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Nitrate Reductase Nanoparticles: Synthesis and Characterization

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ABSTRACT

Nanoparticles of enzyme Nitrate reductase (NaR) a soluble homodimer enzyme of ∼100 kDa polypeptide with cofactors – FAD, heme-molybdopterin (Mo-MPT) and electron donor NAD(P)H, catalyses the reduction of nitrate to nitrite has been synthesised. Nanoparticles of Nitrate reductase enzyme have been prepared by chemical desolvation method including glutaraldehyde cross-linking to form the nanoaggregate. Characterisation of NaR nanoparticles has been made by Transmission Electron Microscopy (TEM), UV-Visible Spectroscopy and by electrochemical Impedance Spectroscopic Study (EIS). TEM study revealed the size of globular aggregated was in the range of 20–30 nm. UV Visible spectroscopic studies depicted that the absorption of NaR NPS is much higher at 560 nm than that of the free enzyme, which showed maximum absorption at 540 nm. NaR NPs aggregates formed were more active, highly stable, have a higher shelf life and can be reused repeatedly. Enzyme nanoparticles with 10-100 nm dimensions and exhibit unique physical, chemical and catalytic properties due to increased surface area. Nitrate reductase nanoparticles can be used as a biochemical tool to increase protein production and grain yield by promoting amino acids production in plants. The synthesised NaR NPs are used in the fabrication of enzyme-based nanosensor in the detection of nitrates in drinking water and serum samples.

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INTRODUCTION

Nitrate reductase (1.6.6.1-3) a soluble homodimer having two identical subunits connected by molybedin cofactor and catalyses the reduction of nitrate (NO^{3-}) to nitrite (NO^{2-}), reflects the activity of nitrogen flux in leaves (Skipper *et al.*, 2001) The enzyme is a molybdoflavo protein (Hewittd and Nicholas, 1964) with an operative sulfhydryl group was first isolated from soybean leaves and Neurospora by (Evans and Nason, 1953). Nitrate reductase (NaR) acts as a central point of metabolism as governing the flux of reduced nitrogen by a regulatory mechanism in plants, algae and fungi. NaR has two active sites joined by internal electron transport pathway from FAD to the Mo-MPT (Shiraishi and Campbell, 1996). Under molybdenum deficient conditions, NaR activity could not be detected. NaR has eight sequence segments: (a) N-terminal "acidic" region;(b) Mo-MPT domain with nitrate-reducing active site; (c) interface domain; (d) Hinge 1 containing phosphorylated serine (e) cytochrome b domain; (f) Hinge 2; (g) FAD domain; and (h) NAD(P)H domain. The electrons are passed from NADH to nitrate (Ratnam *et al.*, 1997) as shown in the given equation (i)

$$NADH \rightarrow FAD \rightarrow Cytochrome\ b5 \rightarrow MoCo \rightarrow Nitrate$$
$$NO_3^- + NADH \rightarrow NO_2^- + NAD^+ + OH^- \quad \ldots\ldots\ldots\ldots (i)$$

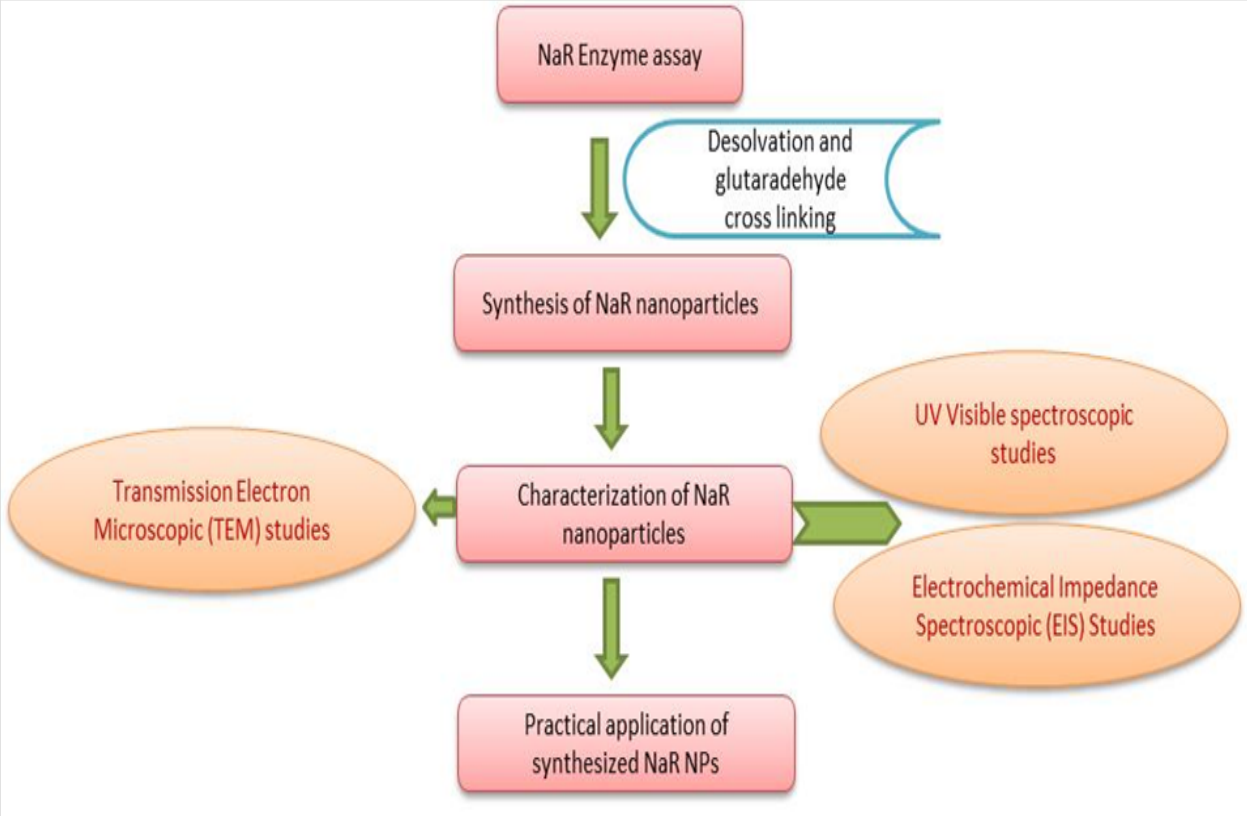
The active site is a Mo ion in +6 oxidation state

bound to four thiolates of two pterin molecules (Lu *et al.*, 1994). Sulfur atom in the coordination sphere protects it from direct attack (Stolz and Basu, 2002). Nitrate binding induces conformational rearrangement in-ring thus reduction of nitrate into nitrite occurs (Su *et al.*, 1996). Nitrate reductase found in cytosol and reduction of nitrate takes place chiefly in green leaves and roots. NaR is labile, and the reduction reaction is affected by oxygen. First nitrate assimilation is initiated by uptake of nitrate from the root system then reduced to nitrite by nitrate reductase and further nitrite is reduced to ammonia by nitrite reductase (Crawford and Arst, 1993). It is a substrate inducible (Beevers and Hageman, 1969) type of enzyme. Its synthesis is induced by NO_3^- in tissues of higher plants. It is synthesised in the cells when its substrate, i.e., NO^{3-} is present and disintegrates when NO^{3-} is absent. Its activity is affected by nitrate conc., ammonium, carbohydrate level, other environmental factors and its reducing equivalents temperature and light (Kaiser and Brendle-Behnisch, 1991). Light stimulates protein phosphatase which dephosphorylates some serine residues and leads to activation of nitrate reductase enzyme.

Under darkness and in the presence of Mg^{++}, a protein kinase (Huber *et al.*, 1992) is stimulated which phosphorylate the same serine residues of the nitrate reductase and causes inactivation of nitrate reductase enzyme. Mainly regulation of nitrate reductase activity occurs through reversible phosphorylation-dephosphorylation process (Mackintosh, 1992) than synthesis and breakdown of this enzyme which takes hours. It is regulated both at transcriptional and translational level (Nussaume *et al.*, 1995) induced by light, nitrate and a negative feedback mechanism. Nitrate reductase is subject to post-translational modulation (Scheible *et al.*, 1997) involving reversible phosphorylation on 543-serine residue (Bachmann *et al.*, 1996) and binding of Mg^{2+} or another divalent cation and an inhibitor protein which have a regulatory effect on nitrate reductase (Kaiser and Huber, 1994). Nitrate reductase improves the plants' tolerance in anoxic conditions (Botrel *et al.*, 1997). Increased activity of nitrate reductase is related to dramatically increased nitrite release in roots showing dephosphorylation of the nitrate reductase in anoxic conditions. In anoxia-tolerant, lower vertebrates have an intrinsic ability to increase intracellular nitrite concentration in tissues such as the heart, red and white skeletal muscles which develop different nitrite levels when exposed to profound hypoxia (Dolomatov *et al.*, 2011). Liver, muscle and heart tissue possess nitrate reductase activity that supplies nitrite to the masses during severe hypoxia. NaR activity decreases during water stress. Nitrate Reductase activation declines in drought leave compared with well-watered controls. The values of NaR activity are considered low in the presence of Mg^{2+} inhibitor (Venkatesan, 2005).

Nanotechnology is a fascinating branch of applied science which is widely used in almost all fields with improved properties of particles at nano scale (Ferrario-Méry *et al.*, 1998). Synthesis of enzyme nanoparticles at larger scale enhances the use of enzymes by removing the only limitation of reusability associated with their use in any reaction. Enzyme nanoparticles are prepared by converting enzyme into nano-aggregates by cross-linking strategy (Unkles, 2001). For this enzyme, the molecule is surrounded by the organic/inorganic network that results in a stable catalyst with no mass transfer limitation (Kim and Grate, 2003). Immobilisation of enzyme nanoparticles resulted in a loss of enzyme activity and denaturation due to change in various physicochemical conditions. The cross-linking method of the enzyme via glutaraldehyde performed reaction with amine residue was initially developed in the 1960s. This method stabilises enzyme by converting it in the form of nanoaggregate and their successful use in biocatalyst industry. There are some drawbacks associated with this cross-linking technology such as low enzyme activity retention, little stability and inferior reproductively (Ruan *et al.*, 1998).

Further, the results could be better obtained by precipitating and cross-linking the enzyme on physical support that helps in the improvement of the technique. Addition of different types of salts, organic solvents, and acid helps in enzyme aggregation and precipitation. These Enzyme nanoparticles are very tiny particles and have different properties than their larger particle counterparts thus possess unique structural and catalytic properties which enhance the rate of reaction. This nanoaggregate are supramolecular structure held by non-covalent interactions with the much higher and increased catalytic activity of then the native enzyme (Haring and Schreier, 1999). Recently, several enzyme nanoparticles have been developed, but till now no nitrate reductase nanoparticles has been reported. Hence in the present study, NaR NPs were synthesised by the glutaraldehyde method and characterised by using Transmission electron microscopy (TEM), UV-visible spectroscopy and Electrochemical Impedance Spectroscopic (EIS).



Graph 1: Gaphical Abstract

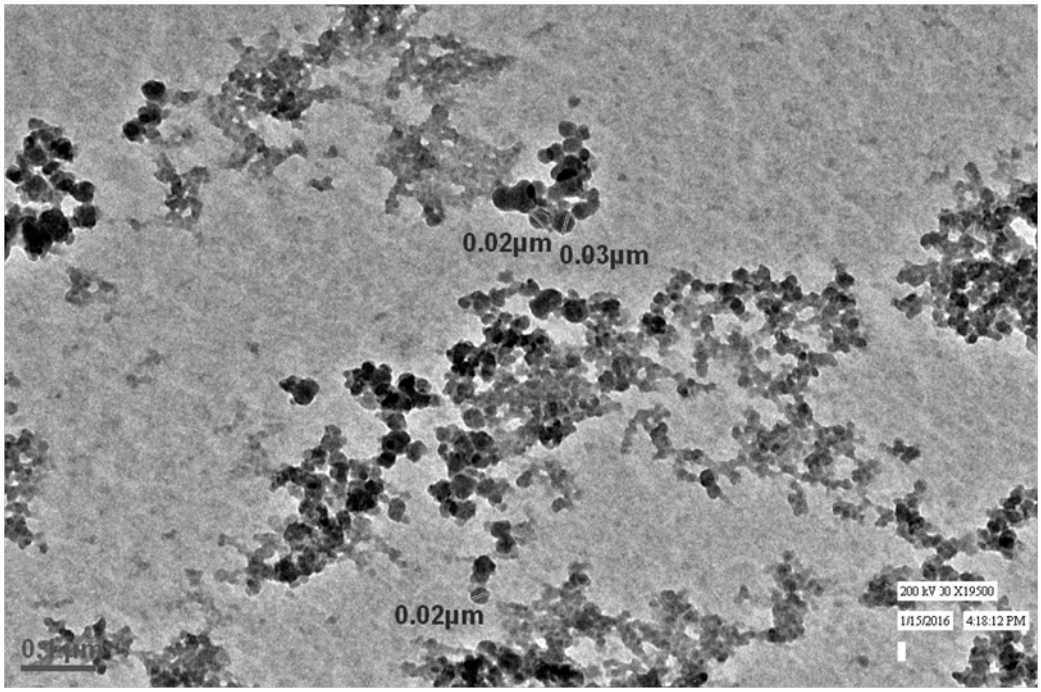


Figure 1: TEM image of prepared NaR Nanoparticles

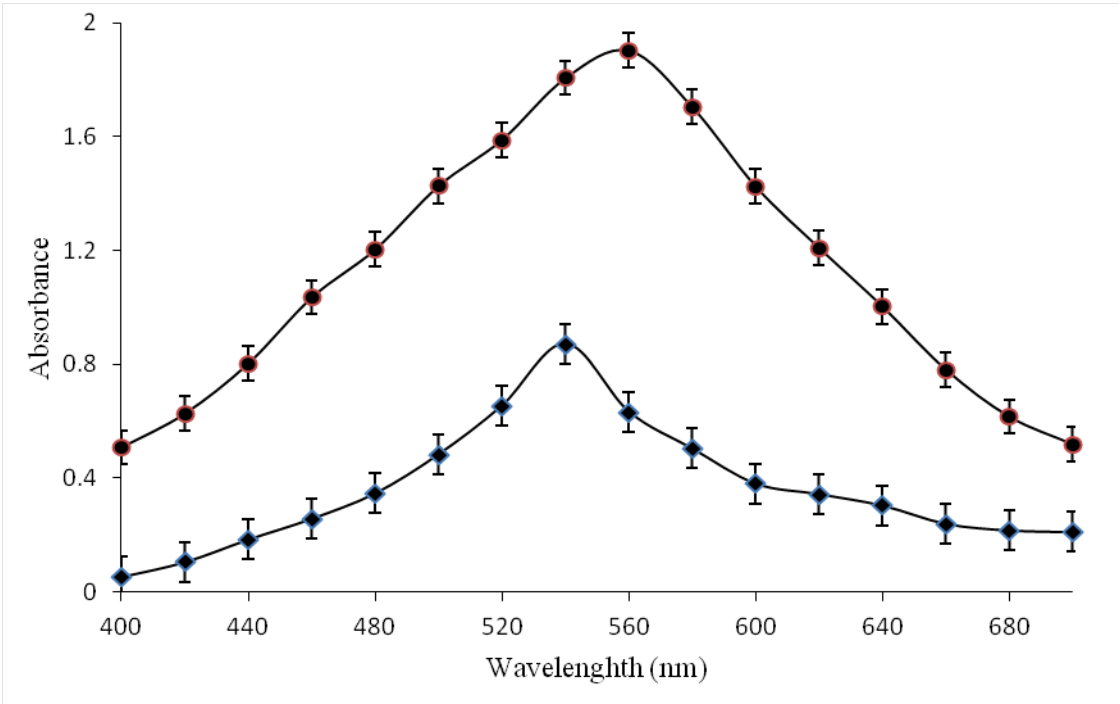


Figure 2: UV visible spectra of free NaR enzyme and NaR Nanoparticles

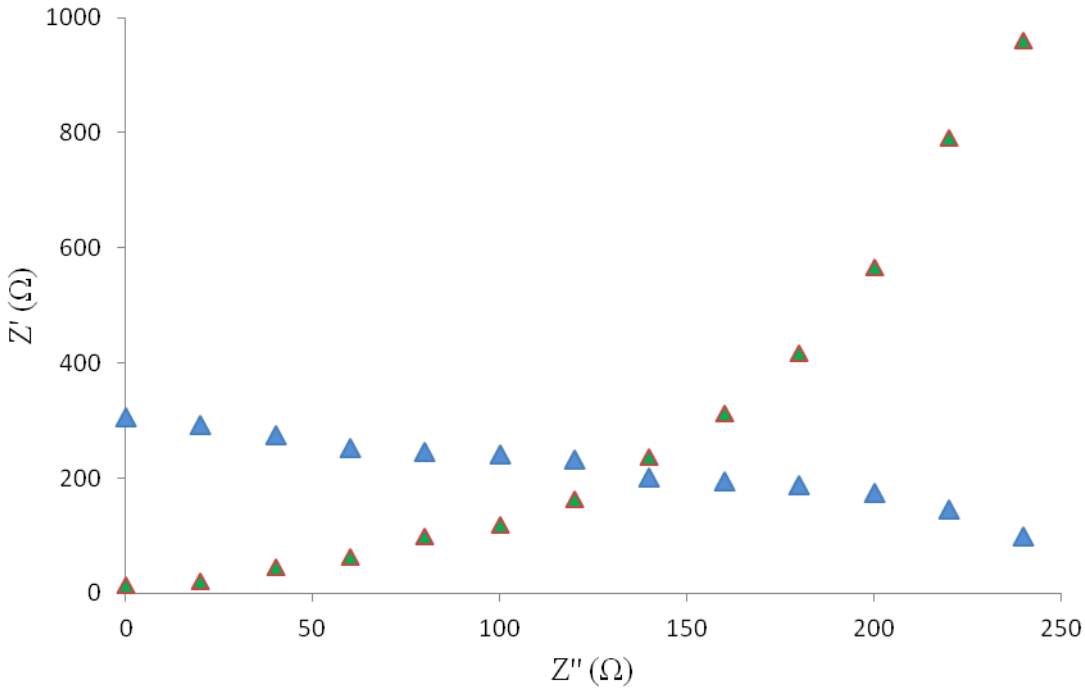


Figure 3: Nyquist plot of Electrochemical Impedence Spectroscopic of free NaR and NaR Nanoparticles coated Au electrode.

MATERIALS AND METHODS

Chemicals

Enzyme Nitrate Reductase from Aspergillus from Sigma–Aldrich, N-ethyl-N-(3-dimethyl amino-propyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), Potassium nitrate, potassium dihydrogen phosphate and dipotassium hydrogen phosphate, N-(1-naphthyl)-ethylenediamine hydrochloride (NED), NADH were purchased from Himedia. All other chemicals were of analytical reagent (AG) grade. Double Distilled water (DW) is used in all experiments. Instruments –Cold centrifuge, stirrer, UV-visible spectroscopy was carried out at Department of M.D. University, Rohtak, Transmission electron microscopy was carried out at AIIMS New Delhi.

Enzyme assay

The enzyme activity was measured as the rate of nitrite production. One unit of NaR is defined as the amount of enzyme required to generate one μ mol of nitrite/min. Assay of NaR was done as described by (Campbell and Smarrelli, 1978) with modification.

Synthesis of NaRNPs Nanoparticles

The reaction mixture contained 1.3 mL of 0.1 PB (pH 7.5), 0.2 mL of 0.2 M KNO_3 and 0.2 mL of 0.4 mM NADH.After incubation at 30° C for 2 minutes, 0.3 ml of the enzyme was added, the tubes were shaken and incubated for 30 minutes. 1 mL of 0.02% N-(1-naphthyl)-ethylenediamine hydrochloride (NED) was added to the test tubes to develop the coloured complex, and A_{540} nm was read against the blank. In blank, the crude enzyme was replaced by the buffer solution. **Synthesis of NaR nanoparticles**

Nitrate reductase NPs were prepared by desolvation method as described by (Kundu *et al.*, 2013). 3 mL of absolute ethanol was added to 1.5 mL of enzyme solution (1mg/mL) at a dropping rate of 0.1 to 0.2 mL/minute under constant stirring at 500 rpm resulting into small particles of nano size. After that, 1.8 mL of 2.5% glutaraldehyde was added to the nanoparticles suspension under continuous stirring at 4ºC for 24h. The amino group were introduced by the addition of 0.12 gm cysteamine dihydrochloride with constant stirring for 5-6 hour, forming enzyme nanoparticles by cross-linking. NaR nanoparticles (NPs) were dispersed in 0.1M phosphate buffer and sonicated stored at 4ºC. The amino group of cysteamine reacts with –CHO group of glutaraldehyde cross-linked to form nanoparticles.

Characterisation of NaR NPs

The aggregated nitrate reductase nanoparticles were studied by Transmission electron microscopy (TEM) at AIIMS New Delhi. Nanoaggregates were immobilised on a gold electrode by dispersing it. UV visible spectroscopy of both free Nitrate Reductase and Nitrate Reductase NPs were measured at different wavelengths in spectronic-20 at Department of Environment Science, Maharishi Dayanand University, Rohtak. EIS study of enzyme immobilised electrode was done at National Physical Laboratory (NPL) New Delhi.

RESULTS AND DISCUSSION

Synthesis and Characterisation of NaR NPs

Transmission Electron Microscopic (TEM) studies

The TEM images and selected area electron diffraction patterns of NaR nanoparticles reveal the spherical particles with an average size of 20 – 30 nm, which is in close agreement with the crystalline structure complexity. It indicates that the synthesised NaR nanoparticles are not a single crystal, instead are the aggregates of several single crystals. The TEM images showed NaR NPs aggregates with an average diameter of about 20-30 nm (Figure 1).

UV Visible spectroscopic studies

UV visible spectra of free nitrate reductase and nitrate reductase nanoparticles were studied separately. Free nitrate reductase showed a characteristic absorption peak at 540 nm, and nitrate reductase nanoparticles showed an absorption peak of 560 nm (Figure 2). The peak is shifted towards higher wavelength, which is indicative of structural conformation of the enzyme after nanoparticles synthesis. Similar reports of shifting in absorption maxima peaks were studied previously by various researchers for the synthesis of other enzyme nanoparticles.

Electrochemical Impedance Spectroscopic (EIS) Studies

EIS study confirms the enzyme nanoparticles formation and their immobilisation on the electrode. At higher frequencies, the diameter of semicircle suggests the charge transfer resistance (RCT). There is the poor electrical conductivity of the enzyme at a lower frequency due to hindrance to electron transfer. The R_ct of enzyme nanoparticles coated electrode was lower due to decreased resistance and higher charge conductivity at higher frequency(Figure 3).

Practical application of synthesised NaR NPs

Enzyme nanoparticles with 10-100 nm dimensions and exhibit unique physical, chemical and catalytic

properties due to increased surface area. Enzyme nanoparticles exhibit good shelf life and stability so could be used again and again without leaching. It is easy to immobilise these aggregates on specific support with a high proportion of active enzyme activity. Their practical utility lies in improving yield and water stress tolerance of plants, to study nitrate transporter and in making improved nanosensor.

CONCLUSIONS

The synthesised NaR NPS enzyme nanoparticles exhibit increased surface area and have better chemical and catalytic action. The thiol group on their surface helps in attachment over the specific surface, thus no denaturation and loss of bioactivity in media. Nitrate reductase nanoparticles can be used as a biochemical tool to increase protein production and grain yield by promoting amino acids production in plants. During nitrogen assimilation, NaR nanoparticles can be used to study the involvement of nitrate transporters in nitrate sensing in plants and fungi. In plants, the importance of regulating nitrate reductase activity by nanoparticles is to limit the amount of nitric oxide being produced during the drought, which has many damaging effects. A rapid and specific method based on nitrate reductase biosensor can be used for the determination of *nitrate* in meat and fishery products. The synthesised NaR NPs are used in the fabrication of enzyme-based nanosensor in the detection of nitrates in drinking water and serum samples. The nanoparticles based improved nanosensor achieves superiority in terms of linear range, sensitivity and response time. Enzyme nanoaggregates provide enhanced shelf life and operational stability to the nanosensor with no leaching action. Thus enzyme nanoaggregate with higher shelf life and stability plays an essential role as an industrial and biomedical catalyst.

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Conflict of Interest

None.

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