Electrochemical technique for cell viability assessment via monitoring of intracellular NADH with a modified double mediator system

A dissertation submitted to the

Graduate School of Innovative Life Science

University of Toyama, Japan

In partial fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

in Biological Information Systems

By

Md. Abdul Alim

March 2022

ABSTRACT

In this thesis, I proposed and developed a new electrochemical technique for cell viability assessment via monitoring of intracellular NADH with a modified double mediator system.

Developing of rapid and precise methods for cell counting and cell viability assessment is very important to evaluate the acute cytotoxicity of fast-acting drugs, pollutants, food additives, fermentation processes in the food processing industry, and in cell biology. There are various kinds of conventional methods have been developed for cell viability assessment. However, some methods such as the fluorescent dye-staining methods take a time to analysis and cannot assess the intracellular metabolic activity. To resolve this problem, electrical methods such as the impedance spectroscopy method and electro-orientation method have been developed. These methods are rapid but still unable to assess an intracellular metabolism as cell viability. On the other hand, conventional MTT or WST assays can assess the total intracellular NADH as a marker of cell viability though it is time-consuming. These colorimetric methods have some other limitations such as cytotoxicity of assay dye, several steps to get results, scattering effect of cells suspended in medium, and therefore cannot evaluate the toxic effect of fast-acting drugs or chemicals.

With this background, the main objective of this study was to develop a rapid, precise, and convenient electrochemical method to evaluate the real cell viability through the monitoring of intracellular NADH which is one of the most important metabolites for not only mammalian cells but also microorganisms.

In chapter 1, I highlighted the background and purpose of this study, and provided some important biological terms related to this study. In chapter 2, I comprehensively explained the required materials and methods for this study.

In chapter 3, I introduced the electrochemical technique with 1-methoxy-5-methylphenazinium methyl sulfate (mPMS) and $[Fe(CN)_6]^{3-}$(FeCN) as a modified double

mediator system to monitor intracellular NADH on mammalian cells. A combination of 10 µM mPMS and 500 µM FeCN was the optimum concentration, and 10 minutes of incubation was enough to monitor intracellular NADH by chronoamperometry at +0.5 V applications. My this mPMS/FeCN system worked as useful as previously reported enzyme-dependent menadione (Mena)/FeCN system. I confirmed that the electron transfer from intracellular NADH to mPMS occurred non-enzymatically, though the cytosolic enzyme catalyzed the electron transfer from intracellular NADH to Mena. Next, I applied my modified double mediator system to count the various kinds of mammalian cells. Here the cell counting results by my method were compared with the results by conventional WST-1 assay. The oxidation current in chronoamperometry after 10 minutes of incubation showed an excellent linear relationship in two times wider cell concentration as compared to the cell concentration detected by conventional WST-1 assay. Furthermore, I applied my method to investigate the acute toxic effect of oxamic acid on metabolic activity in PC12 cells as a model tumor cell by blocking LDH. Recently, lactate dehydrogenase (LDH) inhibition by oxamic acid has taken a lot of attention for the anti-cancer drug. My result demonstrated that the electrochemical technique with the modified double mediator system might be useful for screening of fast-acting drugs to intracellular metabolism.

In chapter 4, I described the application of my method for yeast cell counting and to evaluate the acute cytotoxicity of two antifungal agents in yeast cells. Firstly, I paid attention to itaconic acid that has been especially used to make hydrogels for water decontamination and eco-friendly biodegradable polymer. Itaconic acid is also important as a natural metabolite that acts as a key regulator for the TCA cycle by an inhibitory effect on succinate dehydrogenase (SDH). Itaconic acid with mM concentration interferes the TCA cycle metabolism by direct inhibition of SDH. So, itaconic acid cytotoxicity monitoring is highly important. I succeeded to evaluate the metabolic inhibition effect of itaconic acid in yeast cells by electrochemical monitoring of intracellular NADH with my modified double mediator system. Further, I applied my method to evaluate the toxic effect of nystatin in yeast cells. Nystatin is widely used as an anti-fungal drug. It has been reported that the toxic effect of nystatin at the concentration of µg/mL range was evaluated by the colony counting method on the basis of cell membrane disruption. Here, the toxic effect of nystatin with two order lower concentration was evaluated

by my method. The results obtained by my method demonstrated that 0.01 μg/mL of nystatin induced intracellular NADH decrease to promote apoptosis without cell membrane disruption.

In chapter 5, I concluded this research. Result obtained in this study suggested that my method might be applicable to evaluate the various types of acute cytotoxicity such as inhibition of respiratory chain, protein and DNA synthesis, etc. I believe that my method might be useful as a tool for academic study, cell-based research, medical and pharmaceutical applications. Finally, in the future perspective, I introduced the possibility of controlling intracellular metabolic activity by electrochemical reduction of intracellular NAD^+ to NADH.

ACKNOWLEDGEMENT

At the outset, all praises and utmost thanks to Almighty Allah for giving me strength, patience, self-confidence, and ability to accomplish my research work in time despite my personal problems and dangers.

It's a matter of great pleasure of having the opportunity to express my deepest sense of gratitude, sincere appreciation, and heartfelt admiration to my reverend teacher and research supervisor, Professor **Dr. Hiroaki Shinohara**, Department of Life Science and Bioengineering, Faculty of Engineering, University of Toyama, for his constant guidance and motivation, excellent co-operation, continuous encouragement and inspiration accorded to me throughout the entire period of my work. I have realized that teaching and research is his passion, and he loves his students very much. I am very impressed with his honesty and polite behavior. His inspirations always motivated me how to collaborate the bio-electrochemistry and bio-sensing system with synthetic chemistry. I owe to him the most considering it a rare fortune to work under him.

I would like to express my extreme gratefulness to the honorable teacher, Assistant Professor **Dr. Minoru Suga,** Department of Life Science and Bioengineering, Faculty of Engineering, University of Toyama, for extending laboratory facilities for studying the anti-microbial activity of toxic drugs.

I would like to express my sincere thanks to graduate and M.Sc. students who helped me in my research work and daily life at Toyama University. I am grateful to Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj, for giving me the study leave for my doctoral study in Japan. At the same time, I am also grateful to the government of Japan for providing the MEXT Scholarship because, without this financial support, I would not be able to conduct my doctoral study.

I owe a great deal to my parents and brother, who were the constant source of inspiration during my study and research work.

I would like to express my special thanks to my beloved daughter (Mosammad Tafcira Alim Ameena) and wife (Mst. Tasmina Khatun) for their endless sacrifices in my absence during the period of my doctoral study. Their love and faith in me helped me to complete this study. I am also indebted to my respected teacher Dr. Mohammad Hannan Sheikh and Dr. Mohammad Tofazzal Hossain Tarafder, Department of Chemistry, Rajshahi University, Bangladesh, whose suggestions and inspiration always kept me active during this work.

There is no word to express my feelings except that this thesis is dedicated to my beloved parents.

ABBREVIATIONS

OD	Optical density
AC	Alternative current
EO	Electro-orientation
NBT	Nitroblue tetrazolium
MTT	2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
XTT	sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)- carbonyl]-2H-tetrazolium
MTS	5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfo- phenyl)-2H-tetrazolium inner salt
WST-1	sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H- tetrazolium inner salt
mPMS	1-methoxy-5-methylphenazinium methyl sulfate
FeCN	Ferricyanide $[Fe(CN)_6]^{3-}$
Mena	Menadione
PC12 cell	Rat pheochromocytoma cell
RBL-2H3 cell	Rat basophilic leukemia cell
C6 cell	Rat glioma cell
DMEM	Dulbecco's modified Eagle's medium

EMEM	Eagle minimal essential medium
PBS	Phosphate buffer saline powder
HS	horse serum
FBS	Fetal bovine serum
HBSS	Hanks' balanced salts solution
PI	Propidium iodide
SPCE	Screen-printed carbon electrode
EDTA	Ethylenediaminetetraacetic acid
$K_3[Fe(CN)_6]$	Potassium ferricyanide
DMSO	Dimethylsulfoxide
$[Fe(CN)_6]^{4-}$	Ferrocyanide
LDH	Lactate dehydrogenase
SDH	Succinate dehydrogenase
Db.m	Double mediators

CONTENTS

Chapter 1

Introduction

1.1 Background of this study

Cell viability assessment is very important in cell biology research, the medical diagnosis, pharmaceutical-drug screening, environmental safety monitoring, and food processing industry. There is a growing demand for a new cell viability assessment method that should be rapid, precise for the evaluating cytotoxicity and toxicological properties of a large number of chemicals/drugs to various kinds of cells. Cell viability refers to the percentage of metabolically active cells present in the whole population as compared to dead cells [1]. Monitoring of metabolic activity is an efficient matter to understand the real state of cell or cell viability.

In this chapter, I have highlighted the background and purpose of this study. Furthermore, I have provided some important biological terms related to this study.

There are two types of cell death-mechanisms associated with cell viability assessment; one is based on the death from cell membrane disruption (necrosis), and another is the death from metabolic disorders without cell membrane disruption (apoptosis). To identify the dead and alive cells on the basis of cell membrane disruption, different types of methods have been developed over the past decades. Traditionally, trypan blue dye exclusion method [2–5], resazurin base assay [6–8], and neutral red up take assay [9–11], and fluorometric method with double staining dyes [12–14] are used well for determining living and dead cell on the basis of cell membrane disruption. However, its analysis takes a time and cannot assess the metabolic activity to understand the cell's real health-state.

For yeast and bacterial cell viability assessment, various kinds of methods are available. For example, colony counting [15–17] and growth of inhibition zone methods [18–20] are also on the basis of cell membrane disruption observation. These methods take a long incubation time to get results and cannot assay the cell's real state. $(OD)_{600}$ method is only useful for yeast/bacterial cell proliferation or counting but not suitable for viability assessment [21–25].

Besides this, rapid and non-invasive electrical techniques have been developed using the polarization of cells in an AC electric field without a chemical reagent.

Recently, in our laboratory, Professor Minoru Suga has developed an electro-orientation method whether individual non-spherical cells were dead or alive from electro-orientation positions versus electric field direction depending on the intracellular conductivity [26]. However, this electrical technique is unable to assess an intracellular metabolism because this method based on the integrity of the cell membrane.

Moreover, the impedance spectroscopy method can monitor the growth of microbial cells due to the increase in capacitance [27,28]. This impedance spectroscopy based on the capacitance change is applicable to count the microbial cells in suspension and flow system. In this capacitance based measurement system a quick measurement is possible. But this system is expensive, and unable to evaluate the intracellular metabolic activity. Furthermore, the cellular impedance measurement technique depending on the change of biological cell-electrode surface resistance is applicable to the mammalian cells at adhesive condition. This method is useful for long-time continuous monitoring of cell proliferation assay and cytotoxicity test [29–33]. In this surface resistance based measurement system a rapid measurement is also possible. But this system expensive, and unable to evaluate the intracellular metabolic activity. Because it takes a time to monitor the change of cell attachment state to the electrode surface, this method is not suitable for the quick evaluation of intracellular metabolic activity and acute cytotoxicity test. Another problem in this method is that cells are adhered to the electrode even after they die. In that case, the surface resistance does not decrease accordingly and this result raises the dilemma to evaluate the cell viability.

Cellular respiration measurement by O_2 electrode is also an important method to evaluate the cell metabolism [34]. But when the toxic effect is far from the respiratory chain, which means at very low oxygen consumption change, the O_2 monitoring around cells may be not applicable to evaluate the cytotoxicity. Furthermore, to monitor the cell respiration by O_2 electrode, we need very precise and closed system.

On the other hand, about more than half of the century, the first generation of cell membrane permeable tetrazolium (positive charged) salts, NBT [35–38] and MTT [38–42] was used to measure the total intracellular NADH as cell viability ranging from mammalian to microbial origin. The net positive charge on MTT was reduced by intracellular NADH to produce insoluble formazan and accumulated inside of the cell. This formazan was then extracted by dissolving with an organic solvent such as DMSO or detergent solution and then measured by absorption spectroscopy to determine the intracellular NADH. This MTT dye is not suitable for cell viability testing because its crystalline formazan damages the cell membrane, and it takes a minimum 44 to 52 hours to complete this assay [43].

Recently, a new generation cell membrane-impermeable tetrazolium dye (net negative charged) such as XTT, MTS and WST-1 were developed and conjugated with cell membrane-permeable an intermediate redox mediator mPMS to monitor intracellular metabolic activity through the reduction of such terminal salts to soluble formazan [44–53]. The total amount of formazan produced by reducing intracellular NADH determines the cell viability as metabolic activity. That means the amount of formazan indirectly indicates the extent of intracellular metabolic activity. So, the monitoring of intracellular NADH is an excellent method to evaluate the cell viability or real health-state of cell. These colorimetric methods have some other limitations such as cytotoxicity, several steps to get results, scattering effect of cells suspended in medium, long incubation time (standard incubation time is 2 to 3 hours), and therefore cannot evaluate the toxic effect of fast-acting drugs or chemicals. Therefore, a more rapid measuring system is still requested.

More recently, bio-electrochemical methods receiving increasing attention in cell biology research and cell viability tests. From the literature review, some research groups reported the bio-electrochemical systems to monitor cell viability and intracellular enzyme activity [54–59]. They have been used menadione (Mena) as a lipophilic cell membrane permeable electron mediator and FeCN as a hydrophilic extracellular electron mediator in a double mediator system (Mena/FeCN). NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) enzyme is essential to transfer the electron from intracellular NADH to menadione. That means electron transfer from intracellular NADH to Mena is catalysed by cytosolic NAD(P)H

oxidoreductase [60]. The activity of the NQO1 enzyme depends on the cell cycle or cell stage. Therefore, monitoring of intracellular NADH with Mena/FeCN system depends on the cell cycle or cell state. With this background, the main objective of this study was to develop a universal electrochemical double mediator system to evaluate the real cell viability via monitoring of intracellular NADH as metabolic activity without cell membrane breaking. Intracellular NADH is one of the most important metabolites containing not only in mammalian cells but also in yeast and bacterial cells.

To achieve my goal, I have searched a suitable cell membrane-permeable mediator which can accept electrons from intracellular NADH enzymatically independent. Regarding this, mPMS is an efficient electron mediator. I have confirmed by my experiments that electron transfer from intracellular NADH to mPMS does not depend on the enzymes [61], cell cycle or cell state. Therefore, monitoring of intracellular NADH with the mPMS/FeCN system does not depend on the enzymes and cell cycle. So, mPMS/FeCN system is more universal or ubiquitous. I expect to measure the intracellular NADH in various kinds of cells at any time with this system. This is the specific advantage of my system, which has mentioned in detail in chapter 3.

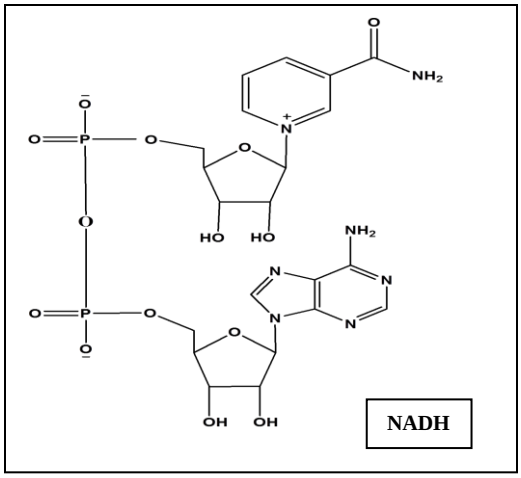
My mPMS/FeCN system has some more advantages, such as mPMS is highly soluble in water [62], photochemically stable [61]. On the other hand, Mena is insoluble in water. To make the menadione solution, it first needed to dissolve in organic solvent and then in water. Furthermore, menadione is more sensitive to light than mPMS. So, one's needs to be more precautions for the use and storing of it. Another important factor, since the redox potential of menadione is more negative than mPMS, I think that reduced form of menadione has high affinity than reduced form of mPMS to transfer the electron to O_2 (dissolve oxygen) for the production of hydrogen peroxide. Hydrogen peroxide is toxic for cells, whatever it is produced inside or outside of the cell. Because of the lower sensitivity of mPMS to O_2 (dissolve oxygen), mPMS is better than Mena to cycle the electron between intracellular NADH to extracellular region or electrode. Of course, to avoid this sensitivity, ferricyanide is used to accept the electron from them.

1.2 Purpose of this study

The purpose of my study was to develop a modified universal electrochemical double mediator system for cell viability assessment via monitoring of intracellular NADH as the metabolic activity without cell membrane breaking. Already, I have published this method in the journal of Electrochemistry [63].

1.3 Explanation of important biological terms

1.3.1 Nicotinamide adenine dinucleotide



Nicotinamide adenine dinucleotide (NAD^+/NADH) is a vital coenzyme worked in living cells. These metabolites are formed in glycolysis, pyruvate decarboxylation and the tricarboxylic acid cycle. They play crucial role to the basic reaction in all living cells that keeps the cells alive. Energy metabolism accompanied by conversion of NAD^+ to NADH in living cell provides attractive information for construction of electrochemical cell viability assay and cell-based biosensor. The cell viability assessment is now reflected as the extent of metabolic activity through the measurement of intracellular NADH without cell membrane disruption. Now a days scientists target the intracellular NADH for cancer treatment with blocking its production by the inhibition of important enzyme in cancer cells.

1.3.2 Tricarboxylic acid cycle in living cell

All living cells possess the tricarboxylic acid cycle where energy metabolism is occurred with producing NADH. The knowledge of TCA cycle metabolism **(Fig. 1.2)** is most important to understand the cell's real health condition at any adverse environment. In this thesis, I have evaluated the toxic effect of oxamic acid in PC12 cell, and itaconic acid and nystatin in yeast cell. The chemical structure of oxamic acid, itaconic acid, nystatin and, some bio-molecule related to this research is shown in **Figs 1.3 and 1.4 respectively.**

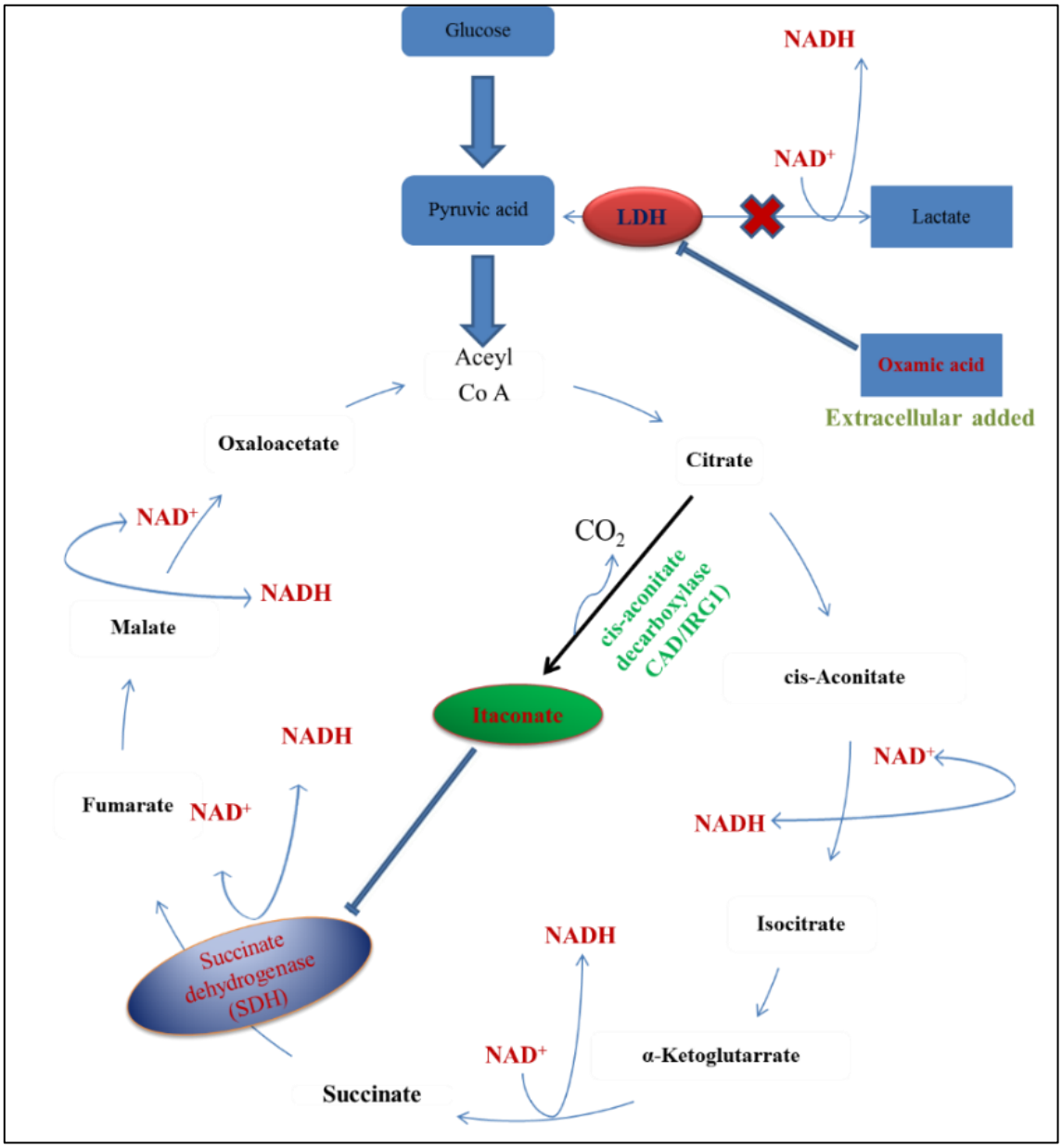
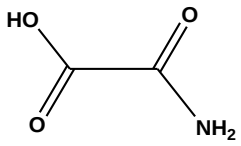


Figure 1.1: Tricarboxylic acid cycle

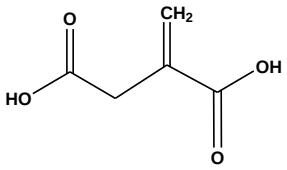
1.3.3 Toxic chemicals used for cytotoxicity test

The following chemicals have been used to evaluate the cytotoxicity in this research. Oxamic acid is a well-known inhibitor of LDH. Oxamic acid affects the transformation of pyruvate to lactate metabolism in cancer cells, and thus used for cancer treatment. On the other

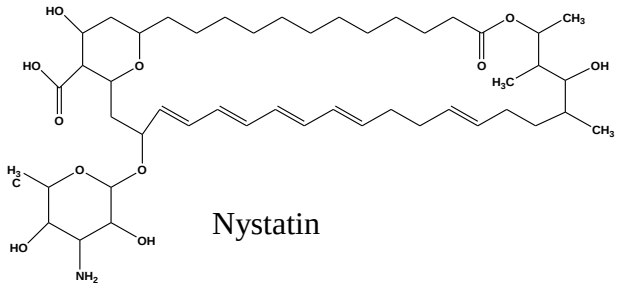
hand, itaconic acid is an anti-fungal agent. It inhibits the activity of SDH in TCA cycle in yeast cell, and thus decreases the cell viability.



Oxamic acid



Itaconic acid

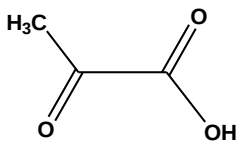


Nystatin

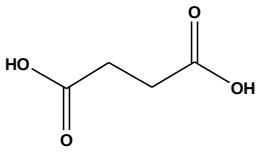
Figure 1.2: Chemical structure of toxic compounds; oxamic acid, itaconic acid and nystatin.

1.3.4 Chemical structures of important biomolecules

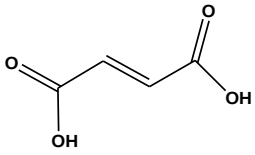
The following bio-molecules are very important related to intracellular metabolism. Isocitrate, succinate to fumarate, malate and NADH are generated stepwise from acetyl co enzyme A to continue the flow of TCA (Tricarboxylic acid) cycle in the cell.



Pyruvic acid



Succinic acid



Fumaric acid

Figure 2.3: Chemical structure of important biomolecules related to this research.

Chapter 2

Materials and methods

2.1 Chemicals and solutions

PC12 cell and C6 cell were obtained from the JCRB cell Bank, National Institute of Biomedical Innovation, Health and Nutrition (Osaka, Japan) and RBL-2H3 cell was obtained from the cell bank of RIKEN Bio Resource Center (Tsukuba, Japan). DMEM, EMEM, Phosphate buffer saline powder PBS, penicillin/streptomycin, and HS were obtained from Gibco (Tokyo, Japan). FBS was obtained from ICN Biomedicals (California, United States). HBS powder was obtained from Sigma–Aldrich (Tokyo, Japan). WST-1 salt obtained from Dojindo (Kumamoto, Japan). mPMS, itaconic acid, nystatin (Biochemical reagent grade), potassium chloride (99.5%), sodium bicarbonate was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). $K_3[Fe(CN)_6]$ (99%) obtained from KANTO Chemical Co.INC. Oxamic acid (97%) obtained from Tokyo Chemical Industry Co. Ltd. PI obtained from Universal Benevolence Chemical Laboratory. SYTO9 was obtained from Invitrogen (Thermo Fisher Scientific-JP). All chemicals were of analytical reagent grade and used without further purification and all solutions were prepared using ultrapure water. HBSS was also prepared by dissolving the HBS in pure water. SPCE were obtained from Shinshin Science Co.

In this thesis three types of mammalian cells, and yeast (*Schizosaccharomyces pombe*) has been used.

2.2 Cell handling procedure

All cells were carefully handled in clean-bench as shown in **Fig. 2.1** maintaining continuously a sterial laminar air flow to avoid the risk of contaminations.

Figure 2.1: Clean-bench maintaining continuously a sterial laminar air flow.

2.2.1 Mammalian cell culturing and passaging

All mammalian cells were cultured in 25 cm^2 polystyrene flask. The PC12 cells were grown in culture DMEM medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum and 1% penicillin–streptomycin. C6 cells were cultured in the same procedure as PC12 cells except horse serum. The RBL-2H3 cells were cultured in EMEM medium supplemented with 10% FBS, 1% penicillin–streptomycin. All cells were maintained at 37 ℃ in a humidified atmosphere and continuously supplying 5% CO_2 in incubator. All culture medium

was prepared in the sterial clean-bench. After preparation, the culture mediums were then filtered with 0.22 μm porus vacuum filter and stored in refrigerator. When bottom of the surface of culture flask became about 70 to 80% covered, cells were washed one time with 5 mL PBS, and detached with 1 mL trypsin-EDTA solution and tapping if needed. 4 mL fresh medium was then added to the flask to stop the action of trypsin-EDTA and transferred to 15 mL centrifuge tube to centrifuge at 500 G for three minutes. After centrifugation, supernatant containing trypsin-EDTA was removed with aspirator and cells were suspended in the fresh medium. From this cells suspension in medium, cells were then used to grow in next flask (passaging), for experimental purpose, and for cryopreservation. For experimental purpose cells were washed with HBSS, serial diluted to adjust the desired concentration and taken in the wells of plate (see in the experimental section).

2.2.2 Preparation of cell suspension

After reaching 70 % confluence, the harvested cells were collected by centrifugation at 500 G for three minutes. To avoid the unwanted electrochemical oxidation of interference compounds such as ascorbic acid (Vitamin C) contained in the culture medium, uric acid released from cells etc, cells were washed with HBSS and suspended in HBSS for electrochemical measurement. In the final step, cells were counted with a hemocytometer and exactly diluted with HBSS to give various density of cell suspension, which was next used for making the calibration curves and other experimental purpose. The flow sheet diagrams of whole experimental methods are shown in **Figs. 2.4 and 2.5**.

2.2.3 Cell cryopreservation

After 70 to 80% confluence, firstly cells were harvested by tripsin-EDTA and centrifuged at 500 G for 3 minutes. The supernatant was removed, keeping 300-60 μL cell pellet in medium. The cell mixture was then suspended in a medium containing 10% DMSO, a cryoprotective agent. Next, the cell mixture was inoculated into 1 mL cryovials and inserted into a Bio Freezing vessel (Nihon freezer Co. Ltd) filled with isopropanol. The freezing vessel was then placed into a - 80 ℃ freezer and allowed to cool for a minimum 24 hours. The freezing container and contents were then taken out from - 80 ℃ fridge and the cryovials were transferred

into a box labelled with cell line, passage number and date. The box was then placed inside a liquid nitrogen storage Dewar, to avoid liquid nitrogen from seeping into the boxes and cryovials. This process reduces the risk of cryovial eruption during the thawing process.

2.2.4 Cell thawing

At first, minimum 15 minutes prior to thawing the cells, a 25 cm^2 T-flask containing 4 mL of medium was placed in an incubator at 37℃ maintaining 5% CO_2. A cryovial was taken out from the liquid nitrogen storage and warmed in a 37℃ water bath until thawed. The cryovial was then thoroughly sprayed with 75% ethanol and wiped down before placing in the laminar safety hood. The whole content (1 mL) was then taken out carefully from the cryovials and taken into a 15 mL centrifuge tube containing 4 mL medium. The centrifuge tube ultimately contained total 5 mL was then centrifuged 2 times for 3 minutes at 500 G following the same technique for removal of DMSO completely. The supernatant was discarded and 1 mL medium was added to resuspend the cell pellet. The centrifuge tube containing cells in medium were then carefully taken into the pre-warmed T-flask. The T-flask was then incubated for 24 hours at 37℃ under 5% CO_2 to allow the cells to adjust, after which time the passage was carried out.

2.2.5 Yeast cell culture and preparation of cell suspension

Firstly, very small amount (just like a single colony) of the yeast cells (strain number: ATCC38399) were pick up with a sharp head of small stick and taken in 1.5 mL micro-centrifuge tube containing 500 µL yeast extract. This tube was them incubated for 24 hours at 30℃. After incubation, from this cell suspension, about 20 µL of was then taken in a L-tube containing 10 mL fresh yeast extract and incubated for 17 hours shaking with 80 rpm at 30℃ to get $(OD)_{600} = 0.8 = 1\times10^7$ cells/mL. After reaching $(OD)_{600} = 0.8$, cells were washed with HBSS (by centrifugation at 1600×*g* for 5 minutes to eliminate the unwanted electrochemical oxidation from the interfering molecules such as ascorbic acid (Vitamin C) contained in the culture medium, uric acid released from cells etc). Cells were then re-suspended in 1 mL HBSS, which ultimately gave the final concentration 1×10^8 cells/mL. I used this (1×10^8 cells/mL) concentration as the initial concentration and serial dilution was performed to give various concentrations to make the calibration curve and other experimental purpose.

Yeast culture medium prepared by following; for 200 mL yeast medium, 2 g yeast extract power was supplemented with 6 g glucose, and autoclaved at 120℃ for 15 minutes. After that, 4 mL (360 mg/50 mL) leucine was added in the previously autoclaved mixture of yeast extract and glucose in the clean bench with continuous laminar air flow at 30℃.

2.3 Conventional WST-1 assay

WSTs assay is based on the measurement of mitochondrial dehydrogenase activity have become the most widely useful to monitor metabolic activity. This method used in cell biology or biochemistry to monitor metabolic activity of cells ranging from mammalian to microbial origin [41,60]. In this assay, oxidised mPMS goes inside of the cell, became reduced and goes outside to transfer the electrons to extracellular hydrophilic mediator WST-1. Hydrophilic mediator accepts electrons and converted into soluble formazan. This amount of formazan is then measured after 2 to 3 hours incubation at 450 nm by microplate reader. The principle of the conventional WST-1 assay is shown in Fig.2.6.

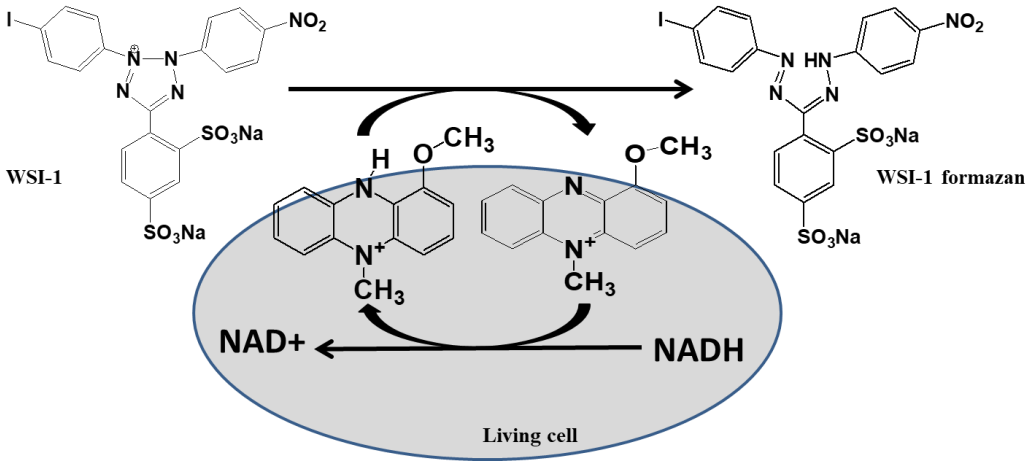


Figure 2.2: Principle of the conventional WST-1 assay.

2.4 Electrochemical technique for cell viability assessment on mammalian cells

To count the mammalian cells various densities of cell suspensions with HBSS was plated in different wells of 96 well plate and FeCN (final conc.500 µM) and mPMS (final conc.10 µM) were added to each well. The total volume of experimental cell suspension was 150 µL. After incubation for 10 minutes, a SPCE **(Fig. 2.3)** connected to computer controlled Potentiostate/Galvanometer (Metrohm Autolab B.V., Multi Autolab cabinet) was immersed into the cell's suspension and chronoamperometric measurement was immediately performed by potential application at + 0.5 V vs Ag/AgCl reference electrode on the SPCE. The used SPCE has a carbon paste working electrode (0.055cm^2), a carbon paste counter electrode and an Ag/AgCl reference electrode. Used SPCE is a custom-made product from ALPS ELECTRIC CO., LTD (Product name is ALADO APE-02)

For the evaluation of toxic effect of oxamic acid, PC12 cells suspension (in HBSS) was taken (3× 10^5 cells/ well) in each well of a 96 well plate and 10 µL oxamic acid/HBSS was added (final conc. 0 to 20 mM). After 1 hour incubation, 15 µL FeCN (final conc. 500 µM) and 15 µL mPMS (final conc. 10 µM) solutions were added into each well. In every case, the total volume of experimental suspension was maintained to 150 µL with HBSS. Additionally, after 10 minutes incubation at 37 ℃ under 5% CO_2, chronoamperometric measurement was performed to evaluate the cytotoxicity of the oxamic acid. The whole experimental procedure is shown **in Fig. 2.4.**

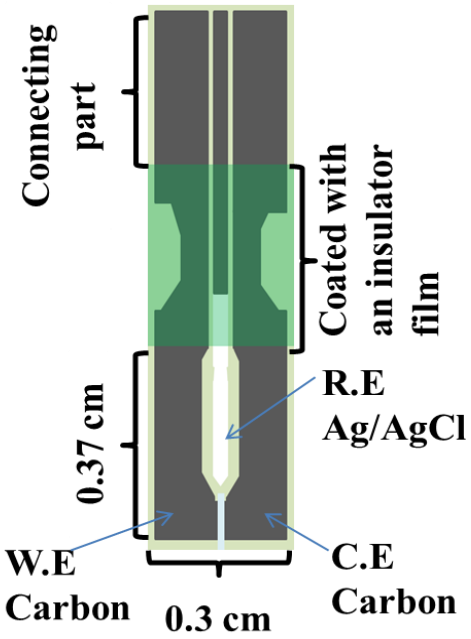


Figure 2.3: Illustration of a disposable screen-printed-carbon electrode.

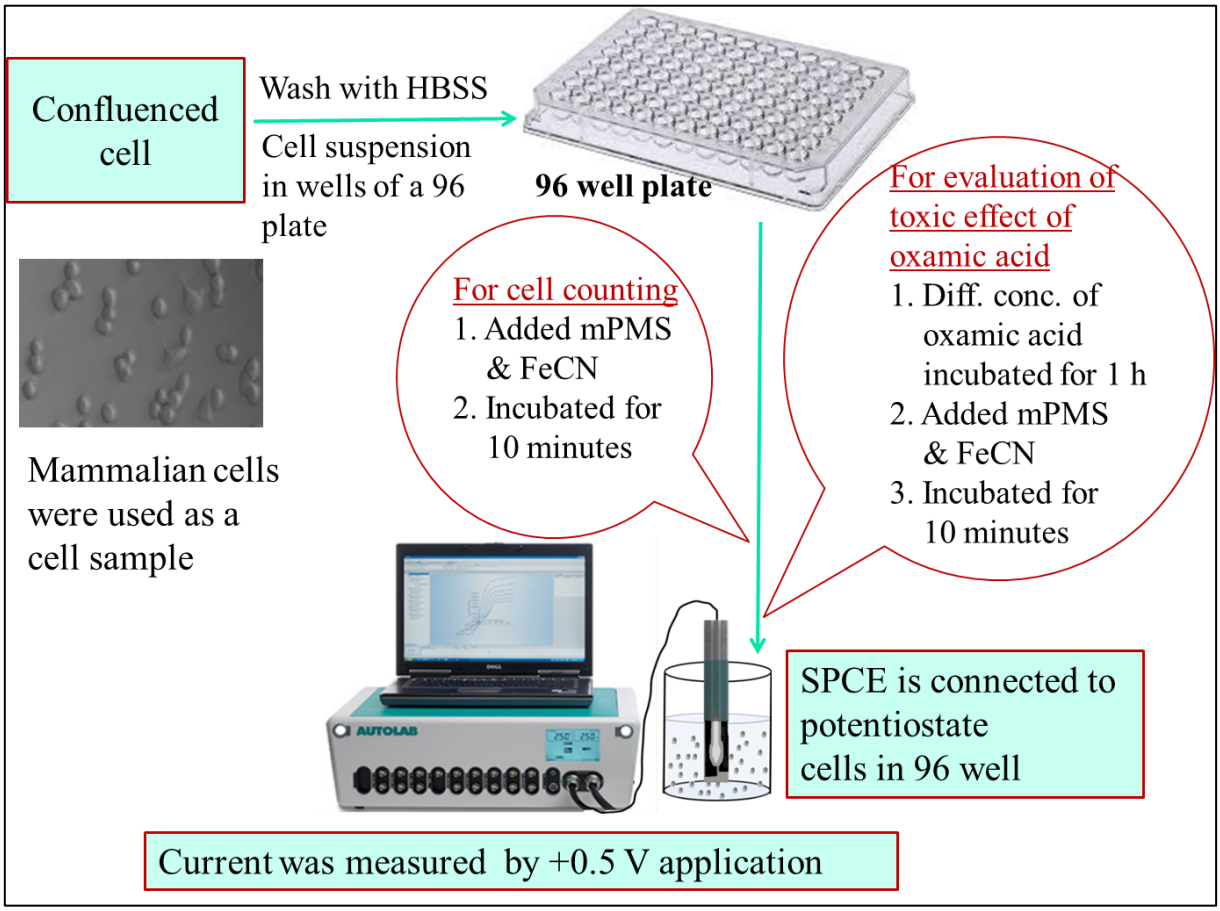


Figure 2.4: Flow sheet diagram of experimental procedure for mammalian cell counting and evaluation of toxic effect of oxamic acid on PC12 cell.

2.5 Electrochemical technique for cell viability assessment on yeast cells

Various densities of yeast cell suspensions were taken in different wells of 96 well plate and FeCN (final conc.500 µM) and mPMS (final conc.10 µM) were added to each well. In every case, the total volume of experimental cell suspension was 150 µL. After incubation for 10 minutes, a SPCE was immersed into the yeast cell suspension and chronoamperometric measurement was immediately performed by potential application at + 0.5 V vs Ag/AgCl reference electrode on the SPCE.

For the evaluation of cytotoxicity of itaconic acid, yeast cell suspension/HBSS (1×10^6 cells/well) was taken in each well of a 96 well plate, and 22.5 µL itaconic acid/HBSS was added (final conc; 0 to 25 mM). In case of nystatin, 10 µL nystatin was added (final conc. 0.01 to 0.16

μg/mL) into each well. After 1 hour incubation for itaconic acid and 45 minutes for nystatin, 15 μL FeCN (final conc. 500 μM) and 15 μL mPMS (final conc. 10 μM) solutions were added into each well. In every case, the total volume of experimental suspension was maintained to 150 μL with HBSS. Additionally, after 10 minutes of incubation at 37 ℃ under 5% CO_2, chronoamperometric measurement was performed to evaluate the cytotoxicity of the itaconic acid. The whole experimental procedure is shown **in Fig. 2.5.**

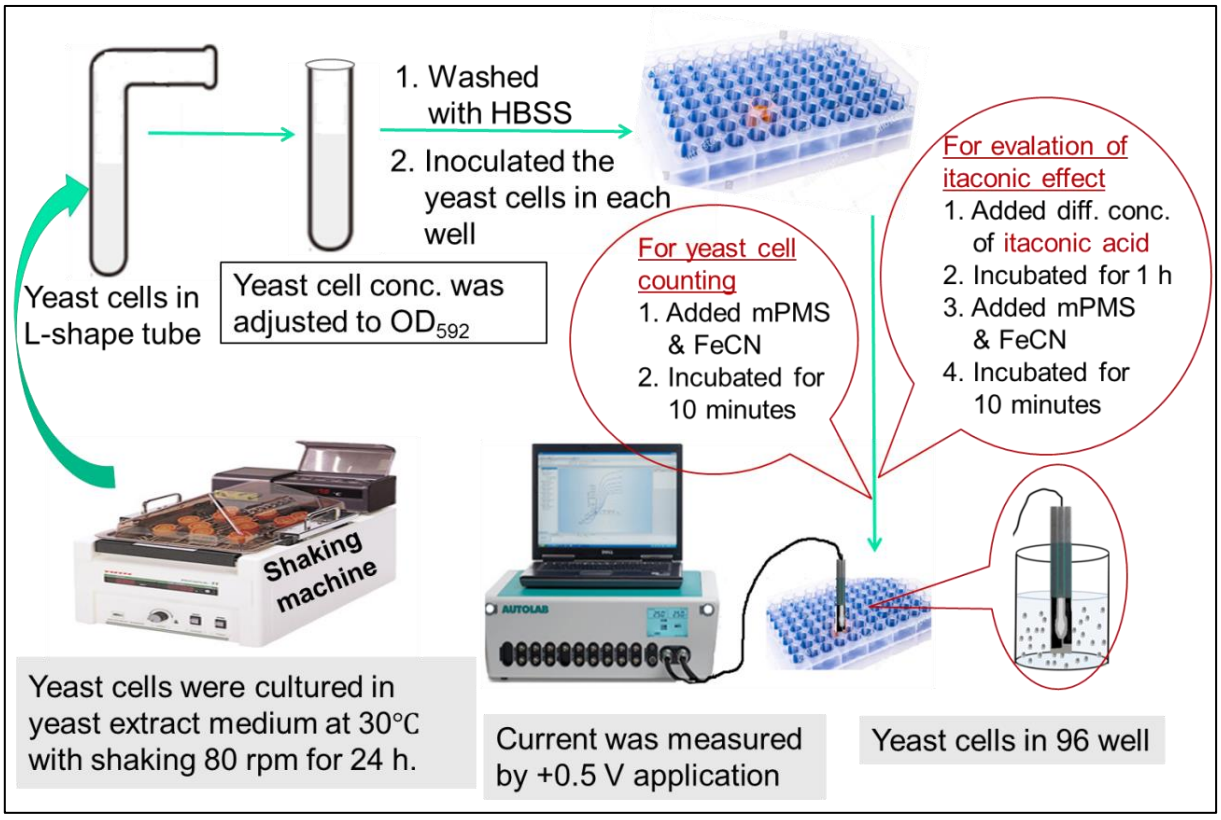


Figure 2.5: Flow sheet diagram of whole experimental procedure for yeast cell counting and evaluation of toxic effect of itaconic acid and/or nystatin on yeast cell.

Chapter 3

Mammalian cell viability assessment via monitoring of intracellular NADH with mPMS/FeCN double mediator system

3.1 Introduction

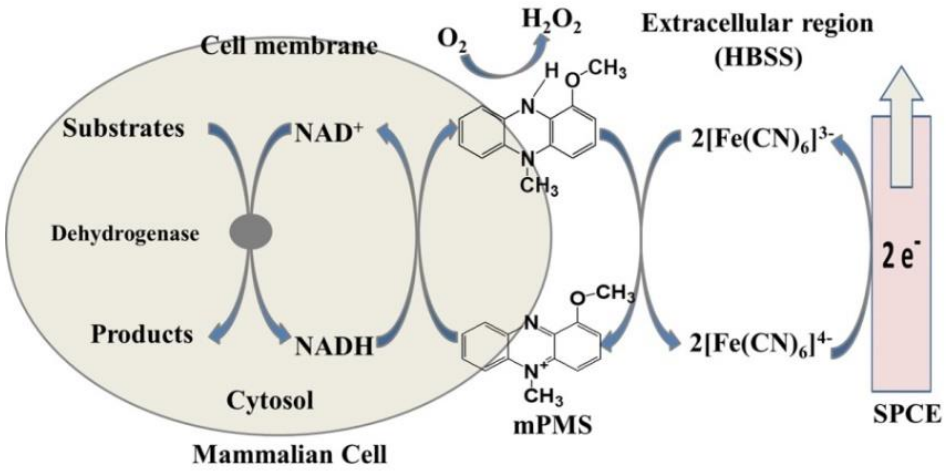
Intracellular NADH is an active electron donor considered to play a crucial role in the mechanism of glycolytic pathway, TCA cycle and respiratory electron transport chain for ATP production making it potential bio-marker to assess the cell viability. Energy metabolism accompanied by conversion of NAD^+ to NADH or NADH to NAD^+ in living cell provides attractive information for the detection of cell viability. Cell viability assessment is now reflected as the extent of metabolic activity through the measurement of intracellular NADH.

Over the past decades, different types of methods are prevalent for dead and alive assay. Traditionally, trypan blue dye exclusion method [2–5], resazurin base assay [7], and neutral red up take assay [11] and fluorometric method with double staining dyes [12] are used for cell viability determination. These methods are based on the principle where the cell membrane is destroyed or not. The simplicity of these methods distinguishes only the living and dead cells in the entire population, cannot to measure the metabolic activity to understand the real state of cell. Previously, the first generation of cell membrane permeable MTT was used to evaluate the cell viability in which MTT was reduced by intracellular NADH to produced insoluble formazan. This formazan was then extracted and measured by absorption spectroscopy to determine the intracellular NADH [42,64] This MTT dye is not suitable for cell viability testing because its crystalline formazan damages the cell membrane, and it takes minimum 44 to 52 hours to complete this assay.

Recently, a new generation cell membrane impermeable tetrazolium dye such as WSTs were developed and conjugated with cell membrane-permeable an intermediate redox mediator mPMS to monitor intracellular NADH through the reduction of such terminal salts to soluble formazan [45,46] These colorimetric assays are easy and useful to count the cell and

cytotoxicity test. But its standard incubation time is two hours which is time consuming [43]. Therefore; a more rapid measuring system is still requested.

Furthermore, some research groups have previously reported the electrochemical systems to monitor cell viability and intracellular enzyme activity [54–59,65] They have been used menadione (Mena) as a lipophilic cell membrane permeable electron mediator and FeCN as a hydrophilic extracellular electron mediator in double mediator system (Mena/FeCN). In this system, electron transfer from intracellular NADH to Mena is catalysed by cytosolic NAD(P)H oxidoreductase [60]. On the other hand, it has also been reported that the electron transfer from intracellular NADH to mPMS occurred by non-enzymatically [60,61] So, I expected that the combination of mPMS and FeCN system might be independent on the cytosolic enzyme. That means, this system might be works as a universal to monitor intracellular NADH of various mammalian cells. The illustration of the measurement principle of mPMS/FeCN system is shown in Scheme 1.



Scheme 1: Illustration of the speculated principle for monitoring of the intracellular NADH with the double mediator (mPMS/FeCN) system. Electron transfer from intracellular NADH to mPMS occurred by non-enzymatically.

Furthermore, mPMS has a few advantages as compare to Mena such as water solubility, lower redox potential and stability under the light.

So, herein I have investigated the usefulness of this new combination of double mediators to monitor intracellular NADH and employed to count various kinds of mammalian cells. Furthermore, I have applied this double mediator system to evaluate the acute cytotoxicity of oxamic acid on cytoma cells (PC12 cell).

3.2 Analytical procedure

3.2.1 Preparation of PC12 cell lysate for WST-1 reduction

After cultivation, PC12 cell were washed with PBS and resuspended with 120 µL PBS (5.5×10^6 cells/mL). Cells were fridged in the refrigerater at -28 ℃ for 30 minutes and just melted in water bath at 37 ℃. This fridging and melting process were done more than three times. Cell lysate was then pipeted, and after the centrifugation, lysate supernatant was collected for the use of WST-1 reduction experiment.

To obseve the WST-1 reduction capability, 10 µL lysate supernatant was used with testing compounds and maintaining the final concentrations of 70 µM (µmol L^{-1}) WST-1, 5 mM (mmol L^{-1}) NADH and either 100 µM Mena or 8.75 µM mPMS in HBSS by modified method of ref 20. In every cases, the total volume of 200 µL was adjusted by adding HBSS in 96 well. Furthermore, to confirm the NADH-dependent reducing activity for mediators of cytosolic NAD(P)H oxidoreductase in the lysate supernatant, lysate supernatant was heated with 90℃ for 7 minutes to induce the enzyme activity loss.

3.2.2 Cell counting by WST-1 cell counting kit (CCkit-1)

Firstly, 5.5 mM WST-1 salt and 2 mM mPMS solution were separately prepared using HBSS as a solvent. Then WST-1 was mixed with mPMS at 9:1(v/v) ratio which contained 5 mM WST-1 and 0.2 mM mPMS [66] to make cell counting kit (CCKit-1) solution.

For cell counting, 100 µL of various density of cell suspension in HBSS (by serial dilution) was taken into each well of a 96 well plate and CCKit-1 was then added into each well. After incubation at 37℃ under 5% CO_2 for 60 minutes, the formation of corresponding formazan was measured by absorption at 450 nm with a micro plate reader (Promega GloMax Multi$^+$ detection system).

3.2.3 Evaluation of cytotoxicity of mPMS and $K_3[Fe(CN)_6]$ by PI staining method

100 µL of cell suspension (3×10^5 cells/ well) was plated to each well of a 96-well plate. 15 µL of different concentration of mPMS was added to all wells. Cells without mPMS exposure (only DMEM or HBSS) were used as a control. After 60 minutes incubation at 37℃ under 5% CO_2, 20 µL PI (final conc.1.5 µM) was added and again incubated for 15 minutes. In every case, the total volume of experimental cell suspension was maintained to 150 µL with DMEM or HBSS. Finally, 10 µL cell suspensions were applied onto the hemocytometer. Taken a photograph of cells under the fluorescence microscopic observation and PI-stained cells (dead cells) were counted for determining the cell viability. The dead cell ratio was calculated as the ratio of number of PI-stained cells against number of total cells.

3.2.4 Electrochemical monitoring of intracellular NADH with single or double mediator systems

All electrochemical measurements were performed at room temperature using a computer controlled Potentiostate/Galvanometer (Metrohm Autolab B.V., Multi Autolab cabinet) to which a SPCE was connected. The used SPCE has a carbon paste working electrode (1×1 mm^2), a carbon paste counter electrode and an Ag/AgCl reference electrode.

For single mediator system, 100 µL cell suspension (3×10^5 cells/well) with HBSS was taken in wells of 96 well plate and added 10 µM mPMS (final conc.). In every case, the total volume of experimental cells suspension was maintained to 150 µL. After incubation for 10 minutes, a SPCE was vertically immersed into the cell suspension and chronoamperometry was immediately performed by the potential application at + 0.1 or + 0.5 V to oxidise reduced form of mPMS.

On the other hand, in case of double mediator system, 100 µL cell suspension (3×10^5 cells/well) with HBSS was taken in wells of a 96 well plate and 15 µL FeCN (final conc. 500 µM) and 15 µL mPMS (final conc. 10 µM) solutions were added in a drop wise to the cell suspension. In every case, the total volume of experimental cell suspension was maintained to 150 µL. After 10 minutes incubation, a SPCE was vertically immersed into the cell suspension and + 0.5 V was applied to sufficiently oxidise the accumulated ferrocyanide. Before starting

every electrochemical measurement, the potential at + 0.5 V was applied for double mediator system (+ 0.1 or + 0.5 V for single mediator) to the carbon working electrode in a cell suspension without mediators for 10 seconds to keep the electrode surface state the same.

3.2.5 Electrochemical cell counting

Various densities of cell suspensions were plated in different wells, and FeCN (final conc. 500 µM) and mPMS (final conc. 10 µM) solutions were added to each well. The total volume of experimental cell suspension was 150 µL. After incubation for 10 minutes, a SPCE was immersed into the cell suspension and chronoamperometric measurement was immediately performed by potential application at + 0.5 V vs Ag/AgCl reference electrode on the SPCE.

3.2.6 Evaluation of cytotoxicity of oxamic acid on PC12 cells by electrochemical method

In this study, PC12 cell suspension (in HBSS) was taken (3×10^5 cells/ well) in each well of a 96 well plate and 10 µL oxamic acid/HBSS was added (final concentration of oxamic acid; 0 to 20 mM). After 1 hour incubation, 15 µL FeCN (final conc. 500 µM) and 15 µL mPMS (final conc. 10 µM) solutions were added into each well. In every case, the total volume of experimental suspension was maintained to 150 µL with HBSS. Additionally, after 10 minutes incubation at 37℃ under 5% CO_2, chronoamperometric measurement was performed to evaluate the cytotoxicity of the oxamic acid.

3.3. Optimization of measurement conditions of mPMS/FeCN double mediator system

3.3.1 Investigation of enzyme-dependency of the reduction of WST-1 by NADH through the mediatation of Mena or mPMS

To check the enzyme-dependency for electron transfer from intracellular NADH to Mena or mPMS, I have used the PC12 cell lysed as an enzyme source. Whole experimental procedure described in experimental section.

Here, produced formazan from WST-1 was measured by absorbance at 450 nm with various conditions described in the figures (LS = Lysate supernatant =10 µL, WST-1 = 70 µM, NADH = 5 mM Mena = 100 µM). In every cases, the total volume of 200 µL was adjusted by

adding HBSS in 96 well. Measurment was done from 3 to 75 minutes after mixing of the compounds. **Fig. 3.1** shows that absorbence of WST-1 formazan was gradually increased inpresence of lysed supernutant with the increased of incubation time, and after 50 minutes became saturation. In case of other conditions, absorbance was not observed.

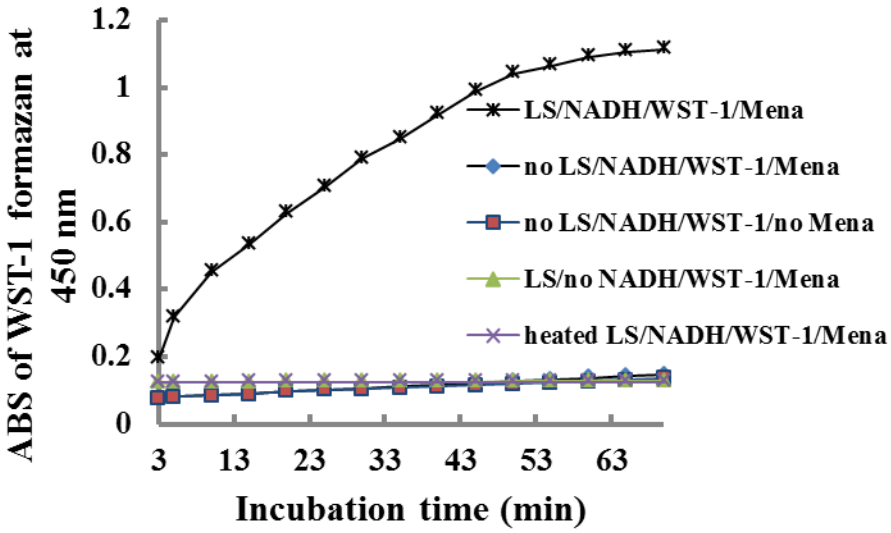


Figure 3.1: Measurment of incubation time dependent absorbance of WST-1 formazan at 450 nm in the presence of lysed supernutant with the mediation of Mena.

Fig. 3.2(A and B) shows a comparison of cytosolic enzyme-dependency for the reduction of 70 µM WST-1 by 5 mM NADH through the mediatation of 100 µM Mena or 10 µM mPMS. In every cases, the total volume of 200 µL was adjusted by adding HBSS in 96 well. In case of Mena in **Fig.3.2A**, no absorbance increase was observed by addition of LS heated for 30 minutes.

The results from **Fig.3.2 (A and B)** demonstrated that cytosolic enzyme was essential for WST-1 reduction by NADH through the mediation of Mena. On the other hand, when mPMS was used as the electron mediator, enough formazan was produced quickly within 3 minutes and kept constantly in both of the absence and the presence of LS as shown in **Fig. 3.2B.** Same amount of formazan was produced in the presence of heated LS. This data demonstrated that WST-1 reduction occurred by non-enzymatically through the mediation of mPMS. From these results, it was confirmed that the electron transfer from NADH to Mena was dependent on

cytosolic enzyme and the reduction rate of Mena was slow incontrast to mPMS which takes electron from NADH enzyme-independenly and very fastly.

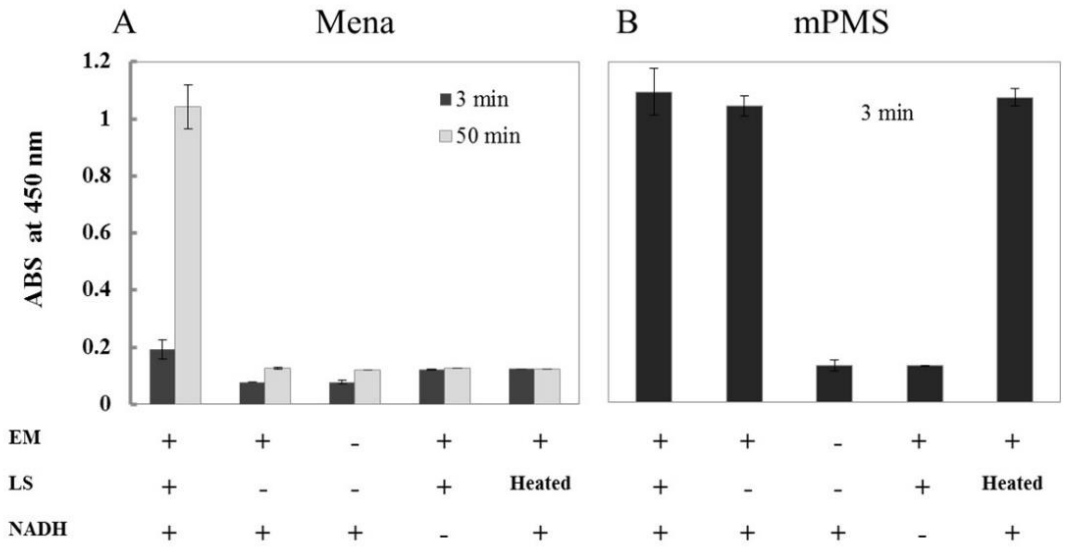


Figure 3.2: Cytosolic enzyme-dependency of the reduction of WST-1 by NADH through the mediatation of Mena or mPMS. Produced formazan from WST-1 was measured by absorbance at 450 nm with various conditions described under the bottom of the figures (EM = Electron mediator, e.i., Mena and mPMS. LS = Lysate supernatant). Measurment was done during 75 minutes after mixing of the compounds. The results are expressed as the mean ± SD from three experiments. (A) Mena was used as the electron mediator. Produced formazan increased as time passed after addition of PC12 cell lysate (LS), however no absorbance increase was observed by addition of LS heated for 30 minutes. This data demonstrated that cytosolic enzyme was essential for WST-1 reduction by NADH through the mediation of Mena. (B) mPMS was used as the electron mediator. Enough formazan was produced quickly within 3 minutes and kept constantly in both of the absence and the presence of LS. Same amount of formazan was produced in the presence of heated LS.

Next, I have also examined the enzyme-concentration dependency for the reduction of NADH by Mena. **Fig. 3.3** shows that absorption of produced formazan decreases with decreasing enzyme-concentration in cell lysed. So, the reduction of NADH by Mena was not only dependented on the enzyme but also its concentration also.

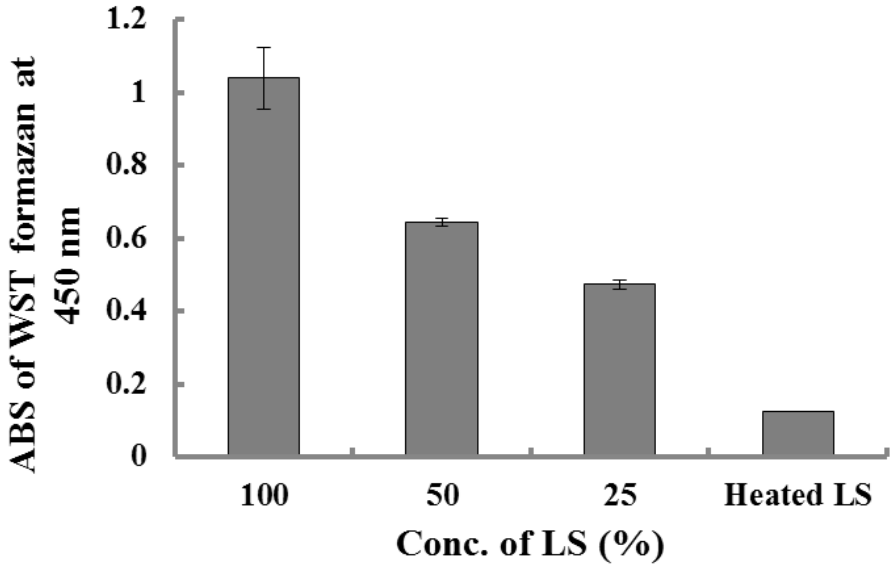


Figure 3.3: Enzyme concentration dependent absorbance of WST-1 formazan at 450 nm. NADH was incubated for 50 minutes with WST-1, Mena, different concentration of LS. The results are expressed as the mean ± SD from three experiments.

3.3.2 Electrochemical monitoring of the intracellular NADH with a single mediator and double mediators

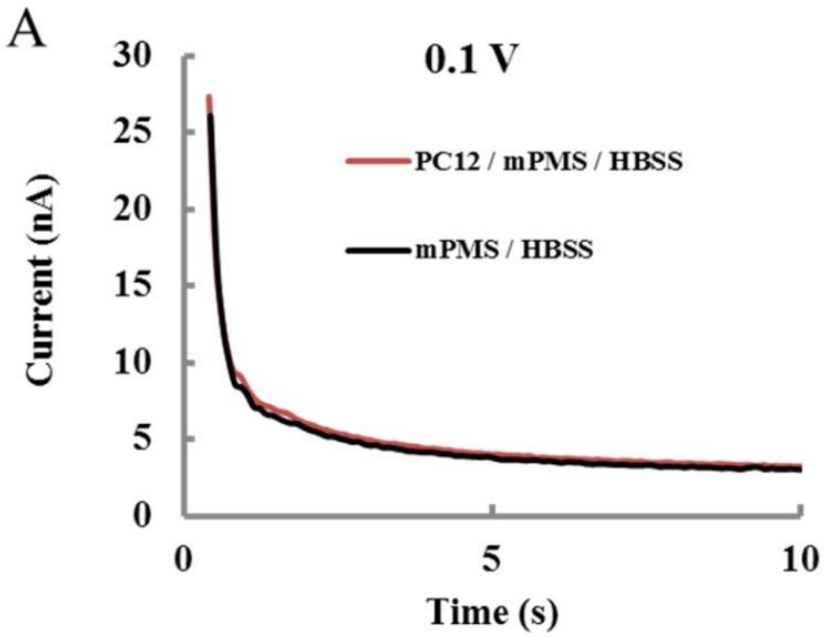
mPMS crosses the cell membrane, takes electron from intracellular NADH non-enzymatically and the reduced form of mPMS goes outside of the cell [60]. In this study, I have confirmed by my results that mPMS takes electron from NADH non-enzymatically as shown in **Fig. 3.2.** So, firstly I have tried to use mPMS as a single mediator. After 10 minutes incubation of PC12 cells (3×10^5 cells/well) with 10 µM mPMS, + 0.1 V was applied to oxidise the reduced form of accumulated mPMS. Since redox potential of mPMS was -0.16 V, + 0.1 V might be enough to oxidise reduce form of mPMS. But the measured oxidation current did not increase as compared to the control (10 µM mPMS, in the absence of cells) as shown **in Fig.3.4A.** I considered that dissolved O_2 easily took electron from the reduced mPMS to produce H_2O_2 and this was the reason why the oxidation current did not increase even though cells existed.

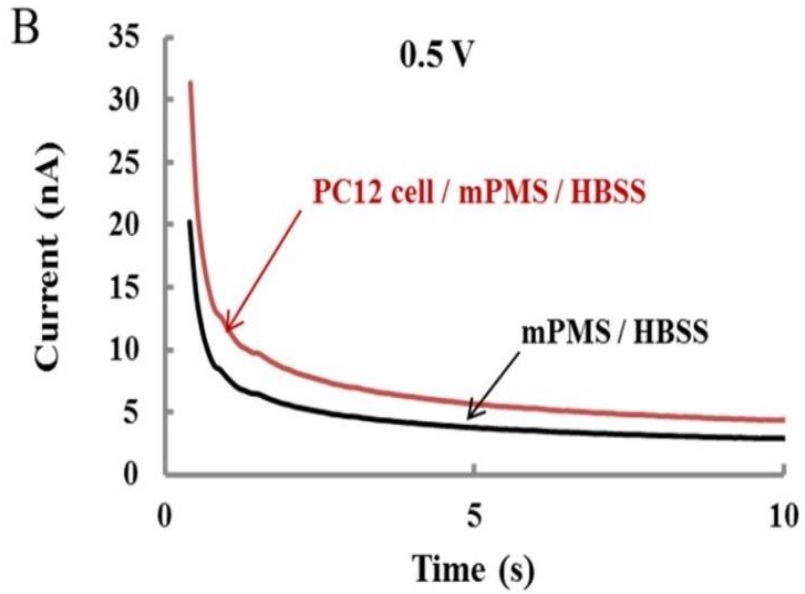
Next, I tried to apply +0.5 V to check H_2O_2 production in this single mediator system. In this case, the oxidation current increased a little as compared to control as shown in **Fig.3.4B.** So, these data suggested that dissolve oxygen interfered effective sensing of intracellular NADH in the single mediator system.

As a way to circumvent this problem, I considered the addition of second mediator FeCN which was useful as same as Mena/FeCN double mediator system [54–57] Enough concentration of FeCN takes electron non-enzymatically from reduced form of mPMS before accepting the electron by dissolved oxygen and as a result, $[Fe(CN)_6]^{4-}$ was accumulated outside of cells as time goes on. I therefore measured the accumulated $[Fe(CN)_6]^{4-}$ by cyclic voltammetry in my double mediator system in the presence of cells and observed the oxidation current increase as compared to control as shown in **Fig. 3.5** which supported that the **Scheme 1** might be suitable to detect the cell viability.

I considered to use chronoamperometry for more effective measurement of the accumulated $[Fe(CN)_6]^{4-}$. From the voltammogram, the redox potential of FeCN was +0.12 V vs Ag/AgCl on SPCE in HBSS. So, I considered more than +0.32 V was necessary to oxidise the accumulated $[Fe(CN)_6]^{4-}$. On the other hand, HBSS does not include electrochemical active interfering compounds except O_2. Therefore, I chose +0.5 V to sufficiently oxidise the accumulated $[Fe(CN)_6]^{4-}$ in HBSS by chronoamperometry. Actually, as shown in **Fig. 3.4C,** the significant increase of oxidation current at +0.5 V was observed in this double mediator system in the presence of cells.

This measured oxidation current only from accumulated $[Fe(CN)_6]^{4-}$indirectly reflects the intracellular NADH concentration and therefore cell viability. After all, the double mediator system was better than the single mediator system and hence using the double mediator system, following all electrochemical measurements were done by chronoamperometric method to monitor the intracellular NADH.


A
0.1 V
PC12 / mPMS / HBSS
mPMS / HBSS
Current (nA)
Time (s)
0
5
10
15
20
25
30


B
0.5 V
PC12 cell / mPMS / HBSS
mPMS / HBSS
Current (nA)
Time (s)
0
5
10
15
20
25
30
35

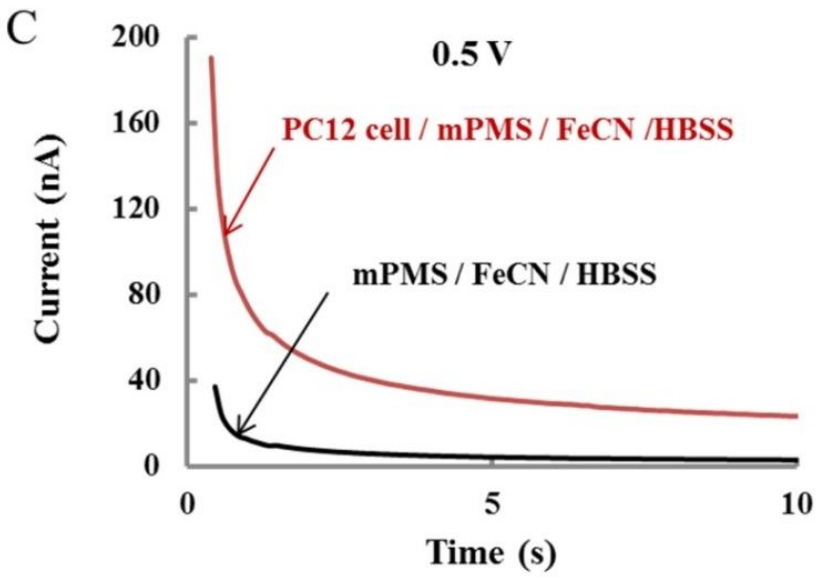


Figure 3.4: Chronoamperograms for monitoring the intracellular NADH in PC12 cells. (A) and (B) Single mediator system; PC12 cells (3×10^5 cells/ well) were incubated for 10 minutes in 10 µM mPMS/HBSS and + 0.1 or + 0.5 V was applied respectively to oxidise reduced form of mPMS. (C) Double mediator system; PC12 cells (3×10^5 cells/ well) were incubated for 10 minutes in 10 µM mPMS and 500 µM FeCN/HBSS and +0.5 V was applied to oxidise accumulated $[Fe(CN)_6]^{3-}$. In both A, B and C, red line is the oxidation current in the presence of cells and black line is the oxidation current in the absence of cells.

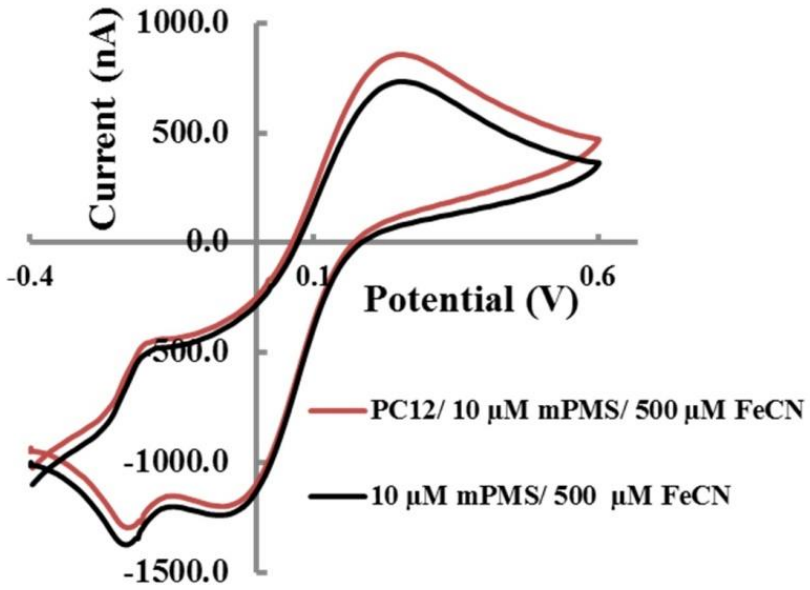


Figure 3.5: Cyclic voltammograms of double mediators in the presence and absence of PC12 cells. The PC12 cells (3×10^5 cells/ well) were incubated with 10 µM mPMS and 500 µM FeCN for 10 minutes under 5% CO_2 at 37℃. After incubation, accumulated ferrocyanide was measured by cyclic voltammetry. Cells with 10 µM mPMS and 500 µM FeCN, the oxidation current was significantly increased as compared to control (without cells). The voltammograms supported that Scheme1 might be suitable to detect the cell viability.

3.3.3. Cytotoxicity assay of mPMS and $K_3[Fe(CN)_6]$

In order to establish the new double mediator system, I examined the cytotoxicity of mediators on PC12 cells by using propidium iodide (PI). The result demonstrated that more than 50 µM mPMS was toxic for cells **(Fig. 3.6A**). I also checked that up to 1000 µM FeCN was not toxic for cells (data is not shown). Besides this, the cytotoxicity of mixed solution of 500 µM FeCN with 10 or 20 µM mPMS was not toxic for PC12 cell **(Fig. 3.6B).**

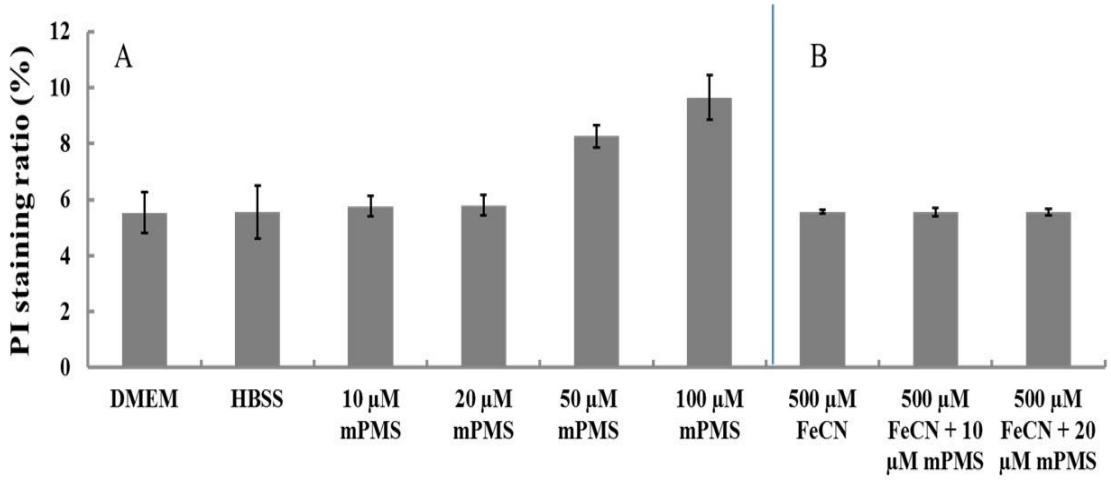
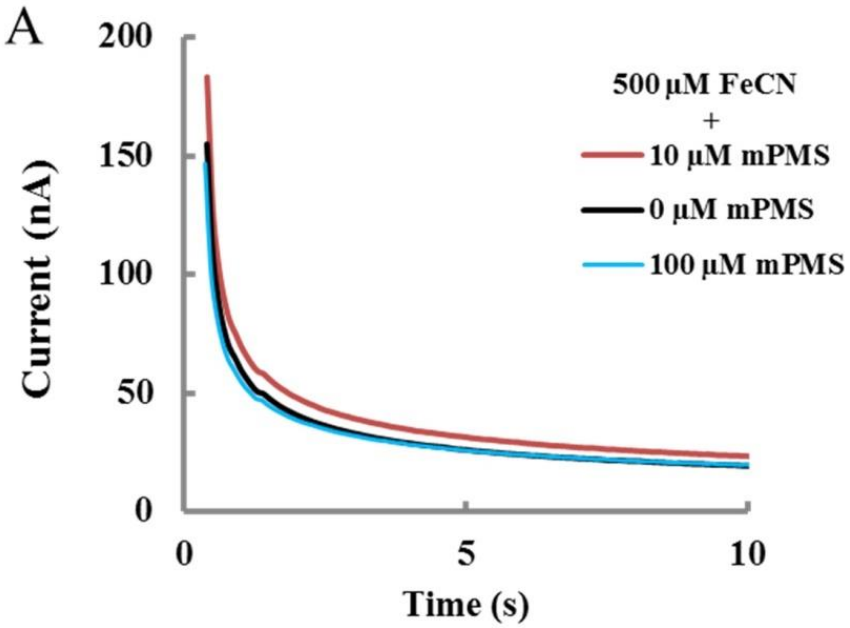


Figure 3.6: Determination of cytotoxicity of mPMS (A) and the mixture of mPMS and $K_3[Fe(CN)_6]$ (B) on PC12 cells. Cells were incubated with mPMS or the mixture of mPMS and 500 µM $K_3[Fe(CN)_6]$ for 60 minutes at 37℃ under 5% CO_2. 20 µL PI (final conc.1.5 µM) was added and again incubated for 15 minutes. Every case, total volume of experimental cell suspension was maintained to 150 µL with DMEM or HBSS. It was observed that more than 50 µM mPMS was toxic on PC12 cells. On the other hand, 10 or 20 µM mPMS coexisting 500 µM $K_3[Fe(CN)_6]$ (or only 500 µM $K_3[Fe(CN)_6]$) was not toxic on PC12 cells. The results were expressed as the mean ± SD from four experiments.

3.3.4. Optimization of mediator's concentration

I optimized the concentration of mPMS to obtain the maximum current based on the effective oxidation of intracellular NADH. The PC12 cells (3×10^5 cells/ well) were incubated in 500 µM (final conc.) FeCN/HBSS solution including various concentrations (final conc. was 0, 10, 20, 50, 100 µM) of mPMS. After 10 minutes of incubation, the oxidation current was measured by the application of + 0.5 V vs Ag/AgCl. All cases, the read point of oxidation current was 5 second. It was observed that in case of 10 and 20 µM mPMS combined with 500 µM FeCN, the oxidation current response was maximum and almost similar **(Fig. 3.7)** because the mixed solution of 10 or 20 µM mPMS with 500 µM FeCN was not toxic for cell. This larger amount of emerging oxidation current with double mediator system compared with control FeCN single mediator system confirmed the mediation of electron by mPMS between intracellular NADH and extracellular FeCN. At higher concentration (50 µM and 100 µM) of

mPMS, the produced oxidation current decreased because of its cytotoxicity. From these data, I assumed that 10 µM mPMS and 500µM FeCN was sufficient to monitor the intracellular NADH for this method. Further I optimized the FeCN concentration. After incubation of PC12 cells (3×10^5 cells/ well) with 10 µM mPMS and various concentrations of FeCN at 37℃ under 5% CO_2 for 10 minutes, the chronoamperometric current was measured. The oxidation current with10 µM mPMS and 500 µM FeCN was **maximum (Fig. 3.8)** and therefore, I decided these concentrations of the double mediators were best combination for the electrochemical measurement of intracellular NADH.



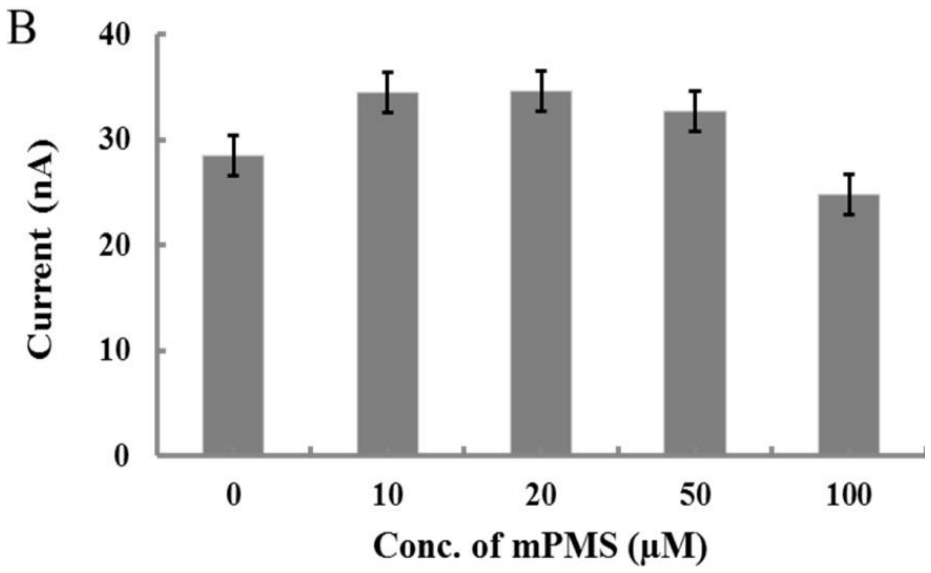


Figure 3.7: Optimization of mPMS concentration for the electrochemical monitoring of intracellular NADH in PC12 cells**.** (A) Oxidation current profiles of double mediator solutions containing various concentrations of mPMS and 500 μM $K_3[Fe(CN)_6]$ for monitoring intracellular NADH. (B) mPMS concentration dependent oxidation current in the chronoamperogram. The PC12 cells (3×10^5 cells/ well) were incubated in the mixed solutions with various concentration of mPMS and 500 μM $K_3[Fe(CN)_6]$. The oxidation current was measured at 5 second after +0.5 V application in chronoamperometry after 10 minutes incubation at 37℃ under 5% CO_2. It assumed that the coupling of 10 μM mPMS and 500 μM $K_3[Fe(CN)_6]$ was most suitable to monitor the intracellular NADH for this assay. The results are expressed as the mean ± SD from four experiments.

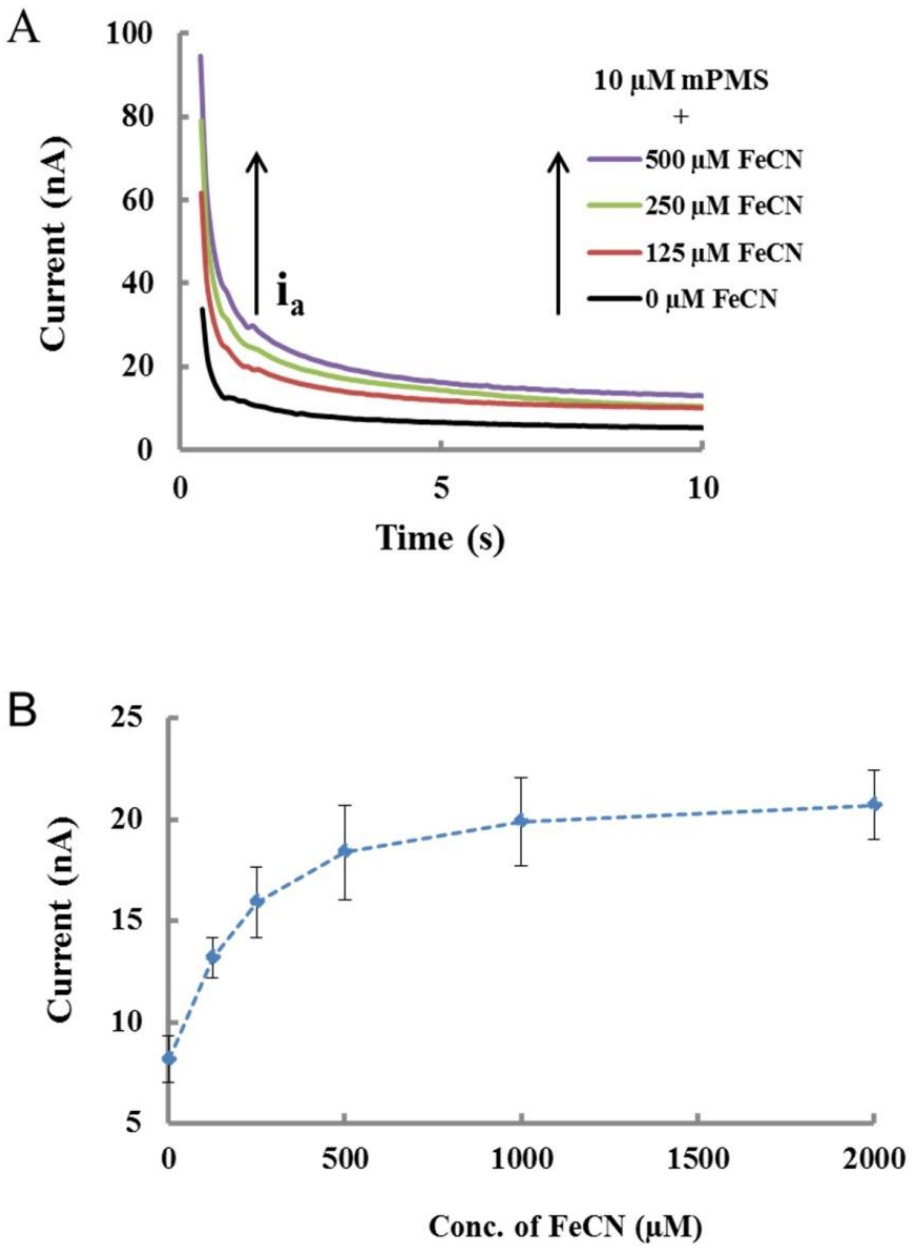
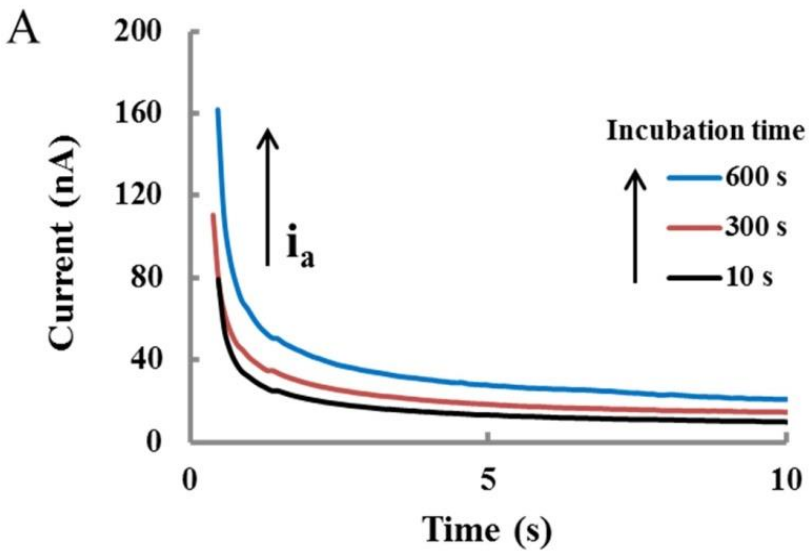


Figure 3.8: (A) $K_3[Fe(CN)_6]$ concentration-dependent chronoamperogram in PC12 cell suspension including the double mediators, (B) $K_3[Fe(CN)_6]$ concentration-dependent oxidation current. PC12 cells (3×10^5 cells/ well) were incubated for 10 minutes at 37℃ under 5% CO_2 in the mixed solutions with 10 µM mPMS and various concentrations of $K_3[Fe(CN)_6]$. Oxidation current in the chronoamperogram was read at 5 second after potential application at + 0.5 V. In case of 10 µM mPMS and 500 µM $K_3[Fe(CN)_6]$, the oxidation current reached maximum and therefore these conditions were used for following all electrochemical measurements.

3.3.5. Optimization of incubation time for cells with the double mediators

The PC12 cells (3×10^5 cells/ well) were taken in wells of a 96 well plate and the double mediators (500 µM FeCN and 10 µM mPMS was the final conc.) were added sequentially from first to last well with 300 seconds intervals. To each well followed by ultimately incubation with different periods (10, 300, 600, 900, 1200 s), the oxidation current was measured by chronoamperometry. The oxidation current was read at 5 second after + 0.5 V potential application. The oxidation current obtained after various seconds of incubation increased as increasing incubation time and became saturation by 600 seconds incubation **(Fig. 3.9).** This data demonstrated that 600s incubation is enough to facilitate mPMS mediated intra and extra-cellular reactions. 10 minutes was therefore chosen as the optimized incubation time and further all electrochemical measurements were done after 10 minutes incubation of cells with the double mediators.



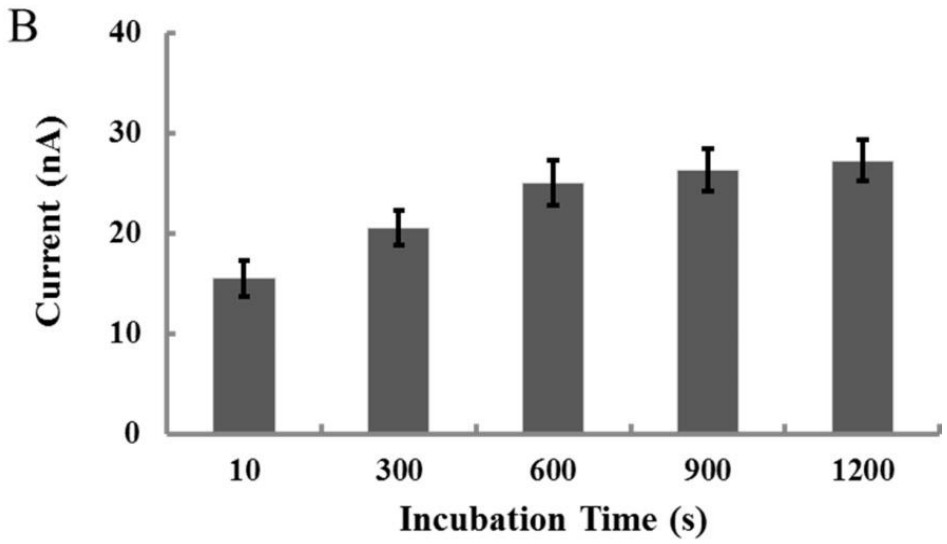
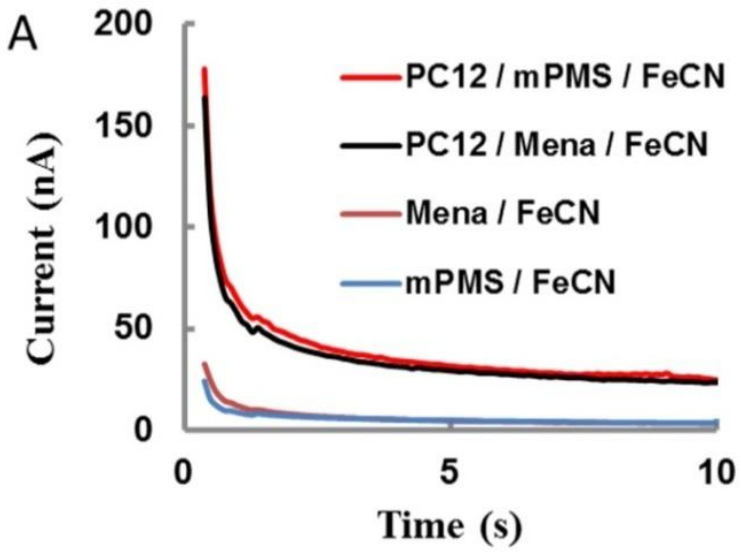


Figure 3.9: Optimization of incubation time before electrochemical measurement. (A) Oxidation current profiles with same double mediator system and different incubation time for monitoring intracellular NADH. (B) Incubation time dependent oxidation current in the chronoamperograms. The oxidation current was read at 5 second after potential application at + 0.5 V. The oxidation current gradually increased by increasing incubation time up to 1200 s and 600 s was enough to facilitate mPMS-mediated intracellular NADH monitoring. These data indicated that 600 s was the optimized incubation time and further all electrochemical measurements were done by 600 s incubation of cells in double mediator solutions.

3.3.6. Comparison of the mPMS/FeCN double mediator system with the Mena/FeCN double mediator system

I have compared my mPMS/FeCN double mediator system with Mena/FeCN double mediator system for monitoring intracellular NADH through the oxidation of accumulated $[Fe(CN)_6]^{4-}$. **From Fig 3.10,** it was observed that the oxidation current response from accumulated $[Fe(CN)_6]^{4-}$ of both systems were almost similar. So, I would like to say my mPMS/FeCN system also works well as same as Mena/FeCN system to monitor intracellular NADH.



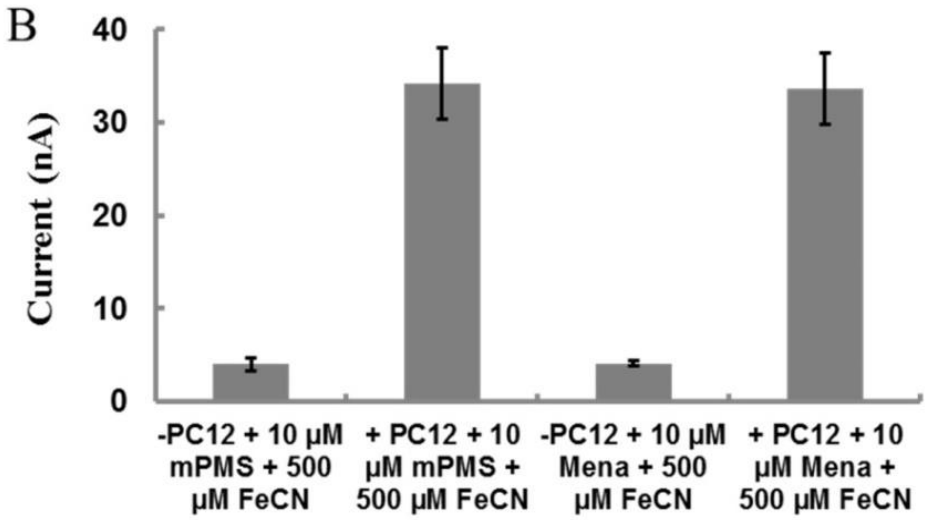
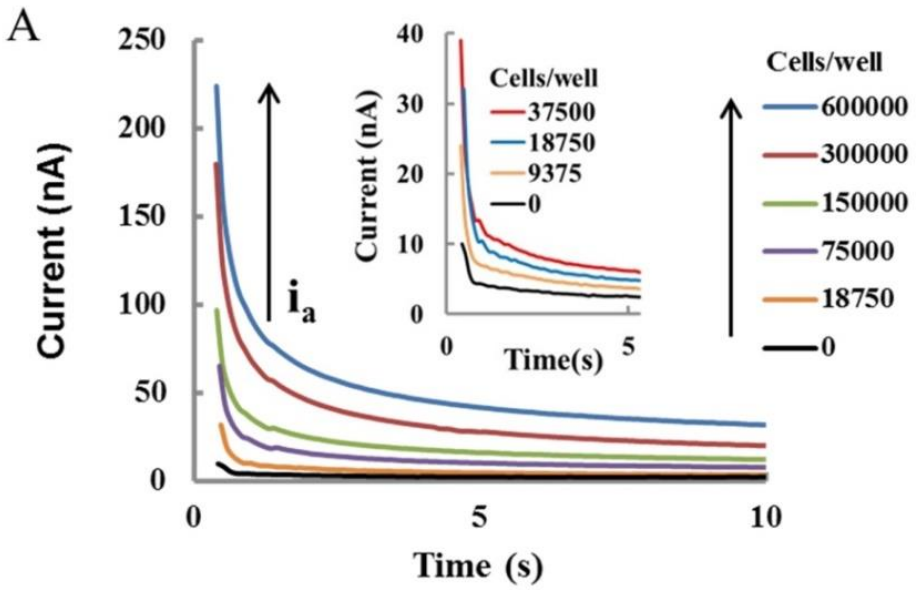


Figure 3.10: Comparison of my double mediator (mPMS/FeCN) system and Mena/FeCN system. (A) Chronoamperograms for monitoring the intracellular NADH in PC12 cells with both double mediator systems. PC12 cells (3×10^5 cells/ well) were incubated in 10 µM mPMS + 500 µM FeCN/HBSS or PC12 cells (3×10^5 cells/ well) were incubated in 10 µM Mena + 500 µM FeCN/HBSS. For both systems, after 10 minutes incubation the chronoamperometric measurement was done by the potential application at +0.5 V. (B) Comparison of the oxidation current at 5 second with mPMS/FeCN system and Mena/FeCN system. The oxidation current value of my double system was almost similar to Mena/FeCN system in the presence and absence of cells. These data demonstrated that my mPMS/FeCN system works well as same as Mena/FeCN system to monitor the intracellular NADH.

3.4 Cell counting by electrochemical method using my double mediator system

At first, cell counting of PC12 cells was performed by electrochemical measurement using 10 µM mPMS and 500 µM FeCN system. **Fig.3.2.1A** shows the chronoamperometric profiles to various density of cell suspension. The oxidation current increased proportionally as increasing number of PC12 cells ranging from 9000 to 600000 cells/well as shown in **Fig. 3.2.1B.**



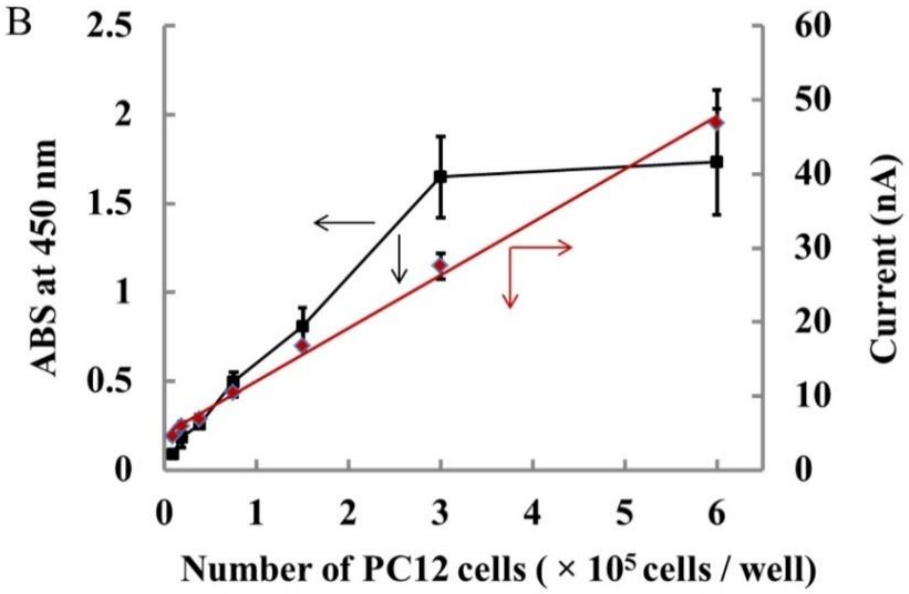


Figure 3.2.1: (A) PC12 cell number-dependent oxidation current profiles of my double mediator system for monitoring intracellular NADH. Inset: Oxidation current profile to lower concentration of cells. (B) Calibration curve for PC12 cell counting. The oxidation current was read at 5 second. Red line is the data of the electrochemical method and black line is the data of the WST-1 assay. The results are expressed as the mean ± SD from four experiments, R^2 = 0.996.

On the other hand, in the case of WST-1 assay, it was clearly seen that PC12 cell number ranging from 9000 to 300000 cells/well showed the linear relationship with absorbance at 450 nm but more than 300000 cells/well, the curve became saturation. Therefore, I concluded that as compared to the WST-1 assay, larger number of the metabolically active PC12 cells could be quickly quantified by the electrochemical measurement with my double mediator system.

To examine the applicability of this double mediator system, I have further applied this method to count the C6 cells and RBL-2H3 cells under same conditions. **Figs. 3.2.2 and 3.2.3** showed the cell counting calibration curves for C6 and RBL-2H3 cells. These figures also revealed an excellent correlation between the measured oxidation current and number of metabolically active cells ranging from 9000 to 600000 cells/ well. From these results, my electrochemical method was considered to be useful as an alternative to the WST-1 assay to

count rapidly various kinds of mammalian cells and therefore to evaluate the cell viability very quickly.

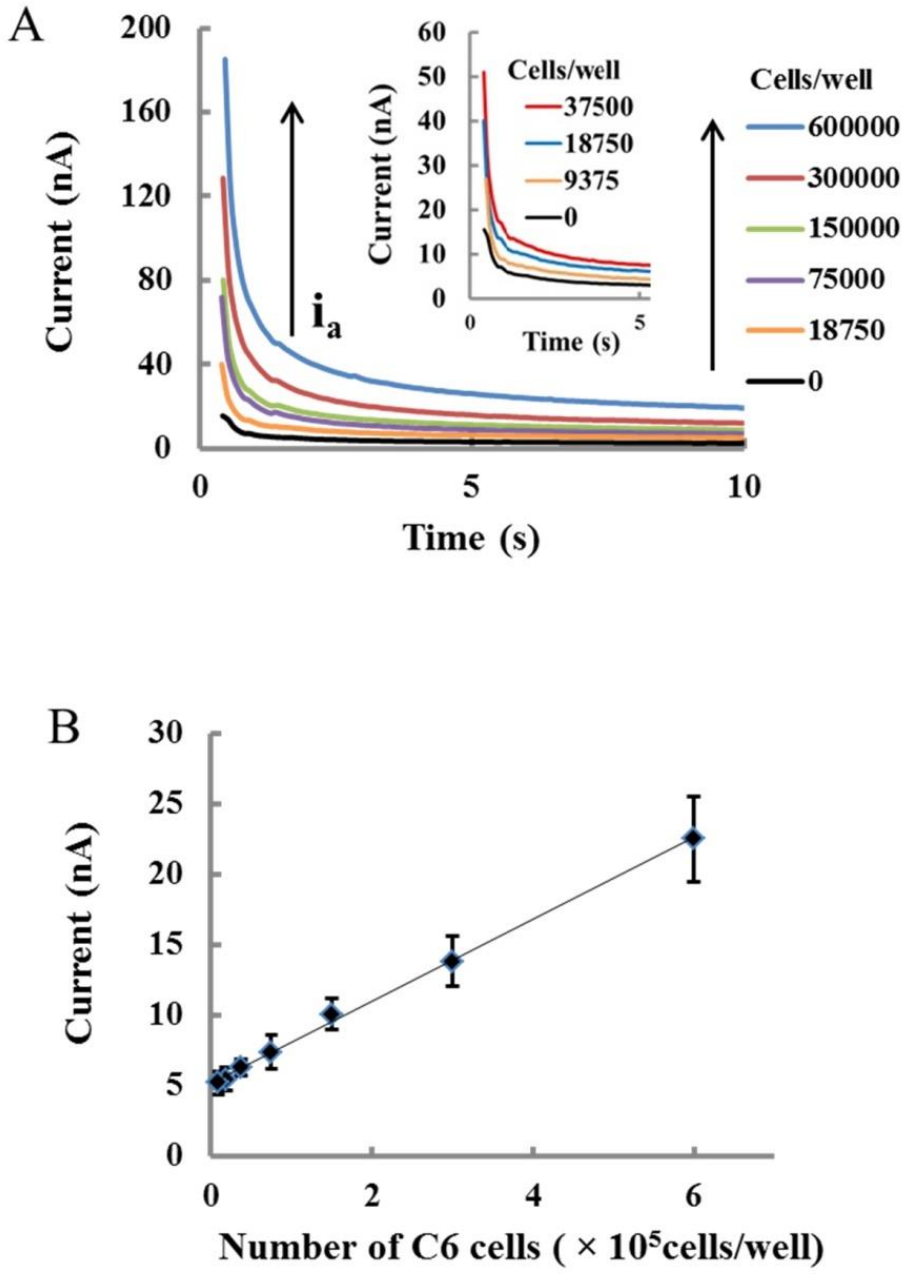


Figure 3.2.2: (A) C6 cell number-dependent oxidation current profiles of double mediator system for monitoring intracellular NADH. Inset: Oxidation current profile to lower concentration of cells. (B) Calibration curve for C6 cell counting. The results are expressed as the mean ± SD from three experiments, $R^2 = 0.998$.

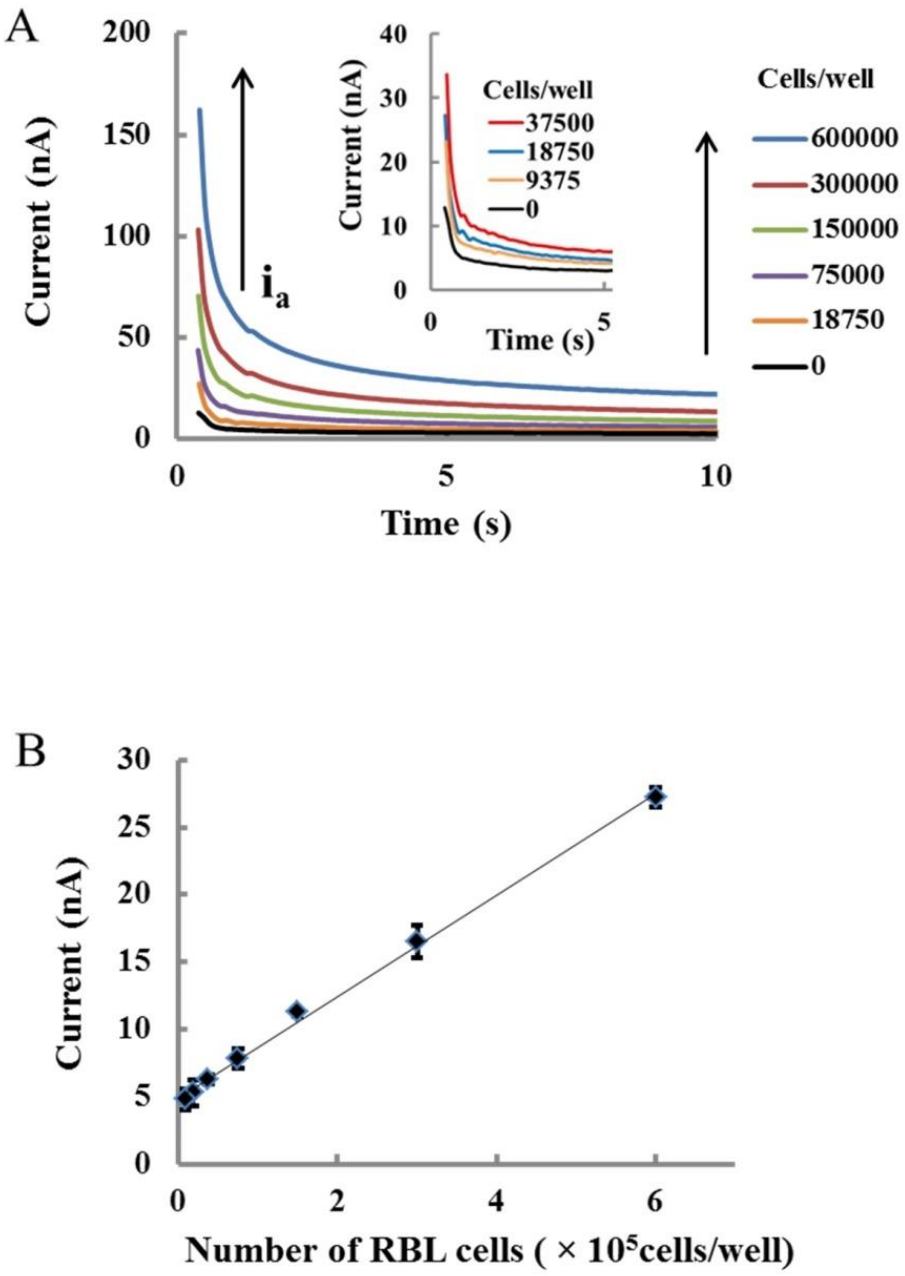


Figure 3.2.3: (A) RBL-2H3 cell number-dependent oxidation current profiles of double mediator system for monitoring intracellular NADH. Inset: Oxidation current profile to lower concentration of cells. (B) Calibration curve for RBL-2H3 cell counting. The results are expressed as the mean ± SD from three experiments, $R^2 = 0.997$.

3.5 Evaluation of acute inhibition of metabolic activity of PC12 cell by oxamic acid with my double mediator system

Oxamic acid is a well-known inhibitor of LDH and thus significantly affects the pyruvate to lactate metabolism [67–70]. LDH is a principal enzyme which catalyses the transformation of pyruvate to lactate linked to redox reaction of dinucleotide coenzyme found in human and all eukaryotic cell. Recently, LDH inhibition by oxamic acid has further taken a lot of attention for anti-cancer drug [69–72]. So, in this study, I applied my electrochemical method to monitor the rapid decrease of intracellular NADH in PC12 cells by LDH inhibition. **Fig. 3.2.4** shows the oxamic acid concentration-dependent oxidation current profiles. The decrease of oxidation current after the treatment of PC12 cells was clearly dependent on the concentration of oxamic acid. It indicated that intracellular NADH level corresponding to the cell viability decreased within 1 hour by oxamic acid treatment. This result corresponded with the report by Zhai et al**.**[70]. They found that oxamic acid with high concentration (20 to 100 mM) significantly decreased the nasopharyngeal carcinoma cells proliferation or cell viability by blocking the LDH activity.

Here I have just investigated the acute toxic effect of oxamic acid on metabolic activity in PC12 cell for one hour by blocking LDH. In near future, I will investigate the incubation-time dependent cytotoxicity of oxamic acid on cytoma cells. Furthermore, I would like to evaluate the various types of acute cytotoxicity such as inhibition of respiratory chain, protein and DNA synthesis etc., by my electrochemical method.

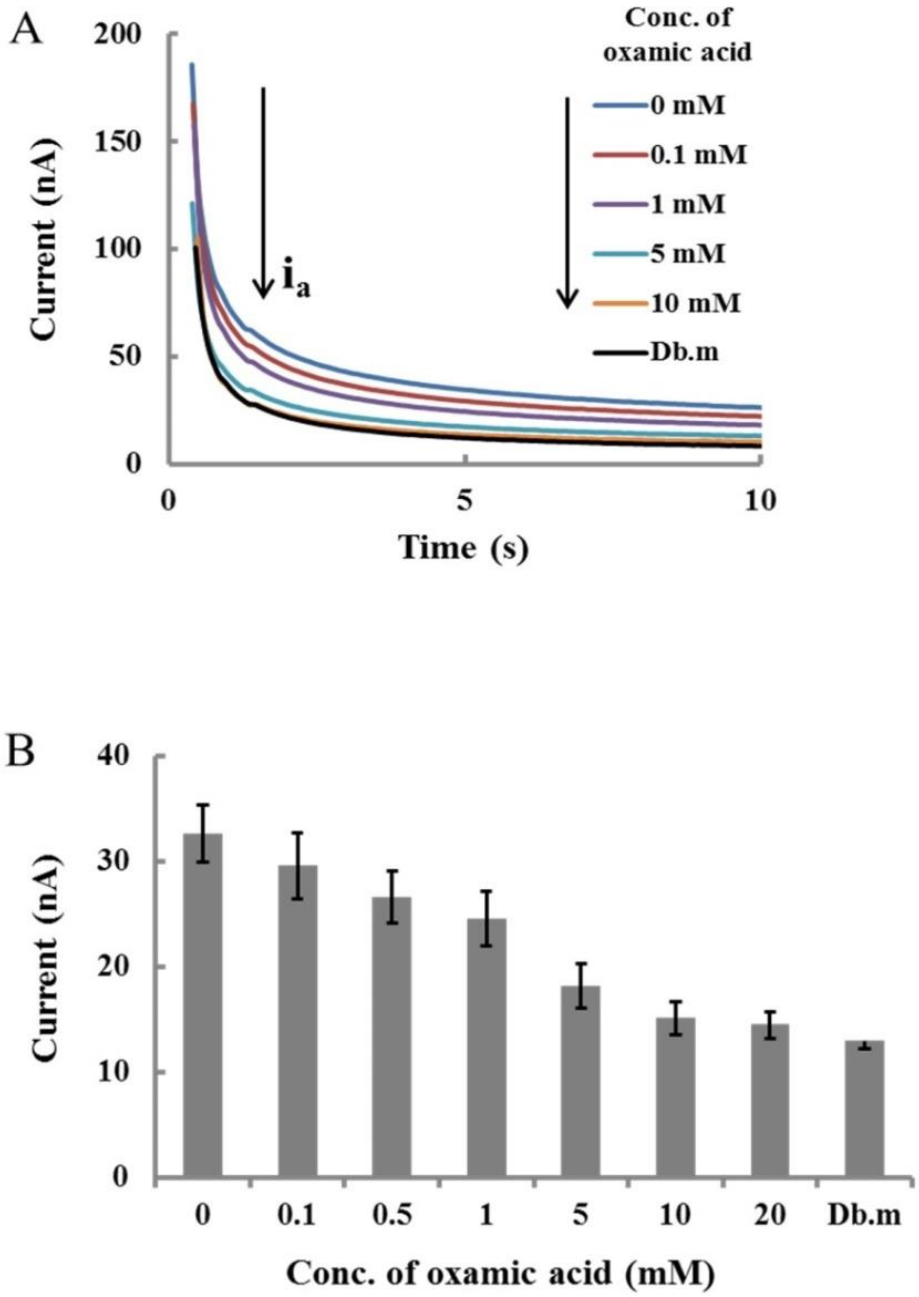


Figure 3.2.4: Acute cytotoxicity test of oxamic acid on PC12 cells. (A) Chronoamperograms of double mediator system for monitoring intracellular NADH after treatment of various concentration of oxamic acid for 1 hour. (B) Decrease of oxidation current depending on the oxamic acid concentration. The oxidation current was read at 5 second after potential application at + 0.5 V. The results are expressed as the mean ± SD from four experiments. Db.m means the double mediator solution in the absence of cells and oxamic acid.

3.6 Conclusion

In this study, I have successfully developed a rapid and highly sensitive double mediator (mPMS/FeCN) system for electrochemical monitoring of intracellular NADH in various mammalian cells. I have optimized the mediator's concentration, incubation time and applied potential for monitoring the intracellular NADH by chronoamperometry. I also confirmed my mPMS/FeCN system worked as well as previously used Mena/FeCN system and my double mediator system was universal for various kinds of mammalian cells due to the cytosolic enzyme-independent electron transfer between NADH and mPMS. As compared to WST-1 assay, my electrochemical method was able to count the mammalian cells with wider range within 10 minutes. It was further demonstrated that my electrochemical method is useful to evaluate the acute cytotoxicity of oxamic acid on cytoma cells.

In near future, this method will be applied to evaluate the cell viability of microorganisms and acute cytotoxicity of anti-fungal and anti-bacterial agents. Furthermore, if I can fabricate multi-plate electrode array system, my method may be alternate to WST assay for rapid and high-throughput cell viability and acute cytotoxicity test.

Chapter 4

Electrochemical monitoring of the viability of yeast cells by using a mPMS/FeCN double mediator system

4.1 Introduction

Yeast cell counting and viability assessment are crucial for industrial processing where yeast cells are used. Yeast is an essential microorganism; it has great importance in food (bakeries, wine, and beers) [73–76], pharmaceutical, and biofuel production industry. On the other hand, human diseases caused by pathogenic yeast have a significant morbidity and mortality rate. So, rapid and early assessment of yeast cell viability is strongly needed for controlling fermentation for the production of the products mentioned above and for evaluating the antimicrobial effect of drugs.

There are various kinds of conventional methods available for the evaluation of yeast cell viability. For example; the most traditional colony counting method [16,77], $(OD)_{600}$ method (useful only for cell counting but not suitable for cell viability test) [24,25], growth of inhibition zone method [19], fluorescent dye stained-based method [78]. Although these methods easily identify living and dead cells in the whole population, it takes a long time to get results and cannot assess the metabolic activity. Besides this, rapid and non-invasive electrical techniques have been developed using the polarization of cells in an AC electric field without a chemical reagent. The impedance spectroscopy method can online-monitor the growth of microbial cells due to the increase in capacitance [28]. Recently, I have developed an electro-orientation method whether individual non-spherical cells were dead or alive from EO positions versus electric field direction depending on the intracellular conductivity [26]. However, these electrical techniques were unable to assess an intracellular metabolism as the viability assays were based on the integrity of the cell membrane.

On the other hand, Bio-electrochemical technique is most reliable and convenient for cell viability test via monitoring the intracellular NADH compared to colorimetric WSTs assay, which is time consuming [46]. Heiskanen et al. pioneeringly reported a bio-electrochemical

double mediator system with Mena and FeCN to monitor intracellular enzyme activity and cell viability [54,55]. In this system, electron transfer from intracellular NADH to Mena is catalyzed by an enzyme such as NADH oxidase (enzymatically dependent, as I discussed in chapter3) [60]. By far, this system has not yet been reported for cell counting and acute cytotoxicity test on yeast cells. On the other hand, my developed electrochemical double mediator system (mPMS / $[Fe(CN)_6]^{3-}$) is not dependent on the intracellular NADH. This is why; I applied my ubiquitous double mediator system for the first time to count the yeast cell and acute cytotoxicity test of itaconic acid and nystatin through the measurement of intracellular NADH.

Here, I have firstly chosen the itaconic acid for its cytotoxicity test because itaconic acid has many industrial applications, such as in the production of acrylic acid, paints, varnishes, and different organic compounds [79]. Recently, itaconic acid has been specially used to make hydrogels for water decontamination and eco-friendly biodegradable polymer [79]. Due to the numerous steps and low production rate in chemical method [80], many industrial companies now paying more attention to produce itaconic acid with the biotechnological method in yeast cell such as *Aspergillus terreus* [81–85], *Ustilago maydis* [86,87] strains and *Saccharomyces cerevisiae* [88]. I know that itaconic acid is a natural metabolite that acts as a key regulator to continue the TCA cycle by an inhibitory effect on SDH [89–91]. If itaconic acid is produced in high concentration interferes/prevents the TCA metabolism by direct inhibition of SDH. As a result, TCA cycle metabolism stops along with dramatically decrease in NADH, as I expected. So, itaconic acid cytotoxicity monitoring is important. In this study, I extracellularly added 0 to 25 mM itaconic acid in the yeast cell suspension, which inhibited the TCA cycle metabolism in the yeast cell.

Further, I have used applied to evaluate the toxic effect of nystatin in yeast cell. Nystatin is a polyene an anti-fungal. By far, toxic effect of nystatin with higher concentrations on yeast cell was evaluated by conventional colony counting method which takes 2 to 3 days [16][77], which is laborious and time consuming**.** Here I have applied my method to check the cytotoxicity with lower concentration of nystatin.

4.2 Analytical procedures

4.2.1 Evaluation of cytotoxicity of mPMS and $K_3[Fe(CN)_6]$ by a mixture of SYTO9 and PI double staining method

100 µL of cell suspension was taken to each well of a 96-well plate. 15 µL of different concentration of mPMS or 30 µL of (different conc. mPMS + 500, 1000, 2000 µM FeCN) was added to all wells. Cells with fresh HBSS (without mPMS or mPMS + FeCN) were used as a control. After 60 minutes of incubation at 37℃ under 5% CO_2, 0.5 µL PI (final conc. 10 µM) and 3 µL SYTO9 (final conc. 12 µM) as added and again incubated for 15 minutes. In every case, the total volume of experimental cell suspension was maintained at about 150 µL with HBSS. Finally, 3 µL cell suspensions were applied onto the glass, taken a photograph of PI and SYTO9 stained cells under the microscopic fluorescence observation for all cases. It was evaluated that 10 or 20 µM mPMS and the mixture of 10 or 20 µM mPMS and 500, 1000, or 2000 µM FeCN was not toxic for yeast cells (data is not shown).

4.2.2 Electrochemical yeast cell counting with mPMS/FeCN double mediator system

Various densities of yeast cell suspension were plated in different wells and, FeCN (final conc. 500 µM) and mPMS (final conc. 10 µM) were added to each well. The total volume of experimental cell suspension was 150 µL. After incubation for 10 minutes, an SPCE was vertically immersed into the yeast cell suspension, and chronoamperometric measurement was immediately performed by a potential application at + 0.5 V vs Ag/AgCl reference electrode on the SPCE to sufficiently oxidise the accumulated ferrocyanide ($[Fe(CN)_6]^{4-}$) outside of the cell. This $[Fe(CN)_6]^{4-}$ was produced from the reduction of FeCN by intracellular NADH with the mediation of mPMS [63].

4.2.3 Yeast cell counting by WST-1 assay

Various densities of yeast cell suspension were plated in different wells and, cell counting Kit-1 (WST-1) was added to each well. Cell counting Kit-1 was prepared by following this article [66]. The total volume of experimental cell suspension was 150 µL. After 2 h incubation, absorption of generated WST-1 formazan was measured by 450 nm.

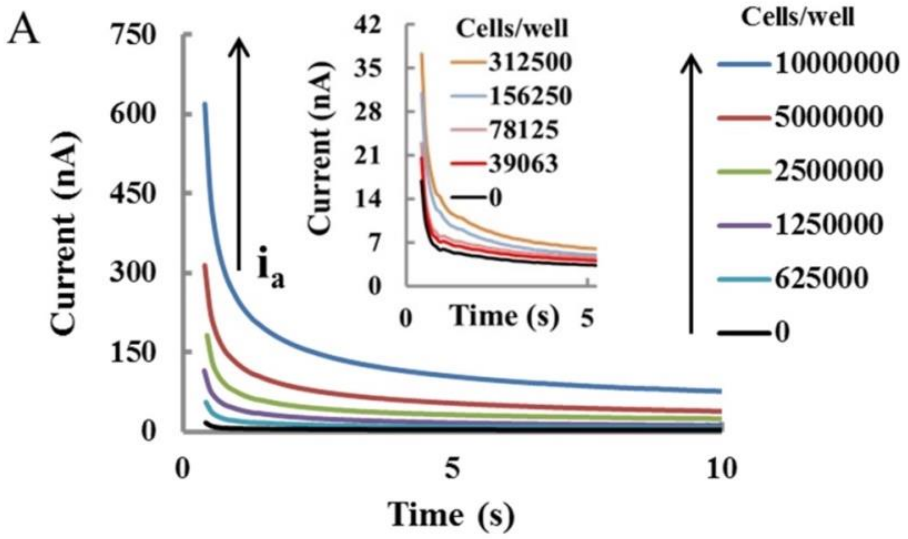
4.2.4 Evaluation of cytotoxicity of itaconic acid and nystatin on yeast cells by electrochemical method

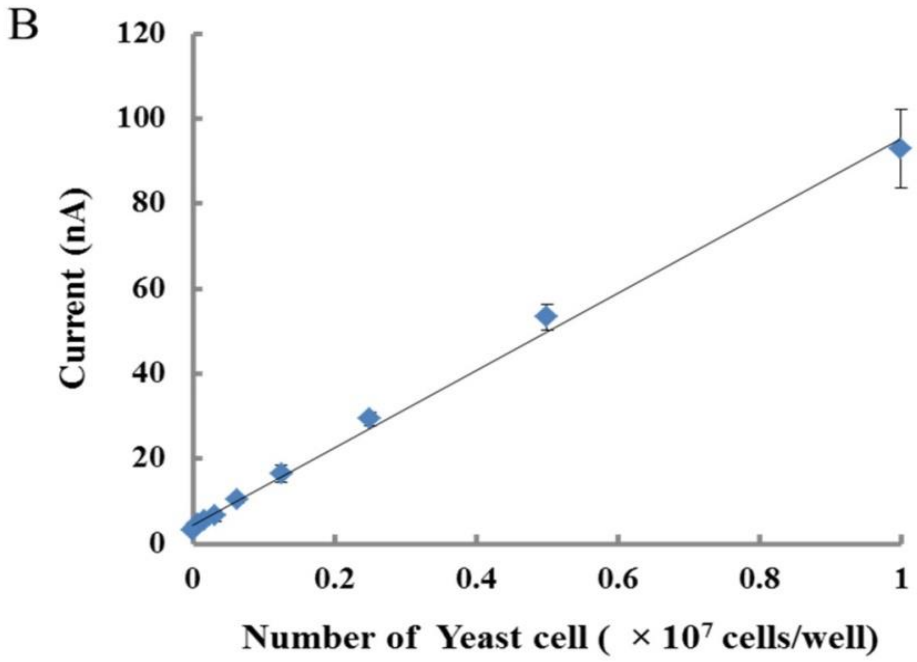
In this study, yeast cell suspension/HBSS (1×10^6 cells/well) was taken in each well of a 96 well plate, and 22.5 µL itaconic acid/HBSS was added (final conc; 0 to 25 mM). In case of nystatin, 10 µL nystatin was added (final conc. 0.01 to 0.16 µg/mL) into each well. After 1 hour incubation for itaconic acid and 45 minutes for nystatin, 15 µL FeCN (final conc. 500 µM) and 15 µL mPMS (final conc. 10 µM) solutions were added into each well. In every case, the total volume of experimental suspension was maintained to 150 µL with HBSS. Additionally, after 10 minutes of incubation at 37℃ under 5% CO_2, chronoamperometric measurement was performed to evaluate the cytotoxicity of the itaconic acid.

4.3 Electrochemical yeast cell counting with the mPMS/$[Fe(CN)_6]^{3-}$ double mediator system

Chronoamperometric profile **(Fig. 4.1A)** demonstrated that the larger and smaller oxidation current was originated from the larger and smaller number of yeast cells. The current was produced from the oxidation of formed $[Fe(CN)_6]^{4-}$ that was generated from intracellular NADH with redox mediation of mPMS. **Fig. 4.1B** represents produced oxidation current showed an excellent linear relationship with the number of viable yeast cells. On the other hand, conventional $(OD)_{600}$ is not suitable for cell counting in high density because of the scattering effect. In **Fig. 4.1C** for WST-1 method, the calibration curve for yeast cell counting is not entirely linear may be due to the scattering effect arising from the high concentration of yeast cells when radiation at 450 nm was absorbed by WST-1 formazan. Although yeast cell counting range (from 4×10^4 to 1×10^7 cells/well) was same in both of methods, the linear range in my method was wider than that of WST-1 assay. That means a wider range in quantity of yeast cell could be counted more precisely by my method.

So, my modified double mediator system was not affected by scattering effect, turbidity of cell suspended medium, which might be better than the conventional $(OD)_{600}$ and WST-1 method for yeast cell counting within short time as I expected. Furthermore, I have applied this system to evaluate the acute cytotoxicity of itaconic acid in yeast cells by monitoring intracellular NADH.


A
Current (nA)
Time (s)
i_a
Cells/well
312500
156250
78125
39063
0
Cells/well
10000000
5000000
2500000
1250000
625000
0


B
Current (nA)
Number of Yeast cell (× 10^7 cells/well)

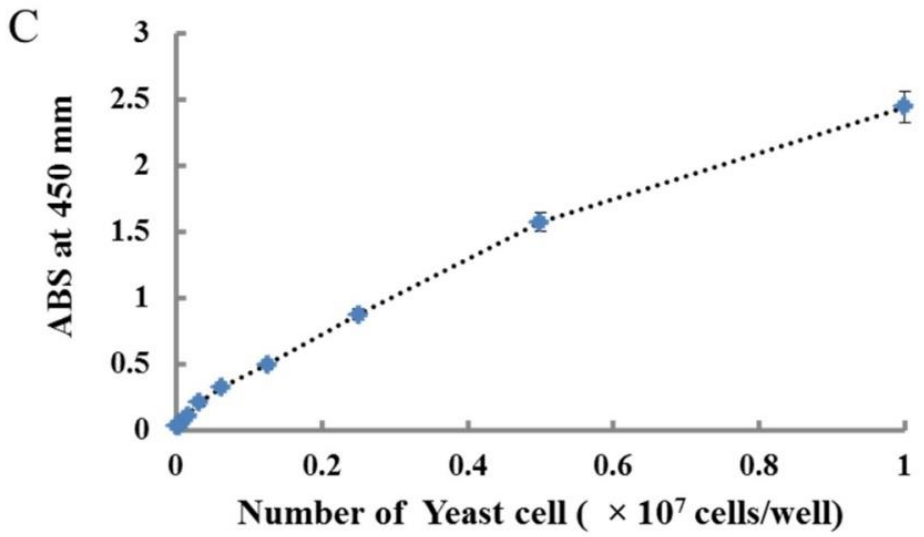


Figure 4.1: (A) Yeast cell number-dependent oxidation current profiles with a new double mediator system for monitoring intracellular NADH. Inset: Oxidation current profiles to lower concentration of cells. (B) Calibration curve for yeast cell counting. The oxidation current was read at 5 second in each chronoamperogram. The results are expressed as the mean ± SD from three experiments, R^2 = 0.997. (C) Calibration curve for yeast cell counting by WST-1 cell counting kit-1.

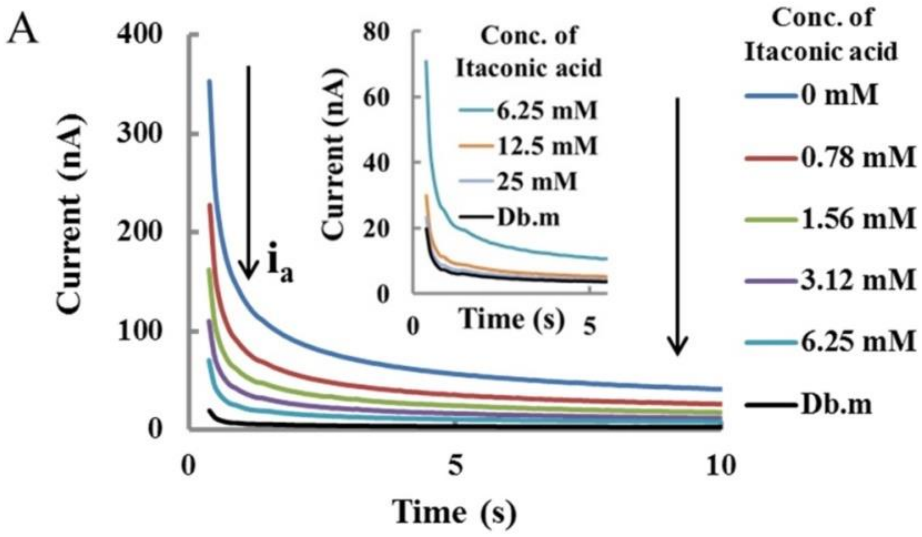
4.4 Evaluation of cytotoxic effect of antifungal drugs in yeast cell

4.4.1: Evaluation of the cytotoxic effect of itaconic acid in yeast cells by mPMS/$[Fe(CN)_6]^{3-}$ double mediator system and comparison with WST-1 assay

Itaconic acid produced from *cis*-aconitate by *cis*-aconitic acid decarboxylase (CAD) or immune response gene-1 via decarboxylation reaction in TCA cycle [91,92]. This endogenously synthesized itaconic acid regulates succinate level and whole metabolism in eukaryotic cell by the inhibitory activity on SDH [89–91]. Jha et al. reported that itaconic acid produced at the first break-point, and this produced itaconic acid directly regulates the SDH in second break-point [90,93].

Although itaconic acid is a metabolite, the naturally produced high concentration of itaconic acid inhibits the SDH; as a result, succinate cannot oxidise to fumarate. On the other

hand, exogenous addition of itaconic acid inhibits isocitrate lyase, a key enzyme of glyoxylate shunt metabolism in bacteria [92,94,95]. However, extra administration of itaconic acid triggers direct inhibition of SDH to cause succinate accumulation in eukaryotic mitochondria [89,90,96,97]. As a result, succinate cannot convert to fumarate, accumulates in the mitochondria, and the TCA cycle stops. As a consequence of this whole interference phenomenon, I expect intracellular NADH also quickly to decreases. **Fig. 4.2** shows that the oxidation current was decreased with increasing itaconic acid concentration after 1 hour exposure. This decrease of oxidation current is due to the inhibition of succinate dehydrogenase, which also quickly decreased the mitochondrial NADH as we expected. My result was similar to the result obtained by T. Cordes et al. They have used 0 to 25 mM itaconic acid on RAW 264.7 macrophages and Bone-marrow-derived macrophages (BMDMs) and reported that a few mM of itaconic acid inhibited SDH along with succinate accumulation [90].



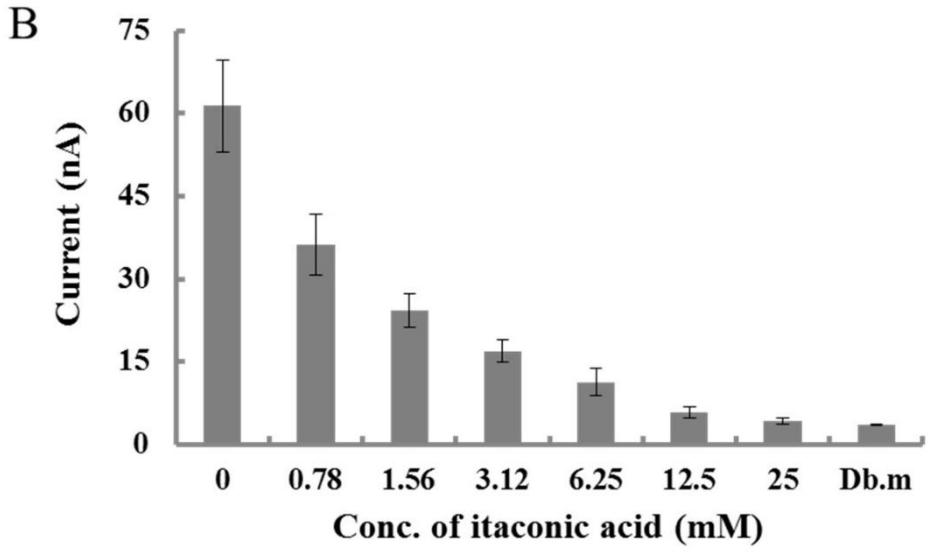


Figure 4.2: Acute cytotoxicity test of itaconic acid on yeast cells. (A) itaconic acid concentration dependent chronoamperograms of double mediator system for monitoring intracellular NADH after 1 hour treatment with various concentrations of itaconic acid. (B) Decrease of oxidation current depending on the itaconic acid concentration. The oxidation current was read at 5 second after potential application at + 0.5 V. The results are expressed as the mean ± SD from three experiments. Db.m means the double mediator including HBSS in the absence of cells and itaconic acid.

Fig. 4.3 shows that the absorption of produced formazan at 450 nm was decreased with increasing itaconic acid concentrations after 1 h exposure and additionally, after 2.5 h of incubation at 30℃. This result was similar to the result obtained from our electrochemical double mediator system. But, at the high concentration of itaconic acid (such as at 12.5 and 25 mM) in **Fig.4.3,** the absorption of produced formazan did not so decrease like as the oxidation current in **Fig.4.2.** This somewhat different result in WST-1 assay from our electrochemical method might be due to the long incubation time in WST-1 assay. It is now difficult for us to tell what happened in intracellular function at long incubation time in WST-1 assay. It should be further elucidated.

So, from these above two results, it was confirmed that the extracellular administration of itaconic acid in the yeast cells decreased the intracellular NADH which was precisely

evaluated by our electrochemical double mediator system as compared to conventional WST-1 assay.

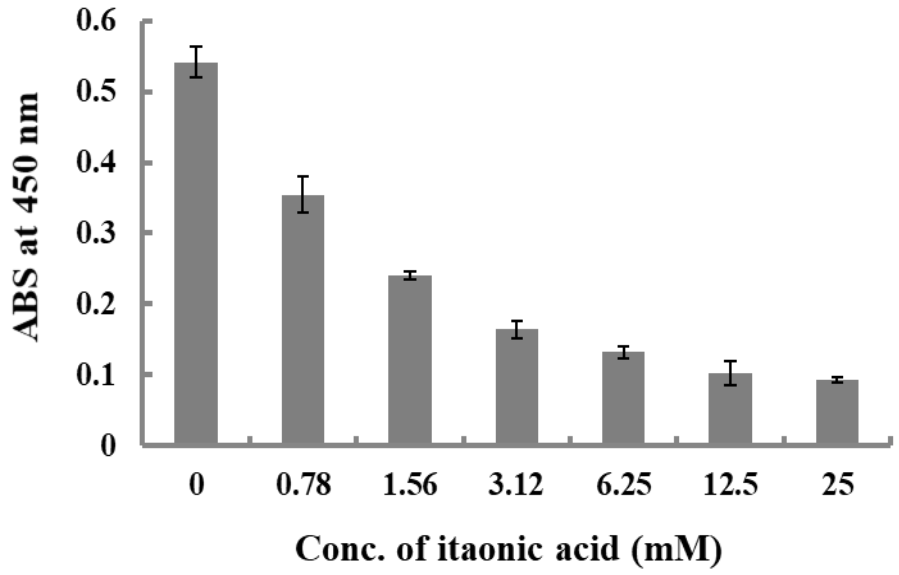


Figure 4.3: Evaluation of cytotoxicity of itaconic acid in yeast cells by WST-1 assay. Yeast cells (1×10^6 cells/well) was incubated for 1 h with different concentration of itaconic acid (final conc; 0 to 25 mM), and after that 10 µL CCK-1 was added into each well. Additionally, after 2.5 h of incubation at 30℃, the absorption of produced formazan was measured at 450 nm by a micro plate reader. The results were expressed as the mean ± SD from three experiments.

4.4.2 Evaluation of toxic effect of nystatin in yeast cells

Nystatin is an anti-fungal polyene, target the cell wall and plasma membrane. Anti-fungal polyene primarily binds to the cytoplasmic ergosterol [98] and inserts into the plasma membrane to form a pore-like structure results the destruction of membrane integrity and leakage of intracellular ion [16,99–103]. It has been reported that the toxic effect of nystatin at the concentration of µg/mL range was evaluated by the colony counting method on the basis of cell membrane disruption [16,77,99,104]. Here, I have used two order lower concentration was evaluated of nystatin to evaluate its cytotoxicity.

To check the membrane was destroyed or not, I have used the mixture of SYTO9 and PI as a double-fluorescent dye. Firstly, yeast cells were incubated for 1 hour with different concentration of nystatin, and additionally incubated with (SYTO9 + PI) for 15 minutes. Finally, a photograph of yeast cells stained with SYTO9 + PI were taken by fluorescence microscope. Fig. 4.3 shows that at low concentration (0.32 µg/mL) of nystatin, cell membrane disruption was not occurred and PI could not permit inside the cell. From this result, it was confirmed that without cell membrane disruption, nystatin penetrated inside of the yeast cell.

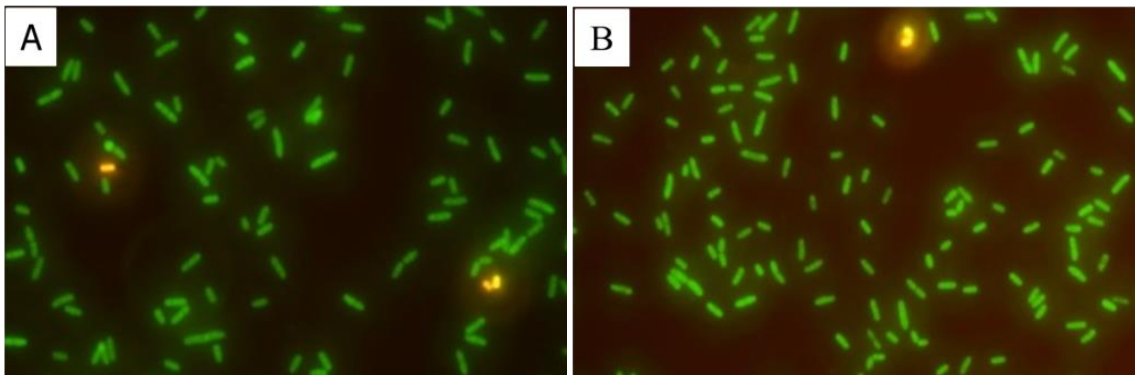


Figure 4.4: Microscopic image of yeast cells after 1 hour incubation with different concentration of nystatin. (A) Control condition; yeast cells without nystatin. (B) Yeast cells with 0.32 µg/mL nystatin.

Next, to evaluate the viability, I have measured the oxidation current at +0.5 V by my double mediator system with same concentrations of nystatin after 60 minutes incubation and additionally 10 minutes for double mediators. Fig. 4.4A shows that the oxidation current decreases with increasing the nystatin concentration ranging from 0.01 to 0.16 µg/mL. I considered that nystatin at this concentration range induced intracellular NADH decrease to promote apoptosis without cell membrane disruption. It has been reported that the pore formation in the membrane by polyene (amphotericin B) is not the sufficient mechanism to induce the cell death [100,105]. Since cell membrane disruption was not occurred, I hypothesised the apoptosis caused by nystatin which should be elucidated. Because, the cell cycle (G_2/M) arrest, abnormal morphology of mitochondria, oxidative damage is the reasons for apoptosis caused by polyene [101,105–108].

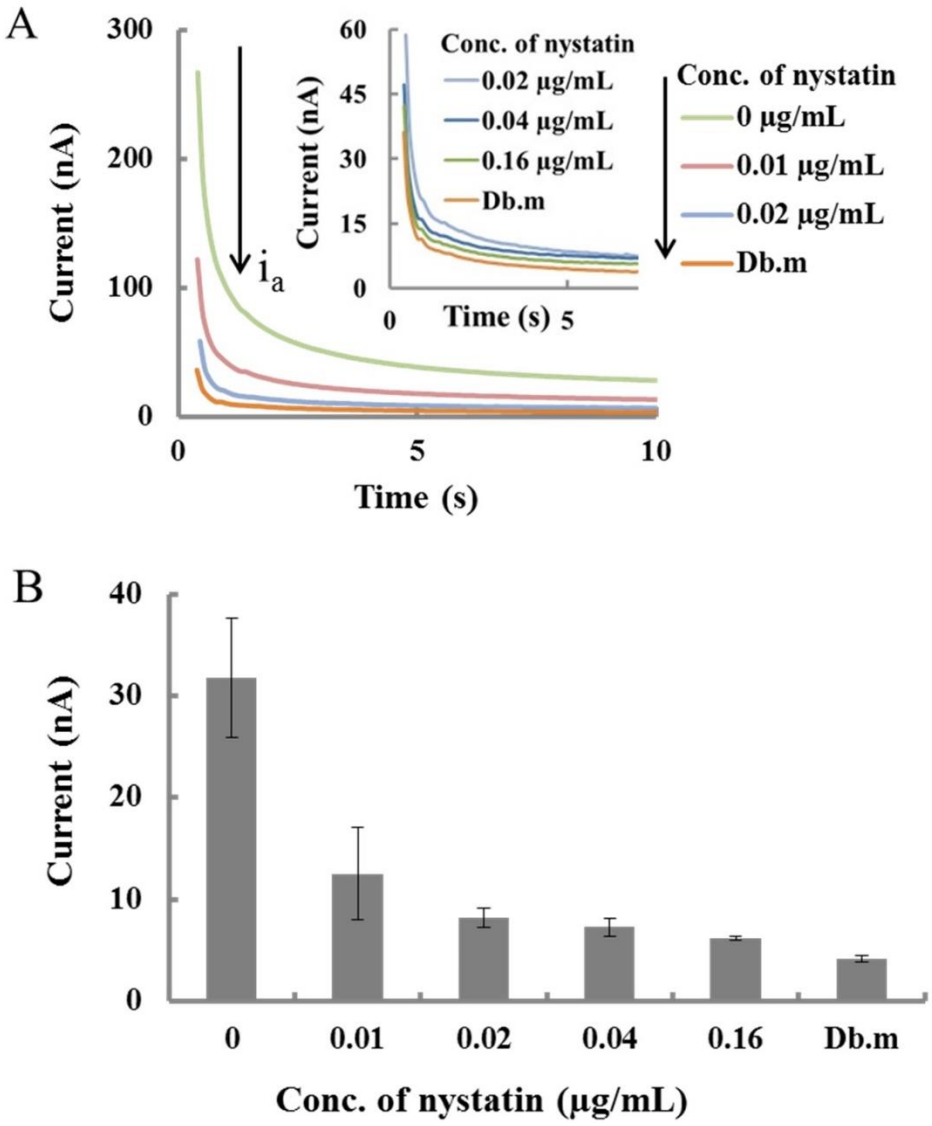


Figure 4.5: Acute cytotoxicity of nystatin in yeast cell. (A) Oxidation current profiles for monitoring the inhibition effect of nystatin on yeast cells. (B) Nystatin concentration dependent oxidation current of double mediator system for monitoring intracellular NADH after 1 hour treatment with various concentrations of nystatin. The decrease of oxidation current depending on the nystatin concentration. The oxidation current was read at 5 second after potential application at + 0.5 V. The results are expressed as the mean ± SD from three experiments. Db.m means the double mediator including HBSS in the absence of cells and nystatin.

4.5 Conclusion

According to the results, a wider range in quantity of yeast cells was precisely counted within 10 minutes by using a double mediator (mPMS)/$[Fe(CN)_6]^{3-}$) system. Furthermore, this method was applicable for the evaluation of acute cytotoxicity caused by fast-acting chemical compounds including drugs and pollutants in yeast cells. Next, I sought to apply this system to bacterial cell counting and viability test.

Chapter 5

Conclusion and future perspective

5.1 Conclusion

In this study, I have successfully developed a rapid and highly sensitive electrochemical modified double mediator (mPMS/FeCN) system for real cell viability assessment through the monitoring of intracellular NADH in various kinds of cells without cell membrane disruption. I have optimized the mediator's concentration, incubation time and applied potential for monitoring the intracellular NADH by chronoamperometry. I have also confirmed my mPMS/FeCN system worked as well as previously used enzyme-dependent Mena/FeCN system and my double mediator system was universal for various kinds of cells due to the cytosolic enzyme-independent electron transfer between NADH and mPMS. As compared to conventional WST-1 assay, my electrochemical method was able to count the mammalian cells with wider range within 10 minutes. It was further demonstrated that my electrochemical method is useful to evaluate the acute cytotoxicity of fast-acting drugs such as anti-cancer effect of oxamic acid in PC12 cell, toxic effect of anti-fungal agent such as itaconic acid and nystatin in yeast cell. However, my electrochemical method overcomes most of the limitations of other optical methods, such as cytotoxicity of assay dye, the scattering effect, turbidity of cells suspended in medium and long incubation time. Results suggested that my method might be applicable to evaluate the various types of acute cytotoxicity such as inhibition of respiratory chain, protein and DNA synthesis etc.

I believe that my method might be useful as a tool not only for academic study but also for the cell-based research, medical and pharmaceutical applications as I expected.

Near future I would like to continue my research to develop a system for cell viability assessment at cell-adhered state with my mPMS/FeCN double mediators.

5.2 Future perspective

My next goal is to develop an electrochemical method to control the intracellular metabolic activity by reduction of intracellular NAD^+ to NADH with redox mediator. It will open a new concept of bio-reactors which can synthesize useful bio-chemical compounds including important drugs by using NADH-dependent enzymatic reactions. I believe this research will be useful for development of green and sustainable society and industry. The principle of the electrochemical reduction for the promotion of cell metabolism is shown in Fig.5.1 schematically.

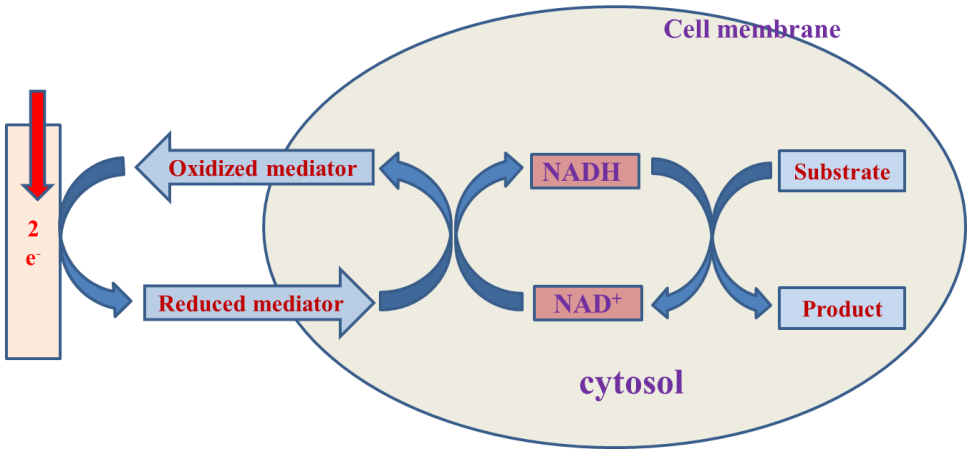


Figure 5.1: Expected principle of the electrochemical reduction for the promotion of cell metabolism.

Appendix

Cell viability

Prior to every experimental treatment, everybody should know the cell's health condition. In this case, viability, proliferation and vitality are closely related term. Viability refers to the percentage of metabolic active cells presents in the whole population as compared to death cell whereas proliferation is a measure of cell division. On the other hand, vitality is the physiological capabilities of cells. Monitoring of the intracellular NADH/metabolic activity is the indication of cell viability.

Electrochemical technique for cell viability assessment

Electrochemical techniques have been used in various fields such as biofuel cell, biosensors, electrochemical analysis and synthesis. In case of biological oxygen demand (BOD) estimation, blood glucose measurement and contaminant determination, the electrochemical technique has been mostly employed. By the definition; electrochemical technique is a process in which the charge transfer takes place between electrode and a redox species in solution. Electrochemical techniques made it possible to rapid monitor the chemical processes inside living cells using electron transfer mediators. Three types of electrodes; reference, working and counter (auxiliary) electrodes are immersed in an analyte solution containing supporting electrolyte. Typically, alkali metal's salt is used as supporting electrolyte but doesn't react with the electrodes. Generally, oxidation or reduction reactions occurred at the working electrode surface and current flows between the working and counter electrode, this current is continuously measured. Potentiostate maintains the constant potential difference between working and reference electrode with changing the applied potential between the working and auxiliary electrodes.

In any electrochemical cell, it involves electrode which is a source of electrons. Considering reduced cofactor, NADH as a source of electrons and the above characteristic reactions, it could be possible to monitor cell viability by the measurement of oxidation current from intracellular NADH with cell membrane permeable mediator. The mediator will accept and

release the electrons between those coenzymes and extracellular region such as FeCN or suitable electrodes.

Using double mediators, chronoamperometry and cyclic voltammetry are most useful method to observe the intracellular reaction inside the cell. In this thesis, I have employed cyclic voltammetric and chronoamperometric technique to measure the accumulated ferrocyanide in extracellular cell suspension for monitoring intracellular NADH with mPMS/FeCN double mediators. The electrochemical measurement of accumulated ferrocyanide is an indication of metabolic activity inside of the cell.

Cyclic voltammetry

In cyclic voltammetry, current is measured as a function of applied potential. The current produced from the reaction on the working electrode is plotted versus the applied potential. As time being passing, the potential applied on the working electrode is started to ramp in forward direction until it reaches in maximum value which was previously set as applied potential. After reaching this potential-value, the potential is ramped in the opposite direction to the initial potential. In this way, varying the applied potential at the working electrode a cyclic voltammogram is generated and repeated one or more times to analysis the electrochemical properties of analyte in the solution. In this thesis, I have measured the accumulated $[Fe(CN)_6]^{4-}$ by cyclic voltammetry in double mediator system in the presence of cells and observed the oxidation current increase as compared to control as shown in Fig. 3.4, which supported that the Scheme 1 might be suitable to detect the cell viability.

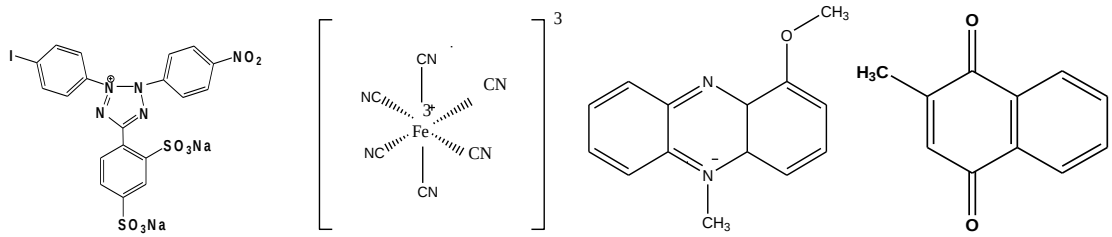
Chronoamperometry

In chronoamperometry, a fixed potential is applied on the working electrode with respect to the reference electrode and produced current from the oxidation or reduction reaction on the working electrode is recorded as a function of time. Chronoamperometry is very useful to monitor the continuous electron transfer. Here, I have measured the accumulated $[Fe(CN)_6]^{4-}$ by chronoamperometry in my double mediator system in the presence of cells and observed the oxidation current increase as compared to control.

Electron mediators

The electron mediators are those agents which transfer the electrons from the active redox canter of the enzyme in the living cell to extracellular region contains terminal electron acceptor or vice versa. Generally, some redox chemical compounds and electrode surface are used as a terminal electron acceptor.

There are two types of redox mediators; hydrophilic and lipophilic mediators. Hydrophilic mediators such as potassium ferricyanide, ruthenium hexamine and carboxymethylferrocenium are water-soluble cannot permit inside of the cell. On the other hand, lipophilic mediators such as phenazine methosulphate, phenazine ethosulphate, dichloroindophenol and neutral red, benzoquinones such as 2-methyl-1,4-naphthoquinone (menadione), benzoquinone, 1,2- naphthoquinone, and benzoamines such as 2,3,5,6-tetramethyl 1,4-phenylenediamine (TMPD) and N, N-dimethyl-p-phenylenediamine are lipid soluble, can cross the cell membrane and access cytoplasm and mitochondria [109,110]. The reduced molecules (nicotinamide adenine dinucleotide NADH /nicotinamide adenine dinucleotide phosphate NADPH) are located in the mitochondrial electron transport chain in the eukaryotic cells and in the plasma membrane of prokaryotic cells gives electron to the oxidized mediator. Oxidised mediator became reduced and goes outside to transfer the electrons to extracellular hydrophilic mediator. Hydrophilic mediator accepts electrons and this hydrophilic mediator is then measured by optical method (WSTs method) or electrochemical methods such as amperometry, voltammetry or coulommetry. The redox reaction of the mediators on the working electrode indirectly provides the intracellular important information producing electrochemical or optical signal.



WST-1 FeCN mPMS Mena

Chemical structure of hydrophilic mediator WST-1, FeCN, and lipophilic mediator mPMS, Mena.

References

[1] M. Kwolek-Mirek, R. Zadrag-Tecza, Comparison of methods used for assessing the viability and vitality of yeast cells, FEMS Yeast Res. 14 (2014) 1068–1079. https://doi.org/10.1111/1567-1364.12202.

[2] S.A. Altman, L. Randers, G. Rao, Comparison of Trypan Blue Dye Exclusion and Fluorometric Assays for Mammalian Cell Viability Determinations, Biotechnol. Prog. 9 (1993) 671–674. https://doi.org/10.1021/bp00024a017.

[3] W. Strober, Trypan blue exclusion test of cell viability., Curr. Protoc. Immunol. Appendix 3 (2001) 2–3. https://doi.org/10.1002/0471142735.ima03bs21.

[4] F. Piccinini, A. Tesei, C. Arienti, A. Bevilacqua, Cell Counting and Viability Assessment of 2D and 3D Cell Cultures: Expected Reliability of the Trypan Blue Assay, Biol. Proced. Online. 19 (2017) 1–12. https://doi.org/10.1186/s12575-017-0056-3.

[5] K.S. Louis, A.C. Siegel, Cell Viability Analysis Using Trypan Blue: Manual and Automated Methods Kristine, 2011. https://doi.org/10.1007/978-1-61779-108-6.

[6] X. Gong, Z. Liang, Y. Yang, H. Liu, J. Ji, Y. Fan, A resazurin-based, nondestructive assay for monitoring cell proliferation during a scaffold-based 3D culture process, Regen. Biomater. 7 (2020) 271–281. https://doi.org/10.1093/rb/rbaa002.

[7] J. O'Brien, I. Wilson, T. Orton, F. Pognan, Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity, Eur. J. Biochem. 267 (2000) 5421–5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x.

[8] R.C. Borra, M.A. Lotufo, S.M. Gagioti, F. de M. Barros, P.M. Andrade, A simple method to measure cell viability in proliferation and cytotoxicity assays, Braz. Oral Res. 23 (2009) 255–262. https://doi.org/10.1590/S1806-83242009000300006.

[9] H. Babich, E. Borenfreund, Applications of the Neutral Red Cytotoxicity Assay to In Vitro Toxicology, Altern. to Lab. Anim. 18 (1990) 129–144. https://doi.org/10.1177/026119299001800116.1.

[10] M. Bacanli, H.G. Anlar, A.A. Başaran, N. Başaran, Fenoliklerin sitotoksisite profillerinin değerlendirilmesi: Farklı hücrelerde farklı zaman aralıklarında nötral kırmızı ve MTT Yöntemlerinin karşılaştırılması, Turkish J. Pharm. Sci. 14 (2017) 95–107. https://doi.org/10.4274/tjps.07078.

[11] G. Repetto, A. del Peso, J.L. Zurita, Neutral red uptake assay for the estimation of cell viability/ cytotoxicity, Nat. Protoc. 3 (2008) 1125–1131. https://doi.org/10.1038/nprot.2008.75.

[12] P. Stiefel, S. Schmidt-Emrich, K. Maniura-Weber, Q. Ren, Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide, BMC Microbiol. 15 (2015) 1–9. https://doi.org/10.1186/s12866-015-0376-x.

[13] N. Atale, S. Gupta, U.C.S. Yadav, V. Rani, Cell-death assessment by fluorescent and nonfluorescent cytosolic and nuclear staining techniques, J. Microsc. 255 (2014) 7–19. https://doi.org/10.1111/jmi.12133.

[14] E. Berdalet, Q. Dortch, New double-staining technique for RNA and DNA measurement in marine phytoplankton, Mar. Ecol. Prog. Ser. 73 (1991) 295–305. https://doi.org/10.3354/meps073295.

[15] M. Rosario, G. Armesto, M. Prieto, A. Otero, Microbiología ^ ggM, (1993).

[16] G. Serhan, C.M. Stack, G.G. Perrone, C.O. Morton, The polyene antifungals, amphotericin B and nystatin, cause cell death in Saccharomyces cerevisiae by a distinct mechanism to amphibian-derived antimicrobial peptides, Ann. Clin. Microbiol. Antimicrob. 13 (2014) 18–21. https://doi.org/10.1186/1476-0711-13-18.

[17] S. Sieuwerts, F.A.M. De Bok, E. Mols, W.M. De Vos, J.E.T. Van Hylckama Vlieg, A simple and fast method for determining colony forming units, Lett. Appl. Microbiol. 47 (2008) 275–278. https://doi.org/10.1111/j.1472-765X.2008.02417.x.

[18] H.S. Bhargav, S.D. Shastri, S.P. Poornav, K.M. Darshan, M.M. Nayak, Measurement of the Zone of Inhibition of an Antibiotic, Proc. - 6th Int. Adv. Comput. Conf. IACC 2016. (2016) 409–414. https://doi.org/10.1109/IACC.2016.82.

[19] M. Balouiri, M. Sadiki, S.K. Ibnsouda, Methods for in vitro evaluating antimicrobial activity: A review, J. Pharm. Anal. 6 (2016) 71–79. https://doi.org/10.1016/j.jpha.2015.11.005.

[20] B. Bonev, J. Hooper, J. Parisot, Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method, J. Antimicrob. Chemother. 61 (2008) 1295–1301. https://doi.org/10.1093/jac/dkn090.

[21] V.R. Krishnamurthi, I.I. Niyonshuti, J. Chen, Y. Wang, A new analysis method for

evaluating bacterial growth with microplate readers, PLoS One. 16 (2021) 1–19. https://doi.org/10.1371/journal.pone.0245205.

[22] S.E. McBirney, K. Trinh, A. Wong-Beringer, A.M. Armani, Wavelength-normalized spectroscopic analysis of Staphylococcus aureus and Pseudomonas aeruginosa growth rates, Biomed. Opt. Express. 7 (2016) 4034. https://doi.org/10.1364/boe.7.004034.

[23] K. Stevenson, A.F. McVey, I.B.N. Clark, P.S. Swain, T. Pilizota, General calibration of microbial growth in microplate readers, Sci. Rep. 6 (2016) 4–10. https://doi.org/10.1038/srep38828.

[24] R. Wang, B. Lorantfy, S. Fusco, L. Olsson, C.J. Franzén, Analysis of methods for quantifying yeast cell concentration in complex lignocellulosic fermentation processes, Sci. Rep. 11 (2021) 1–12. https://doi.org/10.1038/s41598-021-90703-8.

[25] T.A. Mensah, Measurement of Yeast Growth Using, (2018). https://doi.org/10.13140/RG.2.2.15418.03527.

[26] M. Suga, A. Kunimoto, H. Shinohara, Non-invasive, electro-orientation-based viability assay using optically transparent electrodes for individual fission yeast cells, Biosens. Bioelectron. 97 (2017) 53–58. https://doi.org/10.1016/j.bios.2017.05.034.

[27] M.C. Hofmann, M. Funke, J. Büchs, W. Mokwa, U. Schnakenberg, Development of a four electrode sensor array for impedance spectroscopy in high content screenings of fermentation processes, Sensors Actuators, B Chem. 147 (2010) 93–99. https://doi.org/10.1016/j.snb.2010.03.041.

[28] B. Luchterhand, J. Nolten, S. Hafizovic, T. Schlepütz, S.J. Wewetzer, E. Pach, K. Meier, G. Wandrey, J. Büchs, Newly designed and validated impedance spectroscopy setup in microtiter plates successfully monitors viable biomass online, Biotechnol. J. 10 (2015) 1259–1268. https://doi.org/10.1002/biot.201400534.

[29] I. Giaever, C.R. Keese, A morphological biosensor for mammalian cells, Nature. 366 (1993) 591–592. https://doi.org/10.1038/366591a0.

[30] L. Wang, L. Wang, H. Yin, W. Xing, Z. Yu, M. Guo, J. Cheng, Real-time, label-free monitoring of the cell cycle with a cellular impedance sensing chip, Biosens. Bioelectron. 25 (2010) 990–995. https://doi.org/10.1016/j.bios.2009.09.012.

[31] C. Lo, J. Ferrier, Bioimpedance Sensing, Encycl. Nanotechnol. 57 (2016) 257–257.

https://doi.org/10.1007/978-94-017-9780-1_100077.

[32] Y. Xu, X. Xie, Y. Duan, L. Wang, Z. Cheng, J. Cheng, A review of impedance measurements of whole cells, Biosens. Bioelectron. 77 (2016) 824–836. https://doi.org/10.1016/j.bios.2015.10.027.

[33] Q. Hassan, S. Ahmadi, K. Kerman, Recent advances in monitoring cell behavior using cell-based impedance spectroscopy, Micromachines. 11 (2020). https://doi.org/10.3390/MI11060590.

[34] E. Takahashi, Y. Yamaoka, Simple and inexpensive technique for measuring oxygen consumption rate in adherent cultured cells, J. Physiol. Sci. 67 (2017) 731–737. https://doi.org/10.1007/s12576-017-0563-7.

[35] R.J. Aitken, Nitroblue tetrazolium (NBT) assay, Reprod. Biomed. Online. 36 (2018) 90–91. https://doi.org/10.1016/j.rbmo.2017.09.005.

[36] S.C. Hyung, W.K. Jun, Y.N. Cha, C. Kim, A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells, J. Immunoass. Immunochem. 27 (2006) 31–44. https://doi.org/10.1080/15321810500403722.

[37] L. Sandvik, J. Erikssen, E. Thaulow, G. Erikssen, The New England Journal of Medicine Downloaded from nejm.org at MCGILL UNIVERSITY LIBRARY on November 30, 2015. For personal use only. No other uses without permission. From the NEJM Archive. Copyright © 2010 Massachusetts Medical Society. All rights rese, Phys. Fit. as a Predict. Mortal. Men. 328(8) (1993) 2010–2013.

[38] P.D. Kilmer, Review Article: Review Article, Journalism. 11 (2010) 369–373. https://doi.org/10.1177/1461444810365020.

[39] T. Bernas, J.W. Dobrucki, The role of plasma membrane in bioreduction of two tetrazolium salts, MTT, and CTC, Arch. Biochem. Biophys. 380 (2000) 108–116. https://doi.org/10.1006/abbi.2000.1907.

[40] M. V. Berridge, A.S. Tan, Characterization of the Cellular Reduction of 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular Localization, Substrate Dependence, and Involvement of Mitochondrial Electron Transport in MTT Reduction, Arch. Biochem. Biophys. 303 (1993) 474–482. https://doi.org/10.1006/abbi.1993.1311.

[41] J.C. Stockert, R.W. Horobin, L.L. Colombo, A. Blázquez-Castro, Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives, Acta Histochem. 120 (2018) 159–167. https://doi.org/10.1016/j.acthis.2018.02.005.

[42] A. Bahuguna, I. Khan, V.K. Bajpai, S.C. Kang, MTT assay to evaluate the cytotoxic potential of a drug, Bangladesh J. Pharmacol. 12 (2017) 115–118. https://doi.org/10.3329/bjp.v12i2.30892.

[43] P. Ngamwongsatit, P.P. Banada, W. Panbangred, A.K. Bhunia, WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic Bacillus species using CHO cell line, J. Microbiol. Methods. 73 (2008) 211–215. https://doi.org/10.1016/j.mimet.2008.03.002.

[44] C.J. Goodwin, S.J. Holt, S. Downes, N.J. Marshall, Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS, J. Immunol. Methods. 179 (1995) 95–103. https://doi.org/10.1016/0022-1759(94)00277-4.

[45] M. V. Berridge, P.M. Herst, A.S. Tan, Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction, Biotechnol. Annu. Rev. 11 (2005) 127–152. https://doi.org/10.1016/S1387-2656(05)11004-7.

[46] L.M. Yin, Y. Wei, Y. Wang, Y.D. Xu, Y.Q. Yang, Long term and standard incubations of WST-1 reagent reflect the same inhibitory trend of cell viability in rat airway smooth muscle cells, Int. J. Med. Sci. 10 (2012) 68–72. https://doi.org/10.7150/ijms.5256.

[47] J.A. Barltrop, T.C. Owen, A.H. Cory, J.G. Cory, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)te trazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans As cell-viability indica, Bioorganic Med. Chem. Lett. 1 (1991) 611–614. https://doi.org/10.1016/S0960-894X(01)81162-8.

[48] K. Nonami, NII-Electronic Library Service, Chem. Pharm. Bull. 57 (2002) 364–370. http://www.mendeley.com/research/geology-volcanic-history-eruptive-style-yakedake-volcano-group-central-japan/.

[49] A. V. Peskin, C.C. Winterbourn, A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1), Clin. Chim. Acta. 293 (2000) 157–166.

https://doi.org/10.1016/S0009-8981(99)00246-6.

[50] D.A. Scudiero, R.H. Shoemaker, K.D. Paull, A. Monks, S. Tierney, T.H. Nofziger, M.J. Currens, D. Seniff, M.R. Boyd, Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines, Cancer Res. 48 (1988) 4827–4833.

[51] M. V. Berridge, A.S. Tan, Trans-plasma membrane electron transport: A cellular assay for NADH- and NADPH-oxidase based on extracellular, superoxide-mediated reduction of the sulfonated tetrazolium salt WST-1, Protoplasma. 205 (1998) 74–82. https://doi.org/10.1007/BF01279296.

[52] J.C.W. Comley, C.H. Turner, Potential of a soluble tetrazolium/formazan assay for the evaluation of filarial viability, Int. J. Parasitol. 20 (1990) 251–255. https://doi.org/10.1016/0020-7519(90)90107-X.

[53] P.M. Debnam, G. Shearer, Colorimetric assays for substrates of NADP+-dependent dehydrogenases based on reduction of a tetrazolium dye to its soluble formazan, Anal. Biochem. 250 (1997) 253–255. https://doi.org/10.1006/abio.1997.2245.

[54] A. Heiskanen, J. Yakovleva, C. Spégel, R. Taboryski, M. Koudelka-Hep, J. Emnéus, T. Ruzgas, Amperometric monitoring of redox activity in living yeast cells: Comparison of menadione and menadione sodium bisulfite as electron transfer mediators, Electrochem. Commun. 6 (2004) 219–224. https://doi.org/10.1016/j.elecom.2003.12.003.

[55] A. Heiskanen, C. Spégel, N. Kostesha, S. Lindahl, T. Ruzgas, J. Emnéus, Mediator-assisted simultaneous probing of cytosolic and mitochondrial redox activity in living cells, Anal. Biochem. 384 (2009) 11–19. https://doi.org/10.1016/j.ab.2008.08.030.

[56] M. Rahimi, H.Y. Youn, D.J. McCanna, J.G. Sivak, S.R. Mikkelsen, Application of cyclic biamperometry to viability and cytotoxicity assessment in human corneal epithelial cells, Anal. Bioanal. Chem. 405 (2013) 4975–4979. https://doi.org/10.1007/s00216-013-6843-z.

[57] K. Ino, T. Onodera, M.T. Fukuda, Y. Nashimoto, H. Shiku, Combination of Double-Mediator System with Large-Scale Integration-Based Amperometric Devices for Detecting NAD(P)H:quinone Oxidoreductase 1 Activity of Cancer Cell Aggregates, ACS Sensors. 4 (2019) 1619–1625. https://doi.org/10.1021/acssensors.9b00344.

[58] M. Mazloum-Ardakani, B. Barazesh, S.M. Moshtaghioun, M.H. Sheikhha, Designing and

optimization of an electrochemical substitute for the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay, Sci. Rep. 9 (2019) 1–8. https://doi.org/10.1038/s41598-019-51241-6.

[59] G. Gao, D. Fang, Y. Yu, L. Wu, Y. Wang, J. Zhi, A double-mediator based whole cell electrochemical biosensor for acute biotoxicity assessment of wastewater, Talanta. 167 (2017) 208–216. https://doi.org/10.1016/j.talanta.2017.01.081.

[60] K. Stapelfeldt, E. Ehrke, J. Steinmeier, W. Rastedt, R. Dringen, Menadione-mediated WST1 reduction assay for the determination of metabolic activity of cultured neural cells, Anal. Biochem. 538 (2017) 42–52. https://doi.org/10.1016/j.ab.2017.09.011.

[61] V.E. Acceptors, l-Methoxy-5-Methylphenazinium Methyl Sulfate, 1473 (1977) 1469–1473.

[62] P. Kugler, Quantitative dehydrogenase histochemistry with exogenous electron carriers (PMS, MPMS, MB), Histochemistry. 75 (1982) 99–112. https://doi.org/10.1007/BF00492537.

[63] M.A. Alim, M. Suga, H. Shinohara, Rapid and Highly Sensitive Electrochemical Technique for Cell Viability Assay via Monitoring of Intracellular NADH with New Double Mediator System, Electrochemistry. 12 (2021) 2–7. https://doi.org/10.5796/electrochemistry.21-00088.

[64] J.C. Stockert, A. Blázquez-Castro, M. Cañete, R.W. Horobin, Á. Villanueva, MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets, Acta Histochem. 114 (2012) 785–796. https://doi.org/10.1016/j.acthis.2012.01.006.

[65] Y. Matsumae, Y. Takahashi, K. Ino, H. Shiku, T. Matsue, Electrochemical monitoring of intracellular enzyme activity of single living mammalian cells by using a double-mediator system, Anal. Chim. Acta. 842 (2014) 20–26. https://doi.org/10.1016/j.aca.2014.06.047.

[66] H. Tominaga, M. Ishiyama, F. Ohseto, K. Sasamoto, T. Hamamoto, K. Suzuki, M. Watanabe, A water-soluble tetrazolium salt useful for colorimetric cell viability assay, Anal. Commun. 36 (1999) 47–50. https://doi.org/10.1039/a809656b.

[67] L. Fiume, M. Vettraino, M. Manerba, G. Di Stefano, Inhibition of lactic dehydrogenase as a way to increase the anti-proliferative effect of multi-targeted kinase inhibitors, Pharmacol. Res. 63 (2011) 328–334. https://doi.org/10.1016/j.phrs.2010.12.005.

[68] X. Li, W. Lu, Y. Hu, S. Wen, C. Qian, W. Wu, P. Huang, Effective inhibition of nasopharyngeal carcinoma in vitro and in vivo by targeting glycolysis with oxamate, Int. J. Oncol. 43 (2013) 1710–1718. https://doi.org/10.3892/ijo.2013.2080.

[69] S.R. Choi, A.B. Beeler, A. Pradhan, E.B. Watkins, J.M. Rimoldi, B. Tekwani, M.A. Avery, Generation of oxamic acid libraries: Antimalarials and inhibitors of plasmodium falciparum lactate dehydrogenase, J. Comb. Chem. 9 (2007) 292–300. https://doi.org/10.1021/cc060110n.

[70] X. Zhai, Y. Yang, J. Wan, R. Zhu, Y. Wu, Inhibition of LDH-A by oxamate induces G2/M arrest, apoptosis and increases radiosensitivity in nasopharyngeal carcinoma cells, Oncol. Rep. 30 (2013) 2983–2991. https://doi.org/10.3892/or.2013.2735.

[71] R. Moreno-Sánchez, Á. Marín-Hernández, I. Del Mazo-Monsalvo, E. Saavedra, S. Rodríguez-Enríquez, Assessment of the low inhibitory specificity of oxamate, aminooxyacetate and dichloroacetate on cancer energy metabolism, Biochim. Biophys. Acta - Gen. Subj. 1861 (2017) 3221–3236. https://doi.org/10.1016/j.bbagen.2016.08.006.

[72] W.K. Miskimins, H.J. Ahn, J.Y. Kim, S. Ryu, Y.S. Jung, J.Y. Choi, Synergistic anti-cancer effect of phenformin and oxamate, PLoS One. 9 (2014). https://doi.org/10.1371/journal.pone.0085576.

[73] H. Akbari, K. Karimi, M. Lundin, M.J. Taherzadeh, Optimization of baker's yeast drying in industrial continuous fluidized bed dryer, Food Bioprod. Process. 90 (2012) 52–57. https://doi.org/10.1016/j.fbp.2010.12.005.

[74] M. Ciani, F. Comitini, I. Mannazzu, P. Domizio, Controlled mixed culture fermentation: A new perspective on the use of non-Saccharomyces yeasts in winemaking, FEMS Yeast Res. 10 (2010) 123–133. https://doi.org/10.1111/j.1567-1364.2009.00579.x.

[75] E.J. Lodolo, J.L.F. Kock, B.C. Axcell, M. Brooks, The yeast Saccharomyces cerevisiae - The main character in beer brewing, FEMS Yeast Res. 8 (2008) 1018–1036. https://doi.org/10.1111/j.1567-1364.2008.00433.x.

[76] W.A. Elkhateeb, M. Akram, Yeast as Biotechnological Tool in Food Industry Yeast as Biotechnological Tool in Food Industry, (2021). https://doi.org/10.23880/oajpr-16000243.

[77] C.C. Tonon, R.S. Francisconi, E.A.F. Bordini, P.M.M. Huacho, J. de C.O. Sardi, D.M.P. Spolidorio, Interactions between terpinen-4-ol and nystatin on biofilm of Candida

albicans and Candida tropicalis, Braz. Dent. J. 29 (2018) 359–367. https://doi.org/10.1590/0103-6440201802073.

[78] Y. Viability, M. With, Determine yeast concentration and viability at your benchtop, BioProbes. 4 (2012) 28–29.

[79] B.E. Teleky, D.C. Vodnar, Biomass-derived production of itaconic acid as a building block in specialty polymers, Polymers (Basel). 11 (2019). https://doi.org/10.3390/polym11061035.

[80] C.S. Marvel, T.H. Shepherd, Polymerization Reactions of Itaconic Acid and Some of Its Derivatives, J. Org. Chem. 24 (1959) 599–605. https://doi.org/10.1021/jo01087a006.

[81] Á.P. Molnár, Z. Németh, I.S. Kolláth, E. Fekete, M. Flipphi, N. Ág, Á. Soós, B. Kovács, E. Sándor, C.P. Kubicek, L. Karaffa, High oxygen tension increases itaconic acid accumulation, glucose consumption, and the expression and activity of alternative oxidase in Aspergillus terreus, Appl. Microbiol. Biotechnol. 102 (2018) 8799–8808. https://doi.org/10.1007/s00253-018-9325-6.

[82] S. Krull, L. Eidt, A. Hevekerl, A. Kuenz, U. Prüße, Itaconic acid production from wheat chaff by Aspergillus terreus, Process Biochem. 63 (2017) 169–176. https://doi.org/10.1016/j.procbio.2017.08.010.

[83] L. van der Straat, M. Vernooij, M. Lammers, W. van den Berg, T. Schonewille, J. Cordewener, I. van der Meer, A. Koops, L.H. De Graaff, Expression of the Aspergillus terreus itaconic acid biosynthesis cluster in Aspergillus niger, Microb. Cell Fact. 13 (2014). https://doi.org/10.1186/1475-2859-13-11.

[84] M. Okabe, D. Lies, S. Kanamasa, E.Y. Park, Biotechnological production of itaconic acid and its biosynthesis in Aspergillus terreus, Appl. Microbiol. Biotechnol. 84 (2009) 597–606. https://doi.org/10.1007/s00253-009-2132-3.

[85] A.H. Hossain, A. Li, A. Brickwedde, L. Wilms, M. Caspers, K. Overkamp, P.J. Punt, Rewiring a secondary metabolite pathway towards itaconic acid production in Aspergillus niger, Microb. Cell Fact. 15 (2016) 1–15. https://doi.org/10.1186/s12934-016-0527-2.

[86] T. Boruta, M. Bizukojc, Production of lovastatin and itaconic acid by Aspergillus terreus: a comparative perspective, World J. Microbiol. Biotechnol. 33 (2017) 1–12. https://doi.org/10.1007/s11274-017-2206-9.

[87] F. Carstensen, T. Klement, J. Büchs, T. Melin, M. Wessling, Continuous production and recovery of itaconic acid in a membrane bioreactor, Bioresour. Technol. 137 (2013) 179–187. https://doi.org/10.1016/j.biortech.2013.03.044.

[88] Y. Xu, Z. Li, Utilization of ethanol for itaconic acid biosynthesis by engineered Saccharomyces cerevisiae , FEMS Yeast Res. 21 (2021) 1–13. https://doi.org/10.1093/femsyr/foab043.

[89] V. Lampropoulou, A. Sergushichev, M. Bambouskova, S. Nair, E.E. Vincent, E. Loginicheva, L. Cervantes-Barragan, X. Ma, S.C.C. Huang, T. Griss, C.J. Weinheimer, S. Khader, G.J. Randolph, E.J. Pearce, R.G. Jones, A. Diwan, M.S. Diamond, M.N. Artyomov, Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation, Cell Metab. 24 (2016) 158–166. https://doi.org/10.1016/j.cmet.2016.06.004.

[90] T. Cordes, M. Wallace, A. Michelucci, A.S. Divakaruni, S.C. Sapcariu, C. Sousa, H. Koseki, P. Cabrales, A.N. Murphy, K. Hiller, C.M. Metallo, Immunoresponsive gene 1 and itaconate inhibit succinate dehydrogenase to modulate intracellular succinate levels, J. Biol. Chem. 291 (2016) 14274–14284. https://doi.org/10.1074/jbc.M115.685792.

[91] T. Cordes, C.M. Metallo, Itaconate alters succinate and coenzyme a metabolism via inhibition of mitochondrial complex II and methylmalonyl-coa mutase, Metabolites. 11 (2021) 1–14. https://doi.org/10.3390/metabo11020117.

[92] A. Michelucci, T. Cordes, J. Ghelfi, A. Pailot, N. Reiling, O. Goldmann, T. Binz, A. Wegner, A. Tallam, A. Rausell, M. Buttini, C.L. Linster, E. Medina, R. Balling, K. Hiller, Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 7820–7825. https://doi.org/10.1073/pnas.1218599110.

[93] L.A.J. O'Neill, A Broken Krebs Cycle in Macrophages, Immunity. 42 (2015) 393–394. https://doi.org/10.1016/j.immuni.2015.02.017.

[94] B.A. McFadden, S. Purohit, Itaconate, an isocitrate lyase directed inhibitor in Pseudomonas indigofera, J. Bacteriol. 131 (1977) 136–144. https://doi.org/10.1128/jb.131.1.136-144.1977.

[95] B.X.C. Kwai, A.J. Collins, M.J. Middleditch, J. Sperry, G. Bashiri, I.K.H. Leung,

Itaconate is a covalent inhibitor of theMycobacterium tuberculosisisocitrate lyase, RSC Med. Chem. 12 (2021) 57–61. https://doi.org/10.1039/d0md00301h.

[96] C. Nastasi, A. Willerlev-Olsen, K. Dalhoff, S.L. Ford, A.S.Ø. Gadsbøll, T.B. Buus, M. Gluud, M. Danielsen, T. Litman, C.M. Bonefeld, C. Geisler, N. Ødum, A. Woetmann, Inhibition of succinate dehydrogenase activity impairs human T cell activation and function, Sci. Rep. 11 (2021) 1–13. https://doi.org/10.1038/s41598-020-80933-7.

[97] W.W. Ackermann, V.R. Potter, Enzyme Inhibition in Relation to Chemotherapy, Proc. Soc. Exp. Biol. Med. 72 (1949) 1–9. https://doi.org/10.3181/00379727-72-17313.

[98] F.C. Odds, A.J.P. Brown, N.A.R. Gow, Antifungal agents: Mechanisms of action, Trends Microbiol. 11 (2003) 272–279. https://doi.org/10.1016/S0966-842X(03)00117-3.

[99] H. Lee, D.G. Lee, Novel approaches for efficient antifungal drug action, J. Microbiol. Biotechnol. 28 (2018) 1771–1781. https://doi.org/10.4014/jmb.1807.07002.

[100] A. Ogita, K.I. Fujita, T. Tanaka, Enhancing effects on vacuole-targeting fungicidal activity of amphotericin B, Front. Microbiol. 3 (2012) 1–6. https://doi.org/10.3389/fmicb.2012.00100.

[101] S.A.A. Mousavi, G.D. Robson, Oxidative and amphotericin B-mediated cell death in the opportunistic pathogen Aspergillus fumigatus is associated with an apoptotic-like phenotype, Microbiology. 150 (2004) 1937–1945. https://doi.org/10.1099/mic.0.26830-0.

[102] K.I. Fujita, M. Tatsumi, A. Ogita, I. Kubo, T. Tanaka, Anethole induces apoptotic cell death accompanied by reactive oxygen species production and DNA fragmentation in Aspergillus fumigatus and Saccharomyces cerevisiae, FEBS J. 281 (2014) 1304–1313. https://doi.org/10.1111/febs.12706.

[103] B.M.E. Hayes, M.A. Anderson, A. Traven, N.L. Van Der Weerden, M.R. Bleackley, Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins, Cell. Mol. Life Sci. 71 (2014) 2651–2666. https://doi.org/10.1007/s00018-014-1573-8.

[104] X. Lyu, C. Zhao, Z.M. Yan, H. Hua, Efficacy of nystatin for the treatment of oral candidiasis: A systematic review and meta-analysis, Drug Des. Devel. Ther. 10 (2016) 1161–1171. https://doi.org/10.2147/DDDT.S100795.

[105] B. Chudzik, M. Koselski, A. Czuryło, K. Trębacz, M. Gagoś, A new look at the antibiotic

amphotericin B effect on Candida albicans plasma membrane permeability and cell viability functions, Eur. Biophys. J. 44 (2015) 77–90. https://doi.org/10.1007/s00249-014-1003-8.

[106] A.J. Phillips, I. Sudbery, M. Ramsdale, Apoptosis induced by environmental stresses and amphotericin B in Candida albicans, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 14327–14332. https://doi.org/10.1073/pnas.2332326100.

[107] R.S. Al-Dhaheri, L.J. Douglas, Apoptosis in Candida biofilms exposed to amphotericin B, J. Med. Microbiol. 59 (2010) 149–157. https://doi.org/10.1099/jmm.0.015784-0.

[108] B. Almeida, A. Silva, A. Mesquita, B. Sampaio-Marques, F. Rodrigues, P. Ludovico, Drug-induced apoptosis in yeast, Biochim. Biophys. Acta - Mol. Cell Res. 1783 (2008) 1436–1448. https://doi.org/10.1016/j.bbamcr.2008.01.005.

[109] K. Baronian, A. Downard, R. Lowen, N. Pasco, Detection of two distinct substrate-dependent catabolic responses in yeast cells using a mediated electrochemical method, Appl. Microbiol. Biotechnol. 60 (2002) 108–113. https://doi.org/10.1007/s00253-002-1108-3.

[110] M. Lou Fultz, R.A. Durst, Mediator compounds for the electrochemical study of biological redox systems: a compilation, Anal. Chim. Acta. 140 (1982) 1–18. https://doi.org/10.1016/S0003-2670(01)95447-9.

The End