Experimental Study of Apatite Layer Formation on Chitosan/Bioactive Glass Scaffolds for Bone Tissue Regeneration

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Abstract--**In this study, sol-gel derived bioactive glass (BG), in the ternary SiO_2-CaO-P_2O_5 system, was synthesized and added as a reinforcement material in Chitosan (CH)-matrix scaffolds with ratios of CH/BG 70/30, 50/50 and 30/70. The scaffolds were prepared by freeze-drying process for 24 hours. The addition of BG in the CH-matrix improved the composite scaffolds bioactivity, as seen by the precipitation of bone like apatite layer after immersion in simulated body fluid (SBF) for 7 days. The apatite layer formation on the surface of each scaffold was confirmed by Scanning Electron Microscopy (SEM), X-ray Powder Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR). XRD result for BG indicated net formation using alternative calcium and phosphorus sources. SEM, XRD and FTIR results demonstrated that CH/BG scaffolds had higher bioactivity in comparison with CH scaffolds. These results advised that synthesized BG had a potentiality as reinforcement material in CH scaffolds and better bioactive behavior.**

Keywords--**composite scaffold, chitosan, bioactive glass, bioactivity test, apatite**

I. Introduction

Three-dimensional porous structures, named scaffolds, are developed to contribute to formation of functional tissue displaying properties that mimics natural, healthy tissues [1].

In this sense, scaffolds for bone tissue engineering (BTE) are required to be bioactive, biodegradable and provide an appropriate environment for cell attachment, proliferation and differentiation to finally form a new bone tissue [2]. Given that bone tissue is a mineralized composite, it is well know that one biomaterial type is not sufficient for bone tissue regeneration [3]. Therefore, composite scaffolds based on flexible biodegradable biopolymers and inorganic ceramics have been developed.

CH is a linear polysaccharide composed by glucosamine units extracted from crustaceans. Also, this is a biodegradable polymer, which allows osseointegration and presents an intrinsic antibacterial activity [4]. However, CH itself is not the ideal material for BTE due to its low mechanical strength and poor bioactive capabilities [5]. For this reason, the addition of bioactive glasses (BG), as reinforcement in CH scaffolds, could improve mechanical and bioactive properties without decreasing CH qualities. BG capability increase apatite layer formation giving a suitable and safe chemical bonding between composite scaffolds and living bone [6]. The bioactivity has been studied through apatite layer formation on the biomaterial surface after being soaked in SBF solution [7]. In this paper, composite scaffolds made of CH and sol-gel derived BG with three different ratios were analyzed in order to identify bioactive performance by in-vitro tests for 7 days using SBF solution.

II. Materials and Methods

A. *Materials*

Chitosan (medium molecular weight) and glutaraldehyde 25% were purchased from Sigma-Aldrich, Germany. The N-deacetylation degree of chitosan was found to be 80.22%. Tetraethylorthosilicate (TEOS: $C_8H_{20}O_4Si$) and calcium acetate ($Ca(CH_3COO)_2$) were purchased from Merck Inc. P_2O_5 was purchased from Alfa Aesar. Ethanol, Acetic acid and Lactic acid were purchased from Panreac. All chemicals from SBF preparation were obtained from Sigma-Aldrich, Germany.

B. *Bioactive glass synthesis*

Sol-gel prepared BG consisting of $65SiO_2$-$5P_2O_5$-30CaO (based on mol.%) was synthesized and characterized, as it is following described: TEOS was added into an ethanol/distilled water solution, with a molar ratio 1:4 both TEOS/distilled water and TEOS/ethanol according to Vaid, et al. [8]. The mixture reacted for 1 h assisted by agitation. P_2O_5 and an ethanol/distilled water solution with a molar ratio 1:4 both P_2O_5/distilled water and P_2O_5/ethanol were added to react completely in stirring for 45 min. Later on, calcium acetate was added to react for 11 min. Finally, an acetic acid/distilled water solution (6:1) was added into mixture (distilled water/BG 1:4). The final solution was kept in agitation until the gel was formed (gel point).

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BG gel was kept in a container for 3 days at room temperature. The gel was heated at 120ºC for 2 days to remove all water content. The final product was crushed in order to obtain a dry powder.

C. Preparation of the composite scaffolds

With the aim of preparing highly porous and bioactive composite scaffolds, CH was dissolved in 1% (v/v) aqueous lactic acid in order to obtain a CH solution at 2% (w/v). Composite scaffolds with different ratios of CH/BG (70/30, 50/50, 30/70) were elaborated. Stirring for 30 min and sonication for 1h were needed to assure the BG homogenization into CH solution [2]. In addition, Glutaraldehyde (1% w/w, respect to CH amount) was added to the mixture with constant stirring. The mixtures were frozen with dry ice for 24 h. Lastly, the scaffolds were taken to a freeze dryer at -80ºC and 0.02 mbar for 24 h in order to produce 3D porous structure.

D. Sample characterization

The synthesized BG were analyzed by XRD with XPert PANalytical Empyrean Series II diffractometer. This instrument worked with voltage and current settings of 45 kV and 40 mA respectively and used Cu-Kα radiation (1.5405980 Å). Regarding SEM analysis the morphology and microstructure of the composite scaffolds were evaluated using a scanning electron microscope (SEM-JOEL-JSM 6490 LV) that operated at the acceleration voltage of 20 kV. The functional groups present on the composite scaffolds were examined by Fourier Transform Infra-Red (FTIR) analysis with Perkin Elmer Spectrum One model with DTGS detector spectrometer.

E. In vitro bioactivity study in SBF solution

The SBF solution was prepared according to Kokubo et al. [7]. The composite scaffolds were immersed in the SBF solution and incubated at 37 ºC in close tubes for 7 days. Afterwards, the scaffolds were removed from the SBF solution and washed with distilled water to removed adsorbed minerals, and finally they were analyzed by FTIR and XRD.

III. Results and Discussion

A. XRD analysis of the synthesized BG

Fig. 1 presents narrow and differentiable peaks, which are characteristics of augmented crystallinity due to thermal treatment (1050 °C) carried on the XRD analysis. Primary and secondary peaks, even the hump centered close to 2θ =21°, present important consistencies regarding angle and intensity, as it was reported by other authors for comparable systems [9,10].

B. SEM observations

The low and high magnifications from the top view of scaffolds are shown in Fig. 2. According to the observations, the CH scaffolds, 70/30 and 50/50 (CH/BG) scaffolds showed high porosity with lamella structure (Fig. 2.a, c and e). The micrographs of these sort of scaffolds show a network of interconnected pores, having different shapes, in a size range from 100 to 200 µm. This is desirable to achieve osteogenic cell ingrowth (Fig. 2.b, d and f). [11,12].

These results allow to identify the possible bioactive behavior according to its porosity of each scaffold on in vivo conditions. Nevertheless, 30/70 (CH/BG) scaffolds show less porosity and less interconnection due to the high amount of BG incorporated into the composite (Fig. 2.g and h).

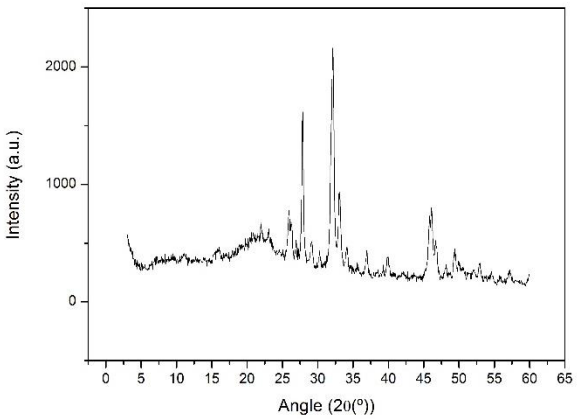

Figure 1. XRD patterns of synthesized BG after 1050ºC treatment.

C. Sample characterization after in vitro assays

The two samples with appropriate pore size were immersed in SBF solution. FTIR and XRD were used for apatite layer study. XRD spectra showed in Fig. 3 and Fig. 4 compare 70/30 and 50/50 (CH/BG) scaffolds before and after soaking in SBF. The main peaks on both samples after SBF immersion match with the main peaks of Hydroxyapatite pattern spectra on HighScore Plus software (Reference code 96-901-3628) as Pourhaghgouy et al [2]

reported. Furthermore, Fig. 5 and 6 depict FTIR spectra which were performed on 70/30 and 50/50 (CH/BG) scaffolds before and after immersion in SBF. As it can be seen on Fig. 6 for 50/50 (CH/BG) scaffold, the IR spectrum after soaking in SBF revealed two phosphate bonds at 576 and 669 cm^{-1} related to P-O bonding vibration on PO_4^{-3} group in calcium phosphate crystalline phases and the decrease of the 794 and 1085 cm^{-1} peaks related to Si-O-Si bonding vibration indicate the Hydroxyapatite layer formation on the surface of the scaffold [13]. However, this same result on 70/30 (CH/BG) scaffold proves the low capacity of forming a mineralized layer due to the poor amount of BG on the composite.

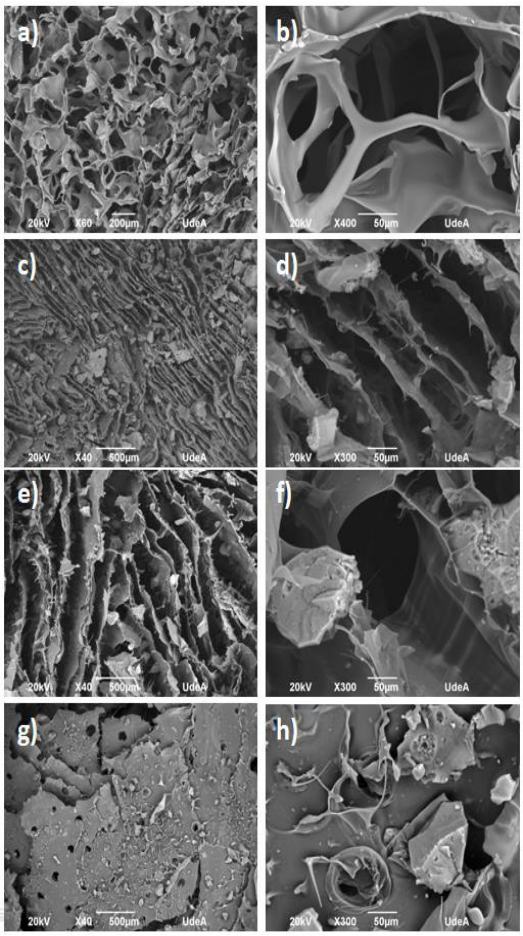

Figure 2. SEM micrographs of CH scaffold (a-b), 70/30 CH/BG scaffold (c-d), 50/50 CH/BG scaffold (e-f) and 30/70 CH/BG scaffold (g-h).

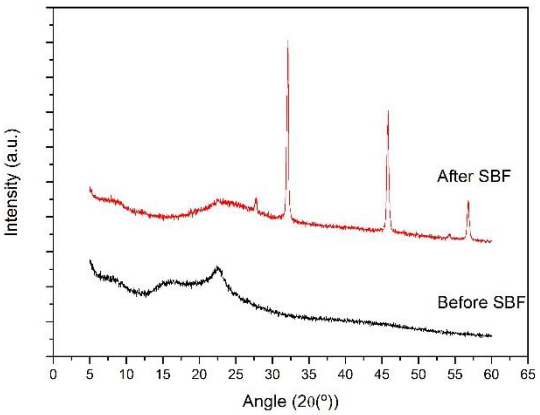

Figure 3. XRD patterns of 70/30 CH/BG scaffolds before and after immersion in SBF.

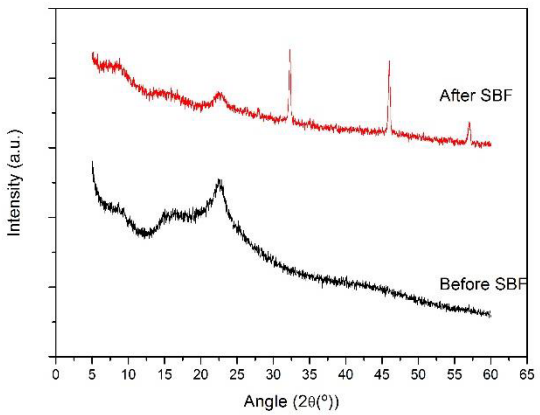

Figure 4. XRD patterns of 50/50 CH/BG scaffolds before and after immersion in SBF.

IV. Conclusions

The synthesized BG demonstrated appropriate network formation for bioactive glasses from SiO_2-CaO-P_2O_5 ternary system and an excellent bioactivity. The BG synthetized with calcium acetate and P_2O_5 (Phosphorus (V) oxide) reagents showed a similar response in comparison of bioactive glasses with tetrahydrate calcium nitrate ($Ca(NO_3)_2.4H_2O$) and TEP (triethyl phosphate). CH/BG scaffolds presented desirable porous morphology and bioactivity. Using BG as reinforcement material CH scaffold improved bioactivity without reducing its ability of forming 3D porous structures.

The pore size of scaffolds decreased due to the large amounts of BG; however, lower quantities decreased scaffold bioactivity. 50/50 scaffolds were proved to be

appropriate for BTE applications due to its pore size and interconnectivity as it shown in Fig. 2.e and 2.f and bioactivity in Fig. 4. This study has shown that desirable pore structure and interconnected porosity of the composite scaffolds can be achieved through procedure and CH/BG ratio control.

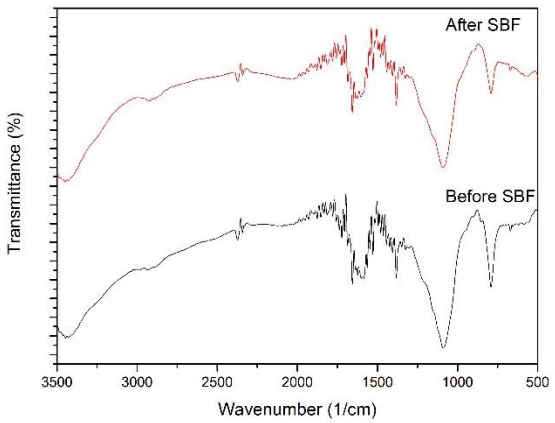

Figure 5. FTIR patterns of 70/30 CH/BG scaffolds before and after immersion in SBF.

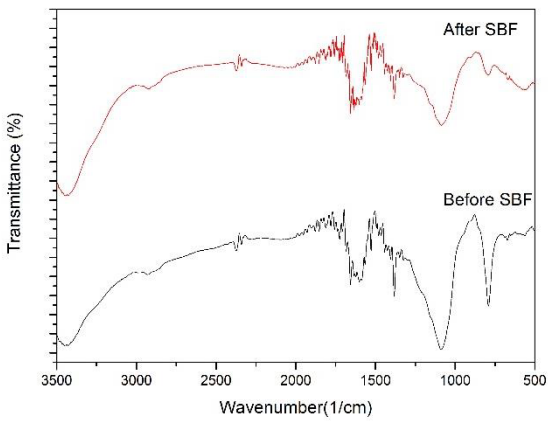

Figure 6. FTIR patterns of 50/50 CH/BG scaffolds before and after immersion in SBF.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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