Compositional Variations in the Calcium Phosphate Layer Growth on Gel Glasses Soaked in a Simulated Body Fluid

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The growth of calcium phosphate layers on the surface of $SiO_2-CaO-(P_2O_5)$ bioactive glasses immersed in a simulated body fluid (SBF) under static and dynamic conditions has been studied by XRD, SEM, EDS, TEM, and XPS. It has been found that the chemical composition of the layer is very heterogeneous and that there is evidence for the presence of a composition gradient across the layer. The Ca/P molar ratio in the layer decreases from a value of 1.6, corresponding to hydroxyapatite in the interior, to a value close to 1 in the outermost surface, where a high population of HPO_4^{2-} groups is found. The chemical composition at the interphase is influenced by the presence of P in the starting glass substrate as well as by the conditions, static or dynamic, used to carry out the bioactivity tests. A continuous recrystallization of the calcium phosphate layer is proposed to account for these results.

Introduction

Glasses that promote bone-tissue formation at their surface and bond to osseous tissues when implanted are usually called bioactive.[1,2] Because of this unusual property, they are widely studied for biomedical applications in areas including orthopedics and dentistry. The bone-bonding mechanism still is not fully understood. However, all bioactive glasses form on their surface a calcium phosphate layer that crystallizes to hydroxycarbonate apatite (HCA) when in contact with physiological fluids.[3] Thus, the formation of a biologically active HCA on the glass surface was proposed as the prerequisite for bonding to the living tissues.[4] The calcium phosphate layer is also formed in vitro when bioactive glasses are soaked in solutions that mimic human plasma. The ability to form the HCA layer in vitro was related to the bone-bonding ability in vivo. Therefore, in vitro studies in the development of new bioactive glasses are frequently used because they allow predictions of the behavior of these glasses inside the human body.

The bioactivity of glasses obtained by a sol−gel procedure has, in general, been related to their chemical composition and surface properties such as surface area and porosity.[5−7] Indeed, these properties are of paramount importance in determining the formation of the apatite-like layer at the initial stages of the process when the glasses are exposed to physiological fluids.[8] However, as the phosphate layer grows and progressively covers the glass surface, it is this layer that determines the nature of the biomaterial/bone interface. Therefore, the response of the organism to the biomaterial after implantation could eventually be determined by the chemical nature of the apatite-like surface.

There are a number of investigations, albeit scarce, that report the alteration of pure hydroxyapatite samples after treatment with aqueous solutions of different compositions.[9−11] In particular, the exposure of hydroxyapatite to low-pH solutions has been shown to deplete the calcium in the surface.[9,11] Therefore, changes in the surface of apatite-like layers growing under in vitro assays might also take place. Following this hypothesis, the surface characteristics of the apatite layer developing on bioactive sol−gel glasses have been studied by using X-ray photoelectron spectroscopy (XPS). This technique allows for the detection of the chemical features of the first atomic layers of the material under study. The growth process of the phosphate layer on the glass surface was also followed by XRD, SEM, EDS, and TEM.

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Table 1. Composition (mol %) of the Prepared Sol−Gel Glasses

	SiO_2	CaO	P_2O_5
80S17C3P	80	17	3
80S20C	80	20	0

Experimental Section

Gel Glasses Synthesis. Gel glasses 80S20C and 80S17C3P with the compositions shown in Table 1 were prepared as previously reported.[12,13] Tetraethyl orthosilicate (TEOS), triethyl phosphate (TEP), and hydrated calcium nitrate [$Ca(NO_3)_2{\cdot}4H_2O$] were used as reactants.[5−7] HNO_3 was used as the catalyst. The molar ratio of (H_2O + HNO_3)/TEOS was 8. Reactants were consecutively added at 1 h intervals with continuous stirring.

The obtained sol was cast in a Teflon container that was sealed and kept at room temperature to allow for the gel to form. Aging was performed at 70 °C for 72 h and drying at 150 °C for 48 h with the lid of the container substituted by another lid featuring a 1-mm-diameter hole that allowed the gases to escape.

Dry gels were ground and sieved, with the 32−68 μm fraction selected for the preparation of the disks (13 mm in diameter and 2 mm in height), which were prepared by compacting the powders with 50 MPa of uniaxial pressure and 150 MPa of isostatic pressure. Finally, the disks were treated for 3 h at 700 °C for nitrate elimination and stabilization. The surface area and the average pore size of the obtained samples were, respectively, 154 m^2/g and 12 nm for 80S20C glass and 286 m^2/g and 36 nm for 80S17C3P glass.

In Vitro Assays in SBF. In vitro assays were performed by soaking the glass disks, vertically mounted in a Pt wire, in a simulated body fluid (SBF) at 37 °C that was buffered at pH 7.30 with tris(hydroxymethyl)aminomethane/HCl. SBF is an acellular solution with an ionic composition (in units of mmol/L, 142 Na^+, 5.0 K^+, 1.5 Mg^{2+}, 2.5 Ca^{2+}, 103.0 Cl^-, 27.0 HCO_3^-, 1.0 HPO_4^{2-}, and 0.5 SO_4^{2-})[8] almost equal to that of human plasma. To avoid surface effects, the ratio of the geometric surface area of the specimen to the solution volume was 0.075 cm^{-1}, as in our previous studies.[12−14] This way, every glass disk was soaked in 45 mL of SBF. The studies were performed following two protocols that have been described elsewhere.[14] (a) Static: SBF solution was not renewed during the assays. (b) Dynamic: SBF was renewed at a rate of 1 mL/min constant flow, with the volume of solution in the glass container (45 mL) kept constant throughout the assays. A schematic depiction of both protocols is presented in Figure 1.

To avoid microorganism contamination, the SBF solution was previously filtered with a 0.22-μm Millipore system, and all of the operations/manipulations were performed in a Telstar AV-100 laminar flux cabinet.

For every soaking time, the disks were taken out of the SBF, gently rinsed with water and acetone, and dried in air to stop further reactions. Then, the Ca, P, and Si ionic concentrations and the pH of the solution were measured, and the glass surface was analyzed. The Ca concentration and pH were determined in an ILyte $Na^+/K^+/Ca^{2+}$/pH system, whereas the P and Si concentrations were determined by complex formation in a Beckman DU-7 UV−vis spectrophotometer. For every soaking time, the results presented are the average of the values obtained for two independent samples.

The glass surface, both before and after being soaked in SBF, was studied by X-ray diffraction (XRD) in a Philips X'Pert MPD instrument, by scanning electron microscopy (SEM) in a JEOL 6400 microscope at 20 kV, by transmission electron microscopy (TEM) and electron diffraction (ED) in a JEOL 2000 FX microscope operating at 200 kV, and by energy-dispersive X-ray spectroscopy (EDS) performed in both an Oxford-LINK Pentafet system connected to the SEM microscope and a LINK AN 10000 system connected to the TEM microscope. For the studies using the transmission electron microscope, samples scraped from the surface of the glass disks were dispersed in *n*-butanol and then transferred to carbon-coated copper grids. Chemical analysis of the surface was performed by X-ray photoelectron spectroscopy (XPS) with an Escalab-210 spectrometer. Binding energies were corrected for charge effects by reference to the carbon 1s peak at 284.9 eV. The EDS analyses of samples were done in both the surface and transverse cross section of the glass piece.

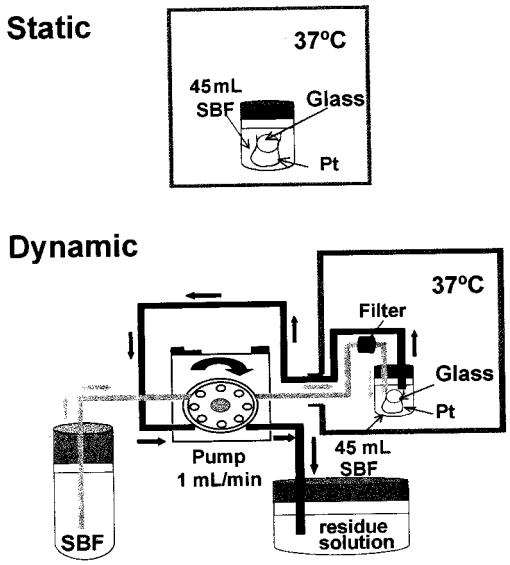

Figure 1. Schematic depiction of the two protocols used for the in vitro tests. Static: The piece of glass is soaked in SBF at 37 °C in a sealed container. Dynamic: The SBF solution is continuously exchanged with a peristaltic pump.

Results and Discussion

The formation of an apatite-like layer on the surface of the glass disks was studied by XRD after the samples had been immersed in the SBF solution. This is illustrated in Figure 2, in which the XRD patterns of the 80S20C and 80S17C3P glasses after 7 days of soaking under the two hydrodynamic conditions, static (without assay solution renovation) and dynamic (with continuous renovation of solution), are shown.

The XRD patterns of 80S20C under dynamic conditions and those of 80S17C3P under both static and dynamic conditions showed diffuse reflections centered at 26°, 32°, 40°, 47°, 50°, 53°, and 64° in 2θ, which can be assigned, respectively, to the (002), (211), (130), (222), (213), (004), and (304) reflections of an apatite-like phase. However, XRD pattern of 80S20C under static conditions did not show this kind of maxima. The general tendency observed was that the presence of phosphorus in the glass and the use of a dynamic protocol increased the intensity of the maxima of the apatite-like phase. The absence of Bragg reflections in the diffractogram of the P-free glass soaked statically has been attributed to the very small size of the calcium phosphate crystals comprising the layer,[13] which can nevertheless be conveniently detected by FTIR spectroscopy. The influence of the in vitro assay conditions on the crystallization of the calcium phosphate phase was discussed elsewhere.[14]

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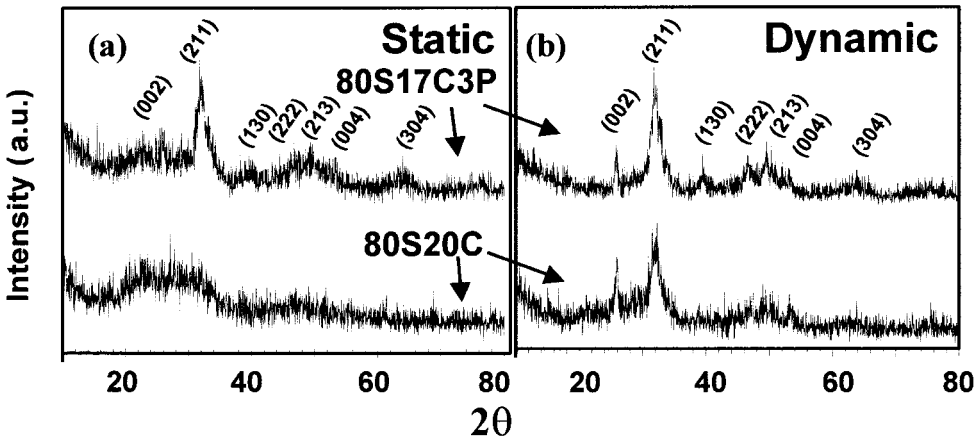

Figure 2. XRD patterns of 80S20C and 80S17C3P after 7-day in vitro assay, under (a) static and (b) dynamic conditions.

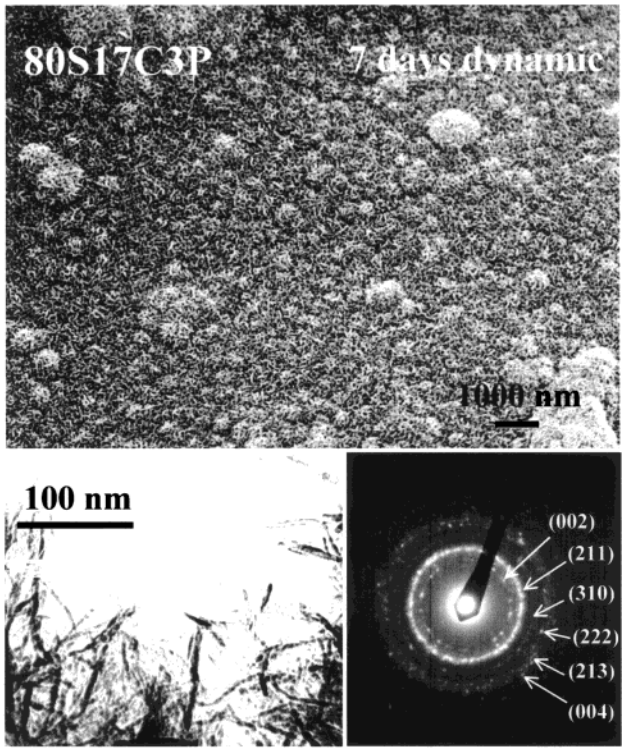

Figure 3. Top: SEM micrograph of 80S17C3P after being immersed for 7 days in SBF under dynamic conditions. Bottom left: TEM micrograph of material scraped from 80S17C3P surface after 7 days in SBF in dynamic. Bottom right: ED pattern.

The full covering of the 80S17C3P substrate by the calcium phosphate layer after a one-week assay is observed by SEM (Figure 3, top), where the characteristic round shaped aggregates of elongated particles are identified. In the bottom left of Figure 3 is presented the TEM micrograph showing that the particles are actually aggregates of tiny needlelike crystals. The electron diffraction pattern (Figure 3, bottom right) can be assigned to an apatite phase.

Despite the similarities in the XRD patterns, which are sensitive to the bulk phosphate layer, the crystallization process takes place under very different chemical environments, as can be observed in Figure 4, where variations in the solution for the 80S20C glass are shown. In the absence of a continuous renewal of the SBF solution, a very sharp increase in the Ca concentration takes place in the very first hours of the test, but it then stabilizes at a value close to 8 mM after one week. The concentration of P follows the reverse trend, and in consequence, a very high Ca/P ratio in solution prevails during the entire calcium phosphate growing process (Ca/P = 25 at 1 week). Indeed, the pH increases during the process, rising to 8 after one week.

In contrast to this behavior, the continuous exchange of the SBF solution kept the ion concentration nearly at the same level as the standard SBF solution, i.e., pH = 7.3 and Ca/P = 2.5.

Taking into account these differences, it would be expected that the chemical composition of the calcium phosphate layer would also change as a function of the chemical environment prevailing in the in vitro test. There is some evidence to support this hypothesis from the chemical composition determined by EDS in a scanning electron microscope (Table 2). For the two glasses, the static assays lead to a calcium phosphate layer with a chemical composition close to that of apatite, namely, a Ca/P molar ratio = 1.67. On the contrary, the continuous flow of SBF decreases this value to 1.20−1.22, well below the value of the calcium-deficient apatite currently described.[15] However, it was reported that apatite-like crystals with Ca/P = 1.60 were detected by TEM/EDS in the bulk of the calcium phosphate layer grown under dynamic conditions, embedded within a poorly ordered phosphate matrix.[14]

These results point to the existence of a strong chemical heterogeneity in the calcium phosphate layer. Indeed, taking into account that this layer is usually several microns thick, the EDS coupled to the SEM would deliver reliable chemical information only on the outer rim of the layer. Therefore, a gradient of chemical composition could exist across the phosphate layer. With the purpose of elucidating this hypothesis, the glasses exposed to the SBF solution for one week were studied by means of XPS.

Representative high-resolution XPS spectra of Ca 2p, P 2p, and O 1s for the glass 80S20C after 7 days in dynamic assays are provided in Figures 5−7, respectively. It is worth mentioning that no silicon is detected on the samples. The Ca 2p spectrum, (Figure 5) shows a doublet with Ca $2p_{3/2}$ at 347.4 ± 0.2 eV and Ca $2p_{1/2}$ at 350.8 ± 0.2 eV, which are characteristic of divalent Ca^{2+} in common salts, calcium phosphates included. The

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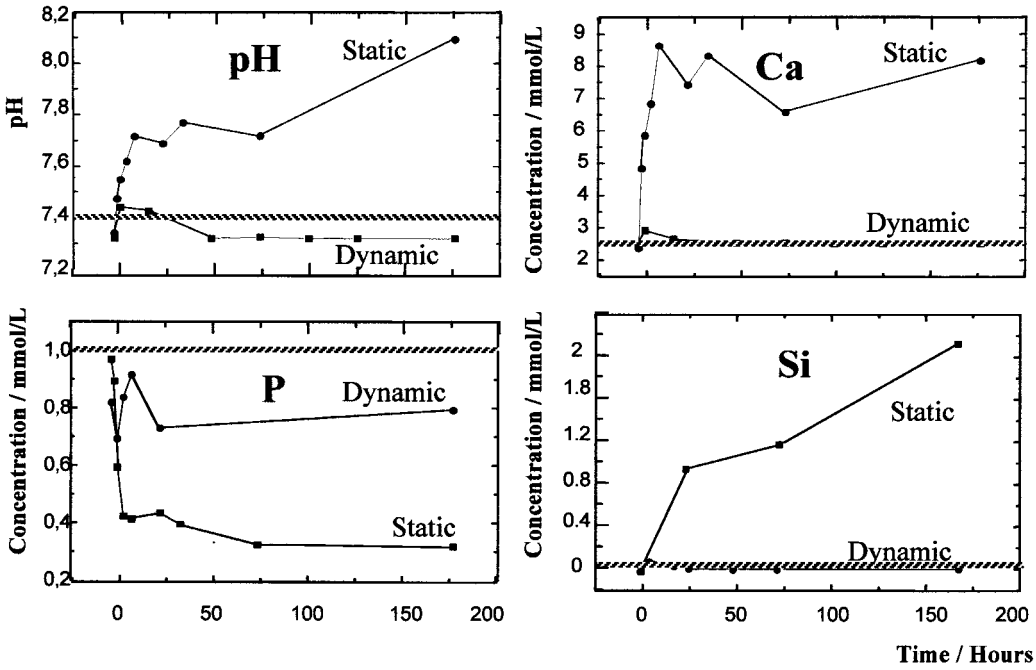

Figure 4. Variation with soaking time in SBF of pH and calcium, phosphorus, and silicon concentrations in solution in dynamic and static assays for 80S20C. A thick line indicates human plasma values.

Table 2. Ca/P Molar Ratio as Determined by EDS and XPS Chemical Analysis of the Glasses after 7 Days of Immersion in SBF

	EDS		XPS	
glass	static	dynamic	static	dynamic
80S17C3P	1.64	1.22	1.44	0.97
80S20C	1.60	1.20	1.58	1.15

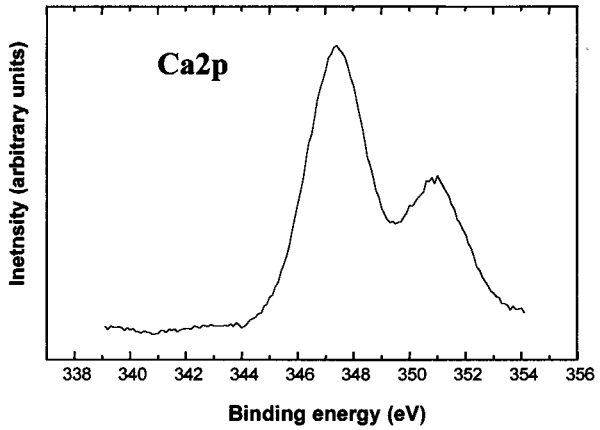

Figure 5. XPS spectrum of Ca 2p of 80S20C after 7 days of being soaked under dynamic conditions.

phosphorus 2p spectrum (Figure 6) consists of one rather symmetric signal centered at 133.3 ± 0.2 eV, and no significant shift in the peak was detected among the several samples examined. The slight asymmetry of this band has been attributed to the $2p_{1/2}-2p_{3/2}$ splitting, which is 1 eV.[16] Indeed, the difference between the P 2p signal of hydroxyapatite (HA), where all the P atoms pertain to PO_4^{3-} groups, and brushite ($CaHPO_4 \cdot 2H_2O$), which contains only HPO_4^{2-}, has been reported to be

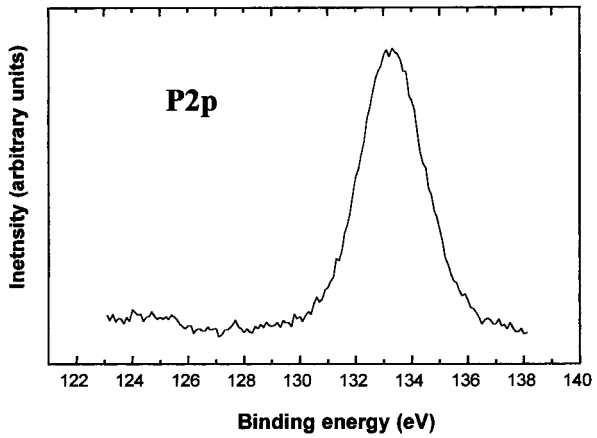

Figure 6. XPS spectrum of P 2p of 80S20C after 7 days of being soaked under dynamic conditions.

only 0.4 eV, for measurements with a standard error of ±0.2 eV.[17] Therefore, the 2p level of phosphorus can be considered to be rather insensitive to changes in its second coordination shell.

The oxygen 1s spectrum in Figure 7 shows a clear asymmetry, and the peak can be deconvoluted into two components, one centered at 531 ± 0.2 eV (the most intense) and one at 533.1 ± 0.2 eV. The first peak appears at the same binding energy as in hydroxyapatite. The high-energy peak has been observed in HA in prolonged contact with a NaCl solution,[9] as well as in monetite, the anhydrous calcium acid phosphate, $CaHPO_4$,[18] where the low-energy component is simultaneously detected. Therefore, the component at 531.1 eV can be assigned to HPO_4^{2-} groups. The presence of this ion on the surface of apatite would arise from the

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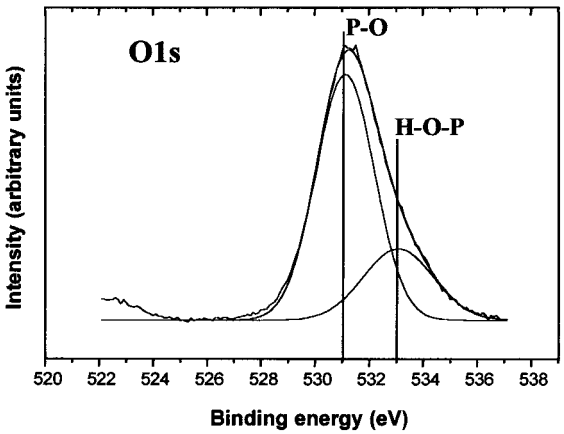

Figure 7. XPS spectrum of O 1s of 80S20C after 7 days of being soaked under dynamic conditions.

partial hydrolysis of some PO_4^{3-} groups. The hydrolysis process should be associated with the loss of calcium ions in order to keep the charge balanced, and therefore, the Ca/P ratio at the surface of the calcium phosphate layer should be lower than that of the stoichiometric apatite. Indeed, this is observed (Table 2). In all cases, the Ca/P ratio in the outermost layer obtained by XPS is lower than 1.6, but the deficiency in calcium is much more pronounced for the sample grown under dynamic conditions. In this case, the Ca/P ratio is close to 1.

The differences in the Ca/P surface ratios between static and dynamic tests can be rationalized in terms of the pH of the solution, as it is expected that the lower pH of the dynamic test enhances the hydrolysis of the PO_4^{3-} surface groups. It is worth mentioning that the incorporation of phosphorus in the glass influences the chemical composition at the surface of the phosphate layer by lowering the Ca/P ratio.

The alteration of the phosphate under assay conditions close to those prevailing in vivo to give HPO_4^{2-} surface groups could have a strong influence in the biological processes leading to bone formation brought about by the biomaterial after implantation. It has been mentioned that the bonding of proteins to the surface can be facilitated by the existence of HPO_4^{2-} groups,[10] whereas the low Ca/P ratio at the bone surface[18] could also improve its binding to the phosphate layer covering the glass.

Quantitative determination of the chemical gradient across the phosphate layer was carried out by analyzing a cross section of this layer by EDS/SEM (Figure 8). As can be seen in this figure, three different regions can be distinguished across the glass disk, according to their chemical compositions. In region I (from the glass core to 20 μm below the surface), silicon is the most abundant element, calcium is also detected and, interestingly, phosphorus is also present in the inner region of the glass. The presence of P in the glass core shows the fast diffusion of the phosphate ions from the solution into the glass immersed in the SBF solution. The transition region II (20−10 μm) is delimited by an abrupt decrease in silicon and a simultaneous increase in Ca and P. At a depth of 10 μm, no silicon is detected, whereas the Ca/P ratio is 1.61. In the pure calcium phosphate region III, the Ca/P ratio decreases continuously outward, and a value of 1.43 is measured at 1 μm below the layer surface. This decrease in the Ca/P ratio will continue

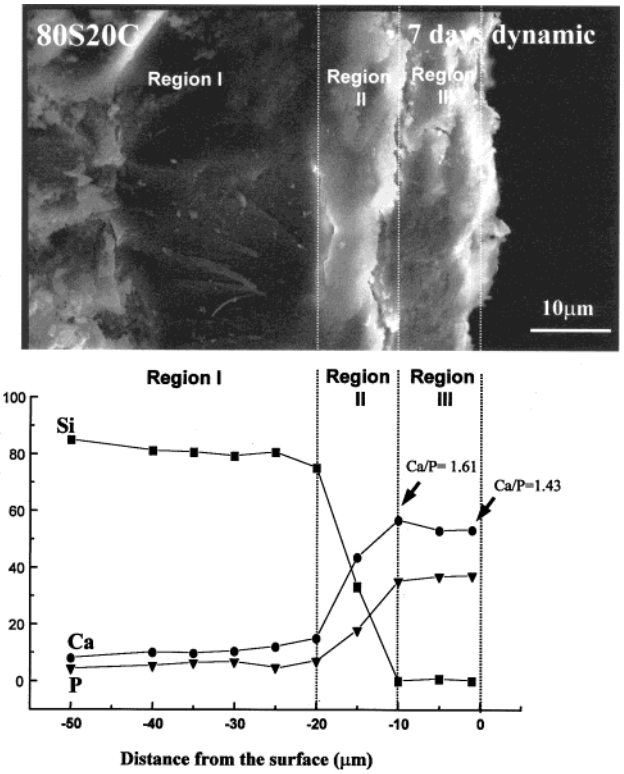

Figure 8. Top: SEM micrograph of a cross section of 80S20C after being soaked for 7 days under dynamic conditions. Bottom: Element distribution obtained by EDS.

to the outermost part of the layer, as is shown by the 1.20 value in Table 2.

The XPS and EDS/SEM results confirm the existence of a composition gradient across the calcium phosphate layer. A continuous reduction of the Ca/P ratio from that corresponding to nearly stoichiometric apatite in the inner portion of the layer to a ratio of 1 in the outermost surface, as determined by XPS, is found. The tiny crystals detected by TEM (Figure 3) would form the apatite region.

The partial hydrolysis of the phosphate groups should take place during the whole growth process of the phosphate layer, at least in the dynamic test. Therefore, a very low Ca/P molar ratio would have been expected across the layer, which is not observed. To reconcile the continuous growth of a calcium-deficient phosphate with the existence of apatite crystals in the bulk layer, a continuous recrystallization of the initially formed calcium phosphate into apatite can be postulated. The recrystallization rate would be lower than the growth rate of the layer, which explains the composition gradient described above. After one week of assays, nearly all of the inner portion of the layer formed at the earlier stages of the process (boundary between regions II and III) has been transformed into apatite crystals. According to this model, the extension of the apatite front inside the phosphate layer would be determined by the relative rates of the recrystallization and calcium deposition on the layer. Eventually, an optimum integration of the biomaterial after implantation could rely on appropriate control of the rates of these two parallel chemical processes.

Conclusions

This study has evidenced the heterogeneous nature of the calcium phosphate layer growing on bioactive glasses under in vitro tests. Differences in the chemical composition of this layer have been found as a function of the chemical composition of the glass and the conditions, static or dynamic, used to perform the in vitro tests.

The XPS study shows the presence of HPO_4^{2-} and PO_4^{3-} groups on the outermost layer of the calcium phosphate with a Ca/P molar ratio lower than that of apatite. Indeed, the deficiency in calcium increases if the in vitro test is carried out with a continuous renewal of the nutrient SBF solution (dynamic conditions). This finding is attributed to the lower pH of the solution as compared with that in the static test. A gradient of Ca and P has been found across the calcium phosphate layer. Apatite crystals constitute the region close to the glass substrate, whereas the calcium concentration decreases from this inner region to the layer surface. A continuous recrystallization process of the calcium phosphate with a low Ca/P ratio compared to that of apatite is proposed to take place in the bulk layer. Thus, the apatite front is progressing from the interior to the outermost part of the layer.

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