Electrochemical Measurement of Attached Animal Cell Density in Hydroxyapatite-pulp Composite Fiber Sheet by Flow Injection Analysis Using 2,3,5-Trimethyl-1,4-benzoquinone

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Abstract

An Amperometric flow injection system to measure the density of attached animal cells in hydroxyapatite-pulp composite fiber (HAPC) sheets was developed. 2,3,5-Trimethyl-1,4-benzoquinone (BQ) was injected into the HAPC sheet module containing animal cells as a mediator. 2,3,5-Trimethyl-1,4-hydroquinone, which was produced by the reduction process of animal cells, was monitored amperometrically with a glassy carbon electrode. The carrier solution used was Dulbecco's phosphate buffered saline. A good linear relationship between the peak current and attached cell density was obtained in a range of 8.3×10^5 - 6.1×10^6 cells/sheet. The propose method can be applied for the measurement of cell density in a range of 8.3×10^5 - 1.6×10^7 cells/sheet. The peak current remained steady for 10 successive injections of 2,3,5-trimethyl-1,4-BQ at a concentration of 10 µM. Cytotoxicity due to 2,3,5-trimethyl-1,4-BQ was hardly observed at a concentration of less than 20 µM. This analytical system can be used for monitoring the cell density in bioreactors during the period in which the cells proliferate in the logarithmic phase.

Key-words: flow injection analysis, electrochemical measurement, animal cell, density, hydroxyapatite, quinone

1. Introduction

Continuous culture with immobilized animal cells is the most common way to reach high cell density and thus high productivity in a bioreactor [1]. In addition, immobilization of animal cells in a three-dimensional environment protects them from shear effect [2], which can improve the stability of the culture. Thus, various microcarriers, hollow-fibers and encapsulations have been developed as supports for cell attachment in the continuous culture with immobilized animal cells [3].

Recently, the hydroxyapatite-pulp composite fiber (HAPC) sheet has been developed and applied to the continuous culture of immobilized animal cells [4,5]. Since the entire surface of the HAPC sheet is coated with hydroxyapatite, the sheet has a high biocompatibility. In addition, the HAPC sheet has a suitable three-dimensional environment for cell growth because there are many spaces 10 - 200 µm in diameter in the sheet. Continuous culture of immobilized animal cells using the HAPC sheet can achieve high cell density and thus high productivity in a bioreactor [6].

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Accurate monitoring and control of processes involving immobilized animal cells are important to reach high cell density and high productivity. Among the parameters to monitor, the cell density is a variable of the highest importance [7,8]. Measurement of the cell density is required to determine all specific rates and to calculate mass balances, and it is used as a variable to establish process control strategies. Further, measurement of the cell density provides additional information on the physiological state of the cells, and thus enables indirect product quality control. For these reasons on-line monitoring of cell density is extremely important for high cell density process control.

Adherent cells are generally quantified by direct or automated counting after their detachment from the supports by trypsinization. Alternatively, cell nuclei may be counted after disruption of cells by citric acid. However, neither method is applicable to on-line measurement purposes.

Recently, techniques based on the metabolism of chemical mediators such as naphthoquinones (NQs) for the measurement of cell density in a plastic dish and a multiwell plate have been reported [9]. Although this method is very convenient and simple, it does not take into consideration the necessity to protect animal cells from the toxicity of quinones during measurement. Therefore, this method is also not applicable to the on-line measurement of cell density in a bioreactor.

We have developed an electrochemical method for

measuring attached cell density in the HAPC sheet using 2,3,5-trimethyl-1,4-benzoquinone (BQ) by a flow injection analysis (FIA) system. 2,3,5-Trimethyl-1,4-BQ was reduced to the corresponding hydroquinone by animal cells, and then the hydroquinone produced was detected amperometrically on a glassy carbon electrode as shown in Fig. 1. The cells are protected from the toxicity of quinones by limiting its concentration and the reaction time. The cytotoxicity of 2,3,5-trimethyl-1,4-BQ is hardly observed at a concentration of less than 20 μM. The proposed FIA system was applied to an off-line measurement of attached cell density in the HAPC sheet in continuous cell cultivation.
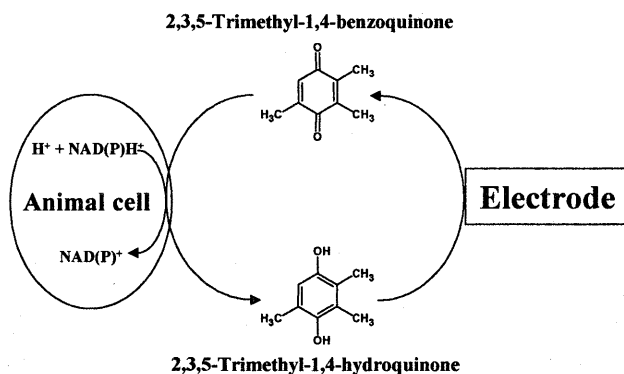
2,3,5-Trimethyl-1,4-benzoquinone

2,3,5-Trimethyl-1,4-hydroquinone

Fig. 1 Electrochemical detection of hydroquinone generated by animal cells.

2. Experimental

2.1. Reagents

1,4-BQ and 2,6-dimethyl-1,4-BQ were bought from Sigma Chemicals (St. Louis, MO, USA). The other BQs and NQs were bought from Tokyo Kasei Co. (Tokyo, Japan). 2,3,5-Trimethyl-1,4-hydroquinone and 2,3-dimethyl-1,4-hydroquinone were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical reagent grade and were used without further purification.

2,3,5-Trimethyl-1,4-BQ and 2,3-dimethyl-1,4-BQ were obtained by oxidation of 2,3,5-trimethyl-1,4-hydroquinone and 2,3-dimethyl-1,4-hydroquinone, respectively, with PbO_2 [10].

2.2. Cell and methods of cell culture

Experiments were performed using a Chinese hamster ovary cell line (CHO-K1, Riken Gene Bank, RGB0285) as the animal cell. The cells were cultured in 60 mm plastic dishes (Nalge Nunc, Rochester, NY, USA) containing Ham's F12 medium (Gibco BRL, Life Technology, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmosphere of 5% CO_2 / 95% air.

2.3. Preparation of the HAPC sheet

HAPC was synthesized by the hydroxyapatite liquid reaction [4,5]. Because the core of the HAPC obtained is pulp fiber, it is easy to prepare sheets from the HAPC by paper-making techniques. The HAPC sheet (0.4 mm thickness) was then cut into small disks (25 mm i.d.) and used for the cultivation of cells.

2.4. Immobilization and cultivation of cells in HAPC sheet

An HAPC sheet was washed with distilled water 3 times and autoclaved in distilled water at 121°C for 20 min. The sheet was then washed with culture medium 3 times and placed in a 60 mm plastic dish for static culture. After inoculating the HAPC sheet with 3 × 10^5 cells, 7 ml of Ham's F12 medium supplemented with 10% FBS was added to the dish. The cells were cultured in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. The culture medium was changed routinely every 2 days by transferring the sheet into a new dish containing 7 ml of fresh culture medium.

2.5. Measurement of attached cell density in HAPC sheet by flow injection analysis system

A schematic diagram of the FIA system is shown in Fig. 2. The carrier solution in a reservoir was propelled by a micro-tube pump (MP-3, Tokyo Rikakikai, Tokyo) through an air-damper, an injector (Select Pro, Alltech, KY, USA), and the HAPC sheet module containing CHO-K1 cells, then transported to an electrochemical flow-through detector (ECD-300, EICOM, Kyoto) and finally to a waste tank. The electrochemical flow-through cell consists of glassy carbon disk (12 mm in diameter), Ag/AgCl, and stainless tube (SUS-316) as working, reference, and counter electrodes, respectively. Surface of the working electrode is polished before the measurement. Dulbecco's phosphate buffered saline was used as the carrier solution. The mediator flow system consisted of another micro-tube pump connected to the injector with a sample loop (500 μl). Amperometric measurements were run by applying each fixed potential for mediators at the working electrode vs. Ag/AgCl. Each applied potential was set at a more positive value than that of the anodic peak observed by cyclic voltammetry experiments. Cyclic voltammetry experiments were run at 100 mV/sec as a potential scan rate (Fig. 3). The potential was set at +250 mV vs. Ag/AgCl for 2,3,5-trimethyl-1,4-BQ.
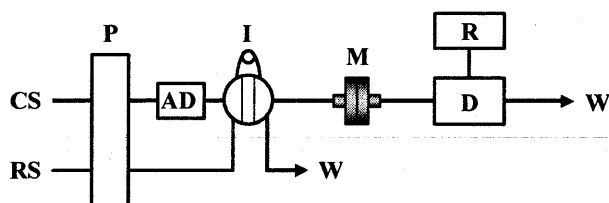

Fig. 2 Schematic diagram of the flow injection system for the measurement of attached animal cells in the HAPC sheet. CS: Dulbecco's phosphate buffered saline; P: micro-tube pump; AD: air-damper; RS: mediator; I: injector; M: module; D: detector; R: recorder; W: waste

2.6. Measurement of cell number and cell viability

CHO-K1 cells in a plastic dish and in the HAPC sheet were detached by treatment with 0.2% trypsin / 0.02% EDTA (Gibco), and suspended in 4 ml of culture medium. Then, cell numbers were determined by counting cells under a phase-contrast microscope.

CHO-K1 cells were incubated in fresh culture medium containing 0 - 100 μM mediators for 24 h in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. The cell viability

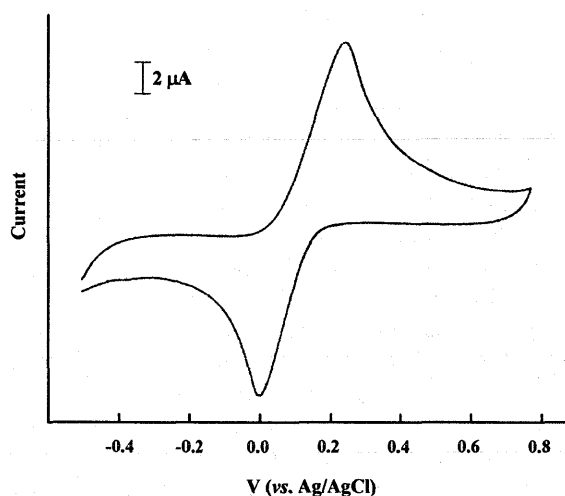was determined with the method developed by Ishiyama *et al.* [11].

Fig. 3 Cyclic voltammograms obtained with a glassy carbon electrode in the presence of 2,3,5-trimethyl-1,4-hydroquinone.

2.7. Bioreactor system

The HAPC sheet is used in a module for the continuous cultivation of animal cells. A stainless steel folder is used as the module. The stainless steel folder provides a structure into which 25 mm diameter circle of HAPC sheet are inserted. The culture medium in a bottle is propelled by a circulation pump through the HAPC sheet from a surface side to another filling sheet inner space, and then transported to silicon tube for the gas exchange, finally returned to the medium bottle (Cell Max, NBS Biologicals Ltd., UK). The CHO-K1 cells can be cultured continuously over a month with the cultivation system [6]. The cell density in the module was ca. 8.0×10^7 cells/sheet at maximum.

3. Results and discussion

3.1. Comparison of mediator

It is well known that quinones are metabolized by animal, plant and microbial cells through the reaction of intracellular DT-diaphorase with NADH [12]. Therefore, various derivatives of BQ or NQ were applied to the measurement of the CHO-K1 cell density. The density of cells attached in the HAPC sheets was measured by the FIA system, as shown in Fig. 2, using 20 μM solutions of various mediators. Mediators were diluted with Dulbecco's phosphate buffered saline. The HAPC sheets containing the cells were prepared by static culture in plastic dishes for a week. These sheets were inserted into a stainless steel folder and then connected to the flow line. Figure 4 shows the effect of mediators on the metabolism efficiency of the cells. Several BQs gave relatively high peak currents; in particular, the maximum peak current was obtained when 1,4-BQ was used. However, the peak currents decreased with the repetition of injection of 1,4-BQ as well as 2-methyl-1,4-BQ. Although the peak currents produced by 2,6-dimethyl-1,4-BQ and 2,3,5-trimethyl-1,4-BQ were smaller than those produced by 1,4-BQ, stable peak currents were obtained. On the other hand, only slight peak currents were obtained when 1,4-NQ or 2-methyl-1,4-NQ was used. This

reason is that naphthohydroquinones produced from NQs by the cells were rapidly oxidized in the presence of oxygen molecules. In contrast, hydroquinones generated from BQs by the cells are relatively stable under aerobic conditions. It appears likely that these differences are related to the structural properties of the reduced forms of these quinones.

3.2. Cytotoxicity

The proposed FIA system can be used for monitoring cell density in bioreactors. Therefore, it is necessary to protect animal cells from the toxicity of quinones during measurement of cell density. We examined the cytotoxicity of several mediators using CHO-K1 cells. 1,4-BQ, 2-methyl-1,4-BQ, 2,6-dimethyl-1,4-BQ, 2,3,5-trimethyl-1,4-BQ, and 2-methyl-1,4-NQ were evaluated as mediators. The cytotoxicity as a function of the concentration of the mediators was quantified by measuring cell viability. After incubation for 24 h, approximately 100% of the cells survived with 2,3,5-trimethyl-1,4-BQ at concentrations up to 20 μM while cytotoxicity was observed with 2-methyl-1,4-NQ, at a concentration as low as 5 μM, as shown in Fig. 5. The mechanism of growth inhibition and cell killing by 2-methyl-1,4-NQ is not well understood. Oxidative stress is considered to be a mechanism of action of quinoid compounds such as 2-methyl-1,4-NQ, because toxic oxygen species can be generated during redox cycling involving the quinoid structures [13]. Cytotoxicity was observed with other BQs at a concentration of 20 μM. Another possible mechanism of toxicity of these BQs is thought to be the direct arylation of cellular thiols resulting in depletion of glutathione and inhibition of sulfhydryl-dependent proteins. Therefore, we decided to use 2,3,5-trimethyl-1,4-BQ as a mediator in subsequent experiments. The final concentration of 2,3,5-trimethyl-1,4-BQ was adjusted to less than 20 μM. The peak current remained steady for 10 successive injections of 2,3,5-trimethyl-1,4-BQ at a concentration of 10 μM. Thus, the cytotoxicity of 2,3,5-trimethyl-1,4-BQ can be ignored during the measurement of cell density.
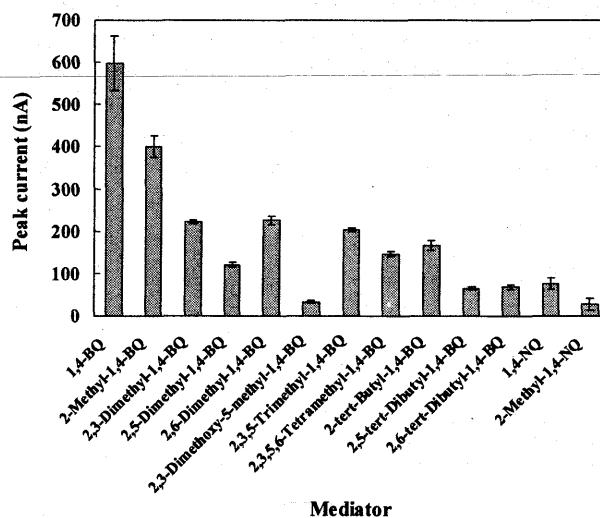

Fig. 4 Effect of mediators on the metabolism efficiency by CHO-K1 cells.
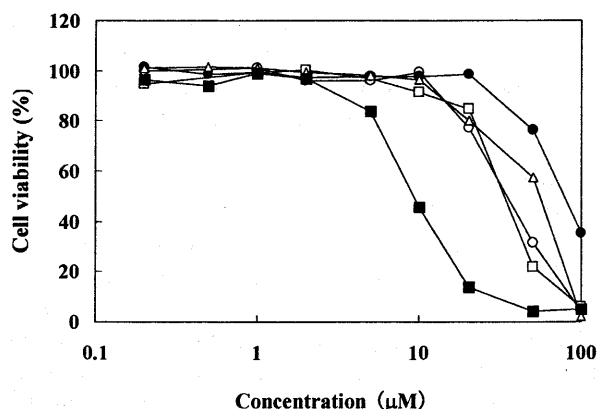BQ: benzoquinone; NQ: naphthoquinone.

Fig. 5 Effect of concentration of mediators on the cytotoxicity in CHO-K1cells.
1,4-BQ: O; 2-methyl-1,4-BQ: □; 2,6-dimethyl-1,4-BQ: △; 2,3,5-trimethyl-1,4-BQ: ●; 2-methyl-1,4-NQ ■.

3.3. Flow rate

The effects of the flow rate on the peak current and the baseline reversion time (95% reversion) were investigated in the range of 0.5 - 2.0 ml/min. The peak current decreased remarkably with increasing flow rate above 1.3 ml/min. The choice of flow rate involves a compromise between sensitivity and sample output rate. A flow rate of 1.3 ml/min was used in this experiment, considering its relatively high current and the short sample output time. The baseline reversion time (95% reversion) was about 5.1 min at this flow rate.

3.4. Measurement of attached cell density in HAPC sheet by flow injection analysis system

The densities of CHO-K1 cells cultured in HAPC sheets for various periods were measured by the FIA system as shown in Fig. 2. The HAPC sheets containing the cells were prepared by static culture in plastic dishes and continuous culture in the bioreactor. These sheets were inserted into the stainless steel folder and then connected to the flow line. 2,3,5-Trimethyl-1,4-BQ was used at a concentration of 10 µM as a mediator and injected into the flow line. The peak current increased after injection of the mediator and returned to the baseline within ca. 7 min (Fig. 6). The peak current was hardly observed when an HAPC sheet without the cells was measured.
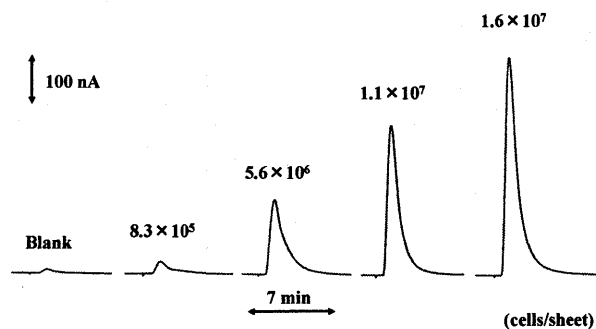

Fig. 6 Flow signals during the measurement of CHO-K1 cells.

A good linear relationship between the peak height of the

oxidative current and the cell density was obtained in a range of 8.3×10^5 - 6.1×10^6 cells/sheet, as shown in Fig. 7. Since CHO-K1 cells grow in the inner space of the HAPC sheet above the density of 8.2×10^6 cells/sheet, it is difficult to detach CHO-K1 cells from the HAPC sheet efficiently and determine cell numbers accurately. Therefore, it is thought that the values of the cell density above 8.2×10^6 cells/sheet contain considerable errors. In consideration of the error of cell density, it is likely that the proposed method can be applied for the measurement of cell density above 8.2×10^6 cells/sheet although the correlation between the peak height of the oxidative current and the cell density in this range was low. The relative standard deviations for three successive injections to each sheet were less than 2.5% above the density of 8.2×10^6 cells/sheet. In the usual way, the relative standard deviation for the plural sheets containing cells at a definite density must be determined. However, it is impossible to prepare the plural sheets containing cells at a definite density because CHO-K1 cells are inoculated on the HAPC sheet and then cultured for a certain period. All sheets have CHO-K1 cells at different cell density. Therefore, the measurement for the sheet containing cells at a definite density can be accomplished only once.

No linearity between the peak current and the cell density was obtained above 2.0×10^7 cells/sheet (data not shown). Above the density of 2.0×10^7 cells/sheet, it is necessary to correlate the peak currents with the cell densities by other functions.
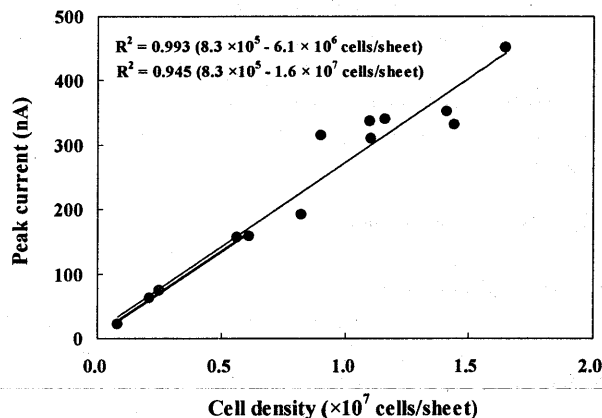

Fig. 7 Cell proliferation measurement of CHO-K1 cells.

This analytical system can be applied to the measurement of the attached cell density less than 1.6×10^7 cells in HAPC sheets and may be used for monitoring the cell density in bioreactors during the period in which the cells proliferate in the logarithmic phase.

References

[1] P. W. Runstadler and S. R. Cernek, "Animal cell biotechnology", Academic Press Limited, London (1988).
[2] T.J. Nikolia and W-S. Hu, Enzyme Microb. Technol., 14, 203 (1992).
[3] B.K. Lydersen, "Large scale cell culture technology", Hanser Publishers, New York (1987).

[4] H. Kawakatsu, *Kinoushi Kenkyukaishi*, **32**, 28 (1993).

[5] H. Kawakatsu, S. Ide, Y. Okuda and S. Shirahata, *Cytotechnology*, 35, 65 (2001).

[6] T. Sakai, Y. Katakura, K. Teruya, H. Kawakatsu, Y. Hirofuji, T. Tsukatani, S. Morishita, and S. Shirahata, in the Proceedings of the annual meeting of the Japanese Association for Animal Cell Technology, p. 41 (2003), Osaka, Japan.

[7] B. Sonnleitner, *Proc. Cont. Qual.*, **2**, 97 (1992).

[8] B. Sonnleitner, *J. Biotechnol.*, **25**, 5 (1992).

[9] F. Nishimoto and S. Yamashoji, *J. Ferment. Bioeng.*, **77**, 107 (1994).

[10] A. R. Surrey, *Org. Synth.*, **III**, 753 (1955).

[11] M. Ishiyama, M. Shiga, K. Sasamoto, M. Mizuguchi and P. He, *Chem. Pharm. Bull.*, **41**, 1118 (1993).

[12] L. Ernster, L. Danielson and M. Ljunggren, *Biochim. Biophys. Acta*, **58**, 171 (1962).

[13] Y. Noshikawa, B. I. Carr, M. Wang, S. Kar, F. Finn, P. Dowd, Z. B. Zheng, J. Kerns and S. Naganathan, *J. Biol. Chem.*, **270**, 28304 (1995).