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# Amperometric immunosensor for ricin by using on graphite and carbon nanotube paste electrodes

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ABSTRACT

Ricin is a scheduled chemical warfare agent and biological warfare agent. Attempts were made for the detection of ricin in water samples by utilizing amperometric immunosensors. These electrodes were made by mixing Paraffin oil with graphite powder and multiwalled carbon nanotubes. The graphite paste electrode (CPE) and multiwalled carbon nanotubes paste electrode (MWCNTPE) were tested for their ability to detect 1-naphthol. A sandwich enzyme linked immunosorbent assay system was used to detect ricin. The detection limit for both electrodes was compared. It was found that the response of amperometric sensor is proportional to the ricin concentration in both the cases and is linear in the range 0.625–25 ng/ml for MWCNTPE and 2.5–25 ng/ml for CPE. The SEM showed that the MWCNTPE has revealed crevices/voids in which the antibodies may get trapped. Spectroscopic experiments proved that MWCNTPE adsorbs antibodies better than CPE. The high sensitivity of MWCNTPE was attributed to its better electrochemical properties rather than to its efficiency to adsorb antibodies.



## 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 to 65 kDa. Ricin consists of two peptide chains (A and B) held together by a disulfide 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 leading to cell death [2]. Ricin inhibits protein synthesis by inactivating ribosomes of eukaryotic cells. Detection of ricin is important as it is a listed chemical warfare agent. In contrast to global scenario very little work has been done in India on the toxin ricin, even though India is the largest producer of castor seeds. In spite of the CWC, ricin can be misused by any country and also by the terrorist due to the wild growth of *R. communis* plant. A portable detection system for detection of ricin in water is desirable for field use.

Several methods were reported in the literature for detection of ricin. These include immunoassay methods and biosensors. Competitive radioimmuno-assay that could detect ricin in blood at concentration of 50–100 pg level has been described [3]. The assay's usefulness was demonstrated with the blood from a patient undergoing treatment with ricin for a tumor. The first enzyme linked immunoassay (ELISA) for ricin was reported [4]. 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]. Immunocytochemical detection of ricin was studied using an immunoperoxidase method on tissue after a ricin injection [6]. A sensitive ELISA method by using chemiluminescence was reported [7]. This was found to be better than conventional ELISA methods developed using alkaline phosphatase (ALP). A fiber optic-based biosensor for the detection of ricin was reported more recently [8]. 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 pg/ml to 250 ng/ml. Detection of multiple toxic agents using a planar array immunosensor [9], micromechanical sensors [10] and luciferase based assay [11] were reported. The detected concentration was as low as 25 ng/ml for ricin and 15 ng/ml of pestis F1 antigen. However these methods require non-portable instrumentation and hence are not applicable for field related studies. Graphite paste electrodes (CPEs) were mostly used in electroanalysis due to their low background current, wide potential window, and low cost. We recently reported on the use of screen printed electrodes (SPEs) for the detection of ricin in water samples [12]. In this paper we used CPE and MWCNTPE for detection of ricin. Recently MWCNTs have come to the fore front of electrochemical research due to their attractive electronic, chemical and mechanical properties [13–15]. The

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tubular form of MWCNT has got appreciable electronic properties which suggest that they might mediate the electron transfer reactions with an electroactive species in solutions and this was tried [16,17]. MWCNTs can also be pretreated with concentrated acids in order to open the nanotubes and create carboxylic groups and this exhibited an intense catalytic activity towards the electrochemical oxidation of phenolic derivative [18]. MWCNT paste electrodes (MWCNTPEs) were recently utilized for biosensor applications [19]. Also SPEs modified with MWCNTs were used for biosensing and immunosensing applications [20]. In our laboratory we modified the SPE with MWCNTs and gold and used in immunosensing of Malaria falciparum [21]. Carbon nanotubes (CNTs) were also used in immunosensing by using transducers such as field effect transistors [22] and antibodies were also attached to CNTs using covalent and non-covalent approach and were investigated [23]. The SPEs were also modified with MWCNTs and used for immunosensing [24]. Recently MWCNTPEs prepared using ionic liquid was reported for biosensing applications [25]. The CNT paste electrodes (CNTPEs) without any support were used for biosensing applications [26,27], but they were not used for immunosensing. In this paper, attempts were made to use cheaper electrodes for sensing ricin. Here we attempted to detect ricin in water by using inexpensive CPE filled with graphite paste and MWCNTPE filled with MWCNT paste and detection limits exhibited by both electrodes were compared. The relative efficiency for adsorption of antibodies by these electrodes was studied by optical density measurements.

## 2. Experimental

### 2.1. Apparatus

Cyclic voltammagram (CVM) experiments and chrono amperometric experiments were performed with an Autolab potentiostat from Ecochemie, model no. PGSTAT 12. The detection was carried out in a 10 ml electrochemical cell, with a working electrode of 4 mm diameter; an Ag/AgCl/Satd. KCl was used as reference electrode with platinum as a counter electrode. The thermogravimetric analysis (TGA) experiments were performed with TA instruments model no. 2950. Spectro nanophotometer Implen serial no. 1257 has been used for finding change in absorbance. Sonication has been done by using Sonics & Materials Inc., model no. VCX750. SEM (Quanta 400ESEM The Netherlands) has been used for scanning electron micrograph (SEM) studies.

### 2.2. Reagents and chemicals

The detection buffer consisted of 0.1 M diethanolamine (DEA) containing 0.02 M of magnesium chloride and 0.1 M sodium chloride (NaCl) with an adjusted pH of 9.8. The antigen/antibody buffer consisted of 0.02 M phosphate buffer with 150 mM NaCl (PBS pH 7.4). The washing buffer consisted of 100 mM of Tris and 100 mM NaCl and adjusted to pH 7.2. It has been reported that pH 7.4 was best optimized for the antibody immobilization [28] and the same was used in our experiments with 20 mM of PBS containing 0.03% bovine serum albumin (BSA) of pH 7.4. Standard solution of the toxin ricin was prepared (25 mg/ml) in PBS of pH 7.4 and the required dilutions were prepared as and when required. Anti-ricin antibody raised in rat (CAb) and anti-ricin antibody raised in rabbit tagged alkaline phosphatase enzyme (AbALP) from a concentration of 0.5 mg/ml were diluted using 100 mM Tris containing 100 mM NaCl with a pH 7.4. The dilutions were prepared freshly before use. Goat antirabbit IgG–HRP conjugate, BSA, OPD, 3,3′-diaminobenzedine (DAB), 4-chloro alpha naphthol, DEA, 1-napthyl phosphate monosodium salt, MWCNT, Tris–HCl, PBS buffer 7.2 pH were purchased from Sigma Chemicals. Other chemicals used were of analytical grade. The CAb and AbALP were raised in our laboratory. The antibody of ricin raised in rabbit was tagged with ALP conjugate as per standard protocols in our laboratory.

### 2.3. Preparation of CPE and MWCNTPE

The details of preparation of CPE were reported earlier [29]. The carbon paste was made by manual mixing and sonicating, graphite or MWCNTs with mineral oil in various proportions. The electrode holder was made up of Teflon cylinder (4 mm inner diameter) which has a screw type of arrangement that can push the paste as and when required. The bore is filled with graphite paste or MWCNT paste. The mixture was tightly packed layer by layer inside the electrode. The conductivity between the brass screw and the tip of the carbon paste material is checked by the multimeter to ensure the connection. Prior to mixing, the MWCNTs (0.05 g) were treated with 60 ml of 2.2 M nitric acid for 24 h in stirring condition and then agitated for 30 min in ultra sonicator. This is washed with the water till the pH is neutralized [30]. Then it is dried for 1 h with nitrogen and then the moisture is removed by keeping the MWCNTs at 110 °C for 3 h [30]. These MWCNTs were mixed with mineral oil for making MWCNTPE. A potential of 1.7 V was applied on these electrodes for 3 min in DEA buffer of pH 9.8 and then these electrodes were used for immobilization of antibodies.

### 2.4. Ricin purification

Ricin was purified in the laboratory as described elsewhere [31]. The affinity chromatography was performed on acid treated Sepharose 4B (0.1 M HCl, 3 h at 500 °C in 0.5 M sodium chloride solution) according to previously published report [32]. Under these conditions lectins bind to the gel matrix (to galatose residue available on the partially acid-hydrolyzed matrix). The matrix bound protein was eluted with β-D-galactose.

These lectins were then separated on the basis of size difference, using Bio-Gel A-0.5 (BioRad, USA). The fractions eluted here was pooled, concentrated and used for all experiments. Native and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and non-reduced conditions were performed to assess the purity of ricin.

### 2.5. Preparation of toxoid and antibody raising

Ricin was treated with 1% formaldehyde for 4 weeks at 370 °C. Unreacted formaldehyde was removed by desalting on Sephadex G-25 before the toxoid was tested for residual toxicity or used for immunization. The toxoid preparation was assessed for residual toxicity using the neutral red viability assay [33]. Antibodies titers were determined by Octerlouny's double immune diffusion and checker board titration. By indirect microplate ELISA as described in literature [34] to give the best condition to detect ricin in aqueous solution.

### 2.6. Steps in immunosensing of ricin

A known quantity of CAb of ricin (10 μl) in PBS buffer was physically adsorbed on the electrode as per Fig. 1. It was left at 37 °C for 1 h and subsequently it was blocked with buffered solution of 3% BSA after washing for 30 min. This is to reduce the nonspecific adsorption effect. The second step is to incubate with the diluted concentrations of ricin in PBS solution for 15 min. This results in selective antigen antibody interaction due to physical adsorption. This was followed by incubating AbALP for 15 min. The secondary antibodies forms a complex with the secondary sites

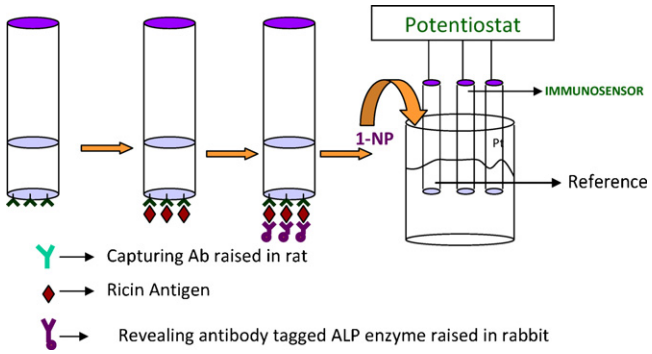


**Fig. 1.** Sandwich immunoassay procedure for immunosensing of *Ricinis communis* using MWCNT paste electrode.

forming a sandwich ELISA assay [35]. After each step the electrode was washed with Tris buffer containing NaCl of pH 7.2 to remove any unbound antigen or antibody. The electrode was finally dipped in an electrochemical cell containing DEA buffer (pH 9.8) and a potential of 0.4 V with respect to reference Ag/AgCl/Satd. KCl electrode was applied. After allowing 3 min for the stabilization of the electrode to achieve constant current the substrate 1-napthyl phosphate was added and the resulting amperometric current was noted. The concentration of the substrate used in our experiment is 11 mM [35]. The output current obtained can be co-related to the concentration of ricin present on the electrodes.

## 3. Result and discussion

### 3.1. Physical characterization of MWCNTs and graphite paste

SEM was used for examining the morphology of MWCNTs and graphite paste. Fig. 2a shows the image of purified and acid treated MWCNTs. They were randomly oriented and with an entangled structure which protrudes outside with a clean surface. The SEM of Fig. 2b shows that the MWCNTs were protruding out of globules when mixed with oil. Also the randomly oriented MWCNTs create voids. These voids or crevices in the randomly oriented MWCNTs will be useful in trapping the antibodies during the process of physical adsorption. In these crevices antibodies may get trapped.

The SEM of graphite paste as shown in Fig. 2c shows a homogenous mixture which is in contrast to MWCNTs paste.

### 3.2. Optimization of the MWCNT/graphite paste material

Different compositions of the MWCNTs paste were made (i.e. 33, 50, 60, 65, 70, 75, and 80) with mineral oil and the electrodes were characterized electrochemically. Cyclic voltammetry experiments were performed with these electrodes in 1 mM $K_3Fe(CN)_6$ in 0.02 M PBS of pH 7.4, by scanning between 0.6 and −0.2 V at a scan rate of 50 mV/s (figure not shown). The relation between voltage difference between the anodic peak and cathodic peak and the current vs. composition is shown in Fig. 3. When the composition was 33/67 for MWCNTs/mineral oil paste, the peak separation was high due to high resistance of mineral oil. The electrochemical response has increased when the mineral oil has been reduced in the composition. At 80/20 composition best reversibility conditions such as lowest peak separation and highest peak current. At 65/35 and 70/30 compositions the electrochemical response is almost similar. However at 70/30 composition the aqueous solution seeps into the electrode when exposed to buffer solution for longer durations. And that resulted in the loss of immunosensing characteristics and hence we used 65/35 composition for further studies with MWCNT paste. This composition was in close agreement with the previous work published for CPE [36] and single walled carbon nanotubes [37].

### 3.3. Electrochemical characterization of MWCNTs paste electrode

The CPEs and MWCNTPEs were characterized by using electrochemical techniques such as amperometry and cyclic voltammetry. These electrodes (CPE and MWCNTPE) were also characterized for their electrochemical property towards 1-naphthol. The details of characterization of CPE were described elsewhere [29,38]. The details of the hydrodynamic conditions for getting maximum current and the potential for oxidation of 1-naphthol were reported

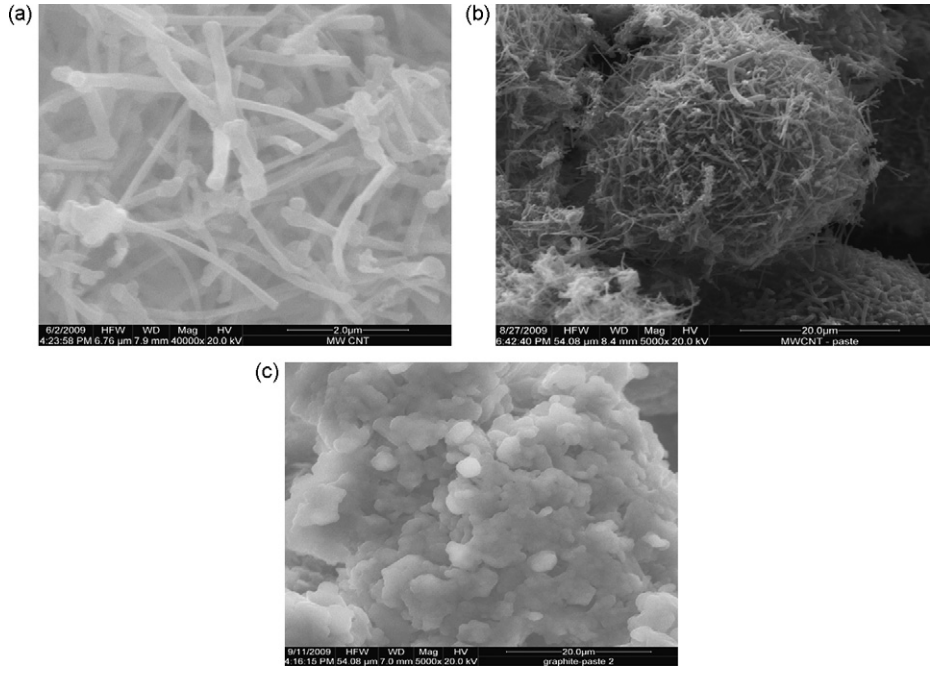


**Fig. 2.** (a) SEM image of MWCNT, (b) SEM of MWCNT/oil mixture, and (c) SEM image of graphite paste/oil mixture.

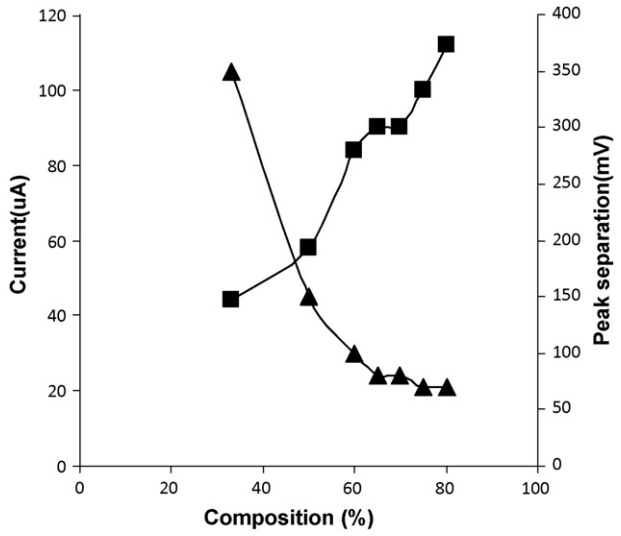


**Fig. 3.** The effect of the paste composition on (a) peak potential separation ($\Delta E_p$) and (b) peak current for CVM of $1 \times 10^{-3}$ M ferricyanide. Working electrode 4 mm diameter and scan rate 50 mV/s (▲). Peak separation with respect to composition (■). Current with respect to composition.

elsewhere [39]. The speed of rotation of the stirrer has been fixed at 700 rpm and the applied potential was fixed at 0.4 V with respect to reference electrode. Fig. 4 shows the calibration plot of the concentration of product 1-naphthol with respect to the current obtained when MWCNTPE was used as the working electrode.

### 3.4. Optimization of the MWCNT oxidation

The TGA analysis showed that the pure MWCNTs had a loss of 0.96% of the weight. These MWCNTs were acid oxidized to create acidic groups and hydroxyl groups. The MWCNTs were treated with 2.2 M of nitric acid, and then washed with water till the pH was neutralized. These MWCNTs were treated with acid for different durations. For 4 h treated MWCNTs the TGA curve showed a loss of 1.2%. When MWCNTs were treated for 16 h with acid the loss was 5.2%. As the time was increased to 24 h the weight loss was 7.4%. And at 24 h it showed best electrochemical activity. This is because after 24 h of oxidation, the smaller MWCNTs will be destroyed and only bigger nanotubes will exist [40]. Further increment in time

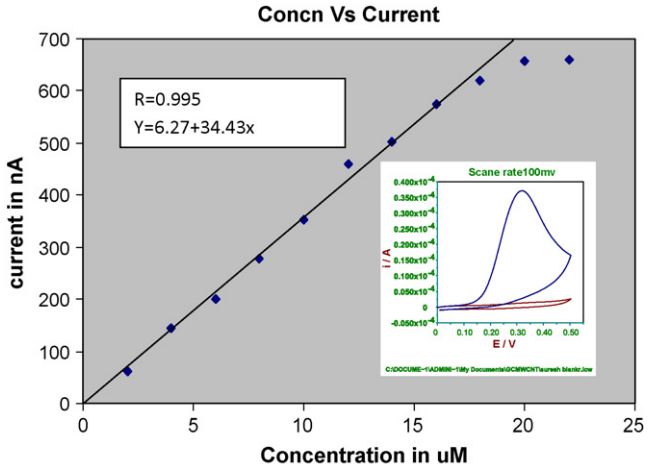


**Fig. 4.** Calibration plot of 1-napthol vs. current. Working electrode is MWCNTPE, reference electrode Satd., Ag/AgCl/KCl, counter electrode is Pt electrode. Applied potential 0.4 V w.r.t. Ag/AgCl. Inset: cyclic voltammagram of 0.1 M diethanolamine of pH 9.8 with and without 1 mM of 1-naphthol.

to 48 h for acid oxidation resulted in poor electrochemical characteristics. This may be due to the reason that the MWCNTs become fragmented with increment in the time of oxidation [40]. So the time was optimized at 24 h for treatment of MWCNTs with nitric acid.

### 3.5. Optimization of revealing antibody (AbALP)

Fig. 5a gives the response of the CPEs and MWCNTPEs after blocking with BSA for 30 min and incubating the electrodes with AbALP for 15 min. Various dilutions of AbALP were incubated and tested for obtaining the blank current. There was an appreciable increase in background current when a dilution of 1/50,000 to 1/5000 was used. This background current is due to nonspecific adsorption of AbALP on the sensing surface. A dilution of 1:40,000 of the AbALP were optimized for further studies. Similarly for the CPE, the antibody dilution was optimized at 1:35,000. The standard deviation of CPE was calculated to be 6.8% ($n = 4$) and for MWCNTPE it was 11% ($n = 4$).

### 3.6. Optimization of capturing antibody (CAb)

For this optimization all the steps except the step of incubation of the antigen were performed. As per Fig. 5b when the dilution was around 1:18,000 for CPEs, amperometric current almost matched with the blank current obtained with 1:35,000 of AbALP. Similarly for MWCNTPEs at a dilution of 1:20,000 the current equalized the background current. So to remove any effect arising due to the CAb–AbALP interaction, this dilution was optimized for detection. The standard deviation of CPE was found to be 7.8% ($n = 4$) and for MWCNTPE was 11.25% ($n = 4$).

### 3.7. Detection of ricin

The electrodes (CPE and MWCNTPE) were initially incubated with CAb at optimized dilution for 60 min. Subsequently they were blocked with 3% BSA for 30 min followed by incubation in ricin at various concentrations. Then these electrodes were incubated with AbALP for 15 min. Each step is followed by washing with washing buffer to remove any unbound antigen or antibodies. These electrodes were dipped in an electrolyte containing DEA buffer. A potential of 0.4 V vs. Ag/AgCl was applied and the amperometric current was measured. When the current reached a steady state value, the substrate is added and the rise in the current was noted. The amperometric response was found to be linear in the range of 625 pg/ml to 25 ng/ml for MWCNTPE (Fig. 5c). The maximum relative standard deviation (RSD) was found to be 11 for MWCNTPE. The amperometric current was found to be linear in the range of 2.5–25 ng/ml for the graphite paste electrode (Fig. 5c). The maximum RSD was found to be 15 for CPE. The repeatability of the results could be correlated by the RSD. The correlation coefficient of CPE was calculated to be $r = 0.9966$ and for MWCNTPE $r = 0.9989$. Sensitivity was enhanced by more than three times when MWCNTPE was used. The enhanced sensitivity is attributed to the better electrochemical properties of MWCNT than bulk graphite material. The limit of detection (LOD) is defined as the lowest concentration of ricin response which is three times higher than the standard deviation of current response in absence of ricin under identical conditions. The LOD was found to be 562 pg/ml for MWCNTPE and in case of CPE it is 1.7 ng/ml. The experiments were repeated at three different times and the results were found to be the same. For a concentration of 10 ng/ml of ricin the RSD of CPE was 4.1 and for MWCNTPE it was 3.2. By using screen printed electrodes [12] the detection of ricin was found to be linear in the range of 40–100 ng/ml. In the present report we used MWCNTs and thereby could achieve linearity in the range of 625 pg/ml to

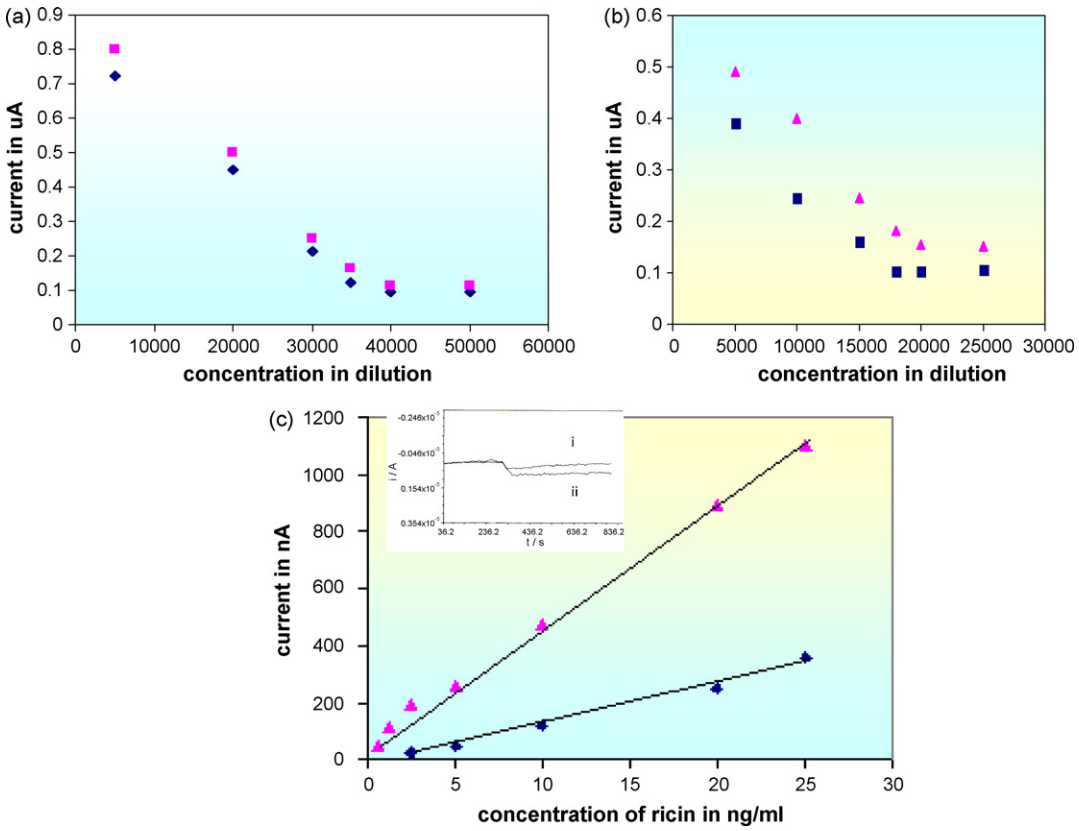


**Fig. 5.** (a) Optimization of antibody tagged ALP enzyme (revealing antibody) concentration given in dilution curve (♦) shows the optimization of MWCNT paste electrodes curve (■) shows the optimization of graphite paste electrodes. (b) Optimization of antibody raised in rat (capturing antibody) concentration given in dilution. Dotted curve (▲) shows the optimization of MWCNTPE. Dotted curve (■) shows the optimization of CPE. (c) Calibration curve for the concentration of ricin vs. current got due to the detection by using multiwalled carbon nanotube paste electrodes (▲) MWCNTPE (■) CPE. Inset: comparative detection of 10 ng/ml of ricin (i) CPE and (ii) MWCNTPE.

25 ng/ml. Recently, by using magneto-elastic ricin immunosensor [41] the lowest concentration of ricin that could be detected with an incubation time of 1 h is 10 ng/ml. In another recent method by using surface plasmon resonance [42] the LOD for ricin achieved was 0.5 ng/ml. And these methods may not be suitable for field related studies and require expensive instrumentation. By using the methods and electrodes described in this paper it is possible to make a cheap portable detection system with better sensitivity.

In order to investigate the reason for higher efficiency of MWCNTPE, the relative adsorption efficiency of MWCNTPEs and CPEs for antibodies were carried out and presented. The AbALP (1:100) was adsorbed on these electrodes for 15 min. After washing to remove any unbound antibody, 2 ml of 4 mM para nitro phenyl phosphate (PNPP) substrate was placed on the electrodes at a pH of 9.8 by a special arrangement on Teflon electrodes. After each measurement, the PNPP solution was returned back to the electrode surface. And the optical density (OD) was observed with respect to time. The results were presented in Fig. 6. The higher OD values for MWCNTPEs can be attributed to its ability to adsorb more amount of AbALP. The reason for higher adsorption of antibody on MWCNTPE can be attributed to (i) the voids or crevices between randomly oriented MWCNTs which can act as traps for the antibodies and (ii) due to interaction between proteins and the CNT sidewall, which can be ascribed to hydrophobic interactions between the exterior fullerene surface and regions of high hydrophobic residue density within the protein tertiary structure [39]. In this work, the amount of enzyme tagged antibody used for adsorption by both electrodes is same. Hence the higher sensitivity for MWCNTPE can be attributed to its better electrochemical properties rather than adsorption capacity of antibodies. Even though the adsorption efficiency of MWCNTPE is better than CPE, the non-specific binding of

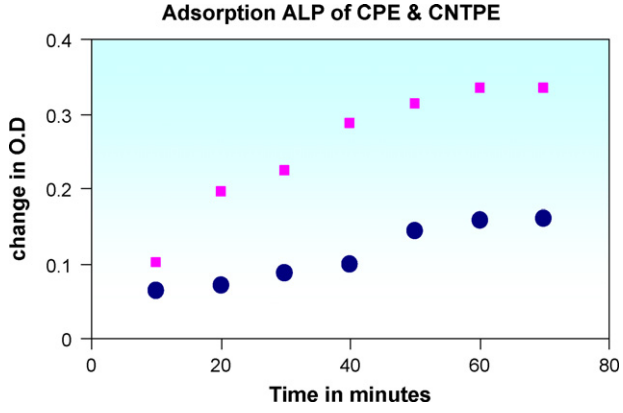


**Fig. 6.** Adsorption of antibody tagged ALP enzyme on CPE and CNTPE electrodes. Electrode size 4 mm. 2 ml of DNPP substrate has been used and the change in the absorbance has been with respect to time. After measuring OD the solution is returned back to electrode surface. (■) Absorption change of MWCNTP electrodes for an antibody dilution of 1:100. (●) Absorption change of CP electrodes for an antibody dilution of 1:100.

antibodies does not allow us to exploit this property. This may not be a problem in biosensor applications using enzymes.

## 4. Conclusions

The MWCNTPE has a higher sensitivity (more than three times) than the CPE for ricin detection. Studies were conducted to find the relative ability of these electrodes with physical adsorption of conjugated antibody. The high sensitivity with MWCNTPE was

attributed to good electrochemical properties rather than its ability to adsorb the antibodies. A portable ricin detection system using any hand held potentiostat can be used in field testing.

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