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Micro-ion beam analysis of physico-chemical reactions at the interface between sol-gel derived glass particles in the SiO₂-CaO system and biological fluids

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Abstract

In this study, we benefited from the sensitivity of ion beam analysis methods to characterize *in vitro* the bioactive glass/biological fluids interface and to perform local measurements of elemental concentrations at the 10⁻⁶g/g level. A glass in the SiO₂-CaO composition was elaborated by sol-gel processing. Samples of glass powders were soaked in biological fluids for periods up to 4 days. The surface changes were characterized using Particle Induced X-ray Emission (PIXE) associated to Rutherford Backscattering Spectroscopy (RBS), which are efficient methods for multielemental analysis. In addition, these ion beam methods permit accurate trace elements quantification. Elemental maps of major and trace elements were obtained at a micrometer scale and revealed the bone bonding ability of the material. The formation of a calcium phosphate-rich layer occurs after a few minutes of interaction and the glass particles are progressively coated with this thin film. Traces of magnesium are proved to be blended into the Ca-P layer. Kinetics of formation of the Ca-P layer shows the high reactivity of this material in contact with a biological medium. However the Ca-P-Mg layer is finally dissolved after a few days of interaction. The absence of P in the initial glass matrix may explain that SiO₂-CaO glass particles encounter great difficulties to achieve the transformation of their peripheral amorphous Ca-P layer into a more stable bone-like apatite phase.

Keywords: PIXE-RBS methods; biomaterials; bioactive glass; sol-gel, biomineralization.

Introduction

The bone-bonding ability of bioactive glasses has led them to be employed as implants in the human body to repair and replace damaged bony tissues. For this purpose, they can be clinically used as filling materials that also present the ability to contribute to the healing process^{1,2}. In contact with living tissues, bioactive glasses are able to establish an enduring interface consisting of a calcium phosphate-rich layer that can crystallize into a bone-like apatitic phase. The bioactivity mechanisms and growth of the layer have been extensively investigated over the past years^{3, 4, 5}. However there is still a lack of quantitative information regarding the physico-chemical reactions occurring at the interface and the ionic exchanges at the surface of the glass.

For this purpose, a SiO₂-CaO bioactive glass was elaborated using the sol-gel method, which permits the synthesis of materials with higher purity and homogeneity at low processing temperature^{6,7}. The reason for choosing a glass in the SiO₂-CaO binary system was first a higher homogeneity in the glass to be expected. Furthermore, the study of the Ca-P layer growth process was easier since the phosphate ions were only coming from the solution. Samples of gel-glass powder were immersed in biological fluids for varying periods. Analyses of major, minor and trace elements present at the biomaterial/biological fluids interface were performed by particle-induced X-ray emission (PIXE) associated to Rutherford back-scattering spectroscopy (RBS). Obtaining PIXE elemental maps at a micrometer scale permits the complete follow-up of the calcium phosphate layer formation along with accurate major and trace element quantification. It allows important evaluation for the *in vitro* bioactivity.

Experimental

Preparation of the bioactive glass samples

Gel-glass powders containing 75wt% SiO₂-25wt% CaO were prepared using the sol-gel process. Tetraethylorthosilicate (TEOS; Si(OC₂H₅)₄) and calcium nitrate Ca(NO₃)₂, 4H₂O were mixed in a solution of ethanol in presence of water and HCl. The prepared sol was then transferred to an oven at 60°C for gelification and aging. Four hours later, the obtained gel was dried at 125°C for 24 hours, then finally grinded to powder and heated at 700°C for 24 hours. The final surface area of the glass was found to be 30 m²/g by nitrogen sorption analysis.

In vitro assays

10 mg of gel-glass powder were soaked at 37°C for 15 min, 30 min, 1, 6 h and 1, 2, 3, 4 days in a standard Dulbecco's Modified Eagle Medium (DMEM, Biochrom AG, Germany), which composition is almost equal to human plasma. The surface area to DMEM volume ratio was fixed at 500 cm⁻¹. After interaction, the samples were removed from the fluids, air dried and embedded in resin (AGAR, Essex, England). Before characterization, the glass particles were cut into thin sections of 1 micrometer nominal thickness using a Leica EM UC6 Ultramicrotome, and laid out on 50 mesh copper grids. The grids were then placed on a Mylar film with a hole of 3 mm in the centre.

PIXE-RBS analysis

Analyses of the biomaterial/biological fluids interface were carried out using nuclear microprobes at the CENBG (Centre d'Études Nucléaires de Bordeaux-Gradignan, France). For PIXE-RBS analyses, we chose proton scanning micro-beam of 1.5 MeV energy and 50 pA in intensity. Such settings resulted in higher ionization cross-sections and an increased sensitivity for the micro-analysis of bioactive glasses composed of light elements ($Z \leq 20$). The beam diameter was nearly 1 micrometer. An 80 mm² Si(Li) detector was used for X-ray detection, orientated at 135° with respect to the incident beam axis and equipped with a beryllium window 12 µm thick. PIXE spectra were treated with the software package GUPIX⁸. Relating to RBS, a silicon particle detector placed 135° from the incident beam axis provided us with the number of protons that interacted with the sample. Data were treated with the SIMNRA code⁹.

Results

Multielemental maps of SiO₂-CaO glass particles after immersion in biological fluids

Elemental maps for each immersion time in DMEM were recorded. Figure 1 represents the elemental distribution of a powder grain after 15 minutes of interaction with biological fluids. The observed distributions correspond to the intensity of X-rays locally emitted by the sample under proton irradiation. Figure 1 shows the high reactivity of SiO₂-CaO glass particles in contact with biological fluids: within 15 minutes, they are able to incorporate phosphorus coming from their environment, leading to the formation of a calcium phosphate enriched layer at their periphery. Four grains are visible on Figure 1. In the centre of the picture, two grains of approximate size 20 microns are in an advanced state regarding the development of the calcium phosphate layer. Both particles are coated with a P-containing layer a

few micrometers thick. Ca is uniformly distributed. The silicate network is partially broken down on the particle located at the right-hand side of the picture. However, greater quantities of Si seem to be located in the regions where growth of the Ca-P rich layer has begun. Two other SiO₂-CaO grains are visible at the right-hand side of the picture, in the upper and lower corners. Here dissolution of Ca is in progress and only low concentrations of P are detected.

Physico-chemical reactions involved in the bioactivity process continue and lead to a very marked distribution of elements after 1 h of interaction between glass particles and biological fluids. The periphery of SiO₂-CaO glass particles is coated with a homogeneous calcium phosphate-rich layer. The core of the grain consists of the primary silicate network in which Ca has migrated from the inner of the particle to the periphery. Traces of Mg are proved to be blended into the glass matrix.

After 6 h of interaction the Ca-P-rich layer surrounds only partially the glass particle. Although this peripheral layer is enduring dissolution, Ca-P enriched regions that contain traces of Mg still remain. The inner of the glass particle is composed of the silicate network. Elemental maps after 1 and 2 days of immersion in biological fluids clearly show that dissolution continues at the periphery of the glass particles. After 1 day soaking, the calcium phosphate precipitates are present in a scattered way at the surface of the particles. After 2 days soaking, Figure 2 shows that the calcium phosphate layer has been almost completely dissolved. The Si-rich core of the particle is the only region remaining since it is more resistant to dissolution. The same observations are reported after 3 and 4 days soaking. SiO₂-CaO glass particles are from now on composed of the core of the grains, that is the silicate network, and only low concentrations of Ca and P are detected at the surface of the particles. No more traces of Mg are detected after 2 days soaking.

Evolution of the elemental concentrations at the periphery of the glass particles

Elemental maps were divided in various regions of interest depending on the distribution of chemical elements. Thanks to Supavisio analysis software¹⁰, we created thin masks of measurement at the periphery of the glass particles, in areas where the Ca-P enriched layer developed. With this methodology, calculation of elemental concentrations was made possible at the periphery of the grains. Results are presented in Figure 3. Each point corresponds to the average of concentrations calculated in several regions of interest. These regions of interest were defined over various samples in order to be ensured of measurements reproducibility.

During the first 15 minutes of interaction between SiO₂-CaO glass particles and biological fluids, Ca and P concentrations increase, as a result of the quick formation of a cal-

cium phosphate layer at the periphery of the material. Therefore Si concentration decreases in this region, as Si, Ca and P oxides concentrations represent nearly 100 % of the glass matrix. After 30 minutes of interaction, an important decrease in Ca concentration is observed at the periphery of the glass particles, due to the growing quantities of Ca that are leached in biological fluids because of the dealkalinisation of the glass surface. As a consequence, important changes in P and Si concentrations are observed at that time. Then, growth of the calcium phosphate layer continues at the periphery of the particles. It is supplied by incorporation of ions coming both from the glass matrix and biological fluids. As a result, it is observed a rapid increase in Ca and P concentrations and a decrease in Si concentration until 6 hours of interaction. After 6 hours soaking, Ca concentration represents 33.4 % of the peripheral layer. The layer contains 9.1 % of P and 12.9 % of Si are still present. Traces of Mg are detected in the order of 0.9 %. However multielemental maps showed that the newly formed calcium phosphate layer is unstable and that it is quickly dissolved by biological fluids; indeed, Ca and P concentrations drop to low values beyond 6 hours of interaction. In the meantime, Si concentration increases up to nearly 40 %. After 4 days of interaction with biological fluids, the periphery of SiO₂-CaO glass particles is composed of 42.3 % Si and 3.8 % Ca. Neither P nor Mg remains at the surface of the glass particles.

Evolution of the elemental concentrations in the core of the glass particles

Using the methodology described above, elemental concentrations in the core of SiO₂-CaO glass particles were calculated for the different times of interaction with DMEM. Figure 4 shows the results. During the first times of interaction, migration and diffusion of ions from the inner of the particles to their periphery lead to the observed fluctuations in the composition of the glass matrix. Then it is noted that the core of the particles grow poorer in Ca and P as the time of interaction with DMEM increases. As a consequence, increasing quantities of Si are detected in the core of the material. After 4 days of interaction, the composition of the core of the particles is close to that of the periphery: Si and Ca concentrations represent 40.2 and 6.1 wt % respectively.

Discussion

The bioactivity process consists of a well-identified group of physico-chemical reactions occurring at the surface of the material. Briefly, the alkaline and alkaline-earth ions present in the glass matrix are first exchanged with H⁺ of the solution, then polycondensation reactions of surface silanols create a high-surface area silica gel. This porous hydrated silica layer provides a large number of sites for the formation and growth of calcium phosphates

that will progressively crystallize into a biologically reactive hydroxycarbonate apatite equivalent to the mineral phase of bone^{11, 12}. Concerning SiO₂-CaO glass particles, dealcalination of the glass matrix and ionic exchanges start very quickly, so that a calcium phosphate-rich layer is formed within minutes at the periphery of the particles. Such a high reactivity is directly linked to the high content of Si and Ca oxides in the glass matrix. In contact with an aqueous medium, Ca is a very soluble alkaline-earth element. SiO₂ represents 75 wt% of the glass composition; the more the glass is composed of Si, the thicker is the hydrated, porous silica-gel layer formed after surface silanols polycondensation. This generates a large active surface area which speeds up both the dissolution process and the ionic exchanges.

Though the Ca-P rich layer is quickly formed, an essential observation is that it is finally dissolved after a few days of interaction. It indicates the formation of a peripheral calcium phosphate layer which composition significantly differs from that of hydroxyapatite. In fact, hydroxyapatite is the most stable and least soluble of all calcium phosphates. Thus our results suggest the formation of amorphous calcium phosphates at the periphery of SiO₂-CaO glass particles. Usually, amorphous calcium phosphates are the first phase precipitated from a supersaturated solution and they are a transient phase during the formation of thermodynamically more stable hydroxyapatite. The absence of P in the initial glass matrix may explain that SiO₂-CaO glass particles encounter great difficulties to achieve the transformation of their peripheral amorphous Ca-P layer into a more stable apatitic phase. It is all the more true that the *in vitro* assays were conducted under static conditions: biological fluids were not renewed and therefore contained only limited quantities of P and Ca.

Conclusion

Micro-PIXE associated to RBS are original methods to specify the role of major and trace elements in physico-chemical reactions occurring at the periphery of bioactive gel-glass particles. A major advantage of these nuclear microprobes is to enable accurate quantitative analyses of the glass particles/biological fluids interface^{13, 14}. For this purpose, we developed a specific preparation protocol that allowed the characterization of porous powders with grains of a few micrometers. The calcium phosphate-rich layer formation and evolution of the glass network are highlighted. Important information concerning the material physico-chemical properties are the incorporation of magnesium at the periphery of the particles as well as the final dissolution of the Ca-P-Mg layer.

It is important to note that the *in vitro* assays were conducted in an acellular biological medium under static conditions. The formation and evolution of the Ca-P-rich layer might be

quite different in a cellular environment, since the release of critical concentrations of soluble Si and Ca could give rise to both intracellular and extracellular responses at the interface of the glass with its cellular environment^{15, 16, 17}. One of our future prospects is therefore to control the dissolution of the material along with the release of critical concentrations of biologically active ions at the rate needed for bone cell proliferation and differentiation.

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Figures

Black-and-white figures (to be published)

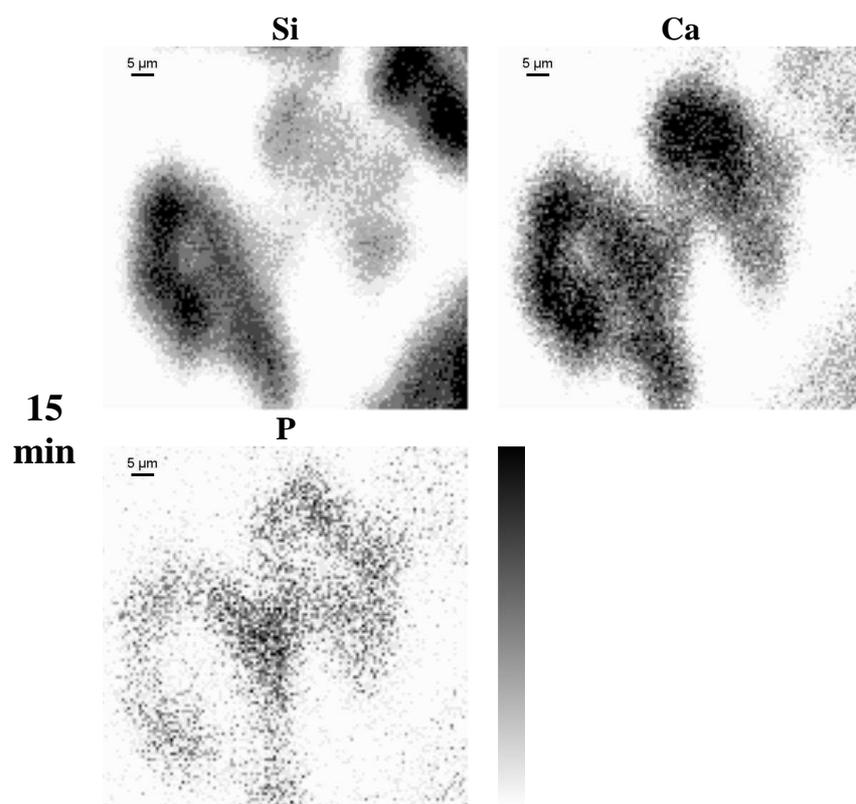


Figure 1: Elemental maps of SiO₂-CaO glass particles after 15 minutes of interaction with biological fluids (80 × 80 μm²).

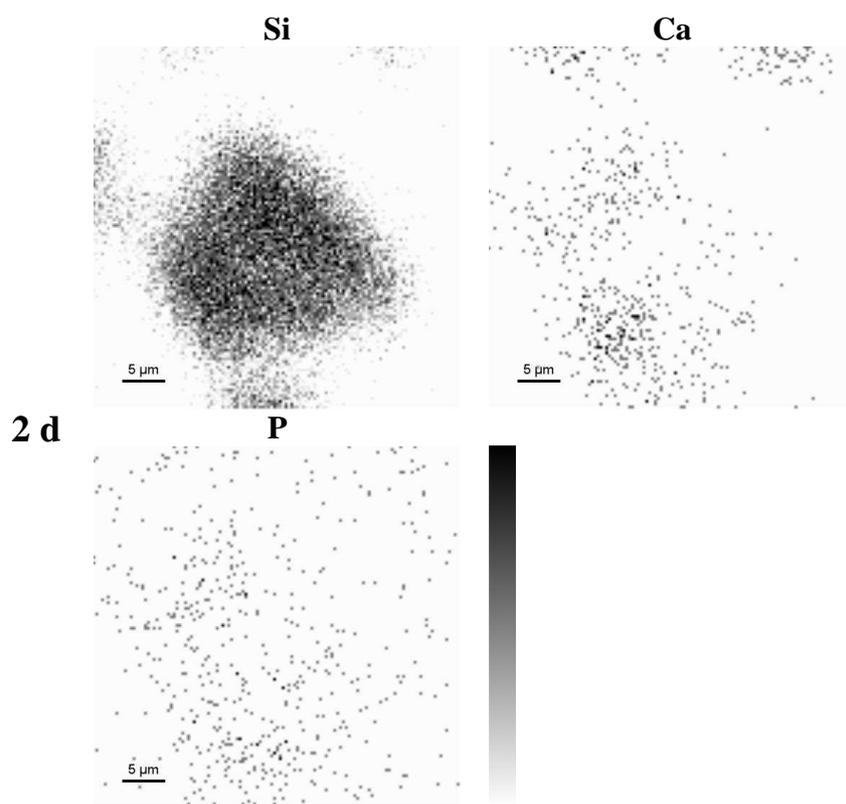


Figure 2: Elemental maps of a $\text{SiO}_2\text{-CaO}$ glass particle after 2 days of interaction with biological fluids ($43 \times 43 \mu\text{m}^2$).

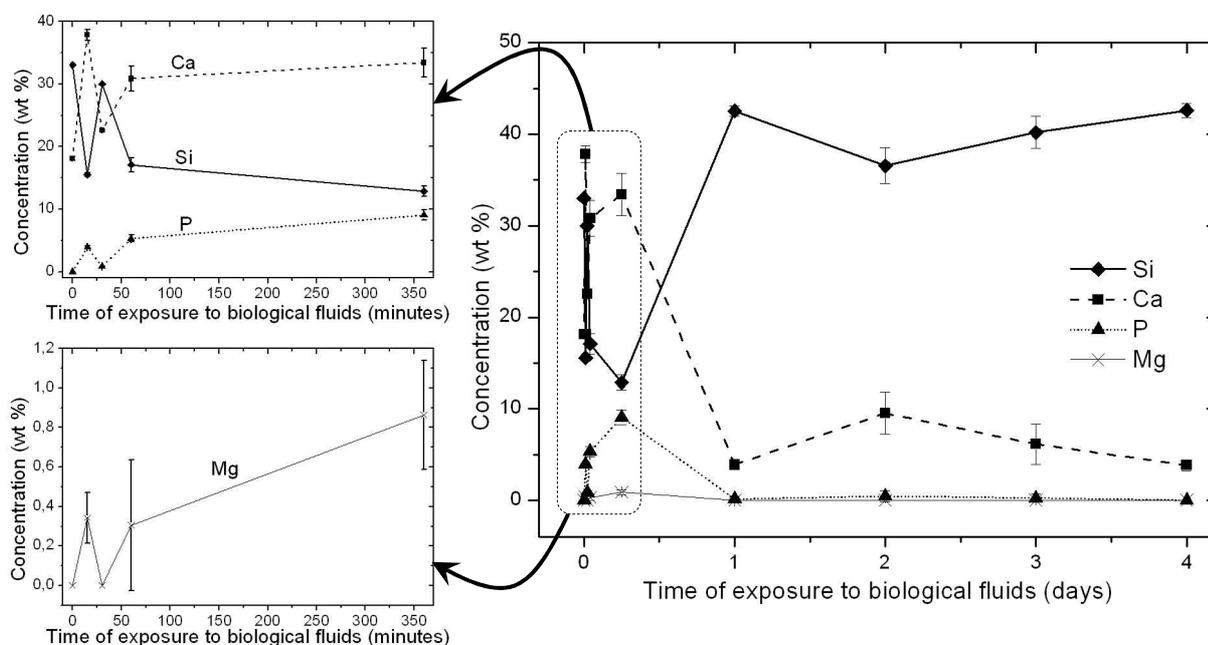


Figure 3: Evolution of elemental concentrations at the periphery of the $\text{SiO}_2\text{-CaO}$ glass particles with time of exposure to biological fluids. Inset: evolution of elemental concentrations at the periphery of the glass particles during the first 6 hours of interaction.

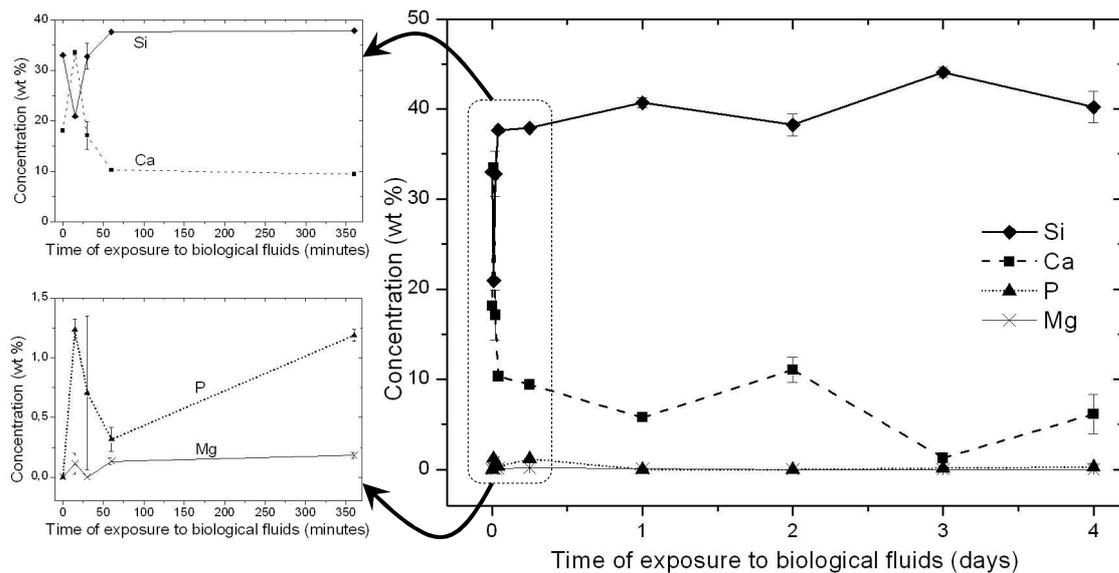


Figure 4: Evolution of elemental concentrations in the core of the $\text{SiO}_2\text{-CaO}$ glass particles with time of exposure to biological fluids. Inset: evolution of elemental concentrations in the core of the glass particles during the first 6 hours of interaction.

Color figures (for the web)

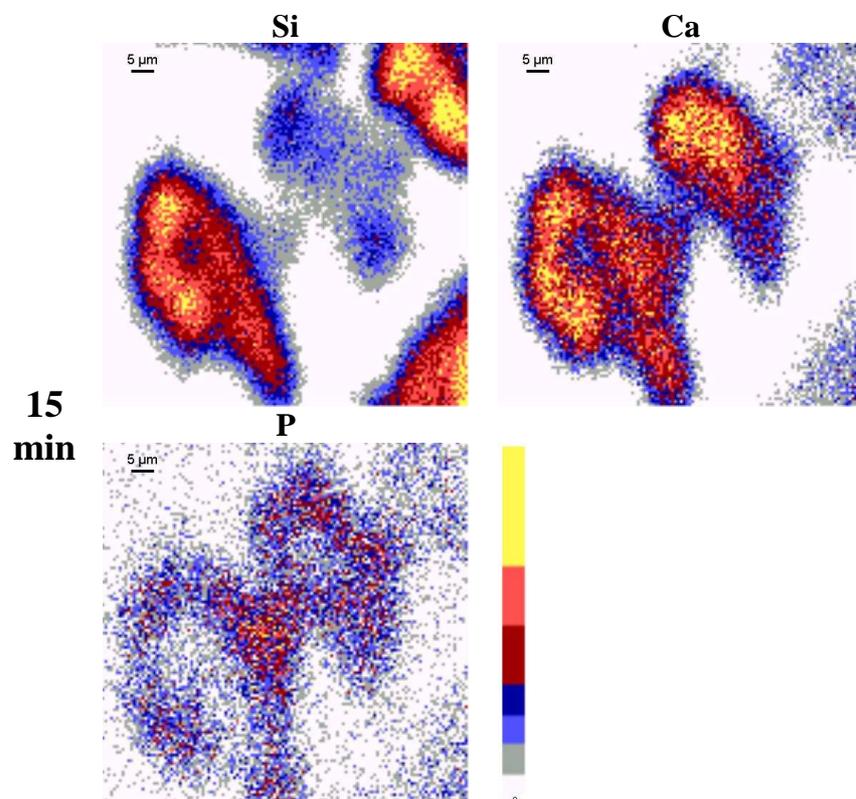


Figure 1: Elemental maps of $\text{SiO}_2\text{-CaO}$ glass particles after 15 minutes of interaction with biological fluids ($80 \times 80 \mu\text{m}^2$).

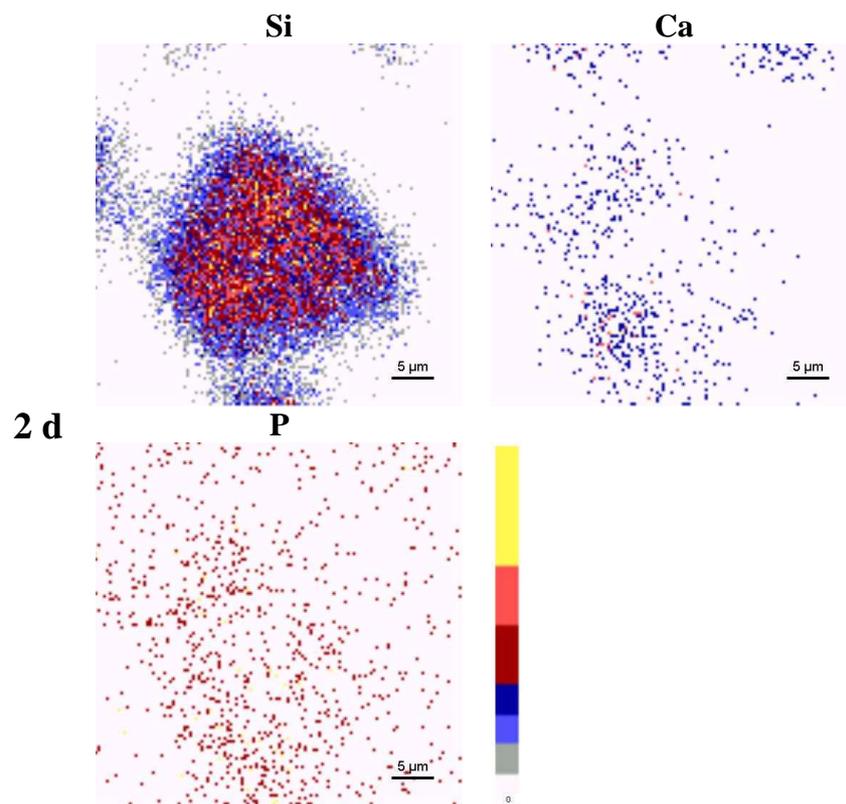


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