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In vitro study of manganese-doped bioactive glasses for bone regeneration

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ABSTRACT

A glass belonging to the system SiO_2–P_2O_5–CaO–MgO–Na_2O–K_2O was modified by introducing two different amounts of manganese oxide (MnO). Mn-doped glasses were prepared by melt and quenching technique and characterized by means of X-ray diffraction (XRD), scanning electron microscopy (SEM) observation and energy dispersion spectrometry (EDS) analysis. In vitro bioactivity test in simulated body fluid (SBF) showed a slight decrease in the reactivity kinetics of Mn-doped glasses compared to the glass used as control; however the glasses maintained a good degree of bioactivity. Mn-leaching test in SBF and minimum essential medium (MEM) revealed fluctuating trends probably due to a re-precipitation of Mn compounds during the bioactivity process. Cellular tests showed that all the Mn-doped glasses, up to a concentration of 50 μg/cm^2 (μg of glass powders/cm^2 of cell monolayer), did not produce cytotoxic effects on human MG-63 osteoblasts cultured for up to 5 days. Finally, biocompatibility tests demonstrated a good osteoblast proliferation and spreading on Mn-doped glasses and most of all that the Mn-doping can promote the expression of alkaline phosphatase (ALP) and some bone morphogenetic proteins (BMPs).

1. Introduction

It is widely known that bioactive glasses can promote in vitro hydroxyapatite precipitation and in vivo bone bonding [1,2]. This ability, commonly known as "bioactivity", is the response that some glasses show after exposure to physiological or simulated body fluids [3]. During the first hours of incubation in a biological environment a complex mechanism of alkaline ions release occurs. The exchange of the released ions with H_3O^+ from the surrounding aqueous solution leads to the formation of the initial reaction film (commonly a hydroxylated surface), which evolves rapidly into a silica-gel layer and, in turn, to a mineralized hydroxyapatite layer.

Starting from this consolidated experimental evidence, many studies have been carried out in order to assess the effect of ion release from bioactive glasses, and the ability of glasses and glass-ceramics to release ions with a therapeutic action has been investigated in depth; a review on metallic ions as therapeutic agents in tissue engineering scaffolds has recently been published [4]. Due to their ability to release specific ions (such as Si^{4+}, Mg^{2+}, Ca^{2+}) bioactive glasses provide a biocompatible surface, which in vivo can be actively mineralized and also stimulates both intracellular and extracellular responses. There is increasing evidence of in vivo promotion of osteogenic stem cell colonization, as well as of angiogenesis in contact with bioactive glasses [5]. For these reasons the "genetic design" of new bioactive glasses [6] by introducing several active elements (such as Sr, Cu, Fe, and Zn) to their formulations is a very promising research field. However, as yet few studies have investigated the role that trace elements could have in some pathways of cellular metabolism, when introduced to composition of a biomaterial.

Among these elements, Mn is recognized as playing a significant role in the metabolism of both muscle and bone [7]. This element is present in small amounts in several tissues and organs, such as the bone (about 2 ppm), hypophysis, pancreas, liver, and intestinal mucosa. Additionally, Mn is essential for normal skeletal development, a well-functioning immune system, the efficient hematopoiesis, the healthy cartilage and bone tissue synthesis [7–11]. This element is an indispensable cofactor of enzymes essential for cell functions, such as superoxide dismutases, RNA polymerase, pyruvate carboxylase and arginase [12,13].

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Among bivalent cations such as Ca^{2+}, Mg^{2+} and Mn^{2+}, Mn^{2+} ions seem to be the most promising ones acting as activators of several integrins, glycoproteins of cellular membrane that are essential for certain cellular processes (e.g. cellular adhesion, proliferation, and spreading on different substrates) as well as mediating interactions between cells and the surrounding environment, which may be other cells or the extracellular matrix (ECM) [14–17].

As previously mentioned, Mn^{2+} ions are involved in the synthesis of ECM proteins and proteins of plasmatic membranes [18]: the most important protein of ECM is collagen, which confers particular structural properties to connective tissues, such as bone, and binds to different types of integrins. In the remodeling of bone tissue collagen fibers are degraded and release their essential constituents, such as prolidase, a metalloprotease that has a specific requirement for manganese [13]. Moreover it has been demonstrated that Mn^{2+} plays an important role in bone mass maintenance, since a reduction of this element has been noted in patients with osteoporosis [9,19].

On the other hand, negative effects of an excessive dosage of Mn^{2+} ions in the human body have also been reported, providing useful information about the tolerance levels [13,20–23].

The presence of Mn^{2+} ions inside biomaterials has been investigated in alumina devices used to favor osteointegration; nevertheless, the in vivo tests performed in the same work did not explained the action of these ions on new bone formation [21]. Furthermore, hydroxyapatite doped with Mn^{2+} ions has been prepared using ion-exchange technique and co-precipitation processes [24–28]. These studies have shown that Mn-doped hydroxyapatite is non-toxic; however, the ability of this element to promote cell differentiation and bone regeneration has not been sufficiently investigated. The influence of Mn^{2+} ions on human osteoblasts has also been investigated in vitro by directly adding different concentrations of $MnCl_2$ to the cells [29]; this study has shown that the effect of Mn^{2+} ions on cellular functions is strongly concentration-dependent and so the incorporation and in turn the release of Mn^{2+} ions from a biomaterial should be adjusted accordingly. Finally, a study involving a biocompatible and bioactive coating of Mn^{2+}-doped β-tricalcium phosphate (β-TCP) confirmed that the coating was not cytotoxic and that β-TCP doped with the highest Mn^{2+} amount showed higher potential for cellular proliferation and better viability when tested in osteoprogenitor cell culture than the same coating material doped with a lower Mn^{2+} content [30].

Glasses and glass-ceramics can have their composition modulated during or after their synthesis (e.g. overworking their surface reactivity by ion-exchange). This allows a focused and controlled insertion of trace elements that is not possible with other materials; also bioactive glasses doped with various ions in a wide range of compositions can be obtained using traditional methods of synthesis (melting and quenching) or sol–gel processes. All these factors permit a controlled release of ions modulating their action with respect to the bone remodeling processes, leading to successful integration and creating bioactive materials for bone reconstruction with a fast and specific action mechanism.

Despite recent interest in the investigation of different formulations of bioactive glasses doped with other active ions, to the authors' knowledge no study concerning the effect of Mn-doped silica-based glass has been reported in literature. The addition of Mn ions into different glass systems has been studied in the past, and the structure of Mn-containing glasses has been documented. For example it was reported in literature that Mn can exist in more than one oxidation state in glasses and, in function of the glass composition, Mn can occupy network forming or modifying positions [31]. Moreover it is known that the manganese ions appear as Mn^{3+} with octahedral coordination in borate glasses and as Mn^{2+} with both tetrahedral and octahedral environments in silicate and germanate glasses [32].

Starting from the abovementioned rationale, the purpose of this work is to evaluate for the first time the effect of Mn-doping on some properties of a bioactive glass (CEL2) successfully used in a previous work [33] to produce 3D glass-ceramic scaffolds. In the previous work CEL2 demonstrated high bioactivity and good biological behavior, assessed by osteoblast proliferation with the synthesis of calcium nodules. In the present work the effect of Mn doping was investigated specifically by the study of the eventual modification of glass reactivity, its biocompatibility and its ability to promote cell proliferation and cellular differentiation.

2. Materials and methods

2.1. Glass sample synthesis and characterization

A bioactive glass (CEL2) [33] with the following molar composition, 45% SiO_2, 3% P_2O_5, 26% CaO, 7% MgO, 15% Na_2O, 4% K_2O, was modified by substituting 0.25 mol% and 0.5 mol% of MgO with MnO. The glasses with 0.25% and 0.5% MnO were named Oliglass 1 and Oliglass 2, respectively (Table 1). The three glasses (CEL2, Oliglass 1 and Oliglass 2) were produced by traditional melting and quenching of the following row products: SiO_2, $Ca_3(PO_4)_2$, $CaCO_3$, $4MgCO_3Mg(OH)_2\ 5H_2O$, Na_2CO_3, K_2CO_3, and $MnCO_3$. Briefly, they were prepared by melting the reactants in a Pt crucible at 1500 °C for 1 h in air and quenching the melt in distilled water, to obtain a frit, and separately in a brass mold to obtain glass bars. The frit was dried in air at room temperature, milled and sieved to obtain powders with particle size below 32 μm, while the bars were annealed at 500 °C for 13 h and cut in sheets of $10 \times 10 \times 1.5\ mm^3$; the sheets were polished with SiC abrasive papers up to 1200 grit. Both powders and sheets have been prepared and used in for the characterizations described below.

In order to evaluate eventual thermal, morphological, compositional or structural variations due to the Mn doping, the glasses were characterized by means of differential thermal analysis (DTA 404 PC NETZSCH), scanning electron microscopy (SEM-FEI, Quanta Inspect 200, The Netherlands), energy dispersion spectrometry (EDAX PV 9900) and X-ray diffraction (X'Pert Philips diffractometer, The Netherlands) using the Bragg Brentano camera geometry and the Cu-Kα incident radiation.

2.2. Bioactivity and in vitro release tests

In vitro tests in simulated body fluid (SBF) [2] were performed to compare the bioactivity of Mn-doped glasses (Oliglass 1 and Oliglass 2) with CEL2. Sheets of the three glasses were immersed in 25 ml of SBF at 37 °C for 1 and 7 days. Afterwards, the samples were analyzed by means of SEM observation and compositional (EDS) and structural (XRD) analyses to assess the growth of hydroxyapatite on their surface; the pH of solution was also monitored during the immersion.

The Mn release was carried out by soaking glass powders (1 g) and sheets in 30 ml of SBF solution for up to 28 days; aliquots of SBF solutions were extracted out at fixed time periods (3 h, 1, 3, 7, 14, 28 days) and analyzed by means of a graphite furnace atomic absorption spectroscopy (GF-AAS) [Perkin Elmer (mod. 4100 ZL), USA]. For comparative purposes fresh SBF has been analyzed and used as a reference. The GF-AAS instrument employed a Zeeman-effect background corrector with an auto sampler (mod. AS/71) programmed to dispense 20 μl of the sample. To obtain

Table 1
CEL2, Oliglass 1 and Oliglass 2 compositions.

	CEL2 mol%	Oliglass 1 mol%	Oliglass 2 mol%
SiO_2	45	45	45
P_2O_5	3	3	3
CaO	26	26	26
MgO	7	6.75	6.5
Na_2O	15	15	15
K_2O	4	4	4
MnO	0	0.25	0.5