Nanomaterials based biosensors for cancer biomarker detection

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Abstract. Biosensors have enormous potential to contribute to the evolution of new molecular diagnostic techniques for patients suffering with cancerous diseases. A major obstacle preventing faster development of biosensors pertains to the fact that cancer is a highly complex set of diseases. The oncologists currently rely on a few biomarkers and histological characterization of tumors. Some of the signatures include epigenetic and genetic markers, protein profiles, changes in gene expression, and post-translational modifications of proteins. These molecular signatures offer new opportunities for development of biosensors for cancer detection. In this context, conducting paper has recently been found to play an important role towards the fabrication of a biosensor for cancer biomarker detection. In this paper we will focus on results of some of the recent studies obtained in our laboratories relating to fabrication and application of nanomaterial modified paper based biosensors for cancer biomarker detection.

Keywords. Biosensor, Nanomaterials, Conducting paper, Cancer biomarker

1. Introduction
Cancer is currently the leading cause of morbidity and mortality worldwide and it is estimated that more than 11 million people are diagnosed with cancer every year and there will be 16 million new cases every year by 2020 [1, 2]. Cancer is defined as abnormal and uncontrolled cell growth due to an accumulation of specific genetic and epigenetic defects, which are either environmental or hereditary in origin [3]. The unregulated cell growth leads to the formation of a tumor mass that over time becomes independent of normal homeostatic checks and balances [4]. As the cancer progresses, the tumor begins to spread beyond the site of origin and metastasizes to other body organs and systems, making it incurable [3]. More than 200 distinct forms of cancer exist which include lung, prostate, breast, ovarian, hematologic, skin, and colon cancer, and leukemia etc [5]. Some cancers like stomach and cervical cancer are strongly associated with bacterial and viral infections, respectively [6]. The conventional techniques for cancer detection such as centrifugation, chromatography, and fluorescence and magnetic-activated cell sorting rely on the expertise and subjective judgment of highly skilled personnel [7]. Diagnosing a cancer via cross sectional imaging (CT scan) and biopsy is an expensive and often uncomfortable approach for patients and yield substantial false-negative rates and a limited potential for early diagnosis of disease [8-10]. Therefore technologies to recognize and understand the signatures of normal cells and how these become cancerous,
promise to provide important insights into the aetiology of cancer that can be useful for early detection, diagnosis, and treatment [11].

The detection of clinical biomarkers plays a crucial role in the early detection of a cancer, design of individual therapies, and to identify underlying processes involved in the disease [12]. Biomarkers are chemical substances related with the elevation of malignant tumors which are found in blood, urine, or body tissues [13]. They are normally produced directly by the embryonic tissue or tumor tissue [14]. Biomarkers indicate changes in the expression of a protein that is correlated to risk or progression of a disease or its response to treatment, and that can be measured in tissues or in the blood [14-15]. As a result, biomarkers can be specific cells, molecules or genes, gene products, enzymes or hormones [14]. An ideal cancer biomarker should have high clinical sensitivity and specificity, quick release in the blood enabling early diagnosis, capability to remain elevated for longer time in the blood, and ability to be assayed quantitatively [16-18]. More than 160 types of biomarkers are identified that are being used for the detection of cancer [19]. The expression of specific biomarkers and their accurate detection can be helpful in the diagnosis, staging and effective treatment of a cancer at an early stage. Various cancer markers are being widely used for diagnosing cancer, but carcinoembryonic antigen (CEA) is known to be a tumor marker associated with colon, lung, ovarian and breast cancer that are responsible for more than half of all cancer deaths each year [20]. CEA is a highly glycosylated cell surface glycoprotein, belonging to a group of substance known as the tumor-associated antigens. Hence, the monitoring of CEA level before and after cancer therapy facilitates early recognition of recurrences or detection of previously unremarked metastases. Determination of CEA in serum can be an interesting alternative for clinical diagnosis and monitoring of a cancer [21].

The quantification of biological or biochemical processes is of utmost importance for medical, biological and biotechnological applications [22]. However, converting the biological information to an easily processed electronic signal is challenging due to the complexity of connecting an electronic device directly to a biological environment. Electrochemical biosensors provide an attractive means to analyze the content of a biological sample due to direct conversion of a biological event to an electronic signal [22-23]. Over the past decades several sensing concepts and related devices have been developed. In this context, paper based electrochemical sensors are known to play an important role in on-going transition towards the POC diagnostic devices [24-25]. These papers based POC can be flat, folded, simple, low cost, flexible, and lightweight. Further, intelligent use of the polymers and nanomaterials (graphene, CNT) on paper may lead to enhanced performances with increased sensitivities and lowered detection limits of several orders of magnitudes. One advantage of utilizing these nanomaterials is the high specific surface thus already enabling the immobilization of an enhanced amount of bioreceptor units.

In this manuscript we discuss some of the research works reported on the development of nanomaterial modified paper substrate for cancer biomarker detection. Efforts have also been made to summarize some of the recent research work relating to CEA detection using CNT and graphene modified paper substrate.

2. Conventional method for CEA biomarker detection

Carcinoembryonic antigen (CEA) was described almost five decades ago by Gold and Freedman and was hypothesized to be an oncofetal antigen that is expressed in normal adult tissue as well [26]. Recently, CEA has been favored as a target antigen for primary and metastatic colorectal detection and other carcinomas of epithelial origin [27]. Owing to technological advancements various methods for CEA detection have been developed over the years ranging from immunohistopathology [28-29] to radioimmuno assays [30-31] (Enzyme linked immune sorbent assay-ELISA) [32-33] and reverse transcriptase polymerase chain reaction (RT-PCR) [34-35] to radioactive tracer fluoro-2-deoxy-D-glucose-Positron Emission Tomography (FDG-PET) [36-37] detection. Goldenberg et al reported a 3-step unlabeled antibody immunocytochemical staining method for CEA detection where horseradish peroxidase stain was used. The immunocytochemical reaction could be used to detect CEA concentrations
above 0.7 μg/g in ethanol fixed and 3.0-5.0 μg/g in formalin fixed specimens. The CEA concentration values detected by this method are considerably high that indicates low sensitivity of the method [28]. Kleist et al used continuous cell line (HT29) from human carcinoma of the colon for synthesizing colon tumor antigen CEA which was detected by immunofluorescence technique. The weaker fluorescence of CEA indicated low sensitivity of the detection method [29]. Goldenberg et al reported a radioimmuno detection technique for CEA detection in which the prepared hyperimmune goat Ig against CEA was radio labeled with $^{133}$I (iodine radioactive isotope) [30]. The detection limit of this method was 2 cm and any tumor below 2 cm was undetectable which a major drawback of this method. RT-PCR based detection of CEA mRNA molecules in peripheral blood of 95 colorectal carcinoma (CRC) patients was carried out by Dong et al [34]. The sensitivity of the method achieved was 2 copies per tube that were high enough to determine very low levels of CEA in peripheral blood. Yonemura et al employed RT-PCR coupled with conventional cytological assay for determination of CEA mRNA in peritoneal washes of 230 patients of gastric cancer [35]. The sensitivity achieved by combining RT-PCR with cytological assay resulted in higher sensitivity over the conventional cytological assay alone.

Positron emission tomography (PET) can be employed to distinguish recurrent tumors from post therapeutic changes. PET is a functional imaging technique relying on physiological changes or metabolic functions for the detection of disease. Fluoro-2-deoxy-D-glucose (FDG) is a radioactive glucose analog used in medical imaging modality PET. The increased glucose metabolism of tumor cells is the base for using FDG-PET in oncology which makes it an important tool for quantitative analysis of malignant tumors. Flanagan et al used FDG-PET for investigating unexplained elevation of plasma CEA levels in 22 colorectal cancer patients post colorectal surgery. PET was more sensitive than conventional imaging methods with a positive predictive value of 89% (15 out of 17) in CRC patients [36]. ELISA has been considered as gold standard technique for detection of proteinaceous molecules and has been traditionally employed for tumor antigen, CEA detection [32]. Various modified ELISA techniques using gold nanoparticles have been used to further improve the sensitivity [33]. Inspite of the reliable detection, ELISA is a time consuming, complex and expensive detection method.

These conventional techniques hold advantage in providing low detection limits but are complex, time consuming and expensive. The requirement of trained personnel and expensive equipments as in case of FDG-PET is another major disadvantage of these techniques. They are not rapid and the diagnosis period ranges from a few hours to a few days making them laborious and time consuming. The success of these techniques has been marred by low sensitivity and specificity. Although a number of groups have modified the fundamental techniques for obtaining better results, like modified fluorescence RT-PCR, combining conventional cytology with RT-PCR, radioimmuno assays ,yet the achieved sensitivity and specificity of these techniques is low and calls for an urgent need for rapid, reliable, specific, and sensitive alternative technique for cancer detection.

3. Role of Biosensors in CEA detection
Early diagnosis of tumor biomarkers is critical for treatment of cancer. The urgent need for a rapid, reliable, specific and sensitive method for diagnosis of cancer markers at an early stage of disease has focused on point of care (POC) diagnostics. Biosensors have a number of potential advantages over other methods of cancer diagnosis, especially reduced assay time, portability, high sensitivity and selectivity, simplicity, miniaturized and flexibility. Biosensor-based diagnostics can assist cancer screening and improve the rates of earlier diagnosis and attendant improved forecast. This technology can be particularly useful for enhanced health care delivery in the public setting and to underserved diaspors. Biosensors have potential for multi-target analyses, automation, and cost effective testing [38].

Biosensor is a bioanalytical device, which converts a biological response into a measureable signal. Biosensor is used to estimate the concentration of desired substances and other parameters of biological interest without using the biological system directly. A biosensor consists of three major components such as a bio-recognition element for selective recognition of an analyte also known as bio-
receptor, an immobilization matrix for the immobilization of a recognition biomolecule and a transducer for conversion of biochemical response into a measurable signal [39]. Bio-receptor and transducer are together referred to as a biosensing membrane. Based on the method of signal transduction biosensors may be classified into six basic groups, i.e. optical, mass, micromechanical, electrochemical, magnetic, and thermal sensors [40]. Among the various biosensors, electrochemical biosensors have received special attention as they allow high sensitivity, lower detection limits, automation, inexpensive testing, and development of disposable devices and methodologies capable of working with very small sample volumes [41].

3.1. Electrochemical Biosensor

An electrochemical biosensor is a small device that can be used for direct measurement of the analyte in the sample matrix. Ideally, such a device is capable of responding continuously and reversibly and does not perturb the sample. Electrochemical biosensors combine the analytical power of electrochemical techniques and the specificity of biological recognition processes. The most commonly used transducers in electrochemical biosensors are amperometric and potentiometric transducers. The analytical information in potentiometric devices is obtained by converting the biological response or biochemical reaction into a potential signal by the use of ionselective electrodes. Amperometric biosensors on the other hand monitor the current generated against applied constant potential by the reduction or oxidation of the electroactive species involved in the biorecognition process. Due to high sensitivity and wide linear range, amperometric biosensors have attracted more attention. The ongoing research on new sensing concepts, combined with numerous technological advancements, has expanded horizons for extensive clinical applications of amperometric devices [42]. The enhanced sensitivity, specificity, simplicity, and inherent miniaturization of modern electrochemical bioassays allow them to compete with the most advanced optical protocols [41]. Feng et al reported an electrochemical biosensor for label free neoplastic cell detection using aptamer and functionalized graphene. The high binding affinity of the aptamer to the overexpressed nucleolin on the neoplastic cell surface enabled the electrochemical aptasensor to detect as low as thousand cells [43]. Chen et al developed a simple, label free electrochemical biosensor for oral cancer detection based on nuclease-assisted target recycling and DNAzyme for the detection of DNA species related to oral cancer in saliva [44].

3.2. Conventional electrodes used for cancer biomarker detection

For the fabrication of electrochemical biosensor for cancer biomarker detection various electrodes such as indium tin oxide, glassy carbon and gold electrodes have been used. Kumar et al used zirconia modified ITO electrode for oral cancer biomarker detection using cyclic voltammetry technique [45]. Zeng et al used nanostructured gold modified ITO electrode for CEA detection using electrochemical impedance spectroscopy [46]. Liu et al used gold electrode for fabrication of impedimetric biosensor for CEA detection [47]. In another study Huang et al used gold nanoparticle, carbon nanotube and chitosan modify glassy carbon electrode for CEA detection using differential pulse voltametry [48]. Lee et al reported a porous silicon antibody immunoassay platform for the detection of total human kallikrein 2, a protein involved the pathology of prostate cancer [49]. The major limitation of using the electrodes is they are costly, hard, brittle, non disposable and is difficult to functionalize. In addition to the aforementioned problems with the conventional electrodes, the requirement of very high temperatures for their processing and expertise makes them all the more undesirable. Therefore, in order to overcome these prevalent problems with the conventional electrodes, the attention has been drawn towards paper based electrode as a potential substrate.

Paper is becoming a suitable substrate for biosensing applications since it is simple, cost-effective, flexible, and are disposable. It is also beneficial in terms of cheap production, renewable raw materials and a porous structure useful for modification of paper substrate for sensing application [50]. Conducting paper (CP) has been considered as an efficient electrochemical platform for conduction of electronic
signal generate during biosensing process [51]. For fabrication of CP both organic and inorganic materials can be used to make paper conducting. While inorganic materials often have a better electrical performance but the drawbacks are the high material cost, processing difficulties and cracks in film during bending/sintering. However, organic materials are preferred due to the mechanical flexibility, low cost, and simple processing. In this context, carbon nanomaterial, conducting polymer and their composite is promising candidate for fabricating a CP. The advantages of paper as a sensor substrate compared to the conventional electrodes are listed in Table 1.

Table 1. Comparison of the properties of the conventional electrodes with the paper electrode.

| S.No | Properties                  | Conventional electrodes (Indium tin oxide and gold coated glass, Glassy carbon, Silicon) | Paper |
|------|-----------------------------|----------------------------------------------------------------------------------------|-------|
| 1    | Cost                        | Very high                                                                              | Very low |
| 2    | Flexibility                 | No                                                                                     | Yes   |
| 3    | Disposability               | No                                                                                     | Yes   |
| 4    | Biocompatibility            | No                                                                                     | Yes   |
| 5    | Modification/Functionalization | Difficult                                                                         | Easy  |
| 6    | Fluid flow                  | Forced                                                                                 | Capillary action |
| 7    | Surface by Volume ratio     | Low                                                                                   | High  |
| 8    | Fabrication and High through put production | No                                                                                   | Yes   |

3.3. Graphene oxide integrated conducting paper for Cancer biomarker detection

Graphene is a flat monolayer of carbon atoms which are tightly packed into a two-dimensional (2D) honeycomb lattice. Graphene, has received much attention due to its unique physicochemical properties such as high surface area, high mechanical strength, and ease of functionalization, excellent conductivity and mass production [52]. Therefore, integration of graphene with conducting paper may perhaps lead to improved paper properties such as enhanced electrochemical activity, sensitivity and stability of paper sensor. Ruecha et al fabricated a paper based cholesterol biosensor with the help of electrospraying, wax printing and screen printing using graphene/polyvinylpyrrolidone/polyaniline nanocomposite for the detection of cholesterol [24]. The detection technique was amperometric and the fabricated electrode was found to be stable for about 2 weeks. Lu et al used graphene-gold nanoparticle for fabrication of paper based electrochemical DNA sensor by modifying paper substrate using Wax printing and screen printing [53]. In another work Xu et al reported an electrochemiluminescence sensor by modifying paper with poly(sodium 4-styrenesulfonate) functionalized graphene/nafion composite for the detection of DNA mismatches [54]. The electrode was fabricated with the help of photolithography and screen printing. Paper based chemiluminescence excited photoelectrochemical aptamer device for adenosine triphosphate measurement based on a gold coated paper electrode modified with a tin dioxide quantum dot/reduced graphene oxide nanocomposite has been fabricated by Wang et al [55]. Moreover, graphene oxide (GO) has been also incorporated in paper matrix for cancer biomarker detection. The dual-signal amplification technique was used by employing graphene oxide-chitosan/gold nanoparticles platform and [4,4’-(2,5-dimethoxy-1,4-phenylene)bis(ethyl-2,1-diyl) dibenzoic acid] (P-acid) functionalized nanoporous silver as signal amplification label [56]. The immunosensor was used to detect prostate specific antigen (PSA) and carcinoembryonic antigen (CEA) using electrochemical impedance spectroscopy. The linear detection
range obtained for CEA was 0.001-10ng/mL with a LOD 0.8 pg/mL. The fabricated immunosensor was also found to be stable for about 4 weeks. Li et al detect CEA on paper platform by electrochemilumininescence based method. The materials used to fabricate the electrode were gold-chitosan hybrids and graphene quantum dots functionalized Au@Pt[57]. Further, Wu et al fabricated a paper based electrochemical immunodevice integrated with amplification-by-polymerization for the ultrasensitive multiplexed detection of cancer biomarkers (CEA, AFP, CA 125, CA 153). The material used in the electrode fabrication was GO and chitosan. The detection range of the fabricated sensor measured using differential pulse voltametry (DPV) was in the range, 0.01-100ng/mL [58].

The above discussed work require wax printing, screen printing and costly conducting ink (carbon paste, silver paste etc) and hold complicated design. To overcome this problem, we have recently fabricated a paper based label free electrochemical immunosensor based on PEDOT:PSS-RGO composite for the detection of CEA by chronoamperometric technique (figure 1) [59]. PEDOT:PSS-RGO modified conducting paper electrode are prepared by simple dip coating method, wherein the conductivity is further improved from ~1.16×10⁻⁴ Scm⁻¹ up to ~3.12×10⁻² Scm⁻¹ on treatment with ethylene glycol. The observed significant increase in electrical conductivity is due to conformational rearrangement in the polymer and is due to strong non-covalent cooperative interaction between PEDOT and the cellulose molecules.

Moreover the variation of relative conductivity of paper electrode with folding angle and folding cycle was studied (figure 2). It is observed that 4% relative conductivity deviation during -180° to 180° folding angle and 20% conductivity deviation after 30 cycle of folding and unfolding (1 cycle = 360° rotation) was observed.

Figure 1. Schematic of PEDOT:PSS-RGO based paper biosensor for CEA detection[59]
Figure 2. (a) Schematic diagram of PEDOT:PSS-RGO paper electrode folding at different deformation angle. (b) The ratio of measured conductivity ($\sigma_{\text{meas}}$) to initial conductivity ($\sigma_{0}$) of PEDOT:PSS-RGO based paper with respect to folding angle. (c) The ratio of measured conductivity ($\sigma_{\text{meas}}$) to initial conductivity ($\sigma_{0}$) of PEDOT:PSS-RGO based paper versus folding cycle (1 cycle = 360°)[59].

Integration of RGO in modified paper results in improved electrochemical activity and signal stability (Figure). Further this platform is used for the immobilization of monoclonal antibody against carcinoenmbryonic antigen (anti-CEA) (anti-CEA/RGO/EG@CP). The chronoamperometric studies were performed in PBS containing 5mM [Fe(CN)$_6$]$^{3-}/-4$ at 2 V. The sensing response of PEDOT:PSS-RGO composite based paper immuno electrode as a function of CEA concentration (figure 3) revealed high sensitivity of 25.8 mAng$^{-1}$mLcm$^{-2}$ in the physiological range of 2–8 ngmL$^{-1}$ and good storage stability (21 days) for label free detection. The response of paper electrode is validated using CEA concentration of serum sample of cancer patient. This simple, low cost, flexible paper sensor can be decomposed by simple incineration and has immense potential as a smart medical diagnostic kit or point of care biosensor.

Figure 3. (a) Electrochemical response studies of anti-CEA immobilized PEDOT:PSS-RGO paper at different concentration of CEA. (b) Calibration plot between the magnitudes of current recorded and CEA concentration (curve i); control experiment in absence of antibody (curve ii)[59].
3.4. Carbon nanotube integrated conducting paper for cancer biomarker detection

Carbon nanotubes (CNTs) have been exploited for the development of electrochemical and biological sensors because of their excellent electrochemical properties, large surface area, ballistic electron transport and high mechanical strength. In addition to enhanced electrochemical reactivity, CNT-modified electrodes can be employed to immobilize biomolecules and to minimize surface fouling effects \[60\]. Moreover, CNT-based electrochemical transducers offer substantial improvements in the performance of amperometric enzyme electrodes, immunosensors and nucleic-acid sensing devices. Recent studies have demonstrated that CNT can be used to enhance the electrochemical reactivity of important biomolecules, and can be utilized to promote the electron-transfer reactions of proteins such as cytochrome c, ascorbic acid, xanthine oxidase, catalase, tryptophan and dopamine \[61\]. Further, CNT’s exceptional properties such as small size, great strength, high electrical and thermal conductivity, and large specific surface area make them excellent amplification platforms to increase the number of signal-generating molecules \[52\].

Carbon nanotubes (CNTs) considered as a suitable candidate to be used in paper based sensing devices due to effective deposition characteristics using carbon nanotubes-based ink and excellent electronic transduction properties. For instance, Shim et al. took advantage of cotton yarns to build a SWCNT based chemiresistor for protein detection \[62\]. Wang et al modified filter paper with carbon nanotube and antibodies for amperometric detection of microcystin-LR toxin \[63\]. Similarly Pozuelo et al fabricated a conductive paper by simply painting paper filter with SWCNT ink. This platform was further utilized for human immunoglobulin G detection \[64\]. Zang et al reported electrochemical Immunoassay on 3D microfluidic paper-based device for detection of various cancer biomarker such as α-fetoprotein, carcinoma antigen 125, carcinoma antigen 199 and CEA \[65\]. For this purpose they modify paper surface with carbon paste, wax, carbon nanotubes, chitosan with the help of wax and screen printing technique. In another work, a battery-triggered microfluidic paper-based multiplex electrochemiluminescence immunodevice based on potential-resolution strategy was demonstrated for the diagnosis of four cancer markers (r-fetoprotein (AFP), CA 153, CA 199 and CEA \[66\]. For this purpose carbon nanotube, chitosan and glutaraldehyde modified paper zone were used for antibody immobilization which are connected with screen printed carbon working electrode and Ag/AgCl auxiliary electrode through the paper channel. Further, Wang et al proposed a microfluidic paper-based analytical device (μPADs). A wax-patterned microfluidic paper-based three-dimensional electrochemical device (3D-μPED) was demonstrated based on the multi-walled carbon nanotubes (MWCNTs) modified μPAD. Using HRP-O-Phenylenediamine- \( \text{H}_2\text{O}_2 \) electrochemical system, a sandwich immunoassay on this 3D-μPED for sensitive diagnosis of two tumor markers simultaneously in real clinical serum samples was developed with a linear range of 0.001–75.0 U mL\(^{-1}\) for cancer antigen 125 and 0.05–50.0 ng mL\(^{-1}\) for CEA \[67\]. In this work different printing technique (screen printing, wax printing etc), costly conducting ink or sophisticated design were used for biomolecule detection.

We used composites of poly(3,4-ethylenedioxythiophene):poly(styrenesulfonate) (PEDOT:PSS) and carbon nanotube (CNT) for fabrication of the conducting paper by simple dip coating method \[68\]. It was found that the conductivity of formic acid treated paper increased by 2 orders of magnitude due to removal of non-conducting PSS molecule from conducting paper surface leading to conformational rearrangement. This fabricated paper is electrochemical active, flexible, efficient conductive and can be easily disposed off by simple incineration (figure 4). Further this platform was utilized for cancer biomarker (carcinoembryonic antigen, CEA) detection by immobilizing monoclonal antibody against CEA (anti-CEA). The PEDOT:PSS-CNT based electrochemical paper immunosensor showed sensitivity \[7.8 \mu\text{A(ng/ml)}^{-1}\text{cm}^2\] in a linear detection range of \( 2–15 \text{ngmL}^{-1} \) (figure 5) and feasibility of paper electrode was also validated with CEA concentration in serum sample of cancer patient. It was observed that incorporation of carbon nanotubes improve heterogeneous electron transfer rate constant and linear detection range of PEDOT:PSS-CNT based conducting paper compare to PEDOT:PSS-RGO based CP.
4. Conclusions

We have discussed the prospects of CP based biosensors for cancer biomarker detection. Compared to the conventional electrode used for detection of CEA, CP based electrochemical biosensors are flexible, cost effective, light weight and can be easily disposed. The studies suggest that paper doped with MWCNT and graphene results in enhanced sensitivity, lower detection limit and wider linear detection range and have a great potential for early cancer detection. This nanomaterial modified paper based platform should be used in the development of medical diagnostics kits, flexible electronics and energy storages devices.
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