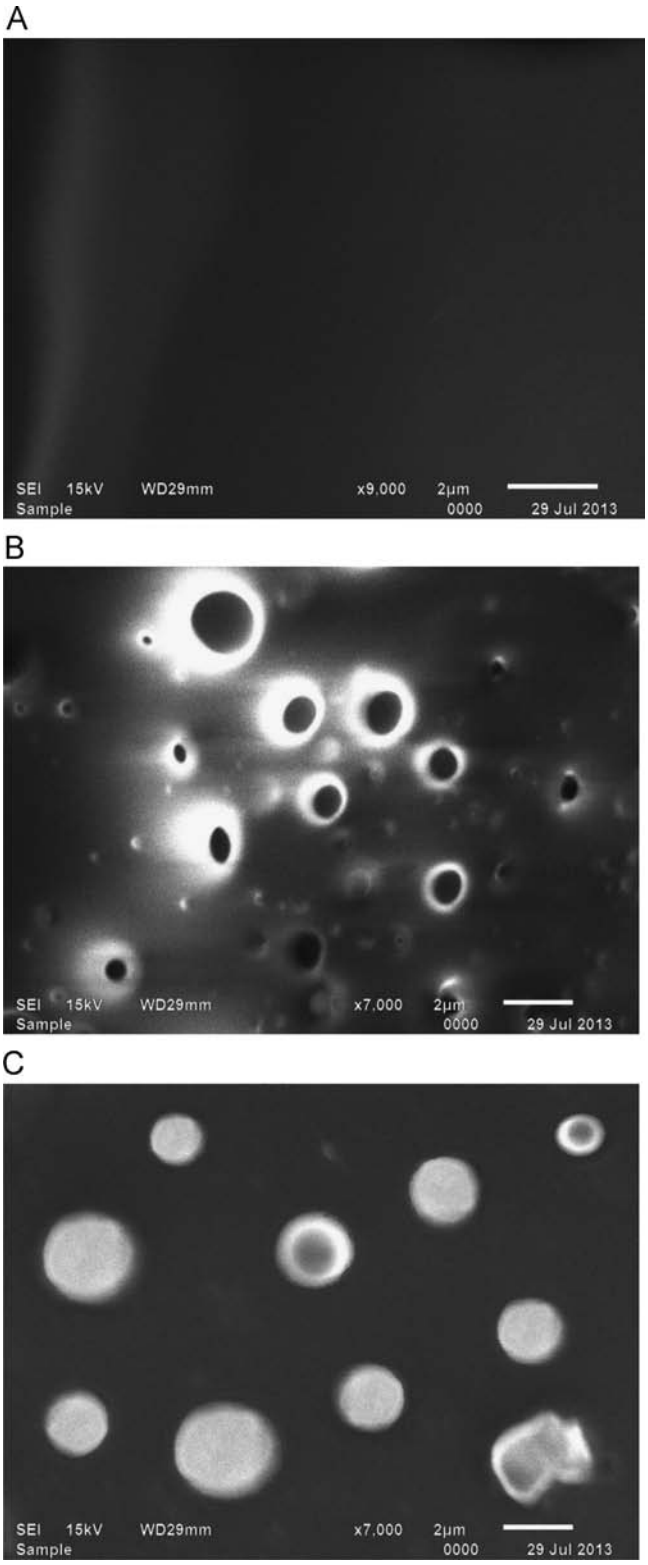


Fig. 4. Surface morphologies of bare (A), AuNPs modified (B) and AuNPs/NR modified (C) epoxy as studied by SEM.

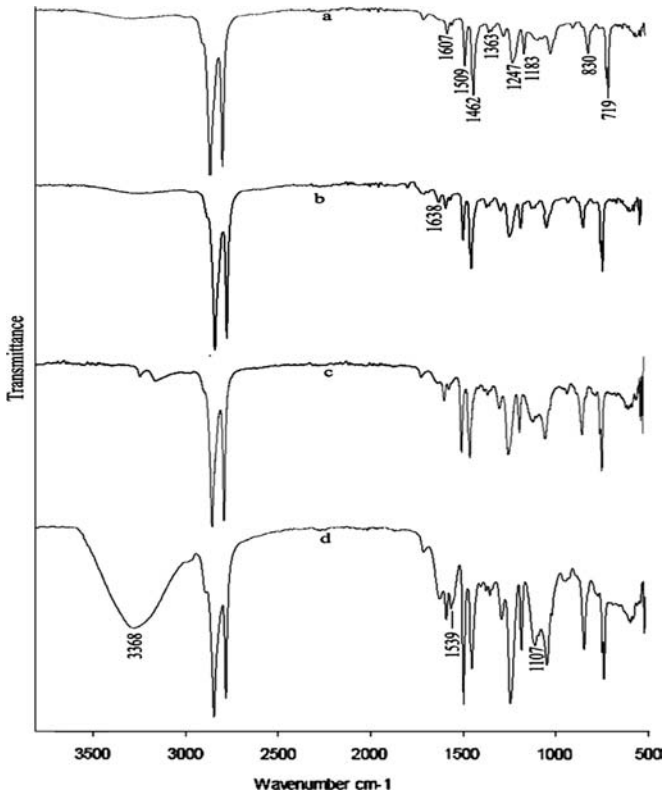


Fig. 5. FTIR spectra of bare epoxy (curve a), epoxy/NR (curve b), epoxy/AuNPs (curve c) and epoxy/AuNPs/NR conjugates (curve d).

Table 1
Immobilization efficiency of epoxy/AuNPs support.

Support	Enzyme added to membrane 5 cm × 5 cm (μg)	Enzyme coupled to membrane 5 cm × 5 cm (μg)	Total activity added (units)[a]	% retention of activity	Conjugation yield (μg/cm^2)
Epoxy/ AuNPs	200	177 ± 0.02	22.0	93.90 ± 0.85	35.40 ± 0.01

[a] One unit of nitrate reductase is defined as the amount of NR required to reduce 1.0 μmole of nitrate into nitrite per minute in the NADH system at 30 °C and pH 7.3.

enzyme was completely inactive but the immobilized enzyme retained about 10% of its initial activity. Activation energy (E_a) for free and immobilized NR was 4.37 and 6.44 kJ/mol, respectively. Reduction in conformational stability after immobilization might account for a higher E_a of bound enzyme.

A hyperbolic relationship was obtained between KNO_3 concentration and NR activity. Free as well as epoxy/AuNPs bound NR showed saturation at 10.0 mM substrate concentration (Fig. 8). The kinetic constants K_m and V_{max} as calculated from the Lineweaver–Burk plot are shown in the inset of Fig. 8. K_m was found to be lower for immobilized NR (1.66 mM) compared to the free enzyme (2.22 mM) but higher compared to the reported results for NR immobilization on pyrrole viologen (0.21 mM) [11], polyviologen (≈ 0.75 mM) [38], methyl viologen (≈ 0.25 mM) [39] and laponite clay gel (0.007 mM) [14]. The V_{max} value of 3.33 μmol (min - mg protein)$^{-1}$ for epoxy/AuNPs bound NR was slightly higher than the V_{max} value of 3.13 μmol (min mg protein)$^{-1}$ for free NR. Changes in V_{max} could be attributed to conformational changes in structure of enzyme after interaction with support and accessibility of the substrate to the active site of the enzyme. The k_{cat} value (94.84 s^{-1}) of free NR was higher than that of epoxy/AuNPs bound NR (56.63 s^{-1}). Catalytic efficiency as judged from k_{cat}/K_m revealed that immobilization decreased the catalytic efficiency of NR, the value of k_{cat}/K_m being 42.72 mM^{-1} s^{-1} for free and 34.11 mM^{-1} s^{-1} for immobilized NR.

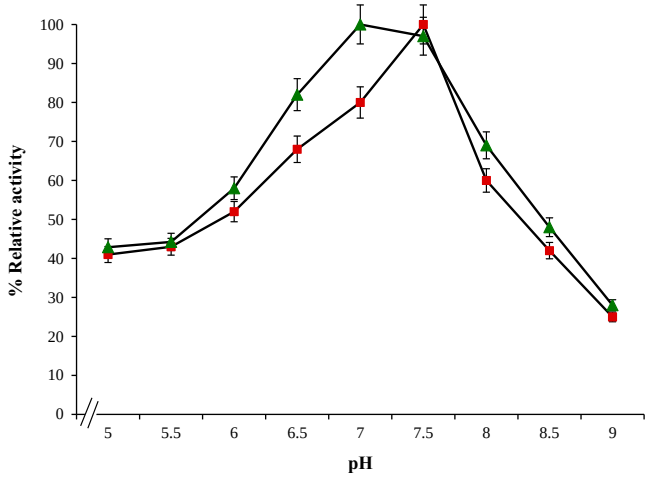


Fig. 6. Effect of pH on the activity of free ■ and epoxy/AuNPs bound NR ▲. Each assay was carried out at final concentrations of 24.0 mM for potassium phosphate, 0.05 mM for EDTA, 9.5 mM for potassium nitrate and 0.10 mM for β-NADH in a 2.0 ml reaction mixture. Temperature was kept constant at 30 °C, while the pH of the reaction buffer was varied from 5.0 to 9.0 as indicated.

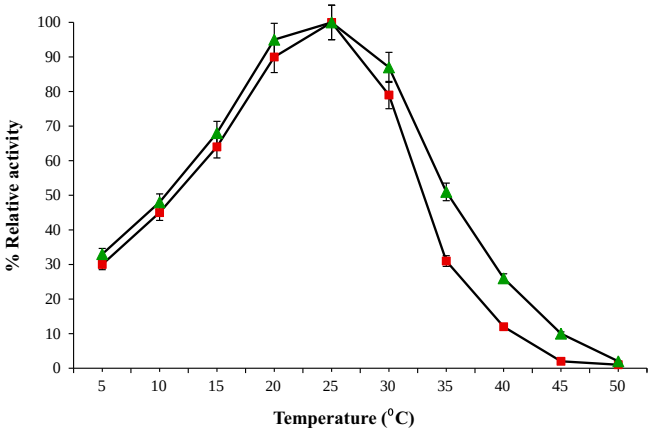


Fig. 7. Effect of temperature on the activity of free ■ and epoxy/AuNPs bound NR ▲. Incubation temperature was varied from 5 to 50 °C and pH of the reaction buffer was 7.5 for free NR and 7.0 for immobilized NR. Other experimental conditions were the same as mentioned in Fig. 6.

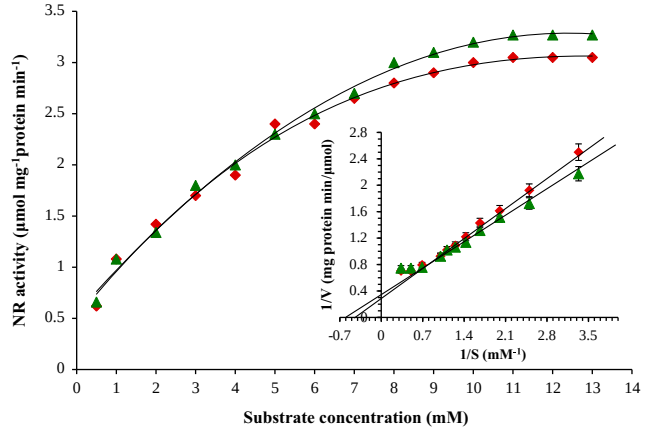


Fig. 8. Michaelis–Menten kinetics and Lineweaver–Burk plot (in the inset) of the free ■ and immobilized NR ▲ catalysis plotted using potassium nitrate as substrate.

3.5. Thermal stability measurements

The residual activity of both the enzyme preparations was determined after 30 min of incubation at temperature ranging from 25 to 50 °C, with an interval of 5 °C. Activity of epoxy/AuNPs/NR conjugates was almost 10% more than the free enzyme between 30 and 50 °C except at 35 °C, where the activity of immobilized enzyme was almost twice the activity of free enzyme. This suggests that AuNPs impart conformational stability to the enzyme and are similar to the observations reported for glucose oxidase/AuNPs bioconjugates [40]. The enhanced thermal stability of immobilized NR may also be attributed to the presence of epoxy layer, which might have increased the heat absorption capacity of the support and hence the thermal stability of the enzyme.

3.6. Storage stability measurements

Storage stability of free NR and epoxy/AuNPs/NR conjugates stored in potassium phosphate buffer (25.0 mM, pH 7.3) at 4 °C was measured by assaying the assay mixture daily for 48 days. Immobilization of NR on epoxy/AuNPs support significantly increased its storage stability as after 24 days free enzyme was completely inactive but the immobilized enzyme retained 30% of its initial activity (Fig. 9). Complete loss of activity for immobilized NR was observed on day 42, which is quite encouraging as compared to the reported results of NR immobilization on other supports, which have been summarized in Table 2. Half-life of free and epoxy/AuNPs/NR conjugates was about 7 and 14 days respectively, clearly suggesting the stabilizing effect of immobilization on the enzyme.

3.7. Determination of water nitrate

Nitrate content of the water samples was determined using epoxy/AuNPs/NR conjugates and analytical comparisons drawn with the reported data on nitrate determination are presented in Table 2. Linearity between nitrate concentration and A_{540} was obtained from 0.1 to 10.0 mM. The minimum detection limit of the present method was 0.05 mM, which is lower compared to the data for NR immobilization on Azure A (0.5 mM) and methyl viologen (0.1 mM) [41] and carbonnanotube/polypyrrole (0.17 mM) [42]. The % recoveries of added nitrates (0.1 and 0.2 mM) in water samples expressed as mean ± S.D. for six samples were 95.23 ± 2.62 and 96.77 ± 1.15 respectively. To check the reproducibility and reliability of the present method, nitrate contents of six water samples were determined in one run (within-day) and after one-week storage at room temperature (between-day). The results showed that nitrate values of these determinations agreed with each other and within-day and between-day coefficients of variations were 1.012% and 3.125%

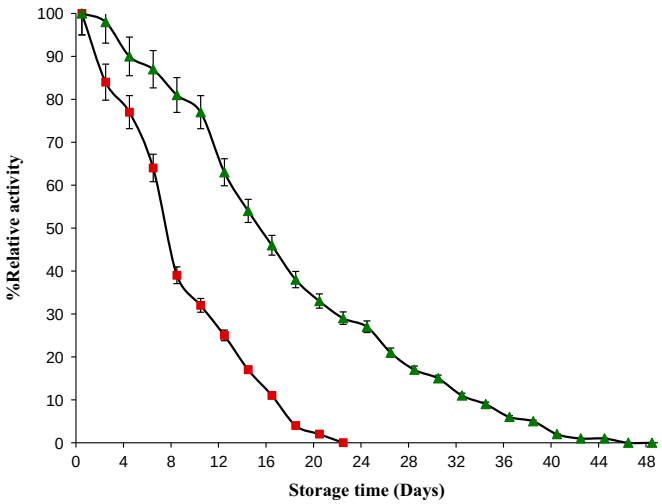


Fig. 9. Storage stability of free ■ and epoxy/AuNPs bound NR ▲ at 4 °C.

Table 2
A comparison of nitrate determination parameters using various immobilized NR preparations.

S. No.	Source of NR	Immobilization matrix	Method of immobilization	Assay pH	Linearity (mM)	Detection Limit (mM)	Storage stability at 4 °C	Reference
1.	*Aspergillus niger*	Epoxy/AuNPs	Covalent binding	7.0	0.1–10.0	0.05	50 % activity retained after 14 days	Present work
2.	*Pseudomonas stutzeri*	Azure A	Adsorption	8.0	NA	4.8×10^{-3}	50 % activity retained after one day	[6]
3.	*Pseudomonas stutzeri*	Methyl viologen	Adsorption	8.0	NA	4.8×10^{-3}	50 % activity retained after two days	[6]
4.	*Escherichia Coli*	Clay-polypyrrole composite	Entrapment	7.5	5.0×10^{-3}–0.163	500.0	20 % activity retained after 5 days	[11]
5.	*Aspergillus niger*	Nafion/methyl viologen	Crosslinking	7.5	0.02–0.25	5.0×10^{-3}	70 % activity retained > 14 days	[12]
6.	Yeast	Polypyrrole films	Entrapment	7.3	0.10–5.0	1.5×10^{-2}	NA	[13]
7.	NA	Laponite clay gel	Entrapment followed by crosslinking	7.5	NA	NA	NA	[14]
8.	*Aspergillus niger*	Polypyrrole/carbon nanotubes	Adsorption followed by crosslinking	7.0	5.0×10^{-6}–10.0	2.0×10^{-4}	89.6 % activity retained after 30 days	[37]
9.	*E. coli*	Azure A	Adsorption followed by crosslinking	6.8	0.5–10.0	0.50	NA	[41]
10.	*E. coli*	Methyl viologen	Adsorption followed by crosslinking	6.8	0.10–10.0	0.10	50 % activity retained after 36 days	[41]
11.	*Aspergillus niger*	Polypyrrole/carbon nanotubes	Covalent attachment	7.5	0.44–1.45	0.17	70 % activity retained after daily usage for 10 days	[42]
12.	*Escherichia Coli*	Viologen polypyrrole films	Entrapment	NA	NA	4.0×10^{-4}	NA	[43]
13.	*Aspergillus niger*	Glassy carbon disk	Adsorption	7.5	2.5×10^{-6}–2.5×10^{-5}	NA	35 % activity retained after three days	[44]
14.	*Aspergillus niger*	Polypyrrole	Entrapment	7.3	0.02–0.50	5.0×10^{-4}	10 hrs with intermittent use	[45]

NA: Not available.

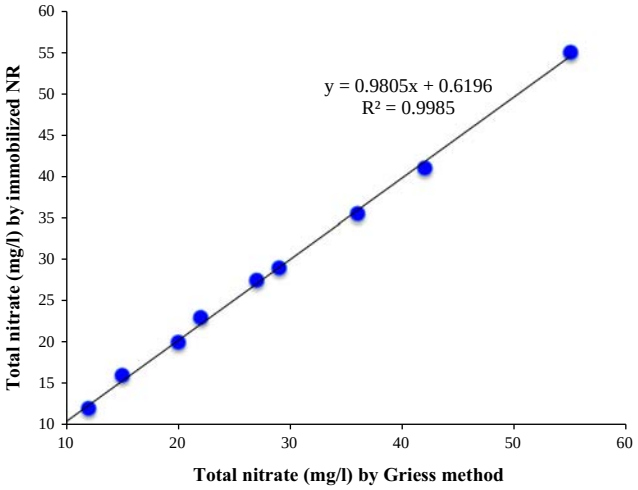


Fig. 10. Correlation between water nitrate values determined by the Griess reaction method and the present method using epoxy/AuNPs immobilized NR.

respectively. To evaluate the accuracy of the present method nitrate concentrations of 10 water samples as determined by the present method were compared with those obtained by the Griess reaction method. Nitrate values in water obtained by the two methods showed good correlation, the value for coefficient of determination (R^2) being 0.998 (Fig. 10). Reusability of immobilized NR was determined at 25 °C and pH 7.3. The immobilized NR retained about 80% and 50% of initial activity after 6 and 16 reuses respectively. At the end of 24th reuse, the activity dropped to 10%.

4. Conclusion

Stabilization of NR via covalent coupling to AuNPs and a simple protocol for easy recovery of AuNP/NR conjugates by using epoxy affixed AuNPs instead of its soluble counterpart has been described. SEM and FTIR results confirmed that epoxy groups did not open in the presence of enzyme and immobilization occurred only on AuNPs. Epoxy polymers provided stability to the matrix and "glue" the AuNPs. Biocompatible nature and high surface area of AuNPs facilitated 88.5% enzyme loading with 93.90 ± 0.85% retention of initial activity, which is quite high considering the complex and multisubunit structure of NR. Optimum pH and K_m values for immobilized NR were lower than that of free enzyme. Epoxy/AuNPs/NR conjugates retained twice the activity of free enzyme when kept at 35 °C for 30 min. Moreover, half-life of NR, when stored at 4 °C, also increased from 7 to 14 days after immobilization. Epoxy/AuNPs/NR conjugates were successfully tested for their ability to determine the water nitrate concentrations. The results for water nitrate determination were consistent, reliable and reproducible. Minimum detection limit (0.05 mM) and linearity (0.1–10.0 mM) were well within the range commonly encountered in real life samples. The main advantage of epoxy/AuNPs support is high immobilization efficiency, increased stability and easy isolation of immobilized NR from the solution. Moreover, nitrate determination using epoxy/AuNPs/NR bioconjugates is a simple alternative to currently used chemical and electrochemical methods. The method does not require a trained person and hence may be adopted for routine measurement of nitrates in various biological and environmental samples. Alternatively, epoxy/AuNPs/NR bioconjugates may be employed for development of nitrate biosensor for rapid online measurements. The protocol may also be adopted for stabilization of other multimeric proteins.

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