Study of Phosphocalcic Glasses from SiO_2-CaO-P_2O_5 System with and Without Silver

II. The bioactivity analysis by FTIR, SEM methods and microbiological study of silver-doped glasses

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The study in vitro of the glass powders bioactivity was performed by soaking them in simulated body fluid for 3 to 21 days at a temperature of 37°C and pH = 7.20. The synthesis de novo of hydroxyapatite, post soaking was confirmed by Fourier Transform Infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). The study of the antimicrobial activity was performed by microbiological examination on two strains of pathogenic bacteria involved in postoperative nosocomial infections.

Keywords: FTIR, SEM, doping silver, antibacterial activity

Because at the global level the average age and life expectancy of the population grew, it became urgent and imperative needed the discovery of new biomaterials types which once implanted in the human body to have a longer duration of use [1, 2].

In recent decades interest in bioactive oxidic materials has increased steadily because research has shown that getting their by modern methods, including sol-gel, shows concrete performances in terms of bioactivity, compared with conventional methods.

The main problem researchers faced was to identify solutions that would lead to the formation of a proper biochemical link at the interface between biomaterial and host tissue [1-3].

The phosphocalcic glasses with bioactive capacity are designed to induce specific biological activity, which involves tissue-implant interaction [4]. This consists in forming a layer of biologically active carbonated hydroxyapatite, with a structure and composition, equivalent with mineral phrase of the bone [5].

Experimental part

Characterization of the samples

The chemical groups that appear in the structure of post soaking glasses in SBF were studied (identified and assigned) by Fourier Transform Infrared spectroscopy (FTIR) using a Bruker Vertex 80 spectrometer, equipped with ATR crystal (attenuated total reflectance). The data were collected at room temperature in the range of wavelengths 4000-400 cm^{-1} and spectral resolution of 2 cm^{-1}.

The morphological study of the glasses before and after soaked was performed by using Field Emission Scanning Electron Microscope - Hitachi SU-70 with resolution of 1.0 nm at 15 kV. XRD, FTIR and SEM determinations were performed at ICSMT - Multidisciplinary Research Institute for Science and Technologies, Valahia University of Targoviste.

The antimicrobial properties of quaternary glass with silver were determined by a microbiological study on two strains of pathogenic bacteria/conditioning pathogenic (Staphylococcus aureus and Escherichia coli). It has used the cultural method of incorporating of the germs on specific nutritional media and incubation of them at temperatures and optimum durations for each microorganism culture. It has been worked in aseptic environment, in microbiological niche with sterile instruments and utensils, plates and incubation was conducted in Nitech adjustable electric thermostat ($\pm 0.1^0$C).

Antimicrobial activity of sol-gel glasses doped with silver

For the microbiological study were purchased two lyophilized pure culture of bacteria from SC Mediclim SA Bucharest.

It was opted for Staphylococcus aureus ATCC 25923-reg Gram positive bacteria and for Escherichia coli ATCC 25922 reg - Gram negative pathogenic conditioning bacteria, both strains being in the top five places in the top of microorganisms involved in post-surgical intra-hospital infections, with bacterial species of the genus Pseudomonas, Proteus and Clostridium [6-8].

The antibacterial activity was determined at various concentrations of the bioglass obtained by decimal dilutions in the same volume of nutritive medium (15 mL) and with an inoculum of bacteria reconstituted by 3×10^8 cells CFU/mL (CFU - colony forming units).

The plaques with nutrient mediums, bioglass and bacterial inoculum were incubated aerobically at specific temperatures and durations for each strain of bacteria.

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Staphylococcus aureus was used specific culture medium - Baird-Parker (ox fibrinogen and rabbit plasma agar), and incubation was conducted for 48 hours at 37^{0}C.

For Escherichia coli was used TBX selective medium (tryptone-ball-glucuronide) and the plates were incubated for 24 hours at 44^{0}C. The bacteria development led to the formation of blue-green colonies of Escherichia coli and colonies of black-gray, shiny and convex for Staphylococcus aureus. In parallel tests were performed for ternary glass, doped with silver.

Results and discussions

The study of bioactivity, i.e. the formation of hydroxyapatite and carbonated hydroxyapatite on the surface of the two bottles was performed also by using IR spectroscopy by Fourier transform (FTIR analysis).

In this context, the formation and development was monitored in a 21-day interval, of the specific molecular groups of glasses of the system SiO_2-CaO-P_2O_5, but also hydroxyapatite.

In this respect, it was considered the formation and development of molecular groups such as Si-O-Si-O-Si or Si-OH structure found in the silicon phosphocalcique glasses structure obtained and groups type carbonate (CO_3^{2-}), phosphate (PO_4^{3-},P-O-, P-O-P) and hydroxyl (HO^-) present in the structure of the stoichiometric hydroxyapatite but also carbonated. The S1 and S2 FTIR spectra of the bioglass are interpreted compared with FTIR spectra of pure hydroxyapatite.

In figure 1 it is presented the FTIR spectra for the sample S1 before and after soaking of the glass powder in the simulated human liquid at 37^{0}C and *p*H = 7.20, for 3, 7, 14 and 21 days (fig. 1 a-e), by comparison with spectrum hydroxyapatite (chart f).

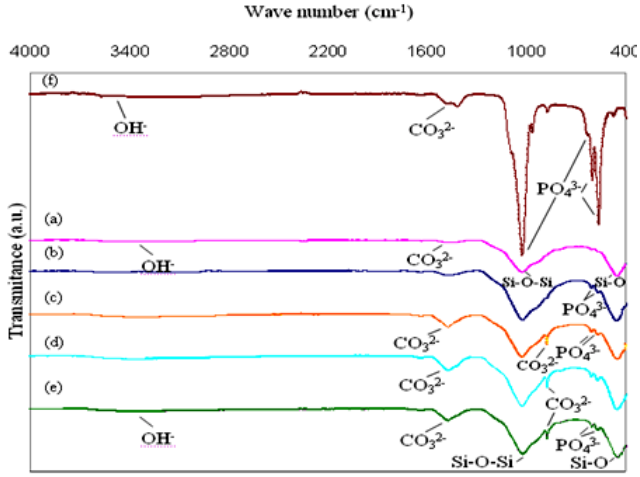


Fig. 1 FTIR spectra of sol-gel glass S1
a) unsoaked; after soaked in simulated body fluid for:
b) 3 days, c) 7 days, d) 14 days, e) 21days and f) hydroxyapatite

The FTIR spectrum in figure 1, for the glass by composition S1 unsoaked highlights the existence of groups type Si-O-Si and Si-O, specific of the silicate network present in the structure. In this respect, the peak located at 454 cm^{-1} is characteristic to bending vibration mode of Si-O-, also peak at 1022 cm^{-1} corresponding to asymmetric stretching vibration of Si-O-Si bridges.

The literature [3, 9, 10] shows that pure hydroxyapatite presenting in the range of 3200-3700 cm^{-1} a specific bearing of vibration band of water molecules associated with this and intramolecular OH groups, bound in the SiO_2-CaO-P_2O_5 glass structure, as shown in graphs a-f in figure 1.

In graphics b–e the peaks located at 564 cm^{-1}, respectively, 605 cm^{-1} end 1025cm^{-1} highlighted the presence of phosphate (PO_4^{3-}) in glass structure.

The first two peaks belong vibration band 567-601 cm^{-1} specific of the P-O vibration, due to unsymmetrical bending of the PO_4^{3-} tetrahedra, and the third is specific of the P-OH vibration from PO_4^{3-} structure. They are present in all postimersion spectra from 3 to 21 days.

At the same time, it emphasized the presence carbonate groups (CO_3^{2-}) in the glass structure, by the peaks located at 879 cm^{-1} and 1465 cm^{-1}.

The peak at 1465 cm^{-1} begins to be apparent from day 3 to the soaked (figure 1b), and that at 879 cm^{-1} is visible from the 7th day (chart c). This confirms that the carbonated hydroxyapatite begins as the XRD analysis revealed. From the graphs d and e is observed that the intensity of training HAp-C increases after the 14th day of soaking, as evidenced by increased amplitude peaks of CO_3^{2-}.

In figure 2 shows the FTIR spectrum S2 doped with silver sample before and after soaking in simulated body fluid liquid glass powder (in the same conditions as glass S1) along with the spectrum of hydroxyapatite (chart f) .

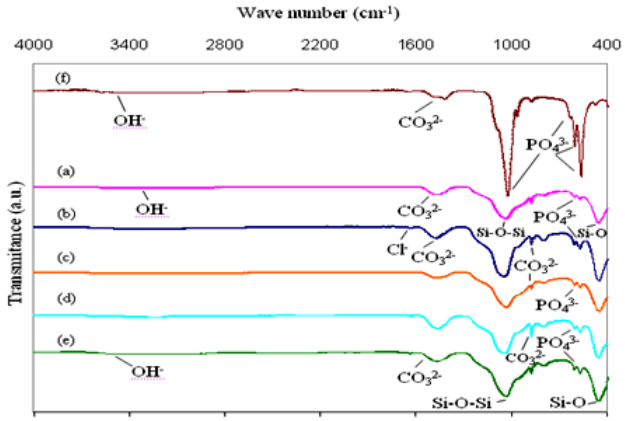


Fig. 2. FTIR spectra of sol-gel glass S2
a)unsoaked; after soaked in simulated body fluid for:
b)3 days, c) 7 days, d) 14 days, e) 21days and f) hydroxyapatite

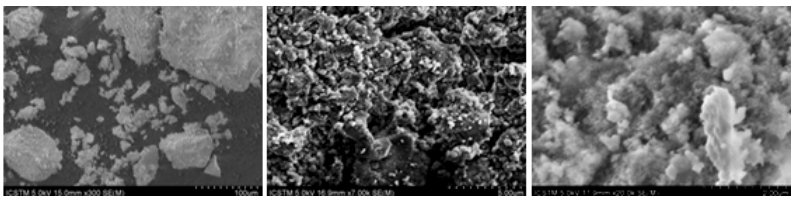
In the chart a of FTIR spectra of glass doped with silver highlights the presence of silanol groups, three-dimensional network- silicate phospho-calcium silicate glasses doped with other metals or not. These are highlighted by peak at 454 cm^{-1} specific groups Si-O- and the 798 cm^{-1} and 1035 cm^{-1} peak typical to the vibrational bands by asymmetrical stretching of the Si-O- bridges or Si-O-Si type.

After 3 days of soaked (chart b) and 7 days (chart c) begin to be evident the formation of the carbonated hydroxyapatite in the presence of CO_3^{2-} peaks at 878 cm^{-1} and 1473 cm^{-1} and the peaks located formations phosphate 570 cm^{-1} (P-O-), 604 cm^{-1} (P-O-P), 861 cm^{-1} (specific to the P-OH vibration valence P-O- groups, $CaCO_3$ and carbonated hydroxyapatite) and 963 cm^{-1} (PO_4^{3-} vibration valence HO- in the structure P-OH).

It is also evident the appearance of the HO- groups from the water structure linked intramolecular to hydroxyapatite after the 7th day of soaking (see chart c) to the peak 1637 cm^{-1} and 3577 cm^{-1}, 3360 cm^{-1} peak - after 14 days (chart d) and 3375 cm^{-1} peak - after 21 days soaking (chart e); these peaks belong to 3200-3700 cm^{-1} vibration band specific to the molecules of water associated with hydroxyapatite.

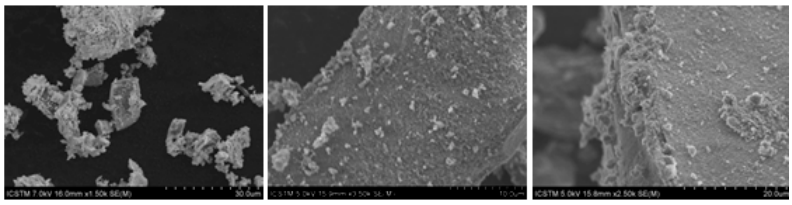
The peaks characteristic to PO_4^{3-} and CO_3^{2-} groups increase in intensity after 7 days of soaking.

Though not very apparent, from day 3 and until day 21 of soaking it can be observed changes in the spectra, in the



(a) unsoaked b) soaked 7 days (c) soaked 14 days

Fig. 3. SEM micrographs for S1 sol-gel glass powders



(a) unsoaked (b) soaked 14 days (c) soaked 21 days

Fig. 4. SEM micrographs for S2 sol-gel glass powders

form of small peaks in the range 1710–1739 cm^{-1}, associated with the vibration of the specific chlorides, here mentioning AgCl, which is part of the vibration band in the range 1600 – 1800 cm^{-1} specific to chlorapatite and chlorine compounds associated glasses doped with after soaking silver phosphocalcic in SBF.

In figure 3 show SEM micrographs of fine glass composition S1. In the first sample (**a**), the unsoaked in simulated human liquid it is highlighted the glass particles of different sizes and shapes. They can be observed particle with sizes ranging between 10 and 100 nm. After soaking in simulated human fluid for 7 days as evidenced by the SEM micrograph shown in figure 3**b**, at magnification of 7.000X, the apatite particles can be observed on the surface of glass powder. In this case, the size of the apatite particles is below 0.5 mm, the shape of these particles is mostly spherical one. SEM micrograph of figure 6**c** shows the formation of clumps of apatite on the surface of glass powder; their size varies between 0.5 mm and 2 mm. There is an increase in the coverage of the glass surface with particles of apatite, something which confirms the formation of hydroxyapatite in such conditions and as evidenced by X-ray diffraction analysis (parts I).

Glass doped with silver sample is studied in morphologically by SEM analysis, figure 4. If the case of the unsoaked sample, figure 4a can be observed particles whose size varies between 3 and 30 nm. At the same time, in terms of morphology it is highlighted particle with an irregular, angular and having a high roughness, specific to materials with large specific surface area. In the case of the sample soaked in simulated human fluid for 14 days, figure 4b, the apatite particles are highlighted on the surface of the glass; rough surface appearance, has facilitated the development of these phosphocalcic formations. Following insertion in simulated human fluid for 21 days (fig. 4c), at the surface glass powder has been observed a decrease of apatite particles as a result of the dissolution, owing to a static soaking method. At the same time, the high specific surface of these glasses, a result of the synthesis process, favoured the formation of clusters of particles, which are ideal for the formation of the interface tissue-implant.

Study of antimicrobial activity in case of sol-gel glasses doped with silver

Embedding ions Ag^+ in the structure of silico- phosphocalcic glass S2 led to confer antimicrobial properties, in accordance with what is known from the literature after 1995 [11-16] when it came to the unanimous conclusion that only ion forms of transition metal (Ag, Cu, Zn) included in the structure of bioactive glasses have antibacterial effect. The presence of silver in the 1^+ oxidation state results in electrostatic attraction between it and the negatively charged cell membrane and subsequent destruction of microorganisms through various biochemical mechanisms [17-19].

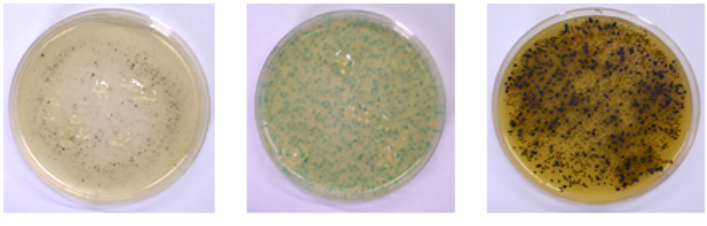
To determine the minimum dose bactericidal of glass S2 doped with silver successive tests were made with various concentrations of bioglass in the same amount of culture medium (15 mL) and the same inoculum of bacteria (3 x 10^8 CFU/mL) the two strains mentioned above.

The first test was carried out with 0.1 g of fine glass mortar S2 directly included in the Petri dish, and over it was distributed the culture medium and inoculum specific to each strain of bacteria. After the temperature was determined it has been found that the boards (dual each bacterial species) have not developed any colony of microorganisms (fig. 5a), or Staphylococcus aureus, or Escherichia coli.

Tests were repeated for bioglass concentrations increasingly smaller, 0.05-0.03 g bioglass, when they obtained the same results - the lack of typical colonies of bacteria, for both genders. This demonstrates that the bioglass has bactericidal concentrations.

For the same concentrations (0.1g, 0.05-0.03 g) were carried out in the same tests for ternary glass S1 – non-doped with silver. In this case, Petri dishes with strains of Staphylococcus aureus and Escherichia coli incubated under identical conditions, as time and temperature, as in the case of S2 glass, colonies were invaded by two specific strains of microorganisms. The colonies could not be counted, as shown in figure 5b and 5c. This leads to the conclusion that glass non-doped with Ag (S1) hasn't bactericidal or bacteriostatic activity.

Therefore until the concentrations mentioned above S2 bioglass has bactericidal properties, resorted to making



(a) (b) (c)

Fig. 5. Glass powders with silver 0.1g/plates (a), control plates without silver for Escherichia coli (b) and Staphylococcus aureus (c)

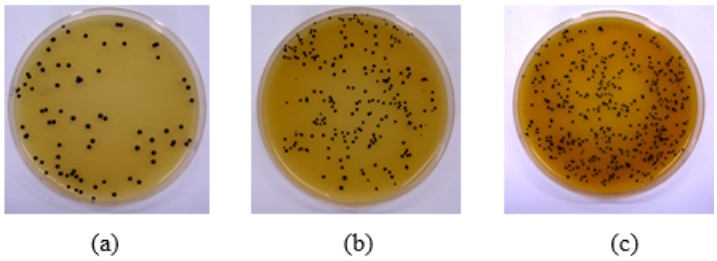


Fig. 6. Glass powders with silver soaked on peptone saline (physiological serum) at different dilutions for Staphylococcus aureus: (a) $0.05 \cdot 10^{-4}$ g/ mL, (b) $0.05 \cdot 10^{-5}$ g/mL,

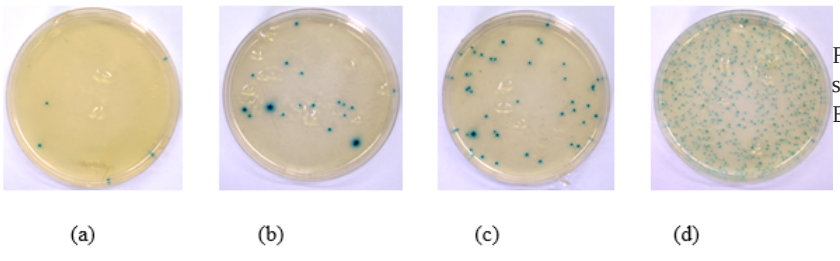


Fig. 7. Glass powders with silver soaked on peptone saline (physiological serum) at different dilutions for Escherichia coli: (a) $0.03 \cdot 10^{-4}$ g/mL, (b) $0.03 \cdot 10^{-5}$ g/mL, (c) $0.03 \cdot 10^{-6}$ g/mL, (d) $0.03 \cdot 10^{-7}$ g/mL

decimal dilutions of glass in peptone saline so: for Staphylococcus aureus was done first decimal dilution (10^{-1}) starting from 0.05 g powder of S2 glass suspended in 9.9 mL peptone saline. This was stirred for 6 h to diffusion of ions (including Ag^+) from the glass structure in the serum, which will be used later for the following decimal dilutions (1 mL in 9 mL of serum dilution 10^{-1} dilution 10^{-2}, etc.) for Escherichia coli, with a emphasized sensitivity for Ag^+ ion has gone from 0.03 g glass powder S2, following the same protocol work From each decimal dilution of S2 bioglass are taken 0.1 mL and is incorporated in Petri dishes on nutrient media–specific to each strains of bacteria, keeping constant the amount of inoculum (0.1 mL) with a concentration of $3\ 10^8$ CFU/mL for both types of bacteria.

The first visible sign of loss of bactericidal activity was observed at a concentration of 0.05×10^{-4} g/mL for Staphylococcus aureus (fig. 6a) and 0.03×10^{-4} g/mL for Escherichia coli (fig. 7a), when the number of colonies begin to grow. The plates in figure 6b and 7b is seen starting to develop a greater number of specific colonies from each strain of bacteria.

Figure 6c and 7c and 7d, the increase in the number of bacterial colonies is observed, with increased tenfold dilution of bioglasses: 0.05×10^{-6} g/mL for Staphylococcus aureus and $0.03 \cdot 10^{-6}$ g/mL for Escherichia coli. Therefore, Gram- negative bacteria (Escherichia coli) is more sensitive to the cytotoxic effect of silver than the positive Gram (Staphylococcus aureus).

In our previous papaer the synthesis of glasses and characterization by WD-XRF and XRD were studied [20].

Conclusions

FTIR analysis of two glasses confirms the presence of PO_4^{3-}, CO_3^{2-} and HO^- groups and specific presence of hydroxyapatite and carbonated hydroxyapatite in the composition of both the SBF after soaked glasses, and also the presence of Si- O- groups, respectively Si-O -Si characteristic to silica phosphocalcique glasses. In the case of S2 composition the presence of AgCl, Ag is observed and also the chlorapatite that clogs the pores of the hydrated silica network and reduce the formation of the carbonated hydroxyapatite stoichiometric and that carbonated specific of the bone tissue.

Surface morphology of both glasses, evaluated by SEM analysis demonstrates the presence of coarse surfaces, some concave pores, enabling the formation of hydroxyapatite and carbonated hydroxyapatite post-implantation in vivo. Glass surfaces S2 presents clogged with AgCl and Ag, XRD and FTIR, however study shows that these compounds do not cancel bioactivity glass, although it is lower compared to that of composition S1.

S1 ternary glass does not have an antibacterial effect, while quaternary glass doped with silver has bactericidal effect for both strains at different concentrations of the bottle: 0.05 g/10 mL (0.005 g/mL) for Staphylococcus aureus and 0.03 g/10 mL (0.003 g/mL) for Escherichia coli.

S2 glass doped in silver has bacteriostatic properties in extremely low concentrations determined after only 6 hours of the bioglass in peptone saline, as follows:

- from 0.005 to $0.005^{\times}10^{-5}$ g/mL for Staphylococcus aureus. At dilutions higher than $0.5^{\times}10^{-7}$ g/mL of bioglass S2 bacteriostatic activity is reduced until it reaches zero.
- from 0.003 to $0.003^{\times}10^{-5}$ g/mL for Escherichia coli. Dilutions above $0.3^{\times}10^{-7}$ g/mL decrease and ultimately lead to the cancellation of antimicrobial activity.

For the conditions of grafting actual in vivo, to benefit from the synergistic action, bioactive and antimicrobial, the two compositions are recommended to use a mixture of the two glasses in favour of S1 or synthesizing a glass containing silver more than 7 wt% (even 1-3% wt). This is justified since the release of silver in phospho- calcium silicate matrix is slow and bactericidal action of the ions Ag^+ is viable even after completion of their release from the implanted material.

On the other hand , reducing the silver content from the composition of the glass reduces the amount of AgCl formed at the contact implant human liquids, compound that reduces the bioactivity of the glass by clogging its pores, thus reducing contact surface material-tissue defining aspect for the formation of the biologically - active interface of the hydroxyapatite and carbonated hydroxyapatite.

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