Ubiquinone modified printed carbon electrodes for cell culture pH monitoring

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1. Abstract

The measurement of pH is important throughout many biological systems, but there are limited available technologies to enable its periodical monitoring in the complex, small volume, media often used in cell culture experiments across a range of disciplines. Herein, pad printed electrodes are developed and characterised through modification with: a commercially available fullerene multiwall carbon nanotube composite applied in Nafion, casting of hydrophobic ubiquinone as a pH probe to provide the electrochemical signal, and coated in Polyethylene glycol to reduce fouling and potentially enhance biocompatibility, which together are proven to enable the determination of pH in cell culture media containing serum. The ubiquinone oxidation peak position (E_{pa}) provided an indirect marker of pH across the applicable range of pH 6 – 9 (R^2=0.9985, n=15) in complete DMEM. The electrochemical behaviour of these sensors was also proven to be robust; retaining their ability to measure pH in cell culture media supplemented with serum up to 20% (v/v) [encompassing the range commonly employed in cell culture], cycled >100 times in 10% serum containing media and maintain >60% functionality after 5 day incubation in a 10% serum containing medium. Overall, this proof of concept research highlights the potential applicability of this, or similar, electrochemical approaches to enable to detection or monitoring of pH in complex cell culture media.

Keywords:

Carbon; cyclic voltammetry; Ubiquinone; carbon nanotubes; cell culture; pH

2. Introduction

Cell culture is a fundamental technique used in drug development and cytotoxicity screening, from small scale R&D to high throughput screening (Astashkina et al. 2012; Macarron et al. 2011; Onoue et al. 2008; Ramm et al. 2016; Zang et al. 2012). This model of cell based analysis allows for the evaluation of a myriad of biomarkers, however current methods for assessing cell state in real time is limited by cost of equipment, methods of measurement (e.g. optical or spectrophotometric) and lack of application to multiwell format due to shape and sizing of instruments (Heux et al. 2011). Stability of pH is of critical importance in cell culture, with the majority of cell lines favouring pH similar to physiological values of ~pH7.2 (Arora 2013). To promote optimal cell propagation, the cell growth solution must be maintained within a constant physiological pH range, which in turn reduces the potential for unforeseen cellular changes to occur. Commonly, culture media contains the pH indicator phenol red (phenolsulfonphthalein). Whilst beneficial to provide a quick guide to pH, is not ideal due to the ambiguity surrounding the determinable pH value of the media and that a linear determination of pH cannot even be accurately assessed by spectrophotometer without potential for discrepancy (Rovati et al. 2012). As several assays require the removal of phenol red due to its potential to act as a potent interferent (Ettinger and Wittmann 2014; Węsierska-Gądek et al. 2007), cell culture media loses pH indicating properties via spectrophotometric analysis.

Conventional pH methods present limitations to the assessment of culture media state in real time; the utilization of electrochemical means would allow for *in situ* analysis of cell culture media in real time. The standard glass potentiometric pH electrodes are limited by size, cost, risk of contamination and suffer from pH drifting, resulting in the need of regular recalibration. Cell culture medium presents a challenge in regards to electrochemical pH determination due to the constituents of the medium; generally being rich in organic salts, vitamins, amino acids, sugars and serum, which greatly increases the potential for adsorption (biofouling) to occur.

Previous attempts to monitor pH via novel electrochemical approaches more generally have been achieved through the use of various quinone moieties (Guin et al. 2011; Kampouris et al. 2009; Mandler et al. 1992), ferrocenes (Lahav et al. 1998), metal oxides(Liao and Chou 2008; Wang et al. 2002) and other redox molecules (Galdino et al. 2015). Sharp (2013) demonstrated the use of the biologically relevant molecule, uric acid, as a means to determine pH. A key facet of the pH probe mediators for voltammetric applications in physiological or cell culture media, is that mediators should be complimentary and compatible to the cell culture environment in case any leaching or interaction occur, and provide a Nernstian shift in E_{pa} with changing pH.

Operationally, a voltammetry-based pH sensor requires a mediator to provide a predictable response (oxidation or reduction peak potential(s)) to shifts in pH. Providing that the mediator can be monitored across a relevant voltage range by voltammetric interrogation, free from interference or consumption, then mediators such as Quinone-derivatives could provide a broad approach to pH monitoring. Such species may present linear shifting of voltammetric redox peak potential (E_p) as pH of the medium shifts as determined by the Nernst equation (Eq1).

$$E_p = E^0_{f_{Ox/Red}} - 2.303\frac{RT}{F}\,pH$$

Eq 1

The novel approach outlined within utilises the coenzyme ubiquinone (Q10) as a probe for pH determination; this is a biologically derived fat soluble molecule and vital component of the electron transport chain within mitochondria (Duberley et al. 2014). Q10 presents in 3 potential redox states, with the fully oxidised form, ubiquinone, being the least biologically prevalent form (Yamamoto and Yamashita 1997), which also demonstrates low potential for absorption as an exogenous supplement without modification (James et al. 2005). Due to the low redox potential of ubiquinone / ubiquinol (Krizman et al. 2012), interaction with other common media constituents is unlikely to occur.

A critical stage in the development of electrochemical sensors for use in biological media is their analytical stability in media rich in potential interfering components such as various amino acids, vitamins and serum, background noise will be elevated resulting in a loss of amperometric peak magnitude (Daggumati et al. 2015). Several commercial membranes currently exist to facilitate the conduction of protons and provide potential adsorption sites, preventing fouling of the working electrode (WE) surface. As reviewed in Hurk and Evoy (2015), several coatings have previously been utilised, such as nitrocellulose, nylon, polycarbonate and other polymers. However, for utilisation in cell culture, the outer protective membrane must be both capable of restricting adsorption at the WE and compatible/safe to be used in culture media.

Outlined herein is the design and development of a pad printed electrode for the determination of pH in complex aqueous media with a high serum content. Through the utilisation of printed electrodes, a compact device for the determination of pH is explored. Printed electrodes are selected as they allow for a variety of sizes and options to be made quickly and affordably over glass and optical counterparts. Printed electrodes can also be modified *in situ*, folded and arranged accordingly to suit the size of the well being utilised. The addition of nanostructure composites is included to explore whether these can aid sensitivity, such single or multi walled nanotubes to increase amperometric peak signal is well documented (Moraes et al. 2009; Pifferi et al. 2014; Pumera 2009) and are incorporated to increase surface area and increased presence of oxygen functionalised groups (Pan and Xing 2008) provides potential for an increase in electrochemical activity.

The study herein documents the fabrication and utilisation of a pad printed, fullerene multi walled carbon nanotubes (MWCNTs), Nafion modified, ubiquinone as a pH probe, and PEG coated electrode (PPCE/MWCNT/Naf/Q10/PEG) for the determination of pH in cell culture media.

3. Method

3.1 Reagents and solutions

All chemicals used were of analytical/ cell culture grade purchased from Alfa Aesar (Haverhill, MO, USA), Sigma Aldrich (St. Louis, MO, USA), Biowest (Rue de la Caille, Nuaillé, FR) and Lonza (Muenchensteinerstrasse, Basel, CH), and used without further purification. All solutions were prepared using deionised water with resistivity of 18.2MΩ cm^3 using a select purewater 300 water purification system (Suez, UK).

Eagle's modified essential medium (EMEM) was supplemented with heat inactivated fetal calf serum (HIFC (10% (v/v))), non-essential amino acids (NEAA) (1% (v/v)) and 1% antibiotic antimycotic (1%(v/v) (herein referred to as complete EMEM (CMEM))).
Dulbecco's modified eagle medium (DMEM) (high glucose) was supplemented with HIFC (10% (v/v)) NEAA (1% (v/v)), 1% antibiotic antimycotic (1%(v/v)) and L glutamine (2mM) (herein referred to as complete DMEM (CDMEM). Both complete formulations are used routinely in cell culturing and molecular assays.

Britton Robinson Buffer (BRB) was prepared in house; a x10 stock solution consisted of boric acid (0.4M), phosphoric acid (0.4M) and acetic acid (0.4M).
Phosphate buffered saline (PBS) was prepared in house; a x10 solution consisted of NaCl (1.37M), KCl (26.83mM), Na_2HPO_4 (0.10M) and KH_2PO_4 (17.64mM) in deionised water.
Working PBS and BRB solutions were prepared by diluting the stock solution 1/10 with deionised water, the pH was adjusted to a physiological pH of 7 using a glass pH meter (Hanna, UK) via the addition of 5M HCl or 3M NaOH.

Stock solutions of ubiquinone (Coenzyme Q10) were suspended in 99.8% EtOH at a concentration of 0.5mM. Solutions containing ubiquinone were protected from light and refrigerated at 4°C, shaken periodically over 3 days and the day prior to use were sonicated to ensure all ubiquinone had solubilised.

3.2 Instrumentation and software

All electrochemical measurements were performed using a Metrohm autolab PGSTAT101 (Metrohm, Utrecht, Netherlands). Experiments were performed using a standard three electrode system; a pad printed carbon working electrode (PPCE), a Ag/AgCl (3M NaCl, ALS) reference electrode, and a platinum wire (ALS) counter electrode. Analysis was performed using Autolab NOVA (version 2.0, Metrohm, Utrecht, Netherlands)).

3.3 Pad printed carbon electrode fabrication

Commercially available carbon/ graphite pastes were used as the base for the carbon working electrode printing ink (C2000802P2, Gwent Electronics UK). The working electrode consisted of a deposition of 5 layers of carbon graphite ink onto a 250 micron thick cellulose acetate sheet, with an average depth of 5μm total for all layers. The mean working area of the PPCEs was ~5.07mm^2 with an average diameter of 2.75mm. The working area was further defined by sealing the body of the electrode in between a sheet of 80 micron laminating film (Ethylene Vinyl Acetate inner, Polyethylene Terephthalate outer (PCUK inks LTD, UK), by passing it through a 110°C laminator 3 times in succession to seal. Electrodes were washed in deionised water and dried under a gentle stream of nitrogen. A highly conductive copper tape (<0.00Ω) was attached to the working track of the electrode before connecting the electrode to the potentiostat.

3.4 Pad printed Carbon electrode modification

A commercially available fullerene MWCNT composite (3-20 nm OD, 1-3 nm ID, 0.1-10 micron long; ID: 44945, Alfa Aesar) was suspended in D521 Nafion (0.05% in EtOH: H_2O (50:50)) at 10mg/mL. This suspension was sonicated for 24 hours in a sealed bijou to produce a homogenous solution. 2 µL of solution was drop cast onto the PPCE and dried at 100°C in a convection oven for 30 minutes. Electrodes were cooled to room temperature, washed with via deionised water and dried under a gentle stream of nitrogen gas. Further purification and analysis of the Fullerene-MWCNT mixture was not performed as primary focus of enhancing electrode functionality was to facilitate an increase in peak height and sharpness. The commercially available mixture was 95% pure MWCNT, therefore the remaining 5% is expected to contain a combination of MWCNT, SWCNT, C_{60}, C_{70} and other carbon allotropes.

3.5 Electrode pre treatment

Electrodes were cycled between -2V and +2V at a rate of 100 mV/s for 10 minutes in 0.05M H_2SO_4. Electrodes were washed with deionised water and dried under a gentle stream of nitrogen gas.

3.6 Preparation of ubiquinone pad printed electrodes (UPPCEs)

The deposition of ubiquinone onto PPCEs was performed using a drop casting method. 2µL of solution containing ubiquinone was dropped onto the surface of the working PPCE and allowed to dry for 30 minutes via evaporation in a dark box protected from light. Modified electrodes were prepared daily for use unless otherwise stated. Ubiquinone casting solutions of up to 500µM (50µM increments) were used for assessment in BRB, and EMEM. This range was chosen due the solubility of Q10 in EtOH peaking at 580µM, restricting to 500µM reduced the likelihood of precipitating out of solution prior to casting.

3.7 Polyethylene glycol coating

Polyethylene glycol (8K) (PEG) was solubilised in deionised water until saturation occurred (~630mg/mL (w/v)). From the saturated solution (100%), a 50% (v/v) solution of PEG was created using deionised water. Electrodes were briefly immersed into the solution to coat the surface and dried at room temperature, then washed with deionised water and dried under a gentle stream of nitrogen.

3.8 Statistics

Data acquired has been subjected to analysis using ANOVA and in conjunction with Tukey HSD to confirm potential observed significance.

4. Results

4.1 Ubiquinone casting optimisation

To explore the relationship between the concentrations of ubiquinone cast onto the surface of the printed carbon electrodes, increasing concentrations of ubiquinone were applied to the carbon electrodes and cycled in BRB (pH7.0). Resulting voltammograms showed a clearly defined oxidation peak ~0.38-0.44V (vs Ag/AgCl). As shown in Figure 1, 500µM concentrations of ubiquinone demonstrated the greatest anodic peak with better precision. Unmodified electrodes demonstrated no observable peak compared to mediated electrodes in both BRB and EMEM.

(Insert Figure 1)

(Insert Figure 1 caption)

4.2 Testing electrodes across a broad pH range

The 500µM UPPCEs were characterised in both BRB and EMEM (the most basic culture media tested) from pH 4 to 10 to assess the anticipated peak potential shift, as shown for BRB in Figure 2, with some degree of linearity [E_{pa}=-0.0823pH+1.0313] (R^2 = 0.9335) across this broad pH range in BRB. Electrodes demonstrate a negative shift in I_{pa} as pH increases as well as a negative shift in E_{pa}, importantly the E_{pa} shift highlights the capacity of ubiquinone to move predictably with changes in pH and therefore has potential as a pH probe for voltammetric pH assessment.

(Insert Figure 2)

(Insert Figure 2 caption)

To assess whether this sensing approach had potential to function in proteinaceous cell culture media, electrodes were cycled in CMEM to determine if potential protein fouling or interferences may affect the applicability of these sensors. A loss of I_{pa} was observed, whilst a shift in E_{pa} was retained it was notably less precise and reliable [y=-0.1953pH+1.8217, R^2 = 0.7604]. Whilst these sensors show some peak shift with changing pH, there is a very low capacity for detection of anodic peaks at >pH9 due to the decreases in peak height.

The majority of cell lines are commonly maintained close to the physiological range of pH 7.0-7.6, hence values for pH 4-6 and 9-10 are very unlikely to present in cell culture. Therefore, electrodes were assessed across pH of 6-9 (in 0.5 increments) in basic EMEM resulting in a greater linear correlation than the larger pH range 4-10, E_{pa}=-0.1006pH+1.0515 (R^2=0.9592).

4.3 Protein fouling of electrodes

Prior to being able to test in more complex culture media, e.g. using 10% heat inactivated foetal calf serum (HIFC) in culture media, UPPCE electrodes were assessed for the severity of impact that fouling of HIFC can have on their electroanalytical performance. As shown in Figure 3, following a large impact on peak magnitude by the introduction of 2.5% serum there are general trends for the progressive fouling of the electrode, reducing activity with the number of cycles performed in serum containing media, and that the greater the HIFC concentration within the media – the earlier this impacts on peak height magnitude and greater the overall impact on peak magnitude.

(Insert Figure 3)

(Insert Figure 3 caption)

4.4 Enhancing electroanalytical sensitivity

Whilst the effect of protein fouling can be mitigated by the incorporation of permselective membranes and antifouling coatings, these can often have a negative impact on the sensitivity through reduced diffusion and surface area of the electrodes. Therefore, the ability of a commercially available fullerene–MWCNT mixture to enhance electroanalytical properties of the printed carbon electrodes was explored through the surface modification with 1-10mg/mL of the fullerene-MWCNT mixture cast in Nafion. Nafion concentrations were tested from 0.01-0.5% (total concentration in solution), a final working concentration of 0.05% Nafion was utilised in electrode development [data not shown]. Figure 4A illustrates a general trend of increasing peak height with increasing quantities of fullerene-MWCNT mixture up to 7.5-10mg/mL where the increase plateaus. In addition to the peak magnitude, as this voltammetric pH sensor has the peak position as its analytical measure, peak sharpness is a key consideration to allow for accurate and precise interpretation of the peak potentials. Figure 4B highlights the half height peak width of the oxidation peaks and indicates that 5mg/mL provides a balance of peak magnitude and peak sharpness. These two phenomena are related, and are attributed to an increased thickness of the fullerene-MWCNT film which impacts its conductivity, stability and results in increased background currents, as per MWCNT modified electrodes reported in literature (Guo et al. 2013; Jain and Sharma 2012). Therefore, 5mg/mL appears to be the optimal load of fullerene-MWCNT that can be cast before these negative characteristics are observed.

(Insert Figure 4 A & B)

(Insert Figure 4 caption)

4.5 Application of PEG to reduce fouling

The final step in developing this electrode was the application of PEG to reduce potential fouling from more complex cell culture media containing e.g. serum. PEG was selected as a well reported anti-fouling agent (Quinn et al. 1997; Yan et al. 2011), which is a bio-inert material capable of preventing the passage and subsequent adhesion / fouling by proteins, by forming a hydration layer on the hydrophilic head of the PEG chain/ molecule.

(Insert Figure 5)

(Insert Figure 5 caption)

Figure 5 displays the shift in I_{pa} as electrodes are cycled in CMEM with and without 10% HIFC. A significant loss of I_{pa} is observed at both plain UPPCE with the electrodes loosing 36% of the initial I_{pa} value in 0% CMEM and 61% in 10% CMEM after 20 scans. However, the addition of PEG maintains the peak magnitude at approximately 100% indicating that it is able to correct for the fouling attributed to the basic MEM solution and when this is spiked with 10% HIFC, as is typically used in cell culture experiments.

4.6 pH sensing in high serum medium

To test the electrodes in an authentic environment, HeLa culture samples grown in CMEM were stressed by means of removing from a CO_2 environment or through the addition of 0.1M HCl. This resulted in a range of cell culture media at different pH values; in comparing the analytical responses of the composite pH sensor presented here with a conventional pH probe.

The correlation between the printed carbon electrode and conventional glass pH electrodes are shown in Figure 6, for CMEM there is a general agreement across the narrow pH range of the authentic samples, however upon

attempts to manually adjust this to test the pH sensing capabilities over a broader range led to instability of the pH of the samples, potentially exceeding the buffering range / capacity of the media. A second, commonly used media, DMEM was also investigated. DMEM is similar to EMEM in that the constituents are almost identical but DMEM has twice the concentration of most amino acids contained in MEM, additional sugars (dextrose) and salts (pyruvic acid), an increase of glucose and 50% more $NaHCO_3$. To explore this media a total of 15 DMEM samples were prepared; 5 measured following normal / authentic growth, but then the pH of 5 was increased through being left open outside of the incubator in ambient conditions and 5 through manual addition of small quantities of 0.1M HCl. Given the greater concentrations of buffer within DMEM it was stable enough to warrant analysis; giving rise to the three groups of 5 points (filled black) in Figure 6. These show a very good linearity and agreement when the new electrodes are compared with the conventional glass electrode used as a gold standard.

Testing of the electrode in CMEM demonstrates a weak linear relationship between oxidation peak position and pH CMEM $y=0.8218x+1.1723$ ($R^2=0.6921$), although as shown in Figure 6 there is some agreement and these values are across relatively narrow range. Due to the limited buffering capacity of CMEM outside of a controlled environment (CO_2 incubator), media removed from this environment fails to maintain pH without manipulation or other buffering agents, such as HEPES (Lelong and Rebel 1998). As our analytical experiments were conducted outside of a controlled environment there is capacity for drift in pH and the weaker buffering capacity also limited the stability of manipulated pH range to more acidic or basic values. When a similar experiment was conducted in the more complex DMEM across a broad pH range; there is much greater linearity and agreement with the identity line shown in Figure 6; The modified electrodes demonstrate a linear relationship for measured pH from range 6-9, with a strong linearity and agreement with the identity line for use in complete DMEM [CDMEM $y=1.0163x-0.1504$ ($R^2=0.9879$)].

(Insert Figure 6)

(Insert Figure 6 caption)

The average deviation for DMEM tests was -0.14; this presents with a greater degree of accuracy than the phenol red indicator, which has a nonlinear shift across pH; unlike phenol red, this method has little/no interference with regards to cell imaging or with homeostatic metabolisms (such as sodium-potassium or certain steroid hormones, (Ettinger and Wittmann 2014; Węsierska-Gądek et al. 2007)).

4.7 Electrode longevity

To explore UPPCE/MWCNT Naf/PEG functionality and capacity for extended periods of time and use in complex media, electrodes were cycled 100 times in CMEM, pH 7.4, from -0.4 – 0.8V at 50mV/s vs Ag/AgCl, resulting in a 13.93% (SD: 8.5%) decrease in I_{pa} and a -0.0186mV shift in E_{pa} (which would equate to approximately +0.2 pH shift). Initially the electrode peak increases, attributed to the electrochemical activation/ cleaning of the electrode. I_{pa} then decreases with each passing cycle, however the rate of decrease does not inhibit pH sensing capability for the electrode as it is the peak position which provides the analytical measure. A slight drift of E_{pa} towards the negative potential is observed, this is due to the change of pH of the media, which likely occurred due to the lack of CO_2 within the immediate environment, resulting in an increase in pH, as expected due to Henrys law.

Electrodes were assessed for their use in culture media without constant cycling. Commonly, culture media is changed once it is considered 'spent', usually denoted by change in media colour (phenol red), or when cells show signs of slow growth or death. Cells were therefore placed in CMEM and monitored on an increasing time scale to assess loss of I_{pa}. It is common practice to change culture media within a conventional working 5 day

week, regardless of cells requiring passaging or if the media is completely exhausted of nutritional components (Masters and Stacey 2007). When the electrodes were incubated in CMEM for 120 hours/ 5 days, 60% of peak magnitude was maintained whilst there was a shift in E_{pa} across this time period from a minimum of 0.13916 to a maximum of 0.234378V, (pH7.76 to 6.70), which may be attributed to the exhaustion of $NaHCO_3$ at 12 hours and subsequent media change at 24 hours. But overall, this suggests that this electrode configuration may offer future potential for longitudinal measurement of pH through embedded pH sensors across a reasonable time and replicate range.

5. Conclusion

The research presented outlines the development and characterisation of a novel sensor assembly for the measurement of pH using a carbon nanotube composite modified, ubiquinone mediated and PEG coated printed carbon electrode. This has shown to be a potential solution to monitoring cell culture *in situ*, in real time. Electrodes can be simply constructed via solvent casting without the need for specialist equipment (such as spin coating) or extensive pretreatments. The immobilised redox molecule ubiquinone facilitates the measurement of pH across the relevant cell culture / physiological range of pH 6-9 with a linear response (R^2 = 0.9917) in buffered solution, a strong agreement with conventional glass pH electrode in cell culture media (CDMEM, R^2 = 0.9879), and were shown to retain function after 100 repeated measurements and when incubated for 5 days in cell culture media. These low cost, disposable electrodes also have potential for further use in a variety of applications, from assay and culture flask monitoring to further miniaturisation and assessment of other biofluids

Whilst this proof-of-concept research has highlighted future potential – further research into the physical development and reproducibility of sensors produced on a larger scale, and more detailed studies into the longer-term ability of the sensing assembly to function immersed in cell culture environment are warranted. Further modification steps to enhance the peak magnitude and sharpness may also contribute to enhancing the analytical capabilities of such sensing systems.

Figures

Figure 1 - Calibration graph of ubiquinone coated printed carbon electrodes; ubiquinone concentration range of 50-500 µM in 50µM increments. Electrodes were cycled from -0.6 – 1.2V in 0.4M BRB at 50mV/s. E_{pa} occur at ~0.38-0.44 V vs Ag/AgCl, (n=3, error bars represent standard deviation).

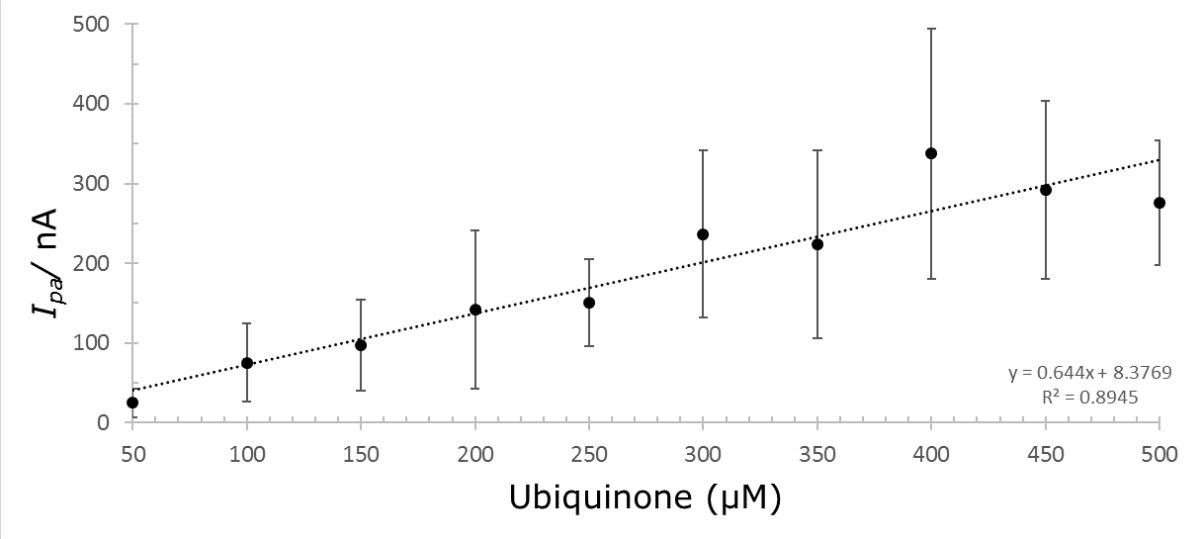


Figure 2 – Cyclic voltammogram of UPPCEs in 0.4M BRB, pH4-10. Electrodes were cycled in BRB at 50mV/s between -0.6 – 1.2V.

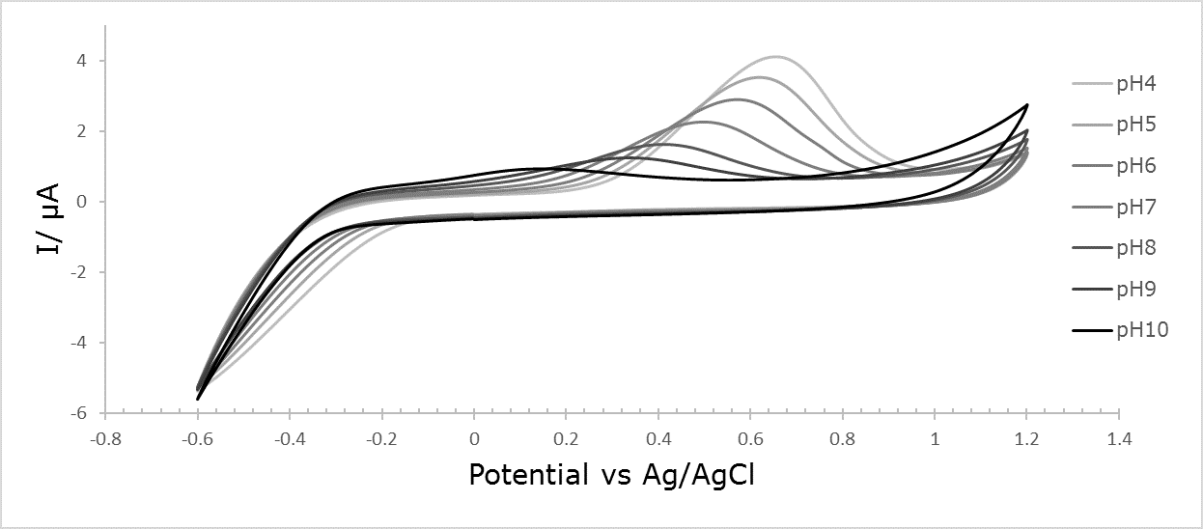


Figure 3 - I_{pa} (A) of 500mM UPPCEs in increasing % HIFCS CMEM (pH6). Electrodes were cycled 50 times at 50mV/s between -0.4 – 0.8V, (n=4).

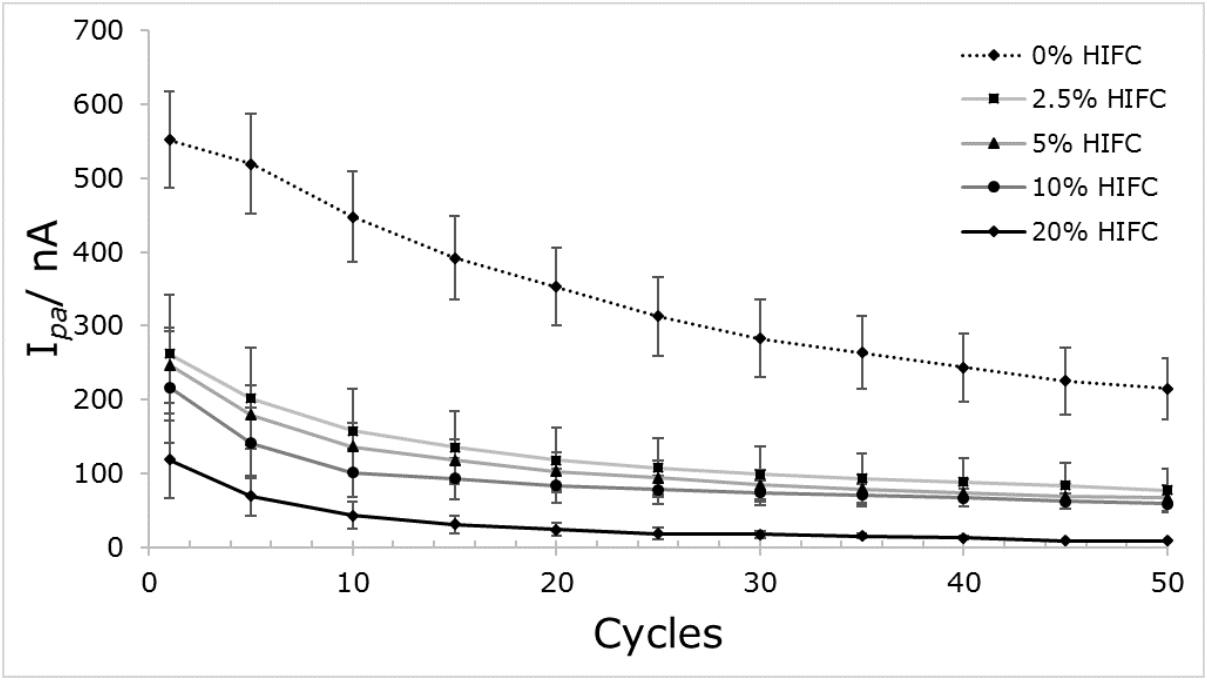
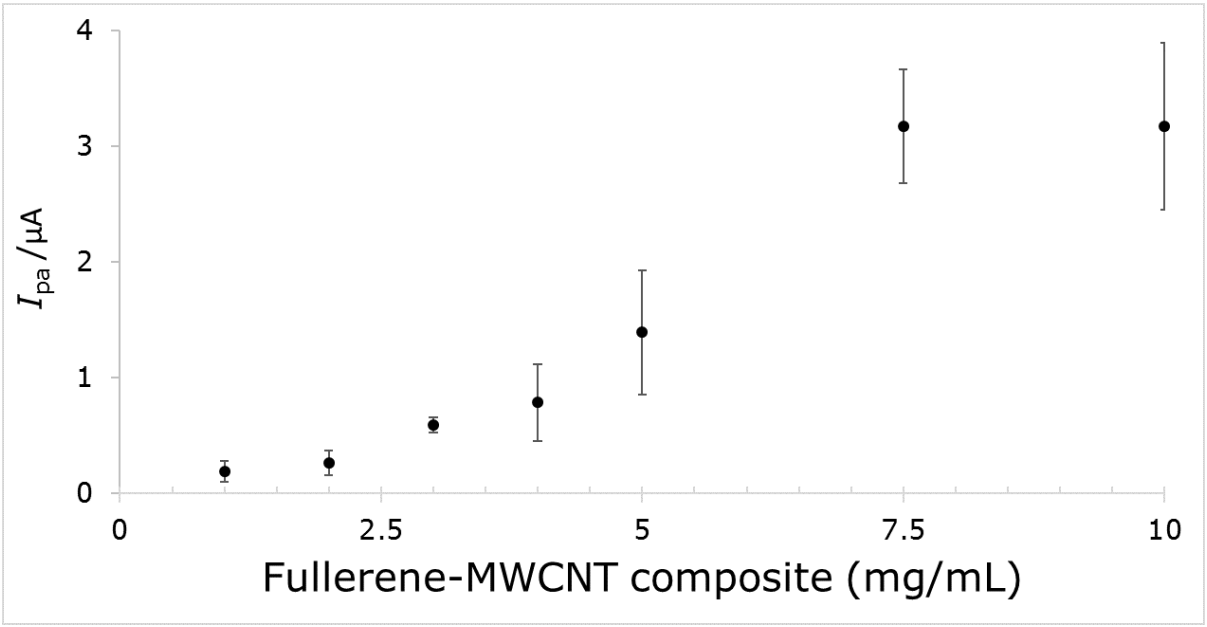


Figure 4 (A&B) –UPPCE/MWCNT Nafion electrodes, increasing concentration of the Fullerene MWCNT mixture vs I_{pa} (A) and half height peak width (B) for modified electrodes cycled in CMEM from -0.6V to 1.2V at 50mV/s(n=5)



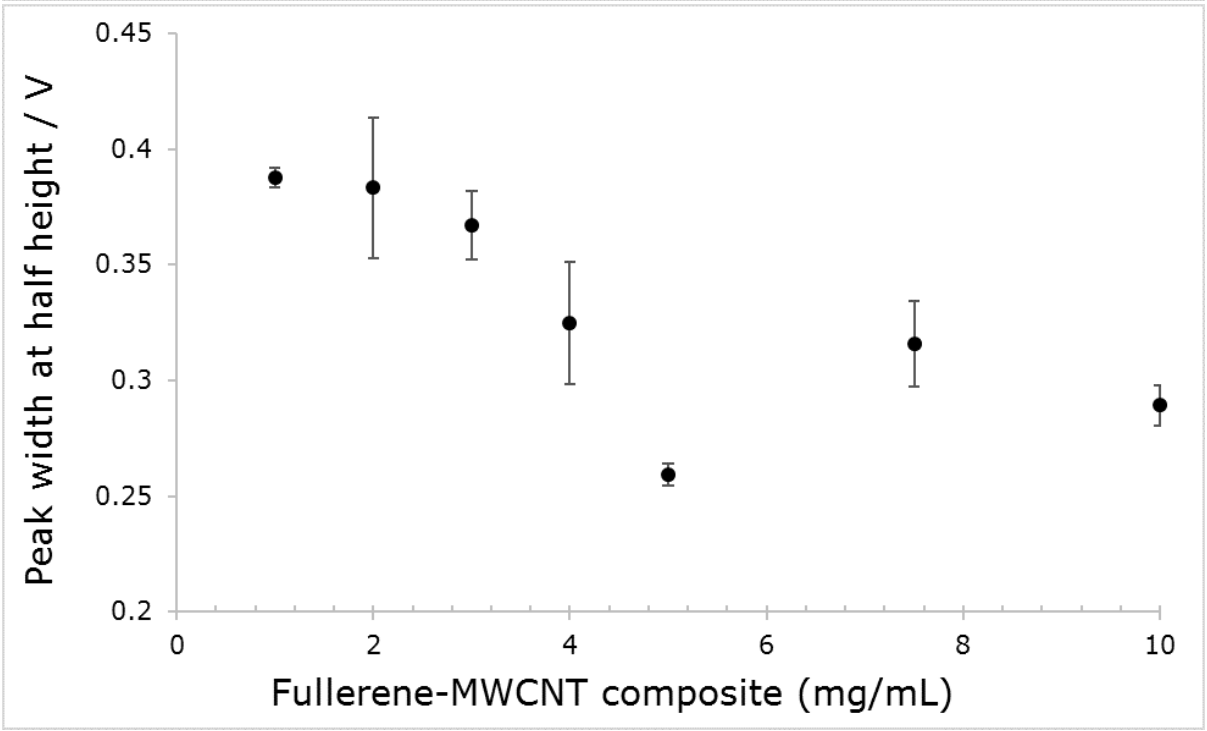


Figure 5 – I_{pa} as percentage of scan 1 for 20 replicate scans in CMEM with 0 or 10% HIFC for plain and PEG modified UPPCE electrodes, scanned between -0.4 – 0.8V, at 50mV/s in CMEM.

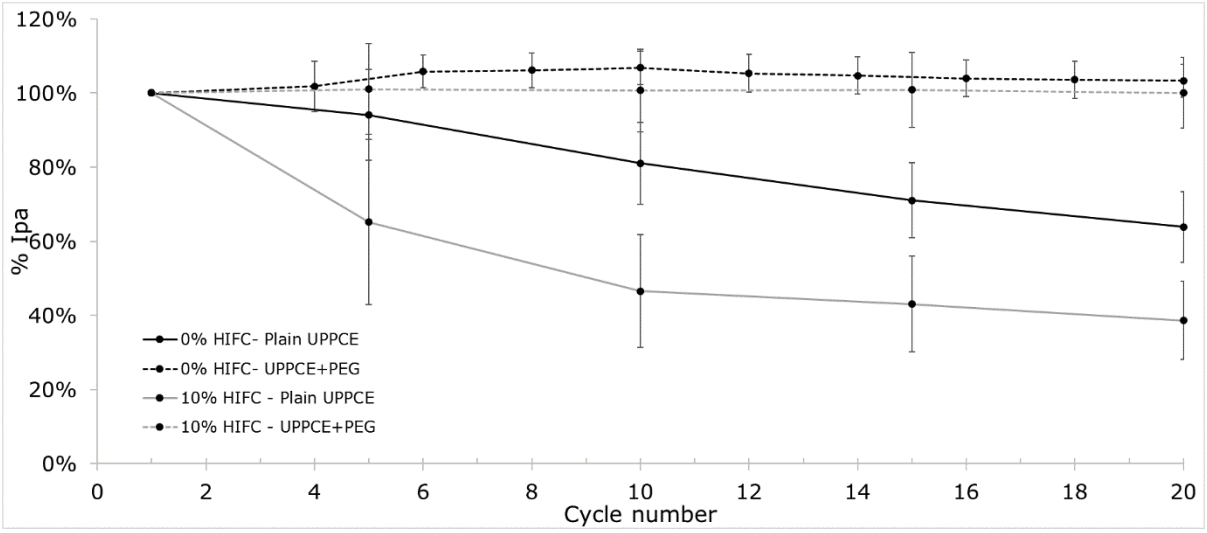
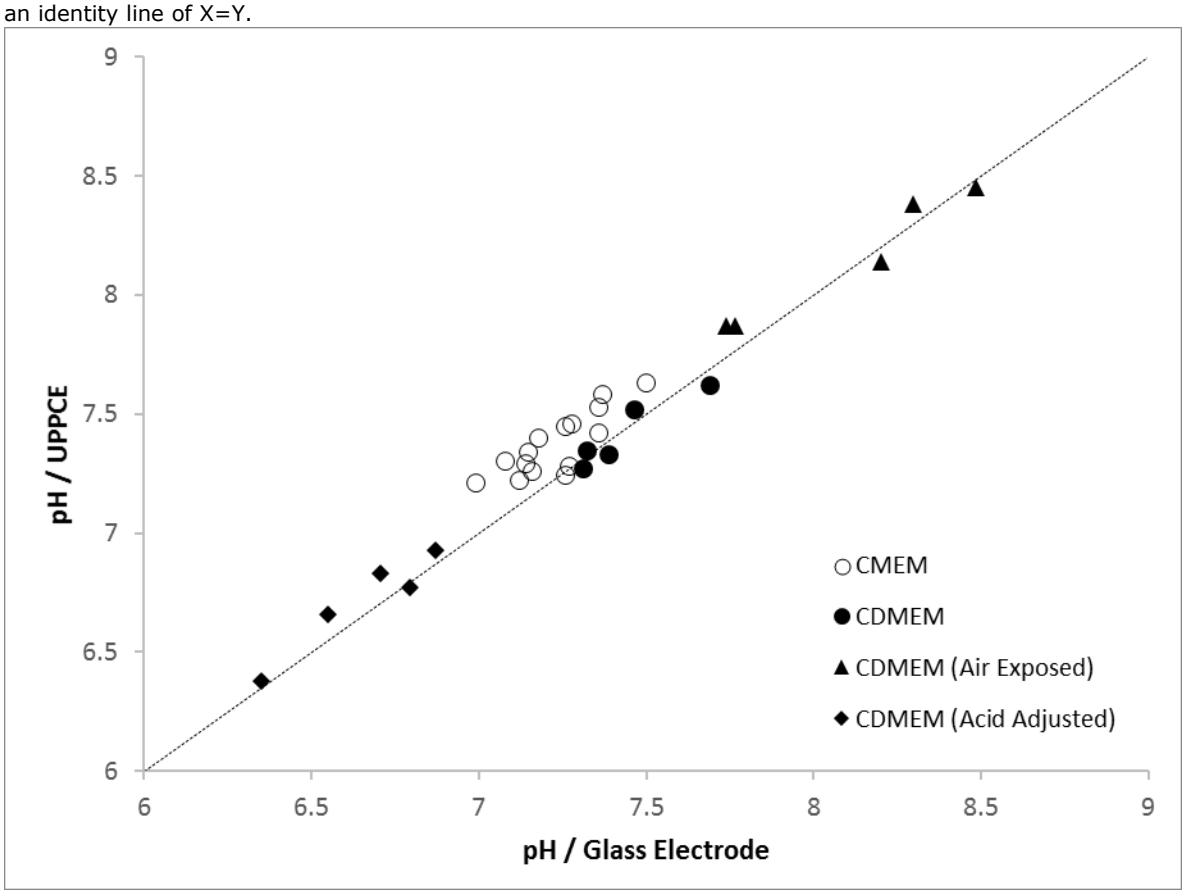


Figure 6 – Relationship between the UPPCE/MWCNT Naf/PEG/ compared to the standard glass electrode in both CMEM and CDMEM (10%). (n=15) after 18 hours growth of HeLa cells in 37°C, 5% CO_2 incubator, compared to an identity line of X=Y.



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