STUDY OF BIOACTIVITY AND ANTIMICROBIAL ACTIVITY IN CASE OF GLASSES FROM SiO_2-CaO -P_2O_5 TERNARY SYSTEM

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Abstract. *This paper presents the study of bioactivity for two compositions of SiO2-CaO-P2O5 glasses by X-ray diffraction analysis. In order to determine the bioactive properties, glass samples were tested by soaking for different times in simulated body fluid. The aim of study was to establish the rate of apatite growth to the surface of the glasses. In order to determine the antimicrobial activity of the samples, a glass doped with silver from the same ternary system was analyzed. The research consisted of testing the antiseptic properties of glass on two reference cultures of bacteria most commonly found in hospital-acquired infections (Staphylococcus aureus and Escherichia coli). It was found that the glass bacteriostatic activity increases with the concentration of silver in its composition.*

Keywords: *bioglasses, phosphocalcic glasses, WD-XRF, XRD, Staphylococcus aureus, Escherichia coli*

1. INTRODUCTION

Obtainig of bioactive glasses has evolved mainly after 2000, with the discovery of new technologies for synthesis, such as the sol-gel process. This method was imposed due to the multiple advantages of technological process, in terms of costs and finding new areas of application in medicine.

The first bioactive glass composition (45% SiO2, Na_2O 24.5%, 24.5% CaO and 6% P_2O_5), known under the trade name of Bioglass, were synthesized by Larry L. Hench [1]. Also, the autor has been defined the concept of bioactive material. The main feature of these materials is the property of forming a strong bond at the interface with the hard tissue (bone), which was grafted. The mechanism of formation of tissue-implant interfacial bond involve some physicochemical and biochemical complex processes and certainly form a bioactive layer of apatite (calcium and phosphorus) at the implant surface [2].

The simulated body fluid used to test bioactivity of glasses, in this study, was first synthesized by Kokubo et al. [3, 4, 5]. Simulated body fluid (SBF) was used for in vitro test of bioactivity since the 1990s. This is a acellular and non-protein solution, which contains various salts that simulates the composition, concentration and pH of human plasma [2, 3] .

Both plasma and simulated body fluid are potentially saturated in hydroxyapatite, because they have in composition enough Ca^{2+} and HPO_4^{2-} to support its de novo synthesis [3].

Doping bioactive glasses structure with silver is a relatively new technique for orthopedic and maxillofacial reconstruction. This method reduce the risk of bacterial contamination nearby implant. This phenomenon is based on the antimicrobial action of Ag^+ ions diffusing from the porous structure of the glass in the adjacent graft area without being toxic to the tissue. [6, 7].

2. EXPERIMENTAL PROCEDURE

2.1. Synthesis of sol-gel glasses

Two glasses from SiO_2-CaO-P_2O_5 system has been synthesized. Composition of synthesized and analyzed bioactive glasses in this study is presented in Table 1.

Table 1. Composition of bioactive glasses

Composition [% wt]	SiO_2	CaO	P_2O_5	Ag_2O
S_1	50	41	9	-
S_2	50	38	9	3

Phosphocalcic glasses of SiO_2-CaO-P_2O_5 ternary system has been synthesized by sol-gel method using as precursors tetraethyl-orthosilicate ($Si(OC_2H_5)_4$ - TEOS), triethylphospate ($(C_2H_5)_3PO_4$ - TEP), calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$) and for silver-doped glass was additionally used as a raw material, silver nitrate ($AgNO_3$).

Synthesis of glasses involves 4 stages: hydrolysis and condensation of the precursors in order to obtain the sol,

gelation and aging of the gel (Fig. 1), drying of gel at temperatures below 180° C, for 48-72 hours (Fig. 1b), followed by stabilization of xerogels by calcination at 600° C for 8 hours. (Fig. 1c). [8, 9].

a b c

Fig. 1 Obtaining of sol-gel glasses: a - aged gels, b - dried gels (xerogels), c - bioglass powder

The structural changes to the glass surface synthesized by sol-gel technique and soaked between 3 and 21 day in simulated body fluid (SBF) has been studied by X-ray diffraction analysis (XRD) [1].

In the case of this study was chosen the static immersion method of the samples in order to stimulate material reactivity. The glass powders are immersed in SBF from 3 to 21 days without change the solution. In the case of dynamic method, SBF solution is periodically changed, in order to maintain high concentrations to ions in solution, where bioactivity is tested [1, 10, 11].

Glass doped with Ag was analyzed in terms of antimicrobial activity by microbiological study on two cultures of bacteria commonly found in postoperative hospital infections.

2.2. Caracterization of the samples

The chemical composition of the oxide (CaO - SiO_2 - P_2O_5 - Ag_2O) was determined by wavelength dispersive X-ray fluorescence spectroscopy (WD-XRF) in accordance with standard methodology of the appliance Advant'X ARL- Thermo Scientific with X-ray tube of 2400 W, 60kV şi 80 mA, using a crystal diffraction LiF 200 and 220 scintillation detector [8].

Structural characterization of bioactive glass powder was made by X-ray diffreaction method by using Rigaku Ultima IV diffractometer. XRD analysis showed the presence of apatite on the surface of synthesized glass after their immersion in simulated body fluid (SBF).

In order to determine the antimicrobial properties of Ag^+ doped glass has been used the method of incorporating cultural germs on nutrient media and incubation of their by using adjustable thermostat Nitech (+/- 0.1 ° C), at different temperature and period of time, base on type of culture used. Handling of bacterial cultures was done by using microbiological niche, Pasteur pipettes, Petri dishes and sterile glassware.

2.3. Preparation of simulated body fluid

Because in vivo tests involves difficulties regarding reproducibility, ethical issues, long period of time and high costs, materials designed for implantation are previously tested in vitro.

Choice of the solution used for in vitro bioactivity study, respectivly, reactions that occur on the surface of the material is very important as to be reproduced as faithfully as real biological conditions post-implantation. The composition of the SBF solution with 1.5 N concentration is shown in Table 2.

Table 2. The composition and ionic strength SBF 1.5 N to 1L

Reagent	Amount [g]	Ion concentration [mmol / l]	
		Cation	Anion
NaCl	11.994	Na^+ 213.0	-
$NaHCO_3$	0.525		HCO_3^- 6.3
KCl	0.336	K^+ 7.5	-
K_2HPO_4	0.342		HPO_4^{2-} 1.5
$MgCl_2$	0.458	Mg^{2+} 2.3	-
HCl 1M	60 cm^3	-	Cl^- 221.7
$CaCl_2$	0.417	Ca^{2+} 3.8	
Na_2SO_4	0.107	-	SO_4^{2-} 0.8
$(CH_2OH)_3$ -C-NH_2	9.086	-	-
HCl 1M	Până la pH= 7.2-7.4	pH= 7.25	

The glass powders were immersed for variable period of time (3-21 days) in the SBF, by static method, at temperature of 37 °C and an initial pH of 7.2.

2.4. The antimicrobial activity of the silver-doped glass

For this study has been selected two strains of bacteria commonly involved in hospital infection, appeared after surgery. They have been purchased as lyophilized pure cultures: Escherichia coli ATCC 25922 Gram-negative bacteria, conditioned pathogens extremely resistant to antibiotics and Staphylococcus aureus ATCC reg. 25923 - pathogenic Gram-positive bacteria, with the highest risk of postoperative infection in prosthetic surgery and bone reconstruction.

The antimicrobial activity has been determined at various concentrations of bioactive glass and various decimal dilutions of cultures of microorganisms. Plates with the nutrient media, powder glass and inoculum of bacteria were aerobically incubated for differint period of time, depending on the type of bacteria: 37 °C, 48 hours - Staphylococcus aureus and, respectively, 44 °C - 24 hours for the Escherichia coli. The volume of culture medium has been constant (15 ml) and concentrations glass powders in range of 0.005-0.03mg / ml.

At the same time tests on glass doped with silver has been performed. This composition did not affect the viability of bacterial cultures. At certain concentrations of silver ions S2 glass has bactericidal properties of both strains of microorganisms [6].

3. RESULTS AND DISCUSSION

3.1. Determination of elemental composition by WD - XRF analysis

The results of WD-XRF analysis and the yield of the synthesis process for both glasses has been shown in Table 3.

Table 3 Chemical composition and yield of the sol-gel glasses

Sample	Oxides [% wt]				The yield [%]
	SiO_2	CaO	P_2O_5	Ag_2O	
S1	49.55	38.87	10.62	-	94.6
S2	53.46	30.76	11.25	4.33	97.2

These results confirm the correctness of the chosen method of synthesis, also reactions yield obtained show that the technological route adopted was correct. In case of hydrolysis and condensation reactions are considered optimal for higher yields of 85% [12].

3.2. X-ray diffraction analysis

As a result of soaking in SBF solution is found that the sol-gel glasses are able to generate nucleation of apatite crystals on the surface thereof, thus confirm their bioactive property [13, 14].

Figure 2 shows the X-ray diffraction patterns diffraction before and after soaking in simulated body fluid at 37 °C and pH = 7.25 for different periods of time, in case of glass S1 (SiO_2-CaO P_2O_5).

S1 sample unsoaked in simulated body fluid (Fig.2a) presents a characteristic spectrum of amorphous materials.

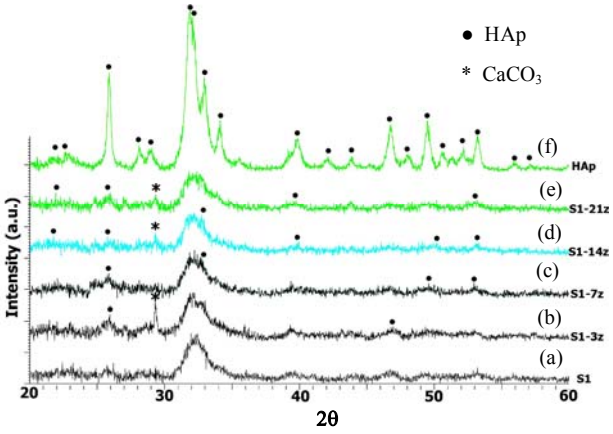


Fig. 2. XRD spectra for S1 sol-gel samples before and after soaking in SBF

After a 3-days of soaking in simulated body fluid (Fig.2b), it can be observed on the glass surface first apatite crystallites, as evidenced by peaks located at 25,92 2θ (3,43Å); 39,31 2θ (2,29 Å); 46,83 2θ (1,93 Å). At the same time, has been highlighted calcium carbonate in the structure of same glass composition by the peak located at 29,28 2θ (3,04 Å).

In case of sample soaked for 7, 14 and 21 days in SBF solutin (Fig.2c, Fig.2d and Fig.2e) rate of apatite formation on the CaO-SiO_2-P_2O5 glass surface is highlighted by the appearance of new peaks located at 21,9 2θ (4.05 Å); 32,96 2θ (2,71 Å); 50,59 2θ (1,8 Å); 53,2 2θ (1,72 Å). Also, it may notice a decrease in the proportion of $CaCO_3$ with increasing time of soaking in alkaline solution.

Figure 3 presents XRD diffraction pattern in case of glass powders doped with silver (CaO-SiO_2-P_2O_5-Ag_2O) before (Fig. 3a) and after immersion in SBF solution between 3 and 21 days (Fig.3b, Fig.3c, Fig.3d and Fig. 3e) under the same conditions as the sample S1.
For the glasses doped with silver, denoted by S2, the rate of apatite formation on the surface of the powder is lower compared to S1 composition.

The presence of apatite on surface of glass powder has been highlighted only after 7 days of immersion in simulated body fluid (Fig. 3c). The phenomenon is confirmed by diffraction peak located at 25,92 2θ (3,43Å). The same analysis confirm slow formation of apatite particles, even after a 21 days of soaking in SBF solution (Fig.3e), by the peaks located at 25,92 2θ (3,43Å) and 39,83 2θ (2,26 Å) in accordance with ICDD-PD2: 00-009-0432.

In the case of the four samples soaked for 3, 7, 14 and 21 days stands out the presence of significant proportions of silver chloride in structure of glass powders analyzed. Thus, has been revealed diffraction lines for AgCl located at 27,83 2θ; 32,24 2θ; 46,23 2θ; 54,82 2θ; 57,47 2θ, in accordance with PDF2: 00-031-1238.

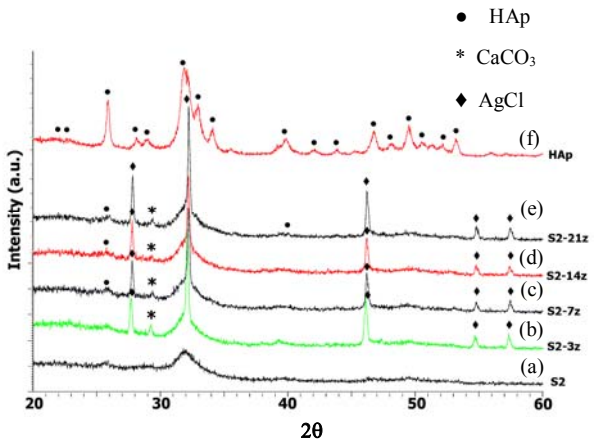


Fig. 3. XRD spestra for S2 sol-gel samples befor and after soaking in SBF

In the case of S2 glass composition, AgCl formation is found in significant quantities from the early days of soaking. This phenomenon can be explained by diffusion of Ag ions in the glass matrix (much easier because Ag is network modifier, linked by weak physical forces) and their reaction with anions of Cl^-, present in extremely high amounts in simulted body fluid (221.7 mmol/l). However, AgCl formed not negatively affected antimicrobial activity of S2 glass.

Also the presence of $CaCO_3$ at the S2 glass surface has been confirmed by X-ray diffraction analysis, by the peak located at 29.28 2θ (3.04 Å) (ICDD PDF-2: 00 - 005 - 586), when the powders have been soaked from 3 to 21 days.

Crystallization of $CaCO_3$ after soaking of samples can be attributed also on the degree of saturation of the SBF with Ca^{2+} and HCO^{3-} ions and decrease of pH, which favors the the precipitation of alkaline compounds.

3.3. Study of antimicrobial activity of glass doped with silver

To promote the optimal development of bacteria chosen for the study, they were incubated aerobically, by using culture media selective, specific to each strains, as follows: for Staph. aureus has been used Baird – Parker medium with bovine fibrinogen and rabbit plasma on agar, specific for coagulase positive Staphylococcus and TBX trypsin medium, bile and glucuronide for E. coli [15, 16, 17, 18, 19].

Pure cultures of bacteria has been reconstituted in compliance with standard conditions, also for analysis an inoculum with initial concentration of $5 \cdot 10^7$ CFU/ml standard for the two strains, has been used.

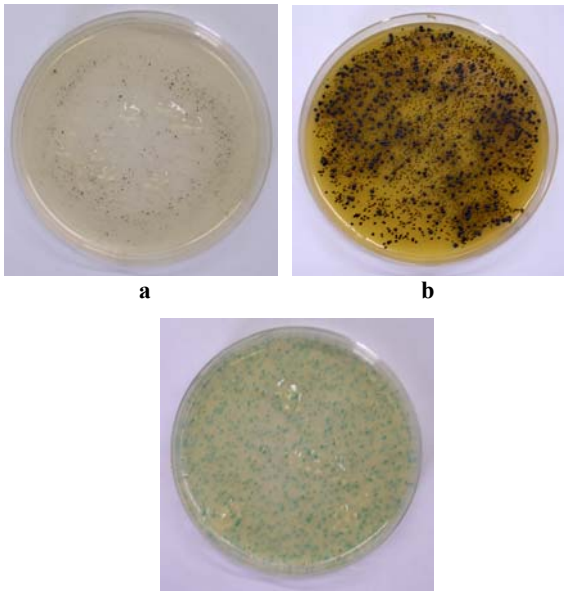
The antibacterial activity of bioactive glasses change with concentration of the silver ions and the dilution of microorganisms.

A first test was carried out with 0.1ml of each pure inoculum culture and 0.1g of silver glass S2.

After heating in the conditions set out above, it was found that the Petri plates not developed any colony of bacteria, for any strain (Figure 4a). The test has been repeated for concentrations increasingly smaller of sol-gel glass, until 0.05-0.03g / ml with the same results - no typical colonies of bacteria - which shows that these concentrations, glass S2 has bactericidal effect.

At the same time, tests has been carried out on the S1 glass without silver. In these plates, bacteria have grown tremendously, in this case the colonies could not be counted (Figure 4b and 4c). In order to set the minimum limits of concentrations for which powder glass has bacteriostatic activity decimal dilutions of the glass powder, have been performed: 0.1g S2 glass and 9.8 ml peptone saline (physiological serum) were stirred 6 hours at room temperature for the diffusion of ions from glass powder, including those silver; in second step 0.1ml pure culture of Staph. from the first decimal dilution aureus bacterial inoculum (10^{-1}) is added and well mixed.

For E. coli was repeated the same methodology. In this case has been started at a concentration of glass at 0.03 g / 9.9 ml peptone saline solution, below its bactericidal activity.



a **b**

c

Fig. 4. Glass powders with silver (a), control plates without silver for Staphylococcus aureus (b) and Escherichia coli (c)

From this mixture are taken 0.1ml and added in Petri dishes on nutrient media, specific to each bacteria. This operation is repeated for each type of culture with the same volume of bacterial inoculum in the same dilution (10^{-1}), but with reduced amounts of sol-gel glass by successive tenfold dilutions.

In Figure 5 is shown a bacteriostatic effect of the S2 glass on the Staphylococcus aureus bacteria depending on the concentration of silver ions, respectively, on the Escherichia coli bacteria, in Figure 6.

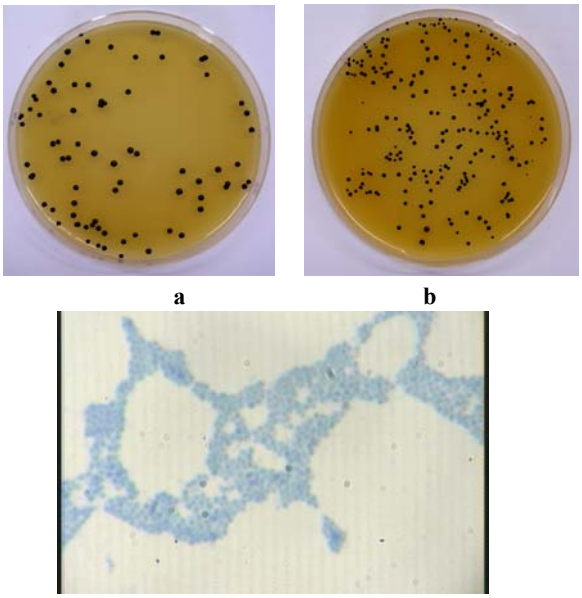


Fig. 5. Glass powders with silver soaked on peptone saline (physiological serum) at different dilutions: (a) $0.01 \cdot 10^{-3}$ g/ ml, (b) $0.01 \cdot 10^{-5}$ g/ml, (c) Staphylococcus aureus

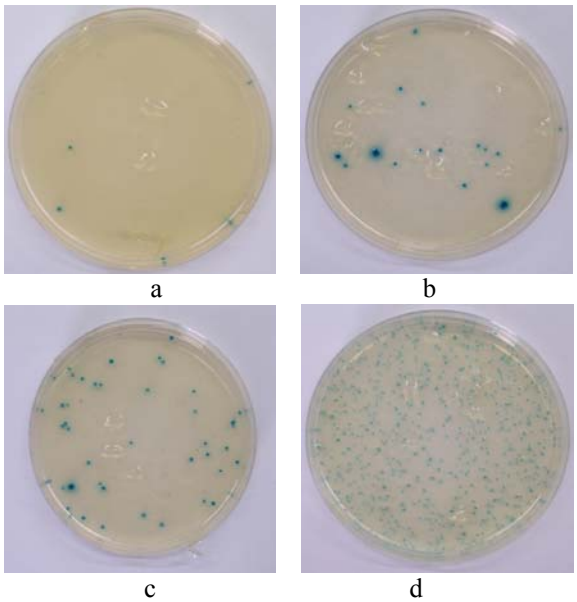


Fig. 6. Glass powders with silver soaked on peptone saline (physiological serum) at different dilutions: (a) $0.03 \cdot 10^{-1}$ g/ ml (b) $0.03 \cdot 10^{-2}$ g/ ml, (c) $0.03 \cdot 10^{-3}$ g/ml, (d) $0.03 \cdot 10^{-4}$ g/ml

Glass doped with silver have bactericidal effect for both strains, at various concentrations of the glass: for Staphylococcus aureus lethal concentration is in the range 0.05-0.1g S2/10 ml, while the concentration of Escherichia coli even down to 0.03g S2/10ml. Therefore, Gram-negative bacteria (Escherichia coli) is more sensitive to the cytotoxic effect of silver by comparison to the Gram positive bacteria.

The glass doped with silver, at extremely low concentrations, has definitely bacteriological properties:

- $0.01\text{-}0.01 \cdot 10^{-3}$ g/ml (10^{-2} - 10^{-5} g/mol) for Staphylococcus aureus. At dilutions of the glass greater than10^{-5}, bactericidal activity is reduced until it reaches zero.
- 0.003 - $0.003 \cdot 10^{-3}$ g / ml ($3 \cdot 10^{-3} – 3 \cdot 10^{-6}$ g/ml).

4. CONCLUSION

This study demonstrated that phosphocalcic glasses from SiO_2-CaO-P_2O_5 ternary system, can be obtained by sol-gel method with good results, by using precursors such as: TEOS, TEP, $Ca(NO_3)_2 \cdot 4H_2O$ and $AgNO_3$ for the doped silver glasses.

X-ray fluorescence spectroscopy, in case of of samples unsoaked in SBF solution confirm chemical composition (Table 3), close to the theoretically values and yield higher than the minimum presented in the literature.

The bioactivity of the glass powder was highlighted by using XRD analysis. Samples has been soaked in SBF solution with pH = 7.25, at 37 °C for 3, 7, 14 and 21 days. In vitro tests has been shown that after 3 days of immersion were identified crystalline apatite formation on the surface of S1 glass. In case of S2 glass - doped with silver apatite was identified after 7 days of soaking. Therefore, from two compositions, a higher degree of bioactivity present S1 glass without Ag^+.

S1 glass without silver did not influence the viability of bacterial strains used in this study. The bactericidal or bacteriostatic activity was not revealed in this case. The colonies developed and become infected plate could not be counted.

The antimicrobial activity of silver is not diminished by the presence of other species of ions (calcium, phosphates, silicates) that diffusing with it in peptone saline solution.
Although the efficiency against infections and contamination of silve is proven, the mechanism by which the silver ions exerts its toxicity against bacteria is not fully understood.

In order to benefit from both characteristics of the glasses studied, bioactivity and antibacterial effect, for medical applications (grafting) can be recommended to use a mixture of the two compositions, by reducing the content of glass S2 up to the bactericidal activity of each strain of the microorganisms.

Based on the same assumption can be synthesized glasses with a silver content much lower, even less than 1%, in order to be able to preserve both properties of the glasses and to promote their synergistic action.

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