



The Effects of Zoledronate on the Structure, Dissolution and Bioactivity of the Bioglass Composite: An Investigation by MAS-NMR and ICP-OES Methods

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Abstract: Composite of bioglass (BG) with 0.1 wt.% of zoledronate (Z) has been elaborated for medical applications as reported in a previous study [1]. The synthetic material has been proven to be bioactive. This study used two physical-chemical methods: MAS-NMR (Magic angle spinning – nuclear magnetic resonance) and ICP-OES (Inductively coupled plasma – optical emission spectrometry) to clarify the effect of zoledronate on the structure, dissolution and bioactivity of BG. The obtained results show that the use of 0.1 wt.% of zoledronate modified the structural network, slowed down the dissolution and stimulated the bioactivity of bioglass.

Keywords: Bioglass, zoledronate, composite, lyophilization, *in vitro*, bioactivity.

1. Introduction

Bioactive glasses (bioglasses - BG) are a group of surface-active ceramic materials used for artificial implants in human body to repair and replace diseased or damaged bones. The main composition of bioglasses consists of SiO₂, CaO, Na₂O and P₂O₅ oxides in which these oxides do not exist independently but bond together to form a 3D continuous random structural network. The bioactivity of bioglasses is the ability to form a hydroxyapatite (HA) layer on their surface

during *in vitro* and *in vivo* experiments. The resulting apatite layer permits an intimate bone-bonding between the artificial implant and the host tissue [2-4].

Bisphosphonates (BPs) are a class of compounds that are widely used to treat some diseases related to bone loss (such as osteoporosis), Paget's disease, fibrous dysplasia, myeloma and bone metastases [5-6]. Bisphosphonates are stable analogues of inorganic pyrophosphate, a naturally occurring polyphosphate present in serum and urine, and can prevent calcification of bone mineral by binding to newly forming crystals of hydroxyapatite. Pyrophosphate has a P-O-P structure, two phosphate groups are linked by

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an oxygen atom while bisphosphonates have a P-C-P structure, a central carbon atom replacing the oxygen. Like pyrophosphate, bisphosphonates have high affinity for bone mineral and they prevent calcification both *in vitro* and *in vivo* experiments [7, 8]. Bisphosphonates have the ability to bind to bone mineral, thus preventing crystallization of tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ and dissolution of hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. The ability of bisphosphonates is enhanced when the R^1 side chain (attached to the central carbon atom of the P-C-P group) is a hydroxyl group [9]. The presence of a hydroxyl group at the R^1 position increases the affinity of these compounds for calcium ions in bone mineral due to the formation of tridentate binding rather than the formation of bidentate binding [10-12]. Furthermore, bisphosphonate have been shown to be an anti-resorptive agent due to their inhibitory capacity to bone resorption by cellular effects on osteoclasts which induce osteoclasts to undergo apoptosis [13].

Zoledronate (Z) - a novel type of bisphosphonate containing an imidazole substituent, has demonstrated more powerful inhibition for osteoclast mediated bone resorption than other bisphosphonates [14, 15]. The formula of zoledronate molecule is shown in the Figure 1.

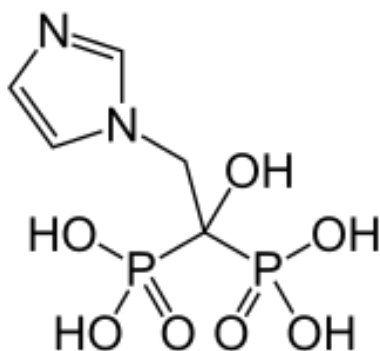


Fig. 1. Molecular structure of Zoledronate.

In previous study [1], we have reported the elaboration of BG-0.1Z composite. The

bioactivity of this biomaterial was confirmed by the formation of hydroxyapatite layer on its surface after *in vitro* experiment. The research also highlighted that the introduction of 0.1 wt.% of zoledronate stimulated the bioactivity of bioactive glass. In this work, two modern methods Solid State NMR and ICP-OES were used to elucidate the effect of the introduction of zoledronate on the structure, dissolution and bioactivity of bioglass.

2. Materials and methods

2.1. Materials

The required chemicals for elaborating the BG and BG-0.1Z composite are listed below:

Calcium metasilicate CaSiO_3 (99% in purity, Aldrich-Sigma), trisodium trimetaphosphate $(\text{NaPO}_3)_3$ (95% in purity, Aldrich-Sigma), sodium metasilicate Na_2SiO_3 (99.9% in purity, Aldrich-Sigma) and zoledronate (Z) (98% in purity, Aldrich-Sigma).

2.2. Elaboration of bioactive glass (BG)

Bioactive glass was elaborated by melting method [1]. After a calculation based on molecular weights and number of moles, a mixture 30 (g) comprising of 14.8524 (g) CaSiO_3 , 2.5281 (g) $(\text{NaPO}_3)_3$ and 12.6195 (g) Na_2SiO_3 was used to synthesize the bioactive glass with the composition of 46% SiO_2 , 24% Na_2O , 24% CaO and 6% P_2O_5 . This mixture was homogenized for 1 hour using the mixer. The mixed powder was melted in a platinum crucible in order to avoid pollution because the melting point of platinum is high ($1768,2^\circ\text{C}$) and the platinum is inert with chemical reactions. The temperature was ramped to 900°C with a rate of $10^\circ\text{C min}^{-1}$. The temperature was kept at 900°C for 1 hour to effectuate the decompose reactions of initial products, and then increased to 1300°C with a rate of $20^\circ\text{C min}^{-1}$. This temperature was maintained for 3 hours to melt the mixture reaction. The melted bioactive glass was poured

into the brass moulds and annealed at the glass transition temperature in a regulated muffle furnace, to remove the residual mechanical constraints. After cooling to room temperature, the bulk glasses were ground and sieved to obtain the glassy particles with the sizes less than 40 μm .

2.3. Elaboration of BG-0.1Z composite

The BG-0.1Z composite was elaborated in our previous research [1]. The first, the zoledronate powder was dissolved in the distilled water to form the zoledronate solution. Then, the bioactive glass particles with the size less than 40 μm were suspended in this solution. The magnetic stirrer was used to mix the bioactive glass particles in zoledronate solution for 24 hours at room temperature. The second, this mixture of bioactive glass particles in zoledronate solution was stirred at 70°C for 4 hours in order to promote the combination between the zoledronate molecules and the powdered bioactive glass. Afterward, the mixture was frozen by the liquid azote for 30 minutes. Finally, the sample was transferred into a freeze-drying (Christ Alpha 1-2 LD plus, version 1.26) at -60°C and around 1 mbar for 24 hours to remove completely water. The bioactive glass/zoledronate composite contained 0.1 wt.% of zoledronate amount was synthesized. It is named: BG-0.1Z composite.

2.4. *In vitro* assays in SBF

The *in vitro* experiments were realized by soaking 250 mg of powder into 50 ml of simulated body fluid (SBF) with pH and mineral composition nearly equal to those of human blood plasma. The SBF solution was prepared by dissolving NaCl, NaHCO₃, KCl, K₂HPO₄.3H₂O, MgCl₂.6H₂O, CaCl₂ and (CH₂OH)₃CNH₂ into deionised water using the method of Kokubo [16]. The powdered samples of BG and BG-0.1Z composite were immersed in SBF solution placed into sealed polyethylene bottles. They were maintained at body temperature (37°C) under controlled agitation

50 rpm (round per minute) during 1, 3, 6, 15 and 30 days. The powder samples were removed from the incubator, filtered, cleaned with deionised water to stop the reaction and then rinsed gently with pure ethanol and dried at room temperature. The dried powders of biomaterials were stored to investigate by using the physico-chemical methods.

2.5. Analysis methods

The Solid-state magic angle spinning nuclear magnetic resonance (MAS-NMR) spectroscopy was used to highlight the effect of zoledronate on the glassy network. The ²⁹Si and ³¹P MAS-NMR spectra were measured on a Bruker Avance 300 spectrometer (7T). Material samples were packed in zirconium rotors with a diameter of 2.5 mm, and spun at the magic angle of 54.7° with a spinning frequency of 15 MHz. The deconvolution of the MAS_NMR spectra was performed on the dmfit2010 software [17]. The elemental concentrations of SBF before and after soaking of biomaterials were measured using inductively coupled plasma optical emission spectrometry (ICP-OES). Sample solution is sprayed (transformed into an aerosol) and carried by a gas carrier (Ar with high purity) through a torch, where a plasma (a gas in which atoms are ionized) is ignited. When sample atoms are ionized, they emit radiation at some specific wavelength. These specific components are selected by a diffracting grating, and converted in electric signals by a photomultiplier. After calibration, it is possible to determine the amount of each element present in solution by analyzing the intensity of the radiation emitted at the specific elemental frequency.

3. Results and discussion

3.1. ²⁹Si NMR investigation

The structural network of a silica glass is based on the chains of SiO₄ tetrahedra linked by one or more summits. The notation Qⁿ describes

SiO₄ tetrahedron in which n is the number of bridging oxygen (Si-O-Si) worn by a tetrahedron [18-19]. In the same way, the structural network of a phosphate glass is formed by PO₄ tetrahedra. The BG is a phosphosilicate composed of 46% SiO₂, 24% Na₂O, 24% CaO and 6% P₂O₅ (wt.%). Its structure consists of SiO₄ and PO₄ tetrahedrons. Thus the measurements of solid state NMR spectra of nucleus of ²⁹Si and ³¹P can evaluate the structure of bioactive glass and also evaluate the effects of zoledronate on the structure of bioactive glass. The measured MAS-NMR spectra were deconvoluted and compared to the scientific references to estimate the P, Si populations in the structure of biomaterials.

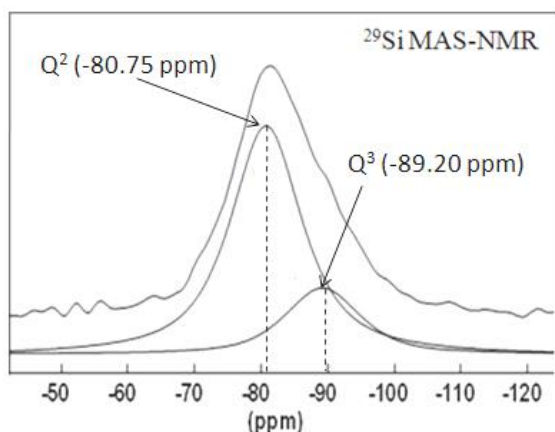


Fig. 2. MAS-NMR ²⁹Si spectrum of BG and its deconvolution.

In the MAS-NMR ²⁹Si spectrum deconvolution of BG, two resonances at -80.75 and -89.20 ppm were observed (Fig. 2). They contributed 78.16% and 21.84% respectively of the SiO₄ tetrahedral population. The resonance at -80.75 ppm assigned to Q² tetrahedra with two bridging oxygens and other one at -89.20 ppm corresponds to Q³ tetrahedra with three bridging oxygens [18, 19]. As regards to the references [19], the chemical neutrality around

the non-bridging oxygens of Q³ tetrahedra is respected by the preferential present of Na⁺ cations, this is presented as Si(OSi)₃(O...Na). The non-bridging oxygens of Q² species are rather combined with Ca²⁺ cations and Na⁺ remaining cations. These two combinations can be expressed as Si(OSi)₂(O₂...Ca) and Si(OSi)₂(O...Na)₂ [19].

In the ²⁹Si deconvoluted spectrum of BG-0.1Z composite, two resonances at -76.50 and -82.20 ppm were identified (Fig. 3). The first at -76.50 ppm assigned to Q¹ tetrahedra with one bridging oxygen. This contribution represents 40.92% of the SiO₄ tetrahedral population. The second at -82.20 ppm corresponds to Q² tetrahedra with two bridging oxygen. This contribution represents 59.08% of SiO₄ population [18, 19]. The characteristic resonance of Q³ species was not shown. Like that, the introduction of zoledronate in BG caused the disappearance of Q³ species and the decrease of Q² species to profit Q¹ species. It can be considered that the zoledronate molecules associate with the glassy network on breaking the Si-O-Si bridging bonds in Q² and Q³ tetrahedra to create Q¹ tetrahedra.

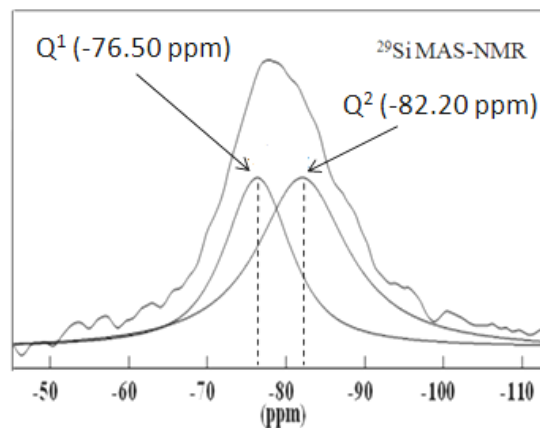


Fig. 3. MAS-NMR ²⁹Si spectrum of BG-0.1Z composite and its deconvolution.

3.2. ^{31}P NMR investigation

The MAS-NMR ^{31}P spectrum deconvolution of BG presented only resonance at 7.62 ppm with a width at half-height at about 8.7 ppm (Fig. 4). It is a typical characteristic chemical shift of phosphorus in an environment of PO_4^{3-} orthophosphates (Q^0) [20, 21]. This chemical shift is included between the chemical shift of phosphorus in Na_3PO_4 environment (10-16ppm) and the one in $\text{Ca}_3(\text{PO}_4)_2$ environment (0-3ppm) [20, 21]. Thus, the orthophosphate groups did not present preferential association with one or the other cations.

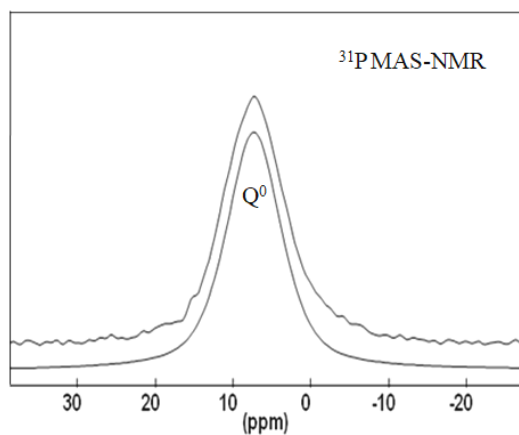


Fig. 4. MAS-NMR ^{31}P spectrum of BG and its deconvolution.

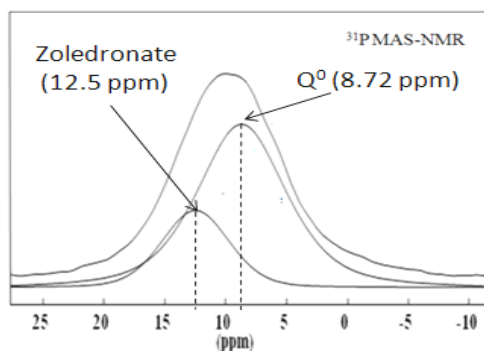


Fig. 5. MAS-NMR ^{31}P spectrum of BG-0.1Z composite and its deconvolution

After deconvolution the ^{31}P spectrum of BG-0.1Z composite, two resonances were

observed at 12.5 ppm (width at half height about 6.5 ppm) and 8.72 ppm (width at half height about 8.65 ppm) (Fig. 5). The resonance at 8.72 ppm has a width at half height which is coincident with the one of the phosphorus resonance in the spectrum of pure bioactive glass. So it is assigned to the orthophosphate environment. As the reference, the NMR ^{31}P spectrum of pure zoledronate shows a peak centered around 15 ppm with a width at half-height around 6.5 ppm [22, 23]. The resonance at 12.5 with width at half height around 6.5 ppm is assigned to phosphorus of zoledronate in the composite structure. The ^{31}P spectrum of BG-0.1Z did not express the characteristic resonance of pure zoledronate. Thus, the zoledronate molecules were not alone on the surface of bioactive glass but combined with bioactive glass particles to form a composite system. The phosphorus initial characteristic resonances of pure zoledronate and pure bioactive glass are 15 and 7.62 ppm respectively. In the ^{31}P spectrum of BG-0.1Z composite, the characteristic resonance of pure zoledronate was transferred from 15 ppm to 12.5 ppm (transfer to negative chemical shift) while the one of 46S6 bioactive glass transferred from 7.62 ppm to 8.72 ppm (transfer to positive chemical shift). This can be explained by the effect of zoledronate to the bioactive glass. The affinity of zoledronate for calcium ions in glassy network causes a transfer of calcium cations toward the zoledronate molecules, consequently decreasing the electronic shielding of the phosphorus in bioactive glass and producing a more positive chemical shift. Conversely, the apparition of calcium ions around phosphorus atoms in zoledronate molecules causes the increasing of electronic shielding around phosphorus atoms; consequently the characteristic resonance of phosphorus of zoledronate is transferred to negative chemical shift.

3.3. ICP-OES analysis

The variations of Si, Ca and P concentrations were presented respectively in

figures 6-8. The release of silicon toward the synthetic physiological liquid (SBF) is coherent with the dissolution of vitreous matrix (Fig. 6). The ICP-OES data demonstrated that the presence of zoledronate in the BG network slowed down the release of silicon concentration. Zoledronate molecules with groups OH maybe interact with soluble silanol groups $\text{Si}(\text{OH})_4$ via hydrogen bonds which can reduce the release of silicon from glassy network to the SBF physiological fluid.

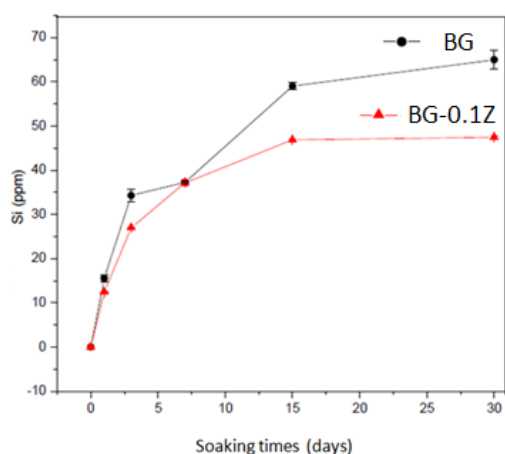


Fig. 6. Behaviour of Si concentration in SBF solution.

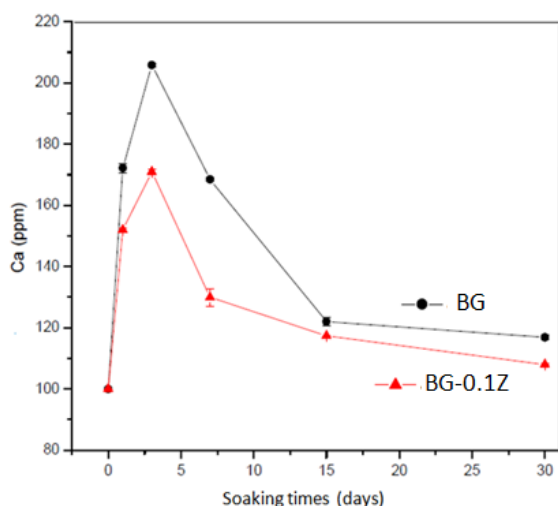


Fig. 7. Behaviour of Ca concentration of in SBF solution.

The calcium and phosphorus concentrations in SBF are correlated to the formation of hydroxyapatite layer on the surfaces of bioactive glass and its composite. Figure 7 shows the variations of calcium ions concentrations in SBF as a function of soaking times. For BG, the behaviour of calcium concentration followed 3 steps: increase, decrease and saturation. First step, calcium concentration in the analyzed SBF increased very strongly from 100 ppm to 172 ppm after 1 day of immersion, this increase is coherent with the release of available calcium content in network of pure bioactive glass, and it is consistent with the mechanism of the desalkalization on the glass surface under effect of physiological environment. After that, the calcium concentration rose reasonable to reach 208 ppm after 3 days of immersion. Second step, the calcium concentration decreased very strongly until 15 days of immersion. This decrease corresponds to the transfer of calcium ions to form the hydroxyapatite layer on the surface of bioactive glass. Third step, the calcium concentration was almost constant from 15 days to 30 days of immersion. This indicates that the precipitation of apatite layer on the surface of bioactive glass was almost completely after 15 days of immersion. At 30 days of immersion, the calcium concentration was 119 ppm, it demonstrated that the BG utilized not totally the available calcium content from glass network to form the apatite layer. Comparing the two evolutions of the calcium concentration for BG and for the BG-0.1Z composite, we find that zoledronate slowed down the release of calcium concentration during the first step and stimulated calcium consumption in the second step. The slowing down of calcium release can be explained by the adherence of zoledronate molecules with Ca^{2+} ions present in the vitreous glassy network which prevents the release of calcium under the effect of physiological fluid. The quick calcium consumption can be attributed to the affinity of zoledronate on the

surface of glass with Ca^{2+} ions present in the liquid SBF. This promotes the rapid transfer of Ca^{2+} ions from the SBF liquid to the surface of the BG-0.1Z composite to precipitate an amorphous layer of calcium phosphate, then a crystallized layer of hydroxyapatite material.

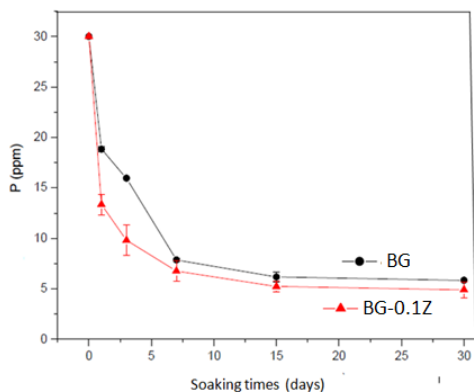


Fig. 8. Behaviour of P concentration in SBF solution

Figure 8 shows the evolution of phosphorus concentration in SBF after different immersion times for the bioglass BG and BG-0.1Z composite. A decrease of phosphorus concentration in SBF solution was observed for both BG and BG-0.1Z. This decrease corresponds to the consumption of phosphorus to form a hydroxyapatite layer on the surface of biomaterials. It is recognized that the phosphorus concentration of BG-0.1Z composite decreases rapidly compared to pure BG. This confirmed that the introduction of zoledronate enhances the formation of apatite layer.

4. Conclusion

BG and BG-0.1Z composite have been successfully developed and investigated by using two modern methods. Solid state NMR has clearly demonstrated that the introduction of zoledronate caused the modification of glassy network. This can be explained by the breaking of Si-O-Si bridging bonds in Q^2 and Q^3 tetrahedra due to the adsorption of zoledronate molecules on the glass surface.

ICP-OES analysis highlighted that the introduction of zoledronate slowed down the dissolution of bioglass and stimulate the bioactivity of bioglass after *in vitro* experiment.

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Ảnh hưởng của zoledronate tới cấu trúc, sự hòa tan và hoạt tính sinh học của vật liệu composite thủy tinh y sinh - Nghiên cứu đánh giá bằng phương pháp MAS-NMR và ICP-OES

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Tóm tắt: Vật liệu composite thủy tinh hoạt tính sinh học chứa 0,1% khối lượng của zoledronate đã được tổng hợp, đánh giá và công bố trong nghiên cứu trước đây. Bài báo này trình bày các kết quả phân tích bằng hai phương pháp MAS-NMR và ICP-OES để làm rõ hơn ảnh hưởng của zoledronate tới cấu trúc, sự hòa tan và hoạt tính sinh học của vật liệu thủy tinh. Kết quả thu được cho thấy sự có mặt của zoledronate trong thành phần của composite đã làm biến đổi cấu trúc, giảm khả năng hòa tan và tăng hoạt tính của thủy tinh y sinh.

Từ khóa: Thủy tinh sinh học, zoledronate, composite, kỹ thuật sấy đông khô, *in vitro*, hoạt tính sinh học.