DEVELOPMENT OF PROTOTYPE REMOTE

SENSING KIT FOR SUBCUTANEOUS AND BRAIN GLUCOSE MONITORING

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DEVELOPMENT OF PROTOTYPE REMOTE SENSING KIT FOR SUBCUTANEOUS AND BRAIN GLUCOSE MONITORING

by

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This theses is dedicated to

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LIST OF ABBREVIATIONS

4VP	:	4-vinylpyridine
MeCN	:	Acetonitrile
ADC	:	analog – digital converter
ANOVA	:	analysis of variance
BBB	:	Blood – brain barrier
CNS	:	Central Nervous System
CV	:	cyclic voltammogram
DAC	:	digital – to – analog converter
EDX	:	Emission dispersive x-ray
EC	:	endothelial cells
FAD	:	flavin adenine dinucleotide
GOx	:	glucose oxidase
GLUT	:	Glucose transporters/hexose transporters
i_{max}	:	maximum current under saturating substrate conditions
MED	:	Mediator
K_m	:	Michaelis – Menten constant
OFN	:	Oxygen-free nitrogen
PBS	:	phosphate – buffered saline
P4VP	:	poly - 4-vinylpyridine
PEG	:	polyethylene glycol tert-octylphenyl ether
KH_2PO_4	:	potassium dihydrogen phosphate

SEM	:	Scanning electron microscope
NaCl	:	sodium chloride
NaOH	:	sodium hydroxide
STZ	:	streptozotocin
TMAC	:	tetramethylammonium chloride
TEER	:	Transendothelial electrical resistance

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DEVELOPMENT OF PROTOTYPE REMOTE SENSING KIT FOR SUBCUTANEOUS AND BRAIN GLUCOSE MONITORING

ABSTRACT

The study of subcutaneous and brain striatum glucose levels employing wireless glucose microsensor was proposed. A new implantable glucose sensor has been fabricated by immobilizing glucose oxidase on a chemically – modified carbon fiber prior to dipping in rat's serum for 3 days for preconditioning. The sensor was inserted subcutaneously and in the brain striatum on a male spraguely rat without any incision. The "preconditioned" microsensor, operated in the amperometric mode with an applied potential of 0.23 V *vs.* Ag|AgCl, was able to directly measure the glucose concentration upon infusion of glucose. The apparent Michaelis – Menten (K_m) value at 5.1 ± 0.5 mM was obtained. The "preconditioned" microsensor shows good stability and reproducibility with constant response spanned over 25 days. Most common interferences in glucose analysis were minimized by the outerlayer Nafion®. The sensitivities of both "clean" and "preconditioned" microsensors were 510 μM and 530 μM respectively. Analysis of glucose by both wired and wireless techniques are comparable with the conventional glucometer kit with R.S.D. value of 1.5%. Hematology examinations showed minimal material – tissue interaction.

The wireless transmission of glucose level was achieved by means of Bluetooth technology, embedded in a microprocessor. As the implanted microsensor was not affected by biocompatibility failures, these mechanical

devices allowed a more refined understanding towards glucose control in diabetic patients. This study has demonstrated that the wireless transmission measurement of subcutaneous and brain striatum glucose is feasible. The measurements of subcutaneous and brain striatum made via wireless sensing showed no significant variation ($p > 0.88$ two – way ANOVA; $n=5$), with measurements made via conventional method and glucometer kit. This indicated a statistically insignificant change in sensitivity.

PEMBANGUNAN KIT PENDERIAAN JARAK JAUH PROTOTAIP BAGI PEMONITORAN GLUKOSA SUBKUTIN DAN OTAK

ABSTRAK

Kajian tentang paras glukosa subkutin and dan otak dengan menggunakan mikropenderia glukosa tanpa wayar telah dicadangkan. Alat mikropenderia untuk tujuan memantau tahap glukosa didalam subkutin and otak telahpun direka dengan mengimobilisasikan enzim glucose oxidase ke atas gentian karbon sebelum ia di rendam di dalam serum tikus untuk tujuan pra-keadaan. Mikropenderia itu dapat dimasukkan ke dalam subkutin and bahagian striatum otak tanpa kesukaran. Mikropenderia dioperasi dalam mod amperometric dengan keupayaan potential +0.23 V vs Ag/AgCl mampu menyukat tahap glukosa dengan mudah. Nilai pemalar Michealis Menten yang diperoleh ialah 5.1 ± 0.5 mM. Mikropenderia "prakeadaan" ini adalah amat stabil and menunjukkan kebolehulangan yang tinggi dengan gerakbalas yang stabil selama 25 hari. Kebanyakan gangguan dalam penentuan glukosa telahpun dikurangkan dengan penggunaan lapisan Nafion®. Kepekaan bagi kedua – dua mikropenderia ialah 510 μM dan 530 μM. Analisis glukosa dengan menggunakan kaedah wayar dan tanpa wayar adalah setanding dengan analisis daripada kit glucometer dengan nilai R.S.D. sebanyak 1.5%. Kajian hematologi menunjukkan interaksi antara bahan dan tisu adalah amat minimal.

Penghantaran tahap glukosa tanpa wayar dijalankan dengan menggunakan teknologi Bluetooth yang digabungkan dalam suatu

pemikroproses. Oleh kerana mikropenderia itu tidak dijejaskan oleh masalah kebioserasian, penggunaan alat mekanikal ini membolehkan kawalan glukosa dalam pesakit diabetik dijalankan dengan lebih berkesan. Kajian ini telah menunjukkan transmisi tanpa wayar tahap glukosa subkutin dan bahagian striatum otak mampu dilakukan. Ukuran glukosa subkutin dan striatum otak dengan menggunakan kaedah tanpa wayar tidak menunjukkan variasi bererti ($p > 0.88$ ANOVA dua hala; $n=5$) dengan pengukuran yang dibuat dengan menggunakan alat lazim dan kit glucometer. Ini menunjukkan tiada perubahan bererti statistik dari segi kepekaan.

1.0 INTRODUCTION

1.1 Relation between Blood Glucose Levels and Diabetes Mellitus

Insulin which is secreted by the islets of Langerhans of the pancreas [1 – 3], contains four types of cell; (i) B – (or β –) cells secrete insulin (ii), A– cells secrete glucagons (iii), D– cells secrete somatostatin and (iv) PP cells secrete pancreatic polypeptide (the function of which is unknown). The core of each islet contains mainly the predominant B–cells surrounded by a mantle of A– cells interspersed with either D– cells or PP cells. The insulin is synthesized as a precursor in the rough endoplasmic reticulum and usually, any insulin that is active in one species is often active in another. Blood glucose concentration is the main factor controlling the synthesis and secretion of insulin. B – cells corresponds both to the rate of change of blood glucose and to the absolute glucose concentration. There is a steady basal release of insulin and also a response to a change in blood glucose. The response to increase in blood glucose has two phases; (i) the initial rapid phase reflecting the release of stored hormone and (ii) the slower phase, reflecting both continued release of stored hormone and new synthesis. Insulin entering the blood circulation greatly enhances the uptake of glucose in certain tissues but with only minimal effect on the uptake of glucose by brain tissues. Hence, the response to the change in blood glucose level becomes abnormal only in diabetes mellitus.

Diabetes mellitus is a complex endocrine metabolic disorder that resulted from a total or partial lack of insulin. The earliest manifestation of the disease is

the loss control of the blood glucose level. There are three different major groups of diabetes i.e., gestational, non – insulin dependant (maturity – onset diabetes) and insulin dependant diabetes (juvenile – onset diabetes). In all, diabetes mellitus occurs upon absolute deficiency of insulin, which is secreted continuously by pancreatic beta cells. The secretion of insulin in response to glucose is rapid and could be triggered even by as low as 2 mM glucose [1]. The secretion is stopped when insulin has brought the glucose level to its basal. This overall mechanism keeps the blood glucose level in check. Thus, the destruction of pancreatic beta cells could lead to severe insulin deficiency. Hyperglycemia is a classic symptom of diabetes mellitus but hyperglycemic condition without other classic symptoms is not dispositive of a diagnosis of diabetes mellitus. Hyperglycaemia is due to uncontrolled hepatic glucose output and reduce uptake of glucose by skeletal muscle with reduce glycogen synthesis. However, hyperglycemia is also an independent medical condition with other causes [2]. As the renal threshold for glucose re-absorption is exceeded, glucose spills over into the urinary tract. Hence, for this very reason, it is important to monitor the glucose level in human blood.

1.2 Glucose Transport to the Brain

1.2.1 The blood – brain barrier

Over 100 years ago, a simple experimental showed that if blue dye was injected into the blood stream of an animal, that tissues of the whole body except the brain and spinal cord could turn blue. These bizarre observations i.e. the

failure of the brain and spinal cord to be stained at autopsy was observed by Ehrlich et al [3 – 6]. To explain this, a barrier, namely the blood – brain barrier (BBB), was thought to exist between the blood vessels and the brain and this barrier prevented materials from entering the brain. The structure of the BBB remains elusive until recent reports [7 – 9] have shown that the BBB consisted of microvascular endothelial cells held together by tight junctions. These endothelial cells were somehow different from other endothelial cells due to the absence of fenestrations and the endothelial cells were linked by tight junctions. These tight junctions prevented molecular communication between the brain or the central nervous system (CNS) and the plasma (Figure 1). Only certain hydrophilic molecules such as monoamines, glucose, acetylcholine and L – amino acids diffused across the BBB while small lipophilic molecules, i.e. O_2 and CO_2, penetrated the barrier freely [10 – 12]. Such a rigid separation imposes at least two or more critical conditions;

1) Cells of the immune system had minimal access to the BBB. The CNS was thought to be excluded from the activities in the immune system and
2) Important therapeutically active compounds had limited access to the brain as well. Only a fraction of the neuroactive drugs penetrate the BBB and was mediated by a transporter, which can act either to facilitate entry into the brain or to diminish it by pumping the compound from the endothelial cell interior back into the bloodstream. Drugs that gain entry in this way includes various sedative histamine antagonists and those were excluded are such as antibacterial and anticancer drugs that are substrate

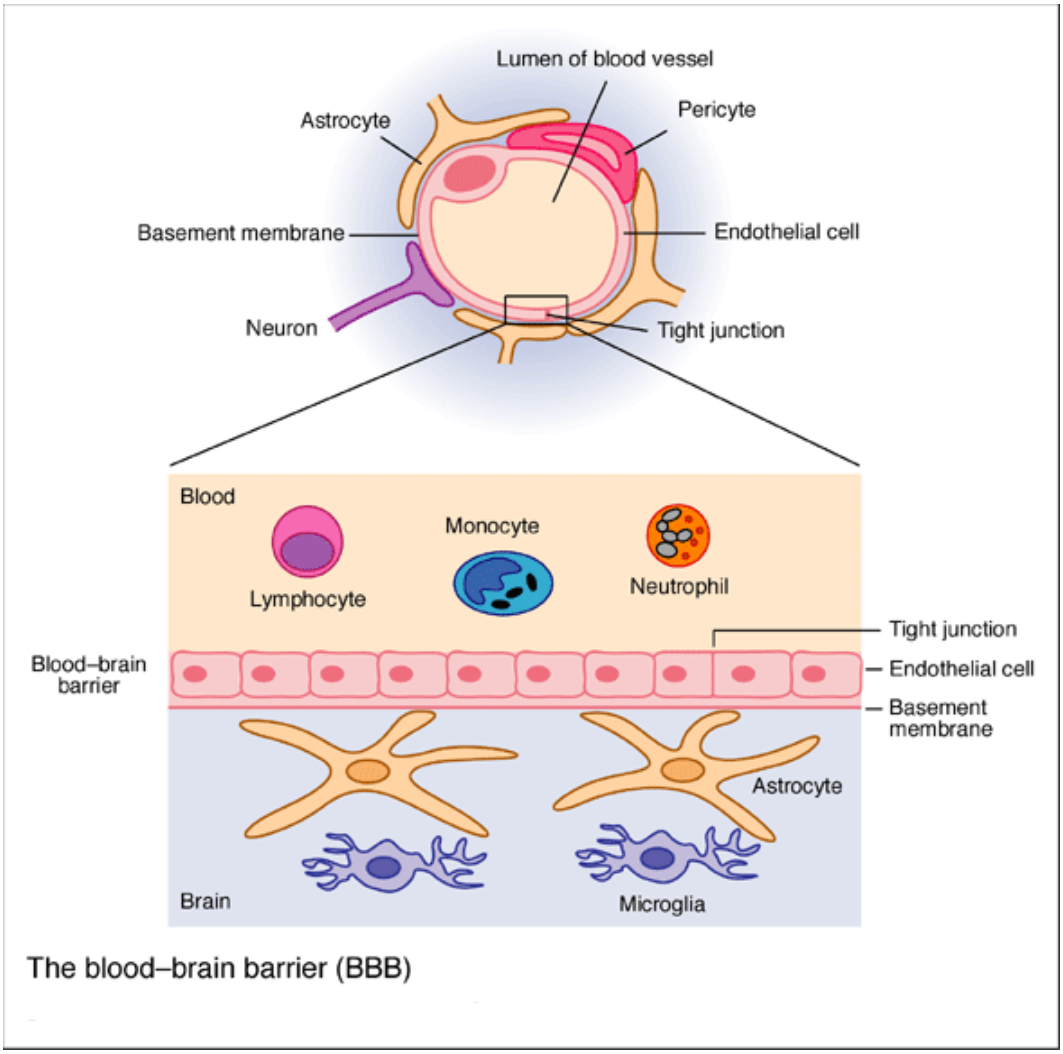

Figure 1: The microvascular structure of the blood – brain barrier.

for the P – glycoprotein transporter [8]. The fully differentiated BBB consists of a complex cellular system of highly specialized endothelial cells, a number of pericytes embedded in the basal membrane perivascular macrophages and astrocytic endfeet.

While the endothelial cells form the forefront of the BBB, the interaction with adjacent cells seems to be a prerequisite for barrier function. Among the features of BBB properties were;

1) Tight junctions are domains of occluded intracellular clefts which formed intramembrane of strands and appeared as a chain of fusion points [13 – 16]. Unlike simple tight junctions, these junctions in BBB provide high electrical resistance and low permeability in concert with a stable morphology
2) The endothelial cells have lower rate of transcytosis in comparison to other peripheral organs and minimal transcellular exchange of solutes and
3) The intake of important molecules into the CNS system was mediated by specific transport and carrier molecules. This mediated diffusion was energy free, as in the case of mediating glucose into the brain. These three features allowed cerebral microcapillaries to form a tight cellular layer with a high transendothelial electrical resistance (TEER) [17 – 22].

1.2.2 Blood – brain barrier glucose transport

The brain uses glucose, largely through oxidative metabolism, as its primary fuel for energy generation. When blood glucose levels are reduced i.e. insulin administration to levels below 3 mmol/L, subtle brain dysfunction occurs in

humans within minutes. There is little short-term flexibility of the brain for facultative use of alternative fuels. Normally, the brain depends upon a continuous supply of glucose from the blood stream. Glucose must be transported into the brain through the endothelial cells of the brain microvasculature using facilitated diffusion by transport carrier proteins.

Glucose transport is one of the most essential and most studied of the BBB transport systems. Tight junctions of the BBB endothelia block solutes, including blood-borne glucose, from crossing between cells into brain extracellular space. Glucose, as a polar, hydrophilic compound, does not sufficiently diffuse either into or out of the intact monolayer of the endothelial cells (ECs) lining brain capillaries. To maintain the brain's high rate of aerobic metabolism and neuronal homeostasis, highly regulated transport of glucose must occur through regionally selective, facilitated transport [23, 24]. In response to this high demand, glucose is transported rapidly via transporter proteins. There are saturable and unsaturable components to the transport. Saturable transport occurs via an energy-independent hexose transporter across the two sides of the cell membrane, while diffusion process occur intra- and paracellularly. Different members of the family of hexose transporters, GLUTs, are found in the brain and throughout the mammalian system [25]. One member, GLUT1, is located on the BBB endothelium, both luminally and abluminally [25]. The 55-kDa GLUT1 isoform, also found in human erythrocytes, has been credited as the main glucose transporter of the BBB endothelia.

1.3 Electrochemical microsensors fabrication

Analytical, biological and clinical chemists have for decades concerned themselves with the development of instruments and techniques capable of determining the identity and concentration of chemical substances that affect living things. Recently, this endeavor has focused on electrochemical biosensors, small portable or disposable sensing devices that can be used to detect biologically important subtances in vivo or in vitro.

Electrochemical amperometric microsensors have become powerful tools for studying fundamental chemical signaling in the body fluid [26 – 31]. The measurement is done by maintaining potential at a constant level sufficient for reduction/oxidation of the species of interest (or a substance electrochemically coupled to it). The current that flows is then proportional to the analyte concentration. In comparison with other analytical techniques, such microsensors possessed simplicity of operation and substrate selectivity of the enzymes. In addition to reducing tissue damage, miniaturization of the cathode area made the microsensor less sensitive to convection. This is important if the microsensor were to be implanted in the brain tissues. When the brain tissues were examined under light microscope after microsensor – based measurements, they bore no evidence that the microsensor has been implanted before. In other words, the "damage track" that the microelectrode left behind was too small [28]. One of the approaches to monitor the glucose concentration in human blood is by means of a mechanical device i.e. glucose sensor. The availability of implantable glucose sensors, first introduced during the early 80s, has improved the monitoring

process [30]. The measurement is possible because of a high transfer rate of glucose from blood vessels to the interstitial space which results in a short lag time between changes of the blood glucose concentration and the subcutaneous tissue. It has also been reported [31, 32] that the subcutaneous glucose concentration is at the same level as blood glucose under certain conditions. Therefore, any glucose sensor for in vivo application would have to be rapid in its response. Several enzyme – based glucose sensors [26 – 28, 30 - 33] have been applied for the in vivo or subcutaneous glucose monitoring with some degree of success (Table 1). As noted, most of these sensors are too large to be suitable for in vivo monitoring if frequent changes of the sensor are required. For such applications, a miniaturized sensor (less than 0.1mm in diameter) is needed that can be conveniently implanted by the patient. A sensor with an outer diameter of less than 0.1mm will be acceptable considering that the needles used for their daily insulin injections are in 0.3mm range. The fabrication of such devices usually requires the deposition of several layers of polymer and enzyme directly to the sensor. The miniaturization of the sensor, therefore, is not trivial. In addition, these sensors, upon implantation have relatively short “implantable life”. The decrease in response over time is, probably, due to an inflammatory reaction which occurs at the electrode – solution interface. The material tissue interaction upon sensor implantation is the biggest obstacle in developing a viable implantable sensor. Biocompability failures, i.e. fibrous encapsulation and membrane biodegradation limited the sensor’s longevity and caused inflammatory skin reaction at the implantation site.

Table 1: Glucose sensors used for subcutaneous glucose monitoring

Shape	o.d., (mm)	Determinant site	in vivo shelf life
Needle type	1.0	H_2O_2	5 days
Needle type	2.5	H_2O_2	8 days
Needle type	1.3	Dimethylferrocene	10 days
Needle type	0.5	Dimethylferrocene	14 days
Needle type	0.35	H_2O_2	14 days
planar	1.0 (width)	H_2O_2	14 days

The application of these glucose microsensors normally requires electrical communication between the active centre of the enzyme and the electrode surface [32 – 35]. Two buried redox active centers, flavin adenine dinucleotide (FAD), are found in 1 unit of glucose oxidase (GOx). These FAD centers are oxidized by O_2 which diffuses through the insulating protein shell. The overall enzymatic process could be illustrated as in the following equation,

$$GOx_{ox} + \text{glucose} \rightarrow GOx_{red} + \text{gluconolactone} \quad (1)$$

$$GOx_{red} + O_2 \rightarrow GOx_{ox} + H_2O_2 \quad (2)$$

From equations (1) and (2), the amount of glucose can be determined by detection of either H_2O_2 or O_2. However, both of these approaches have caused analytical difficulties as O_2 concentration usually fluctuates in the microenvironment of the brain. Thus, redox mediators have been added to shuttle the electron from FAD centers and electrode surfaces. In such systems, equation 2 is replaced by the following,

$$GOx_{red} + MED_{ox} \rightarrow GOx_{ox} + MED_{red} + H^+ \quad (3)$$

$$MED_{red} \rightarrow MED_{ox} + e^- \quad (4)$$

In this process, the mediator completes the redox cycle. The electrooxidization of the MED is utilized in the analytical detection of glucose.

Even though the use of chemicals as mediator(s) improved electron transfer, they are always costly. Sometimes they are not very sensitive at very low analyte concentration, and their compability with the enzyme concerned is

also questionable. Hence, the need to design biosensors which do not use mediators i.e. mediatorless. In recent years, various conducting polymers were employed in the fabrication of mediatorless sensors. One such polymer whch is extensively studied is poly – 4 vinylpyridine (P4VP) whose solution and metallocomplexes have been used in the preparation of chemically modified electrodes [34, 37, 39]. Isolation and purification of these complexes are normally complicated, expensive and time consuming. The P4VP layer so produced also tend to dissociate if an inert is not used together with low ionic strength solution [34, 37]. The direct polymerization of P4VP onto carbon paste electrodes for the detection of glucose has been reported [39]. However, the paste in such electrodes is difficult to reproduce, leading to irreproducible sensors. In addition to this, the immobilized enzyme, usually, suffers premature denaturation around the highly lipophilic environment of the paste. Thus, carbon fibre was chosen as it is typically carbon with acceptable conductivity and minimal cost [35].

1.4 In vitro glucose sensors: blood glucose test strips

Blood glucose test strips (Figure 2) rely on the coimmobilization of GOx and dimethylferrocene on a screen printed carbon surface [36 – 44]. The ferrocene in oxidized form acts as an artificial electron acceptor that shuttles electrons between the enzyme and carbon surface. The following reaction sequence occurs;

- Enzymatic reaction: glucose diffusing towards the electrode is oxidized to gluconic acid by the enzyme immobilized in a suitable matrix on the electrode surface

  $Glucose + GOx_{red} \rightarrow gluconolactone + GOx_{ox}$ (5)

- Mediator reaction: the oxidized form of the enzyme is regenerated by oxidation by the ferricinium ion, which thus acts as a cofactor for the enzyme

  $GOx_{ox} + 2FeCp_2^{+} \rightarrow GOx_{red} + 2FeCp_2 + 2H^{+}$ (6)

- Amperometric reaction: ferrocene is electrochemically oxidized at the electrode surface at the electrode surface with a reversible $E_{1/2}$ at +165mV versus SCE

  $2FeCp_2 \rightarrow 2FeCp_2^{+} + 2H^{+}$ (7)

Equation 5 is kinetically controlled and governed by the diffusion of substrate towards and through the immobilized enzyme layer where glucose is gradually consumed by oxidation of the enzyme. Equation 6 is virtually assumed to be quantitative. Equation 7 which yields the desired analytical signal, besides depending on reaction 5, that feeds it, is also governed by diffusion of ferrocene through the immobilization layer towards the carbon electrode. A schematic diagram of a test strip is shown in Figure 2.

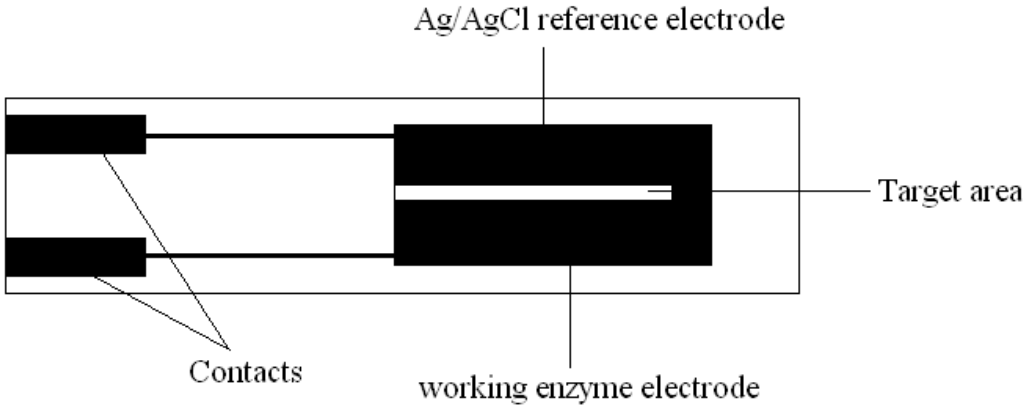

Figure 2: Schematic representation of a blood glucose test strip

1.5 In vivo glucose sensors

At the time of writing, there are three commercially available in vivo glucose sensors, which make use of different technologies (i) Minimed – Medtronic System (MMS), (ii) Glucowatch and (iii) Glucoday system [45, 46]. However, only MMS will be reviewed as it is more widely used by clinicians. The sensor is based on the long – established technology of GOx immobilized at a positively charged base electrode, with electrochemical detection of hydrogen peroxide production;

$$\text{Glucose} + O_2 + \text{GOx} \rightarrow H_2O_2 + \text{gluconic acid} \quad (8)$$

$$H_2O_2 \rightarrow O_2 + 2e^- \quad (9)$$

H_2O_2 is detected at 700mV vs SCE. Because the sensor is implanted in the subcutaneous tissue, it measures interstitial fluid glucose concentrations, which

are similar to blood glucose levels in the steady state but lag behind when glycaemia change rapidly, for example after a meal. The magnitude of this lag has been variously recorded in numerous studies with needle type enzyme electrodes (Table 1). Aside from the lag, two other major problems with MMS are unpredictable drift and impaired responses in vivo, which necessitate repeated calibration against finger-prick capillary blood glucose concentrations. As in the case of MMS, the calibration was performed four times daily. There has been considerable speculation as to the reason for lowered and drifting responses of sensors implanted in subcutaneous tissue, including protein and cell coating, low and varying pO_2 or a wound response. The fact that the sensor sensitivity remains low after implantation and recovers after repeated washing indicates a reversible accumulation of unknown substance on the sensor, rather than tissue – related phenomena. In a recent study, MMS profiles were compared to finger – prick blood glucose self – monitoring. There were many times when episodes of hyperglycemia or hypoglycemia in these patients were missed by intermittent testing but detected by continuous sensing. This particularly applied to postprandial hyperglycemia and nocturnal hypoglycemia, though the mean and oscillations in blood glucose concentrations were about equally assessed by MMS and intermittent testing. The accuracy of MMS using the much – used (and much criticized) Clarke grid error is apparently good, with about 90% of non – calibration paired blood and sensor values falling in the clinically acceptable zones. However, in clinical practice important disagreements between sensor

and reference blood glucose are not uncommon. This has been reported in the past [45, 46].

1.6 Wireless sensing

In the early 1990s, a two – way voltammetry system based on a wireless interconnection via infrared (IR) transmission between the laboratory animal and measuring devices was proposed [47 – 50]. The ramp was then sent to the working electrode via IR (one way). The consequent oxidation signal was sent back yet again via IR to the analyzing system (second way). In vivo recording of voltammetric recordings of ascorbic acid obtained by conventional system and IR method were well defined and almost identical in shape. Other in vivo experiments revealed that the IR could allow the simultaneous recordings of neuronal activity. However, dangerous variations of tension at the level of working electrode due to occasional, random interruption of the first way IR channel appeared to be one of the major problems. In addition, another major downside of employing such techniques could be incapability of controlling the mobile unit by the fixed station. This is because it operates over short range distances and at only medium range bandwith.

The use of biosensors on awake animals and free of cable tethers i.e. wireless transmission would be significant in measuring analyte in physiological fluid. Recent advances in wireless networking have opened up new opportunities in a variety of applications [51] including healthcare systems [52, 53]. The advent of Bluetooth allowed seamless interconnectivity between the animal laboratory

and the measuring equipment [51]. Bluetooth system does not require the laboratory animal to be confined to the measuring equipment. It also allows the animal to move around freely within a specific distance from the measuring equipment. Although susceptible to minimal noise interference, the data transmission was reliable at 99.9% [54, 55]. The uniqueness of wireless communications systems is that it enables ad hoc networking among devices without the need for infrastructure such as base stations or access points.

In developing remote sensing for an amperometric microsensor, a fast data acquisition from low amplitude signals exposed to noise artifacts is an essential. Although recent developments in digital telemetry hold great promise, it is only data rate limiting and its practical application is questionable [55]. Besides, an onboard microprocessor is needed for digitizing the analog signals obtained from the sensor. The capacity of such a microprocessor in performing several tasks simultaneously is doubtful [55, 69 – 71]. Also, measurements obtained using remote sensing should be free of electrical interference as conventional measurements are, normally, performed without a Faraday cage.

1.7 Objective

The aim of this study is to monitor subcutaneous and brain striatum glucose level in a diabetic and hyperglycemia rat employing “cableless” amperometric glucose microsensor. The first part of this study is to fabricate glucose microsensors for subcutaneous and brain striatum monitoring. The fabricated microsensor is then dipped in rat’s serum (“preconditioned”

microsensor) and its performance is compared with the microsensor without dipping in the rat's serum ("clean" microsensor). The "preconditioned" microsensor is used for subcutaneous glucose monitoring while the "clean" microsensor is used for brain striatum glucose monitoring. The second part of the study is to implant the microsensors both subcutaneously and in the brain striatum. For subcutaneous glucose studies, hematological aspect on each group of rats after the implantation for both types of microsensors are also examined and compared. The third part of this study is to establish the viability of the remote sensing of amperometric signals from a glucose microsensor. The final part involves wireless transmission of subcutaneous and brain striatum glucose level. The amperometric current produced from the GOx – glucose reaction is transmitted to the home – base unit in a personal computer. Comparisons of conventional wired system and glucometer with wireless measurement are also made. Taken together, the results obtained establish the proof of wireless transmission of amperometry at a glucose microsensor.

2.0 Methodology

2.1 Materials

The 4-vinylpyridine (4VP) monomer (~96%), mercury (Hg, RdH 10008) (99.99%) and tetramethylammonium chloride, (TMAC), were from Fluka Chemika, Switzerland and used without further purification. Acetonitrile (MeCN) from Romil Chemicals, England; Araldite® epoxy resin from Ciba Geigy, Switzerland; Triton® X-100, i.e. polyethylene glycol tert-octylphenyl ether (PEG), 10% (w/v) and glucose oxidase (GOx) from *Asperigillus niger* (EC 1.1.3.4, catalogue no. 646 431, grade II lyophilised, 100,000 i.u.), obtained from Boehringer Mannheim GmbH, Germany were used as received. Bovine serum albumin (Fraction V, 98 – 99% albumin), ethyl carbamate, bovine serum, β-D-glucose, glutaraldehyde and mineral oil were obtained from Sigma Chemicals, USA and were used as received. Oxygen-free nitrogen (OFN) was obtained from Nissan-IOI, Malaysia. A stock solution of Nafion® (0.5 % w/v) was prepared in methanol. Nafion® (5 %w/v) (equivalent mass, 1100 g) in a mixture of low molecular weight alcohols and 10 % water was obtained from Aldrich (Milwaukee, WI, USA). The composite graphite powder from pencil lead 2B, Mars Lumograph 100, was from Staedtler, Germany. All other reagents were of the highest grade available and were used as received.

Bovine serum was first diluted to 80% in phosphate – buffered saline (PBS) solution at pH 7.4 prior use. The stock solution of β-D-glucose was also prepared in PBS and allowed to mutarotate and left overnight at 4°C before use.

This was to establish equilibrium concentrations between α and *β* anomers. The glucose concentrations were reported as total glucose concentrations. The phosphate buffered saline (PBS) solution was prepared, daily, by dissolving 2.754 g of NaCl, 2.081 g of KH_2PO_4 and 0.477 g of NaOH in 1000 mL of distilled water and then pH adjusted to 7.4 with 0.1 M NaOH. Pure water from a Milli-Q Plus of Millipore Corp., USA was used for preparation of all aqueous solutions.

2.2 Instrumentation

Most electrochemical measurements and analysis were done using either the Potentiostat/Galvanostat (Model 273A, Princeton Applied Research, USA) controlled by an IBM PS/2 model 480 PC or Autoranging Microvolt DMM (Model 197A, Keithley Ins., Cleveland, Ohio, USA). The pH measurement was obtained using benchtop pH/ISE meter (model 720A Orion, Boston, USA). Teflon coated antimagnetic stainless steel tweezers style #4 (110 mm) and style #3 (120 mm) of Sigma Chemicals, St. Louis, USA, was used in the preparation of microelectrode. Magnification of microelectrode was seen under a stereomicroscope (model SMZ – 2T, Nikon, Japan) and photos were taken with an attached camera (model F – 601, Nikon, Japan). The micropippete puller (PB – 7) and micropippete grinder (EG – 4) was from Narishige, Japan. The rat's blood glucose level was also determined using glucometer (Accu – Chek® Advantage II, with test strips and complete meter, Roche Diagnostics, GmbH, Mannheim, Germany).

2.3 Microsensors Fabrication

The carbon fiber (8 μm in diameter, 6.4 mm long,) was from Lot # 20590, Johnson Matthey Electronics, (MA, USA) was inserted into the glass capillary (1 x 90 mm GD – 1, Narishige, Japan) using tweezers and was then pulled by a micropipette puller ((PB – 7, Narishige, Japan) with maximum weight. The protruding carbon fiber was trimmed so that the remaining of the fiber was inside the glass capillary. The microelectrodes were then polished at an angle of 45° on the micropipette grinder (EG – 4, Narishige, Japan) on an extra – fine diamond abrasive plate to produce elliptical carbon disk. Electrical contact was established by filling the capillary with mercury (0.5 mL) and then with composite graphite paste. Preparation of the composite graphite paste was mentioned elsewhere [56 – 58]. A copper wire was then inserted into the paste. The upper part of the capillary was finally sealed with Araldite® epoxy resin. The microelectrode was left sitting at room temperature overnight and was then cured at 70°C for two days. It was then washed with acetone, later rinsed with pure water, and finally dried in a cold air stream prior to polymer deposition.

The preparation of P4VP from 4VP was described elsewhere [56 – 58]. Briefly, the electrochemical cell was, initially, purged with oxygen – free – nitrogen (OFN) gas for 10 minutes. Later P4VP film was anodically electrodeposited from the 4VP solution onto the carbon fiber electrode at potential from -0.4 V to +0.6 V vs Ag/AgCl and at scan rates of 50 mVs^{-1}, respectively with supporting electrolyte either TMAC or TBAP. The pH of the solution ranges from 2.9 to 3.5 adjusted by dropwise addition of 0.1M acetic acid

or 0.1M NaOH. Upon completion of deposition time, the microelectrode was remove carefully and washed with a mixture of acetonitrile and water. The P4VP – modified electrode was left to dry under reduced pressure for 30 minutes. The GOx was then immobilized onto the polymer modified microelectrodes by immersing the microelectrode in 2.0 mL of PBS solution containing 200 $i.u.mL^{-1}$GOx and glutaraldehyde for a period of 30 min. The dropwise addition of 10μL of stock Nafion® solution was applied onto the GOX/P4VP electrode. The resulting microelectrode was allowed to dry under reduced pressure for 5 minutes. The final assembly of Nafion® - coated GOX/P4VP microelectrode was washed, conditioned in PBS and kept at 4°C in the refrigerator for future use. The later was referred as a "clean" microelectrode. To minimize inflammatory skin reaction, the microsensor was dipped in the rat's serum for 3 days and was referred as the "preconditioned" microelectrode. During this period, the amperometric responses of the microsensor towards 10 mM glucose in PBS solution at pH 7.0 were evaluated for every 12 h. Prior to dipping, blood samples were collected from the rat's tail and enough amount of heparin was added to eradicate coagulation before being were centrifuged at 500 r.p.m. As a result, a process similar to membrane biofouling and fibrous encapsulation started even before implantation. The so called "passivation" layer was to further eradicate the process of membrane biofouling and fibrous encapsulation upon microsensor implantation. "Preconditioned" microsensors, after dipping in rat's serum for 72 hours were then used for further investigation. The amperometric responses towards increasing glucose concentration for both "clean" and "preconditioned"

microsensors were investigated at E_{app} = +0.23 V vs Ag/AgCl. “Preconditioned” microsensor will only be used on subcutaneous glucose monitoring while for brain striatum glucose monitoring, “clean” microsensor will be used.[§]

The pseudo Ag/AgCl reference electrode was prepared from a silver wire (0.01 mm o.d. 10 cm length). The electrode was dipped in a stirred 0.1 M HCl and a current of 0.4 mA cm^{-2} was passed for a period of 60 min until the silver wire was coated with AgCl. The final assembly was then rinsed using pure water. A potential of 50 mV was produced when the resulting pseudo Ag/AgCl reference electrode was scanned in 0.1 M NaCl at 37°C vs Ag/AgCl (3 M KCl). The reference electrode was then sealed to the glass capillary.

2.4 Subcutaneous glucose measurements

Healthy male Sprague – Dawley rats (n=15; 350±50g) were anaesthetized by injection of chlorolase/urethane (ethyl carbamate, 1500 mg kg^{-1}, i.p.). Injection of anesthesia tends to lower the rat’s body temperature. Thus, a light beam from a tungsten lamp (120 W) was used to keep the body temperature within the range of 35 - 37°C. The rat’s body oxygen level was maintained by continuously supplying of O_2 – air. The “clean” microelectrode and the reference electrode were implanted without any incision subcutaneously into the dorsal region whereas the “preconditioned” micrelectrode and the reference electrode were implanted at the back of the neck of the rat. The sensors were then connected to

[§] The term “stain” in reference [35] was replaced by “preconditioned” for this thesis

Autoranging Microvolt DMM. The measurements were made at an applied constant potential of 0.23 V *vs* Ag/AgCl. The sensors were calibrated before and after the in vivo experiment. The rat's blood sugar level was increased by injecting glucose (10 mM glucose min^{-1} kg^{-1}). In order to check the blood sugar level, blood samples were taken at a regular interval in the ear vein (vena auricularis posterior). Calibration curve was constructed prior to implantation of the microsensor by measuring the amperometric current of prepared glucose standard solutions [28, 31]. The one-point calibration procedure was performed a posteriori by taking into account the capillary blood glucose concentration determined immediately before microsensors implantation infusion, whatever their level, and the concomitant sensor output. This procedure was performed either once per day (before before sensors implantation, the value of glycemia and sensor output being those used in the two-point calibration procedure), twice per day or three times per day.

Capillary blood glucose concentration was determined using glucometer. If blood glucose was lower than 5 mM, the rat was given glucose infusion (10 mM glucose min^{-1} kg^{-1}). If blood glucose was higher than 5mM, the implanted sensors were disconnected from the measuring devices until the capillary glucose had fallen to a stable lower level. A second capillary blood glucose measurement was then performed. The two sets of values of capillary blood glucose concentration and concomitant sensor output were used to calibrate the sensor. For the other days of the trial, the procedure was repeated a posteriori by taking into account

the capillary blood glucose concentration observed immediately before glucose infusion.

The output from the sensor was obtained as the experiments proceeded. Between measurements (day interval), the microsensor was electrically disconnected from the rat, which was not anaesthetized during this period. At the end of the course, blood samples were taken from the dorsal region and the back of the neck of the rat, i.e. sites implanted with “clean” microsensor and “preconditioned” microsensor for hematology examinations [59, 60]. For each rat anaesthetized for in – vivo measurement, a new pair of “clean” and “preconditioned” microsensor was used. All experiments involving rats have been conducted according to approved ethical guidelines [61].

2.5 Brain glucose measurements

Diabetes was induced in an 285 ± 5 g adult male Sprague – Dawley rat, ten days before study, by injecting intravenously STZ (50 mg kg^{-1} body weight in 200 mL citrate buffer, pH 4.3) in the penis vein. Four days after STZ infusion, the rats received daily injections of protamine zinc insulin at a dosage sufficient enough to maintain body weight but insufficient to prevent chronic hyperglycemia. The control non – diabetic rats received either saline infusion (euglycemia, n = 5) or a variable rate of glucose infusion (20mM glucose min^{-1} kg^{-1} each time) to raise and maintain plasma glucose at diabetic level. Chronically hyperglycemic rats received a variable glucose infusion to maintain them at their morning glucose level in the range of 22 - 24 mmol L^{-1} (n = 5). In