Electrochemical biosensors for real-time detection of angiogenesis

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**ELECTROCHEMICAL BIOSENSORS FOR
REAL-TIME DETECTION OF ANGIOGENESIS**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2014

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School of Chemical and Biomedical Engineering

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Table of Contents

Acknowledgements	ii
List of Figures.....	ix
List of Tables	xviii
Abstract.....	xix
1 Introduction	1
1.1 Background	1
1.2 Motivations	5
1.3 Objective and specific aims	6
2 Review of literature	8
2.1 Key regulators of angiogenesis.....	8
2.1.1 Hypoxia inducible factors (HIFs).....	8
2.1.2 Vascular endothelial growth factor (VEGF) family	11
2.1.3 Delta-like ligand 4 and Notch signalling.....	13
2.1.4 Angiopoietins (Ang) and Tie receptors	15
2.1.5 Platelet-derived growth factor family (PDGF).....	16
2.2 Extracellular metabolic (O ₂ , CO ₂ , pH, glucose, lactate) and signalling (NO) markers of angiogenesis	17
2.3 Electrochemical detection	19
2.3.1 Electrochemical cell.....	19
2.3.2 Mass transport	19
2.3.3 Steps in a simple electrochemical reaction.....	20
2.3.4 Nernst equation	21
2.3.5 Cyclic voltammetry	22
2.3.6 Amperometry.....	25
2.3.7 Electrochemical impedance spectroscopy	27
2.4 Nanomaterials for electrochemical biosensors	29
2.4.1 Graphene - properties and preparation methods	30
2.4.2 Iridium oxide - properties and preparation methods.....	32
2.4.3 Poly(methylene blue) (PMB) - properties and preparation methods	34

2.5	Development of electrochemical biosensors	37
2.5.1	Nitric oxide biosensors	37
2.5.2	O ₂ biosensors	39
2.5.3	pH biosensors	40
2.5.4	Glucose biosensors.....	41
3	Highly sensitive nitric oxide sensing using three-dimensional graphene/ ionic liquid nanocomposite†.....	44
3.1	Abstract	44
3.2	Introduction.....	45
3.3	Experimental methods.....	47
3.3.1	Materials	47
3.3.2	Preparation of 3-D graphene material.....	48
3.3.3	Preparation of nanocomposite gel electrode.....	48
3.3.4	Preparation of saturated NO in PBS	49
3.3.5	Characterisations and electrochemical measurements.....	50
3.4	Results and discussion.....	50
3.4.1	Morphology and composition characterisation of samples	50
3.4.2	Electrochemical behaviour of 3-D graphene/IL nanocomposite electrode.....	53
3.4.3	Electrocatalysis of 3-D graphene/IL nanocomposite towards sensitive NO detection.....	56
3.5	Conclusion	62
4	RGD-peptide functionalised graphene biomimetic live-cell sensor for real-time detection of nitric oxide†.....	63
4.1	Abstract	63
4.2	Introduction.....	64
4.3	Experimental methods.....	65
4.3.1	Materials	65
4.3.2	Preparation of pyrenebutyric acid-functionalised graphene.....	65
4.3.3	Preparation of biomimetic graphene film.....	65
4.3.4	Cell culture and manipulation	66
4.3.5	Characterisation and electrochemical measurements	68

4.3.6	Real-time detection of NO released by HUVECs.....	68
4.4	Results and discussion.....	69
4.4.1	Characterisations of biomimetic graphene film	69
4.4.2	Cell adhesion on biomimetic graphene film.....	71
4.4.3	Electrochemical behaviour of biomimetic graphene film	73
4.4.4	Flexibility and reproducibility of the biomimetic graphene film	76
4.4.5	Real-time detection of NO released by HUVECs on the film	77
4.5	Conclusion	79
5	Iridium oxide microelectrode for the real-time physiological pH measurements of porcine aortic endothelial cells	80
5.1	Abstract	80
5.2	Introduction.....	81
5.3	Experimental methods.....	84
5.3.1	Chemicals	84
5.3.2	Instruments	84
5.3.3	Preparation of AEIROFs on MEA chip.....	85
5.3.4	pH sensor characterisation.....	86
5.3.5	pH measurements of PAECs in real-time.....	87
5.4	Results and discussion.....	89
5.4.1	FESEM characterisation of AEIROF coated with PEI and fibronectin.....	89
5.4.2	Electrochemical characterisation of PEI-coated AEIROFs.....	91
5.4.3	Light microscopy images of control chip and chip with PAECs before and after cell experiments.....	91
5.4.4	Effect of fibronectin coating on pH responses of sensors in cell culture medium.....	95
5.4.5	Real-time measurements of extracellular pH.....	96
5.5	Conclusion	103
6	O ₂ and glucose biosensors based on a novel electropolymerised redox polymer	104
6.1	Abstract	104
6.2	Introduction.....	105

6.3	Experimental methods.....	108
6.3.1	Chemicals	108
6.3.2	Instruments	109
6.3.3	Preparation of dissolved O ₂ and glucose biosensors.....	109
6.4	Results and discussion.....	110
6.4.1	FESEM characterisation of O ₂ biosensor	110
6.4.2	Electrochemical characterisation of O ₂ biosensor in N ₂ -purged PBS.....	111
6.4.3	Effect of pH on O ₂ biosensor in N ₂ -purged PBS	115
6.4.4	Electrochemical impedance spectroscopy of O ₂ biosensor in N ₂ -purged PBS	119
6.4.5	Electrochemical characterisation of O ₂ biosensor in atm O ₂ PBS ...	123
6.4.6	Effect of pH on O ₂ biosensor in atm O ₂ PBS	125
6.4.7	Electrochemical impedance spectroscopy of O ₂ biosensor in atm O ₂ PBS.....	126
6.4.8	CV of O ₂ biosensor in different concentrations of dissolved O ₂	130
6.4.9	Amperometric detection of dissolved O ₂	132
6.4.10	FESEM characterisation of glucose biosensor.....	137
6.4.11	Electrochemical characterisation of glucose biosensor in N ₂ -purged PBS.....	138
6.4.12	Effect of pH on glucose biosensor in N ₂ -purged PBS and direct electron transfer of glucose oxidase.....	141
6.4.13	Direct electron transfer of glucose oxidase in N ₂ -purged glucose solutions.....	143
6.4.14	Electrochemical characterisation of glucose biosensor in atm O ₂ PBS... ..	145
6.4.15	Effect of pH on glucose biosensor in atm O ₂ PBS.....	148
6.4.16	CV of glucose biosensor in different concentrations of glucose.....	149
6.4.17	Amperometric response of glucose biosensor.....	150
6.5	Conclusion	155
7	Overall conclusion and perspectives	156
	List of Abbreviations	162
	References.....	168

Publications.....	186
Book chapter.....	186

List of Figures

Figure 1.1 (a) In sprouting angiogenesis, a new vascular channel is formed by fusing two primary vascular channels. (b) In non-sprouting angiogenesis, each primary vascular channel divides to form two new vascular channels..... 2

Figure 2.1 Crosstalk exists between EC metabolism and angiogenesis (Chapter 2.2). The EC utilises glycolysis to generate its energy. Glucose is transported into the EC by a glucose transporter (GLUT) and is converted into pyruvate via glycolysis. The H^+ produced during glycolysis are pumped out of the cell by the $H^+/ATPase$, causing extracellular acidification. Pyruvate is then converted to lactate. The presence of lactate and the lack of O_2 during hypoxia causes PHD-2 to be inhibited, allowing HIF-1 and HIF-2 to enter the nucleus and bind to DNA. HIF-1 causes the expression of inducible nitric oxide synthase (iNOS) and VEGFA while HIF-2 results in the expression of endothelial nitric oxide synthase (eNOS) and VEGFR2. The enzymes eNOS and iNOS produce NO, which increases cell permeability. Low concentrations of NO inhibit cytochrome c oxidase (CcO) in the mitochondrion to allow the redistribution of O_2 within the EC. 9

Figure 2.2 Binding of VEGFA to VEGFR2 triggers several intracellular signalling pathways in the EC, which produces NO, as well as proteins needed for cell survival, proliferation and migration. 13

Figure 2.3 (a) The EC expresses VEGFR2 when the intracellular domain of the Notch1 receptor is together with its extracellular domain at the cell membrane. The extracellular domain of the Notch1 receptor comes into contact with Dll4 of an adjacent cell. (b) The Notch1 receptor is cleaved by an enzyme into its two

domains. The extracellular domain is uptaken by the adjacent cell while the intracellular domain enters the nucleus and binds to DNA, inhibiting the expression of VEGFR2.....	14
Figure 2.4 (a) The binding of Ang1 to the Tie2 receptor causes the recruitment of pericytes. (b) In the absence of VEGFA, the binding of Ang2 to the Tie2 receptor causes cell death for vascular regression. (c) In the presence of VEGFA, the binding of Ang2 to the Tie2 receptor leads to cell survival, proliferation and migration for the growth of blood vessels.	16
Figure 2.5 (a) A potential ramp with $v = 0.05 \text{ Vs}^{-1}$ and (b) the resulting CV curve showing current response/ μA against potential/V for 10 mM of $[\text{Fe}(\text{CN})_6]^{4-}$ in 1 M aqueous KCl solution at a Au electrode. The experimental value of ΔE_p is 0.065V.	23
Figure 2.6 (a) The Au microelectrode was stepped from $E_i = 0.75\text{V}$ to $E_s = 0\text{V}$ to $E_f = 0.75\text{V}$. (b) The corresponding current response of the electrode to the applied potential steps in 10 mM ferric ammonium sulphate in 0.5 M aqueous HClO_4 solution.....	26
Figure 2.7 (a) The equivalent circuit for a simple electrochemical reaction $\text{O} + ne \leftrightarrow \text{R}$. (b) The corresponding Argand diagram.....	28
Figure 2.8 (a) Chemical structure of MB^+ and (b) its electrochemical reduction...	35
Figure 2.9 Electrochemical reduction of poly(MB^+) to poly(LMB).....	36
Figure 3.1 Schematic representation of the fabrication of the 3-D graphene/IL nanocomposite as well as its photograph.....	49

Figure 3.2 FESEM images of 3-D graphene material with (a) low and (b) high magnification and of 3-D graphene/IL nanocomposite with (c) low and (d) high magnification.....	51
Figure 3.3 FTIR spectra of 3-D graphene material and 3-D graphene/IL nanocomposite.....	53
Figure 3.4 (a) CVs of electrochemical behaviour of the 3-D graphene/IL nanocomposite in 10 mM of $[\text{Fe}(\text{CN})_6]^{3-}$ in 1 M aqueous KCl using scan rates of 0.01 V s^{-1} to 1.00 V s^{-1} . (b) Plot of peak current/ μA against scan rate/ V s^{-1} (black circles) and square root of scan rate/ $\text{V}^{0.5} \text{ s}^{-0.5}$ (hollow circles).	55
Figure 3.5 CVs of 0 mM of NO in PBS using 3-D graphene/IL electrode (curve 1), and 0.2 mM of NO in PBS using 3-D graphene/IL (curve 2), 3-D graphene/mineral oil (curve 3), MWCNTs/IL (curve 4), graphite/IL (curve 5) and GC electrode (curve 6) ($\nu = 0.05 \text{ V s}^{-1}$).....	57
Figure 3.6 Plot of peak potential/ V against scan rate/ V s^{-1} for CVs of 0.2 mM of NO in PBS using the 3-D graphene/IL nanocomposite. The inset shows the CVs.	58
Figure 3.7 (a) Amperometric response of the 3-D graphene/IL nanocomposite over time following injections of $0.8 \mu\text{mol L}^{-1}$ of NO in PBS. (b) Plot of current response/ μA against NO concentration/ $\mu\text{mol L}^{-1}$	60
Figure 4.1 Schematic illustration of preparation of the free-standing biomimetic film and its live-cell assay. (a) Chemical reduction of graphene oxide and pyrenebutyric acid functionalisation. (b) Preparation of pyrenebutyric acid-functionalised graphene film by filtration of the solution of pyrenebutyric acid-functionalised graphene. (c) Fabrication of the biomimetic graphene film by covalently bonding RGD-peptide onto the surface of the pyrenebutyric acid	

functionalised graphene film via EDC/NHS coupling. (d) The functional biomimetic graphene film as both cell adhesion and sensing matrix.	67
Figure 4.2 (a) Photograph of biomimetic RGD-peptide covalently bonded graphene film. The inset of panel (a) is an AFM image of pyrenebutyric acid-functionalised graphene sheets. (b) High magnification side-view, (c) low magnification side-view and (c) top-view SEM images of the biomimetic graphene film. (d) RA-IR spectra of (1) pyrenebutyric acid functionalised graphene film and (2) biomimetic graphene film. (f) Fluorescence staining of biomimetic graphene film and the inset is fluorescence staining of graphene film without RGD-peptide. The scale bars represent 2 mm.	70
Figure 4.3 Microscopy images of HUVECs cultured on (a) plain graphene film and (b) biomimetic graphene film. (c) The number of adhered cells and (d) the average cell length of HUVECs on (1) plain graphene film and (2) biomimetic graphene film.	72
Figure 4.4 (a) CV curves of the biomimetic graphene film in culture medium in the absence (1) and presence (2) of 10 μM NO ($v = 0.02 \text{ V s}^{-1}$). (b) The logarithmic plot of the response current vs. NO concentration. Insets of panel B are a typical DPSCA used to obtain the calibration curve and the calibration curve for low concentration of NO. (c) Selectivity of the biomimetic graphene film for 0.3 μM NO against 25 μM of interfering species (NO_2^- , K^+ , Na^+ , NO_3^- , SO_4^- , ascorbic acid).....	75
Figure 4.5 (a) Photographs of the relaxed and bent biomimetic graphene film. (b) Current responses/ μA of the biofilm subjected to bending.	76

Figure 4.6 (a) Schematic drawing showing the setup for the live-cell assay. (b) Real-time monitoring of NO released by the HUVECs on the biomimetic graphen film in cell culture medium. The drug was added at the time indicated by the arrow. (c) The current responses of the biomimetic graphene film with cultured HUVECs towards different drugs. Ach is a model drug to stimulate cell NO release and L-NAME is a specific NO inhibitor.....	78
Figure 5.1 The MEA chip.....	85
Figure 5.2 Experimental setup for real-time PAECs pH measurement. (a) The MEA chip was placed in a chip holder and (b) the leak-free Ag AgCl reference electrode was positioned into the well of the chip. (c) 5% CO ₂ was blown across the top of the chip through a hole in the rubber tubing. The chip holder was connected to the CHI 1030 electrochemical workstation.....	88
Figure 5.3 FESEM image of AulAEIROF microelectrode coated with PEI and fibronectin.....	89
Figure 5.4 FESEM image of 20 µg mL ⁻¹ fibronectin coating on silicon nitride chip surface.	90
Figure 5.5 FESEM image of 5% PEI coating on silicon nitride chip surface.	90
Figure 5.6 Light microscopy images (40X) of the control chip without PAECs (a) after removing from the incubator and (b) after the experiment. Inverted light microscopy images (67X) of control chip without PAECs (c) after removing from the incubator and (d) after the experiment. Electrode with PEI-coated AEIROF circled in red.	93
Figure 5.7 Light microscopy images (40X) of chip with PAECs (a) after removing from the incubator and (b) after the second dose of PD98059. Light microscopy	

images (100X) of showing morphology of PAECs on chip (c) after removing from the incubator and (d) after the second dose of PD98059. Inverted light microscopy images (67X) of chip with PAECs (e) after removing from the incubator and (f) after the experiment. Electrode with PEI-coated AEIROF circled in red.	94
Figure 5.8 OCP/V vs. time/ks measurements of the addition of reagents to (a) control chip and (b) chip with PAECs. Measurements were done at an average room temperature of 22.0 °C and gassed with 5% CO ₂	97
Figure 6.1 (a) Low magnification FESEM image (2200X) and (b) high magnification FESEM image (35 000X) of PMB-PPy.....	111
Figure 6.2 CV of AulPMB-PPy in N ₂ -purged PBS with different scan rates.	112
Figure 6.3 Plot of $\log(-i_{p,c})/A$ against $\log v/V \text{ s}^{-1}$ (red circles) and plot of $\log(i_{p,a})/A$ against $\log v/V \text{ s}^{-1}$ (blue squares) for AulPMB-PPy in N ₂ -purged PBS.	113
Figure 6.4 Plot of $E_{p,c}/V$ (red circles) and $E_{p,a}/V$ (blue squares) against $v/V \text{ s}^{-1}$ for AulPMB-PPy in N ₂ -purged PBS.	113
Figure 6.5 Effect of pH on AulPMB-PPy in N ₂ -purged PBS ($v = 0.05 \text{ V s}^{-1}$).	116
Figure 6.6 Chemical structure of MB ⁺ dimer.....	117
Figure 6.7 Electrochemical reduction of poly(MB ⁺) to poly(LMB).....	118
Figure 6.8 (a) Chemical structure of MB ⁺ . (b) The electrochemical reduction of MB ⁺ to LMB.....	118
Figure 6.9 EIS of (a) AulPMB-PPy and (b) bare Au with amplitude of 0.005 V from 0.01 Hz to 1.00 MHz in N ₂ -purged PBS from pH 4 to 8.....	120
Figure 6.10 Plot of $R_{ct} \times 10^5/\Omega$ against pH for AulPMB-PPy (red circles) and bare Au (blue squares) in N ₂ -purged PBS.	121
Figure 6.11 CV of AulPMB-PPy in atm O ₂ PBS with different scan rates.....	123

Figure 6.12 Plot of $\log(-i_{p,c})/A$ against $\log v/V \text{ s}^{-1}$ for AuPMB-PPy in atm O ₂ PBS.	124
Figure 6.13 Plot of $ i_{p,c} v^{-0.5} / \mu\text{A V}^{-0.5} \text{ s}^{0.5}$ against $v/V \text{ s}^{-1}$ for AuPMB-PPy in atm O ₂ PBS.	124
Figure 6.14 Effect of pH on AuPMB-PPy in atm O ₂ PBS.	126
Figure 6.15 EIS of (a) AuPMB-PPy and (b) bare Au with amplitude of 0.005 V from 0.01 Hz to 1.00 MHz in atm O ₂ PBS from pH 4 to 8.	127
Figure 6.16 Plot of $R_{ct} \times 10^5 / \Omega$ against pH for AuPMB-PPy (red circles) and bare Au (blue squares) in atm O ₂ PBS.	128
Figure 6.17 CV of (a) AuPMB-PPy and (b) bare Au in N ₂ -purged PBS (red), atm O ₂ PBS (blue) and O ₂ -saturated PBS (brown) ($v = 0.05 \text{ V s}^{-1}$).	131
Figure 6.18 Amperometric response of AuPMB-PPy (red) and bare Au (blue) to successive additions of 100 μL O ₂ -saturated PBS to 8 mL N ₂ -purged PBS with magnetic stirring, which corresponds to 0.014 mM dissolved O ₂ per addition. The first addition was at 100 s (arrow) and additions were made every 50 s thereafter ($V = -0.300 \text{ V}$). The insets show the current response/ μA against time/s for the addition of 0.014 mM dissolved O ₂ to (a) bare Au and (b) AuPMB-PPy. The 90% response time of both electrodes is 12 s.	133
Figure 6.19 Calibration plot of increase in cathodic current/ μA against dissolved O ₂ concentration/mM for (a) AuPMB-PPy (n=3) and (b) bare Au. i is the increase in cathodic current in μA and c is the concentration of dissolved O ₂ in mM.	134
Figure 6.20 CV of AuPMB-PPy in atm O ₂ PBS on the 1st day (red) and 18th day (blue) ($v = 0.050 \text{ V s}^{-1}$).	135
Figure 6.21 FESEM image (2200X) of PMB-PPy-GOD.	137

Figure 6.22 CV of AuPMB-PPy-GOD in N ₂ -purged PBS with different scan rates.	138
Figure 6.23 Plot of $\log(-i_{p,c})/A$ against $\log v/V \text{ s}^{-1}$ (red circles) and plot of $\log(i_{p,a})/A$ against $\log v/V \text{ s}^{-1}$ (blue squares) for AuPMB-PPy-GOD in N ₂ -purged PBS.....	139
Figure 6.24 Plot of $E_{p,c}/V$ (red circles) and $E_{p,a}/V$ (blue squares) against $v/V \text{ s}^{-1}$ for AuPMB-PPy-GOD in N ₂ -purged PBS.....	139
Figure 6.25 Effect of pH on AuPMB-PPy-GOD in N ₂ -purged PBS ($v = 0.05 \text{ V s}^{-1}$).	141
Figure 6.26 CV of AuPMB-PPy-GOD in PBS and different concentrations of glucose purged with N ₂ ($v = 0.05 \text{ V s}^{-1}$).	144
Figure 6.27 CV of AuPMB-PPy-GOD in atm O ₂ PBS with different scan rates.	146
Figure 6.28 Plot of $\log(-i_{p,c})/A$ against $\log v/V \text{ s}^{-1}$ for AuPMB-PPy-GOD in atm O ₂ PBS.....	147
Figure 6.29 Plot of $li_{p,c} v^{-0.5} / \mu\text{A V}^{-0.5} \text{ s}^{0.5}$ against $v/V \text{ s}^{-1}$ for AuPMB-PPy-GOD in atm O ₂ PBS.....	147
Figure 6.30 Effect of pH on AuPMB-PPy-GOD in atm O ₂ PBS ($v = 0.05 \text{ V s}^{-1}$).	148
Figure 6.31 CV of AuPMB-PPy-GOD in PBS and different concentrations of glucose at atm O ₂ ($v = 0.05 \text{ V s}^{-1}$).	149
Figure 6.32 Amperometric response of AuPMB-PPy-GOD to successive additions of 10 μL 250 mM glucose to 10 mL of atm O ₂ PBS with magnetic stirring, which corresponds to 0.25 mM glucose per addition. The first addition was at 50 s (arrow) and additions were made every 30 s thereafter ($V = -0.360 \text{ V}$). The inset shows the	

current response/ μA against time/s of the AulPMB-PPy-GOD to the addition of 0.25 mM glucose. The 90% response time is 7 s.	151
Figure 6.33 Calibration plot of decrease in cathodic current/ μA against glucose concentration/mM for AulPMB-PPy-GOD in atm O_2 (n=3). i is the decrease in cathodic current in μA and c is the concentration of glucose in mM.	152
Figure 6.34 Lineweaver-Burk plot corresponding to the linear range of 0.25 mM to 1.49 mM glucose. i_{ss} is the decrease in cathodic current in μA and c is the concentration of glucose in mM.	152
Figure 6.35 Current response of AulPMB-PPy-GOD to 0.25 mM glucose vs. the interfering species, 10 mM ascorbic acid (AA) and 10 mM uric acid (UA) (n=3).	153
Figure 6.36 CV of AulPMB-PPy-GOD in atm O_2 PBS on the 1st day (red) and 29th day (blue) ($v = 0.050 \text{ V s}^{-1}$).	153

List of Tables

Table 3.1 Comparison of sensitivity and detection limit for NO of different sensors.	61
Table 6.1 Values of formal potential (E^0)/V and peak separation (ΔE_p)/V against scan rate/V s ⁻¹ for Au/PMB-PPy in N ₂ -purged PBS with different scan rates.	114
Table 6.2 Values of ΔE_p /V against pH of Au/PMB-PPy in N ₂ -purged PBS.	117
Table 6.3 Values of $R_{ct} \times 10^5/\Omega$, $C_{dl}/\mu F$ and $j_0/\mu A\ cm^{-2}$ against pH for Au/PMB- PPy and bare Au in N ₂ -purged PBS.....	122
Table 6.4 Values of $R_{ct} \times 10^5/\Omega$, $C_{dl}/\mu F$ and $j_0/\mu A\ cm^{-2}$ against pH for Au/PMB- PPy and bare Au in atm O ₂ PBS.....	129
Table 6.5 Comparison of O ₂ biosensor performances	136
Table 6.6 Values of E^0 /V and ΔE_p /V against scan rate/ V s ⁻¹	140
Table 6.7 Comparison of glucose biosensor performances.....	154

Abstract

Electrochemical biosensors have been made to detect the metabolic markers, pH, O₂ and glucose, and nitric oxide (NO), the signalling molecule involved in angiogenesis. A novel three-dimensional (3-D) graphene/ionic liquid (IL) nanocomposite demonstrates highly sensitive detection of NO in phosphate buffered saline (PBS). An arginine-glycine-aspartic acid (RGD) peptide-functionalised biomimetic graphene film has been used as both a cell culture and sensing matrix to detect NO released by human umbilical vein endothelial cells (HUVECs) in real-time under acetylcholine (Ach) stimulation. The amount of NO released is dose-dependent and inhibited by NG-nitro-L-arginine methyl ester (L-NAME). A poly(ethyleneimine) (PEI)-coated anodically electrodeposited iridium oxide film (AEIROF) exhibiting super-Nernstian response to pH functions as miniature pH sensor for detecting acute changes in extracellular pH due to the interaction of porcine aortic ECs (PAECs) with fibronectin and thrombin. Thrombin causes dissolution of fibronectin, extracellular acidification of PAECs and a change in cell morphology from stretched to round cells. O₂ and glucose biosensors based on a novel electropolymerised redox polymer are developed and prepared by one-step electropolymerisation of methylene blue (MB⁺) and pyrrole for the O₂ biosensor, with the addition of glucose oxidase (GOD) for the glucose biosensor. The O₂ biosensor demonstrates superior sensitivity towards dissolved O₂ at atmospheric (atm) O₂ concentrations and below and is insensitive to pH. The glucose biosensor exhibits direct electron transfer (DET) and is insensitive to pH from pH 6 to 8 in N₂-purged PBS and from pH 4 to 8 in atm O₂ PBS.

1 Introduction

1.1 Background

In this chapter, references [1] and [2] are a book chapter and review paper, respectively. They are used to describe the process of angiogenesis and its implications in pathophysiological conditions as they provide an excellent summary of the relevant literature from the 1990s to 2000s.

Vasculogenesis and angiogenesis are key processes of vascular development which occur sequentially in embryos [1]. During an early phase of embryonic development, the cells of the early embryo are partitioned into three layers called the ectoderm, mesoderm and endoderm [3]. A subset of the mesodermal cells differentiates into endothelial cells (ECs), which assemble to form blood islands [1]. The blood islands then connect to form a primary vascular network and this process is called vasculogenesis [4]. The primary vascular network expands to form new vessels by two distinct mechanisms - sprouting angiogenesis and non-sprouting angiogenesis (Figure 1.1) [5]. In sprouting angiogenesis, ECs of existing primary vascular channels form elongated features called sprouts which invade the proximal space, interact with other sprouts and fuse together to form new vascular channels [1]. During non-sprouting angiogenesis, mesenchymal cells surrounding each EC of an existing vascular channel push the EC inwards, causing the vascular channel to be divided into two, forming two new vascular channels [1].

Figure 1.1 (a) In sprouting angiogenesis, a new vascular channel is formed by fusing two primary vascular channels. (b) In non-sprouting angiogenesis, each primary vascular channel divides to form two new vascular channels.

These two processes synergistically give rise to complex vascular channels, which undergo pruning, remodelling and maturation to form blood vessels [5]. These blood vessels are then infiltrated by pericytes and smooth muscle cells which provide mechanical support and ability of these blood vessels to contract [6].

Transforming growth factor β is considered a major regulator of vasculogenesis by mediating the production of extracellular matrix (ECM) macromolecules such as fibronectin and laminin [7]. Little is known about other factors which regulate vasculogenesis [1]. Angiogenesis has been more extensively studied. Fibronectin [8], laminin [9] and integrins [10] have been suggested to play major roles in ECM remodelling during the formation of new vascular channels. A soluble factor, vascular endothelial growth factor (VEGF), and its family of

receptors have been identified as one of the key regulators of angiogenesis [11]. During oxygen deprivation (hypoxia), hypoxia inducible factors (HIFs) cause the production of VEGF in ECs to promote angiogenesis to increase the supply of O₂ to neighbouring cells [12]. There are other factors which mediate vascular channel formation. These include the so-called fibroblasts growth factor (FGF) [13, 14], Delta-like ligand 4 (Dll4) and the Notch1 receptor [15, 16], as well as angiopoietins (Ang) and the Tie2 receptor family [17, 18]. The platelet-derived growth factor (PDGF) family is found to be responsible for the recruitment of pericytes and smooth muscle cells to provide structural integrity and contraction ability in the blood vessels [19, 20].

The crosstalk between EC metabolism and angiogenesis was highlighted by Fraisl et al. [21]. The extracellular environment presents stimuli which affect the metabolism of the cell, triggering various series of intracellular events which influence angiogenesis. For example, hypoxia increases anaerobic glycolysis in ECs [21]. H⁺ produced during glycolysis are pumped out of the cell to maintain intracellular pH, thereby causing extracellular pH to fall. In addition, HIFs are activated and nitric oxide (NO), a potent signalling molecule of angiogenesis, is produced. NO modulates the expression of VEGF and its receptors within the cell. VEGF is released and diffuses to the neighbouring ECs, where it elicits effects on ECs with VEGF receptors present on their cell membrane. These ECs respond to the VEGF gradient and migrate towards it, eventually forming new blood vessels [22].

Perturbation of angiogenesis disrupts the delicate balance of blood flow, metabolism, as well the structural integrity of tissues [2]. Angiogenesis is thus implicated in diverse pathophysiological conditions such as chronic wounds [23], rheumatoid arthritis [24] and cancers [25]. ECs in chronic wounds express high amounts of protease, which damage the ECM macromolecules, thereby delaying wound-healing [23]. In arthritis, the growth of blood vessels and nerves at the normally vessel-free joint leads to inflammation and chronic pain [24]. A protein called "oncogenic Ras" plays a critical role in tumour angiogenesis in cancers [26]. Ras can be activated by NO which is produced during hypoxia, as well as during tumour acidosis and signals through two independent pathways to upregulate VEGF production. In addition, Ras induces the expression of cyclooxygenase-2 in tumours, which induces the upregulation of pro-angiogenic factors such as FGF-2, PDGF and VEGF. The increase in these pro-angiogenic factors by tumour cells and/or host cells enhances the growth of new blood vessels and persistent angiogenesis results in defective blood vessels which are disorganised and leaky [6]. The morphology of these new blood vessels differ in different carcinomas, suggesting that different angiogenic factors were involved [2].

1.2 Motivations

Angiogenesis is a multistage process involving the formation of blood vessels from pre-existing ones. It is a highly complex, dynamic and heterogeneous process which involves the interaction of multiple pathways and its mechanisms may differ in various developmental stages in different organs [1]. Arterial, venous, lymphatic and haemogenic ECs, organ-specific ECs and circulating endothelial progenitor cells are some of the members of the heterogeneous EC population [1, 27], making the study of angiogenesis complicated. Studies on the mechanisms of angiogenesis have rarely been achieved in real-time, so the proposed mechanisms are highly speculative and inconclusive.

Angiogenesis is also implicated in diverse pathophysiological conditions such as chronic wounds [23], rheumatoid arthritis [24] and cancers [25]. Assessments of vascular density by magnetic resonance imaging [28], Doppler ultrasound [29], nuclear medicine imaging [30], perfusion computed tomography [31] and the immunodetection of circulating biomarkers VEGF and FGF-2 [32] are some of the techniques which have been applied to identify tumour angiogenesis *in vivo*. However, these approaches may not yield conclusive results due to the complexity of factors involved and variations in their temporal, heterogeneous release during different stages of a disease [2]. It is also difficult to determine if the upregulation of angiogenic factors causes tumour progression, or the other way around. Hence, these studies have provided little clue to the mechanisms for pathophysiological angiogenesis.

The extracellular environment of ECs and its intracellular mechanisms control the crosstalk between angiogenesis and EC metabolism [21]. External stimuli such as growth factors, changes in O₂ levels and drugs cause ECs to become activated, eliciting acute cellular responses which often involve a change in EC metabolism. Hence, level of signalling molecules such as NO and metabolic markers such as O₂, CO₂, glucose, lactate and pH are expected to become affected. However, these changes are under-investigated. Since there is crosstalk between EC metabolism and angiogenesis [21], comparing the metabolism of ECs during physiological conditions and under the influence of ECM macromolecules, soluble factors, drugs or pathophysiology can provide some insights on how these influences affect angiogenesis in order to elucidate their individual and combined effects. In particular, there is an urgent need to study these complex interactions in real-time in order to understand the short-term causes and effects on angiogenesis, which has been difficult to achieve using conventional biological techniques.

1.3 Objective and specific aims

Electrochemistry was selected as the experimental approach in order to meet the objective of acquiring real-time, quantitative data in a non-destructive manner to understand the effects of external stimuli on the metabolism of ECs and their relationships to angiogenesis. Nanomaterials were used to construct electrochemical biosensors suitable for the real-time, quantitative detection of extracellular metabolic markers, pH, O₂ and glucose, as well as the signalling

molecule, NO. This is the first step towards establishing the interactions of soluble factors, ECM macromolecules, EC metabolism and angiogenesis in real-time.

Different platforms were used for ECs studies, which were subjected to the availability of resources at each university. Hence, the specific aims were set for the experiments which could be performed at each university.

At NTU, the aim is to develop high performance novel graphene-based nanostructured materials for the detection of NO. This involves the use of a three-dimensional (3-D) graphene/ionic liquid (IL) nanocomposite to fabricate a NO biosensor. In addition, it includes the construction of a biomimetic graphene film for the cell culture of human umbilical vein endothelial cells (HUVECs) and detection of NO released by these cells under the stimulation of acetylcholine (Ach).

At Imperial College London, microelectrode array (MEA) chips are available and the aim is to use anodically electrodeposited iridium oxide film (AEIROF) as a miniature pH biosensor on a chip to study the acute changes in extracellular pH of porcine aortic endothelial cells (PAECs) cultured on fibronectin due to the stimulation of thrombin.

A collaborative effort between the two universities involves the development of O₂ and glucose biosensors based on a novel electropolymerised redox polymer film, with the intention of miniaturising these biosensors on a chip in future.

2 Review of literature

2.1 Key regulators of angiogenesis

Cells experiencing hypoxia need to restore their supply of O₂ in order maintain basic cell functions like cellular respiration. Most human cells, including endothelial cells (ECs), contain at least one type of hypoxia inducible factor (HIF), an intracellular O₂ sensor, which is activated during hypoxia [12]. In ECs, this leads to the production and release of vascular endothelial growth factor (VEGF) [33]. VEGF binds to its receptors on neighbouring ECs, resulting in intracellular cascades which trigger angiogenesis. ECs proliferate and migrate towards the source of the VEGF and pericytes and smooth muscles cells are recruited for the formation of new blood vessels for the restoration of O₂ supply [6].

2.1.1 Hypoxia inducible factors (HIFs)

The HIF transcription factors HIF-1 and HIF-2, each of which consists an α and β subunit, upregulate angiogenesis independently during hypoxia (Figure 2.1) [12]. Hydroxylation of the α subunit is catalysed by protein hydroxylase domain containing protein 2 (PHD-2) in the presence of O₂ [12]. The hydroxylated α subunit is transcriptionally inactive and ubiquitinated for proteolytic degradation [34].

Figure 2.1 Crosstalk exists between EC metabolism and angiogenesis (Chapter 2.2). The EC utilises glycolysis to generate its energy. Glucose is transported into the EC by a glucose transporter (GLUT) and is converted into pyruvate via glycolysis. The H^+ produced during glycolysis are pumped out of the cell by the $H^+/ATPase$, causing extracellular acidification. Pyruvate is then converted to lactate. The presence of lactate and the lack of O_2 during hypoxia causes PHD-2 to be inhibited, allowing HIF-1 and HIF-2 to enter the nucleus and bind to DNA. HIF-1 causes the expression of inducible nitric oxide synthase (iNOS) and VEGFA while HIF-2 results in the expression of endothelial nitric oxide synthase (eNOS) and VEGFR2. The enzymes eNOS and iNOS produce NO, which increases cell permeability. Low concentrations of NO inhibit cytochrome c oxidase (CcO) in the mitochondrion to allow the redistribution of O_2 within the EC.

During hypoxia, PHD-2 becomes inactive due to the lack of O₂. In addition, lactate produced during anaerobic respiration also inhibits PHD-2 (Chapter 2.2). Therefore, the α subunit of HIF is stabilised, with HIF-1 α requiring a higher degree of hypoxia than HIF-2 α to be stabilised [35]. The α subunit then dimerises with its β subunit. The stabilised HIF-1 enters the nucleus of the EC and binds to deoxyribonucleic acid (DNA), upregulating the expression of vascular endothelial growth factor A (VEGFA) [36] and inducible nitric oxide synthase (iNOS), which catalyses the reaction between O₂ and L-arginine, producing NO [37]. The binding of HIF-2 to DNA results in the expression of a VEGF receptor called VEGFR2 (Chapter 2.1.2) [38]. While inhibiting NO production via the expression of arginase (an enzyme which breaks down L-arginine), HIF-2 fosters the generation of NO through endothelial nitric oxide synthase (eNOS), which enhances EC permeability for VEGFA-induced angiogenesis [37].

The concentration of endothelial NO affects the stability of HIF-1 α and therefore cell signalling [12]. NO has higher affinity than O₂ towards cytochrome c oxidase (CcO), which is found in the mitochondrion [39]. During hypoxia, the concentration of endothelial NO increases and below 400 nM, NO inhibits CcO and therefore cellular respiration [39, 40]. This leads to redistribution of O₂ within the cell. As intracellular O₂ increases, the activity of O₂-dependent enzymes, including PHD-2, is restored and HIF-1 α is destabilised [41]. However, at higher concentrations of endothelial NO, such as that seen in pathophysiology, HIF-1 α is stabilised by NO via S-nitrosylation [42], irrespective of O₂ concentration [40].

2.1.2 Vascular endothelial growth factor (VEGF) family

The VEGF family consists VEGFA, VEGFB, VEGFC, VEGFD, VEGFE and placental growth factor, which are key regulators of angiogenesis [43]. VEGFA is a positive regulator of angiogenesis [6]. The gene encoding human VEGFA can undergo alternative splicing to give rise to 4 different isoforms, namely VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆, where the numbers in subscript indicate the amino acids forming each of the VEGF isoforms following the removal of the signal peptide [44, 45]. VEGF₁₈₉ and VEGF₂₀₆ are able to bind to heparin and are retained in the ECM. In contrast, VEGF₁₂₁ is not bound to the ECM as it does not bind to heparin and diffuses freely to interact with other cells. VEGF₁₆₅ has properties intermediate of the other types of VEGF, with some of it being secreted and the bulk of it remaining bound to the ECM [46].

2.1.2.1 Receptors of VEGF family

There are three different receptors on the surface of ECs, namely VEGFR1, VEGFR2 and VEGFR3, to which various members of the VEGF family can bind to trigger intracellular signalling cascades [11, 43]. VEGFR1 and VEGFR2 are involved in angiogenesis and interact with VEGFA while VEGFR3 participates in the formation of lymphatic vessels from pre-existing ones and interacts with VEGFC and VEGFD [6]. These receptors are members of the receptor tyrosine kinase superfamily and are transmembrane proteins. 7 immunoglobulin-like (antibody-like) units, Ig I to Ig VII, form the extracellular domain of the VEGFR and two tyrosine kinase fragments, TK-1 and TK-2, make up the intracellular

domain of VEGFR [47]. When the ligands bind to their corresponding receptors, the receptor dimerises and its intracellular domain possessing tyrosine kinase activity undergoes transphosphorylation or autophosphorylation [6].

VEGFR1 primarily functions as a negative regulator of the activity of VEGFA on VEGFR2 [6, 43]. The soluble form of VEGFR1 binds to VEGFA and hence reduces the binding of VEGFA to VEGFR2, which mediates most of the angiogenic effects of VEGFA on ECs [48]. Pigment epithelium-derived factor (PEDF) also inhibits angiogenesis by enhancing the γ -secretase activity of ECs, resulting in increased cleavage of the transmembrane domain of VEGFR1 [49].

VEGFR2 is the primary mediator of VEGFA's activity for angiogenesis and its activation stimulates EC proliferation, survival and migration [2, 6]. The activation of VEGFR2 elicits several intracellular signalling pathways (Figure 2.2). The protein kinase C (PKC)/extracellular receptor kinase (Erk) is initiated by the autophosphorylation of the tyrosine residues, Tyr1175, of the intracellular domain of VEGFR2 [50]. Erk translocates to the nucleus and phosphorylates c-Jun, a transcription factor. C-fos is transcribed and causes EC proliferation [2]. The activation of the VEGFR2-dependent phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) inhibits caspase activity by upregulating anti-apoptotic proteins A1 and Bcl-2, leading to cell survival [51]. EC migration is facilitated by the p38 mitogen-activated protein kinase (MAPK) pathway and focal adhesion kinase (FAK) which phosphorylates paxillin, causing cytoskeleton reorganisation [52]. In addition, the activation of GTP-binding protein Rac by phosphatidylinositol (3,4,5)P₃ (PIP₃) also aids in cell migration [53]. The activation of VEGFR2 also

stimulates eNOS to produce NO, which enhances EC proliferation and migration [54].

Figure 2.2 Binding of VEGFA to VEGFR2 triggers several intracellular signalling pathways in the EC, which produces NO, as well as proteins needed for cell survival, proliferation and migration.

2.1.3 Delta-like ligand 4 and Notch signalling

Notch receptors are a family of transmembrane, heterodimeric proteins [55]. Notch1 and Notch4 receptors and their transmembrane ligands, Jagged1 and 2 and Delta-like ligand 1 (Dll1) and Dll4, are expressed in vascular ECs [6]. The intercellular contact formed by the binding of the ligand to the extracellular domains of Notch receptor (Figure 2.3a) results in the proteolytic cleavage of the receptor at two sites [56]. Firstly, the extracellular domain of the Notch receptor is

cleaved and uptaken by an adjacent cell which also expresses the ligand (Figure 2.3b) [6]. Secondly, intracellular domain of the Notch receptor is cleaved and translocates to the nucleus, where it binds to DNA and alters gene expression [57].

Figure 2.3 (a) The EC expresses VEGFR2 when the intracellular domain of the Notch1 receptor is together with its extracellular domain at the cell membrane. The extracellular domain of the Notch1 receptor comes into contact with DII4 of an adjacent cell. (b) The Notch1 receptor is cleaved by an enzyme into its two domains. The extracellular domain is uptaken by the adjacent cell while the intracellular domain enters the nucleus and binds to DNA, inhibiting the expression of VEGFR2.

DII4 and the Notch1 receptor are involved in regulating the activation of tip cells, a group of ECs at the leading site of new blood vessels [58]. The tip cells express high levels of VEGFR2 and respond to the VEGFA gradient by sprouting

elongated structures called filopodia and growing towards it [59]. ECs expressing the Notch1 receptor are activated by Dll4 and the signalling cascade prevents the activation of these cells into tip cells by limiting the expression of VEGFR2 (Figure 2.3), causing a decrease in sensitivity towards VEGFA [58]. However, ECs in the growing capillary branch are stimulated to proliferate by the binding of VEGFA to VEGFR2, highlighting that VEGFA is able to independently control the migration of tip cells as well as the proliferation of ECs [59].

2.1.4 Angiopoietins (Ang) and Tie receptors

Angiopoietin1 (Ang1) and Ang2 are glycoproteins which are ligands of the Tie1 and Tie2 tyrosine kinase receptors [60]. These receptors are expressed exclusively in ECs. The role of the Tie1 receptor, while distinct from that of the Tie2 receptor [17], is still not well understood [60]. None of the ligands bind to the Tie1 receptor. It interacts with the Tie2 receptor and is activated when Ang1 binds to the Tie2 receptor [61]. Ang1 is expressed by mesenchymal cells and smooth muscle cells and it binds to and activates the Tie2 receptor expressed on ECs, leading to the recruitment of pericytes and giving structural support to the blood vessels (Figure 2.4a) [62]. Ang2 functions as a competitive inhibitor of Ang1 and binds to the Tie2 receptor, but does not result in the phosphorylation of the receptor unlike Ang1 [63]. However, Ang2 elicits different biological actions in the absence and presence of VEGFA, leading to vascular regression (cell death) (Figure 2.4b) and angiogenesis (cell survival, proliferation and migration) (Figure 2.4c), respectively, for vascular remodelling [62]. The inhibition of the Ang1/Tie2

signalling by Ang2 causes the destabilisation of capillaries without the recruitment of pericytes, which in turn makes the ECs hypersensitive to the pro-angiogenic effect of VEGFA [63].

Figure 2.4 (a) The binding of Ang1 to the Tie2 receptor causes the recruitment of pericytes. (b) In the absence of VEGFA, the binding of Ang2 to the Tie2 receptor causes cell death for vascular regression. (c) In the presence of VEGFA, the binding of Ang2 to the Tie2 receptor leads to cell survival, proliferation and migration for the growth of blood vessels.

2.1.5 Platelet-derived growth factor family (PDGF)

The PDGF family comprises four different PDGF strands which can form the homodimers PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD or heterodimer

PDGF-AB [64]. The PDGF receptors, homodimers PDGFR α and PDGFR β and heterodimer PDGFR $\alpha\beta$, are also tyrosine kinase receptors. PDGF-AA binds exclusively to PDGFR α while PDGF-BB binds with high affinity to PDGFR β but with lower affinity to PDGFR α and PDGFR $\alpha\beta$ [65]. PDGF-CC binds to both PDGFR α and PDGFR β while PDGF-DD binds to PDGFR $\alpha\beta$ only. During angiogenesis, the expression of PDGFB is high in tip cells and this recruits pericytes and vascular smooth muscle cells expressing PDGFR β on their surfaces to form the walls of new blood vessels and these blood vessels undergo maturation [59]. PDGFR β is not expressed in ECs so PDGFB does not have any effect on them [66].

2.2 Extracellular metabolic (O₂, CO₂, pH, glucose, lactate) and signalling (NO) markers of angiogenesis

O₂, CO₂, pH, glucose and lactate are key extracellular metabolic markers while NO is a signalling molecule of angiogenesis. Metabolic rate of the cell can be measured by the rate of uptake of reactants such as O₂ and glucose, or by the rate of release of products like CO₂ and lactate, which contribute to extracellular acidification [67]. Fraisl et al. had highlighted the crosstalk between EC metabolism and angiogenesis [21]. In normoxic healthy tissue, ECs exist as quiescent cells. However, they are also adapted with metabolic mechanisms which enable them to survive hypoxia during sprouting angiogenesis. During normoxia, resting ECs utilise primarily glycolysis instead of oxidative phosphorylation to generate adenosine triphosphate (ATP), allowing most of the O₂ to diffuse through

them to the surrounding tissue. A little of the pyruvate produced enters the mitochondrion and the bulk of it is converted to lactate, which inhibits PHD-2 (Figure 2.1) [12]. This causes HIFs to be active and results in a positive feedback loop that favours glycolysis since the intracellular O_2 concentration is low.

During hypoxia, migrating ECs also generate ATP through glycolysis and pyruvate does not enter the mitochondrion and is converted to lactate (Figure 2.1) [12]. HIFs are also active since PHD-2 is inhibited by both hypoxia and lactate, thereby greatly accelerating glycolysis. While oxidative phosphorylation of ECs are inhibited under hypoxia, they still consume O_2 as O_2 is needed for the production of NO, which increases cell permeability [37] and stimulates cell proliferation and migration [54] during VEGFA-induced angiogenesis. In addition, more H^+ are present intracellularly as a result of increased glycolysis. These are removed via the H^+ /ATPases to maintain intracellular pH, causing a decrease in extracellular pH [21]. Hence, we can see that the interplay of metabolic markers and NO is important in angiogenesis.

2.3 Electrochemical detection

2.3.1 Electrochemical cell

An electrochemical cell is typically made up of an ion-conducting electrolyte solution and three electrodes [68]. The electrolyte solution e.g. aqueous KCl has high ionic conductivity which minimises solution resistance. The three electrodes are called the reference electrode, working electrode and counter electrode. The reference electrode has constant potential and can be a Ag|AgCl or saturated calomel electrode. The electrochemical reaction occurs at the working electrode-solution interface. The counter electrode allows the current to flow from the working electrode but does not interfere with the reaction at the working electrode.

2.3.2 Mass transport

Mass transport of the electroactive species (dissolved in the electrolyte solution) to the working electrode's surface can occur by three different ways or a combination of these ways [69]. These include diffusion (movement of the species down its concentration gradient), migration (movement of charged species due to an electric field) and convection (movement of species due to a density gradient or stirring). The one-dimensional flux, $J(x)$ ($\text{mol cm}^{-2} \text{s}^{-1}$), of an electroactive species to the electrode's surface can be described by the Nernst-Planck equation [69] as

$$J(x) = -D \frac{\partial C(x)}{\partial x} - \frac{zF}{RT} DC \frac{\partial \phi(x)}{\partial x} + Cv(x)$$

where D ($\text{cm}^2 \text{s}^{-1}$) is the diffusion coefficient of the electroactive species, $C(x)$ (mol cm^{-3}) is its concentration, z is its charge and $v(x)$ (cm s^{-1}) is the rate of movement of the volume element in the solution. R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) and F is Faraday's constant (96485 C mol^{-1}). $\frac{\partial C(x)}{\partial x}$ is the concentration gradient and $\frac{\partial \phi(x)}{\partial x}$ is the electrical potential gradient. The first term of the equation describes diffusion of the electroactive species, the second term the migration of the charged electroactive species, and the third term the convection of the solution. Migration of charged electroactive species in an electric field is prevented by using a dilute concentration of electroactive species e.g. $10 \text{ mM } [\text{Fe}(\text{CN})_6]^{4-}$ and a high concentration of electrolyte solution e.g. 1 M aqueous KCl solution.

2.3.3 Steps in a simple electrochemical reaction

For a simple electrochemical reduction reaction, we begin with a solution of inert electrolyte containing an electroactive species (O) in oxidised state. The working electrode is held at a potential sufficiently negative from the equilibrium potential to reduce O . O diffuses from the bulk solution to the working electrode. Electron transfer occurs where the number of e^- transferred per mole of O is given by n as O is reduced to R . R diffuses from the surface of the electrode and into the bulk solution.

The diffusive flux, J ($\text{mol cm}^{-2} \text{s}^{-1}$), of O to the electrode surface is related to the current, i (A), where n is the number of e^- transferred, F is Faraday's constant (96485 C mol^{-1}) and A is the area of the electrode (cm^2) [70, 71].

$$J = \frac{i}{nFA}$$

When the species O and R are near the electrode surface, they can adsorb on the electrode surface and/or react with species before or after electron transfer.

2.3.4 Nernst equation

The Nernst equation [69] describes the equilibrium potential, E (V), of an electrochemical cell with fast kinetics and reversible reaction at the surface of the working electrode as

$$E = E^0 + \frac{RT}{nF} \ln \frac{a_O}{a_R}$$

where E^0 (V) is the standard potential, R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the temperature (K), F is Faraday's constant (96485 C mol^{-1}), n is the number of e^- transferred, a_O (mol L^{-1}) is the activity of the oxidised species and a_R (mol L^{-1}) is the activity of the reduced species.

If the activity coefficients of both the oxidised and reduced species are unity, the Nernst equation can be simplified as

$$E = E^{0'} + \frac{RT}{nF} \ln \frac{c_O}{c_R}$$

where $E^{0'}$ is the formal potential and c_R and c_O are the bulk concentration of the reduced and oxidised species (mol L^{-1}), respectively.

2.3.5 Cyclic voltammetry

Cyclic voltammetry (CV) is an electroanalytical technique in which the working electrode potential is ramped (Figure 2.5a) and the resulting current is measured and current is plotted against the potential (Figure 2.5b). The magnitude of the electron transfer current rises as potential initially increases as the rate is exponentially-dependent on the potential and reaches a maximum at the peak potential, as current is limited by mass transport and electron transfer, the concentration gradient becomes steeper [72]. The magnitude of the anodic peak current is $i_{p,a}$ and that of the cathodic peak current is $i_{p,c}$. The magnitude of the current decreases beyond the peak potential and attains a concentration gradient that is constant at high overpotential due to natural convection [72]. The scan rate (ν) also affects the current response, with higher ν resulting in a higher current due to greater capacitive charging and increase of redox current proportional to $\nu^{1/2}$ since less time is available for the surface concentrations as a result of a complex function of current to time-dependent change of potential [72].

Figure 2.5 (a) A potential ramp with $\nu = 0.05 \text{ Vs}^{-1}$ and (b) the resulting CV curve showing current response/ μA against potential/V for 10 mM of $[\text{Fe}(\text{CN})_6]^{4-}$ in 1 M aqueous KCl solution at a Au electrode. The experimental value of ΔE_p is 0.065V.

For a redox-active species such as $[\text{Fe}(\text{CN})_6]^{4-/3-}$ with reversible 1 e^- transfer, a pair of peaks is observed in the CV (Figure 2.5b). On the forward scan from -0.1V to 0.7V vs $\text{Ag}|\text{AgCl}$, an anodic peak at $E_{p,a}$ corresponding to the oxidation of $[\text{Fe}(\text{CN})_6]^{4-}$ to $[\text{Fe}(\text{CN})_6]^{3-}$ is observed and on the reverse scan from 0.7V to -0.1V , a cathodic peak at $E_{p,c}$ for the reduction of $[\text{Fe}(\text{CN})_6]^{3-}$ to $[\text{Fe}(\text{CN})_6]^{4-}$ is seen. The peak separation (ΔE_p) of the reversible reaction is given by $E_{p,a} - E_{p,c}$ or $\frac{0.059\text{V}}{n}$ at 298 K , where n is the number of e^- transferred. The formal potential ($E^{0'}$) can be estimated as $\frac{E_{p,a} + E_{p,c}}{2}$ if the diffusion coefficients of the oxidised and reduced species are similar. In addition, $\left| \frac{i_{p,a}}{i_{p,c}} \right| = 1$ and $E_{p,a}$ and $E_{p,c}$ are independent of scan rate. The Randles-Sevcik equation [73] describes the anodic peak current, $i_{p,a}$ (A), of a diffusion-controlled, reversible reaction at a planar disk electrode as

$$i_{p,a} = 2.69 \times 10^5 A c_R n^{3/2} D_R^{1/2} \nu^{1/2}$$

where A is the area of the electrode (cm^2), c_R is the bulk concentration of the reduced species (mol cm^{-3}), n is the number of e^- transferred, D_R is the diffusion coefficient of the reduced species ($\text{cm}^2 \text{s}^{-1}$) and ν is the scan rate (V s^{-1}).

However, for a quasi-reversible or irreversible reaction, electron transfer is sluggish [73]. A larger overpotential needs to be applied to obtain the same rate of electron transfer, causing the peaks to broaden and ΔE_p to be larger than $\frac{0.059\text{V}}{n}$ at 298 K , where n is the number of e^- transferred [69]. In addition, $\left| \frac{i_{p,a}}{i_{p,c}} \right| \neq 1$. The equation for the anodic peak current (A) becomes

$$i_{p,a} = 2.99 \times 10^5 A c_R n(\alpha n_a)^{1/2} D_R^{1/2} \nu^{1/2}$$

where α is the transfer coefficient and n_a is the number of e^- involved in the rate-limiting charge transfer step. For a totally irreversible reaction, the cathodic peak is not present. $E_{p,a}$ will shift positively by $\frac{1.5RT}{\alpha nF}$ or $\frac{30}{\alpha}$ mV at 298 K per ten-fold increase in ν [74].

2.3.6 Amperometry

In amperometry, the working electrode is stepped from a potential (E_i) of zero faradaic current to a potential (E_s) where the kinetic of charge transfer is so fast that the surface concentration of the electroactive species O is effectively zero and the rate of reaction becomes limited by mass transport (Figure 2.6) [69]. The potential is either maintained at E_s or stepped to a final potential (E_f) as in double potential step chronoamperometry [75]. The resulting mass transport-limited current (i_l) is given by

$$i_l = nFAmC_o = \frac{nFAD_oC_o}{\delta}$$

where n is the number of e^- involved in the rate-limiting charge transfer step, F is Faraday's constant (96485 C mol⁻¹), A is the area of the electrode (cm²), m is the mass transport coefficient (cm s⁻¹), C_o is the bulk concentration of the electroactive species O (mol cm⁻³), D_o is the diffusion coefficient of the electroactive species O (cm² s⁻¹) and δ (cm) is the thickness of the Nernst diffusion layer.

Figure 2.6 (a) The Au microelectrode was stepped from $E_i = 0.75\text{ V}$ to $E_s = 0\text{ V}$ to $E_f = 0.75\text{ V}$. (b) The corresponding current response of the electrode to the applied potential steps in 10 mM ferric ammonium sulphate in 0.5 M aqueous HClO_4 solution.

In an unstirred solution, the current measured at the planar disk electrode decays over time according to the Cottrell equation [69]

$$i(t) = \frac{nFAD_o^{1/2}C_o}{\pi^{1/2}t^{1/2}}$$

where t is time in seconds.

2.3.7 Electrochemical impedance spectroscopy

In electrochemical impedance spectroscopy (EIS), a small amplitude, sinusoidal potential $E = \Delta E \sin 2\pi ft$ is applied over a range of frequencies (f), typically from 10^{-2} to 10^4 Hz, to the working electrode and the resulting current $i = \Delta i \sin (2\pi ft + \phi)$, where ϕ is the phase shift, is measured [68]. The impedance $Z = \frac{E}{i}$ is a complex quantity and the imaginary part of Z is plotted against its real part in an Argand diagram.

A simple electrochemical reaction $O + ne^- \leftrightarrow R$ can be modelled as a circuit (Figure 2.7a) consisting an uncompensated resistance (R_u), charge transfer resistance (R_{ct}), Warburg impedance and double layer capacitance (C_{dl}) and its Argand diagram (Figure 2.7b) is shown on the next page

Figure 2.7 (a) The equivalent circuit for a simple electrochemical reaction $\mathbf{O} + ne^- \leftrightarrow \mathbf{R}$. (b) The corresponding Argand diagram.

A semi-circle is observed at high frequencies where the kinetics of electron transfer is rate-limiting and a straight line is seen at low frequencies where the reaction is diffusion-limited. $R_u (\Omega)$ is given by the intersection of the curve with the Z_{real} axis at high frequency and $R_{ct} (\Omega)$ is the diameter of the semicircle. $C_{dl} (F)$ is obtained from the maximum of the semicircle where

$$f = \frac{1}{2\pi R_{ct} C_{dl}}$$

The exchange current density, j_0 ($A\ cm^{-2}$), can be calculated by the equation

$$j_0 = \frac{RT}{nFR_{ct}}$$

where R is the gas constant ($8.314\ J\ mol^{-1}\ K^{-1}$), T is the temperature (K), n is the number of e^- involved in the rate-limiting charge transfer step, F is Faraday's constant ($96485\ C\ mol^{-1}$).

2.4 Nanomaterials for electrochemical biosensors

Electrochemical biosensors function by the principles of electrochemistry and the specificity of biological interactions. The biotransducer catalyses an electrode reaction involving the analyte, leading to a quantitative electrical response proportional to the concentration of the analyte, thereby allowing the real-time detection of the biological event [73]. Electrochemical biosensors are divided into two categories – biocatalytic devices which employ immobilised enzymes, cells or tissues as their biocomponents and affinity sensors which utilise antibodies,

membrane receptors or nucleic acids [73]. This dissertation focuses on the first type of biosensors, which are discussed in greater detail in the following sections.

Conventional electrodes are made up of bulk material such as Pt, Au or glassy carbon (GC). However, the rise of nanomaterials in recent years has opened the exploration of novel electrode materials with superior performances with high sensitivities, low detection limits and excellent selectivities. These properties are attributed to nanomaterials having high surface area-to-volume ratio and tunable electron transport which promote direct and efficient electron transfer [76]. Surface modification of nanomaterials alters their functional groups, which can improve their properties and/or allow them to be combined with other nanomaterials. Combining different nanomaterials produces nanocomposites with synergistic effects and exciting new properties and enhanced performances not found with individual nanomaterials alone [77]. Nanomaterials utilised in this dissertation, namely graphene, iridium oxide (IrOx) and poly(methylene blue) (PMB), are introduced along with their properties and preparation methods below.

2.4.1 Graphene - properties and preparation methods

Graphene is a unique two-dimensional (2-D) carbon nanomaterial, which has garnered tremendous interest over the past few years. Graphene use has seen exponential growth in its applications related to electrochemical biosensors. A single layer of sp^2 -bonded carbon atoms is arranged in a honeycomb lattice [78]. Graphene boasts a high surface area-to-volume ratio with exceptionally high electron mobility and ballistic electron transport [79]. It holds the potential to

outperform other carbon nanomaterials such as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). Already, the advantages of graphene have been demonstrated in its excellent electrocatalytic activity and electrical conductivity, which are superior to that of carbon nanotubes (CNTs) [80, 81]. Furthermore, graphene has a wide electrochemical window and low charge transfer resistance as compared to graphite or GC electrodes [80, 82]. These advantages of graphene have been exploited for enzymatic glucose [83] and H_2O_2 [84] biosensors by achieving direct electron transfer (DET) with enzymes. In addition, graphene has also been applied for the electrocatalytic sensing of H_2O_2 [85] and dopamine [86].

The properties of different forms of graphene vary according to the preparation method. Graphene is commonly prepared by two approaches [87]. In the first approach, graphene is exfoliated by mechanical cleavage from graphite or reduction of exfoliated graphene oxide (GO). The second approach involves growing graphene directly on a surface by using chemical vapour deposition (CVD) or epitaxial growth. Mechanical cleavage of graphite gives pristine graphene but suffers from low throughput and difficulty in transferring the single-layer graphene [88].

Reduction of GO is the most attractive method as it is cost effective and suitable for mass production of graphene. GO disperses well in water and many other solvents and can be reduced via chemical [89], electrochemical [90], photochemical [91] or thermal methods [92], which offer opportunities for surface functionalisation of the reduced graphene oxide (rGO). rGO contains many defects

in the carbon lattice, has numerous reactive sites at its edges and is more electrochemically active than pristine graphene [93]. CVD allows large graphene films, doped with heteroatoms such as nitrogen or boron, to be grown on nickel or copper substrates [94].

CVD-grown graphene contains defects and impurities so its properties also differ from pristine graphene. Epitaxially-grown graphene is formed by the high temperature decomposition of silicon carbide [95]. However, these two methods have drawbacks of low throughput and difficulty in transferring the graphene film to another substrate [96].

2.4.2 Iridium oxide - properties and preparation methods

IrOx, a semi-conductor metal oxide, is electrochromic and displays Nernstian to super-Nernstian responses to pH [97]. The electrochromism of IrOx arises from changes in its oxidation state, from colourless in Ir(III) to blue in Ir(IV) [98] and this property has found applications in IrOx film-based display devices [99] and optical storage devices [100]. The reduction of IrOx film is H⁺-dependent, with anhydrous films demonstrating Nernstian responses while hydrated films exhibiting super-Nernstian responses to pH [97]. The Nernstian response to pH is described by the following equation for the Ir(IV) to Ir(III) redox transition [101],

where one electron is transferred per proton. The super-Nernstian response to pH can be explained by complex equilibrium shown in the following equation [102],

where $0.12 < x < 0.25$ according to the degree of film hydration and gives -0.073 V pH^{-1} to -0.081 V pH^{-1} according to the equation [102],

$$E = E_{M,MO,H^+}^0 - 2.303 \frac{RT}{nF} \text{pH}$$

The potential (E) of the metal/metal oxide electrode is the result of the equilibrium between the sparingly soluble oxide and its saturated solution. E_{M,MO,H^+}^0 is the standard potential which includes the ionisation product of water and solubility product of the metal oxide. R is the gas constant ($8.3144 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the temperature in Kelvin, F is Faraday's constant (96485 C mol^{-1}) and n is the number of electrons transferred.

In addition to its sensitivity to pH, its stability in aqueous solution, fast response time and low impedance make IrOx film an appealing nanomaterial for the fabrication of potentiometric pH sensors [102, 103]. The response of the film to pH is affected by its preparation method and resulting degree of film hydration. IrOx films can be prepared by electrochemical growth in aqueous H_2SO_4 solution [104], electrodeposition from a solution of iridium oxalate [99, 105], sputtered deposition [103] and thermal treatment [102]. The first two methods yield hydrated films while the latter two methods result in anhydrous films. In the first method, an Ir wire is placed in aqueous H_2SO_4 solution and oxidised by potential cycling and the amorphous film formed is known as anodic IrOx film (AIROF) [104]. Electrodeposition of IrOx is an attractive approach for microelectrode array

modification and it is cheaper than the first method as the Ir wire is expensive. AEIROF is formed by anodically electrodepositing the IrOx on the electrode by applying a constant current [99] or potential [105] to a solution of iridium oxalate. Sputtered IrOx film (SIROF) is prepared by reactive sputtering from an Ir target using oxygen plasma onto the electrode [103]. However, the Ir target is expensive. Thermal preparation of IrOx film can be done by oxidising an Ir wire by immersion in KNO₃ melt at 420 °C [106] or wetting it with aqueous NaOH solution and then oxidising it at 800 °C [102]. Anhydrous IrOx films need to be wetted by soaking in de-ionised (DI) water for several days before use [102]. Chloride is known to form complexes with Ir(IV), leading to dissolution of the IrOx. This dissolution can be reduced by coating the IrOx with Nafion. Although Nafion does not prevent chloride access to the electrode, it can entrap the chloroiridate complex anions and decrease mass transport [102].

2.4.3 Poly(methylene blue) (PMB) - properties and preparation methods

Phenothiazine contains a tricyclic ring aromatic with sulphur and nitrogen atoms and its derivatives have substituents at either the 2 and 10 or 3 and 7 positions [107]. Methylene blue (MB⁺) is a phenothiazine derivative with substituents at the 3 and 7 positions (Figure 2.8a). MB⁺ is electrochromic and appears blue in its oxidised state, hence its name. MB⁺ can undergo a series of electron transfers and proton transfers depending on the pH to give leuco methylene blue (LMB), which is colourless (Figure 2.8b) [108, 109]. Other common phenothiazine derivatives such as new methylene blue, methylene green,

azur A, toluidine blue and thionine [110, 111] are also electroactive and water-soluble [112]. They can be adsorbed or immobilised onto the electrode to function as redox mediators for enzymatic electrochemical biosensors [113-116].

Figure 2.8 (a) Chemical structure of MB^+ and (b) its electrochemical reduction.

However, these mediators tend to leach over time due to their solubility in water, resulting in poor biosensor performance. Electropolymerisation of a phenothiazine derivative yields a conductive polymer film and this improves the stability of the mediator and biosensor. The electropolymerisation can occur at physiological ranges of pH, which is important for maintaining the activity of enzymes. It is attractive to immobilise the enzyme together with the polymer onto the electrode via one-step electropolymerisation in solution [117].

PMB is electroactive and its electrochemical reaction is shown in Figure 2.9 [118]. It exhibits diffusion-limited behaviour due to the rate-limiting diffusion of

counterions into or out of the film [119]. In addition to functioning as a redox mediator in glucose biosensors [117], PMB is found to improve the electrocatalytic oxidation of nicotinamide adenine dinucleotide (NADH) [117], ascorbic acid, dopamine and epinephrine [120], as well as reduction of O₂ [121] and has recently been used to achieve the DET of glucose oxidase (GOD) [122].

Figure 2.9 Electrochemical reduction of poly(MB⁺) to poly(LMB).

PMB is prepared by the electro-oxidation of MB⁺ monomers by an electrode in solution containing a catalyst such as SO₄²⁻ [123]. At high overpotential, the MB⁺ monomers are irreversibly oxidised to radical cations, which combine as oligomeric species and precipitate, forming PMB on the electrode [118].

The growth of the film depends on six factors. First, the type of electrode material affects the electropolymerisation. All conditions being the same, the CV of electropolymerising MB⁺ on Pt foil and GC electrode was reported to be different [124]. Second, increased adsorption of MB⁺ at metal electrode occurred at higher MB⁺ concentration, and upon reduction, resulted in the formation of more

LMB, which has poor conductivity. Hence, the resulting film is less conductive than that formed with lower concentrations of MB^+ [125]. Third, the choice of buffer and supporting electrolyte is important to prevent MB^+ precipitation before electropolymerisation. ClO_4^- and BF_4^- are not used since they cause MB^+ to precipitate [126]. Suitable buffers include phosphate buffered saline (PBS) [117, 118, 123, 125] and borate buffered saline [117, 124, 126, 127] and the supporting electrolytes include aqueous KCl [117, 124, 126] and Na_2SO_4 [123, 125]. Fourth, the pH of the solution affects the growth rate of PMB, which was found to increase in basic pH [126]. Fifth, the range of potential applied affects the surface roughness of the PMB film [127]. Smooth films are formed when the film is not over-oxidised. However, over-oxidation results in rough films, with MB^+ monomers adsorbing onto the film. Sixth, increasing the number of CV cycles enhances the thickness of the film, which affects the film's capability to exchange ions during the redox reaction and makes it sensitive to the size, concentration and degree of hydration of the anions in solution [125].

2.5 Development of electrochemical biosensors

2.5.1 Nitric oxide biosensors

NO was named 'Molecule of the Year' in 1992 and since then, its biological role as a signalling molecule has been extensively studied. It plays an important role in the regulation of cell function of the nervous, vascular and immune systems [128], such as acting as a neurotransmitter [129], modulating vasodilation [130] and the action of macrophages [131], respectively. It is also

implicated in the pathogenesis of these systems as seen in tumour angiogenesis [132] and Parkinson's disease [133]. Electrochemical sensing offers the advantages of simplicity and quantitative real-time detection of NO *in vitro* [134-139], *in situ* [140-142] and *in vivo* [143, 144] with excellent sensitivity through the use of nanomaterials and nanocomposites, as well as selectivity by using membranes such as Nafion which could prevent interfering species from reacting at the electrode [145].

The earliest NO sensor was a Clark type NO electrode by Shibuki in 1990, which detected the direct electro-oxidation of NO on the electrode surface [141]. The electrode comprised a Pt working electrode and Ag counter electrode inserted into a glass micropipette which was filled with aqueous NaCl and HCl solutions and sealed with a chloroprene rubber membrane [146]. However, its sensitivity was low and the electrode was fragile [146]. Improvements were made to this amperometric NO sensor by incorporating a Pt wire disk as the working electrode and a Ag|AgCl reference electrode within a Faraday-shielded stainless steel sleeve and this gave rise to the first commercial NO sensor in 1992 [147]. Meanwhile, surface-modified carbon fibre NO microelectrodes were explored, such as that reported by Malinski and Taha in 1992. Their electrode was coated with tetrakis porphyrin and Nafion [145], which allows the elimination of interfering species such as dopamine and ascorbic acid [146]. Recently, the use of nanomaterials and nanocomposites has tremendously enhanced the performances of NO sensors. These include SWCNTs/room temperature IL composite gel [134], MWCNTs [135] and myoglobin/MWNCTs [136] and Au nanoparticles (NPs) immobilised on

various substrates [137-139], which exhibit nanomolar limits of detection, high sensitivities above $100 \text{ nA } \mu\text{M}^{-1}$ and fast response times.

2.5.2 O_2 biosensors

O_2 is critical for cell survival as it functions as the final electron acceptor in the electron transport chain, which creates a H^+ gradient that is needed to drive the oxidative phosphorylation of ATP in the mitochondria during aerobic cellular respiration [3]. When the cell is lacking in O_2 , it switches over to anaerobic cellular respiration. However, prolonged O_2 deprivation will kill the cell and the measurement of dissolved O_2 or O_2 tension is clinically important for detecting and monitoring ischemia [148], particularly for patients with traumatic brain injuries [149].

The first amperometric O_2 biosensor was developed by Clark in 1954, which came to be known as the Clark electrode [150]. It consists a Pt microelectrode and a Ag|AgCl reference electrode in a chamber sealed with a Teflon membrane and containing aqueous KCl solution. O_2 in solution diffuses across the membrane and is electrochemically reduced at the Pt microelectrode. The use of nanomaterials and nanocomposites have tremendously improved the electrocatalytic detection of dissolved O_2 . Metallophthalocyanines, metalloporphyrins and their nanocomposites [151, 152] are popular choices for O_2 reduction despite difficulty in electropolymerising them [153], since they often demonstrate 4 e^- transfer for the reduction of dissolved O_2 [151, 152]. O_2 biosensors based on these materials include layer-by-layer (LBL) iron(III) tetra-(N-

methyl-4-pyridyl)-porphyrin (FeT4MPyP) and iron tetrasulfonated phthalocyanine (TeTsPc) [153], SiO₂/SnO₂/Mn(II) phthalocyanines [154], mesoporous SiO₂/C/cobalt(II) phthalocyanine (Si/C/CoPc) [155] and cobalt tetrasulphonated phthalocyanine/ poly(L-lysine) (PLL) [156]. Anthraquinone-derivative functionalised MWCNTs nanowires (HOOC-2-AQ/AMWCNTs) [157], indigotetrasulfonate/PLL [158] and PMB-doped SiO₂ NPs [121] have also been used as O₂ biosensors. These nanocomposite-based O₂ biosensors have achieved detection limits on the order of 10 µg L⁻¹, linear ranges over a few mg L⁻¹ and excellent sensitivities on the order of µA mg⁻¹ L cm⁻².

2.5.3 pH biosensors

pH is an important indicator of cell metabolism. For example, H⁺ are formed during glycolysis and the Krebs cycle [3]. Enhanced glycolysis increases intracellular H⁺ concentration and the excess H⁺ are removed by the H⁺/ATPase, causing extracellular pH to fall [21]. Increased lactate and CO₂ production by the cell also lead to a decrease in extracellular pH [67]. The secretion of H⁺, such as that by the parietal cells of the gastric glands, is important in maintaining the acidity of the stomach [105]. The levels of extracellular and intracellular pH are therefore important indicators of cellular metabolic rates [67]. The measurement of pH is also critical in diagnosing diseases such as gastroesophageal reflux disease [159] and myocardial ischemia [160].

The traditional pH sensor is the glass electrode [161]. A Galvanic potential difference arises at the glass-solution phase boundary and is a function of the

proton activity of the solution, allowing pH to be potentiometrically measured [70]. However, it suffers from slow response time, has high impedance and is mechanically fragile [102]. Metal-metal oxide electrodes such as the Ir/IrO_x film electrode [102], Au/IrO_x film microelectrode [105, 162, 163] and tungsten-tungsten oxide film nanoelectrode [164] show improved stability, fast response time and low impedance towards *in vivo* [162] and *in situ* pH measurements [102, 105, 163, 164]. Notably, hydrous IrO_x films exhibit super-Nernstian response of -0.073 V pH⁻¹ to -0.081 V pH⁻¹ and therefore high sensitivity to pH [97]. Together with their ease of preparation through electrochemical growth or electrodeposition, hydrous IrO_x film remains an attractive nanomaterial for potentiometric pH sensors. Other nanomaterials such as processible poly(aniline) [165], carboxyl group-functionalised SWCNTs [166] and solution-gated epitaxial graphene [167] have also shown excellent performances for pH measurements.

2.5.4 Glucose biosensors

Glucose is the key biomolecule involved in glycolysis, which is needed to drive the generation of ATP in the cell [3]. Hence, the rate of glucose uptake is indicative of the energy demands of the cell. Diabetic patients suffer from hyperglycemia and the development of glucose biosensors has been centred around the need for these patients to monitor their blood glucose concentration [168].

The first generation enzymatic glucose biosensor was proposed by Clark and Lyons in 1962 by entrapping a thin layer of glucose oxidase (GOD) over an O₂ electrode using a dialysis membrane, which would detect glucose levels by

measuring the consumption of O₂. [169]. The reaction of glucose with O₂ catalysed by GOD is as follows [170],

The design of the glucose biosensor was improved by Updike and Hicks, who added a second O₂ electrode as a control [171]. Guilbault and Lubrano developed a glucose biosensor based on the amperometric detection of H₂O₂ liberated by the reaction of glucose and O₂ catalysed by GOD [172]. However, the sensitivity of the first generation glucose sensor is low due to the insulative shell of GOD, which impedes electron transfer from its redox centre to the electrode surface [73]. Since the 1990s, the use of electron mediators such as ferrocene derivatives and osmium compounds in second generation enzymatic glucose biosensors improved the transfer of electrons to the electrode and enhanced the sensitivity of these biosensors [73, 168]. However, mediators often leach out from the electrode over time.

Third generation enzymatic glucose biosensors no longer require electron mediators and instead capitalise on DET from the flavin adenine dinucleotide (FAD) redox centre of GOD to the electrode surface through the use of nanomaterials and nanocomposites with high electrical conductivity and large surface area-to-volume ratio. These include IL/mesoporous carbon/GOD [173], chemically rGO/GOD [82], nanostructured TiO₂/GOD [174], carbon decorated-zinc oxide nanowire array/GOD [175], NiO hollow spheres/poly(3,4-ethylenedioxythiophene)/GOD [176] and graphene/Au NPs/GOD/chitosan [177].

The recent development of non-enzymatic glucose biosensors have involved the use of Cu nanowires [178], PtAu-MnO₂ binary nanocomposite [179], free-standing SWCNT film [180], Pt-Pb alloy NPs/MWCNTs [181], PtNi NPs/graphene [182] and 3-D graphene/cobalt oxide [183] for the direct oxidation of glucose. These two classes of glucose biosensors display impressive performances with detection limits on the micromolar to nanomolar scale, millimolar linear ranges and sensitivities with an order of 10 to 100 $\mu\text{A mM}^{-1} \text{cm}^{-2}$, which can be practically applied for the detection of glucose levels of diabetic patients *in vitro*.

3 Highly sensitive nitric oxide sensing using three-dimensional graphene/ionic liquid nanocomposite†

3.1 Abstract A nanocomposite gel with a uniform porous structure and well-controlled compositions prepared by mixing 3-D graphene material with an IL, 1-butyl-3-methylimidazolium hexafluorophosphate, is used for NO detection. It shows a fast response of less than 4 seconds, an excellent sensitivity of $11.2 \mu\text{A cm}^{-2} (\mu\text{mol/L})^{-1}$ and an extremely low detection limit of 0.75 nM with a signal-to-noise ratio of 3 ($S/N = 3$), a performance superior to that of reported works based on CNTs and Au NPs. The high sensitivity is attributed to the large electroactive surface area of the graphene gel nanocomposite towards NO oxidation. The electrochemical behaviour of the gel nanocomposite is investigated and explained.

† This paper is cited as reference [184]. Reprinted (adapted) with permission from S.R. Ng, C.X. Guo, C.M. Li., Highly sensitive nitric oxide sensing using three-dimensional graphene/ionic liquid nanocomposite. *Electroanalysis*, 23(2): 442-448. Copyright (2011) WILEY-VCH.

3.2 Introduction

NO is a biological signalling molecule and plays an important role in the regulation of angiogenesis. During hypoxia, the production of NO is increased in the endothelial cells (ECs) (Chapter 2.1.1) [37]. It binds to cytochrome c oxidase (CcO) in the mitochondrion to inhibit cellular respiration to allow O₂ redistribution within the cell and also enhances cell permeability [39, 40]. The binding of vascular endothelial growth factor A (VEGFA) to its receptor on the cell membrane triggers intracellular events which lead to increased NO production that stimulate the cell to proliferate and migrate in response the VEGFA gradient [54].

The levels of NO are critical for the proper functions of ECs while abnormal levels of NO are associated with diseases such as cancer [185] and Parkinson's disease [133]. Hence, the sensitive detection of NO is imperative to understanding its spatial and temporal release profile to elucidate both its physiological and pathological actions, as well as to provide a platform for practical applications such as clinical diagnosis. Indirect techniques such as fluorescent probes [186, 187], X-ray photoelectron spectroscopy [188] and reverse-phase high performance liquid chromatography [189] have been developed to detect NO. However, these methods are complicated and provide little clue to the real-time release profile of NO. In contrast, electrochemical sensing possesses superior simplicity and allows the direct quantitative assessment of NO [190-195].

Since the first NO electrochemical sensor fabricated by Shibuki in 1990 [196], various approaches have been explored to enhance the performances and robustness of the amperometric NO sensors. These include the tetraakis porphyrin

and Nafion-modified carbon fibre electrode [197] and a platinized platinum anode, which achieve nanomolar limits of detection [198]. Recently, functional nanomaterials have opened up a window of opportunity in electrochemistry due to their high surface area-to-volume ratio and tunable electron transport which promote efficient electron transfer [199-201]. Nanomaterials such as SWCNTs [202], MWCNTs [136, 203, 204], IL [205] and Au NPs [206-208] have been widely used in electrochemical NO sensors to enhance sensitivities and push down detection limits. However, the real-time detection of NO remains a challenge due to the trace level of the endogenous NO, and more critically, its short half-life of 5 seconds in the presence of O₂ [190, 209, 210]. Therefore, the development of NO sensors with high sensitivity and fast response is still greatly needed.

Graphene, a single layer of carbon atoms arranged in a honeycomb lattice, has attracted great attention lately [211, 212]. It is highly advantageous for use in electrochemical sensing due to its large 2-D electrochemically active surface area which maximises exposure to analytes and boosts sensitivity [211, 213], as well as its exceptionally high carrier mobility which promotes electrical conductivity [79, 214]. Graphene also has a wide electrochemical window for detection, exhibits low charge transfer resistance [82, 215] and demonstrates excellent electrocatalytic activity [215, 216]. IL is a liquid electrolyte at temperatures below 100 °C which consists a small anion and a bulky organic cation such as imidazolium and pyridinium [217]. IL also possesses an intrinsically high ionic conductivity and electrochemical stability [202, 217-219], which makes it a candidate for incorporation into electrochemical sensors for the detection of gaseous analytes.

Very recently, IL has been extensively used as a binder to prepare carbon paste electrodes for broad electrochemical applications [220-225]. IL composite electrodes fabricated from SWCNTs [202], MWCNTs [226] and mesoporous carbon [218] have been developed and their electrocatalytic behaviours have been demonstrated. However, the use of graphene with IL for the electrochemical detection of NO has not been explored until now.

In this work, a novel carbon paste electrode was prepared by combining a 3-D graphene material and an IL, 1-butyl-3-methylimidazolium hexafluorophosphate [217, 218]. The 3-D graphene material was first synthesised by thermal exfoliation. The IL was then grinded with the 3-D graphene material to form a nanocomposite gel. The morphology, composition and electrochemical properties of the nanocomposite were characterised. Electrochemical NO sensing by using the nanocomposite gel was explored.

3.3 Experimental methods

3.3.1 Materials

The IL, 1-butyl-3-methylimidazolium hexafluorophosphate (BMIMPF₆) was purchased from Fluka. All other chemicals were acquired from Sigma-Aldrich and used without purification. 0.1 M phosphate buffered saline (PBS) (pH 7.0) was prepared by mixing 0.1 M NaH₂PO₄ (39 v%) and 0.1 M Na₂HPO₄ (61 v%). The DI water used was purified using a Millipore Milli-Q system.

3.3.2 Preparation of 3-D graphene material

Graphite powder was added into a mixture of concentrated aqueous H_2SO_4 , $\text{K}_2\text{S}_2\text{O}_8$ and P_2O_5 of weight ratio 5:1:1 and heated at $80\text{ }^\circ\text{C}$ for 4.5 h with magnetic stirring. The mixture was cooled and diluted with DI water, then filtered and washed with DI water to remove the residual acid. The residue was dried at room temperature overnight and added to concentrated aqueous H_2SO_4 solution in an ice bath and stirred for 15 min. KMnO_4 was gradually added and the mixture was heated at $35\text{ }^\circ\text{C}$. It was diluted with DI water in an ice bath every 2 h for 6 h. Aqueous H_2O_2 solution (30 v%) was added and the mixture was stirred for 30 min. The mixture was diluted with DI water, centrifuged and washed with 10 v% aqueous HCl solution several times. Thereafter, the mixture was washed and centrifuged with DI water repeatedly to remove the acid. The mixture was filtered and dried in vacuum at $60\text{ }^\circ\text{C}$ to obtain graphite oxide. The graphite oxide was then placed in a glass bottle under vacuum conditions and heated at $150\text{ }^\circ\text{C}$ for 45 min. After cooling down, a loose black powder of graphene was collected.

3.3.3 Preparation of nanocomposite gel electrode

The process to prepare the 3-D graphene/IL nanocomposite gel is illustrated in Figure 3.1. 16 mg of as-prepared 3-D graphene (or MWCNTs as a comparison) was mixed with 1.6 mL of IL in an agate mortar for 30 min, forming a homogenous black paste. The same procedures were carried out to prepare the 3-D graphene/mineral oil nanocomposite, with the exception of using mineral oil as the binder. The nanocomposite was dispersed in acetonitrile and sonicated for 1 h to

prepare a 70% solution, which was then cast onto a glassy carbon (GC) electrode with an apparent surface area of 0.071 cm^2 . Subsequently, $5 \text{ }\mu\text{L}$ of 10% Nafion was immobilised on the electrode surface to eliminate interfering species such as nitrite and ascorbic acid [202].

Figure 3.1 Schematic representation of the fabrication of the 3-D graphene/IL nanocomposite as well as its photograph.

3.3.4 Preparation of saturated NO in PBS

NO was prepared according to the procedures reported (Caution: NO is toxic above a concentration of 100 ppm and the preparation should be conducted in a fume hood) [202]. Briefly, 2 M aqueous H_2SO_4 solution was released drop-wise from a column into a round bottom flask containing saturated NaNO_2 solution, which was stirred using a magnetic stirrer to ensure a homogenous reaction. The NO produced was passed sequentially through saturated aqueous KOH, 10% (w/v) KOH and 2.5% (w/v) KOH solutions to remove any nitrogen oxides which might have been produced in the reaction of H_2SO_4 with NaNO_2 . Saturated NO was finally collected in 0.1 M PBS. The concentration of the NO-saturated solution was

1.8 mM at room temperature according to the reported procedure [202]. The saturated NO solution was stored in a N₂-protected environment for further use.

3.3.5 Characterisations and electrochemical measurements

The morphology and microstructure of the samples were investigated by field emission scanning electron microscopy (FESEM, JSM-6700F, Japan). Fourier transform infrared (FTIR) spectra were collected by Magna-IR 500 spectrometer (Nicolet Instruction, USA). Electrochemical measurements were carried out using CHI 760B electrochemical workstation (CH Instruments Inc., Texas, USA) with a three-electrode system, employing a Pt coil (0.393 cm²), a Ag|AgCl electrode (0.552 cm²) containing saturated aqueous KCl solution (-0.039 V with respect to saturated calomel electrode) and the graphene/IL nanocomposite electrode as the counter, the reference and the working electrode respectively. The average of three measurements for each of the four electrodes was used to calculate the standard deviations. All experiments were conducted at a room temperature of 25 °C.

3.4 Results and discussion

3.4.1 Morphology and composition characterisation of samples

The morphologies of the 3-D graphene material and 3-D graphene/IL nanocomposite were characterised by FESEM. As seen in Figure 3.2a, the prepared graphene material has porous structures. Its high magnification image (Figure 3.2b) shows loose nanosheets packed with each other. The nanocomposite gel produced

by grinding 3-D graphene and IL has a uniform structure (Figure 3.2c, d). The uniform nanocomposite may be formed by the good interaction of 3-D graphene with IL through cation- π and/or π - π interactions [227], which have also been observed for IL with other carbon nanomaterials such as mesoporous carbon [228] and MWCNTs [229]. The porous structure of the nanocomposite is evident from the high magnification image (Figure 2d). It has recently been reported that nanomaterials with porous structures have fast electron transfer kinetics because they can be stoichiometric electron acceptors and hosts for electron-donating guest species [200]. In addition, the nanocomposite is stable and retains its physical gel-like property under ambient conditions for at least three weeks.

Figure 3.2 FESEM images of 3-D graphene material with (a) low and (b) high magnification and of 3-D graphene/IL nanocomposite with (c) low and (d) high magnification.

The composition of the nanocomposite was examined by FTIR spectroscopy and the result is presented in Figure 3.3. The absorption band at 1566 cm^{-1} is attributed to the skeletal vibration of the graphene sheets. The absorption band around 1725 cm^{-1} for the C=O group is observable for the 3-D graphene material, which are ascribed to the acid moieties on the edges of the graphene sheet but they should not impede the conductive properties of graphene [230]. Furthermore, the bands around 1412 cm^{-1} for carboxyl group and around 1232 cm^{-1} for epoxy group totally disappear, indicating the successful preparation of graphene [231]. The FTIR spectrum changes greatly after mixing graphene with the IL. Apart from the bands of graphene, additional bands are observed. Bands at 1572 and 1263 cm^{-1} correspond to the stretching vibrations of the C-N bonds in the aromatic system of imidazole, while a band at 837 cm^{-1} matches the stretching vibrations of P-F inside the IL. The peak at 1725 cm^{-1} for C=O is also attenuated. These results clearly demonstrate the successful fabrication of the 3-D graphene/IL nanocomposite.

Figure 3.3 FTIR spectra of 3-D graphene material and 3-D graphene/IL nanocomposite.

3.4.2 Electrochemical behaviour of 3-D graphene/IL nanocomposite electrode

The electrochemical behaviour of the nanocomposite electrode was characterised with 10 mM of $[\text{Fe}(\text{CN})_6]^{3-}$ in 1 M aqueous KCl solution. Figure 3.4a shows the CV curves obtained by varying the scan rate from 0.01 to 1.00 V s^{-1} . All these CVs show a well-defined redox reaction with an almost unit ratio of the reverse-to-forward peak currents, indicating that a fast and reversible reaction occurred on the nanocomposite. The plots of peak current against the scan rate (v) and its square root ($v^{1/2}$) are presented in Figure 3.4b. The blue line represents the plot of the peak current i_P against the square root of the scan rate $v^{1/2}$. At low scan rates below 0.40 V s^{-1} , i_P shows a good linear relationship with $v^{1/2}$. However, at

higher scan rates, i_p deviates positively from the straight line displayed at lower scan rates. In contrast, the red line representing the plot of the peak current i_p against the scan rate v displays a linear relationship with v for all scan rates. The electrochemical behaviour of the nanocomposite electrode can be well explained by modelling it against the following equation [134],

$$i_p = i_D + i_T = 2.69 \times 10^5 A c_R n^{3/2} D^{1/2} v^{1/2} + \frac{n^2 F^2 V c_R v}{4RT} = K_D v^{1/2} + K_T v$$

The peak current i_p (A) is the sum of the peak current i_D at a planar disk electrode and the thin-layer cell current i_T formed in the inner porous nanocomposite. It is known that i_D is proportional to $v^{1/2}$ and i_T is proportional to v [74]. A is the area of the electrode (cm^2), c_R is the bulk concentration of the reduced species (mol cm^{-3}), n is the number of e^- transferred, D_R is the diffusion coefficient of the reduced species ($\text{cm}^2 \text{s}^{-1}$), v is the scan rate (V s^{-1}), F is Faraday's constant, (96485 C mol^{-1}), V is the volume of the thin-layer cell (cm^3), R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) and T is the temperature (K). K_D (C V^{-1}) and K_T (C V^{-1}) are constants for the planar disk electrode and thin-layer cell, respectively.

Figure 3.4 (a) CVs of electrochemical behaviour of the 3-D graphene/IL nanocomposite in 10 mM of $[\text{Fe}(\text{CN})_6]^{3-}$ in 1 M aqueous KCl using scan rates of 0.01 V s^{-1} to 1.00 V s^{-1} . (b) Plot of peak current/ μA against scan rate/ V s^{-1} (black circles) and square root of scan rate/ $\text{V}^{0.5} \text{ s}^{-0.5}$ (hollow circles).

The 3-D graphene/IL nanocomposite electrode has porous structures (Figure 3.2c, d) and its inner porous surface area should be much larger than its outermost surface area. At low scan rates, the diffusion layer thickness is large enough to fully use up the entire inner porous electrode surface and the electrode behaves like a planar electrode with an expanded surface area. Thus, the linear relationship of i_p with $v^{1/2}$ is observed. With increasing scan rate, the diffusion layer path becomes shorter, but the main porous volume is still filled with reactant/electrolyte and this forms a thin-layer cell inside the porous nanocomposite [134]. This is why i_p deviates from the relation of i_p vs. $v^{1/2}$ at higher scanning rates and eventually becomes proportional to v as shown in Figure

3.4, giving rise to the typical electrochemical behaviour of a thin-layer cell. This result is also in good agreement with the electrochemical behaviour of powder microelectrodes [232, 233].

3.4.3 Electrocatalysis of 3-D graphene/IL nanocomposite towards sensitive NO detection

The nanocomposite was used to detect NO and the CV curves are presented in Figure 3.5. Curve 1 shows that the control using PBS solution without NO has no defined redox peak in the entire potential range, hence indicating the broad electrochemical window of the nanocomposite. In contrast, a large oxidation peak is observed on curve 2 for 0.2 mM NO in PBS. During the reaction, NO loses an electron and forms NO^+ , which is rapidly converted to NO_2^- via a chemical reaction [134],

Figure 3.5 CVs of 0 mM of NO in PBS using 3-D graphene/IL electrode (curve 1), and 0.2 mM of NO in PBS using 3-D graphene/IL (curve 2), 3-D graphene/mineral oil (curve 3), MWCNTs/IL (curve 4), graphite/IL (curve 5) and GC electrode (curve 6) ($\nu = 0.05 \text{ V s}^{-1}$).

The CV curves also indicate that the oxidation of NO on the electrode is a totally irreversible reaction since no reduction peak current (reduction peak) is observed from curve 2. The performance of the 3-D graphene/IL nanocomposite (curve 2) for NO detection was compared with that of a 3-D graphene/mineral oil nanocomposite (curve 3), MWCNTs/IL nanocomposite (curve 4), graphite/IL nanocomposite (curve 5) and GC electrode (curve 6) as shown in Figure 3.5. The 3-D graphene/mineral oil nanocomposite does not show any peak due to a high contact resistance, indicating that it is not a suitable material for the electrocatalytic oxidation of NO. The 3-D graphene/IL nanocomposite has a peak current density

of $669 \mu\text{A cm}^{-2}$, which is higher than that of the MWCNTs/IL nanocomposite ($176 \mu\text{A cm}^{-2}$), graphite/IL nanocomposite ($117 \mu\text{A cm}^{-2}$) and GC electrode ($64.8 \mu\text{A cm}^{-2}$). The higher current response of the 3-D graphene/IL nanocomposite could be due to its large electrochemically active surface area. In addition, NO could adsorb onto the IL and partition into it, leading to pre-concentration of NO on the IL.

The electrochemical behaviour of NO on the nanocomposite electrode was investigated with respect to the scan rate. It is observed that the peak current potential shifts positively with an increase in scan rate due to the irreversible reaction of NO as expected [74].

Figure 3.6 Plot of peak potential/V against scan rate/ V s^{-1} for CVs of 0.2 mM of NO in PBS using the 3-D graphene/IL nanocomposite. The inset shows the CVs.

The oxidation of NO using the nanocomposite was explored for an amperometric NO sensor. Figure 3.7a displays the amperometric responses measured at 0.8 V vs. Ag/AgCl in solutions containing different concentrations of

NO by successive injections of $0.8 \mu\text{mol L}^{-1}$ of NO in stirred PBS solution. The injected NO caused a stepped increase in oxidation current. From the steady-state measurements, a response time of less than 4 s was determined when the responding current reaches 95% of the steady-state current after injection of $0.8 \mu\text{mol L}^{-1}$ of NO into the tested samples. The response time includes the mass transport time after the injection of NO to achieve a uniform solution and it should be actually shorter than 4 s for *in situ* or *in vivo* experiments. The calibration curve of the current response against the concentration of NO is presented in Figure 3.7b and it shows a linear range of 0.8 μM to 16 μM with a detection limit of 0.75 nM ($S/N = 3$). The sensitivity of the nanocomposite electrode calculated from the slope of the calibration curve is $11.2 \mu\text{A cm}^{-2} (\mu\text{mol L}^{-1})^{-1}$. Both the sensitivity and detection limit are much more superior to CNTs [136, 202, 203] and Au NPs-based [207, 208] NO sensing platforms listed in Table 3.1. The higher sensitivity, low detection limit and fast response towards NO detection can be attributed to the unique properties of the graphene component in the nanocomposite such as the large electrochemically active surface area and fast charge transfer capability. The notably good response of the nanocomposite towards NO is useful in solving problems associated with *in situ* NO detection such as its short half-life in the presence of O_2 and the low concentration of NO released by endothelial cells (ECs).

Stability is critical for the sensing application of a nanocomposite. The stability of the nanocomposite was evaluated through its amperometric response by continuous testing for two hours per day in 0.1 M PBS solution. The nanocomposite electrode was stored under ambient conditions at room temperature

when it was not in use. Even after three weeks, it could still retain 95% of its initial amperometric response towards the same concentration of NO. This demonstrates the good long-term shelf-life of the nanocomposite-based NO sensor and its great potential for the *in situ* sensing of NO released by ECs during angiogenesis.

More work needs to be done to evaluate the biocompatibility of the nanocomposite electrode to ECs in order to use the electrode simultaneously as a growth matrix for ECs and NO biosensor to detect NO released by these cells during angiogenesis. The sensitivity, limit of detection and response time of the nanocomposite electrode for the detection of NO in the presence of endothelial cells and the cell culture medium need to be characterised.

Figure 3.7 (a) Amperometric response of the 3-D graphene/IL nanocomposite over time following injections of $0.8 \mu\text{mol L}^{-1}$ of NO in PBS. (b) Plot of current response/ μA against NO concentration/ $\mu\text{mol L}^{-1}$.

Table 3.1 Comparison of sensitivity and detection limit for NO of different sensors.

Electrode materials	Sensitivity based on apparent surface area of electrode/ $\mu\text{A cm}^{-2}(\mu\text{mol/L})^{-1}$	Detection limit/ nM	Ref.
3-D Graphene/IL	11.2	0.75	This work
Au NPs/ITO	0.02	560	[208]
Au NPs	4.6	27	[207]
MWCNTs	5.8	80	[203]
MWCNTs/Biomolecule	3.1	80	[136]
SWCNTs/IL	2.1	100	[202]

3.5 Conclusion

3-D graphene/IL nanocomposite gel with a homogeneous, porous structure was prepared and characterised. The nanocomposite gel electrode has a large electroactive surface area and behaves as a combination of a planar disk electrode and a thin-layer electrochemical cell demonstrating a fast response of less than 4 s, a high sensitivity of $11.2 \mu\text{A cm}^{-2} (\mu\text{mol L}^{-1})^{-1}$ and a low detection limit of 0.75 nM, much more superior to that of other NO sensing platforms based on CNTs and Au NPs. The high performance of the gel composite may be primarily attributed to the porous graphene material with a high specific surface area and superior conductivity. Furthermore, the nanocomposite-based NO sensor possesses a large dynamic response range and excellent stability. These results demonstrate that the 3-D graphene/IL nanocomposite provides a novel platform for sensitive NO detection.

4 RGD-peptide functionalised graphene biomimetic live-cell sensor for real-time detection of nitric oxide[†]

4.1 Abstract

A free-standing biomimetic NO biosensor was fabricated by covalently bonding arginine-glycine-aspartic acid (RGD)-peptide on the surface of pyrenebutyric acid-functionalised graphene film. The resulting graphene film sensor comprises a well-packed layered nanostructure, in which the RGD-peptide component provides desired biomimetic properties for superior human umbilical vein endothelial cells (HUVECs) attachment and growth on the film's surface to allow the real-time detection of NO, an important signal yet short-lived molecule released by the attached cells under acetylcholine (Ach) stimulation. The film sensor exhibits good flexibility and stability by retaining its original response after 45 bending/relaxing cycles and high reproducibility from its almost unchanged current responses after 15 repeated measurements. It possesses high sensitivity, good selectivity against interferences often present in biological systems and demonstrates real-time quantitative detection capability towards NO released by HUVECs.

[†] This paper is cited as reference [234]. Reprinted (adapted) with permission from C.X. Guo, S.R. Ng, S.Y. Khoo, X.T. Zheng, C. Peng, C.M. Li. RGD-peptide functionalized graphene biomimetic live-cell sensor for real-time detection of nitric oxide molecules, *ACS Nano*, 6(8): 6944-6951. Copyright (2012) American Chemical Society.

4.2 Introduction

In the previous chapter, the 3-D graphene/IL nanocomposite was shown to sensitively detect NO dissolved in phosphate buffered saline (PBS) [184]. However, the same experimental setup is unsuitable for detecting NO released by endothelial cells (ECs) since the time taken for diffusion of NO from the cells to the electrode will be too long. The best way is to grow the cells directly on electrode surface so that the NO molecules can be detected almost as soon as they are released by the cells. Thus, the electrode surface should possess excellent biomimetic properties for good cell growth and adhesion, since these processes are very complex, highly regulated and requires ligation of specific surface receptors for the maintenance of cellular functions [235-237].

Herein, a novel approach to construct a smart functional biomimetic film sensor by covalently bonding RGD-peptide onto the surface of graphene to significantly boost cell adhesion and growth for the real-time electrochemical detection of NO released by HUVECs under Ach stimulation is reported. RGD-peptide is selected because its peptide moiety mimics the cell-binding sequence of extracellular matrix proteins and through ligation of integrins on the outer surface of the cell membrane, promotes cell adhesion [238, 239].

4.3 Experimental methods

4.3.1 Materials

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated, and used without further purification. DI water was purified with Millipore Milli-Q system.

4.3.2 Preparation of pyrenebutyric acid-functionalised graphene

Graphite oxide was prepared in the same method as described in Chapter 3.3.2. 0.1 mg mL⁻¹ graphite oxide aqueous solution was exfoliated by sonicating it at ambient conditions for 60 min, resulting in a dispersion of graphene oxide. 100 mg of NaOH was added to 100 mL of the graphene oxide dispersion, followed by 150 mg of pyrenebutyric acid to prepare the pyrenebutyric acid-functionalised graphene oxide dispersion. The mixture was reduced with 250 µL of hydrazine monohydrate at 80 °C overnight. The dispersion was centrifuged to yield a black supernatant.

4.3.3 Preparation of biomimetic graphene film

The preparation of the biomimetic graphene film is illustrated in Figure 4.1. The pyrenebutyric acid-functionalised graphene dispersion was filtered through a cellulose acetate membrane (pore size of 0.22 µm) to produce a graphene film. The film was repeatedly washed with DI water and dried under vacuum. Then, the film was immersed in a solution of 4 mM of ethyl(dimethylaminopropyl) carbodiimide

(EDC) and 10 mM of N-hydroxysulfosuccinimide (NHS) for 1 h. After the activated film was gently rinsed with DI water, 1 mg mL⁻¹ RGD-peptide solution was added to its surface and incubated for 2 h to yield the biomimetic graphene film with covalently bonded RGD-peptide, which was then rinsed with pH 7.4 PBS to remove the excess RGD-peptide.

4.3.4 Cell culture and manipulation

Culture medium was prepared by mixing MCDB 131 medium, sodium bicarbonate, heparin, bovine brain extract (Hammond) and fetal bovine serum (Bio-Industries) in autoclaved DI water and filtered. HUVECs were cultured in the as-prepared culture medium at 37 °C in a humidified incubator (95% air with 5% CO₂). To culture cells on the graphene films, HUVECs were seeded with a density of ~5000 cells cm⁻² and allowed to stay at 37 °C in the humidified incubator (95% air with 5% CO₂) for different durations to optimise the time for cell adhesion and growth. The optimised time for cell adhesion and growth was found to be 18 h.

Figure 4.1 Schematic illustration of preparation of the free-standing biomimetic film and its live-cell assay. (a) Chemical reduction of graphene oxide and pyrenebutyric acid functionalisation. (b) Preparation of pyrenebutyric acid-functionalised graphene film by filtration of the solution of pyrenebutyric acid-functionalised graphene. (c) Fabrication of the biomimetic graphene film by covalently bonding RGD-peptide onto the surface of the pyrenebutyric acid functionalised graphene film via EDC/NHS coupling. (d) The functional biomimetic graphene film as both cell adhesion and sensing matrix.

4.3.5 Characterisation and electrochemical measurements

Pyrenebutyric acid-functionalised graphene sheets were characterised by atomic force microscopy (AFM) (Nanoman, Veeco, Santa Barbara, CA) using the tapping mode. The morphologies of surface-modified graphene films were investigated by scanning electron microscopy (SEM) (JSM-6700F, Japan). Fluorescent images for fluorescein isothiocyanate mixed isomer (FITC) labelled graphene films were acquired with a proteomic imaging system (Perkin Elmer) with excitation wavelength of 494 nm and emission wavelength of 518 nm. Phase-contrast images of cells cultured on graphene films were taken with an inverted microscope (Olympus IX71). Electrochemical measurements were performed in a three-electrode system using CHI-760D electrochemical workstation (CH Instruments Inc. USA) with the biomimetic graphene film (with or without cultured cells), Pt wire (0.473 cm^2) and Ag|AgCl electrode (0.552 cm^2) containing saturated aqueous KCl solution (-0.039 V with respect to saturated calomel electrode) as working, counter and reference electrodes, respectively.

4.3.6 Real-time detection of NO released by HUVECs

The setup of the device for real-time detection of NO released by HUVECs was the same as that for electrochemical measurement. The diameter of the low columniform is 2 mm and height is 20 mm. 2 mL cell culture medium was used as the supporting electrolyte. NO released by the cells was measured using double potential step chronoamperometry (DPSCA).

4.4 Results and discussion

4.4.1 Characterisations of biomimetic graphene film

Figure 4.2a shows that the RGD-peptide functionalised graphene film has a free-standing structure, which comprises a well-packed layered structure (Figure 4.2b) with a thickness of $\sim 8\mu\text{m}$ (Figure 4.2c). The surface of the film has a wave-like morphology, as seen from the top-view SEM image in Figure 4.2d. The surface properties of different graphene-based films were investigated by reflection absorption infrared spectroscopy (RA-IR). Figure 4.2e shows a strong peak at 1586 cm^{-1} for the pyrenebutyric acid-functionalised graphene film before the attachment of RGD-peptide (curve 1), which can be assigned to the stretch mode of a carboxyl group [240]. In contrast, plain graphene without pyrenebutyric acid functionalisation displays almost negligible peaks at 1586 cm^{-1} (not shown). These observations suggest the successful functionalisation of graphene with pyrenebutyric acid. After attachment of RGD-peptide to the pyrenebutyric acid-functionalised graphene film, the resulting film clearly exhibits two new peaks at 1645 and 1533 cm^{-1} , corresponding to the characteristic amide I and amide II of the RGD-peptide [241], whereas the peak at 1586 cm^{-1} assigned to the carboxyl group becomes weak but is still observable (curve 2). These results indicate that RGD-peptides are effectively covalently bonded to the pyrenebutyric acid-functionalised graphene film and the resulting film still has free carboxyl groups on its surface.

Figure 4.2 (a) Photograph of biomimetic RGD-peptide covalently bonded graphene film. The inset of panel (a) is an AFM image of pyrenebutyric acid-functionalised graphene sheets. (b) High magnification side-view, (c) low magnification side-view and (c) top-view SEM images of the biomimetic graphene film. (d) RA-IR spectra of (1) pyrenebutyric acid functionalised graphene film and (2) biomimetic graphene film. (e) Fluorescence staining of biomimetic graphene film and the inset is fluorescence staining of graphene film without RGD-peptide. The scale bars represent 2 mm.

The distribution of RGD-peptide on the film was visualised using FITC that is highly reactive towards primary amine groups of peptides and proteins. The biomimetic graphene film shows a strong signal and a uniform colour distribution, while the control experiment of FITC-stained graphene film without RGD-peptide conjugation displays a negligible fluorescence signal (Figure 4.2f), suggesting the uniform distribution of RGD-peptide on the graphene film. It is noted that the biomimetic graphene film can keep its structure intact in aqueous solution under mild ultrasonication, exhibiting very good mechanical strength for potential solution-based manipulations and testing.

4.4.2 Cell adhesion on biomimetic graphene film

The surface property of the sensing matrix must be suitable for cell adhesion in order to detect biomolecules released by the attached cells in real-time. As shown in Figure 4.3a, HUVECs cultured on plain graphene film appear stretched with pseudopodia, suggesting such a film is a biocompatible platform for cell attachment and growth. It is known that cell adhesion and growth on a substrate depend not only on the surface properties of the substrate, but also on the type of proteins which adsorb onto its surface (from culture medium) and/or secreted by the cells [239], which may contribute to the observed cell adhesion and growth on plain graphene film. The HUVECs' attachment and growth are significantly improved in the presence of RGD-peptide, as clearly indicated by more than 2-fold increase in cell density and nearly 50% increase in cell length over that of plain graphene film (Figure 4.3c, d). The peptide moiety of the RGD-

peptide mimics the cell-binding sequence of ECM proteins [238], which provides the graphene film its biomimetic property and greatly promotes cell adhesion and growth.

Figure 4.3 Microscopy images of HUVECs cultured on (a) plain graphene film and (b) biomimetic graphene film. (c) The number of adhered cells and (d) the average cell length of HUVECs on (1) plain graphene film and (2) biomimetic graphene film.

4.4.3 Electrochemical behaviour of biomimetic graphene film

The biomimetic graphene film (2 cm²) was used to detect NO in cell culture medium. From Figure 4.4a, when NO was absent (curve 1), the biomimetic graphene exhibits only capacitive behaviour caused by its double layer over the entire potential range (0.0 to 1.0 V vs Ag|AgCl). In contrast, a large oxidation peak with peak potential at 750 mV is observed in the CV of the film in cell culture medium containing 10 µM of NO (curve 2), which shows good electrocatalysis towards the oxidation of NO. During oxidation, NO loses an electron to form NO⁺ followed by its chemical reaction which forms NO₂ as follows [134, 198],

Double potential step chronoamperometry (DPSCA) is an electrochemical technique which obtains the current response with high S/N ratio in a heterogeneous solution. Thus, changes in NO concentration can be monitored by using DPSCA with a double-pulse potential at a regular interval. The typical DPSCA recorded for NO in cell culture medium is shown in the inset of Figure 4.4b. The current response is defined as the difference between the first potential (650 mV, onset potential of NO oxidation) and the second potential (750 mV, the peak potential of NO oxidation). The logarithmic plot of the current response vs. NO concentration is shown in Figure 4.4b, with two linear regions associated with the increment of the NO concentration from nanomolar to micromolar. The detection limit is around 25 nM (S/N = 3). With HUVECs on the sensing matrix, the response of the biomimetic graphene films towards NO is around 1/30 of that

without cells and the detection limit is around 80 nM. The selectivity of the sensing matrix towards NO was investigated using interfering species such as nitrite, ascorbic acid and various ions. As seen in Figure 4.4c, the biomimetic graphene biofilm displays good selectivity towards NO and avoids the interferences from these species including nitrite, which has a similar oxidation potential to NO. The good selectivity of the sensing matrix may come from its retained surface carboxyl groups (as seen in Figure 4.2e) that bear negative charges to repel the negatively charged nitrite and ascorbic acid.

Figure 4.4 (a) CV curves of the biomimetic graphene film in culture medium in the absence (1) and presence (2) of 10 μM NO ($\nu = 0.02 \text{ V s}^{-1}$). (b) The logarithmic plot of the response current vs. NO concentration. Insets of panel B are a typical DPSCA used to obtain the calibration curve and the calibration curve for low concentration of NO. (c) Selectivity of the biomimetic graphene film for 0.3 μM NO against 25 μM of interfering species (NO_2^- , K^+ , Na^+ , NO_3^- , SO_4^- , ascorbic acid).

4.4.4 Flexibility and reproducibility of the biomimetic graphene film

In order to examine the flexibility and stability of the biomimetic graphene film, its responses to bending and relaxing cycles were investigated. As shown in Figure 4.5, the film (2 cm^2) almost retains its original current response after 45 bending/relaxing cycles, showing excellent flexibility and stability, which is attributed to the good mechanical stability of graphene and strong integrity of the layered film. The good flexibility of the biomimetic graphene is advantageous for emerging applications in *in vivo* NO biosensor [242]. The response of the sensor is also highly reproducible as indicated from its stable responses after 10 measurements. The deviation of the current responses for six biomimetic graphene films prepared by the same procedure is less than 7.6% (relative standard deviation).

Figure 4.5 (a) Photographs of the relaxed and bent biomimetic graphene film. (b) Current responses/ μA of the biofilm subjected to bending.

4.4.5 Real-time detection of NO released by HUVECs on the film

During *in situ*, real-time NO detection, the close proximity between the NO-releasing cells and the sensing matrix is a critical factor for the effective detection of NO, since NO has a limited diffusion distance and short half-life in the presence of O₂. Since the HUVECs are cultured on the biomimetic graphene biofilm, the NO released by the cells can be efficiently detected in real-time with good sensitivity. The setup is shown in Figure 4.6a. Ach is selected as a model drug to stimulate the cells for NO release because the signalling pathway of Ach to NO generation by the cell is well understood. Ach binds to the cell and triggers a signalling cascade which activates the Ca²⁺-calmodulin complex and leads to the production and release of NO [243, 244]. NG-nitro-L-arginine methyl ester (L-NAME), a NO inhibitor, was used as a model drug to inhibit the release of NO. It is noted that the additions of Ach (1 mM) or L-NAME (1 mM) into the cell culture medium does not cause any significant current change on the biomimetic graphene film (without cultured cells) during the electrochemical measurements, clearly indicating that both Ach and L-NAME are not electrochemically active over the range of potentials used.

The dynamic responses of the HUVECs cultured on the biomimetic graphene film towards the drugs were investigated. The DPSCA responses are shown in Figure 4.6b. The stimulating and inhibiting agents were added as indicated by the arrow. Both 1 mM and 0.5 mM Ach stimulations cause significant current responses which are concentration-dependent. The current response of 1 mM Ach stimulation is more than two times than that of 0.5 mM Ach stimulation

after a period of 2 min as shown in Figure 4.6b, c. In contrast, no response is observed for the cells under the stimulation of a mixture of 1 mM ACh and 1 mM L-NAME, and this can be attributed to the specific inhibiting behaviour of L-NAME towards NO release. Hence, this experiment demonstrates the capability of the biomimetic graphene film as a matrix for the culture of HUVECs and the real-time, quantitative detection of NO released by these cells under ACh stimulation.

Figure 4.6 (a) Schematic drawing showing the setup for the live-cell assay. (b) Real-time monitoring of NO released by the HUVECs on the biomimetic graphene film in cell culture medium. The drug was added at the time indicated by the arrow. (c) The current responses of the biomimetic graphene film with cultured HUVECs towards different drugs. ACh is a model drug to stimulate cell NO release and L-NAME is a specific NO inhibitor.

4.5 Conclusion

A facile approach to construct a biomimetic NO biosensor by covalently bonding RGD-peptide onto the surface of pyrenebutyric acid-functionalised graphene film is demonstrated in this study. RGD-peptide modification gives the film its biomimetic characteristics for the attachment and growth of HUVECs. The biomimetic graphene film exhibits good flexibility and stability by retaining its original current response after 45 bending/relaxing cycles, and high reproducibility as indicated from its almost unchanged current responses after 10 repeated measurements, while showing high sensitivity and good selectivity towards the detection of NO. The attached HUVECs were stimulated by Ach and released NO, which was selectively detected by the film, thereby demonstrating its real-time quantitative detection capability for NO. This study not only reports an approach to fabricate a biomimetic graphene film, but also provides a powerful platform to build next-generation biomedical devices for live-cell assay and drug effect screening.

5 Iridium oxide microelectrode for the real-time physiological pH measurements of porcine aortic endothelial cells

5.1 Abstract

Miniature pH sensors were fabricated by anodically electrodepositing iridium oxide films (AEIROFs) onto microelectrodes on a chip and coated with 5% poly(ethyleneimine) (PEI) for mechanical stability. The modified electrodes demonstrate super-Nernstian response to pH. The surface of the chip was coated with fibronectin for the cell adhesion and growth of porcine aortic endothelial cells (PAECs). Thrombin induces the dissolution of fibronectin, causing the pH to fall. Thrombin also causes extracellular acidification by PAECs and a change in cell morphology from stretched to round cells. The use of PD98059, a mitogen-activated protein (MAP) kinase inhibitor, reduced extracellular acidification, indicating that the action of thrombin involves a MAP kinase-dependent pathway. The effects of thrombin on PAECs and fibronectin are significant for the activation of PAECs. Thrombin causes ECs to lose their anchorage on the extracellular matrix and acutely increases cell metabolism and cytoskeleton reorganisation, likely in preparation of cell proliferation and migration in thrombin-mediated angiogenesis.

5.2 Introduction

The rate of endothelial cell (EC) metabolism is influenced by many external stimuli. These include the pH, concentrations of dissolved O₂ and glucose, interactions with soluble factors and the extracellular matrix. Alterations in the rate of EC metabolism during angiogenesis can manifest as changes in the concentrations of metabolic markers such as pH, lactate and CO₂ (Chapter 2.2). Measuring extracellular pH is a good indicator of cell metabolism since an increase in energy metabolism increases glycolysis and CO₂ production and results in extracellular acidification [67].

The measurement of pH can be achieved using potentiometric pH sensors. The traditional pH sensor is the glass electrode [161]. However, it has high impedance, suffers from slow response time and is mechanically fragile. In contrast, metal/metal oxide electrodes such as the Ir|IrOx film electrode [102], Au|IrOx film microelectrode [105, 162, 163] and tungsten/tungsten oxide film nanoelectrode [164] showed improved stability, fast response time and low impedance towards *in vivo* [162] and *in situ* pH measurements [102, 105, 163, 164]. The potential (E) of the metal/metal oxide electrode is the result of the equilibrium between the sparingly soluble oxide and its saturated solution and changes according to the following equation [102],

$$E = E_{M,MO,H^+}^0 - 2.303 \frac{RT}{nF} pH$$

where E_{M,MO,H^+}^0 is the standard potential which includes the ionisation product of water and solubility product of the metal oxide, R is the gas constant (8.314 J mol⁻¹

K^{-1}), T is the temperature in Kelvin, F is Faraday's constant ($96485C\ mol^{-1}$) and n is the number of electrons transferred.

In as early as 1947, the use of Ir as a pH electrode was proposed by Perley and Godshalk [245]. Between the 1970s and 1980s, investigations into the formation of IrOx during CV studies of an Ir electrode in acid were of great interest [246], particularly about their bulk and surface properties [247] and charge storage reactions [248]. This led to their applications as pH sensors [101, 103], electrocatalysts [249] and electrochromic display devices [250]. In order to exploit the electrochromic properties of IrOx as display devices, the electrodeposition of IrOx from solution onto non-metal substrates was investigated [251, 252]. The use of acidic sulfoiridate (III, IV) complexes to electrodeposit IrOx films from solution by periodic reverse current electrolysis was proposed but the solution was unstable [251]. Yamanaka found that the anodic electrodeposition of IrOx films from alkaline iridium oxalate produced smooth, lustrous films [252], and the reaction is given by [105],

where the oxidation of oxalate ligand forms CO_2 and the hydrated IrOx is electrodeposited. His method has been used for the fabrication of pH sensors from AEIROFs on Au substrates [102, 105, 253-257].

The anodic electrodeposition of IrOx from iridium oxalate has been carried out using cyclic voltammetry [102, 247, 248, 258], applying a constant current

density [252, 254], constant potential [105, 259] or pulsed potential [257, 260]. The varying degrees of hydration depending on the electrodeposition conditions give rise to Nernstian to super-Nernstian response ranging from -0.061 V pH^{-1} to -0.090 V pH^{-1} [101, 256], taking into account an error of 5 Kelvin in temperature measurement. The super-Nernstian behaviour can be explained by the equation where more than one proton is transferred per electron [101, 102, 105],

where x varies according to the degree of film hydration. The super-Nernstian response is desirable as it offers a higher sensitivity to pH measurements, particularly for studies with cells, where the changes in pH might be small.

Thrombin, a coagulation factor and protease, has been shown to activate porcine ECs during tissue inflammation [261]. Thrombin also induces an increase in energy metabolism in HUVECs, leading to extracellular acidification [262]. Fibronectin, an extracellular matrix glycoprotein, has been used for the cell culture of ECs on microelectrode array (MEA) chips [184, 263, 264] and prevents electrode biofouling with minimal effects on the catalytic property and active area of the electrode [265]. However, the interaction of thrombin, porcine aortic endothelial cells (PAECs) and fibronectin, and its acute effects on extracellular pH have not been studied in real-time until now. It is of interest to study this interaction to gain insights to thrombin-mediated angiogenesis. In this work, AEIROFs were miniaturised onto MEA chips, which are then coated with 5% PEI for mechanical stability. The surface of the chip was then coated with fibronectin

for the cell adhesion and growth of PAECs. The extracellular pH is measured in real-time to investigate the interaction of thrombin, PAECs and fibronectin.

5.3 Experimental methods

5.3.1 Chemicals

5 M H_2SO_4 and 85% H_3PO_4 were purchased from Fluka. 37% HCl , 70% HNO_3 and 5 N NH_4OH , KH_2PO_4 , iridium (IV) chloride (IrCl_4), 50 wt% poly(ethyleneimine) (PEI) ($M_w \sim 750,000$) were acquired from Sigma-Aldrich. NaOH and KCl , as well as ethanol, were obtained from Fischer Scientific. Anhydrous K_2CO_3 was bought from Fisons Scientific Apparatus, oxalic acid dehydrate ($(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$) and 30 w/w% H_2O_2 from Analar and Decon90 from Decon Laboratories Limited. DI water with a resistivity of 15 $\text{M}\Omega \text{ cm}$ from Purite was used in all experiments.

5.3.2 Instruments

The microelectrode array (MEA) chips and chip holder were fabricated by Aleria Biodevices. Each chip has a Au pseudo-reference electrode, 14 Au working electrodes (25 μm diameter), as well as a U-shaped Au counter electrode (Figure 5.1). The pseudo-reference electrode was not used. A leak-free $\text{Ag}|\text{AgCl}$ reference electrode (IJ Cambria) (-0.052 V with respect to saturated calomel electrode) was used instead. A CHI 1030 electrochemical workstation (CH Instruments, Inc.) was employed to input the experimental parameters and acquire the data. A S20

SevenEasyTM glass pH meter (Mettler Toledo) with temperature display was used to measure the pH of the phosphate buffers and the room temperature. All values of the sensors' sensitivity to pH presented here are adjusted to 25.0 °C, unless stated otherwise. Field emission scanning electron microscopy (FESEM) images were captured by a LEO 1525 Gemini. Leica microscopes were used to take the light and inverted light microscopy images.

Figure 5.1 The MEA chip.

5.3.3 Preparation of AEIROFs on MEA chip

The MEA chips were cleaned sequentially with Decon90, hot DI water, ethanol, 5 M HNO₃ and DI water. The working electrodes, situated in a 1 μm recess, were electrochemically cleaned in 0.5 M H₂SO₄. AEIROF was electrodeposited by applying constant current density of 1.70 mA cm⁻² for 15 min to the iridium oxalate solution, which was prepared according to the method described by Yamanaka [252]. After electrodeposition, the chips were filled with DI water to let the AEIROFs equilibrate overnight. The next day, the chips were dried with N₂ and coated with 20 μL of 5% PEI to improve the mechanical stability of the AEIROF, since the film grew out of the recess. It is unsuitable to coat the chip with fibronectin directly as the presence of Cl⁻ in the cell culture medium used

to suspend fibronectin will react with the AEIROF to form a chloroiridate complex [102] and cause the film to dissolve. The chips were left to dry overnight, then rinsed to remove the excess PEI and filled with DI water and allowed to rehydrate over 1 day. pH calibrations were done for these chips from pH 4.0 to 7.7 using chloride-free phosphate buffers as described in the next section. The chips were then coated with 20 μL of 20 $\mu\text{g mL}^{-1}$ fibronectin in Dulbecco's Modified Eagle's Medium (DMEM) and left to dry overnight in the biological safety cabinet. The excess fibronectin was rinsed off with Dulbecco's phosphate buffered saline (DPBS).

5.3.4 pH sensor characterisation

CV was found to be unsuitable for characterising the AEIROFs since the CVs are irreproducible due to the increasing build up of the less conductive Ir(III) oxide with each scan. pH calibrations of the sensors were performed in phosphate buffers of pH 4.0, 5.0, 6.0, 7.0 and 7.7 made up of 1 M H_3PO_4 and 1 M NaOH at open circuit potential (OCP). This range of pH was investigated as the purpose of this project was the measurement of physiological pH of PAECs. Thus, lower and higher pH values were excluded from the study. The OCP vs. time was measured for 5 min in each solution from low to high pH using the CHI 1030. The pH calibration curve was obtained by plotting the OCP at the end of run against pH. The sensitivity is given by the slope of the best fit line and the formal potential, the intercept, values of which are adjusted to 25.0 $^{\circ}\text{C}$. A two-point calibration using pH 6.5 and pH 7.5 cell culture medium gassed with 5% CO_2 was performed for the

fibronectin-coated chips before the cells were seeded. Lower pHs were not used to prevent the denaturation of the fibronectin.

5.3.5 pH measurements of PAECs in real-time

PAECs of passage 8 were used. The cell culture medium contained DMEM supplemented with 1 mg mL⁻¹ glucose, sodium bicarbonate, 5 µg mL⁻¹ endothelial cell growth factor, 90 µg mL⁻¹ heparin, 5 mM L-glutamine, 10% foetal bovine serum, 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, 2.5 µg mL⁻¹ amphotericin and 50 µg mL⁻¹ gentamycin. The cells were cultured to confluence in T75 flasks. They were washed with DPBS, trypsinised and spun down at 1000 rpm for 5 min and finally resuspended in the cell culture medium. 30 000 cells were seeded on each chip and incubated at 37 °C and 5% CO₂ overnight.

After 17 h, the chip was removed from the incubator and placed in the chip holder and continuously gassed with 5% CO₂ (Figure 5.2c), which is crucial in getting meaningful results. HCO₃⁻ acts as a buffer in the cell culture medium and without a constant supply of CO₂, the small volume of cell culture medium will quickly lose its buffering capacity and the pH will rise, turning the cell culture medium from red to bright pink due to the presence of the phenol red indicator in the cell culture medium. If the pH of the cell culture medium were too high, it will kill the PAECs.

The chip was allowed to equilibrate at room temperature and the OCP was recorded for 30 min using CHI 1030. Reagents were added to the chip at 30 min

interval in the following order – two doses of 5 U mL^{-1} thrombin from human plasma (T6884) in 0.1% (w/v) bovine serum albumin (BSA), two doses of $50 \text{ }\mu\text{M}$ PD98059 (a MAP kinase inhibitor) (Figure 2), $5.88 \text{ mM H}_3\text{PO}_4$ and $11.11 \text{ mM H}_3\text{PO}_4$. There was around a 5 min lag time between each step as the chip was disconnected from the chip holder and the 5% CO_2 supply and transferred to the biological safety cabinet located in a different laboratory for the reagents to be added and for observation under the microscope. At the end of the experiment, the chip was rinsed thrice with cell culture medium and a two-point calibration with pH 6.5 and 7.5 cell culture medium was similarly performed.

Figure 5.2 Experimental setup for real-time PAECs pH measurement. (a) The MEA chip was placed in a chip holder and (b) the leak-free Ag|AgCl reference electrode was positioned into the well of the chip. (c) 5% CO_2 was blown across the top of the chip through a hole in the rubber tubing. The chip holder was connected to the CHI 1030 electrochemical workstation.

5.4 Results and discussion

5.4.1 FESEM characterisation of AEIROF coated with PEI and fibronectin

Figure 5.3 shows a FESEM image of the Au/AEIROF microelectrode after the surface of the chip was coated with 5% PEI, followed by $20\text{ }\mu\text{g mL}^{-1}$ fibronectin. The PEI coating cannot be observed in this case as the fibronectin coating is thick and forms patches on the silicon nitride surface of the chip. Fibronectin at high magnification appears as tightly packed clusters with a cobblestone morphology as seen in Figure 5.4. In contrast, PEI on silicon nitride consists tiny particles which are loosely packed (Figure 5.5). The difference in surface morphology in addition to integrin ligation may explain why PAECS did not attach onto 5% PEI alone but did so in the presence of fibronectin.

Figure 5.3 FESEM image of Au/AEIROF microelectrode coated with PEI and fibronectin.

Figure 5.4 FESEM image of 20 $\mu\text{g mL}^{-1}$ fibronectin coating on silicon nitride chip surface.

Figure 5.5 FESEM image of 5% PEI coating on silicon nitride chip surface.

5.4.2 Electrochemical characterisation of PEI-coated AEIROFs

The super-Nernstian response to pH is observed for the PEI-coated AEIROFs ranging from -0.063 V pH^{-1} to -0.077 V pH^{-1} , with an average of $-0.069 \pm 0.006 \text{ V pH}^{-1}$ ($n=5$). The formal potential ranged from 0.540 V to 0.661 V , with an average of $0.585 \pm 0.050 \text{ V}$. Calibration curves with good linear response ($R^2 > 0.980$) were obtained on the 2nd or 3rd day of calibration over the dynamic range of pH 4.0 to 7.7. The average $t_{95\%}$ ($n=5$) are 0.3 s for pH 4.0, 19.4 s for pH 5.0, 49.6 s for pH 6.0, 102.4 s for pH 7.0 and 190.2 s for pH 7.7. It is possible that at lower pH, reduction of Ir(IV) oxide causes the build-up of the less conductive Ir(III) oxide, resulting in slower charge transfer during the oxidation process at an alkaline pH.

5.4.3 Light microscopy images of control chip and chip with PAECs before and after cell experiments

Both the control chip and the chip with PAECs were coated with 5% PEI and $20 \mu\text{g mL}^{-1}$ fibronectin. Figures 5.6 shows the images for the control chip and Figures 5.7 the chip seeded with 30 000 PAECs.

In Figure 5.6a, the surface of the control chip appears dotted with clear round particles, which are likely fibronectin. After the experiment, long clear particles are observed instead (Figure 5.6b), which might indicate a change in the fibronectin structure during the course of the experiment. When the chip was reused and seeded with PAECs, the cells failed to attach, suggesting that the fibronectin had been denatured or lost from the chip. However, the dark blue

AEIROF was present on the control chip both before (Figure 5.6c) and after (Figure 5.6d) the control experiment.

Figure 5.7a shows the chip seeded with 30 000 PAECs and a higher magnification image (Figure 5.7c) confirms that the PAECs were healthy with their characteristic stretched, polygonal morphology at sub-confluent levels. However, after the first addition of thrombin, the PAECs were observed to become round and did not recover after the addition of PD98059 to inhibit the action of thrombin as seen in Figure 5.7d. Similarly, the AEIROF was visible on the chip before (Figure 5.7d) and after (Figure 5.7f) the PAECs experiment.

Figure 5.6 Light microscopy images (40X) of the control chip without PAECs (a) after removing from the incubator and (b) after the experiment. Inverted light microscopy images (67X) of control chip without PAECs (c) after removing from the incubator and (d) after the experiment. Electrode with PEI-coated AEIROF circled in red.

Figure 5.7 Light microscopy images (40X) of chip with PAECs (a) after removing from the incubator and (b) after the second dose of PD98059. Light microscopy images (100X) of showing morphology of PAECs on chip (c) after removing from the incubator and (d) after the second dose of PD98059. Inverted light microscopy images (67X) of chip with PAECs (e) after removing from the incubator and (f) after the experiment. Electrode with PEI-coated AEIROF circled in red.

5.4.4 Effect of fibronectin coating on pH responses of sensors in cell culture medium

The preliminary data acquired for the control experiment and PAECs experiment are presented in Figure 5.8a and Figure 5.8b, respectively. Curve smoothing was carried out using a Savitzky Golay filter with second order polynomial fitting with 301 points for each of the 30 min run and 51 points for the 5 min calibration runs. While the data has been presented in a continuous time format to facilitate visualisation and comparison of results, there is an approximately 5 min lag time in between each step and the first two-point calibration was performed a day after the fibronectin had been coated. The chip was also disconnected from the 5% CO₂ supply for the reagents to be added, during which the cell culture medium would be susceptible to losing some of its buffering capacity. All values for sensitivity reported here are corrected to 25.0 °C.

Prior to coating with fibronectin, the PEI-coated AEIROF control chip has a sensitivity of -0.073 V pH⁻¹ and formal potential of 0.548 V in phosphate buffer. After coating with fibronectin, the control chip gives a sensitivity of -0.061 V pH⁻¹ and formal potential of 0.847 V when calibrated in pH 6.5 and 7.5 cell culture medium. The other chip to be seeded with PAECs has a sensitivity of -0.068 V pH⁻¹ and formal potential of 0.540 V before coating with fibronectin. With fibronectin, it has a sensitivity of -0.066 V pH⁻¹ and formal potential of 0.649 V. A paired two tailed t-test ($\alpha = 0.05$) for the sensitivity gives $t = 1.25$, which lies within $-12.71 \leq t \leq 12.71$, so there is no significant difference in the mean sensitivity before and after coating with fibronectin. The paired two-tailed t-test (α

= 0.05) for the formal potential gives $t = 2.15$, which lies within $-12.71 \leq t \leq 12.71$, so there is no significant difference in the mean formal potential before and after coating with fibronectin. The changes in sensitivity and formal potential to each chip could be due to the fibronectin, adsorption of proteins in the cell culture medium onto the film or that insufficient time has passed for the rehydration of the AEIROFs since coating the chip with fibronectin requires drying the chip.

5.4.5 Real-time measurements of extracellular pH

Following incubation overnight, both the control chip and chip with PAECs show a large drop of around 0.25 V and 0.30 V in OCP relative to the calibration value for pH 7.5, respectively. An earlier control experiment using the glass pH meter to measure the pH of cell culture medium without cells before and after it was placed in incubator overnight showed that the pH of the cell culture remained at 7.62. While looking at the data alone suggests that the pH had risen to pH 12, the colour of the cell culture medium containing phenol red indicator still appeared red and had neither turned bright pink nor yellow. Therefore, the pH must still be above 6.8 and below 8.2. Furthermore, the PAECs look healthy from the light microscopy images (Figures 5.7a, c) and they would otherwise have died and rounded up or detached if the pH had fallen drastically. This suggests that incubation process, possibly the heat, had modified the ratio of Ir(III) oxide to Ir(IV) oxide, resulting in a fall in formal potential, and the OCP measured immediately after removing the chip from the incubator was much lower than expected.

(a)

(b)

Figure 5.8 OCP/V vs. time/ks measurements of the addition of reagents to (a) control chip and (b) chip with PAECs. Measurements were done at an average room temperature of 22.0 °C and gassed with 5% CO₂.

In the control experiment shown in Figure 5.8a, the addition of 5 U mL⁻¹ human plasma thrombin introduces a rise in OCP of 0.113 V (or a decrease of ~2 pH units) at the end of 30 min. The 0.1% (w/v) BSA used to suspend the thrombin was pH 5.30, which could have a minor contribution towards increasing the OCP. The bulk of the decrease in pH is thought to have come from the dissolution of fibronectin into the solution following exposure to thrombin. Galdal et al. reported that 2 NIH U mL⁻¹ bovine thrombin induced the loss of extracellular fibronectin fibrils from HUVECs in 30 min [266]. Furthermore, when the control chip was reused for the PAECs experiment, the PAECs surprisingly did not attach and appeared round, suggesting that the fibronectin had been denatured or lost during the control experiment.

A second dose of thrombin did not appear to have an appreciable effect on changing the OCP on the control chip. The two doses of PD98059 also did not change the OCP much with an average change of ± 0.018 V, which made it ideal for studying any effects the inhibitor would have on the PAECs.

The concentrations of the aqueous H₃PO₄ solution added after the two doses of PD98059 are carefully chosen to be within the buffering capacity of the cell culture medium and also to prevent further denaturation of the fibronectin. The addition of the acid would demonstrate that had the buffering capacity of the cell culture medium been exceeded due to the action of the reagents, an obvious change in the OCP should have been recorded. The addition of 5.88 mM H₃PO₄ causes the OCP to fall by 0.031 V (or increase by ~0.5 pH unit) instead of increasing the OCP. This could be due to the H⁺ combining with the HCO₃⁻ in the cell culture medium

to form CO_2 and hence adding to the buffer capacity of the solution. Upon giving 11.1 mM H_3PO_4 , a very steady OCP is observed, perhaps indicating that the $\text{HCO}_3^-/\text{CO}_2$ equilibrium has been re-established. The two-point calibration after the control experiment shows that the super-Nernstian performance of the sensor is retained with a sensitivity of -0.080 V pH^{-1} and formal potential of 0.658V, which confirms that the OCP measurements are valid.

As seen in Figure 5.8b for the chip with PAECs, the first dose of thrombin induces rapid extracellular acidification of PAECs as expected [262]. The local pH decreased by close to 7 pH units within minutes and 2 pH units can be attributed to the dissolution of fibronectin. Therefore, there is a net drop in 5 pH units due the action of 5 U mL^{-1} thrombin on the cells. This is followed by a fast, steady decline in OCP, which might be associated with a reduction in extracellular acidification as the PAEC morphology changed from a polygonal phenotype to a contracted phenotype by the end of 30 min. Such a change in cell shape had been reported by Galdal et al. after they exposed confluent monolayer cultures of human umbilical vein endothelial cells (HUVECs) to $0.5 \text{ NIH U mL}^{-1}$ human α -thrombin for 30 min [267]. They also found that thrombin altered the HUVEC integrity and the cell membrane appeared to be more permeable [267]. The increase in membrane permeability could be due to the rapid disassembly of claudin-5 from the tight junctions of endothelial cells under thrombin stimulation as reported by Kondo et al. [268]. Clearly, more in depth studies would be required to understand the mechanism of thrombin on both the change in cell morphology and extracellular

acidification and how the two are related. The sudden rapid change in pH could also shock the cells and result in cell death.

During the second dose of thrombin, the PAECs give a more steady OCP than during the first dose and the steady decline in OCP was not observed, probably because the rate of change of PAECs from the polygonal phenotype to contracted phenotype has reached its maximum and was quite stable. The pH is still about 4.6 units lower than the pH value after equilibration to ambient conditions. Taking into account the 2 pH units from the dissolution of fibronectin, 10 U mL^{-1} thrombin continues to have a net effect of lowering the pH by 2.6 units.

The addition of $50 \text{ }\mu\text{M}$ PD98059 caused a reduction in extracellular acidification [262], resulting in a decrease in the OCP as expected and the pH falls by about 0.8 pH unit. A second dose of PD98059 causes the OCP to fall further by almost 1 pH unit as the extracellular acidification was further reduced. However, the PD98059 did not cause the cells to recover to their polygonal phenotype for the duration at which the experiment was performed.

When 5.88 mM H_3PO_4 was added, a gradual increase in OCP corresponding to a decrease in 1 pH unit is observed. This clearly shows that the buffer capacity of the cell culture medium had been depleted during the previous steps. Further addition of H_3PO_4 to a final concentration of 11.11 mM induces an initial decrease in pH by 0.8 unit but the pH rose by the same amount at the end of 30 min. This could possibly be the same phenomenon as in the control experiment when the addition of acid appears to be increasing the buffer capacity of the chip. The two-point calibration after the experiment shows that the sensor preserved its

super-Nernstian response to pH with a sensitivity of -0.075 V pH^{-1} and formal potential of 0.748V.

In both the control chip and chip with PAECs, the two-point calibration in cell culture medium after the experiment shows that the super-Nernstian sensitivities of the AEIROFs are retained. A paired two tailed t-test ($\alpha = 0.05$) for the sensitivity gives $t = -1.06$, which lies within $-12.71 \leq t \leq 12.71$, so there is no significant difference in the mean sensitivity before incubation (calibration in cell culture medium for fibronectin-coated chips) and after the experiment. The paired two tailed t-test ($\alpha = 0.05$) for the formal potential gives $t = -0.31$, which lies within $-12.71 \leq t \leq 12.71$, so there is no difference in the mean formal potential before incubation (calibration in cell culture medium for fibronectin-coated chips) and after the experiment. In addition, the presence of the AEIROFs were confirmed from the inverted light microscopy images taken after the experiment. Thus, the changes in pH measured from the OCP are valid.

However, the formal potential is different from the expected value assuming that the pH should be $\sim \text{pH } 7.6$ at the end of the equilibration run. This could be due to a change in the ratio of the Ir(III) oxide to Ir(IV) oxide during the course of the experiment and insufficient time had passed to allow for the species to equilibrate. Hence, while the sensors cannot be used to accurately determine the pH of the solution without knowing the values to expect, their high sensitivities are extremely advantageous to measuring acute pH changes in real-time.

The interaction of thrombin, PAECs and fibronectin and its acute effects on extracellular pH are studied in this work. Thrombin induces the dissolution of

fibronectin and PAECs exhibits a change in cell morphology as it loses its attachment to fibronectin [266]. Thrombin also causes acute extracellular acidification of the PAECs through a MAP kinase-dependent pathway [262], which was partially inhibited by the addition of the PD98059 inhibitor. The increase in extracellular acidification may be attributed to increased glycolysis and Na^+/H^+ exchange by the PAECs [262], due to an increase in cell metabolism. Such effects of thrombin on PAECs and fibronectin are significant for the activation of PAECs. Thrombin causes ECs to detach from the extracellular matrix and increases their metabolism and causes cytoskeleton reorganisation, likely in preparation for proliferation and migration in thrombin-mediated angiogenesis [262].

5.5 Conclusion

AEIROFs were miniaturised onto MEA chips and coated with 5% PEI and the resulting film demonstrated super-Nernstian responses to pH. The chip was then coated with fibronectin for the on-chip culture of PAECs. The changes in extracellular pH of the PAECs exposed to fibronectin were investigated in real-time. Thrombin dissolves fibronectin and causes the pH to fall. Thrombin also induces the acute extracellular acidification by the PAECs, which likely indicates an increase in cell metabolism. The morphology of the cells also changed from a stretched phenotype to a round phenotype. PD98059, the MAP kinase inhibitor, reduces extracellular acidification. This shows that the effect of thrombin on extracellular acidification of the cells requires a MAP kinase-dependent pathway. This effects of thrombin on PAECs and fibronectin is significant for the activation of PAECs. Thrombin causes ECs to lose their anchorage on the extracellular matrix and acutely increases cell metabolism and cytoskeleton reorganisation to prepare for cell proliferation and migration in thrombin-mediated angiogenesis.

6 O₂ and glucose biosensors based on a novel electropolymerised redox polymer

6.1 Abstract

An O₂ biosensor and a glucose biosensor based on a novel electropolymerised redox polymer film are developed. To prepare the O₂ biosensor, 20 mM methylene blue (MB⁺) and 1 mM pyrrole were mixed together as a precursor solution to electrodeposit a film on a Au electrode by cyclic voltammetry (CV). The glucose biosensor was electrochemically fabricated in the solution prepared by adding 4 mg mL⁻¹ glucose oxidase (GOD) to the precursor solution described above. The presence of pyrrole prevents the formation of MB⁺ monomers in the polymer film, giving rise to the electrochemical, followed by catalytic (EC') mechanism of the film and enhances the stability of the polymer film. The O₂ biosensor (Au/PMB-PPy) has a superior sensitivity of 244 $\mu\text{A mM}^{-1} \text{cm}^{-2}$, linear range of 0.014 mM to 0.254 mM and detection limit of 0.597 μM (S/N = 3) for dissolved O₂. Notably, it is pH-insensitive in both N₂-purged and atmospheric (atm) O₂ PBS from pH 4 to 8 and is promising for use in physiological conditions where pH fluctuations are expected. The glucose biosensor (Au/PMB-PPy-GOD) exhibits direct electron transfer (DET) in N₂-purged PBS and glucose solutions. In atm O₂, it possesses a sensitivity of 6.06 $\mu\text{A mM}^{-1} \text{cm}^{-2}$ in the linear range of 0.25 mM to 1.49 mM glucose with a detection limit of 29.7 μM glucose (S/N = 3). It is pH-insensitive in N₂-purged PBS from pH 6 to 8 and atm O₂ PBS from pH 4 to 8, highlighting its potential for use in physiological pH conditions.

6.2 Introduction

The levels of O_2 and glucose play key roles in regulating angiogenesis as explained in Chapters 2.1 and 2.2. Protein hydroxylase domain containing protein 2 (PHD-2) is inhibited during hypoxia and in the presence of lactate, a by-product of glucose metabolism [12]. This in turn allows the hypoxia inducible factors (HIFs) to enter the nucleus and NO and vascular endothelial growth factor A (VEGFA), which upregulate angiogenesis, are produced [36, 37]. Hence, the real-time detection of extracellular levels of dissolved O_2 and glucose of endothelial cells (ECs) are important for the study of angiogenesis.

The miniaturisation of electrodes onto lab-on-a-chip devices is an attractive approach for the real-time detection of biomolecules uptaken or released by cells or tissues on the chip. An electrochemical cytosensorTM microphysiometer capable of measuring extracellular dissolved O_2 , glucose, lactate and acidification rate using modified Pt microelectrodes has been reported [269]. Dissolved O_2 was measured by electrochemical reduction a Nafion-coated Pt microelectrode, glucose and lactate by oxidising H_2O_2 produced by the corresponding first generation enzymatic biosensors, and acidification rate by potentiometry. However, there have been limited examples of nanomaterials-based electrochemical microphysiometer for the simultaneous detection of metabolic markers. The primary difficulty lies in designing an electrode modification method which allows each microelectrode on the chip to be individually modified. This can be overcome through the use of electrochemical growth techniques such as electrodeposition or electropolymerisation.

In this work, O₂ and glucose biosensors were prepared by one-step electropolymerisation so that they can be independently miniaturised onto lab-on-a-chip devices in future to investigate the effects on which the degree of hypoxia has on the glucose metabolism of ECs. Amperometric O₂ biosensors based on direct electrochemical reduction of O₂ suffer from the interference of solution pH, since its reaction involves H⁺ and makes it pH-sensitive. This problem was addressed by indirectly detecting dissolved O₂ by coupling it to an electrochemical reaction that is not pH-sensitive.

The second challenge is that the glucose biosensor must be able to operate under hypoxia for our purposes. First generation enzymatic biosensors require O₂ to function while second generation enzymatic biosensors suffer from the leaching of mediators [170]. In the third generation glucose biosensors, nanomaterials or nanocomposites enable the direct electron transfer (DET) of the flavine adenine dinucleotide (FAD) active centre of the glucose oxidase (GOD) [176], allowing the reagentless glucose biosensor to operate without O₂, making them the best approach to the problem.

So far, there have been few examples of one-step electropolymerised third generation glucose biosensors. Many third generation glucose biosensors require multiple preparation steps and GOD is often adsorbed [83, 173, 175, 176, 270] onto the nanomaterials or nanocomposites, but such methods are unfeasible for modifying individual microelectrodes on a chip. Polypyrrole (PPy) is a popular choice for the one-step electropolymerisation with GOD for glucose biosensors since it forms a conductive polymer at physiological pH and preserves the activity

of GOD [271, 272]. However, PPy has poor stability in aqueous solution due to solution attack, which results in the loss of electronic communication between the PPy molecules [273]. Phenothiazines such as methylene blue (MB^+) have been used as soluble mediators in enzymatic biosensors. Interestingly, electropolymerisation of MB^+ within physiological pH yield conductive poly(methylene blue) (PMB), making it attractive for one-step electropolymerisation with GOD. Recently, two step-electropolymerisation was used to prepare PMB-doped silica nanocomposites on a glassy carbon electrode, which showed electrocatalytic activity towards the reduction of dissolved O_2 [121] and GOD cast onto the PMB-doped silica nanocomposites showed DET [122], highlighting the feasibility of using PMB for the detection of O_2 and for making a third generation glucose biosensor.

It is found that PMB electropolymerised on a Au electrode (Au/PMB) has poor stability (Figure S6), perhaps due to the bulky size of the MB^+ monomer, which makes the formation of continuous polymer chains difficult. To overcome this problem, pyrrole was introduced to stabilise the polymer formed. The synergistic effects of PMB with PPy are described in this work.

Figure S6 CV of AulPMB in atm O₂ PBS on the 1st day (red) and 9th day (blue) ($v = 0.050 \text{ V s}^{-1}$).

6.3 Experimental methods

6.3.1 Chemicals

MB⁺, pyrrole, GOD from *Aspergillus niger*, β-D-glucose, H₂SO₄, Na₂SO₄, KOH and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. Na₂HPO₄ and KH₂PO₄ were obtained from Fisher Scientific. HCl was acquired from Analar and ethanol from Aik Moh Chemicals. DI water was used for all experiments. Pyrrole was distilled and stored under N₂-protection in the dark at 4 °C when not in use. PBS of acidic pH was prepared by adding HCl and that of alkaline pH was made by adding KOH. 0.01M PBS (pH 7.4) was bubbled with N₂ or O₂ at least 20 min for experiments in N₂-purged PBS and O₂-saturated PBS,

respectively. Glucose solutions were prepared in PBS at least one day in advance to allow time for the mutarotation of the glucose molecules.

6.3.2 Instruments

A CHI 660D electrochemical workstation (CH Instruments, Inc.) was used. A three-electrode setup was employed for all electrochemical experiments with a Ag|AgCl reference electrode (0.552 cm^2) containing saturated aqueous KCl solution (-0.039 V with respect to saturated calomel electrode), Au (2 mm diameter) working electrode and Pt coil (0.393 cm^2) as the counter electrode. A Hanna pH meter (Sigma-Aldrich) was used to measure the pH of the solutions. For FESEM imaging, the polymer films were grown on Au electrodes prepared by physical vapour deposition on indium tin oxide glass and imaged using Jeol JSM-6700F FESEM.

6.3.3 Preparation of dissolved O_2 and glucose biosensors

The Au electrode was polished with $0.05\text{ }\mu\text{m}$ alumina slurry, sonicated in ethanol, followed by DI water. It was then electrochemically cleaned in 0.5 M H_2SO_4 by CV (-0.5 V to 1.7 V , 0.5 V s^{-1} , 25 cycles), held at -0.5 V for 5 min to reduce any oxide formed, then rinsed with DI water and dried by N_2 . A pH 7.3 buffer solution containing 0.1 M Na_2HPO_4 , 0.025 M KH_2PO_4 and 0.1 M Na_2SO_4 was prepared. Fresh 20 mM MB^+ and 1 mM pyrrole were added to the buffer solution as the precursor to fabricate the O_2 biosensor, and an additional 4 mg mL^{-1}

GOD was added for the construction of the glucose biosensor. SO_4^{2-} is a catalyst for the electropolymerisation of MB^+ and the electropolymerisation is more efficient at alkaline pH [119]. The electrode was quickly immersed into the precursor solution and CV (-0.6 V to 1.2 V, 0.1 V s^{-1} , 50 cycles) was used to electropolymerise the monomers without or with GOD onto the surface of the electrode, forming Au/PMB-PPy and Au/PMB-PPy-GOD as the O_2 and glucose biosensors, respectively. After electropolymerisation, the electrode was soaked in 0.01 M PBS several times to remove the excess MB^+ . This was followed by potential cycling in PBS (-0.5 V to 0.2 V, 0.1 V s^{-1} , 10 cycles) for anion exchange and the electrode was stored in PBS at 4°C overnight before use.

6.4 Results and discussion

6.4.1 FESEM characterisation of O_2 biosensor

Figure 6.1 shows the FESEM images of PMB-PPy. The PMB-PPy appears flat and is dotted with spots (Figure 6.1a), which are likely salt crystals and small clumps of polymer as seen in the high magnification image (Figure 6.1b). The generally flat surface of the film implies that it is not over-oxidised, since rough films are formed when the film is over-oxidised [127].

Figure 6.1 (a) Low magnification FESEM image (2200X) and (b) high magnification FESEM image (35 000X) of PMB-PPy.

6.4.2 Electrochemical characterisation of O₂ biosensor in N₂-purged PBS

Au|PMB-PPy was characterised using different scan rates by CV in N₂-purged PBS. As seen in Figure 6.2, a pair of redox peaks are observed around -0.34 V and -0.26 V. The Au|PMB-PPy demonstrates diffusion-limited behaviour since the gradient of both curves in Figure 6.3 are close to 0.5, which implies that the cathodic ($i_{p,c}$) and anodic ($i_{p,a}$) peak currents scale almost proportionally with the square root of scan rate ($v^{0.5}$). This behaviour is in agreement with that reported by Barsan et al. for PMB [119], who proposed that the diffusion of counterions into or out of the polymer film is rate-limiting. From Figure 6.4, the anodic peak

potential ($E_{p,a}$) remains constant with different ν and this is characteristic of a reversible reaction, while the cathodic peak potential ($E_{p,c}$) shifts negatively with increasing ν . The formal potential (E^0) was estimated by $\frac{E_{p,a} + E_{p,c}}{2}$ and the peak separation (ΔE_p) was calculated by ($E_{p,a} - E_{p,c}$) and given in Table 6.1. From Table 6.1, ΔE_p suggests that the reaction is a highly reversible 1 e^- transfer at 0.025 V s^{-1} , but becomes quasi-reversible at higher ν .

Figure 6.2 CV of Au/PMB-PPy in N₂-purged PBS with different scan rates.

Figure 6.3 Plot of $\log(-i_{p,c})/A$ against $\log v/V s^{-1}$ (red circles) and plot of $\log(i_{p,a})/A$ against $\log v/V s^{-1}$ (blue squares) for AuPMB-PPy in N_2 -purged PBS.

Figure 6.4 Plot of $E_{p,c}/V$ (red circles) and $E_{p,a}/V$ (blue squares) against $v/V s^{-1}$ for AuPMB-PPy in N_2 -purged PBS.

Table 6.1 Values of formal potential (E^0)/V and peak separation (ΔE_p)/V against scan rate/V s⁻¹ for Au/PMB-PPy in N₂-purged PBS with different scan rates.

Scan rate/ V s ⁻¹	E^0 / V	ΔE_p / V
0.005	-0.259	0.047
0.010	-0.260	0.049
0.025	-0.264	0.059
0.050	-0.269	0.075
0.075	-0.270	0.079
0.100	-0.274	0.082
0.200	-0.280	0.096
0.300	-0.285	0.097
0.400	-0.286	0.105
0.500	-0.288	0.106

6.4.3 Effect of pH on O₂ biosensor in N₂-purged PBS

Since it is of interest to utilise the O₂ biosensor under physiological conditions, the effect of pH on the AulPMB-PPy is investigated (Figure 6.5). As seen in Table 6.2, ΔE_p increases as pH decreases. The value of ΔE_p at pH 8 implies a 1 e⁻ transfer reaction, which becomes increasingly sluggish as pH falls and it appears kinetically more difficult to reoxidise the polymer. The loss of e⁻ needs to be accompanied by the expulsion of positively charged counterions to maintain charge neutrality within the film. Since there are more H⁺ in the solution as pH falls, it becomes harder for these counterions to be expelled. This, however, does not affect the amperometric detection of dissolved O₂ using the AulPMB-PPy since a constant cathodic potential will be applied. An extra cathodic peak is observed around -0.10 V in pH 4. This may be due to Cl⁻ de-doping from the polymer film since the largest amount of HCl was added to prepare the pH 4 PBS.

The negligible effect of pH on PMB-PPy (Figure 6.5) is interesting as it differs from the expected pH-dependent electrochemistry of PMB alone (Figure 6.7) [118], where parallels can be drawn to that of MB⁺ (Figure 6.8) [108, 109]. Dimers of MB⁺ form at the N with the methyl groups, with longer chains giving rise to PMB (Figure 6.6) [119]. The N at the centre of the ring has a lone-pair and can accept a H⁺. Hence, the electrochemical reduction of poly(MB⁺) to poly(LMB) is expected to be pH-dependent (Figure 6.7). However, the reduction of PMB-PPy appears to be pH-independent (Figure 6.5). It could be that during electropolymerisation, pyrrole reacts with MB⁺ and binds to the N in the centre of the ring and the resulting PMB-PPy becomes unable to accept H⁺. The following equation is proposed for the electrochemistry of AulPMB-PPy in N₂-purged PBS,

Figure 6.5 Effect of pH on AulPMB-PPy in N₂-purged PBS ($\nu = 0.05 \text{ V s}^{-1}$).

Table 6.2 Values of $\Delta E_p/V$ against pH of Au/PMB-PPy in N₂-purged PBS.

pH	$\Delta E_p/V$
4	0.135
5	0.116
6	0.098
7	0.094
8	0.070

Figure 6.6 Chemical structure of MB⁺ dimer.

Figure 6.7 Electrochemical reduction of poly(MB^+) to poly(LMB).

Figure 6.8 (a) Chemical structure of MB^+ . (b) The electrochemical reduction of MB^+ to LMB.

6.4.4 Electrochemical impedance spectroscopy of O₂ biosensor in N₂-purged PBS

The electrochemical impedance spectroscopy (EIS) of the Au/PMB-PPy (Figure 6.9a) was performed with an amplitude of 0.005 V from 0.01 Hz to 1.00 MHz at the respective cathodic half-peak potential in N₂-purged PBS of different pH to obtain the charge transfer resistance (R_{ct}) and exchange current density (j_0) in the kinetically controlled region of the CV and compared to that of a bare Au electrode (Figure 6.9b). At low frequencies, the data points deviate from the expected straight line due to natural convection. However, the maximum of each semicircle is visible and given by $f = \frac{1}{2\pi R_{ct} C_{dl}}$ and R_{ct} is twice the radius of the semicircle. The exchange current density is given by $j_0 = \frac{RT}{nFR_{ct}}$. As seen in Figure 6.10, the R_{ct} of the Au/PMB-PPy and bare Au follow different trends and change with pH. However, they are still on the same order of magnitude, indicating that the presence of the film does not severely alter the R_{ct} . From Table 6.3, the j_0 of the Au/PMB-PPy are also similar to those of the bare Au. The double-layer capacitance (C_{dl}) of the Au/PMB-PPy is smaller than that of the bare Au as expected, indicating the presence of the film on the electrode.

Figure 6.9 EIS of (a) AuPMB-PPy and (b) bare Au with amplitude of 0.005 V from 0.01 Hz to 1.00 MHz in N_2 -purged PBS from pH 4 to 8.

Figure 6.10 Plot of $R_{ct} \times 10^5 / \Omega$ against pH for AulPMB-PPy (red circles) and bare Au (blue squares) in N_2 -purged PBS.

Table 6.3 Values of $R_{ct} \times 10^5/\Omega$, $C_{dl}/\mu F$ and $j_0/\mu A \text{ cm}^{-2}$ against pH for Au/PMB-PPy and bare Au in N_2 -purged PBS.

Electrode	pH	$R_{ct} \times 10^5/\Omega$	$C_{dl}/\mu F$	$j_0/\mu A \text{ cm}^{-2}$
Au/PMB-PPy	8	2.917	3.069	2.802
	7	5.247	3.675	1.558
	6	4.199	4.593	1.947
	5	3.563	4.467	2.294
	4	1.970	3.095	4.149
Bare Au	8	2.654	7.264	3.079
	7	2.158	7.374	3.787
	6	1.944	8.185	4.204
	5	3.023	9.364	2.704
	4	4.241	9.796	1.927

6.4.5 Electrochemical characterisation of O₂ biosensor in atm O₂ PBS

The effect of PPy on the AulPMB-PPy (Figure 6.11) is apparent when compared to the CV of AulPMB (Figure S6). The presence of PPy causes the pair of peaks around 0.1 V and -0.1 V, which likely belongs to the MB⁺ monomer (Figure S6) to disappear. In addition, no anodic peak is observed for the AulPMB-PPy, indicating a catalytic reaction. The cathodic reaction of AulPMB-PPy shows diffusion-limited behaviour as deduced from Figure 6.12, since the gradient is close to 0.5. $E_{p,c}$ shifts negatively with increasing ν and the plot of $|i_{p,c}|/\nu^{0.5}$ against ν (Figure 6.13) reveals an electrochemical followed by catalytic reaction (EC') mechanism since $|i_{p,c}|/\nu^{0.5}$ falls with ν [72]. It appears that PMB-PPy is electrochemically reduced and then rapidly reoxidised by dissolved O₂.

Figure 6.11 CV of AulPMB-PPy in atm O₂ PBS with different scan rates.

Figure 6.12 Plot of $\log(-i_{p,c})/A$ against $\log v/V \text{ s}^{-1}$ for Au|PMB-PPy in atm O_2 PBS.

Figure 6.13 Plot of $|i_{p,c}|v^{-0.5}/\mu A V^{-0.5} s^{0.5}$ against $v/V \text{ s}^{-1}$ for Au|PMB-PPy in atm O_2 PBS.

6.4.6 Effect of pH on O₂ biosensor in atm O₂ PBS

pH is found to have negligible effects on the AulPMB-PPy (Figure 6.14), thereby fulfilling the aim of making a pH-insensitive O₂ biosensor. If the cathodic peak were to belong to the electrochemical reduction of dissolved O₂, the reaction would be pH-dependent since O₂ reduction requires H⁺. Since this is not so, the cathodic peak must belong to the electrochemical reduction of PMB-PPy described in equation 6.1 and this supports the EC' mechanism proposed earlier. The following equations describe the EC' mechanism of AulPMB-PPy in atm O₂ PBS, where PMB-PPy is first electrochemically reduced (equation 6.2a), then chemically reoxidised by dissolved O₂ in a catalytic reaction (equation 6.2b),

The pH-independence of AulPMB-PPy in N₂-purged and atm O₂ PBS is attributed to the synergistic effects of PMB and PPy, making it a promising material for detecting dissolved O₂ even with fluctuating pH.

Figure 6.14 Effect of pH on AulPMB-PPy in atm O₂ PBS.

6.4.7 Electrochemical impedance spectroscopy of O₂ biosensor in atm O₂ PBS

The EIS was performed with an amplitude of 0.005 V from 0.01 Hz to 1.00 MHz at the respective cathodic half-peak potential in atm O₂ PBS of different pH for the AulPMB-PPy (Figure 6.15a) and bare Au electrode (Figure 6.15b). From Figure 6.16 and Table 6.3, the R_{ct} and j_0 of the AulPMB-PPy and bare Au are found to be similar. The C_{dl} of the AulPMB-PPy is consistently smaller than the bare Au as expected. Comparing Table 6.4 to Table 6.3, R_{ct} appears to decrease in the presence of O₂.

Figure 6.15 EIS of (a) AulPMB-PPy and (b) bare Au with amplitude of 0.005 V from 0.01 Hz to 1.00 MHz in atm O₂ PBS from pH 4 to 8.

Figure 6.16 Plot of $R_{ct} \times 10^5 / \Omega$ against pH for AulPMB-PPy (red circles) and bare Au (blue squares) in atm O_2 PBS.

Table 6.4 Values of $R_{ct} \times 10^5/\Omega$, $C_{dl}/\mu F$ and $j_0/\mu A \text{ cm}^{-2}$ against pH for Au/PMB-PPy and bare Au in atm O_2 PBS.

Electrode	pH	$R_{ct} \times 10^5/\Omega$	$C_{dl}/\mu F$	$j_0/\mu A \text{ cm}^{-2}$
Au/PMB-PPy	8	0.969	1.989	8.432
	7	0.940	2.051	8.693
	6	1.004	2.326	8.138
	5	1.045	2.236	7.822
	4	1.044	2.237	7.827
Bare Au	8	0.885	4.696	9.240
	7	1.045	4.818	7.824
	6	0.907	4.582	9.016
	5	0.987	5.103	8.286
	4	0.939	5.363	8.709

6.4.8 CV of O₂ biosensor in different concentrations of dissolved O₂

AuPMB-PPy (Figure 6.17a) and bare Au (Figure 6.17b) give comparable cathodic peak currents to different concentrations of dissolved O₂, albeit at different peak potentials. In atm O₂ PBS, the AuPMB-PPy produces a single cathodic peak by the EC' mechanism while the bare Au gives 2 cathodic peaks as electrochemical reduction of O₂ occurs as a 2-step reaction. In O₂-saturated PBS, a peak at 0.04V is observed for the Au electrode, which might be the oxidation of H₂O₂. However, no such peak is observed for the AuPMB-PPy. Although the cathodic peak potentials of AuPMB-PPy are more negative than bare Au, the bare Au requires electrochemically cleaning between each measurement to give reproducible results while the AuPMB-PPy does not. Bare Au is also susceptible to the adsorption of ions in the solution which causes its electrocatalytic properties to fall over time, while the AuPMB-PPy is able to give consistent CVs when left soaked in PBS. Hence, the ability of AuPMB-PPy to give peak currents comparable to bare Au in all three different concentrations of dissolved O₂ makes it a promising O₂ biosensor.

Figure 6.17 CV of (a) Au/PMB-PPy and (b) bare Au in N₂-purged PBS (red), atm O₂ PBS (blue) and O₂-saturated PBS (brown) ($\nu = 0.05 \text{ V s}^{-1}$).

6.4.9 Amperometric detection of dissolved O₂

The amperometric response of AulPMB-PPy and bare Au to increasing dissolved O₂ concentrations are compared (Figure 6.18). The concentration of saturated O₂ in water is reported as 1.1 mM [274]. At 25 °C and atm pressure, the solubility of O₂ in water is 8.24 mg L⁻¹ [275] and the concentration of dissolved O₂ is therefore 0.258 mM. From Figure 6.19a, the sensitivity of the AulPMB-PPy is found to be 244 $\mu\text{A mM}^{-1} \text{ cm}^{-2}$ (7.63 $\mu\text{A cm}^{-2} \text{ mg}^{-1} \text{ L}$) over a linear range of 0.014 mM (0.448 mg L⁻¹) to 0.254 mM (8.13 mg L⁻¹) with a detection limit of 0.597 μM (0.0191 mg L⁻¹) (S/N = 3) and has 90% response time of 12 s. In comparison, the bare Au has a sensitivity of 254 $\mu\text{A mM}^{-1} \text{ cm}^{-2}$ over a linear range of 0.014 mM to 0.174 mM as seen in Figure 6.19b and has a 90% response time of 12 s. The AulPMB-PPy outperforms the bare Au in terms of its linear range. The AulPMB-PPy also has superior sensitivity over other electrode materials listed in Table 6.5. The ability of AulPMB-PPy to cover a linear range up to atm O₂ outtrivals that of other electrode materials and is highly important for physiological applications of the AulPMB-PPy as an O₂ biosensor. The AulPMB-PPy was stable in storage at 4 °C in PBS over 18 days (Figure 6.20), which is superior to that of AulPMB (Figure S6) and it retained 97% of its initial current response in atm O₂ PBS.

Figure 6.18 Amperometric response of Au/PMB-PPy (red) and bare Au (blue) to successive additions of 100 μL O_2 -saturated PBS to 8 mL N_2 -purged PBS with magnetic stirring, which corresponds to 0.014 mM dissolved O_2 per addition. The first addition was at 100 s (arrow) and additions were made every 50 s thereafter ($V = -0.300$ V). The insets show the current response/ μA against time/s for the addition of 0.014 mM dissolved O_2 to (a) bare Au and (b) Au/PMB-PPy. The 90% response time of both electrodes is 12 s.

Figure 6.19 Calibration plot of increase in cathodic current/ μA against dissolved O_2 concentration/ mM for (a) AulPMB-PPy ($n=3$) and (b) bare Au. i is the increase in cathodic current in μA and c is the concentration of dissolved O_2 in mM .

Figure 6.20 CV of AulPMB-PPy in atm O₂ PBS on the 1st day (red) and 18th day (blue) ($\nu = 0.050 \text{ V s}^{-1}$).

Table 6.5 Comparison of O₂ biosensor performances

Electrode materials	Sensitivity/ $\mu\text{A cm}^{-2}$ $\text{mg}^{-1} \text{L}$	Linear range/ mg L^{-1}	Detection limit/ mg L^{-1}	Ref.
PMB-PPy	7.63	0.45-8.13	0.019	This work
Mesoporous SiO ₂ /C/cobalt (II) phthalocyanine	2.16	0.50-6.60	0.010	[155]
Layer-by-layer (iron(III) tetra-(N-methyl-4-pyridyl)- porphyrin/tetrasulfonated phthalocyanine)	4.12	0.20-6.40	0.060	[153]
Anthraquinone-derivative functionalised multi-walled carbon nanotubes nanowires	5.00	0.20-6.80	0.020	[157]

6.4.10 FESEM characterisation of glucose biosensor

The FESEM image of the PMB-PPy-GOD film is shown in Figure 6.21. This is different from the flat surface observed for PMB-PPy and the change arises from the presence of GOD. The resulting PMB-PPy-GOD has a wrinkled texture and the film appears interconnected, which might facilitate the movement of electrons across the polymer film.

Figure 6.21 FESEM image (2200X) of PMB-PPy-GOD.

6.4.11 Electrochemical characterisation of glucose biosensor in N₂-purged PBS

The AulPMB-PPy-GOD exhibits a pair of redox peaks around -0.34 V and -0.24 V (Figure 6.22), which show diffusion-limited behaviour as inferred from Figure 6.23, since the gradients are close to 0.5. This behaviour is different from the surface voltammetry behaviour observed when GOD is adsorbed on the surface of the electrode [176]. As only one pair of peaks is observed, it is likely that the redox peaks of GOD(FAD/FADH₂) are fused together with those of PMB-PPy. Like the AulPMB-PPy, $E_{p,a}$ remains rather constant, indicating a reversible reaction. $E_{p,c}$ shifts negatively with increasing ν (Figure 6.24), causing an increase in ΔE_p (Table 6.6) and this may imply a quasi-reversible reaction.

Figure 6.22 CV of AulPMB-PPy-GOD in N₂-purged PBS with different scan rates.

Figure 6.23 Plot of $\log(-i_{p,c})/A$ against $\log v/V \text{ s}^{-1}$ (red circles) and plot of $\log(i_{p,a})/A$ against $\log v/V \text{ s}^{-1}$ (blue squares) for Au/PMB-PPy-GOD in N_2 -purged PBS.

Figure 6.24 Plot of $E_{p,c}/V$ (red circles) and $E_{p,a}/V$ (blue squares) against $v/V \text{ s}^{-1}$ for Au/PMB-PPy-GOD in N_2 -purged PBS.

Table 6.6 Values of E^0/V and $\Delta E_p/V$ against scan rate/ $V\ s^{-1}$.

Scan rate/ $V\ s^{-1}$	E^0/V	$\Delta E_p/V$
0.005	-0.273	0.065
0.010	-0.272	0.071
0.025	-0.273	0.074
0.050	-0.276	0.078
0.075	-0.277	0.081
0.100	-0.277	0.085
0.200	-0.281	0.090
0.300	-0.281	0.102
0.400	-0.286	0.103
0.500	-0.290	0.113

6.4.12 Effect of pH on glucose biosensor in N₂-purged PBS and direct electron transfer of glucose oxidase

pH has an interesting effect on the peaks (Figure 6.25). A pair of peaks is observed from pH 6 to 8 but these split into two pairs of peaks at pH 4 and 5. From the pH behaviour of AulPMB-PPy (Figure 6.5), we can deduce that the pair of peaks at more negative potential at pH 4 and 5 belong to PMB-PPy since they display sub-Nernstian response towards pH, while the extra pair of peaks seen at less negative potentials must belong to the direct electron transfer (DET) of GOD. The DET of GOD refers to the ability of GOD(FADH₂) to be electrochemically oxidised to GOD(FAD) [176], which is a key feature of third generation glucose biosensor and important for its operation under hypoxic conditions.

Figure 6.25 Effect of pH on AulPMB-PPy-GOD in N₂-purged PBS ($\nu = 0.05 \text{ V s}^{-1}$).

Indeed, $E_{p,c}$ shifts negatively by -0.062 V pH^{-1} from pH 4 to 5, which is very close to that expected for the Nernstian behaviour of GOD(FAD/FADH₂) towards pH. In addition, the ΔE_p for this pair of peaks is 0.053V at pH 4 and 0.037V at pH 5, indicating that the redox reaction involves more than 1 e⁻ transfer. The following equations describe the redox behaviour of PMB-PPy-GOD.

From pH 6 to 8:

From pH 4 to 5:

From pH 6 to 8, the electrochemical reduction of PMB-PPy-GOD occurs as a single step and shows sub-Nernstian response to pH (equation 6.3). However, at pH 4 and 5, the GOD(FAD) is first electrochemically reduced (equation 6.4a), followed by the electrochemical reduction of PMB-PPy (equation 6.4b).

The sub-Nernstian behaviour of PMB-PPy-GOD towards pH from pH 6 to 8 makes it attractive for use under physiological conditions where the pH is not expected to fall below 6. Such unique behaviour for a third generation glucose biosensor is attributed to the use of the pH-insensitive PMB-PPy. It is possible that

the positively charged PMB and PPy and their monomers interact electrostatically with the negatively charged GOD during electropolymerisation and establish an electrically conductive path to the FAD/FADH₂ redox centre of GOD in the resulting PMB-PPy-GOD, giving rise to the DET of GOD.

6.4.13 Direct electron transfer of glucose oxidase in N₂-purged glucose solutions

The addition of glucose produces a decrease in $i_{p,c}$ and increase in $i_{p,a}$ as expected (Figure 6.26), demonstrating that the pair of peaks involve the DET of GOD(FAD/FADH₂) and verifies that the AuPMB-PPy-GOD is a third generation glucose biosensor. The reaction of the AuPMB-PPy-GOD in the N₂-purged glucose solutions can be described as such,

The PMB-PPy-GOD reacts enzymatically with glucose and GOD(FAD) is reduced (equation 6.5a). The PMB-PPy component of the PMB-PPy-GOD may exist as mixture of oxidised and reduced states if they contribute electrons towards the enzymatic reaction. During the negative scan of the CV, PMB-PPy-GOD is electrochemically reduced according to equation 6.3. $i_{p,c}$ decreases with increasing

glucose concentration since more of the PMB-PPy-GOD are already in their reduced state after the enzymatic reaction with glucose. Therefore, on the positive scan of the CV, $i_{p,a}$ increases with higher glucose concentration due to the greater amount of reduced PMB-PPy-GOD. This demonstrates the DET of GOD, where reduced GOD is reoxidised according to equation 6.5b.

Figure 6.26 CV of Au|PMB-PPy-GOD in PBS and different concentrations of glucose purged with N_2 ($v = 0.05 \text{ V s}^{-1}$).

6.4.14 Electrochemical characterisation of glucose biosensor in atm O₂ PBS

The CV of AulPMB-PPy-GOD is shown in Figure 6.27. AulPMB-PPy-GOD shows diffusion-limited behaviour, as inferred from Figure 6.28, since the gradient is close to 0.5. $E_{p,c}$ shifts negatively with increasing v and AulPMB-PPy-GOD is found to exhibit the EC' mechanism since $i_{p,c}v^{-0.5}$ falls with increasing v (Figure 6.29). The EC' mechanism of AulPMB-PPy-GOD can be described by the following equations, where PMB-PPy-GOD is electrochemically reduced (equation 6.6a) and then catalytically regenerated by dissolved O₂ (equation 6.6b).

Figure 6.27 CV of Au|PMB-PPy-GOD in atm O₂ PBS with different scan rates.

Figure 6.28 Plot of $\log(-i_{p,c})/A$ against $\log v/V \text{ s}^{-1}$ for Au/PMB-PPy-GOD in atm O_2 PBS.

Figure 6.29 Plot of $i_{p,c}|v|^{-0.5}/\mu A V^{-0.5} \text{ s}^{0.5}$ against $v/V \text{ s}^{-1}$ for Au/PMB-PPy-GOD in atm O_2 PBS.

6.4.15 Effect of pH on glucose biosensor in atm O₂ PBS

The effect of pH on AulPMB-PPy-GOD is different in atm O₂ PBS (Figure 6.30) and N₂-purged PBS (Figure 6.25). Here, AulPMB-PPy-GOD appears insensitive to pH, as expected from equation 6.6a. The presence of dissolved O₂ clearly alters the electrochemical behaviour of the AulPMB-PPy-GOD. The extra pair of peaks belonging to GOD(FAD/FADH₂) at pH 4 and 5 in N₂-purged PBS (Figure 6.25) are not seen here. While a small peak is observed at around -0.1V, this is similar to the one for AulPMB-PPy (Figure 6.14) and likely arises from the PMB-PPy and not the GOD. The insensitivity of AulPMB-PPy-GOD to pH in atm O₂ PBS highlights its great potential for use even with fluctuations in physiological pH levels as long as the supply of atm O₂ is maintained.

Figure 6.30 Effect of pH on AulPMB-PPy-GOD in atm O₂ PBS ($\nu = 0.05 \text{ V s}^{-1}$).

6.4.16 CV of glucose biosensor in different concentrations of glucose

The behaviour of Au/PMB-PPy-GOD to different concentrations of glucose at atm O₂ is investigated (Figure 6.31). The reaction still appears to follow the EC' mechanism. $i_{p,c}$ decreases while $i_{p,a}$ increases when glucose concentration is higher. PMB-PPy-GOD first reacts enzymatically with glucose according to equation 6.5a and is reduced. During the negative scan of the CV, any remaining PMB-PPy-GOD is electrochemically reduced. When glucose concentration is high, most of the PMB-PPy-GOD is already reduced following the enzymatic reaction, therefore $i_{p,c}$ is smaller in magnitude. The reduced PMB-PPy-GOD is then catalytically regenerated by its reaction with dissolved O₂ according to equation 6.6b.

Figure 6.31 CV of Au/PMB-PPy-GOD in PBS and different concentrations of glucose at atm O₂ ($v = 0.05 \text{ V s}^{-1}$).

6.4.17 Amperometric response of glucose biosensor

The amperometric detection of glucose using the AuPMB-PPy-GOD was carried out at atm O₂ and achieved a 90% response time of 7 s (Figure 6.32). The calibration plot is given in Figure 6.33 and the Lineweaver-Burk plot is shown in Figure 6.34. The Lineweaver-Burk expression [176] of the Michaelis-Menten equation is given by the equation

$$\frac{1}{i_{ss}} = \frac{K_M^{app}}{i_{max}} \frac{1}{c} + \frac{1}{i_{max}}$$

where i_{ss} is the steady-state current, K_M^{app} is the apparent Michaelis-Menten constant, c is the substrate concentration and i_{max} is the maximum current.

From Figure 6.32, the decrease in cathodic current prior to the first addition of glucose represents the consumption of dissolved O₂ to regenerate the reduced PMB-PPy-GOD in the absence of glucose, since PMB-PPy-GOD is also sensitive to dissolved O₂ concentrations. Hence, the baseline current for the calibration plot is taken at the foot of the current spike due to the first addition of glucose. From Figure 6.33, the sensitivity is 6.06 $\mu\text{A mM}^{-1} \text{cm}^{-2}$ from 0.25 mM to 1.49 mM glucose with a detection limit of 29.7 μM glucose ($S/N = 3$) and the K_M^{app} is 9.60 mM (Figure 6.34). As the concentration of glucose increases further, there are fewer free active sites of GOD to catalyse the reaction of glucose to gluconolactone, hence the sensitivity falls beyond 1.49 mM glucose. Nonetheless, AuPMB-PPy-GOD still offers higher sensitivity and lower detection limit over most of the electrode materials listed in Table 6.7.

From Figure 6.35, 10 mM ascorbic acid (AA) and 10 mM uric acid (UA) show negligible interferences on the average current responses of 0.25 mM glucose. The AulPMB-PPy-GOD was stable in storage at 4 °C in PBS over 29 days (Figure 6.36).

Figure 6.32 Amperometric response of AulPMB-PPy-GOD to successive additions of 10 μL 250 mM glucose to 10 mL of atm O₂ PBS with magnetic stirring, which corresponds to 0.25 mM glucose per addition. The first addition was at 50 s (arrow) and additions were made every 30 s thereafter ($V = -0.360$ V). The inset shows the current response/μA against time/s of the AulPMB-PPy-GOD to the addition of 0.25 mM glucose. The 90% response time is 7 s.

Figure 6.33 Calibration plot of decrease in cathodic current/ μA against glucose concentration/mM for AulPMB-PPy-GOD in atm O_2 ($n=3$). i is the decrease in cathodic current in μA and c is the concentration of glucose in mM.

Figure 6.34 Lineweaver-Burk plot corresponding to the linear range of 0.25 mM to 1.49 mM glucose. i_{ss} is the decrease in cathodic current in μA and c is the concentration of glucose in mM.

Figure 6.35 Current response of Au/PMB-PPy-GOD to 0.25 mM glucose vs. the interfering species, 10 mM ascorbic acid (AA) and 10 mM uric acid (UA) (n=3).

Figure 6.36 CV of Au/PMB-PPy-GOD in atm O₂ PBS on the 1st day (red) and 29th day (blue) ($\nu = 0.050 \text{ V s}^{-1}$).

Table 6.7 Comparison of glucose biosensor performances

Electrode materials	Sensitivity/ $\mu\text{A mM}^{-1} \text{cm}^{-2}$	Linear range/ mM	Detection limit/ μM	Ref.
PMB-PPy-GOD	6.06	0.25-1.49	29.7	This work
Poly(N[3-(trimethoxysilyl)propyl]aniline)/horse radish protein-GOD	0.16	1.00-20.00	100	[276]
TiO ₂ /carbon nanotubes/ Pt nanoparticles/GOD	0.24	0.006-1.50	5.70	[277]
Layer-by-layer (Au nanoparticles/thionine)/ GOD/horse radish protein	3.80	0-3.00	35.0	[114]

6.5 Conclusion

An O_2 biosensor, AulPMB-PPy, and a glucose biosensor, AulPMB-PPy-GOD, were prepared by one-step electropolymerisation of MB^+ and pyrrole, with the addition of GOD to prepare the glucose biosensor. The presence of pyrrole not only prevents the formation of MB^+ monomers in the polymer film and gives rise to its EC' mechanism, but also improves the stability of the polymer film. The O_2 biosensor (AulPMB-PPy) demonstrates a superior sensitivity of $244 \mu A mM^{-1} cm^{-2}$ over a linear range of 0.014 mM to 0.254 mM and has a detection limit of 0.597 μM ($S/N = 3$). It has a 90% response time of 12 s and good stability of 18 days. Notably, the AulPMB-PPy is pH-insensitive in N_2 -purged and atm O_2 PBS from pH 4 to 8 and is highly promising for use in physiological conditions where pH fluctuations are expected. The glucose biosensor (AulPMB-PPy-GOD) exhibits DET of GOD in N_2 -purged PBS and glucose solutions. It possesses a sensitivity of $6.06 \mu A mM^{-1} cm^{-2}$ in the linear range of 0.25 mM to 1.49 mM glucose with a detection limit of 29.7 μM glucose ($S/N = 3$). It has a 90% response time of 7 s and stability over 29 days. The AulPMB-PPy-GOD is pH-insensitive in N_2 -purged PBS from pH 6 to 8 and atm O_2 PBS from pH 4 to 8, highlighting its potential for use in physiological pH conditions. The two biosensors prepared here are feasible for future miniaturisation on lab-on-a-chip to probe the effect of the degree of hypoxia on the glucose uptake of ECs in real-time.

7 Overall conclusion and perspectives

Four works have been presented in this dissertation, which encompasses the design of nanomaterials-based electrochemical biosensors capable of the real-time, quantitative detection of the signalling molecule, NO, and metabolic markers, pH, O₂ and glucose, which are involved in angiogenesis. These biosensors are useful for studying the acute cellular responses of endothelial cells (ECs) to external stimuli, to gain a better understanding of angiogenesis. In addition, some of these biosensors have been shown to be biocompatible for the culture of ECs and are able to operate in cell culture medium.

In the first work, 3-D graphene/IL nanocomposite shows highly sensitive detection of NO in phosphate buffered saline (PBS) and its performance is superior to that of gold nanoparticles (AuNPs) and carbon nanotubes (CNTs).

In the second work, an arginine-glycine-aspartic acid (RGD)-peptide-functionalised biomimetic graphene film which functions as both a cell culture and sensing matrix is developed. Human umbilical vein endothelial cells (HUVECs) were cultured on the biomimetic graphene film and the NO released by these cells under acetylcholine (Ach) stimulation and NG-nitro-L-arginine methyl ester (L-NAME) inhibition were detected in real-time in cell culture medium. Ach increased the amount of NO released in a dose-dependent manner while L-NAME inhibited the release of NO, thereby providing insights to the real-time interactions between HUVECs and soluble drug molecules and the resulting release of NO.

In the third work, poly(ethyleneimine) (PEI)-coated anodically electrodeposited iridium oxide film (AEIROF) on a chip functions as a miniature

potentiometric pH sensor and exhibits super-Nernstian response to pH. The interaction of thrombin, porcine endothelial cells (PAECs) and fibronectin and their resulting changes to extracellular pH was investigated in real-time on-chip in cell culture medium. The pH falls as thrombin cause dissolution of fibronectin and extracellular acidification of PAECs, changing their morphology from stretched to round cells. PD98059, a mitogen-activated protein (MAP) kinase inhibitor, reduced extracellular acidification, indicating that thrombin acts through a MAP kinase-dependent pathway in PAECs to increase extracellular acidification. Thrombin not only causes ECs to lose their anchorage, but also acutely increases EC metabolism and cytoskeleton reorganisation, likely in preparation of cell proliferation and migration in thrombin-mediated angiogenesis.

Lastly, a novel electropolymerised redox polymer prepared from a precursor solution of methylene blue (MB^+) and pyrrole was used to fabricate an O_2 biosensor, with the addition of glucose oxidase (GOD) to the precursor solution for a glucose biosensor. The O_2 biosensor (Au/PMB-PPy) demonstrates superior sensitivity towards the detection of dissolved O_2 at atm O_2 concentration and below. Notably, it is insensitive to pH in both N_2 -purged and atm O_2 PBS from pH 4 to 8. The glucose biosensor (Au/PMB-PPy-GOD) exhibits direct electron transfer (DET) of GOD and is insensitive to pH from pH 6 to 8 in N_2 -purged PBS and from pH 4 to 8 in atm O_2 PBS. Since these biosensors can operate without O_2 , they are potentially useful for probing EC metabolism changes in relation to hypoxia during angiogenesis. In addition, their insensitivity to pH make them advantageous for studying EC metabolism studies where changes in physiological pH are expected.

Their ease of miniaturisation allow their potential application for the simultaneous detection of O₂ and glucose of ECs on lab-on-a-chip devices.

Despite the overall good performances of these biosensors, each biosensor has its own limitations and improvements need to be made to overcome these problems. The 3-D graphene/IL nanocomposite on a glassy carbon electrode is unsuitable for detecting NO released by ECs on a cell culture dish as it will take too long for NO to diffuse from the ECs to the electrode since the diffusion coefficient of NO is likely to be on the order of $10^{-6} \text{ cm}^2 \text{ s}^{-1}$. This can be overcome by screen-printing the nanocomposite on a chip and culturing ECs on it so that any NO released by the ECs is almost immediately detected by the electrode. The biocompatibility of the 3-D graphene/IL for EC attachment and growth also needs to be evaluated.

The surface area used for the detection of NO released by the HUVECs on the RGD-peptide-functionalised biomimetic graphene film was limited by the diameter of the columniform. It will be better to increase the surface area used for detecting NO released by HUVECS by using a customised columniform of larger diameter. This will give a more reliable average value for the concentration of NO released by HUVECs per unit area since cell distribution may vary across the film.

The PEI-coated AEIROF was unable to measure the exact pH value of the cell culture medium unlike in phosphate buffer due to the unexpected change in formal potential following coating with fibronectin and incubation. This limited its use in the cell experiments to measuring changes in pH. The cause of this problem needs to be investigated. The incubator can be set to 25 °C to minimise any impact

that the heat could have on the film but the viability and attachment of the PAECs to the chip at 25 °C would also need to be evaluated. The experimental setup can be improved with inlets for the addition of chemicals to the chip so that the chip does not have to be disconnected from the setup for the chemicals to be added.

The linear range of the glucose biosensor can be improved by adding more GOD to the precursor solution, so that there will be more GOD present on the biosensor to catalyse the reaction of glucose to gluconolactone at higher concentrations of glucose. The calibration of the glucose biosensor needs to be done in different levels of O₂ below atm O₂ in order to use the O₂ and glucose biosensors simultaneously. The biocompatibility of the O₂ biosensor and glucose biosensor for EC culture have not been evaluated.

For future works, the addition of a lactate biosensor will allow a more comprehensive investigation into the rate of EC metabolism since lactate is a by-product of glucose metabolism in ECs. The lactate biosensor can be fabricated using a procedure similar to the glucose biosensor by adding lactate oxidase to the precursor solution of MB⁺ and pyrrole.

The biosensors in this dissertation have been used individually. However, it would be even more meaningful to use two or more of them simultaneously so as to build a more comprehensive picture of the crosstalk between EC metabolism and angiogenesis. One way is to use lab-on-a-chip devices to miniaturise these sensors by screen-printing, electrodeposition or electropolymerisation, such as that proposed for the O₂ and glucose biosensors. Thereafter, ECs are cultured on these

electrodes, which simultaneously detect the various metabolic markers and NO as the cells are exposed to drugs or soluble factors.

Since shear stress also affects angiogenesis [278], its effects can be investigated along with soluble factors and the extracellular matrix (ECM) macromolecules to achieve a greater understanding of angiogenesis in real-time. This can be done by varying the flow rate of cell culture medium to the chip and observing the morphology of the ECs and measuring the levels of metabolic markers.

More work needs to be done to having a better understanding of both the spatial and temporal uptake and release of soluble factors and signalling molecules by the ECs, respectively. The use of fluorescently tagged soluble factors can enable visualisation of when these factors bind to ECs. The time between their binding to the cells to the detection of signalling molecules can also be studied. The signalling molecules can be detected on various locations on a chip in real-time by using an array of microelectrodes.

The autocrine and paracrine effects of these factors and molecules and how they alter EC metabolism and angiogenesis in real-time also need to be investigated. Autocrine effects (effects which biomolecules have on the cell which produces them) can be studied by inhibiting enzymes involved in the biochemical reactions downstream of the intracellular signalling pathway of interest. Paracrine effects (effects which biomolecules produced by a cell have on its neighbours) can be investigated by separating the ECs into colonies and inhibiting receptors

involved in the uptake of the biomolecule of interest by the neighbouring colony of ECs.

In conclusion, the fabrication of nanomaterials-based electrochemical biosensors for NO, pH, O₂ and glucose with good performances have been accomplished in this dissertation. I have shown how some of these electrochemical biosensors are biocompatible and useful for studying the interactions of soluble factors, ECs and ECM macromolecules in cell culture medium. Through the use of drugs and inhibitors whose actions are well understood, the intracellular pathways through which the soluble factor elicits an acute metabolic response from the ECs can be probed, and this is significant in gaining insights to the processes involved in angiogenesis. I have also discussed the limitations and improvements to be made to these biosensors, which will enable us to gain a better understanding of angiogenesis in the near future. These biosensors also have the potential for application to studying the acute cellular responses of other cell types to external stimuli.

List of Abbreviations

a	Activity
AA	Ascorbic acid
Ach	Acetylcholine
AEIROF	Anodically electrodeposited iridium oxide film
AFM	Atomic force microscopy
AIROF	Anodic iridium oxide film
Ang	Angiopoietins
Atm	Atmospheric
ATP	Adenosine triphosphate
c	Bulk concentration
CcO	Cytochrome c oxidase
C_{dl}	Double layer capacitance
CNTs	Carbon nanotubes
CoPc	Cobalt(II) phthalocyanine
CV	Cyclic voltammetry
CVD	Chemical vapour deposition
δ	Thickness of the Nernst diffusion layer
D	Diffusion coefficient
DET	Direct electron transfer
DI	De-ionised
DII4	Delta-like ligand 4

DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DPSCA	Double potential step chronoamperometry
ΔE_p	Peak separation
E^0	Standard potential
$E^{0'}$	Formal potential
EC'	Electrochemical followed by catalytic reaction
ECs	Endothelial cells
ECM	Extracellular matrix
EDC	Ethyl(dimethylaminopropyl) carbodiimide
EIS	Electrochemical impedance spectroscopy
eNOS	Endothelial nitric oxide synthase
E_p	Peak potential
$E_{p,a}$	Anodic peak potential
$E_{p,c}$	Cathodic peak potential
Erk	Extracellular receptor kinase
f	Frequency
F	Faraday's constant
FAD	Flavin adenine dinucleotide
FADH ₂	Reduced flavin adenine dinucleotide
FAK	Focal adhesion kinase
FESEM	Field emission scanning electron microscopy

FGF	Fibroblasts growth factor
FITC	Fluorescein isothiocyanate mixed isomer
FTIR	Fourier transform infrared
GC	Glassy carbon
GO	Graphene oxide
GOD	Glucose oxidase
HIF	Hypoxia inducible factor
HOOC-2-AQ/AMWCNTs	Anthraquinone-derivative functionalised MWCNTs nanowires
HUVECs	Human umbilical vein endothelial cells
IL	Ionic liquid
iNOS	Inducible nitric oxide synthase
i_{max}	Maximum current
i_p	Peak current
$i_{p,a}$	Anodic peak current
$i_{p,c}$	Cathodic peak current
IrOx	Iridium oxide
i_{ss}	Steady-state current
J	Flux
j_0	Exchange current density
K_M^{app}	Apparent Michaelis-Menten constant
LBL	Layer-by-layer
LMB	Leuco methylene blue

L-NAME	NG-nitro-L-arginine methyl ester
m	Mass transport coefficient
MAPK	Mitogen-activated protein kinase
MB ⁺	Methylene blue
MEA	Microelectrode array
MWCNTs	Multi-walled carbon nanotubes
n	Number of electrons
n	Number of repeats
NADH	Nicotinamide adenine dinucleotide
NHS	N-hydroxysulfosuccinimide
NO	Nitric oxide
NPs	Nanoparticles
OCP	Open circuit potential
$\emptyset$	Phase shift
PAECs	Porcine aortic endothelial cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PD98059	MAP kinase inhibitor
PEDG	Pigment epithelium-derived factor
PEI	Poly(ethyleneimine)
PHD-2	Protein hydroxylase domain containing protein 2
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol (3,4,5)P3

PLL	Poly(L-lysine)
PKB	Protein kinase B
PKC	Protein kinase c
PMB	Poly(methylene blue)
PPy	Poly(pyrrole)
R	Gas constant
R_{ct}	Charge transfer resistance
RA-IR	Reflection absorption infrared spectroscopy
Ref.	Reference
RGD	Arginine-glycine-aspartic acid
rGO	Reduced graphene oxide
R_u	Uncompensated resistance
SIROF	Sputtered iridium oxide film
S/N	Signal-to-noise ratio
SWCNTs	Single-walled carbon nanotubes
t	Time
T	Temperature
TeTsPc	Iron(III) tetra-(N-methyl-4-pyridyl)-porphyrin
FeT4MPyP	Iron tetrasulfonated phthalocyanine
UA	Uric acid
ν	Scan rate
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Z	Charge
2-D	Two-dimensional
3-D	Three-dimensional

References

1. Sato, T.N. and S. Loughna, *Vasculogenesis and angiogenesis*, in *Mouse Development: Patterning, Morphogenesis, and Organogenesis*, J. Rossant and P.P.L.T. Tam, Editors. 2002, Gulf Professional Publishing. p. 211-233.
2. Walsh, D.A., *Pathophysiological mechanisms of angiogenesis*. Advances In Clinical Chemistry, 2007. **44**: p. 187.
3. Campbell, N. and J. Reece, *Biology*. 6th ed. 2002, San Francisco, CA: Benjamin Cummings.
4. Risau, W. and I. Flamme, *Vasculogenesis*. Annual Review of Cell and Developmental Biology, 1995. **11**(1): p. 73-91.
5. Ribatti, D. and E. Crivellato, "*Sprouting angiogenesis*", a reappraisal. Developmental Biology, 2012. **372**(2): p. 157-165.
6. Karamysheva, A.F., *Mechanisms of angiogenesis*. Biochemistry (Moscow), 2008. **73**(7): p. 751-762.
7. Goumans, M., et al., *Transforming growth factor-beta signalling in extraembryonic mesoderm is required for yolk sac vasculogenesis in mice*. Development, 1999. **126**(16): p. 3473.
8. George, E., H. Baldwin, and R. Hynes, *Fibronectins are essential for heart and blood vessel morphogenesis but are dispensable for initial specification of precursor cells*. Blood, 1997. **90**(8): p. 3073.
9. Avraamides, C.J., B. Garmy-Susini, and J.A. Varner, *Integrins in angiogenesis and lymphangiogenesis*. Nature Reviews Cancer, 2008. **8**(8): p. 604-617.
10. Francis, S., et al., *Central roles of $\alpha\beta 1$ integrin and fibronectin in vascular development in mouse embryos and embryoid bodies*. Arteriosclerosis, thrombosis, and vascular biology, 2002. **22**(6): p. 927.
11. Ferrara, N., H.-P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors*. Nature Medicine, 2003. **9**(6): p. 669-676.
12. Fraisl, P., *Crosstalk between oxygen-and nitric oxide-dependent signaling pathways in angiogenesis*. Experimental Cell Research, 2013. **319**(9): p. 1331-1339.
13. Schelling, M., *FGF mediation of coronary angiogenesis*. New York Academy Sciences Annals, 1991. **638**: p. 467-469.
14. Seghezzi, G., et al., *Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis*. Journal of Cell Biology, 1998. **141**(7): p. 1659.
15. Shutter, J., et al., *Dll4, a novel Notch ligand expressed in arterial endothelium*. Genes & Development, 2000. **14**(11): p. 1313.
16. Krebs, L., et al., *Notch signaling is essential for vascular morphogenesis in mice*. Genes and Development, 2000. **14**(11): p. 1343.
17. Sato, T., et al., *Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation*. Nature, 1995. **376**: p. 70-74.
18. Suri, C., et al., *Requisite role of angiopoietin-1, a ligand for the Tie2 receptor, during embryonic angiogenesis*. Cell, 1996. **87**(7): p. 1171-1180.

19. Hungerford, J., et al., *Identification of a novel marker for primordial smooth muscle and its differential expression pattern in contractile vs noncontractile cells*. Journal of Cell Biology, 1997. **137**(4): p. 925.
20. Lee, S., et al., *Proliferation and differentiation of smooth muscle cell precursors occurs simultaneously during the development of the vessel wall*. Developmental Dynamics, 1997. **209**(4): p. 342-352.
21. Fraisl, P., et al., *Regulation of angiogenesis by oxygen and metabolism*. Developmental Cell, 2009. **16**(2): p. 167-179.
22. Carmeliet, P. and R.K. Jain, *Molecular mechanisms and clinical applications of angiogenesis*. Nature, 2011. **473**(7347): p. 298-307.
23. Greaves, N.S., et al., *Current understanding of molecular and cellular mechanisms in fibroplasia and angiogenesis during acute wound healing*. Journal of Dermatological Science, 2013. **72**(3): p. 206-217.
24. Konisti, S., S. Kiriakidis, and E.M. Paleolog, *Hypoxia - a key regulator of angiogenesis and inflammation in rheumatoid arthritis*. Nature Reviews Rheumatology, 2012. **8**: p. 153-162.
25. Folkman, J., *Angiogenesis in cancer, vascular, rheumatoid and other disease*. Nature Medicine, 1995. **1**(1): p. 27-30.
26. Kranenburg, O., M. Gebbink, and E. Voest, *Stimulation of angiogenesis by Ras proteins*. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 2004. **1654**(1): p. 23-37.
27. Cines, D.B., et al., *Endothelial cells in physiology and in the pathophysiology of vascular disorders*. Blood, 1998. **91**(10): p. 3527-3561.
28. Delorme, S. and M.V. Knopp, *Non-invasive vascular imaging: Assessing tumour vascularity*. European Radiology, 1998. **8**(4): p. 517-527.
29. Taylor, P., *VEGF and imaging of vessels in rheumatoid arthritis*. Arthritis Research, 2002. **4**(Suppl 3): p. S99 - S107.
30. Haubner, R. and H.-J. Wester, *Radiolabeled tracers for imaging of tumor angiogenesis and evaluation of anti-angiogenic therapies*. Current Pharmaceutical Design, 2004. **10**(13): p. 1439-1455.
31. Miles, K.A., *Perfusion CT for the assessment of tumour vascularity: Which protocol?* The British Journal of Radiology, 2014. **76**(suppl_1): p. S36-S42.
32. Dirix, L.Y., et al., *Elevated levels of the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients*. British Journal of Cancer, 1997. **76**(2): p. 238-243.
33. Semenza, G.L., *HIF-1: Mediator of physiological and pathophysiological responses to hypoxia*. Journal of Applied Physiology, 2000. **88**(4): p. 1474-1480.
34. Pugh, C.W. and P.J. Ratcliffe, *Regulation of angiogenesis by hypoxia: role of the HIF system*. Nature Medicine, 2003. **9**(6): p. 677-684.
35. Alexander, P., *Hypoxia inducible factor-2 α in cancer*. Cell Cycle, 2007. **6**(8): p. 919-926.
36. Holmes, K., et al., *Vascular endothelial growth factor receptor-2: Structure, function, intracellular signalling and therapeutic inhibition*. Cellular Signalling, 2007. **19**(10): p. 2003-2012.
37. Branco-Price, C., et al., *Endothelial cell HIF-1 α and HIF-2 α differentially regulate metastatic success*. Cancer Cell, 2012. **21**(1): p. 52.

38. Elvert, G., et al., *Cooperative interaction of hypoxia-inducible factor-2 (HIF-2 α) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1)*. Journal of Biological Chemistry, 2003. **278**(9): p. 7520-7530.
39. Brown, G.C., *Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase*. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 2001. **1504**(1): p. 46-57.
40. Mateo, J., et al., *Regulation of hypoxia-inducible factor-1 α by nitric oxide through mitochondria-dependent and-independent pathways*. Biochemical Journal, 2003. **376**: p. 537-544.
41. Hagen, T., et al., *Redistribution of intracellular oxygen in hypoxia by nitric oxide: Effect on HIF1 α* . Science Signaling, 2003. **302**(5652): p. 1975-1978.
42. Li, F., et al., *Regulation of HIF-1 α stability through S-nitrosylation*. Molecular Cell, 2007. **26**(1): p. 63-74.
43. Stuttfeld, E. and K. Ballmer-Hofer, *Structure and function of VEGF receptors*. IUBMB Life, 2009. **61**(9): p. 915-922.
44. Houck, K., et al., *The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternative splicing of RNA*. Molecular Endocrinology, 1991. **5**(12): p. 1806.
45. Tischer, E., et al., *The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing*. Journal of Biological Chemistry, 1991. **266**(18): p. 11947.
46. Houck, K., et al., *Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms*. Journal of Biological Chemistry, 1992. **267**(36): p. 26031.
47. Terman, B., et al., *Identification of a new endothelial growth receptor tyrosine kinase*. Oncogene, 1991. **6**: p. 1677-1683.
48. Kendall, R. and K. Thomas, *Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor*. Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(22): p. 10705.
49. Cai, J., et al., *Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1*. Journal of Biological Chemistry, 2006. **281**(6): p. 3604-3613.
50. Takahashi, T., et al., *A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC- and DNA synthesis in vascular endothelial cells*. The EMBO journal, 2001. **20**(11): p. 2768-2778.
51. Mavria, G., et al., *ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis*. Cancer Cell, 2006. **9**(1): p. 33-44.
52. Rousseau, S., et al., *p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells*. Oncogene, 1997. **15**(18): p. 2169.
53. Cross, M., et al., *VEGF-receptor signal transduction*. Trends in Biochemical Sciences, 2003. **28**(9): p. 488-494.

54. Hood, J., et al., *VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells*. American Journal of Physiology: Heart and Circulatory Physiology, 1998. **274**(3): p. H1054.
55. Le Borgne, R., A. Bardin, and F. Schweisguth, *The roles of receptor and ligand endocytosis in regulating Notch signaling*. Development, 2005. **132**(8): p. 1751-1762.
56. Le Borgne, R., A. Bardin, and F. Schweisguth, *The roles of receptor and ligand endocytosis in regulating Notch signaling*. Development, 2005. **132**(8): p. 1751.
57. Fortini, M., *Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling*. Nature Reviews Molecular Cell Biology, 2002. **3**(9): p. 673-684.
58. Hellström, M., et al., *Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis*. Nature, 2007. **445**(7129): p. 776-780.
59. Gerhardt, H., et al., *VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia*. Journal of Cell Biology, 2003. **161**(6): p. 1163.
60. Figg, W. and J. Folkman, *Angiogenesis: An integrative approach from science to medicine*, W. Figg and J. Folkman, Editors. 2008, Springer Science+Business Media, LLC. p. 601.
61. Saharinen, P., et al., *Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2*. Journal of Cell Biology, 2005. **169**(2): p. 239-243.
62. Ramsauer, M. and P.A. Amore, *Getting Tie(2)d up in angiogenesis*. The Journal of Clinical Investigation, 2002. **110**(11): p. 1615-1617.
63. Maisonpierre, P., et al., *Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis*. Science, 1997. **277**(5322): p. 55.
64. Fredriksson, L., H. Li, and U. Eriksson, *The PDGF family: Four gene products form five dimeric isoforms*. Cytokine & Growth Factor Reviews, 2004. **15**(4): p. 197-204.
65. Betsholtz, C., *Insight into the physiological functions of PDGF through genetic studies in mice*. Cytokine & Growth Factor Reviews, 2004. **15**(4): p. 215-228.
66. Lindahl, P., et al., *Pericyte loss and microaneurysm formation in PDGF-B-deficient mice*. Science, 1997. **277**(5323): p. 242.
67. Owicki, J. and W. Parce, *Biosensors based on the energy metabolism of living cells: The physical chemistry and cell biology of extracellular acidification*. Biosensors and Bioelectronics, 1992. **7**(4): p. 255-272.
68. Pletcher, D., *A first course in electrode processes*. 2009, Cambridge, UK: RSC Publishing.
69. Zoski, C.G., *Handbook of electrochemistry*, ed. C.G. Zoski. 2007, Netherlands: Elsevier B. V.
70. Scholz, F., *Electroanalytical methods: Guide to experiments and applications*. 2nd ed. 2010, Berlin: Springer. 359.
71. Albery, W.J., *Electrode kinetics*, ed. P.W. Atkins, J.S.E. Holker, and A.K. Holliday. 1975: Oxford University Press.
72. Pletcher, D., et al., *Instrumental methods in electrochemistry*. 2001: Horwood Publishing.
73. Wang, J., *Analytical electrochemistry*. 2006: Wiley-VCH.

74. Bard, A.J. and L.R. Faulkner, *Electrochemical methods: Fundamentals and applications*. 1980, Pennington, NJ: John Wiley & Sons.
75. Kissinger, P.T. and W.R. Heineman, *Laboratory techniques in electroanalytical chemistry*. 1996, New York: Marcel Dekker, Inc.
76. Wang, G. and J. Li, *Interfacial bioelectrochemistry: Fabrication, properties and applications of functional nanostructured biointerfaces*. The Journal of Physical Chemistry C, 2006. **111**(6): p. 2351-2367.
77. Kachoosangi, R., et al., *Carbon nanotube-ionic liquid composite sensors and biosensors*. Analytical Chemistry, 2008. **81**(1): p. 435-442.
78. Shan, C., et al., *Electrochemical determination of NADH and ethanol based on ionic liquid-functionalized graphene*. Biosensors and Bioelectronics, 2009. **25**(6): p. 1504-1508.
79. Geim, A.K. and K.S. Novoselov, *The rise of graphene*. Nature Materials, 2007. **6**: p. 183-191.
80. Shao, Y., et al., *Graphene based electrochemical sensors and biosensors: A review*. Electroanalysis, 2010. **22**(10): p. 1027-1036.
81. Alwarappan, S., et al., *Probing the electrochemical properties of graphene nanosheets for biosensing applications*. The Journal of Physical Chemistry C, 2009. **113**(20): p. 8853-8857.
82. Zhou, M., Y. Zhai, and S. Dong, *Electrochemical sensing and biosensing platform based on chemically reduced graphene oxide*. Analytical Chemistry, 2009. **81**(14): p. 5603-5613.
83. Shan, C., et al., *Direct electrochemistry of glucose oxidase and biosensing for glucose based on graphene*. Analytical Chemistry, 2009. **81**(6): p. 2378-2382.
84. Lu, Q., et al., *Direct electrochemistry-based hydrogen peroxide biosensor formed from single-layer graphene nanoplatelet-enzyme composite film*. Talanta, 2010. **82**(4): p. 1344-1348.
85. Fang, Y., et al., *Self-assembly of cationic polyelectrolyte-functionalized graphene nanosheets and gold nanoparticles: a two-dimensional heterostructure for hydrogen peroxide sensing*. Langmuir, 2010. **26**(13): p. 11277-11282.
86. Huang, K.-J., et al., *Enhanced sensing of dopamine in the presence of ascorbic acid based on graphene/poly(p-aminobenzoic acid) composite film*. Colloids and Surfaces B: Biointerfaces, 2011. **88**(1): p. 310-314.
87. Guo, C.X., S.R. Ng, and C.M. Li, *Graphene-based electrochemical biosensors*, in *Biosensors based on nanomaterials and nanodevices*, L.N. Jun, Wu, Editor. 2012, CRC Press, Taylor & Francis Group.
88. Novoselov, K., et al., *Electric field effect in atomically thin carbon films*. Science, 2004. **306**(5696): p. 666-669.
89. Li, D., et al., *Processable aqueous dispersions of graphene nanosheets*. Nature Nanotechnology, 2008. **3**(2): p. 101-105.
90. Shao, Y., et al., *Facile and controllable electrochemical reduction of graphene oxide and its applications*. Journal of Materials Chemistry, 2010. **20**(4): p. 743-748.

91. Li, X.H., et al., *A green chemistry of graphene: Photochemical reduction towards monolayer graphene sheets and the role of water adlayers*. ChemSusChem, 2012. **5**(4): p. 642-646.
92. Guo, C.X., et al., *Ionic liquid–graphene composite for ultratrace explosive trinitrotoluene detection*. Electrochemistry Communications, 2010. **12**(9): p. 1237-1240.
93. Liu, Y., X. Dong, and P. Chen, *Biological and chemical sensors based on graphene materials*. Chemical Society Reviews, 2012. **41**(6): p. 2283-2307.
94. Reina, A., et al., *Large area, few-layer graphene films on arbitrary substrates by chemical vapor deposition*. Nano Letters, 2008. **9**(1): p. 30-35.
95. Al-Temimy, A., C. Riedl, and U. Starke, *Low temperature growth of epitaxial graphene on SiC induced by carbon evaporation*. Applied Physics Letters, 2009. **95**(23): p. 231907.
96. Mattevi, C., H. Kim, and M. Chhowalla, *A review of chemical vapour deposition of graphene on copper*. Journal of Materials Chemistry, 2011. **21**(10): p. 3324-3334.
97. Yao, S., M. Wang, and M. Madou, *A pH electrode based on melt-oxidized iridium oxide*. Journal of the Electrochemical Society, 2001. **148**(4): p. H29-H36.
98. Mailley, S., et al., *Electrochemical and structural characterizations of electrodeposited iridium oxide thin-film electrodes applied to neurostimulating electrical signal*. Materials Science and Engineering: C, 2002. **21**(1): p. 167-175.
99. Yamanaka, K., *Anodically electrodeposited iridium oxide films (AEIROF) from alkaline solutions for electrochromic display devices*. Japanese Journal of Applied Physics, 1989. **28**(part 1): p. 632-637.
100. Sato, Y., et al., *Reflectance and structure changes of iridium oxide films by reduction*. Journal of The Electrochemical Society, 1989. **136**(3): p. 863-866.
101. Burke, L.D., J.K. Mulcahy, and D.P. Whelan, *Preparation of an oxidized iridium electrode and the variation of its potential with pH*. Journal of Electroanalytical Chemistry, 1984. **163**(1-2): p. 117-128.
102. O'Hare, D., K. Parker, and C. Winlove, *Metal-metal oxide pH sensors for physiological application*. Medical Engineering & Physics, 2006. **28**(10): p. 982-988.
103. Katsube, T., I. Lauks, and J.N. Zemel, *pH-sensitive sputtered iridium oxide films*. Sensors and Actuators, 1981. **2**: p. 399-410.
104. Burke, L.D. and D. Whelan, *A voltammetric investigation of the charge storage reactions of hydrous iridium oxide layers*. Journal of Electroanalytical Chemistry and Interfacial Electrochemistry, 1984. **162**(1): p. 121-141.
105. Bitziou, E., D. O'Hare, and B.A. Patel, *Simultaneous detection of pH changes and histamine release from oxyntic glands in isolated stomach*. Analytical Chemistry, 2008. **80**(22): p. 8733-8740.
106. Dobson, J.V., P.R. Snodin, and H.R. Thirsk, *EMF measurements of cells employing metal—metal oxide electrodes in aqueous chloride and sulphate*

- electrolytes at temperatures between 25–250°C.* *Electrochimica Acta*, 1976. **21**(7): p. 527-533.
107. Karpinska, J., B. Starczewska, and H. Puzanowska-Tarasiewicz, *Analytical properties of phenothiazine derivatives.* *Analytical sciences*, 1996. **12**: p. 161.
 108. Nicolai, S.I.H.d.A., et al., *Electrochemical and spectroelectrochemical (SERS) studies of the reduction of methylene blue on a silver electrode.* *Journal of Electroanalytical Chemistry*, 2002. **527**(1–2): p. 103-111.
 109. Li, J., et al., *Adsorptive and stripping behavior of methylene blue at gold electrodes in the presence of cationic gemini surfactants.* *Electrochimica Acta*, 2005. **51**(2): p. 297-303.
 110. Albery, W.J., et al., *The kinetics of electron transfer in the thionine-coated electrode.* *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, 1982. **133**(1): p. 135-145.
 111. Albery, W.J. and A.R. Mount, *Ring-disc electrodes. Part 24.-Studies of counterion fluxes at a thionine-coated electrode.* *Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases*, 1989. **85**(11): p. 3717-3724.
 112. Karyakin, A.A., E.E. Karyakina, and H.L. Schmidt, *Electropolymerized azines: A new group of electroactive polymers.* *Electroanalysis*, 1999. **11**(3): p. 149-155.
 113. Liu, H., et al., *A reagentless biosensor highly sensitive to hydrogen peroxide based on new methylene blue N dispersed in Nafion gel as the electron shuttle.* *Journal of Electroanalytical Chemistry*, 1996. **417**(1): p. 59-64.
 114. Sun, Y., et al., *Controlled multilayer films of sulfonate-capped gold nanoparticles/thionine used for construction of a reagentless bienzymatic glucose biosensor.* *Electrochimica Acta*, 2007. **52**(25): p. 7352-7361.
 115. Kang, X.B., et al., *Study on a hydrogen peroxide biosensor based on horseradish peroxidase/GNPs-thionine/chitosan.* *Electrochimica Acta*, 2012. **62**: p. 327-334.
 116. Xu, J.Z., et al., *An amperometric biosensor based on the coimmobilization of horseradish peroxidase and methylene blue on a carbon nanotubes modified electrode.* *Electroanalysis*, 2003. **15**(3): p. 219-224.
 117. Silber, A., N. Hampp, and W. Schuhmann, *Poly (methylene blue)-modified thick-film gold electrodes for the electrocatalytic oxidation of NADH and their application in glucose biosensors.* *Biosensors and Bioelectronics*, 1996. **11**(3): p. 215-223.
 118. Pfaffen, V., et al., *On the pH dependence of electroactivity of poly (methylene blue) films.* *Electrochimica Acta*, 2010. **55**(5): p. 1766-1771.
 119. Barsan, M.M., E.M. Pinto, and C. Brett, *Electrosynthesis and electrochemical characterisation of phenazine polymers for application in biosensors.* *Electrochimica Acta*, 2008. **53**(11): p. 3973-3982.
 120. Yogeswaran, U. and S.-M. Chen, *Multi-walled carbon nanotubes with poly(methylene blue) composite film for the enhancement and separation of electroanalytical responses of catecholamine and ascorbic acid.* *Sensors and Actuators B: Chemical*, 2008. **130**(2): p. 739-749.

121. Xiao, X., et al., *Poly (methylene blue) doped silica nanocomposites with crosslinked cage structure: Electropolymerization, characterization and catalytic activity for reduction of dissolved oxygen*. *Electrochimica Acta*, 2011. **56**(27): p. 10055-10063.
122. Xiao, X., et al., *An reagentless glucose biosensor based on direct electrochemistry of glucose oxidase immobilized on poly (methylene blue) doped silica nanocomposites*. *Sensors and Actuators B: Chemical*, 2012. **165**(1): p. 126-132.
123. Kertész, V., J. Bácskai, and G. Inzelt, *Monitoring of formation and redox transformations of poly (methylene blue) films using an electrochemical quartz crystal microbalance*. *Electrochimica Acta*, 1996. **41**(18): p. 2877-2881.
124. Liu, J. and S. Mu, *The electrochemical polymerization of methylene blue and properties of polymethylene blue*. *Synthetic Metals*, 1999. **107**(3): p. 159-165.
125. Damos, F.S., R. Luz, and L.T. Kubota, *Study of poly (methylene blue) ultrathin films and its properties by electrochemical surface plasmon resonance*. *Journal of Electroanalytical Chemistry*, 2005. **581**(2): p. 231-240.
126. Karyakin, A., et al., *The electrochemical polymerization of methylene blue and bioelectrochemical activity of the resulting film*. *Bioelectrochemistry and Bioenergetics*, 1993. **32**(1): p. 35-43.
127. Kaplan, İ.H., K. Dağcı, and M. Alanyalıoğlu, *Nucleation and growth mechanism of electropolymerization of methylene blue: The effect of preparation potential on poly (methylene blue) structure*. *Electroanalysis*, 2010. **22**(22): p. 2694-2701.
128. Michel, T. and O. Feron, *Nitric oxide synthases: Which, where, how, and why?* *Journal of Clinical Investigation*, 1997. **100**(9): p. 2146.
129. Zhou, L. and D.Y. Zhu, *Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications*. *Nitric Oxide*, 2009. **20**(4): p. 223-230.
130. Napoli, C., et al., *Nitric oxide and atherosclerosis: An update*. *Nitric Oxide*, 2006. **15**(4): p. 265-279.
131. MacMicking, J., Q. Xie, and C. Nathan, *Nitric oxide and macrophage function*. *Annual Review of Immunology*, 1997. **15**(1): p. 323-350.
132. Thejass, P. and G. Kuttan, *Allyl isothiocyanate (AITC) and phenyl isothiocyanate (PITC) inhibit tumour-specific angiogenesis by downregulating nitric oxide (NO) and tumour necrosis factor- α (TNF- α) production*. *Nitric Oxide*, 2007. **16**(2): p. 247-257.
133. Kavya, R., et al., *Nitric oxide synthase regulation and diversity: Implications in Parkinson's disease*. *Nitric Oxide*, 2006. **15**(4): p. 280-294.
134. Li, C.M., et al., *Electrochemical detection of nitric oxide on a SWCNT/RTIL composite gel microelectrode*. *Electroanalysis*, 2006. **18**(7): p. 713.
135. Wu, F.H., G.C. Zhao, and X.W. Wei, *Electrocatalytic oxidation of nitric oxide at multi-walled carbon nanotubes modified electrode*. *Electrochemistry Communications*, 2002. **4**(9): p. 690-694.

136. Zhang, L., D.B. Tian, and J.J. Zhu, *Direct electrochemistry and electrochemical catalysis of myoglobin-TiO₂ coated multiwalled carbon nanotubes modified electrode*. Bioelectrochemistry, 2008. **74**(1): p. 157-163.
137. Thangavel, S. and R. Ramaraj, *Polymer membrane stabilized gold nanostructures modified electrode and its application in nitric oxide detection*. The Journal of Physical Chemistry C, 2008. **112**(50): p. 19825-19830.
138. Li, Y.J., et al., *Large-scale self-assembly of hydrophilic gold nanoparticles at oil/water interface and their electro-oxidation for nitric oxide in solution*. Journal of Electroanalytical Chemistry, 2008. **622**(1): p. 103-108.
139. Zhang, J. and M. Oyama, *Gold nanoparticle arrays directly grown on nanostructured indium tin oxide electrodes: Characterization and electroanalytical application*. Analytica Chimica Acta, 2005. **540**(2): p. 299-306.
140. Borgmann, S., et al., *Electrochemical high-content screening of nitric oxide release from endothelial cells*. ChemBioChem, 2006. **7**(4): p. 662-668.
141. Shibuki, K., *An electrochemical microprobe for detecting nitric oxide release in brain tissue*. Neuroscience Research, 1990. **9**(1): p. 69-76.
142. Isik, S., et al., *Cell-compatible array of three-dimensional tip electrodes for the detection of nitric oxide release*. Biosensors and Bioelectronics, 2005. **20**(8): p. 1566-1572.
143. Park, J., et al., *In vivo nitric oxide sensor using non-conducting polymer-modified carbon fiber*. Biosensors and Bioelectronics, 1998. **13**(11): p. 1187-1195.
144. Heinzen, E. and G. Pollack, *Use of an electrochemical nitric oxide sensor to detect neuronal nitric oxide production in conscious, unrestrained rats*. Journal of Pharmacological and Toxicological Methods, 2002. **48**(3): p. 139-146.
145. Malinski, T. and Z. Taha, *Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor*. Nature, 1992. **358**: p. 676-678.
146. Zhang, X., H. Ju, and J. Wang, *Electrochemical sensors, biosensors and their biomedical applications*. 2008: Academic Press.
147. Zhang, X., *Real time and in vivo monitoring of nitric oxide by electrochemical sensors - From dream to reality*. Frontiers in Bioscience, 2004. **9**: p. 3434-3446.
148. Doppenberg, E., et al., *Determination of the ischemic threshold for brain oxygen tension*, in *Intracranial Pressure and Neuromonitoring in Brain Injury*. 1998, Springer. p. 166-169.
149. Haitzma, I.K. and A.I. Maas, *Advanced monitoring in the intensive care unit: Brain tissue oxygen tension*. Current Opinion in Critical Care, 2002. **8**(2): p. 115-120.
150. Clark JR, L.C. and E.W. Clark, *A personalized history of the Clark oxygen electrode*. International Anesthesiology Clinics, 1987. **25**(3): p. 1-29.
151. Zhou, Q., et al., *Template-synthesized cobalt Porphyrin/polypyrrole nanocomposite and its electrocatalysis for oxygen reduction in neutral*

- medium*. The Journal of Physical Chemistry C, 2007. **111**(30): p. 11216-11222.
152. Zhou, Q., et al., *Electrocatalysis of template-electrosynthesized cobalt-porphyrin/polyaniline nanocomposite for oxygen reduction*. The Journal of Physical Chemistry C, 2008. **112**(47): p. 18578-18583.
 153. Duarte, J.C., et al., *A highly sensitive amperometric sensor for oxygen based on iron(II) tetrasulfonated phthalocyanine and iron(III) tetra-(N-methyl-pyridyl)-porphyrin multilayers*. Analytica Chimica Acta, 2008. **612**(1): p. 29-36.
 154. Santos, L.S., R. Landers, and Y. Gushikem, *Application of manganese (II) phthalocyanine synthesized in situ in the SiO₂/SnO₂ mixed oxide matrix for determination of dissolved oxygen by electrochemical techniques*. Talanta, 2011. **85**(2): p. 1213-1216.
 155. Rahim, A., et al., *Dissolved O₂ sensor based on cobalt (II) phthalocyanine immobilized in situ on electrically conducting carbon ceramic mesoporous SiO₂/C material*. Sensors and Actuators B: Chemical, 2012. **177**: p. 231-238.
 156. Luz, R.d.C.S., et al., *Dissolved oxygen sensor based on cobalt tetrasulphonated phthalocyanine immobilized in poly-L-lysine film onto glassy carbon electrode*. Sensors and Actuators B: Chemical, 2006. **114**(2): p. 1019-1027.
 157. Tiwari, I., et al., *Electroanalytical properties and application of anthraquinone derivative-functionalized multiwalled carbon nanotubes nanowires modified glassy carbon electrode in the determination of dissolved oxygen*. Materials Research Bulletin, 2012.
 158. Tsai, T.-H., S.-H. Wang, and S.-M. Chen, *Electrodeposited indigotetrasulfonate film onto glutaraldehyde-cross-linked poly-L-lysine modified glassy carbon electrode for detection of dissolved oxygen*. Journal of Electroanalytical Chemistry, 2011. **659**(1): p. 69-75.
 159. Johnsson, F., B. Joelsson, and P. Isberg, *Ambulatory 24 hour intraesophageal pH-monitoring in the diagnosis of gastroesophageal reflux disease*. Gut, 1987. **28**(9): p. 1145.
 160. Marzouk, S., et al., *Electrodeposited iridium oxide pH electrode for measurement of extracellular myocardial acidosis during acute ischemia*. Analytical Chemistry, 1998. **70**(23): p. 5054-5061.
 161. Carter, N., et al., *Measurement of intracellular pH of skeletal muscle with pH-sensitive glass microelectrodes*. Journal of Clinical Investigation, 1967. **46**(6): p. 920.
 162. Marzouk, S., et al., *Electrodeposited iridium oxide pH electrode for measurement of extracellular myocardial acidosis during acute ischemia*. Anal. Chem, 1998. **70**(23): p. 5054-5061.
 163. Ges, I., et al., *Differential pH measurements of metabolic cellular activity in nl culture volumes using microfabricated iridium oxide electrodes*. Biosensors and Bioelectronics, 2007. **22**(7): p. 1303-1310.
 164. Yamamoto, K., et al., *Solid-state pH ultramicrosensor based on a tungstic oxide film fabricated on a tungsten nanoelectrode and its application to the*

- study of endothelial cells*. *Analytica Chimica Acta*, 2003. **480**(1): p. 109-117.
165. Karyakin, A.A., et al., *Processible polyaniline as an advanced potentiometric pH transducer. Application to biosensors*. *Analytical Chemistry*, 1999. **71**(13): p. 2534-2540.
 166. Weber, J., A. Kumar, and S. Bhansali, *Novel lactate and pH biosensor for skin and sweat analysis based on single walled carbon nanotubes*. *Sensors and Actuators B: Chemical*, 2006. **117**(1): p. 308-313.
 167. Ang, P., et al., *Solution-gated epitaxial graphene as pH sensor*. *Journal of the American Chemical Society*, 2008. **130**(44): p. 14392-14393.
 168. Li, C.M., C.S. Cha, and M. Variation, *Porous carbon nanocomposite/enzyme glucose microsensor*. *Frontiers in Bioscience*, 2004. **9**: p. 3324-3330.
 169. Clark, L.C. and C. Lyons, *Electrode systems for continuous monitoring in cardiovascular surgery*. *Annals of the New York Academy of Sciences*, 1962. **102**(1): p. 29-45.
 170. Wang, J., *Glucose biosensors: 40 years of advances and challenges*. *Electroanalysis*, 2001. **13**(12): p. 983-988.
 171. Updike, S. and G. Hicks, *The enzyme electrode*. *Nature*, 1967. **214**: p. 986-988.
 172. Guilbault, G.G. and G.J. Lubrano, *An enzyme electrode for the amperometric determination of glucose*. *Analytica Chimica Acta*, 1973. **64**(3): p. 439-455.
 173. Sun, W., et al., *Ionic liquid/mesoporous carbon/protein composite microelectrode and its biosensing application*. *Electrochemistry Communications*, 2009. **11**(11): p. 2105-2108.
 174. Bao, S.J., et al., *New nanostructured TiO₂ for direct electrochemistry and glucose sensor applications*. *Advanced Functional Materials*, 2008. **18**(4): p. 591-599.
 175. Liu, J., et al., *Carbon-decorated ZnO nanowire array: A novel platform for direct electrochemistry of enzymes and biosensing applications*. *Electrochemistry Communications*, 2009. **11**(1): p. 202-205.
 176. Guo, C.X. and C.M. Li, *Direct electron transfer of glucose oxidase and biosensing of glucose on hollow sphere-nanostructured conducting polymer/metal oxide composite*. *Physical Chemistry Chemical Physics*, 2010. **12**(38): p. 12153-12159.
 177. Shan, C., et al., *Graphene/AuNPs/chitosan nanocomposites film for glucose biosensing*. *Biosensors and Bioelectronics*, 2009.
 178. Zhang, Y., et al., *Ultrasensitive and selective non-enzymatic glucose detection using copper nanowires*. *Biosensors and Bioelectronics*, 2012. **31**(1): p. 426-432.
 179. Xiao, F., et al., *Growth of coral-like PtAu-MnO₂ binary nanocomposites on free-standing graphene paper for flexible nonenzymatic glucose sensors*. *Biosensors and Bioelectronics*, 2013. **41**: p. 417-423.
 180. Wang, J., et al., *Nonenzymatic glucose sensor using freestanding single-wall carbon nanotube films*. *Electrochemical and Solid-State Letters*, 2007. **10**(5): p. J58-J60.

181. Cui, H.F., et al., *Selective and sensitive electrochemical detection of glucose in neutral solution using platinum-lead alloy nanoparticle/carbon nanotube nanocomposites*. *Analytica Chimica Acta*, 2007. **594**(2): p. 175-183.
182. Gao, H., et al., *One-step electrochemical synthesis of PtNi nanoparticle-graphene nanocomposites for nonenzymatic amperometric glucose detection*. *ACS Applied Materials & Interfaces*, 2011. **3**(8): p. 3049-3057.
183. Dong, X.-C., et al., *3D graphene-cobalt oxide electrode for high-performance supercapacitor and enzymeless glucose detection*. *ACS Nano*, 2012. **6**(4): p. 3206-3213.
184. Trouillon, R., D. O'Hare, and S.I. Chang, *An electrochemical functional assay for the sensing of nitric oxide release induced by angiogenic factors*. *Biochemistry and Molecular Biology Reports*, 2011. **44**(11): p. 699-704.
185. Thejass, P. and G. Kuttan, *Allyl isothiocyanate (AITC) and phenyl isothiocyanate (PITC) inhibit tumour-specific angiogenesis by downregulating nitric oxide (NO) and tumour necrosis factor-alpha (TNF-alpha) production*. *Nitric Oxide*, 2007. **16**(2): p. 247-257.
186. Zhang, X., et al., *Development of a fluorescent probe for nitric oxide detection based on difluoroboradiazas-indacene fluorophore*. *Analytica Chimica Acta*, 2003. **481**(1): p. 101-108.
187. Lim, M. and S. Lippard, *Fluorescence-based nitric oxide detection by ruthenium porphyrin fluorophore complexes*. *Inorganic Chemistry*, 2004. **43**(20): p. 6366-6370.
188. Dubey, M., S. Bernasek, and J. Schwartz, *Highly sensitive nitric oxide detection using X-ray photoelectron spectroscopy*. *Journal of the American Chemical Society*, 2007. **129**(22): p. 6980-6981.
189. Huang, K.J., et al., *Sensitive determination of ultra-trace nitric oxide in blood using derivatization-polymer monolith microextraction coupled with reversed-phase high-performance liquid chromatography*. *Analytica Chimica Acta*, 2007. **591**(1): p. 116-122.
190. Zhang, X., H. Ju, and J. Wang, *Electrochemical sensors, biosensors and their biomedical applications*. 2008: Elsevier. 593.
191. Gutierrez, A.P., et al., *Hybrid materials from carbon nanotubes, nickel tetrasulfonated phthalocyanine and thin polymer layers for the selective electrochemical activation of nitric oxide in solution*. *Electroanalysis*, 2009. **21**(21): p. 2303-2310.
192. Kroning, S., et al., *Myoglobin-clay electrode for nitric oxide (NO) detection in solution*. *Electroanalysis*, 2004. **16**(4): p. 253-259.
193. Krylov, A.V. and F. Lisdat, *Nickel hexacyanoferrate-based sensor electrode for the detection of nitric oxide at low potentials*. *Electroanalysis*, 2007. **19**(1): p. 23-29.
194. Shim, H.H., H. Do, and Y. Lee, *Simple fabrication of amperometric nitric oxide microsensors based on electropolymerized membrane films*. *Electroanalysis*, 2010. **22**(3): p. 359-366.
195. Sivanesan, A. and S.A. John, *Highly sensitive electrochemical sensor for nitric oxide using the self-assembled monolayer of 1,8,15,22-*

- tetraaminophthalocyanatocobalt(II)* on glassy carbon electrode. *Electroanalysis*, 2010. **22**(6): p. 639-644.
196. Shibuki, K., *An electrochemical microprobe for detecting nitric oxide release in brain tissue*. *Neurosci. Res.*, 1990. **9**(1): p. 69-76.
 197. Malinski, T. and Z. Taha, *Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor*. *Nature*, 1992. **358**: p. 676-378.
 198. Lee, Y., B.K. Oh, and M.E. Meyerhoff, *Improved planar amperometric nitric oxide sensor based on platinized platinum anode. 1. Experimental results and theory when applied for monitoring NO release from diazeniumdiolate-doped polymeric films*. *Analytical Chemistry*, 2004. **76**(3): p. 536-544.
 199. Wang, G. and J. Li, *Interfacial bioelectrochemistry: Fabrication, properties and applications of functional nanostructured biointerfaces*. *J. Phys. Chem. C*, 2006. **111**(6): p. 2351-2367.
 200. Guo, C.X., et al., *High-performance biofuel cell made with hydrophilic ordered mesoporous carbon as electrode material*. *Journal of Power Sources*, 2010. **195**(13): p. 4090-4097.
 201. Guo, C.X., et al., *Layered graphene/quantum dots for photovoltaic devices*. *Angewandte Chemie International Edition*, 2010. **49**(17): p. 3014-3017.
 202. Li, C.M., et al., *Electrochemical detection of nitric oxide on a SWCNT/RTIL composite gel microelectrode*. *Electroanal.*, 2006. **18**(7): p. 713.
 203. Wu, F.H., G.C. Zhao, and X.W. Wei, *Electrocatalytic oxidation of nitric oxide at multi-walled carbon nanotubes modified electrode*. *Electrochem. Commun.*, 2002. **4**(9): p. 690-694.
 204. Zhang, L., et al., *A nitric oxide biosensor based on myoglobin adsorbed on multi-walled carbon nanotubes*. *Electroanalysis*, 2005. **17**(7): p. 630-634.
 205. Zhang, Q., W. Wei, and G.C. Zhao, *Direct electrochemistry of myoglobin on a room temperature ionic liquid modified electrode and its application to nitric oxide biosensing*. *Electroanalysis*, 2008. **20**(9): p. 1002-1007.
 206. Thangavel, S. and R. Ramaraj, *Polymer membrane stabilized gold nanostructures modified electrode and its application in nitric oxide detection*. *J. Phys. Chem. C*, 2008. **112**(50): p. 19825-19830.
 207. Li, Y.J., et al., *Large-scale self-assembly of hydrophilic gold nanoparticles at oil/water interface and their electro-oxidation for nitric oxide in solution*. *J. Electroanal. Chem.*, 2008. **622**(1): p. 103-108.
 208. Zhang, J. and M. Oyama, *Gold nanoparticle arrays directly grown on nanostructured indium tin oxide electrodes: Characterization and electroanalytical application*. *Anal. Chim. Acta*, 2005. **540**(2): p. 299-306.
 209. Calabrese, V., et al., *Nitric oxide in the central nervous system: Neuroprotection versus neurotoxicity*. *Nature Reviews Neuroscience*, 2007. **8**(10): p. 766-775.
 210. Moncada, S. and J.P. Bolanos, *Nitric oxide, cell bioenergetics and neurodegeneration*. *Journal of Neurochemistry*, 2006. **97**(6): p. 1676-1689.
 211. Shan, C., et al., *Electrochemical determination of NADH and ethanol based on ionic liquid-functionalized graphene*. *Biosens. Bioelectron.*, 2009. **25**(6): p. 1504-1508.

212. Zhou, K.F., et al., *Electrocatalytic oxidation of glucose by the glucose oxidase immobilized in graphene-Au-Nafion biocomposite*. *Electroanalysis*, 2010. **22**(3): p. 259-264.
213. Schedin, F., et al., *Detection of individual gas molecules absorbed on graphene*. *Nature Materials*, 2007. **6**: p. 652-655.
214. Rao, C., et al., *Some novel attributes of graphene*. *The Journal of Physical Chemistry Letters*, 2010. **1**(2): p. 572-580.
215. Shao, Y., et al., *Graphene based electrochemical sensors and biosensors: A review*. *Electroanal.*, 2010. **22**(10): p. 1027-1036.
216. Alwarappan, S., et al., *Probing the electrochemical properties of graphene nanosheets for biosensing applications*. *J. Phys. Chem. C*, 2009. **113**(20): p. 8853-8857.
217. Wei, D. and A. Ivaska, *Applications of ionic liquids in electrochemical sensors*. *Anal. Chim. Acta*, 2008. **607**(2): p. 126-135.
218. Sun, W., et al., *Ionic liquid/mesoporous carbon/protein composite microelectrode and its biosensing application*. *Electrochem. Commun.*, 2009. **11**(11): p. 2105-2108.
219. Wang, G. and J. Li, *Interfacial bioelectrochemistry: Fabrication, properties and applications of functional nanostructured biointerfaces*. 2006.
220. Ganjali, M.R., et al., *Room temperature ionic liquids (RTILs) and multiwalled carbon nanotubes (MWCNTs) as modifiers for improvement of carbon paste ion selective electrode response: A comparison study with PVC membrane*. *Electroanalysis*, 2009. **21**(19): p. 2175-2178.
221. Haghighi, B. and R. Nikzad, *Prussian blue modified carbon ionic liquid electrode: Electrochemical characterization and its application for hydrogen peroxide and glucose measurements*. *Electroanal.*, 2009. **21**(16): p. 1862-1868.
222. Safavi, A., et al., *Simultaneous electrochemical determination of glutathione and glutathione disulfide at a nanoscale copper hydroxide composite carbon ionic liquid electrode*. *Analytical Chemistry*, 2009. **81**(18): p. 7538-7543.
223. Sun, W., et al., *Direct electrochemistry and electrocatalysis of hemoglobin on gold nanoparticle decorated carbon ionic liquid electrode*. *Talanta*, 2010. **80**(5): p. 2177-2181.
224. Terasawa, N. and I. Takeuchi, *Electrochemical property and actuation mechanism of an actuator using three different electrode and same electrolyte in air: Carbon nanotube/ionic liquid/polymer gel electrode, carbon nanotube/ionic liquid gel electrode and Au paste as an electrode*. *Sensors and Actuators B: Chemical*, 2010. **145**(2): p. 775-780.
225. Zhu, W.L., Y. Zhou, and J.R. Zhang, *Direct electrochemistry and electrocatalysis of myoglobin based on silica-coated gold nanorods/room temperature ionic liquid/silica sol-gel composite film*. *Talanta*, 2009. **80**(1): p. 224-230.
226. Rahimi, P., et al., *Ionic-liquid/NH₂-MWCNTs as a highly sensitive nano-composite for catalase direct electrochemistry*. *Biosensors and Bioelectronics*, 2009. **25**(6): p. 1301-1306.

227. Wei, D. and A. Ivaska, *Applications of ionic liquids in electrochemical sensors*. *Analytica Chimica Acta*, 2008. **607**(2): p. 126-135.
228. Sun, W., et al., *Ionic liquid/mesoporous carbon/protein composite microelectrode and its biosensing application*. *Electrochemistry communications*, 2009.
229. Rahimi, P., et al., *Ionic-liquid/NH₂-MWCNTs as a highly sensitive nano-composite for catalase direct electrochemistry*. *Biosensors and Bioelectronics*, 2009.
230. Si, Y. and E.T. Samulski, *Synthesis of water soluble graphene*. *Nano Letters*, 2008. **8**(6): p. 1679-1682.
231. Lu, W., et al., *Low-temperature exfoliated graphenes: vacuum-promoted exfoliation and electrochemical energy storage*. *ACS Nano*, 2009. **3**(11): p. 3730-3736.
232. Cha, C.S., et al., *Powder microelectrodes*. *Journal of Electroanalytical Chemistry*, 1994. **368**(1-2): p. 47-54.
233. Guo, C.X., et al., *Direct electrochemistry of hemoglobin on carbonized titania nanotubes and its application in a sensitive reagentless hydrogen peroxide biosensor*. *Biosensors and Bioelectronics*, 2008. **24**(4): p. 819-824.
234. Guo, C.X., et al., *RGD-peptide functionalized graphene biomimetic live-cell sensor for real-time detection of nitric oxide molecules*. *ACS Nano*, 2012. **6**(8): p. 6944-6951.
235. Ito, A., et al., *The effect of RGD peptide-conjugated magnetite cationic liposomes on cell growth and cell sheet harvesting*. *Biomaterials*, 2005. **26**(31): p. 6185-6193.
236. Guo, C.X., et al., *Biointerface by cell growth on layered graphene-artificial peroxidase-protein nanostructure for in situ quantitative molecular detection*. *Advanced Materials*, 2010. **22**(45): p. 5164-5167.
237. Kam, N.W.S., E. Jan, and N.A. Kotov, *Electrical stimulation of neural stem cells mediated by humanized carbon nanotube composite made with extracellular matrix protein*. *Nano Letters*, 2008. **9**(1): p. 273-278.
238. Hwang, D.S., S.B. Sim, and H.J. Cha, *Cell adhesion biomaterial based on mussel adhesive protein fused with RGD peptide*. *Biomaterials*, 2007. **28**(28): p. 4039-4046.
239. Uchida, M., et al., *Biomimetic coating of laminin-apatite composite on titanium metal and its excellent cell-adhesive properties*. *Advanced Materials*, 2004. **16**(13): p. 1071-1074.
240. Tang, L., et al., *DNA-directed self-assembly of graphene oxide with applications to ultrasensitive oligonucleotide assay*. *ACS Nano*, 2011. **5**(5): p. 3817-3822.
241. Zhong, X., et al., *A reusable interface constructed by 3-aminophenylboronic acid-functionalized multiwalled carbon nanotubes for cell capture, release, and cytosensing*. *Advanced Functional Materials*, 2010. **20**(6): p. 992-999.
242. He, Q., et al., *Centimeter-long and large-scale micropatterns of reduced graphene oxide films: Fabrication and sensing applications*. *ACS Nano*, 2010. **4**(6): p. 3201-3208.

243. Cohen, R.A. and P.M. Vanhoutte, *Endothelium-dependent hyperpolarization beyond nitric oxide and cyclic GMP*. *Circulation*, 1995. **92**(11): p. 3337-3349.
244. Asakawa, H., K. Mochitate, and T. Haruyama, *Seamless signal transduction from live cells to an NO sensor via a cell-adhesive sensing matrix*. *Analytical Chemistry*, 2008. **80**(5): p. 1505-1511.
245. Perley, G.A. and J.B. Godshalk, *Cells for pH measurements*, U.S.P. Office, Editor. 1947, Leeds and Northrup Company.
246. Rand, D. and R. Woods, *Cyclic voltammetric studies on iridium electrodes in sulphuric acid solutions: Nature of oxygen layer and metal dissolution*. *Journal of Electroanalytical Chemistry*, 1974. **55**(3): p. 375-381.
247. Mozota, J. and B.E. Conway, *Surface and bulk processes at oxidized iridium electrodes - I. Monolayer stage and transition to reversible multilayer oxide film behaviour*. *Electrochimica Acta*, 1983. **28**(1): p. 1-8.
248. Burke, L.D. and D. Whelan, *A voltammetric investigation of the charge storage reactions of hydrous iridium oxide layers*. *Journal of Electroanalytical Chemistry*, 1984. **162**(1-2): p. 121-141.
249. Beni, G., et al., *Electrocatalytic oxygen evolution on reactively sputtered electrochromic iridium oxide films*. *Nature*, 1979. **282**: p. 281-283.
250. Gottesfeld, S., et al., *Electrochromism in anodic iridium oxide films*. *Applied Physics Letters*, 1978. **33**(2): p. 208-210.
251. Yoshino, T., N. Baba, and K. Arai, *Electrochromic IrOx thin films formed in sulfatoiridate (III, IV) complex solution by periodic reverse current electrolysis (PRIROF)*. *Japanese Journal of Applied Physics*, 1987. **26**(9): p. 1547-1549.
252. Yamanaka, K., *Anodically electrodeposited iridium oxide films(AEIROF) from alkaline solutions for electrochromic display devices*. *Japanese Journal of Applied Physics*, 1989. **28**(4): p. 632-637.
253. Pasztor, K., et al., *Iridium oxide-based microelectrochemical transistors for pH sensing*. *Sensors and Actuators B: Chemical*, 1993. **12**(3): p. 225-230.
254. Mailley, S., et al., *Electrochemical and structural characterizations of electrodeposited iridium oxide thin-film electrodes applied to neurostimulating electrical signal*. *Materials Science and Engineering: C*, 2002. **21**(1-2): p. 167-175.
255. Bezbaruah, A.N. and T.C. Zhang, *Fabrication of anodically electrodeposited iridium oxide film pH microelectrodes for microenvironmental studies*. *Analytical Chemistry*, 2002. **74**(22): p. 5726-5733.
256. Elsen, H.A., C.F. Monson, and M. Majda, *Effects of electrodeposition conditions and protocol on the properties of iridium oxide pH sensor electrodes*. *Journal of The Electrochemical Society*, 2009. **156**(1): p. F1-F6.
257. Carroll, S. and R.P. Baldwin, *Self-calibrating microfabricated iridium oxide pH electrode array for remote monitoring*. *Analytical Chemistry*, 2010. **82**(3): p. 878-885.
258. Hitchman, M.L. and S. Ramanathan, *Evaluation of iridium oxide electrodes formed by potential cycling as pH probes*. *Analyst*, 1988. **113**(1): p. 35-39.

259. Wipf, D.O., et al., *Microscopic measurement of pH with iridium oxide microelectrodes*. Analytical Chemistry, 2000. **72**(20): p. 4921-4927.
260. Olthuis, W., et al., *pH sensor properties of electrochemically grown iridium oxide*. Sensors and Actuators B: Chemical, 1990. **2**(4): p. 247-256.
261. Chen, D., et al., *Human thrombin and FXa mediate porcine endothelial cell activation; modulation by expression of TFPI-CD4 and hirudin-CD4 fusion proteins*. Xenotransplantation, 2001. **8**(4): p. 258-265.
262. Fan, Y., et al., *Metabolic responses induced by thrombin in human umbilical vein endothelial cells*. Biochemical and Biophysical Research Communications, 2002. **293**(3): p. 979-985.
263. Trouillon, R., et al., *Electrochemical study of the intracellular transduction of vascular endothelial growth factor induced nitric oxide synthase activity using a multi-channel biocompatible microelectrode array*. Biochimica et Biophysica Acta (BBA) - General Subjects, 2010. **1800**(9): p. 929-936.
264. Trouillon, R.I., et al., *Angiogenin induces nitric oxide synthesis in endothelial cells through PI-3 and Akt kinases*. Biochemistry, 2010. **49**(15): p. 3282-3288.
265. Trouillon, R., et al., *Comparative study of the effect of various electrode membranes on biofouling and electrochemical measurements*. Electrochemistry Communications, 2009. **11**(7): p. 1409-1413.
266. Galdal, K.S., S.A. Evensen, and E. Nilsen, *The effect of thrombin on fibronectin in cultured human endothelial cells*. Thrombosis Research, 1985. **37**(5): p. 583-593.
267. Galdal, K.S. and S.A. Evensen, *Effects of divalent cations and various vasoactive and haemostatically active agents on the integrity of monolayers of cultured human endothelial cells*. Thrombosis Research, 1981. **21**: p. 273-284.
268. Kondo, N., et al., *Thrombin induces rapid disassembly of claudin-5 from the tight junction of endothelial cells*. Experimental Cell Research, 2009. **315**(17): p. 2879-2887.
269. Eklund, S.E., et al., *A microphysiometer for simultaneous measurement of changes in extracellular glucose, lactate, oxygen, and acidification rate*. Analytical Chemistry, 2004. **76**(3): p. 519-527.
270. Guo, C.X., et al., *Thin-walled graphitic nanocages as a unique platform for amperometric glucose biosensor*. ACS Applied Materials & Interfaces, 2010. **2**(9): p. 2481-2484.
271. Umana, M. and J. Waller, *Protein-modified electrodes. The glucose oxidase/polypyrrole system*. Analytical Chemistry, 1986. **58**(14): p. 2979-2983.
272. Sung, W.J. and Y.H. Bae, *A glucose oxidase electrode based on electropolymerized conducting polymer with polyanion-enzyme conjugated dopant*. Analytical Chemistry, 2000. **72**(9): p. 2177-2181.
273. Li, C., et al., *Electrochemical thin film deposition of polypyrrole on different substrates*. Surface and Coatings Technology, 2005. **198**(1): p. 474-477.

274. Rowinski, P., et al., *Electrodes modified with monoolein cubic phases hosting laccases for the catalytic reduction of dioxygen*. Analytical chemistry, 2004. **76**(2): p. 283-291.
275. Boyd, C.E.T., Craig S., *Pond aquaculture water quality management*. 1998: Kluwer Academic Publisher.
276. Manesh, K.M., et al., *One-pot construction of mediatorless bi-enzymatic glucose biosensor based on organic-inorganic hybrid*. Biosensors and Bioelectronics, 2010. **25**(7): p. 1579-1586.
277. Pang, X., et al., *An amperometric glucose biosensor fabricated with Pt nanoparticle-decorated carbon nanotubes/TiO₂ nanotube arrays composite*. Sensors and Actuators B: Chemical, 2009. **137**(1): p. 134-138.
278. Gee, E., M. Milkiewicz, and T.L. Haas, *p38 MAPK activity is stimulated by vascular endothelial growth factor receptor 2 activation and is essential for shear stress-induced angiogenesis*. Journal of Cellular Physiology, 2010. **222**(1): p. 120-126.

Publications

1. L. Yu, **S.R. Ng**, Y. Xu, H. Dong, Y.J. Wang, C.M. Li. 2013. Advances of lab-on-a-chip in isolation, detection and post-processing of circulating tumour cells. *Lab Chip*. 13: 3163-3182
2. C.X. Guo, **S.R. Ng**, S.Y. Khoo, X.T. Zheng, C. Peng, C.M. Li. 2012. RGD-peptide functionalized graphene biomimetic live-cell sensor for real-time detection of nitric oxide molecules. *ACS Nano*, 6(8): 6944-6951
3. C.X. Guo, X.T. Zheng, **S.R. Ng**, Y. Lai, Y. Lei, C.M. Li. 2011. In situ molecular detection of ischemic cells by enhanced protein direct electron transfer on horseradish peroxidase-Au nanoparticles-polyaniline nanowires biofilm. *Chemical Communications*, 47(9): 2652-2654
4. **S.R. Ng**, C.X. Guo, C.M. Li. 2011. Highly sensitive nitric oxide sensing using three-dimensional graphene/ionic liquid nanocomposite. *Electroanalysis*, 23(2): 442-448

Book chapter

C.X. Guo, S.R. Ng, C.M. Li. 2012. "Graphene-based electrochemical biosensors" in "Biosensors based on nanomaterials and nanodevices". CRC Press, Taylor & Francis Group.