Synthesis and characterization of new bioceramic/antibiotic composites

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Silica calcium phosphate bioactive glass with $60SiO_2 \cdot 30CaO \cdot 10P_2O_5$ (mol %) composition was synthesized by sol-gel technique, converted by annealing to a glass-ceramic containing apatite and wollastonite nanocrystalline phases, and loaded with three different antibiotics considered to decrease the risk of infection at implantation sites. The antibiotics used were tetracycline, streptomycin and ampicillin. The loading efficiency was assessed by ultraviolet-visible (UV-Vis) and Fourier transformed infrared (FTIR) spectroscopy. The specific surface areas and the mean pore volume of the samples were investigated before and after antibiotics loading using Brunauer, Emmet and Teller (BET) method. *In vitro* bioactivity of unloaded and antibiotics loaded glass samples was tested in simulated body fluid. The antimicrobial ability of the bioactive glass/antibiotic composites was determined on *Escherichia coli* bacteria.

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1. Introduction

The technological progress achieved in the recent years in field of biomaterials, biotechnology and tissue engineering has resulted in a significant increase in the use of materials for medical applications. Biomaterials such as calcium phosphate glasses or bioceramics are currently used in hard tissue replacement [1] due to their excellent biocompatibility and osteoconductivity. Numerous studies show that bone-like apatite materials favour bone formation and regeneration, having optimal surface characteristics for osteoblast cells to adhere, proliferate and differentiate [2].

In case of surgery, however there is always the risk of infection. Infections related to implants mostly occur from bacterial adhesion, followed by biofilm formation [3]. To lower the risk of infection, local drug delivery shows promising perspectives [4, 5]. By keeping a long term antibiotic release at the implantation site, it is aimed to prevent bacterial adherence to the surface and, at the same time, to diminish the side effects and systemic toxicity compared to other administration procedures [6, 7].

In this study the $60SiO_2 \cdot 30CaO \cdot 10P_2O_5$ bioactive glass-ceramic was loaded with three different antibiotics: tetracycline, streptomycin and ampicillin, in order to evaluate the affinity of these antibiotics towards the bioactive glass matrix.

The loading capability of the antibiotics was estimated by visible (UV-Vis) and Fourier transformed infrared (FTIR) spectroscopies. To assess the effect of antibiotic loading on the surface area, porosity and bioactivity of the glass matrix, surface area analysis was carried out on the bioactive glass before and after loading with antibiotics. X-ray diffraction (XRD) and FTIR spectroscopy were used to test the *in vitro* bioactivity of unloaded and antibiotics loaded glass samples after 14 days immersion in simulated body fluid (SBF). The inhibition of *E-coli* strains was used to address the antimicrobial effect of all samples.

2. Materials and methods

The bioactive glass of $60SiO_2 \cdot 30CaO \cdot 10P_2O_5$ (mol %) composition was synthesised by sol–gel method. The precursors used were reagent grade tetraethyl orthosilicate (TEOS), calcium nitrate $Ca(NO_3)_2.4H_2O$ and triethyl phosphate (TEP). The sol was prepared by stirring TEOS with water and ethyl alcohol in a 1:2:1 weight ratio catalysed by HNO_3 and heated to 80 ºC for about 30 min in a closed recipient in order to achieve a complete hydrolysis without lowering the pH below 3,5. $Ca(NO_3)_2.4H_2O$ and TEP previously dissolved in water were then added to the cooled down silica sol and stirred together for another 30 min and then left to gel undisturbed at room temperature. The formed gel was dried at 110 ºC for couples of hours followed by heat treatment at 550 ºC for 2 hours. The resulting material was milled to a powder and sieved to obtain a maximum grain size of about 40 μm.

For antibiotics loading 2 g of bioactive glass powder were immersed for 4 hours in 10 ml of phosphate buffer (PBS) with pH 7.4 to which tetracycline, streptomycin and ampicillin was added, respectively. For all three PBS solutions, the antibiotic concentration was 10 mg/ml. After 4 hours the PBS solution was removed and kept for further

analysis, and the bioactive glass was washed three times with fresh PBS to remove the unbound antibiotic. The washed glass powder was then dried in air.

To prove the antibiotic attachment on the bioactive glass matrices the withdrawn solutions were analysed by UV-Vis spectroscopy and the loaded powders were analysed by FTIR spectroscopy. UV-Vis measurements were performed on a Jasco V-670 UV-Vis-NIR spectrometer with a slit width of 2 nm and 1 nm spectral resolution. For FTIR measurements identical amounts of glass before and after loading as well as pure antibiotics were mixed with KBr in order to obtain thin pellets with a thickness of about 1 mm. The FTIR spectra were recorded at room temperature in the 350-2500 cm^{-1} range on a 6100 Jasco spectrometer with a resolution of 0.5 cm^{-1}.

The surface analyses were performed by nitrogen adsorption at 77 K, using Qsurf Series M1 surface area analyser, based on the Brunauer, Emmet and Teller (BET) method. During the measurements the experimental conditions were kept constant, with 30% N_2 and 70% He gas mixtures.

The bioactivity of the glass before and after antibiotics loading was determined by immersion for 14 days in simulated body fluid (SBF) with ion concentration close to that of human blood plasma [8]. Hydroxyapatite formation was evaluated from XRD patterns and FTIR spectra recorded before and after immersion in SBF. The XRD patterns were recorded on a Shimadzu X-ray Diffractometer XRD-6000 with 2˚/min in the 10˚-80˚ interval.

The *in vitro* antimicrobial properties of the composites containing tetracycline, streptomycin and ampicillin against potential human pathogen were evaluated based on the zone of inhibition method for the gram-negative *Escherichia coli* (*E. coli)* strain ATCC 25922, with a concentration of colony-forming units (CFU) of $3*10^8$ CFU/ml. For antimicrobial tests the composites were mixed with distilled water in 1:3 ratio and introduced in identical holes carved in the culture medium.

For simplicity, the bioceramic/antibiotic composites will be further called as BC-TCL, BC-STR and BC-AMP to denote the bioceramic (BC) loaded with tetracycline (TCL), streptomycin (STR) and ampicillin (AMP), respectively.

3. Results and discussion

To evidence the antibiotics loading into the porous bioactive glass-ceramic, the UV-Vis spectra of the initial PBS solutions with antibiotics were compared with the UV-Vis spectra of the solutions retrieved after 4 hours of immersion. The difference in intensities on all three samples (Fig. 1) shows that part of the antibiotics is missing from the initial PBS solution indicating a successful attachment on the bioactive glass-ceramic surface. The difference in intensities is more pronounced for the samples containing tetracycline and less observed for the ampicillin loaded sample. While tetracycline has a high affinity and capacity to form chemical bonds with Ca^{2+} ions from the bioactive matrix [9], the ampicillin has a reduced affinity towards bioactive glass surfaces due to charge effects, with the negatively charged ampicillin and BC repelling each other as reported by El-Fiqi et al. [10]. The streptomycin affinity towards this bioactive glass-ceramic matrix was found to be between the other two.

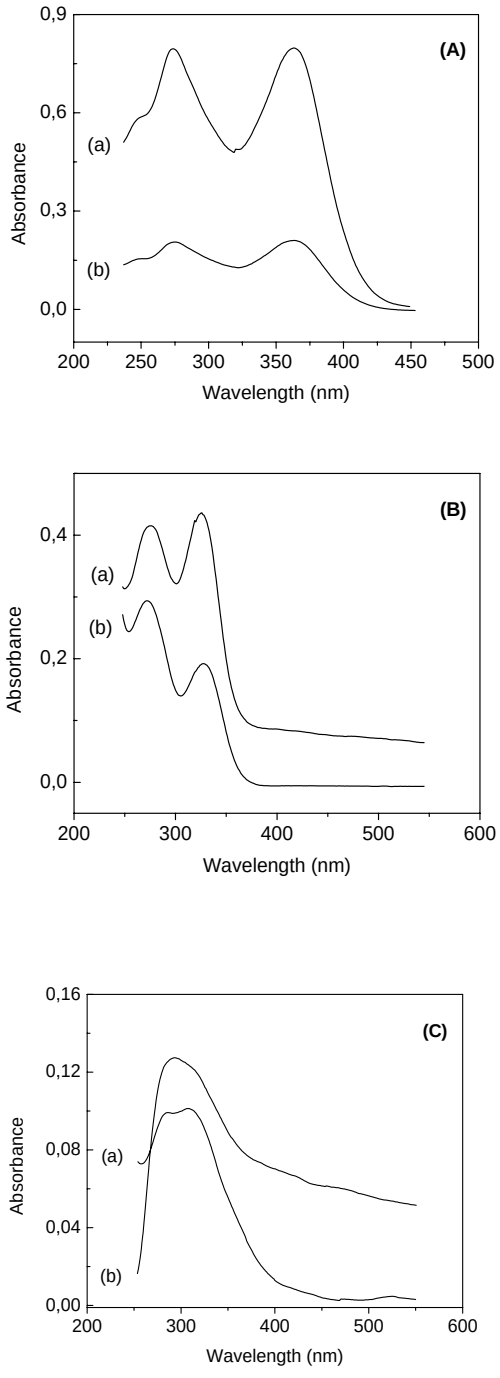

Fig. 1. UV-Vis spectra of BC-TCL (A), BC-STR (B), BC- AMP (C). (a) Initial antibiotic concentration 7 mg/ml; (b) Antibiotics concentration in the remained solutions, collected after loading.

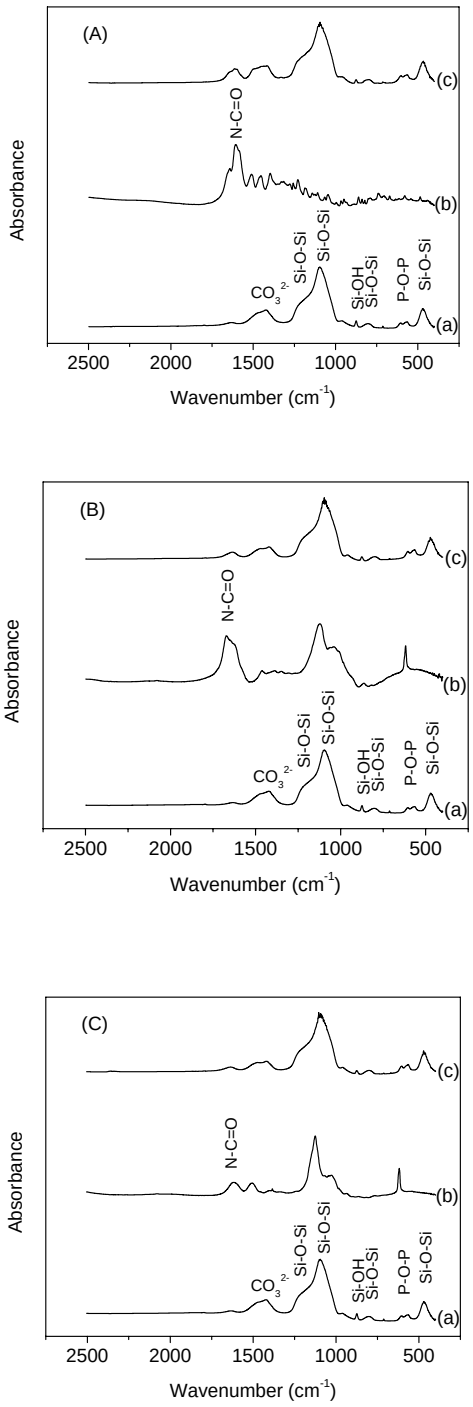

Fig. 2. FTIRspectra of: (a) bioactive glass-ceramic matrix; (b) pure antibiotics; (c) bioactive glass-ceramic loaded with TCL (A), STR (B), AMP(C).

The antibiotic attachment was further verified by FTIR spectroscopy. The FTIR spectra of the antibiotics loaded samples were compared with the FTIR spectra of pure antibiotic and bare bioactive glass-ceramic (Fig. 2). The strong absorption bands at 1209 and 1093 cm^{-1}, as well as that at 803 and 469 cm^{-1} can be attributed to the Si-O-Si bonds in the BC network. The peaks around 952 cm^{-1} are assigned to the vibration of Si-OH bonds. P-O bending vibrations were associated with the bands around 560 cm^{-1}, while the absorption bands at 1421 and 1472 cm^{-1} could arise from C-O stretching vibrations [11] as well as from ring vibrations in antibiotics [12, 13]. These bands are observed in the FTIR spectra of both bare bioactive glass-ceramic and antibiotics loaded ones. For the pure antibiotics a major feature that is not overlapping with the aforementioned adsorption bands is the 1600-1690 cm^{-1} adsorption band corresponding to amide I. This dominant feature of the pure antibiotic appears also on all loaded samples and proves the antibiotic attachment.

Bioactivity of the sol-gel glasses is determined by several factors including the glass composition and its textural properties, i.e. specific surface area and porosity [14], thereby keeping a high surface area after loading is an important factor to be considered. The nitrogen adsorption/desorption studies based on BET method show that the specific surface area of the loaded samples increases for all three composites (Table 1). The increased surface area can be attributed to the extra surface roughness provided by the attached antibiotics [15] and represents a proof of bioactive glass-ceramic surface modification, following the antibiotic adsorption process.

Table 1. The specific surface area and the mean pore volume values for the bioactive glass-ceramic matrix (BC) before and after loading with TCL, STR and AMP.

	BC	BC-TCL	BC-STR	BC-AMP
Specific surface area (m^2/g)	53	68	70	68
Mean pore volume (ml/g)	0.11	0.33	0.29	0.35

The fact that antibiotic loading does not decrease the bioactivity of the samples is further shown by XRD and FTIR results. The X-ray diffractogram (Fig. 3) of the bare bioactive glass-ceramic sample obtained after heat treatment at 550 ^oC for 2 hours show wollastonite ($CaSiO_3$) and apatite ($Ca_{10}(PO_4)_6(OH)_2$) like peaks at 29.1^o and 32^o , respectively [16-19]. The size of the developed crystallites was estimated from the full width at half-maximum of the diffraction peaks using the Sherrer equation, and the average size was between 30 and 50 nm. The presence of both hydroxyapatite and wollsatonite is desirable for bioactive materials considered for scaffolds for bone tissue engineering [20].

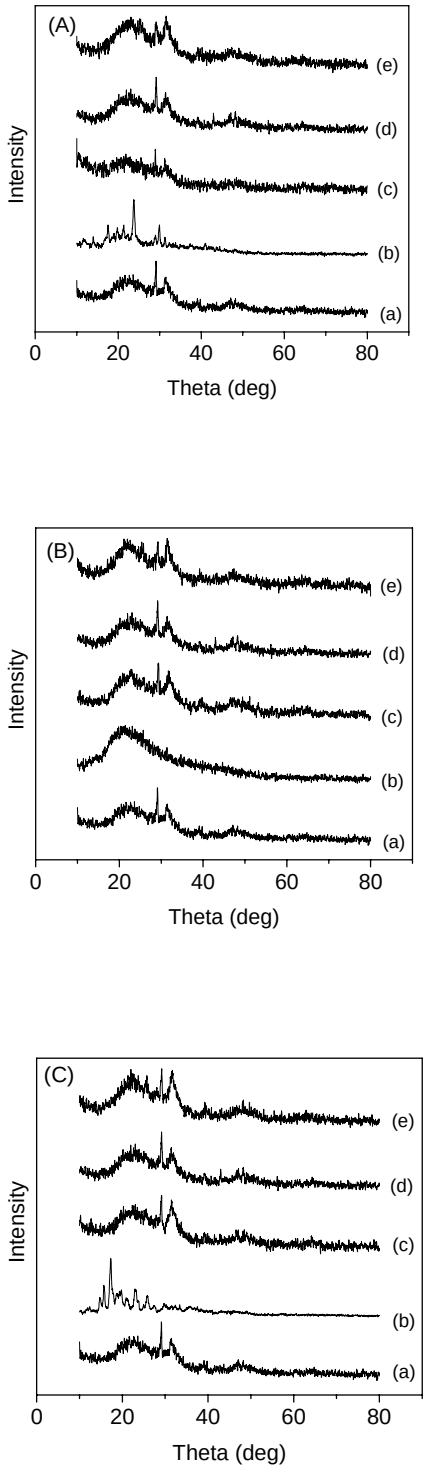

Fig. 3. XRD spectra of bare bioactive glass-ceramic (a), pure antibiotics (b), and bioactive glass-ceramic loaded with antibiotic (c) bioactive glass-ceramic after immersion in SBF (d), and bioactive glass with antibiotic after immersion in SBF (e), for TCL (A), STR (B) and AMP (C).

The antibiotic specific peaks are not recorded from the antibiotic loaded samples very probably due to the low amount of crystallised antibiotic present in the loaded samples, but the apatite like peak is still present, and in the SBF immersed sample we can see a clear increase and narrowing of the peaks indicating further the apatite formation capabilities of the samples.

Fig. 4 presents the FTIR spectra recorded for the bare bioactive glass-ceramic and for the sample loaded with antibiotics before and after immersion in SBF for 14 days. It is observed a doublet around 560-600 cm^{-1}, increasing in intensity as a result of SBF immersion, attributed to vibrations of $[PO_4]$ units of the hydroxyapatite phase [21]. The doublet is evident also for the bare sample which strengths the previous statement according to the bioactive glass-ceramic contains an appetite like phase even before SBF immersion. The FTIR results together with the XRD data prove that the loading of the bioactive glass-ceramic matrixes with antibiotics will not hamper the samples bioactivity.

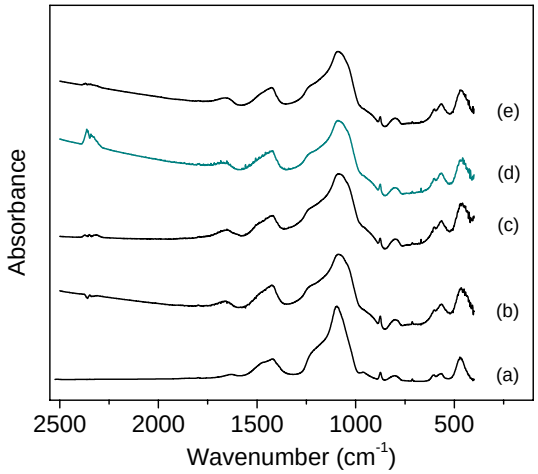

Fig. 4 FTIR spectra of bioactive glass-ceramic before (a) and after immersion in SBF (b), bioactive glass-ceramic loaded with TCL (c), STR (d), and AMP (e) after immersion in SBF.

Antimicrobial tests on the *E-coli* show a strong antimicrobial effect for both tetracycline and streptomycin loaded samples, which indicate that the released antibiotics retain their efficacy after being released from the composites. No inhibition effect for ampicillin loaded or blank bioactive glass-ceramic matrix (Table 2) was evidenced. The lack of inhibitory effect in case of the sample loaded with ampicillin could be associated with the poor binding capacity to the bioactive glass-ceramic matrix, as shown by the fast release curve for this drug.

Table 2. Microbial inhibition zones around bioceramic/antibiotic composites tested on E-coli.

Plate	BC (mm^2)	BC–TCL (mm^2)	BC–STR (mm^2)	BC–AMP (mm^2)
1	0	16	21	0
2	0	21	16	0
3	0	26	24	0
4	0	20	22	0
5	0	20	18	0
Average	0	20.6	20.2	0

Tetracycline, streptomycin and ampicillin antibiotics loading into $60SiO_2 \cdot 30CaO \cdot 10P_2O_5$ bioactive glass-ceramic was proven, and ampicillin showed the smallest affinity towards the glass matrix due to its negatively charged surface. The enhanced loading of tetracycline thanks to the binding capability of its Ca^{2+} ions was shown using UV-Vis

4. Conclusions

A porous bioactive glass-ceramic containing hydroxyapatite and wollsatonite nanocrystalline phases was obtained by calcination of the $60SiO_2 \cdot 30CaO \cdot 10P_2O_5$ sol-gel derived glass sample. Antibiotics loading into this bioactive glass-ceramic was investigated for tetracycline, streptomycin and ampicillin using UV-Vis and FTIR analysis. The spectroscopic results showed the smallest loading affinity in case of ampicillin, due to its negatively charged surface. The enhanced loading of tetracycline was correlated with the binding capability of its Ca^{2+} ions. Streptomycin affinity towards this bioceramic matrix was slightly less, but it showed an increased inhibition effect compared to tetracycline. Ampicillin has a low binding rate, a very fast release and no inhibition effect. The bioactivity test evidenced that the antibiotics loading do not hamper the bioactivity of these new bioceramic/antibiotic composites.

Acknowledgments

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