Antibacterial effect of morphous (poly-crystalline) and amorphous (glass) nano-bioactive glass 45S5 on *Streptococcus mutans*

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Abstract

Objective: Bioactive glass 45S5 is a surface reactive glass-ceramic biomaterial, developed in 1969. BAG 45S5 with particle size of 20-60 nm has the ability of bone regeneration, broad spectrum antibacterial effect, repairs and replaces diseased or damaged bone. The aim of this study was to evaluate the antibacterial activity and determine MIC and MBC values of nano-BAG45S5 on *Streptococcus mutans*.

Methods: In this study the *in vitro* Antibacterial activity of polycrystalline and glass forms of nano-BAG 45S5 was evaluated. Bacterial susceptibility to test materials was examined by antibiogram test. Afterwards MIC and MBC assays were conducted via broth dilution, disc diffusion and colony count methods.

Results: Despite amorphous nano-BAG 45S5, poly-crystalline form had antibiogram negative test result. In broth dilution test, the optical absorbance of test dilution of 50mcg/ml and higher concentrations were equal to negative control's optical absorbance and their inhibitory zone diameter were measured 10.0mm in disc diffusion test. No colony was observed on the culture media of test dilution of 200mcg/ml and higher concentrations.

Conclusion: *Streptococcus mutans* (ATCC 35668) is not susceptible to poly-crystalline nano-BAG45S5. Amorphous nano-BAG45S5 is bacteriostatic against *Streptococcus mutans*. MIC and MBC values for amorphous nano-BAG45S5 were 50 ppm and 200 ppm, respectively.

Key words: MBC, MIC, Morphology, Nano-BAG45S5, *Streptococcus mutans*.

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Introduction:

Bioactive glass is a surface reactive glass-ceramic biomaterial which was first developed by Larry Hench and colleagues at university of Florida in 1969. Bioactive glass is widely being used as implant material in damaged bone replacement and repair due to its high bio-compatibility (1). Bioactive glass 45S5 (BAG45S5) is an implant material produced in 1971 which can attach to bone and soft tissues (2) and induce healing process of the lesions in soft tissues (3, 4). The recent studies revealed that bioactive glass which was synthesized via sol-gel method had considerable antimicrobial activity against classic clinical pathogens (5, 6). Studies carried on micron BAG45S5 showed that the material had a considerable antibacterial effect on sub- and supra-gingiva pathogens (7). Furthermore, it causes a decrease in colonization of some of the oral (8) and skin (9) pathogens. Bioactive glass of SiO_2-Na_2O-CaO-P_2O_5 structure releases basic ions like sodium, calcium and silica (10) in aqueous medium

which results in increased pH (11). Moreover, Hu *et al.* (2009) showed that BAG particles can destroy the bacteria cell wall by attaching to it (12). Therefore, a decrease in BAG particle size enhances the exchange surface area with the surrounding aqueous medium leading to an increase in basic ion release (10), which results in enhancement in antimicrobial efficacy.

An study done by Waltimo *et al.* (2007) on *Enterococcus faecalis* compared nano-BAG45S5 (20-60 nm) with micron BAG45S5 and revealed that nano-BAG45S5 had a greater antibacterial activity because smaller particle size increases the release of basic ions due to the increased exchange surface area and showed that a direct relationship exists between antimicrobial activity of BAG45S5 and its particle size (13).

Previous studies evaluated the antibacterial effect of micron sized BAG45S5 on some oral and non-oral pathogens, none was conducted on *Streptococcus mutans*. In just one study the impact of reduction in particle size to nanometric was evaluated on *Enterococcus faecalis* while the antibacterial potency was not measured. The impact of morphologic shape which is responsible for basic ion release and affects the efficacy and potency of antimicrobial characteristics of the material (1) has not been studied. In the present study antimicrobial activity of 2 morphologic forms of nano-bioactive glass 45S5 (poly crystalline and glass forms), with particle size of 20-60 nm, which was synthesized for the first time by Amir Kabir University, through a special method, was evaluated on *Streptococcus mutans*, one of the most important oral pathogens responsible for dental carries, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays were conducted in order to measure the antimicrobial potency.

Methods:

The powder of morphous and amorphous nano-BAG45S5 of SiO_2-Na_2O-CaO-P_2O_5 structure and standard species of *Streptococcus mutans* (ATCC 35668) were used. The standard population was solid cultures containing standard species of *Streptococcus mutans*. The sampling method was goal-based and the cultures were randomly appointed to 9 groups and 9 cultures were randomly selected.

Antibiogram Test:

Suspensions of test materials with concentration of 200 mcg/ml were prepared in phosphate buffer (pH=7.2). After stirring, paper discs which had been soaked in supernatant of each sample were placed on the Muller Hilton Agar (MHA) plate which had been cultured with *Streptococcus mutans* prior to the test and incubated in 37°C for 24 hours. Amoxicillin paper disc of 25 mcg was used as positive control.

MIC Assay via Broth Dilution:

The supernatant of the material, with antibiogram positive test result, was added to tubes containing Muller Hilton Broth (MHB) to prepare serial dilutions of 200 mcg/ml, 50 mcg/ml, 25 mcg/ml, 12.5 mcg/ml, 6.25 mcg/ml, 3.125 mcg/ml, 1.56 mcg/ml, 0.78 mcg/ml.

20 µl of *Streptococcus mutans* fresh suspension was added to the test and positive control tubes to make turbidity equal to 0.5 McFarland. Tubes were incubated a long with negative control (containing MHB and phosphate buffer) in 37°C for 24 hours. The turbidity of tubes, which is an indication of bacterial growth, was measured with spectrophotometer in 600 nm wave length (14) and MIC value was determined.

MIC Assay via Disc Diffusion:

Paper discs had been soaked in serial dilutions of test materials, and Amoxicillin paper disc of 25 mcg, as positive control, were placed on the MHA, which had been cultured with *Streptococcus mutans* prior to the test, and incubated in 37°C for 24 hours. The inhibitory zone diameters were measured (14).

MBC Assay:

Clear tubes with no bacterial growth in broth dilution test were cultured on MHA and colonies were counted after incubation in 37°C for 24 hours.

All the tests were carried out 3 times. Antimicrobial activity of the test materials was evaluated via MIC, MBC assays and MBC/MIC ratio, and reported in ppm (14).

Data analysis and the linearity of relationship between optical absorbance and serial dilutions, inhibitory zone diameter and serial dilutions were examined by two-way ANOVA test, and LSD test in case of significant difference.

Results:

In the present study, the antibacterial activity of morphous (poly-crystalline) and amorphous (glass) forms of nano-BAG45S5, synthesized by Amir Kabir University in Tehran, was evaluated on *Streptococcus mutans* via broth dilution and disc diffusion methods after 24 hours of incubation.

There was no inhibitory zone in antibiogram test for poly-crystalline nano-BAG45S5 (200 mcg/ml) (Figure 1) while inhibitory zone diameter for amorphous nano-BAG45S5 and positive control were 12.0 mm and 25.0 mm respectively (Figure 2).

The turbidity of tubes declined as concentration of amorphous form increased in a linear pattern (Diagram 1), in broth dilution test. The results are shown in table 1.

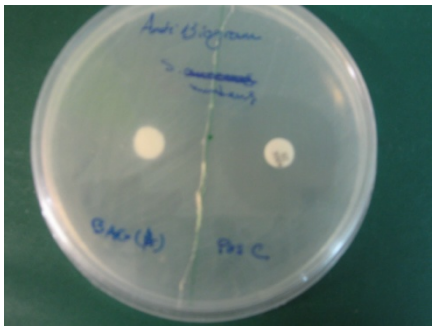

Figure 1- Antibiogram Test, Poly-crystalline nano-BAG45S5 (left) and Amoxicillin (right)

Data analysis by two-way ANOVA test showed a significant statistical difference between antimicrobial activities of the test material in various dilutions. Further analysis by LSD test approved the result (p<0.05). MIC value of amorphous nano-BAG 45S5 for *Streptococcus mutans* was 50 ppm.

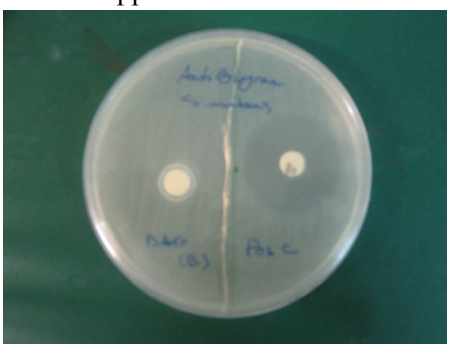

Figure 2- Antibiogram Test, Amorphous nano-BAG45S5 (left) and Amoxicillin (right)

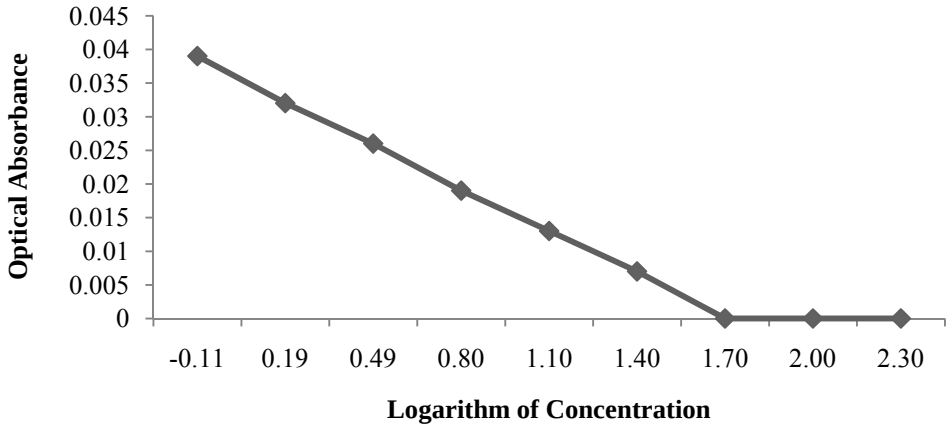

Diagram 1- Optical absorbance vs. logarithm of concentration. r= -0.99, p-value< 0.05

Table 1- Mean of absorbance of serial dilutions. p-value< 0.05 r= -0.99,

Concentration mcg/ml	Optical absorbance
Negative Control	0
200	0
100	0
50	0
25	0.007
12.5	0.013
6.25	0.019
3.125	0.026
1.56	0.032
0.78	0.039
Positive Control	0.112

In disc diffusion test, inhibitory zone diameters were reported in mm. Data were analyzed by two-way ANOVA and LSD tests. There was a significant statistical difference between the inhibitory zone diameters of various dilutions (p<0.05) and linear relationship pattern existed (Diagram 2). The results are shown in table 2.

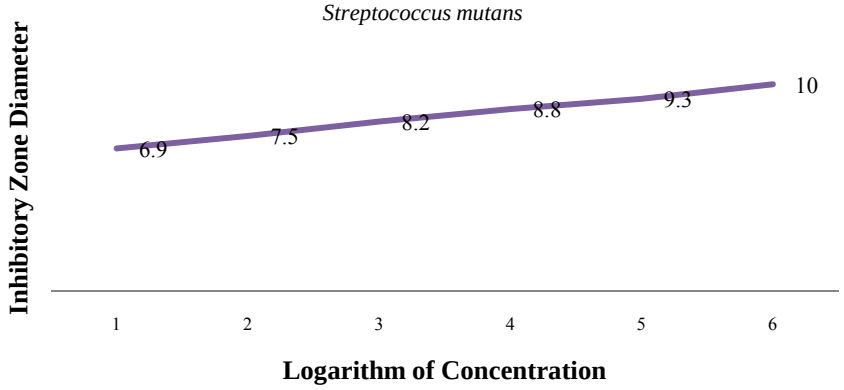

Diagram 2- Inhibitory zone diameter vs logarithm of concentration after 24h of incubation. r= 0.9, p< 0.05

Table 2- Mean of inhibitory zone diameters after 24 hours of incubation. r = 0.9, p-value< 0.05

Concentration mcg/ml	Inhibitory zone diameter
200	10.0
100	10.0
50	10.0
25	9.3
12.5	8.8
6.25	8.2
3.125	7.5
1.56	6.9
0.78	
Positive Control	25.0

Inhibitory zone diameter of positive control (Amoxicillin 25 mcg) was measured 25.0 mm. Inhibitory zone diameter of test dilution of 50 mcg/ml and higher concentrations were 10.0 mm (Figure 3).

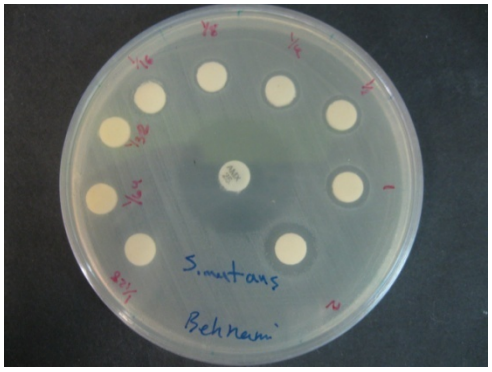

Figure 3- MIC Assay, Disc Diffusion Method

Results from colony count showed that as the concentration of test material increased, the number of colonies on the solid culture medium declined significantly. MBC value of amorphous nano-BAG45S5 for *Streptococcus mutans* was obtained 200 ppm. There was no colony on the culture of negative control while the positive control's culture had the most colonies (Table 3) (Figure 4).

Table 3- Colony count results

Concentration mcg/ml	Colonies
200	0
100	175
50	735
25	1540
12.5	
Negative Control	0

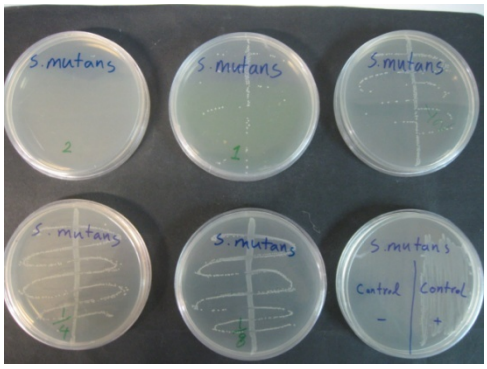

Figure 4- MBC Assay, Colony Count Method

Discussion:

Bioactive glass is a novel material with significant antimicrobial activity against some known oral, skin, etc. pathogens that destroys the cell wall by increasing the pH of aqueous environment through releasing basic ions (5-9). Adding silver to the material enhances its antimicrobial efficacy (15). It has been revealed that decrease in particle size from micron to nanometric increases the antimicrobial efficacy due to increase in exchange surface area between the material and surrounding environments and as a result the release of basic ions increases (10).

Several studies have been conducted in order to evaluate the antimicrobial activity of micron sized BAG45S5 against a broad spectrum of oral and non-oral pathogens (5-12).

Waltimo *et al.* (2007) studied and compared the antibacterial properties of nanometric and micron BAG on *Enterococcus faecalis*, an important oral pathogen. Results showed a dramatic increase in anti-bacterial activity of nano-BAG (13).

Most of the studies carried out on BAG45S5 aimed to evaluate the antimicrobial activity of micron particle size while in the present study the antimicrobial potency of nano-BAG45S5 (20-60 nm) against *Staphylococcus mutans* – one of the most important dental carries pathogens (16) – was evaluated along with the impact of crystalline morphology on its antimicrobial activity. For this purpose, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration were measured. MBC/MIC ratio was calculated to determine whether the material is bactericidal or bacteriostatic.

Results from antibiogram test show that poly-crystalline nano-BAG45S5 synthetized through a special sol-gel method by Amir Kabir University of Tehran, has no antibacterial effect on *Streptococcus mutans* due to no inhibitory zone on the culture medium which makes

potency evaluation, MIC and MBC assays unessential. On the other hand, the glass nano-BAG45S5 had an inhibitory zone comparable to the positive control's inhibitory zone which approves its antimicrobial activity against *Streptococcus mutans*.

Positive result of antibiogram test made glass form of nano-BAG45S5 a subject to Broth Dilution, Disc Diffusion and Colony Count Methods in order to determine MIC and MBC values. The obtained data indicates that MIC and MBC values of amorphous nano-BAG45S5 against *Streptococcus mutans* are 50 and 200 ppm, respectively. Therefore, MBC/MIC ratio is 4 which means that amorphous nano-BAG45S5 is bacteriostatic (17) against *Streptococcus mutans*.

In previous studies, the impact of particle size and basic ion exchange surface area which affects the pH of the aqueous environment was studied and a direct relationship between the surface area and the antimicrobial activity was discovered (13). In the present study, the impact of morphologic shape of nano-BAG45S5 was evaluated. The results shows that the antimicrobial activity of nano-BAG45S5 can be dramatically influenced by its crystalline morphologic form in a way that shifting from glass form to poly-crystalline form can make the material inactive against bacteria. Further studies are required to be conducted to discover the relying reasons of this experiment.

Conclusion:

The results obtained in this study showed that *Streptococcus mutans* is not susceptible to polycrystalline nano-BAG45S5 whereas the glass form (amorphous form) of nano-BAG45S5 is bacteriostatic against *Streptococcus mutans***.**

Conflict of Interest: "None Declared"

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