



# Haemoglobin immobilized on nafion modified multi-walled carbon nanotubes for O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> and CCl<sub>3</sub>COOH sensors

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## ABSTRACT

A conductive biocomposite film (MWCNTs-NF-Hb) containing multi-walled carbon nanotubes (MWCNTs) incorporated with entrapped haemoglobin (Hb) in nafion (NF) has been synthesized on glassy carbon electrode (GCE), gold (Au), indium tin oxide (ITO) and screen printed carbon electrode (SPCE) separately by potentiostatic methods. The presence of both MWCNTs and NF in the biocomposite film enhances the surface coverage concentration ( $\Gamma$ ), and increases the electron transfer rate constant ( $K_s$ ) to 132%. The biocomposite film exhibits a promising enhanced electrocatalytic activity towards the reduction of O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> and CCl<sub>3</sub>COOH. The cyclic voltammetry has been used for the measurement of electrocatalysis results of analytes by means of biocomposite film-modified GCEs. The MWCNTs-NF-Hb-modified GCEs' sensitivity values are higher than the values obtained for other film modified GCEs. The surface morphology of the biocomposite films which have been deposited on ITO has been studied using scanning electron microscopy and atomic force microscopy. The studies have revealed that there was an incorporation of NF and immobilization of Hb on MWCNTs. Finally, the flow injection analysis has been used for the amperometric studies of analytes at MWCNTs-Hb and MWCNTs-NF-Hb film modified SPCEs. The amperometric study results have shown higher slope values for MWCNTs-NF-Hb biocomposite film.

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## 1. Introduction

A wide variety of applications of matrices made of carbon nanotubes (CNTs) for the detection of bioorganic and inorganic compounds such as insulin, ascorbic acid, etc. were already reported [1–4]. The rolled-up graphene sheets of carbon, i.e., CNTs, exhibits a  $\pi$ -conjugative structure with a highly hydrophobic surface. This property of CNTs allows them to interact with some organic aromatic compounds through  $\pi$ - $\pi$  electronic and hydrophobic interactions [5–7]. These interactions were used for preparing composite sandwiched films for electrocatalytic studies [8] and in the designing of nanodevices with the help of non-covalent adsorption of enzyme and proteins on the side walls of CNTs. This method resulted in the development of CNT-based nanostructures, which contain biochemical units in them [9]. Some attempts were also made to prepare hydrophilic surface CNTs to overcome the dispersion problems in aqueous medium for bio-electrochemical applications [10]. Electrodes which are modified with composite films are widely used not only in the preparation of capacitors, batteries, fuel cells, chemical sensors and biosensors but also in the field of material science and photoelectrochemistry [11–13].

Even though the electrocatalytic activity of the CNTs with protein matrices individually shows good results; some properties like mechanical stability, sensitivity for different techniques and electrocatalysis for multiple compound detections are poor. Besides CNTs and proteins, there are other interesting polymer materials, and one such material is nafion (NF). Due to the presence of a sulfonated group in NF and its strong acidic character, it has been used as a solid catalyst in organic synthesis. Extensive studies have been already carried out using hybrid thin films of CNTs-NF. These film-modified electrodes are being used for the electrocatalytic oxidation of biochemical compounds [14–16].

Haemoglobin (Hb) consists of four subunits of polypeptide enzymes. A haem (iron porphyrin) group in each subunit acts as an active center. Haemoglobin usually shows slow rates of electron transfer on a bare metal electrode surface. The idea of enhancing the electron transfer rate of haemoglobin using mediators and solid electrodes have been explored, for example, using methylene green [17]. The enhancement of the electron transfer rate of haemoglobin were reported using polyion surfactants of dihexadecylphosphate and poly(diallyldimethylammonium) (DHP and PDDA) [18,19], didodecyldimethylammonium bromide (DDAB) [20,21], poly(ester sulfonic acid) [22], dimyristoylphosphatidylcholine (DMPC) [23], clay films [24,25], etc. [26–30]. Interestingly, the catalytic activity towards different compounds using direct electron transfer between Hb-modified electrode from aqueous

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solution were already investigated [31,32]. These reports are concerned with analytical applications and the properties of haemoglobin during the electrocatalytic oxidation or reduction of electroactive haemoglobin on electrode surfaces. Further, the literature survey reveals that there were no previous attempts made for the synthesis of biocomposite film composed of CNTs, NF and Hb for sensor applications. In this paper, we report a novel biocomposite film (MWCNTs-NF-Hb) made of multi-walled carbon nanotubes (MWCNTs) incorporated with NF and Hb, its characterization and its enhancement in functional properties, peak current and electrocatalytic activity. We have reported also, its application in the electrocatalysis of  $O_2$ ,  $H_2O_2$  and  $CCl_3COOH$ . The film formation process involves the modification of glassy carbon electrode (GCE) with uniform well dispersed MWCNTs on it and drying it. Then electrodeposition of NF and immobilization of Hb from neutral aqueous solution on the MWCNTs modified GCE have been done subsequently.

## 2. Experimental

### 2.1. Apparatus

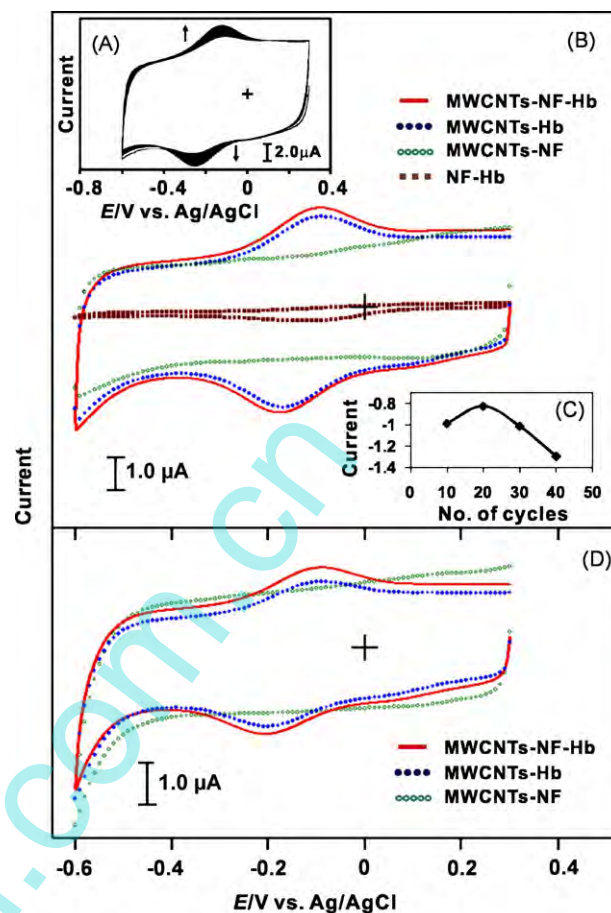
Cyclic voltammetry (CV) was performed in an analytical system model CHI-611, CHI-400 and CHI-1205A potentiostat. A conventional three-electrode cell assembly consisting of an Ag/AgCl reference electrode and a Pt wire counter electrode were used for the electrochemical measurements. The working electrode was either an unmodified GCE or GCEs modified with NF-Hb, MWCNTs-NF, MWCNTs-Hb or MWCNTs-NF-Hb biocomposite films. In these experiments, all the potentials have been reported versus the Ag/AgCl reference electrode. The gold working electrode was an 8 MHz AT-cut quartz crystal coated with a gold plating. The diameter of the quartz crystal was 13.7 mm; the gold electrode diameter was 2.4 mm. The flow injection analysis (FIA) of the analytes at screen printed carbon electrode (SPCE) were done using Alltech 426 HPLC pump containing an electrochemical cell. The morphological characterizations of the films were examined by means of SEM (Hitachi S-3000H) and atomic force microscopy (AFM) (Being Nano-Instruments CSPM4000). All the measurements were carried out at  $25^\circ\text{C} \pm 2$ .

### 2.2. Materials

Hb, MWCNTs (OD = 10–20 nm, ID = 2–10 nm and length = 0.5–200  $\mu\text{m}$ ), NF and potassium hydroxide obtained from Aldrich and Sigma–Aldrich were used as received. All other chemicals used were of analytical grade. The preparation of aqueous solution was done with twice distilled deionized water. Solutions were deoxygenated by purging with pre-purified nitrogen gas. Buffer solutions were prepared from  $H_2SO_4$  for the pH 2.5 aqueous solution.

### 2.3. Preparation of MWCNTs and MWCNTs-NF-Hb-modified electrodes

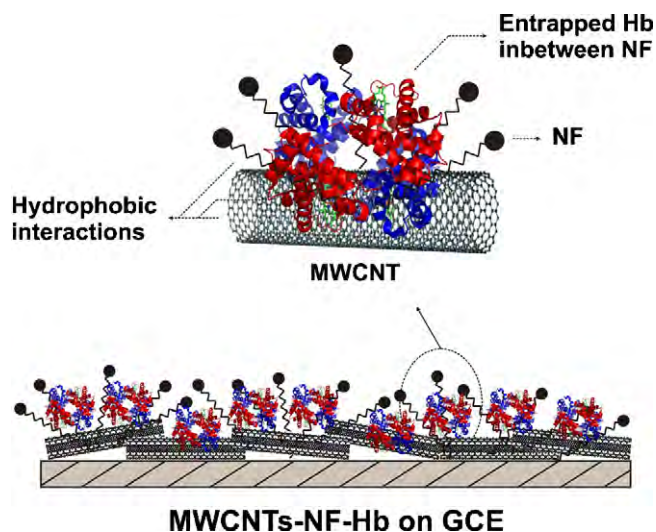
There was an important challenge in the preparation of MWCNTs. Because of its hydrophobic nature, it was difficult to disperse it in any aqueous solution to get a homogeneous mixture. Briefly, the hydrophobic nature of the MWCNTs was converted in to hydrophilic nature by following the previous studies [10,33]. This was done by weighing 10 mg of MWCNTs and 200 mg of potassium hydroxide in to a ruby mortar and grained together for 2 h at room temperature. Then, the reaction mixture was dissolved in 10 ml of double distilled deionized water then precipitated many times in to methanol for the removal of potassium hydroxide. Then, the obtained MWCNTs in 10 ml water were ultrasonicated for 6 h to get a uniform dispersion. This functionalization process of MWCNTs is to get a hydrophilic



**Fig. 1.** (A) Consecutive CVs of MWCNTs-NF GCE modified from 0.1 mM Hb present in pH 2.5  $H_2SO_4$  aqueous solution, scan rate at  $50\text{ mV s}^{-1}$ . (B) CVs of GCE modified from NF-Hb, MWCNTs-NF, MWCNTs-Hb and MWCNTs-NF-Hb film in pH 2.5  $H_2SO_4$  aqueous solution, scan rate at  $20\text{ mV s}^{-1}$ . (C) Plot of Hb peak current at MWCNTs-NF-modified GCE vs. number of cycles of NF growth by consecutive CVs. (D) CVs of gold electrode modified from MWCNTs-NF-Hb, MWCNTs-Hb and MWCNTs-NF films in pH 2.5  $H_2SO_4$  aqueous solution, scan rate at  $20\text{ mV s}^{-1}$ .

nature for the homogeneous dispersion in water. This process not only converts MWCNTs to hydrophilic nature but this helps to breakdown larger bundles of MWCNTs in to smaller ones too. This was confirmed using SEM, which is not shown in the figures. A homogeneous 0.005 wt% NF solution was used for depositing NF on the electrodes.

Before starting each experiment, GCEs were polished by BAS polishing kit with 0.05  $\mu\text{m}$  alumina slurry and rinsed and then ultrasonicated in double distilled deionized water. The GCEs studied were uniformly coated with  $75\text{ }\mu\text{g cm}^{-2}$  of MWCNTs then dried. The concentration of homogeneously dispersed MWCNTs was exactly measured using a micro-syringe. The electrochemical deposition of NF on MWCNTs modified GCE was performed from the 0.005 wt% NF solution in pH 2.5 by consecutive CV over a suitable potential region of  $-0.6$  to  $0.5\text{ V}$ , 40 cycles and scan rate at  $100\text{ mV s}^{-1}$  (figure not shown). Then, the modified MWCNTs-NF electrode was carefully washed with double distilled deionized water and dried. The immobilization of Hb on the MWCNTs-NF-modified electrode was performed from 0.1 mM Hb in pH 2.5  $H_2SO_4$  aqueous solution by consecutive CV over a suitable potential region of  $-0.6$  to  $0.3\text{ V}$ , 30 cycles and scan rate at  $50\text{ mV s}^{-1}$ . Then, the modified MWCNTs-NF-Hb electrode was carefully washed with double distilled deionized water to perform other studies. Solutions were deoxygenated by purging with pre-purified nitrogen gas for about 1 h. For a detailed comparison of electrocatalysis reac-



**Scheme 1.** Schematic representation of possible interaction between MWCNTs, NF and Hb in the formation of MWCNTs-NF-Hb biocomposite film-modified electrodes.

tions, we studied different types of modified electrodes such as MWCNTs-NF, MWCNTs-Hb and MWCNTs-NF-Hb. In all these experiments, the electrodes were first modified by MWCNTs and then electrodeposited with NF and immobilized with Hb. These comparative characterization studies were done to reveal the obvious necessity of the presence of MWCNTs in the NF-Hb biocomposite film.

The electrocatalytic reduction studies of  $O_2$  were carried out using  $O_2$  saturated aqueous solutions (pH 2.5  $H_2SO_4$ ) at various electrodes mentioned in Section 2.1. The concentration of  $O_2$  in the aqueous solution was varied by de-aerating with pre-purified  $N_2$  gas. The variation of  $O_2$  concentration for each experiment was measured using  $O_2$  meter (Oxi 323-A/set WTW 82362, Germany). In these studies, electrochemical cells were properly sealed with ventilation, and a continuous flow of  $N_2$  gas was maintained over the solution during the electrochemical measurements to avoid the  $O_2$  interference from atmosphere. Similarly, the electrocatalysis studies of  $H_2O_2$  and  $CCl_3COOH$  were also carried out in  $N_2$  atmosphere.

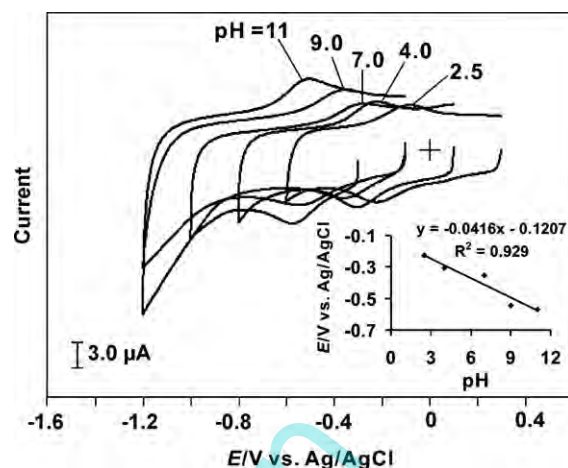
### 3. Results and discussions

#### 3.1. Electrochemical synthesis and its characterization of MWCNTs-NF-Hb biocomposite film

The immobilization of Hb (from 0.1 mM Hb) on MWCNTs-NF modified GCE using pH 2.5  $H_2SO_4$  aqueous solution has been performed by consecutive CVs as shown in Fig. 1(A). In this figure, the redox couple found growing on subsequent cycles, which indicates that during the cycle the immobilization of Hb took place on MWCNTs-NF-modified GCE at suitable potential range of 0.3 to  $-0.6$  V. In the following experiments, each newly prepared biocomposite film on GCE has been washed carefully in deionized water to remove the loosely bounded Hb on the modified GCE, and then

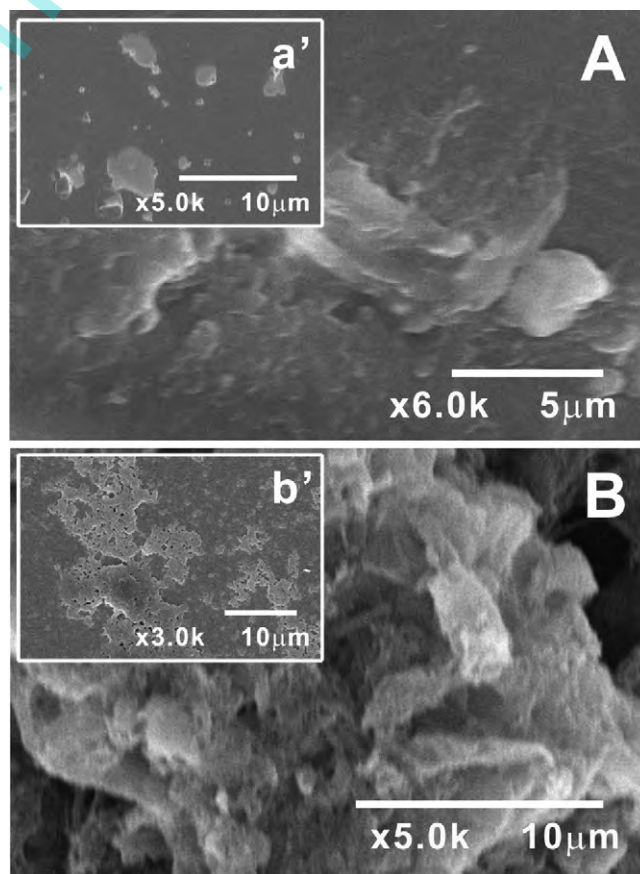
**Table 1**  
Surface coverage concentrations ( $\Gamma$ ) of Hb at different types of modified electrodes using CV technique in  $H_2SO_4$  aqueous solution (pH 2.5).

Electrode type	Modified film	$\Gamma$ (pmol $cm^{-2}$ )
GCE	NF-Hb	96.2
	MWCNTs-Hb	155
	MWCNTs-NF-Hb	169
Gold	MWCNTs-Hb	252
	MWCNTs-NF-Hb	318



**Fig. 2.** CVs of MWCNTs-NF-Hb film on GCE synthesized at similar conditions and transferred to various pH solutions; scan rate:  $50 \text{ mV s}^{-1}$ . The inset shows the formal potential vs. pH (2.5–11), the slope  $-41 \text{ mV/pH}$  is almost nearer to Nernstian equation for non-equal number of electrons and protons transfer.

transferred to pH 2.5 aqueous solutions for other electrochemical characterizations. Fig. 1(B) shows the electrochemical signal of Hb at  $20 \text{ mV s}^{-1}$  on NF modified GCE with irreversible cathodic peak current at  $E_{pc} = -0.19 \text{ V}$  versus Ag/AgCl in pH 2.5  $H_2SO_4$  aqueous solution. Whereas in MWCNTs and MWCNTs-NF, reversible redox peaks at formal potential  $E^{0'} = -0.13 \text{ V}$  has been obtained for  $Fe^{III/II}$  redox reaction corresponding to the immobilized Hb. Among MWCNTs and MWCNTs-NF, MWCNTs-NF modified GCE shows higher peak current for  $Fe^{III/II}$  redox reaction of the immobilized Hb. The



**Fig. 3.** SEM images of (A) NF-Hb and (B) MWCNTs-NF-Hb biocomposite film on ITO electrode. The insets are (a') NF and (b') Hb on ITO electrode.

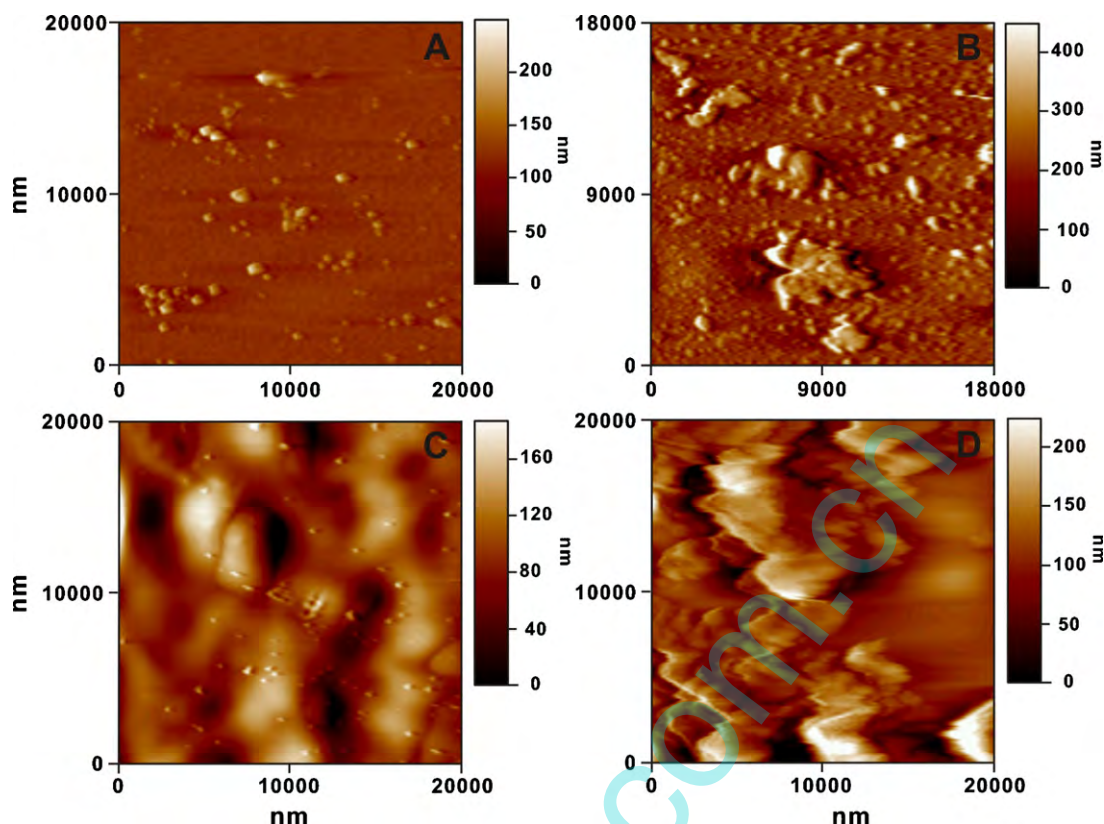


Fig. 4. AFM images of (A) NF, (B) Hb, (C) NF-Hb and (D) MWCNTs-NF-Hb biocomposite film on ITO electrode.

Fig. 1(B) shows no redox peaks at MWCNTs-NF in the absence of Hb. The optimization of the number of cycles for NF deposition has been done using the plots shown in Fig. 1(C). Where, different MWCNTs-NF-Hb films have been synthesized using different cycling of NF deposition, and then the CVs of MWCNTs-NF-Hb have been obtained using pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solution and the peak currents have been plotted against number of cycles. It is obvious that, above and below 20 cycles, the peak current decreases, which shows that the optimum deposition of NF took place at 20 cycles. Similarly, the optimization of NF concentration has also been studied, in which the deposition of NF from 0.005 wt% solution shows higher redox peak current for Hb (figures not shown). The possible interaction between MWCNTs, NF and Hb in the formation of MWCNTs-NF-Hb biocomposite film is given in Scheme 1.

From the CVs in Fig. 1(B), the surface coverage concentration ( $\Gamma$ ) values have been calculated and given in Table 1. In this calculation, the charge involved in the reaction ( $Q$ ) has been obtained from CVs and applied in the equation  $\Gamma = Q/nFA$  where, the number of electron transfer involved in the Hb redox reaction has been assumed as two (one electron for each heme group, that is two Hb molecules) [34]. These values indicate that the presence of MWCNTs increases the surface area of the electrode, which in turn increases the  $\Gamma$  of Hb. The different scan rate studies of MWCNTs-Hb and MWCNTs-NF-Hb biocomposite films on GCE using pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solution (see supplementary data) shows that the redox peak currents of both the films increases linearly with the increase of scan rate. The results demonstrated that the redox process is not controlled by diffusion. Plots from different scan rate results (supplementary data) show that MWCNTs-NF-Hb possesses faster electron transfer rate than MWCNTs-Hb film. Further, the electron transfer rate constant ( $K_s$ ) have been calculated from these different scan rate results based on Laviron theory [35]. Where,  $K_s = 0.08$  and  $0.19 \text{ s}^{-1}$  for MWCNTs-Hb and MWCNTs-NF-Hb bio-

composite film, respectively. From these  $K_s$  values the increase in the ability of electron transfer between the electrode surface and the Hb in presence of NF is  $\approx 132\%$ . These results too show that, there is an enhancement in the functional properties of the biocomposite film at lower scan rates in presence of both NF and MWCNTs [36]. Gold electrodes have also been used to characterize the biocomposite films in pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solution. Fig. 1(D) represents the redox peak of Hb on MWCNTs-NF-Hb, MWCNTs-Hb and MWCNTs-NF-modified gold electrode, where MWCNTs-NF-Hb shows higher redox peak current for Hb than other two films. These results revealed the importance of the presence of both MWCNTs and NF in the biocomposite film, where MWCNTs enhances the electron transfer on different electrodes which in turn widens the sensor-based applications. Fig. 2 shows the CVs of MWCNTs-NF-Hb on GCE in various pH aqueous buffer solutions without the presence of Hb. This shows that the film is stable in the pH range between 2.5 and 11, and the values of  $E_{pa}$  and  $E_{pc}$  depends on pH value of the buffer solution. The inset in Fig. 2 shows the formal potential of MWCNTs-NF-Hb plotted over a pH range of 2.5–11. The response shows a slope of  $-41.6 \text{ mV/pH}$ , which is close to that given by the Nernstian equation for two electron one proton transfer [37].

Four different films; Fig. 3(a') NF, (b') Hb, (A) NF-Hb and (B) MWCNTs-NF-Hb have been prepared on the indium tin oxide (ITO) with similar conditions and potential as that of GCE and were characterized using SEM. From Fig. 3, it is significant that there are morphological differences between all these four films. The morphological structure in (a') shows uniform patches of NF has been formed on ITO. The same uniform patch morphological structure can be seen in (A) and (B). Similarly, (b') shows Hb film with pores as reported previously [38]. When compared (b') with (A) and (B) almost similar morphology could be seen. The presence of MWCNTs is obvious in (B) with highly uneven surface. Comparing all the images, the immobilization of Hb and the incorporation of NF

**Table 2**

Comparison of Epc and Ipc of analytes in electrocatalysis reactions using CV technique at different types of modified electrodes in pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solution.

Analytes	Reaction type	pH	Ep (mV)		Ip (μA)	
			Hb <sup>a</sup>	Hb <sup>b</sup>	Hb <sup>a</sup>	Hb <sup>b</sup>
O <sub>2</sub>	Reduction	2.5	−190	−200	4.5	10.6
H <sub>2</sub> O <sub>2</sub>	Reduction	2.5	−210	−200	4.2	5.1
CCl <sub>3</sub> COOH	Reduction	2.5	−240	−250	9.6	11.6

<sup>a</sup> MWCNTs-modified GCE.

<sup>b</sup> MWCNTs-NF-modified GCE.

on MWCNT modified ITO in (B) shows both uniform NF and Hb film pores. It is a well-known fact that the prolonged exposure to the electron beam will damage the Hb films, so, at most care has been taken in measuring these images. The same modified ITO electrodes have been used to measure the AFM topography images, and these measured morphological structures are similar to that of SEM. Fig. 4(A) shows the patches of NF, whereas (B) shows Hb film with pores, (C) and (D) show the NF-Hb and MWCNTs-NF-Hb biocomposite films, respectively. These SEM and AFM results have revealed the formation of a MWCNTs-NF-Hb biocomposite film.

### 3.2. Electrocatalysis studies of oxygen at MWCNTs-NF-Hb biocomposite film

The electrocatalytic reduction of O<sub>2</sub> has been carried out at bare GCE, MWCNTs-NF, MWCNTs-Hb and MWCNTs-NF-Hb biocomposite film modified GCEs at pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solutions. Fig. 5(A) shows the electrocatalytic reduction of O<sub>2</sub> at different modified electrodes. In all the modified electrodes, the cathodic peak current of Hb at the potential of about 0.2 V increases noticeably, as the concentration of O<sub>2</sub> increased. However, for bare GCE the electrocatalytic reduction peak current of O<sub>2</sub> is at −0.6 V. These results show that the MWCNTs-NF-Hb biocomposite film possess higher peak current for O<sub>2</sub> reduction when comparing all other modified GCEs (Ipc and Epc values given in Table 2). The above-mentioned film modified electrodes catalytic phenomenon must be because of the presence of Hb in the modified electrode. From the slopes of the linear calibration curves, the sensitivity of the biocomposite modified GCEs and their correlation coefficient have been calculated and are given in Table 3. From this table, it is clear that the sensitivity of MWCNTs-NF-Hb biocomposite film is higher for O<sub>2</sub> reduction. The electrochemical reduction of the O<sub>2</sub> to H<sub>2</sub>O by Fe<sup>II</sup> present in the MWCNTs-NF-Hb biocomposite film could be given by

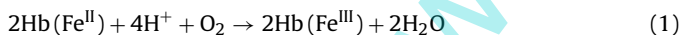
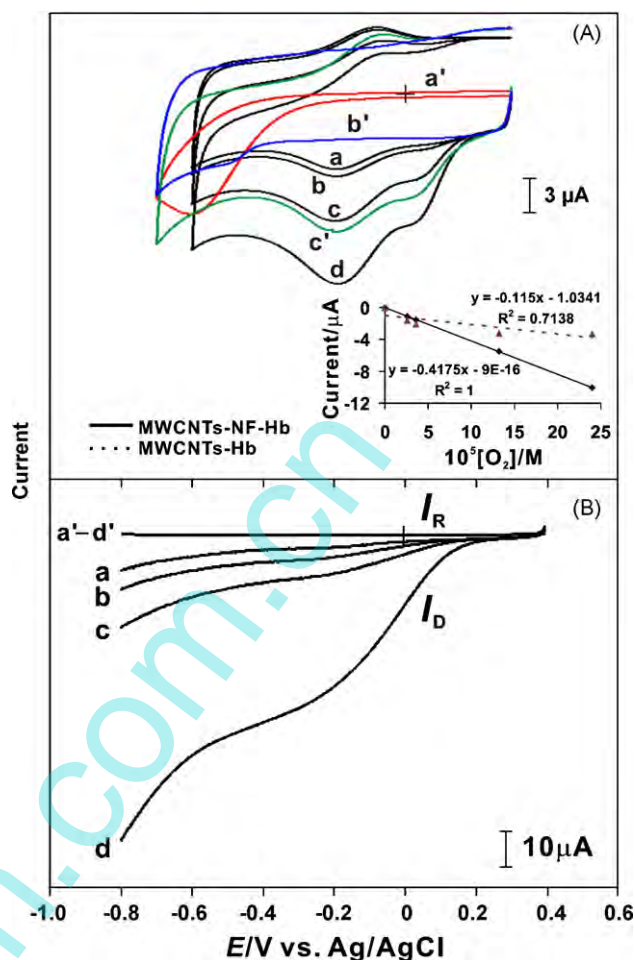


Fig. 5(B) shows the electrochemical reduction of O<sub>2</sub> by MWCNTs-NF-Hb biocomposite film-modified GCE disk electrode (at 0.2 V) and electrochemical oxidation of H<sub>2</sub>O<sub>2</sub> by bare platinum ring electrode (at 0.75 V) in an aqueous buffer solution at pH 2.5. The increase in concentration of O<sub>2</sub> increases the disk current (I<sub>D</sub>). However, the platinum ring current (I<sub>R</sub>), does not show obvious increase during the increase of O<sub>2</sub> concentration, which shows that the reaction in Eq. (2) does not occur at ring electrode. This result shows that H<sub>2</sub>O<sub>2</sub> is not formed during the reduction of O<sub>2</sub>. From this argument, the reduction mechanism of O<sub>2</sub> at the MWCNTs-NF-Hb biocompos-

**Table 3**

Sensitivity and correlation co-efficient of different modified electrodes for various analytes in CV technique.

Analytes	Reaction type	Sensitivity (mA mM <sup>−1</sup> cm <sup>−2</sup> ) [Slope's correlation coefficient]	
		MWCNTs-Hb	MWCNTs-NF-Hb
O <sub>2</sub>	Reduction	−0.14 [0.7138]	−0.52 [1]
H <sub>2</sub> O <sub>2</sub>	Reduction	−0.67 [0.9341]	−0.76 [0.9964]
CCl <sub>3</sub> COOH	Reduction	−0.92 [0.9741]	−1.02 [0.995]



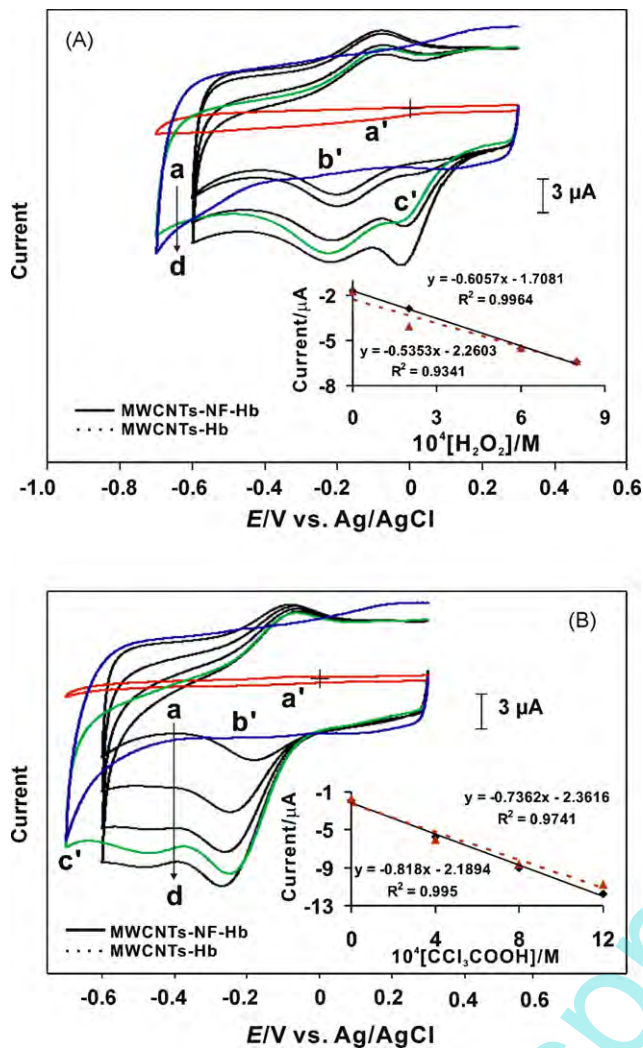
**Fig. 5.** (A) CVs of MWCNTs-NF-Hb biocomposite film in pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solution with various concentration of O<sub>2</sub> = (a) 25.7, (b) 36.2, (c) 131.9 and (d) 240 μM. Where, (a') bare GCE, (b') MWCNTs-NF and (c') MWCNTs-Hb in the highest concentration of the analyte, scan rate at 50 mV s<sup>−1</sup>. The inset in (A) shows the plot of current vs. different concentration of O<sub>2</sub>. (B) RRDE voltammograms of MWCNTs-NF-Hb biocomposite film (at 1600 rpm) in pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solution with various concentration of O<sub>2</sub> = (a) 6.1, (b) 25.3, (c) 48 and (d) 240 μM; scan rate at 15 mV s<sup>−1</sup>. Where, I<sub>D</sub> and I<sub>R</sub> are GC disk electrode and platinum ring electrode currents (E<sub>R</sub> = 0.75 V vs. Ag/AgCl), respectively.

ite could be proposed by Eq. (3).



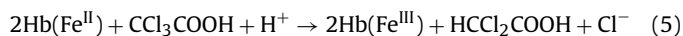
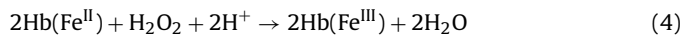
### 3.3. Electrocatalysis studies of H<sub>2</sub>O<sub>2</sub> and trichloroacetic acid at MWCNTs-NF-Hb biocomposite film

Fig. 6(A) and (B) shows the electrocatalytic reduction of H<sub>2</sub>O<sub>2</sub> and trichloroacetic acid (CCl<sub>3</sub>COOH), respectively. The electrolytes used for the electrocatalytic reactions were pH 2.5 H<sub>2</sub>SO<sub>4</sub> aqueous solutions. The CVs have been recorded at the constant time interval of 1 min with nitrogen purging before the start of each experi-

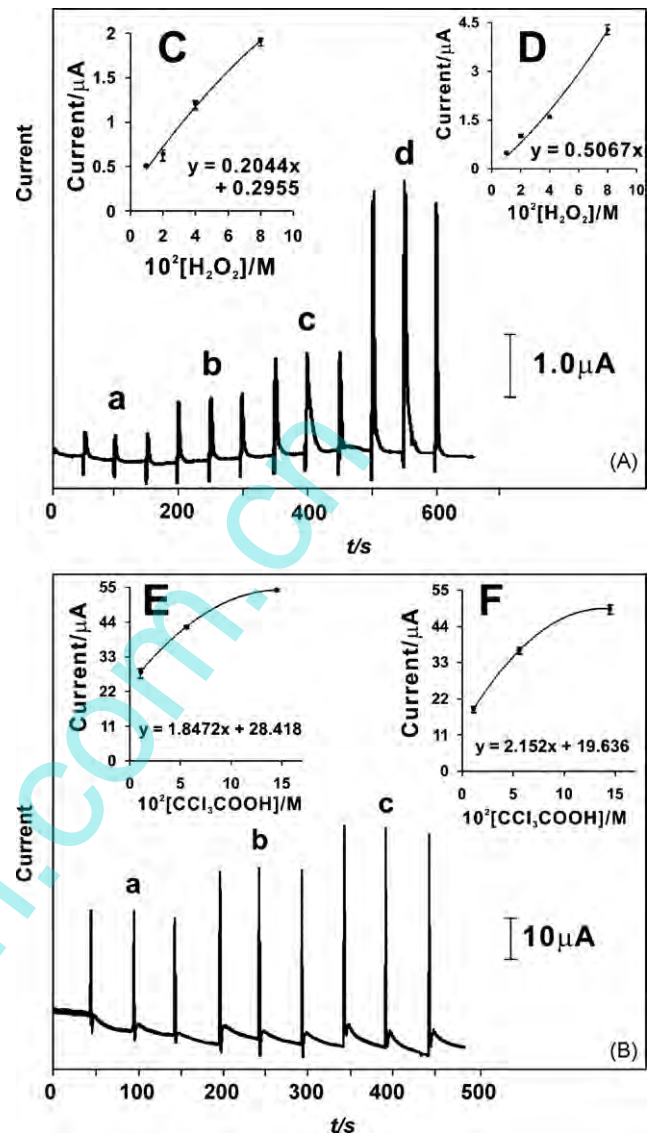


**Fig. 6.** CVs of MWCNTs-NF-Hb biocomposite film in pH 2.5  $\text{H}_2\text{SO}_4$  aqueous solution with various concentration of (A)  $\text{H}_2\text{O}_2$  = (a) 0.0, (b) 0.2, (c) 0.6 and (d) 0.8 mM; (B)  $\text{CCl}_3\text{COOH}$  = (a) 0.0, (b) 0.4, (c) 0.8 and (d) 1.2 mM. Where (a') bare GCE, (b') MWCNTs-NF and (c') MWCNTs-Hb in the highest concentration of the analytes; scan rate at  $50 \text{ mV s}^{-1}$ . The insets in (A) and (B) show the plot of current vs. different concentration of  $\text{H}_2\text{O}_2$  and  $\text{CCl}_3\text{COOH}$ , respectively.

ments. The scan rate used for these electrocatalysis experiments was  $20 \text{ mV s}^{-1}$ . In both the sections of Fig. 6, a' represents bare GCE, b' represents MWCNTs-NF film and c' represents MWCNTs-Hb film-modified GCEs. The CVs of MWCNTs-NF-Hb in Fig. 6(A) and (B) exhibits a reversible redox couple for Hb ( $\text{Fe}^{\text{III/II}}$ ) in the absence of analytes, upon the addition of analytes a new growth in the reduction peak of  $\text{H}_2\text{O}_2$  and  $\text{CCl}_3\text{COOH}$  appeared at  $E_{\text{pc}} = -0.2$  and  $-0.25 \text{ V}$ , respectively. These peak currents show that electrocatalytic reduction of both the analytes took place at Hb ( $\text{Fe}^{\text{II}}$ ) and could be represented by the following equations:



During the electrocatalysis experiments, an increase in the concentration of analytes simultaneously produced a linear increase in the reduction peak currents of the analytes with good film stability as shown in the insets in Fig. 6(A) and (B). It is obvious that, the MWCNTs-NF-Hb shows higher peak current for both the analytes when comparing to all other modified GCEs. The values of  $I_{\text{pc}}$  and  $E_{\text{pc}}$  for the analytes at different films are given in Table 2. From the slopes of the linear calibration curves, the sensitivity of



**Fig. 7.** FIA signal of MWCNTs-NF-Hb biocomposite film with various concentration of (A)  $\text{H}_2\text{O}_2$  = (a) 10, (b) 20, (c) 40 and (d) 80 mM; (B)  $\text{CCl}_3\text{COOH}$  = (a) 11, (b) 56 and (c) 145 mM. The potential applied was  $-0.2 \text{ V}$  for  $\text{H}_2\text{O}_2$  and  $-0.25 \text{ V}$  for  $\text{CCl}_3\text{COOH}$ , and the carrier stream used was pH 2.5  $\text{H}_2\text{SO}_4$  aqueous solution; flow rate =  $0.03 \text{ ml s}^{-1}$  and injected volume =  $30 \mu\text{l}$ . (C) and (D) show the plot of current vs. different concentration of  $\text{H}_2\text{O}_2$  at MWCNTs-Hb and MWCNTs-NF-Hb biocomposite films, respectively. Similarly, (E) and (F) show the plot of current vs. different concentration of  $\text{CCl}_3\text{COOH}$  at MWCNTs-Hb and MWCNTs-NF-Hb biocomposite films, respectively.

the biocomposite modified GCEs and their correlation co-efficient have been calculated and given in Table 3. It is clear that, the sensitivity of MWCNTs-NF-Hb is higher for both the analytes and there is no electrocatalysis reaction at MWCNTs-NF-modified GCE. These results clearly show that, the MWCNTs-NF-Hb biocomposite film can be efficiently used for the detection of  $\text{H}_2\text{O}_2$  and  $\text{CCl}_3\text{COOH}$ .

#### 3.4. Flow injection analysis of $\text{H}_2\text{O}_2$ and $\text{CCl}_3\text{COOH}$ at MWCNTs-NF-Hb biocomposite film

Fig. 7(A) and (B) shows the FIA studies of  $\text{H}_2\text{O}_2$  and  $\text{CCl}_3\text{COOH}$ , respectively at MWCNTs-NF-Hb biocomposite films, which have been synthesized on SPCE at similar conditions to that of GCE. Before the start of each experiment, the modified SPCE electrodes have been washed carefully with deionized water to remove the loosely bounded Hb on the modified SPCE. The carrier stream used was pH 2.5  $\text{H}_2\text{SO}_4$  aqueous solution with the flow rate of  $0.03 \text{ ml s}^{-1}$

and the volume of analytes injected at each cycle was 30  $\mu\text{l}$  at the time interval of 50 s. Fig. 7(A) represents the successive addition of  $\text{H}_2\text{O}_2$  in the concentration range from 10 mM to 80 mM at the potential of  $-0.2\text{V}$ ; these are the optimized experimental conditions obtained from CV studies. Similarly, Fig. 7(B) represents the successive addition of  $\text{CCl}_3\text{COOH}$  in the concentration range from 11 mM to 145 mM at the potential of  $-0.25\text{V}$ . In both cases, the rapid amperometric response of the MWCNTs-NF-Hb biocomposite film is proportional to the respective analyte concentrations. For comparative study, MWCNTs-Hb modified SPCEs have also been used for  $\text{H}_2\text{O}_2$  and  $\text{CCl}_3\text{COOH}$  FIA (figures not shown). The results obtained from all the FIA have been plotted as shown in Fig. 7(C–F), where (C) and (D) represents  $\text{H}_2\text{O}_2$  at MWCNTs-Hb and MWCNTs-NF-Hb biocomposite films respectively, whereas (E) and (F) represents  $\text{CCl}_3\text{COOH}$  at MWCNTs-Hb and MWCNTs-NF-Hb biocomposite films, respectively. The slopes obtained from these above-mentioned insets show that MWCNTs-NF-Hb biocomposite film has higher values for both  $\text{H}_2\text{O}_2$  and  $\text{CCl}_3\text{COOH}$  than that of MWCNTs-Hb film. These insets also represent error bars, where each concentration of every analyte was studied three times. These error bars show that the MWCNTs-NF-Hb biocomposite films have good reproducibility for both the analytes.

#### 4. Conclusions

The developed biocomposite material using MWCNTs, NF and Hb (MWCNTs-NF-Hb) at GCE, Au, ITO and SPCE electrodes shows good electrochemical properties in pH 2.5  $\text{H}_2\text{SO}_4$  aqueous solutions. The MWCNTs-NF-Hb biocomposite film for the electrocatalysis combines the advantages of ease of fabrication, high reproducibility and sufficient long-term stability. The SEM and AFM results show the differences between NF-Hb, MWCNTs-Hb, MWCNTs-NF and MWCNTs-NF-Hb biocomposite film's morphological data. Further, it is observed that the MWCNTs-NF-Hb biocomposite film has excellent functional properties with good electrocatalytic activity on  $\text{O}_2$ ,  $\text{H}_2\text{O}_2$  and  $\text{CCl}_3\text{COOH}$ . The experimental methods of CV and FIA with biocomposite film sensor integrated into the GCE and SPCE which are presented in this paper provide an opportunity for a qualitative and quantitative characterization. Therefore, this work establishes and illustrates, in principle and potential, a simple and novel approach for the development of voltammetric and amperometric sensor based on the modified GCE, ITO, Au and SPCE electrodes.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2008.12.063.

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