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# Carbon nanotube-based ultrasensitive multiplexing electrochemical immunosensor for cancer biomarkers

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ABSTRACT

A multiplexing electrochemical immunosensor was developed for ultrasensitive detection of cancer related protein biomarkers. We employed disposable screen-printed carbon electrode (SPCE) array as the detection platform. A universal multi-labeled nanoprobe was developed by loading HRP and goat-anti-rabbit IgG (secondary antibody, $Ab_2$) onto multiwalled carbon nanotube (MWNT). This universal nanoprobe was available for virtually any sandwich-based antigen detection and showed superiority in several areas. By using the SPCE array and the universal nanoprobe, we could detect as low as 5 pg $mL^{-1}$ of prostate specific antigen (PSA) and 8 pg $mL^{-1}$ of Interleukin 8 (IL-8) with the electrochemical immunosensor. We also demonstrated simultaneous detection of two protein biomarkers with this platform. With these attracted features, our immunoassay system shows promising applications for in-field and point-of-care test in clinical diagnostics.



## 1. Introduction

Early diagnosis of cancer is a challenge facing scientists from all over the world which is very important for cancer therapy. In early diagnosis of cancer, accurate detection of certain protein biomarkers is critical but difficult as there is only trace protein biomarker in serum of early cancer patients (Kulasingam and Diamandis, 2008; Ludwig and Weinstein, 2005; Pepe et al., 2001; Welsh et al., 2003). Traditional assay methods such as enzyme-linked immunosorbent assay (ELISA) (Butler, 2000), radioimmunoassay (Bolton and Hunter, 1973), fluorescence immunoassay (Goldman et al., 2002), electrophoretic immunoassay (Bao, 1997), mass spectrometric immunoassay (Diamandis and van der Merwe, 2005), and immune-polymerase chain reaction (PCR) assay (Widjojoatmodjo et al., 1992) often have some disadvantages, resulting in the increasing demand for operationally simple, ultrasensitive and easily automated device. Considerable efforts have been made to develop rapid, sensitive and selective immunosensors (Akram et al., 2006; Chen et al., 2008; Dill et al., 2004; Mani et al., 2009; Tang et al., 2008; Wu et al., 2008). Electrochemical immunosensors, with the inherent advantages of high sensitivity, low cost, low power requirement and potential of automation, have been applied for clinical diagnosis (Ghindilis et al., 1998; Warsinke et al., 2000).

With the aim of ultrahigh sensitive biosensors, various signal amplification strategies using nanostructured materials have been developed (Cao, 2008; Grodzinski et al., 2006; Song et al., 2010), such as gold nanoparticles (Nam et al., 2003; Wang et al., 2008; Yan et al., 2010; Zhang et al., 2006), quantum dots (Hu et al., 2009), magnetic nanoparticles (Yigit et al., 2008) and carbon nanotubes (Lin et al., 2005). In the area of ultrasensitive electrochemical immunosensing, nanomaterials can be directly used as electroactive labels (Das et al., 2006; Liu et al., 2004) or used as carriers to load a large amount of electroactive labels (Mani et al., 2009; Tang et al., 2008). Using nanomaterials as electroactive labels, Ho' group has reported a novel electrochemical immunosenor. Monoclonal capture antibody was adsorbed on polyethylene glycol-modified disposable screen-printed electrode as the detection platform, while polyclonal signal antibody and gold nanoparticle (AuNP) conjugates were used as electrochemical signal probes (Ho et al., 2010). The electrochemical signal from the bound AuNP congregates was obtained after oxidizing them in 0.1 M HCl at 1.2 V for 120 s, followed by the reduction of $AuCl^{4-}$ in square wave voltammetry (SWV) mode. Using nanomaterials as carriers for signaling and biorecognition have also attracted attentions from scientists. Wang et al. reported carbon nanotubes (CNTs) carrying numerous enzyme tracers for dramatically amplifying enzyme-linked electrical detection of proteins and DNA (Zhao et al., 2009). A novel strategy of electrochemical

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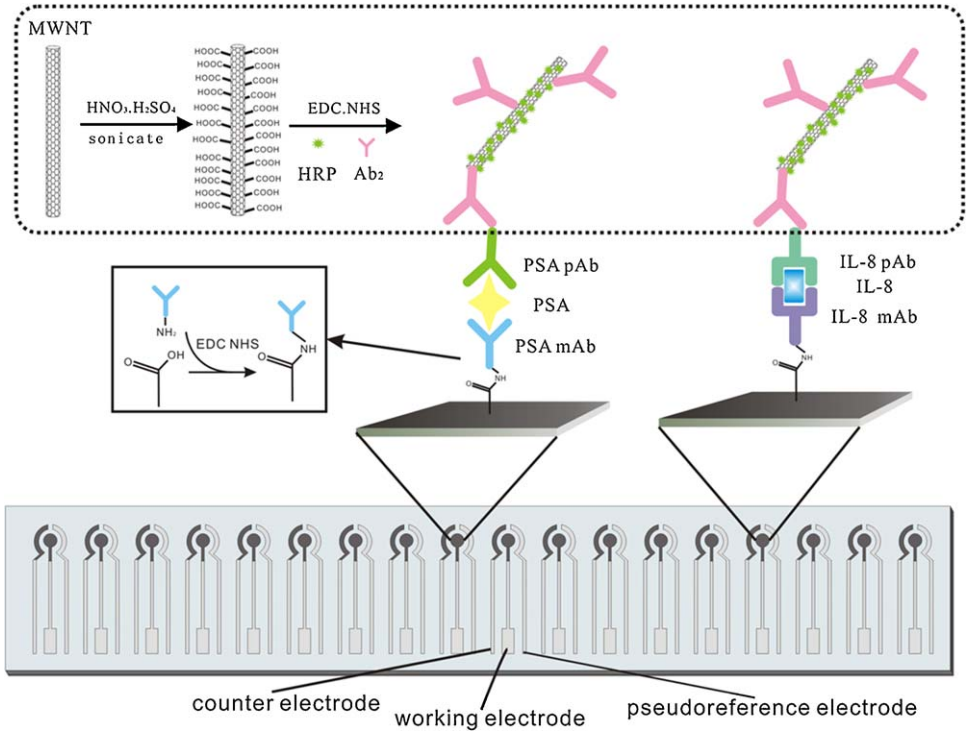


**Scheme 1.** Schematic demonstration for the "sandwich" type strategy electrochemical immunosensor. A 16 channel screen-printed carbon electrode (SPCE) array was employed as the detection platform, each containing a three electrode system: carbon working electrode, a carbon counter electrode and a silver pseudoreference electrode. The capture antibodies were immobilized on the working electrode by a three step protocol: electrochemical activation was first taken to generate carboxylic acid groups on the working electrode and then the EDC/NHS were used to activate the carboxylic acid groups which was then removed. After that, capture antibodies (PSA mAb or IL-8 mAb) were immobilized by using the amine residues on the proteins. The target antigen (PSA or IL-8) and the signal antibody (PSA pAb or IL-8 pAb) formed a "sandwich" type complex with the capture antibody, leading to the binding of the universal nanoprobe to the electrode that can be transuded to the catalytic amperometric readout. The process of the universal nanoprobe preparation was as following: the pristine MWNTs were first sonicated in the $HNO_3$ and $H_2SO_4$ to generate carboxylic acid groups which were then activated by EDC/NHS. After removal of free EDC/NHS, $Ab_2$/HRP mixture in an optimized ratio was added and the universal nanoprobe was achieved.

immunoassays based on the utilization of encapsulated electrochemical signal-generating microcrystals was reported (Mak et al., 2005). The electrochemical signal was achieved by the release of a large amount of ferrocene after sandwich immuno-binding. Rusling's group has achieved greatly enhanced sensitivity using carbon nanotubes (CNTs) carrying horseradish peroxidase (HRP) labels and antibodies for immunodetection of the prostate specific antigen (Jensen et al., 2009; Yu et al., 2006). The limit detection of this CNT amplified immunosensor was low to 4 pg $mL^{-1}$. Nanomaterials have been demonstrated to be excellent carriers in the amplification strategies.

Despite advances in nano-amplification technologies, there are still challenges faced by researchers such as complicated assembly process and stability of nanomaterials. Especially, different antibodies have different electrostatic properties so that the assembly conditions of different antibodies with same nanomaterials are variant very often. When encountering with simultaneous detection of panels of tumor markers in clinical diagnosis of cancers (Liu et al., 2004; Wilson, 2005), several different nanomaterial based bioconjugates were demanded. However, the processes were complicated. As a result, it is necessary to develop a simple approach which can overcome these obstacles and give a total solution for this problem. Herein we proposed an electrochemical immunosenor using a disposable sixteen channel screen-printed carbon electrode (SPCE) array combined with a universal multilabel nanoprobe for the simultaneous detection of cancer biomarkers: prostate specific antigen (PSA) and Interleukin 8 (IL-8). The immobilization of capture antibodies on this SPCE was considerable simple, which was conducted by first electrochemical activating the carbon working electrode. This process generated carboxylate groups to bind to the amine residues on capture antibodies. This covalent binding was proved to be very efficient. A universal multilabel nanoprobe was fabricated by consistent loading of HRP and goat-anti-rabbit IgG (secondary antibody, $Ab_2$) on multiwalled carbon nanotube (MWNT). As $Ab_2$ can bind to rabbit antibodies for any antigen, this multilabel nanoprobe is available to the detection of any target antigen by using unlabelled rabbit polyclonal antibody serving as a bridge. Then the universal nanoprobe can be attached to biosensing surface to generate electrochemical signals. Combining this universal nanoprobe with disposable SPCE array, we provide a promising future in clinical applications with simultaneous immunoassay of multiple protein biomarkers.

## 2. Experimental

### 2.1. Materials

PSA antigen, mouse monoclonal anti-PSA antibody (PSA mAb, clone no. M701042) and rabbit polyclonal signal anti-PSA antibodies (PSA pAb) were purchased from Fitzgerald (U.S.). IL-8 antigen, mouse monoclonal anti-IL-8 antibody (IL-8 mAb, clone no. 500-M08) and rabbit polyclonal anti-IL-8 antibodies (IL-8 pAb) were purchased from Peprotech Canada. Inc. (Ottawa, ON, Canada). Multiwalled Carbon nanotube (MWNT) was purchased from Shenzhen Nanotech Port Co. Ltd (NTP, china). TMB substrate (TMB = 3, 3′, 5, 5′ tetramethylbenzidine; Neogen K-blue low activity substrate) was purchased from Neogen (U.S.). $Ab_2$, HRP (MW 44,000 Da), HRP labeled $Ab_2$ ($Ab_2$-HRP), lyophilized 99% bovine serum albumin (BSA), and Tween-20 were from Sigma Aldrich. The buffer solutions involved in this study are as follows: immunoreagents were dissolved in pH 7.2 0.1 M phosphate saline (PBS) buffer (0.01 M phosphate, 0.14 M NaCl, 2.7 mM KCl).