A NOVEL D-ALANINE SENSOR USING SURFACE PLASMON RESONANCE PROPERTY OF SILVER NANO PARTICLES FOR MICRO DETECTION IN SIMULATED BODY FLUID

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Abstract: High D-alanine concentration is implicated in Alzheimer's disease and blood plasma of patients with renal disorders. A novel, simple D-alanine sensor was developed using the localized surface plasmon resonance property of silver nanoparticles (SNP). The amount of decrease in surface plasmon resonance band of SNP due to H_2O_2 produced during the D-amino acid oxidase reaction corresponded to the concentration of D-alanine which could be directly monitored using the spectrophotpmetric methods. The sensor was used for the detection of D-alanine in human blood plasma mimicking fluid i.e. simulated body fluid which showed linear detection in the range of 0.1mM to 5mM D-alanine concentration.

Keywords: D-alanine; D-amino acid oxidase; silver nanoparticles; sensor

1. Introduction:

D-amino acids have been recognized as candidates of novel physiologically active substances and/or marker molecules of disease. High D-alanine concentration in gray matter of brain is an indicator of Alzheimer's disease [1]. The ratio of free form of D-alanine to the total amino acid concentration has been observed to be up to 20 % in the blood plasma of patients with renal disorders, whereas the ratio is reported as less than 2 % in healthy subjects [2]. D-alanine, which modulate the N-methyl-D-aspartate (NMDA) mediated receptor neurotransmission are found in the Central Nervous System, such as cerebral cortex and hippocampus and the amounts of these amino acids are closely related to diseases with NMDA dysfunctions, such as schizophrenia [3] and depression [4].

Various detection techniques have been in use to determine D-amino acids. These include gas chromatography, HPLC, High-performance capillary electrophoresis and enzymatic methods utilizing D-amino acid oxidase (D-aao) [5]. In recent years, many research groups have reported development of biosensors that specifically detect D-amino acids based on immobilization of D-aao in various supports like 'amberzyme oxirane' support [6], carboxylated mutliwalled carbon nanotube-copper nanoparticles-polyalinine hybrid film electrodeposited on gold electrode [7] and poly (indole-5-carboxylic acid)-zinc sulfide nanoparticles hybrid film [8]. Although these methods of detection are quite reliable and reproducible, they are expensive and time consuming.

Silver nanoparticles that can be easily detected by their characteristic absorption maximum in the visible range, have a wide array of applications such as photosensitive components; catalysts and in chemical analysis [9, 10, 11]. A unique property of nano structured silver is its ability to exhibit localized surface plasmon resonance (LSPR). Briefly, as the size of the silver metal decreases from the bulk-scale to the nano-scale, the movement of electrons through the internal metal framework is restricted. As a result, silver nanoparticles display specific extinction bands in the UV–Vis spectra when the incident light resonates with the conduction band electrons on their surfaces. These charge density oscillations are defined as LSPR [12].

In the present study, a novel, simplified, low cost D-alanine sensor is proposed based on the LSPR property of silver nanoparticles for photometric detection of D-alanine. The reaction between D-aao and its substrate, D-alanine results in the production of H_2O_2 which causes degradation of silver nanoparticles thereby causing a

significant change in the LSPR absorbance strength. The D-alanine sensor was used for the detection of D-alanine in human blood plasma mimicking fluid i.e. simulated body fluid with a very low detection limit of 1mM with a perspective for its use in clinical diagnosis for detection of various diseases associated with elevated levels of D-alanine.

2. Materials and Methods:

2.1. Chemicals and Reagents:

Silver nitrate and sodium borohydrate of analytical reagent grade was purchased from Merck, India. Pig kidney D-amino acid oxidase (1.4.3.3) was purchased from Sigma-Aldrich, India (Product code A5222). All other laboratory chemicals were of the best grade available and used without further purification.

2.2. Synthesis and characterization of silver nanoparticles:

To a 30mL solution of $NaBH_4$ (0.002 M), kept in a magnetic strirrer (Spinot Magnetic Stirrer, Tarsons, India) in ice cold condition, 10 mL of $AgNO_3$ (0.001 M) solution was added dropwise. Yellow coloured colloidal silver nanoparticles (SNP) were formed. UV-Vis spectroscopic analysis of the SNP was done in the range from 300 to 600 nm using 'UV-10' spectrophotometer (Thermo Scientific, USA).

2.3. Preparation of simulated body fluid:

An acellular simulated body fluid (SBF) that has inorganic ion concentrations similar to that of human extracellular fluid and human blood plasma was prepared by the method of Kokubo et al. [13]. The following ion concentrations in SBF mimic that of human blood plasma: Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, HCO_3^-, HPO_4^{2-}, SO_4^{2-}. The pH of SBF was adjusted to 7.25 with 1N HCl.

2.4. Enzyme assay of pig kidney D -amino acid oxidase:

D-amino acid oxidase (D-aao) activity (o-phenelenediamine method) was determined spectrophotometrically in a 96 well plate by measuring the absorbance increase accompanying the oxidation of o-phenylenediamine [14]. Two concentrations of D-alanine i.e.1mM and 1µM were tested. A 50 µL assay mixture contained 1 mM or 1µM D-alanine, 0.03% (w/v) o-phenylenediamine, 5 U/ml horseradish peroxidase (HiMedia, India) and 0.2 units of D-aao in 0.05M sodium phosphate buffer, pH 7.25 or SBF, pH 7.25. The absorbance increase was monitored at 453 nm for 1 min. and the H_2O_2 produced was determined based on the H_2O_2 calibration curve. One unit of enzyme activity was defined as the production of 1mmole of H_2O_2 per minute at 25° C.

2.5. H_2O_2 calibration curve:

Standard curve of H_2O_2 was prepared by taking different concentrations of H_2O_2 ranging from 0.00055 µmoles to 0.04895 µmoles and thereafter reading the absorbance at 453 nm.

2.6. SNP reaction with SBF, stabilization with Tween 20:

SNP 250 mL was reacted with 50 mL of simulated body fluid and thereafter the UV visible spectroscopy in the range of 300 to 500 nm was analyzed. The disintegration of SNP was recovered by addition of Tween 20 (25%), 1mL and the wavelength scan was done in a UV vis spectrophotometer in the range from 300-500nm.

2.7. SNP reaction with H_2O_2:

The reaction of SNP with H_2O_2 was analyzed by adding 5mL H_2O_2 (0.0055mmoles) to a 300mL solution of SNP. The UV vis spectroscopy was analyzed in the range from 300-500nm.

2.8. Sensing experiments:

The D-aao reaction was carried out in 96 well plates with two concentrations of the substrate, 1mM and 1mM. Briefly, to a 250 mL solution of SNP, 1mM or 1mM D-alanine was added. 0.2 Units of D-aao was added for the reaction to occur. The final reaction volume was 300mL which was adjusted by adding 0.05 M sodium

phosphate pH 7.25 or simulated body fluid (SBF), pH 7.25. When the reaction was done in SBF, 1mL Tween 20 (25%) was added for stabilization of SNP from disintegration. The decrease in the surface plasmon resonance band of SNP was monitored in a UV vis spectrophotometer from 300-500 nm.

2.9. Effect of different D-alanine concentration:

The linearity of the sensing experiments was established by performing the D-aao reaction in SNP and simulated body fluid with different concentration of D-alanine i.e. 0.1mM, 1mM, 0.1mM, 1mM, 5 mM. The change in absorbance between the blank (without the D-aao) and test was recorded.

3. Results and Discussion:

3.1. Synthesis of Silver nanoparticles and its reaction with H_2O_2:

As shown in Fig. 1 colloidal silver nanoparticles was formed with sharp peak at 387 nm and yellow appearance [15]. The yellow coloration formed was due to the plasmon absorption band in the range of 380–430 nm [16]. Upon the reaction of SNP with 0.0055 mmoles H_2O_2, the absorbance value decreases due to slight disintegration of the SNP as shown in Fig. 1. The generated H_2O_2 was able to oxidize SNPs to free Ag^+ ions which causes a decrease in the surface plasmon resonance of SNP [17].

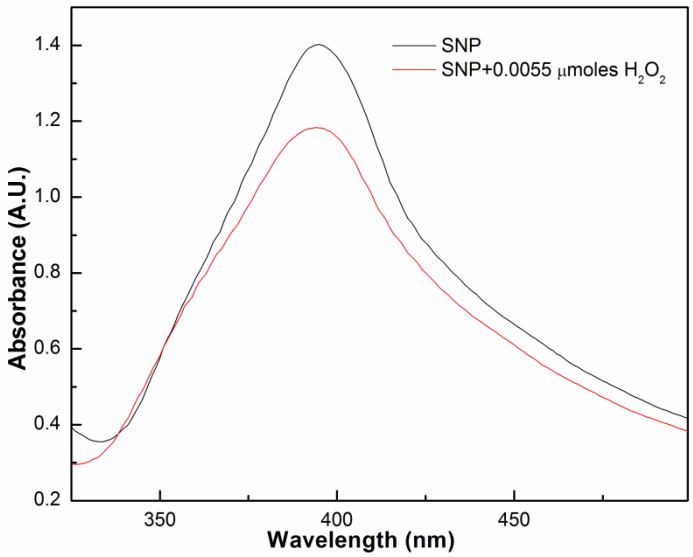

Figure 1: Synthesis of silver nanoparticles (SNP) and the effect of H2O2 on SNP. A decrease in the surface plasmon resonance band of SNP is seen.

3.2. D-aao activity assay:

The D-aao reaction in 0.05 M sodium phosphate buffer, pH 7.25 with 0.2 units of pig kidney D-aao yielded 0.0157 mmoles and 0.00934 mmoles H_2O_2 with 1mM and 1mM D-alanine respectively while the amount of H_2O_2 obtained on D-aao reaction in Simulated body fluid, pH 7.25 with 1mM and 1mM D-alanine was 0.00722 and 0.00326 mmoles respectively as calculated from the calibration curve of H_2O_2. According to the calibration curve of H_2O_2 1 OD equals to 0.0244 µmoles H_2O_2.

3.3. Stabilization of SNP aggregation with Tween 20 upon reaction with SBF:

As shown in Fig. 2 aggregation of SNP results upon reaction with SBF. Various ions in SBF namely, $Ca^{2+}$and Cl^- are known to cause aggregation of SNP [18]. This is clearly shown by the loss of the plasmon resonance peak of SNP. This aggregation of the SNP colloidal suspension can be protected by addition of Tween 20 (25%) that protects the SNP from the various ions present in SBF. Thus tween 20 stabilizes the SNP and causes a shift in the peak from 377nm to 399nm as compared to SNP alone. Marzan and Tourino, 1996 showed that optical properties of the colloidal SNP are influenced by the surfactant molecules like Tween 80 and help in stabilizing them [19].

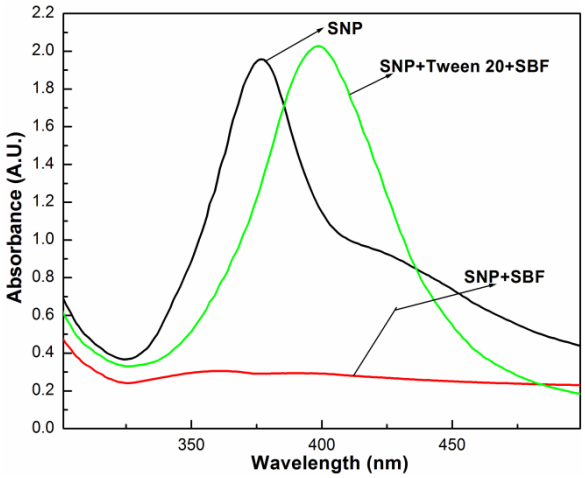

Figure 2: Reaction of SNP with simulated body fluid (SBF) shows the loss of its surface plasmon resonance band. Upon the addition of Tween 20 (25%), complete protection of the SNP can be seen.

3.4. Sensing of D-alanine by D-aao activity in buffer and SBF:

D-aao activity with D-alanine as substrate at concentrations of 1mM and 1mM was performed in SNP and 0.05M sodium phosphate buffer for upto 3 minutes. As shown in Fig. 3a and 3b, the LSPR absorbance strength of SNP peak was affected due to H_2O_2 produced during the Daao reaction with its substrate D-alanine. H_2O_2 was able to oxidize SNPs to free Ag^+ ions which causes a decrease in the surface plasmon resonance of SNP [17]. Ammonia and pyruvic acid are the other two products of D-aao reaction along with H_2O_2. Ammonia produced during the reaction has a moderating effect and stabilizes the SNP [20].

This analysis was done in simulated body fluid with an aim to validate the use of the sensor for detection of D-alanie in human blood plasma mimicking fluid i.e. SBF. As different ions present in SBF causes disintegration of SNP, Tween 20 (25%) was added for stabiliziation of the SNP [19]. The linearity of the sensing was tested by performing the reaction with different D-alanine concentrations. A shown in Fig 3 c d, changes in the plasmon resonance peak of SNP could be seen as compared to respective controls. The amount of decrease in surface plasmon resonance band of SNP corresponded to the concentration of D-alanine which could be directly monitored using the spectrophotpmetric methods. Fig 4 shows the difference in absorbance at 399nm of blanks as against the test at different concentrations. The decrease in surface plasmon resonance band of SNP increased linearly from 0.1mM to 1mM and then gradually decreased from 0.1mM to 5mM.

This shows that the D-alanine sensor can be used for detection of D-alanine in biological samples like blood plasma of patients.

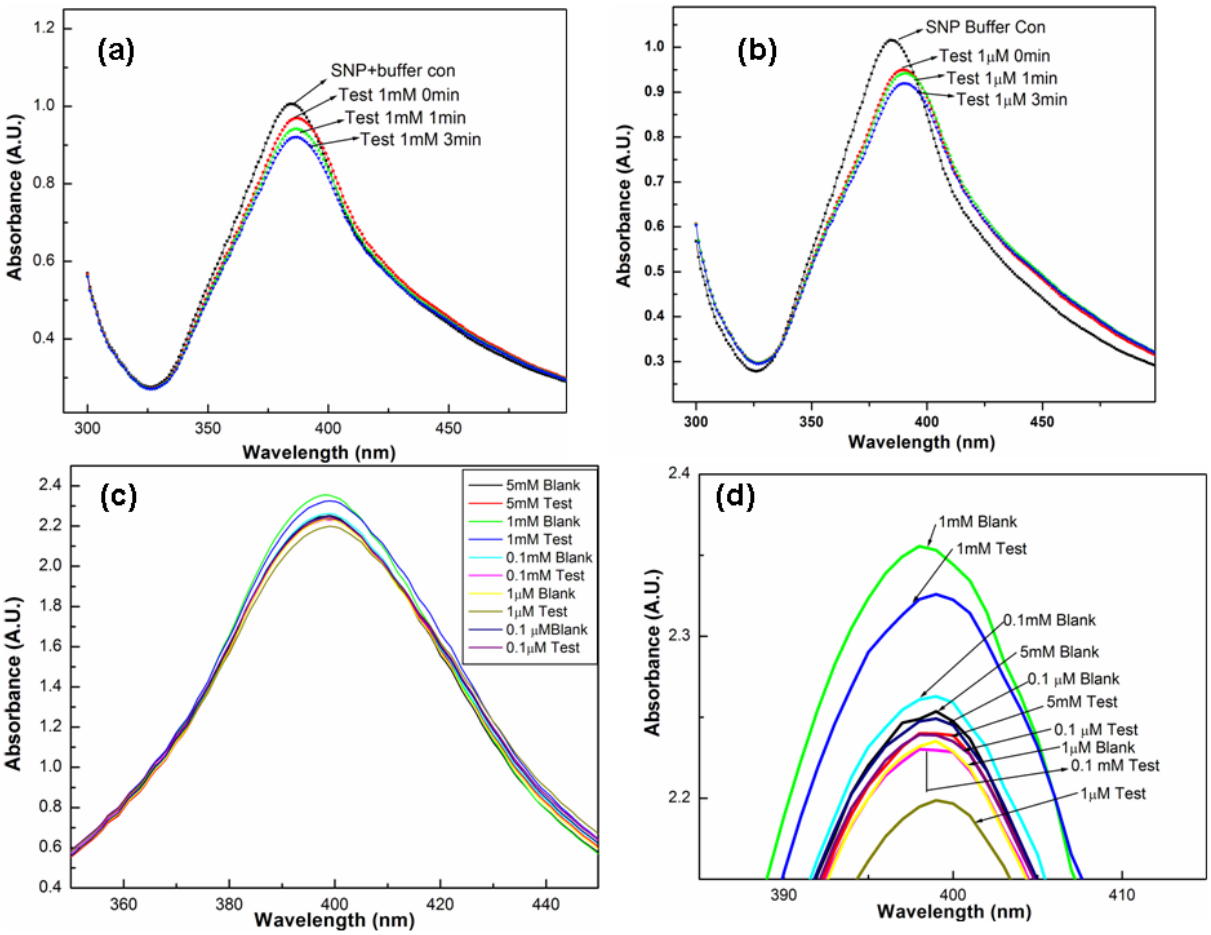

Figure 3: (a) D-amino acid oxidase (D-aao) activity in SNP with [1mM] D-alanine in 0.05M sodium phosphate, pH 7.25 for 3 minutes. (b) D-aao activity in SNP with [1mM] D-alanine. The LSPR absorbance strength of SNP peak was affected due to H2O2 produced during the reaction. (c) D-aao activity in SNP with different concentrations of D-alanine in SBF. (d) The amount of decrease in surface plasmon resonance band of SNP as compared to respective controls.

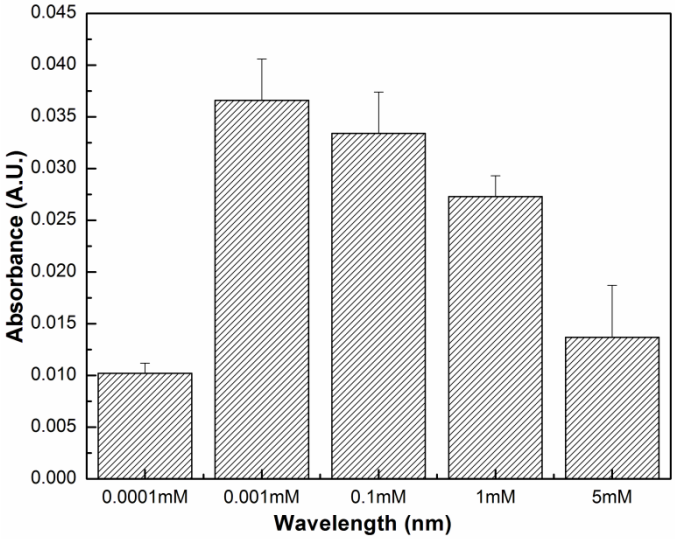

Figure 4: The difference in absorbance at 399nm of blanks as against the test at different concentrations of D-alanine. The decrease in surface plasmon resonance band of SNP increased linearly from 0.1mM to 1mM and then gradually decreased from 0.1mM to 5mM.

4. Conclusion:

High D-alanine concentration is implicated in certain diseases for which their detection is important. Among the many biosensors that have been developed for their detection, our approach is simplified and easy to use. A novel D-alanine sensor using the surface plasmon resonance property of silver nanoparticles was established in simulated body fluid with a liner range of detection from 0.1mM to 5mM and the detection limit was 1mM. This sensor can be used for detection of D-alanine in human blood plasma. The sensor can be validated for detection of other D-amino acids.

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