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Investigation of the Hydroxyapatite Growth on Bioactive Glass Surface

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Abstract

Coating of dental ceramics with a bioactive glass resulted in the formation of a stable and well bonded ceramic substrate thin layer. After immersion in a solution with ion concentrations similar to those of human blood plasma the development of hydroxy-carbonate apatite layer on the surface of bioactive glass may be observed. The objective of this study was to investigate structural surface changes of bioactive glass, after exposure in a simulated body fluid for different number of days. The roughness and topography of the hydroxyapatite surface were investigated by Confocal Scanning Laser Microscopy. The chemical composition was analyzed by Energy Dispersive Spectroscopy measurements and phase characterization was made by Fourier Transform Infrared Spectroscopy.

Keywords: bioactive glass, hydroxyapatite, confocal laser microscopy.

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1 INTRODUCTION

Obtaining a perfect marginal fit is one of the goals in modern restorative dentistry. The restoration of osseous defects also needs the promotion of growing of soft tissues on bone surfaces [1]. For these reasons, the dental restorations and the prosthesis implants used bioactive materials such as glasses and glass-ceramics which proved their powerful implication in soft tissue attachment [2]. Investigations on the bonding mechanism demonstrated its association with the development in a short time after insertion into a biological environment [3-5] of a carbonate-containing hydroxyapatite layer.

Hydroxyapatite (HA) is a naturally occurring mineral and the predominant mineral component of vertebrate bone and tooth enamel. Naturally-occurring bone mineral is made of nanometer sized, poorly-crystalline calcium phosphate with hydroxyapatite structure. However, speaking of the ideal stoichiometric crystalline hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ with atomic Ca/P ratio 1.67 [6], the composition of bone mineral is significantly different and may be represented by the following formula:

$Ca_{8.3}\ (PO_4)_{4.3}(HPO_4, CO_3)_{1.7}(OH, CO_3)_{0.3}$

Bone mineral non-stoichiometry is primarily due to the presence of divalent ions, such as CO_3^{2-} and HPO_4^{2-} which are substituted for the trivalent PO_4^{3-} ions. Substitution by CO_3^{2-} and HPO_4^{2-} ions produce a change of Ca/P ratio, resulting in the Ca/P ratio which may vary between 1.50 and 1.70 depending of the age and bone site [7]. Synthetic calcium phosphate materials have been prepared and studied extensively *in vitro* and *in vivo.* Although some materials used have been called "hydroxyapatite" by some investigators, they actually vary widely in composition Ca/P ratios ranging from 2.0 to as low as 1.3.

The growing layer studied in this work, consists of non-stoichiometric biological apatite (Ca/P molar ratio $\neq$ 1.67) and provides an excellent connection with living tissue [5]. The most efficient procedure –*in vitro*- to form this type of biological apatite on the surface of bioactive materials is the immersion of samples in solutions that simulate body fluid (SBF) under various

time periods [8]. The process of apatite formation consists of a set of reactions like dissolution, precipitation and ion exchange [9]. If the bioactive ceramic is immersed in SBF, a partial dissolution of the surface begins immediately and this is followed by the release of Ca^{2+}, HPO_4^{2-} and PO_4^{3-} ions, thus increasing the supersaturation of SBF regarding to apatite. The layer of hydroxy-carbonate apatite (HCAp) begins to grow up by the calcium and phosphate ions uptake from SBF and by the incorporation of other electrolytes such as CO_3^{2-} and Mg^{2+} [10, 11].

The aim of this study was the investigation of the surface structure changes of ceramic-porcelain mixture used in material-ceramic restoration, coated with commercially available bioactive glass, after exposure in a simulated body fluid for different number of days, in order to evaluate any possible differences which could lead to stable bioactive layer on these conventional ceramics. Roughness and morphology of the hydroxyapatite surface were investigated by Confocal Scanning Laser Microscopy (CLSM). The chemical composition was analyzed by Energy Dispersive Spectroscopy (EDS) measurements and phase characterization was made by Fourier Transform Infrared Spectroscopy (FTIR).

2 MATERIALS AND METHODS

Our investigations were made on ceramic disks prepared using the procedure [3, 4] based on the mixing of dental porcelain powder (IPS Margin, Ivoclar, Schaan, Liechtenstein) with modeling liquid (Modeling liquid for shoulder porcelain, Ivoclar, Schaan, Liechtenstein) and placing the mixture into waxed 1x6 mm disk models (Dental modeling wax, Anutex, Kemdent, UK) fabricated and placed on impression silicon (IPS Margin, Ivoclar, Schaan, Liechtenstein) to form soft molds. The obtained disks with the same dimensions (a diameter of 6 mm and a height of 1 mm) were removed by gentle hand pressure, placed on a fire proof base, and then passed through the recommended thermal cycle, following the manufacturer's instructions (Furnance Programmat P95, Ivoclar, Schaan, Liechtenstein): final T=950^{o}C, heating rate t=80^{o}C/min, vacuum. The next step was the fabrication of a fine powder with a particle size range of 20-63 μm. This was achieved by sieving the bioactive glass powder (Bioglass 45S5), under commercial name PerioGlas® (Bioglass® Synthetic Bone Graft Particulate, US Biomaterials) with particle size range of 90-710 μm and a composition of SiO_2 45 wt%, P_2O_5 6 wt%, CaO 24.5 wt% and Na_2O 24.5 wt% [9], pulverized in ceramic mortar. A specific pre-weighted amount of this powder was spread on the surface of prefabricated ceramic disks. The coated disks were exposed to a second similar thermal cycle, and then every specimen was polished with diamond paste (STRUERS A/S, Denmark) of 3 μm for 1 minute, with rotation speed 150 rpm, using a STRUERS A/S polishing device. Upon fabrication, specimens were washed in pure acetone, rinsed with distilled water and air-dried. Then, the specimens were prepared for soaking in a solution with a composition (Table 1) that simulates human blood plasma [8, 12].

Table 1: Ionic concentration of SBF solution (mMol/l)

Na^+	K^+	Mg^+	Ca^+	Cl^-	HCO_3^-	HPO_4^{-2}
142	5	1.5	2.5	147.8	4.2	1

After immersing in SBF solution with a specific surface to volume ratio (equal to 0.1 cm^{-1}) and storing in an incubator at 37^{o}C for a time period of 1-11 days, EDS, FTIR analysis and CLSM investigations were performed on every specimen.

To establish the evolution of surface hydroxyapatite related to its chemical characteristics, X-rays microanalysis was performed with an Energy-Dispersive X-ray Analyzer connected to a FEI/Philips XL 30 FEG ESEM. All disks were investigated under the same microscopical conditions.

The characterization of bioglass-coated specimens by the FTIR spectra, for the selected test conditions, was made with a Bruker IFS 113v, FTIR spectrometer, in reflectance mode in MIR region (5000-400 cm^{-1}). Due to the low quality of the pellet's surface for each spectrum, 256 consecutive scans were recorded with a 2 cm^{-1} resolution.

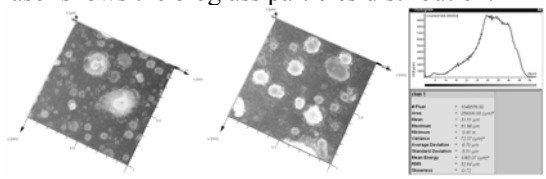
The presence of carbonate groups in the apatite layer of the bioglass-coated disks was emphasized by additional measurement in transmittance mode on pellets produced by following a simple method: a small quantity from the surface layer of the specimens was removed with a sharp tip of a microscalpel and mixed with KBr.

The assessment of topography and uniformity of hydroxyapatite layer was accomplished by CLSM. This method was recommended for many studies on the interaction of human osteoblasts with bioactive ceramics [13]. This investigation process was based on sequential exploration of the sample by a laser beam and acquirement of resulting interaction effects between light and material for surface or spatial imaging. The resolution was improved as the confocal microscope eliminated optical influences from neighbor domains. For non-destructive investigation of specimens by CLSM, a Leica TCS SP system equipped with an Ar-ion laser having a wavelength of 488 nm and a set of PL Fluotar (20X, 40X, NA 0.7) objectives was used. The objective resolution was limited by diffraction and for used wavelengths, it was about 200 nm. The system was performing in reflection mode and data processing and displaying were made by Leica software.

3 RESULTS AND DISCUSSION

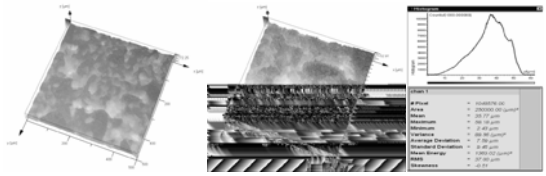
Bioglass-coated specimens were examined before and after each day of the 11 days range of immersion time in SBF. The initial specimen after heat-treatment consisted of bioglass particles with sharp edges and vacancies randomly distributed on the surface. In figure 1, a CLSM image achieved by scanning of initial

specimen with the 488 nm wavelength of the ion argon laser shows the bioglass particles distribution.



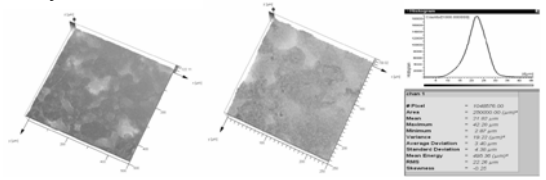
a b c

Figure 1: Bioglass-coated specimen after heat-treatment (non-immersed) –CLSM image (a – 20X objective; b – 40X objective; numerical aperture NA=0.7; c – histogram for corresponding specimen on 500 μm x 500 μm surface and 20X objective)



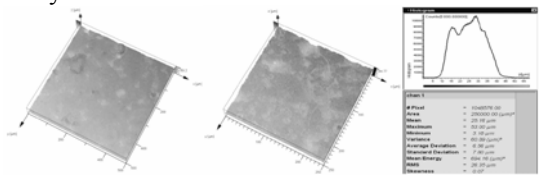
a b c

1 day in SBF



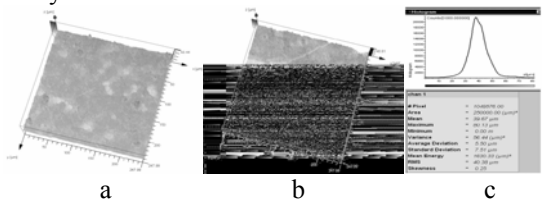
a b c

5 days in SBF



a b c

9 days in SBF



a b c

11 days in SBF

Figure 2: Bioglass-coated specimen after different time immersing in SBF –CLSM image (a – 20X objective; b – 40X objective; numerical aperture NA=0.7; c – histogram for corresponding specimen on 500 μm x 500 μm surface and 20X objective)

The scanned surface dimensions were relatively big in order to distinguish the majority of uniformity types appearing from the bioglass coating process. This reference specimen had a lot of uncovered vacancies on the bioglass surface with the largest having an approximate diameter of 0.25 mm. Surface CLSM images of immersed specimens were presented in figure 2 to demonstrate the gradual change related to time of soaking. As seen in the CLSM pictures in figure 2, after soaking for 1 day in SBF, bioactive glass particles were still distinguished, and the number of vacancies became smaller. One may see a reduction in the dimensions of the vacancies with the largest lacuna around 150 μm. The scanned zone, which was smaller (500 μm x 500 μm) presented a lower height variation on the z-axis than non-immersed specimen (72.25 μm against 78.35 μm).

In the following three days (2-nd, 3-rd, 4-th days) the increased non-uniformity could be due to apparition of Ca-P concentrations (see EDS analysis described in this chapter). On the 5-th day, a plane surface with several protuberances (approximately 50 μm against average height on z-axis) was observed in certain zones. The edges of different height domains seem completely round off and this demonstrated that Ca-P aggregates were big and dense enough. After 9 days of immersion time, a plane deposition was observed especially in image b, obtained with the 40X objective. The surface non-uniformities had almost spherical shapes, which were reflection images of Ca-P aggregates. The z-axis dimensions of these shapes demonstrated that their diameter increased with increasing immersion time in SBF. After 11 days of immersion in SBF solution, an increasing sample surface non-uniformity was observed due to the increasing Ca-P depositions on the layer surface. In the image obtained by 40X objective, several spherical shapes situated on a plane surface were observed. The investigated zone was rowed over by two perpendicular cracks of 2 μm width, and their clear visibility demonstrated the CLSM efficiency in surface defects analysis.

One important facility of CLSM was the ability to explore topography analysis. This was very useful in characterizing the development of apatite layer formation on the z-axis as showed in figure 2 (histograms marked c). For timing assessment of hydroxyapatite layer, a spatial z-series on each specimen, after each day of immersion in SBF was generated by CLSM method. The histograms presented in figure 2c show the variation of the number of photons emitted from the specimens' surface in reflection process on laser scanning period. The spatial step on the z-axis was 1 μm in depth and the basic parameters for roughness study were standard deviation and skewness. When number of counted photons increased, the corresponding surface was planer. Thus, there was a clear dependence between the photons counted for every specimen and the roughness.

To establish the roughness evolution on the investigation period (11 days), the variation of standard deviation and skewness was presented in figure 3.

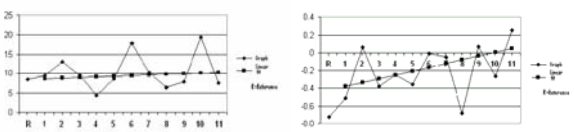


Figure 3: The variation of the standard deviation and the skewness for counted photons emitted from each specimen surface (reference and 1 to 11 days): a – standard deviation; b – skewness

It was observed that the standard deviation increased and reached a maximum on the 10-th day. This variation signified the increased roughness with the increasing number of days of immersion in SBF. The skewness also had an increasing trend, and its value (which changes its sign at 10-th day) demonstrated that the distribution of "craters" on the sample surface do not keep its first configuration. The number of non-uniformities became bigger as the skewness decreased and the number of immersion days in SBF increased. From figure 3, it could be seen that the increased roughness contributed to the extension of contact surface between hydroxyapatite layer and the surrounding tissues. Roughness mainly improves mechanical attachment but it is possible that increases the ability of surface to adsorb organic molecules like proteins thus improving the biocompatibility, although the correlation of this event – increasing roughness – with cell attachment remains unknown [14].

As with the above mentioned, the apparition of Ca-P formations after 1 day of immersion in SBF was established by EDS procedure, as revealed by pictures from figure 4.

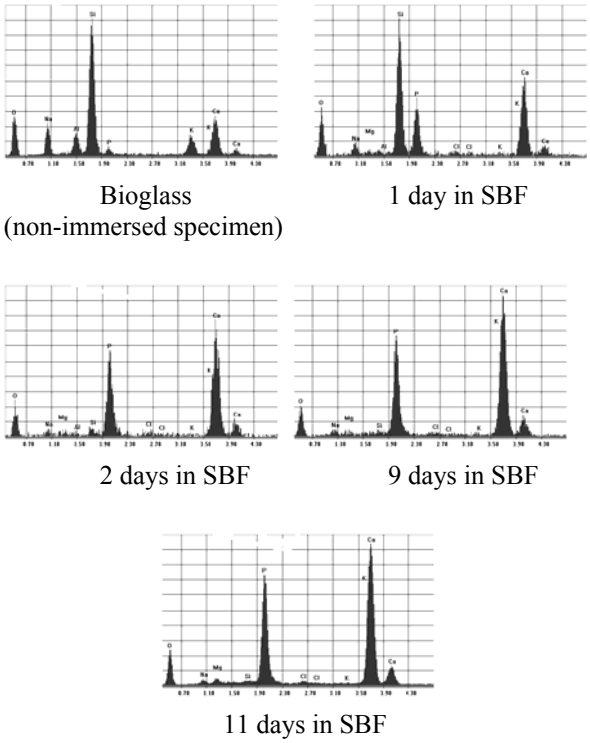


Figure 4: EDS analysis: establishing of Ca/P ratio along immersing of specimens in SBF solution

After 1 day in SBF solution, EDS analysis showed an increase in Ca and P (21.14 and 11.7 wt% respectively) and decrease in Si (21.48 wt%). After 2 days in SBF, there was an obvious decrease in Si and the molar Ca/P ratio developed to a value which corresponded to non-stoichiometric biological apatite. After 9 days, the molar Ca/P ratio ranged from 1.6 to 1.8 (nonstoichiometric hydroxy-carbonate apatite – HCAp) [14]. After 11 days, the whole surface of the specimen was covered with spherical Ca-P particles which created a dune-like apatite layer. The disappearance of Si in later days was attributed to the formation of a thicker fully grown layer on the surfaces of the respective specimens. Results from EDS analysis revealed the gradual development of hydroxy-carbonate apatite on the surfaces of coated ceramic specimens after immersion for various times in SBF. Figure 4 shows that even after 2 days in SBF, the Ca/P ratios were in accordance to non-stoichiometric biological apatite i.e. Ca/P molar ratio < or > 1.67.

In order to fully characterize the bioglass-coated specimens, FTIR spectroscopy was used. The results of measurements were presented in figure 5. The spectral regions where the most important characteristic bands arise for 950 C Bioglass (non-immersed bioglass coated specimens) are 400-500 cm^{-1}, that was attributed to Si-O-Si bending, 550-650 cm^{-1}, was attributed to P-O bending vibrations and 1000-1050 cm^{-1} was characterized by very strong asymmetric vibrations Si-O-Si.

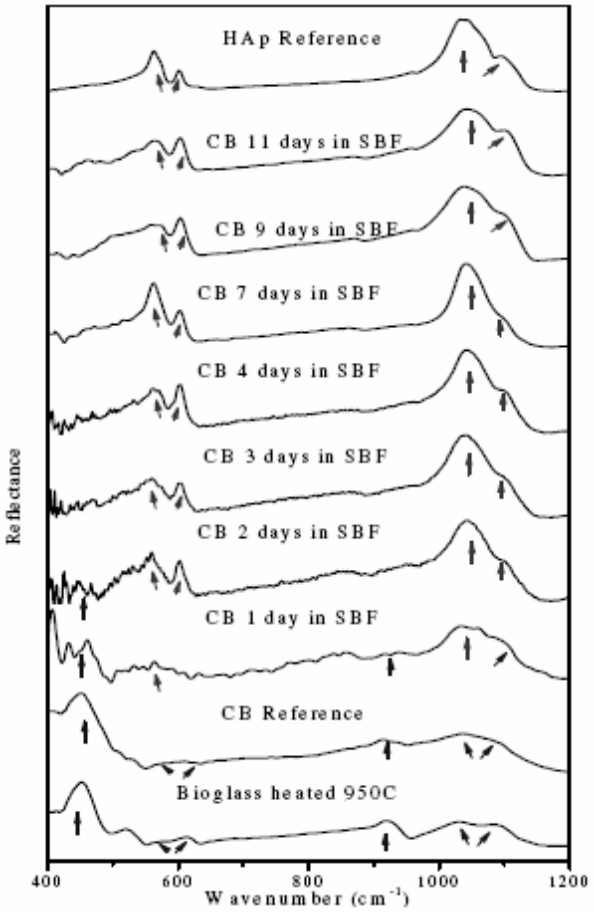


Figure 5: FTIR reflectance spectra of coated specimens before and after immersion for 1, 2, 3, 4, 7, 9, and 11 days in SBF

The peak at 920 cm^{-1} could be attributed to the Si-O stretching mode. The above reference FTIR spectra

offered the characteristics of the silica glass. The spectrum of HAp (hydroxyapatite) reference show three main bands attributed to PO_4 group: 1035 cm^{-1} assigned to P-O stretching vibration and the two bands at 610-600 cm^{-1} and 560-550 cm^{-1}, assigned to P-O bending vibration. Thus, the most important features in the spectra of the reacted specimens observed in the vibrational frequency range were associated with the PO_4 group of HCAp, in accordance with the literature [15]. The formation of biological HAp – that is HCAp, hydroxy-carbonate apatite – on the surfaces of the specimens was observed in the spectra, the appearance of two bands at 603 and 561 cm^{-1} was attributed to PO_4 bending vibration and the strong band at 1035 cm^{-1} was caused by PO_4 symmetric stretching vibration [10]. Thus, the development of HCAp layer caused the weakening and finally the disappearance of the bands attributed to Si-O-Si vibration from bioactive glass. The spectrum was dominated by bands generated by PO_4 group vibrations. The goal of FTIR measurement was to compare reflectance spectra of bioglass-coated specimens with the FTIR spectrum of crystalline HCAp reference. The reflectance spectrum from the surface of the specimens that were immersed for 1 day in SBF presented – beyond the strong spectroscopic characteristics of SiO_2 rich layer – a very small participation of amorphous calcium phosphate. This could be seen from the weak band at 560-610 cm^{-1}. The characteristic bands of HCAp start to appear in the FTIR spectra of the surface specimens after 2 days in SBF and they were confirmed with the increase of incubation time. After the third day of immersion in SBF, the disappearance of the Bioglass bands were observed. Furthermore, as the days of immersion in SBF increased, the phosphate bands became sharper and shifted. This indicated a powerful growth of more crystalline biological HAp (HCAp) [12, 13]. The characteristics bands of the crystalline HCAp were clearly revealed after 7 days immersion time.

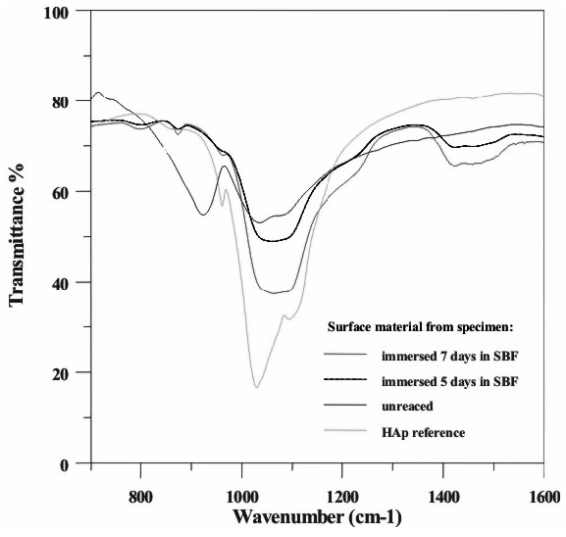


Figure 6: FTIR transmittance spectra of coated specimens and reference specimen

An important impediment in examining the apparition of CO_3 group from the reflectance spectra was the weak bands in the spectral area of 1250-1600 cm^{-1} due to the roughness of the surface of the forming apatite layer. The carbonate group was distinguished in transmittance mode of FTIR spectra (figure 6).

The presence of carbonate group was observed in two locations on the curve corresponding to 7 days immersion of the SBF specimen: one peak at 1530-1400 cm^{-1} was assigned to C-O stretching vibration and a second peak at 878 cm^{-1} was assigned to C-O out-of-plane bending vibration [17-19].

The inclusion of carbonate into apatite structure modified the morphology [20] as well as the structure [21, 22] of the apatite and it was possible to reduce biological reactivity of the bone mineral [23-25].

The apparition of carbonate group on FTIR spectra was confirmed from the molar Ca/P ratios which had values corresponding to non-stoichiometric biological apatite (Ca/P molar ratio 1.67) [16].

4 CONCLUSIONS

The dental ceramics coated with a bioactive glass layer heat-treated at 950^oC proved to be stable and well bonded with the growing layer of carbonate apatite on their surface after immersion in a simulated body fluid for 1-11 days. The investigation of carbonate apatite layer on its morphology and topography by CLSM show an increased surface roughness which was very convenient for a faster connection with organic tissues. Chemical analysis investigated by EDS show that Ca/P ratio was variable around values obtained in the bone cases. The FTIR measurements demonstrated the presence of CO_3 group and hence the HCAp apparition and complete stabilization of its growing after 9 days immersion in SBF. The EDAX analysis showed that the Ca/P ratio between 9-th and 11-th days of immersion in SBF reached its values corresponding to carbonate hydroxyapatite (1.6-1.8). Taking these reports into consideration, we concluded that CLSM method was a complementary method for SEM and ESEM methods used by majority of investigators on bioactive materials. CLSM technique has some advantages on SEM method, like a larger array scanned on the sample surface under normal environment conditions and no obligatory preparation of samples before investigations.

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