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Original Research Article

In Vitro Evaluation of Manganese-Containing Glass-Ceramic in Quaternary SiO_2-CaO-Na_2O-P_2O_5 System

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ABSTRACT

In this research, sol-gel experimental conditions are imposed to prepare a new Mn-containing SiO_2-based bioactive glass. The current study primarily aims to investigate the impact of the presence of manganese ion on the glass structure, bioactivity, and cytotoxicity. The obtained glass-ceramics were characterized using a X-Ray Diffractometer (XRD). According to the observations, crystallization of silicorhenanite and calcite phases in the manganese-containing sample were inhibited before and after soaking in the simulated body fluid (SBF), respectively. In vivo bioactivity evaluation confirmed the bioactive nature of the obtained powders. Finally, the cellular test was carried out, the results of which demonstrated non-cytotoxicity of the samples towards human Bone Marrow Stromal Cells (hBMSCs) cells up to 7 days.

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1. INTRODUCTION

The term biomaterial refers to man-made materials used for repairing or restoring body functions after they have been injured or damaged. To be effective as bone tissue replacement, a biomaterial must be non-toxic with the ability to form a hydroxyapatite (HA) layer on its surface to decrease the rejection potential [1]. Generally, biomaterials can be divided into three groups: bioinerts (non-toxic and biologically inactive), bioactives (both nontoxic and biologically active), and biodegradables or bioresorbables (dissolved and replaced by the surrounding tissues, called the third-generation materials) [1,2]. Bioactive glasses (BGs) are nontoxic biomaterials that exhibit bioactivity in orthopedics through their interactions with body fluids [3-5].

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Recently, application of biologically active ions has gained more significance than ever to enhance the biological and physical effectiveness of BGs and develop multifunctional biomaterials with a wide range of biomedical applications [6-8]. In addition to their essential role in human health, metallic ions can be a cost-effective alternative to pharmaceuticals [9-11]. Incorporation of metallic ions into BGs has been extensively studied in recent years [7,10,12-14]. Manganese plays a critical role in both bone and muscle metabolism [15,16]. Manganese present in the human body help prevent bone loss caused by free radicals and therefore, its prolonged deficiency can cause osteoporosis. Low manganese levels can be detected in osteoporotic patients [10,17]. Luthen et al. [18] investigated the effects of different manganese ion on the cellular functions like spreading, proliferation as well as gene expression in human osteoblasts by directly introducing different concentrations of $MnCl_2$ to the cell suspension (0.01–0.5 mM). Their result showed a strongly concentration-dependent effect of manganese cations on the cell functions, which should be adjusted through incorporation to different biomaterial. Manganese-containing bioactive glasses have also been investigated in recent years [12,16,19-25]. Compared to Mn-free glasses, those glasses that contain manganese in their composition enjoy an increase in the osteoblast differentiation, bone mineralization, Alkaline Phosphatase (ALP), and bone morphogenetic protein expression [16]. It was found that Mn-doped samples exhibited significant bioactivity, given the formation of HA after only a few hours and their complete coverage after fourteen days [25]. Further, the antibacterial properties of these glasses were identified [26]. The research findings confirmed that 1.6 ppm of Mn^{2+} in basal Dulbecco's Modified Eagle Medium (DMEM) stimulated the osteoblast proliferation without impairing cell viability [23]. Based on these studies, Mn incorporation into the bioactive glass networks can provide superior bone regeneration materials. However, in most studies, the presence of alkaline or alkaline earth elements has been completely neglected or small amount of these ions have been investigated. Considering the advantages of these elements, the current study aimed to synthesize and evaluate the bioactive glass containing significant amounts of alkaline and alkaline earth ions along with manganese ion, which can be considered an innovation in academic milieu. This research aimed to produce a sol-gel manganese-containing SiO_2-based bioactive glass with high alkali and alkaline earth oxide content ($52SiO_2.(30\text{-}x)CaO.14Na_2O.4P_2O_5.xMnO$, x = 2 (mol %)) to obtain a potential biomaterial for bone tissue regeneration. In addition, it evaluated the effect of Mn incorporation on the sample structure, cytotoxicity, and in-vitro bioactivity.

2. MATERIALS AND METHODS

2.1. Bioactive Glass Powder Synthesis

All chemicals used in this study were purchased from Merck and Sigma-Aldrich and used as received. Calcium-nitrate-tetrahydrate ($Ca(NO_3)_2.4H_2O$), manganese nitrate-tetrahydrate ($Mn(NO_3)_2.4H_2O$), sodium nitrate ($NaNO_3$), tetraethyl-orthosilicate alkoxides (TEOS, $Si(OC_4H_9)_4$), and triethyl phosphate (TEP, $P(C_2H_5O)$) were used as precursors. Deionized water and absolute ethanol were used as solvents, and 0.05 M citric acid was used as the sol-gel reaction catalyst. The sol-gel process was used to prepare Mn-free and Mn-containing glass powders as discussed in our previous work [27]. First, TEOS and TEP were diluted in ethanol and then, $NaNO_3$ and ($Ca(NO_3)_2.4H_2O$) were dissolved in an acidic solution, respectively. In the next step, the former solution was gradually added to the latter. The resultant solution was stirred for three hours until complete hydrolysis was achieved. Next, the solution was sealed and left at room temperature for gel formation. The obtained gel was aged at 70 °C for 24 h and dried at 110 °C in an oven for 24 h. Finally, the dried sample was calcined at 650 °C for an hour (3 °C/min). To obtain Mn-containing glass (2Mn-BG), Mn was introduced into the glass by partial replacement of the calcium content. For this purpose, a similar process to BG synthesis was applied except that ($Mn(NO_3)_2.4H_2O$) was added to the acidic solution before ($Ca(NO_3)_2.4H_2O$) dissolution.

2.2. Powder Characterization

The as-prepared products were analyzed using X-Ray Diffraction (XRD, DRON-8, Bourevestink, Russia, CuKα, 40 Kv, λ = 1.5418 Å). Scanning was carried out from 15 to 100° with a step size of 0.026° per step at step time of 49.2 s.

2.3. In Vitro Bioactivity Assessment

The bioactivity of the synthesized powders was evaluated by immersing them in Simulated Body Fluid (SBF), according to the approved Kokubo protocol [28]. For this purpose, 15 mg of glass powders were immersed in 15 ml of SBF in polyethylene bottles and kept in an incubator (Memmert GmbH-CokG, Germany) at 37 °C for 14 days. The initial pH of SBF was kept at 7.4. The HA layer formation was confirmed by the EDX and XRD analyses after 14 days of immersion in the SBF. At each time, the point samples were removed from SBF, rinsed with distilled water, and dried in an oven at 60 °C. To study the dissolution process of the synthesized powders, the pH variation of the samples was also recorded. The pH variations for the SBF-soaked sample were measured at the intervals of 1, 3, 7, and 14 days by an electronic pH meter (BEL, PHS3-BW, Italy).

2.4. Biological Tests

2.4.1. Cytotoxicity

Cell viability was investigated by conducting MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, a calorimetric method for quantification of tetrazolium compounds reduction into a water-soluble purple formazan by viable cell mitochondria. The indirect assay was then carried out following the 10993-5 States [29]. The ionic products of the samples were obtained as follows: the powder samples (BG, 2Mn-BG) were first sterilized by UV radiation for an hour and then, the samples were suspended in basal DMEM culture medium at the concentration of 1 mg/ml for 24 h at 37 °C. To obtain a better understanding of cell viability, the cells were treated with different concentrations of powders for prolonged periods. Consequently, the extracts were diluted using DMEM to achieve 1000, 100, and 10 µg/mL final concentrations and filtered (0.22 µ), and the test was carried out at different time points (1, 3, and 7 days). Meanwhile, human Bone Marrow Stromal Cells (hBMSCs) were seeded in a 96-well plate containing 200 µl DMEM supplemented with 15 % FBS and 1 % antibiotic with the density of 5 × 103 cells/well. The cultured cells were incubated at 37 °C with 5 % CO_2 for 24 h. Subsequently, the cell culture media were removed and replaced by the extracted media and incubated for 1, 3, and 7 days. During 1 and 3 days, the powders suspension added at the beginning was used and after 5 days, the culture media was replaced by the new aliquots of extracts. The cells grown without sample extracts were next used as control. At the predetermined time, the extracts were removed and replaced with the 80 µl fresh medium and 20 µl of MTS solution (2 mg/ml). These cells were incubated for 3-4 h at 37 °C in a humidified atmosphere of 5 % CO_2. Finally, the absorption was determined using a spectrophotometer (Synergy HT, BioTek, U.S.A.) at 490 nm wavelength. The cell viability is determined using Equation (1):

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance of samples}}{\text{Mean absorbance of control}} \times 100 \quad (1)$$

All materials used in the cell culture process were obtained from the Gibco brand (Thermo Fisher Scientific, USA).

2.4.2. Statistical Analysis

All experiments were conducted in triplicate and presented in means ± Standard Deviation (SD). Statistical differences of these values were evaluated using one-way Analysis of Variance (ANOVA) using prism software. Here, $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Phase Analysis

Figure 1 shows the diffraction patterns of the synthesized powders stabilized at 650 °C for one hour at the heating rate of 3 °C/min. The BG diffraction pattern confirms the presence of combeite $Na_2Ca_2Si_3O_9$ (PDF#075-1687) and pseudo-apatite crystalline phase of silicorhenanite $Na_2Ca_4(PO_4)_2SiO_4$ (PDF#032-1053) without any traces of nitrate phases related to the used precursors. Apparently, the synthesized powders are glass-ceramic. Addition of manganese oxide caused a change in the powder color from white to light brown. A comparison of the diffraction patterns of BG and 2Mn-BG powder confirmed the diminishing of the peaks attributed to the silicorhenanite phase. Therefore, it can be concluded that the presence of manganese prevented the crystallization of this phase. Calcium content in the 52S4 glass, combeite, and silicorhenanite phase is approximately 21, 23, and 33 wt. %, respectively. It should be noted that formation of the silicorhenanite crystalline phase requires a higher calcium content. In this respect, that silicorhenanite formation was hindered by addition of Mn instead of CaO is acceptable. The results from evaluating the effect of manganese on the structure of 45S5 glass-ceramic confirmed that the presence of manganese caused a decrease in the degree of powder crystallinity [30]. In addition, amorphous phases proved to show higher bioactivity than the crystallized samples with the same composition [31].

The XRD results revealed that the presence of MnO could not guarantee the formation of any new phase, and no peak attributed to the primary manganese precursor was observed in the diffraction pattern, indicating that Mn^{2+} was embedded in the glass-ceramic structure.

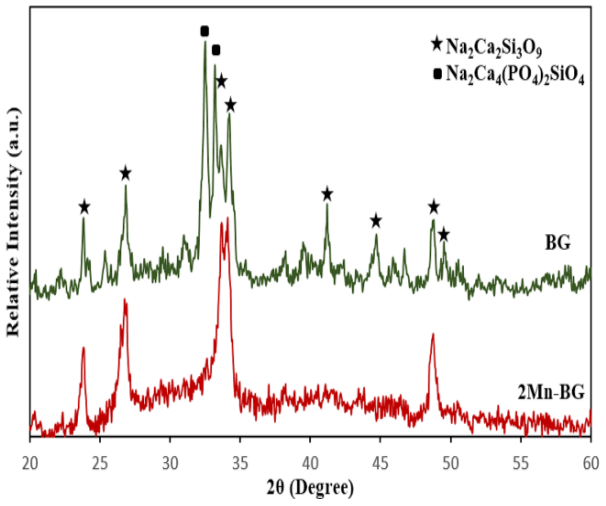


Figure 1. XRD patterns of BG and 2Mn-BG powders stabilized at 650 °C for 1 h with a heating rate of 3° C/min

3.2. In Vitro Bioactivity

The in vitro HA layer formation on a material surface is indicative of the in-vivo bioactivity of the material [4,32]. The formation of the HA phase requires the ionic interaction of biomaterial and SBF; therefore, different factors including the material composition and its degradation rate affect the thermodynamics and kinetics of the reactions [33].

Figure 2 shows the pH variations during the SBF soaking for BG and 2Mn-BG. As observed, the pH values increased in the first three days of immersion and then up to day 7, these values decreased until they finally reach a rather constant value up to day 14. The pH variations in the SBF result from the exchange of ions between the SBF and the samples. As accepted, after the glass-ceramic immersion in the SBF, alkaline, and alkaline earth ions, like Na^+ and Ca^{2+}, enter the SBF and react with the hydroxyl groups forming bases that increase the pH, such as $Ca(OH)_2$ and NaOH. In the next step, Ca^{2+} ions are adsorbed onto the surface (CaO-P_2O_5-rich layer precipitation) and once again, the pH value is reduced. The constant pH values at longer times indicate a balance between these two processes, namely ion release into the solution from the material surface along with reabsorption of these ions from SBF and precipitation of CaO-P_2O_5-rich layer on the surface [16,34]. The above-mentioned trend was detected for both samples, and no significant difference in the pH values was observed for the BG and 2Mn-BG samples while being soaked in the SBF environment.

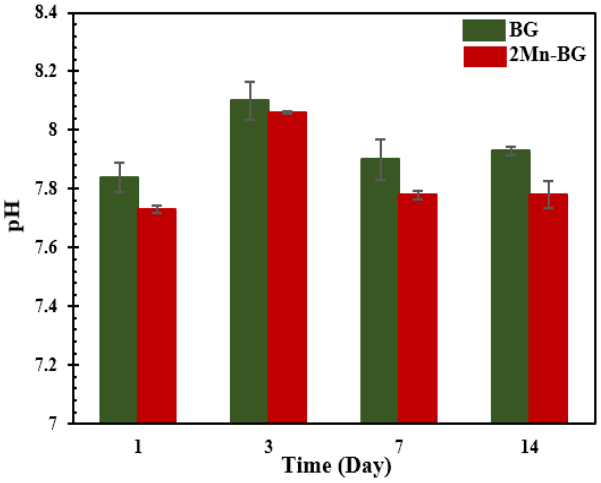


Figure 2. The pH value in the synthesized powders up to 14 days immersion in SBF

Table 1 shows the elemental analysis of the powders before and after in vitro test in SBF after 14 days. A decrease in the Ca and an increase in the Si content were observed on the surface of both samples, thus confirming the release of Ca^{2+} ions into the SBF and formation of a silicon-rich layer on the surface of samples.

TABLE 1. Ion concentration of synthesized powders according to EDS analysis before and after immersion in SBF

Elements	BG		2Mn-BG	
	Before soaking (wt. %)	After soaking (wt. %)	Before soaking (wt. %)	After soaking (wt. %)
Si	13.27	28.32	13.90	21.31
Ca	20.29	13.15	16.56	7.49
Na	11.74	0.13	12.76	2.52
P	0.73	3.01	0.97	1.60
Mn	-	-	3.5	2.95

Figure 3 shows the XRD pattern of the samples after immersion in the SBF. The patterns confirm the formation of HA phase ($Ca_{10}(PO_4)_6(OH)_2$; PDF#09-0432) with the main peak at around 32 degrees in both samples, confirming the bioactive nature of the powders.

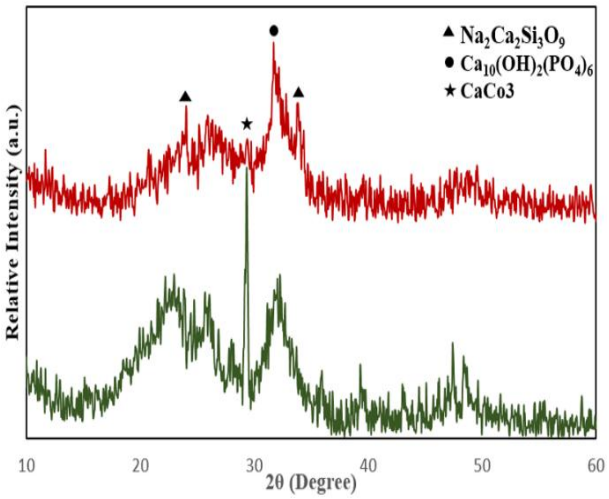


Figure 3. XRD patterns of the BG and 2Mn-BG samples after 14 days of immersion in SBF

The major peak for calcite at about 29° ($CaCO_3$; PDF#00-001-837) appeared in the BG diffraction pattern after immersion. This peak, however, almost disappeared in the diffraction pattern of the manganese-containing sample. The reaction between the high contents of Ca^{2+} and $(CO_3)^{2-}$ ions, released from glass powder particles and SBF solution, respectively, was the main reason for calcite phase precipitation [35-37].

The higher amounts of calcium oxide in the Mn-free sample could be the reason for calcite precipitation [38,39].

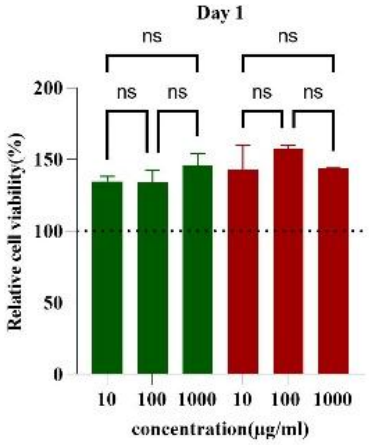
In addition, the presence of Mn in the glass would enhance its durability by creating stronger Mn–O–Si bonds than those created by Ca–O–Si [35,30]. The stronger bonds in turn reduce the Ca release rate into the

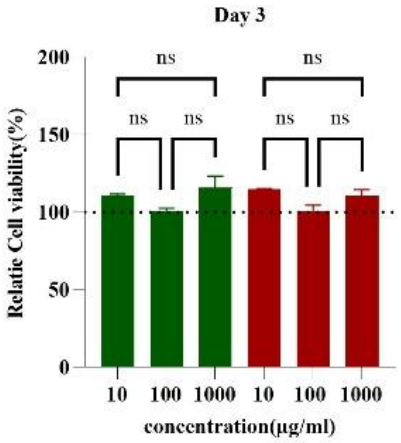
SBF and inhibit calcite precipitation. The peaks related to the combeite phase can be observed in the 2Mn-BG powders pattern. Based on the obtained results, it can be concluded that the presence of manganese stopputs an end in the ped calcite deposition without any negative effect on bioactivity.

3.3. Cytotoxicity Evaluation

The cytotoxicity was evaluated by hBMSCs exposure to the ionic dissolution of the synthesized powders. As indicated in Figure 4, no cytotoxic effect was observed with the dissolution products of the prepared samples at three different concentrations of 1000, 100, and 10 µg/ml. Of note, an increase in the cell proliferation was observed in the hBMSCs exposed to the conditioned media for up to seven days at higher concentrations.

It can be concluded that the dissolution products of the synthesized powders can enhance the proliferation potential of cells and produce higher levels of cell function, compared to the control group, meaning that Mn-containing samples are not cytotoxic under the evaluated conditions.





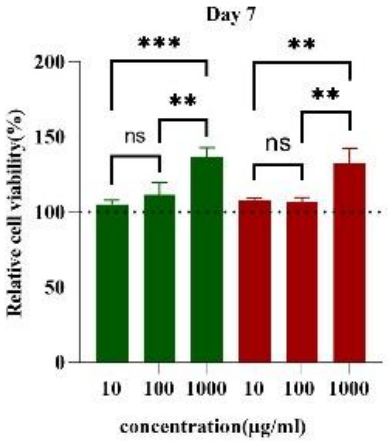


Figure 4. Viability of hBMSCs in the presence of increasing concentrations of synthesized powders (10, 100, and 1000 µg/ml). Green: BG, Red: 2Mn-BG. Data presented as mean ± SD of three independent sample, n=3. (ns indicates not significant differences, ** indicates $p < 0.05$, and *** indicates $p < 0.02$)

4. CONCLUSION

In this study, manganese-free and manganese-containing glass-ceramic in the quaternary system SiO_2–CaO–Na_2O–P_2O_5 were synthesized using the sol-gel route. The results indicated that combeite and silicorhenanite were crystallized in the Mn-free sample followed by heat treatment at 650 °C for one hour. However, the Mn ion addition inhibited the crystallization of the silicorhenanite phase. The in-vivo bioactivity test on both BG and 2Mn-BG samples confirmed their noticeable bioactivity. Further, calcite precipitation was diminished in the 2Mn-BG sample. The MTS test results demonstrated the nontoxicity of BG and 2Mn-BG samples towards the hBMSCs cells such that an improvement in the mitochondrial activity of these cells was observed until 7 days.

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