

**DEVELOPMENT OF GLUCOSE MEASUREMENT SYSTEM FOR
AUTOMATIC TISSUE ENGINEERING CELL CULTURE**

CHANYANUT WONGSA

**MASTER OF ENGINEERING
IN BIOMEDICAL ENGINEERING**

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**GRADUATE SCHOOL
CHIANG MAI UNIVERSITY
JUNE 2023**

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**A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF ENGINEERING
IN BIOMEDICAL ENGINEERING**

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THIS THESIS HAS BEEN APPROVED TO BE A PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF ENGINEERING
IN BIOMEDICAL ENGINEERING

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9 June 2023

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To my late grandfather.



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Chanyanut Wongsas

หัวข้อวิทยานิพนธ์	การพัฒนาระบบตรวจวัดกลูโคสเพื่อการเพาะเลี้ยงเซลล์อัตโนมัติ สำหรับงานวิศวกรรมเนื้อเยื่อ	
ผู้เขียน	นางสาว ชัญญา นุช วงษา	
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	ศ.ดร.เกตุ กรุดพันธ์	อาจารย์ที่ปรึกษาร่วม

บทคัดย่อ

งานวิจัยนี้นำเสนอการพัฒนาระบบสำหรับการตรวจวัดกลูโคสในการเพาะเลี้ยงเซลล์แบบใหม่สำหรับงานวิศวกรรมเนื้อเยื่อ โดยการใช้อนุภาคนาโนทองคำ ผสมกับท่อนาโนคาร์บอน กลูโคสออกซิเดสเอนไซม์ และสารละลายไคโตซาน (AuNP-SWCNT-GOD-CS) เพื่อปรับเปลี่ยนบนพื้นผิวของคาร์บอนอิเล็กโทรด (SPE) ได้เป็นเซนเซอร์สำหรับตรวจวัดกลูโคสแบบใช้เอนไซม์ เซนเซอร์ที่พัฒนาขึ้นได้ถูกนำไปเชื่อมต่อกับระบบสูบน้ำและผสมสารละลายอัตโนมัติ เพื่อการวิเคราะห์ตามลำดับ (SIA System) ระบบตรวจวัดกลูโคสที่พัฒนาขึ้นสามารถนำไปประยุกต์ใช้ในการประเมินข้อมูลจากการเพาะเลี้ยงเซลล์ภายในสภาพแวดล้อมเดียวกัน แต่ภายใต้เงื่อนไขที่แตกต่างกันได้มาก สูงสุดถึง 6 แบบ ระบบนี้สามารถใช้ในการวิเคราะห์สารละลายที่มีความเข้มข้นสูงได้ เนื่องจากมีส่วนประกอบของช่องผสมสารจากระบบไมโครฟลูอิดิก ทั้งยังช่วยลดเวลาที่ใช้ในการตรวจวิเคราะห์ลดปริมาณสารตัวอย่าง และลดความเสี่ยงจากการปนเปื้อน ระบบที่พัฒนาขึ้นสามารถตรวจวัดกลูโคสในปริมาณเชิงเส้นได้สูงสุดถึง 3.8 mM ในสารอาหารที่ใช้เพาะเลี้ยงเซลล์ ประสิทธิภาพของระบบตรวจวัดกลูโคสที่พัฒนาขึ้นได้ถูกประเมินโดยการนำไปตรวจวิเคราะห์หาค่าความเข้มข้นของกลูโคสในสารอาหารที่ใช้เลี้ยงเซลล์ (L929 fibroblast cell line) เปรียบเทียบกับวิธีตรวจวัดกลูโคสด้วยสีแบบมาตรฐาน ผลการทดลองพบว่าระบบที่พัฒนาขึ้นมีความถูกต้องในการตรวจวัดกลูโคสได้ในระดับที่ยอมรับได้ นอกจากนี้ระบบตรวจวัดกลูโคสที่พัฒนาขึ้นยังแสดงให้เห็นถึงศักยภาพที่สามารถจะนำไปประยุกต์ใช้ในการเพาะเลี้ยงเซลล์อัตโนมัติ สำหรับการตรวจวัดสารสำคัญอื่นได้ในอนาคต

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ABSTRACT

The study presents a novel system for monitoring glucose in cell culture, incorporating the use of gold nanoparticles, single-walled carbon nanotubes, glucose oxidase enzyme, and chitosan solution (AuNP-SWCNT-GOD-CS) modified on a commercial carbon screen-printed electrode (SPE). This system is integrated with a sequential injection analysis (SIA) system, which includes a flow cell, syringe pump, selective valve, mixing channel, holding coil, and a six-well plate for cell inoculation. The system allows for the operation of six separate cell culture units under different conditions within the same environment. The modified glucose measurement system offers automated in-line sampling and sample dilution through a mixing unit. This ensures rapid analysis and reduces the risk of contamination. Additionally, the utilization of 3D-printed microfluidic components, a mixing channel, and a flow cell helps streamline operations and minimize sample volume. The developed system can detect glucose linearly up to a concentration of 3.8 mM in cell culture media. In cases where higher glucose concentrations are present, in-line sample dilution can be implemented. The effectiveness of the developed glucose measurement system was verified through successful testing with mouse fibroblast (L929) cell culture, producing results that aligned with those obtained using the traditional colorimetric method. This research showcases significant potential for further advancement and application in the field of biomedical research, particularly for in vitro cell-based experiments.

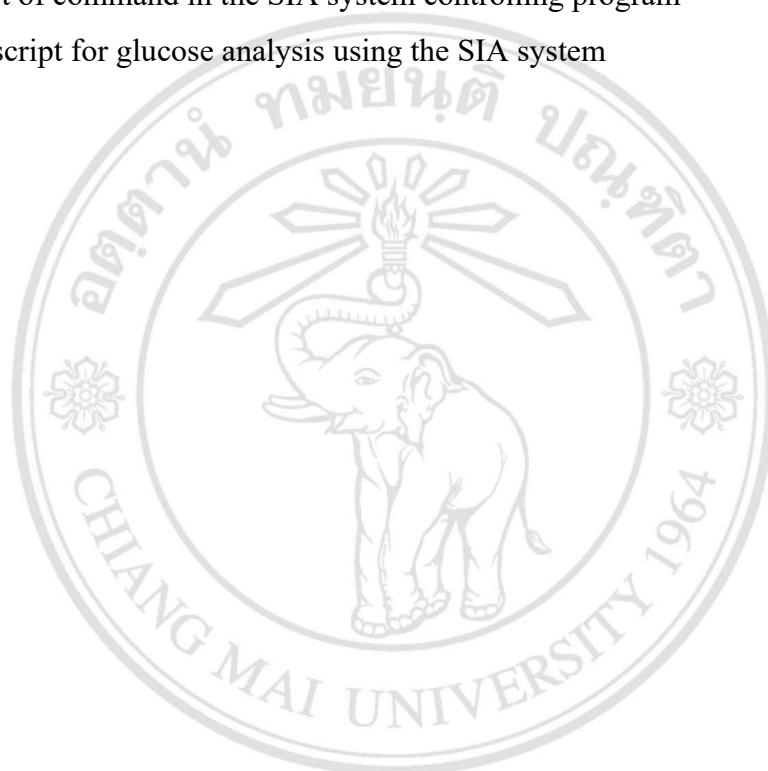
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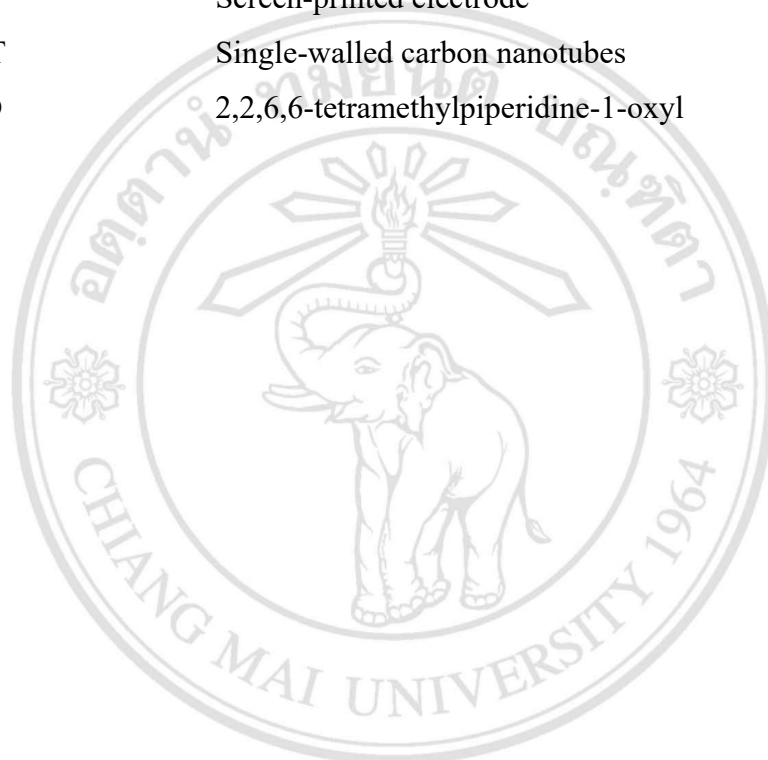
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LIST OF ABBREVIATIONS

AgNP	Silver nanoparticle
AuNP	Gold nanoparticle
BSA	Bovine serum albumin
C-SnS	Carbon-coated Nano tin sulfide
CHO	Chinese hamster ovarian
CNCs	Cellulose nanocrystal
CNT	Carbon nanotubes
CS	Chitosan
CV	Cyclic voltammetry
CVD	Chemical vapor deposition
DMEM	Dulbecco's Modified Eagle Medium
e ⁻	Electron
EIS	Electrochemical impedance spectroscopy
FAD	Flavin adenine dinucleotide
FADH ₂	Flavin adenine dinucleotide hydroquinone form
FEP	Fluorinated Ethylene Propylene
FIA	Flow injection analysis
GCE	Glassy carbon electrode
GDH	Glucose dehydrogenase enzyme
GOD	Glucose oxidase enzyme
HRP	Horseradish peroxidase
ITO	Indium tin oxide
LOD	Limit of detection
LOQ	Limit of quantification
MTOS	Methyltrimethoxysilane
MWCNT	Multiwalled-carbon nanotubes
OPD	O-phenylenediamine
PBS	Phosphate buffered saline

PLC	Programmable logic controller
PTFE	Polytetrafluoroethylene
PtNP	Platinum nanoparticles
RPM	Revolution per minute
RT-GM	Real-time monitoring of the glycosylation change
SIA	Sequential injection analysis
SPE	Screen-printed electrode
SWCNT	Single-walled carbon nanotubes
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl



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CHAPTER 1

Introduction

1.1 Background

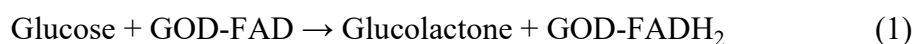
Mammalian cell culture has been significant in the field of biomedical research and has been utilizing in various applications including protein expression, biomaterial testing, and drug discovery. With recent developments in regenerative medicine technologies such as tissue engineering and stem cell therapy, the demand for large-scale cell cultivation has increased. Precise monitoring and control of the bioreactor conditions where cells are cultured, are necessary to produce a considerable number of mammalian cells. For the past decade, the development of integrated measurement tools or sensors for monitoring cell metabolism during the cultivation process has been extensively studied [1-6]. The parameters that are typically considered for monitoring cell growth include the pH level of the cell culture environment, lactate production, and oxygen and glucose consumption. Several publications have focused on creating sensors for monitoring pH, oxygen levels, glucose, and lactate in cell culture. These sensors differ in their designs, techniques, and modified materials used [1-10]. In this study, the development of a glucose measurement system was fabricated by incorporating a modified glucose sensor with sequential injection analysis to determine the glucose concentration of L929 cell line culture media, aiming to be a monitoring system prototype for tissue engineering utilization.

1.2 Principal and Theory

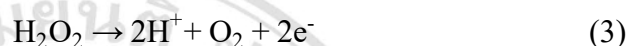
1.2.1 Principle of Enzymatic Glucose Sensor

Biosensor is a device employing isolated enzymes, immunosystems, tissues, organelles, or whole cells to detect chemical compounds typically through electrical, thermal, or optical signals based on specific biochemical reactions

[11]. For the enzymatic glucose biosensor, the mechanism is basically based on the redox interaction of glucose and glucose oxidase (GOD) enzyme, following the equations as below.



where Flavin adenine dinucleotide (FAD) is a redox cofactor.



There are three methods commonly employed for electrochemical sensing of glucose: oxygen consumption measurement, hydrogen peroxide measurement, and the utilization of a mediator to facilitate electron transfer to the electrode [12]. Currently, there have been advancements in the development of enzymatic glucose sensors, categorized into three generations. The first generation primarily relies on detecting hydrogen peroxide production and measuring oxygen consumption. In this method, the decrease in oxygen concentration corresponds to the glucose concentration. However, achieving high selectivity in hydrogen peroxide measurement necessitates a high operational potential. The second generation of enzymatic glucose sensors improved by utilizing a non-physiological electron acceptor, known as a redox mediator, instead of measuring hydrogen peroxide. However, a significant drawback of this approach is the high toxicity associated with the use of the mediator. As a result, the third generation of enzymatic glucose sensors was developed to address the limitations of the previous generations as shown in Figure 1.1. This approach involves directly monitoring the currents of electron exchange between the biological system and the electrode, eliminating the need for a redox mediator [12].

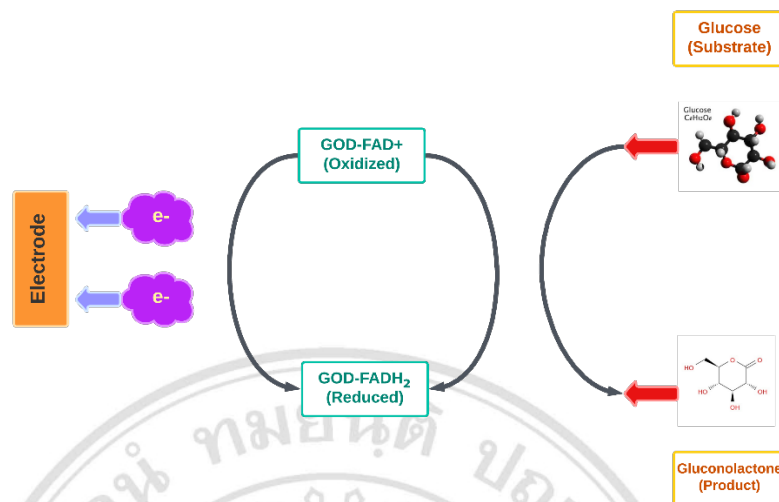


Figure 1.1 The schematic of the third-generation glucose sensor modified from [13]

1.2.2 Glucose Enzymatic Colorimetric Assay

The enzymatic colorimetric assay for glucose has become the established method for measuring glucose concentrations. In this procedure, glucose reacts with the glucose oxidase enzyme, resulting in the release of hydrogen peroxide. The hydrogen peroxide then undergoes a redox reaction with a peroxidase reagent. This reaction triggers the oxidative condensation of 4-aminoantipyrine and phenol, leading to the formation of a quinoneimine dye as shown in Figure 1.2 [14]. The intensity of the dye's color is directly proportional to the concentration of glucose present and can be measured by a spectrophotometer. The accuracy and precision of this method made it suitable to be adopted as a standardized protocol for glucose quantitation. Nevertheless, the colorimetric approach encounters an issue with color interference, particularly when using a pH indicator like phenol red in cell culture media solution, which can potentially disrupt data interpretation. Additionally, this method involves the use of chemical reagents and requires a 30-minute enzyme incubation period, resulting in lengthy processing time, increased waste generation, and a higher risk of contamination.

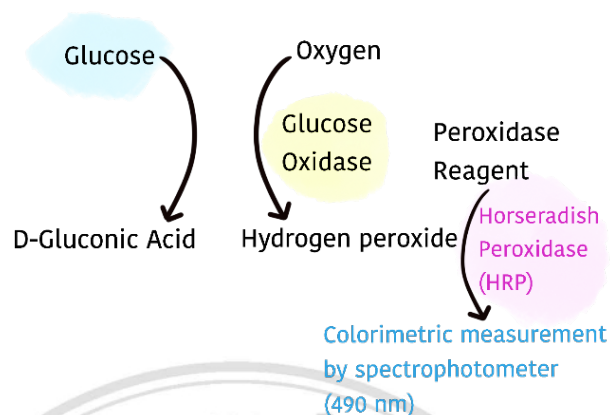


Figure 1.2 The schematic of glucose enzymatic colorimetric assay modified from [15]

1.2.3 Principle of Sequential Injection Analysis

Sequential Injection Analysis (SIA) is an automated analytical technique that is used for performing a series of chemical analyses. It is a modification of the flow injection analysis (FIA) method and shares similar principles. SIA involves injecting discrete sample volumes or segments into a flowing carrier stream, which sequentially passes through various analytical stages or zones within the instrument. The injected sample segments are separated from each other by air bubbles or carrier solution segments, ensuring sequential analysis without sample cross-contamination [16].

The key components of a typical SIA system include a peristaltic pump or syringe pump for generating the flow, a series of valves or microfluidic switches for directing the flow path, and various detectors for measuring the analyte concentration. The system is controlled by a computer or programmable logic controller (PLC), allowing for precise control and automation of the analysis process. The analytical stages in SIA can include various operations such as sample pretreatment, reagent addition, mixing, reaction, and detection. The specific configuration and design of the SIA system depend on the analytical requirements of the assay being performed [16].

1.3 Purpose of The Study

To develop a system for in-line measurement of glucose concentration in culture medium for automatic tissue engineering cell culture.

1.4 Research Scope

1.4.1 The system will be developed to determine glucose concentration in the cell culture medium.

1.4.2 The system will be optimized and characterized under related physico-chemical conditions in cell culture.

1.4.3 The application of the developed system will be demonstrated with a cell line cell culture.

1.5 Educational Advantages

This research attempts to develop a glucose measurement system that not only serves as a versatile model for monitoring a wide range of nutrients and metabolites in culture mediums across diverse tissue engineering cell cultures but also offers numerous other significant benefits. By providing a flexible framework, this system can be adapted to monitor and regulate the levels of various vital substances necessary for optimal cell growth and function.

Furthermore, the implementation of this model system holds potential for remote-controlled cell culture systems in the future. This innovative approach allows for real-time monitoring and precise control of nutrient and metabolite concentrations, irrespective of working location. As a result, researchers in the tissue engineering field can benefit from improved efficiency, reduced time requirements, and minimized risks of contamination during the cell culture process.

By embracing this advanced glucose measurement system, enhanced automation and remote control capabilities not only streamline experimental procedures but also enable the optimization of cell culture conditions, leading to accelerated research progress and increased reproducibility of results. Ultimately, these advancements contribute to the

development of more effective tissue engineering strategies, promoting better outcomes for advancements in regenerative medicine.



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CHAPTER 2

Literature Review

2.1 Tissue Engineering and Cell Culture

Tissue Engineering is an interdisciplinary area of study that merges engineering, technology, and living science to create biological replacements for repairing, maintaining, or enhancing tissue or organ function [17]. The early step of producing such replacement tissues involves cell expansion typically called cell cultivation as shown in Figure 2.1 [18].

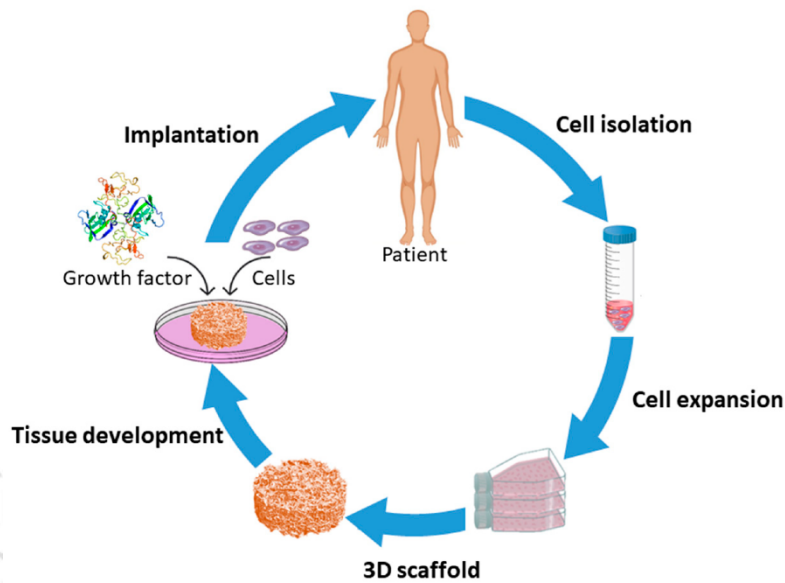


Figure 2.1 The schematic of replacement tissues production [18]

To establish a system for monitoring cell cultures, it is essential to comprehend the three main types of cell cultivation. These include primary cell explantation, in which cells are obtained directly from donor tissue; general cell culture, which uses immortalized cell lines; and organotypic culture [19]. Conversely, research on cell culture monitoring is classified into three categories: 2D static cell culture for adherent cell culture in static conditions, 2D dynamic cell culture for adherent cell culture in dynamic conditions, and

3D cell culture for suspension cells [20]. Most cell culture types need the same supplementary which is cell culture medium contains necessary nutrients for cell growth and sustaining cell lifespan. Real-time monitoring and optimal nutrient offset control could lead to effective tissue or organ production in tissue engineering.

2.2 Glucose and Glucose Sensor

Glucose is a critical nutrient in cell culture media. To study cellular metabolism, it is important to measure the rate of glucose uptake in cell cultures under different experimental conditions. During cell production, changes in glucose levels can significantly affect cell growth [21].

Biosensors are analytical devices that combine immobilized biochemical recognition elements with physiochemical signal transducers. They typically consist of three components: the biological recognition elements, which detect target molecules; the transducer or converter, which transforms the recognition elements into a measurable electrical signal; and the signal processing system, which may include electronic signal amplification [12, 22].

Glucose sensors employ various types of transducers, including electrochemical, optical, thermometric, piezoelectric, and magnetic. However, electrochemical biosensors are the most prevalent type due to their superior sensitivity and reproducibility, ease of maintenance, and affordability. Enzymatic amperometric glucose biosensors are the most frequently employed devices to measure the current of electrons exchanged between electrodes and biological systems [12].

Numerous research studies have aimed to enhance the efficiency of glucose sensors by incorporating nanomaterials, such as carbon nanotubes, platinum nanoparticles, silver nanoparticles, and gold nanoparticles, as well as hybrid nanomaterials that consist of glucose oxidase enzyme (GOD) coatings on the electrode surface [1, 2, 6-10]. The preference for GOD over alternative glucose sensing enzymes, such as various cofactor glucose dehydrogenases (GDH), stems from its exceptional specificity to glucose, effectively reducing the occurrence of inaccurate glucose readings. Additionally, the use of GDH enzymes often requires an electron mediator, which can potentially introduce

toxic effects [23]. Furthermore, several studies have reported advancements in the modification of glucose sensors, transitioning from the commercial screen-printed electrode (SPE) [1, 2, 6].

The early period of glucose sensor development focused on the clinical application to measure the concentration of glucose in human serum samples, beneficial to diabetes patients. For example, a biocompatible hybrid nanomaterial was produced by mixing methyltrimethoxysilane (MTOS) with platinum nanoparticles (PtNP), multiwalled-carbon nanotubes (MWCNT), and chitosan (CS) solution to become a PtNP-MWCNT-CS/silica composite [7], the modified material was used as a matrix of glucose oxidase enzyme (GOD) to immobilized into the glassy carbon electrode (GCE) due to their feature, high surface to volume ratio. Then, the sensor was tested by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) to investigate sensor performance. The result from CV shows a sensor sensitivity of $2.08 \mu\text{A mM}^{-1}$, a linear range of $1.2 \mu\text{M}$ to 6 mM , and a low detection limit (LOD) of $0.3 \mu\text{M}$. Then, the sensor was satisfyingly applied to detect glucose concentration in human serum compared to those detected by the standard protocol in the hospital.

Another approach to fabricate glucose biosensor is multilayer film method. For example, a multilayer glucose biosensor was fabricated by mixing carbon nanotubes (CNT), chitosan (CS), and gold nanoparticles (AuNP) to immobilize glucose oxidase enzyme (GOD) with cross-linking through glutaraldehyde and bovine serum albumin (BSA) on the glassy carbon electrode (GCE) [9]. The effect of film layer number was tested. The results show that the increase in current response was induced by the increase of the layer number until reached 8 layers. The sensor was characterized and calibrated to detect glucose in phosphate buffer solution then the result showed the working range linearly from $6 \mu\text{M}$ to 5 mM and a detection limit of $3 \mu\text{M}$. The stability of the sensor was found at 81.3% retaining in 10 days. Then, the sensor was applied to glucose detection of human plasma samples and provided a satisfactory result close to the result from the standard analyzer.

In the another study, horseradish peroxidase (HRP) and glucose oxidase enzyme were embedded in silver nanoparticle(AgNP)-carbon nanotubes(CNT)-chitosan(CS) film

using a layer-by-layer technique [8]. A mediator O-phenylenediamine (OPD) was co-immobilized with HRT in the inner layer. The layer of material film was coated into the indium tin oxide (ITO) electrode. The modified sensor was characterized by cyclic voltammetry (CV) and provided a sensitivity of $135.9 \mu\text{A mM}^{-1}$, a linear range from 0.5 to 50 μA , and a detection limit of 0.1 μA . For clinical application, the sensor was employed to detect glucose in human serum samples and compared with the hospitalized method. The result shows an acceptable accuracy of the sensor.

A carbon-coated nano tin sulfide (C-SnS) was prepared into two steps hydrothermal and chemical vapor deposition (CVD) [10]. The material was used to immobilize glucose oxidase enzyme on a glassy carbon electrode. The CV profile of the sensor was assessed to demonstrate sensor performance in phosphate buffer saline. The Amperometric response was conducted to detect 0.1 mM glucose and 0.1 mM interference of citric acid (CA) and uric acid (UA) for specificity testing of the sensor. The effect of scan rate and the effect of pH were investigated. The result shows the linear range from 0.03 to 0.7 mM, a sensor sensitivity of $43.9 \text{ mA M}^{-1} \text{ cm}^{-2}$. The redox current has a linear correlation with the scan rate. The optimal current response occurred at pH = 7. Although the result of interference testing is unclear the sensor selectivity to glucose still shows an acceptable response range.

Although glucose biosensors have been well-developed and applied in clinical-used to detect blood glucose level, there have been some research studies about integrating a glucose biosensor in a cell culture monitoring system for biomedical work.

A transparent microfluidic multi-sensor system was developed for drug screening and cancer research [4]. The system includes oxygen and pH sensors as well as biosensors that were fabricated using thin-film processing. Glucose and lactase oxidase were immobilized in a UV-curable enzyme membrane to create biosensors. The glucose biosensor was calibrated using RPMI-1640 cell culture medium with an amperometric measurement at 450 mV. The calibration showed a glucose sensitivity of approximately $3.3 \text{ nA mM}^{-1} \text{ mm}^{-2}$, linearity up to 10 mM, and a detection limit of 75 μM . Human glioblastoma multiforme T98G brain cancer cells were cultivated on the chip for 48 hours, and glucose and lactate measurements were conducted at stop times of 3-5 minutes

alternating with flow times of 5-15 minutes. In the drug screening experiment, the researchers used Cytochalasin B to inhibit glucose metabolism inside the cell and found that the lactate production decreased after the addition of the drug, but was recovered by adding fresh medium proportional to glucose concentration. The addition of drug compounds did not affect the sensor performance.

An in-situ biosensor array was designed, developed, and validated for monitoring metabolites in cell cultures [1, 2]. The micro biosensor was created using thin film technologies by electrodeposition of a mixture of carbon nanotubes, oxidases, and chitosan on commercial gold screen-printed electrodes. The biosensors were calibrated in Phosphate Buffered Saline (PBS) and cell culture media, with the glucose biosensor showing a sensitivity of $410 \pm 50 \text{ nA mM}^{-1} \text{ mm}^{-2}$ in PBS and $4.7 \pm 1.3 \text{ nA mM}^{-1} \text{ mm}^{-2}$ in cell culture medium. Detection limits of $14 \mu\text{M}$ and 1.4 mM were obtained in PBS and cell culture medium, respectively. Long-term stability testing showed that the biosensors retained activity of over 80% for the first 10 days and 60% after 20 days. For the cell culture experiment, U937 cells were seeded in three cell culture flasks at different densities, and glucose concentration was measured every 16, 40, 64, and 88 hours after seeding for all three densities. Cell viability and pH were also checked at each time point. Microdialysis probes were used for online sampling, and a current-to-voltage conversion was used to read the current flowing on the counter electrode. The voltage was digitized and stored in a microcontroller, and the values were sent to a portable device via Bluetooth. The system was kept in an incubator to reduce the risk of contamination. The results showed that glucose consumption was greater for higher cell density, and after 40 hours of cell cultivation, the glucose concentration availability remained at 50%.

A microsensor array chip was capably developed to measure multiple cell biomarkers in parallel, including glucose and lactate [3]. The chip was fabricated using micro-electro-mechanical system (MEMS) based optical lithography and dry etching processes and featured screen-printed glucose biosensors calibrated in phosphate buffer pH 7.3. The biosensors exhibited a sensitivity of approximately 6 nA mM^{-1} and linearity up to 5 mM , with a working range of approximately 10 mM . In a cell culture experiment, five microwells were coated with collagen and contained cell culture medium with added

glucose, three of them containing mammalian cells and two controls. The microsensor system was placed at the bottom of microwells, and glucose biosensors were operated in an amperometric mode. The results showed that the glucose concentration of the wells containing mammalian cells rapidly decreased in 2 hours and reached the baseline in 8 hours, while the control microwells remained unchanged for 15 hours. The researchers suggested that the cell culture medium should be removed and exchanged after 8 hours of cultivation due to cytotoxicity raised from direct contact between the microsensor array chip and the cells. However, the study was limited by this cytotoxicity issue.

A disposable optical biosensor was developed for continuous in-line glucose monitoring in animal cell culture [5]. The biosensor was based on a commercial oxygen sensor that was modified with glucose oxidase enzyme (GOD). The biosensor was covered with a polymerizing enzyme solution containing GOD, bovine serum albumin (BSA), glycerol, and glutaraldehyde, and a hydrophilic perforated membrane with two different permeabilities (high and low) was used to tune the dynamic range of the biosensor. The biosensor was sterilized by irradiation and calibrated in PBS buffer solution. The biosensor was found to have a cross-sensitivity to the oxygen of $\leq 1.4\%$ when measured at 5 mM glucose, and the limit of detection (LOD) and limit of quantification (LOQ) were around 0.45 mM and 1.5 mM, respectively. The dynamic range was found to be 0-20 mM, and the functional stability was greater than 52 days. For cell culture experiments, the biosensors were mounted onto optic fibers, and each fiber was placed in a 125 mL shake flask containing Chinese hamster ovarian (CHO) cells. The glucose level was measured for 11 days and compared with the stimulated biosensor response with manually added glucose on days 4, 7, 8, and 10. The biosensor was recalibrated on day 7, and the results showed that the glucose level monitored from experiments was strongly related to the stimulated biosensor response in both high and low-permeable membrane types. Overall, this study demonstrates the potential of an optical biosensor for continuous in-line glucose monitoring in animal cell culture, which can provide real-time information and improve the efficiency of bioprocess control.

A biocompatible and inert nanomaterial: cellulose-based material, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) oxidized-cellulose nanocrystal (CNCs) was used

as an enzyme scaffold for immobilize and stabilize glucose oxidase enzyme on screen-printed electrode instead of the standard method, glutaraldehyde crosslinking due to their special carrier matrix property [6]. The modified sensor was calibrated in standard glucose solution in phosphate buffer to investigate sensor performance. The calibration provided the sensor sensitivity of $5.7 \pm 0.3 \mu\text{A mM}^{-1} \text{cm}^{-2}$, a linear range of 0.1-2 mM, and the limit of detection is 0.004 mM. The sensor was employed in glucose detection of low glucose DMEM medium in NIH 3T3 fibroblast cell culture. To avoid the matrix effect in the cell culture medium, the medium was diluted 1:4 (v/v) in phosphate buffer before conducting the measurement process. The successful glucose monitoring was shown as a result of glucose concentration decreasing during the period of cell culture.

The summary of developed enzymatic glucose biosensor studies is shown in Table 2.1

Table 2.1 The summary of the developed enzymatic glucose biosensors

Electrode	Modified materials	Sensitivity	Working range	Limit of detection	Cell culture	In-line measurement	Ref
GCE	MWCNT, CS, PtNP, GOD, MTOS	$2.8 \mu\text{A mM}^{-1}$	1.2 μM –6 mM	0.3 μM	No	No	[7]
GCE	CNT, AuNP, CS, GOD	n/a	6 μM –5 mM	3 μM	No	No	[9]
ITO	AgNP, CNTs, CS, GOD	$135.9 \mu\text{A mM}^{-1}$	0.5–50 μM	0.1 μM	No	No	[8]
GCE	C-SnS-NPs, GOD	$43.9 \text{ mA M}^{-1} \text{cm}^{-2}$	0.03–0.7 mM	n/a	No	No	[10]
PtE	pHEMA, GOD	$3.3 \text{ nA mM}^{-1} \text{mm}^{-2}$	up to 10 mM	75 μM	human glioblastoma multiforme (T98G) brain cancer cells	Yes	[4]

Table 2.1 The summary of the developed enzymatic glucose biosensors (continued)

Electrode	Modified materials	Sensitivity	Working range	Limit of detection	Cell culture	In-line measurement	Ref
Au-SPE	MWCNT, CS, GOD	4.7 ± 1.3 nA mM ⁻¹ mm ⁻²	5–25 mM*	1.4 mM	human myeloid leukemia (U937) cells	Yes	[1, 2]
Carbon ink-Pt-SPE	CoPC, GOD	6 nA mM ⁻¹	Up to 10 mM*	n/a	human choriocarcinoma (BeWo) cells	Yes	[3]
Oxygen sensor	GOD, BSA, glycerol, glutaraldehyde	n/a	0-20 mM	0.45 mM	Chinese hamster ovarian (CHO) cells	Yes	[5]
CB-PB SPE	TEMPO, GOD, CNCS	57 ± 3 nA mM ⁻¹ mm ⁻²	0.1-2 mM	4 μM	NIH 3T3 fibroblast cells	No	[6]

* indicating glucose concentration in cell culture media.

2.3 Sequential Injection Analysis

Sequential injection analysis (SIA) is a flow analytical technique based on a multi-position selective valve, allowing the operation of multiple samples separately, as illustrated in Figure 2.2. [16]. Over the past few decades, this method has been extensively enhanced and applied in various fields, including biopharmaceutical studies, monitoring fermentation processes in bioreactors, and conducting cell culture experiments.

The SIA system has previously been employed in the biopharmaceutical process to study interactions with different types of cells in vitro. These applications include monitoring drug release, conducting release tests, and assessing product quality [24-28]. In addition, there is also found application in fermentation bioreactors for real-time monitoring of metabolites and products, including lactic acid, ammonia, glycerol, glucose, and formaldehyde [29, 30].

In recent times, the SIA system has been utilized in mammalian cell culture to monitor bioreactor conditions and ensure product quality. These applications involve integrating the SIA system with different analytical techniques.

For example, the online near real-time SIA with capillary electrophoresis was used to monitor extracellular lactate in a human embryonic kidney cell line (HEK293) culture flasks [31], The μ SI incorporating an ultra-performance liquid chromatography system was used for real-time monitoring of the glycosylation change (RT-GM) by manganese-induced in Chinese hamster ovary (CHO) cell culture [32], and the sequential injection analysis electronic tongue (SIA-ET) was used for study pharmacodynamics effect of Diclofenac drug in normal human lung fibroblasts (MRC-5) and human lung epithelial carcinoma (A549) cell line base on an electrochemical analysis [33].

The overall outcome has shown the SIA system to be advantageous for real-time analysis in bioprocesses, providing benefits such as reduced processing time, automation, and the capability to perform multiple assays within a single system [34].

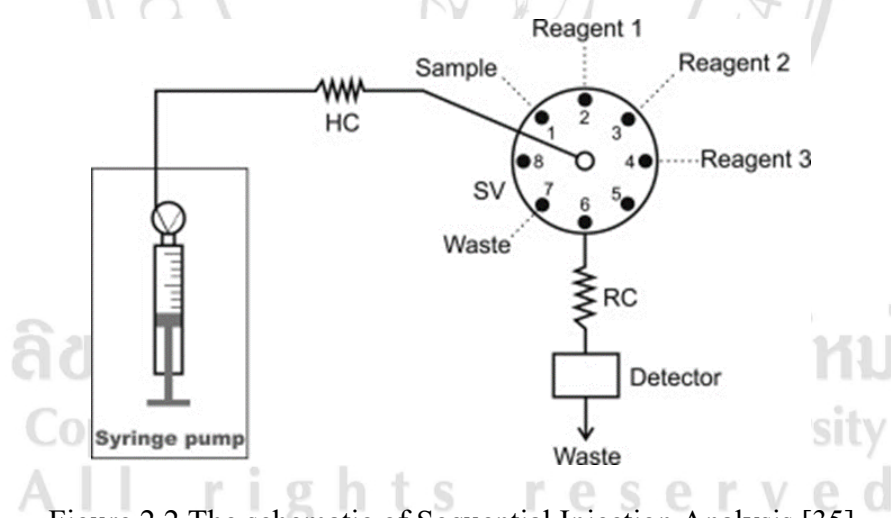


Figure 2.2 The schematic of Sequential Injection Analysis [35]

CHAPTER 3

Materials and Method

This research consists of three main parts: system preparation, system verification, and application in real samples. The research workflow is described in Figure 3.1.

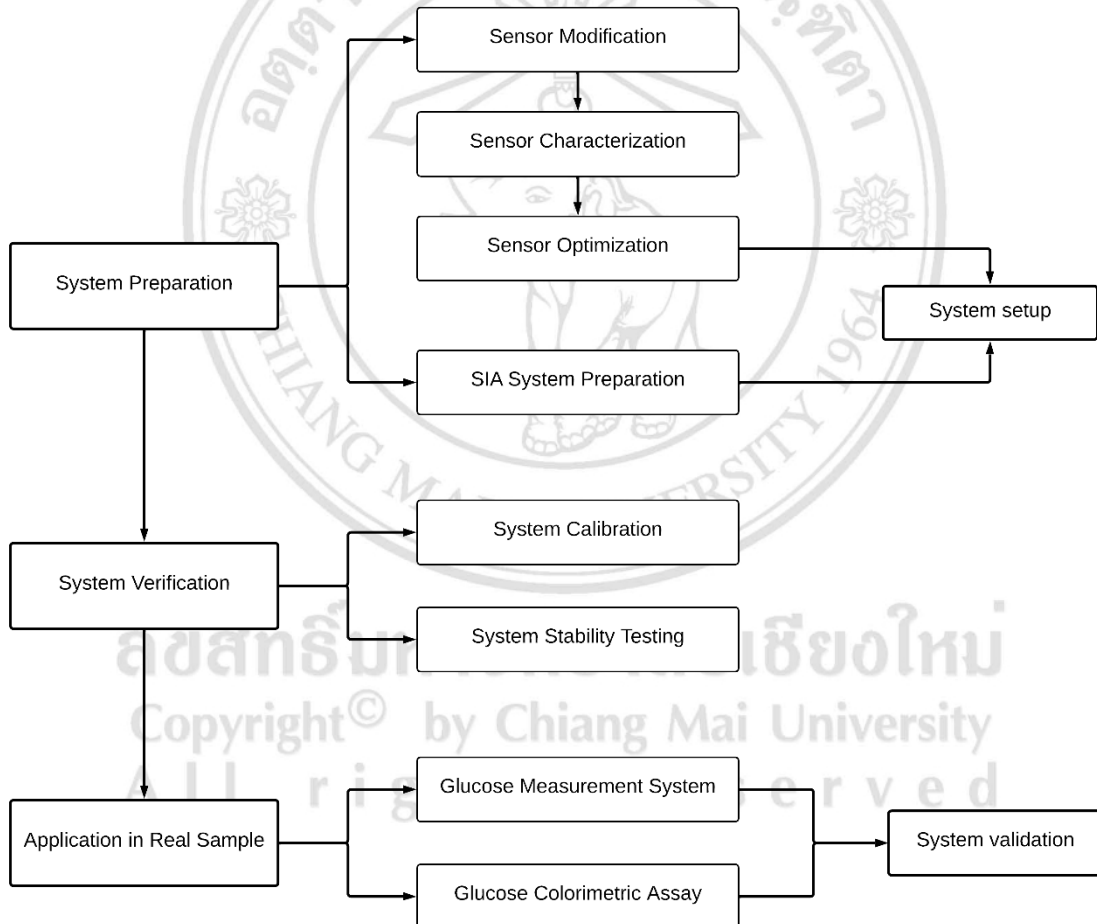


Figure 3.1 The schematic of research workflow

3.1 Materials and Chemicals

Carbon screen-printed electrode was purchased from Dropsens, Spain.

A 10 mM PBS solution pH 7.4 was prepared by dissolving one tablet of phosphate buffered saline (PBS, 1X) (Amresco Inc, Solon, OH, USA) in 100 mL of MilliQ water.

A 1000 U mL⁻¹ glucose oxidase (GOD) stock solution was prepared by dissolving 4 mg of Glucose oxidase from *Aspergillus Niger*, recombinant (269 U mg⁻¹, Merck, Darmstadt, Germany) in 1 mL of PBS solution.

A 10 mM glucose standard stock solution was prepared by dissolving 0.9008 g of D-(+)-glucose (C₆H₁₂O₆, Sigma-Aldrich, Saint Louis, MO, USA) in 50 mL of PBS solution.

A single-walled carbon nanotube (SWCNT, dispersion in H₂O, Sigma-Aldrich, Saint Louis, MO, USA) was sonicated 10 minutes before use.

A colloidal gold (20 nm, Kestrel Bio Science, Bangkok, Thailand) was used as-received.

A chitosan solution was prepared by dissolved chitosan pellets (chitosan oligomer 100 mesh, Taming Enterprise, Beijing, China) in 1% acetic acid and neutralized by dialysis.

A glucose-free Dulbecco's Modified Eagle Medium (DMEM) (11966, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) was used as-received.

3.2 System Preparation

To fabricate the glucose measurement system, it comprises the electrochemical detection unit and the sequential injection system (SIA). The fabrication process begins with the electrochemical detection unit, where the enzymatic glucose sensor is fabricated, optimised, and characterized using electrochemical measurement. Subsequently, the SIA system is prepared, which involves the preparation of the circulating pump, selective valve, flow cell, holding coil, and mixing channel. After the preparation step is completed, the system fabrication will be initiated by combining the electrochemical detection unit together with the SIA system.

3.2.1 Sensor Fabrication

Four commercial carbon screen-printed electrodes were activated by performing cyclic voltammetry using a 100 μL of 0.5 M sulfuric acid (H_2SO_4) solution with 50 cycles at a scan rate of 100 mV s^{-1} , as depicted in Figure 3.2. The first electrode was left as a bare electrode. The second electrode was coated with a 10 μL of glucose oxidase solution (GOD), the third electrode was coated with a single-walled carbon nanotubes (SWCNT) mixed with GOD, and the last one was coated with a colloidal gold nanoparticles (AuNP) mixed with SWCNT and GOD. Later, all electrodes were coated with a 10 μL of chitosan solution as top layer. This process is depicted in Figure 3.3, and the electrodes were then left semi-dried for 24 hours at 4°C temperature.

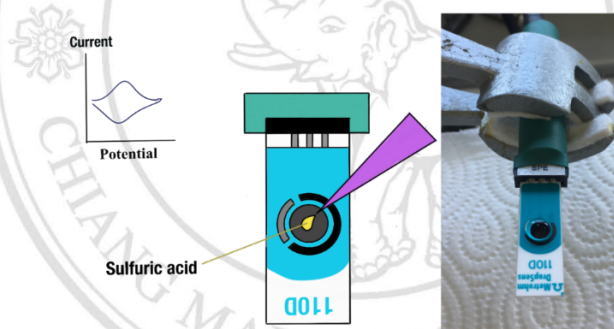


Figure 3.2 The electrode activation

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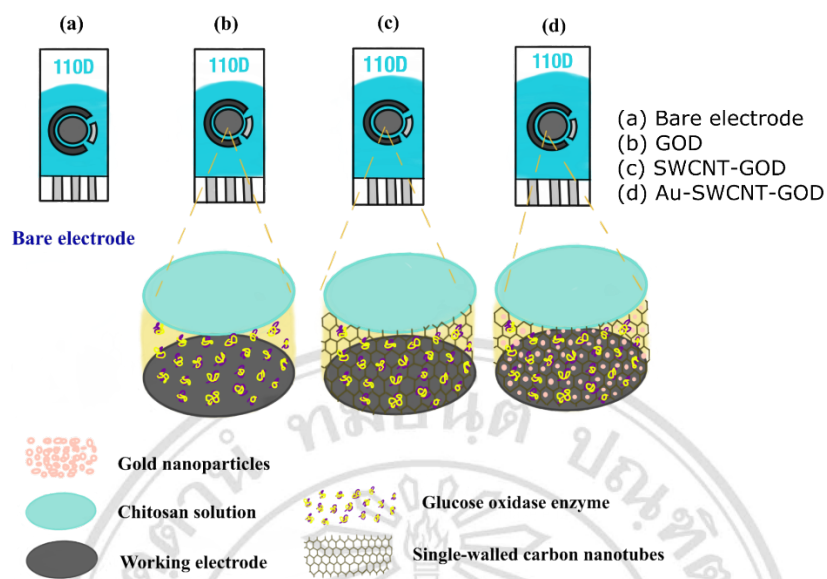


Figure 3.3 The schematic of four different modified electrodes

3.2.2 Sensor Characterization

The cyclic voltammetry of four sensors were performed in PBS solution to compare the sensor characteristic. The scan rate was set at 50 mV s^{-1} , as illustrated in Figure 3.4.

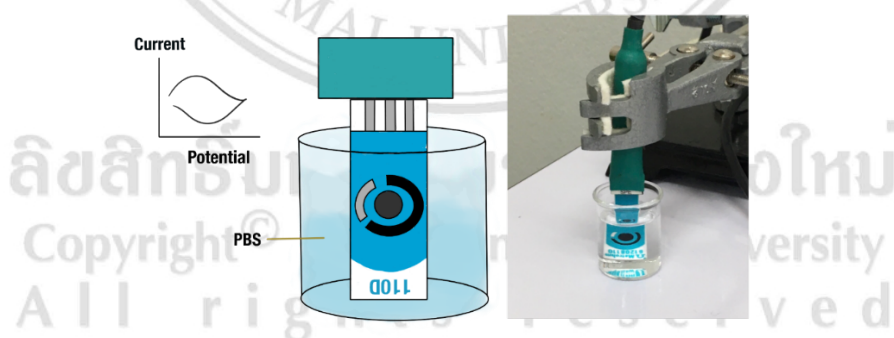


Figure 3.4 The Cyclic Voltammetry in PBS

The selected sensor was subjected to cyclic voltammetry in the glucose-free DMEM with and without the addition of a glucose standard solution, as shown in Figure 3.5.

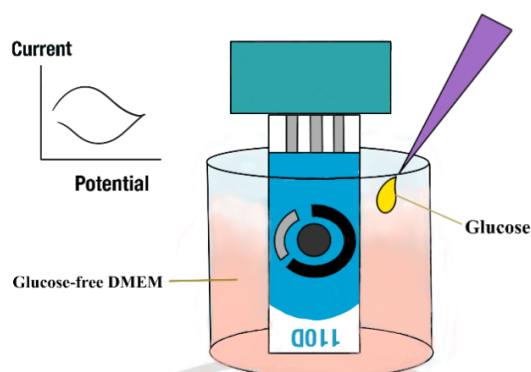


Figure 3.5 The Cyclic Voltammetry in DMEM

3.2.3 Sensor Optimization

The sensor was tested by varying the glucose oxidase units from 100 U to 1000 U to obtain the optimal concentration of GOD solution suitable for the glucose sensor in the system.

A 1000 U glucose oxidase stock solution was diluted to 250 U and 100 U by adding PBS solution, resulting in the final concentrations. Three commercial carbon screen-printed electrodes were activated by performing cyclic voltammetry using 100 μL of a 0.5 M sulfuric acid (H_2SO_4) solution with 50 cycles at scan rate of 0.1 V s^{-1} .

Each individual activated electrode was coated with a 10 μL of the mixing solution: 100U, 250U, and 1000U of glucose oxidase solution, respectively, mixed with SWCNT and AuNP in a 10:1:1 ratio of GOD-SWCNT-AuNP. All modified electrodes were coated with a 10 μL of chitosan solution as the top layer. This process is depicted in Figure 3.6, and the electrodes were then left semi-dried for 24 hours at 4°C temperature.

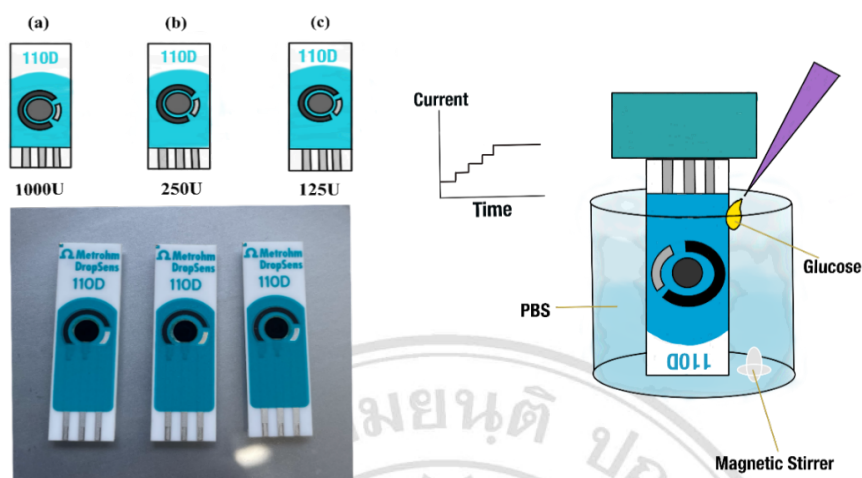


Figure 3.6 The GOD glucose sensors coated with various GOD amount and amperometric measurement in PBS

All sensors were tested in the PBS solution by performing amperometric measurement of glucose at an applied potential of +0.8 V to investigate the sensor performance and response to glucose.

3.2.4 SIA System Preparation

The preparation of the SIA (Sequential Injection Analysis) system involves several steps. First, the flow cell is prepared, which is the location of the glucose sensor detection unit. Next, the pump is set up to circulate the solution through the measurement system. Additionally, the selective valve is installed to choose and enable the solution to flow through each separate channel. The holding coil is included to temporarily store the sample solution before it is aspirated into the detection unit. Lastly, a mixing channel is incorporated to facilitate the mixing of the solution with the dilution.

a) Flow cell

The flow cell was designed and fabricated using LCD-based 3D printing at the BMEi, CMU laboratory. The size of the flow

cell was carefully considered to ensure a perfect fit with the dimensions of the carbon screen-printed electrode. The flow cell was designed to have connection ends that allow the sample solution to flow through the detection area via a Polytetrafluoroethylene (PTFE) connector and ultimately be directed to the waste.

b) Circulated pump

To find a suitable circulation pump for the system, a comparison was made between the peristaltic pump and the syringe pump, both of which were fabricated at the BMEi, CMU laboratory.

For accuracy testing, the weight of water was measured, corresponding to different flow rates. The flow rate of the pump was set at 0.05, 0.1, 0.5, and 1 mL per minute. The weight of the water was measured for 1 minute at each flow rate, assuming that the weight of 1 g of water is equivalent to 1 mL of water.

Finally, the selected pump was considered and designed to connect with the other parts of the system.

c) Selective valve

In this research, the selective valve was designed to be suitable for a six-well plate of cell culture, allowing for individual conditions in all six wells under the same incubation environment. There are two selective valve models with different designs were fabricated at the BMEi, CMU laboratory. Both models were demonstrated and applied to the system in order to compete and determine the appropriate one.

d) Holding coil

The holding coil was fabricated by using a length of Fluorinated Ethylene Propylene (FEP) tube rolled up onto the

plastic core. There are two connections of the holding coil connected to the pump and the selective valve.

e) Mixing channel

The mixing channel was designed and fabricated at the BMEi, CMU laboratory to facilitate the mixing of solution dilutions, allowing for compatibility with high concentrations or reducing the need for multiple ports for standard solutions.

3.3 System Verification

The glucose measurement system was established by integrating the glucose sensor detection unit with the SIA system, as shown in Figure 3.7. In order to validate the system, calibration was conducted using amperometric measurements in DMEM. Subsequently, the system's stability was evaluated through triple calibration using a single glucose electrochemical sensing detection loop, indicating the sensor's shelf life. Furthermore, the calibration of an individual sensor was assessed over a three-day period to observe its stability specifically during the cell culture phase.

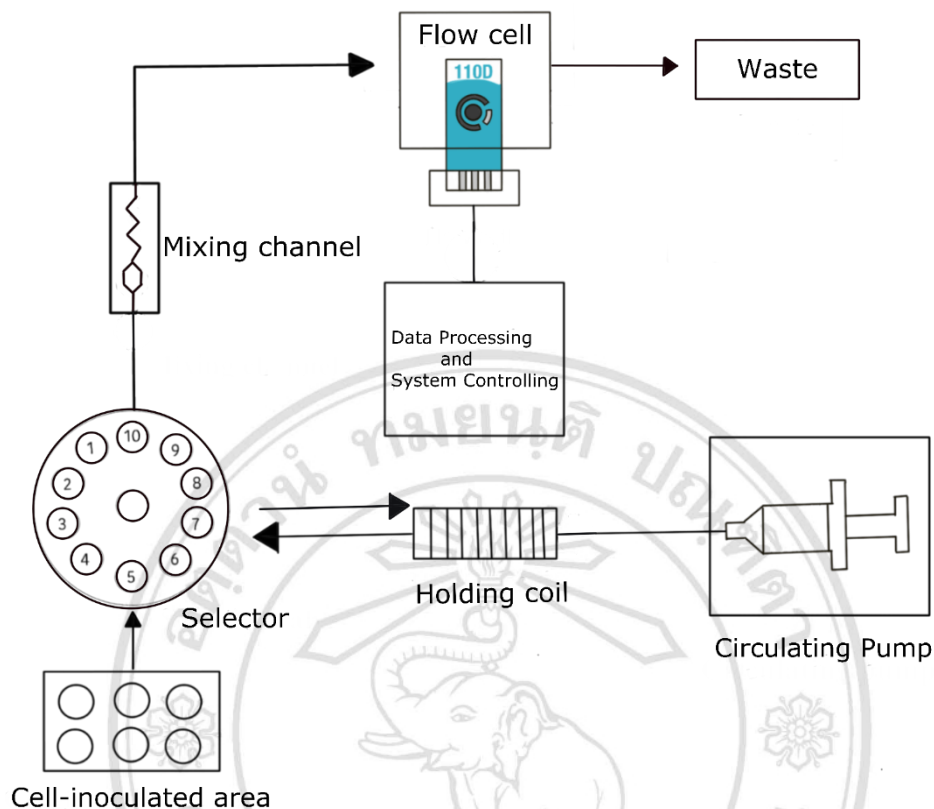


Figure 3.7 The assembly of glucose measurement system

3.3.1 System Calibration

A glucose-free Dulbecco's Modified Eagle Medium (DMEM) was diluted 1:4 (v/v) with a PBS solution. A 10 mM glucose standard stock solution was added to the diluted DMEM to yield final concentrations of 0.5 mM, 1 mM, 2 mM, 3 mM, and 4 mM. A glucose standard solution in DMEM, including 0 (blank), 0.5 mM, 1 mM, 2 mM, 3 mM, and 4 mM, was connected to the selective valve channel, as shown in Figure 3.8.

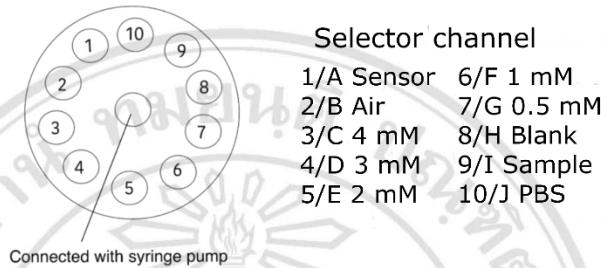
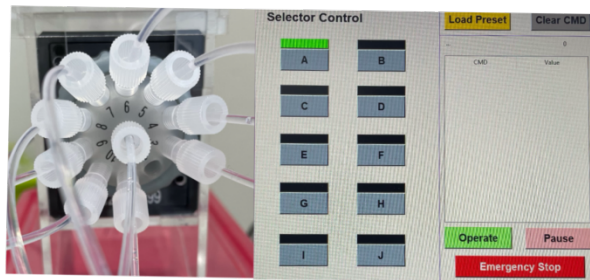


Figure 3.8 The connection of selector channel

The sequence of the selector started from port C, followed by ports D, E, F, G, and H, respectively corresponding with high to low concentrations of glucose, as shown in Figure 3.9.

Sequential profile for system calibration

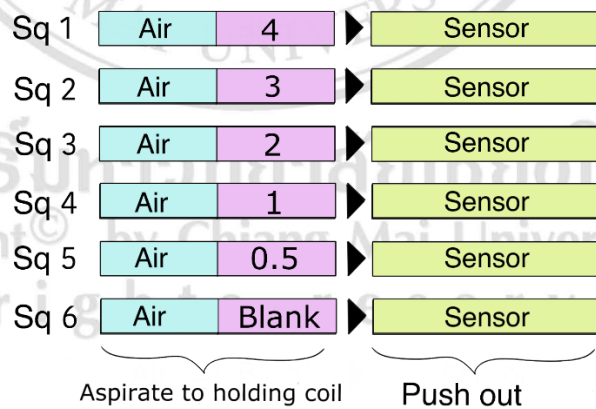


Figure 3.9 The sequential profile of system calibration

The pump control was set to control the volume of solution, either filling in or draining out, as shown in Figure 3.10.

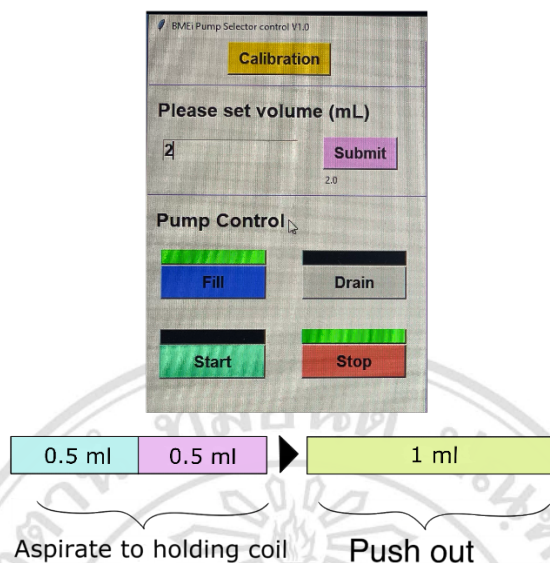


Figure 3.10 Pump controlling and volume setting

Amperometric measurement was performed with an applied potential of +0.8 V. Subsequently, a calibration curve was plotted. From the calibration curve, the sensitivity, working range, and limit of detection of the sensor were calculated.

3.3.2 System Stability Testing

a) System Stability Testing

To demonstrate the system stability, the amperometric measurement of a glucose sensor was triple calibrated within a single electrochemical detection loop. The current response was observed during the calibration process to predict the sensor's shelf life. The calibration sequence follows low to high concentrations of glucose, as shown in Figure 3.11

Sequential profile for system calibration

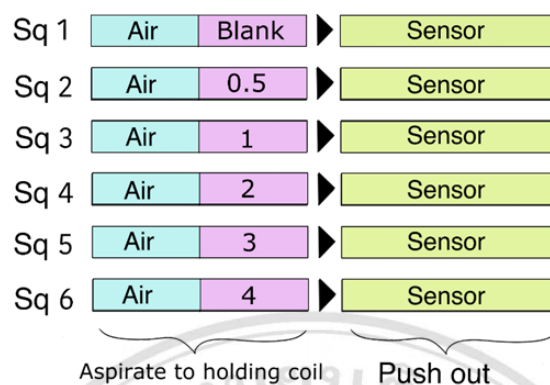


Figure 3.11 The sequential profile of system stability setting

b) System Stability Testing in three days

Amperometric measurements of the glucose sensor were conducted in 0.5, 2, 3, and 4 mM glucose concentrations in glucose-free DMEM. These measurements were repeated after 3 days to assess the stability of the sensor, coinciding with the cultivation period of the L929 cell line. The difference in current response between each glucose concentration on day 1 and day 3 was calculated to determine the remaining sensor response.

3.4 Application in Cell Culture Media

The modified system was applied to measure the concentration of glucose in DMEM samples used in the L929 cell culture sampled at different incubation times: 24 hours (Day 1) and 72 hours (Day 3). The off-line glucose measurement using a glucose colorimetric assay was also performed parallelly. Finally, the results from both processes were compared.

3.4.1 Glucose Measurement System

Amperometric measurements were employed to detect the glucose concentration in waste DMEM on day 1 and day 3. The applied potential was set at +0.8V. The DMEM were diluted 1:4 (v/v) with PBS solution as sequential profile of sample detection shows in Figure 3.12 (see Appendix A). The current response was

calculated as a concentration by substituting the data of the current response peak for each concentration into the linear equation obtained from the calibration curve.

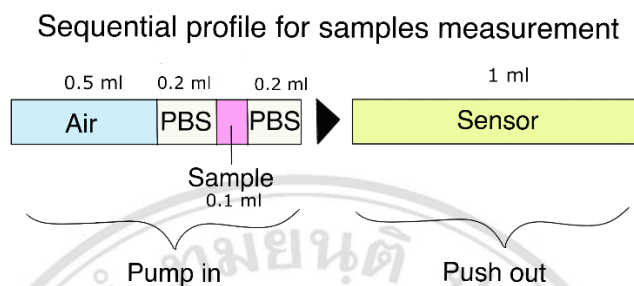


Figure 3.12 The sequential profile of sample measurement

3.4.2 Glucose Colorimetric Assay

To investigate the accuracy performance of the system, the results of glucose measurement in DMEM using the modified glucose measurement system were compared with the results of glucose measurement in DMEM from the standard glucose colorimetric assay. A 100 μ l of each standard and sample was mixed with 4 μ l of glucose oxidase enzyme and incubated at 37°C for 30 minutes. Then, a 2 μ l aliquot of each reaction solution was mixed with 200 μ l of peroxidase reagent from horseradish, as depicted in Figure 3.13. Colorimetric measurement was performed at 490 nm wavelength, as shown in Figure 3.14. All samples were run in triplicate.

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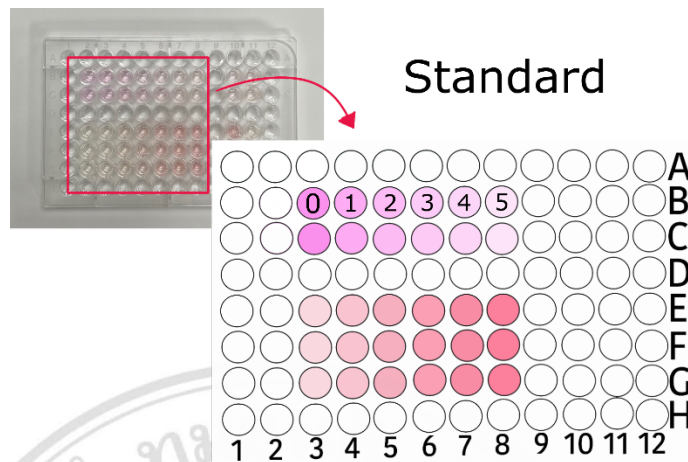


Figure 3.13 Glucose colorimetric assay for standard

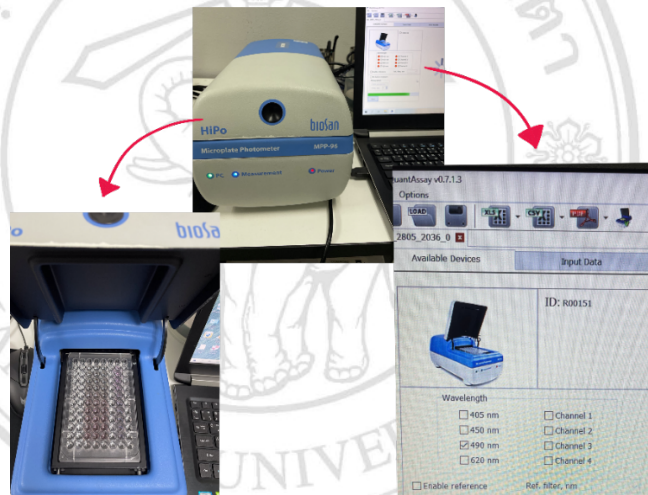


Figure 3.14 The spectrophotometry

3.5 Statistical Analysis

The glucose concentrations of DMEM cell culture media were calculated using either the current response from glucose measurement system or the absorbance from glucose colorimetric assay. The results from both procedures were compared at 24- and 72- hour cell incubation time points. The statistical paired t-test was performed with a significance level of $\alpha = 0.05$.

CHAPTER 4

Results and Discussion

4.1 The Result of System Preparation

4.1.1 The Result of Sensor Characterization

a) Sensor Characterization in PBS

A comparison was conducted to assess the current response using cyclic voltammetry among three different modified sensors and a bare carbon screen-printed electrode, as shown in Figure 4.1. The results obtained from cyclic voltammetry (CV) testing of four different sensors in PBS indicated that incorporating nanoparticles onto the electrode surface increases the charging current compared with the current response of bare electrode (dash line). This enhancement is attributed to the increased surface area and conductivity facilitated by the negatively charged SWCNT and Au nanoparticles. Additionally, chitosan creates a positively charged three-dimensional structure that effectively immobilizes enzymes and nanoparticles on the electrode's surface. These findings suggest the formation of an electric double layer at the electrode interface, indicating a modification in the electrical properties of the surface. Notably, the Au-SWCNT-GOD-CS (pink line) modification on the carbon screen-printed electrode (SPE) exhibited a highest current response compared to the SWCNT-GOD-CS (green line) and GOD-CS (yellow line) modified SPE, respectively. As a result, the Au-SWCNT-GOD-CS modified carbon SPE was selected to test its response for glucose detection in DMEM.

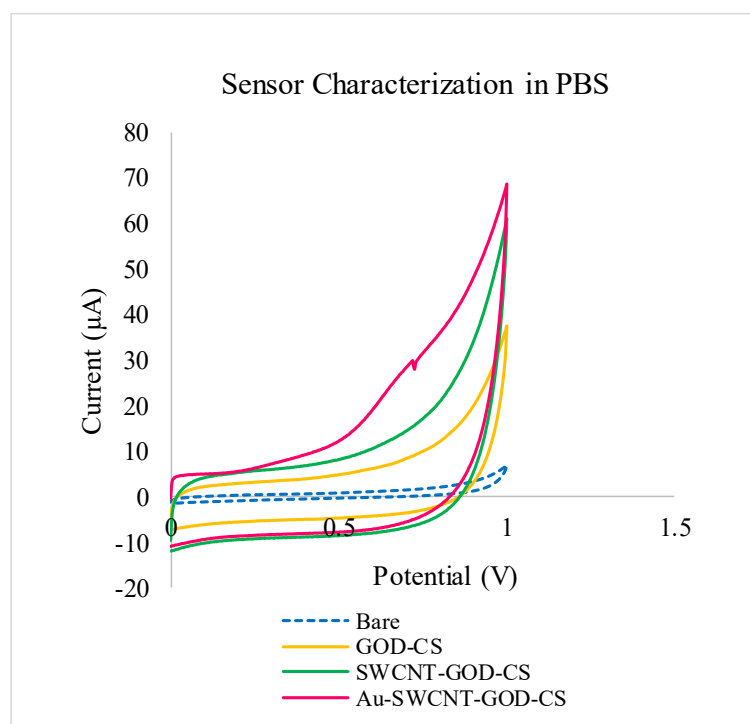


Figure 4.1 Cyclic Voltammetry of three different modified sensors compare to bare carbon-SPE in PBS solution at the scan rate of 50 mVs^{-1}

b) Sensor Characterization in DMEM

The Au-SWCNT-GOD-CS modified carbon screen-printed electrode (SPE) was subjected to cyclic voltammetry testing in glucose-free DMEM and then with the addition of 10 mM glucose, as shown in Figure 4.2. The results demonstrated a higher current response at the high potential starting from +0.8 V in the presence of 10 mM glucose compared to its absence. This oxidative current arise from catalysis of glucose oxidation by GOD. First, the redox reaction taking place on the first hydroxyl group of the GOD enzyme, which acts at the reducing end of glucose. Next, the enzyme utilize oxygen as an external electron acceptor, resulting in the release of hydrogen peroxide, which is then detected by the working electrode. These findings indicate the enzyme activity of GOD in the presence of glucose in DMEM. Therefore, the potential at +0.8 V was chosen for detection of glucose in the further experiment.

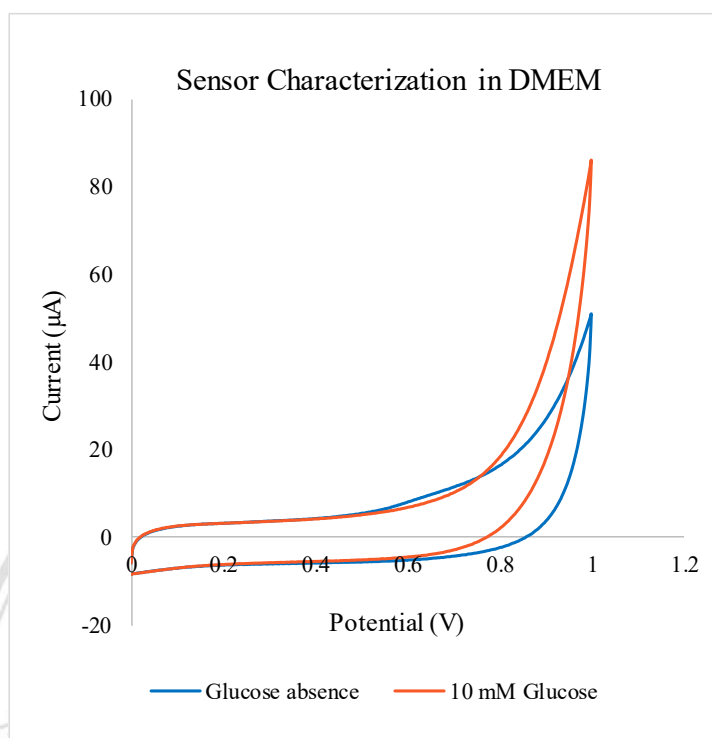


Figure 4.2 Cyclic voltammetry of Au-SWCNT-GOD-CS modified SPE in DMEM at the scan rate of 50 mVs⁻¹

4.1.2 The Result of Sensor Optimization

The Au-SWCNT-GOD-CS modified carbon screen-printed electrode (SPE) was optimized by coating the glucose oxidase enzyme unit at three different levels: 1000 units, 250 units, and 100 units. The three different modified sensors underwent amperometric measurements with a glucose bulk solution of PBS. The results showed in Figure 4.3. Additionally, a calibration curve was plotted, illustrating the relationship between the current response and the glucose concentration, as depicted in Figure 4.4.

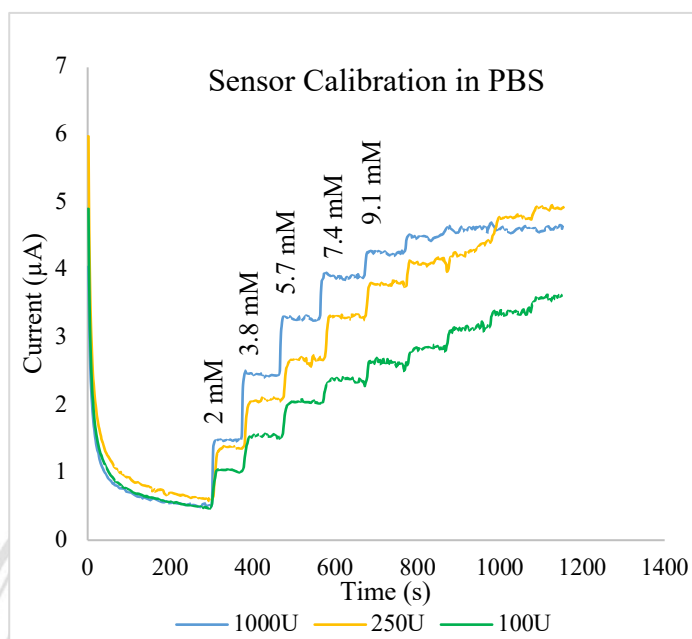


Figure 4.3 Amperometric measurement of 1000U / 250U / 100U GOD modified glucose sensor in PBS solution at the applied potential of +0.8V and at the stirring speed of 300 RPM

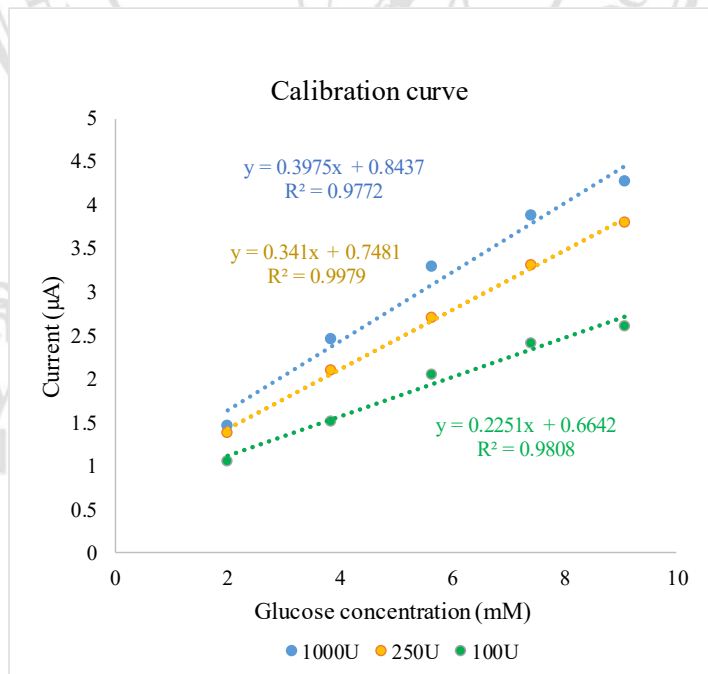


Figure 4.4 The calibration curve of 1000U / 250U / 100U GOD modified glucose sensor in PBS solution

The results obtained from the calibration curve demonstrate that the current response showed a linear increment as the glucose concentration increased within the working range of up to 9.1 mM for all sensors. However, when comparing the different sensors, the one equipped with 250 U GOD enzyme unit exhibited compromised characteristics in terms of sensitivity and an appropriate working range for glucose detection. For 250 U GOD, a linear equation was obtained as current response = 0.34 [glucose] + 0.75, $R^2 = 0.99$. As a result, this optimised sensor was chosen to be employed as the detection unit for the glucose measurement system.

4.1.3 The Result of SIA System

a) Flow cell

The flow cell was first designed and fabricated using acrylic resin-based 3D printing, as shown in Figure 4.5. The size of the flow cell chamber was a perfect fit with the dimensions of the carbon screen-printed electrode. The flow cell was designed to have two silicone tube connections located on the top lid, allowing the sample solution to flow through the detection area with a volume of less than 100 μ l. Unfortunately, this design still has a problem of solution leakage, as well as residual solution volume or air bubbles remaining in the chamber.

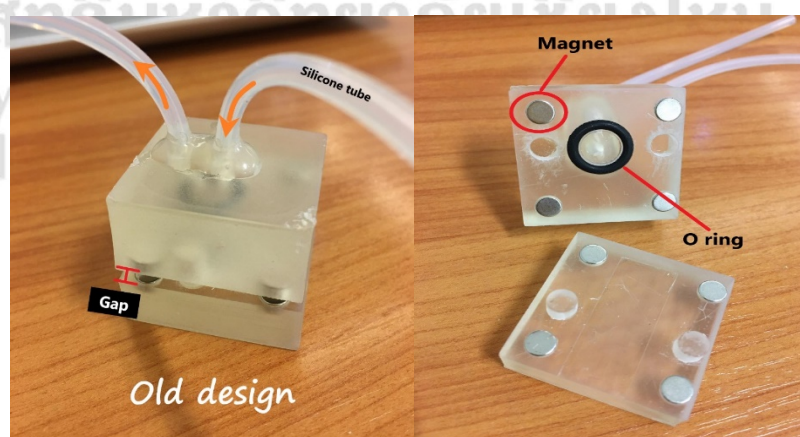


Figure 4.5 The first design of the flow cell

The problem with the initial design model was fixed by replacing the silicone tube with a Polytetrafluoroethylene (PTFE) connector, which was positioned on the opposite side of the flow cell, as illustrated in Figure 4.6. The second model underwent testing to address solution leakage and eliminate the issue of air bubbles. Ultimately, this model was successfully implemented in the system.

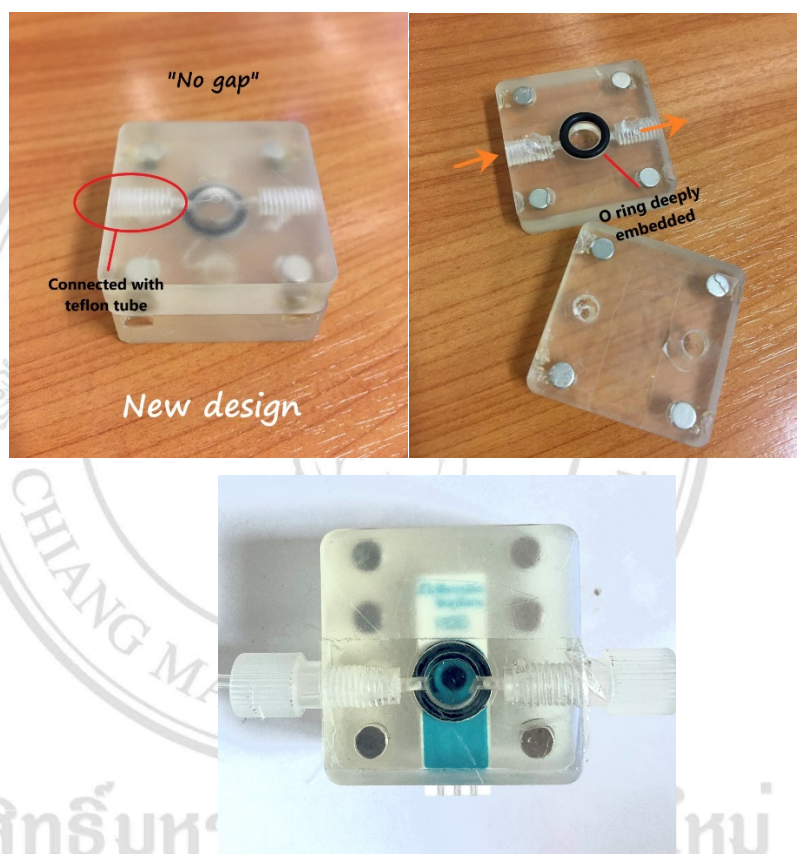


Figure 4.6 The second design of the flow cell

b) Circulated pump

The results of comparing the peristaltic pump (Figure 4.7) and the syringe pump (Figure 4.9), based on measuring the weight of water at different flow rates, are presented in the calibration curve shown in Figure 4.8 and Figure 4.10. The relationship between the weight of water and the flow rate was observed by the slope of the equation from calibration curve: y (weight of water) = slope. x (flow rate). The

results show that the syringe pump demonstrates high accuracy in measuring water weight corresponding to flow rates, with the calibration slope close to 1, indicating that the weight of 1 g of water is equivalent to 1 mL of water. In contrast, the peristaltic pump shows a deviation with a calibration slope of 0.7. Additionally, the syringe pump exhibits a strong correlation between volume and flow rate, with an R^2 value of 0.999. Another useful feature of the syringe pump is its ability to aspirate both forward and backward in a single operation, which helps streamline the system. Consequently, the syringe pump was selected for use in the glucose measurement system

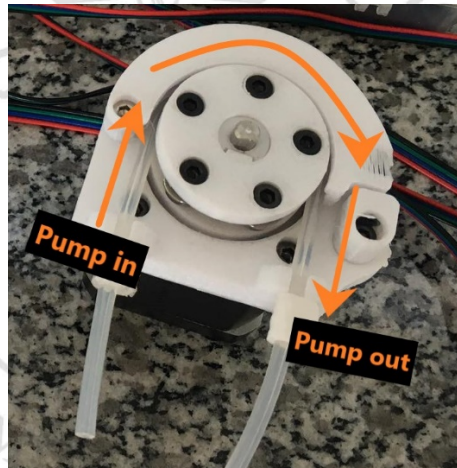


Figure 4.7 The peristaltic pump

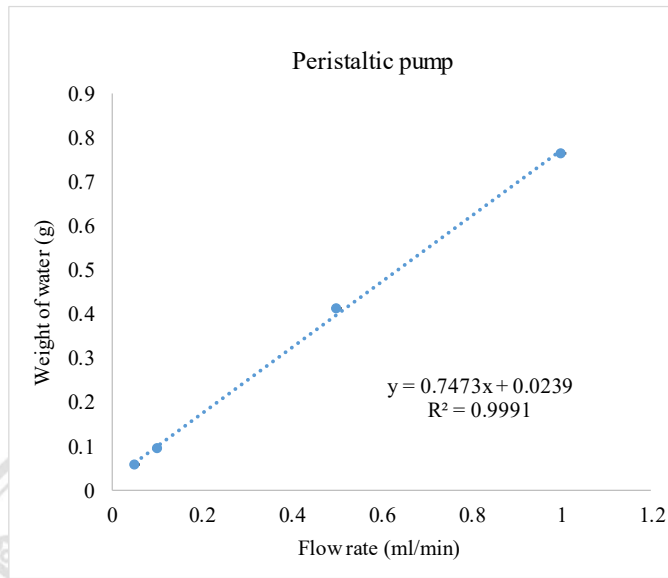


Figure 4.8 The calibration of peristaltic pump, $n = 3$

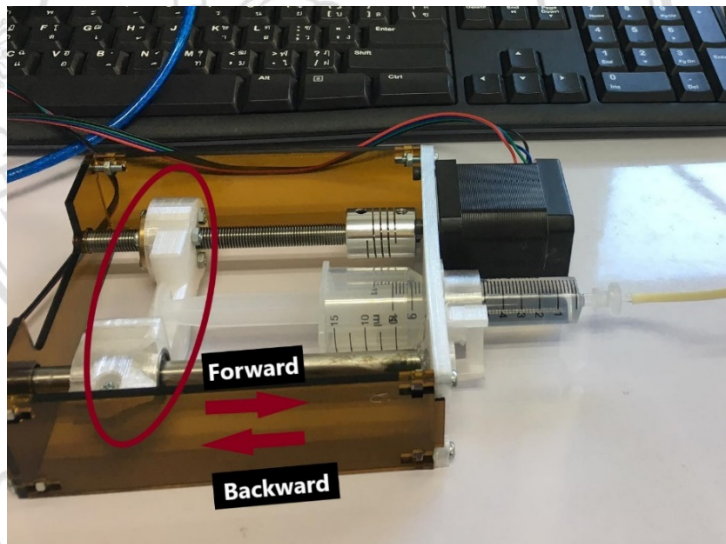


Figure 4.9 The syringe pump

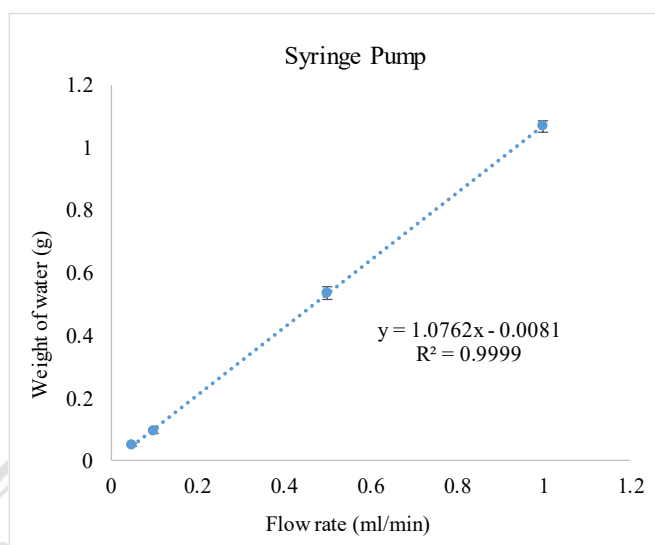


Figure 4.10 The calibration of syringe pump, n = 3

c) Selective valve

The first model of the selective valve was designed to be suitable for a six-well plate of cell culture and fabricated using acrylic resin-based 3D printing, employing the same material as the flow cell. The six units of the selector valve were designed to connect with the cell-inoculated area, as shown in Figure 4.11 and Figure 4.12. However, this model has a problem with solution leakage and a limitation on the number of working ports.

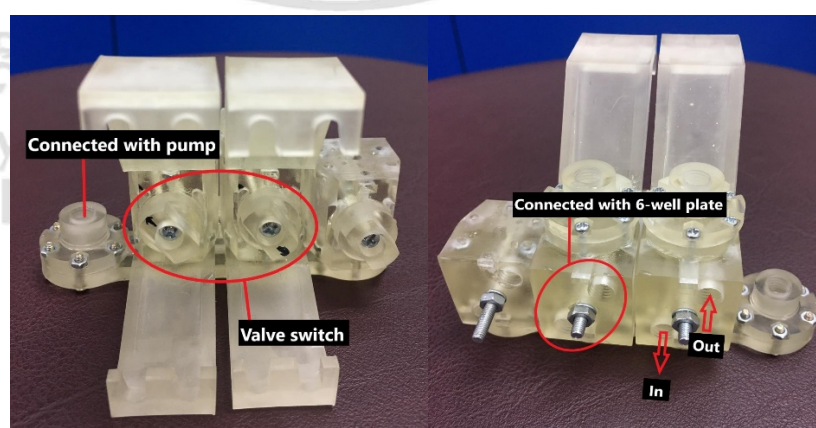


Figure 4.11 The first design of the selective valve

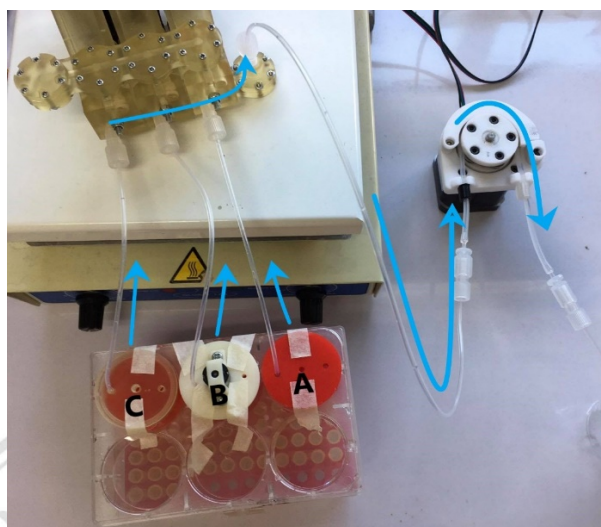


Figure 4.12 The selector connected with six-wells plate

To overcome the number of working port issue, the commercially available selector, with 10 ports for working channels, was utilized as shown in Figure 4.13. This model was chosen to be implemented in the system.

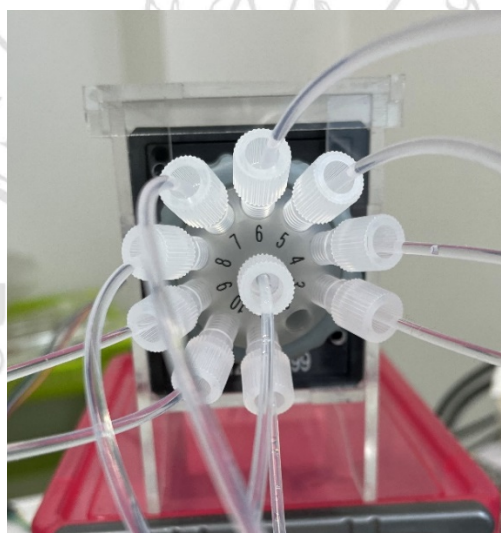


Figure 4.13 The final design of the selective valve applied to the system

d) Holding coil

The holding coil, which serves as the solution reservoir before it is injected into the electrochemical detection unit, was fabricated using

a length of Fluorinated Ethylene Propylene (FEP) tube rolled up onto a plastic core. There are two connections for the holding coil, one connected to the pump and the other connected to the selective valve, as shown in Figure 4.14.



Figure 4.14 The holding coil

e) Mixing channel

The mixing channel was created using the same material and fabrication technique employed for the flow cell. It features a 45-degree zig-zag shape, as shown in Figure 4.15, specifically designed to enhance the mixing of solution dilutions.

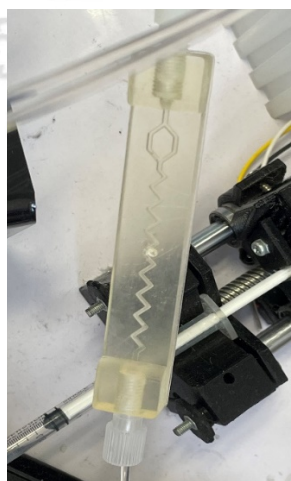


Figure 4.15 The mixing channel

4.2 The Result of System Verification

4.2.1 The Result of System Calibration

When the electrochemical detection unit was integrated into the SIA system, a range of standard glucose solutions with varying concentrations were added to glucose-free DMEM, and Amperometric measurements were performed. The results displayed a series of increasing current responses corresponding to the glucose concentration, as depicted in Figure 4.16. A calibration curve was plotted, demonstrating a strong correlation with an R^2 value of 0.9863 and a sample size of $n=3$, as shown in Figure 4.17. The system exhibited a linear working range of up to 3.8 mM for glucose concentration. The glucose concentration could be determined by utilizing the equation derived from the calibration curve: I (Peak of current) = $0.8351(\text{glucose conc.}) + 1.7124$. The limit of detection was calculated to be 0.3 mM (3σ , [36]). The sensitivity per electrode area was measured as $66.8 \text{ nA mM}^{-1} \text{ mm}^{-2}$.

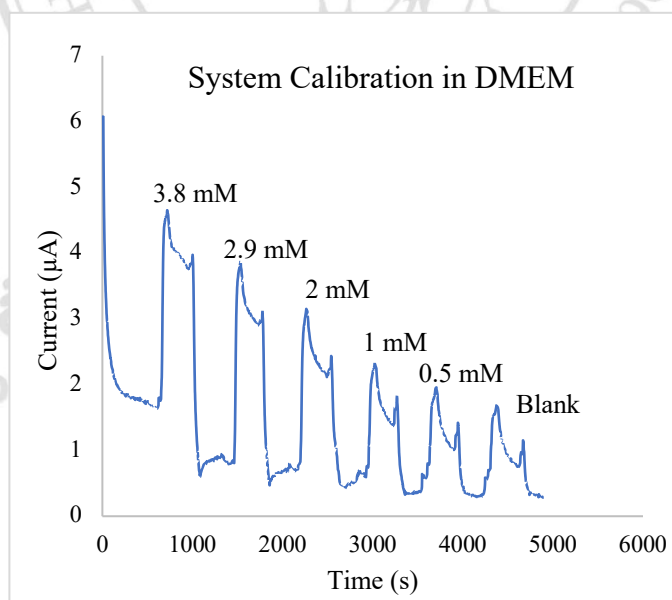


Figure 4.16 The Amperometric measurement of the modified system in DMEM at the applied potential of +0.8 V

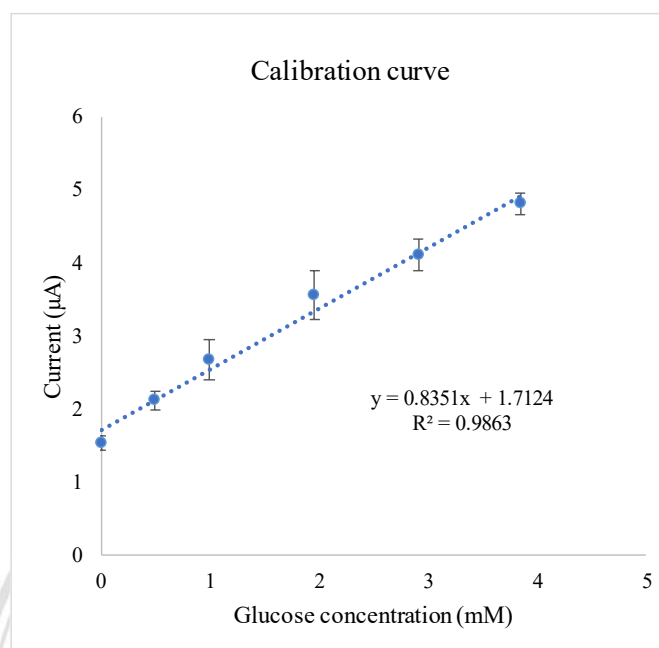


Figure 4.17 The calibration curve of the modified system in DMEM, n = 3

4.2.2 The Result of System Stability

a) The Result of System Stability Testing in Three-Cycle Loop

An individual Au-SWCNT-GOD-CS modified sensor was integrated into the system, and Amperometric measurements of standard glucose solutions in DMEM were conducted in three-cycle loop to assess the long-term stability of the system. The calibration curve was constructed using the average current peak obtained from the three loops. The results demonstrate a linear relationship between each current response and the corresponding concentration of glucose within each individual loop as shown in Figure 4.18. Despite a decrease in sensitivity to $46.95 \text{ nA mM}^{-1} \text{ mm}^{-2}$ when considering the average current peak from the three-loop measurement, the correlation remains strong with an R^2 value of 0.9545, as depicted in Figure 4.19. This suggests that the system is capable of maintaining stability over a three-time cycle period.

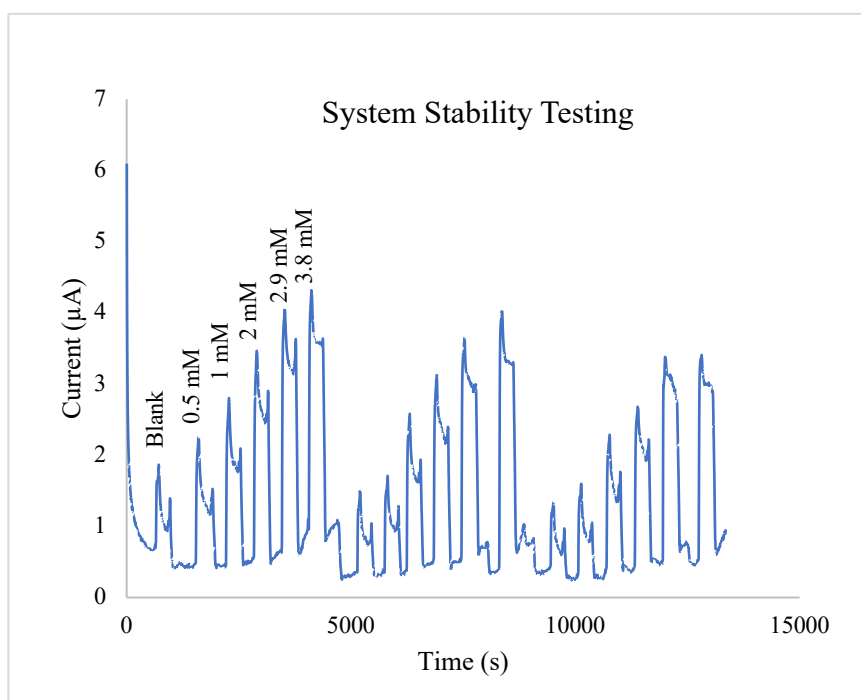


Figure 4.18 The Amperometric measurement of the modified system in DMEM with repeated in three cycle loop

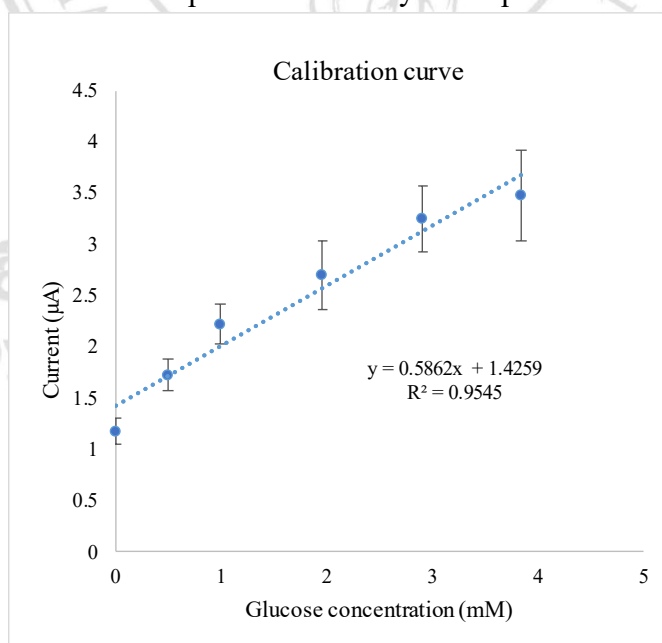


Figure 4.19 The Calibration curve of the modified system in DMEM with repeated in three cycle loop

b) The Result of System Stability Testing in Three Days

An individual Au-SWCNT-GOD-CS modified sensor was incorporated into the system, and Amperometric measurements of standard glucose solutions in DMEM were performed over a three-day period, coinciding with the duration of L929 cell culture. The calibration curve was plotted as shown in Figure 4.20. The results indicate that the glucose sensor maintained a current response ranging from 81.04% to 93.76% compared to the initial current response on day 1 as shown in Table 4.1. The decreasing of the sensor stability might be occurring by the destruction of GOD structure due to H₂O₂ product from redox reaction. Additionally, all three calibration curves exhibited a strong correlation between the glucose concentration and the current response. These findings suggest that the glucose sensor is suitable for implementation in the cultivation of the L929 cell line over a three-day period.

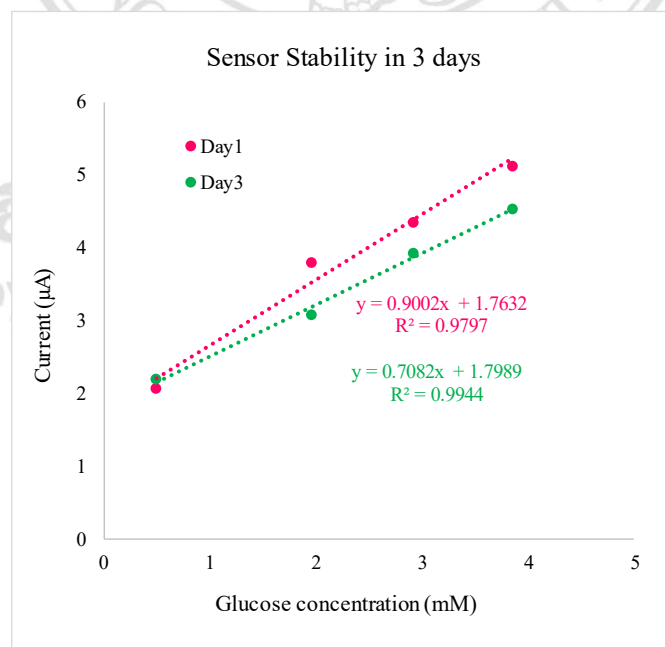


Figure 4.20 The calibration curve of the modified system in DMEM with repeated measurement in three days

4.3 The Result of Cell Culture Media Application

4.3.1 The Result of Glucose Enzymatic Colorimetric Assay

The concentration of glucose in DMEM from L929 cell cultivation at 24 hours (Day 1) and 72 hours (Day 3) of incubation was determined using a standard glucose enzymatic colorimetric assay. The glucose concentration in the DMEM at Day 1, as measured by the standard assay protocol (see Appendix B), was determined to be 15.48 mM, while the glucose concentration at Day 3 was found to be 1.06 mM.

4.3.2 The Result of Glucose Measurement System

The concentration of glucose in the DMEM from L929 cell cultivation at 24 hours (Day 1) and 72 hours (Day 3) of incubation was determined using the glucose measurement system. The glucose concentration in the DMEM at Day 1, as measured by the modified glucose measurement system, was determined to be 13.47 mM, while the glucose concentration at Day 3 was found to be 2.46 mM.

Finally, the glucose concentrations in the DMEM at 24 and 72 hours of incubation during L929 cell cultivation using two different methods: the standard glucose colorimetric assay and the modified glucose measurement system were compared, as depicted in Figure 4.21. A statistical paired t-test was conducted to evaluate the efficiency of the system compared to the standard protocol. The results showed that the p-value was greater than 0.05 at a standard significance level, indicating that there was no significant difference between the standard glucose enzymatic colorimetric assay and the modified glucose measurement system.

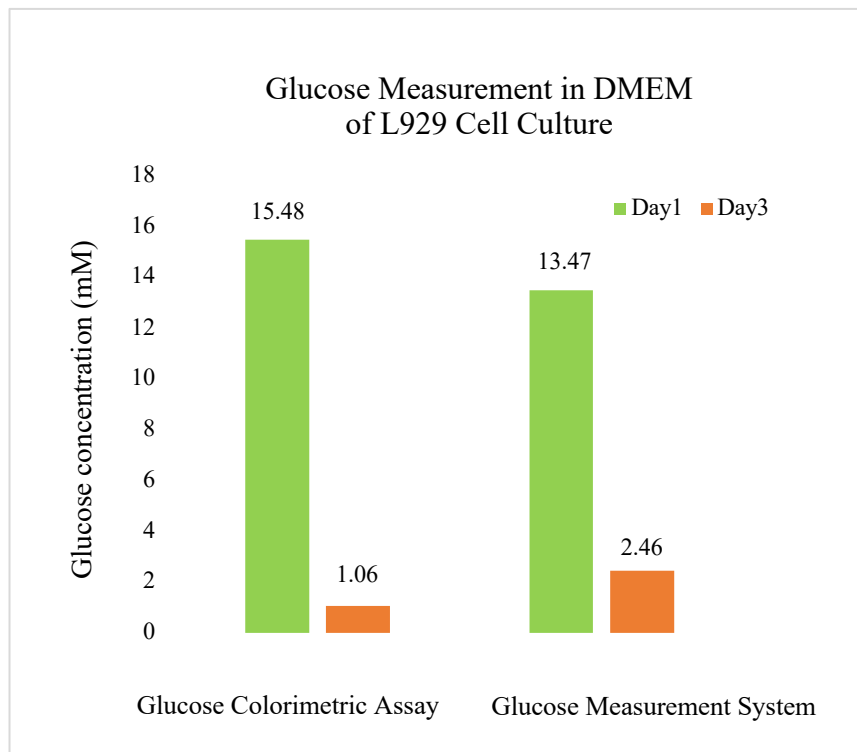


Figure 4.21 The comparison of Glucose measurement in DMEM using glucose colorimetric assay and modified glucose measurement system

The developed glucose measurement system utilizing electrochemical detection has demonstrated rapid, straightforward, and superior performance compared to the traditional colorimetric assay. The system is capable of processing and preparing samples, conducting detection, and presenting results within approximately 5 minutes per sample, whereas the conventional method necessitates an enzyme incubation period of at least 30 minutes. An additional advantage of the electrochemical approach is its immunity to interference from the color and turbidity of the culture media. For instance, commonly used pH indicator phenol red in various commercial media formulations can disrupt the colorimetric method. Furthermore, there is no requirement for sample and reagent preparation for each measurement, thus reducing both working time and waste generation.

CHAPTER 5

Conclusion and Suggestion

A comprehensive system was created to monitor glucose levels in cell cultures in real-time. It combines an amperometric detector and an SIA system. The system allows for separate manipulation and treatment conditions for each culture, with six individual cell-inoculated areas. Automation features are incorporated for tasks like in-line sampling, sample dilution, and solution mixing. The detection unit utilizes an Au-SWCNT-GOD-CS modified SPE and a 3D-printed flow cell to ensure accurate and efficient glucose detection. The system's performance was successfully demonstrated using L929 fibroblast cell culture and compared to the standard glucose measurement assay.

Furthermore, the system has potential for future development to simultaneously monitor changes in metabolites across multiple cell types or simulated conditions, accommodating up to six types/conditions. It can be seamlessly integrated into automatic cell culture systems, simplifying processes like automatic media or solution feeding. The system's versatility makes it applicable to various areas of biomedical research, including cellular metabolism studies for drug and cancer therapy, as well as in vitro cytotoxicity tests for biomaterial and tissue engineering applications.

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APPENDIX A

Table A1 List of command in the SIA system controlling program

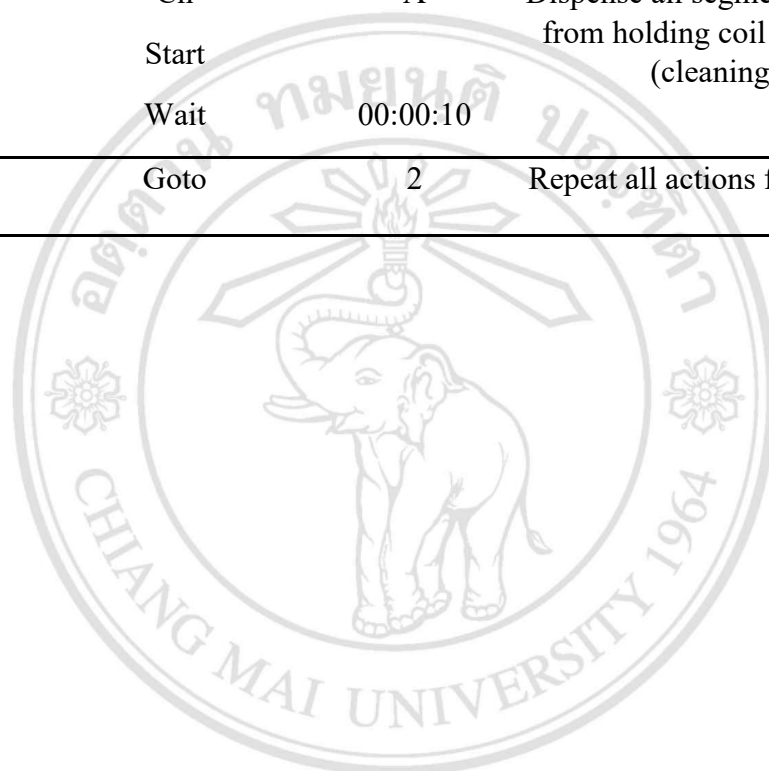
Command	Value	Description
Loop	Number	Set number of scripts to repeat
Pump	Number	Set volume of syringe pump to aspirate or dispense (mL)
Goto	Number	Jump to a line number that set of the script
Wait	hh:mm:ss	Set delay time
Dir	F or B	Set syringe pump direction (forward or backward)
Ch	Selection valve	Select selection valve position
Start	(A or B or C or else)	Starting syringe pump
Msgbox	Text	Displays the specified text in the message box

Table A2 A script for glucose analysis using the SIA system

Line number	Command	Value	Action
1	Loop	3	Repeat all actions for 3 times
2	Pump	0.5	
3	Dir	B	
4	Ch	B	Aspirate air 500 μ l into holding coil
5	Start		
6	Wait	00:00:10	
7	Pump	0.2	
8	Dir	B	
9	Ch	J	Aspirate PBS 200 μ l into holding coil
10	Start		

Line number	Command	Value	Action
11	Wait	00:00:10	
12	Pump	0.1	
13	Dir	B	
14	Ch	I	Aspirate sample 100 μ l into
15	Start		holding coil
16	Wait	00:00:10	
17	Pump	0.2	
18	Dir	B	
19	Ch	J	Aspirate PBS 200 μ l into
20	Start		holding coil
21	Wait	00:00:10	
22	Pump	1	
23	Dir	F	
24	Ch	A	Dispense all segment 1000 μ l
25	Start		from holding coil to sensor
26	Wait	00:00:10	
27	Pump	0.5	
28	Dir	B	
29	Ch	B	Aspirate air 500 μ l into
30	Start		holding coil (cleaning)
31	Wait	00:00:10	
32	Pump	0.5	
33	Dir	B	
34	Ch	J	Aspirate PBS 500 μ l into
35	Start		holding coil (cleaning)

Line number	Command	Value	Action
36	Wait	00:00:10	
37	Pump	1	
38	Dir	F	
39	Ch	A	Dispense all segment 1000 μ l from holding coil to sensor (cleaning)
40	Start		
41	Wait	00:00:10	
42	Goto	2	Repeat all actions for 3 times



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APPENDIX B

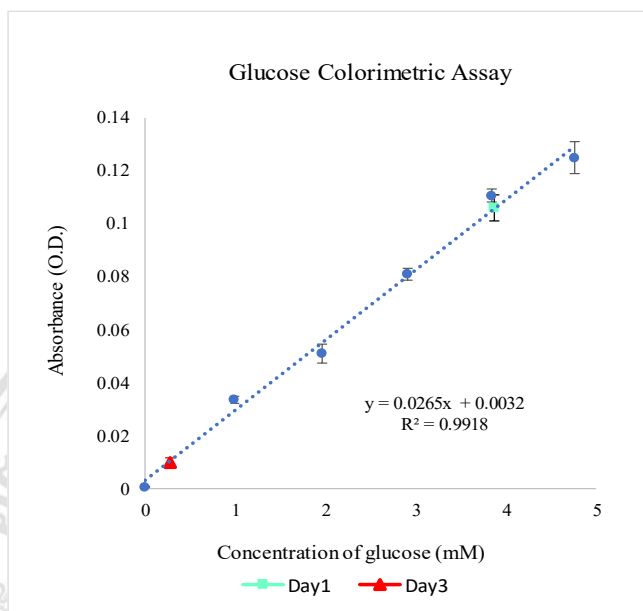


Figure B1 The result of glucose measurement in DMEM with 24 hrs and 72 hrs incubation using standard glucose enzymatic colorimetric assay

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