Chemical Engineering Science 66 (2011) 570–581

Contents lists available at ScienceDirect

Chemical Engineering Science

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

The release of single cells from embryoid bodies in a capillary flow device

Ioannis Papantoniou, Mike Hoare, Farlan S. Veraitch *

The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK

ARTICLE INFO

Article history:
Received 20 May 2010
Received in revised form
18 October 2010
Accepted 21 October 2010
Available online 3 November 2010

Keywords:
Embryonic stem cells
Embryoid bodies
Shear
Capillary flow
Bioprocessing
Differentiation

ABSTRACT

The recovery of intact cells from embryoid bodies (EBs) is an essential stage in the development of many cell-based therapies. The use of a capillary device is explored whereby a suspension of EBs is exposed to controlled flow for the purpose of their break up and the release of single cells. The device was designed to avoid exposure of the released cells to shear stresses greater than 50 N m^{-2} at the wall or entrance; this shear stress level is a reported value above which loss of cell integrity may occur.

The disaggregation of the EBs leads to a mixed population of intermediate bodies and single cells. The break-up process was described using a first-order relationship with both the rate and final extent of loss of EBs being a function of the flow rate and the total time of exposure to shear stress as determined by capillary length and number of passes. The release of single cells is shown to be related to the loss of EBs and hence again a first-order relationship may be used. The overall relationship developed is used to predict successfully the release of single cells in a capillary device configuration suited to cell preparation for therapy, i.e. for defined sterile operation with the use of long capillaries with a low number of passes.

The break up of the EBs was achieved without the use of reagents such as trypsin to degrade the extracellular matrix that links the cells together and where there may be concern of the loss of key components at the cell surface. 60% of the cells released by capillary shear retained their cell wall integrity. Analysis of the phases of break up identified the release of cells from the EBs as being the point of loss of integrity. Exposure of already released cells to shear stress within the capillary led to no further loss of integrity.

The potential of this method for releasing cells for therapy is discussed.

1. Introduction

Embryonic stem (ES) cells are isolated from early embryos. They have the ability to be converted into almost every adult cell type and can be grown continuously in culture (Evans and Kauffman, 1981; Martin, 1981; Thomson et al., 1998). These two properties have generated a significant amount of interest in ES cells as they represent a potentially unlimited supply of any adult cell type. Cells generated from ES cells could have a number of possible clinical applications (Zhang et al., 2001; Vats et al., 2005). For example, ES cells can be converted into the cells lost during Parkinson's disease, diabetes, myocardial injury and in degenerative diseases of the retina (Roy et al., 2006; Jiang et al., 2007; Passier and Mummery, 2005; Lamba et al., 2006). Achieving these clinical outcomes will be heavily reliant upon the development of scalable, robust, reproducible and efficient processes.

ES cells are commonly expanded in tissue culture flasks in an attached format maintaining their characteristics in the presence of anti-differentiation factors (such as leukaemia inhibitory factor (LIF) in the case of mouse ES cells). In the absence of anti-differentiation factors ES cells can be converted into adult cells, a process known as differentiation. The most common technique to induce ES cell differentiation is via the formation of cell aggregates in the form of non-adherent spheroids referred to as embryoid bodies (EBs) (Doetschman et al., 1985; Hopfl et al., 2004). EBs can be grown in suspension and are held together by an extracellular matrix (ECM) (Itskovitz-Eldor et al., 2000; Schroeder et al., 2005). These properties mean that EBs can be successfully cultivated in scalable suspension systems such as stirred tank reactors. These reactors provide a significant advantage over the two dimensional surface attached format (Schroeder et al., 2005; Cameron et al., 2006; Cormier et al., 2006; Gilbertson et al., 2006; Kehoe et al., 2010). By controlling the EB culture environment during its growth, cell differentiation can be directed according to the media composition used (Schuldiner et al., 2000) or culture methods followed (Hopfl et al., 2004). One major processing challenge associated with the culture of EBs is the disaggregation of these tightly bound, compact aggregates prior to subsequent culture, purification or transplantation steps.

Abbreviations: ES cells, embryonic stem cells; EB, embryoid body; GFP, green fluorescent protein; EBF, embryoid body fragment; SC, single cell; ECM, extracellular matrix; LIF, leukaemia inhibitory factor

* Corresponding author. Tel.: +44 20 7679 2648; fax: +44 20 7209 0703.
E-mail address: f.veraitch@ucl.ac.uk (F.S. Veraitch).

0009-2509/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ces.2010.10.026

Currently the majority of methods for growing EBs use static suspension cultures with uncontrolled growth. However, methods are emerging to control the final size of the EBs. For example, eddies formed under agitation, in a stirred tank, were seen to control the size of EBs by removing cells lying on the outer surface of the EBs and by preventing EB–EB aggregation (Fok and Zandstra, 2005; Schroeder et al., 2005). In these studies care was taken to ensure that the impeller-generated shear stress was below the level that would be damaging to the cultured cells. The critical shear rate for the expansion of ES cell aggregates has been shown to be 0.78 N m^{-2} (Cormier et al., 2006) although for short exposure times ES cells have been seen to maintain their viability and number when flown through capillaries and exposed to wall shear stresses of 8 N m^{-2} (Veraitch et al., 2008). Similar methods have been used for the control of human ES cell derived EBs (Cameron et al., 2006) and mammalian neural precursor cell aggregates (Sen et al., 2002; Gilbertson et al., 2006). Earlier studies using mammalian cell lines have shown that aggregate size is proportional to energy dissipation rate in a stirred reactor with power dependencies of -0.25 for BHK cell aggregates (Moreira et al., 1995) and of -0.56 for neural stem cell aggregates (Sen et al., 2002). A number of other methods have been explored to control the initial size of EB aggregates. The hanging drop technique (Maltsev et al., 1993) and the pelleting of aggregates in customised V-bottomed 96-well plates (Burridge et al., 2007; Moeller et al., 2008) can be used to tightly control the initial number of cells per EB. However, these methods require further development before they can be used for the production of EBs on a scale suitable for use in therapy.

The disaggregation of EBs into smaller aggregates or a single cell suspension is required before these cells can be further processed in subsequent cell culture, cell sorting or engraftment protocols. Currently the standard method used for the dissociation of the EBs exposes aggregates to a concentrated solution of detachment enzymes. A widely used enzyme in cell culture for this purpose is trypsin that is derived from animal sources. The use of animal-derived products could introduce a number of undesirable contaminants into the process and efforts are underway to replace them during the derivation and culture of ES cells (Ludwig et al., 2006). In addition, enzymatic dissociation methods can cause cell death in primary adult and embryonic cells (Gori, 1964; Heng et al., 2007), affect subsequent adhesion events (Brown et al., 2007), impose selective pressure during the culture of neuronal cells (Tholey et al., 1985) and possibly lead to the selection of karyotypically abnormal stem cells during serial passaging (Mitalipova et al., 2005). More importantly, harvesting cells with trypsin can disrupt the activity of trans-membrane proteins and may irreversibly attenuate the function of ES-derived cellular therapies such as dopaminergic neurons (Wersinger et al., 2004).

This study investigated whether forces generated by fluid flow in a capillary were capable of dissociating EBs into single cell suspensions with particular focus on the physical characterisation of EB breakage. Such a system, especially if it can be restricted to a single pass operation, does provide the opportunity for low residence time operation to help minimise both time dependent degradation of the cells and any variation in the cell nutrient growth environment, e.g. oxygen tension, pH, etc.

2. Materials and methods

2.1. Mouse embryonic stem cells

46C mouse ES cells were kindly donated by Stem Cell Sciences (Cambridge, UK). This cell line, previously generated by gene targeting, expresses the green fluorescent protein (GFP) under the control of endogenous *Sox1* regulation (Ying et al., 2003). *Sox1* is the earliest known marker for neuroectoderm in the mouse embryo (Wood and Episkopou, 1999). Therefore, GFP expression can be used as a convenient means to check the number of early neuroectoderm cells formed during differentiation.

2.2. Cell culture

46C mouse ES cells were cultured on a 150 cm^2 Iwaki tissue culture treated plasticware (SLS, Nottingham, UK), pre-coated with 2 mL of 0.1% w/v gelatin (Sigma, Poole, UK) for 10 min. The culture medium for undifferentiated cell growth consisted of Glasgow modified essential medium (GMEM, Sigma) supplemented with 0.1 mM 2-mercaptoethanol (VWR, Leicestershire, UK), 1% v/v of 100× MEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% v/v foetal calf serum (all Invitrogen, Paisley, UK) and 1000 units mL^{-1} leukaemia inhibitory factor (LIF, Millipore, Watford, UK). Cells were routinely passaged every 2 days with 2 mL of 0.025% v/v porcine derived trypsin (Invitrogen) supplemented with 0.372 mg mL^{-1} ethylenediaminetetraacetic acid (EDTA) and 1% v/v chick serum (both Sigma) at 37 °C for 2–3 min. The enzymatic activity of the trypsin was quenched with 10 mL of culture medium before centrifugation (280*g*, 3 min) in an Eppendorf 5810R centrifuge (Eppendorf, Cambridge, UK) and re-suspension in 10 mL of fresh culture medium. Cell suspensions were diluted in ratios between 1:6 and 1:10 in fresh growth medium before being inoculated into pre-gelatinised tissue culture treated plasticware. Cultures were seeded at a sufficiently low density (3×10^4 cells cm^{-2}) to ensure that no more than 80% of the growth surface was covered with cells after 2 days of cultivation.

2.3. Embryoid body culture

Initiation of EB formation was achieved by differentiation of ES cells towards the neuroectodermal lineages using a modified version of previously published protocols (Bain et al., 1995; Stavridis and Smith, 2003). Cells from a 150 cm^2 flask containing 2×10^5 cells cm^{-2} were detached using trypsin as shown above. The trypsin was quenched with 10 mL culture medium this time in the absence of LIF (denoted as LIF-Medium). The cells were centrifuged as before and re-suspended in 40 mL of fresh LIF-Medium. This cell suspension (10 mL) was divided equally between four 100 mm diameter bacterial grade Petri dishes (SLS) resulting in each plate containing 7.5×10^5 cells ml^{-1} each. The day of inoculation was defined as day 0 of differentiation. The medium was replaced every 2 days by allowing the aggregates to settle at the bottom of a 25 mL universal tube (SLS) for 5 min before carefully removing the spent medium and adding fresh medium. On day 2 the medium was replaced with 10 mL of fresh LIF-Medium. On day 4 the medium was replaced with 10 mL of LIF-Medium containing 5×10^{-7} M retinoic acid (Invitrogen). On day 6 the EBs were re-suspended in 10 mL of N2B27 medium (Stem Cell Sciences). On day 8 the EBs were re-suspended in 10 mL of fresh N2B27 medium. The EB suspension was gently centrifuged (50*g* for 1 min) to form a sediment before carefully removing the supernatant. The EB volume was measured with a wide bore pipette and a fixed volume of supernatant was added back in order to achieve the desired solid vol%. A wide bore pipette was used to gently resuspend the EBs. The resultant suspension contained 4% v/v of EBs. Size distribution measurements before and after the re-suspension step ensured that the sample preparation had not affected the initial size distribution of the EB population and were used to verify the vol% of EBs. This provided the material for the capillary studies.

2.4. Capillary shear device

The device configuration chosen for the purpose of single cell (SC) release from the embryoid bodies (EBs) was a capillary