Osteoinduction and Antiosteoporotic Performance of Hybrid Biomaterial Chitosan-Bioactive Glass Graft: Effects on Bone Remodeling

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Abstract

Osteoinductive and antiosteoporotic phenomena could be created by using synthetic biomaterials for applications in bone surgery. In the present study, CH-based bioactive glass (BG-CH) with 17 wt% chitosan was elaborated by a freeze-drying process. BG-CH was implanted in the muscle and in the femoral condyles of ovarioctomised rats. Grafted tissues were carefully removed for physico-chemical and histological analysis. Several physic-chemical techniques (XRD, FT-IR, MEB, ICP-OES and NMR) were employed to highlight the effects of chitosan on the glass matrix before and after implantation.

The results of the study show that despite the non-additional osteogenic cells or agents, BG-CH is endowed with an osteoinductive property. After 8 weeks, 13C NMR spectra showed the characteristic signals of γ-carbons of the hydroxyproline (71 ppm) abundant in collagen.

Keywords: Bioactive glass; Chitosan; Composite; Grafting; Osteoinduction; Anti osteoporosis; MAS-NMR; Bone regeneration

Introduction

Recent studies demonstrated that bioglass can be considered as a promising way of promoting tissue repair. It shows a superior in vitro apatite-forming ability, and is able to directly bond to living bone in vivo [1]. Although the bioactive glass has osteoconductive ability, it does not possess any osteoinductive property [2,3]. In addition, even though it apparently interesting in terms of bone repairs and regeneration, it does not seem optimal in terms of therapeutic properties. The explosion in tissue engineering research has highlighted the need for new classes of biodegradable polymers. Chitosan (CH) and some of its complexes have been studied for use in a number of biomedical applications. These include wound dressings [4], drug delivery systems [5] and bone graft [6]. It is recognized that some biomaterials do not have an osteoinductive character in the absence of additional osteoinductive agents, such as Bone Morphogenic Proteins (BMPs) [7]. However, these molecules are complicated by a rapid and burst release that provides relatively short-term control [8]. Multiple functional properties such as drug delivery and low immunogenicity have provided ample opportunities for their potential applications. For that, BG-CH with 17 wt% CH has been synthesized and studied as a versatile composite for tissue-engineering. Recently, it has been demonstrated that CH matrix remains porous and acts as a sponge and trapping the growth factors [9]. In our previous study, the specific surface and porosity were determined. BET analysis was performed on the BG-CH biocomposite. The mesoporous material structure was revealed by adsorption isotherm [10]. In addition BG-CH contained a high rate of porosity equal to 81% which can play an important role of vascularization. In fact, during bone healing, it is well-known that the cells synthesize a number of growth factors. It could act as a putative local regulator of bone remodeling and thus, the idea is that CH having a microstructure may act as a chelating agent of growth factors and cytokines, which promote the recruitment, proliferation and differentiation of cells. The overall purpose of the BG-CH-based tissue engineering approach is to induce bone formation at extra-skeletal sites without the need of additional osteogenic cells or growth factors. In order to assess the bone quality, several biophysical tools have been developed and applied to characterize natural and tissue-formed bone. Our pervious study aimed to evaluate the performance of BG-CH implanted in the femoral condyl of an ovarioctomized rat by using several physico-chemical techniques [11]. In this work, NMR spectroscopy was used for monitoring the development of the major components of the organic bone matrix, in particular collagen [12-14]. In the present study, 13C NMR represents a very useful tool to track the bone organic component. Although previous NMR studies on collagen in hard and soft tissues had to rely on selective isotopic

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labeling [15-18], techniques have now advanced to study the 3°C in the native environment with BG-CH bone graft. The aim of this study is to describe and discuss the osteoinduction mechanism and anti-osteoporosis phenomenon. Moreover, we aim to analyze the properties of the de novo formed bone after 8 weeks of implantation into the femoral condyle rat bone using histology, ICP-OES, SEM and NMR spectroscopy.

Materials and Methods

Biomaterials synthesis

First, the bioactive glass particles were synthesized via the melting process [10,19,20]. To synthesize BG-CH biocomposite, a concentration (m/v) of chitosan solution (0.4 g of CH mixed in 40 ml of acetic acid) equal to 1% was used. The CH polymer with a medium molecular weight was dissolved in 1% acetic acid aqueous solution (Sigma, France) during 2 h at room temperature. Mixture of bioactive glass particles and CH polymer was stirred during 2 h at room temperature using magnetic stirring at 1,200 rpm (round per minute). After eliminating surplus solution, the mixture was frozen by liquid azotes and placed into a freeze-drying during 24 h to remove the solvent. The obtained composite with dimension size between 40 and 60 µm was immersed in 10% NaOH solution for two hours and washed several times with deionized water in order to neutralize the residues of acetic acid. Finally, the composite was frozen by liquid azotes and freeze-dried again for 24 h to completely remove water. The prepared implants were sterilized by y-irradiation from a 60Co source. Simple dilutions 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 kHz. Powdered samples of about 100 mg were necessary to fill up the rotor of the NMR probe. The deconvolution of the MAS-NMR spectra was performed on the dmfit 2010 software [19].

ICP-OES analyses – kinetic of ossification: Implanted muscles and femoral condyles were harvested, fixed in Burdack (formalin) (Sigma, France) and refrigerated. Specimens were dehydrated, using graded solutions of alcohol increasing from 70 to 100% ETOH (Siphat, Tunisia). The specimens were then infiltrated with methylmethacrylate and embedded (polymerized) before making the inclusion in a mixture of methylmethacrylate (MMA) (PROLABO, France) and glycolmethacrylate (GMA) (PROLABO, France) without prior decalcification. Sections 6 to 7 µm thick were dissected along a transverse plane using a sliding microtome (Reichert-Jung). Same histology stains are only used to study very specific types of biological tissue.

Physico-chemical analyses

XRD analyses – structural studies: Obtained compounds, before and after "in vivo" experiments were analyzed by using X-ray Diffraction. Data were collected using a Philips PW3710 diffractometer (45 kV, 40 mA and Cu-K = 1.540598 Å). The crystalline structures of pure glass (BG) and composite glass-chitosan (BG-CH) were highlighted versus time of implantation in femoral condyle.

NMR measurements: The characterization of the organic part of bone mineral was done using 13C solid-state NMR spectroscopy. The measurements 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 kHz. Powdered samples of about 100 mg were necessary to fill up the rotor of the NMR probe. The deconvolution of the MAS-NMR spectra was performed on the dmfit 2010 software [19].

ICP-OES analyses – kinetic of ossification: Femoral condyls were dried for 24 h at 65°C. Dry femurs were weighed accurately and placed in 25-mL tubes. The 2 ml of nitric acid were added. One milliliter of 30% H2O2 (Sigma, France) was placed in the tube after 10 min. The volume of the mixture was made up to 500 ml with distilled water. Standard solutions of Ca, P, Na, Sr, Si and Fe were used to prepare the working standard solution, and a blank solution. The element concentrations were measured using inductively coupled plasma optical emission spectrometry ICP-OES (Ciros; Spectro Analytical Instrument, Germany).

SEM – study of the morphologies: The Scanning Electron Microscopy (SEM) (Jinlul SM 6301F) was used to identify morphological changes between bone, BG and BG-CH. The collected samples were prefixed with 2.5% glutaraldehyde solution PBS (phosphate buffer solution, pH 7.4) (Sigma, France) overnight, and then washed with phosphate buffer solution (PBS), pH 7.4. Then, the samples were post fixed with 2% osmic acid solution (Sigma, France) (PBS, pH 7.4) for 90 min and dehydrated with alcohol. The samples were then freeze-dried with a freeze-dryer (JFD-300 Electron Optic Laboratory), and were carried out with a vapor deposition system (JFC-1200).

Statistical analysis

Statistical analysis was performed using Mann-Whitney U-test with SPSS software. P<0.05 was accepted as statistically significant.

Results

XRD characterization of specimens after the in vivo assays

After 4 days of BG implantation, X-ray diagram didn’t shows any crystallization peak. This halo is highly related to the amorphous structure of BG biomaterial. It didn’t exhibit any modification or
sign of degradation. Four days after surgery, X-ray diagrams of BG-CH-bone suggested the presence of 2 peaks with low intensity at 19.5° (2θ) in (001) orientation plane and a new one at 29° (2θ). This new peak highlights the interactions between bioactive glass and the chitosan polymer. An association between the orthorhombic system of chitosan and the amorphous structure of the glass to form a new structure of glass-chitosan composite was suggested [10]. There is a characteristic broad hump at 31.5° (2θ). This broad diffraction pattern is an indication of the predominantly amorphous form of BG-CH. However, 7 days after surgery, peaks at 19.5° and 29° (2θ) disappeared. This transformation exhibited BG-CH fast resorption and degradation behavior. In this stage, bone tissue can be viewed as a fine layer of organic matrix without deposition of mineral crystals. XRD analysis permits to determine the formation of HAP as an indication of bone consolidation. In fact, the mineral phase of bone consists of small crystals containing calcium and phosphate, called hydroxyapatite [14]. After 15 days of implantation, we noted the presence of new sharp diffraction peaks that are the result of new formation at 25.7° (2θ) in (002) plane orientation. These peaks show that bone healing is emphasized to provide a stable surface on which osteoblasts and/or their precursor cells may migrate and secrete bone matrix. After 30 days of BG-CH exposure, minerals were deposited within collagen fibers in which large amounts of solid mineral crystals were deposited and the registered peaks were more developed. After 60 days, the evaluation of the crystallinity was performed especially by the analysis of (002) and (211) line profile in implanted bone. The implanted bone was presented in the same way as normal bone and the degrees of calcification seemed to be enhanced. XRD analysis indicates that the formation of HAP was more accentuated in BG-CH when compared with BG especially 15 days after surgery (Figure 1).

NMR measurements

The organic moiety of the bone Extra Cellular Matrix (ECM) was investigated in detail by 13C solid-state MAS NMR. Figure 2 reports the 13C spectrum and the relative assignments of the native bone and BG-CH/bone after implantation. The NMR spectroscopy showed 13C spectra of native femoral condyle bone, where the typical spectral signature of collagen was observed. Most of the signals in the 13C spectra could be assigned by reference to the chemical shifts of the amino acid residues constituting type I collagen. The signal assignments which agreed with literature were attributed. The 182 and 175 ppm signals were consistent with carboxylate/carbonyl carbons which could belong to proteins. The γ-carbons of the hydroxyproline (71 ppm) were abundant in collagen. γ-carboxyglutamate (55 ppm), occurs in several other bone proteins like osteocalcin. The β-carbon of phosphoserine (55 ppm), was found in other bone phosphate carrier proteins. After 4 weeks of BG-CH implantation, 13C spectrum derived essentially from CH as observed in Figure 2. In fact, at 102.5 ppm, the resonance of the carbon C(1) assigned to the glucosamine unit is observed, while at 95 ppm a much less intense signal due to C(1) of the acetylglucosamine unit is shown. At 171.3 ppm, weak resonances respectively due to the carbonyl C(7) and C(1) are observable. The NMR spectra obtained after 8 weeks of implantation could be described as a superposition of the NMR spectra of the de novo generated collagen which represents the most ECM abundant organic component. In fact, characteristic signals from all collagen amino acids are obtained with a unique signal at 71.1 ppm, which can be assigned to the γ-carbon of hydroxyproline (Hyp), an amino acid that is not found in other proteins. This was a clear indication that BG-CH degradation had taken place during an implantation time of 8 weeks.

Histological examination

Muscle implantation and assessment of the osteo-inductive

Figure 1: X-ray diffraction from bones implanted by bioglass (BG, in left) and bioglass-chitosan (BG-CH, in right) after 4, 7, 15, 30 and 60 days compared with hydroxyapatite reference spectrum.
Figure 2: NMR spectra of native rat bone with all detected at a resonance frequency of Ala: Alanine; Arg: Arginine; Glu: Glutamic acid; Gly: Glycine; Hyp: Hydroxyproline; Pro: Proline. Implant BG-CH after 4 weeks and implant BG-CH after 8 weeks.
observed that BG-CH had an average pore size of approximately 10 μm in diameter. The pores could be expected to afford space for bone in growth. In the beginning of the experiment, there was no cell invasion on the BG or on the BG-CH implanted groups (Figure 5A and 5B). After 4 weeks, BG-CH was shown to encourage cell spreading. In fact, the bone surrounding BG-CH presented a highly cellular layer, more advanced ossification and a much larger stimulation of osteoregeneration than those of the BG group. However, only few cells were observed on the surface of BG biomaterial group (Figure 5C and 5D). By the end of the experiment, BG-CH offered a remarkable bone maturation that it could not be readily differentiated from the normal one (Figure 5E). Moreover, Figure 5F can show the BG-CH biodegradation and the mineral crystals disturbance among the bone collagen fibrils.

ICP- OES characterization of newly -formed bone

As results of ovariectomy, the contents of Ca, P and Na were significantly decreased when compared to those of control group (P<0.01) as illustrated in Table 1. As regards, Sr and Si, Na and Fe, no significant differences are observed among control and OVX groups. The implanted group with BG-CH showed that the general reduction of Si over time concomitant with the change in the release rate of Ca concentrations. In fact, Si concentration was decreased from 71 to 50 μg/g at 15 days and 30 days, respectively. However, Na content exhibited 9.00 mg/g measurements whose investigation shows 139 and 145 mg/g after 15 and 30 days, respectively. However, Na content exhibited 9.00 mg/g and 10 μm in diameter. The pores could be expected to afford space for bone in growth. In the beginning of the experiment, there was no cell invasion on the BG or on the BG-CH implanted groups (Figure 5A and 5B). After 4 weeks, BG-CH was shown to encourage cell spreading. In fact, the bone surrounding BG-CH presented a highly cellular layer, more advanced ossification and a much larger stimulation of osteoregeneration than those of the BG group. However, only few cells were observed on the surface of BG biomaterial group (Figure 5C and 5D). By the end of the experiment, BG-CH offered a remarkable bone maturation that it could not be readily differentiated from the normal one (Figure 5E). Moreover, Figure 5F can show the BG-CH biodegradation and the mineral crystals disturbance among the bone collagen fibrils.

Discussion

Osteinduction property

The osteoinductive capacity of chitosan-based bioglass (BG-CH)
in Wistar rat has been investigated. BG-CH biomaterials with particle sizes ranging from 40 to 63 μm were implanted into the muscles and the bone of the rats for 12 weeks. Our method of characterization by NMR spectroscopy was of great interest to determine the chemical transformation of the newly-formed bone in molecular rearrangement structure, developed in contact with BG-CH composite. To our knowledge, no data is available concerning local response of BG-CH as an osteoinductive material, 13C NMR characterization and anti-osteoporotic analysis in an ovariectomized rat model.

In the present work, BG-CH biomaterials demonstrated the ability to induce bone after 12 weeks in muscular sites without the addition of osteogenic cells or bone growth factors prior to implantation. CH is considered as a bioactive molecule related to the specific interactions of their surface with the extracellular fluids and cells, ionic exchanges, and cellular activity [21,22]. How the BG-CH microstructure functioned to produce this osteoinductive property was not clear, but some explanations could be found and many hypotheses could be postulated i) Through a dissolution–precipitation process, the development of a calcium phosphate-rich layer might initiate bone formation either by mimicry with the bone mineral structure or by the presence of osteogenic compounds contained naturally in body fluids that might have concentrated at the newly formed mineral layer [23]. When the osteoinduction of calcium phosphate ceramic was reported, the micropore was also reported [24,25], ii) An excellent porosity of bioactive materials signifying more proteins could be absorbed on the surface. The larger surface area could also make easy ion exchanges and bone-like apatite surface formation by dissolution and re-precipitation process [26]. During bone repair, chitosan played an important role in providing cell anchorage site, and structural guidance due to their porosity [10]. They also provided the interface to respond to physiological and biological changes, and to remodel the extracellular matrix to integrate with the surrounding native tissues [27]. Therefore, it was critical to promote cell attachment and migration to regenerate new tissues [28]. To accelerate the repair process, chitosan is implied to incorporate growth factors that are particularly important because it could directly or indirectly promote most cellular processes associated with wound healing, including chemotaxis, stimulation of cell proliferation, and extracellular matrix production [27]. More proteins absorbed on pore surface and the more easily formed apatite layer may facilitate bone formation. The importance of the microstructure in BG-CH-induced osteogenesis does not mean that the microstructure acts as the osteogenic agents in osteoinduction. It may act as a carrier or absorbent of osteogenic agents and then as a release agent [27-30]. Thus, BG-CH biomaterial may function as a solid-phase domain for the anchorage of BMP. After BG-CH swelling in the biological

*Figure 4:* (A) Disconnected trabecular bone in the OVX at 60 days after ovariectomy. (B) A new woven bone in the BG implanted group. (C) A gel layer adhesion to the new bone in the BG-CH implanted group. (D) The osteoid tissue in BG implanted group. (E) The mineralized tissue in the BG-CH implanted group. (F, G) A reduced inter-trabecular space and a higher trabecular thickness. (H) Osteoblast cells. (I) The osteocyte enclosed a space called the lacuna room. (J) Osteoclastic cells in the Howship’s lacunae-like. Goldner's trichrome staining (A, B, C, D, E) (10 x objective). Hematoxylin and eosin stain (F, G,(x10) H,(x100), (J,x40)).

*Figure 5:* (A, B) Absence of cells invasion on the BG or on the BG-CH implanted groups. (C, D) Highly cellular layer, more advanced ossification and a larger stimulation of osteoregeneration surrounding BG-CH compared with those of the BG group. (E) Degree bone maturation after BG-CH implantation. (F) BG-CH biodegradation and mineral crystals disturbance among the collagen fibrils of bone.
medium, the gel matrix acts as a sponge through which the fluid accumulates various growth factors ensuring the osteoinductive property. In fact, CH processes a chain of amino groups along the CH structure [31,32]. Many researchers are now seeking the ability of this amino group to adsorb metal and to chelate ions. This cationic nature is primarily responsible for the electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged molecules. This property is of great interest because a large number of cytokines/growth factors are linked to GAG [33-36]. The BG-CH composite may retain and concentrate growth factors secreted by colonizing cells. From the results presented herein, the addition of the CH to the biogases materials could be considered as an important factor for triggering osteoinduction.

**Anti osteoporotic phenomenon**

BG-CH is endowed with another attractive biological property. In fact, an anti-osteoporotic performance was attributed to CH. In a previous study, this molecule was found to inhibit osteoclastic activity, and is thus widely used to treat patients with osteoporosis [37]. One study demonstrated that incorporation of chitosan to the cement prevents the osteoclastic resorption of the composite biomaterial compared to the cement alone [38]. This inhibitory effect of chitosan on osteoclast resorption activity has important implications on bone formation and bone remodeling after in vivo implantation. Moreover, it is endowed with mitogenic activity toward various types of mesenchymal cells, including osteoblasts [39]. Therefore, it may also enhance bone formation and aid in the differentiation of osteoprogenitor cells [40]. In the present study, during the first 3 days, blood invaded all of the empty spaces between the original bone and the implant. Thereafter, at the end of the first week of implantation, callus and mesenchymal tissues entirely replaced blood while host bone resorption started. Finally, callus, mesenchymal tissues, and host bone have gradually disappeared in favor of newly formed bone while bone remodeling took place [7]. In the current study, CH induced new bone formation in the distracted zone, and the amount of new bone formation was similar to that in control. These findings suggest that these combined responses to oxidative stress in ovariectomised rats. Ann Pharm Fr 71: 234-242.

**References**


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