

Bicomponent Composite Electrochemical Sensors for Sustained Monitoring of Hydrogen Peroxide in Breast Cancer Cells

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ABSTRACT

Hydrogen peroxide (H_2O_2) is a molecule that plays an important role in cancer. Low concentrations in H_2O_2 have an important physiological role in signalling whilst higher concentrations can induce cell death. Current sensors for cellular monitoring of H_2O_2 are fragile and widely used for recording over a duration of minutes. Our study focused on developing a robust sensing device that can be used for sustained cellular monitoring of H_2O_2 to provide vital insight into its role in cancer. We made composite electrodes using varying compositions of multiwall carbon nanotubes (MWCNTs) and platinum black (PtB). These electrodes were investigated using different electroanalytical approaches, scanning electron microscopy and energy dispersive X-ray spectroscopy. We found that a composition containing 15 % MWCNT and 20 % PtB was the most sensitive for H_2O_2 detection. This electrode had a limit of detection of 17 nM and a sensitivity of $73.3 \pm 1.1 \text{ A M}^{-1} \text{ cm}^{-2}$ when normalised for the conductive surface area. The composite electrode was able to provide stable current responses over 200 minutes. To evaluate the sensor, measurements in breast cancer cells were conducted, where administration of the pro-oxidant tert-Butyl hydroperoxide increased H_2O_2 levels. This response was validated using a reactive oxygen species sensitive dye and confocal imaging. Our findings showcase that MWCNT PtB composite electrodes have excellent sensitivity and provide the ability to conduct sustained measurement. This approach towards sensor development can be suitable for monitoring different reactive species which are of paramount importance in complex biological environments.

Keywords

Multi-walled carbon nanotubes, platinum black, electrochemistry, conductivity, hydrogen peroxide, cancer

1. Introduction

Hydrogen peroxide (H_2O_2) is an important reactive oxygen species (ROS) that has a multitude of biological functions. It serves an important role in normal cellular functions and oxidative stress [1]. Various studies have highlighted that H_2O_2 plays an important role in cancer [2-5]. At low cellular concentrations H_2O_2 play a physiological role in signalling, however with increasing concentrations of H_2O_2 is associated with the carcinogenesis process and even higher levels of H_2O_2 can produce cell death.

H_2O_2 is a reactive molecule that is not very stable, making it a difficult molecule to monitor in biological systems. Given the changes in the roles H_2O_2 play with changes in concentration, techniques that can offer the ability to monitor temporal changes in concentration over a sustained period are attractive approaches. Conventional approaches for H_2O_2 determination such as fluorescence and chemiluminescence methods [6, 7] are complex and time consuming. In comparison, electroanalytical methods can offer simple and rapid approach for the determination of H_2O_2 [8].

Direct oxidation of H_2O_2 is often difficult to conduct due to slow kinetics observed on carbon electrodes. Therefore, electrodes are either chemically or biologically modified for H_2O_2 . Although biological modified sensors, namely with superoxide dismutase offer excellent selectivity, but have greater variability [9-11]. More commonly has been the use of chemical modified sensors [12-16]. One of the most successful approaches has been to film coat carbon fiber microelectrodes with platinum black for determination of H_2O_2 and other reactive species [17-21]. These electrodes are very fragile and have been used mainly for single cell measurements for monitoring over a few minutes. To be able to monitor H_2O_2 levels from multiple cells and tissues over a sustained period, a

mechanically robust electrode is needed. Composite electrodes can be made using any combination of conductive materials and have been shown to be stable over a wide range of experimental conditions, are mechanical robust electrodes and can be made to any geometry [22-28].

Within this paper we aimed to develop a bicomponent composite electrode made using multi-walled carbon nanotube (MWCNT) as a conductive base material and platinum black (PtB) as the catalytic mediator that facilitates electron transfer for the monitoring of H₂O₂. Polydimethylsiloxane (PDMS) was utilised as the insulating binder. The percentage of MWCNT and PtB was modified to identify the best composition for sensitive determination of H₂O₂. The surface composition of the electrode was explored using scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) spectroscopy. Finally, the electrode was characterized for analytical performance before conducting measurements of H₂O₂ following administration of a to a breast cancer cellular matrix.

2. Experimental

2.1. Fabrication of bicomponent composite electrodes

Electrodes were fabricated from MWCNTs 30-50nm (inner diameter nm and specific surface area of 60 m²/g) (Cheap Tubes Inc., VT, USA) with Dow Sylgard 184 polydimethylsiloxane (PDMS; Ellsworth adhesives Ltd, Scotland, UK). PDMS was made at a ratio of 10-parts elastomer base: 1-part elastomer hardener. Platinum black powder (<20µm, ≥99.95% trace metals basis) was purchased from Sigma-Aldrich-UK. To make the varying compositions of composite electrodes, the components were mixed for 20 minutes to form a paste. This composite was then packed into hollow electrodes, 3mm in

diameter (IJ Cambria Scientific, UK), and left to set overnight at room temperature. We opted for a larger electrode as our vision was to track measurements from large population of cells rather than single cells and thus enhance our sensitivity. Additionally, given the approach to manufacturing the electrode, this could be tailored to any size to match applications of interest.

2.2. Electrochemical characterisation and assessment of bicomponent electrodes

Cyclic voltammetry (CV) measurements were carried out using a three-electrode system, with the composite electrodes as the working electrode, a platinum wire as the auxiliary electrode and Ag|AgCl (3.0 M KCl) as the reference electrode (IJ Cambria Scientific, UK). All experiments were carried out using a CHI 760E or CHI 1009A potentiostat. 1 mM $\text{Ru}(\text{NH}_3)_6^{2+/3+}$ was prepared in 0.5 M KCl. The $\text{Ru}(\text{NH}_3)_6^{2+/3+}$ waveform was 0.1 V to -0.5 V. All waveforms were performed at a scan rate of 0.1 V s⁻¹. Hydrogen peroxide was prepared in 0.1 M PBS buffer. For all CV electrochemical measurements 10 cycles were completed for each electrode at a scan rate of 0.05 V s⁻¹. Calibration and stability measurements were carried out using amperometry at 0.65 V. Measurements were performed inside a 37°C and 5% CO₂ incubator. For studies in cells, multi-step amperometry was utilised, where 40 s were used to establish a baseline at 0 V followed by a step to 0.65 V for 60 s. Measurements were performed continuously every 150 s.

2.3. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) spectroscopy

The composite electrodes were imaged using a Zeiss SIGMA field emission gun SEM equipped with an Everhart-Thornley detector operating in secondary electron detection mode, using 20 kV accelerating voltage, a 20 μm aperture, and 8.2 mm working distance. All SEM and EDX imaging and measurements were performed in replicates of 3.

2.4. Electrochemical and fluorescent imaging studies of breast cancer cells

MDA-MB-231 human breast cancer cells were purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) culture medium (Gibco), supplemented with 10% foetal bovine serum (FBS) (Atlanta Biologicals), with environmental conditions of 37 °C, 5% CO₂. For experiments sensing hydrogen peroxide from cultured cells, MDA-MB-231 cells were seeded in Sarstedt 6 well plates at a density of 400,000 cells per well. The following day, cells were treated with pro-oxidant tert-butyl hydroperoxide (tBHP) or left untreated and electrochemical readings were obtained by placing the sensors directly into the culture medium. For comparative imaging, MDA-MB-231 cells were seeded at the same density on glass coverslips within Sarstedt 6 well plates. Glass coverslips were sterilised using 70% ethanol and washed with phosphate buffered saline (PBS) prior to seeding. The following day, cells were treated with tBHP for 2 hours, then incubated with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Invitrogen) for 30 minutes and washed with PBS. Coverslips were mounted onto glass slides using Fluoroshield™ with DAPI (Sigma) for immediate imaging on an LSM800 Confocal Microscope (Zeiss).

2.5. Data analysis

To compare between the different composite electrodes, the current was normalised by the volume fraction of the conductive components present within the composite electrode. This would provide important insight if the enhanced current response was solely due to increasing amounts of the conductive components or due to additional factors such as enhanced electrochemical activity from the chemical conductive components. The volume fraction provides the fraction of the total area of the electrode that is occupied by conductive particles [27, 29] and was calculated using the equation:

$$\text{Volume fraction} = \frac{(m_{\text{MWCNT}}/m_{\text{PDMS}})(1/\rho_{\text{MWCNT}})}{(m_{\text{MWCNT}}/m_{\text{PDMS}})(1/\rho_{\text{MWCNT}}) + (1/\rho_{\text{PDMS}})} + \frac{(m_{\text{PtB}}/m_{\text{PDMS}})(1/\rho_{\text{PtB}})}{(m_{\text{PtB}}/m_{\text{PDMS}})(1/\rho_{\text{PtB}}) + (1/\rho_{\text{PDMS}})}$$

Where m_{MWCNT} is the mass fraction of MWCNT, m_{PtB} is the mass fraction of the PtB, m_{PDMS} is the mass fraction of PDMS, ρ_{MWCNT} is the density of MWCNT (2.1 g/cm³), ρ_{PtB} is the density of the PtB (0.7 g/cm³), and ρ_{PDMS} is the density of PDMS (0.965 g/cm³). The volume fractions for the varying compositions of electrodes made are shown in Table 1. These values were used to normalise the current data generated.

Table 1. Volume fraction of the conductive area of different sensor permutations that contain two conductive components

Pt Black (%)	MWCNT (%)	
	15	20
0	0.075	0.103
5	0.159	0.193
10	0.239	0.281
20	0.394	-

All measurements were reported as the mean \pm the standard deviation. Statistical comparisons were performed using t-test, one-way ANOVA with Tukey *post hoc* tests and two-way ANOVA with Tukey *post hoc* tests and Dunnett *post hoc* tests. Statistical analysis was completed on Graphpad Prism 8.0. For electrochemical measurements conducted during cellular studies, the current difference between 0.65 V and 0 V was obtained and presented against time.

3. Results and Discussion

3.1. Investigating the most suitable percentage of MWCNT within the composite

Within our bicomponent composite electrode, the base conductor we utilised was MWCNT, which we have previously shown to be a sensitive carbon allotrope that delivers sufficient conductivity with low weight percentage within the composite electrode [23, 30, 31]. We compared MWCNT from 10 to 25 %, in which the cyclic voltammogram responses to outer sphere redox couple $\text{Ru}(\text{NH}_3)_6^{2+/3+}$ are shown in **Figure 1A**. There was a significant linear increase in the cathodic peak current (I_pC) with increasing percentage of MWCNT ($p < 0.001$, one-way ANOVA, $n=5$, **Figure 1B**). This is expected as with increased conductive material within the composite, the number of conductive pathways present is improved. The peak separation (ΔE) which provides insights into the kinetics of electron transfer at the electrode surface was measured and results are shown in **Figure 1C**. There was a significant decrease in ΔE as the percentage of MWCNT present in the composite material increased ($p < 0.001$, one-way ANOVA, $n=5$). There was a significant reduction in the ΔE from composite electrodes made using 10 % MWCNT to those made using 15, 20 and 25 % ($p < 0.001$, $n=5$). There was a significant reduction in ΔE in the composite electrodes made using 15 % MWCNT when compared to 20 and 25 % MWCNT ($p < 0.001$, $n=5$). However, no difference in ΔE was observed between 20 and 25 %. Although there are marked improvements in the ΔE with an increase in the percentage of MWCNT within the composite electrode, the ΔE is still large, which is expected given the nature of the composite electrode is not a fully conductive sensing material.

From the percentage range of MWCNT explored, we were unable to reach a percolation threshold as it was not possible to add more conductive material and still have the composite that can set and form an electrode. Although the best composite is 25 % MWCNT, it is not ideally suited towards making a bicomponent composite electrode. This is because there was no scope for further modification of this electrode by adding other conductive components, as there would be insufficient PDMS for binding. Therefore, 15 and 20 % MWCNT electrodes were chosen for subsequent studies they showed good conductivity and had potential to be further modified by adding PtB to tailor the electrodes towards detection of H_2O_2 .

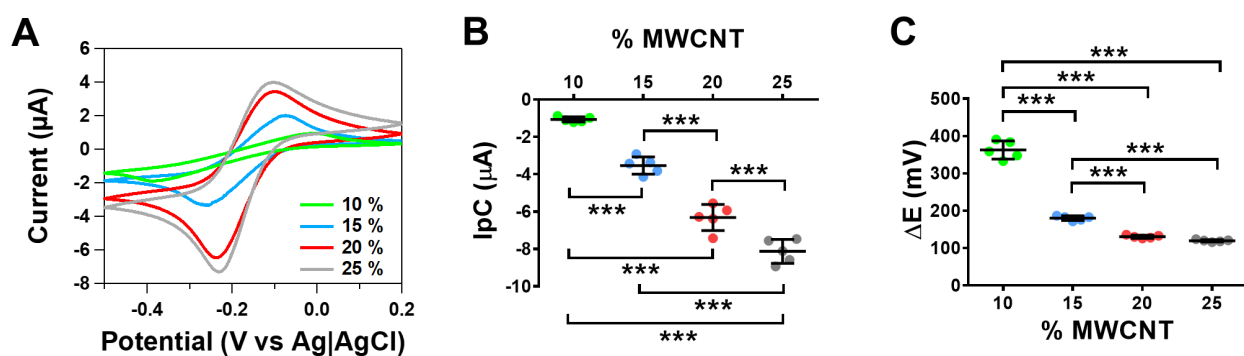


Figure 1. Electrochemical responses in composites with varying MWCNT percentage. (A) Representative cyclic voltammograms of 10, 15, 20, 25% MWCNT in 1 mM $Ru(NH_3)_6^{2+/3+}$ at 0.05 $V s^{-1}$. (B) Cathodic peak current (I_{pC}) and (C) ΔE . Data shown as mean \pm SD, $n=5$, *** $p<0.001$

3.2. Understanding how variations in PtB composition can influence the determination of H_2O_2 .

To tailor the electrode towards determination of H_2O_2 , we added varying compositions of PtB to 15 and 20 % MWCNT composite electrodes. PtB has been shown to provide

enhanced sensitivity for the determination of H_2O_2 [17, 18, 32]. For the 15 % MWCNT electrode, the maximum amount of PtB that could be added to still allow the composite to bind was 20 %, whilst for the 20 % MWCNT electrode, 10 % PtB was the maximum amount that could be added to still allow the composite to bind to form an electrode. **Figure 2A&B** shows cyclic voltammograms of 15 % and 20 % MWCNT electrodes with varying weight percentages of PtB. The addition of PtB, significantly enhanced the current and improved peak shape. Given our composite electrodes contain 2 different conductive components, we calculated the volume fraction of the composite that was conductive (**Table 1**) and used this to normalise the current response observed in the composite electrodes. This approach was taken to understand if enhanced performance of the sensors was due to the increased amount of conductive material or due to the enhanced activity of the combined conductive components. **Figure 2C**, shows a significant reduction in normalised current, on both 15 and 20 % MWCNT composite sensors, when the percentage of PtB is increased ($p < 0.01$, 2-way ANOVA, $n=5$). When considering the composite electrode containing 15 % MWCNT, there was a significant decrease in the normalised current when 20 % PtB was added when compared to 0 % PtB ($p < 0.001$, $n=5$). A similar trend was observed in the 20 % MWCNT composite, where when 10 % PtB was added, the normalised current was significantly reduced to when no PtB was present ($p < 0.05$, $n=5$, **Figure 2C**). These results highlight that although there is an increase in the amount of conductive material present through the addition of PtB, this reduces the normalised current obtained for the outer sphere redox probe. This suggests that electron transfer of the outer sphere probe is more favourable on MWCNT sites than PtB sites on the electrode surface. The expected current for the electrode using the Randles-Sevcik

equation for a purely semi-infinite linear diffusion system is 12.6 μA (see supplementary information for equation and value parameters used), and thus the current observed on the electrodes (3.4 μA for 15 % and 6.4 μA for 20 % MWCNT) is significantly higher when factoring in the percentage of conductive material present within the composite electrode, suggestive that the electrode is behaving as a "continuous" conductor due to overlapping diffusion layers.

Figure 2D shows the change in ΔE with increasing amounts of PtB in both 15 and 20 % MWCNT electrodes. There was a significant decrease in ΔE with increasing percentage of PtB added to the MWCNT composite electrode ($p < 0.001$, $n=5$). For the 15 % MWCNT electrode, there was a significant decrease in ΔE when 10 and 20 % PtB was added when compared to 0 % PtB ($p < 0.001$, $n=5$, **Figure 2D**). For the 20 % MWCNT electrode, there was a significant decrease in ΔE when 10 % PtB was added when compared to 0 % PtB ($p < 0.001$, $n=5$, **Figure 2D**). The ΔE for 15 % MWCNT 20 % PtB was similar to 20 % MWCNT 10 % PtB. The addition of PtB significantly enhances the conductivity of the composite electrode and thus improves electron transfer kinetics.

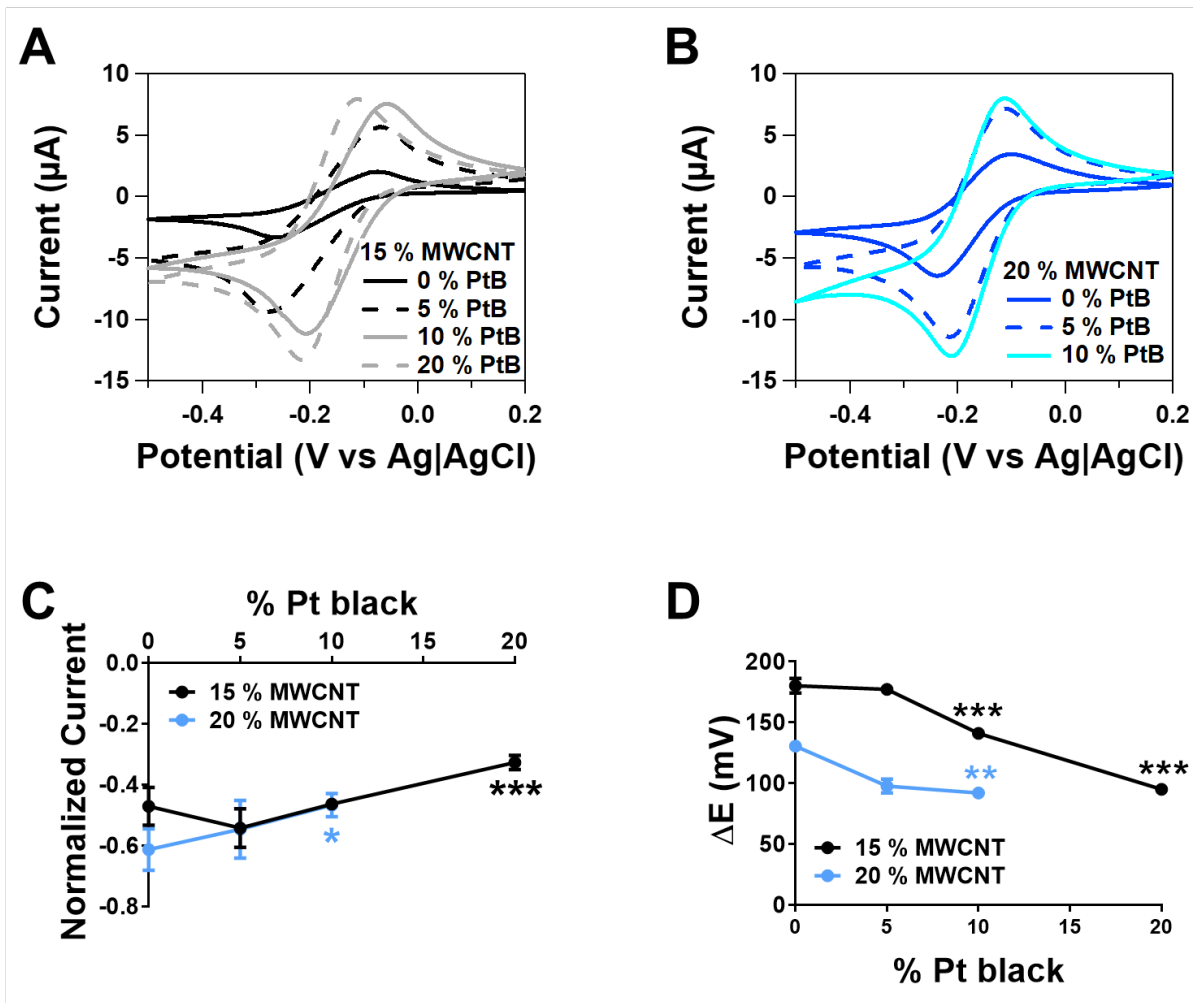


Figure 2. Effect of adding PtB on responses of an outer sphere redox probe. Representative cyclic voltammograms of (A) 15 and (B) 20 MWCNT with increasing percentage of PtB in 1 mM $\text{Ru}(\text{NH}_3)_6^{2+/3+}$ at 0.05 V s^{-1} . (C) Cathodic peak current (I_{pC}) normalised by the volume fraction of the electrode and (D) ΔE . Data shown as mean \pm SD, $n=5$, $*p<0.05$, $**p<0.01$, $***p<0.001$

To further examine the bicomponent composite electrodes, we evaluated which composite electrode had the greatest sensitivity to determine H_2O_2 . Using the 15% MWCNT 20% PtB composite electrode, differential pulse voltammogram of $50 \mu\text{M}$ H_2O_2 was conducted to identify the oxidation peak potential to conduct amperometric studies.

From the differential pulse voltammogram response shown in **Figure 3A**, 0.65 V was utilised for amperometric determination of H₂O₂. **Figure 3B** shows amperometric responses at 0.65 V, where 50 µM H₂O₂ was added after 40 s. The current difference before and after addition of 50 µM H₂O₂ was monitored for each composite composition. The current response obtained was normalised with the volume fraction and is shown in **Figure 3C**. There is a significant increase in the normalised current observed with increasing percentage of PtB ($p < 0.001$, $n = 5$, 2-way ANOVA). For the 15 % MWCNT sensors, the normalised current was significantly greater in electrode containing 10 % ($p < 0.01$) and 20 % ($p < 0.001$) PtB when compared to electrode without PtB. For composite electrodes made using 20 % MWCNT there was a significant increase in the normalised current for electrode containing 5 % ($p < 0.05$) and 10 % ($p < 0.001$) when compared to electrodes that did not contain PtB (**Figure 3C**). The normalised current for the 15 % MWCNT 20 % PtB electrode was slightly higher than 20 % MWCNT 10 % PtB ($p < 0.05$). When comparing all the different compositions, the electrode with the greatest percentage of PtB was shown to have the greatest performance, indicating that PtB is essential to enhance the electrodes' sensitivity for H₂O₂ detection. These findings highlight that adding PtB powder into a composite electrode can be used as an effective alternative approach to coating electrodes with a layer of PtB to make electroanalytical sensors for monitoring reactive species [18].

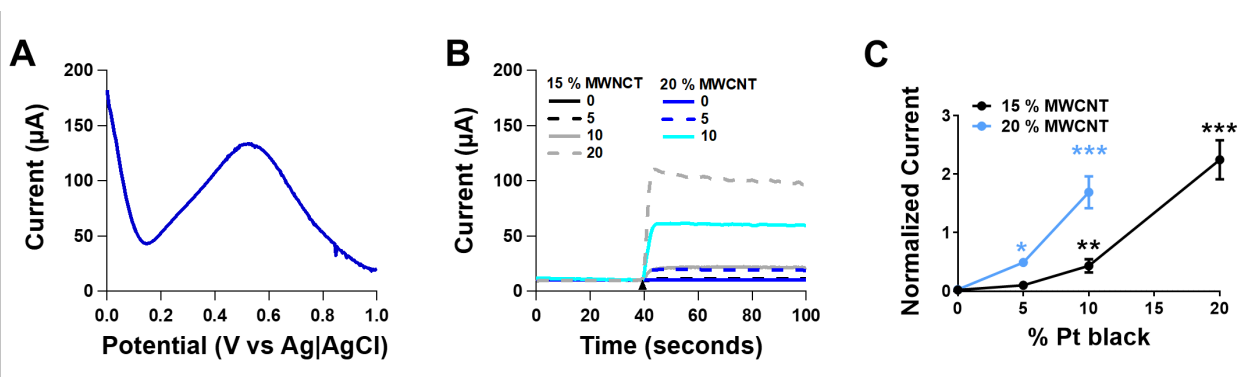


Figure 3. Determination of H_2O_2 on varying MWCNT and PtB composite electrodes. (A) shows a differential pulse voltammogram response of $50 \mu\text{M}$ H_2O_2 on a 15 % MWCNT 20 % PtB electrode. (B) Amperometric responses at 0.65 V, where $50 \mu\text{M}$ H_2O_2 was added after 40 s. The difference in the current from baseline to when H_2O_2 was added. (C) The current response to H_2O_2 obtained on the different composite electrodes. The response was normalised by the volume fraction. Data shown as mean \pm SD, $n=5$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.3. Microscopy and spectroscopy analysis to evaluate differences in electrode surface structure

To understand the improved sensitivity of the 15% MWCNT 20% PtB composite electrode when compared to the 20% MWCNT 10% PtB composite electrode, characterisation of the electrode surface was performed using SEM and EDX spectroscopy. **Figure S1** shows SEM and EDX images of the two conductive components, MWCNT and PtB. The PtB powder is present as small, clustered particles. The pie charts show the elemental composition of the 3 components that were used to make the composite electrodes. There is the presence of carbon impurities in PtB, whilst trace amounts of nickel and silicon were present in MWCNT. The amount of nickel present is not sufficient to make

contribution to the electrocatalytic oxidation of H_2O_2 as shown in **Figure 3B** there was no observed current response on solely MWCNT composite electrodes. For the PDMS, the elemental analysis reveals the presence of oxygen, carbon, and silicon. From the analysis of the elemental compositions found within the material it was possible to differentiate between the 3 materials used to make the composite electrode. From the EDX images the presence of PtB is represented by Pt, the presence of MWCNT is represented by C and for the presence of PDMS is represented by Si. This provides insight into the surface arrangement of the two conductive materials and non-conductive material.

Figure 4 shows the SEM and EDX response of the two most sensitive electrodes (15% MWCNT 20% PtB and 20% MWCNT 10% PtB composite electrode) for detection of H_2O_2 . There are clear differences in the structural arrangement of the PtB between the two composite electrodes. Within the 15% MWCNT 20% PtB composite electrode, there are larger less-disperse structures of the PtB on the electrode surface. Whilst on the 20% MWCNT 10% PtB composite electrode, there are smaller clusters of PtB present in a more dispersive fashion. Overall, the 20% MWCNT 10% PtB composite electrode looks more homogenous than the 15% MWCNT 20% PtB composite electrode and thus we would have been expected this electrode to be more sensitive towards detection of H_2O_2 . However, the larger sites of PtB provided the enhanced sensitivity towards H_2O_2 determination. Having larger pockets and a greater fraction of the electrode surface covered by PtB may help facilitate electron transfer, given these surface sites might be more accessible on the electrode surface compared to the small dispersive clusters observed on the 20% MWCNT 15 % PtB electrode. **Figure S2** shows the SEM and EDX spectroscopy responses from all other composites, where electrode which have 15 %

MWCNT electrodes seem to have larger structures of Pt, whilst smaller clusters are observed in electrodes which have 20 % MWCNT. These results indicate that the amount of MWCNT within the electrode has a key influence on the structure of PtB present on the electrode surface.

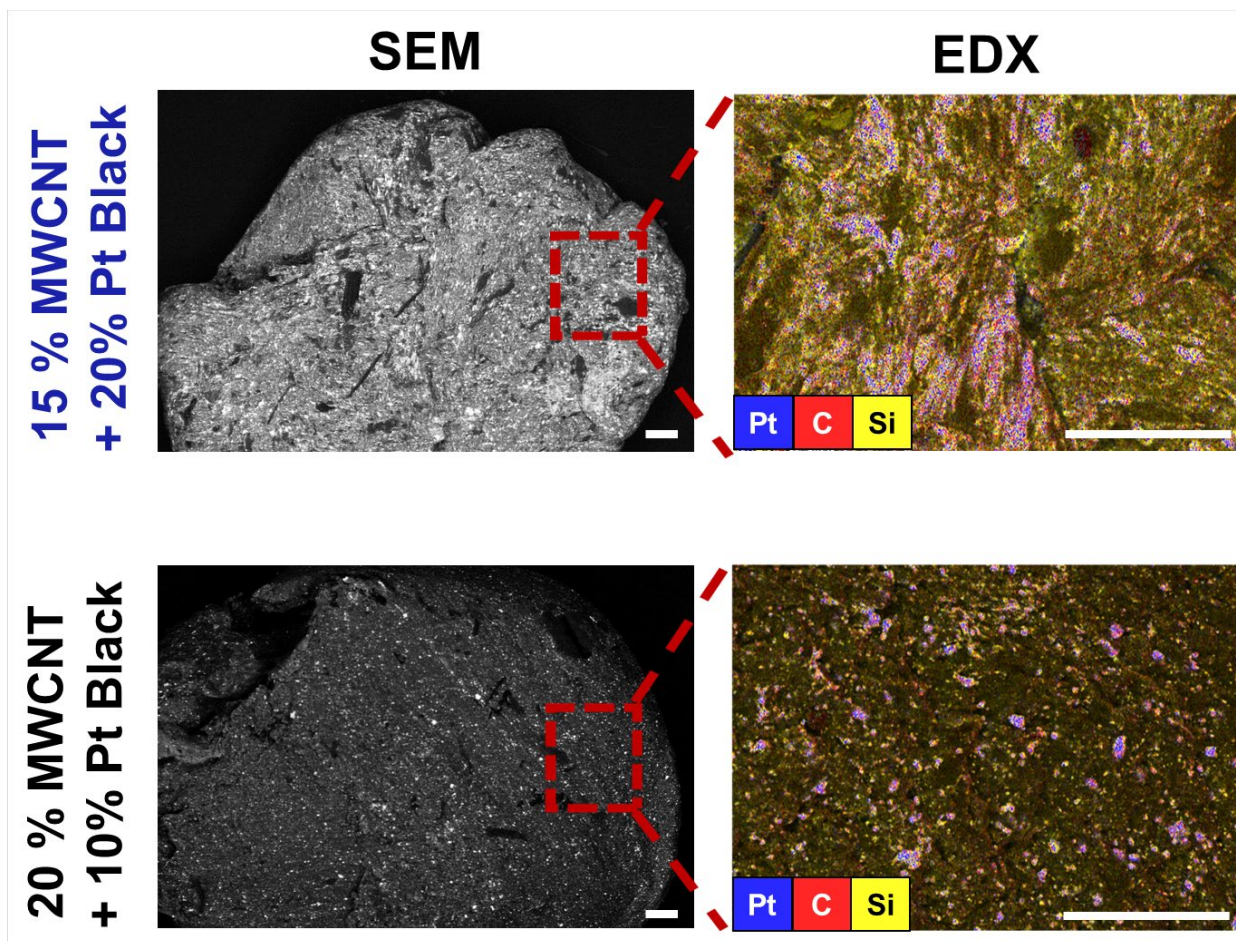


Figure 4. Representative SEM and EDX spectroscopy images of different compositions of MWCNT PtB composite electrodes. Scale bar is 100 μm .

3.4. Evaluation of the sensitivity, stability, and selectivity for monitoring H₂O₂

Based on the evaluation of the different compositions of composite electrodes, 15 % MWCNT and 20 % PtB was determined to be the composition that had the greatest response to H₂O₂. Studies to explore the sensitivity and stability of the electrode were conducted. **Figure 5A** shows amperometric responses at 0.65 V, where at 40 s fixed concentration of H₂O₂ were added. There was increase in the current with increasing concentration. The calibration response is shown in **Figure 5B** for a physiological concentration range. Experiments were carried out DMEM/F12 culture media under 37°C and 5% CO₂ to mimic the conditions used for cellular measurements. The sensitivity was $28.6 \pm 0.4 \text{ A M}^{-1} \text{ cm}^{-2}$ when considering the geometric area of the electrode, however if the conductive fraction of the electrode area is factored by using the volume fraction, then the sensitivity significantly increased to $73.3 \pm 1.1 \text{ A M}^{-1} \text{ cm}^{-2}$ (n=6). The limit of detection (LOD) was 17 nM. This MWCNT PtB composite electrode has a slightly higher LOD, but significantly greater sensitivity than PtB film coated microband electrodes (LOD of 10 nM and sensitivity of $6.02 \text{ A M}^{-1} \text{ cm}^{-2}$) that were used for monitoring H₂O₂ [18].

One limitation of currently used electrode for cellular monitoring is often they are used for short term monitoring in the duration of minutes. This is mainly due to the fragility of microelectrodes and PtB film coating. **Figure 5C** shows amperometric current responses of 5 μM H₂O₂ monitored over a period of 200 minutes. The responses observed every 10 minutes up to 200 minutes. **Figure 5D** shows the current response over the 200 minutes was normalised to the initial current response. There was no significant difference in the current over the duration of 200 minutes, suggesting that the electrode is highly stable for the sustained measurement of H₂O₂.

Lastly, we investigated the selectivity of the sensor against common biological interferences. **Figure 5E** shows amperometric traces at +0.65 V, where a clear response to 5 μM H_2O_2 was observed but minimal response was observed for other chemical interferants. **Figure 5F** shows that our MWCNT PtB composite electrode was able to selectively detect H_2O_2 when compared to 5 μM dopamine, ascorbic acid, norepinephrine, and tryptophan. This may be due to the overpotential for these interferants being higher on the component electrode and thus limiting the degree of interference.

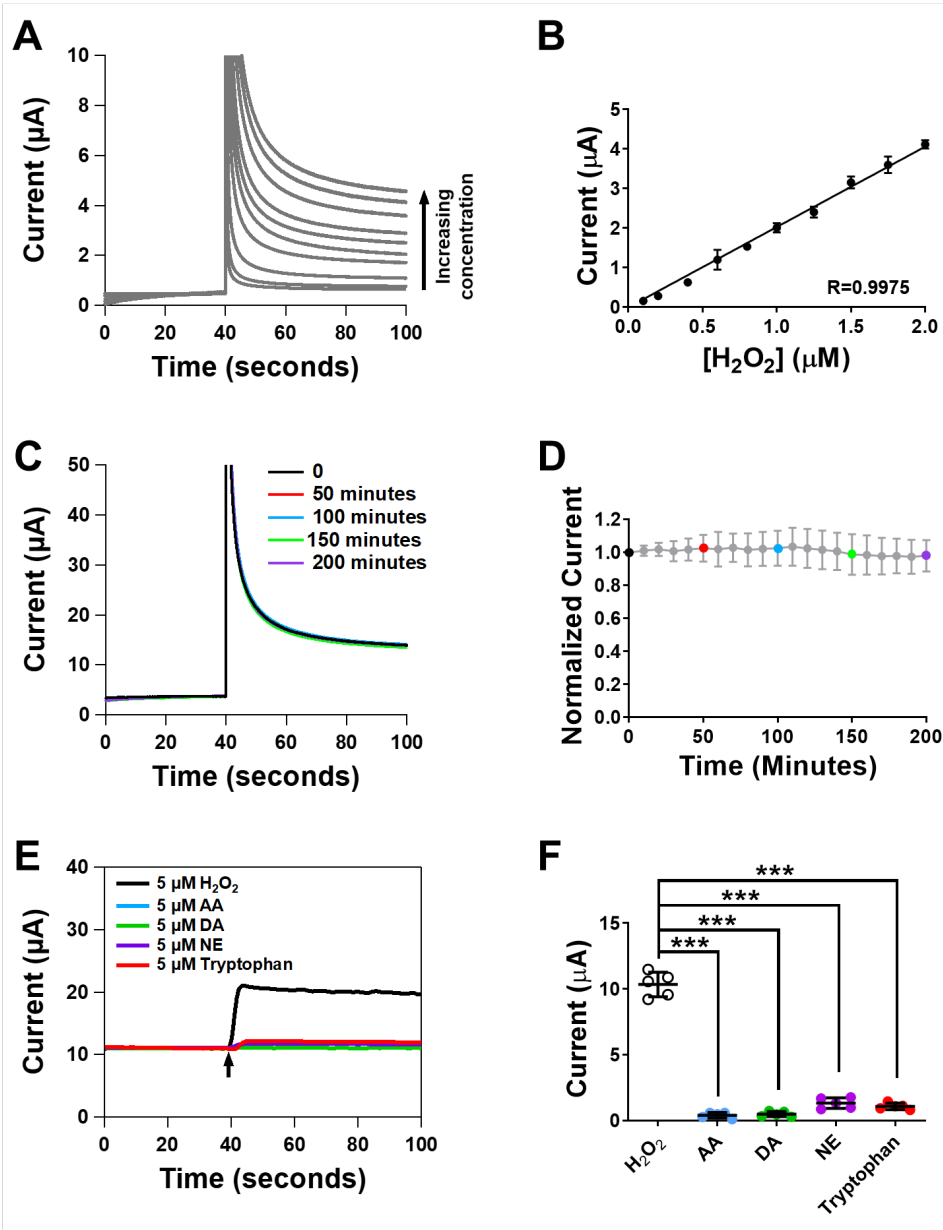


Figure 5. Evaluation of sensitivity and stability of the 15 % MWCNT 20 % Pt composite electrode. (A) shows amperometric traces at 0.65 V, where varying concentration of H_2O_2 were added on 40 s. (B) H_2O_2 Calibration response. (C) Amperometric traces obtained in 5 μM , where the voltage is changed from 0 V to 0.65 V at 40 seconds and repeated measurements were conducted over 200 minutes. (D) The current monitored over the 200 minutes was normalised to the initial response monitored on the electrode. The coloured markers match those traces shown in (C). (E)

Selectivity studies where the response of H₂O₂ and common biological interferences are shown.

(F) Responses of 5 μM H₂O₂ when compared to 5 μM ascorbic acid (AA), dopamine (DA), norepinephrine (NE) and tryptophan. Data shown as mean ± SD, n=6, ***p<0.001.

3.5. Continuous detection of H₂O₂ from breast cancer cells

To determine the ability of the 15 % MWCNT 20 % PtB composite sensor to continuously monitor changes in H₂O₂ in a cellular matrix, we performed pharmacological studies using MDA-MB-231 human breast cancer cells. To biologically induce H₂O₂, cells were treated with tBHP, a pro-oxidant known to induce cellular oxidative stress during its metabolism by either cytochrome p450, resulting in the production of peroxy or alkoxy radical species [33], or, more generally, via glutathione peroxidase and the oxidation of glutathione (GSH). GSH pools necessary for maintaining cellular redox balance are depleted by tBHP, impairing cellular antioxidant capacity [34], and allowing accumulation of other ROS, such as H₂O₂, as a result.

For electrochemical measurements, chronoamperometry was utilised, where the current was monitored at 0 V for 40 s before being stepped to 0.65 V for 60 s. The current difference was utilised as the response of H₂O₂. Chronoamperometry responses are shown for cells treated with tBHP in **Figure 6A**, where the dashed line response corresponds to the same time duration as the confocal imaging. Responses were monitored from untreated cells and those treated with tBHP. There was no change in cells that were untreated for the entire 200 minutes that measurements were conducted for.

There is a clear significant increase in the concentration of H_2O_2 observed from 110 to 200 minutes when comparing the cells treated with tBHP to untreated ($p < 0.001$, $n = 6$, **Figure 6B**). Validation of this increase in cellular ROS-production observed using our composite electrode was carried out using confocal imaging. Imaging of tBHP-treated cells showed increased fluorescence, and therefore elevation in cellular ROS levels when compared to untreated cells (**Figure 6C**). These results indicate that this composite electrode has the capabilities of providing sustained stable sensing of H_2O_2 from complex cellular matrices.

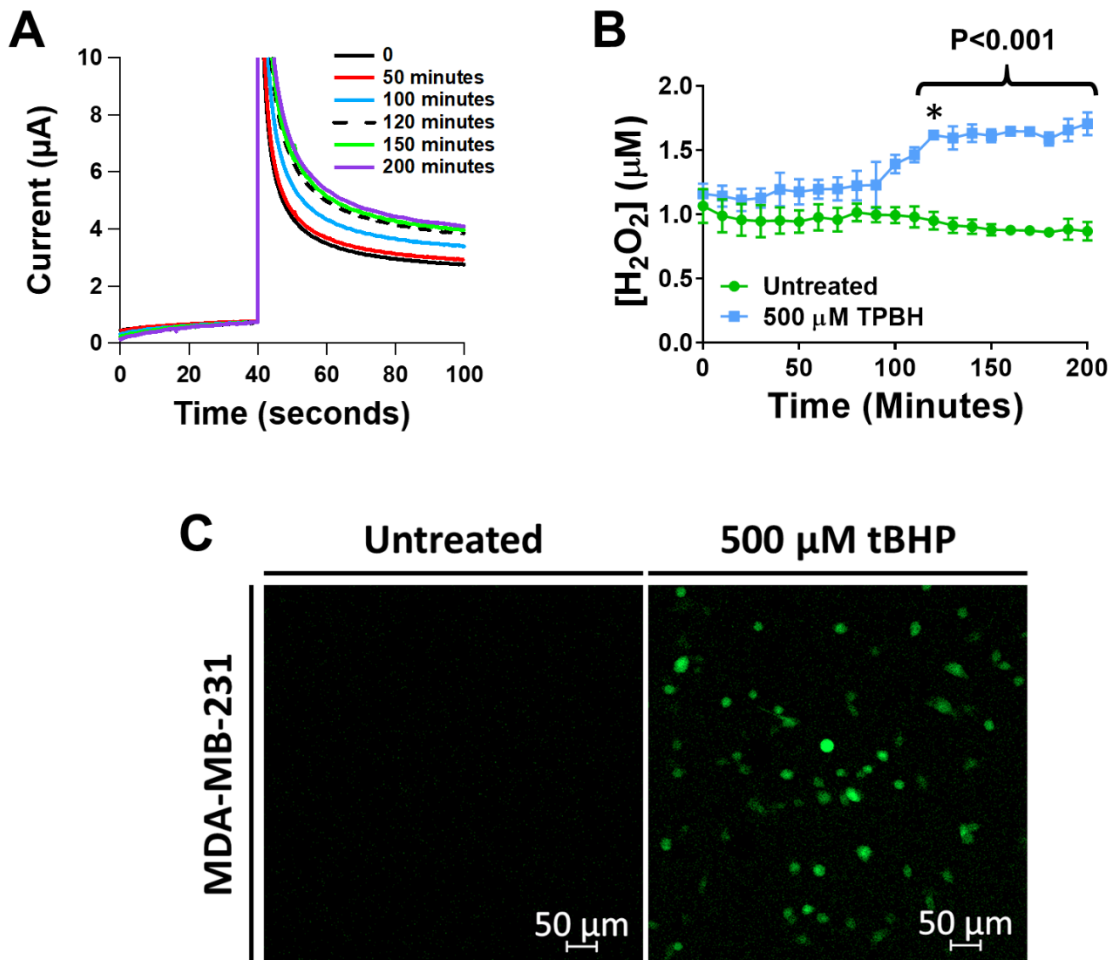


Figure 6. Determination of H_2O_2 production from MDA-MB-231 cells. Electrode was placed in cell culture media during measurements. (A) chronoamperometric traces obtained in cells which were treated with tBHP where the voltage was switched from 0 V to 0.65 V at 40 s. The average current in the last 5 seconds of the response at 0.65 V was subtracted from that obtained at 0 V. Traces shown from 0 to 200 minutes, where the dashed trace at 120 minutes corresponds to confocal microscopy images. (B) Changes in the concentration of H_2O_2 for untreated and treated with tBHP MDA-MB-231 cells. Treatment was administered at 0 minutes after stable baseline was determined (n=6). Measurements performed at 37°C and 5% CO_2 with current measured shown for every 10 minutes. Where * corresponds to the confocal microscopy images. (C) Representative confocal microscopy images showing untreated and treated cells after incubation in H_2DCFDA for 30 minutes. Cellular tBHP treatment was administered for 2 hours prior to staining. Data shown as mean \pm SD, n=6.

4. Conclusion

Sustained measurements to understand concentration changes in H_2O_2 are critical to functional role in cancer biology. Within our study we developed a composite electrode made using 15 % MWCNT and 20 % PtB, which was highly sensitive and stable towards sustained monitoring of H_2O_2 in a cellular environment. Our approach towards sensor fabrication showed greater sensitivity than electrodes made using film coating of PtB. During cellular studies, our electrode was able to clearly track changes in H_2O_2 concentrations in the presence of a pro-oxidant which were validated with confocal imaging. This study highlights that bicomponent composite electrodes can provide an alternative and more suitable strategy for fabrication of electrodes, which can have the potential to conduct long-term sustained measurement of important biological analytes.

Acknowledgments

The authors like to thank CRUK ESPRC Multidisciplinary Project Award (C57783/A24316; NS/A000065/1) for funding. They would like to thank Dr Marcus Dymond for valuable discussions.

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