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LSI-based amperometric sensor for real-time monitoring of embryoid bodies

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ABSTRACT

A large scale integration (LSI)-based amperometric sensor is used for electrochemical evaluation and real-time monitoring of the alkaline phosphatase (ALP) activity of mouse embryoid bodies (EBs). EBs were prepared by the hanging drop culture of embryonic stem (ES) cells. The ALP activity of EBs with various sizes was electrochemically detected at 400 measurement points on a Bio-LSI chip. The electrochemical measurements revealed that the relative ALP activity was low for large EBs and decreased with progress of the differentiation level of the ES cells. The ALP activity of the EBs was successfully monitored in real time for 3.5 h, and their ALP activity in a glucose-free buffer decreased after 2 h. To the best of our knowledge, this is the first report on the application of an LSI-based amperometric sensor for real-time cell monitoring over 3 h. The chip is expected to be useful for the evaluation of cell activities.

1. Introduction

Embryonic stem (ES) cells have the capacity for long term cell renewal and differentiate into a variety of cell lineages, such as neurons (Abranches et al., 2009) and cardiomyocytes (Park et al., 2007; Choi et al., 2010). Therefore, ES cells have received a great deal of interest from researchers studying tissue replacement and regenerative medicine (Rippon and Bishop, 2004). The differentiation of ES cells into specific cell lineages is strictly controlled with differential changes in gene expression, which is mediated by the three-germ layers (Hwang et al., 2009). When ES cells are cultured under appropriate conditions, they tend to form embryoid bodies (EBs), which are 3D cell aggregates that generally enable differentiation of ES cells (Khoo et al., 2005). Significant progress has been made to optimize the differentiation of ES cells into specific lineages by empirical investigation of manipulation. To elucidate the events that lead to undifferentiated self-renewal and differentiation of ES cells, biomarkers such as alkaline phosphatase (ALP) are used. Previous studies show that even after months of culturing, undifferentiated ES cells express a high level of ALP (Pease et al., 1990), which can be detected using both optical (Ivanova et al., 2006) and electrochemical methods (Obregon et al., 2012). To date, a number of cell microarrays have been developed for bioimaging and analysis of cells (Ino et al., 2012b, 2008; Tuleuova et al., 2010). Even though the optical method is the most widely used technique for analysis (Li et al., 2010), it has some disadvantages, such as fluctuations due to quenching or emission from non-target molecules, shielding by turbid solutions, and the need for a label that may have a toxic effect on the cells. As an alternative, the electrochemical method has been proposed. The electrochemical method, which is amenable to the miniaturization process, provides a number of advantages, such as rapidity, simplicity and sensitivity. However, the conventional electrochemical method is inferior to the optical method in terms of imaging or comprehensive measurements of biomaterials. Recently, several electrochemical measurement systems have been developed to realize multipoint measurements and bioimaging. The most commonly used tool for bioimaging is scanning electrochemical microscopy (SECM) (Takahashi et al., 2009; Şen et al., 2012a; Murata et al., 2009), which measures local electrochemical behavior through scanning, although this technique may suffer from temporal resolution. To achieve higher temporal resolution for the detection of electrochemical reactions at different locations, we have previously reported two new different types of bio-chips; the local redox cycling-based electrochemical (LRC-EC) chip device (Ino et al., 2012b; Şen et al., 2012b; Ino et al., 2012a, 2011) and Bio-LSI (Inoue et al., 2012).

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With the LRC-EC system, 1024 electrochemical sensors were successfully incorporated with a pitch of 200 μm using only 64 connecting pads (Ino et al., 2011). The ALP activity of EBs was also successfully detected using the LRC-EC chip device (Ino et al., 2012b, 2012a). Bio-LSI has been developed based on a technology of complementary metal-oxide-semiconductor (CMOS) to achieve higher temporal resolution for the detection of electrochemical reactions at different locations (Inoue et al., 2012). The Bio-LSI chip comprises 400 sensors with a pitch of 250 μm. The diameter of each sensor point is 50 μm. The Bio-LSI chip has a dynamic range from ±1 pA to ±100 nA with very low electronic noise (Inoue et al., 2012) compared to other CMOS-based sensor array platforms (Rothe et al., 2011; Kruppa et al., 2010). The Bio-LSI chip realizes an electrochemical image from 400 sensors that can be acquired within 200 ms; therefore, real-time monitoring can be achieved for the detection of biosamples, such as proteins, DNA and cells. Real-time monitoring of an enzymatic reaction was successfully detected using the Bio-LSI chip (Inoue et al., 2012).

In the present study, the ALP activity of different sized EBs was detected to evaluate their differentiation levels from the amperometric responses of the Bio-LSI chip. The ALP activities of EBs with various sizes were successfully and simultaneously obtained within 200 ms using the Bio-LSI chip, including the data processing time. In addition, real-time measurement was also performed for up to 3.5 h to monitor the EB behavior under different conditions. To our knowledge, this is the first report of long multipoint electrochemical-based real-time monitoring for cell analysis.

2. Materials and methods

2.1. EB formation

Strain 129/SvEv, passage 11 was purchased from DS Pharma Biomedical Co., Ltd. (Japan) and used for the formation of EBs. The ES cells were cultured using Stem Medium (DS Pharma Biomedical Co., Ltd.) with 1000 U/mL of mouse leukemia inhibitory factor (LIF) and 1 mM β-mercaptoethanol to maintain the undifferentiated state. The medium was changed every day and the ES cells were passaged every 3 days. The hanging drop method was adopted to form EBs through cell aggregation (Lee et al., 2011; Tripathi et al., 2011; Tung et al., 2011). After detachment of the ESs using Accutase solution (Millipore, USA), the cells were placed in a 35 mm diameter dish as 20 μL droplets of containing either 1000 or 2000 cells/drop to obtain EBs with diameters between 200 and 500 μm. After 3 days of incubation at 37 °C in a humidified atmosphere of air containing 5% CO_2, single EBs were formed inside the droplets to be used for electrochemical detection of ALP activity.

2.2. Chip fabrication

The LSI chip was designed by Toppan Technical Design Center Co., Ltd. (Japan) and implemented in Semiconductor Manufacturing International Corporation (SMIC, China) on an 8-inch wafer. The wafer with open contact holes delivered by the foundry was diced into $25 \times 25\ mm^2$ squares to obtain more than 32 dies ($10 \times 10\ mm^2$) for Bio-LSI fabrication. A positive photoresist (OFPR-800LB-200cp, Tokyo Ohka Kogyo Co., Ltd., Japan; LOR 15A, MicroChem Corp., USA) was used for the electrode patterns. Each die was first sputter-deposited with Ti (15 nm) and then Pt (35 nm), and lastly with a 600 nm thick Au layer (L-332S-FH, Avelva Corp., Japan) The photoresist was subsequently removed with acetone and 502A stripper (Tokyo Ohka Kogyo Co., Ltd., Japan) and then, a 5 μm thick insulator layer was fabricated using a negative photoresist (SU-8 3005, MicroChem Corp., USA) to define the electrode area. Each die was then diced and the diced chip was fixed onto a Au wire patterned ceramic substrate (1.0 mm thick, 632.0 mm wide, 646.0 mm long) using Ag paste. After electrically connecting the I/O pads of the LSI and the Au wires on the substrate with gold bonding wires, a well for the sample solution was fabricated on the chip using polydimethylsiloxane (PDMS). Detailed information regarding fabrication and system setup is provided in our previous paper (Inoue et al., 2012).

2.3. Electrochemical detection

The performance of the Bio-LSI chip was investigated with respect to the response of *p*-aminophenol (PAP) oxidation. Briefly, Ag/AgCl and Pt electrodes were used as reference and counter electrodes, respectively, and chronoamperometry was performed by stepping the voltage from −0.30 to 0.30 V vs. Ag/AgCl to oxidize PAP.

For evaluating ALP activity of EBs, Tris–HCl (pH 9.5) buffer solution containing 4.7 mM *p*-aminophenyl phosphate (PAPP) was introduced into the Bio-LSI chip. The EBs were then placed onto the detection area using a capillary tube. Ag/AgCl and Pt electrodes were used as reference and counter electrodes, respectively. Chronoamperometry was performed by stepping the voltage from −0.30 to 0.30 V vs. Ag/AgCl to oxidize PAP, a product of the ALP-catalyzed hydrolysis of PAPP on the EB surface, into *p*-quinine imine (PQI) (Fig. 1A) (Obregon et al., 2012). Potential stepping and data acquisition were conducted using a Bio-LSI control system (Inoue et al., 2012). After electrochemical detection, the EBs were collected for further analysis.

2.4. ATP content analysis

The ATP content of the EBs immediately after electrochemical detection was determined using an ATPlite kit (PerkinElmer Life Sciences, USA) to correlate the ALP activity and ATP content. After electrochemical detection, EBs of different sizes were lyzed using a mammalian cell lysis solution to reveal the ATP content, which is hydrolyzed into AMP by luciferase, after which light is emitted and measured using chemiluminescence detection methods (Fluoroskan Ascent, Thermo Fisher Scientific, USA).

2.5. Real-time monitoring

Two different solutions were used for the real-time monitoring; 3 mL aliquots of glucose and glucose-free HEPES solutions (150 mM NaCl, 4.2 mM KCl, 11.2 mM glucose, 10 mM HEPES, 2 mM $MgCl_2$) at pH 7.0. Prior to measurements, a buffer solution containing 4.7 mM PAPP was introduced into the Bio-LSI chip, followed by the placement of small and large EBs onto the Bio-LSI chip. After stabilization for 40 s at −0.30 V, the input potential was stepped from −3.0 to 0.30 V to oxidize PAP into PQI and the potential was then kept at 0.30 V throughout the monitoring process. During real-time monitoring, electrochemical images consisting of 400 points were acquired every 4 s to avoid excessive data storage. Real-time monitoring was continued for 3.5 h.

3. Results and discussion

3.1. Characterization of the chip

The Bio-LSI chip contained 400 electrochemical sensors of Au disk microelectrodes (diameter, 50 μm) (Fig. 1B), an Ag/AgCl electrode and a Pt counter electrode, which were used for electrochemical detection (Fig. S1). When the potential of the sensor points was stepped from −0.30 to 0.30 V, the individual points produced typical current–time curves with the change in the PAP concentration (Fig. 2A). Fig. 2B shows the relationship