Characterization in vitro studies and antibacterial properties on a sol-gel derived silver incorporated bioglass

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Abstract. The SiO2-CaO, SiO2-CaO-P2O5 and SiO2-CaO-P2O5-Ag2O glass systems were synthesized by the sol-gel technique and characterized with different techniques such as X-ray diffraction (XRD), Fourier Transform Infrared spectroscopy (FTIR), and Environmental Scanning Electron Microscopy (ESEM). In vitro bioactivity tests were performed in Simulated Body Fluid (SBF). The antibacterial action of 65S5Ag (65%SiO2 + 24%CaO + 6%P2O5 + 5% Ag2O) is attributed exclusively to the leaching of Ag+ ions from the glass matrix. The activity of SiO2-CaO-P2O5-Ag2O was compared with that of its binary and ternary counterpart glass system. The concentrations of Ag-bioglass, in the range of 0.05 mg/mL of culture medium, were found to inhibit the growth of these bacteria.

1. Introduction

Bioactive glasses are special glass systems which are generally composed of SiO2, CaO, P2O5, and Na2O. They can be produced by the traditional melting process or by the more versatile sol-gel process [1, 2]. The bioactive behavior of these glasses is defined as the ability to bond to soft and hard tissues by means of complex chemical and biochemical reactions, which produces strong and compliant interface between the glass and the tissue [1]. Due to their high level of tissue integration and regeneration, bioactive glasses have been used clinically in a variety of situations [2, 3]. Numerous studies have been performed on them to try to improve their properties, for instance addition of other elements [4-11], sol-gel preparation [12-15], or porous bioactive glasses [16, 17].

Sol-gel processing has been intensely studied as an alternative method for preparing ceramics and glasses for a wide variety of applications including the field of bioceramics. This is because of the potential advantages of higher purity and homogeneous materials that are produced at lower processing temperatures. Li et al. showed that CaO-P2O5-SiO2 powders produced by this technique are more bioactive than the melt-derived glasses of the same composition [18]. The high bioactivity of the sol-gel derived materials is related to the textural features of the gels, i.e., pore size and pore volume associated with the large surface area, higher rate of dissolution, and the negative surface charge [19].
Among the problems encountered the microorganism adhesions on implant surfaces represent an initial crucial step in infection and lead to the formation of biofilm, in which microorganisms are more resistant antimicrobial agents [20]. To solve the problem of contamination, it has been proposed to use antimicrobial agents such as antibiotics, fluorine and biocides metal ions [21-23]. The antibacterial activity of silver ions and their biological impact have been demonstrated by many works [24-28]. Silver-containing bioactive glasses have been produced in different ways: sol-gel [29, 30], melting, ion-exchange [31], and their antibacterial activity have been investigated by biological tests using different bacterial stocks.

The main aim of the presented work was to evaluate the in vitro bioactivity and antimicrobial action of a novel sol-gel derived SiO$_2$–CaO–P$_2$O$_5$–Ag$_2$O. The properties of the silver-incorporated bioglass were compared with other glasses: SiO$_2$–CaO, SiO$_2$–CaO–P$_2$O$_5$ silver-free bioglass system. The *Escherichia coli* (gram negative bacteria) species was chosen for the preliminary investigation because it was found in very high concentration at biomaterials-related infection sites [32-38].

2. Materials and method

2.1. Preparation of bioactive glasses

The three sol-gel glasses: SiO$_2$–CaO, SiO$_2$–P$_2$O$_5$–CaO, and SiO$_2$–P$_2$O$_5$–CaO–Ag$_2$O were synthesized as follows: initially, tetraethoxysilane (TEOS; Aldrich) was added into 0.1 M nitric acid and the mixture was allowed to react for 60 min for the acid hydrolysis of TEOS. Table 1 lists the nominal composition of the prepared glasses. The following reagents were added in the following sequences, allowing 45 min for each reagent to react completely: triethylphosphate (TEP; Aldrich), calcium nitrate tetrahydrate (Aldrich), and silver nitrate (Aldrich). After the final addition, mixing was continued for 1h to allow for the completion of hydrolysis. The resultant solution was kept in a sealed Teflon container for 3 days at ambient temperature conditions for gelation. The gel formed was placed in a sealed container and heated at 70°C for an additional 3-days period. The water was removed and small holes were created on the lid to allow the leakage of gases and heat treated at 150°C for 2 days to remove all the free water. The dried gel was then calcined for 3h at 700°C to stabilize the glass and to eliminate residual nitrates.

| Glasses  | SiO$_2$ | CaO | P$_2$O$_5$ | Ag$_2$O |
|----------|---------|-----|------------|---------|
| 65S35C   | 65      | 35  | 0          | 0       |
| 65S6P    | 65      | 29  | 6          | 0       |
| 65S5Ag   | 65      | 24  | 6          | 5       |

2.2. Materials

2.2.1. XRD measurements

X-ray diffraction spectra were obtained using a Discover model equipped with a monochromatized Cu-K$_\alpha$ radiation ($\lambda= 1.5418$ Å). Scans were performed with a step size of 0.02° over an angular range 2θ from [20-80°].

2.2.2. FT-IR absorption measurements
Fourier transform infrared (FTIR) spectroscopy was performed by FTIR using an IR Bruker VERTEX 70 spectrometer. The FTIR spectra were recorded from 400 to 4400 cm\(^{-1}\). All measurements were recorded with a resolution of 4 cm\(^{-1}\).

2.2.3. SEM study

The microstructure of the surface of the samples was observed on a scanning electron microscope (ESEM Quanta 200 FEI Company), with energy dispersive X-ray (EDX) analysis.

2.2.4. pH measurements

The pH changes of the SBF during the dissolution of the undoped and silver-doped glasses were measured at every hour and up to 6 h using IQ microprocessor pH-meter (210 pH)-meter (IQ Inc. USA).

2.2.5. Antibacterial activity test

Antimicrobial activities of samples were investigated against yeast O128B12. The bacteriostatic effect of 65S5Ag was determined by performing a viable count after exposure of the bacterial colony to 65S5Ag for 24 h. A single bacterial colony was used to inoculate a 50 mL starter culture. The initial concentration was approximately 2×10\(^8\) colony forming units/mL (CFU/mL). Assay mixtures containing 65S35C, 65S6P, and 65S5Ag in concentrations ranging from 0.05 to 2 mg/mL were prepared in three replicates. Negative control assay mixtures, containing only the cell inoculum in growth medium, were cultured in triplicate. This concentration range was chosen in consideration with the elution of Ag\(^+\) from 65S5Ag during the course of the dissolution experiment. Following inoculation, the cultures were incubated for 24 h at 37°C. After the incubation period, a 1 mL sample was taken and serial dilution was plated onto the growth medium employed in the liquid culture. The number of colonies was counted after overnight incubation. Approximate minimal bactericidal concentration (MBC) was estimated as the concentration of 65S5Ag leading to a 99% reduction in viability.

3. Results and discussion

3.1. Crystalline phase in 65S3, 65S6, and 65S5Ag

X-ray diffraction and FTIR evidenced the formation of an apatite-like layer on the glass surface after soaking in SBF. This mechanism could be explained through the following steps: (a) rapid exchange of protons H\(^3\)O\(^+\) from the physiological solution with Ca\(^{2+}\) and Na\(^+\) ions in bioglass to form the Si-OH groups, (b) loss of soluble silica as Si(OH)\(_4\) by breaking of Si-O-Si bridging links and subsequent formation of surface silanol groups in the process, (c) condensation and repolymerization of surface silanols to form SiO\(_2\)-rich surface layer, (d) migration of Ca\(^{2+}\) and PO\(_4\(^{3-}\) ions through the surface silica-rich layer and formation of a Ca-P rich layer on the surface of the bioglass, (e) incorporation of OH\(^-\) and CO\(_3\)\(^{2-}\) ions from the solution and subsequent crystallization of the Ca-P layer to form HCA [39]. An XRD study of as-received samples confirmed that all of them were amorphous, except 65S5Ag, the crystalline phase in this bioglass was identified to be CaAg(PO\(_4\)). X-ray diffraction patterns obtained for the binary and ternary glasses after 4 days soaking showed one wide diffraction maxima 32\(^\circ\), corresponding to (211) reflections of an apatite-like phase. For quaternary glass 65S5Ag, after just 4h, the (200), (211) and (310) reflections of the apatite phase appeared owing to the increase in its crystallinity. Data obtained by XRD show that an apatite-like phase is formed on glass surface. The formation of the apatite was also confirmed by FTIR and SEM/EDX examination.
Figure 1. XRD spectra of the sol-gel derived bioglasses sintered at 700°C for 3h before and after immersion in SBF for different times. (a): 65S35C, (b): 65S6P and (c): 65S5Ag.

Figure 2 shows FTIR spectra. All glasses after soaking in SBF solution for different times reveal P-O bending (amorphous) (560 cm\(^{-1}\)) bands. Emerging of P-O bending (crystalline) (475 cm\(^{-1}\)) bands reveals the formation of hydroxycarbonated apatite (HCA) layer. Presence of O-H stretching (1630-3453 cm\(^{-1}\)), C-O stretching (800 cm\(^{-1}\)) and (1426 cm\(^{-1}\)) bands shows the crystalline nature of HCA layer.

Figure 2. FTIR spectra of the sol-gel derived bioglasses sintered at 700°C for 3h.
Figure 3. SEM micrographs of the bioglasses samples before and after soaking into SBF.

3.2. pH measurements and concentration of Ca and P

Figure 4 shows the variation of pH of the SBF with immersion times, this variation is similar for all glasses. The cumulative variation of Ca and P ionic concentrations and variation of pH with the soaking time in SBF for samples 65S35C, 65S6P, and 65S5Ag. As can be observed in all cases, pH variation with time was also similar for the three samples, increasing up to 9 during the first 10 hours and to 10 after 96 hours of immersion and then no significant change took place. However, variation of Ca and P concentration in SBF was different for each composition of glass. For 65S35C, Ca concentration reached a maximum after 4 days and did not change significantly until the end of the test, while for 65S6P and 65S5Ag, Ca and P concentrations, gradually decreased up to 6 hours and 4 hours successively.

Figure 4. Variation of pH in the SBF with soaking time, for samples 65S35, 65S6P, and 65S6Ag.
Figure 5. Ca and P concentration with immersion time in SBF for gel glass samples: (a) 65S35, (b) 65S6P, and (c) 65S5Ag
3.3. Antibacterial activity test

The MBC, MIC was estimated for 65S5Ag as 0.06 mg/mL for *E. coli*. The silver-free bioglasses (65S35C and 65S6P) show neither bacteriostatic nor bactericidal effects on the micro-organism studied. As a matter of fact, the silver free bioglass did not affect the viability of the *E. coli* bacterial strain under investigation. Comparing the results obtained for 65S5Ag and 65S35C, 65S6P, it can be inferred that the silver released from 65S5Ag is instrumental in the antibacterial action of the 65S5Ag. Further, it can be concluded that the other ionic species, such as Ca, Si and P released along with Ag⁺ from the glass, do not alter the antibacterial property of the 65S5Ag.

4. Conclusion

Glasses 65S35C, 65S6P, and 65S5Ag were obtained successfully by the sol-gel method. They are bioactive by forming a hydrated carbonate apatite layer on their surface on exposure to SBF. The glasses have been characterized by XRD, FTIR and SEM. An *in vitro* antimicrobial activity test has shown that the introduction of silver to the three-component system, SiO₂-P₂O₅-CaO inhibits the growth of the *E. coli* bacteria. The dissolution study has confirmed that the material is capable of releasing silicate species, which is very important for its mitogenic effect *in vivo*. The rate of dissolution of silver ions is relevant to eventual clinical applications, since the prerequisite of an effective, topical antimicrobial agent is an immediate and concentrated release of that agent.

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