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# Detection of the osteogenic differentiation of mesenchymal stem cells in 2D and 3D cultures by electrochemical impedance spectroscopy

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ABSTRACT

Human mesenchymal stem cells are promising candidates for cell-based therapies since they have the capacity to differentiate into a variety of cell types. However, the acceptance of hMSCs for clinical applications as well as *in vitro* tissue models will depend on strategies for standard characterisations. Impedance spectroscopy is a proven and powerful tool for non-invasive monitoring of cellular processes. The aim of this study was to prove the hypothesis, that the process of osteogenic differentiation can be monitored non-invasively and time-continuously by using impedance spectroscopy.

This hypothesis was examined for 2D cell layers of hMSCs by continuous impedance spectroscopy employing a planar electrode-based chip and for 3D aggregates of hMSCs after 21 and 25 days of osteogenic treatment by using a capillary measurement system.

The impedance spectra of osteogenic treated hMSCs reported a significant increase of the magnitude of impedance compared to controls cultivated in normal growth medium. The osteogenic status of the cells was determined by alkaline phosphatase expression and von Kossa staining. In respect to that finding it is concluded that impedance spectroscopy is an appropriate method for non-invasive characterisation of osteogenic differentiation of hMSCs, which is relevant for quality control of cell-based implants and cell-based test systems for drug development.



## 1. Introduction

Mesenchymal stem cells (MSC) have the capacity to differentiate into a variety of cell types, such as osteocytes, chondrocytes, adipocytes, myocytes and tenocytes (Owen and Friedenstein, 1988; Caplan, 1991; Prockop, 1997; Mackay et al., 1998; Young et al., 1998; Pittenger et al., 1999). Since MSCs can easily be isolated from the bone marrow and expanded *in vitro*, they provide a source of cells that can be employed in a wide range of applications, such as tissue engineering, cell therapy or in drug screening.

The use of human mesenchymal stem cells (hMSC) in multiple clinical approaches is successfully demonstrated. Mesenchymal stem cells are used in spinal fusion approaches, in the repair of segmental and craniotomy defects (Krebsbach et al., 1998; Quarto et al., 2001; Muschler et al., 2003) and hMSC are used to create bone grafts for orthopaedic surgery and reconstructive medicine. This is of particular interest due to the high number of bone lost in consequence of trauma, inflammation or cancer (Bruder et al., 1998; Casabona et al., 1998; Shang et al., 2001). Promising results have been reported in animal experiments and first clinical studies (Delécrin et al., 2000; Kon et al., 2000; Marcacci et al., 2007). In addition a variety of common skeletal disorders such as osteoporosis and arthritis cannot be treated successfully with conventional drugs and therefore the requirement for appropriate bone substitutes increases. Another objective is the application of hMSC to improve the healing process after hip or knee replacement by preseeding the implants with osteocytes (Livingston et al., 2002; Holtorf et al., 2005).

Recently, it was shown that the treatment of the genetic bone disorder osteogenesis imperfecta with hMSC provide clinical benefits to patients, either by infusion of bone marrow derived hMSCs or as carriers for gene therapy approaches (Horwitz et al., 1999, 2001, 2002; Chamberlain et al., 2004). Further research into new treatment strategies for these disorders as well as the development of new drugs and toxicological screenings can be aided by standardized *in vitro* models based on hMSCs. Due to the unique proliferation and multipotent differentiation properties of MSC the realisation of tissue models based on human cells for a wide range of applications is imaginable and first *in vitro* model based on hMSC for chondrogenesis and osteogenesis were already described (Johnstone et al., 1998; Pound et al., 2007).

The acceptance of hMSCs as cell source for cell-based therapies or *in vitro* tissue models will depend on the availability of well-characterised cells and cell aggregates. Hence, standard characterisations and quality control following GxP (e.g. Good Manufacturing Practise, Good Clinical Practise) guidelines and

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regulations are necessary and mandatory for cell cultivation. Current methods for examining the osteogenic status of hMSCs by specific markers, e.g. alkaline phosphatase (ALP), collagen-1 or mineralization of the extracellular matrix (ECM), are invasive and samples have to be prepared and destroyed just for quality control procedures (Kern et al., 2006). Therefore, the development of non-invasive methods for online monitoring of the cell culture conditions and proliferation and differentiation processes is sorely needed.

Impedance spectroscopy of cells and tissues provides physiological and morphological information by measuring the frequency dependence of their electrical properties (Schwan, 1993; Malmivuo and Plonsey, 1995) and has been employed to state living cells *in vitro* (Reininger-Mack et al., 2002). Microcapillary setups were used to record the impedance spectra of tumour models to evaluate therapeutic approaches (Thielecke et al., 2001; Bartholomä et al., 2005). Further applications of electrode-based impedance spectroscopy are the analysis of electrophysiological cells and tissues such as heart-muscle cells and neuronal cells, cellular migration studies (Giaever and Keese, 1991, 1993; Linderholm et al., 2006), monitoring of cell growth (Luong et al., 2001) or apoptosis (Arndt et al., 2004). Moreover, a quantitative monitoring of cells during cytotoxification was described (Xiao et al., 2002).

Osteogenic differentiation processes of hMSCs are characterized by morphological changes and the expression of bone specific ECM, e.g. collagens, adhesion proteins such as osteocalcin, osteopontin and osteonectin, and finally the mineralization of the ECM.

It was shown recently that the induced differentiation of hMSC into adipocytes can be monitored by impedance spectroscopy (Cho et al., 2009). It is therefore assumed that the changes in cellular properties during osteogenic differentiation of hMSCs are also accessible by impedance spectroscopy.

The aim of this study is to prove the hypothesis, that the process of osteogenic differentiation can be monitored non-destructively and time-continuously by using impedance spectroscopy. This hypothesis will be examined for 2D cell layer of hMSC by using a planar electrode-based chip and for 3D aggregates of hMSC by using a capillary measurement system.

## 2. Materials and methods

### 2.1. Material

Cell culture materials were all purchased from Greiner Bio-one GmbH (Frickenhausen, Germany). Phosphate buffered saline (PBS), alpha MEM (without desoxyribonucleotides or ribonucleotides, with GlutarMAX), penicillin/streptomycin, trypsin/EDTA solution and fetal bovine serum (FBS Gold) were from PAA Laboratories GmbH (Pasching, Austria). HistoBond positive charged slides were from Paul Marienfeld GmbH & Co. KG (Bad Mergentheim, Germany). Cell proliferation reagent WST-1 was from Roche (Mannheim, Germany) and Mikrozid AF Liquid was from Schuelke (Norderstedt, Germany). Vectorshield mounting medium was purchased from Vector Laboratories (Peterborough, UK). The leukocyte alkaline phosphatase staining kit (#86), Methylcellulose (#274429) and all other chemicals were purchased from Sigma (Steinheim, Germany).

### 2.2. Isolation and cultivation of hMSCs

Human MSCs were obtained from femoral heads of three different donors ranging in age from 71 to 79. The bone marrow was abrased, dissected and suspended in alpha MEM, then the mononuclear cell mass was isolated with a ficoll density gradient centrifugation at $1000 \times g$ for 30 min and resuspended in complete medium (alpha MEM, 15% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin). All cells were plated out in 12 ml of medium in a T175 flask and incubated at 37 °C, 5% $CO_2$ in a humidified atmosphere (BB 6220, Heraeus-Christ, Hanau, Germany). Non-adherent cells were removed by changing medium after 24 h and adherent cells were kept in culture while fresh complete medium was replaced every 2–3 days. When reaching confluence, cells were harvested with trypsin/EDTA for 3 min at 37 °C and recovered cells were seeded out into new culture flasks at a density of $2.5 \times 10^3$ cells/cm$^2$ for further expansion. All cell populations were tested for expression of surface markers (CD73+, CD90+, CD105+, CD31−, CD34−, CD45−) by flow cytometry (FACSCalibur, Becton Dickinson GmbH, Heidelberg, Germany) and osteogenic potential in monolayer cultures. For experiments only passages 5–12 from cell populations with a suitable marker profile and a proven osteogenic potential were used.

### 2.3. Osteogenic differentiation of hMSC 2D cultures

Cells were kept at 37 °C, 5% $CO_2$ in a humidified atmosphere. The osteogenic differentiation was initialised by treatment with 100 nM dexamethasone, 50 μg/ml l-ascorbic acid and 5 mM β-glycerophosphate (Pittenger et al., 1999) whereas hMSC were held in growth medium for control. The medium was replaced every 2–3 days.

### 2.4. Spheroid formation of hMSCs and osteogenic differentiation of 3D aggregates

For spheroid formation of hMSCs, a cell suspension at a concentration of $2 \times 10^4$ cells per 100 μl was cultured in 96-well, U-bottom, non-adhesive plates (Greiner, #650185). To prevent cell adhesion 0.1% methylcellulose was added to the culture medium additionally. Cellular aggregation was achieved using a rotation platform (Heidolf Rotamax 120, Schwabach, Germany) within 2 days by 75 rpm. Afterwards, spheroids were cultured in the presence of 100 nM dexamethasone, 50 μg/ml l-ascorbic acid and 5 mM β-glycerophosphate for 21 days to induce osteogenic differentiation. Control spheroids were cultivated in complete growth medium. The medium was refreshed every 2–3 days by exchanging 50% of the liquid. Spheroids were used for analysis after reaching a cross-sectional mean diameter of approximately $330 \pm 30$ μm (day 21) and $390 \pm 30$ μm (day 25) to exclude size depending effects.

#### 2.4.1. Induction of necrosis in spheroids during osteogenic differentiation

Spheroids were treated from day 5 on with Mikrozid liquid to induce necrosis for detection of necrotic areas using impedance spectroscopy.

### 2.5. Impedance measurements and data analysis

#### 2.5.1. Impedance monitoring of 2D hMSC layers during osteogenic differentiation

The impedance of the 2D hMSC layers during osteogenic differentiation was monitored using a planar electrode-based chip (Fig. 1A) (Cho et al., 2007; Cho and Thielecke, 2008). In brief, the planar and circular platinum electrodes had a diameter of 1 mm with a distance between the middle of electrodes >2 mm and were electrically connected with an impedance analyzer (1260, Solartron Analytical, Farnborough, UK). For the impedance measurement, the alternating potential was set to 10 mV with the frequency range of 100 Hz to 1 MHz. For the impedance analysis of the hMSC layer, an equivalent circuit model consisting of serially connected constant phase elements for electrode impedance (de Boer and van Oosterom, 1978), impedance of cell layer, and medium resistance