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Regular Article

# Effects of a mesoporous bioactive glass on osteoblasts, osteoclasts and macrophages

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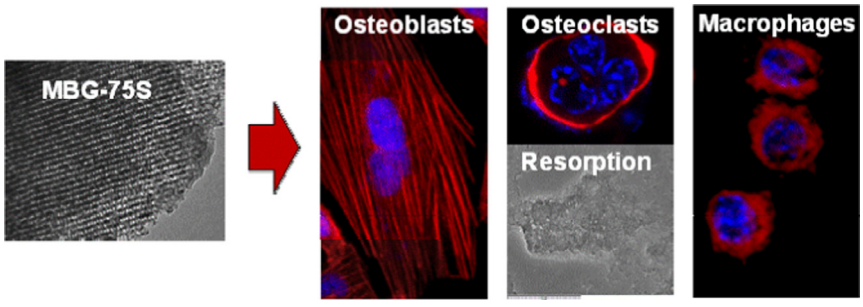
## GRAPHICAL ABSTRACT

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## ABSTRACT

A mesoporous bioactive glass (MBG) of molar composition $75SiO_2$-$20CaO$-$5P_2O_5$ (MBG-75S) has been synthetized as a potential bioceramic for bone regeneration purposes. X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), nitrogen adsorption studies and transmission electron microscopy (TEM) demonstrated that MBG-75S possess a highly ordered mesoporous structure with high surface area and porosity, which would explain the high ionic exchange rate (mainly calcium and silicon soluble species) with the surrounded media. MBG-75S showed high biocompatibility in contact with Saos-2 osteoblast-like cells. Concentrations up to 1 mg/ml did not lead to significant alterations on either morphology or cell cycle. Regarding the effects on osteoclasts, MBG-75S allowed the differentiation of RAW-264.7 macrophages into osteoclast-like cells but exhibiting a decreased resorptive activity. These results point out that MBG-75S does not inhibit osteoclastogenesis but reduces the osteoclast bone-resorbing capability. Finally, *in vitro* studies focused on the innate immune response, evidenced that MBG-75S allows the proliferation of macrophages without inducing their polarization towards the M1 pro-inflammatory phenotype. This *in vitro* behavior is indicative that MBG-75S would just induce the required innate immune response without further inflammatory complications under *in vivo* conditions. The overall behavior respect to osteoblasts, osteoclasts and macrophages, makes this MBG a very interesting candidate for bone grafting applications in osteoporotic patients.



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## 1. Introduction

Mesoporous bioactive glasses (MBGs) are bioceramics intended for bone tissue regeneration purposes. Discovered in 2004 by Zhao et al. [1], MBGs mean a significant upgrade respect to the conventional sol-gel bioactive glasses prepared by Li et al. in 1991 [2]. Similarly, to sol-gel bioactive glasses, MBGs are commonly prepared in the ternary system $SiO_2$-CaO-$P_2O_5$ [3–5] and, in the last decade, different research groups have incorporated different ions with potential therapeutic properties [6–11]. In the case of MBGs, the incorporation of a structure directing agent (SDA) to the synthesis results in the formation of an ordered mesophase by the self-organization of the SDA into micelles. Soluble silica, phosphate and calcium species condensates around this organic template, which leads to a mesoporous structure after calcination, thus providing higher textural properties compared to conventional sol-gel bioactive glasses [12,13].

The primary consequence on the biological behavior is a faster and more intense ionic exchange (mainly $Ca^{2+}$ and silica species) between the MBG and the surrounding fluids [14]. In fact, some MBGs have shown the fastest *in vitro* bioactive behavior when soaked in simulated body fluid, in terms of the nucleation and growth of a carbonate nanocrystalline apatite on their surface, very similar to the biological one found in bones [15]. However, the MBG surface reactivity is not their only action mechanism. The ions released from MBG also stimulate the expression of several genes of osteoblastic cells and induce angiogenesis both *in vitro* and *in vivo* [16,17]. Recent studies suggest that these ions could also regulate immune responses by altering the ionic microenvironment between the implants and hosts [18]. The importance of the immune response during biomaterial-mediated osteogenesis makes necessary the evaluation of the osteoimmunomodulatory properties of biomaterials for bone tissue [19].

Recently, the *in vivo* response to these materials has been studied in different animal models, evidencing certain advantages respect to other bioceramics. Due to their potential bone regeneration capabilities, MBGs are being considered as bone grafts in the case of osteoporotic patients. Osteoporosis is produced by the bone remodeling disruption that is due to either increased bone resorption by osteoclasts or decreased new bone formation by osteoblasts or both [20]. The biomaterials most commonly employed for treatment of osteoporotic bone and bone regeneration have been designed to stimulate the osteogenesis process and bone formation by osteoblasts. For this reason, osteoblasts are commonly used for the *in vitro* evaluation of bone materials [21] but few studies are focused on the effects of these biomaterials on bone resorbing osteoclasts [22]. Osteoclasts are multinucleated giant cells which differentiate from hematopoietic stem cells of the monocyte/macrophage lineage through sequential steps [23] regulated by several growth factors and cytokines expressed by different bone cell types [24,25]. Osteoclasts can also differentiate *in vitro* from macrophages by stimulation with the macrophage/mono cyte-colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor kappa-B ligand (RANKL) [22]. These agents induce the fusion of pre-osteoclasts, which become multinucleated cells, and the formation of "ruffled membrane", critical for bone resorption, that involves the tight attachment of osteoclasts to the bone surface to create the "sealing zone" rich in F-actin [26]. During bone resorption, osteoclasts isolate the resorptive space from the surrounding bone and release matrix-degrading enzymes, hydrogen ions and chloride ions inside the sealing zone, producing the bone matrix degradation and the dissolution of the bone mineral component, respectively [27].

The effects of MBGs on osteoblasts have been widely evaluated by different research groups [7,12,13,38,41], whereas the effects on other cell types involved in bone remodeling are practically unknown. The present study is focused on the effects of a potential mesoporous bioactive glass for bone regeneration, with molar composition 75$SiO_2$-20CaO-5$P_2O_5$ (MBG-75S), on osteoclast differentiation, bone resorption activity and macrophage activation towards pro-inflammatory M1 phenotype. CaO plays a fundamental role in the biological properties of $SiO_2$-CaO-$P_2O_5$ MBGs. However, previous works demonstrated that compositions with higher CaO content led to disordered mesoporous structures [4]. The composition MBG-75S was chosen with the aim of ensuring enough CaO content while keeping the highly ordered mesoporous structure. Previously, the dose-dependent action of this powdered material on osteoblasts has been evaluated through the analysis of cell cycle, morphology, size, complexity and apoptosis after the treatment with different doses of MBG-75S.

## 2. Materials and methods

### 2.1. Synthesis and characterization of MBG-75S

Mesoporous bioactive glass MBG-75S with molar composition 75$SiO_2$-20CaO-5$P_2O_5$ was prepared by EISA method and using Pluronic F127 as structure directing agent. For this purpose, 32 g of Pluronic F127 was dissolved in an ethanol-HCl (0.5 M) solution. Thereafter, 61.3 ml of tetraethylorthosilane (TEOS), 6.28 ml of triethylphosphate (TEP) and 17.6 mg of $Ca(NO_3)_2{\cdot}4H_2O$ were gradually added in 3 h intervals. The mixture was stirred for 24 h, poured into Petri dishes (9 cm in diameter) and introduced in an incubator at 30 °C for 7 days, until solvent evaporation and gelling. The transparent membranes so obtained were calcined at 700 °C for 3 h under air atmosphere. The resultant powder was gently milled in dry conditions and sieved, collecting the grain fraction below 40 μm. Chemicals of highest purity available have been used in the present study.

X-ray diffraction experiment was carried out in a Philips X'Pert diffractometer equipped with a Cu Kα radiation (wavelength 1.5406 Å). The patterns were collected between 0.5 and 6.5 2θ° angle using a Bragg-Brentano geometry. Fourier-transform infrared spectroscopy was done using a Nicolet Magma IR 550 spectrometer and using the attenuated total reflectance (ATR) sampling technique with a Golden Gate accessory.

Nitrogen adsorption/desorption isotherm was obtained with an ASAP 2020 equipment. The MBG-75S was previously degassed under vacuum for 15 h, at 150 °C. The surface area was determined using the Brunauer-Emmett-Teller (BET) method. The pore size distribution between 0.5 and 40 nm was determined from the adsorption branch of the isotherm by means of the Barret-Joyner-Halenda (BJH) method. The surface area was calculated by the BET method and the pore size distribution was determined by the BJH method using the adsorption branch of the isotherm.

Scanning electron microscopy (SEM) was carried out using a JEOL-6335F microscope, operating at 15 kV. Transmission electron microscopy (TEM) was carried out using a JEOL-1400 microscope, operating at 300 kV (Cs 0.6 mm, resolution 1.7 Å). Images were recorded using a CCD camera (model Keen view, SIS analyses size 1024 X 1024, pixel size 23.5 mm × 23.5 mm) at 60,000× magnification using a low-dose condition.

### 2.2. Soluble species release from MBG-75S to the culture medium

The levels of soluble calcium, phosphates and silica species in the culture medium were measured by inductively coupled plasma (ICP) spectroscopy, after soaking MBG-75S in Dulbeccós Modified Eaglés Medium (DMEM, Sigma Chemical Company, St. Louis, MO, USA) (1 mg/ml) for 3 and 7 days.