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.

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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

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 multi-walled 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