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# *In vitro* cell response to Co-containing 1393 bioactive glass

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ABSTRACT

Cobalt ions are known to stimulate angiogenesis *via* inducing hypoxic conditions and hence are interesting agents to be used in conjunction with bioactive glasses (BGs) in bone tissue engineering approaches. In this work we investigated *in vitro* cell biocompatibility of Co releasing 1393 BG composition (in wt.%: 53$SiO_2$, 6$Na_2O$, 12$K_2O$, 5MgO, 20CaO, and 4$P_2O_5$) derived scaffolds with osteoblast-like cells (MG-63) and human dermal microvascular endothelial cells (hDMECs).
Cell viability, cell number and cell morphology of osteoblast-like cells in contact with particulate glass and 3D scaffolds were assessed showing good biocompatibility of 1393 reference material and with 1 wt.% CoO addition whereby 5 wt.% of CoO in the glass showed cytotoxicity. Furthermore for 1393 with 1 wt.% of CoO increased mitochondrial activity was measured. Similar observations were made with hDMECs: while 1393 and 1393 with 1 wt.% CoO were biocompatible and the endothelial phenotype was retained, 5 wt.% CoO containing BG showed cytotoxic effects after 1 week of cell culture. In conclusion, 1 wt.% Co containing BG was biocompatible with osteoblast like cells and endothelial cells and showed slightly stimulating effects on osteoblast-like cells whereas the addition of 5 wt.% CoO seems to exceed the vital therapeutic ranges of Co ions being released in physiological fluids.



## 1. Introduction

The clinical demand for engineered bone tissue has been growing in recent years in direct relation to the increasing human population [1]. Tissue engineering (TE) is one of the approaches being investigated to tackle this problem by regenerating bone tissue using a combined cell/material therapy [2–5]. In common TE strategies a three-dimensional porous structure, termed "scaffold", fabricated from a suitable artificial or natural material is used [6–9]. Ideally, these scaffolds should not only provide a passive structural support for bone cells, but they should also stimulate bone formation by inducing osteoblastic cell proliferation and differentiation. Furthermore, it has become evident that a functional vascularization of the engineered bone construct is essential for their successful clinical application in bone regeneration [10,11].

Bioactive glasses (BG) such as 45S5 Bioglass® or "1393" BG are known to be bioactive, a behavior which is characterized by formation of a carbonated hydroxyapatite layer (HCA) when exposed to biological fluid [12], while also showing osteoinductive behavior. Hence, BGs are being widely considered in bone regenerating approaches [7]. In order to enhance the biological performance of BGs towards a specific cell response the addition of therapeutic metal ions and bioinorganics into the glass matrix, which can be released in a controlled manner upon scaffold degradation, has emerged as a common approach [13,14].

Cobalt ions have been considered as important agents in the context of bone physiology [15–18] since Co is known to induce hypoxia conditions and to stabilize hypoxia-inducible factor 1 (HIF-1) [19,20]. Hypoxia conditions, in turn, are known to activate several pro-regenerative processes in human body [21] *via* regulation of the HIF-1. Furthermore, HIF-1 activation has been shown to result in accelerated bone ingrowth whereby the HIF-1a pathway has been identified as being critical for angiogenesis and skeleton regeneration [22] and is important for development of angiogenesis, stem cell differentiation and fracture repair [23,24]. Furthermore, HIF-1 is stabilized under hypoxic conditions and regulates several genetic pathways relevant for skeleton repair [25].

Hence Co-releasing bioactive glasses have been proposed as hypoxia mimicking material [26] to be used for artificial stabilization of HIF-1. These potential benefits of $Co^{2+}$ ions have been further tested *in vitro*

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and *in vivo*. For instance, Cobalt was shown to promote angiogenesis *via* activation of hypoxia inducible factor 1 (HIF-1) in a rat remnant kidney *in vivo* model when Co was applied by subcutaneous injections [18]. Also in a rat bladder model Co was shown to enhance the hypoxia response, cell growth and angiogenesis indicated by stimulated expression of HIF-1α and vascular endothelial growth factor (VEGF) [15]. Furthermore, bone marrow derived stem cells (BMSCs) pre-treated with $CoCl_2$ induced higher degree of vascularization and enhanced osteogenesis within collagen scaffolds implanted *in vivo* [27].

However, Co is also known to be toxic when applied at high dosage [20,28]. Hence, for applications in tissue engineering an (inorganic) matrix is needed for the controlled release of Co ions into physiological environment. In this context BGs have been shown to be suitable carriers for therapeutic ions [13,29–33].

Previously, we reported on the structure and acellular bioactivity of Co-releasing bioactive glass (Co-BG) derived scaffolds [30]. In this study, the cellular biocompatibility of the Co-BG powder and the scaffolds was assessed by means of cellular response of osteoblast-like cells (MG-63) and human dermal micro endothelial cells (hDMECs). The commercial use of bioactive glasses includes the application of particulate glass as powder or granules as bone filling material (Novabone®, BonAlive®) [7]. Also inclusion of bioactive glass particles as filler in polymer/BG composites has been widely investigated [9]. Hence, in this study we considered the behavior of osteoblast-like cells in contact with particulate Co-containing 1393 glass in order to test the biocompatibility of this novel glass composition. Further, the response of osteoblast-like cells seeded directly on 3D scaffolds was assessed and the vitality of endothelial cells when exposed to ionic dissolution products from Co-BG scaffolds was evaluated.

## 2. Experimental

### 2.1. BG fabrication

Melt derived Co releasing 1393 bioactive glass and respective 3D scaffolds were fabricated by melt derived route and foam replica method as described elsewhere [30]. Three glass compositions based on 1393 BG containing Co were used: termed 1393, 1393-1Co and 1393-5Co, containing 0 wt.%, 1 wt.% and 5 wt.% CoO, respectively.

### 2.2. Cell culture

#### 2.2.1. Osteoblast-like cells

Osteoblast-like (MG-63) cells (Human osteosarcoma cell line, Sigma-Aldrich, Germany) were cultured at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$, in DMEM (Dulbecco's modified Eagle's medium, Gibco, Germany) containing 10 vol.% fetal bovine serum (FBS, Sigma-Aldrich, Germany) and 1 vol.% penicillin/streptomycin (Sigma-Aldrich, Germany). Cells were grown for 48 h to confluence in 75 $cm^2$ culture flasks (Nunc, Denmark), washed with phosphate buffered saline (PBS) and harvested using Trypsin (Sigma, Germany). Cells were counted by a hemocytometer (Roth, Germany) and diluted to a final concentration of 100,000 cells $ml^{-1}$.

Initial cytotoxicity tests of the glasses were carried out by culturing bioactive glass (BG) glass powders in direct contact with MG-63 cells. The BG powder was dispersed in DMEM and ultra-sonicated for 5 min to break agglomerates. MG-63 cells were cultured in direct contact with BG particles at concentrations from 0.1 to 1000 μg $ml^{-1}$ in 600 μl DMEM for 48 h.

Additionally, in order to test the interaction of cells and the scaffold surface the cells were directly seeded on the BG derived scaffolds. Prior to cell seeding the scaffolds were immersed in DMEM for 24 h. The cells were applied on the scaffolds ($5 * 5 * 5$ $mm^3$) surface at a concentration of 100,000 cells/ml.

#### 2.2.2. Endothelial cells

Human dermal microvascular endothelial cells (hDMECs) and the specialized endothelial cell growth media (EGM MV2) were obtained from PromoCell GmbH, Heidelberg, Germany. Passage 5 cells were used for all experiments. hDMECs were exposed to ionic dissolution products released from 1393 derived scaffolds. The scaffolds were placed in permeable inserts in 24 cell culture well plates. Cells were seeded in a concentration of 100,000/ml.

### 2.3. Cell characterization

#### 2.3.1. Osteoblast cells

*2.3.1.1. Mitochondrial activity.* The mitochondrial activity was assessed applying a WST-8 assay (Sigma-Aldrich). After the time point of interest the culture medium was removed from the respective well culture plate and the cells were washed with PBS. After addition of the WST-8 reagents (1 vol.% in DMEM) in each well, the plates were incubated for 1.5 h. Subsequently, the supernatant of all samples was transferred to a 98-well plate (100 μl in each well) to measure the absorbance at 450 nm and 650 nm with an ELISA-Reader (Perkin Elmer, Multilabel Reader Enspire 2300, Germany).

*2.3.1.2. LDH-activity.* Lactate dehydrogenase (LDH-) activity provides a measurement of the amount of attached cells on the samples. A commercially available LDH-activity quantification kit (TOX7, Sigma-Aldrich) was used to quantify cell number by the LDH enzyme activity in the cell lysate. Cells were washed with PBS and lysed with lysis buffer for 30 min (1 mL/well). Lysates were centrifuged (5 min, 2500 rpm) and 100 μL from the supernatant solutions were transferred to 1 mL cuvettes. 100 μl of master-mix containing equal amounts of substrate solution, dye solution, and cofactor solution for LDH assay were added to each cuvette. After 30 min reaction in the dark the reaction was stopped with 300 ml 1 M HCl per cuvette. The dye concentration was measured on a spectrophotometer (Specord 40, Jena Analytik, Germany) at 490 nm.

*2.3.1.3. Vybrant™ cell-labeling.* To analyze the adherent growth and distribution of cells on the BG scaffold samples, commercially available Vybrant™ cell-labeling solution (Molecular Probes, The Netherlands) was used. After 48 h of incubation, cell culture medium was removed and staining solution (5 μl dye labeling solution to 1 ml of growth medium) was added and incubated for 15 min. Afterwards the solution was removed, the samples were washed with PBS (phosphate buffered saline, Gibco) and cells on the BG samples were fixed by 3.7 vol.% paraformaldehyde. The samples were prepared for confocal scanning laser microscopy (CSLM, Leica TCS SP5 II, Germany) to analyze cell morphology and distribution.

#### 2.3.2. Endothelial cells

*2.3.2.1. Cell morphology and cell number.* Cell morphology of the hDMECs was assessed after 2 weeks cultivation time with a light microscopy (LM, Nikon Eclipse TE 2000-U, Japan).

Cell number of hDMECs was determined by trypsinization of the cells and counting the cell number using a Neubauer chamber.

*2.3.2.2. Flow cytometry analysis (FACS).* The human endothelial cell surface markers (Cluster of differentiation, CD31; von Willebrand factor, vWF, Vascular endothelial growth factor 2, VEGFR2) were stained at $5 \times 10^4$ cells for each antigen. CD31 is a known platelet adhesion protein which is expressed at cell connecting junctions and is found in early and mature endothelial cells and is known to be involved in activation of integrins. CD31 was stained by mouse anti-human CD31-Biotin followed by a second staining step with Streptavidin PerCP-eFluor® 710 (both from eBioscience, San Diego, CA, USA). vWF is an important platelet