

(21) Application No: 0918135.5
(22) Date of Filing: 15.10.2009
(30) Priority Data:
(31) 10090026353 (32) 27.03.2009 (33) KR

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(51) INT CL:
C12Q 1/00 (2006.01)

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(58) Field of Search:
INT CL C12Q
Other: WPI, EPODOC, BIOSIS, MEDLINE

(54) Title of the Invention: **Sensor for detecting stem cell differentiation based on electrochemical methods**
Abstract Title: **Alkaline phosphatase sensor**

(57) A sensor for detecting stem cell differentiation, including (a) an electrode; and (b) a substrate of an alkaline phosphatase. Alkaline phosphatase is a stem cell differentiation marker; the phosphorylation or dephosphorylation of the substrate may be measured using an electrical signal in the present sensor and enables the electrical determination of stem cell differentiation.

Fig. 1

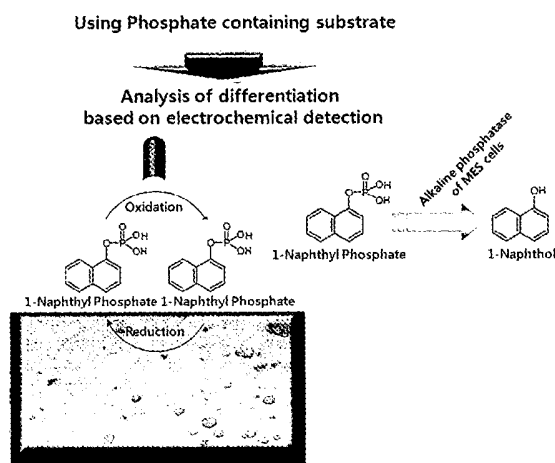


Fig. 1

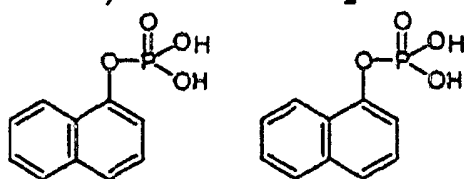
Using Phosphate containing substrate



Analysis of differentiation
based on electrochemical detection

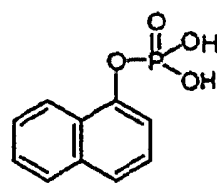
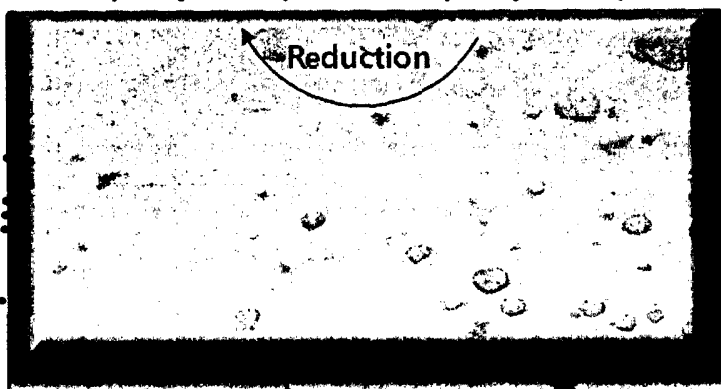


Oxidation



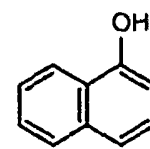
1-Naphthyl Phosphate 1-Naphthyl Phosphate

Reduction



1-Naphthyl Phosphate

Alkaline phosphatase
of MES cells



1-Naphthol

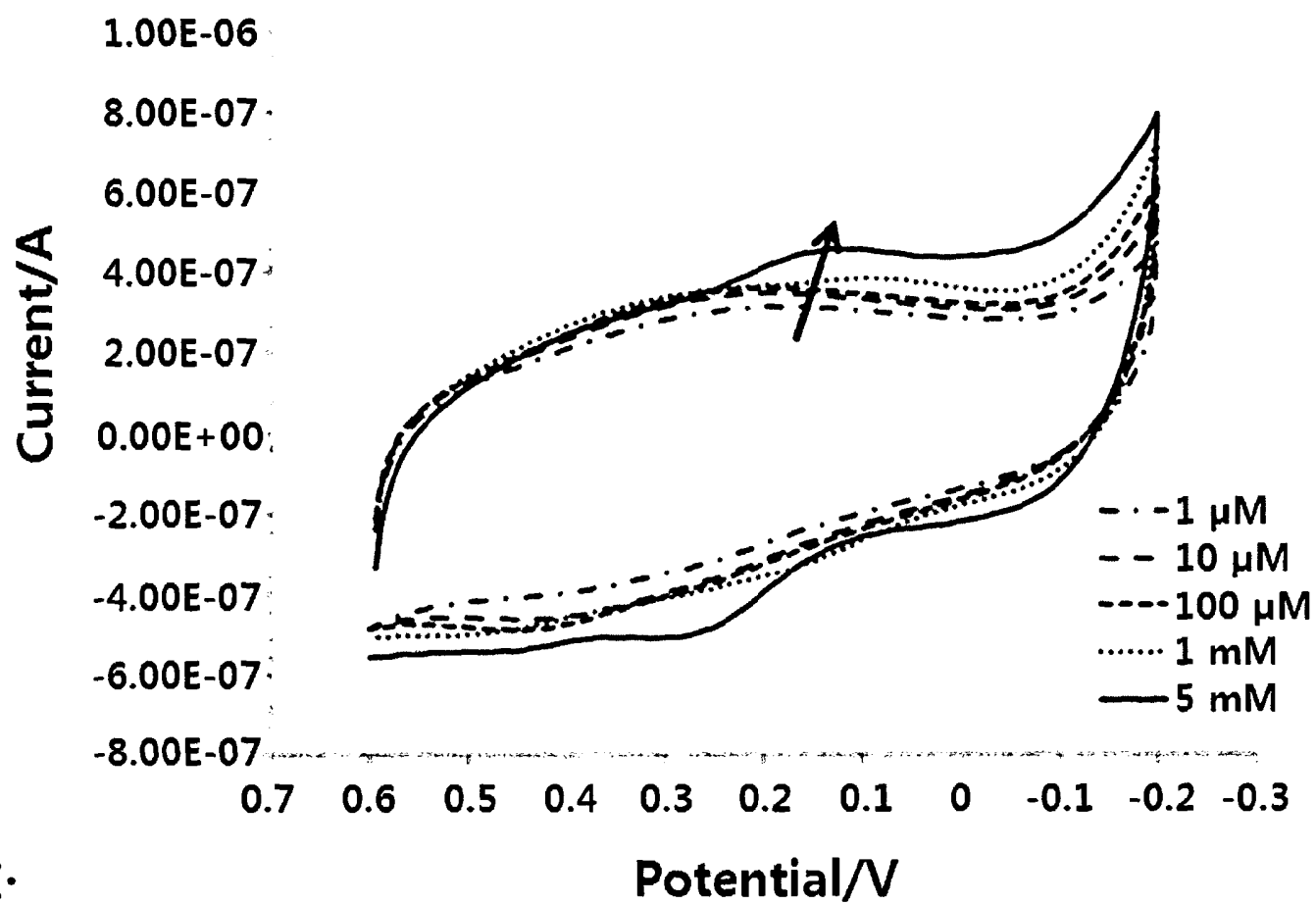
Fig. 2a

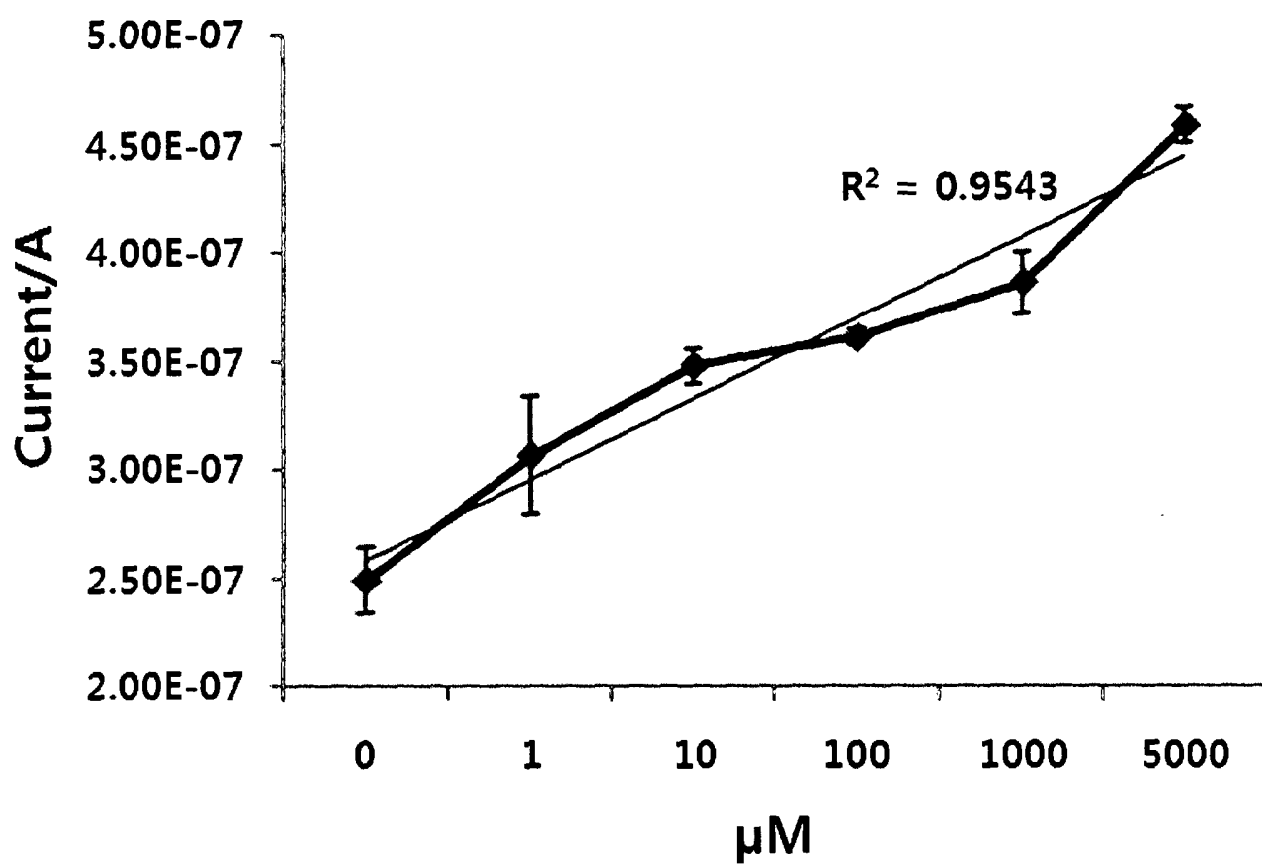
Fig. 2b

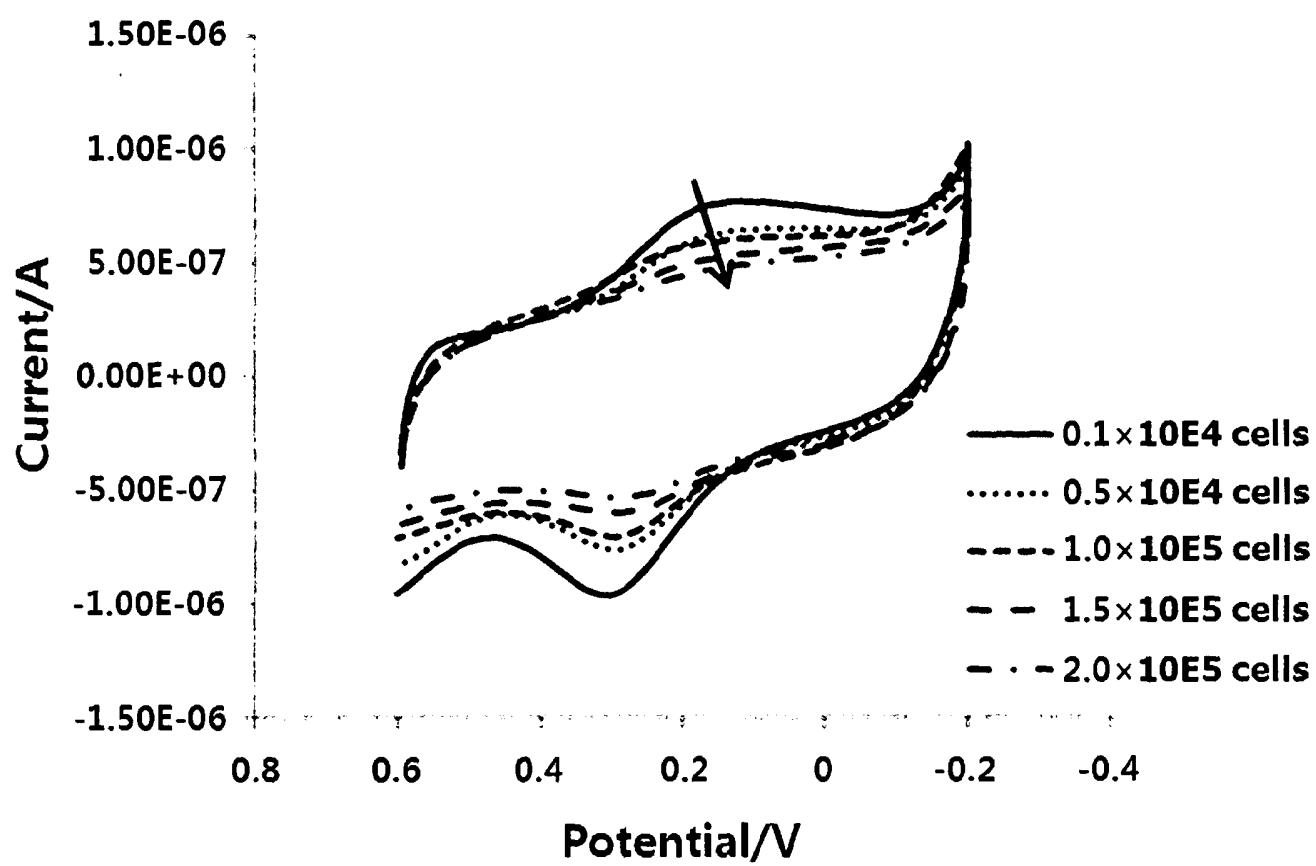
Fig. 3a

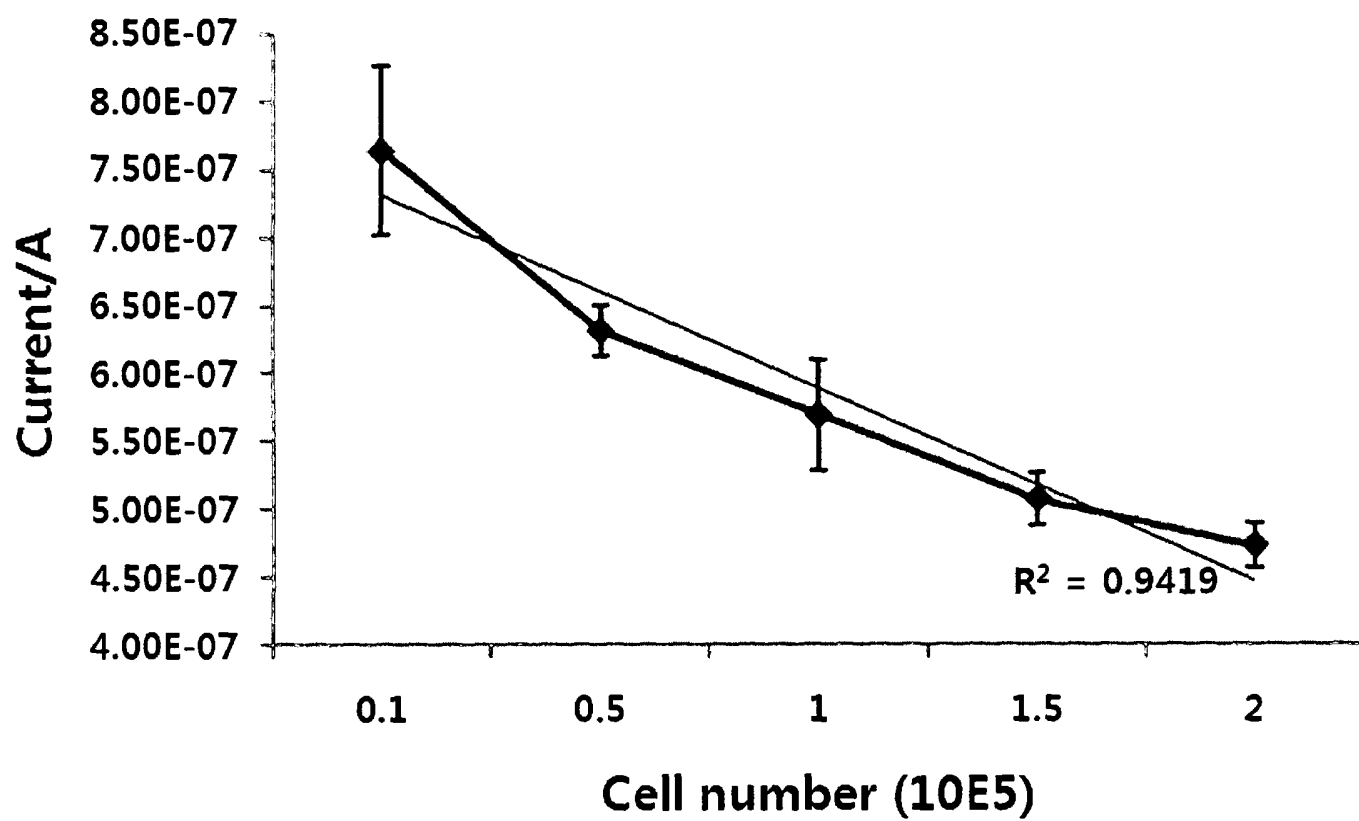
Fig. 3b

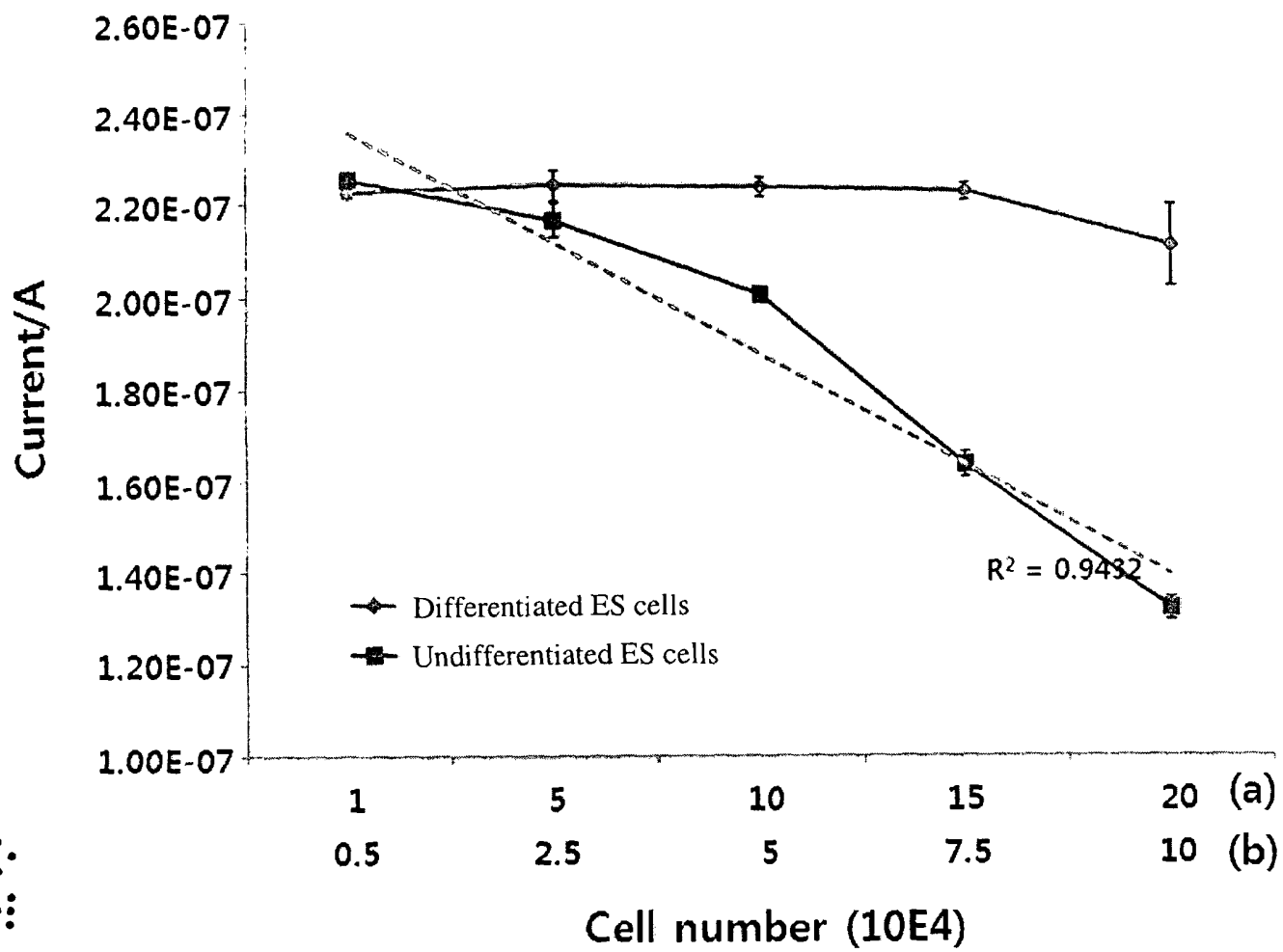
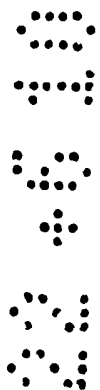
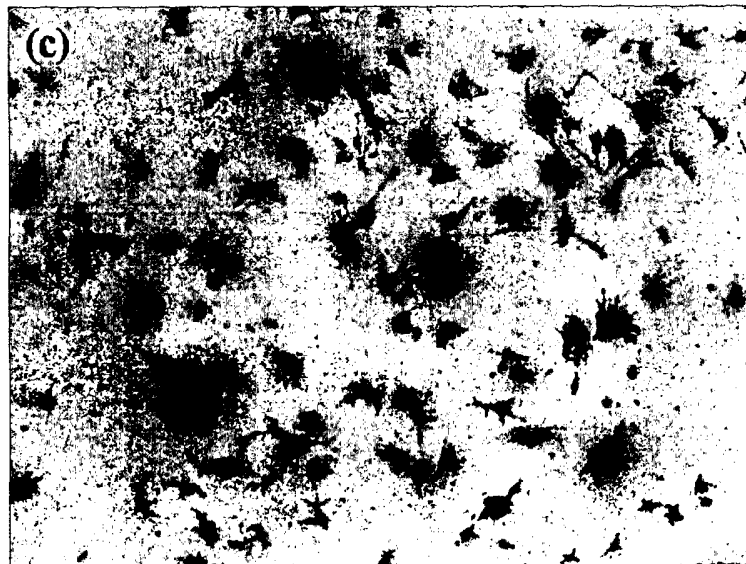
Fig. 4



Fig. 5b



Fig. 5c



SENSOR FOR DETECTING STEM CELL DIFFERENTIATION BASED ON ELECTROCHEMICAL METHODS

BACKGROUND OF THE INVENTION

5 FIELD OF THE INVENTION

The present invention relates to a sensor for detecting a stem cell differentiation, including: (a) an electrode; and (b) a substrate for an alkaline phosphatase.

10 DESCRIPTION OF THE RELATED ART

Cell chip technology is a promising tool for utilization in cell based assays. There are two kinds of cell detection systems for these chips. These systems are based on optical detection and electrical (electrochemical) detection respectively. Optical systems allow one to observe visual changes of the cells and have high
15 sensitivity and selectivity. Optical systems are limited by size of the instrument and the process of transforming the optical signal into an electrical signal [1-2].

While electrical cell detecting systems are relatively less developed than optical systems, they have the ability to be miniaturized and the signals are easily analyzed. There have been some attempts to analyze living cells as electrochemically
20 dynamic systems by detecting electron generation and electron transfer at the interface [3].

Living cells have been studied by many electrochemical situations such as electron transfer at electro active centers in cells, open circuit potential at the cell/sensor interface, electric cell-substrate impedance sensing (ECIS), scanning
25 electrochemical microscopy (SECM) to obtain images of the respiratory activity of collagen-embedded living cells, electrochemical impedance spectroscopy (EIS), and activation of an oxygen electrode [4-11].

However, there has been no attempt to detect the differentiation of MES cells using electrical or electrochemical systems.

Throughout this application, various publications and patents are referred and citations are provided in parentheses. The disclosures of these publications and patents in their entities are hereby incorporated by references into this application in order to fully describe this invention and the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

The present inventors have intensive studies to develop a novel sensor and method for detecting a stem cell differentiation based on electrochemical methods. As results, we have discovered that the phosphorylation or dephosphorylation of the substrate for alkaline phosphatase as a stem cell undifferentiation marker which dephosphorylates its substrate could be measured using an electrical signal to determine a stem cell differentiation in an electrochemical manner.

Accordingly, it is an object of the invention to provide a sensor for detecting a stem cell differentiation.

It is another object of this invention to provide a method for determining a stem cell differentiation.

Other objects and advantages of the present invention will become apparent from the detailed description to follow taken in conjugation with the appended claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 schematically represents the electrochemical detection system in which 1-naphthyl phosphate (NP) as a substrate of an alkaline phosphatase (AP) involved in the sensor of the present invention is changed into 1-naphtol by the alkaline phosphatase of a mouse embryonic stem cell undifferentiated.

Fig. 2a represents a cyclic voltammogram of 1-NP used as a substrate in the sensor of the present invention and Fig. 2b represents a linear plot of reduction current peak as increasing concentration of 1-NP.

Fig. 3a represents a cyclic voltammogram of mouse embryonic stem cells according to addition of 1-NP and Fig. 3b represents a linear plot of reduction current peak as increasing concentration of MES cells. Data are presented as the mean $\pm$ standard deviation of three different experiments. Arrow indicates increasing cell number.

Fig. 4 is a linear plot of reduction current peak as increasing concentration of (a) differentiated MES cells and (b) undifferentiated MES cells. The scan rate was 100 mV/s. The temperature was $37 \pm 0.5^\circ\text{C}$ and the CO_2 was 5%. Data are presented as the mean $\pm$ standard deviation of three different experiments.

Fig. 5a represents a viability test according to 1 mM 1-NP treatment. Data are presented as the mean of three different experiments. Fig. 5b-5c represent AP staining assay of (b) undifferentiated MES cells and (c) MES cells treated with 1-NP.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of this invention, there is provided a sensor for detecting a stem cell differentiation, including: (a) an electrode; and (b) a substrate for an alkaline phosphatase.

The present inventors have intensive studies to develop a novel sensor and method for detecting a stem cell differentiation based on electrochemical methods. As results, we have discovered that the phosphorylation or dephosphorylation of the substrate for alkaline phosphatase as a stem cell undifferentiation marker which dephosphorylates its substrate could be measured using an electrical signal to determine a stem cell differentiation in an electrochemical manner.

The sensor for detecting the stem cell differentiation of the present invention is constituted of a conventional three-electrode system known to those ordinarily skilled in the art, which includes a working electrode, a counter electrode and a reference electrode.

5 According to a preferable embodiment, the electrode involved in the sensor of the present invention is selected from the group consisting of a gold, copper, glass carbon, platinum and Ag/AgCl electrode. More preferably, the electrode includes a gold, copper or glass electrode as the working electrode, a platinum electrode as the counter electrode, and a Ag/AgCl electrode as the reference electrode, and most
10 preferably a gold electrode as the working electrode, a platinum electrode as the counter electrode, and a Ag/AgCl electrode as the reference electrode.

The alkaline phosphatase used in the present invention refers to a marker representing an undifferentiation status of stem cells.

The substrate for alkaline phosphatase contained in the sensor of the present
15 invention may include various compounds containing a phosphate group known to those ordinarily skilled in the art, preferably 1-naphthyl phosphate, calcium phosphate, phenyl phosphate, 2-aminophenyl phosphate, 4-aminophenyl phosphate, p-aminophenyl phosphate, 3-indoxyl phosphate, 5-bromo-4-chloro-3-indoxyl phosphate, 4-methylumbelliferyl phosphate, 6-chloro-3-indoxyl phosphate or
20 hydroquinone diphosphate, more preferably 1-naphthyl phosphate, phenyl phosphate, 2-aminophenyl phosphate, 4-aminophenyl phosphate or 3-indoxyl phosphate, and most preferably 1-naphthyl phosphate.

In particular, 1-naphthyl phosphate as a substrate for alkaline phosphatase is a compound in which the phosphate group is linked to two benzene rings (Fig. 1),
25 and is dephosphorylated by alkaline phosphatase present in undifferentiated stem cells so as to be changed into 1-naphtol. It is demonstrated that the electrochemical characteristics of the modified substrate have quite different to those of the pre-modified substrate (Fig. 2).

According to another preferable embodiment, the stem cell used in the sensor of the present invention is an embryonic stem cell or a germ stem cell, and more preferably an embryonic stem cell.

5 According to still another preferable embodiment, the stem cell used in the sensor of the present invention is a human or mouse-derived stem cell, and more preferably a mouse-derived stem cell.

According to further still another preferable embodiment, the stem cell used in the sensor of the present invention includes an alkaline phosphatase where the stem cell is an undifferentiated stem cell; and the stem cell includes no alkaline
10 phosphatase where the stem cell is a differentiated stem cell.

According to another preferable embodiment, the sensor of the present invention measures the phosphorylation or dephosphorylation of alkaline phosphatase substrate using an electrical signal to determine the differentiation of the stem cell.

15 According to still another preferable embodiment, the substrate dephosphorylated by alkaline phosphatase in the present invention includes 1-naphtol, calcium ion, phenol, 2-aminophenol, 4-aminophenol, p-aminophenol, 3-indoxol, 5-bromo-4-chloro-3-indoxol, 4-methylumbelliferol, 6-chloro-3-indoxol or hydroquinone, more preferably 1-naphtol, phenol, 2-aminophenol, 4-aminophenol or
20 3-indoxol, and most preferably 1-naphtol.

In another aspect of this invention, there is provided a method for determining a stem cell differentiation, comprising the steps of: (a) preparing the sensor for detecting the stem cell differentiation; (b) contacting cells of interest to
25 the sensor; and (c) measuring the phosphorylation or dephosphorylation of a substrate for alkaline phosphatase on the cells through an electrical signal.

To examine whether the stem cells are differentiated or not, the method according to the step of this invention is minutely explained as follows:

(a) The step preparing the sensor for detecting the stem cell differentiation.

The sensor for detecting a stem cell differentiation containing an electrode; and a substrate for an alkaline phosphatase is prepared.

5 Since the electrode of the sensor and the substrate for the alkaline phosphatase are described above, the common descriptions between them are omitted in order to avoid undue redundancy leading to the complexity of this specification.

(b) The step contacting cells of interest to the sensor.

10 And then, the stem cells of interest are contacted to the sensor for verifying a differentiation status.

(c) The step measuring the phosphorylation or dephosphorylation of the substrate for alkaline phosphatase on the cells through an electrical signal.

15 The substrate is dephosphorylated by alkaline phosphatase in undifferentiated stem cells, and the substrate dephosphorylated may be measured using a cyclic voltammetry because the modified substrate has quite different electrochemical properties compared to the pre-modified substrate.

20 Data for undifferentiated stem cells may be quantitated by tracing the electrochemical signal of the alkaline phosphatase substrate using its electrochemical characteristics, and the signal of the alkaline phosphatase substrate changed according to differentiation may be detected using an electrochemical method to determine the differentiation of the stem cell.

25 Likewise, the electrochemical signal of the alkaline phosphatase substrate in the undifferentiated stem cells is quantitated according to the amounts of stem cells, and the electrochemical signal of the alkaline phosphatase substrate in the differentiated stem cells is also quantitated according to the amounts of stem cells.

And then, these results are compared to determine the extent of the stem cell differentiation.

In a view of identifying the differentiation of mouse embryonic stem (MES) cells as a preferable embodiment, differentiated MES cells show an independent relationship between electrical signal and increasing cell number, while undifferentiated MES cells represent a linear correlation between electrical signal and increasing cell number. R^2 value representing the linearity of undifferentiated MES cells is significantly calculated as 0.9432, indicating that the cell status could be identified using this electrochemical method and the detection limit is 50,000 undifferentiated MES cells (Fig. 4).

As described above in detail, the present invention provides a sensor for detecting a stem cell differentiation, including (a) an electrode; and (b) a substrate for an alkaline phosphatase. The phosphorylation or dephosphorylation of the substrate for an alkaline phosphatase as a stem cell undifferentiation marker which dephosphorylates its substrate may be measured using an electrical signal in the present sensor. Therefore, the sensor of the present invention enables to electrically detect a stem cell status in a high-throughput manner and to determine the stem cell differentiation.

The present invention will now be described in further detail by examples. It would be obvious to those skilled in the art that these examples are intended to be more concretely illustrative and the scope of the present invention as set forth in the appended claims is not limited to or by the examples.

EXAMPLES

Experimental Materials and Methods

2.1 Materials

1-Naphthyl phosphate and phosphate buffered saline (PBS) (pH 7.4, 10 mM) solution was purchased from Sigma-Aldrich. All other chemicals that are used in this study were obtained commercially as reagent grade.

5 2.2 Undifferentiated MES Cell Culture and Embryonic Body formation

J1 cells, mouse embryonic stem (ES) cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, 1mM sodium pyruvate, 10^{-4} M 2-mercaptoethanol, $1 \times$ nonessential amino acids, and 1,000U of leukemia inhibitory factor (LIF) per ml at 37°C and 5% CO₂. EB formation was performed with J1 ES cells as previously described [13]. ES cells (2×10^6 cells) were seeded onto the bacterial-grade Petri dishes in DMEM containing 10% FBS without LIF. After 2 days of suspension culture, EBs were collected and trypsinized. A total of 1.37×10^6 cells were replated on 0.2% gelatin-coated 6-well plates containing the same medium. The number of cells was counted after 3 days.

15

2.3 Fabrication of Electrochemical Cell

The culture chamber is a regular square chamber and each side is 2mm long. We used the working gold electrode (CHI101), the Ag/AgCl reference electrode (CHI111), and the platinum counter electrode (CHI115). The J1 cells were transferred into the chamber at a known cell density by infusion with fresh culture medium. The number of cells was determined by a trypan blue dye method using a hemacytometer.

20

2.4 Electrochemical Sensing of MES Cells

The cyclic voltammetry experiments were performed using a CHI660A machine controlled by a general electrochemical system software. A three-electrode system was designed which was comprised of the gold as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl cell as the reference

25

electrode. Measurements were carried out to study the electrical properties of MES cells as well as detect the differentiation of the mouse embryonic stem cells by the interaction between 1-NP and differentiation marker alkaline phosphatase. PBS (10 mM, pH 7.4) was used as an electrolyte at a scan rate of 0.1 V/s. Before the experiment occurs, the culture media was removed and the 1-NP containing PBS was injected into the chamber. The experiment was started 15 min after buffer changing, and the measurement conditions were maintained at 37°C, 5% CO₂.

2.5 Alkaline Phosphatase Staining Immunoassay

Alkaline Phosphatase (AP) staining immunoassay kit was purchased from Sigma-Aldrich. First, the cells were fixed using a citrate-acetone-formaldehyde fixative solution. After fixation, an alkaline-dye mixture which is composed of sodium nitrate, FRV-alkaline solution, and naphthol AS-BI alkaline solution was added to the cells in a dark room and allowed to stain for 15 min. Finally, the cells were counterstained using hematoxylin solution.

3. Results

3.1 Signal Detecting of 1-Naphtyhyl Phosphate

We quantified the 1-NP signal as follows. The concentration of 1-NP was ranged from 1 μ M to 5 mM in an electrolyte buffer of 10mM PBS (Figs. 2a-2b). Fig. 2a describes the electrochemical property of the 1-NP. The peak was observed around 0.1 V and the potential slowly changed to negative voltage as the concentration of 1-NP decreased. Fig. 2b shows the direct, linear, relationship between 1-NP peak current and concentration of 1-NP. Linear regression analysis was performed which proved the reliability of the electrochemical quantifying method using cyclic voltammetry ($R^2=0.9543$). These results indicate that 1-NP can be quantified by this cyclic voltammetry assay.

3.2 Electrochemical Response of Undifferentiated MES Cells

By incorporating 1-NP into the substrate of the MES cells, alkaline phosphatase (an embryonic stem cell marker) can be tracked by its dephosphorylating action on 1-NP. When the dissolved 1-NP changes to 1-Naphthol there is a corresponding change in electrical signal as 1-Naphthol cannot be seen by the boundary conditions set up to detect 1-NP. The detection was performed in 15 minutes after the 1-NP was injected. Fig. 3a shows the electrochemical property of MES cells after addition of 1-NP. The entire potential was slightly changed to positive direction, but the reduction peak current was decreased with an increasing concentration of MES cells. The reason for this potential change could include the media, because media has a lot of different materials. The linear plot is showed in Fig. 3b and the linear regression analysis is represented by R^2 value, 0.9419. These results indicate good correlation between cell number and CV reduction current peak which is estimated to 1-NP.

3.3 Detecting Differentiation of MES Cells

In order to detect the differentiation of MES cells, we compared electrochemical signals from undifferentiated MES cells and differentiated MES cells. Each group of cells was seeded into a culture chamber to a specific cell number. Because the undifferentiated MES cells have better adhesion and growth than differentiated MES cells, we seeded two times more differentiated cells than the undifferentiated MES cells.

Fig 4 shows the relationship between undifferentiated MES cells and differentiated MES cells. Differentiated MES cells showed an independent relationship between electrical signal and increasing cell number, while undifferentiated MES cells showed a linear relationship between signal and cell number. The linearity of undifferentiated MES cells is represented by a high R^2 value of 0.9432. The P-value for reliability between undifferentiated MES cells and differentiated MES cells is

0.095, 0.001, 0.006 and 0.002 respectively for each point starting from the second data point of the cell number. Other than the lowest two cell numbers, all other points were significantly different at a 5% confidence level. That means the third point of cell number is the detection limit to identify the differentiation of MES cells.

5 These results indicate that we could identify the cell status using this electrochemical method and the detection limit is 50,000 undifferentiated MES cells.

3.4 1-Naphthyl Phosphate Effect to Mouse MES Cells

10 To ensure that the presence of 1-NP is not affecting our tests by altering MES cell viability or differentiation, an Alkaline Phosphatase (AP) staining assay was performed. Fig. 5a shows the result of the viability test after treating the cells with 1mM 1-NP. The cell viability was 99.65% as compared with control. Figs. 5b-5c show the results of AP staining before NP treated stem cells and after NP treated stem cells. Each figure shows well stained stem cells, which means that 1-NP does not
15 affect the differentiation of stem cells.

4. Discussion

In this paper, we propose a cell based sensor that could detect differentiation of MES cells based on electrochemical tools. Electrochemical cell based sensors have
20 been investigated before, but most of them are used for detecting cell viability. These kinds of cell based sensor have been applied to drug screening of cancer cells or to environmental toxicant effect to cells [12].

Electrochemical cell based sensors can be divided into two groups based on measuring techniques. One technique is based on measuring impedance [14-16].
25 Cells are generally insulative. If cells cover a large area of the electrode, there is a relatively high level of impedance. In order for this technique to work the cells must be able to attach to the electrode surface. This limits this technique's usefulness to cells which are the type to be adherent. This technique is based on the assumption

that the surface attached cells are alive and detached cells are dead.

Another type of electrochemical cell based sensor utilizes an amperometric technique [6]. Amperometric technique involves detecting electrical current with a fixed potential. Sometimes CV technique is introduced as a means to determine the potential for an amperometric technique. The amperometric technique measures the amount of oxygen and the amount of dopamine around cells to determine cell viability. Recently, the signal of HeLa cell was quantified by CV and Potential Stripping Analysis (PSA) techniques [12]. However, to date there has been no attempt to measure the differentiation of stem cells using electrical or electrochemical tools.

In summary, we report the successful demonstration of identifying and quantifying ES cell differentiation utilizing a novel technique base on 1-NP measured via an electrochemical method. We have shown that measuring the 1-NP is a feasible means to determine ES cell differentiation, and the presence of this indicator did not affect the viability and differentiation of these cells. For this experiment, we tested 1-NP as a substrate for detecting the differentiation of MES cells. We have successfully quantified the 1-NP by an electrochemical method, and this technique was applied to MES cells to detect the differentiation status of the cells. The peak current of 1-NP increases only with undifferentiated MES cell number indicating that 1-NP only reacts with undifferentiated MES cells. Finally, we compared the signal of differentiated MES cells and undifferentiated MES cells, and we determined the detection limit of undifferentiated MES cell number to be $\geq 50,000$. The proposed electrochemical measurement system can be applied to electrical stem cell chip for diagnosis, drug detection and on-site monitoring.

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Having described a preferred embodiment of the present invention, it is to be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this invention is to be determined by appended claims and their equivalents.

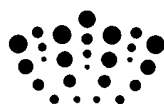
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What is claimed is:

1. A sensor for detecting a stem cell differentiation, comprising: (a) an electrode; and (b) a substrate for an alkaline phosphatase.
- 5 2. The sensor according to claim 1, wherein the electrode is selected from the group consisting of a gold, copper, glass carbon, platinum and Ag/AgCl electrode.
3. The sensor according to claim 1, wherein the substrate is selected from the group consisting of 1-naphthyl phosphate, calcium phosphate, phenyl phosphate, 2-aminophenyl phosphate, 4-aminophenyl phosphate, p-aminophenyl phosphate, 3-10 indoxyl phosphate, 5-bromo-4-chloro-3-indoxyl phosphate, 4-methylumbelliferyl phosphate, 6-chloro-3-indoxyl phosphate and hydroquinone diphosphate.
4. The sensor according to claim 1, wherein the stem cell is an embryonic stem cell or a germ stem cell.
- 15 5. The sensor according to claim 1, wherein the stem cell comprises an alkaline phosphatase where the stem cell is an undifferentiated stem cell; and the stem cell comprises no alkaline phosphatase where the stem cell is a differentiated stem cell.
- 20 6. The sensor according to claim 1, wherein the sensor measures the phosphorylation or dephosphorylation of the substrate for alkaline phosphatase on the stem cell through an electrical signal to determine the differentiation of the stem cell.
- 25 7. The sensor according to claim 6, wherein the dephosphorylated substrate is selected from the group consisting of 1-naphtol, calcium ion, phenol, 2-aminophenol, 4-aminophenol, p-aminophenol, 3-indoxol, 5-bromo-4-chloro-3-indoxol, 4-

methyumbelliferol, 6-chloro-3-indoxol and hydroquinone.

8. A method for determining a stem cell differentiation, comprising the steps of:
- (a) preparing the sensor for detecting the stem cell differentiation according to claim 1;
 - (b) contacting cells of interest to the sensor; and
 - (c) measuring the phosphorylation or dephosphorylation of a substrate for alkaline phosphatase on the cells through an electrical signal.



Application No: GB0918135.5

Examiner: Dr Jeremy Kaye

Claims searched: 1-8

Date of search: 8 February 2010

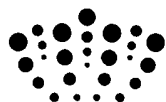
Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
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X	1-8	Electroanalysis, Vol.16, 2004, Castano-Alvarez, M. et al., "Gold electrodes for detection of enzyme assays...", pp.1487-1496
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