Rapid and Highly Sensitive Electrochemical Technique for Cell Viability Assay via Monitoring of Intracellular NADH with New Double Mediator System

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ABSTRACT

It is very important to assess cell viability rapidly and sensitively for the cell biology research, medical and pharmaceutical application. Compared to conventional methods, we have established a new rapid and sensitive bio-electrochemical system using small screen-printed carbon electrode (SPCE) and 1-methoxy-5-methylphenazinium methyl sulfate (mPMS)/[Fe(CN)6]3−(FeCN) as double electron mediators for monitoring cell viability through the measurement of intracellular NADH. A combination of mPMS and FeCN system works as useful as previously reported menadione (Mena)/FeCN system. We confirmed that the electron transfer from intracellular NADH to mPMS occurred non-enzymatically, though the electron transfer from intracellular NADH to Mena was catalyzed by cytosolic enzyme. We applied our system to count the three kinds of mammalian cells. The illustration of the measurement principle of mPMS system is shown in Scheme 1. Furthermore, we have applied this method to evaluate the acute cytotoxicity of oxamic acid on cytoma cells. Only 10 min incubation and high sensitivity embellished this method. These results strongly supported that our electrochemical method might be potent to alternate to WST assay for cell viability and acute cytotoxicity test.

1. Introduction

Rapid, precise quantitative estimation of cell viability is the prerequisite for evaluation of cytotoxic and toxicological properties of a large number of chemicals/drugs to various cells as an alternative to animals. Over the past decades, different types of methods are prevalent for dead and alive assay. Traditionally, trypan blue dye exclusion method,1–4 resazurin base assay,5 and neutral red up take assay6 and fluorometric method with double staining dyes7 are used for cell viability determination. These methods are based on the principle where the cell membrane is destroyed or not. On the other hand, the cell viability assessment is also carried out through the measurement of intracellular metabolic activity. Intracellular NADH is an important indicator for sensing metabolic activity. Previously, the first generation of cell membrane permeable methyl-thiazolyl-tetrazolium (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.8,9

Recently, a new generation cell membrane impermeable tetrazolium dye such as water soluble tetrazolium salts (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.10,11 In this case, hydrophobic phenazinium cation works as a electron shuttle across the cell membrane. These colorimetric assays are easy and useful to count the cell and cytotoxicity test. But its standard incubation time is two hours (h) which is time consuming.12 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.13–19 They have been used menadione (Mena) as a lipophilic cell membrane permeable electron mediator and [Fe(CN)6]3−(FeCN) as a hydrophilic extracellular electron mediator in double mediator system (Mena/FeCN). In this system, electron transfer from intracellular NADH to Mena is catalyzed by cytosolic NAD(P)H oxidoreductase.20 On the other hand, it has also been reported that the electron transfer from intracellular NADH to mPMS occurred by non-enzymatically.20,21 So we 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.

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

So, herein we 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, we have applied this double mediator system to evaluate the acute cytotoxicity of oxamic acid on cytoma cells (PC12 cell).

2. Experimental

2.1 Materials

PC12 (rat pheochromocytoma) cell and C6 (rat glioma) cell were...
obtained from the JCRB cell Bank, National Institute of Biomedical Innovation, Health and Nutrition (Osaka, Japan) and RBL-2H3 (rat basophilic leukemia) cell was obtained from the cell bank of RIKEN Bio Resource Center (Tsukuba, Japan). Dulbecco’s modified Eagle’s medium (DMEM), Eagle minimal essential medium (EMEM), Phosphate buffer saline powder (PBS), penicillin/streptomycin, and horse serum (HS) were obtained from Gibco (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from ICN Biomedicals (California, United States). Hanks’ balanced salts (HBS) powder was obtained from Sigma–Aldrich (Tokyo, Japan). 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 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°C under 5% CO₂ for 60 min, the formation of corresponding formazan was measured by absorption at 450 nm with a micro plate reader (Promega GloMax Multi+ detection system).

2.5 Cell counting by WST-1 cell counting kit (CCKit-1)

Firstly, 5.5 mM WST-1 salt and 2 mM mPMS solution were prepared. After WST-1 was added, 0.2 mM mPMS was added and again incubated for 15 min. The ratio of number of PI stained cells against number of total cells was calculated as the ratio of number of PI stained cells against number of total cells.

2.6 Evaluation of cytotoxicity of mPMS and K₃[Fe(CN)₆] by PI staining method

100 µL of cell suspension (3 × 10⁵ 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 min incubation at 37°C under 5% CO₂, 20 µL PI (final conc.1.5 µM) was added and again incubated for 15 min. Every case, total volume of experimental cell suspension was maintained to 150 µL with DMEM or HBSS. Finally, 10 µL cell suspensions were applied on the hemocytometer. A photograph of cells under the fluorescence microscopic observation and PI stained cells (dead cells) were counted for determining the cell viability.

2.7 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 mm²), a carbon paste counter electrode and an Ag/AgCl reference electrode.

For single mediator system, 100 µL suspension (3 × 10⁵ cells/well) with HBSS was taken in wells of a 96 well plate and added 10 µM mPMS (final conc.). In every case, total volume of experimental cell suspension was maintained to 150 µL. After incubation for 10 min, a SPCE was vertically immersed into the cell suspension and chronoamperometry was immediately performed by the potential application at +0.1 V to +0.5 V to observe the WST-1 reduction capability.

To observe 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 case, 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 cytotoxic NADPH oxidoreductase in the lysate supernatant, lysate supernatant was heated with 90°C for 7 min to induce the enzyme activity loss.

After reaching 70% confluence, the adherent cells were detached from the bottom of the culture flask by 0.05% trypsin-EDTA treatment. The harvested cells were collected by centrifugation. To avoid the unwanted electrochemical oxidation of interference compounds such as Vitamin C contained in the culture medium, uric acid released from cells etc., cells were washed with HBSS and resuspended 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.

After cultivating, PC12 cell were washed with PBS and resuspended with 120 µL PBS (5.5 × 10⁶ cells/mL). Cells were frozen in the refrigerator at −28°C for 30 min and just melted in water bath at 37°C. This freezing 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.

For single mediator system, 100 µL cell suspension (3 × 10⁵ cells/well) with HBSS was taken in wells of a 96 well plate and added 10 µM mPMS (final conc.). In every case, total volume of experimental cell suspension was maintained to 150 µL with DMEM or HBSS. Finally, 10 µL cell suspensions were applied on the hemocytometer. 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.

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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 s to keep the electrode surface state the same.

2.8 Electrochemical cell counting

Various densities of cell suspensions were plated in different wells and final concentration of 500 µM FeCN and 10 µM mPMS solutions were added to each well. Total volume of experimental cell suspension was 150 µL. After incubation for 10 min, 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.

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

In this study, PC12 cell suspension (in HBSS) was taken (3 × 10⁵ 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 h incubation, 15 µL FeCN (final conc. 500 µM) and 15 µL mPMS (final conc. 10 µM) solutions were added into each well. Every case, total volume of experimental suspension was maintained to 150 µL with HBSS. Additionally, after 10 min incubation at 37 °C under 5% CO₂, chronoamperometric measurement was performed to evaluate the cytotoxicity of the oxamic acid.

3. Results and Discussion

3.1 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.²⁵ In this study, we have confirmed by our results that mPMS takes electron from NADH non-enzymatically as shown in Fig. S1B. So, firstly we have tried to use mPMS as a single mediator. After 10 min incubation of PC12 cells (3 × 10⁵ 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. 1A. We considered that dissolved O₂ easily took electron from the reduced mPMS to produce H₂O₂ and this was the reason why the oxidation current did not increase even though cells existed.

Next, we tried to apply +0.5 V to check H₂O₂ production in this single mediator system. In this case, the oxidation current increased a little as compared to control as shown in Fig. 1B. So, these data suggested that dissolve oxygen interfere effective sensing of intracellular NADH in the single mediator system.

As a way to circumvent this problem, we considered the reduction of second mediator FeCN which was useful as same as Mena/FeCN double mediator system.¹³⁻¹⁶ Because of high the stability and enough concentration of [Fe(CN)₆]⁴⁻, the electron transfer reaction between reduced form of mPMS and [Fe(CN)₆]⁴⁻ might be faster than between reduced form of mPMS and dissolved O₂. As a result, ferrocyanide, [Fe(CN)₆]⁴⁻ was accumulated outside of cells as time goes on. We therefore measured the accumulated [Fe(CN)₆]⁴⁻ by cyclic voltammetry in our double mediator system in the presence of cells and observed the oxidation current increase as compared to control as shown in Fig. S2 which supported that the Scheme I might be suitable to detect the cell viability.

We considered to use chronoamperometry for more effective measurement of the accumulated [Fe(CN)₆]⁴⁻. From the voltammo-

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Figure 1. Chronoamperograms for monitoring the intracellular NADH in PC12 cells. (A) and (B) Single mediator system; PC12 cells (3 × 10⁵ cells/well) were incubated for 10 min 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 × 10⁵ cells/well) were incubated for 10 min 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.
ments were done by chronoamperometric method to monitor the intracellular NADH.

3.2 Optimization of measurement conditions with the double mediator system

3.2.1 Cytotoxicity assay of mPMS and K₃[Fe(CN)₆]₆

In order to establish the new double mediator system, we examined the cytotoxicity of mediators on PC12 cells by using PI. The result demonstrated that more than 50 µM mPMS was toxic for cells (Fig. S3A). We 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. S3B).

3.2.2 Optimization of mediator’s concentration

We optimized the concentration of mPMS to obtain the maximum current based on the effective oxidation of intracellular NADH. The PC12 cells (3 × 10⁵ 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 min 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 s. 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. S4) 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, we assumed that 10 µM mPMS and 500 µM FeCN was sufficient to monitor the intracellular NADH for this method. Further we optimized the FeCN concentration. After incubation of PC12 cells (3 × 10⁵ cells/well) with 10 µM mPMS and various concentrations of FeCN at 37 °C under 5 % CO₂ for 10 min, the chronoamperometric current was measured. The oxidation current with10 µM mPMS and 500 µM FeCN was maximum (Fig. S5) and therefore, we decided these concentrations of the double mediators were best combination for the electrochemical measurement of intracellular NADH.

3.2.3 Optimization of incubation time for cells with the double mediators

The PC12 cells (3 × 10⁵ cells/well) were taken in wells of a 96 well plate and the double mediators (final conc.: 500 µM FeCN and 10 µM mPMS) was added sequentially from first to last well with 300 s 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 s 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 s incubation (Fig. S6). This data demonstrated that 600 s incubation is enough to facilitate mPMS mediated intra and extra-cellular reactions. 10 min was therefore chosen as the optimized incubation time and further all electrochemical measurements were done after 10 min incubation of cells with the double mediators.

3.2.4 Comparison of mPMS/FeCN double mediator system with Mena/FeCN double mediator system

We have compared our mPMS/FeCN double mediator system with Mena/FeCN double mediator system for monitoring intracellular NADH through the oxidation of accumulated [Fe(CN)]₄³⁻. From Fig. 2, it was observed that the oxidation current response from accumulated [Fe(CN)]₄³⁻ of both systems were almost similar. So, we would like to say our mPMS/FeCN system also works well as same as Mena/FeCN system to monitor intracellular NADH.

3.3 Cell counting by electrochemical method using our double mediator system

At first, cell counting of PC12 cells was performed by electrochemical measurement using 10 µM mPMS and 500 µM FeCN system. Figure 3A shows the chronoamperometric profiles to various densities of cell suspensions. The oxidation current increased proportionally as increasing number of PC12 cells ranging from 9000 to 600 000 cells/well as shown in Fig. 3B.

On the other hand, in the case of WST-1 assay, it was clearly seen that PC12 cell number ranging from 9000 to 300 000 cells/well showed the linear relationship with absorbance at 450 nm but more than 300 000 cells/well, the curve became saturation. Therefore, we 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 our double mediator system.

To examine the applicability of this double mediator system, we have further applied this method to count the C6 cells and RBL-2H3 cells under same conditions. Figures S7 and S8 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 600 000 cells/well. From these results, our 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.
3.4 Evaluation of acute inhibition of metabolic activity of PC12 cell by oxamic acid

Oxamic acid is a well-known inhibitor of lactate dehydrogenase (LDH) and thus significantly affects the pyruvate to lactate metabolism.23–26 LDH is a principal enzyme which catalyzes the transformation of pyruvate to lactate linked to redox reaction of dinucleotide coenzyme found in human and all eukaryotic cells. Recently, LDH inhibition by oxamic acid has further taken a lot of attention for anti-cancer drug.26–28 So in this study we applied our electrochemical method to monitor the rapid decrease of intracellular NADH in PC12 cells by LDH inhibition. Figure 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 h by oxamic acid treatment. This result corresponded with the report by Zhai et al.26 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 we have just investigated the acute toxic effect of oxamic acid on metabolic activity in PC12 cell for one h by blocking LDH. In near future, we will investigate the incubation-time dependent cytotoxicity of oxamic acid on cytoma cells. Furthermore, we would like to evaluate the various types of acute cytotoxicity such as inhibition of respiratory chain, protein and DNA synthesis etc., by our electrochemical method.

4. Conclusion

In this study, we have successfully developed a rapid and highly sensitive double mediator system for electrochemical monitoring of intracellular NADH in various mammalian cells. We have optimized the mediator’s concentration, incubation time and applied potential for monitoring the intracellular NADH by chronoamperometry. We also confirmed our mPMS system worked as well as previously used Mena/FeCN system and our 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, our electrochemical method was able to count the mammalian cells with wider range within 10 min. It was further demonstrated that our 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 we can fabricate multi-plate electrode array system, our method may be alternate to WST assay for rapid and high-throughput cell viability and acute cytotoxicity test.

Authors Contribution

Mohammad Abdul Alim: This is my Ph.D. research work. (Lead) Minoru Suga: Data curation (Supporting) Hiroaki Shinohara: Supervision (Lead)
Data Availability Statement

The data that support the findings of this study are openly available under the terms of the designated Creative Commons License in J-STAGE Data at https://doi.org/10.50892/data.

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