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Sol–gel-derived bioactive glass containing SiO_2–MgO–CaO–P_2O_5 as an antibacterial scaffold

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Abstract: Bioactive glass (BG) composites with a base of SiO_2–Na_2O–CaO–P_2O_5 are biocompatible biomaterials. The assessment of their abilities for medical applications has interested researchers. We produced a BG-containing SiO_2–MgO–CaO–P_2O_5 by the sol–gel method. To determine the antibacterial effects, we analyzed the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) properties of this product on three microorganisms, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, known causative agents for biofilm formation on implant surfaces. In addition, we performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to study the cytotoxic effects of our composite on animal cells. Our results demonstrated that our BG product inhibited the growth of bacteria in a concentration-dependent manner without any cytotoxic effects. Therefore, our BG product can be utilized as an appropriate implant for treating bone and tooth defects. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2012.

Key Words: bioactive glass, antibacterial activity, sol–gel process, aerobic bacteria, nasocomial infection

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INTRODUCTION

Bioactive glass (BG) composites are biodegradable and biocompatible biomaterials[1] with based containing different percentages of SiO_2–Na_2O–CaO–P_2O_5[2]; they are extensively under review for medical applications in the dental and orthopedic fields. The study of BG was started four decades ago by Hench and Paschall.[3] According to their report, these composites can be attached to any kind of tissue (soft or hard) through chemical bonds. The main mechanism in hard tissues is the formation of a hydroxycarbonate apatite layer on a surface of the BG scaffolds along with release of calcium ions and silica to the surrounding tissue. In a soft tissue, in addition to precipitating a hydroxycarbonate apatite layer on collagen fibrils and the BG surface, these fibrils can attached to a silica layer through ionic, electrostatic, and/or hydrogen chemical bonds.[3]

Further study showed that this BG was capable of interacting with a tissue surface in many ways, thus causing integration and regeneration of tissues. Hence, they are utilized for bone restoration resulting from periodontal disease and alveolar ridge resorption, as well as conductive tissue defects and filling defects caused by surgery.[4,5]

Increasing infection risk is the main problem after implant applications in humans. Direct contamination of implants is the most frequent route; it occurs during surgery and hospitalization. The "Race for the surface" is the term used to indicate the frequency of biofilm formation and integrity of tissue cells on the surface of implants.[6] When more tissue cells adhere on the implant surfaces, fewer biofilms form. Results from studies performed on infected implants has demonstrated that *Staphylococcus epidermidis* and *Staphylococcus aureus*, together with *Escherichia coli* and *Pseudomonas aeruginosa*, are the most commonly isolated pathogens.[6,7] The percentaged of infected catheters, artificial joints, and heart valves contaminated by *S. epidermidis* and *S. aureus* are reported to be about 50% and 23%, respectively.[8] Additionally, *P. aeruginosa* is an important pathogen in nasocomial infections that causes 12% of urinary tract infections, 10% of bloodstream infections, and 7% of hip joint infection in patients residing at hospitals.[9] Therefore, the usage of components with antimicrobial characteristics is necessary. The antibacterial behavior of BG was noted in several previous studies and is an appropriate characteristic for medical applications. Allan et al.[10]

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observed the antibacterial effects of particular bioglass on microorganisms present in the oral cavity. In addition, BG S53P4 was shown to have growth inhibitory effects on several aerobic[11] and anaerobic[12] bacteria *in vitro*. Recently, Mortazavi et al.[13] reported antibactericidal activity of BG 58S at low concentration against *E. coli*, *P. aeruginosa*, *Salmonella typhi*, and *S. aureus*.

In this study, we synthesized BG nanopowder based on SiO_2–MgO–CaO–P_2O_5 by the sol–gel-derived method and assessed its antibacterial efficacy on *S. aureus*, *E. coli*, and *P. aeruginosa* by minimal bactericidal concentration (MBC) and minimal inhibitory concentration (MIC). To evaluate the cytotoxicity effects of BG on mammalian cells, we performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and the trypan blue staining assay on Cho cells exposed to 20 mg/mL BG nanopowder; the level was chosen as a concentration used in animal bodies.

MATERIALS AND METHODS

BG nanopowder synthesis

To prepare BG powder composed of SiO_2, MgO, CaO, and P_2O_5, the sol–gel method was used. This method includes the techniques which have been applied to synthesize oxides.[14,15] Sol–gel processing is a soft-chemistry method for achieving functional materials at low temperatures. All the chemical materials were purchased from Merck, Germany. Table I lists amounts of each compound used in this product. In brief, tetraethylorthosilicate (TEOS) was added to 0.1 *M* HNO_3 (30 mL) as a catalyst and stirred for 30 min at room temperature to allow hydrolyzing. After 45 min, triethyl phosphate (TEP), calcium nitrate tetra-hydrate, and magnesium nitrate hexa-hydrate were added to mixture and stirred for an hour at room temperature to complete hydrolysis reaction. To form gelatinization products and condensation, the resulted sol was stored in an isolated Teflon container for 10 days. The gel was first dried at 70°C for 3 days and then at 120°C for 2 days. The dried gel was passed through a 90-μm pore size filter (170 meshes). To stabilize the glass structure, the product was heated at 700°C for 24 h. Finally, the BG nanopowder was prepared by ball milling (SVD15IG5-1, LG Company, Germany) for 30 min.

BG nanopowder characterization and analysis

The crystal structure of the nanopowder was assayed by X-ray diffraction (XRD) technique with Cu Kα = 1.54 Å wavelength (Philips, Germany). The resident time and size of each step were 1 s and 0.02°, respectively. The particle sizes of the BG nanopowder were analyzed using transmission electron microscopy (TEM) (Philips CM120operated at 100 kV). A mixed suspension of these particles was prepared in ethanol (0.1 g/10 mL). The particles were deposited on a Cu grid that supports a carbon film.

The morphology and microstructure of the synthesized BG and nanocomposite samples and measurement of pore size were evaluated using scanning electron microscope (SEM). The nanocomposite samples were coated with a thin layer of gold (Au) by sputtering (EMITECH K450X, England), and then their morphology was observed on a SEM (SEM-Philips XL30) that operated at the acceleration voltage of 15 kV.

TABLE I. Amounts of Compounds Used for the Preparation of BG Nanopowder

Chemicals	Amounts
TEOS (SiO_2)	13.13 g
TEP (P_2O_5)	0. 91 g
$Ca(NO_3)_2{\cdot}4H_2O$(CaO)	6.14 g
$Mg(NO_3)_2{\cdot}6H_2O$	1.28 g
HNO_3	30 m*L* (0.1 *M*)

Microorganisms and culture medium

S. aureus (ATCC 43300), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) were selected as representative gram positive, gram negative, and resistant bacteria, respectively. All of the strains were aerobic and were grown at 37°C. Mouler Hinton (MH) broth and MH agar (Merck, Germany) were used as a media to test the susceptibility of the bacteria.

Antimicrobial activity test

To detect the antibacterial concentration of BG, we performed MIC and MBC tests according to Clinical and Laboratory Standards Institute guidelines.[16] Ten different concentrations, including 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, and 1.59 mg/mL of broth, were prepared by adding appropriate amounts of MH broth. One milliliter of each concentration was transferred to a test tube. Then 1 mL of one of three different suspensions with the inoculum concentration equivalent to 0.5 McFarland (almost1 $\times 10^8$ CFU/mL) was added to the appropriate test tube. After 24 h at 37°C, the turbidity of each sample was determined. To evaluate the MBC concentration, 1 mL of each incubated test tube was cultured on an MH agar plate at 37°C for 24 h. Then the bacterial growth was assessed. Each test was performed in triplicate. Tubes without BG nanopowder as a positive control and without bacteria as a negative control were also examined.

Cytotoxicity effects analysis

To evaluate the cytotoxicity effects of BG nanopowders on mammalian cells, we utilized the Chinese hamster ovary cell (Cho cell line), which was purchased from the Pasteur Institute of Iran. These cells were cultured in RPMI1640 medium (Invitrogen) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO_2. After the cells reached 90% confluency, the viable cells were counted with the trypan blue (0.4%, w/v; Sigma–Aldrich, Germany) stain; then 10^4 cells were transferred to each well of the 24-well plates. After 24 h, the supernatant of each well was replaced with the medium containing BG nanopowder with the concentration of 20 mg/mL. To measure the cell viability and proliferation, the MTT assay and trypan blue staining were performed. After 24, 48, or 72 h incubation, the medium of each well was removed, and the wells were washed with phosphate-buffered saline three times. The cells were incubated with 20 μL/well of MTT

(Sigma–Aldrich, Germany) reagent for 4 h at 37°C to allow the mitochondrial enzyme of viable cells, succinate dehydrogenase, to convert the tetrazolium ring to blue formazan. Then the supernatants were replaced with 150 μL dimethyl sulfoxide (Sigma–Aldrich, Germany). The plate was shaken to dissolve the formazan crystals. The optical density of each well was read by Elisa Reader (Tecan, Switzerland) at 540 nm. All the tests were done in triplicate.

When staining the viable cells with trypan blue, the cells were first incubated with 20 mg/mL of BG nanopowder. Then they were collected using trypsin/EDTA and suspended in the new medium. The trypan blue dye was added to the cell suspension at a ratio of 1:1. The numbers of dead and viable cells were counted using a hemocytometer slide.[17]

Statistical analysis

The data from the MTT assay were evaluated by the nonparametric Mann–Whitney test usage SPSS.15 (SPSS, Chicago, IL). Results with a p value less than 0.05 were statistically accepted as significant outcomes.

RESULTS

BG nanopowder characterization

Figure 1 illustrates a graph obtained from XRD analysis of BG nanopowders. The lack of any peaks in this graph is an indicator of the amorphous structure of BG. These data confirm that the sol–gel technique is able to form noncrystalline glasses. The particles of the BG powders, as determined by TEM, had rodlike to spherical features. As shown in Figure 2, the size range of these particles was 20–70 nm. These results confirmed that the synthesized BG nanoparticles were in the nanorange.[18]

The images captured from the surfaces of the porous nanocomposites with SEM (Fig. 3) indicate a network of interconnected pores with a fairly uniform spherical shape in top view and a deformed elliptical shape in the lateral view of the layered nanocomposites. The diameter of these pores has a narrow range that varies between 300 and 500 μm, which is desirable for bone cell growth.

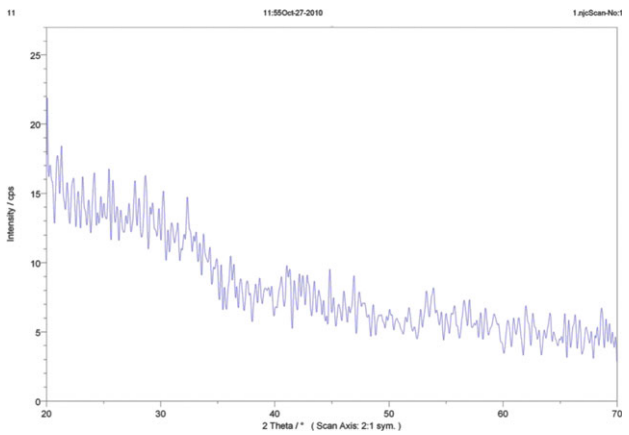

FIGURE 1. X-ray diffraction features of BG nanopowders. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

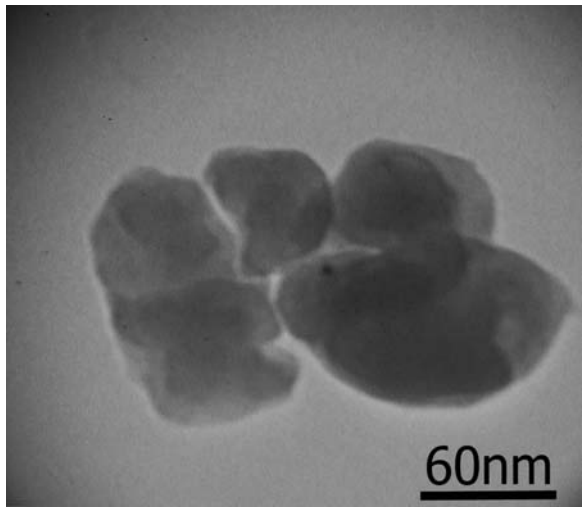

FIGURE 2. TEM images of BG glass nanopowders.

Antibacterial effects of BG nanopowder

The antibacterial effects of BG nanopowder were assessed using three strains of bacteria exposed to different concentrations of this component. The 15.62, 31.25, and 250 mg/mL broths with nanopowders showed bactericidal effects on *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively. This means that, in these concentrations, BG can scarify the bacteria in the test tube (MBC). The BG nanopowders at a concentration of 15.62 mg/mL of broth had inhibitory effects on *P. aeruginosa* and *S. aureus* and completely prevented their growth (MIC). Additionally, we observed no turbidity in tube containing 7.8 mg/mL of broth for *E. coli*. The results are summarized in Table II.

Cytotoxic effects of BG nanopowder

Figure 4 illustrates the results from the cytotoxicity evaluation of 20 mg/mL BG nanopowder as a concentration tested in animal bodies in our previous studies.[19,20] No significant difference was seen between results from the tests and control after 24, 48, or 72 h ($p = 0.05$).

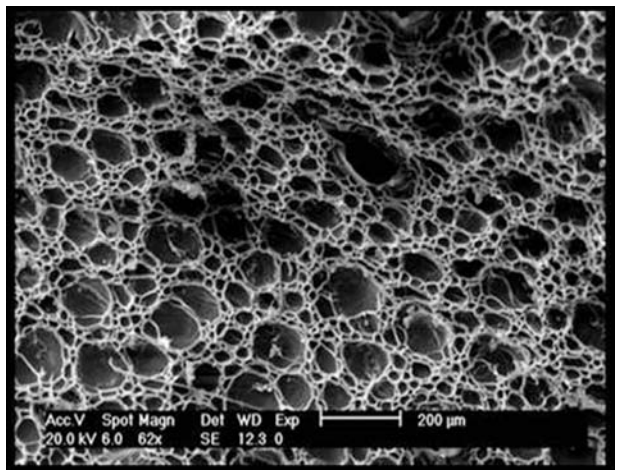

FIGURE 3. SEM micrograph of the lateral view of the modeled BG scaffold.

TABLE II. Results Obtained from Three Strains of Bacteria Exposed to Different Concentrations of BG Nonopowders

Bioactive Glass Con. (mg/mL)	MIC			MBC		
	P. aeruginosa	*E. coli*	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. aureus*
1000	Clear	Clear	Clear	NG	NG	NG
500	Clear	Clear	Clear	NG	NG	NG
250	Clear	Clear	Clear	NG	NG	G
125	Clear	Clear	Clear	NG	NG	G
62.5	Clear	Clear	Clear	NG	NG	G
31.25	Clear	Clear	Clear	G	NG	G
15.62	Clear	Clear	Clear	G	G	G
7.8	Turbid	Clear	Turbid	G	G	G
3.9	Turbid	Turbid	Turbid	G	G	G
1.95	Turbid	Turbid	Turbid	G	G	G

NG, no growth; G, growth.

Trypan blue can pass from the membrane of dead cells and stains them, but the membrane integrity of living cells prohibits the entry of dye. As shown in Figure 5, the viable cells are colorless, but the dead cells are blue. The results from the trypan blue test detected no cytotoxicity effects of our products at 20 mg/mL and confirmed the results obtained from the MTT assay.

DISCUSSION

In this study, we assessed the antibacterial and cytotoxic behavior of some BG nanopowders prepared by the sol–gel method. As described before, hydrolysis and polycondensation are the two main steps of this method; they are performed at temperatures lower than the transition temperature required for forming glass in other methods.[21] Nanopowder preparation is flexible; the nanosize amorphous bodies with micron pores and highly specific surfaces are advantages of this method that can increase the versatility and bioactivity of BG.[22] Results from our XRD and TEM analysis confirmed that our product had a nanosize amorphous structure with a range of 20–70 nm.

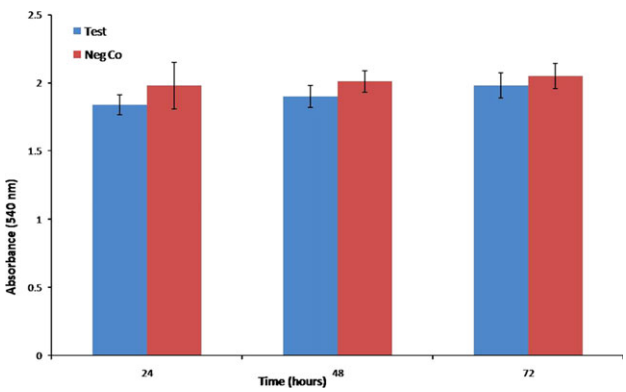

FIGURE 4. MTT analysis results after 24, 48, and 72 h. The cell viability percentage is directly proportional to the absorbance value at 540 nm. The negative control (Neg Co) is a sample without the bioactive nanoglass. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

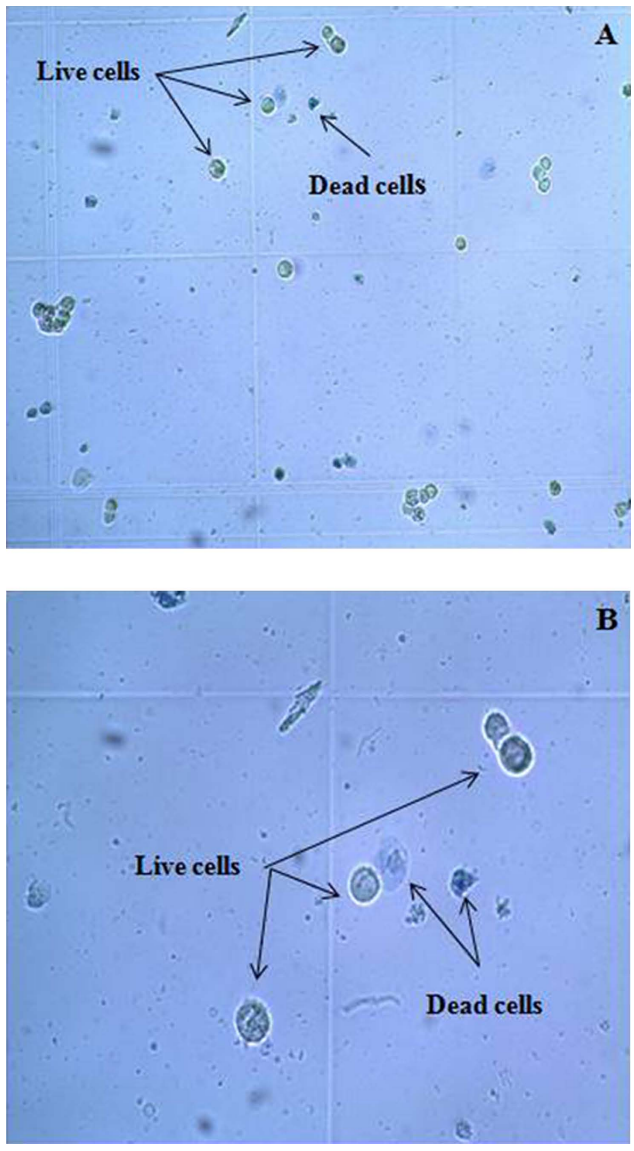

FIGURE 5. Images from the trypan blue staining test. The viable cells are colorless, but the dead cells are blue. (A) An image at ×10. (B) An image at ×20. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

SiO_2, Na_2O, CaO, and P_2O_5 are the main compounds in BG composites with varying weight percentages. Different studies have used diverse metals to assess the characteristics and behaviors of these new composites. Using silver for medical applications is toxic for cells because releasing a high level of silver ions in tissues near implants has negative effects on wound healing and bone integration and restoration.[23,24] MgO is an unconventional compound for synthesizing BG us intended for biological applications. Saboori et al.[25] reported that MgO can enhance bone cell proliferation and differentiation and had no effect on the preparation and thermal processing steps. The usage of MgO substitutions for CaO can improve nanobioglass stability and mechanical properties.[26] The presence of MgO can inhibit osteoporosis and bone brittleness and enhance bone restoration.[25,27] Munukka et al. and Lepparanta et al. have made several BG composites containing differing amounts of MgO (up to 6%) and have evaluated their antimicrobial effects on broad-spectrum bacteria. They found less efficacy in antibacterial behavior.[11,12]

Despite the similarity of the MgO amount in our product and these previous studies, our product demonstrated intensive antibacterial effects even in the absence of silver. In agreement with the previous studies, the antibacterial effects of our product were dose dependent.[10–13] The BG nanopowder concentrations, we selected in this study, were lower than in previous studies. For instance, in the studies performed by Stoor et al.[28] and Allan et al.,[10] 1.67 g/mL liquid and even higher concentrations were evaluated. Moreover, in contrast to results reported by Catauro et al.,[29] our product had growth inhibitory properties without they addition of silver. Catauro et al. stated that the presence of silver in the BG structure was necessary for antibacterial activity. Our results show that silver is not an essential component for inhibiting bacterial growth. It is possible that the release of any ions present in composites is able to create free radicals or increase the pH value, which in turn can cause bacterial death. Further analyses are necessary to verify this assumption.

As described above, *S. aureus*, *E. coli*, and *P. auroginosa* are all microorganisms that can lead to dangerous and resistant nasocomial infections following the contamination of implants and external device surfaces pre- and postoperatively.[6] Constructing antimicrobial structures with nontoxic materials is essential for medical applications, because they are able to limit biofilm formation, decrease hospital infections, and morbidity. In our study, data obtained from MTT analysis showed that there were no cytotoxic effects on the viability and proliferation properties of cells within 72 h. The ability of our composite to inhibit growth of the three tested microorganisms at low concentrations is a significant advantage for improving treatment conditions.

Substantial differences were seen in bactericidal effectiveness between tests done on *S. aureus* as a gram positive bacterium and *E. coli* and *P. auroginosa*. These results are similar to the findings reported by Hu et al.[30] The presence of high amounts of peptidoglycan in the cell wall of gram positive bacteria as a firm layer can create a barrier to the entrance of BG nanopowders. Of course, further assessment must be done for confirming this result.

In conclusion, the sol–gel method can produce BG nanopowders with an appropriate nanosize and noncrystalline structure. In addition to the antibacterial properties in low concentrations of the described product, the omission of silver ions in its composition and the lack of a toxic effect on mammalian cells make this product a desirable implant base for repairing bone and tooth defects.

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