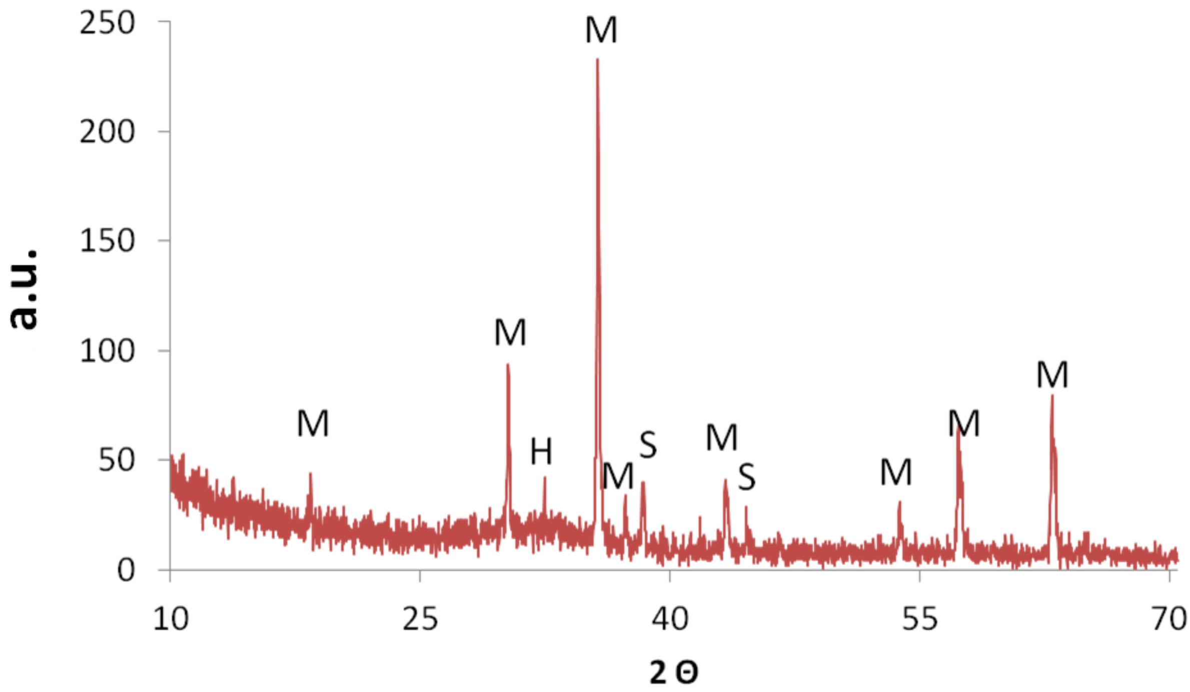

Figure 7.95: XRD analysis of SC45 Ag200-4W

The XRD pattern in figure 7.95 of SC45 Ag200-4W does not present any calcium phosphate crystalline phase. The peaks are the same of the sample before soaking in SBF. This confirms the extreme slowness of the kinetic reactivity of these particular glass ceramics.

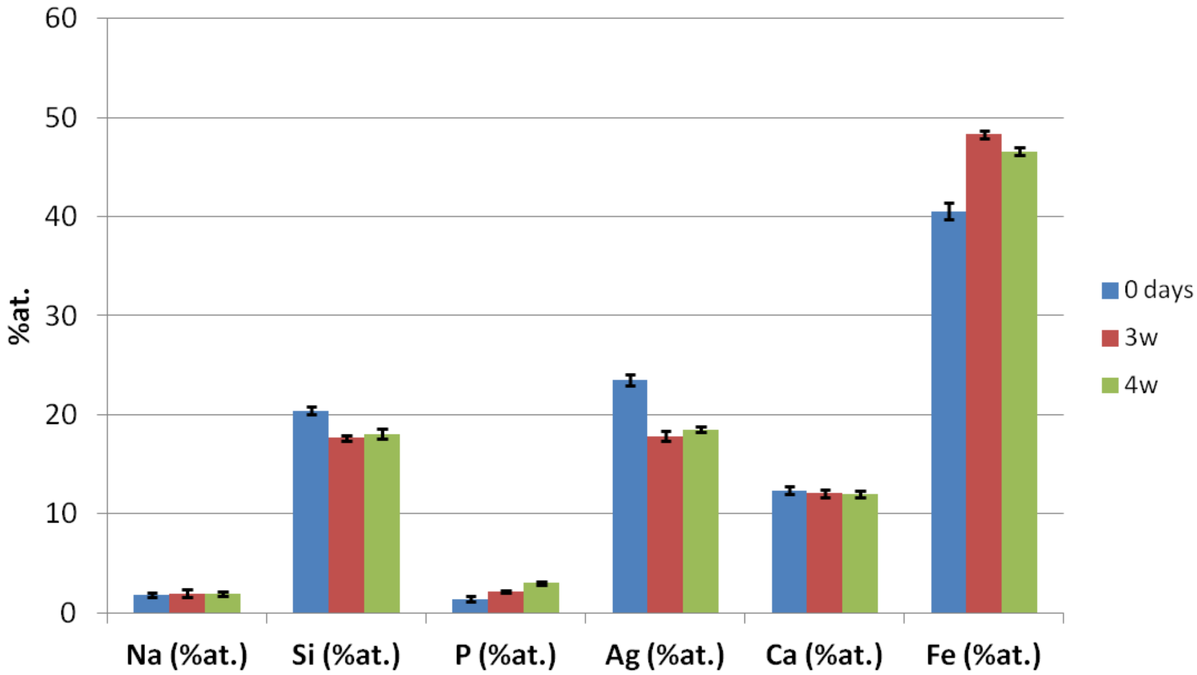

Figure 7.96: % atomic variation of the elements on the SC45 Ag 20 surface before and after in vitro bioactivity test.

The EDS analysis of the elements on SC45 Ag20 before and after SBF is reported in figure 7.96. Each value represents a mean of three values in different EDS analysis on the sample surface. It can be observed that sodium does not change significantly at each time control. Silicon decreases after three weeks and then become constant. Phosphorus increases for all the time points (with a trend similar to those of SC45, SC45 Ag200, SC45 Ag2000). Silver decreases after three weeks and then slightly increases. Calcium becomes constant and iron increases after three weeks and then decreases (similar trend of SC45) .

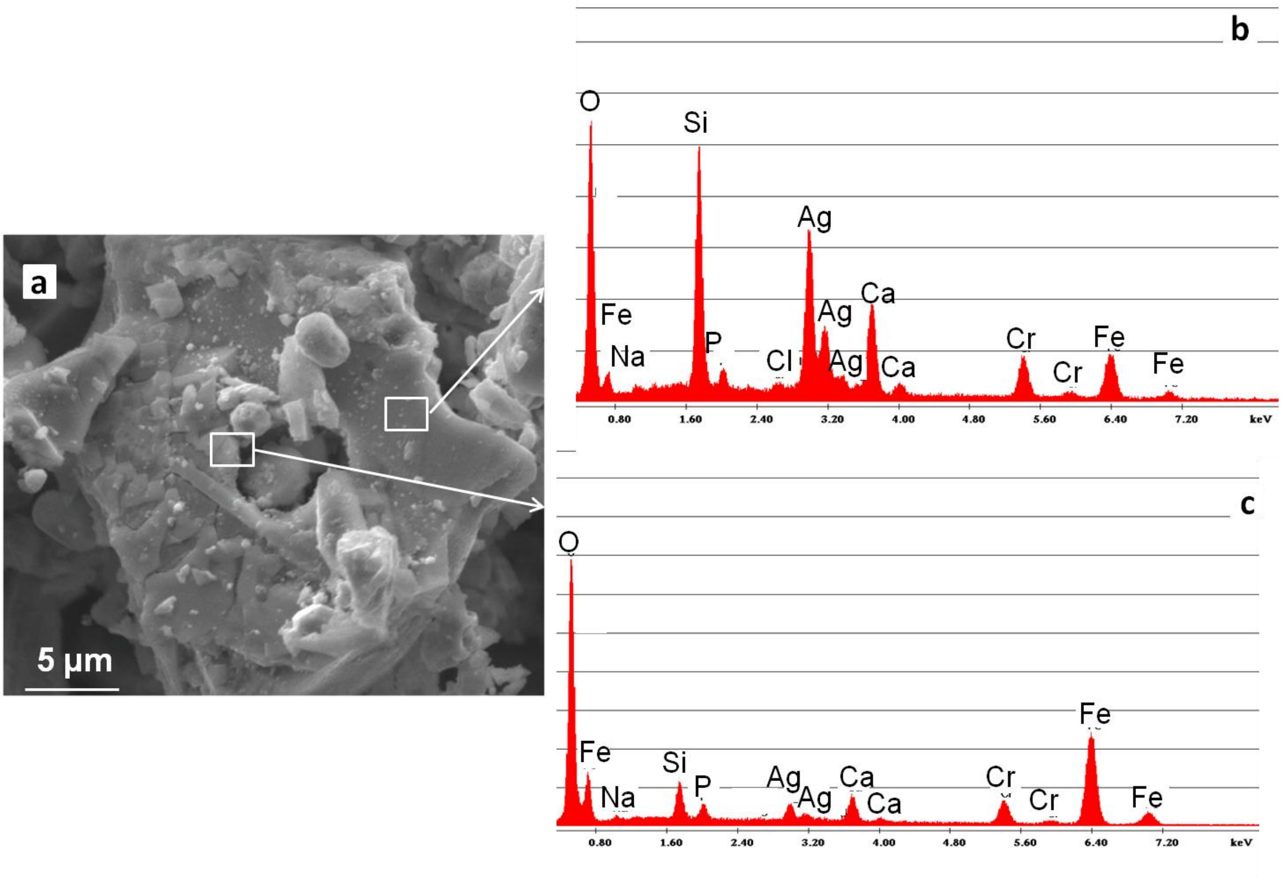

Figure 7.97: SEM morphology and EDS analysis on SC45 Ag 20 particles after 4 weeks in SBF

Figure 7.97 reports the morphology of a SC45 Ag20-4W particle (a) and two local EDS analysis in two different areas of sample (b, c). 7.97b presents the compositional analysis of a smooth surface where the value of silicon is higher than the control (SC45) in figure 7.83. A little amount of chloride confirms the possible formation of silver chloride precipitates.

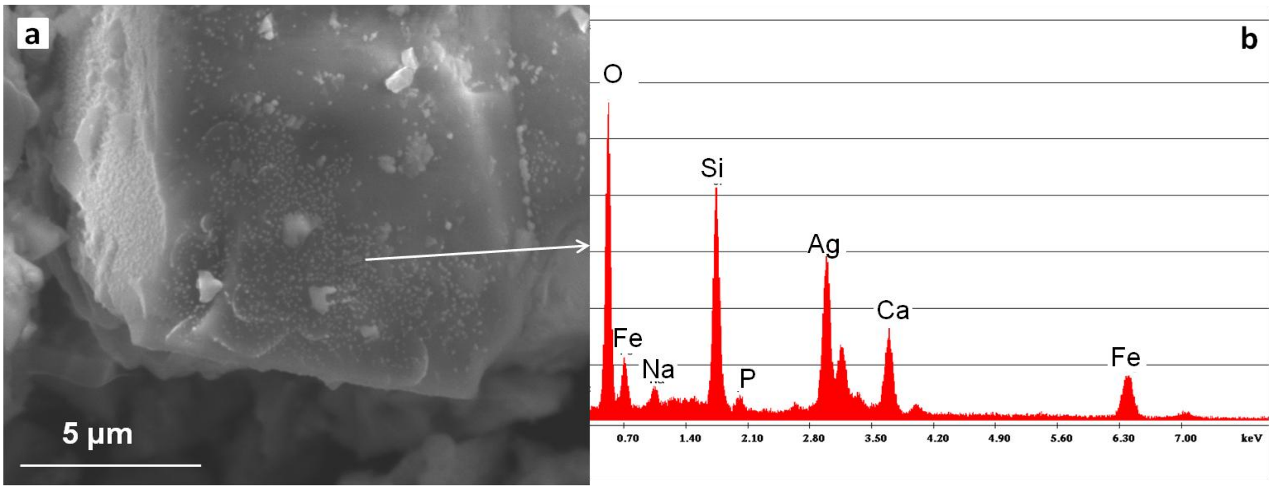

Figure 7.98: SEM morphology and EDS analysis on SC45 Ag 20 particles after 3 weeks in SBF

Figure 7.98 reports a morphological and compositional analysis of SC45 Ag20 after three weeks in SBF. Very small nanoparticles are visible. Probably a first surface modification connected with bioactivity phenomena occurred. The EDS analysis evidences high peaks of silicon and calcium but also of silver.

The silver introduction does not modify the bioactivity process of the glass ceramic.

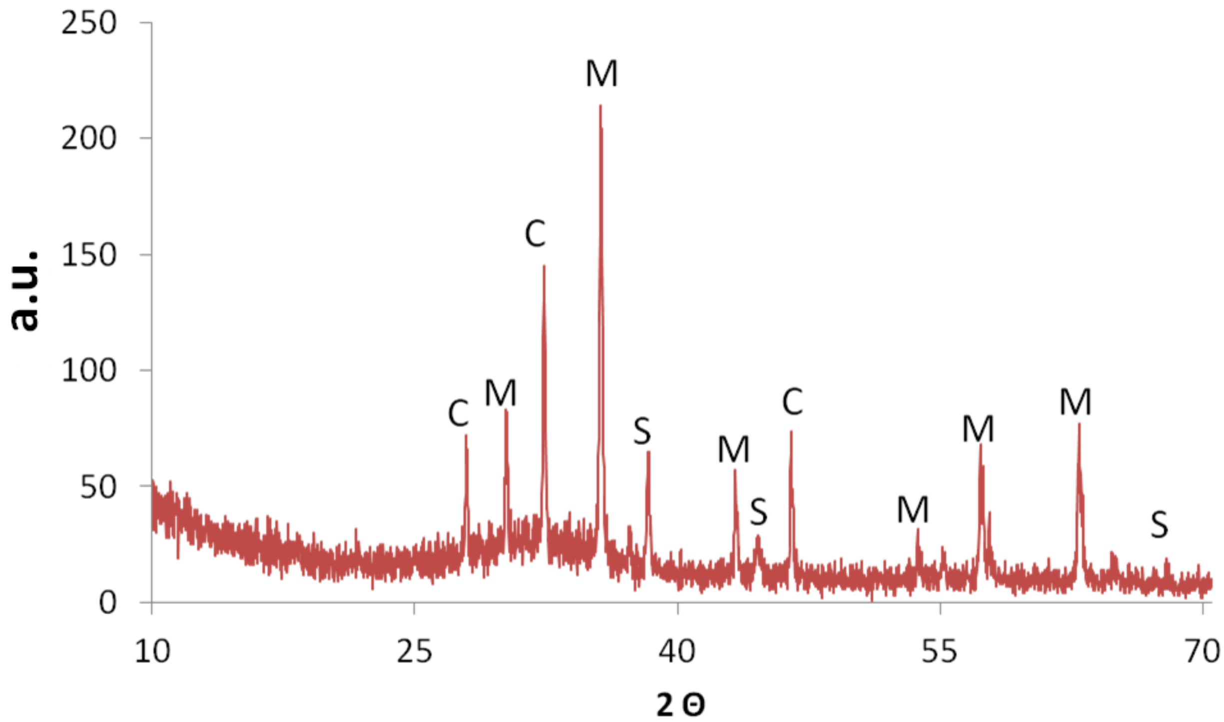

Figure 7.99: XRD analysis on SC45 Ag20-4W

After four weeks of soaking in SBF the peaks of magnetite (M), metallic silver (S) and silver chloride AgCl (C) can be detected, the latter seeming higher than in spectrum registered after three weeks.

7.2.10.9.3. Bioactivity of SC45 doped with copper

The bioactivity of the glass ceramic doped with copper was investigated after 3 and 4 weeks of immersion in SBF, as for silver-doped glass-ceramics, by means of a morphological analysis, in order to detect eventual calcium phosphate precipitates on glass surface, and EDS analysis, for the evaluation of the compositional modification on the sample surface with soaking time. In the following section the most significant results of this characterization will be reported.

Figure 7.100 reports the trend of the pH, obtained by interpolating the average values for each sample and taking into account the respective standard deviations.

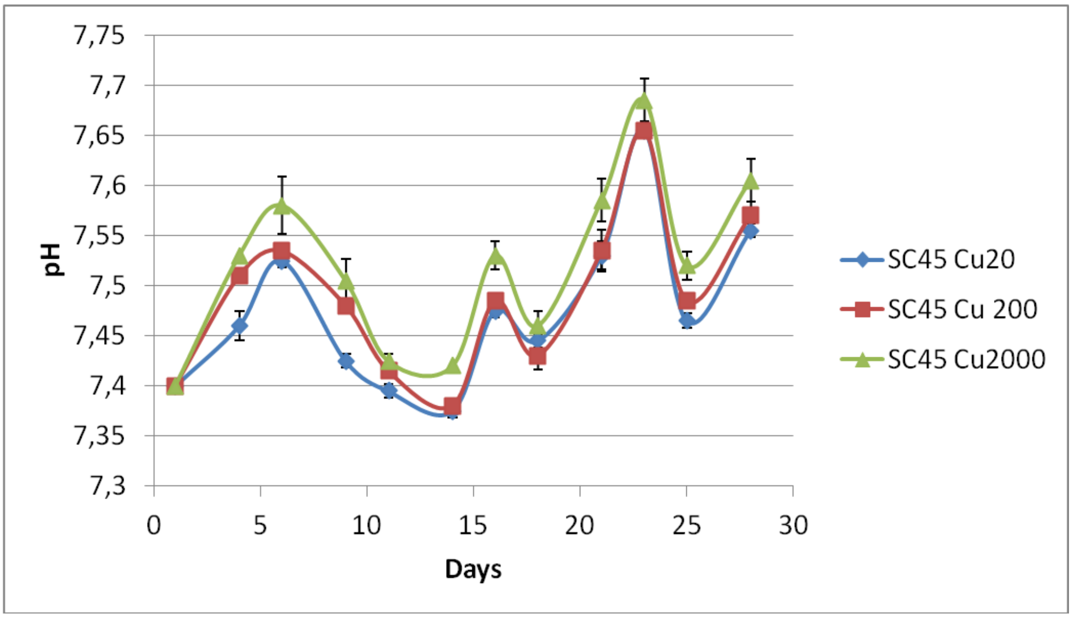

Figure 7.100: Trend of pH for SC45 doped with copper

The values are quite similar for the various glass-ceramic compositions and they oscillate in the physiological range between 7,4 and 7,8 at different time point.

Due to the fact that the trend of the pH for samples doped with copper are very similar to the trend of the pH sample doped with silver, it can assume that the fluctuation is connected to the bioactivity of the amorphous phase of the glass ceramic material and not to the presence of copper and silver ions.

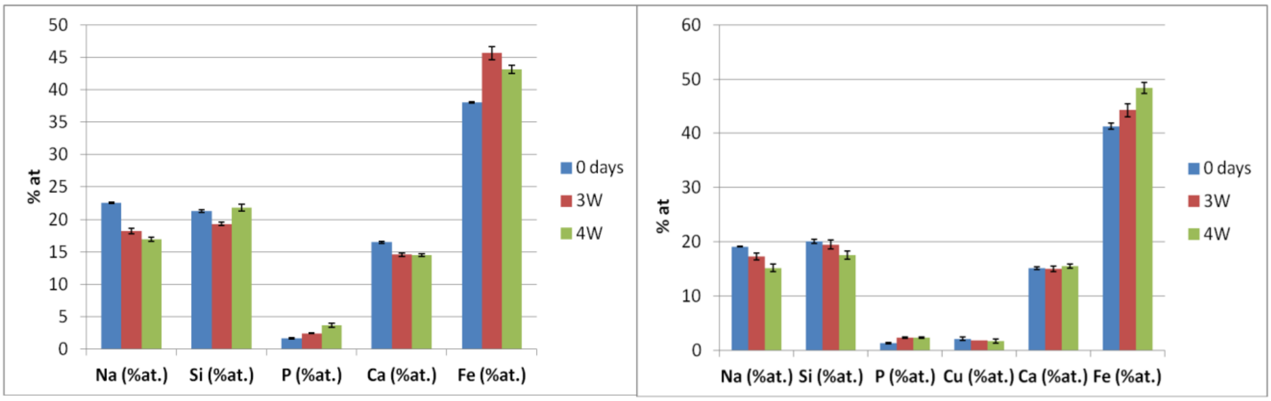

Figure 7.101: % atomic variation of the elements on the SC45 respect to SC45 Cu2000 before and after in vitro bioactivity test

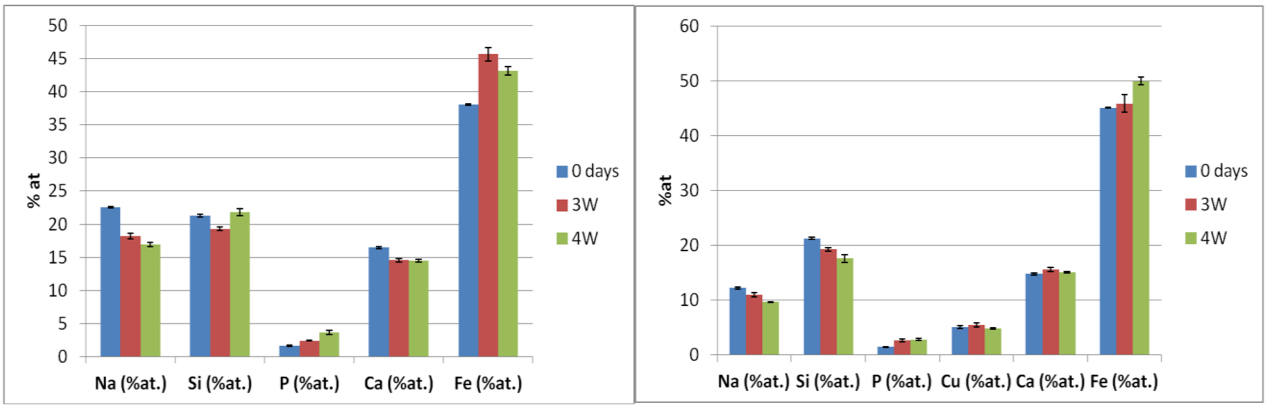

Figure 7.102: % atomic variation of the elements on the SC45 respect to SC45 Cu200 before and after in vitro bioactivity test

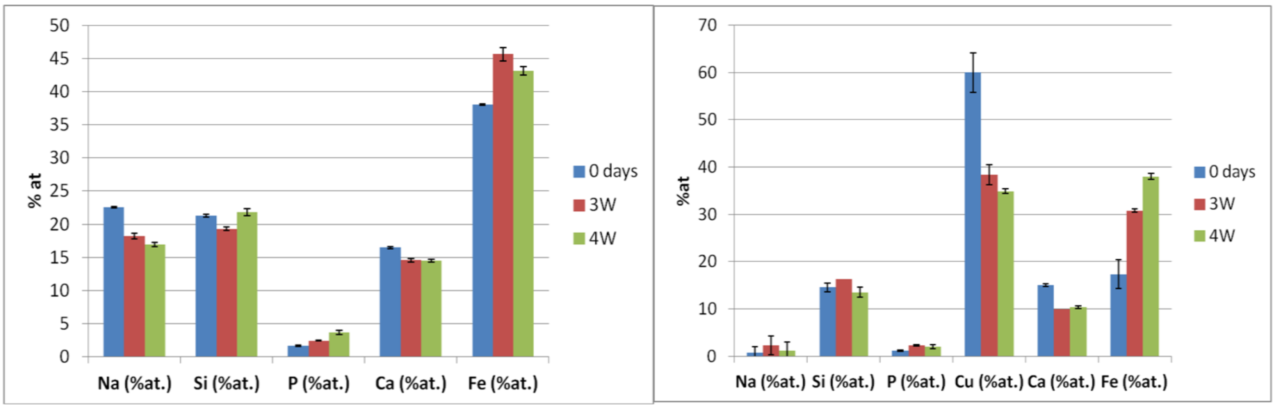

Figure 7.103: % atomic variation of the elements on the SC45 respect to SC45 Cu20 before and after in vitro bioactivity test

Figures 6.101-6.102-6.103 reports the EDS analysis of copper-doped glass ceramics compared to the control (SC45) at 0, 3 and 4 weeks in SBF. The reported values are a mean of three analysis performed on different surface points of each sample.

The trend is quite similar in all formulation: sodium (Na) decreases at 3 and 4 weeks, which confirms the activation of bioactivity mechanism. Silicon decreases for SC45Cu2000 and

SC45Cu200, while it remains almost stable for SC45Cu20. Phosphorus increases for all formulations, probably due to the presence of a phosphate amorphous layer that can be identify as precursors of hydroxyapatite. Calcium remains almost stable at each time point, revealing the slow reactivity of this glass ceramic. Iron increases gradually at 3 and 4 weeks for all glass ceramics. The trend is influenced by the gradual dissolution of the amorphous matrix which allows the exposition of magnetite on the sample surface. Copper does not undergo significant variations in any sample, except for SC45Cu20, where a sensible decrease is seen from 0 to three weeks.

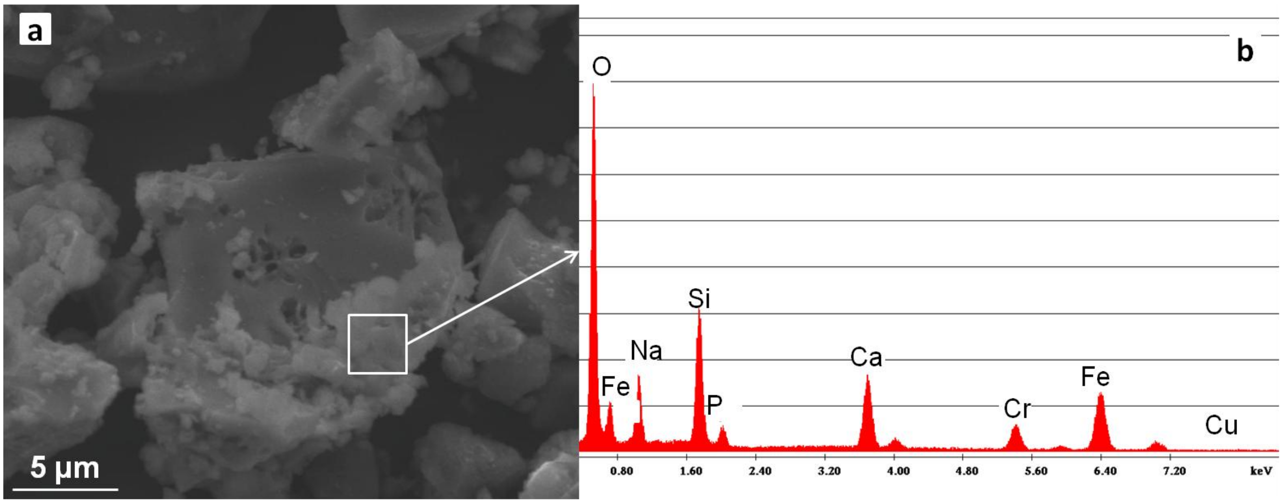

Figure 7.104: SEM morphology (a) and local EDS analysis (b) of SC45 Cu 2000 particles after 3 weeks in SBF

Figure 7.104a reports a morphology of a SC45Cu2000-3W particle, partially cover by some precipitates. The comparison of the peaks of silicon and calcium presented in the local EDS, respect to the same peaks of the EDS of area performed on the same sample before soaking in SBF, evidences an increase of these two elements

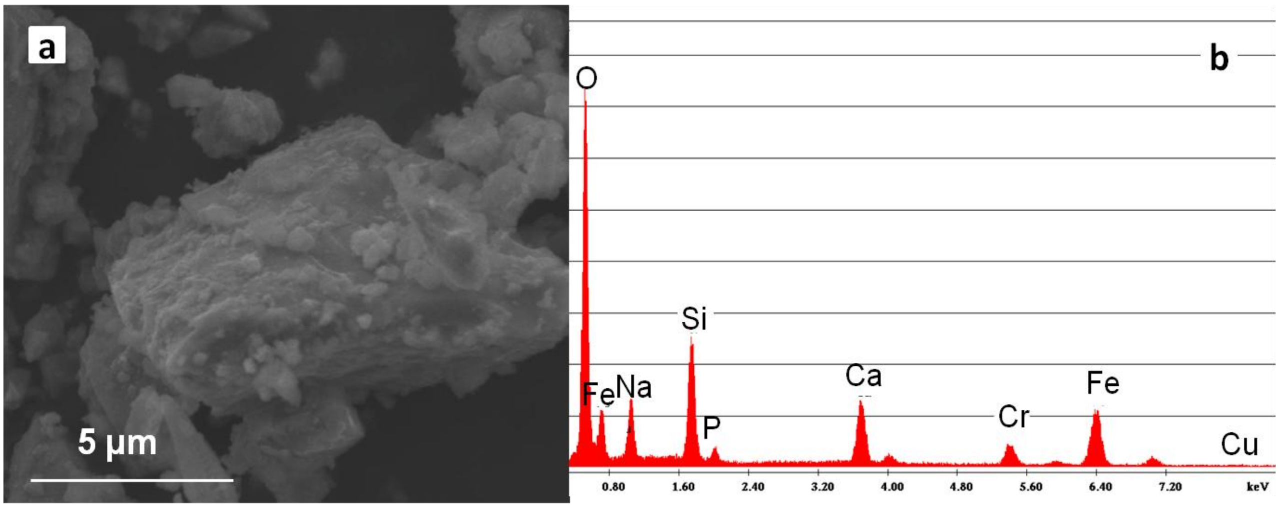

Figure 7.105: SEM morphology (a) and local EDS analysis (b) of SC45 Cu2000 particle after 3 weeks in SBF

Figure 7.106 reports another example of the reactivity of a particle of SC45 Cu2000-3W. On the surface some precipitates are visible and the Si/Ca ratio is 1,55 higher than 1,24 which is the mean ratio of the quantitative area analysis at three weeks. Probably the bioactivity process has produced the precipitation of calcium phosphate precursor but without crystallized into HAp.

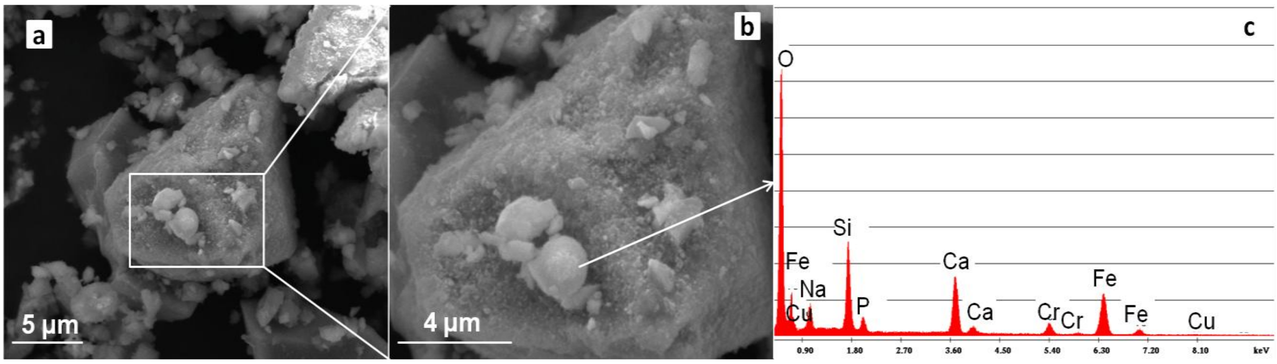

Figure 7.106 : SEM morphology (a-c) and local EDS analysis (b) of SC45 Cu200 particle after 3 weeks in SBF

Figure 7.106a reports the morphology of a SC45 Cu200-3W particle: the scanning electron microscopy shows a layer of reactivity attributable to a silica gel layer that coated glass particle. Magnification of the particle coated with a silica gel is reported in figure 6.113c. Even the EDS analysis presents a high peak of silicon which confirms the presence of coated silica-based layer.

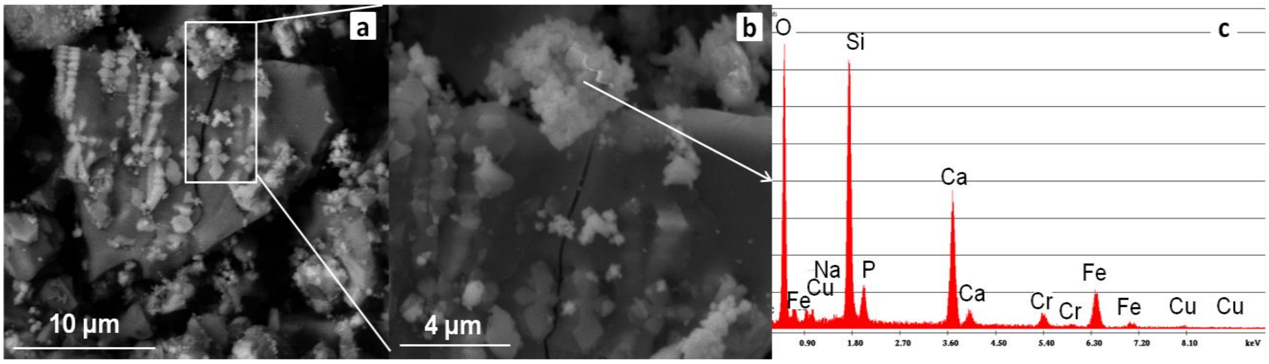

Figure 7.107: SEM morphology (a-b) and local EDS analysis (c) of SC45 Cu 20 particle after 3 weeks in SBF

The morphological analysis of figure 7.107 reports a glass particle of SC45 Cu20 after 3 weeks in SBF with a crack in the middle. The octaedric structure of the magnetite crystals embedded into the glass matrix is evident. The EDS shows a high peak of silicon. This could confirm the presence of silica gel on the particle as presented in the magnification in figure 7.107c.

XRD investigation has not detected any characteristic peaks of one or more calcium phosphate crystalline phase.

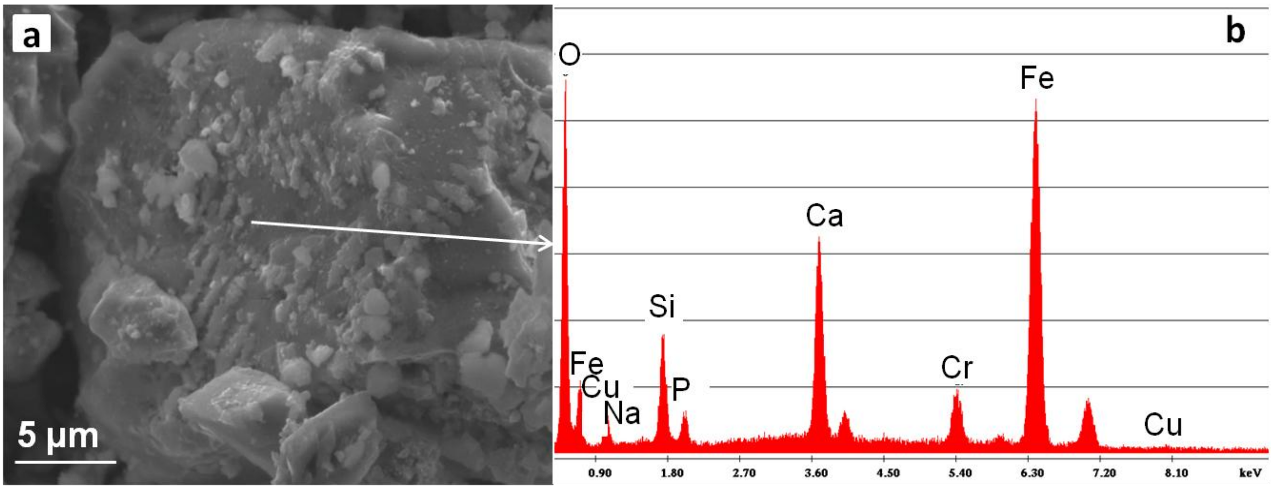

Figure 7.108: SEM morphology (a) and local EDS analysis (b-) of SC45 Cu 2000 particle after 4 weeks in SBF.

Figure 7.108a shows the SC45 Cu2000-4W sample where some crystals of magnetite with columnar shape embedded in the glass matrix are visible. The local EDS analysis presents a higher peak of calcium after soaking in SBF respect to the same sample before this treatment. On the SC45 Cu2000-4W sample some calcium precipitates are present as confirmed by SEM analysis (see arrows).

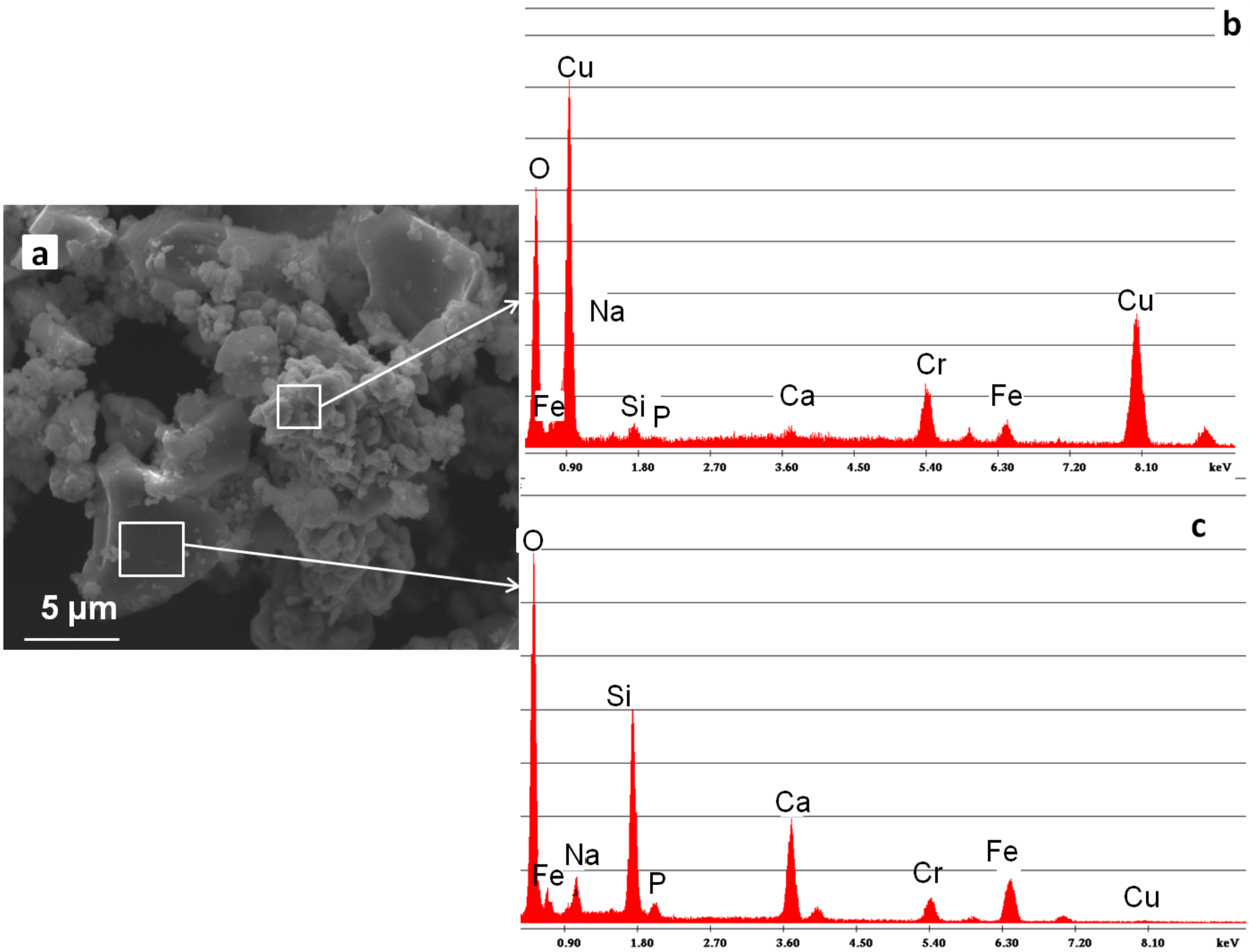

Figure 7.108 : SEM morphology (a), local EDS analysis (b-c) of SC45 Cu 20 particles after one month in SBF.

Figure 7.108a reports the morphology of SC45 Cu20-4W particles. In the EDS analysis in figure 7.108b a high peaks of copper and oxygen are observed. Then, probably, the precipitates observed in figure .108a are made of copper oxide. In figure 6.108c the local EDS of a smooth particle shows an increase of silicon and calcium peaks respect to the EDS of a control sample. Even in this case we can assume that a calcium-rich silica gel precipitated on the glass-ceramic particle without producing crystalline hydroxyapatite.

The x-ray investigation on SC45 Cu2000, SC45 Cu200 and SC45Cu20 after one month in SBF did not evidence any calcium phosphate phase.

In conclusion, the bioactivity investigation performed on SC45 doped with copper and silver by ion exchange molten salt technique did not modify the reactivity of the glass ceramic which remains in any case quite low.

7.3. Reference

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Chapter 8

Conclusion and future developments

The final object of the research work is the development of a multifunctional composite bone cement, with ferrimagnetic, bioactive, antibacterial and structural properties for an innovative approach to hyperthermic treatment of bone tumors.

The main results of this research can be divided in two parts. The first set of results is related to the development of a bioactive and ferrimagnetic composite bone cement for the care of bone tumors, exploiting magnetic hyperthermic treatment, obtained by the dispersion, in a polymethly methacrylate (PMMA) matrix, of different amounts of bioactive and ferromagnetic glass-ceramic particles. The second one concerns the approach to the modification of such composite bone cement by doping the bioactive glass-ceramic with silver and copper, in order to give to the final product a further added value, such as the antibacterial properties. The aim of this further investigation is connected with the bone tumor pathology, which presents an high rate of infections development.

The PMMA matrix used in this research is a commercial product, while the bioactive and ferromagnetic glass ceramic, as well as the approach to the enrichment with antibacterial specie, were developed at the DISAT laboratories of Politecnico di Torino. This glass ceramic, SC45, belongs to the system Na_2O–CaO–SiO_2–P_2O_5–FeO–Fe_2O_3, and previous studies demonstrated its bioactivity and biocompatibility. It contains magnetite as main crystalline phase, which nucleates and crystallizes into the glass matrix during the cooling step of its synthesis, from the row materials melting temperature to room temperature.

Three different weight percentages of SC45 (10, 15 and 20%) were added into the polymeric matrix and all tests were performed on these three formulations (named P10, P15 and P20 respectively), in order to select the best formulation, which could guarantee bioactivity, good mechanical properties, good handling properties and the ability of heating the surrounding environment during the application of an external magnetic field.

The results collected during three years of activity allowed the following conclusions:

X-ray diffraction and magnetic characterizations confirmed that the SC45 glass-ceramic contains 28% wt of magnetite, rather than the stoichiometric amount (45%) expected by the row materials composition. Iron was distributed between the crystalline phase (magnetite) and the residual amorphous phase as solid solution .

The nucleation and crystallization of magnetite into the glass ceramic was evidenced by SEM and FESEM microcopies.

The morphological and compositional analysis of all the composites presented a good homogeneity and dispersion of SC45 particles without agglomeration phenomena, verified by SEM and FESEM observations as well as by micro computed tomography (Micro CT).

Compression and four point bending tests were performed in agreement with ISO 5833-2002 and the results respected the standard requirements for all the tested samples. Only P20 presented a bending strength very close to the lower limit imposed by the normative. This could be due to a too high amount of glass ceramic in the polymer, which weakened the material. This result assessed that P20 is a threshold sample. The addition of higher amounts of glass ceramic particles to the PMMA matrix would strongly decrease the mechanical properties to values not in agreement with the standard specifications.

The setting time test, performed following the ISO 5833-2002, revealed the same behavior of the different composites respect to the commercial bone cement. Thus, the addition of SC45 did not modify the hardening process of the polymer and even the maximum temperature of polymerization remained constant at about 50 °C for all tested samples.

Magnetic hysteresis cycles confirmed a ferrimagnetic behavior of the composite as a function of magnetite amount. Calorimetric measurements, implemented in different ways and with different magnetic devices, recorded a temperature increase, which can be modulated in function of exposure time, intensity of the magnetic field and the applied frequency in the range of kHz. This characterization let assume that the composite bone cements are extremely flexible to different magnetic stimulation therapy protocols.

Impedance measurements assessed the absence of ferrimagnetic resonance in the range of kHz up to 10 MHz, and so it could be assured that the heat generation and absorption of electromagnetic energy became in static conditions comparing hysteresis and calorimetric evaluations.

The power losses quantification evidenced an energy generation proportional to the amount of magnetic material into the polymer. This term varies from 1,20 W/g for a P10 to 5,27 W/g for a P20, using a maximum alternate magnetic field of 39 mT.

In order to investigate the bioactive behavior, the composite bone cements were soaked in simulated body fluid (SBF) up to one month, refreshing the solution every three days, and their surface was analysed with SEM-EDS in order to observe the growth of hydroxyapatite (the principal inorganic component of bone). Hydroxyapatite or its precursors were detected on all sample, demonstrating their potential osteoinductive behavior.

Leaching test, carried out on P20 samples, did not show any significant iron release respect to the limit of instrument detection and respect to concentrations, which are known to cause cytotoxic effect in the human body.

The biological performance of the composite bone cements, expressed by cells morphologies and viability test, was investigated with human osteosarcoma cells, Mg63, both by direct and indirect tests. All the tested samples (P10-P15-P20) enriched with the bioactive glass-ceramic resulted as highly cytocompatible with human osteoblasts. In particular, in the indirect assay cells viability resulted in a range of 95 to 97% for all the chosen time-points. In the direct contact test cell viability did not decrease. Moreover, P10 obtained the highest viability value at three time points respect to the control and other sample formulations. This results will be object of further investigations, but the particular surface roughness and the different amount of exposed glass-ceramic particles could be related to this behaviour.

Adhesion, density and morphology of cells were evaluated, evidencing their ability to develop bridge-like structures, as well as three-dimensional multilayers. Such behaviour was assumed as an example of elevate biocompatibility and positive interaction material-cells. A combined effect between the osteoinductive glass ceramic phase and cell mineralization process was noticed in the formation of HAp crystals on the cement surface, at very early stages, on all samples. Biological characterizations highlighted P10 as the best sample in term of bioactivity, biocompatibility and cell viability.

In vitro magnetic induction cell heating was evaluated on P10 sample before and after seeding different cell lines: Mg63, simulating the tumoral cells, and Human foetal osteoblasts (Hfob) simulating healthy one. After 30 minutes of heating the tumoral cells viability decreased of 97% . While the healthy cells maintained 70% of viability without any significant variation under the effect of alternating magnetic field. The annexin-5 tests showed a 70% of Mg63 cells in apoptosis after 30 minutes of heating. It has been evaluated that they had an higher heat sensibility respect to the other and so an easy capacity to death under the effect of and external heat source.

The results of the characterization performed on SC45 doped with silver or copper by melting and quenching technique, produced the following conclusions:

- Morphological and compositional analyses evidenced the presence of silver both in ionic form inside the amorphous network of the glass ceramic and as micrometric metallic precipitates.
- X-ray diffraction presented as main crystalline phase magnetite with traces of hematite and metallic silver .
- Quantification of magnetite samples treated with silver did not show any sensible variation respect to the control in the same form.
- The magnetic characterizations at 34 kA/m did not present significant differences respect to the reference material. The addition of silver did not modify the magnetic properties of samples.
- The calorimetric results showed a behaviour in agreement with the undoped samples, thus the introduction of silver did not alter the material heating ability.
- Antibacterial test, performed in accordance with NCCLS normative using a standard *Staphylococcus aureus* strain, evidenced the formation of a weak inhibition halo (about 1 mm). Even if the silver amount is remarkable, probably its presence in micrometric particles allow only a poor ionic release.

Copper doped SC45: samples were produced in the same way of SC45 doped with silver.

- Morphological and compositional analyses evidenced the presence of copper in ionic form into the amorphous phase of the glass ceramic, as well as nano-metric sized precipitates, which potentially could express the antibacterial effect. No metallic copper was seen in these samples.
- X-ray diffraction investigation evidenced, in addition to magnetite and hematite, two different crystalline phases, sodium iron silicon oxide ($NaFeSiO_2$ in non-stoichiometric ratio) and, copper iron oxide ($CuFeO_2$). These phase were unexpected and heavily influenced the magnetic and calorimetric properties.
- The copper doped glass-ceramic powders presented the lowest value of magnetic phase, hysteresis energy losses and heating ability. The introduction of ionic copper influenced the magnetic properties of magnetite by the magnetic spin interaction between copper and magnetite. The presence of non-saturated bonds of copper with a consequently high molecular reactivity favoured the correlation with iron ion generating an antiferromagnetic material.

- In the copper doped glass-ceramic bulk the magnetite amount, hysteresis losses and heating ability were not completely recovered. The annealing thermal treatment provided enough energy to stabilize the effect of copper on the magnetic response of the glass-ceramic. The formation of a new crystalline phase, $CuFeO_2$, extinguished the antiferromagnetic effect because all the copper bonds were saturated.
- The antibacterial test did not evidenced any positive result in all the synthetized samples. No relevant differences were noticed between bulk and powder samples. The increasing of specific area and consequently the reactivity from bulk to powder did not modify the antimicrobial behaviour.

The last part of research produced some relevant results concerning SC45 doped with silver and copper by ion exchange in a molten salts solution of $NaNO_3$ and $AgNO_3$. Three different molar concentration was used for doped SC45 with silver (SC45 Ag2000 , SC45 Ag200, SC45 Ag20).

- The morphological and compositional analysis on all samples evidenced the presence of silver sub-micrometric metallic precipitates in different amounts.
- X-ray diffraction investigation revealed the constant presence of metallic silver in each pattern. Any other crystalline phase was detected.
- The calorimetric measurements presented a linear increasing trend with time for all samples. A light decreasing respect to the control was observed for SC45 Ag2000.
- Positive results came from the antibacterial characterization for all silver doped glass ceramics. The samples presented a very wide inhibition zone where no bacteria grown and proliferated, with a very good performance for the SC45 Ag20 samples, which produced a uniform circular halo of 7 mm diameter. Respect to the melting and quenching techniques the SC45 doped with silver by ion exchange in molten salts produced a very wide antibacterial effect due to the preponderance of ionic form and nano-metric size of the particles.
- Bioactivity characterization was performed on the samples at three and four weeks of soaking in SBF. The kinetics reactions of these glass ceramics were quite slow and even after four weeks of treatment no hydroxyapatite was observed on a surface of the glass ceramics. However the presence of a thin layer of silica gel was seen in different samples. Compositional analysis recorded local increase of calcium and phosphorus respect to the

control sample, but without any structural modification of the surface. Even the XRD did not present any calcium phosphate crystalline phase.

The same characterizations were performed on copper doped glass ceramic obtained by ion exchange in molten salt solution of $NaNO_3$ and $Cu(NO_3)_2$. Three different sets of samples were synthesized : SC4 Cu2000, Sc45 Cu200, SC45 Cu20.

- Morphological and compositional investigations showed the presence of copper in different chemical form : ionic in the glass ceramic matrix and as micrometric precipitate of copper oxide, as evidenced by XRD.
- Calorimetric test on Cu samples did not show any significative difference respect to the control
- Antibacterial tests performed on these samples did not show any antimicrobial effects. No inhibition halo was developed around the samples after 24 hours at 37°C of incubation. Cu20 expressed a wide blue zone which confirm, as for meting and quenching techniques, a diffusivity of copper on a agar plate but without any toxic effect on bacteria.
- Regarding bioactivity tests, copper doped samples presented the same results of those doped with silver, in fact after three and four weeks of soaking in SBF, without solution refresh, any hydroxyapatite precipitate was seen on the glass ceramics particles. However, a thin layer of silica gel was seen even on these samples.

In conclusion, an optimization and a complete characterization of innovative magnetic and bioactive composite bone cements was performed. The best formulation was identified in P10 sample. It provided a good trade off between mechanical, chemical, biocompatible and heating cells abilities. This should be the best choice for a future *in vivo* animal model investigation.

The antibacterial characterization on a SC45 still presents some questions that are need to be investigate. From an antibacterial point of view the ion exchange in molten salts seem to be the best technology because all the silver doped material showed a large inhibition halo. The positive result has been influenced by the presence of a certain amount of atomic silver that was penetrate in the glass ceramic matrix and to the nanometric spherical particles that increased the silver specific reactivity. A biocompatibility evaluation of the antibacterial formulation is needed . The next step of the research will be the introduction of the best antibacterial glass ceramic into the polymer matrix in order to develop a completely tailored magnetic, bioactive and antibacterial bone cement. A complete *in vitro* and *in vivo* test will be necessary for future pre-clinical investigations.