

Conducting paper based sensor for cancer biomarker detection

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Abstract. We report results of studies relating to the fabrication of a paper based impedimetric biosensor using poly(3,4-ethylenedioxythiophene):poly(4-styrene sulfonate) (PEDOT:PSS) modified Whatman paper. The layer-by-layer assembly of PEDOT:PSS deposited on the paper substrate results in the formation of a stable, conductive and homogenous film. The film has been characterized using Fourier transfer Infrared spectroscopy and scanning electron microscopy. This conducting paper platform has been used for the conjugation of anti-carcinoembryonic antigen (CEA) protein for quantitative estimation of CEA, a cancer biomarker that is frequently used in detection and monitoring of cancer. The results of the electrochemical impedimetric response studies indicate that the fabricated paper electrode can be used to estimate CEA in the range from 6-20 ng mL⁻¹, has sensitivity of 3.6 Ω mL ng⁻¹ with a lower detection limit of 2.68 ng mL⁻¹.

1. Introduction

The availability of reliable diagnostic tools is an essential requirement in both the developing and developed world [1,2]. In this context there has been an emerging trend for evolution of new methods in nanotechnology, biotechnology and materials science for the development of point-of-care diagnostics (POC) devices. POC devices are analytical platforms that can be used to obtain rapid and accurate result at reasonable cost for biochemical analysis near the vicinity of patient [3,4].

Cancer is a serious concern and medical threat to the world because millions of people die due to uncontrolled cell growth every year. Carcinoembryonic antigen (CEA) is a glycoprotein involved in cell adhesion that is normally produced in gastrointestinal tissue and their level increases in serum of colorectal, lung and breast cancer. CEA determination is mostly used for diagnosis and to monitor patients who suffer from cancer which produce CEA [5-7]. Therefore detection of CEA is very important for diagnosis of cancer or more specifically monitoring of patient after and before getting chemotherapy, surgery, radiation or combination of all.

Paper is composed of cellulose fibre, which is a unit of the un-branched glucose. Due to flexibility, light weight, low cost, easily stored, transported, combustible, biocompatibility and biosafety management paper substrate can be advantageous for development of POC devices [8,9]. Conducting paper has been recently explored for developing innovative techniques for printed basic electronics components and inexpensive sensing kits [10,11]. Kumar *et. al.* fabricated conducting paper electrode by screen printing of silver paste over filter paper. These conducting paper electrodes were later used for



electrochemical deposition of polyaniline to bind anti-sIL2R α for detection of cancer [12]. *Ge et. al.* fabricated electrochemiluminescent immunodevice by chitosan grafted over screen printed conducting carbon paste on paper substrate and subsequent labelling of antibody with ruthenium complex for multiplexed detection of cancer biomarker [13]. These conducting polymers require a conducting substrate for electrochemical detection. Among the various polymer PEDOT: PSS has been found to have high ductility, thermal stability, easy handling of making film and ability to replace the traditionally used electrodes for flexible electronics [14,15].

In the present work a simple approach was used for the fabrication of conducting paper strips by progressively making a film of PEDOT:PSS (Poly(3,4ethylenedioxythiophene)/poly(4-styrene sulfonate)) directly over paper substrate. Electrochemical impedance spectroscopy (EIS) was used to study the interaction of CEA at the conducting paper electrode.

2. Experimental section

2.1. Material and Chemicals

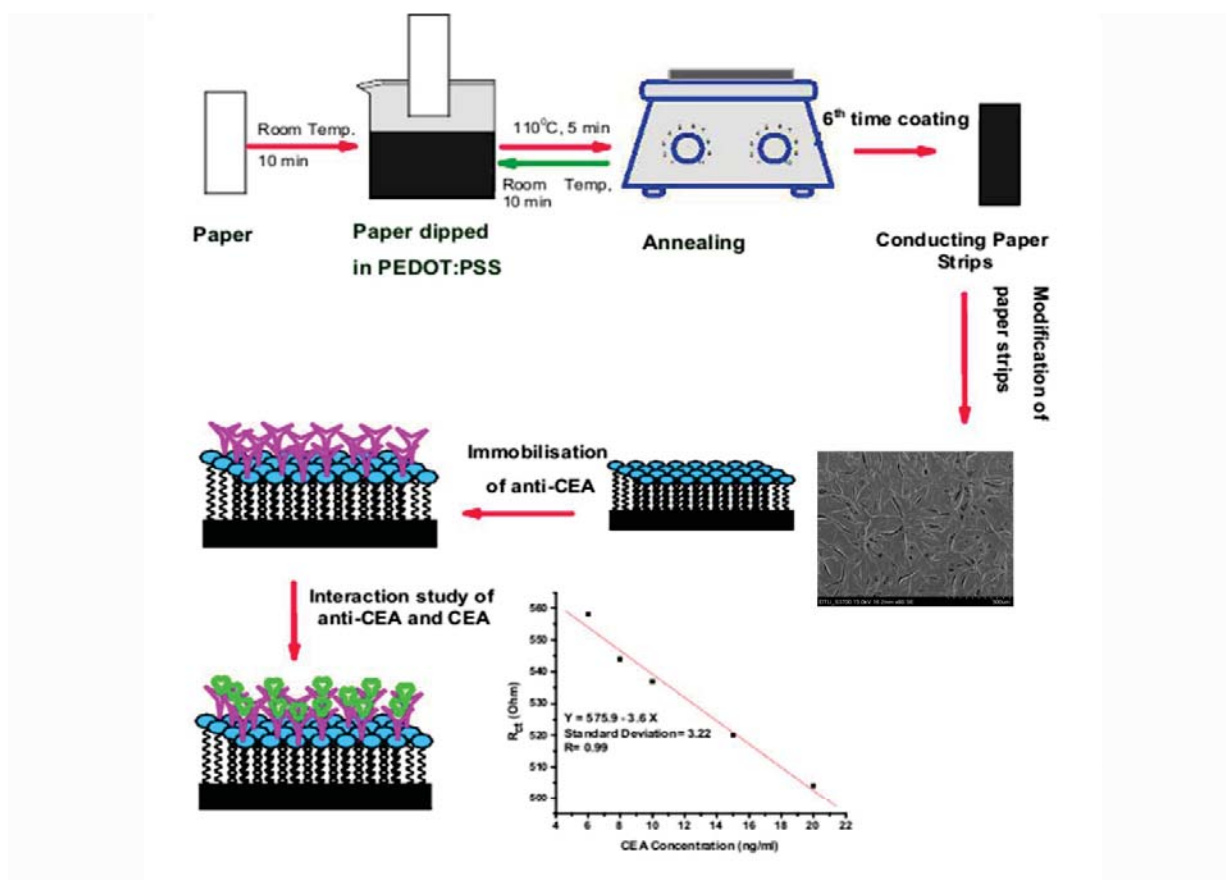
Poly (3, 4-ethylenedioxythiophene)-poly(styrenesulfonate) 1.3 wt %, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Carcinoembryonic Antibody Monoclonal (anti-CEA). Bovine serum albumin (BSA) and carcinoembryonic antigen (CEA) were purchased from Sigma Aldrich, India. Whatman Filter papers #1 was used as a substrate and all other chemicals were of analytical grade. The PEDOT:PSS solution was ultrasonically treated prior to being use. All other chemicals were of analytical grade and were used without further purification. Deionized water (Millipore) was used in all the buffer and solution preparations.

2.2. Fabrication of conducting and hydrophobic paper strips

Whatman filter paper #1 was cut into dimensions of 1cm \times 3cm strips which were used as substrate. The paper strips treated with 2-3 drops of Tween 80 (detergent) in distilled water (DW) were washed with copious amount of water. The strips were further dipped into ethanol solution and sonicated for 15 min, followed by rinsing with DW and keep it in an oven at 60°C till dry. The paper strips were then dipped into PEDOT: PSS solutions for 10 min and then annealed at 110°C for 5 min. These consecutive cycles were repeated until desirable electrical parameter and integrity or mechanical strength of paper was obtained. The number of cycles corresponds to the number of PEDOT:PSS layer deposited on to the paper substrate. The color change from white to black clearly shows (Fig 1) the deposition of PEDOT:PSS and with increase in the deposition cycle (6 cycle) conducting paper strips was fabricated. These conducting paper strips were further treated with methanol at 100°C for 5 min to remove any artifacts from surface and to maintain the homogeneity on surface.

2.3. Sensor strips fabrication

1% (v/v) solution of (3-aminopropyl) triethoxysilane (APTES) solution (in toluene) was used to functionalize conducting paper strips. These modified conducting paper strips were immobilized with CEA (monoclonal) using EDC (0.2 M) as the coupling agent and NHS (0.05M) as activator (Scheme 1), for 2 h in dark. The modified paper strips was washed several time with phosphate buffer (PBS. 50 mM. pH 7.4, 0.9% NaCl) to remove any unbound antibodies. Bovine serum albumin (BSA) solution (2 mg/mL) was used to block non-specific active sites of the electrode. The BSA/anti-CEA/PEDOT:PSS paper electrodes were then washed with PBS and stored at 4°C, when not in use.



Scheme 1. Stratagem used for fabrication of Disposable sensor strips.

2.4. Measurement and characterizations

The electrochemical impedance studies (EIS) were performed using Autolab Potentiostat/Galvanostat (Metrohms, Netherlands) with frequency impedance analyzer. These experiments were carried out using a conventional three-electrode cell with disposable sensor strip as a working electrode, platinum as auxiliary electrode and Ag/AgCl as a reference electrode in phosphate buffer saline (PBS, 50 mM, pH 7.4) containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at a 0.01V potential over the frequency range from 100KHz to 10Hz. Fourier transform infrared (FTIR) studies of conducting paper strips and APTES functionalized conducting paper strips were recorded by Thermo-Scientific Nicolet 380 spectrometer in reflectance mode in the region of 600-4000 cm^{-1} . The surface morphology of the conducting paper and APTES functionalized conducting paper was investigated by scanning electron microscope (Hitachi S-3700N). The contact angle measurements (DSA100, DSA/V 1.9, Kruss Germany) was done to study the surface properties of the modified electrode.

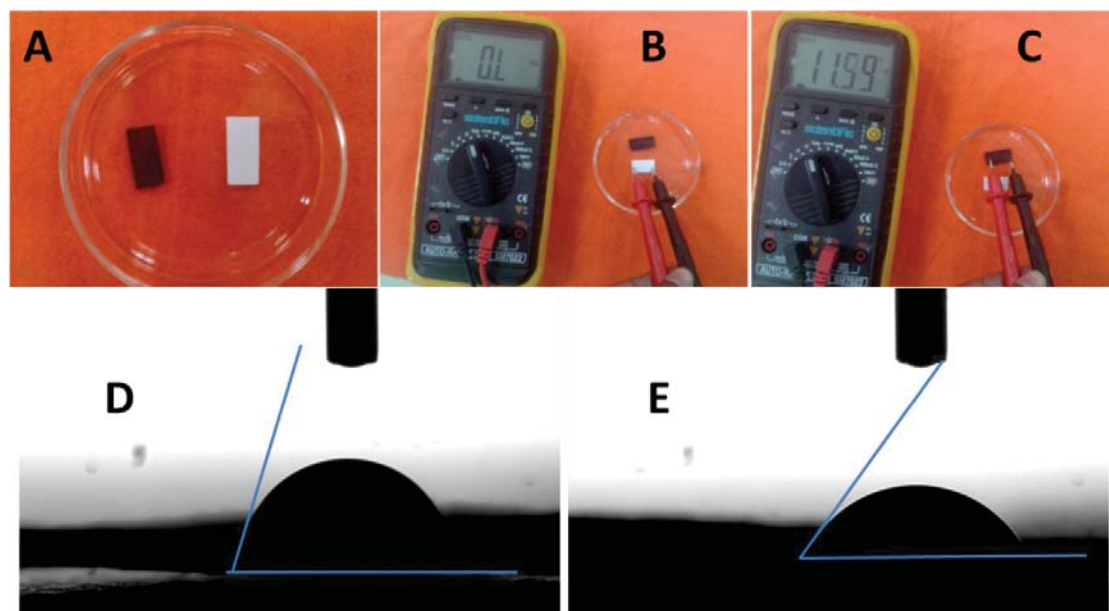


Figure 1. Optical image of paper (white) and conducting paper strips (black) [A,B,C]. Contact angle measurement of conducting paper strips [D] APTES functionalized Conducting paper Strips [E]

3. Results and discussion

The optimization of conducting paper strips was analyzed using EIS. Impedance measurements were done by applying sinusoidal AC voltage of different frequencies for interfacial studies of electrolyte and paper electrodes. EIS plot represented as a complex no. $Z = Z' + jZ''$ where Z is the real part plotted as a X-axis and Z'' is an imaginary part plotted as a Y-axis called Nyquist plot. In the Nyquist plot each point represents impedance at one frequency whereas a portion of semicircle observed at high modulation frequency describes the faradic electron transfer process between electrode and solution [16]. Data analysis after various stages of modification with best fitted equivalent electrical circuit model $R_s(R_pC_{dl})$ by fit and simulation software (NOVA 1.9), where R_s is solution phase resistance, R_{ct} is the charge transfer resistance and C_{dl} is double-layer capacitance that forms at the interface. The diameters of semicircle in Nyquist plot were utilized to observe the change in charge transfer resistant (R_{ct}) at electrode surface [17]. As a control experiment EIS studies for plane paper was also conducted. As the paper is dipped into electrolyte PBS (pH 7.4, containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$), an electrical contact is established due to hydrophilic nature of cellulose fibers. The Nyquist plot obtained for the paper shows a distorted semicircle indicating that paper behaves as a membrane and forms dielectric layer with leakage (data not shown) [16,18]. Controlling the noise by increasing the deposition cycle leads towards the hydrophobic nature of paper strips that restrict the wicking ability of paper and block the pores, which are most appropriate for measurement for the interfacial study between paper strip and solution. As the deposition cycle increases R_{ct} value decreases and heterogenous electron transfer rate constant increases (Table 1). Charge transfer resistance of conducting paper at each step controls the electron transfer kinetics of the redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The heterogeneous electron transfer rate constant (K_{ct}) between conducting paper electrode and the solution can be calculated using Eq(1) [19]:

$$K_{ct} = \frac{RT}{n^2 F^2 A R_{ct} [S]} \dots \dots \dots (1)$$

where R is the gas constant, T is absolute temperature, F is Faraday constant, A is the electrode area (cm^2), $[S]$ is the concentration of redox probe (mol cm^{-3}) and n is the number of transferred electrons per molecule of redox probe. We selected 6 times coated paper strips for further studies because there was no significance change in R_{ct} value after further deposition (desirable electrical parameter obtained).

Table 1. Optimization of paper strips coated with PEDOT: PSS

Paper Strip Coating	R_{ct}	K_{ct}
4 th times	92 K-Ohm	58 cm s^{-1}
5 th times	24 K-Ohm	222 cm s^{-1}
6 th times	15 K-Ohm	355 cm s^{-1}

3.1. Hydrophobic nature of paper

Paper is physically hydrophilic in nature because hydroxyl groups present in cellulose fiber have strong attraction with water and fluid flow through capillary action. Therefore the contact angle (CA) value of the paper approaches to zero. The wicking properties of paper are controlled by enclosing the channel of fibers by annealing with PEDOT: PSS. Conducting paper strip has a water contact angle of 77° confirming hydrophobic surface of conducting paper strips. The surface of conducting paper strips was functionalized by amine ($-\text{NH}_2$) group by treating it with 3-(aminopropyl)triethoxysilane and CA value decrease to 67° attributed to hydrophilic NH_2 group present in APTES (Fig 1).

3.2. Impedance study of conducting paper strips

In case of the conducting paper electrode, impedance at high frequencies under kinetic control and diffusion is negligible but at lower frequencies conducting paper electrode, distorted semicircle is observed because of the large pores and non-uniform layer (Fig 2A). Circuit was further improved by treatment of PEDOT: PSS coated paper with methanol. Treatment with methanol makes the film homogenous that reduces the surface- to- volume ratio of PEDOT: PSS coated paper. The charge transfer resistance increases up to $160\text{K}\Omega$ (curve b) and distortion in the semicircle is reduced. Deposition of APTES at PEDOT:PSS coated paper strips was used for immobilization of biomolecule that acts as a molecular wire for faster transfer of charge. The decrease in the value of R_{ct} after APTES deposition found as $75\text{K}\Omega$ (curve c) is attributed to enhanced transfer of charge from solution to paper electrode due to polarization of terminal $-\text{NH}_2$ group of APTES, resulting in net positive charge on surface attracting the redox mediator $[\text{Fe}(\text{CN})_6]^{-3/4}$.

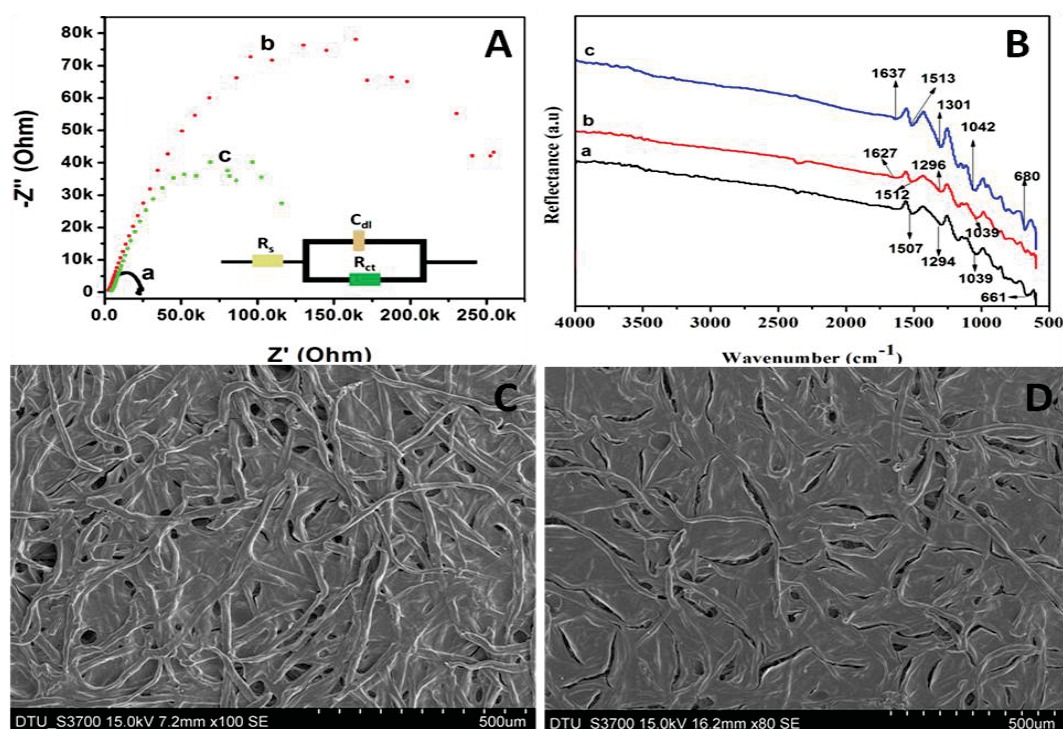


Figure 2. A) Electrochemical impedance spectra of a) Conducting paper strips b) Methanol treated conducting paper strips c) APTES functionalized surface, Inset showing equivalent circuit diagram. B) FTIR spectra of a) conducting paper strips b) Methanol treated conducting paper strips c) APTES functionalized surface. SEM image of C) conducting paper strips D) Methanol treated conducting strips.

3.3. FTIR studies

The FTIR spectra of fabricated paper based on PEDOT:PSS, methanol functionalized and APTMS modified PEDOT:PSS was carried out in the range $600\text{--}4000\text{ cm}^{-1}$ and is shown in Fig 2B. The characteristic FTIR band appears at 1507 cm^{-1} ($\text{C}=\text{C}$), 1294 cm^{-1} ($\text{C}-\text{C}$), 1039 cm^{-1} ($\text{S}-\text{O}$), and 661 cm^{-1} ($\text{C}-\text{S}$) corresponds to the thiophene ring in PEDOT:PSS [20]. The positions and relative bands of the transmittance peak of the PEDOT:PSS (curve a) and methanol functionalized PEDOT:PSS (curve b) are in agreement with literature. The IR spectra confirm that all regions have essentially the same chemical composition (curve a & b), where the most characteristic bands for PEDOT:PSS polymers can be clearly seen. The slight shifting of band at higher wavelength from 1507 cm^{-1} to 1512 cm^{-1} corresponds to $\text{C}=\text{C}$, which may perhaps be due to surface modification of PEDOT:PSS by methanol. The IR bands of PEDOT:PSS overlap with the IR band arises by APTMS modified PEDOT:PSS (curve c). The APTMS modified PEDOT:PSS shows the prominent peak at 680 and 833 cm^{-1} for Si-O symmetric stretching and bending modes, respectively. The band arises at 1042 cm^{-1} for (Si-O-Si) stretching and 1513 cm^{-1} belongs to NH_2 deformation of primary amine present in APTMS modified PEDOT:PSS paper [21].

3.4. Scanning Electron Microscopy measurement

Surface morphology of PEDOT:PSS and methanol treated PEDOT: PSS are further probed through SEM analysis. It is observed (Fig. 2C) that PEDOT:PSS conducting polymer is adsorbed at cellulose fibers wherein the deposition is clearly visible between cellulose fibers of paper. In contrast, surface morphology of methanol treated with PEDOT: PSS becomes more homogenous along with a few pores compared to the untreated PEDOT: PSS strips (Fig. 2D).

3.5. Application of disposable sensor strips

EIS is a sensitive technique that can be used to measure the interaction of biomolecule at the electrode surface. The apparent heterogeneous electron-transfer rate constants (K_{ct}) of disposable paper strips as found to be 1706 cm s^{-1} reveals improvement in electrochemical behavior owing to the immobilization of anti-CEA antibody. Several models of equivalent circuits were used to analyze the obtained plots, which are appropriate for a PEDOT: PSS film. After examining several possible equivalent circuit diagrams, the best fitting over the frequencies ranging from 100 KHz-10 Hz using an equivalent circuit diagram depicted in Fig. 2A (inset), was obtained.

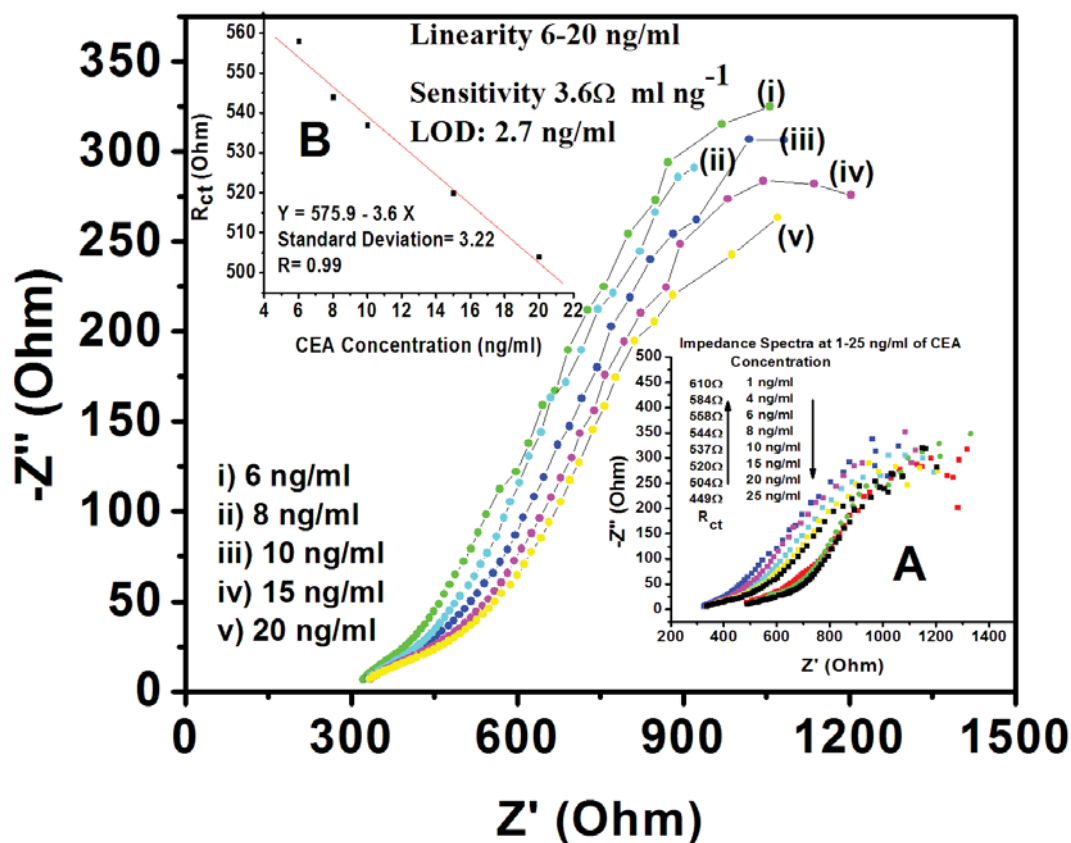


Figure 3. Impedance analysis of the disposable paper strips as a function of different concentration (1-25 ng/ml) of carcinoembryonic antigen (Inset A). Inset B showing a calibration plot between charge transfer resistance (R_{ct}) and the CEA concentration (B).

The change in charge transfer resistance on addition of CEA from human fluids (human-CEA) was due to formation of antigen-antibody complex at the electrode surface. Immobilized antibodies at the electrode surface are worked with more CEA antigen as the concentration increases and correspondingly change in the parameter of impedance spectrum. It was observed that, there is a steady decrease in impedance as antigen concentration increases towards a concentration, 1-25 ng/ml (Fig 3). A linear relationship is found between R_{ct} and CEA concentration between 6-20 ng/ml with correlation coefficient of 0.9900, the relative change in R_{ct} value observed at the different concentration of human-CEA and best fitted using equivalent circuit model. The linear regression equation is $R_{ct}(\Omega) = 576 \Omega - 3.6 \Omega \text{ ml ng}^{-1} \times [\text{CEA concentration (ng/ml)}]$ with a sensitivity of $3.6 \Omega \text{ ml ng}^{-1}$ and detection limit = 2.68 ng/ml calculated from the expression $3S_{y/x}/\text{sensitivity}$, where $S_{y/x}$ is standard deviation.

This immunosensor shows good sensor to sensor and batch to batch reproducibility and time taken for each measurement was ~5 min. It was observed that repeated runs on the same electrode gives reproducible results with RSD's less than 4% and the electrode-to-electrode reproducibility of responses have RSD less than 5% in a series of trials.

4. Conclusions

An electrochemical impedance based immunsensor has been fabricated using conducting poly (3,4ethylenedioxythiophene):poly(4-styrene sulfonate) modified paper. This paper based immunosensor has been used for selective detection of cancer biomarkers, where the inputs have been implied for the rapidity, sensitivity and stability of electrode. This simple approach does not require any highly sophisticated fabrication technique and conducting substrate such as gold, silver, ITO for impedance analysis. The results indicate promising application of this conducting paper in area of electronics, microfluidics and energy storage device.

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