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Electrochemical Detection of Cardiac Biomarkers Utilizing Electrospun Multiwalled Carbon Nanotubes Embedded SU-8 Nanofibers


1 Introduction

The focus of the present decade is towards achieving smart and intelligent health care, which is possible only with the development of reliable, low cost, robust, selective and sensitive biosensors. For a large set of diseases such as cancer, genetic disorders, vascular diseases etc., ultrasensitivity is paramount as the concentration of targeted analytes is in the range of few nano-gram/ml to few pico-gram/ml [1–3]. Apart from being ultrasensitive, the transduction mechanism should be extremely selective. One of the robust ways to achieve high selectivity is to use immunoassay techniques. These techniques rely on highly specific molecular recognition between antigens and antibodies and are integral part of all the main analytical methods in clinical diagnostics. Techniques such as Enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay, radio immunoassay, Fluoroiimmunoassay are being routinely deployed in the clinics for identifying various biomarkers [4–7]. However the equipment associated with these techniques are costly, bulky, time consuming and need skilled manpower to operate them. Electrochemical immunoassay, a viable alternative, offers several advantages such as high sensitivity, fast analysis, simple pre-treatment, small analyte volume, simple instrumentation, ease of miniaturization, to name a few [8–10]. In this technique, immunoassay reactions occurring at the electrode/electrolyte interface induce changes in the electrochemical kinetics which can be analysed using standard electroanalytical techniques.

Immobilization of antibodies onto the electrode surface is one of the critical steps in the development of an electrochemical immunosensor. An effective and simple immobilization method enhancing the amount of antibodies onto the electrode surface is of a great value addition as it not only results in high sensitivity but also detection of targeted antigen over a wide range of concentrations. The sensitivity and selectivity can be boosted manifold by developing novel materials which perform the dual role of immobilization as well as transduction.

Nanohybrid materials are frontrunners for these applications. As a proof concept, the detection of cardiac biomarkers, Myoglobin (Myo), cardiac Troponin I (cTn I) and Creatine Kinase MB (CK-MB) is demonstrated. The synthesized nanofibers were functionalized with the antibodies of the biomarkers and the detection was carried using Electrochemical Impedance Spectroscopy, an excellent technique for understanding the adsorption kinetics. A minimum detection limit of nano-gram/ml is demonstrated using this nanobiosensor platform.

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Biocompatibility is one of the critical requirements for developing biomedical diagnostic devices. Several bio-compatible polymers such as polystyrene (PS), polycarbonate (PC), Poly(ethylene glycol) (PEG), Poly(acrylic acid) (PAA), Polydimethylsiloxane (PDMS), epoxy-based negative photoresist (SU-8), chitosan [12–15] have been explored for developing miniaturized biosensors. One such biocompatible material whose utilization in the fabrication of biomedical devices increased drastically over the past few years SU-8. It is an epoxy based negative photoresist. Apart from its regular utilization in microfabrication and biocompatibility, the ease of functionalization comes in handy for developing highly selective biosensors. Several well developed, straight forward protocols for functionalization of different groups onto SU-8 have been reported [16]. For developing nanobiosensors, SU-8 is preferable as synthesis of SU-8 based nanofibers using electrospinning technique is well explored for different applications [17–20]. Electrostatic spinning or electrospinning is a very simple, robust technique to create nanofibers through uniaxial stretching of electrically charged jet of viscoelastic polymer solution. The diameters of the fibers obtained are in range of 10 μm to 10 nm [21]. In order to utilize SU-8 nanofibers for electrochemical/electrical applications, it is essential to increase the conductivity of the fibers as they are insulating in nature. Multiwall carbon nanotubes (MWCNTs) find wide range of applications in developing sensors including electrochemical sensors because of their excellent electrical, mechanical properties. Though they have excellent transduction properties, surface functionalization of MWCNTs is an issue for developing robust biosensors. Well established protocols can functionalize MWCNTs easily, however, minimal percentage of functional groups and their randomness may result in repeatability issue. Thus the primary focus of this work is to demonstrate the applicability of MWCNTs embedded SU-8 hybrid nanofibers for ultrasensitive biosensing applications as both materials complement each other by overcoming their respective shortcomings viz., minimal functional groups for MWCNTs and lack of conductivity for SU-8. By embedding MWCNTs into SU-8, highly conductive nanofibers that are biocompatible, amenable for functionalization, were synthesized. As a proof of concept, we demonstrate electrochemical detection of three cardiac biomarkers: Myoglobin (Myo), cardiac Troponin I (cTn I) and Creatine Kinase MB (CK-MB) using the synthesized nanofibers. The schematic shown in the Figure 1 illustrates detailed protocol of achieving the same. The rest of the sections focus on explaining these steps in detail.

2 Experimental

2.1 Materials

Standard multi-walled carbon nanotubes (MWCNTs) with diameter range of 5–20 nm was purchased from Reinste Nano Ventures Pvt. Ltd (New Delhi, India). SU-8 (2015) was purchased from MicroChem Crop (USA). Myoglobin from equine skeletal muscle, Troponin I from human heart, Creatine Kinase MB fraction from human heart and Indium Tin Oxide (ITO) Coated Substrates were purchased from Sigma-Aldrich (USA). Monoclonal anti-Myoglobin antibody, monoclonal anti-cardiac Troponin I antibody, monoclonal anti-Creatine Kinase MB antibody, cross-linker molecules N-Hydroxysuccinimide
sodium salt (NHS) and 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide (EDC) were purchased from Abcam biochemical (UK). Chloroform (CHCl₃), Toluene, potassium ferrocyanide (K₃Fe(CN)₆), potassium ferricyanide (K₄Fe(CN)₆) were purchased from MERCK, India. Phosphate buffer solution (PBS) were purchased from Sigma-Aldrich (USA) and all other reagents used were analytical grade. All solutions were prepared with deionized water of 18 MΩ-cm purified from a Milli-Q purification system.

2.2 Apparatus

Synthesis of MWCNTs embedded SU-8 nanofibers was carried out using Electrospinning set up (E-Spin Nano Pvt. Ltd, India). For structural and physical characterization of these synthesized nanofibers X-Ray Diffraction analysis (XRD) (X Pert PRO,USA), Raman Spectroscopy (Senterra, Bruker, UK), Scanning Electron Microscopy (SEM)/Quanta 200, FEI, Frankfurt am Main, Germany; SUPRA 40 VP, Gemini, Carl Zeiss, Oberkochen, Germany), Transmission Electron Microscopy (TEM) (Philips, CM200) were carried out. All the electrochemical experiments were carried out using CH660E Electrochemical Workstation (CH Instruments, USA).

2.3 Synthesis of MWCNTs Embedded SU-8 Nanofibers

Electrospinning is a simple, robust, low cost technique to produce nanofibers at large scale. This technique involves application of a very high electric field is applied between a syringe containing a polymer solution and a cathode which typically is grounded. Sub-micron fibers jet out of the syringe at a critical field when electrostatic forces overcome surface tension forces and are collected onto cathode which also serves as the collector of fibers. The morphology of fibers can be precisely controlled by optimizing the electrospinning parameters. In this work, MWCNTs embedded SU-8 is used as polymer solution. SU-8 is an epoxy based negative photoresist marketed by Microchem, USA and is available in different viscosities. We have used SU-8 2015 as it meets the desired viscosity requirements. Prior to spinning, desired weight percentage of MWCNTs were dispersed in an organic solvent and then mixed with SU-8 2015 and probe sonicated for an hour. The weight percentage of MWCNTs dictated the conductivity of the composite as SU-8 is insulating in nature. It is necessary to have very high conductivity in order to use this as an electrode material for carrying electrochemical impedance spectroscopy. In our previous work, we have proven that 11 % w/w dispersion provides best conductivity and it is not possible to increase the concentration of MWCNT dispersion beyond 13 % as it clogs the syringe thereby precluding the synthesis of uniform nanofibers. Hence 11 % w/w MWCNTs dispersion was used in this experimentation [22]. The solution was immediately loaded into a 26-gauge needle (internal diameter of 0.26 mm) and electrospinning process was carried out with a clean ITO substrate as collector for almost 30 min. The resultant mat of nanofiber was extracted from ITO substrate and was used for further experimentation.

2.4 Functionalization of GCE with MWCNTs Embedded SU-8 Nanofibers and Monoclonal Antibodies

GCE was polished with 0.05 μm alumina and washed thoroughly with DI water. MWCNTs/SU-8 dispersion was prepared by dispersing 1 mg of synthesized MWCNTs/SU-8 nanofibers in 1 ml of toluene and sonicated for 30 min. The dispersion was then drop cast onto the polished electrode and was allowed to dry for 2 hours. Depending on the target analyte, the respective antibody was chemically attached onto this modified electrode using the following protocol.

A mixture of EDC/NHS along with monoclonal antibodies of cardiac biomarkers was sonicated for 20 min. This mixture was then drop cast onto modified electrode and was allowed to dry for 2 hours. The electrode was rinsed thoroughly with DI water to remove any physisorbed monoclonal antibodies. In the subsequent sections, “modified GCE” refers to MWCNTs/SU-8 nanocomposite coated GCE and “antibody modified GCE” refers to MWCNTs/SU-8 nanocomposite modified GCE functionalized with monoclonal antibodies.

2.5 Functionalization of GCE with MWCNTs Embedded SU-8 Nanofibers and Monoclonal Antibodies

Electrochemical studies were performed using an electrochemical analyser (CH660E, CH Instruments, USA) employing a three-electrode system comprising a 0.5 mm platinum wire as the counter-electrode, Ag/AgCl (saturated, 0.1 M KCl) as the reference electrode and Glassy Carbon Electrode (GCE) as the working electrode with the dimension of 3 mm diameter. All the electrodes were purchased from CH instruments Inc, USA. EIS measurements were carried out in 0.1 mM phosphate buffer solution (pH 7.0, 5 ml volume) containing 2.5 mM each of K₃Fe(CN)₆ and K₄Fe(CN)₆. Antibody modified GCE was used as working electrode. A potential of +0.2 V was applied between the working electrode and Ag/AgCl reference electrode. This is the formal potential of the redox couple. The impedance offered by the modified working electrode for the electron transfer was measured between the working and platinum counter electrode. The frequency ranges used for the measurement was from 25 mHz to 1 MHz with a sine wave amplitude of 5 mV. Cyclic Voltammetry measurements were carried out specifically for myoglobin detection and fresh pH 7 phosphate buffer was used as electrolyte in those experiments.
3 Results and Discussion

3.1 HRTEM Analysis

To understand the internal structure of the MWCNT/SU-8 nanocomposite, TEM analysis was carried out. Nanocomposite extracted from ITO substrate dispersed into ethanol and then drop coated onto the TEM grid. The dispersion process resulted in tiny nanoparticles of the synthesized nanofibers. The high resolution images of the same is shown in Figure 2. The Figure 2 (a) shows that tubular-shaped MWCNTs are well dispersed in SU-8 polymer. The length range of the MWCNTs is several tens of micrometers, and they have an external diameter of approximately 10–50 nm. The presence of MWCNTs is confirmed with the help of selected area electron diffraction (SAED) pattern which shows graphitic (002) and (004) reflections which are key signature features of MWCNTs (Figure 2 (b)). The high-resolution image of the individual MWCNTs shows outer diameters of 34 nm. The Figure 2 (c) shows higher magnification of Figure 2 (a). From the atomic scale image of the MWCNTs/SU-8 (Figure 2 (d)), it can be concluded that MWCNTs in the nanofiber retains its crystalline nature, with an interlayer spacing of 3.41 Å (d002 for MWCNT, shown in the sub-figure inset). These results clearly reveal a successful formation of the MWCNT embedded SU-8 nanofibers.

3.2 Detection of Cardiac Biomarkers

The interaction of cardiac biomarkers with monoclonal antibody functionalized, MWCNTs/SU-8 nanocomposite is shown in Figure 1 (b). When antibody modified GCE is used as working electrode the corresponding antigen gets immobilized onto the electrode surface through immunosassay reaction. The charge transfer resistance \( R_{ct} \) of standard redox couple \[ \text{Fe(CN)}_6^{3-}/\text{Fe(CN)}_6^{4-} \] depends on the electrode surface. Different electrodes have different charge transfer resistance and it depends on the surface condition of the electrode.

Any adsorption on the surface of an electrode acts as an inhibition to the electron transfer process thereby increasing the charge transfer resistance. This principle can be used to study the adsorption behaviour at an electrode interface and is applied to detect cardiac biomarkers.

3.2.1 Myoglobin

a) Electrochemical Impedance Spectroscopic Study

The Nyquist plots for different concentrations of Myoglobin on anti-myoglobin modified GCE are shown in Figure 3. The X-axis represents the real part of the impedance and the Y-axis represents the imaginary part of the impedance. The charge transfer resistance \( R_{ct} \) is found to increase with increase in the concentration of Myoglobin. This confirms that there is an adsorption on the electrode surface which is inhibiting the electron transfer rate of the redox couple \[ \text{Fe(CN)}_6^{3-}/\text{Fe(CN)}_6^{4-} \] thereby increasing the charge transfer resistance. The higher is the concentration of Myoglobin, the more is the extent of adsorption leading to higher charge transfer resistance. The control test for the same is carried out by repeating the same experiment with modified GCE. In this case MWCNTs/SU-8 nanocomposite was coated on GCE and they were not functionalized with anti-myoglobin. In such case, myoglobin cannot get adsorbed onto the electrode surface owing to absence of its corresponding antibodies.

![Fig. 2. HRTEM images: (a) MWCNTs embedded SU-8 nanocomposite; (b) an individual MWCNT (inset: the SEAD pattern of the MWCNT); (c) illustrates a good dispersion of MWCNTs in SU-8 (d) an atomic-scale of a MWCNT/SU-8.](image-url)

![Fig. 3. EIS Nyquist plots obtained for anti-myoglobin immobilized, MWCNTs/SU-8 nanocomposite modified GCE before and after the addition of Myoglobin; inset image (a) Variation of standardized charge transfer resistance with respect to concentration of myoglobin.](image-url)
No change in the charge transfer resistance should occur in this case. From the resultant Nyquist plot shown in Figure 4, it can be clearly inferred that there is no change in charge transfer resistance when the electrode surface was not functionalized with antibodies.

b) Cyclic Voltammetry Study

After the EIS experiments were completed, the working electrode was rinsed with DI water to remove any physically adsorbed myoglobin. Fresh phosphate buffer solution was taken and cyclic voltammetry measurements were carried out with this working electrode, with Pt and Ag/AgCl as counter and reference electrodes respectively. These experiments were carried out to validate the chemisorption of Myoglobin onto the modified GCE electrode and strengthen the claim that the change in charge transfer resistance is due to the chemisorption of myoglobin, not physisorption. Since myoglobin is a redox active species containing \( \text{Fe}^{2+} \), adsorbed species of the same onto a working electrode should show redox behaviour even in a pure buffer solution. Furthermore, the peak current should be proportional to the scan rate as in the case of any adsorbed redox species [23]. Figure 5 shows cyclic voltammograms of the same at different scan rates. Myoglobin is showing an irreversible behaviour with a cathodic peak at \(-0.35\) V. This is because of the reduction of \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \). The peak current Vs scan rate is linear (inset Figure 5), indicating the process is an adsorption process as opposed to a diffusion based mass transport process wherein the peak current is proportional to the square root of the scan rate [23]. Ideally, any redox active couple like \( \text{Fe}^{2+}/\text{Fe}^{3+} \) would should both anodic and cathodic peaks at the same voltage [23]. Any shift in the peaks or appearance can be attributed to the nature of the electrode/adsorbed species interface. The electron tunneling distance is the key in this case. Lower the electron tunneling distance, the system would be closer to the ideal behavior. As the electron tunneling distance increases, the kinetics decreases resulting in a quasireversible or irreversible behavior. In this case, the irreversible behaviour in this adsorbed state is expected because the heme group in myoglobin is embedded deep inside the protein. The electron tunnelling distance from the electrode surface is large owing to the presence of the nanocomposite and monoclonal antibody. This results in reduction in kinetics thus making the process irreversible. Cyclic Voltammetry results indicate affirmatively that Myoglobin was adsorbed onto the electrode surface and was detected successfully using EIS.

\( R_{ct} \) is calculated from Nyquist plots by fitting the curve with parameters in the modified Randles circuit shown in Figure 6 (a). This circuit is modified to effectively capture mass transport and kinetic processes that occur in this measurements. A typical Randles circuit comprises of a double layer capacitor \( C_{dl} \) in parallel with a series combination of charge transfer resistance \( R_{ct} \) and Warburg impedance \( W \). This parallel combination is in series with the solution resistance \( R_s \) [23]. However Randles circuit cannot accurately model all the behaviours especially when there is a non-uniformity of the electrode and there is a change in the environment near the electrode/electrolyte interface. Frequency dispersion is a predominant effort and is taken care of by using a constant phase element [24]. In the proposed circuit a parallel constant phase element \( (Q) \) is connected in parallel with solution resistance \( R_s \) to capture the effects of frequency dispersion on the solution resistance. An additional capacitor along with a constant phase element is added parallel to the double layer capacitor \( C_{dl} \). This captures the effect of surface roughness, non-uniform surface modification and variations in the double layer capacitance due to adsorption. \( R_{ct} \) and \( W \) represent the charge transfer resistance and the Warburg impedance respectively. Randles circuit
with these modifications incorporated accurately fits in the model with an error of < 2% as opposed to the original Randles circuit wherein the error is > 10%. Figure 6 (b). The values of $R_n$ with Myoglobin were standardized to the reference value of $R_n$ without Myoglobin and are denoted by $R_{norm}$. The variation of $R_{norm}$ with respect to the logarithmic concentration of Myoglobin is shown in Figure 3 (a) and it is linear with a correlation coefficient of 0.999 with a CV of less than 5%. The linearity range in this case is from 1 ng/ml to 50 ng/ml. The limit of detection was 0.1 ng/ml. This is calculated using standard sigmoidal binding curve analysis [25].

3.2.2 Troponin I and Creatine Kinase-MB

The procedure adapted for detecting cardiac Troponin I and Creatine Kinase-MB is same but for the antibody that was functionalized to the modified GCE. Their respective monoclonal antibodies were functionalized onto the surface to carry out the detection. Figure 7 and Figure 8 shows the EIS plots for cTn I and CK-MB respectively. The inset shows the linearity range for these biomarkers. These markers are not electroactive. However, since the antibody-antigen reactions are highly specific, the selectivity is not a concern. In these cases also, the coefficient of variation across experiments is less than 5%. The limit of detection for cTn I and CK-MB as calculated using standard sigmoidal analysis were found to be 0.1 ng/ml and 1 ng/ml respectively. In the case of cTn I the linearity range was found to be 0.1 ng/mL–10 ng/mL whereas in CK-MB, we observed a wider range of linearity from 10 ng/mL–10 μg/mL.

4 Conclusions

Synthesis and characterization of MWCNTs embedded SU-8 electrospun nanofibers and their application towards ultrasensitive detection of cardiac biomarkers using Electrochemical Impedance spectroscopy (EIS) is demonstrated. The synthesized nanohybrid composite combined excellent electrical and transduction properties of MWCNTs and ease of functionalization and biocompati-

Fig. 6. (a). Modifies Randles electrical equivalent circuit and (b) Experimental and simulated (modified Randles circuit and original Randles circuit) fit impedance data obtained for Myo modified GCE.

Fig. 7. EIS Nyquist plots obtained for anti-cardiac troponin I immobilized, MWCNTs/SU-8 nanocomposite modified GCE before and after the addition of cardiac troponin I; inset image (a) Variation of standardized charge transfer resistance with respect to concentration of cardiac troponin I.

Fig. 8. EIS Nyquist plots obtained for anti- Creatine Kinase-MB immobilized, MWCNTs/SU-8 nanocomposite modified GCE before and after the addition of Creatine Kinase-MB; inset image (a) Variation of standardized charge transfer resistance with respect to concentration of Creatine Kinase-MB.
bility of SU-8. Electrochemical detection of cardiac biomarkers, Myoglobin (Myo), cardiac Troponin I (cTn I) and Creatine Kinase MB (CK-MB) is demonstrated. The proposed system is label-free. This reduces the complexity manifold as labelling is a complex process. Furthermore developing three electrode system using screen printed process is very well established and the entire process is amenable for point of care applications. A minimum detection limit of nano-gram/ml is demonstrated using this nanobiosensor platform for all the biomarkers with a wide range of linearity.

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