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# A novel inhibition based biosensor using urease nanoconjugate entrapped biocomposite membrane for potentiometric glyphosate detection

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ABSTRACT

A potentiometric biosensor based on agarose-guar gum (A-G) entrapped bio-nanoconjugate of urease with gold nanoparticles (AUNps), has been reported for the first time for glyphosate detection. The biosensor is based on inhibition of urease activity by glyphosate, which was measured by direct potentiometry using ammonium ion selective electrode covered with A-G-urease nanoconjugate membrane. TEM and FTIR analysis revealed nanoconjugate formation and its immobilization in A-G matrix respectively. The composite biopolymer employed for immobilization yields thin, transparent, flexible membrane having superior mechanical strength and stability. It retains the maximum activity (92%) of urease with negligible leaching. The conjugation of urease with AUNps allows improvement in response characteristics for potentiometric measurement. The biosensor shows a linear response in the glyphosate concentration range from 0.5 ppm–50 ppm, with limit of detection at 0.5 ppm, which covers maximum residual limit set by WHO for drinking water. The inhibition of catalytic activity of urease nanoconjugate by gyphosate was confirmed by FTIR analysis. The response of fabricated biosensor is selective towards glyphosate as against various other pesticides. The biosensor exhibits good performance in terms of reproducibility and prolonged storage stability of 180 days. Thus, the present biosensor provides an alternative method for simple, selective and cost effective detection of glyphosate based on urease inhibition.



## 1. Introduction

Glyphosate (N-(phosphonomethyl)glycine) is recognized as the world's most widely used weed killer. It is broad-spectrum, non selective herbicide, used for control of long grasses, broad-leaved weeds etc. and has been used in excess amount for the majority of crops just before harvest [1,2]. Glyphosate was regarded as a controversial ingredient in Monsanto's Roundup herbicide on the basis of its toxicity. Later on, in 2015 it is classified as a probable carcinogen by the International Agency for Research on Cancer [3,4]. The recent studies also showed glyphosate as a potent endocrine disruptor and linked to birth defects in laboratory animals [5,6]. The literature describes the escalating use of glyphosate in the past few years; making it the most widely and heavily applied weed-killer in the history of chemical agriculture [7,8]. Considering its rate of consumption, widespread applications and toxic effect on human being there is an urgent need for the detection of glyphosate in drinking water and for agricultural products such as crops, fruits and vegetables. Further, the estimation of this herbicide at the levels established by the Environmental Protection Agency (EPA) and World health organization (WHO) is essential though challenging.

Various analytical methods using ion exchange chromatography, gas chromatography, liquid chromatography (HPLC), capillary electrophoresis and different types of spectroscopy have been described for the detection of glyphosate which possess certain advantages and few limitations [9–14]. The development of biosensor represents a potential alternative to these techniques in order to offer advantages such as ease of operation, lesser detection time, low cost and on-site monitoring as well.

Enzymes exhibit number of features that make their use advantageous. Foremost among them are a very high level of catalytic efficiency and a high degree of specificity that allows them to discriminate between the substrate and inhibitors. Additionally, enzymes generally operate at mild conditions of temperature

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and pH with appreciable reaction rates. For pesticide detection enzyme inhibition based biosensors are well reported [15–20]. These biosensors are based on specific inhibition of enzyme activity by target analyte resulting in the decrease of signal output, proportional to the target analyte. For glyphosate detection only few reports are available on enzyme inhibition based biosensor. In this context, only peroxidase inhibition based biosensors have been reported for glyphosate. Songa et al. have constructed a biosensor by immobilizing horseradish peroxidase electrochemically on the surface of the gold electrode modified with poly(2,5-dimethoxyaniline)-poly(4-styrenesulfonic acid) composite film [21]. Oliveira et al. have fabricated glyphosate biosensor using peroxidase (from atemoya, a hybrid fruit) immobilized on nanoclay along with graphite powder and multiwalled carbon nanotubes [22]. Both biosensors exhibited good performance in terms of sensitivity at very low concentration of glyphosate though it involves complicated synthesis process, complex enzyme electrode assembly and sophisticated measurement system. The highly selective immunosensor is also reported for glyphosate detection. However, the selectivity is shadowed by high material cost [23]. Chemiluminescence sensor based on acrylamide molecular imprinted polymer was developed for glyphosate detection by P. Zhao et al. [24]. The nanocomposite of copper oxide and multiwalled carbon nanotube was used to construct fluorescence sensor for glyphosate estimation. The detection principle is based on inhibition of peroxidase-like catalytic activity exhibited by the nanocomposite, which was measured using amplex red that tend to produce florescent product on oxidation by nanocomposite. In the presence of glyphosate the oxidation of amplex is hampered [25]. Both the methods require spectrophotometer for estimation of emitted light intensity. In view of this, biosensor development is essential for providing a cost effective estimation of glyphosate with acceptable sensitivity and selectivity. It should also have the simplicity of detection, construction and operation along with portability for on-site monitoring.

In the present report, an attempt has been made to fabricate glyphosate biosensor based on urease inhibition. Urease is ubiquitously found in number of plants and vegetables. It is reported to be thermostable and shows activity over a wide pH range. Most importantly, the quantification of enzymatic product is relatively simple by direct potentiometry or photometry. The miniaturized, portable and low cost devices are well documented for urease based biosensor in detection and estimation of urea; therefore fabrication of glyphosate sensor based on urease inhibition may have an advantage in terms of its practical utility [26,27].

The most critical step in the construction of a biosensor is immobilization of enzyme in suitable matrix, which determines the performance of the biosensor. The naturally available biopolymers are preferable for enzyme immobilization as against synthetic polymers due to their biocompatible and eco-friendly nature. Agarose (A) and guar gum (G) are highly hydrophilic and non-toxic biopolymers which provide a natural environment for the enzyme after immobilization. The formation of composite between agarose and guar gum allows modulation of porosity, which is essential for proper confinement of enzyme with minimum leaching. Moreover, the agarose-guar gum composite (A-G) can form thin, transparent, self supported membrane with appreciable mechanical stability. Because of these characteristics, the A-G biocomposite was successfully utilized by our group, for immobilization of tyrosinase and invertase in the construction of biosensors [28,29]. This has led to grow our interest in using agarose-guar gum composite for urease immobilization, and biosensor fabrication.

The interfacing of nanoparticles with biomolecules such as enzymes for sensing is a newer approach for enhancing the performance of biosensor due to the synergistic effect of the two components [30]. Nanoparticles can offer many advantages, such as large surface-to-volume ratio, high surface reaction activity and strong adsorption ability for biomolecules [31]. Gold nanoparticles, in particular, have been widely used to construct biosensors because of their chemical and biological inertness. Many types of biosensors, such as enzyme sensor, immunosensor and DNA sensor, with improved analytical performances have been prepared based on the application of gold nanoparticles [32–36]. Gold nanoparticles (AuNPs) are not only better electrical conductor, but also offer good microenvironment for retaining the activity of the enzyme [37]. They can bind directly to enzymes without disrupting its biological recognition properties [38]. Nowadays, it is revealed that AUNPs also exhibit excellent catalytic effect on many important chemical reactions [39]. In addition, AUNPs are able to reduce the insulating effect of the protein shell and thus enhance electron transfer in the reaction processes [40].

In view of this, the formation of nano-bioconjugate of urease with gold nanoparticles was carried out and immobilized in agarose-guar gum matrix, which was employed for biosensing purpose. Further, the urease and urease nanoconjugate entrapped membranes were checked for potentiometric response towards urea. Depending upon the analytical performance the appropriate membrane was chosen to design a biosensor for glyphosate detection based on urease inhibition.

## 2. Experimentation

### 2.1. Chemicals and reagents

Urease (EC 3.5.1.5) was extracted from the seeds of *Dolichos biflorus* in 0.1 M phosphate buffer (pH 7) and partially purified by ammonium sulfate fractionation (30–70%) in our laboratory which was used as a source of enzyme. Agarose having medium EEO was obtained from Sigma Aldrich and guar gum was obtained from SRL-India. Urea, gold (III) chloride trihydrate, sodium citrate were purchased from Sigma Aldrich. Technical grade glyphosate (95% pure) and other pesticides were obtained from Crop Life Science Ltd., India. All other chemicals were of analytical grade and were used as required without further purification.

### 2.2. Methods

#### 2.2.1. Synthesis of gold nanoparticles

Gold nanoparticles were prepared as described by Kimling et al. [41]. The solution of $HAuCl_4$ (0.25 mM in 50 ml of mili-Q water) was heated on a hot plate, with continuous stirring. As the solution began to boil, 0.5 ml of sodium citrate (1%) was added dropwise until the color changed from yellow to deep red, indicating formation of gold nanoparticles (AuNps). It was further heated for approximately 20–25 min, until the solution turned wine red. This solution of AUNps [2.54 nM, as determined by UV–vis spectra [42]] was allowed to cool and stored at room temperature for further use. The formation of gold nanoparticles was confirmed by UV–vis spectrophotometer, which showed a characteristic absorption peak around 525 nm corresponding to AUNps.

#### 2.2.2. Formation of enzyme nanoconjugate

Urease nanoconjugate was obtained by mixing 0.2 ml of partially purified enzyme extract with 0.5 ml of AuNps solution (2.54 nM), followed by incubation for 1 h at 37 °C. To confirm the conjugate formation, transmission electron microscopy (TEM) was carried out for AuNps with and without urease. The TEM images were taken with FEI model (Techanai G2-20) using a copper grid. Dynamic light scattering analysis was carried out using Ultrafine Particle Analyzer from Leeds and Northrup instrument. The enzymatic activity of urease and urease nanoconjugate were determined spectrophotometrically, using Phenol hypochlorite assay at 660 nm [43]. The

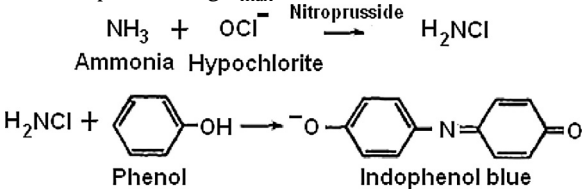
activity of the enzyme was expressed as μmoles of ammonia formed/min/ml of enzyme.

### 2.2.3. Immobilization of urease and urease nanoconjugate in agarose-guar gum matrix

The immobilization of urease and urease nanoconjuagate was achieved by an entrapment method in agarose-guar gum matrix. For immobilization of enzyme, aqueous solution of agarose (3% W/V) and guar gum (1% W/V) were mixed in 1:1 proportion (2 ml), with the appropriate amount of urease [180 units + 0.5 ml buffer] and urease nanoconjugate [180 units + 0.5 ml of AuNp solution (2.54 nm)]. The mixture was spread uniformly on a thick plastic sheet (3.75cm × 1. 25 cm), and kept overnight in dust-free hood for drying. The thin, flexible, self supported enzyme membranes obtained after drying were stored at 4 °C under dry environment for further use. The blank membranes (without enzyme) were prepared under identical conditions for comparison.

### 2.2.4. Determination of entrapment efficiency

For the determination of activity and leaching after enzyme immobilization, biopolymer membrane containing urease was taken and rinsed with assay buffer to remove enzyme molecules adhered to the surface of membrane or loosely attached to carrier matrix. For leaching studies, a small piece of immobilized urease membrane ($1 \times 1\,cm^2$) was kept in the assay buffer for 20 min. After that the buffer was decanted to another tube and activity of decanted solution was determined. While, the enzyme activity of immobilized membrane was checked by incubating small piece of membrane ($1 \times 1\,cm^2$) with substrate (0.2 mM urea) and 0.1 M of phosphate buffer (0.8 ml, pH 7.5). Ammonia generated due to enzymatic reaction was determined spectrophotometrically using Phenol hypochlorite assay. In this assay ammonia reacts with hypochlorite and phenol in presence of catalyst (sodium nitroprusside) under alkaline conditions to produce indophenols, a blue coloured complex, having $\lambda_{max}$ at 660 nm.

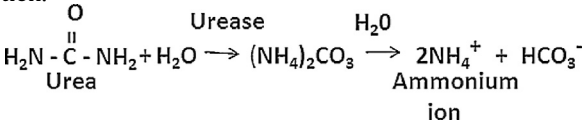



The entrapment efficiency of membrane with urease alone and urease nanoconjugate was determined in terms of percent retention of activity as compared to the soluble enzyme. The biochemical characteristics and kinetic parameters (Km and Vmax) of immobilized urease nanoconjugate were also determined spectrophotometrically by Phenol hypochlorite assay, in order to study the effect of conjugation of urease with AUNps and further its immobilization in agarose-guar gum matrix.

### 2.2.5. Potentiometric measurement of urea

**a. Experimental setup**

Potentiometric measurement of urea was carried out in a small reaction cell with commercially available ammonium ion selective electrode (Elite 8051) and double junction Ag/AgCl reference electrode, with outer reference solution of 1 M $CaCl_2$. The schematic of experimental setup is shown in Fig. 1. Ammonium ion selective electrode was used for the measurement of ammonium ions formed by the catalytic action of urease on urea according to following reaction.




The ion selective electrode was made sensitive to urea by attaching urease immobilized membrane ($1.5 \times 1.5\,cm^2$) on the tip of the electrode, by using an O-ring. An operational amplifier was designed to amplify the sensor output. At the first stage a low offset voltage op-amp (CA 3140 EZ) was used as a unity gain follower. The potential difference developed between ammonium ion selective electrode and reference electrode was amplified 10 times, with additional op-amp inverter (UA 741 CP), at the second stage. The electronic circuit diagram of the amplifier is shown in Fig. 1. The output from the amplifier was measured with the help of a digital multimeter.

**b. Measurement of urea with biosensor assembly**

The enzyme membrane attached to an ammonium ion selective electrode was allowed to equilibrate in buffer for 15 min. For biosensor measurement the electrode with immobilized membrane was immersed in 2 ml of 0.1 M Tris buffer (Tris-HCl, pH 7.0), for 2–3 mins in order to achieve baseline potential. The response time of biosensor with urease and urease nanoconjugate immobilized membrane attached to an ion selective electrode was determined by measuring the output voltage as a function of time upto 15 min with regular interval of 1 min, at urea concentration of 0.1 mM.

The calibration curve was established with the addition of standard urea solution in the concentration range of $5 \times 10^{-6}$ M to $1 \times 10^{-1}$ M. The response in terms of change in the output voltage was recorded for ion selective electrode covered with urease and urease-nanoconjugate immobilized membrane.

### 2.2.6. Glyphosate detection

The preliminary experiment with photometry using Phenol hypochlorite assay was carried out in order to study inhibitory effect of various pesticides on urease activity. For this purpose a small piece of urease immobilized membrane ($0.5 \times 0.5\,cm^2$) was allowed to incubate with 50 ppm of different pesticides (0.05 ml) and 0.1 M of phosphate buffer (0.850 ml, pH 7.5) for 20 min. Thereafter, 0.1 ml of urea was added (2 mM) and further incubated at 37 °C (20 min) for the enzymatic reaction. The ammonia formed after the enzymatic reaction in presence and in absence of pesticide was estimated by Phenol hypochlorite assay. The percent inhibition of urease by pesticides was calculated using the equation $(\%I) = [E_o\text{-}E_p/E_o] \times 100$. Where, $E_o$ is the absorption intensity at 660 nm in absence of an inhibitor and $E_p$ is the absorption intensity at 660 nm in presence of 50 ppm of different pesticides.

The percent inhibition was found to be maximum for glyphosate. Hence, we have evaluated feasibility of present biosensor for glyphosate detection based on urease inhibition. Also, in order to increase the sensitivity of detection of glyphosate the potentiometric transducer was employed rather than using photometry.

Glyphosate detection capability of the fabricated urea biosensor was investigated by measuring the inhibitory effect of this herbicide on urease activity at different concentrations of glyphosate (0.5 ppm–100 ppm). The degree of inhibition was determined potentiometrically. The inhibition measurements were performed with response time for 5 min at 0.1 mM urea and 15 min preincubation with glyphosate. The potential difference corresponding to ammonia formation decreases with preincubation with glyphosate. The percentage inhibition was calculated as $I\% = (V_0\text{-}V_i)/V_0 \times 100$, where $V_0$ is the potential difference in the absence of inhibitor, while $V_i$ is the potential difference obtained after incubation with glyphosate.

### 2.2.7. Type of inhibition

In order to study the kinetics and the mode of inhibition, the response of the biosensor to various concentrations of urea (0.01 mM–5 mM) was measured potentiometrically, using 0.1 M Tris

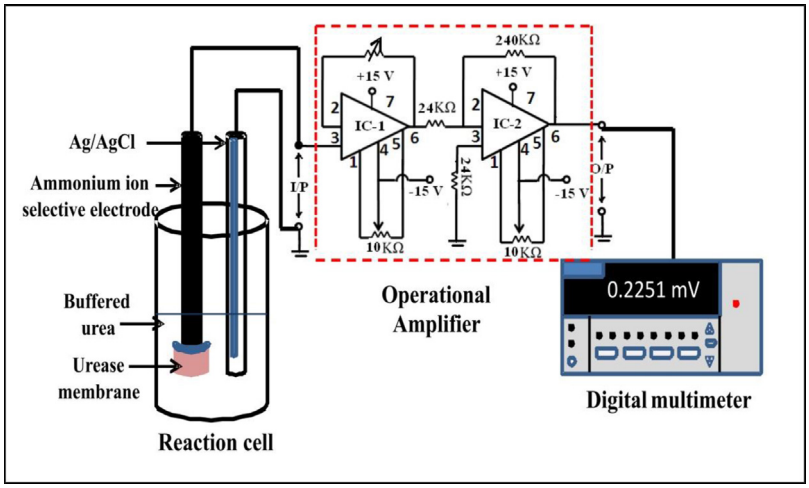



**Fig. 1.** Schematic representation of experimental set-up for potentiometric measurement with circuit diagram of the amplifier.

buffer (pH 7) in absence and presense of glyphosate (20 ppm and 50 ppm). A graph of 1/potential difference (ΔmV) Vs 1/[S] was plotted for each concentration of inhibitor added. From this electrochemical version of Lineweaver-Burk plot (L-B plot), the type of inhibition was determined.

#### 2.2.8. FTIR analysis

The FTIR analysis was carried out to understand the inhibitory effect of glyphosate on urease catalyzed reaction, using Jasco FTIR-6100. FTIR spectra of urease nanoconjugate in presence of urea, glyphosate and urea along with glyphosate were carried out in KBr pellet.

#### 2.2.9. Biosensor response characteristics

**a. Selectivity towards other pesticides**

The selectivity of sensor was established by measuring the potentiometric response of fabricated biosensor with different pesticides like dichlorvos, dimethoate, 2–4 dichlorophenoxyacetic acid (2–4D), hexaconazole, paraquat dichoride at a concentration of 50 ppm. The magnitude of % inhibition with all these pesticides were calculated and compared with glyphosate.

**b. Reproducibility and storage stability of the biosensor**

The reproducibility of the sensor was investigated by making potentiometric measurements with eight membranes obtained from different batches. One membrane from each batch prepared under identical conditions was selected for reproducibility study. The potentiometric measurements were carried out in standard urea solution (0.1 mM) using same experimental setup. For determining the storage stability, the potentiometric response of urease nanoconjugate membrane was measured with standard urea solution (0.1 mM) at regular interval of 30 days for 210 days, where membranes were preserved under dry conditions at 4 °C.

#### 2.2.10. Analysis of water samples

The applicability of the biosensor was tested for the analysis of spiked water samples. The tap water was fortified with three different concentrations of glyphosate (20, 40, 60 ppm). The magnitude of inhibition in these samples was measured and correlated with calibration graph. The percent accuracy for glyphosate detection by using proposed biosensor was determined for real sample analysis.

## 3. Results and discussion

### 3.1. Urease nanoconjugate and its immobilization

The formation of nano-bioconjugate of urease with gold nanoparticles was carried out and further immobilized in agarose-guar gum matrix for biosensing purpose. TEM was performed to ascertain the formation of urease nanoconjugate. TEM image of only gold nanoparticles showed free particles with size ranging from 14 to 23 nm (Fig. 2a,b) while Fig. 2c,d shows the formation of enzyme conjugate with gold nanoparticles. This observation was further confirmed by dynamic light scattering (DLS) studies (Supplementary information Fig.S1 a,b,c). Enzyme nanoparticle conjugate is obtained by interaction of nanoparticles with protein molecule by adsorption process which may involve hydrophobic or electrostatic interactions between them [44–47]. Further, conjugate formation between nanoparticles and protein depends upon size, shape, material of nanoparticles and also depends on secondary and tertiary structures of a protein [48,49]. Hence, in this process the orientation of enzyme (protein) and nanoparticles is very difficult to predict and needs detailed study.

The enzymatic activity of urease nanoconjugate was determined by Phenol hypochlorite assay. It was reported that the complex formation between jack been urease with silver nanoparticles resulted in drastic decrease in the enzyme activity [50]. In contrast with this, we have observed that urease nanoconjugate with AUNps ($896 \pm 20$ Units/ml) exhibits comparable activity with that of free urease enzyme ($900 \pm 23$ units/ml) thereby suggesting that the urease activity is not inhibited by gold nanoparticles.

Thin, transparent membranes with superior mechanical strength [tensile strength = $26.9 \pm 5$ MPa [51]] and stability were obtained by immobilization of urease and urease nanoconjugate in agarose-guar gum composite matrix. These urease membranes were used as a sensing component in the construction of the biosensor. The immobilization efficiency reported in the present study is much higher as compared with other reported biopolymers like agar, agarose, gelatin, alginate, DEAE-cellulose and chitosan used for urease immobilization [52–57].

The higher retention of enzyme activity with negligible leaching is probably due to fine tuning of porosity achieved by mixing two polymers in the desired proportion to form biopolymer composite. The thin, porous membrane allows the easy accessibility of immobilized enzyme towards substrate and the subsequent release of the product. The immobilization efficiency of urease was found to be 84%, while urease nanoconjugate showed effi-

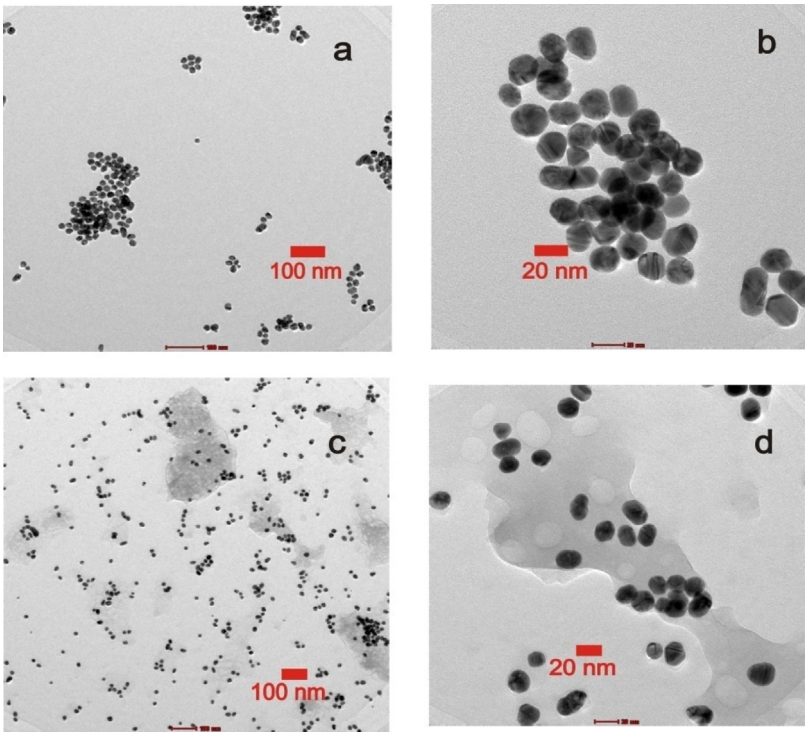



**Fig. 2.** TEM analysis of AUNps (a,b) and Urease-AUNps conjugate (c,d).

ciency of 92%, with negligible leaching. Similar observation has been reported for immobilization of acetyl cholinesterase in fenugreek hydrogel-agarose composite with gold nanoparticles [58]. Further, the entrapped urease nanoconjugate showed comparable values of Km and Vmax and other biochemical characteristics with that of free urease and urease entrapped in A-G matrix (Supplementary information Table S1), indicating no change in the affinity of the enzyme towards the substrate after conjugation. It also suggests that, there is no change in the microenvironment of the enzyme after conjugate formation of urease with AUNps and subsequent immobilization in the agarose-guar gum matrix.

The FTIR analysis was used to ascertain the presence of urease in both immobilization systems. The appearance of an additional peak at 1735 $cm^{-1}$ corresponding to amide-I vibration of protein in both urease and urease nanoconjugate immobilized agarose-guar gum membranes confirms the successful immobilization of urease in biopolymer matrix [59] (Supplementary information Fig.S2). Several other bands related to protein are overlapping with bands of biopolymers.

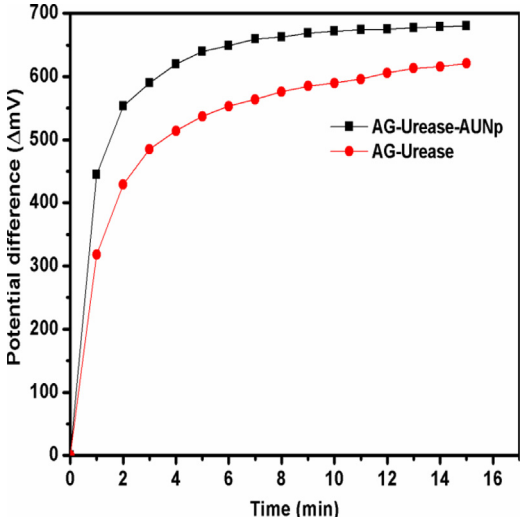



**Fig. 3.** Time response of biosensor with immobilized urease and urease nanoconjugate membrane attached to an ion selective electrode.

### 3.2. Potentiometric response of urease and urease nanoconjugate

The response time for an ion selective electrode covered with urease and urease nanoconjuagte membranes when studied as a function of time, 90% of response was achieved in 3–4 min for enzyme nanoconjugate while urease membrane took 7–8 min (Fig. 3). Urease nanoconjugate membrane needed lesser time for stabilization as compared with urease immobilized membrane.

The potentiometric response of the electrode was checked at the optimum response time for different urea concentrations to obtain a standard curve for urease and urease nanoconjugate membrane, attached to an ion selective electrode. There was a significant difference in potentiometric response of the two membranes (Fig. 4). In both the cases, linearity was observed in the range of $5 \times 10^{-5}$ M to $1 \times 10^{-1}$ M, for urea. The urea concentration up to $5 \times 10^{-6}$ M (S/N = 4 mV) can be detected by electrode covered with immobilized urease nanoconjugate, while for electrode with immobilized urease the detection limit was $1 \times 10^{-5}$ M (S/N = 5 mV). The magnitude of potentiometric response of an ion electrode with urease nanoconjugate membrane was found to be more as compared to urease immobilized membrane at all concentrations of urea (Fig. 4). The sensitivity of electrode with urease nanoconjugate was found to be 280 mV/decade ($R^2 = 0.976$). While, the sensitivity with urease entrapped membrane was 266 mV/decade ($R^2 = 0.970$).

Better response characteristics of the electrode with immobilized urease nanoconjugate may be due to the presence of gold nanoparticles, which are known for providing an active surface for electron transfer in the enzymatic reaction and also enhancing the sensitivity in electrochemical detection [60,61]. Therefore, based on these observations, urease nanoconjugate membrane attached

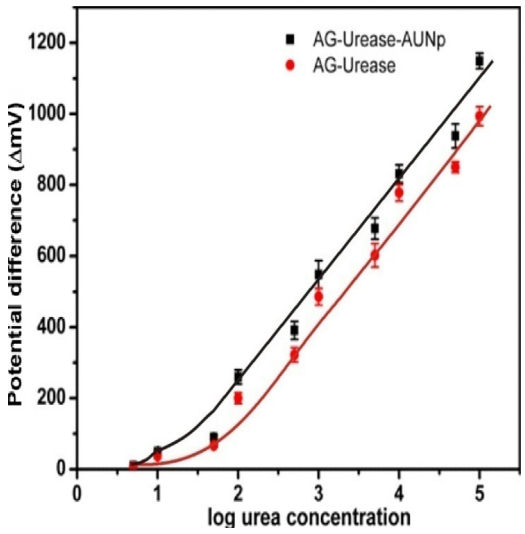



**Fig. 4.** Potentiometric response of an ion electrode with immobilized urease and urease nanoconjugate at different concentrations of urea.

to an ion selective electrode was chosen to design a biosensor for pesticide detection.

### 3.3. Detection of glyphoste by urease inhibition

A preliminary experiment with photometry assay was carried out in order to study the inhibitory effect of various pesticides on urease activity. Among the various other pesticides tested glyphosate showed maximum inhibition of urease activity (supplementary information Table.S2). While, for other pesticides no inhibition or relatively less inhibition was observed as compared with glyphosate. Therefore, urease inhibition based biosensor was developed for the detection of this herbicide.

For glyphosate detection, the initial potentiometric response of urease to urea, corresponding to initial activity of urease nanoconjugate was measured; followed by the measurement of potentiometric response after incubation with different concentrations of glyphosate. The potentiometric response in presence of glyhposate was found to be less as compared with the response without glyphosate. The calibration plot of percentage inhibition of urease activity corresponding to glyphosate concentration was constructed to obtain the working range and limit of detection. It was observed that the potentiometric response of biosensor decreases with increasing concentration of glyphosate. The proposed biosensor showed the linear dynamic range from 0.5 ppm to 50 ppm of glyphosate (Fig. 5), with a limit of detection at 0.5 ppm. The concentration of glyphosate showing 50% inhibition ($ID_{50}$) of urease activity was found to be around 45 ppm. The fabricated biosensor is capable of detecting glyphosate below the permissible value (0.9 ppm) set by World Health Organization (WHO) in drinking water [62].To the best of our knowledge, we are reporting for the first time the development of potentiometric biosensor for the estimation of glyphosate, based on urease inhibition.

### 3.4. Mode of inhibition

In order to study the type of inhibition exhibited by glyphosate on urease, the potentiometric response of biosensor to various concentrations of urea in absence and in presence of glyphosate was determined. The apparent Michaelis–Menten constant (Km) which is related to the enzyme affinity and Vmax ie. the maximum potential difference for the enzyme catalyzed reaction was obtained from the electrochemical version of Lineweaver–Burk plot in presences

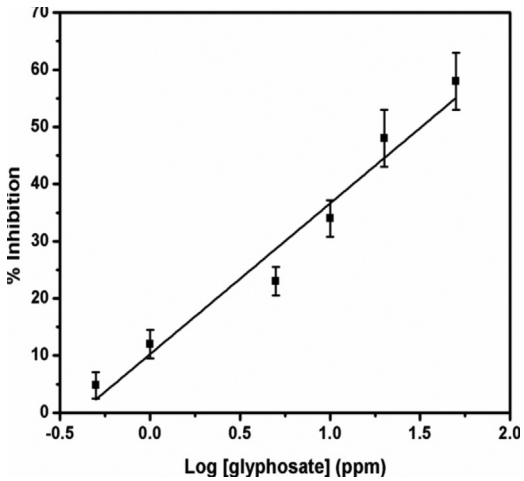



**Fig. 5.** Calibration plot showing the linear dynamic range from 0.5–50 ppm of glyphosate.

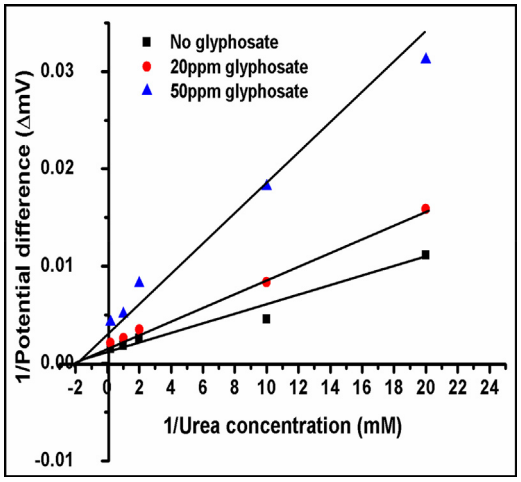



**Fig. 6.** L-B plot in absence and presence of 20 ppm and 50 ppm of glyphosate by potentiometric measurements.

and absences of the inhibitor (Fig. 6). The Vmax value in the absence of glyphosate is 1666 mV, while at 20 ppm of glyphosate Vmax is 757 mV and with 50 ppm Vmax is 312 mV. The Km was found to be 0.5 mM, which is same experiments carried out with and without glyphosate. Thus, indicating the inhibition of urease by glyphosate is non-competitive type. In general, it is observed that in non-competitive type of inhibition the inhibitor does not change the Km of enzyme, while it decreases the Vmax [63]. In this type of inhibition, inhibitor does not have any effect on the substrate binding affinity of the enzyme, but interaction with the inhibitor decreases the catalytic activity of the enzyme.

### 3.5. FTIR analysis

The FTIR analysis of urease nanoconjuagte in presence of urea, glyphosate and urea along with glyphosate was carried out to study the effect of glyphosate on enzymatic reaction. Urease nanoconjuagte has a peak at a 1649 $cm^{-1}$, corresponding to characteristic C=O stretching vibration of amide-I in protein (Fig. 7(c)) [59]. Sample containing urease nanoconjugate with urea shows peak at 1669 cm−1, corresponding to C=O stretching vibration, peak at 1621 $cm^{-1}$ for N—H stretching vibration and peak at 1455 $cm^{-1}$ is related to C—N stretching in urea [64]. Along with characteristic peaks of urea, the additional peak at 1400 $cm^{-1}$ corresponds

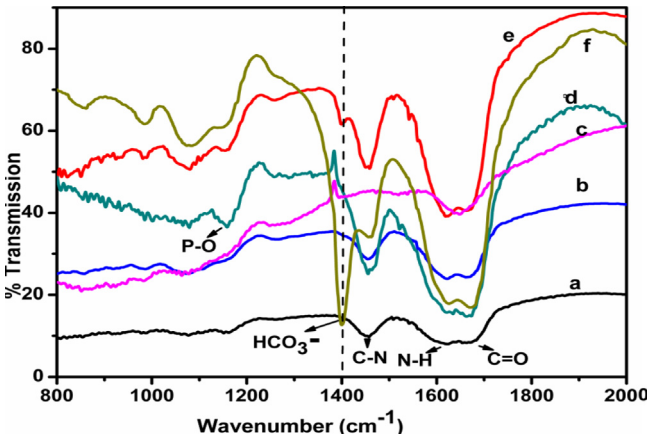



**Fig. 7.** FTIR spectra of (a) urea (b) glyphosate (c) urease-AUNps. Urease nanoconjugate after its interaction with (d) glyphosate (e) urea and (f) urea along with glyphosate in KBr pellet.

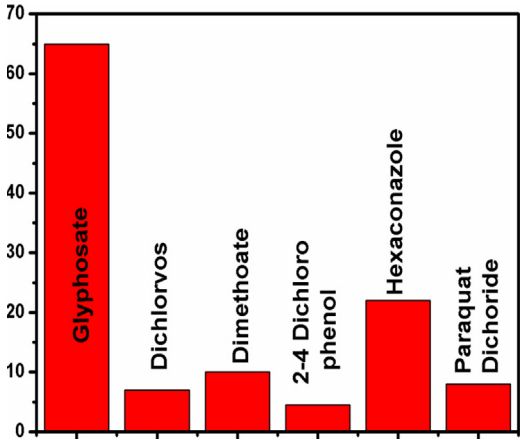



**Fig. 8.** Selectivity of fabricated biosensor for different pesticides.

to bicarbonate ions, which is product of urease reaction on urea (Fig. 7(e)), was also observed in this sample [65]. The sample having urease nanoconjugate with both urea and glyphosate show peaks corresponding to characteristic of both urea and glyphosate (Fig. 7(f)) [66]. The only difference was noted in the peak intensity of the product. The transmission intensity at 1400 $cm^{-1}$ corresponding to bicarbonate ions is significantly more as compared with urease nanoconjugate with urea alone. This clearly indicates the formation of product is comparatively less in the presence of glyphosate. Thus, FTIR analysis confirms the inhibition of catalytic activity of urease in the presence of glyphosate.

It is well known that phosphorodiamidates and imidazoles are typical mechanisms based inhibitors of urease [67]. Phosphorodiamidate having P=O bond that tends to interact with the nickel ion in the active center of urease, which further decreases the binding of urea to urease, leading to inhibition of urease activity [68]. Glyphosate ($OH_2$-PO-NH-COOH) being structurally similar to phosphorodiamidates ($OH_2$-PO-NH-R) may exhibit the inhibition of urease by a similar mechanism.

### 3.6. Selectivity studies

In order to determine selectivity of proposed biosensor for the detection of glyphosate, the potentiometric response was obtained with 50 ppm of glyphosate and other pesticides like dichlorvos, dimethoate, 2–4D, hexaconazole, paraquat dichloride. The result is shown in Fig. 8, indicating the higher inhibition of urease activity

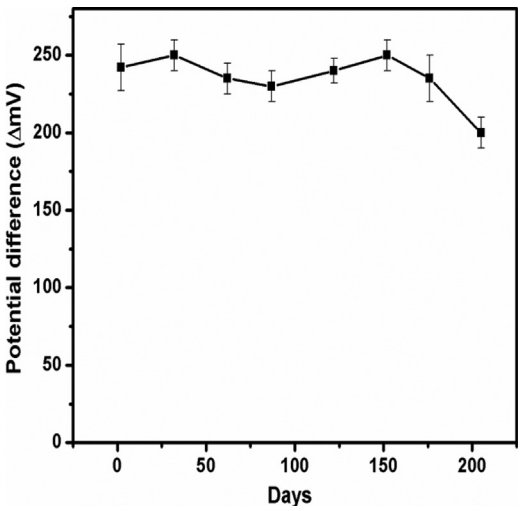



**Fig. 9.** Storage stability studies of urease potentiometric biosensor.

with glyphosate as compared to other pesticides. Then each pesticide has been mixed with glyphosate and extent of inhibition was studied. There was no significant change in degree of inhibition in case of dichlorvos, dimethoate, paraquate dichloride when mixed with glyphosate, while slightly higher inhibition (78%) was observed in mixed sample of glyphosate and hexaconazole as compared with glyphosate alone (66%).

Further, the selectivity factor (% inhibition for glyphosate/% inhibition with other pesticides at 50 ppm) was calculated for each pesticide. In case of dichlorvos, dimethoate, paraquate dichloride the selectivity factor ranges from 6 to 9.2, while for hexaconazole it is around 2.9. Thus, indicating higher selectivity of the proposed biosensor towards glyphosate.

### 3.7. Reproducibility and stability of the biosensor

The reproducibility of proposed biosensor was evaluated by potentiometric measurements in urea, using eight urease nanoconjugate membranes from different batches prepared under identical conditions (Supplementary information, Fig. S3). The potential difference of these measurements was almost comparable with a standard deviation of 6%.

The stability of the biosensor was evaluated by measuring the potentiomertic response for urea at interval of 30 days for 210 days. Almost similar response was obtained upto 180 days (Fig. 9), indicating prolong stability of urease nanoconjugate.

The longer shelf life is due to the biocompatible nature of agarose-guar gum composite matrix, which provides a natural environment for the enzyme after immobilization. The storage stability of urease nanoconjugate membrane is comparable to only urease immobilized membrane, indicating that the formation of conjugate of nanoparticles with urease does not have any effect on stability of the enzyme.

### 3.8. Quantification of glyphosate from spiked water samples

The constructed biosensor was further tested for the detection of glyphosate in tap water samples. Glyphosate concentration in tap water fortified with three different levels (20 ppm, 40 ppm and 60 ppm) was estimated with developed biosensor and percent accuracy was estimated (Supplementary information, Table S4). The values obtained using fabricated biosensor for spiked water samples showed an average accuracy of 86 ± 6% for glyphosate detection.

The linear range, limit of detection, response time and storage stability of previously reported enzyme inhibition based glyphosate biosensors and our results are summarized in Table.S3 (Supplementary information). The glyphosate detection based on urease nanoconjugate provides reasonable sensitivity in wider concentration range with appreciable selectivity. It is able to detect glyphosate below the maximum residual limit (MRL) set by Environmental Protection Agency (EPA, 0.7 ppm) and World Health Organization (WHO, 0.9 ppm) in drinking water. The additional advantage of the present biosensor is its prolonged storage stability (180 days), in comparison with earlier reported peroxidase based biosensor (60 days) [22]. The ubiquitous nature of urease provides relatively easy availability of the enzyme for cost effective fabrication of biosensor. Further, the detection principle (direct potentiometry) being simple allows the ease of miniaturization. The design of measurement system is simple and compact; involving low cost amplifier and digital multimeter. These features have a potential to provide portability to the present biosensor.

## 4. Conclusions

The foregoing results demonstrated that, agarose-guar gum entrapped urease nanoconjugate can be used successfully for glyphosate detection, based on urease inhibition. The formation of bio-nanoconjugate of urease with gold nanoparticles improves the response characteristics of potentiometric measurement which is desirable for glyphosate detection. The better performance was observed by conjugation of gold nanoparticles with urease. The immobilization of urease nanoconjugate using non-toxic and biocompatible agarose-guar gum matrix provides an ideal sensing component for biosensor fabrication. The biocomposite matrix of agarose-guar gum is retaining urease enzyme in its native form with excellent entrapment efficiency. The fabricated urease inhibition based biosensor is highly specific towards glyphosate as against various other pesticides. The response for glyphosate concentration was linear between 0.5–50 ppm, with a detection limit of 0.5 ppm, which is below the maximum residual limit set by EPA and WHO. The fabricated biosensor was found to be reproducible with excellent storage stability of 180 days. The average accuracy of glyphosate detection from spiked tap water samples was 86%, thus demonstrating its practical applicability. Moreover, the detection principle being simple, may allow the ease of miniaturization for low cost and portable device in future.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijbiomac.2017.11.136.

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