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SHORT COMMUNICATION

Detection of Ricin in Water Samples using Disposable Screen-printed Electrodes

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ABSTRACT

Ricin is a highly toxic plant toxin, which is extracted from the beans of the castor plant, *Ricinus communis*. Ricin is thousand times more poisonous than cyanide and thirty times more potent than nerve gases. The toxin (ricin) could be used to contaminate food or water, causing panic. Attempts were made for the detection of ricin in water samples by utilising amperometric immunosensors. Single-use screen-printed electrodes were made using polystyrene and graphite. These electrodes were tested for their ability to detect 1-naphthol which is the product of the reaction between 1-naphthyl phosphate and the enzyme alkaline phosphatase conjugate. An indirect enzyme-linked immunosorbent assay (ELISA) system was used to detect ricin. First, ricin antigen was incubated on the screen-printed electrode. This was followed by blocking with BSA and incubation with antibody raised against ricin in rabbit. The last step wass the incubation with anti-antibody of rabbit conjugated to enzyme alkaline phosphatase. This electrode is inserted in an electrochemical cell containing diethanolamine buffer and a potential of 0.4 V wrt reference electrode (*Ag/AgCl*) was applied using a potentiostat. Various experiments were carried out for optimising the conditions like substrate concentration, amount of antibody raised against ricin, anti-antibody alkaline phosphatase conjugate, and blocking agents. It was found that the response of amperometric sensor is proportional to the logarithmic of ricin concentration from 100 ng/ml to 3200 ng/ml. Using traditional methods, it is possible to detect ricin concentration up to 300 ng/ml in 18 h, while with amperometric immunosensor, one can detect ricin as low as 40 ng/ml within 90 min. The details of making the screen-printed electrodes, characterisation, optimisation of various conditions for the highest sensitivity have been discussed.

Keywords: Screen-printed electrode, amperometric immunosensor, ricin, potentiostat, plant toxin, ELISA

1. INTRODUCTION

Ricin is a toxic lectin present in the seeds of *Ricinus communis*, commonly known as castor plant. Ricin is a glycoprotein. The molecular weight of the toxin varies from 60 kDa to 65 kDa. Ricin consists of two peptide chains (A and B) held together by a disulphide bond[1]. The A chain is having *N*-glycosidase activity and contains the physiologically active site of the molecule. The B chain is a galactose-specific lectin and is essential for the binding of the toxin to the cell surface, and helps in the entry of ricin molecule into the cell. Ricin inhibits protein synthesis by inactivating ribosomes of eukaryotic cells, leading to cell death[2]. Mankind has used toxins for homicidal, suicidal and for malicious mischief. Bulgarian broadcaster Georgi Ivanov Markov was assassinated by ricin- loaded platinum/iridium pellet. Ricin is also included in Schedule I of Chemical Weapons Convention (CWC)

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due to its extremely toxic nature. Castor plant is distributed widely and grown in various geographical locations. Also, extraction of toxin from castor seed is not very difficult. Ricin could be a poor man's weapon of mass destruction.

The detection of ricin is important. In contrast to global scenario, a little work has been done in India on the toxin ricin, even though India is the third largest producer of castor seeds. In spite of the CWC, ricin can be misused by any country and also by the terrorists due to wild growth of *Ricinus communis* plant. Hence, a quick detection system is very much required.

Several methods were reported in the literature for the detection of ricin. These include: immunoassay methods and biosensors. Godal[3], *et al.* described competitive radio-immunoassay that could detect ricin in blood at concentration of 50-100 pg level. The assay's usefulness was demonstrated using the blood from a patient undergoing treatment with ricin for a tumor. Koja[4], *et al.* reported first ELISA for ricin detection. An ELISA for ricin was developed to assay ricin in tissue after a ricin injection. Subsequently, ELISA was reported for plasma and tissue extract[5]. Griffiths[6], *et al.* studied immunocytochemical detection of ricin using an immunoperoxidase method on tissue after a ricin injection. Ricin (2 µg) could be detected at the injection site, but was not found by this method in lymphoid tissue, even though radio-labelled ricin was detected in these tissues. The sensitivity of this sandwich method was about 60 ng in rabbit body fluids. Poli[7], *et al.* reported improved sensitivity using chemiluminescence as an indicator instead of the alkaline phosphatase or O-phenylenediamine substrate used in the conventional ELISA. Narang[8], *et al.* recently reported fibre optic-based biosensor for the detection of ricin. They have used a sandwich immunoassay scheme to detect ricin. The linear dynamic range of detection for ricin in buffer using the avidin-biotin chemistry is 100-250 ng/ml. The detection of multiple[9] toxic agents using a planar array immunosensor[9] is reported. Concentrations as low as 25 ng/ml for ricin, 15 ng/ml of pestis F1 antigen and 5 ng/ml of SEB could be measured. However, these methods require complete non-portable instrumentation difficult to be used in field-related studies. Here, an attempt to detect ricin in water using inexpensive screen-printing electrode is made.

2. MATERIALS AND METHODS

2.1 Chemicals

1-Naphthyl phosphate, the enzyme-linked anti-rabbit alkaline phosphatase conjugate (D 0487) were obtained from the Dako. The dielectric ink and silver ink were obtained from the Eltecks, Bangalore. Other chemicals used were of analytical grade.

2.2 Ricin Purification

Ricin was purified in the laboratory[10]. The affinity chromatography was performed on acid-treated sepharose 4B (0.1 M *HCl*, 3 h at 50 °C in 0.5 M *NaCl*) according to Griffiths[11], *et al*. Under these conditions, lectins bind to the gel matrix (to galactose residues available on the partially acid-hydrolised matrix). The matrix-bound protein were eluted with β-*D*-galactose. These lectins were then separated on the basis of their size difference, using Bio-gel A-0.5 (BioRad, USA). The fractions eluted here were pooled, concentrated, and used for all experiments. SDS-PAGE under reduced and non-reduced conditions were performed to assess the purity of ricin.

2.3 Preparation of Toxoid and Antibody Raising

Ricin was treated with formaldehyde (1 %) for 4 weeks at 37 °C. Unreacted formaldehyde was removed by desalting on Sephadex G-25 before the toxoid was tested for residual toxicity or used for immunisation. The toxoid preparation was assessed for residual toxicity using the neutral red viability assay[12]. Antibodies titers were determined by Octerlouny's double immune diffusion and checker board titration by indirect microplate ELISA as described by Engvall and Perlmann[13] to give the best condition to detect the ricin in aqueous solution.

2.4 Preparation of Screen-printed Electrodes

The screen-printed electrodes were prepared using an ink made of graphite, polystyrene, and

mesitylene as shown in Fig. 1. These electrodes were made by a three-layer structure. The first layer was made by silver paste, the second layer was made of polystyrene and the graphite ink, and the third layer was made using a dielectric ink. These electrodes were characterised[14] for their electrochemical properties.

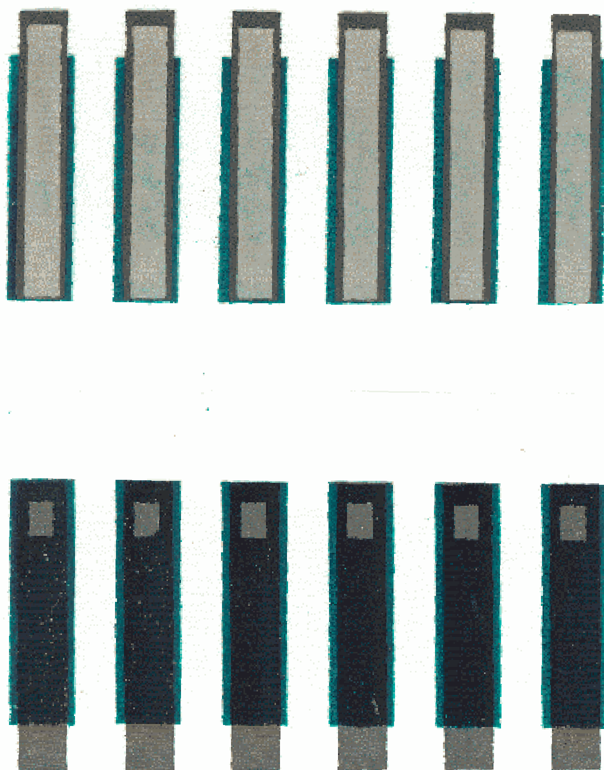


Figure 1. Screen-printed electrodes.

3. IMMUNOSENSING OF RICIN

A known quantity of ricin was physically adsorbed on the electrode. It was left at 37 °C for 1 h and subsequently it was blocked with buffered solution of BSA (3 %) for 15 min. This was followed by incubating antibody of ricin raised in rabbit and finally incubated with enzyme-linked alkaline phosphatase anti-antibody of rabbit. The electrode was finally dipped in an electrochemical cell containing EDA buffer (p*H* 9.8) and a potential of 0.4 V wrt *Ag/AgCl* reference electrode was applied and the resulting amperometric current was noted after the addition of the substrate. The output current was directly proportional to the amount of ricin present on the electrode.

4. RESULTS AND DISCUSSION

The screen-printing electrode was prepared on a three-layer basis. The first layer was printed with silver ink to increase the conductivity of the electrodes. The second layer was printed with graphite carbon ink and a binder. Different polymers like polyvinyl chloride, polystyrene, and polymethyl methacrylate were tried and different compositions were tested. A 60:40 ratio of graphite and polystyrene in mesitylene was optimised. Later, these were characterised using electrochemical techniques such as ammperometry, cyclic voltammetry. The details were reported[14].

Other important parameters like stirring speed of the motor for getting maximum current, maximum amount of antibody conjugate, the amount of substrate to be added, the time of incubation and the potential for oxidation of 1-naphthol were optimised. The relation between stirring speed of solution response of electrode was studied. It was found that the response increases with stirring speed and becomes constant at a rotation speed of 700 rpm. For detection of ricin, an indirect ELISA method was adopted. The blank experiments with various dilutions of antibody raised against ricin and the antibody conjugate were performed. Table 1 gives the effect of using the blocking agents like BSA and skimmed milk on the response of the electrode for the conjugate. The data show that the BSA (3 %) has better blocking ability than the skimmed milk. Hence, further experiments were conducted using BSA (3 %).

Various concentrations of ricin were adsorbed on the screen-printed electrode. The various steps

Table 1. Effect of using the blocking agents on the response of the electrode

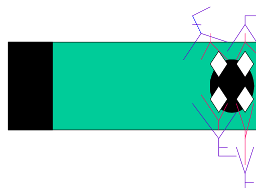
Blocking agent	Concentration of antibody	Concentration of conjugate	Response	Ratio
	-	-	0.006	-
	-	1:5000	0.006	-
BSA (3 %)	1:1000	1:5000	0.075	1.20
	1:2000	1:5000	0.006	1.00
	-	1:5000	0.08	-
Skimmed milk powder	1:1000	1:5000	0.11	1.83
	1:2000	1:5000	0.08	1.33

IMMUNO SENSOR PROCEDURE FOR DETECTION OF RICIN

Step I: Immobilisation of ricin (sample) on Electrode Surface followed by blocking with 3 % BSA

Step II: Incubation of antibody *Y* developed in rabbit

Step III: Incubation of anti body of rabbit conjugated to alkaline phosphatase on electrode



Step IV: The electrode is connected to potentiostat. Substrate is added. Product formed due to enzymatic reaction is oxidised and this oxidation current is output

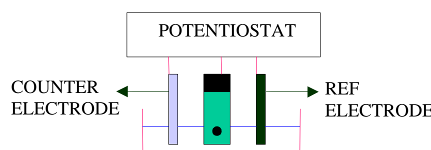


Figure 2. Various steps for performing the immunoassay.

undertaken for performing the immunoassay are presented in Fig. 2. The first step was the adsorption of ricin on the working electrode area of the screen-printed electrode. For this, the agent was spiked with the aqueous buffer and was placed on the electrode surface for 15 min. The agent was physically adsorbed on the surface of the electrode. After this step, the electrode was washed with buffer solution to avoid the current arising due to non-specific binding. Then, it was incubated with BSA (3 %) solution, followed by antibody raised against ricin and rabbit anti-antibody conjugate in a sequential manner. Finally, the electrode was dipped into an electrochemical cell containing EDA buffer. A potential (0.4 V) wrt *Ag/AgCl* reference electrode was applied on the electrode. After waiting for 2 min, the substrate 1-napthyl phosphate was added to the solution. Due to the enzymatic reaction of alkaline phosphatase conjugate with the substrate, the product 1-naphthol was generated. The product got oxidised and gave the response current.

Figure 3 shows the response of immunosensor to the concentration of ricin. The Y-axis shows the corrected amperometric response. The response obtained in blank experiments was 75 nA and the standard deviation (SD) was calculated and found to be 10 nA. The experiments were repeated thrice under the identical conditions and the SD was calculated. Any response above blank + 3xSD can be taken as an indication for the presence of ricin in the water sample. Thus, it is possible to detect ricin as low as 40 ng/ml. However, a linearity exists between logarithmic concentration of ricin (100 ng/m to 3200 ng/ml) and amperometric current.

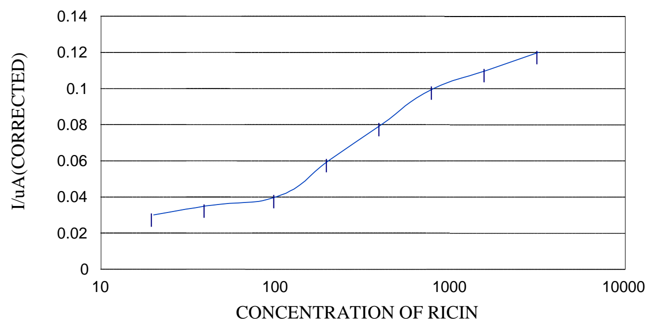


Figure 3. Plot between concentration versus amperometric response (In these experiments antibody 1:1000 dilution and antibody conjugate1:5000 dilution were used).

5. CONCLUSION

A sensor based on disposable screen-printed electrode was made and characterised by the electrochemical methods. The conditions for obtaining maximum response were optimised. It is possible to detect as low as 40 ng/ml of ricin in 1.5 h, while the traditional ELISA method takes as long as 24 h for detecting 300 ng/ml of ricin.

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