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# Adhesion and growth of human bone marrow mesenchymal stem cells on precise-geometry 3D organic–inorganic composite scaffolds for bone repair

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ABSTRACT

Engineering biomaterial scaffolds that promote attachment and growth of mesenchymal stem cells in three dimensions is a crucial parameter for successful bone tissue engineering. Towards this direction, a lot of research effort has focused recently into the development of three-dimensional porous scaffolds, aiming to elicit positive cellular behavior. However, the fabrication of three-dimensional tissue scaffolds with a precise geometry and complex micro- and nano-features, supporting cell in-growth remains a challenge. In this study we report on a positive cellular response of human bone marrow-derived (BM) mesenchymal stem cells (MSCs) onto hybrid material scaffolds consisting of methacryloxypropyl trimethoxysilane, zirconium propoxide, and 2-(dimethylamino)ethyl methacrylate (DMAEMA). First, we use Direct fs Laser Writing, a 3D scaffolding technology to fabricate the complex structures. Subsequently, we investigate the morphology, viability and proliferation of BM-MSCs onto the hybrid scaffolds and examine the cellular response from different donors. Finally, we explore the effect of the materials' chemical composition on cell proliferation, employing three different material surfaces: (i) a hybrid consisting of methacryloxypropyl trimethoxysilane, zirconium propoxide and 50 mol% DMAEMA, (ii) a hybrid material comprising methacryloxypropyl trimethoxysilane and zirconium propoxide, and (iii) a purely organic polyDMAEMA. Our results show a strong adhesion of BM-MSCs onto the hybrid material containing 50% DMAEMA from the first 2 h after seeding, and up to several days, and a proliferation increase after 14 and 21 days, similar to the polystyrene control, independent of cell donor. These findings support the potential use of our proposed cell–material combination in bone tissue engineering.



## 1. Introduction

A common objective in bone tissue engineering research is the design of biomaterial scaffolds that support cell and tissue growth [1]. Many synthetic structures have been designed to impart bulk properties to the construct, such as adequate mechanical strength and sufficient transport properties for cell infiltration and tissue organization. Although the majority of these structures possess similar macroscopic properties as those of the native tissue, the constructs have failed prior to full healing [2,3]. An important parameter identified in the failure of tissue-engineered constructs is insufficient tissue regeneration around the biomaterial directly after implantation. Since the interaction of cells with the biomaterial is a vital element in the evaluation of a scaffold, a lot of research effort focuses on designing biomaterial structures that facilitate favorable interactions and enhance tissue regeneration.

Moreover, the scaffolds designed for tissue engineering applications must be three-dimensional, highly porous and interconnected to support cell attachment and proliferation [4,5]. The role of the scaffold porosity in bone regeneration has been investigated by Kuboki et al. using a rat ectopic model and solid porous particles [6]. Pores are necessary for bone tissue formation because they allow migration and proliferation of osteoblasts and mesenchymal cells, as well as angiogenesis and vascularization. In addition, a porous surface improves mechanical interlocking between the implant biomaterial and the surrounding natural bone, providing greater mechanical stability at this critical interface [7]. The minimum pore size required to regenerate mineralized bone is generally considered to be 100 μm after the study of Hulbert and Proc

*Abbreviations:* BM, bone marrow; MSCs, mesenchymal stem cells; DMAEMA, 2-(dimethylamino)ethyl methacrylate; ZPO, zirconium propoxide; MAPTMS, methacryloxypropyl trimethoxysilane; 3D, three-dimensional; DLW, Direct Laser Writing; FCS, fetal calf serum; SEM, scanning electron microscopy; ANOVA, analysis of variance; P, passage.

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[8]. Materials used for fabricating scaffolds for bone tissue-engineering application must also have sufficient structural integrity matching the mechanical properties of native tissue, and should offer an ideal and critical micro-environment to function as an artificial extra-cellular matrix (ECM) onto which cells attach, grow, and form new tissues [9,10]. Hybrid materials and composites with tunable mechanical, chemical, and biological properties exhibit advantageous features and have attracted particular research attention in recent years [11].

Most available scaffold fabrication methods, such as solvent casting, fiber bonding, phase separation, gas induced foaming, and salt leaching, are either limited to producing scaffolds with simple geometry, or depend on an indirect casting method for scaffold fabrication [12]. They are therefore insufficient for the manufacturing of scaffolds with complex, accurate structural architectures, and rather result in structures of random internal architecture and great structural variation.

Direct Laser Writing (DLW) by multi-photon polymerization (MPP) [13] enables the design and fabrication of fully three-dimensional (3D), readily-assembled microstructures with sub-100 nm resolution [14] and complex internal and external architectures, thereby allowing precise micro-topography control [15,16,41]. Control over characteristics such as scaffold porosity, pore size, and permeability may enhance cell infiltration and mass transport of nutrients and metabolic waste throughout the scaffold. Several authors have discussed the advantages of the DLW technique currently in use [17–21].

Recent advances in stem cell research have provided the theoretical background for the development of cell-based therapies for bone repair. Over the last years, mesenchymal stem cells (MSCs) have emerged as promising candidates for orthopedic applications. Originally isolated from the bone marrow (BM), MSCs are considered as the progenitors for skeletal tissues, and both preclinical and clinical data support the notion that these cells have a great potential for bone regeneration [22–24]. Cells with similar characteristics to BM-MSCs have also been isolated from various other tissues [25]. Yet BM-MSCs represent the most extensively studied population of adult MSCs and are still considered as the gold standard for MSC clinical application.

In a previous study we showed the positive cellular response of the pre-osteoblastic cell line MC3T3-E1 on a hybrid material comprising methacryloxypropyl trimethoxysilane (MAPTMS), zirconium propoxide (ZPO), and 50 mol% 2-(dimethylamino)ethyl methacrylate (DMAEMA), and its favorable mechanical properties for scaffold fabrication [11]. In this work, we explore the interactions of BM-MSCs with precise, complex-geometry 3D scaffolds fabricated by the technology of DLW and consisting of a hybrid material containing MAPTMS, ZPO and 50% DMAEMA. For this, we investigate the initial adhesion of BM-MSCs on the 3D structures within the first 2 h post-seeding, and up to 7 days. To explore the influence of the materials' chemical composition on the cell proliferation, we use three materials, (i) a hybrid consisting of MAPTMS, ZPO and 50 mol% DMAEMA, (ii) a hybrid comprising MAPTMS and ZPO, and (iii) a purely organic polyDMAEMA, to fabricate films on glass substrates, and we quantify the cell proliferation of BM-MSCs on them. Finally, we report on the variability of the proliferation of BM-MSCs derived from three different donors.

## 2. Materials and methods

### 2.1. Hybrid-50% DMAEMA material

The material used for the fabrication of 2D films and 3D structures is an organic–inorganic composite comprising MAPTMS (99%), ZPO (70% in propanol) and DMAEMA (>99%) [11]. 4,4-Bis(diethylamino) benzophenone (BIS) was used as the photoinitiator. DMAEMA was copolymerized with MAPTMS upon photopolymerization, whereas ZPO and the alkoxysilane groups of MAPTMS served as the inorganic network forming moieties.

### 2.2. Sample fabrication: thin films and 3D structures

Three types of specimens, all prepared on round glass substrates with a diameter of 15 mm and a thickness of 100 μm, were employed in this study: (i) two-dimensional films for the quantification of the cell viability and proliferation; (ii) 3D square blocks with dimensions 200 × 200 × 10 μm (l × w × h) for the investigation of cell adhesion and cell morphology by immunocytochemical staining and scanning electron microscopy (SEM); and (iii) 3D scaffolds with a high precision cubic geometry made from horizontally and vertically ordered ring structures for the investigation of cell adhesion by immunocytochemistry and SEM.

For the two-dimensional coatings we used three material compositions: (a) a hybrid material consisting of MAPTMS and ZPO (referred to as 'hybrid'); (b) a hybrid organic–inorganic material of the above two components supplemented with 50 mol% of DMAEMA (referred to as 'hybrid-50% DMAEMA'); and (c) a purely organic material based on DMAEMA (referred to as 'organic') (see Table 1).

Material specimens used for the adhesion, viability and proliferation experiments were incubated for 1 h in ethanol, air-dried under sterile conditions in a laminar flow and rinsed briefly with DMEM cell culture medium prior to cell seeding.

#### 2.2.1. Thin film preparation

Thin films of the hybrid and hybrid-50% DMAEMA materials were prepared by drop-casting or spin-coating onto 100 micron-thick silanized glass substrates. The samples were heated in an oven at 50 °C for 5 min before the photopolymerization, which led to the condensation of the alkoxide groups and the formation of the inorganic matrix. Next, the methacrylate moieties were polymerized using a KrF excimer laser, operating at 248 nm, resulting in the formation of irreversible and fully saturated aliphatic C–C covalent bonds that further increase the connectivity of the material. Finally, the samples were developed for 30 min in a 50:50 solution of 1-propanol:isopropanol to remove the non-polymerized material.

Polymer thin films based on DMAEMA were also prepared on glass slides using surface initiated atom transfer radical polymerization (ATRP). First, a self-assembled monolayer of the in-house synthesized initiator 3-(2-bromoisobutyramido)-propyl(triethoxy) silane was formed by immersing the glass substrates in a THF solution of the initiator for 24 h. Next, the substrate was rinsed extensively with THF and ethanol, dried under a stream of nitrogen and transferred to the polymerization flask. The polyDMAEMA chains were synthesized by ATRP on the initiator-functionalized glass substrates in a 4:1 methanol:water mixture. The polymerization was allowed to proceed at room temperature for 5 h, after which the substrate was removed and rinsed thoroughly with water and methanol and was dried under a nitrogen flow.

#### 2.2.2. Fabrication of 3D hybrid blocks and scaffolds by Direct fs Laser Writing

The experimental setup employed for 3D structure fabrication has been described previously [11,26]. A Ti:Sapphire femtosecond laser (Femtolasers Fusion, 800 nm, 75 MHz, <20 fs) was focused into the photopolymerizable composite using a microscope objective lens (20×, N.A. = 0.65 and 40× N.A. = 0.95, Zeiss, Plan Apochromat). Sample movement was achieved using piezoelectric and linear stages, for fine and step movement, respectively (Physik Instrumente GmbH, Germany). The whole DLW setup was computer-controlled using the 3DPoli software.

### 2.3. Isolation and expansion of BM-MSCs

Bone marrow was aspirated from the posterior iliac crest of (n = 3) of individuals undergoing orthopedic surgery. Institutional ethics committee approval was granted prior to the study and informed consent