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THE EFFECTIVE ANTI-BACTERIAL ACTIVITY OF SOME COPPER CONTAINING BORATE GLASSES

EFECIENȚA ANTIBACTERIANĂ A UNOR STICLE PE BAZĂ DE BOR CU CONȚINUT RIDICAT DE CUPRU

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ABSTRACT

During the last years, the researchers' interest in glass has been continuously increasing, due to the fact that it was used as inorganic support in different composite biomaterials designed as scaffolds in tissue engineering. Therefore, several inorganic materials such as glasses have shown good bioactive and bacteriostatic activity. In this study, we investigated eight oxide glasses with different concentrations of oxides such as copper, strontium, aluminium, and boron, in terms of structural, bioactivity, and antimicrobial properties. Before the antibacterial assay, we measured the dissolution rate in the water of the glass system in order to test its local chemical durability. For the antimicrobial test, the following reference strains were used: *Escherichia coli* 25922, *Staphylococcus aureus* 6538P, *Salmonela eneritidis* 13076, and *Bacillus cereus* 11778. Our results show that the addition of copper oxide increased the dissolution gradient of the glass samples. Furthermore, the bacteriostatic effect manifested by the radius of the inhibition zone increased proportionally with the addition of CuO. The bacteriostatic effect of the samples was observed in both gram negatives and gram positives strains, with more significant effect on *Staphylococcus aureus* strain. The present study highlights the effectiveness of these copper containing glasses against some of known bacterial species capable of causing different types of soft or hard tissue infections in animals and humans.

Keywords: inorganic materials, glass samples, bioactivity, antimicrobial effect, copper

REZUMAT

În ultimii ani, sticla oxidică a fost în centrul preocupărilor cercetătorilor și i s-a acordat o atenție deosebită, deoarece a fost inclusă frecvent ca suport anorganic al biomaterialele compozite concepute ca structuri suport în ingineria tisulară. Astfel, mai multe materiale anorganice cu diferite compoziții sticloase au fost identificate ca având o bună bioactivitate și activitate bacteriostatică. Cu toate acestea, încă există multe neclarități cu privire la interacțiunea țesut-biomaterial și apariția posibilelor infecții. În acest studiu au fost investigate opt probe de sticlă bioactivă cu concentrații diferite de oxid de cupru, stronțiu, aluminiu și bor în scopul determinării bioactivității, proprietăților structurale și antibacteriane. Premergător examenului bacteriologic, a fost înregistrată rata de disoluție a sistemului de sticlă în vederea testării durabilității chimice a acestuia la nivel local. Pentru testarea activității antibacteriene au fost utilizate următoarele tulpini: *Escherichia coli* 25922, *Staphylococcus aureus* 6538P, *Salmonela eneritidis* 13076 și *Bacillus cereus* 11778. Rezultatele obținute evidențiază o creștere în rata de disoluție a sistemului de sticlă odată cu adăugarea oxidului de cupru. Mai mult, efectul bacteriostatic al probelor de sticlă, evidențiat prin măsurarea diametrului zonelor de inhibiție (mm) a crescut proporțional cu adăugarea oxidului de cupru. Cu toate că efectul bacteriostatic al probelor de sticlă a fost evidențiat atât în cazul tulpinilor negative, cât și a celor pozitive,rezultate mai bune au fost semnalate la tulpina de *Staphylococcus aureus.* Studiul de față reliefează eficacitatea probelor de sticlă investigate împotriva unor tulpini bacteriene cunoscute, capabile să provoace diferite tipuri de infecții la nivelul țesuturilor moi sau țesuturilor dure la animale și oameni.

Cuvinte cheie: materiale anorganice, probe de sticlă, bioactivitate, efect antimicrobian, cupru

In recent years, greater attention has been paid to the utilization of bioactive glasses with different antimicrobial and regenerative properties in order to resolve public health concerns as soft and hard tissues regeneration (8). Therefore, available literature witnesses that bioactive glasses have been mainly used in the clinic for the partial replacement of living tissues and as porous implants for bone regeneration (3). One of the risks associated with the use of bioactive glasses is the apparition of biomaterial-centred infection produced by different types of microorganisms that are

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capable of forming a biofilm on their surface (5, 10). In most instances, these biofilm-forming bacteria not only are resistant to the body's defence mechanisms but also display decreased susceptibilities to antibiotics (4). Concerning the antibioresistance to bacteria, nowadays more and more studies claim the resistance of both Grams positive and negative bacteria to current classes of antibiotics used in clinical practice as penicillin G, followed by amoxicillin with clavulanic acid and colistin sulphate and tetracycline (7). Therefore, there is now an acute clinical need for developing new antimicrobials capable of inhibiting both types of bacteria (Grams positive and negative), while repairing certain damaged tissues. It has been found that by adding some antibacterial ions like copper, zinc, or silver in the composition of bioactive glasses one can obtain antibacterial bioactive glasses with some special properties which can be extremely interesting for many biomedical applications (8, 17, 21). Among these explored therapeutic ions, the role of copper ions is particularly important in human metabolism due to their angiogenesis, osteogenesis, and antibacterial properties (14). Concerning the mechanism by which copper causes microbial death, the studied literature highlights that the microorganism degradation process is strongly related to the copper's ability to destroy the bacteria cell membrane (9). In several studies, this step is seen as a primary mechanism that leads to the destruction of bacteria (19). Following this primary step, once inside the cell, copper produces reactive oxygen species damaging the proteins, lipids, and eventually all the genetic material, including genomic and plasmid DNA (11,20). Another unique glass property associated with the antimicrobial effect is represented by the rate of degradation. Once placed at the site of infection, glass can act as a localized antibacterial delivery system; where different ions with different properties could be released to obtain a well-targeted action (1). Due to the aforementioned reasons, in the present work we aimed to study the effect of adding CuO on the structural and antibacterial properties of eight bioactive glasses belonging to the system (100-x) $B_2O_3 \cdot 5SrO \cdot 5Al_2O_3 \cdot xCuO$ (mol %) with x= 0; 0.25; 0.5; 1; 1.5; 2; 4; 8, for potential applications in tissue regeneration. The antibacterial properties of this glass system were studied against *Staphylococcus aureus, Escherichia coli, Bacillus cereus,* and *Salmonella enteritidis* strains.

MATERIALS AND METHODS

Samples synthesis

The antibacterial effect and the dissolution behaviour of eight bioactive glasses belonging to the system (100-x) $B_2O_3 \cdot 5SrO \cdot 5Al_2O_3 \cdot xCuO$ (mol %) with x= 0; 0.25; 0.5; 1; 1.5; 2; 4; 8 were studied. Starting oxides used for the glass preparation were mixed in analytical grades using the melt-quenching technique. The mixture of each composition was transferred to corundum crucibles, which were then placed into a furnace at 1260ºC for 15 min. After reaching the wanted temperature, the molten glass was poured onto an aluminium plate and left to cool to room temperature. Finally, the obtained glasses were ground to a fine powder using a mechanical press and a mortar and pestle.

Dissolution tests in distilled water. Microdilution method

In order to identify the dissolution rate of the glass samples, we performed the Maçon's protocol (13). Before starting Macon's protocol, we have measured for each glass sample the actual particle size distribution by using a sieve of 0.075 mm. Therefore, for this protocol, we sized the glass particles of each sample by sieving and used only the particles that had a diameter less than 0.075 mm. According to this protocol, we added 75 mg of glass powders in an airtight polyethylene container containing 50 ml of purified water. Further, the composition was placed in an incubating orbital shaker (120 rpm), at a constant temperature of 37°C, a condition that mimics the *in-vivo* environment as closely as possible. The samples were kept in the incubator for 5 (series 1) and 10 days (series 2), further the glass samples were filtered using filter papers, glass funnels, and Erlenmeyer glasses. The mass of each solid glass sample that remained on the filter paper was let to dry and finally was measured and registered. Finally, the obtained data were graphically represented with Microsoft Excel.

Antibacterial activity. Disk diffusion susceptibility test

In order to test the susceptibility of bacteria to tested antimicrobials, we used the Kirby-Bauer disk diffusion susceptibility test protocol. For each glass sample, we studied the antimicrobial activity against both Gram-positive (*Staphylococcus aureus* reference strain 6538P, *Salmonella enteritidis* reference strain 13076, and *Bacillus cereus* reference strain 11778) and Gram-negative strains (*Escherichia coli* reference strain 25922). The tested microorganisms were obtained from the Department of Microbiology, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania. The pathogenic organisms were grown on Mueller-Hinton (MH) agar with the following composition: 2.0 g bovine extract, 17.5 g casein hydrolysate, 1.5 g starch, 17.0 g agar. In order to obtain the growth of target organisms we incubated the Mueller-Hinton (MH) agar plate at a temperature of 37°C for 24h. Concerning the obtainment of glass disks suitable for this protocol, we used a manual hydraulic pellet press (Specac, USA) which was able to provide a maximum pressure of 10 tons. Therefore, we transferred 3 grams of glass powder into the cavity of the pellet press and further, we applied pressure of 10 tons for 3 minutes. Treating all the glass samples in this manner, we obtained eight glass disks with a diameter of 0.8 cm (Fig. 1A). After the inoculation of glass disks on the MH agar plate (Fig. 1B) at a temperature of 37°C for 24 h, we have measured the diameter of the inhibition zone. For the comparison, we used amoxicillin which was placed in the centre of the agar plate. The 3D graph representation of data was made with the Origin Pro Version 8.5.1 software (OriginLab Corporation, Northampton, MA, USA).

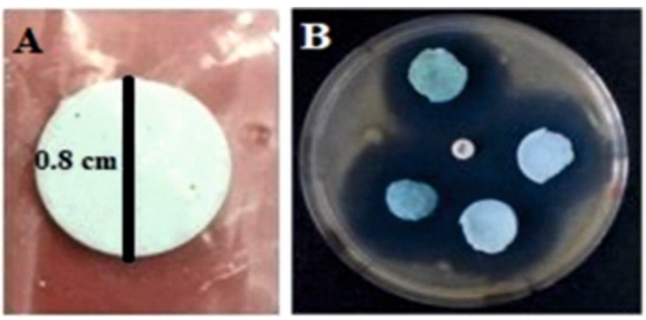

Fig. 1. The obtained glass disk by using the hydraulic press (A); The placement of glass disks on the Mueller-Hinton agar plate (B).

RESULTS AND DISCUSSIONS

Pre-test glass samples characterization

The particle size distribution of the obtained glass samples belonging to the system (100-x) B_2O_3·5SrO·5Al_2O_3·xCuO (mol %) with x= 0; 0.25; 0.5; 1; 1.5; 2; 4; and 8, prepared by the melt quenching technique was smaller than 0.075 mm and their colour was green (Fig. 2). The glass samples were identified by a code number depending on their copper oxide concentration (1→x=0; 2→x=0.25; 3→x=0.5; 4→x=1; 5→x=1.5; 6→x=2; 7→x=4; 8→x=8). The code was used on all data tables and graphs. Furthermore, we noticed that the colour of investigated samples varied with the amount of copper oxide content introduced in the batch (Fig 3). In fact, with the increase in CuO content, the resulting colour is changing, becoming progressively more intense. The colour of a glass is strongly influenced by both coordination and composition of the glass, the presence of Cu^{2+} ions induce the apparition of some special colours like ruby, blue, and emerald green (19). Other researchers have found a positive association between the colour of glass samples and the size of Cu_2O crystals (15).

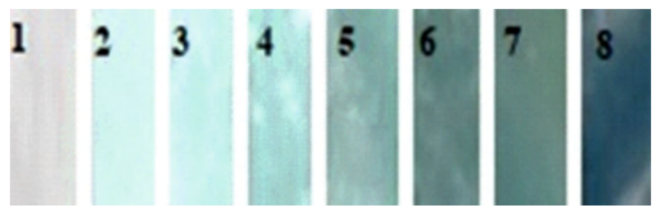

Fig. 3. The color variation of the investigated glass samples

Dissolution test

In order to evaluate the chemical durability of the investigated glass samples, we measured their rate of degradation in purified water.

As we can see in Fig. 4, the dissolution degree is strongly stimulated by the addition of new oxides and respectively, by the exposure time. Therefore, an increase in dissolution rate is observed with increasing CuO content of glass samples (Fig. 4). A dissimilar trend was observed in the study of Neel et al. (2005), in which the phosphate-based glass fibres with varying amounts of CuO presented a decreased degradation as the CuO content increased (1).

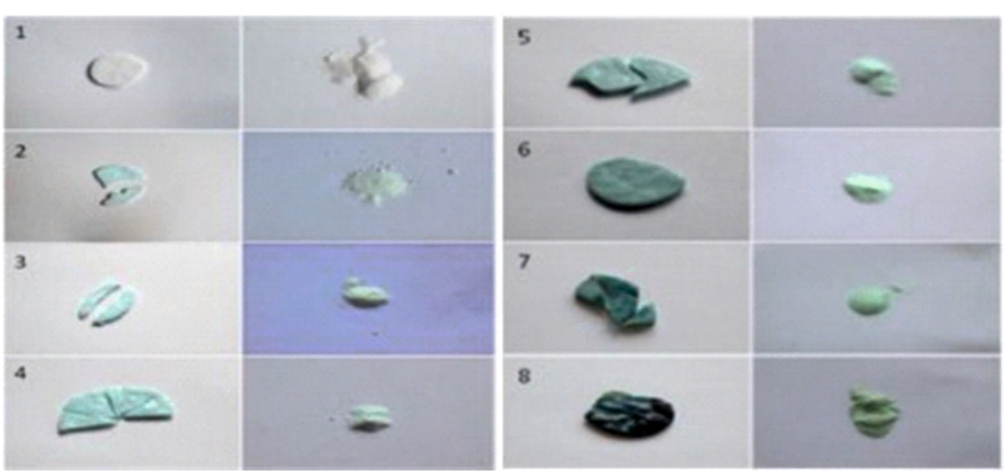

Fig. 2. The glass samples (solids and powder) of the system (100-x)B_2O_3·5SrO·5Al_2O_3·xCuO obtained by the melt quenching technique

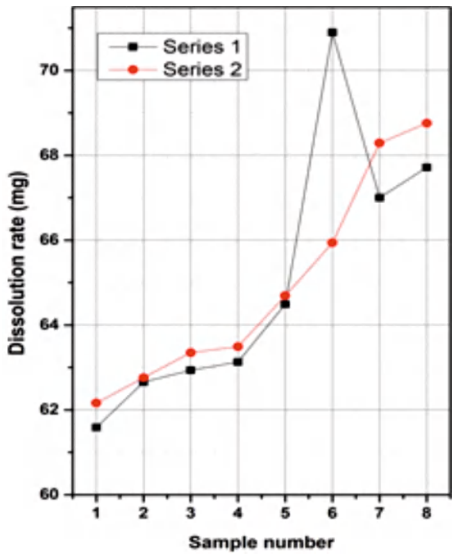

Fig. 4. The graphical representation of the dissolution rate of the investigated glass samples of the system (100-x) B_2O_3·5SrO·5Al_2O_3·xCuO

However, the same article showed that the rate of degradation can be strongly influenced by the glass fibers diameter as with the smaller diameter fibers the authors obtained an increase in the rate of degradation (1).

Therefore, the diameter of glass samples represents an important characteristic that can influence the ion release kinetics and the biological performance of the system.

In our study, the diameter of the investigated glass samples was smaller than 0.075 mm suggesting a possible positive influence on the rate of degradation and implicitly on the biologic properties of the material. Moreover, the glass exposure time to purified water positively influenced the dissolution process. Thus, the dissolution rate was increased in the case of glass samples maintained ten days in purified water (series 2) comparatively with the samples kept only for five days (series 1) (Fig. 5).

Concerning the dissolution process, it was stated that the ion release control from inorganic structures such as glasses favorably influences the materials' bioactive properties and subsequently can increase the possibility of using them in tissue engineering applications (12).

Therapeutic ions such as strontium, copper, and zinc are frequently added to the composition of glasses in order to enhance the material's bioactivity and to induce a positive cell response to degradable materials (16).

Antibacterial test

The antibacterial activity of the glass samples was studied upon *Escherichia coli, Enterococcus faecalis, Bacillus cereus, Staphylococcus aureus, Salmonella enteritidis,* in particular because these bacterial species are capable of determining essential public health concerns in many countries (2, 4). The presence of copper ions in the glass samples determines the apparition of some important biocidal properties. Thus, copper is recognised as a disruptor of bacterial membrane integrity which inevitably leads to loss of cell viability (6).

In order to test the antibacterial effect of the investigated glass samples, we used the disk-diffusion method (fig. 5). In the above view, the antibacterial effect of the investigated glass samples was more significant against *S. aureus* than *E. coli, B. cereus,* and *S. enteritidis* strains. Our results showed that the radius of the inhibition zone is influenced by the concentration of copper oxides in the glass samples. Therefore, we showed that the radius of the inhibition zone of bacterial growth is larger in the case of glass samples with a copper content of 4 and 8 mol% CuO (Fig. 6).

However, the antibacterial effect was evident only in the case of milled glass samples while in the case of dissoluted samples the effect was negative, probably due to insufficient concentration of the active substance.

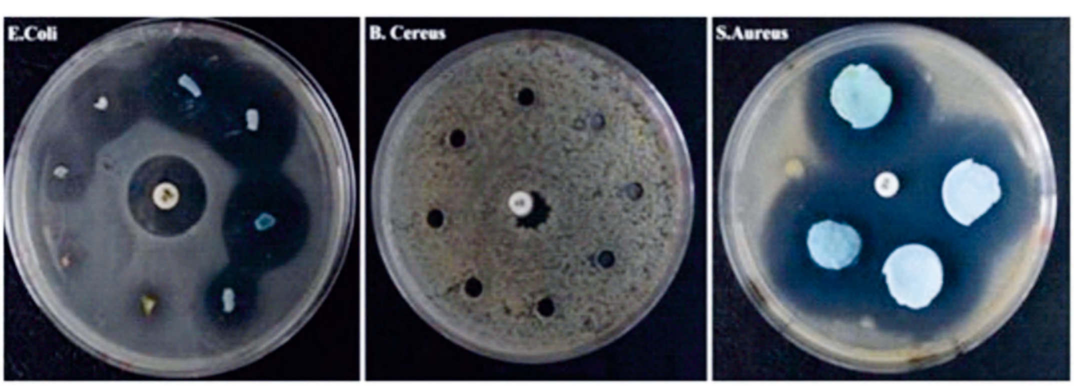

Fig. 5. The disk-diffusion method of glass samples against *E. coli*, *B. cereus,* and *S. aureus* as tested microorganisms

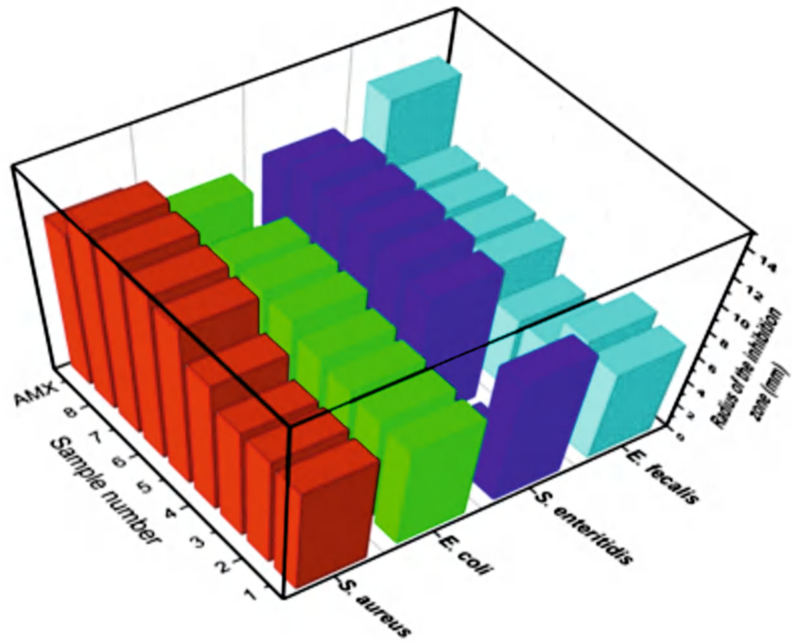

Fig. 6. The results of the disk-diffusion test by measuring the radius of the inhibition zone (mm) of the investigated glass samples

CONCLUSIONS

By using the melt-quenching technique we obtained the $H_2BO_3 \cdot 5SrO \cdot 5Al_2O_3$ glass system with eight different concentrations of copper oxide (0; 0.25; 0.5; 1; 1.5; 2; 4; 8 mol%) in order to find a suitable material with both good structural and antibacterial characteristics that can be used for further *in-vivo* trials involving soft or hard tissues regeneration. The composition of glass and implicitly the content of CuO influences the antibacterial properties of glass as follows. This study also concluded that the inhibitory effect of the investigated glass samples was more evident on *S. aureus* than *E. coli, B.cereus,* and *S.enteritidis* strains. Furthermore, the glass samples with 4 and 8 mol% CuO presented a good antibacterial activity while for the other samples the effect was not so evident. Based on all these findings we can declare that the obtained glass system offers a good perspective for further *in-vivo* trials.

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