Appendix

Published Paper and Conference Presentations

Interact 2004 Conference Proceedings. p95

IN VIVO DETECTION OF THE NEUROTRANSMITTER DOPAMINE AT A 2,4-DINITROANILINE MODIFIED CARBON ELECTRODE

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There has long been vigorous work in gaining a better understanding of the functions of neurotransmitters in mammalian brain systems. In particular, a great deal of attention has been directed towards the neurotransmitter dopamine owing to its related role to drug addictions and mental illnesses such as Parkinson's disease and schizophrenia. Electrochemical detection at physically small electrodes can offer several advantages in the *in vivo* analysis of neurotransmitters including real time measurements, accurate spatial resolution and minimal tissue damage during implantation. Equally important, electrochemistry is suited to the detection of neurotransmitters because of their ease of oxidation at a relatively low potential. However, a major interference effect in the electrochemical detection of dopamine arises from ascorbic acid, which can be oxidised at a similar oxidation potential to that of dopamine. Moreover, the much higher concentration of ascorbic acid (2.5 mM) in the extracellular fluid compared to that of dopamine (1 μM) often masks the presence of dopamine, making it very challenging in the detection of dopamine *in vivo*. In this paper, we will report our development of a surface modification strategy to enhance the sensitivity and selectivity of dopamine at physically small carbon electrodes *in vivo*. Briefly, this involves an anodic attachment of 2,4-dinitroaniline to the electrode surface in dichloromethane; in addition, the ability of this strategy in reducing fouling of electrodes by high molecular weight proteins during *in vivo* detection will also be discussed.

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FEATURE

Amperometric detection of dopamine release

Emerging electrochemical techniques are producing electrodes not only miniature in surface area, but also tiny in physical dimensions. Danny Wong and colleagues describe some of their efforts to manufacture physically small carbon cylinder electrodes and preliminary application of these to the real-time detection of the neurotransmitter dopamine in mammalian cells.

A neurotransmitter called dopamine

The determination of neurotransmitters in vivo has attracted considerable interest in analytical chemistry and neuroscience. These important chemical substances are released into the synapse from a nerve terminal to either excite or inhibit adjacent nerve cells. A particular interest lies in understanding the role of dopamine (3-hydroxytyramine) in the brain because of the close association of abnormal dopamine neurotransmission (release from nerve terminals) with a range of human brain disorders, including Parkinson's disease, schizophrenia and various forms of drug addiction and attention deficit disorders. Countless behavioural, pharmacological and physiological studies in animals have implicated dopamine release in the brain in various aspects of motivated behaviours, including feeding, drinking and sexual behaviours.[1] As such, neuroscientists have been exploiting considerable advancements in the development of specific analytical techniques for the detection and quantification of neurotransmitter release in the brains of both anaesthetised and awake behaving animals.

Shrink the electrodes!

In conjunction with anatomical, physiological and pharmacological evidence, electrochemistry is increasingly becoming a sensitive, real-time detection method for neurotransmitters in vivo.[2] This arises from several attributes of electrochemistry including selectivity, sensitivity, rapid response time and extremely small dimensions of electrochemical probes. Selectivity is important because the matrix (neuronal tissue) in which the measurements will be performed is very complex. Sensitivity is often required because the analyte of interest is present only in trace amounts in vivo. Biochemical events frequently take place on a millisecond time scale (e.g. neuronal firing) and, hence, a rapid response time is necessary. Further, there are emerging techniques in fabricating electrodes with a small physical dimension. The use of these ultramicroelectrodes permits minimal tissue damage upon implantation and, of equal importance, permits very careful selection of the region of tissue where measurements can be performed.

Because of their small surface area, microelectrodes exhibit several advantages including enhanced mass transport, reduced ohmic potential drop and diminished double layer charging effects, making them well suited for use in the detection of neurotransmitters in vivo. Hence, there is still a continuing effort in the manufacture of electrodes with characteristically small dimensions, even though development in this area started nearly 25 years ago.[3–5] To date, submicrometre-sized electrodes with different geometries including disc, band and ring have been prepared. Further, emerging technologies have facilitated the construction of electrodes not only miniature in surface area but also tiny in their physical dimensions. In several reports, electrodes with submicrometre- and nanometre-sized tip diameters were manufactured.[6–9]

In our laboratory, we routinely exploit a methodology involving the pyrolysis of hydrocarbon gases to construct structurally small carbon electrodes. In this methodology, quartz capillaries are initially pulled down to a small tip. In our earlier work, methane was forced through the pulled capillary while it was being pyrolysed.[10] By prolonging the pyrolysis time, a carbon deposit was formed at the tip of the pulled capillary to yield a carbon disc electrode with tip diameters approaching 100 nm. More recently, by adopting a similar procedure and setup, we pyrolysed acetylene in a pulled capillary placed in a nitrogen atmosphere.[11] In such work, by controlling the flow rate and flow direction of nitrogen and acetylene, carbon electrodes ranging from a disc geometry to an approximately cylindrical geometry can be fabricated. The latter electrode geometry exhibits similar appreciable surface area characteristics of carbon fibre electrodes but with an added bonus of enhanced mechanical strength. The appreciable surface area precludes the use of sophisticated instruments to measure

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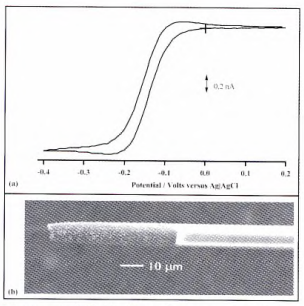

Figure 1 (a) Cyclic voltammogram for the reduction of 0.1 mM $Ru(NH_3)_6^{3+}$ in 50 mM KCl supporting electrolyte at a carbon cylinder electrode. Scan rate = 100 mV/s. This electrode was fabricated in the presence of a nitrogen supply flowing in a parallel direction as the acetylene gas stream. **(b)** Scanning electron micrograph of the carbon cylinder electrode used in (a).

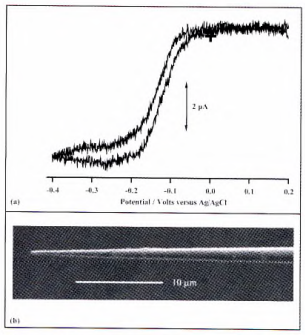

Figure 2 (a) Cyclic voltammogram for the reduction of 2.0 mM $Ru(NH_3)_6^{3+}$ in 50 mM KCl supporting electrolyte at a carbon cylinder electrode. Scan rate = 5 mV/s. This electrode was fabricated using the same conditions as in Figure 1 but a much more finely pulled quartz capillary. **(b)** Scanning electron micrograph of the carbon cylinder electrode used in (a).

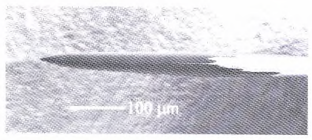

Figure 3 A scanning electron micrograph of a carbon cylinder electrode with a larger surface area, which can be fabricated in the presence of a nitrogen supply flowing in a counter-direction to the acetylene gas stream.

the otherwise tiny currents generated in an in vivo experiment. For characterisation purposes, we shall focus on carbon cylinder electrodes with a larger dimension in the following discussion.

Assessing electrode integrity

Very often, cyclic voltammetry is a convenient technique for assessing the integrity of fabricated electrodes. Figure 1(a) depicts a cyclic voltammogram for the reduction of 0.1 mM $Ru(NH_3)_6^{3+}$ in 50 mM KCl supporting electrolyte at a carbon cylinder electrode. This system was employed because of its insensitivity to the chemistry of the carbon surface. In this experiment, the current arising from the reduction of $Ru(NH_3)_6^{3+}$ was measured while the potential was scanned from 0.2 to –0.4 V against a Ag|AgCl electrode. In this voltammogram, a sigmoidal-shaped signal with minimal background charging current between the forward and reverse scan is used to indicate that the carbon deposit is a relatively non-porous structure which adheres sufficiently onto the wall of quartz capillaries.

Figure 1(b), a scanning electron micrograph (SEM) of the carbon electrode used in Figure 1(a), shows that a continuous carbon film (obtained in parallel streams of nitrogen and acetylene) was deposited at the tip and on the shank of the electrode. From chronoamperometric results, the radius and the length of the carbon cylinder were estimated to be 6 and 53 µm, respectively. (Note that the radius here represents the average radius of the cylindrical structure, not the tip dimension.) These values agree with those visually estimated from the SEM (6 µm in radius and 60 µm in length).

Using the same experimental conditions, but a much more finely pulled quartz capillary, our fabrication technology is capable of producing electrodes with tip diameters (electrochemically estimated by assuming a disc geometry) as small as 14 nm . The cyclic voltammogram and SEM obtained at such an electrode are shown in Figure 2. In spite of the noisy characteristics in this voltammogram, there is still a distinct sigmoidal-shaped signal obtained. However, with this small dimension, we are unable to ascertain the geometry around the tip of the electrode.

Tailor-made surface areas

We deliberately introduced nitrogen gas in a flow direction counter to that of acetylene. Figure 3 shows an SEM of an electrode fabricated in this manner. Compared to Figure 1(b), apart from a continuous film deposited on the electrode, a longer carbon cylinder (~300 µm in length) with a jagged edge toward the larger end of the electrode was obtained. The increased length is expected as the acetylene was being transported further up the shank by the counter-flow of nitrogen. Meanwhile, turbulence arising from the counter-flow pattern of nitrogen and acetylene was likely to be responsible for the formation of a jagged edge in the carbon film.

Dopamine autoreceptor function

A prominent dopamine neuronal system of interest to neuroscientists and clinicians alike is the mesoaccumbens dopamine system. As shown in Figure 4, neuronal cells in the ventral tegmental area of the mammalian (e.g. rat) midbrain contain dopamine. These cells send projections to neurons in the forebrain nucleus accumbens. The arrival of an action potential to dopamine nerve terminals engages dopamine release into the synaptic cleft. Upon release, dopamine diffuses across the synapse to interact with receptors on the opposing neuron, thus completing information transfer from one neuron to another. Dopamine activation of these post-synaptic receptors initiates behaviours critically involved in motivation and drug reinforcement. However, dopamine also acts on receptors located on dopamine terminals themselves. These presynaptic 'autoreceptors' act to decrease subsequent action potential-mediated dopamine release. Thus, dopamine autoreceptors permit fine-tuning of dopamine release at any point in time.

Studies in rats have shown that selective activation of these autoreceptors with certain drugs inhibits dopamine release and locomotor

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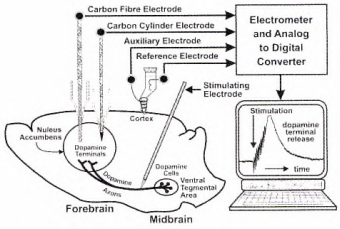

Figure 4 Cross-section of the rat brain depicting dopamine cells in the midbrain ventral tegmental area that send axonal projections to terminate on neurons in the forebrain nucleus accumbens. A conventional carbon fibre or carbon cylinder microelectrode was implanted in the nucleus accumbens, together with a Ag|AgCl reference and auxiliary electrode combination onto the surface of the cortex to complete the three-electrode electrochemical circuit. An electrical stimulating electrode was implanted into dopamine axons to directly stimulate dopamine release from its terminals. An electrometer applied a continuous fixed electrical potential to the carbon microelectrodes and the resulting oxidation current signal monitored on a computer in real time.

activity. In contrast, certain drugs of abuse (amphetamine and heroin) release large amounts of dopamine and increase locomotor activity. Autoreceptors stimulated by dopamine released by drugs of abuse initially oppose further increases in dopamine release. However, their continued use is thought to lead to a dysfunction in autoreceptors involving a loss of ability to counter dopamine release and, in turn, an amplification of their behavioural effects. This condition is termed drug-induced behavioural sensitisation (the most well established animal model of human drug dependence). Thus, the ability to assess dopamine autoreceptor regulation of dopamine release in real time is of significant importance in any effort directed to understanding the underlying neural mechanisms involved in drug addiction.

Electrode implantation

In conjunction with physically small electrodes as described here, fixed-potential amperometry is a suitable electrochemical method for the analysis of dopamine autoreceptor function because it offers a rapid sampling rate and hence enables sub-millisecond detection of dopamine neurotransmission.[12] This is employed here to evaluate the sensitivity and response characteristics of our cylindrical carbon electrodes, compared to conventional carbon fibre electrodes.

Carbon fibre electrodes were fabricated by pulling down a glass capillary containing a single carbon filament (10 µm outside diameter) with a length of 500 µm protruding from the microcapillary tip. A similar radial diffusion profile was expected around this electrode compared to a proportionally smaller carbon cylinder electrode.

Individual rats were anaesthetised with urethane before being placed in a stereotaxic apparatus (see Fig. 5). All adopted procedures and experiments were approved by Macquarie University Animal Ethics Committee (NSW, Australia) and were carried out in strict compliance with the Australian Code of Practice. Both types of electrodes were implanted into the nucleus accumbens and a bipolar electrical stimulating electrode placed within dopamine axons projecting to the nucleus accumbens (see Fig. 4). A Ag|AgCl and auxiliary electrode combination was placed in contact with the surface of the brain to complete the three-electrode circuit. The arrangement of the stimulating electrode with respect to the carbon microelectrodes and reference-auxiliary electrode is shown in Figure 4 and close-up overhead view of these electrodes in the rat's brain in Figure 6. Upon electrode implantation, a potential of +0.4 V was applied to the recording electrodes and the resulting amperometric current digitised (10 000 samples/s) and monitored continuously.

Brief electrical stimulations (5 monophasic pulses, 0.5 ms duration, 50 pulses/s) were applied to the dopamine axons every 30 s to determine the sensitivity and time characteristics of each of the carbon microelectrodes. Both electrodes yielded remarkably comparable responses to electrical stimulation of dopamine axons, as shown in Figure 7. The increase in the amperometric current corresponded to the period of electrical stimulation (100 ms) and was followed by a recovery back to pre-stimulus baseline levels within 500 ms of termination of stimulus, as recorded from both electrodes. The signal increase corresponds to dopamine release evoked by axonal stimulation and to a lesser extent presynaptic uptake clearance of dopamine and diffusion of dopamine away from the recording electrode surface. The subsequent decrease in the signal after stimulation corresponds to efficient presynaptic uptake, and to a lesser extent a combination of diffusion and dopamine metabolism.

Based on the smaller area of the carbon cylinder electrode, the amplitude of response was smaller than the carbon fibre electrode by an order of magnitude. Most significantly, the signal-to-noise ratio for the carbon cylinder electrode was also similar, despite the smaller dimensions. These response differences are consistent with the difference in surface areas of each electrode, suggesting that the carbon cylinder electrode may exhibit enhanced signal-to-noise ratio at even smaller dimensions. Overall, the profile and magnitude of the response can be considered a direct real-time measure of dopamine release.

To assess the contribution of dopamine autoreceptors to the evoked response, subsequent lower frequency stimulations were applied to the dopamine axons. The rationale here was to stimulate just enough dopamine release prior to the test stimulation to activate dopamine autoreceptors. As observed in Figure 7, a series of electrical stimulations of dopamine axons at a lower frequency (20 pulses at 15 pulses/s) before a second test stimulation (5 pulses at 50 pulses/s) markedly attenuated the test stimulus-evoked response. As

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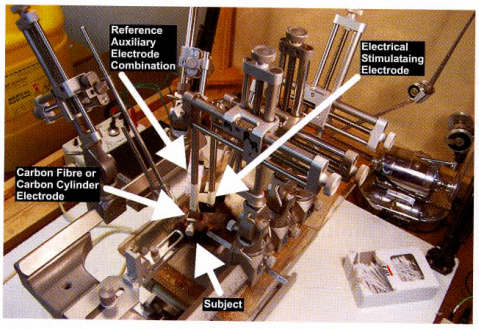

Figure 5 Side view of the experimental setup used to electrochemically record dopamine release in the nucleus accumbens in response to electrical stimulation of dopamine axons. The anaesthetised rat is placed in the stereotaxic frame to permit accurate positioning of the carbon recording electrodes into the accumbens, a reference-auxiliary electrode onto the cortex, and an electrical stimulating electrode into dopamine axons.

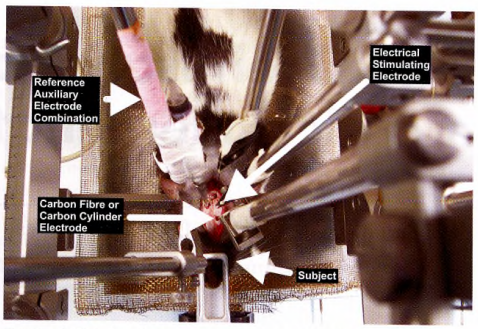

Figure 6 Overhead view depicting the positioning of the carbon recording electrodes into the rat accumbens, a reference-auxiliary electrode onto the cortex, and an electrical stimulating electrode into dopamine axons.

expected, in the presence of this autoreceptor inhibition, dopamine release evoked by the subsequent test stimulation was significantly reduced.

What lies ahead?

There are emerging techniques to construct physically small electrodes, opening up opportunities for analytical chemists and neuroscientists to study the chemistry and mechanisms involved in neurotransmission, which will enhance our understanding of the complex mammalian brain system. In this report, we have outlined a methodology involving the pyrolysis of acetylene gas to fabricate physically small carbon electrodes suitable for the amperometric detection of dopamine release evoked by electrical stimulation of dopamine axons in vivo.

Our preliminary results demonstrated that carbon cylinder electrodes with comparable dimensions to carbon fibre electrodes exhibit an improved signal-to-noise ratio. An added bonus of this methodology is that it allows better control in fabricating carbon cylinder electrodes with a range of physical dimensions including down to nanometres. Improved signal-to-noise ratios at these smaller electrodes will permit analyses with a great degree of spatial accuracy and reliability, as required in the brains of animals as small as the common laboratory rat or mouse. Presently, we are utilising the carbon cylinder electrodes to quantify changes in electrically stimulated dopamine release in the forebrain of the rat following exposure of the animal to drugs commonly abused by humans, such as cocaine and heroin. These in vivo electrochemical studies will allow us to identify the origin of excitatory inputs to dopamine cells in the midbrain and the neurotransmitter receptor mechanisms that underlie 'drug abuse'-induced changes in dopamine neurotransmission in the forebrain.

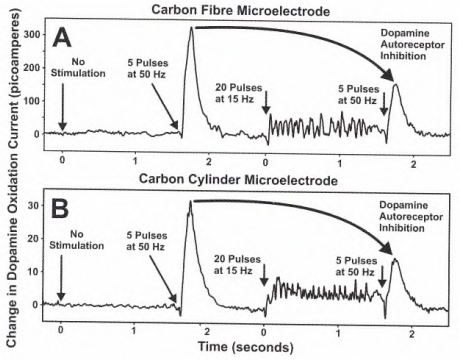

Figure 7 Comparison of the electrochemical responses of carbon fibre (A) and carbon cylinder (B) microelectrodes to brief electrical stimulation (5 pulses at 50 Hz alone) of dopamine axons alone. A series of 20 pulses of electrical stimulation applied at a lower frequency is sufficient to stimulate dopamine terminal autoreceptors such that an attenuation is observed in subsequent test stimulations of dopamine axons.

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Molecular Architecture of an *In Vivo* Dopamine Sensor

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Abstract - An Carbon cylinder electrodes with small tip diameters (≤2 μm) and an appreciable surface area are ideal for the detection of such neurotransmitters *in vivo*. However, owing to the close half-wave potential and much higher extracellular concentration of ascorbic acid, it is often a challenge in the electrochemical detection of the neurotransmitter dopamine in the presence of ascorbic acid. In this paper, enhanced detection of the cationic dopamine, relative to the anionic ascorbic acid, was achieved by exploiting anodically functionalised negatively charged 2,4-dinitrodiphenyl amine (in ethanol and 1,8-diazabicyclo-[5.4.0]undec-7-ene) on carbon cylinder electrodes. This resulted in the formation of an amine linkage and, speculatively, an ethanol linkage on the carbon surface. A significant feature of such a molecular architecture is that such linkages on the electrode surface are expected to be resistant to hydrolysis in aqueous solutions, further prolonging the lifetime of the electrochemical sensors. The voltammetric behaviour of both ascorbic acid and dopamine at amine/ethanol-modified carbon cylinder electrodes is presented in this paper.

1. Introduction

The determination of neurotransmitters (dopamine, noradrenaline, serotonin, etc.) *in vivo* has attracted considerable interest in analytical chemistry and neuroscience. In conjunction with anatomical, physiological, and pharmacological evidence, electrochemistry is increasingly becoming a sensitive, real-time detection method for neurotransmitters *in vivo* [1,2]. This arises from several attributes of electrochemistry including selectivity, sensitivity, rapid response time, and extremely small dimensions of electrochemical probes. Selectivity is important because the matrix (neuronal tissue) in which the measurements will be performed is very complex. Sensitivity is often required because the analyte of interest is present only in trace amounts *in vivo*. Biochemical events frequently take place on a millisecond time scale eg. neuronal firing and hence a rapid response time is necessary. Further, there are emerging techniques in fabricating electrodes with a small physical dimension. The use of such electrochemical sensors permits minimal tissue damage upon implantation and, of equal importance, permits very careful selection of the region of tissue where measurements can be performed.

Our laboratory has previously reported a simple methodology involving an *in situ* pyrolysis of acetylene resulting in the deposition of carbon at the tip and on the shank of pulled quartz capillaries to fabricate structurally small (≤2 μm tip diameters) cylindrical carbon electrodes with an appreciable surface area [3,4]. Notably, the quartz substrate provides the necessary mechanical strength during implantation of the electrode into a specimen, while the appreciable surface area enhances the detection sensitivity and negates the use of sophisticated instruments in measuring the otherwise tiny currents generated. Our work revealed that the characteristics of these electrodes were intermediate between a highly oriented pyrolytic graphite surface and a hydrogenated carbon structure. With an added bonus of low capacitance (~5 μF

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cm^{-2}), the electrodes are most suitable for the detection of neurotrasmitters *in vivo*.

The neurotransmitter dopamine (DA) is of particular interest to us. However, the detection of DA is often challenging owing to its similar half-wave potential to that of ascorbic acid (AA), and the much higher concentration of AA in extracellular fluid [5]. This has prompted a continuing effort in exploiting chemical modification methods to improve on the selectivity of the sensor surface towards DA relative to AA [6-10]. Under physiological pH conditions, DA exists as a cation and AA an anion. Therefore, a great deal of work has focussed on the immobilisation of a negatively charged film on an electrode surface in order to selectively enhance the detection of DA relative to AA. A common and convenient technique involves the immobilisation of the negatively charged ionomer, Nafion, onto electrode surfaces by repeatedly air drying electrodes that have been dipped into a Nafion solution. However, there is very little control of the polymer coating thickness on an electrode surface using this technique. This is of particular concern to such physically small electrodes as those fabricated in our laboratory. Also, the diffusion coefficient of dopamine through Nafion is known to be approximately 1,000 times smaller than that in aqueous solutions [11]. This is expected to cause a significant delay in the electrochemical measurement of events taking place. Hence, many researchers have investigated other chemical modification methods that provide better control of the film thickness while achieving the required selectivity towards DA [6-10].

Following the successful fabrication of carbon cylinder electrodes with a small physical dimension, we will report in this paper our attempts in immobilising a negatively charged organic film on these electrodes to further improve on their detection capability of DA. Clearly, this is a necessary consideration in the fabrication of an electrochemical sensor for the detection of dopamine *in vivo*. In this work, we have anodically bonded an amine (functionalised with nitro groups) to the pyrolysed acetylene surface, followed by further oxidation of an alcohol to yield possibly an anionic carboxylic group on the electrode surface. The electrochemical behaviour of these modified electrodes is presented in this paper.

2. Experimental

2.1 Chemicals and Reagents

Dopamine (DA), ascorbic acid (AA), methanol, 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), 2,4-dinitrodiphenyl amine (BDH, Sydney, Australia), potassium phosphate decahydrate, phosphoric acid, citric acid (all purchased from Sigma Aldrich), and ethanol (Shell Chemicals) were used without purification. Nafion perfluorinated ion-exchange powder supplied as a 5 wt% mixture in lower aliphatic alcohols and 10% water was purchased from Sigma Aldrich. All aqueous solutions were prepared using Milli-Q water. The pH 7.4 citrate/phosphate buffer was prepared by mixing appropriate amount of potassium phosphate decahydrate and citric acid, followed by adjustment with phosphoric acid if necessary. Fresh ascorbic acid and dopamine solutions were prepared with deoxygenated pH 7.4 buffer prior to use.

2.2 Apparatus

Carbon cylinder electrodes were fabricated by *in situ* pyrolysis of acetylene on pulled quartz capillaries, as reported by us previously [3,4]. Voltammetric experiments were conducted using an EDAQ Picostat capable of measuring picoamperes of current (EDAQ, Sydney, Australia). This potentiostatic system was operated using EChem v1.51 software (ADInstruments P/L, Sydney, Australia) *via* a MacLab 4e interface. The electrochemical cell was a conventional three-electrode arrangement with a Ag|AgCl (3 M KCl) reference electrode (in aqueous systems), or a platinum pseudo reference electrode (in non-aqueous systems), and a platinum coil as an auxiliary electrode. The cell was mounted on a rubber mat in an aluminium Faraday cage. All the solutions were purged with nitrogen for 10 min and then kept under a blanket of

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nitrogen. A period of 3 min was allowed for the solution to become quiescent prior to voltammetry.

2.3 Electrode modification

In this work, the electrochemical behaviour of physically small carbon cylinder electrodes in the presence of dopamine and ascorbic acid was examined. Here, electrodes either coated with either Nafion or chemically modified with 2,4-dinitrodiphenyl amine and ethanol were used. In the former, the electrodes were immersed in a 5 wt% Nafion solution for 10 s and then air dried for 15 min, followed by a further 10 min drying in an oven at 100°C. The entire immersion and drying procedure was then repeated two more times. On the other hand, chemical modification of the electrodes initially involved rinsing of electrodes in methanol. The electrodes were then anodised by conducting ten cyclic voltammetric scans between 0 and 1,500 mV in a mixture of 0.1 mM 2,4-dinitrodiphenyl amine and 0.1 mM DBU in methanol. The electrodes were then rinsed in ethanol and then scanned fifteen times between 0 and 1,500 mV in 0.1 mM DBU in ethanol. Finally, the electrodes were rinsed in Milli-Q water.

3. Results and Discussion

3.1 Electrochemical deposition of an anionic film on carbon cylinder electrodes

Based on our previous work in developing a simple procedure for the fabrication of physically small carbon cylinder electrodes and an understanding of the chemistry of the carbon surface [3,4], these electrodes are ideal for the detection of neurotransmitters *in vivo*. However, in order to improve on their selectivity towards DA, it is necessary for us to further investigate possible molecular architecture of an immobilised film on the electrode surface to enhance their detection capability. Such a film on an electrochemical sensor surface often relies on the well-known fact that DA is cationic and AA is anionic under physiological pH. In this respect, we intend to anodically bond 2,4-dinitrodiphenyl amine to the pyrolysed acetylene surface, followed by further oxidation of ethanol to yield an anionic carboxylic group on the electrode surface. Similar approaches based on the use of either an amine or an alcohol have previously been demonstrated to be an effective way for the enhancement of DA detection in the presence of AA oxidation [12]. However, in this work, both an amine and an alcohol were employed to compensate for the lack of edge planes on the carbon cylinder electrodes compared to a glassy carbon surface as reported by us previously [3,4].

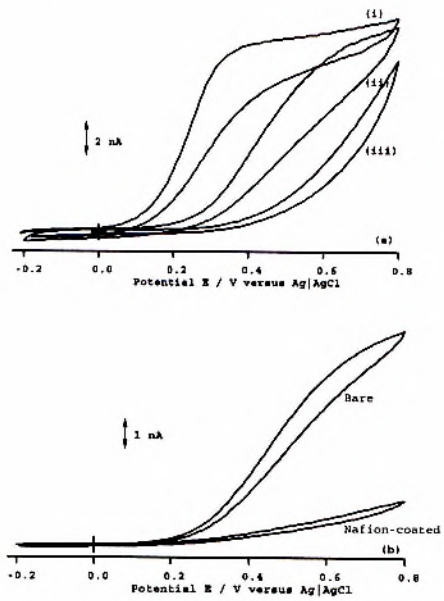

Figure 1 Cyclic voltammetry of (a) 0.1 mM 2,4-dinitrodiphenyl amine and 0.1 mM of DBU in methanol, (b) 0.1 mM DBU alone and ethanol at a carbon cylinder electrode. The cross in each voltammogram indicates the position of zero current. Scan rate = 100 mV s^{-1}.

In order to anodically deposit a film of 2,4-dinitrodiphenyl amine, we have initially conducted ten cyclic voltammetric scans at a carbon cylinder electrode in a solution containing 0.1 mM 2,4-dinitrodiphenyl

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amine and 0.1 mM of a strong organic base, DBU, in methanol between 0 and 1,500 mV. This was followed by fifteen additional cyclic voltammetric scans in 0.1 mM DBU alone and ethanol. The first and last voltammetric scans in these two series of cyclic voltammograms are shown in Figures 1(a) and (b), respectively. In both experiments, a decrease in oxidation current was observed as the number of scans was increased. According to Deinhammer et al [13], such an observation provides evidence for reactions involving 2,4-dinitrodiphenyl amine and ethanol at the carbon surface. Specifically, this decrease in current was attributed to the oxidation of 2,4-dinitrodiphenyl amine in solution, forming NR_2–linkages on the electrode surface [13]. On the other hand, we speculate that the oxidation of ethanol in the second series of cyclic voltammetric scans has promoted the formation of ethanol-carbon bonds on the electrode surface. Further work is being conducted to ascertain this particular aspect of our work.

Note that methanol was employed as the solvent in the first series of cyclic voltammetric scans involving 2,4-dinitrodiphenyl amine and DBU (Figure 1(a)), but ethanol was used in the second series of scans involving DBU (Figure 1(b)). This is because we observed in subsequent experiments carbon surface oxidised in the presence of ethanol exhibited more suppression of AA voltammetric response than that in methanol. Note also that all voltammetric scans above were conducted in the absence of a deliberately added supporting electrolyte, a unique feature associated with microelectrodes. In the experiments, we speculate that DBU was assisting the attachment of both an amine and alcohol but its exact role is currently being investigated in detail. A significant aspect of the molecular architecture developed in this work is that both the NR_2–linkage and ethanol-carbon bonds are expected to be resistant to hydrolysis during use in aqueous solutions. This will aid in producing a stable surface over an extended period of time.

3.2 Cyclic voltammetry of AA and DA at NR_2–ethanol–modified and Nafion-coated carbon electrodes

In this work, cyclic voltammetry of 0.1 mM AA (in pH 7.4 citrate/phosphate buffer solution) at an NR_2–ethanol–modified carbon cylinder electrodes was investigated. The results obtained are depicted in Figure 2(a). Initially, as shown in trace (i), a typical sigmoidal-shaped response was observed for the oxidation of 0.1 mM AA at a bare carbon cylinder electrode. However, trace (ii) shows a less sigmoidal AA oxidation voltammogram at an electrode anodised in the presence 2,4-dinitrodiphenyl amine, implying a less diffusion-controlled oxidation of AA at the electrode surface longer. When anodic oxidisation of ethanol was carried out on the same electrode, further distortion of the sigmoidal response of AA was observed in trace (iii). Hence, a barrier effect to the diffusion of AA appears to have gradually emerged as a result of each of the surface modification steps. In addition, the combination of the oxidation

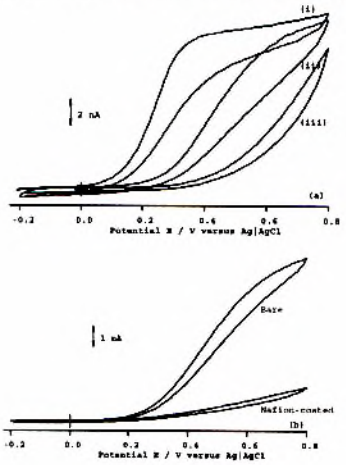

Figure 2 (a) Cyclic voltammetry of 0.1 mM ascorbic acid in a pH 7.4 citrate/phosphate buffer solution at (i) a bare carbon cylinder electrode, (ii) the electrode in (i) was oxidised in 2,4-dinitrodiphenyl amine, and (iii) the electrode in (ii) was oxidised in ethanol; (b) Cyclic voltammetry of 2.5 mM ascorbic acid in a pH 7.4 citrate/phosphate buffer solution at a bare and then Nafion-coated carbon cylinder electrode. Scan rate = 100 mV s^{-1}.

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of 2,4-dinitrodiphenyl amine and ethanol in trace (iii) appears to be capable of providing an additive effect in shifting the oxidation potential of ascorbic acid to a higher value compared to that at a bare electrode in trace (i).

In this work, the anodic detection of AA at Nafion–coated electrodes was also investigated and the results are displayed in Figure 2(b). Note that the bare carbon electrode was coated with Nafion three times in order to obtain a similar degree of suppression compared to that observed at an NR_2–/ethanol–modified electrode.

Similarly, the anodic behaviour of 0.1 mM DA in a pH 7.4 citrate/phosphate buffer was examined at a Nafion–coated electrode and an NR_2–ethanol–modified electrode. The results are shown in Figure 3. While these electrodes still display steady-state voltammetric responses, a 25% decrease in limiting current and a positive shift of 150 mV in the half-wave potential compared to a bare carbon electrode were observed. However, two different barrier effects are expected to be responsible for the diminished limiting current at NR_2–ethanol–modified and Nafion–coated electrodes. In

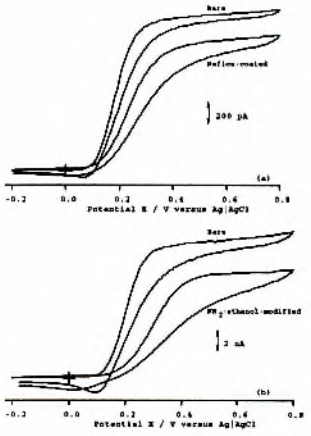

Figure 3 Cyclic voltammetry of 0.1 mM DA in pH 7.4 citrate/phosphate buffer at (a) a bare and then Nafion-coated carbon cylinder electrode, and (b) a bare and then NR_2-ethanol-modified carbon electrode. Scan rate = 100 mV s^{-1}.

the former, it is likely that there was a decrease in the electrochemically active sites as a result of the formation of NR_2–linkages and ethanol-carbon on the electrode surface. In contrast, there was a physical blockage of electrochemically active sites by Nafion on the electrode surface.

During the *in vivo* chronoamperometric detection, the released dopamine being oxidised at an electrode surface can often be reduced by the ascorbic acid in the extracellular fluid. This gives rise to a recycling process of dopamine close to the electrode surface, leading to an enhancement of the dopamine oxidation signal. As the chronoamperometric responses are very small (typically in the picoampere range), this feature can be crucial in detecting the release of dopamine, particularly when there is a significant level of background noise. To examine the performance of the NR_2–ethanol–modified electrodes, cyclic voltammetry was carried out in a solution containing 0.1 mM DA and 2.5 mM AA in a citrate phosphate buffer. For comparison, similar experiments were also conducted at Nafion-coated carbon electrodes. Note that the AA concentration represents the typical concentration found in physiological fluid in the brain. The results of these cyclic voltammetric scans obtained at Nafion–coated and NR_2–ethanol–modified electrodes are shown in Figure 4. Despite the same DA concentration used, the oxidation current obtained in the presence of DA + AA solution was clearly higher than that expected in a DA solution alone. This was very likely to be caused by the recycling of DA described above, where a portion of the DA oxidation product was reduced back to DA by AA, contributing to the current measured in the DA + AA solution. Indeed, an enhancement factor of approximately 2.5 in the DA limiting current was determined from these results, and hence a similar degree of suppression of AA voltammetric response was exhibited by both Nafion-coated and NR_2–ethanol–modified electrodes. It is important to note that all these results were obtained *in vitro*. While the NR_2–ethanol–modified electrodes appear to perform similarly to Nafion-

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coated electrodes, the latter suffer from a few drawbacks in *in vivo* experiments, as mentioned previously. Hence, the performance of these NR_2–ethanol–modified electrodes will need to be examined in *in vivo* systems for further assessment.

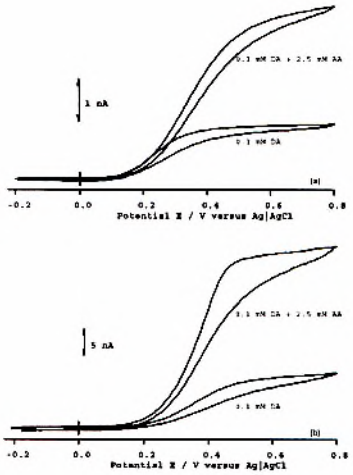

Figure 4 Cyclic voltammetry of 0.1 mM DA + 2.5 mM AA or 0.1 mM DA alone in pH 7.4 citrate/phosphate buffer at (a) Nafion-coated, (b) NR_2-ethanol-modified carbon cylinder electrodes. Scan rate = 100 mV s^{-1}.

4. Conclusion

Following the fabrication of physically small carbon electrodes by *in situ* pyrolysis of acetylene, the carbon surface was modified for the detection of DA in the presence of AA. This was achieved by the oxidation of 2,4-dinitrodiphenyl amine and ethanol at the carbon electrodes. These electrodes showed similar electron-transfer enhancement towards DA and electron-transfer suppression towards AA to those coated with three layers of Nafion. These electrodes will be implanted into rat brains so that their usefulness as an *in vivo* DA sensor can be further assessed.

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TOWARDS A STRUCTURALLY SMALL DOPAMINE-SELECTIVE PROBE

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Several neurotransmitters have been found to oxidise easily at an electrode, making electrochemical detection with structurally small electrodes an excellent *in vivo* technique for real-time analysis in mammalian brains. In our laboratory, a simple methodology involving an *in-situ* pyrolysis of acetylene in pulled quartz capillaries is routinely used to fabricate structurally small (≤5 μm tip diameters) carbon cylinder electrodes with an appreciable surface area. We found that the carbon surface exhibits characteristics intermediate between a highly oriented pyrolytic graphite surface and a hydrogenated carbon structure. With an added bonus of low capacitance (~5 μF cm^{-2}), the electrodes are most suitable for use in detection of neurotransmitters *in vivo*. Dopamine (DA) is a neurotransmitter of particular interest to us. However, the detection of DA is often challenging owing to its close half-wave potential to that of ascorbic acid (AA), and the much higher concentration of AA in extracellular fluid. This has prompted a continuing effort in improving the selectivity of *in vivo* probes for DA relative to AA. Common modification methods involve coating a Nafion or a conducting polymer film to yield an anionic membrane, which acts as a barrier to the oxidation of AA (existing as an anion at physiological pH's) at the electrode surface. However, such procedures are not readily adoptable in our work owing to the undesirably large capacitance increase arising from the additional layer on the small carbon electrodes. In this study, we have anodically bonded an amine (functionalised with carboxylic groups) to the pyrolysed acetylene surface, followed by further oxidation in phosphate buffer to yield an anionic carboxylic acid membrane on the electrode surface. A sigmoidal-shaped cyclic voltammogram was obtained for DA oxidation at these modified carbon electrodes, while the AA oxidation signal was significantly suppressed. Notably, the amide formed will provide a stable structure on the carbon surface, and the hydrophilic carboxylic acid group will aid in reducing biochemical fouling. Equally important, the procedure resulted in only minimal change in double layer capacitance (5 to 10 μF cm^{-2}). The features above will contribute further to the development of a sensitive and selective dopamine probe.