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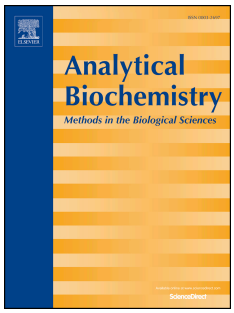

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Enzyme Nanoparticles and their Biosensing Applications: A Review

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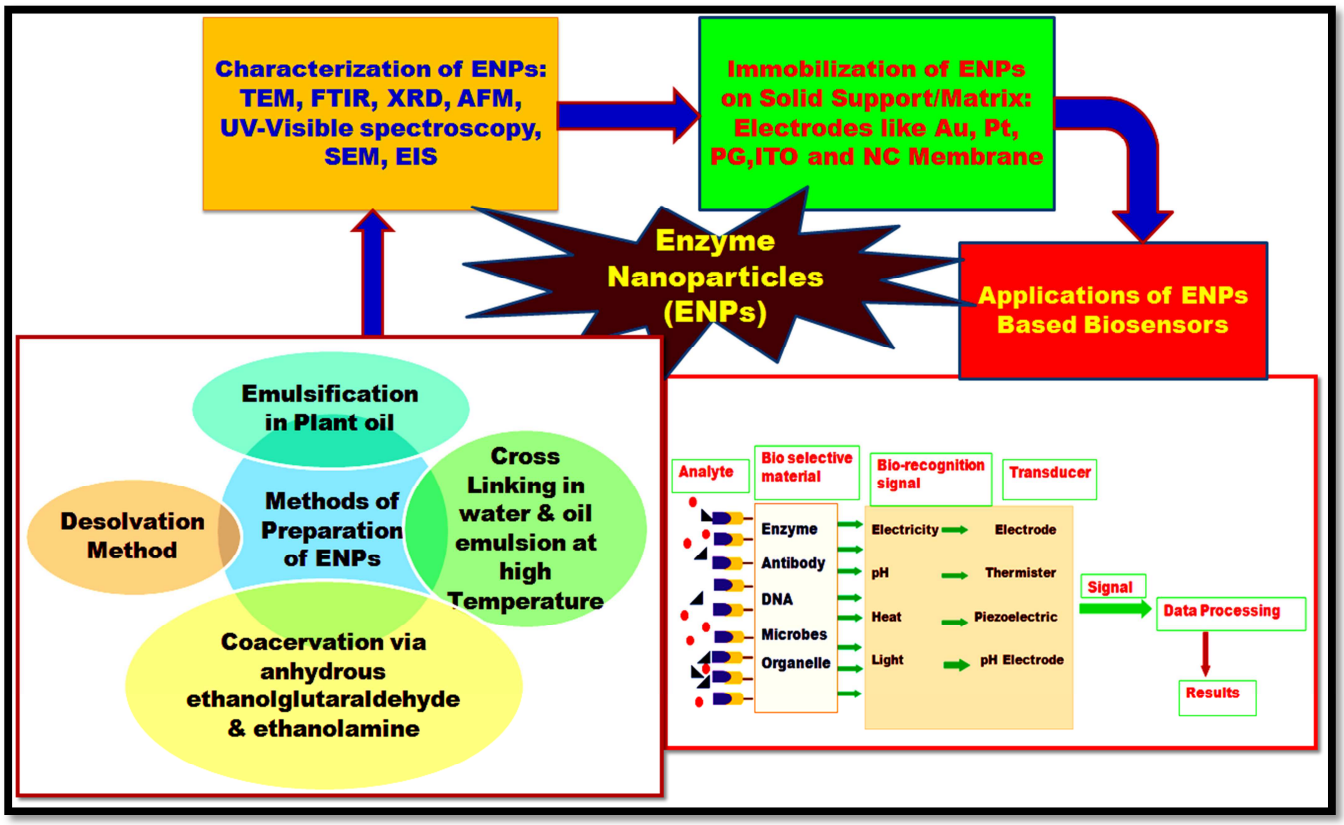

Enzyme Nanoparticles and their Biosensing Applications: A Review

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Abstract

Enzyme nanoparticles (ENPs) are the aggregates of enzymes in the nano scale (10-100nm). These ENPs have been characterized by transmission electron microscopy (TEM), fourier transformation infrared spectroscopy (FTIR), UV-Visible spectroscopy, X-ray diffraction (XRD), atomic force microscopy (AFM), scanning electron microscopy (SEM) and electrochemical impedance spectroscopy (EIS). Enzymes are the biocatalysts exhibiting substrate specificity, catalytic activity, regulator of various metabolic reactions under mild conditions and hence, used as industrial catalysts efficiently. Due to these extraordinary properties of enzymes, they have been used for the diagnosis and treatment of various diseases. However, direct application to enzymes has increased the chances of degradation and thus lowers the performance of analytical devices. Therefore these limitations have been overcome by synthesizing the nanoparticles (NPs) of enzyme. As ENPs have exhibited extraordinary

properties such as large surface to volume ratio, unique optical, thermal and chemical than native enzymes. ENPs based biosensor work optimally within 2-300s, pH range 5.0-6.0 and temperature 3.5-45°C. The linear range of ENPs based biosensor varies between 0.0001-100000 μM and detection limit 0.0002-550 μM. These biosensors have been used for the diagnosis of various diseases such as cardiovascular diseases, renal disorders, diabetes, environment monitoring, and biochemical engineering and reused upto 8-200 times over a period of 60-240 days while stored dry at 4°C. Therefore, the future research should be focused to understand the interaction of ENPs with their analyte and further improvement and commercialization of ENPs based biosensor are discussed. Present review article deciphers the various methods of ENPs preparation, their characterization and ENPs based biosensors.

Keywords: Enzyme Nanoparticles; Immobilization of ENPs; Characterization of ENPs

Abbreviation: Enzyme nanoparticles (ENPs), Transmission electron microscopy (TEM), Fourier transformation infrared spectroscopy (FTIR), UV-Visible spectroscopy, X-ray diffraction (XRD), Atomic force microscopy (AFM), Scanning electron microscopy (SEM), Electrochemical impedance spectroscopy (EIS), Nanoparticles (NPs), Activation energy (Ea), Horse radish peroxidase (HRP), Glucose oxidase (GOD), Cholesterol oxidase (ChOx), Single enzyme nanoparticles (SENs), Cholesterol esterase (ChE), Creatinine (CA), Creatine (CI), Sarcosine oxidase (SOx), Michaelis-Menten constant (K_m), Velocity (v), Bovine serum albumin (BSA), Glycerol kinase (GK), Width at half maximum (FWHM), Observed broadening ($B_{o)}$, Instrumental broadening (B_i), Distilled water (DW), Hydrogen sulphuric acid (H_2SO_4), Nitrocellulose membrane (NC), Solution resistance (Rs), Charge transfer resistance (Rct), Direct current (dc), Glycerol-3-phosphate oxidase (GPO),Glassy carbon electrode (GCE), Pencil graphite electrode (PGE),Lactate dehydrogenase (LDH), Hydrogen peroxide (H_2O_2), Pyruvate oxidase (POx), Graphene oxide (GrO), Cycic voltammetry (CV), Ion-selective field effect transistor (ISFET), Field effect transistor (FET), Ammonium ion selective electrode (AISE), Limit of detection (LOD)

1. Introduction

Enzymes are green and primary biocatalysts which participate in regulation of diverse physiological processes in standard conditions [1] [2]. In nature a large number of enzymes are found which we use in day to day life. These enzymes may be in either free or in immobilized form. Enzymes have been used in diverse applications such as in various metabolic reactions (of plant, animals, microbes), research for e.g. DNA polymerase, enzymes of recombinant DNA technology, food industries, pharmaceutical industries and textile industries [3]. These are globular proteins excluding ribozymes which faster the rate of reaction about 10^8 times without using themselves via lowering the activation energy (Ea) [4]. Almost each and every physiological reaction requires enzymes in minute amount. Immobilized enzymes have offered significant advantages such as the improved stability, better performance for separation and reusable and also provide defense from protease digestion. Though, monomeric enzymes can be used only once, hence more expensive and less stable. These limitations can be overcome by immobilizing enzyme onto solid matrix [5]. In addition to this immobilization of native enzymes onto supporting materials via several covalent or non-covalent bonds has restricted the conformational movement or partial denaturation [6] [7]. Enzyme nanoparticles expose functional groups for example $–SH_2$, $-NH_2$,-COOH, -OH etc. in the side chain of amino acids like cysteine, aspargine, aspartic acid, serine and so on. The type of linkage formed by functional groups of ENPs with different functional groups such as imidazole, indolyl, phenolic hydroxyl, etc. present on the matrix surface depends on extent of interactions. Beside, covalent interactions several different types of non covalent interactions like electrostatic, H-bond, van der waal and hydrophobic interactions such as adsorption have also been used for the binding of ENPs to their support material. These covalent and non covalent interactions formed during immobilization of

ENPs provide binding stability with their matrix consequently better electrochemical reactions at the matrix surface.

Stability and sensitivity of enzymes depends upon the type of solid support used and process of immobilization. Support material can be organic and inorganic however, these support materials increases the risk of leaching from the immobilized enzyme, expensive, sometime susceptible to microbes attack thereby reducing the matrix stability. Furthermore, due to steric hindrance which prevents the formation of enzyme and substrate conjugate as a result insists for new support matrix [8]. Thus, it necessitates designing of such efficient materials and facile strategies which can improve the performance of enzymes. Enzymes can be employed for modification, degradation, preparation of nanoparticles and their immobilization for the construction of biosensor [9].

Enzyme nanoparticles (ENPs) are the aggregated forms of enzymic molecules in an appropriate configuration in nano scale ranges between 10-100 nm [4]. These ENPs have exhibited various characteristics properties including optical, electronic, electrical, thermal, chemical, mechanical, catalytic and large surface area. These excellent features of ENPs have enhanced the functional activities of enzyme based devices such as biosensors. Direct confining of monomeric proteins or enzymes onto nanoparticles causes denaturation and hence enzymes loss their activity. These issues can be overcome by preparing ENPs and confined onto a matrix after their cross linking. Biosensor employing ENPs have exhibited better analytical performance such as limit of detection, working range, reproducibility as well as repeatability [10]. Liu and co-workers have firstly prepared the nanoparticles of horse radish peroxidase (HRP) [11]. Afterwards various other ENPs based amperometric biosensors were developed glucose oxidase (GOD) [10], cholesterol oxidase (ChOx) [12] and uricase [13].

Unlike ENPs, single enzyme nanoparticles (SENs) are comprised of monomeric enzyme coated with organic or inorganic macromolecules of nano range. Sensors employing SENs have increased the electrochemical activity and stability at high temperature. For example: SENs of trypsin have been used for thermal consistency while SENs of chymotrypsin were used for increasing the cellulose degradation [14-16].

Significance of ENPs

Electrochemical biosensors based on enzymes nanoparticles have exhibited novel fascinating features including exceptional selectivity, analytical signaling, and sensitivity, high surface area, better electrical properties, unique optical properties, and nanoscale structures. These ENPs based biosensors have shown high sensitivity and detection of specific compounds timely, whereas conventional detection techniques including chromatography are not easy to operate and time consuming. Various types of enzyme nanoparticles have been used for the construction of biosensors for the detection of specific analyte. Present article comprehensively describe the distinct ENPs based biosensors, such as glucose oxidase (GOx) NPs, horseradish peroxidase (HRP) NPs, uricase NPs, cholesterol esterase (ChE) NPs, cholesterol oxidase (ChOx) NPs, urease NPs, creatinine (CA)/creatine (CI) and sarcosine oxidase (SOx) NPs.

Like enzymes, the function of ENPs depends upon its characteristics kinetic parameters like ideal pH, temperature, incubation time, Michaelis-Menten constant (K_m). The binding site of ENPs is known as active site [17]. Generally, ENPs show hyperbolic relationship between their activity and substrate and follow Michaelis-Menten kinetics during the reaction viz.

$$\mathbf{v=V_{max}[S]/K_m+[S]}$$

where v, is initial velocity of reaction and V_{max} is the maximum velocity. Performance, of an enzyme can be raised or reduced with the help of some compounds called as activators/enhancer or inhibitors. ENPs in conjugation with activators get accelerated the rate of reaction while in combination with inhibitors, decreased the rate of reaction. Inhibitory action of ENPs can be mediated by either reversibly or irreversibly. Reversible inhibition of an enzyme can be attained by :

(i) Competitive inhibition

In this type of inhibition both substrates as well as ENPs compete for the same active site of the ENPs.

(ii) Non-competitive inhibition

In this inhibition, inhibitor first binds with the ENPs to form ENPs-inhibitor (substrate) complex either on the active site or other than active site of ENPs.

(iii) Uncompetitive inhibition

This type of inhibition can be achieved by binding of an inhibitor to ENPs-substrate complex only.

Like enzymes, ENPs require non-proteinous compounds other than proteins for their improved activities they are known as co-factors. They can be organic or inorganic or both. Organic co-factors are also called as co-enzymes from which vitamins are derived. ENPs convert a substrate into its product by involving suitable proximity, configuration, distinct forms of catalysis viz. acid-base, covalent, electrostatic and metal catalysis and finally they released [4].

ENPs exhibit specific activity which is ratio between activity and protein (mg). The activity of ENPs can be regulated by restricted proteolysis, phosphorylation, dephosphorylation,

adenylation, ratio of end product ADP+AMP and ATP, induction and repression. Therefore present study discusses the various methods of ENPs preparation which are as:

2. Methods for ENPs preparation

NPs of soluble proteins/enzymes can be synthesized by their aggregation into NPs which has been achieved by following methods (Figure 1):

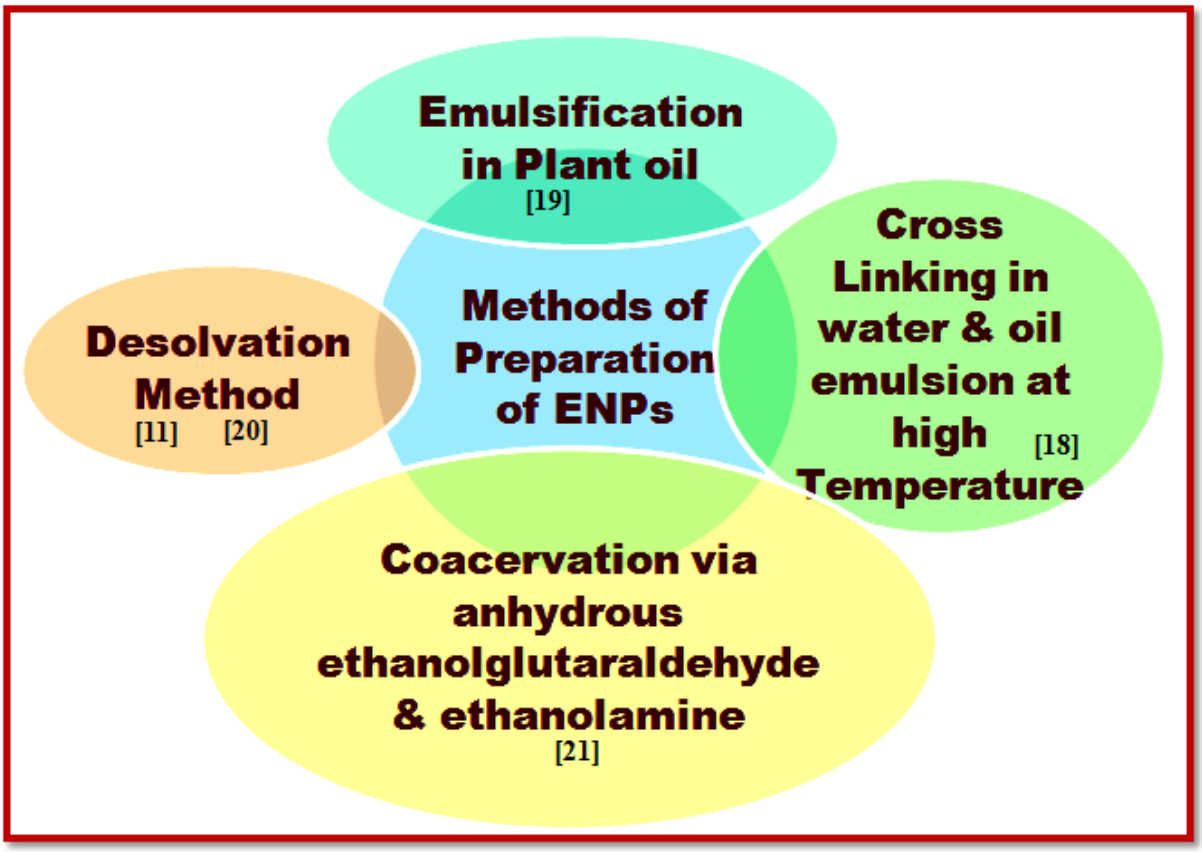


Figure 1: Diagrammatic representation of various methods for ENPs preparation

Desolvation method for ENPs preparation includes four steps viz. desolvation, cross-linking, functionalization and purification [11] [22] [23]. Emulsification method is used for the preparation of NPs of enzyme like albumin [19]. Using emulsification method, plant oil in the

aqueous solution of albumin is converted into an emulsion. This so formed emulsion is mixed into preheated oil at >120°C; thus, fast evaporation of existing water and followed by their irrevocable aggregation between albumin molecules [19]. Simple coacervation method method is used for synthesis of NPs of bovine serum abumin (BSA). In this method, NPs of BSA preparation requires addition of anhydrous ethyl alcohol into 150 ml BSA (5mg/L), 10mM Tris/HCl having 0.02% sodium azide at pH 7.5 till the solution become opaque followed by addition of 150µl of glutaraldehyde (25%) that assists the cross-linking between NPs of BSA. [21]. Cross-linking in w/o (water-in-oil) emulsion is used for emulsification of BSA or human serum albumin (HAS) or hydrated solution of protein by homogenizing at elevated temperature in oil. This emulsion is transferred into preheated oil and maintained at > 100°C under stirring to evaporate and denature and aggregate the protein molecules in aqueous environment [18].

3. Characterization of ENPs

ENPs differ in their characteristics features which are characterized by various techniques which are described below:

3.1 Transmission electron microscopy (TEM)

The TEM images are used to study shape and size of ENPs. ENPs have exhibited characteristics spherical shape and their diameter ranges from 100-200 nm whereas the diameter of native enzyme molecules varies between 2-18 nm (Table 1).

Table 1: Assessment of mean diameter of ENPs and monomeric enzyme using TEM

ENPs	Diameter of ENPs (nm)	Diameter of free enzyme	Reference
Horseradish peroxidase	100	14.2	[11]
Cholesterol oxidase	100-200	-	[12]
Uricase	100	14-18	[13]
GK	144-329	-	[24]
GPO	22-68	-	[24]
Lipase	83-218	-	[24]
Creatininase/Creatinase/Sarcosine oxidase	2-100	-	[25]
ChE	35-40	-	[26]
ChOx	0.06		[26]
Glucose oxidase	117	2-8	[27]
Urease	20-100	13	[28]

3.2 Colorimetric method

ENPs aggregates exhibit characteristics colour for example; ENPs of HRP are white in colour. The ENPs aggregates acquire the colour as that of its free or ionized state.

3.3 FTIR spectra

Infrared spectroscopy provides the prospection to analyze the diverse interatomic bond vibrations at varying frequencies. The analysis of IR absorption spectra exhibits what types of bonds exist in the given sample. Table 2 shows the vibration stretching of different functional groups of ENPs.

Table 2: Illustration of position of different functional groups of ENPs and their corresponding peaks

Types of ENPs	Stretching vibration of different functional groups	Appearance of Peak	Reference
GODNPs	C=N	1673.20 cm^{-1}	[27]
	C=O	1700-1600 cm^{-1}	
	N-H and C-N	1600-1500 cm^{-1}	
Lipase/GK/GPO NPs	OH and -NH	1630 cm^{-1}	[24]
	-C-O	1080^{-1}	

3.4 X-ray diffraction (XRD)

XRD is an efficient characterization technique which displays the characteristic properties like crystal size, strain, configuration and XRD pattern of ENPs. In nanocrystalline materials,

crystals are distributed randomly that cause widening of diffraction peaks due to lack of entire constructive and destrucutive interference of X-rays in a restricted sized lattice. Size and strain of a crystal can be determined by estimating the mean of crystallite size that can be calculated from the full width at half maximum (FWHM) of a diffraction peak using the Scherrer equation as follows:

$$\mathbf{d_{XRD} = K\lambda/B\cos\theta}$$

where, d is crystallite size; λ is diffraction wavelength; B is corrected FWHM; ɵ is the diffraction angle and K is constant that is near to unity [29].

B is calculated from observed FWHM by convoluting Gaussian profile which models the specimen broadening Br, which can be calculated by following equation:

$$\mathbf{B^2_r = B^2_o - B^2_i}$$

Where, B_o is observed broadening and B_i is instrumental broadening. Diffraction pattern of ENPs is based on the constructive interference of monochromatic X-rays with the ENPs that can be detected by the detector. These incident monochromatic X-ray radiations generate a Bragg peak after reflections from the different planes. For constructive inference, phase shift is multiple of 2π then according to Bragg's law equation:

$$\mathbf{n\lambda = 2d\sin\theta}$$

where *n* is an integer, λ is the wavelength of incident wave, *d* is the distance between the planes in the atomic lattice and sinɵ is the angle between the incident ray and the scattering planes.

3.5 Atomic force microscopy (AFM)

AFM is a highly advanced and ideal characterization technique which is capable of visualizing the 3-D structure of enzyme nanoparticles (ENPs). It is a qualitative as well as quantitative aspect of physical characteristics of ENPs such as size, morphology, outer texture as well as roughness. Various statistical parameters of ENPs like size, distribution of surface area and volume can also be investigated [30-32]. ENPs can be characterized in distinct environment such as ambient air, restricted medium and fluid dispersal. It has been reported that NPs can be used as calibration standard for the atomic force microscope [33].

4. Immobilization of ENPs

ENPs immobilization refers to confinement of ENPs at the surface of inorganic/organic matrix either physically, chemically or both so that their function remains persist without any harm and can be reused. Methods like adsorption, entrapment, microencapsulation, covalent binding, aggregated by bi-functional reagents and metal binding have been used for the immobilization of ENPs on support. Covalent immobilization of functional ENPs onto either organic or inorganic matrix produce better results and can be used for long duration. For instance, Au electrode has been used for the confinement of diverse ENPs viz. HRP [11], ChO_X [26], Uricase [13] and many more. Moreover, NPs of GOD extracted from *Aspergillus niger* confined on Pt electrode and nitrocellulose (NC) membrane. Procedure for immobilization of various enzyme nanoparticles onto Au electrode or NC membrane has been described below:

4.1 GOD NPs immobilization onto Pt electrode

GOD NPs were immobilized on Pt electrode covalently [10]. Dipped the cleaned Pt electrode into boiled H_2SO_4 for 30 minutes and then washed with distilled water (DW). Now, Pt

electrode was functionalized with amino (-NH_2) group by adding 10 mg/ml cysteamine dihydrochloride in dark for 2 h. GOD NPs were immobilized onto Pt electrode into ENPs suspension for 10 h at 4°C. Finally, washed the Au electrode immobilized with GOD NPs with DW and dried in air and stored the resulting Pt electrode in 0.1 M PB (pH 7.4) at 4°C.

4.2 GOD NPs immobilization at NC membrane

Kundu et al. (2012) have covalently immobilized GOD NPs onto NC membrane. Firstly, washed the NC, membrane (1x1.5cm^2) by DW to avoid the accumulation of debris and dried in air for 30 minutes. Introduced –NH_2 group onto NC membrane by dipping it in 0.5% chitosan in 2% acetic acid at room temperature. Washed the NC membrane further with 10% methanol to remove the unbound –NH_2 group and dried in air for 30 minutes. This functionalized NC membrane dipped in 2.5% glutaraldehyde (0.1 M PB, pH 6.0) for 2h at room temperature and washed the membrane thoroughly with 0.1 M PB (pH 6.0). Dispersed the 0.5 ml GOD NPs suspension on functionalized NC membrane and kept the membrane overnight at 4°C for effective covalent immobilization and washed with 0.1 M PB (pH 6.0). Discarded the GOD NPs aggregates and checked both NC membrane and discard for enzyme activity. Mounted GOD NPs immobilized onto NC membrane on sensing part of combined electrode of DO meter with a parafilm and connected this NC membrane to the DO meter [27].

4.3 Immobilization of ChOx [13,] ChENPs and ChOx NPs [25], Lipase/GK NPs/GPO NPs [24] on Au electrode

These nanoparticles were confined on Au electrode via thiolated (covalent) bond. Firstly, cleaned the Au electrode with piranha solution containing concentrated H_2SO_4 and H_2O_2 in the 3:1 ratio and rinsed the Au electrode with DW. Now, polished the Au electrode with alumina

slurry for 20 minutes and dipped the Au electrode in absolute alcohol for 30 minutes to sterilize and expose the hydroxyl group. Immersed the resulting Au electrode into suspensions of ENPs in 0.1 M PB, pH 7.0 and kept it for overnight for immobilization. Finally, rinse the working Au electrode in buffer to eliminate the non bounded ENPs to the functional electrode. Stored the ENPs bound Au electrode at 4°C until it is used.

4.4 Immobilization of HRP NPs aggregates onto Au electrode

Very firstly Liu et al. [11] had immobilized the HRP NPs aggregates at the surface of Au electrode. Firstly, scanned the bare Au electrode at the potential of 0.5-1.5 V in 0.2M H_2SO_4 until the characteristics voltammogram was appeared. Now, dipped the Au electrode in HRP NPs suspension at 4°C for 12 h under gentle stirring to form self-assembled HRP NP layer. Finally, rinsed the HRP NPs/AuE with 0.1 M phosphate buffer (pH 7.4) and stored at 4°C in phosphate buffer (PB) pH 7.4 and stored at 4°C in PB buffer until use.

4.5 Immobilization of uricase NPs aggregates onto Au electrode

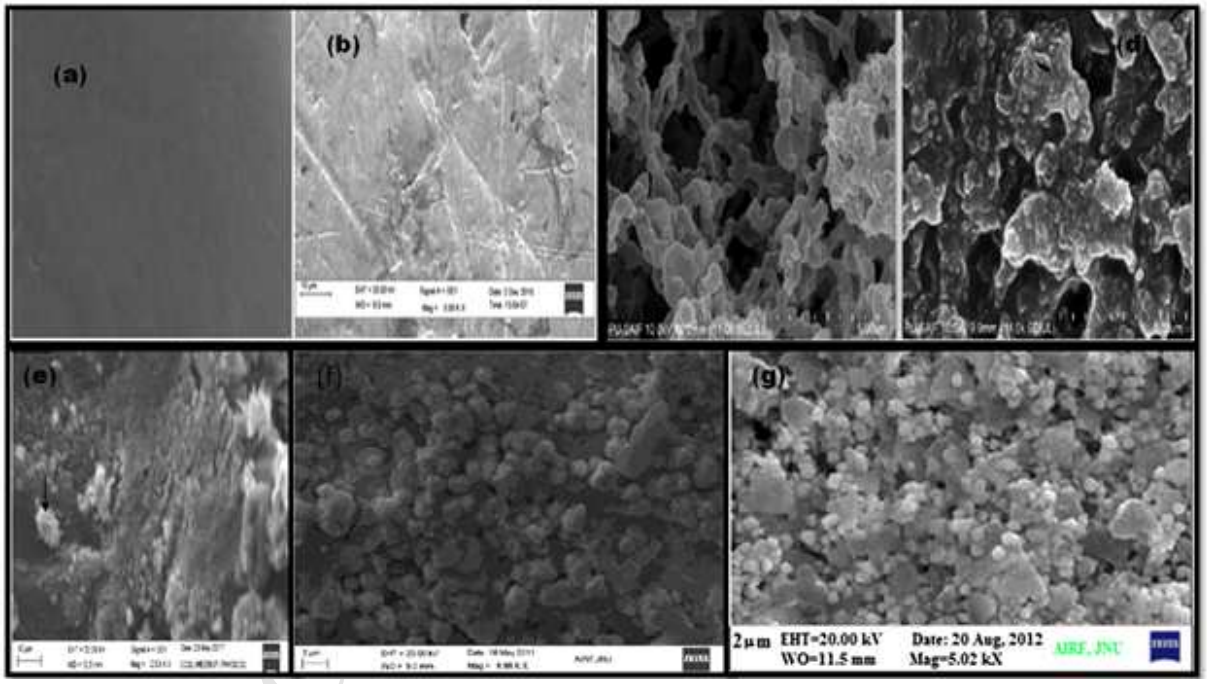
Chauhan et al. (2014) have immobilized the uricase NPs by thiolate bond onto Au electrode. Rinsed the bare Au electrode and scanned at 0.5-1.5 V in freshly prepared 0.2 M H_2SO_4 until characteristic voltammogram is appeared. Immersed this Au electrode in suspension of uricase NPs under stirring at 4°C to form self-assembled uricase NPs. Washed the uricase NPs immobilized on Au electrode with 50000 μM of PB, pH 7.4 and stored at 4°C in same buffer.

5. Characterization of ENPs immobilized onto matrix

ENPs confinement at their support material and support without confinement of ENPs aggregates has been confirmed by using scanning electron microscopy (SEM) and electrochemical impedance spectra (EIS).

5.1 Characterization of ENPs aggregates onto electrode by SEM

Morphological characteristics of modified working electrode are the key attributes to provide the characteristics of surface of functional electrode. SEM has been used to examine the confinement of NPs onto working electrode. SEM images of bare electrode or membrane have exhibited identical or homogenous surface while electrode with ENPs reveals spherical, beaded pattern and cluster of aggregated ENPs on intact surface of working electrode that validates the efficient immobilization of ENPs onto electrode/membrane. Supplementary Figure 1(a) shows the distinct SEM images of different types of nanoparticles.



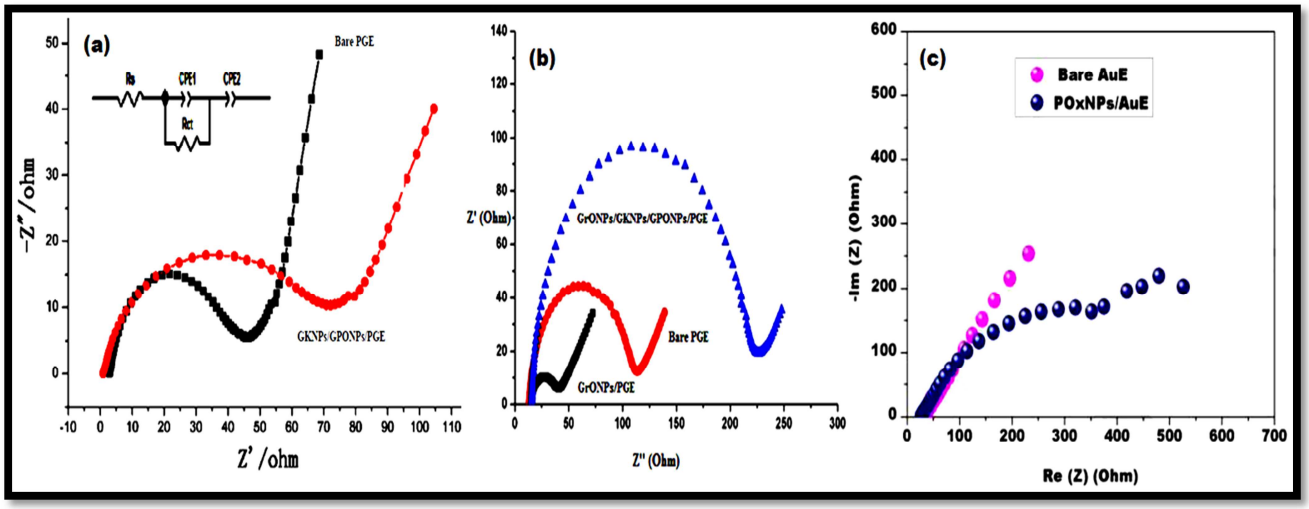
Supplementary Figure 1(a): SEM images of (a) bare Au electrode [24] (b) Lipase NPs/GKNPs/GPO NPs/AuE [24] (c) bare NC membrane (d) Urease NPs onto NC membrane [28] (e) CA NPs/Cl NPs/SOx NPs/GCE [25] (f) Uricase NPs/AuE [13] and (g) ChOx NPs/AuE [12]

5.2 Characterization of ENPs aggregates onto electrode by EIS

EIS is another technique for surface characterization of working electrode to confirm whether the ENPs are immobilized onto working electrode or not. In this technique, electrolyte solution is examined to determine the impedance behavior such as ohmic resistance, capacitance, constant phase element and Warburg impedance, solution resistance (Rs), charge transfer resistance (Rct) and double layer capacitance (C_{dl}). Rct can be determined by using Nyquist plot. The double layer capacitance can be calculated by using that frequency at which optimum semicircle arc is formed:

$$W=2\pi f=1/Rct\ Cdl$$

Capacitance of biological recognition reaction is measured in the absence of Faradaic currents that increased the electrical field on the biological recognition reaction to be investigated, by applying varying direct current (dc) working potentials [34]. This applied dc potential charge the interacting components and also influences the interaction between the biorecognition component and the analyte including their direction. For example in Supplementary Figure 1(b) the Rct value of GKNPs/GPONPs/PGE, GrONPs/AuE, GKNPs/GPONPs/AuE and POxNPs were 70 Ω, 40 Ω, 225Ω and 370 Ω respectively [35], [36], [37].



Supplementary Figure 1(b): EIS spectra of GKNPs/GPONPs/PGE [35], GrONPs/AuE, GKNPs/GPONPs/AuE [36] and POxNPs [37]

6. Kinetic properties of ENPs

Generally, enzymes show enhanced activity at their characteristic kinetic factors including ideal pH, temperature, K_m value, working range and response time. Hence, these kinetic factors of ENPs have been investigated after their immobilization covalently on Au /Pt or artificial membrane e.g. nitro-cellulose membrane as a support materials

6.1 Effect of optimum pH

ENPs based biosensors have shown optimum activity at a particular pH that pH is known as optimum pH of ENPs based biosensor. Every biosensor works at its optimum pH at which it performs better (redox reactions) consequently, obtaining enhanced analytical signal. Table 3 shows the optimum pH of various ENPs based biosensor.

6.2 Effect of optimum temperature and thermostability

The most favorable temperature of ENPs based biosensors has augmented their activity than immobilization of monomeric enzyme. For instance, on elevating the temperature 10°C and 5°C, the performance of uricase and glucose oxidase NPs augmented considerably [13] and [27]. For example ChENPs/ChOxNPs/AuE has maximally performed at 40°C [26] while Lipase NPs/GKNPs/GPONPs/AuE has worked best at 35°C [24].

In comparison to immobilization of free enzyme, ENPs based biosensors have exhibited better thermal consistency due to inter and intramolecular covalent interactions which inhibit the configural modifications of the ENPs on raising temperature.

6.3 Response time

In comparison to free enzyme, ENPs immobilization lowers the response time. The response time of ChENPs/ChOxNPs/AuE was 5 s [26], LipaseNPs/GKNPs/GPONPs/AuE had shown maximum outcomes within 5 s [24], UricaseNPs/AuE was 7 s [13] and ChOxNPs/AuE had 8 s [12]. The response time of various ENPs based biosensors have been given in Table 3.

6.4 K_m value

Immobilization of ENPs onto solid support has decreased the K_m value than free enzyme or protein. For instance immobilized GOD NPs has reduced the K_m value twice that deciphers the cross linking and immobilization of GOD NPs, as a result showed high affinity for its analyte than independent enzyme. Lineweaver-Burke plot between reciprocal of uric acid concentration versus reciprocal of biosensor response in current (mAmp) was used to calculate the K_m value of immobilized uricase NPs on Au electrode which is analogous to monomeric enzyme [13].

6.5 Linear range

The linear range of immobilized ENPs was wide as compared to free enzyme. Therefore, immobilized ENPs are used for determination of various analytic parameters as compared to free enzymes. The working range of various biosensors has been depicted in Table 3.

6.6 Storage stability

Storage stability of immobilized ENPs has shown better storage stability in comparison to monomeric free enzyme because of effective bonding interaction between ENPs aggregates and matrix material. Thus, ENPs are more stable than free enzyme Table 3.

6.7 Reusability

The immobilized ENPs onto their matrix have shown better reusability in comparison to native enzymes. The working electrode comprised of immobilized ENPs can be used 100-200 times; this makes the use of ENPs cost-effective and can be used for a number of applications.

7. Biosensor

The very first biosensor was developed by L.L. Clark an American biochemist in 1950 by immobilizing glucose oxidase (GOD) enzyme at Clark electrode. This biosensor was used for the investigation of blood glucose level by measuring the oxygen level in the blood. The electrode used for fabrication of this biosensor was known as ‘Clark electrode’ or ‘oxygen electrode’.

Principle

Biosensors are known as quantitative analytical devices which contain recognition elements including enzyme, antibodies, phages, aptamers or single stranded DNA and connected to a suitable transducer that may be physicochemical, optical, thermometric, piezoelectric and magnetic [38-40] (Figure 2(a)). The electric signals in the form of current/potential are quantified

by electrochemical sensors, which is directly proportional to analyte concentration. There are mainly two types of electrochemical biosensors that include amperometric and potentiometric. However, electrochemical sensors can be employed in the distinct sample investigation to detect analyte like thermally processed foods, diagnosis and treatment of different diseases.

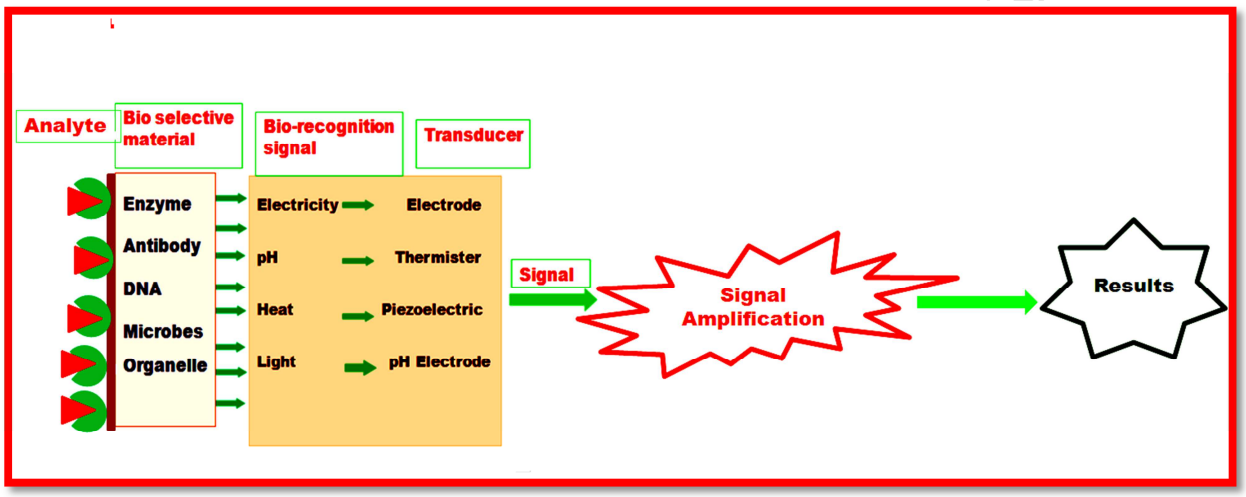


Figure 2(a): Schematic depiction of principle and working of a biosensor

There are several fundamental parameters depicted in Figure 2(b) that must be considered before constructing a biosensing device [41].

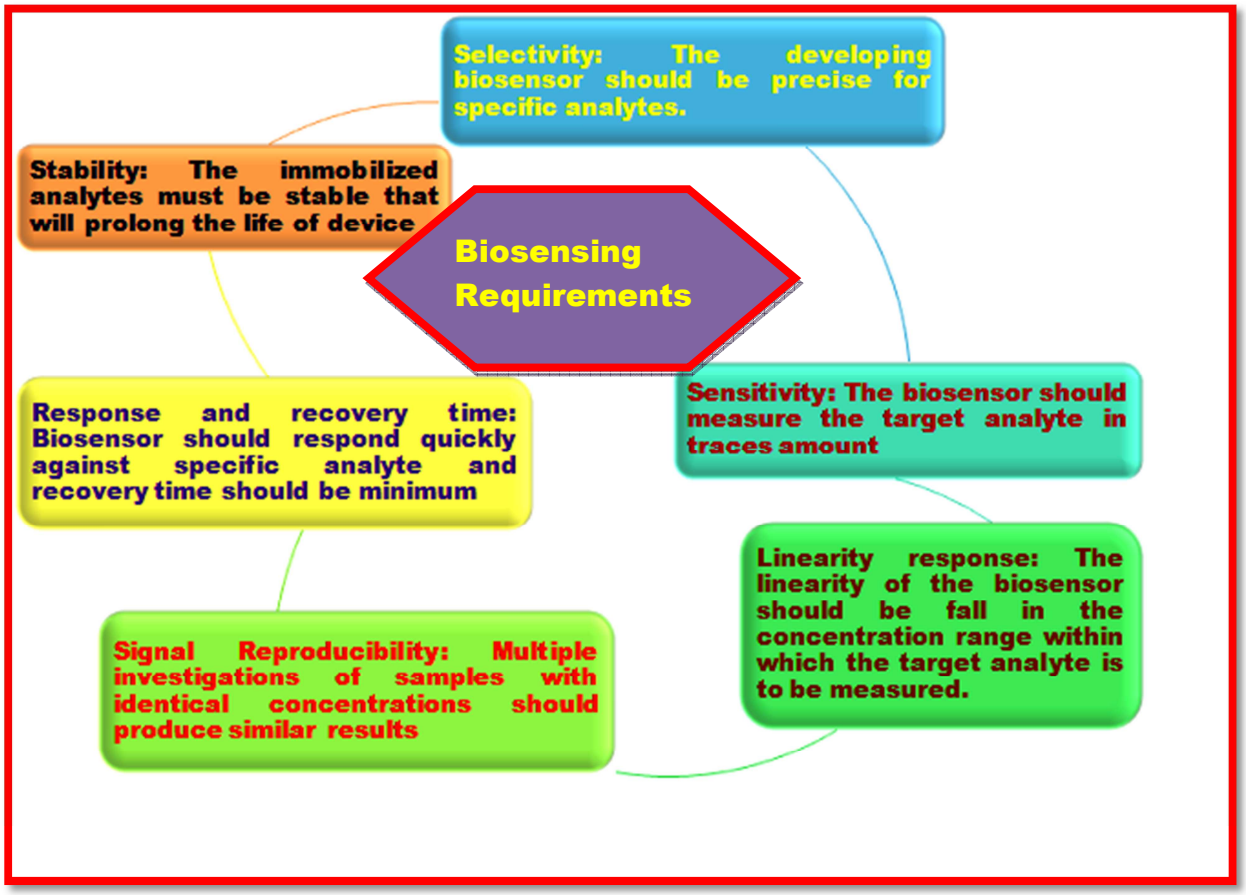


Figure 2(b): Diagrammatic illustration of essential requirements required for the development of ENPs based biosensor

7.1 Classification of biosensors based on ENPs

The electrochemical ENPs based biosensors have been classified into amperometric, potentiometric and impedimetric biosensors. Figure 2(c) depicts the concept of electrochemical biosensors based on ENPs.

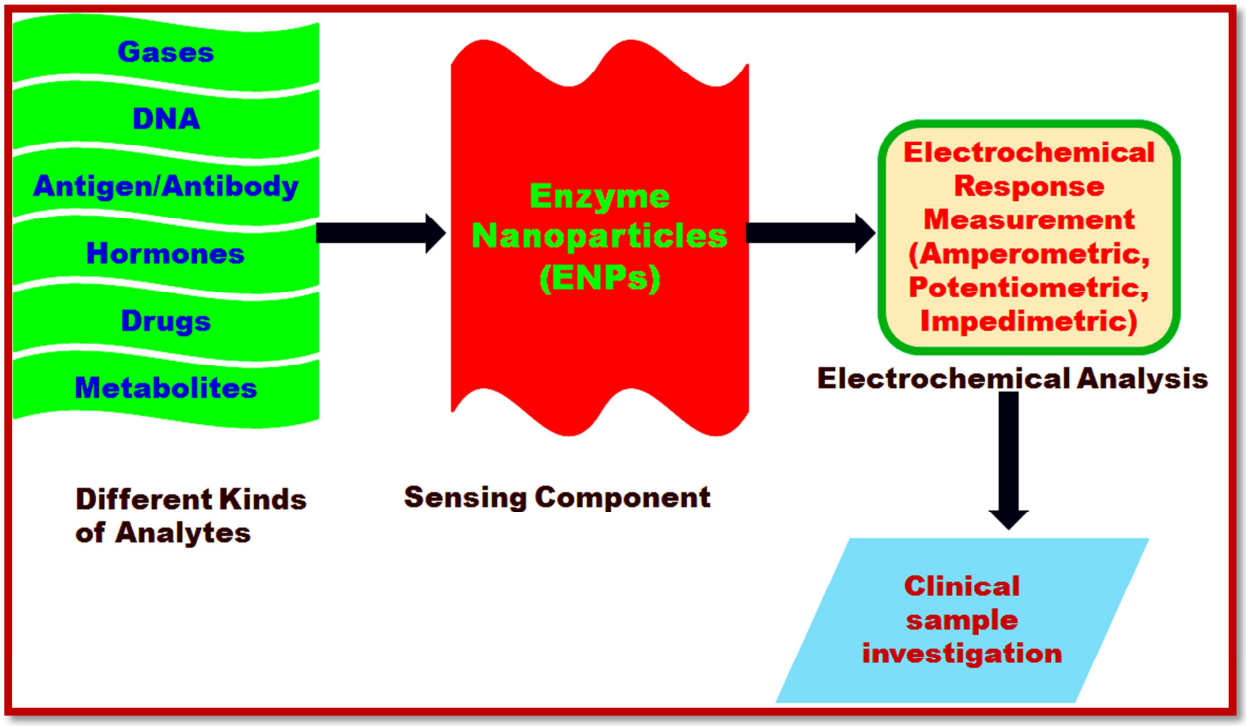


Figure 2(c): Schematic illustration of ENPs based biosensors

7.1.1 Electrochemical biosensor

Electrochemical ENPs biosensors are sensitive devices that are based on the electrochemical reactions between ENPs and reaction mixture. The resultant signals of the electrochemical reactions can be measured in the form of current, voltage or conductance [42] depending upon the type of transducer used. An electrochemical ENPs based biosensor is a three electrode system comprising a counter, reference, and working electrode. Electrochemical biosensors based on ENPs deposited on electrodes have aroused the interest of scientific communities because of their immense applications in diverse fields. A number of strategies have been used for immobilization on support materials includes adsorption, entrapment, covalent bonding and cross linking. These electrochemical biosensors have been employed to improve the health of people, food industries and environment monitoring [43]. Among these

aforementioned areas, health sector is the chief area of biosensing applications which include determination of blood glucose levels in diabetes persons using glucose biosensors, accurate analysis of urea in patients suffering from kidney diseases, detection of cholesterol, triglycerides level, glycerol level and pyruvate level in cardiac patients. Enzyme based electrochemical biosensors have been used for industrial applications including detection of hazardous components in food products, investigation of fermentation broths or food processing steps by analyzing the amount of glucose and additional fermentative end products. Furthermore, biosensors have also played a significant role in sensitive detection of phenolic compounds which are today's most serious concern for environmentalist. Because these phenolic compounds and other toxic compounds mostly exist in diverse source such as agriculture and effluents released from several industries. These compounds have been found to be highly toxic for living beings; consequently, responsible for causing various fatal diseases [43]. Therefore, these environmental issues can be resolved by nano based approaches which are simple, sensitive, economic, fast and reliable [44].

7.1.1.1 Electrochemical detection techniques

Several electrochemical detection techniques including cyclic voltammetry (CV), chronoamperometrically, chronopotentiometrically, impedance spectroscopy and field effect transistor based approaches have been used for target analyte detection in the given sample [42]. These electrochemical techniques have been described as follows:

(i) Cyclic voltammetry (CV)

Cyclic voltammetry is widely used electro-analytical approach and provide information about the redox potential and electrochemical reaction rates of analyte solutions. In this technique, the potential is swept between two values at a constant rate. As the potential attains V2 the scan is inverted and the potential is swept return to V1. The scan rate, (V2-V1)/(t2- t1), is an important aspect, because the extent of scan provide enough time for a given electrochemical reaction to occur. Variation in the scan rates, produce correspondingly varied results. The potential is calculated between the reference electrode and the working electrode, whereas the current is measured between the working electrode and the auxillary electrode. The results obtained are measured by plotting the graph between current *vs.* potential. These plots are known as voltammogram. With increase in potential toward the electrochemical reduction potential of the analyte, the current will also augment [42].

(ii) Chronoamperometry and chronopotentiometry

In chronoamperometry, a square-wave potential is applied to the working electrode and a constant state current is analysed as a function of time. Variation in the current depends on the extension or decrease of the diffusion layer at the electrode. The confined analyte concentration reaches to zero at the electrode surface, diffusion regulate the movement of high concentration of analyte from the solution to the electrode. As a result, concentration gradient develops away from the electrode surface. In the bulk solution the concentration of analyte is maintained at a value of $c0$ by convective transfer. Chronoamperometry is based on Cottrell equation that defines current-time dependence for linear diffusion control at a planar electrode

$$I = nFAc0\sqrt{D/\pi t}$$

where, I is current depends on F Faraday's constant, *n* the number of electrons transferred per molecule, *A* is the area of electrode, c0 is concentration of analyte, D is diffusion coefficient and t is time. Therefore, according to this Cottrell equation, flow of current is proportional to the diffusion rate of analyte to the electrode. While in chronopotentiometry the potential is studied as a function of time in response to a constant or square-wave current [42].

(iii) Electrochemical impedance spectroscopy (EIS)

EIS is a useful tool for the fabrication and investigation of materials for biosensor transduction. During electrochemical sensing, impedance techniques are used for monitoring the changes in electrical properties due to biorecognition events occurring at the surfaces of modified electrodes [42].

(iv) Field effect transistor (FET)

The FET uses an electric field to control the conductivity of a region with depleted charge carriers between source and drain electrodes in a semiconducting material. Control of the conductivity can be attained at gate electrode (third electrode) by changing the electric field potential, relative to the source and drain electrode. Configuration and doping of the semiconducting material contain sufficient positive or negative potential at the gate electrode that either attract or repel the charge carriers in the conduction region. As a result, conduction region will either fill up or drain the depletion region of charge carriers (i.e. electrons). Hence, this

either creates or distorts the efficient electrical magnitude of the conducting conduit and thereby controls the conductance between the source and drain electrodes [42].

Advantages of electrochemical biosensors

These electrochemical devices are acquiescent for miniaturization and exhibited better compatibility. Moreover, these electrochemical biosensors have offered unique sensitivity, selectivity, rapid and easy to operate [45]

Disadvantages of electrochemical biosensors

Deprived interactions between biorecognition elements and electrochemical transducers lower the selectivity, sensitivity and stability of electrochemical biosensors.

7.1.1.1.1 ENPs based Amperometric biosensors

Principle

Amperometric biosensors based on ENPs measure the signal of interest in the form of current which is directly proportional to the concentration of analyte binding with biorecognition component. In these ENPs based devices, the electroactive species are electrochemically oxidized or reduced (redox reactions) onto inert support materials when a constant potential is applied [46] [47]. During these redox reactions, electrons are released from the analyte to the working electrode immobilized with ENPs or vice-versa. As a result, analytical current peaks are formed which are taken as basis of measurement of electrochemical response. The schematic representation of amperometric biosensors based on ENPs has been shown in Figure 2(d).

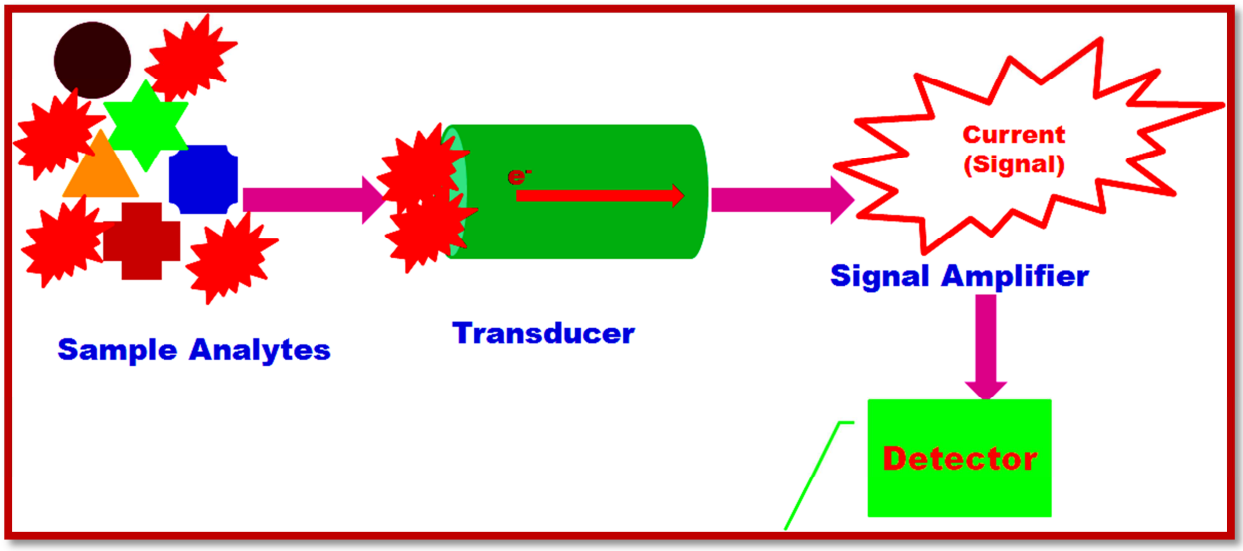


Figure 2(d): Schematic representation of ENPs based amperometric biosensor

7.1.1.1.1 (a) Various generations of ENPs based amperometric biosensor

Amperometry is a sensitive and specific electrochemical technique in which the signal of analyte is current which is directly proportional to the binding of precise analyte by using a steady bias voltage. An amperometric biosensor comprised with two (bi) or three (try) electrodes. In two electrode system, one of the electrodes acting as a reference while other as a functional electrode. Use of biosensors having two electrodes is restricted, as at elevated current, it becomes very difficult to maintain the potential. Alternatively, in three electrode system, the third electrode is acting as an auxiliary counter electrode that provide more surface area to facilitate the better redox reactions. This increases the flow of current, between the auxiliary and the working electrodes, while voltage runs between the working and the reference electrodes [48]. Depending upon the electron kinetics, reduction of acrylamide occurs in three ways which

are referred to as the first, the second and the third generation acrylamide biosensors Figure 2(e) which are explained below:

(i) First generation ENPs based amperometric biosensor

In these amperometric devices oxygen is acting acts as a mediator between the electrode and the ENPs. In the presence of suitable analyte, oxygen is reduced to form hydrogen peroxide accompanied by flavin adenine dinucleotide (FAD), a prosthetic group of enzyme, and $FADH_2$ redox couple. The decreased in oxygen level is directly proportional to the analyte concentration. This deceased oxygen level can be measured by measuring the elevated amount of hydrogen peroxide or deprived oxygen level [49].

(ii) **Second generation ENPs based amperometric biosensor**

Second generation ENPs biosensors involves the use of synthetic electron mediators like ferro/ferricyanide, hydroquinone, ferrocene, and redox organic dyes between the electrode and ENPs. These mediators facilitate electron kinetics between electrode and ENPs and also act as way around the sensing system, when reduced oxygen pressure was noticed from the first generation acrylamide sensor [50].

(iii) **Third generation ENPs based amperometric biosensor**

These biosensors incorporate the direct immobilization of ENPs at the electrode surface and generate an amperometric signal [51]. This accelerates the electron kinetics of ENPs and hence improves the sensing activity [52]. These ENPs based biosensors have offered better biocompatibility and stability [53].

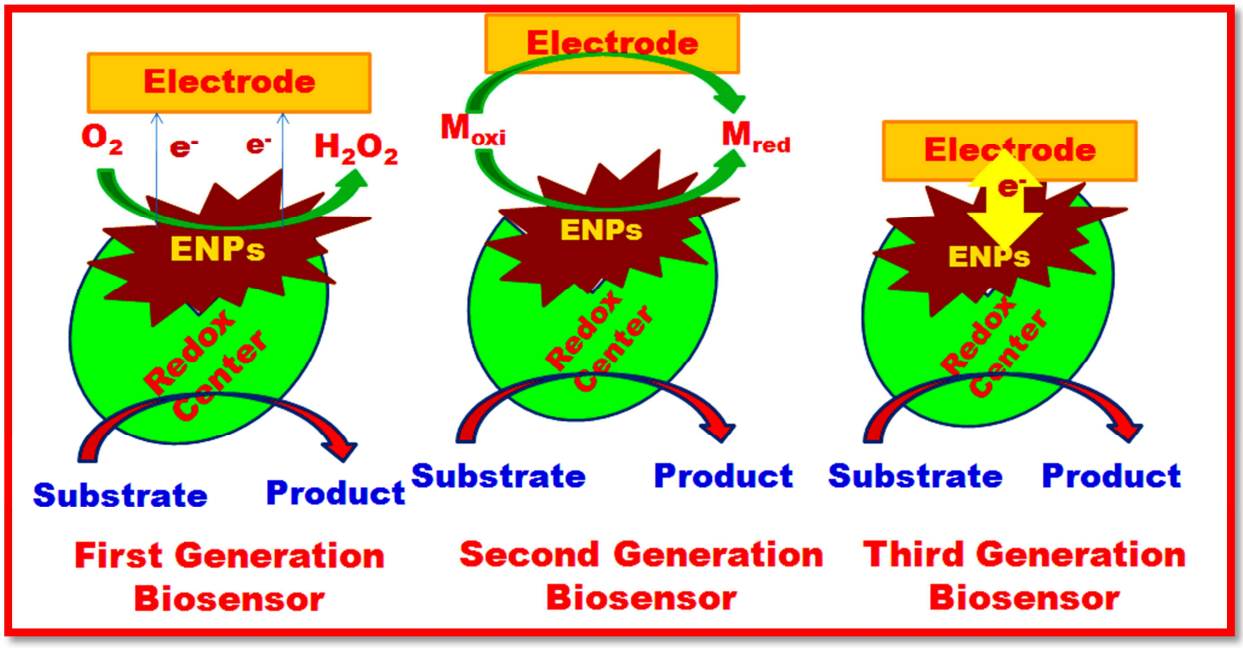


Figure 2(e): Diagrammatic demonstration of first, second and third generation of ENPs based amperometric biosensor

Researchers have developed a number of ENPs based amperometric biosensors viz. ChENPs/ChOxNPs/AuE [26], Lipase/GK/GPO/AuE [24], HRPNPs/AuE [11], GODNPs/AuE [27], ChOxNPs/AuE [12], CA/CI/SOx/GCE [25], GK/GPO/PGE [35], LDHNPs/AuE [54] and SOxNPs/AuE [55]. These ENPs based amperometric biosensors have opened a new platform for the diagnosis of several diseases such as cancer, heart attack, diabetes, gout, problems related to renal, thyroid and muscles. The schematic and electrochemical reactions of aforementioned ENPs based amperometric biosensors have been explained as follows:

Biosensing applications of ENPs based amperometric biosensor

Thus, these biosensors have attracted the interest of researchers in various area as they are small in size, not expensive, rapid with low detection limits and better performance [56]. A

variety of enzyme/protein nanoparticles based amperometric and potentiometric biosensors are explained below:

7.1.1.1.1 Fabrication of cholesterol biosensor base on cholesterol esterase and cholesterol oxidase nanoparticles

An amperometric biosensor was constructed by covalent immobilizing NPs of cholesterol esterase (ChENPs) and cholesterol oxidase (ChOxNPs) on Au electrode for the quantification of cholesterol in the serum sample (Figure 3(a)). These NPs were prepared by desolvation method and immobilized on Au electrode. The biosensor exhibited optimum response within 5 s at pH 5.5, temperature 40 °C at 0.25 V vs Ag/AgCl [26]. Schematic representation of immobilization of ChENPs and ChOxNPs has been shown below:

Evaluation of analytical parameters of ChENPs/ChOxNPs/AuE

The biosensor has been evaluated for its several analytical parameters. The working range of ChENPs/ChOxNPs/AuE was 0.26-18.102 mmol/L. The analytical recovery of known quantity of added cholesterol concentration of 2.59 and 5.18 mmol/L were 94.73% and 96.33% respectively, within and between co-efficient of variations were <2% and <3%. Working electrode can be stored for 60 days at 4°C [26].

Medical implication of ChENPs/ChOxNPs/AuE

The above constructed biosensor was used for the determination of cholesterol level in the sera of apparently healthy and diseased persons [26]. As high concentration of cholesterol in serum causes atherosclerosis, cardiovascular diseases, myxedema, jaundice, diabetes mellitus, nephritis and cerebral thrombosis [57]. Hence, its determination in serum sample is necessary.

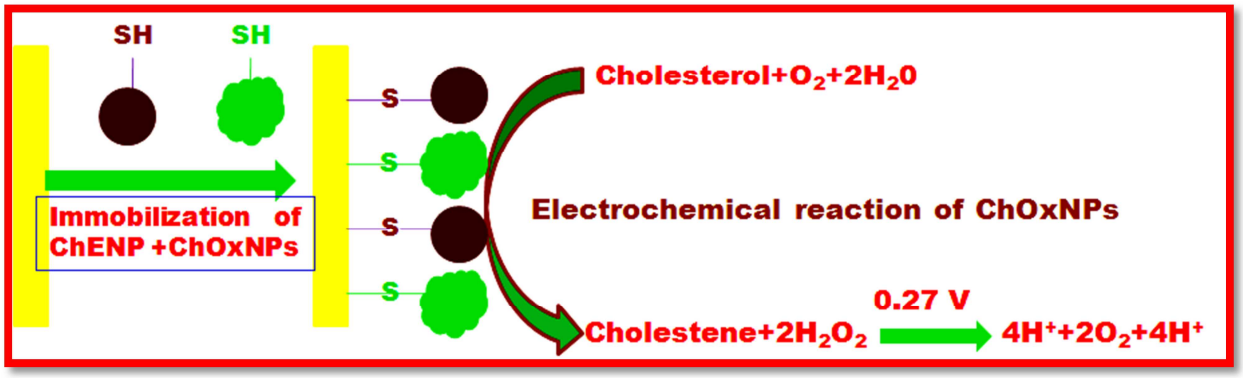


Figure 3(a): Diagrammatic illustration of immobilization and functioning of cholesterol biosensor using ChENPs and ChOxNPs (Source: [26])

7.1.1.1.1.2 Fabrication of triglyceride biosensor based on lipase, glycerol kinase and glycerol-3-phosphate oxidase nanoparticles

Pundir and Aggarwal, (2017) have prepared NPs of lipase extracted from Porcine pancreas; glycerol kinase (GK) extracted from *cellulomonas* sp. and glycerol-3-phosphate oxidase (GPO) from *Aerococcus viridanss* by desolvation method [24]. These NPs were characterized by transmission electron microscopy (TEM) and Fourier transforms infra red (FTIR) spectroscopy and immobilized on Au electrode. Schematic illustration of confinement of Lipase, GK and GPO NPs and their electrochemical reaction occurring at Au electrode has been illustrated in Figure 3(b). The developed Lipase/GK/GPO/AuE biosensor worked best at pH 6.5 and temperature 35°C when applied a potential of 1.2 V as a working potential of biosensor and response time 5s [24].

Evaluation of analytical parameters of Lipase/GK/GPO/AuE

The working range of lower and higher concentration of Lipase/GK/GPO/AuE was 0.01-1.13 mmol/L and 1.13-5.65 mmol/L respectively, **detection limit 1.0 µg/ml,** recovery of added

known concentration of 0.56 mmol/L and 1.13 mmol/L was 95.2% and 96.0% respectively. Within-and-between batch co-efficient of variations were 2.33% and 2.15% respectively. The storage stability of Lipase/GK/GPO/AuE electrode was 90 days when stored at 4 °C [24].

Medical implication of Lipase/GK/GPO/AuE

The above proposed biosensor was used for measuring the triglyceride level in the sera of both apparently healthy and diseased persons [24]. Abnormal level of triglyerides in human causes atherosclerosis and various other heart diseases, pancreatic diseases, puffy liver and spleen [58] [59].

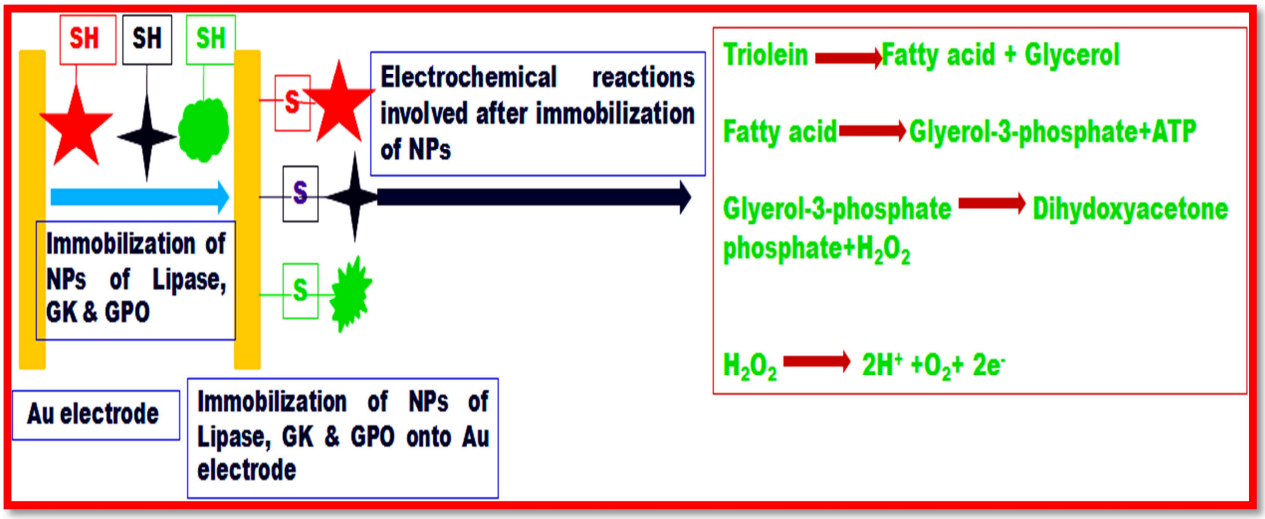


Figure 3(b): Schematic representation of Lipase, Glycerol kinase (GK) and Glycerol-3-phosphate oxidase (GPO) NPs immobilization and electrochemical reaction occurring on Au electrode [24].

7.1.1.1.1.3 Fabrication of H_2O_2 biosensor based on horseradish peroxidase (HRP) nanoparticles

The very first enzymatic amperometric biosensor was constructed by confining HRP NPs covalently on Au electrode [11]. The schematic representation of immobilized HRPNPs and electrochemical reaction occurring at the surface of Au electrode has been illustrated in Figure 3(c). The biosensor has shown optimum at pH 7.4 when applied a potential of 0.16 V.

Evaluation of analytical parameters of HRPNPs/AuE

The working range of HRPNPs/AuE was 1-9 µM with 0.1 µM limit of detection. The % co-efficient of variation was % CV 2.54% and <5 s response time [11].

Medical implication of HRPNPs/AuE

This HRP NPs/AuE biosensor was employed for the measurement of H_2O_2 level in the sera of normal as well as diseased person by Liu et al., (2005). As increased quantity of H_2O_2 may cause diseases like atherosclerosis, ageing and diabetes.

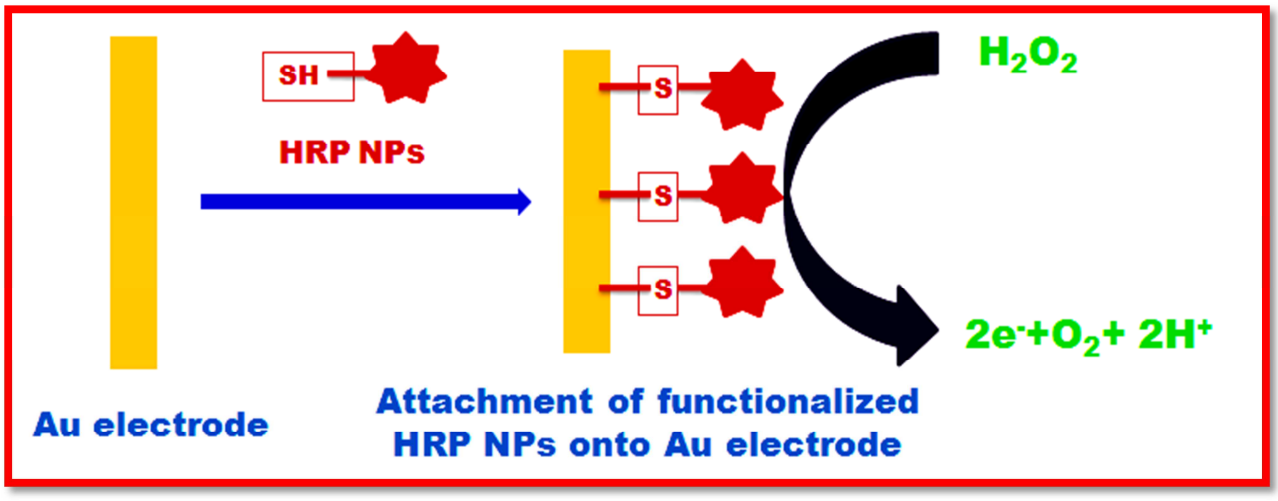


Figure 3(c): Schematic representation of Horseradish peroxidase (HRP) NPs immobilization and electrochemical reaction occurring on Au electrode [11].

7.1.1.1.1.4 Fabrication of glucose biosensor based on glucose oxidase (GOD) nanoparticles

Glucose oxidase (GOD) NPs has been used for the development of two types of biosensors i.e. DO metric [27] and amperometric biosensor [10]. Amperometric biosensor based on GOD NPs was employed for the electrochemical measurement of H_2O_2 produced from glucose whereas GOD NPs based DO metric biosensor was used for measuring the dissolved O_2 (in mg/I) during aerobic oxidation. GOD NPs/AuE works best at pH 7.4, temperature 27 °C; while DO metric biosensor works at pH 6.0, temperature 35°C. Schematic depiction of immobilization of GOD nanoparticles and their electrochemical reaction onto Au electrode has been illustrated in Figure 3(d).

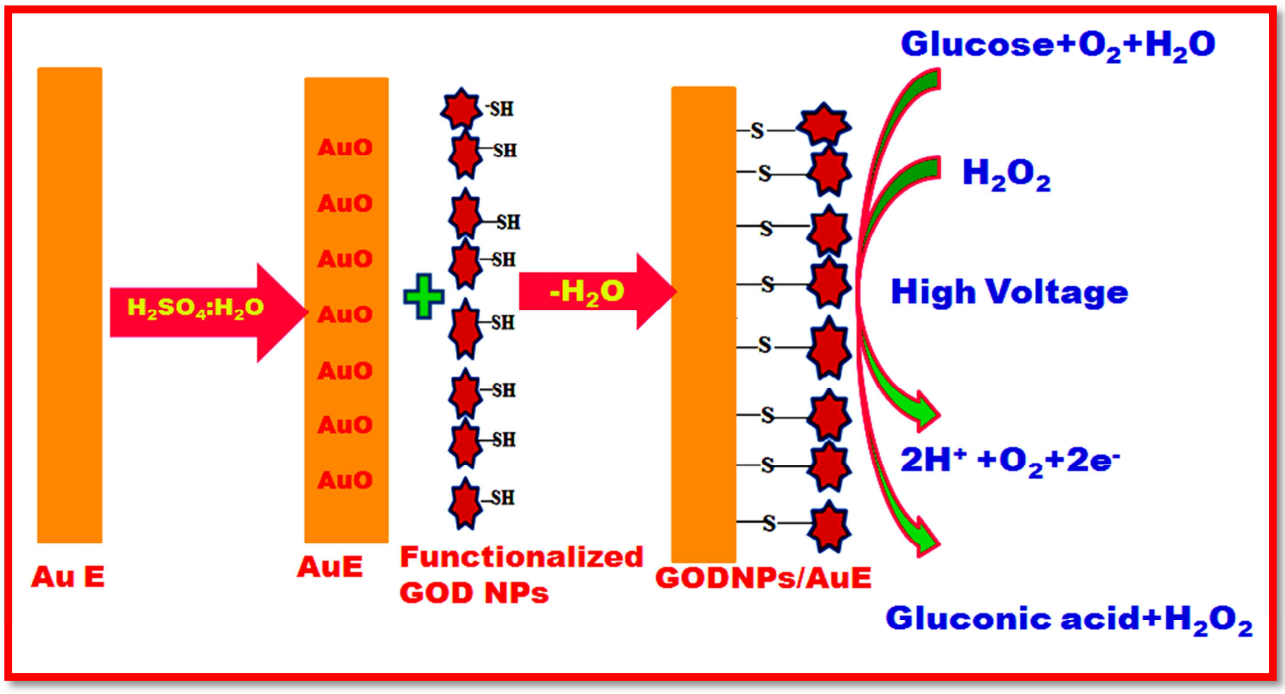


Figure 3(d): Diagrammatic illustration of immobilization of functionalized GODNPs and electrochemical reaction occurring on Au electrode [27].

Evaluation of analytical parameters GODNPs/AuE

The working range of amperometric and Do metric biosensor was 1.9 mmol/L and 0.06-13.9 mmol/L respectively. The limit of detection of GOD NPs/AuE was 18 µM and for DO metric biosensor was 10 µM.

Medical implication of GODNPs/AuE

GODNPs/AuE was used to measure the glucose level in the serum samples of both healthy as well as in diabetic patients whereas DO metric biosensor has measured the dissolved metabolic oxygen.

7.1.1.1.1.5 Fabrication of cholesterol biosensor based on cholesterol oxidase (ChOx)nanoparticles

Chawla et al. (2013) have designed an amperometric biosensor by immobilization cholesterol oxidase NPs (ChOxNPs) covalently onto Au electrode. Schematic representation of ChOx NPs immobilization and their electrochemical reaction at the surface of Au electrode has been depicted in Figure 3(e). The optimum response of ChOxNPs/AuE was at pH 6.0, temperature 35°C, by applying potential 0.27 V vs Ag/AgCl [12].

Evaluation of analytical parameters of ChOxNPs/AuE

The ChOxNPs/AuE has shown 0.32-18.13 mmol/L working range with 0.04 mmol/L LOD. This biosensor can be stored for 90 days at 4 °C.

Medical implication of ChOxNPs/AuE

ChOxNPs/AuE has been used for the quantification of cholesterol level for the diagnosis of hypercholesterlaemia, atherosclerosis, cardiovascular diseases, coronary artery diseases and transient ischaemic heart attacks in both normal and diseased persons [12]. Therefore, it is

necessary to measure the cholesterol level in the humans to prevent the attacks of these fatal disorders.

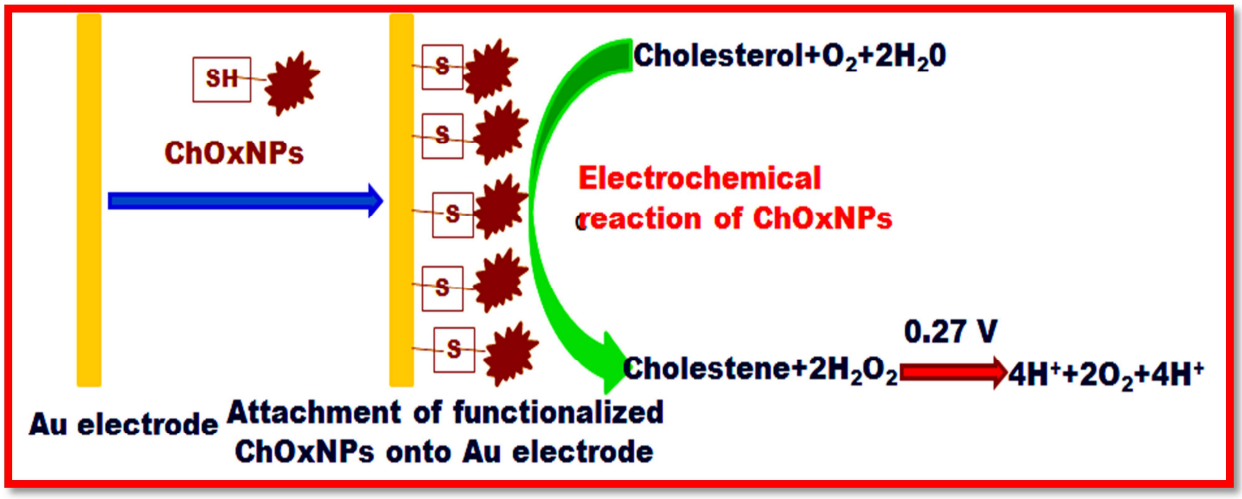


Figure 3(e): Diagrammatic illustration of immobilization of functionalized ChOxNPs and their electrochemical reaction onto Au electrode [12].

7.1.1.1.1.6 Fabrication of uric acid biosensor based on uricase enzyme nanoparticles

Uric acid biosensor was designed amperometrically by the covalent immobilization of uricase NPs [13]. The size of uricase NPs was calculated by taking the average of diameter of TEM images. Schematic illustration of immobilization of uricase NPs and their electrochemical reaction on Au electrode has been illustrated in Figure 3(f). These uricase NPs were deposited electrochemically onto Au electrode. The modified Au electrode with uricase NPs was investigated by SEM, FTIR and EIS. Uricase NPs/AuE had detected the uric acid in blood within 7 s and pH of biosensor was optimized by using different reaction buffers (50 mM) namely sodium succinate buffer pH ranges from 4.0-5.5, sodium phosphate buffer pH ranges from 6.0-8.5 and tris-HCL buffer pH ranges from 8.5-9.0. The optimum pH of the biosensor was 8.5 and temperature was 40°C.

Evaluation of analytical parameters of Uricase NPs/AuE

The working range of this uric acid biosensor was 0.005-0.8 mM, analytical recovery of added uric acid concentration at 0.59 mmol/L and 1.73 mmol/L was 99.57% and 98.77% accordingly, extraordinary sensitivity 0.003 mA $\mu M^{-1}cm^{-2}$ and longer storage stability 210 days when stored at 4°C. The precision of Uricase NPs/AuE was 5.6% and 4.7%.

Medical implication of Uricase NPs/AuE

Uricase NPs/AuE amperometric biosensor was used to investigate the uric acid in blood or urine because high concentration of uric acid in humans is the indication of kidney and metabolic disorders. Anomalous level of uric acid in human beings causes several disorders like gout, hyperuricaemia, Lesch-Nyhan syndrome, cardiovascular as well as renal disorders.

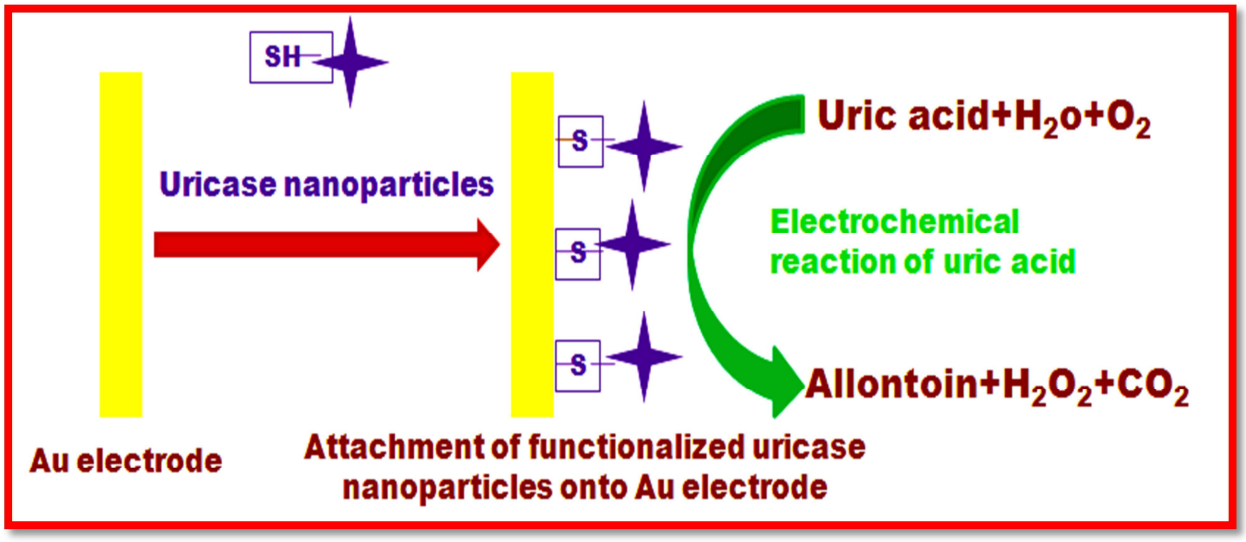


Figure 3(f): Diagrammatic depiction of immobilization of uricase NPs and electrochemical reaction at Au electrode [13].

7.1.1.1.1.7 Fabrication of creatinine biosensor based on creatinine (CA), creatinase (CI) and sarcosine oxidase (SOx) nanoparticles

Creatinine (CA), creatinase (CI) and sarcosine oxidase (SOx) NPs have been synthesized by desolvation method and their morphological aspects were studied by TEM and FTIR. These characterized nanoparticles were immobilized onto glassy carbon electrode (GCE) (Figure 3(g)). The immobilization onto GCE was confirmed by SEM, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) at distinct stages. CA/CI/SOx/GCE works optimally within 2 s, pH 6.0 at 25°C [25].

Analytical evaluation of CA/CI/SOx/GCE

CA/CI/SOx/GCE has showed 0.01-12 μM broad working range with 0.01 μM limit of detection and 97.97% and 98.76% analytical recovery at 0.1 mM and 0.15 mM creatinine concentration. The % CV was 2.06% and 3.09% and can be stored for 240 days at 4°C [25].

Medical implication of CA/CI/SOx/GCE

CA/CI/SOx/GCE was used to measure creatinine level in sera samples of apparently healthy and diseased persons. Because elevated level of creatinine causes renal, muscular and thyroid disorders. The creatinine level in sera of healthy and diseased was 78.69 μM and 298.41 μM respectively [25].

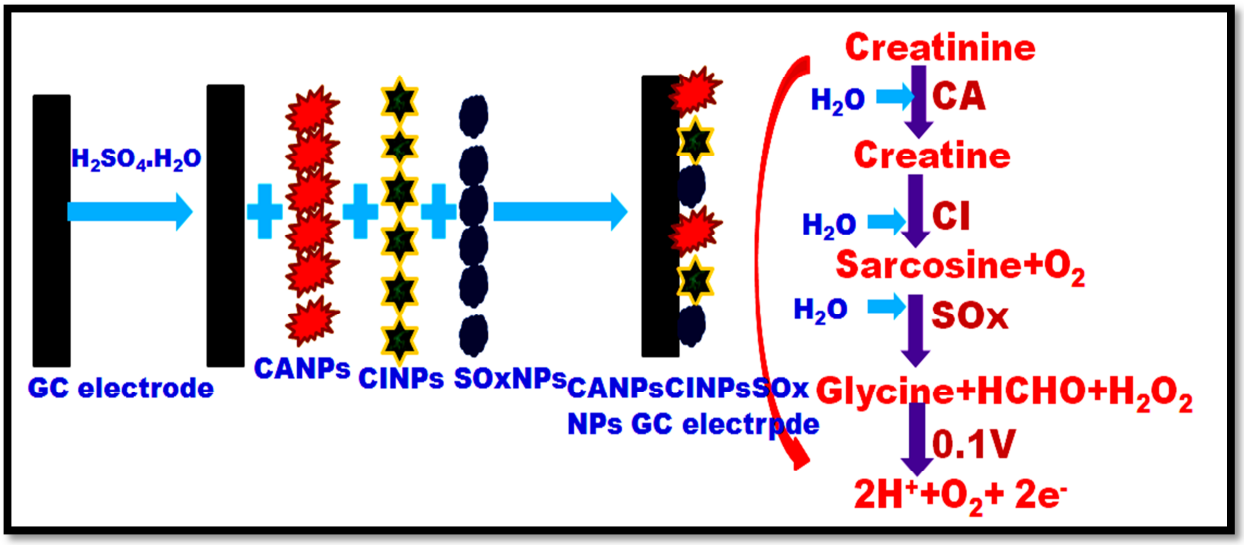


Figure 3(g): Schematic illustration of CA/CI/SOxNPs immobilization and electrochemical reaction at GC electrode [25].

7.1.1.1.1.8 Fabrication of glycerol biosensor based on Glycerol kinase (GK) and glycerol-3-phosphate (GPO) nanoparticles

An improved amperometric biosensor was developed based on immobilization of GK and GPO at PG electrode (Figure 3(h)) [60]. The modified working electrode GK/GPO/PGE was investigated by SEM, EIS and CV. The maximum performance of the GK/GPO/PGE was obtained at pH 7.0 and temperature 30°C when applied a potential of -0.3 V.

Analytical evaluation of GK/GPO/PGE

The response time of the biosensor was 2.5 s. The GK/GPO/PGE biosensor showed better sensitivity i.e. 7.24 μA/mM/cm^2 with a limit of detection 0.0001 μM and broad linear range 100-45000 μM. The analytical recovery of added concentration of glycerol was found to be 98.73 %. The precision of GK/GPO/PGE biosensor was determined by calculating the co-

efficient of variation for with-in and between batch, which was 0.105 % and 0.14 % respectively. The values obtained by GK/GPO/PGE biosensor were correlated with standard immune kit method using regression equation. The storage stability of the biosensor was 180 days [60].

Medical implication of GK/GPO/PGE

The above constructed biosensor was employed for the investigation of glycerol level in serum sample of healthy as well as cardiac patients. The glycerol level in healthy person was found in the range of 1.3-3.2 µM while in diseased patient it was in the range of 8.2 -31.0 µM [48].

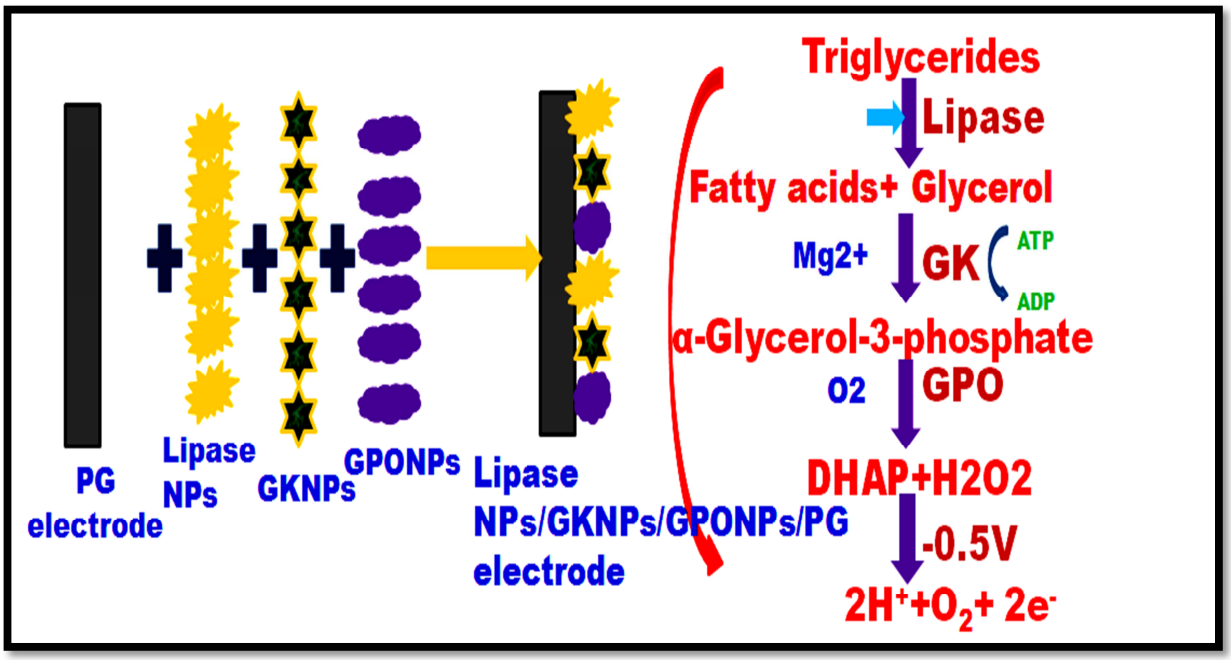


Figure 3(h): Schematic representation of attachment of functionalized Lipase NPs/GKNPs/GPONPs and electrochemical reaction on pencil graphite electrode [56]

7.1.1.1.1.9 Fabrication of lactic acid biosensor based on lactate dehydrogenase nanoparticles

Narwal et al. (2018) have prepared the NPs of lactate dehydrogenase extracted from muscles of rabbit by desolvation method. These LDHNPs were characterized by TEM, FTIR and UV-spectroscopy and were used for the fabrication of an enhanced amperometric biosensor by immobilizing them on Au electrode (Figure 3(i)). The surface of LDHNPs/AuE was studied by SEM, EIS and CV. The LDH biosensor responded optimally at pH 7.0 and 35°C temperature when applied a potential of 0.10 V [61].

Analytical evaluation of LDHNPs/AuE

The percentage recovery of biosensor was 98.61% and within-and-between coefficient of variation was 1.38% and 1.03% respectively. The LDHNPs/AuE exhibited 0.01μM LOD and wide working range 0.01-55000 μM. The sensitivity of LDHNPs/AuE was 3.45 $\mu Acm^{-2}mM^{-1}$ and response time 2.5 s. The results obtained by this LDH biosensor were compared with standard colorimetric method and showed better correlation. The LDHNPs/AuE can be stored for 210 days.

Medical implication of LDHNPs/AuE

The above constructed biosensor was used to measure the lactic acid level in real samples as elevated level of lactic acid caused cardiovascular disorders. The LDH biosensor measured the lactic acid level in range of 600-1360 μM in healthy person while in cardic patients it was in the range of 17000-22200 μM [57].

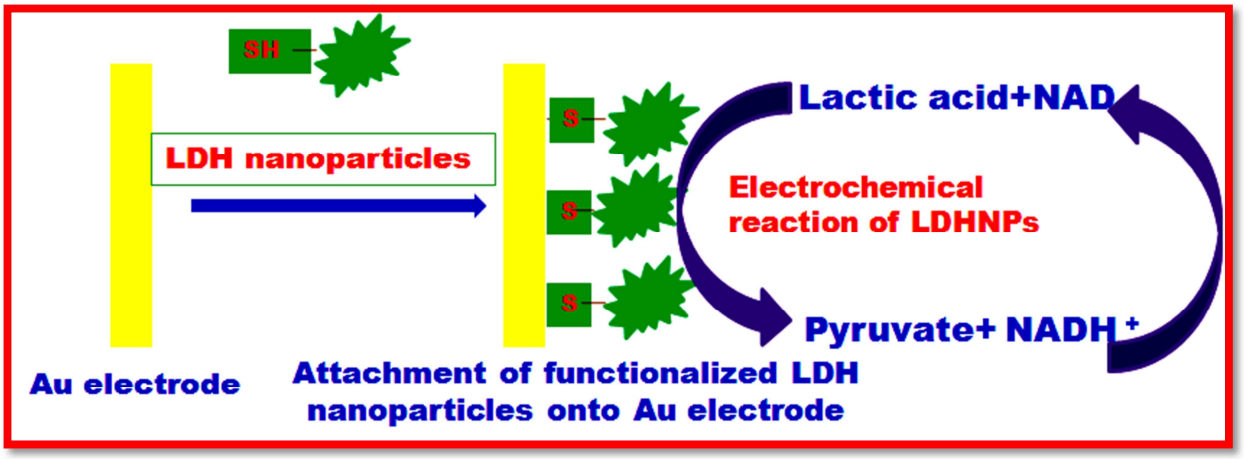


Figure 3(i): Schematic representation of functionalized LDHNPs immobilization and electrochemical reaction at Au electrode [61].

7.1.1.1.1.10 Fabrication of sarcosine biosensor based on sarcosine oxide (SOx) nanoparticles

An improved amperometric sarcosine biosensor was developed by immobilizing NPs of sarcosine oxide (SOx) (Figure (j)) [55]. SOxNPs were characterized by various techniques including SEM, FTIR and EIS. SOx biosensor worked optimally at pH 6.5, temperature 35 °C, 2 s response time when applied a potential of 0.1 VAg/AgCl (Figure 3(j)).

Analytical evaluation of SOxNPs/AuE

The working range of SOxNPs/AuE was 0.1-100 µM with 0.01 µM LOD. The within and between batch of coefficient of variation of SOx biosensor was 0.083% and 0.067% and analytical recovery of added 0.5 µM and 1.0 µM sarcosine concentration was 94.22 % and 97.81% respectively. The storage stability of SOxNPs/AuE was 180 days [55].

Medical implication of SOxNPs/AuE

SOxNPs/AuE was used for the measurement of sarcosine level in the patients suffering from prostate cancer. The above constructed biosensor measured the sarcosine level ranges from 1.2-3.2 µM in normal person and 9.8-21.7 µM in patients of prostate cancer [55].

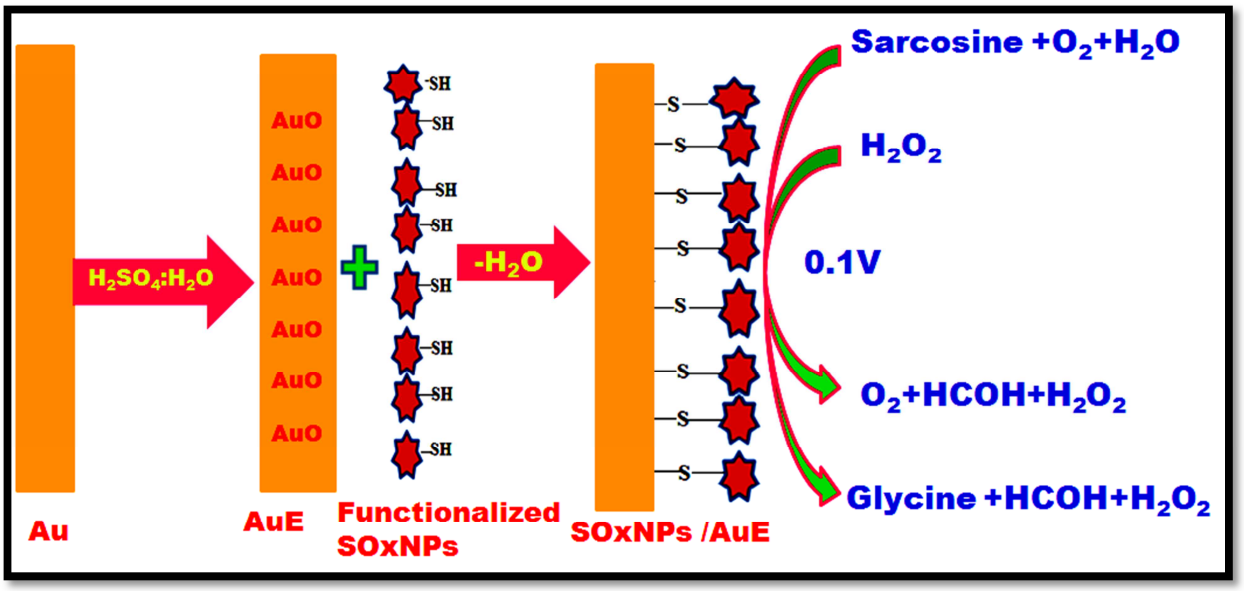


Figure 3(j): Diagrammatic illustration of functionalized SOxNPs immobilization and electrochemical reaction at Au electrode [55]

7.1.1.1.1.11 Fabrication of pyruvate biosensor based on pyruvate oxidase (POx) nanoparticles

Malik et al. (2019) have developed an amperometric pyruvate biosensor by immobilizing the NPs of pyruvate oxidase (POx) (Figure 3(k)). Techniques like TEM, FTIR and UV-spectroscopy was used for the characterization of POx NPs. These POxNPs were then immobilized on Au electrode to form the working electrode. The morphological investigation of working electrode was carried out by SEM and EIS. The developed biosensor was also studied

electrochemically by CV. The optimum pH and temperature of this biosensor was found to be 5.5 and 35°C respectively [37].

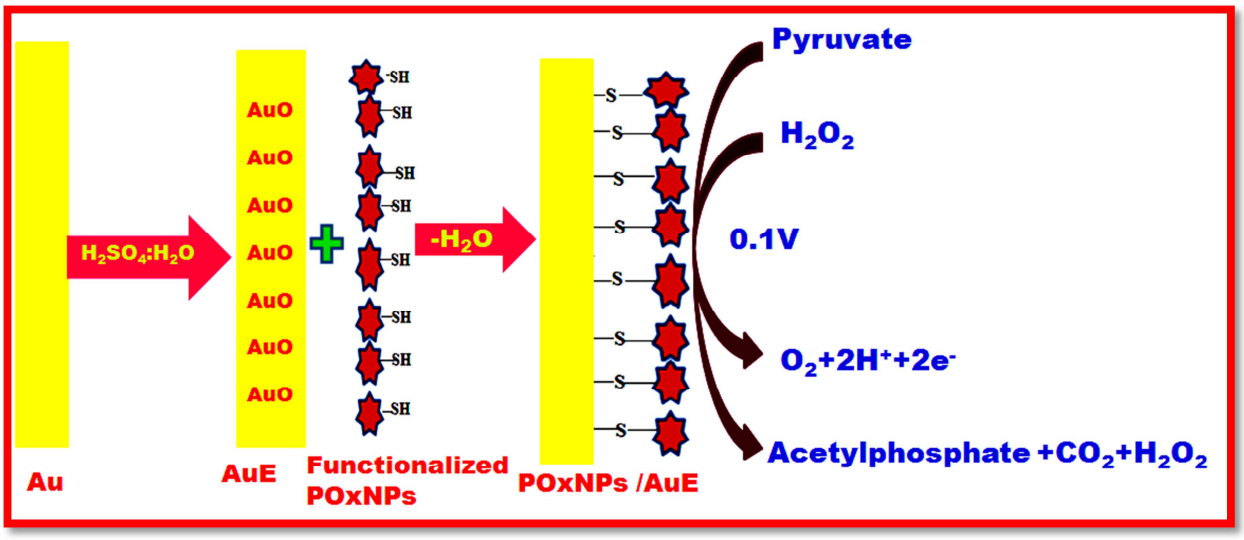


Figure 3(k): Diagrammatic illustration of functionalized POxNPs immobilization and electrochemical reaction at Au electrode

Analytical evaluation of POx NPs/AuE

The working range of POx NPs/AuE was noticed between 0.001-5000 µM. The developed biosensor exhibited the 0.67 µM LOD and 7.5 s response time. The analytical recovery of added known pyruvate concentration (1.0 mM and 2.0 mM) of POx NPs/AuE was 99% and 99.5%. Within-and-between coefficient of variation of this biosensor was 0.045% and 0.040% respectively. The stability of POx NPs/AuE was 240 days when stored at 4°C [37].

Medical Implication of POx NPs/AuE

The abovementioned biosensor was used for the determination of pyruvate level in real samples. Because abnormal level of pyruvate in serum raised the health issues like cardiovascular diseases, autism, coronary infarction and diabetes.The POx NPs/AuE measured the puruvate concentration in range of 147.2-212.4 µM in cardic patients [37]

7.1.1.1.1.12 Fabrication of glycerol biosensor based on glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO) and graphene oxide (GrO) nanoparticles

An amperometric glycerol biosensor was constructed by co immobilizing NPs of GK, GPO, Graphene oxide (GrO) onto pencil graphite electrode (Figure 3(l)) [36]. These enzymic NPs were investigated by TEM and UV spectroscopy. Functional pencil graphite electrode was designed by immobilization of GK NPs, GPO NP and GrO NPs and characterized by using SEM, EIS and CV experiments. The developed biosensor work optimally in reaction buffer with pH 8.0 and 35°C when applied a working potential of 0.45 V [36].

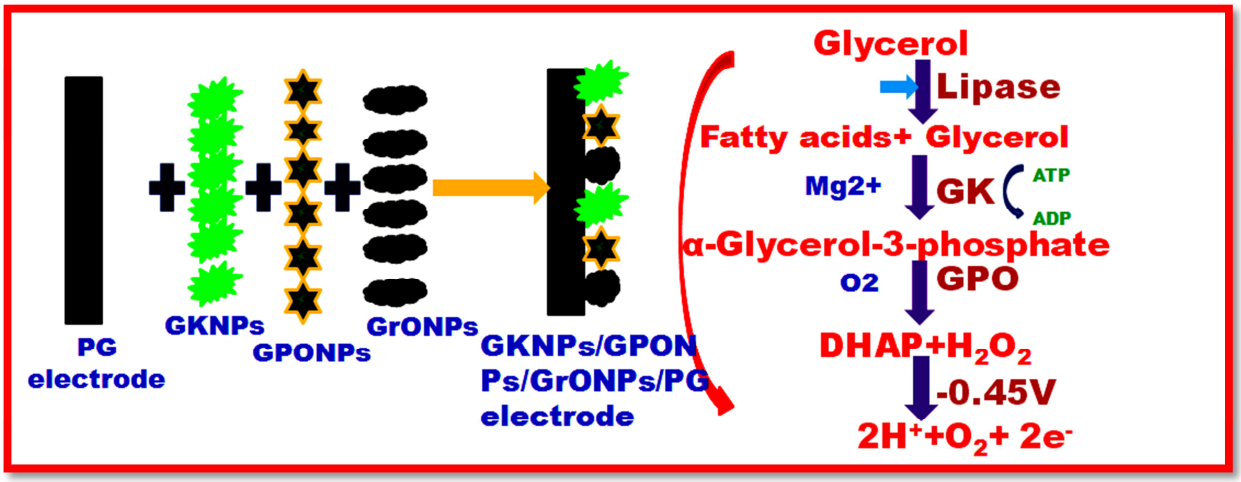


Figure 3(l)): Diagrammatic illustration of functionalized immobilization of GKNPs, GPONPs and GrONPs and electrochemical reaction at PG electrode [36]

Analytical evaluation of GKNPs/GPONPs/GrONPs/PGE

The analytical recovery of GKNPs/GPONPs/GrONPs/PGE was 98.5% and 96.3% at 0.5 mM and 1.0 mM glycerol concentration. The response time of developed GKNPs/GPONPs/GrONPs/PGE was very less i.e. 2 s, with 0.002 µM LOD and storage stability

210 days. Within-and between-batch coefficient of variation was 0.098% and 0.101% respectively. The sensitivity of this biosensor was 121.45 μAmM^{-1}. The working range of GKNPs/GPONPs/GrONPs/PGE was observed between 0.001-60 mM [36].

Medical implication of GKNPs/GPONPs/GrONPs/PGE

The aforementioned biosensor was employed for the analysis of glycerol. Glycerol is an important marker of cardiac diseases. This biosensor was used for the sample investigation for glycerol concentration in sera samples of both healthy as well as person with cardiovascular diseases. GKNPs/GPONPs/GrONPs/PGE was measured the glycerol concentration in the range of 40-70 μM for healthy individuals and 1500-12400 μM for diseased individuals [36].

Advantages of ENPs based amperometric biosensor

These ENPs based amperometric biosensors have shown unique sensitivity, stability, fast, cost-effective, better analytical performance and disposable in comparison to conductometric and potentiometric biosensors [62] [63].

Disadvantages of ENPs based amperometric biosensor

These biosensors have exhibited poor selectivity and working of these devices depends on the reaction environment i.e. pH and temperature.

7.1.1.1.2 ENPs based potentiometric biosensors

Potentiometric biosensors based on ENPs measure the voltage difference between the reference electrode and the functional/working electrode at zero current flow (Figure 4(a)). This difference in voltage relies on analyte concentration. In these biosensors, a steady potential is produced by reference electrode, while the working electrode generates variable potential

depending on analyte concentration [64]. They measure the altered ionic species using ion selective electrodes such as pH, ammonia selective and CO_2 selective electrodes which during electrochemical reactions either release or absorb the H^+ or NH_4^+ ions.

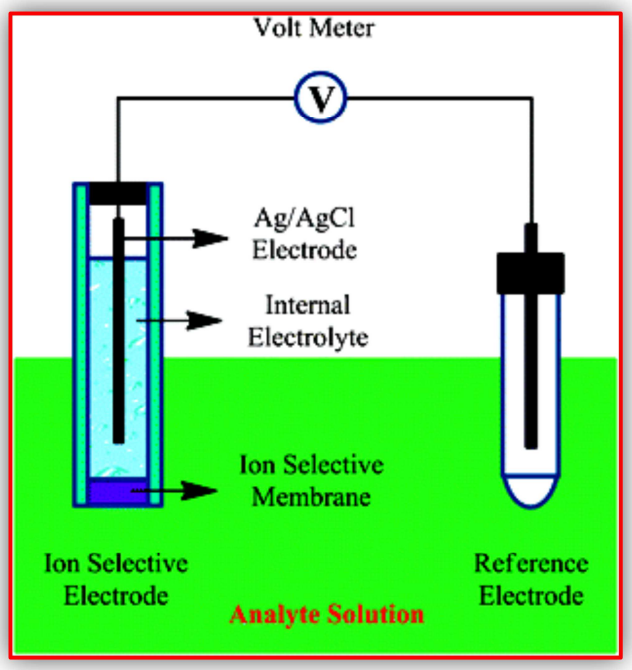


Figure 4(a): Diagrammatic depiction of ENPs based potentiometric biosensors [65]

Principle

Potentiometric ENPs biosensors employ ion-selective electrodes for transducing the biological reaction into an electrical signal. They consist of an immobilized enzyme membrane surrounding the probe from a pH-meter, where during redox reactions generate or absorb H^+ ions. The reaction on thin sensing glass membrane alters the pH which is directly monitored from the pH-meter. Ion-selective field effect transistor (ISFET) is one of robust platform for developing potentiometric biosensors. Approaches like covalent attachment or polymer entrapment have been employed for the immobilization of ENPs to the gate surface of a FET.

These ISFET devices are small in size and low weight and hence can be used for designing a portable monitoring system. The selectivity and analytical performance of ENPs based potentiometric biosensors can be enhanced by developing a nanoscale device and eliminating the nonspecific molecular adsorption. Researchers have electronically analysed the biomolecules by monitoring the charge density variation by using ISFETs [66].

7.1.1.1.2.1 Biosensing applications of ENPs based potentiometric biosensors

Potentiometric ENPs based biosensors have also been used medically. Though, reports related to ENPs are very limited. Some of ENPs based potentiometric biosensor is here under:

7.1.1.1.2.1.1 Fabrication of urea biosensor based on urease nanoparticles

Jakhar and Pundir, 2018 have prepared NPs of urease enzyme extracted from jack beans *Canavalia ensiformis* by desolvation method. The structural investigation of ENPs was carried out by TEM, UV and FTIR and immobilized onto nitrocellulose (NC) membrane functionalized with chitosan by cross-linking with glutaraldehyde. UV-absorption of monomeric urease shows absorption maxima at 280 nm which is due to absorption by peptide bond and aromatic amino acids while the absorption maxima of urease NPs was at 230 nm. This NC membrane with ENPs was mounted onto ammonium ion selective electrode (AISE) and linked to digital pH meter to fabricate the potentiometric urea biosensor (Figure 4(b)). This urea biosensor produced optimum result within 10 s at pH 5.5 and temperature 40°C [28].

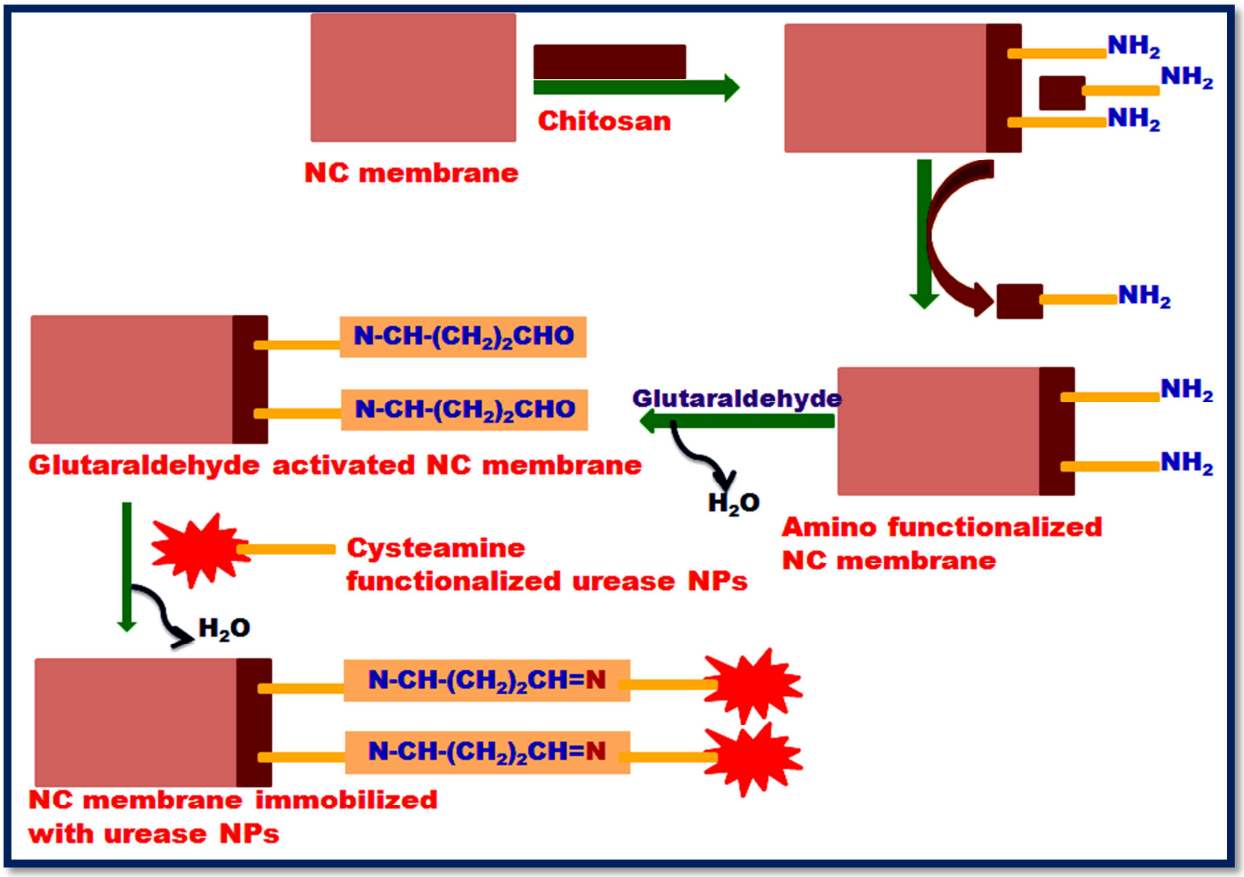


Figure 4(b): Schematic illustration of confining of functionalized urease NPs and potentiometric reaction onto NC membrane [28].

Evaluation of analytical parameters of Urease NPs/NC membrane

The detection limit of urea biosensor was 1 µM/L, analytical recovery 106.33%, within and between co-efficient of variation were 0.18% and 0.15% respectively. The storage stability of urea biosensor was 120 days at 4°C.

Medical implication of Urease NPs/NC membrane

The urea biosensor was used to analyze urea in serum sample of both healthy and diseased patients which was found to be 5.3-13.3 mmol/L and 99.6-147.18 mmol/L respectively. Normal

range of urea in human body is 2500-7500 μM [28]. Abnormal urea level in urine and blood is important indicator to monitor the function of kidney and liver, consequently rapid catabolism of protein components, person may undergo congestive heart failure/attack, requirement of elevated protein diet, malnutrition, shock and stress [28].

7.1.1.1.2.1.2 Fabrication of glyphosate biosensor based on urease nanoconjugates

A potentiometric biosensor was fabricated for the investigation of glyphosate by immobilizing the nanoconjugates of urease enzyme and Au NPs onto NH_4^+ selective electrode. These urease nanoconjugates were characterized by TEM and FTIR spectroscopy [67].

Evaluation of analytical parameters of Urease nanoconjugates on ammonium ion selective electrode

Urease nanoconjugates based potentiometric biosensor showed 0.5 ppm LOD and a working range between 0.5-50 ppm. Sensitivity of aforementioned biosensor was 280 mV/decade with storage stability of 180 days [66].

Implication of urease nanoconjugates

This biosensor was used for the monitoring of glyphosate herbicide in spiked water samples. Glyphosate is a well known nonselective herbicide. Introduction of glyphosate at its higher concentration causes damage to endocrine system of living organisms. The concentration of glyphosate measured by this biosensor was ranges between 20-60 ppm [67].

Advantages of potentiometric biosensors

These ENPs biosensors have shown good linear range, less response time, rapid, stable, no use of analyte and contamination free. It remains unaffected by color or turbidity.

Disadvantages of potentiometric biosensors

The ion selective electrodes used in these potentiometric devices reduce the reproducibility of the sensor. Moreover, the electrodes are more susceptible for spoilage by proteins or other organic solutes consequently, intervention caused by other ions. Electrodes used in these biosensors are very week with minimum life period.

1 **Table 3: Comparison of various kinetic and analytical properties of ENPs based biosensors**

Characteristics	**Glucose Oxidase NPs**	**Cholesterol Oxidase NPs**	**Uricase NPs**	**Creatinine (CA), Creatinase (CI) and Sarcosine oxidase (SOx)NPs**	**Urease NPs**	**Lipase/ Glycerol kinase (GK) / Glycerol-3-phosphate (GPO)NPs**	**Glycerol kinase (GK)/Glycerol-3-phosphate (GPO) NPs**	**Lactic acid dehydrogenase (LDH)NPs**	**Sarcosine Oxidase (SOx) NPs**	**Horseradish peroxidase (HRP)NPs**	**Cholesterol esterase/ Cholesterol oxidase NPs**
Property	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs	Immobilized NPs
Support used	NC membrane	Au wire	Au wire	Glassy carbon electrode	NC membrane	Au electrode	Au electrode	Au electrode	Au electrode	Au electrode	Au electrode

Method of immobilization	Covalent	Covalent	Covalent	Covalent	Covalent	Covalent	PGE	Covalent	Covalent	Covalent	Covalent
Type of electrode	DO metric	Amperometric	Amperometric	Amperometric	Potentiometric	Amperometric	Amperometric	Amperometric	Amperometric	Amperometric	Amperometric
Optimum pH	6.0	6.0		6.0	5.5	6.5	7.0	7.0	6.5	7.4	5.5
Optimum temperature (°C)	35-45	35	20	25	40	35	30	35	35	NA	40
Incubation time (s)	60	8	2	2	10	5	2.5	2.5	2	<5	5

K_m (mM)	4.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Linearity (mM)/mg/dl	0.056-1.39	0.69-38.88	$1e^{-7}$-100mM	0.00001-0.012	0.002-0.08	1.0-100	0.01-45	0.00001-55	0.0001-0.1	0.001-0.009 micromolar	10-700
Limit of detection (µM)	0.55	0.04	$1e^{-7}$	0.01	1.0	1000000	0.0001	0.01	0.01	0.1	0.002
Half life at 4°C in days	180	90	210	240	120	90	180	210	180	NA	60
No. of reuses	150	180	200	20	8-9	NA	NA	NA	NA	NA	200

Analytical Recovery in %	NA	NA	99.57 and 98.77	97.97 and 98.76	106.33	95.2 and 96.0	98.73	98.61	94.22 and 97.81	NA	94.73 and 96.33
Precision in %	NA	NA	5.6 and 4.7	2.06 and 3.09	0.18 and 0.15	2.33 and 2.15	0.105 and 0.14	1.38 and 1.03	0.083 and 0.067	2.54	<2 and <3
Application	Determination of dissolved O_2 level	Determination of cholesterol level in serum samples	Determination of uric acid in serum samples	Determination of creatinine level in serum samples	Determination of urea in serum samples	Determination of triglyceride in serum samples	Determination of glycerol level in serum samples	Determination of lactic acid level in serum samples	Determination of sarcosine in serum samples	Determination of H_2O_2 level in serum samples	Determination of cholesterol level in serum samples
References	[28]	[13]	[14]	[25]	[29]	[25]	[60]	[54]	[55]	[11]	[26]
Abbreviation: NA-Not available; DO-Deoxymetric; GC- Glassy carbon; PG- Pencil graphite; NC- Nitrocellulose; ITO-Indium tin oxide											

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8. Future Prospects

The exploitation of paper based devices in portable biosensors has aroused the interest of medical and environment sector. The electrochemical microfluidic devices are not only disposable but also offer a number of extraordinary characteristics such as facile, economic, huge production, sensitive, specific, low limit of detection and minimum electricity requirement. Therefore, use of electrochemical paper analytical devices act as an ideal platform for the diagnosis of number of diseases [23] [68]. However, to the best of our knowledge there is no lab on chip based on ENPs has been reported for the diagnosis of various serious dissorders so far. Hence, future research should be focused on for the fabrication of such bioelectronic and lab on paper chip to form entire automatic portable devices which could be employed on the bedside of the patient. Microfluidic devices have exhibited several fascinating advantages viz. requirement of minimal sample, less laborious and facile approach, economic and quick response. These attractive characteristics of lab on chip and bioelectronic devices would facilitate the detection of various analytes in biological samples. In our opinion use of ENPs based nanostructures development by dip-pen nanolithography act as a blueprint and enzymes as templates for the designing of nanostructures, which can opened vast opportunities in the future nano techniques. Furthermore, miniaturization and development of wearable ENPs based biosensors could be more beneficial for the better health of mankind.

9. Summary and conclusion

In conclusion, biosensing techniques have been considered more superior than conventional colorimetric, enzymatic colorimetric, spectrophotometric, chromatographic and fluorescence techniques for the investigation of analytes as these techniques have offered simple

fabrication approach, more sensitive and selective, disposable, work with inclusive computerized and give fast outcomes. Present review deciphers the comprehensive report of successfully developed ENPs based biosensors which use optimum parameters such as choice of enzyme, method of immobilization and transduction mode. This review explain the apparent research for the fabrication of ENPs based biosensors with prominence on methods of ENPs preparation, numerous techniques of characterization, methods of ENPs immobilization and the distinct types of transducers used. The comparative analytical features of ENPs based biosensors have been summarized in Table 3. Present study reveals the significance of ENPs biosensors encircling its area of application from investigation of clinical samples. Immobilization of ENPs onto a matrix is essential for the development of electrochemical biosensor. A number of electrode materials such as Au electrode, nitrocellulose membrane, glassy carbon electrode and pencil graphite electrode have been used to obtain ENPs based biosensors. Recently, ENPs or composite ENPs have been used for the development of amperometric or potentiometric biosensors. Hence, without any suspicion, several ENPs biosensors have been documented to date and have various constructive impacts for the analysis of real samples. The ENPs based biosensor reported so far work ideally within 2-300 s, pH range 5.0-6.0, temperature 3.5-45°C and linear range 0.1 nM-100 mM with 0.2 nM-0.55 mM limit of detection. These biosensors have been employed for the diagnosis of a number of serious ailments such as cardiovascular diseases, renal disorders, diabetes, environment monitoring, and biochemical engineering and reused upto 8-200 times while stored for 60-240 days dry at 4°C.

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Highlights

- Review illustrates the various methods of enzyme nanoparticles (ENPs) preparation, characterization techniques, methods of immobilization and applications of various ENPs based biosensor along with their analytical evaluation and medical implication.
- ENPs based biosensors exhibited novel fascinating properties such as exceptional selectivity, sensitivity, optical and electrical conductivity, large surface area.
- ENPs based biosensors work optimally within 2-300s, in pH range 5.0-6.0 and temperature 3.5-45°C, linear range 0.1nM-100mM with 0.2nM-0.55mM detection limit.
- ENPs based biosensors have been employed for the diagnosis of various diseases such as cardiovascular diseases, renal disorders, diabetes, environment monitoring, and biochemical engineering and reused upto 8-200 times over a period of 60-240 days while stored dry at 4°C.
- The future research could be focused on designing of electronic chip for ENPs based biosensors to develop an entire automatic portable device, which can be used at the beside of patients.