Biochemical Engineering Journal 123 (2017) 95–109

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Enzyme catalyzed electrostimulation of human embryonic stem cell-derived cardiomyocytes influence contractility and synchronization

Soon-Jung Park[a,1], Ki-Hoon Kim[b,1], Won-Yong Jeon[b,1], Joseph Seo[a], Jung-Min Han[a], Jong-Soo Kim[a], Hyung-Min Chung[a], Jae-Ho Lee[e,*,2], Sung-Hwan Moon[c,*,2], Hyug-Han Kim[d,*,2]

[a] Department of Stem Cell Biology, Konkuk University School of Medicine, Seoul, 143-701, Republic of Korea
[b] Department of Nanobiomedical Sciences and BK21 PLUS NBM Global Research Center for Regenerative Medicine, Dankook University, Cheonan 330-714, Republic of Korea
[c] Department of Medicine, Konkuk University School of Medicine, Seoul, 143-701, Republic of Korea
[d] Department of chemistry, College of Natural Science, Dankook University, Anseo-dong, Cheonan, 330-714, Republic of Korea
[e] Department of Biomedical Science, College of Life Science, CHA University, Pochen, Gyeonggi-do, 11160 Republic of Korea

ARTICLE INFO

Article history:
Received 2 December 2016
Received in revised form 16 March 2017
Accepted 18 March 2017
Available online 6 April 2017

Keywords:
Electrostimulation
Glucose oxidase
Human embryonic stem cells
Cardiomyocytes
Synchronization

ABSTRACT

Electrical stimulation is a major physiological mediator that has long been implicated by electrophysiologists to have therapeutic benefits. Here, we investigated electrostimulation as a promising mean for micro-scale electrical stimulation to enhance human embryonic stem cell-derived cardiomyocytes. This study demonstrated an easy-to-use bioelectrical stimulation system that could operate without a power unit. It utilizes enzymes to generate electron rich or poor conditions by exploiting the oxidation-reduction capability of glucose and oxygen. Glucose oxidase (−1.03 to −1.31 μA/cm^2) stimulated cardiomyocytes showed increased calcium levels as a result of increased gap junctions which lead to faster synchronization and contractility within 12 days. The simplicity and biocompatibility of this approach could hasten drug screening, cell therapy, and electrophysiological research by making studies much more practical through the enhancement of functional human embryonic stem cell-derived cardiomyocytes.

1. Introduction

Heart disease has been documented to be a primary cause of death worldwide [1]. Damaged cells within cardiac tissues are unable to recover in adult patients due to their limited regenerative capability. For this reason, researches have conducted extensive studies with cardiomyocytes and other cells to advance cell-based therapies for cardiac regeneration [2].

Human embryonic stem cells (hESCs) are capable of indefinite proliferation, making them ideal candidates to acquire cardiomyocytes [3,4]. However, cardiomyocytes cultured *in vitro* maintain immature phenotype. In some cases, they may lose their functionality when cultured over an extended period of time. Therefore, it is necessary to continuously characterize differentiated cardiomyocytes through electrophysiological functionality tests and cardiac specific marker expression analysis [5,6].

Electric field stimulation approach can be applied to hESCs and heart tissue derived cardiomyocytes to induce differentiation while preserving their characters [7,8]. Previous reports have documented that electric field stimulation can improve cell contraction, amplify synchronization, stabilize action potential, and increase calcium current peaks. In addition, the increase of gap junction protein connexin43 can help maintain contractility [9,10]. Such electrical stimulation is being implemented into systems that use a wide range of currents and voltages to obtain more biologically native cardiomyocyte activation [11–14]. While such methods

Abbreviations: GOx, glucose oxidase; BOD, bilirubin oxidase; hESC, human embryonic stem cell; CM, cardiomyocyte.

* Corresponding authors.
E-mail addresses: drjaeho@gmail.com (J.-H. Lee), sunghwanmoon@kku.ac.kr (S.-H. Moon), hankim@dankook.ac.kr (H.-H. Kim).

[1] SJ Park, KH Kim, and WY Jeon contributed equally to this paper as first authors.
[2] SH Moon, JH Lee, and HH Kim contributed equally to this paper as corresponding authors.

http://dx.doi.org/10.1016/j.bej.2017.03.013
1369-703X/

appear promising, clinical usage is very limited in terms of application because most power sources are sizable devices that utilize inorganic materials with low biocompatibility, thereby making it inconvenient for therapeutic purposes [15,16].

Enzyme engendered electrostimulation is very promising in this regard as its components are relatively inexpensive and biocompatible [17]. Mediators involving osmium (Os) polymers play important roles because they possess high redox potentials with increased electron mobility. The use of a cross-linker such as poly (ethylene glycol) diglycidyl ether (PEGDGE) allows for the binding of enzyme, mediator, and electrode to function as a unit to generate electro-physical energy [18,19]. Our previous study has demonstrated such stimulation in C2C12 muscle precursor cells which significantly increased cell proliferation and migration. Furthermore, electrical stimulation can promote the differentiation of cells into myotube [20].

In this study, electrostimulation was applied to human cells by creating electron rich or poor environments through the addition of glucose into cell culture media to generate a current using this enzyme conductive system. Stimulation was operated at an optimal enzyme concentration of 80 μg/mL. It was applied to hESC-derived cardiomyocytes under three different stimulatory conditions: i) Glucose oxidase (GOx; anode only); ii) Bilirubin oxidase (BOD; cathode only); iii) GOx-BOD full (anode and cathode) all groups include the Os polymer and PEGDGE. Under these settings, the effects of stimulation on cardiomyocyte synchronization, contractility, and marker expression were investigated. This electrostimulation approach is advantageous because it can improve and preserve cardiomyocyte functionality with simplicity because enzyme activation is controlled by glucose in culture media. This may be useful for processes such as drug screening, action potential measurements, and cell therapy research aiming to cure heart failure.

2. Materials and methods

2.1. Chemicals

Glucose oxidase (GOx) from *Aspergillus niger* (219 U/mg) was purchased from Amano Enzyme Inc. (Japan). Bilirubin oxidase (BOD) from *Myrothecium verrucaria*, ammonium hexachloroosmate (IV), 4,4′-dimethoxy-2,2′-bipyridine, poly(ethylene glycol) diglycidyl ether (PEGDGE), sodium hydrosulfite, 1-vinylimidazole, and azobisisobutyronitrile (AIBN) were purchased from Aldrich (Milwaukee, WI). Phosphate-buffered saline (PBS, 4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4, and 140 mM NaCl, pH 7.4) and other solutions were prepared using deionized Milli-Q water (Millipore, Bedford, MA). All chemicals used were of analytical grade [21].

2.2. Synthesis of redox polymer PVI-[Os(dmo-bpy)$_2$Cl]$^{+/2+}$

PVI-[Os(dmo-bpy)$_2$Cl]$^{+/2+}$-(PVI-Os) was prepared as described previously [22]. As shown in Fig. 1A, preparation of [Os(dmo-bpy)$_2$Cl$_2$]$^{/+}$ was carried out under nitrogen gas. Briefly, 29.55 mg of dmo-bpy and 30 mg $(NH_4)_2OsCl_6$ were mixed with 10 mL ethylene glycol. The solution was degassed with nitrogen gas and heated for 30 min at 180 °C. Then 2.4 mL sodium hydrosulfate (1 M) was added to the solution at 4 °C and incubated for 30 min. Synthesized crystals were filtrated using a 0.45 μm nylon membrane filter (Whatman, Clifton, NJ, USA) and washed sequentially with cold distilled water (DW). These crystals were dried in the oven for 1 day. Preparation of poly(vinylimidazole) (PVI) was conducted by heating 6 mL of 1-vinylimidazole and 0.5 g AIBN at 70 °C for 2 h under nitrogen gas (Fig. 1B). A dark yellow precipitate was formed soon after heating. After the reaction mixture was cooled down, the precipitate was dissolved in methanol and added in droplets to a strongly stirred solution of acetone. Filtered precipitate was a pale yellow hygroscopic solid. Lastly, [Os(dmo-bpy)$_2$Cl]$^{/+}$ was allowed to react with PVI polymer in 20 mL ethylene glycol and heated to 180 °C for 80 h. After the 80 h reaction, the solution was added to 500 mL diethyl ether with vigorous stirring. Re-precipitated powder was melted in DW and filtrated using a 10 kDa regenerated cellulose membrane filter (Millipore, Billerica MA, USA) to complete the synthesis (Fig. 1C).

2.3. Preparation of enzyme electrodes

The loading solution of both anodic and cathodic catalysts consisted of aqueous stock solutions of PVI-Os (1 mg/mL), various concentrations (8 μg/mL, 80 μg/mL, 800 μg/mL) of enzyme, and PEGDGE (5 mg/mL) mixed in required ratio of 4:4:1. Then 10 μL aliquots of the mixture was placed on screen printed carbon electrodes (SPCEs) printed by a screen printing machine (BS-860AP, Bando, Korea) [23]. The electrodes were dried overnight (>12 h) in a desiccator. To prevent dust contamination, electrodes were sterilized using an EO gas Sterilizer (Person-EO50, Person medical, Korea). Prepared electrodes were attached onto Overhead Projector (OHP) films (Electrodag 423SS, Acheson, USA).

2.4. Electrochemical characterization

For electrochemical measurements, two modified-SPCEs were glued onto the edge of a 35 mm culture dish with medium containing 25 mM glucose dissolved in 0.1 M PBS buffer (pH 7.4). The anodic or cathodic electrode was used with 3.5 mm-diameter working electrodes (active area of 9.62 mm^2) on a flexible polyester film. The two-electrode electrochemical cells were then attached to CHI 660 B potentiostat/galvanostat (Austin, TX, USA) for open circuit potential and i-t curve analysis. A 0.5-mm-diameter platinum wire and an Ag/AgCl micro-electrode (3.0 M KCl saturated with AgCl, Cypress, Lawrence, KS, USA) were used as counter and reference, respectively. Current density was monitored over time. Power density and polarization curves (open circuit potential) were obtained with cyclic voltammetry [24]. The experiment was conducted in ambient air conditions at room temperature.

2.5. hESCs culture, embryonic body formation, and purification of contracting cardiomyocytes

Undifferentiated hESCs H9 (Wicell, Madison, WI) [25] were grown on inactivated mouse embryonic fibroblasts (MEF) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12, 50:50%; Gibco BRL, Gaithersburg, MD) supplemented with 20% serum replacement (Gibco), 1% nonessential amino acids (Gibco), 1 mM L-Glutamine (Gibco), 100 mM beta-mercaptoethanol (Gibco), and 4 ng/mL basic FGF (R&D System, Inc. Minneapolis, MN). Culture media was changed daily and hESCs were transferred to fresh feeder cells every 6–7 days with dissecting pipettes. To induce embryoid body (EB) formation, hESCs were detached from feeder cells with dispase (Gibco) then transferred to ultra-low attachment plates for suspension in basic FGF-free hESC culture medium supplemented with 20 ng/mL BMP4 for 2 days [26].

Subsequently, EBs were cultured in DMEM supplemented with 20% fetal bovine serum (FBS) (Hyclone) for 12 days to differentiate into contracting EBs. They were then plated onto 0.1% gelatin-coated plates and cultured in DMEM-high glucoses supplemented with 10% FBS for 8 days. Beating clusters were isolated and dissociated to a single-cell suspension by 0.25% trypsin-EDTA treatment for 5–10 min. Subsequently, cells were re-plated onto a dish that was coasted with a mixture of matrigel and 0.1% gelatin solution at a ratio of 1:100. Cells were cultured in low glucose DMEM supple-