 DOI: 10.21608/adjalexu.2021.57041.1152

ANTIBACTERIAL CHARACTERIZATION OF NANOPARTICLES BIOACTIVE GLASS COATING ON TITANIUM SURFACE FOR BIOMEDICAL APPLICATIONS (IN VITRO STUDY)

Maged A. Gad[1]* *BDs* Rahaf M. Darwish[1] *BDS,* Ahmed M. Alshimy[2] *PhD,* Mohamed A. Gepreel[3] *PhD,* Mona K. Marei[4] *PhD*

ABSTRACT

INTRODUCTION: Titanium implants are used vastly in dentistry owing to their durability, biocompatibility and mechanical strength making them convenient for high mechanical load implementations. However, titanium surfaces are not bioactive and do not provide effectual bonding with the host bone resulting in loosening and subsequent failure at the bone-implant interface. The coatings of bioactive glass (BG) are used to alter the implant surface chemistry to boost cell functions that promote better osseointegration.

OBJECTIVES: This study aimed to evaluate the antibacterial activity of BG nanoparticles coating deposited to modify titanium surface to enhance osseointegration.

Methodology: Twenty-four titanium-alloy (Ti-6Al-4V) substrates were sandblasted, ultrasonically cleaned and divided into 4 equal groups (A, B, C and D). A and B were the control groups while C and D were coated with 70 mol% silica oxide –30 mol% calcium oxide (70S30Ca) BG nanoparticles using electrophoretic deposition technique (EPD). The BG was prepared by modified sol-gel technique then milled into nanoparticles. The antibacterial action of the obtained coatings against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) bacteria was characterized 24 h following incubation at 35°C using well diffusion susceptibility test.

RESULTS: After incubation, both control and test specimens were showing normal bacterial growth with no observed inhibition zones for both types of bacteria.

CONCLUSIONS: The deposited 70S30Ca BG coating on titanium-alloy substrates using EPD was showing no antibacterial effect against S. aureus and E. coli.

KEY WORDS: Bioactive glass, Bioactive coating, Nanoparticles, Titanium Implant, Electrophoretic deposition.

RUNNING TITLE: Antibacterial activity of bioactive glass coated titanium surface.

---

1. Instructor of Prosthodontics, Researcher at tissue engineering laboratories, Faculty of Dentistry, Alexandria University, Alexandria, Egypt
2. Professor of Prosthodontics, Faculty of Dentistry, Alexandria University, Alexandria, Egypt
3. Professor of Materials Science and Engineering, Egypt-Japan University of Science and Technology (E-JUST), Alexandria, Egypt
4. Professor of Prosthodontics, Head of tissue engineering laboratories, Faculty of Dentistry, Alexandria University, Alexandria, Egypt

* *Corresponding Author:*
Email: Maged.gad@alexu.edu.eg

INTRODUCTION

The most commonly used materials for the fabrication of bone implants under biomechanical loading conditions are titanium and its alloys owing to their biocompatibility, minimal toxicity, excellent mechanical and corrosion resistance properties (1). However, the bio-inert nature of titanium and the surrounding tissue inflammation are main issues for their clinical outcomes. Being bioinert, titanium cannot have interactive osteointegration with the host tissue due to its low bioactive characteristics (2). The orthopedic and dental implants outcome is affected by several factors, including bone-implant osseointegration and the amount of bacteria aggregated around the implants. Past studies have implied that inflammatory disorders affecting dental implants may result in peri-implantitis, loss of bone support and, consequently, decrease in long-term implant survival. Infected implants with their sequential loosening are most frequent in titanium implants and still an issue in dental practice (3).

For this reason, surface modifications of titanium have gained attention for the past years to improve biological surface properties and enhance the osseointegration process (4). They include either, subtractive or additive surface modification. Subtractive methods include titanium surface itself to be changed chemically (e.g., acid etching), physically (e.g., plasma etching), or mechanically (e.g., sandblasting). In comparison, additive methods or what is called surface coating include adding a new material to the substrate surface, resulting in combined advantages of both the substrate and the coating material. The main benefit of the subtractive approach is the absence of the chance of separation as the surface does not have any material applied. However, in contrast to the

coating process, its effects are more minimal on surface properties as a wider range of surface properties can be provided with different coating materials (5).

Although several materials are used for titanium implant coating, bioactive glass (BG) promotes direct bond at the tissue/implant interface with the absence of scar formation. Unlike hydroxyapatite, which degrades slowly, BG can be tailored to be degraded at a rate similar to that of tissue formation. They exhibit significant osteoconductive (suitable surface for bone growth), and osteoinductive (induction of osteoblasts differentiation) properties (6).

Based on processing technique, BGs are classified into two main types: melt quench and sol-gel derived glasses. The later consists of an amorphous structure with nanoporosity that provides greater surface area of the glass, hence enhanced bioactivity. Many sol-gel BGs were developed according to their chemical composition. Modified sol-gel 70S30Ca BG (70 mol% silica oxide –30 mol% Calcium oxide) is being a point of interest as it demonstrated interconnected pores in the macro and nano size ranges, which are favoured by the cells that lead to enhanced cellular response and tissue biocompatibility (7, 8). In addition, 70S30Ca BG was found to provide the highest level of bioactivity among the CaO–SiO2 (binary BG) system, (9).

The higher the surface area of the glass, the greater the dissolution rate and the greater the bioactivity will be. Reducing the size of the material into nanoparticles is one of the ways to increase its surface area. This allows for material shaping versatility and permits the material to be internalized by various cell types. For this reason, BG nanoparticles (20-100 nm) are currently considered a very attractive material for surface coating (10).

In recent years, nano-surface alteration has been applied to enhance the effectiveness of titanium implants, however, minimal in-vitro research provides knowledge regarding their biological capability. Titanium surfaces with improved nanoscale surface characteristics showed enhanced osteoblastic cell proliferation, attachment, alkaline phosphatase activity and upregulated gene expression of bone-related proteins, relative to surfaces with micro-characteristic features (11).

Several requirements are necessary for a successful implant coating, it should be biocompatible with host tissues not to cause a harmful tissue reaction. In addition, high bioactivity of a coating material is required for rapid osseointegration to enable early loading of dental implants. Also, biodegradability of the coat should be at a rate which is similar to that of bone formation to ensure a strong bone implant contact after degradation. Moreover, it should have an antibacterial effect for a sterile osseointegration process. Finally, it should produce an environment that is favourable by the cells to adhere and differentiate such as, hydrophilicity and surface roughness (12).

Among various developed coating techniques, electrophoretic deposition (EPD) technique has superior properties such as: its low cost, simple methodology, ability to produce coatings with controllable thicknesses and the ability to coat irregularly shaped or porous objects uniformly. However, the main disadvantage of EPD is the need for post-deposition thermal processing to densify the coat (13).

The purpose of the current study was the evaluation of antibacterial activity of the deposited sol-gel derived 70S30Ca nanoparticles BG coating on titanium substrate using EPD technique.

The null hypothesis of this study was that the obtained BG coat would not have antibacterial effect.

MATERIALS AND METHODS

The protocol of this study was reviewed and approved by the staff members of the Prosthodontic Department and Research Ethics Committee at the Faculty of Dentistry, Alexandria University (IRB NO: 00010556 – IORG 0008839). The study was an in-vitro (laboratory-based) research and was conducted in Alexandria University and Egypt Japan University of Science and Technology (E-JUST), Alexandria, Egypt.

Nano-bioactive glass powder and suspension preparation (Fig. 1):

70S30Ca BG was prepared using modified sol-gel technique following the technique published before (14). The following chemicals were used in the preparation: Polyethylene Oxide **(Sigma-Aldrich, Egypt)**, Acetic Acid **(Sigma-Aldrich, Egypt),** Urea **(Loba Chemie, Mumbai, India),** Tetramethyl Orthosilicate **(Sigma-Aldrich, Egypt),** Calcium Nitrate Tetrahydrate **(Loba Chemie, Mumbai, India),** Hydrofluoric Acid **(Loba Chemie, Mumbai, India)** and Ammonia **(El-Gomhouria Co., Egypt).** The prepared BG was then milled into nanoparticles (<30 nm) using ball-milling for 40 h **(Planetary Ball Mill PM 400 - RETSCH, Germany)**. The prepared BG nano-powder in addition to pure ethanol **(Alalamia Co., Egypt)** were used to prepare nonaqueous suspension of BG nanoparticles (1 g glass particles / 60 mL ethanol).

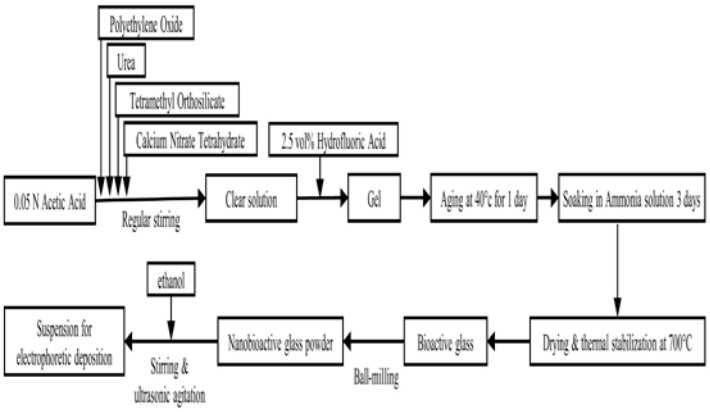

Figure 1: Synthesis of the modified sol-gel derived bioactive glass nanoparticles and the suspension preparation.

Characterization of the prepared BG nanoparticles:

The particle size of the obtained powder was characterized using transmission electron microscope (TEM) **(JEM-2100F, JEOL Ltd., Japan)**.

Functional groups were determined by using Fourier transform infrared (FTIR) spectroscopy analysis. The analysis was conducted in the range of 450–4000 cm^{-1} using a FT-IR BXII spectrometer ***(Perkin-Elmer, USA)***.

Titanium substrates preparation and sample grouping (Fig. 2):
A total of 24 machined titanium substrates (Ti-6Al-4V) were prepared in the shape of discs with a diameter of 10 mm and 1.5 mm thickness, sandblasted and ultrasonically cleaned in ethanol for 10 mins then divided into four equal groups (2 control and 2 test) of 6 specimens each which were all assessed for antibacterial activity as showed in (Fig. 2).

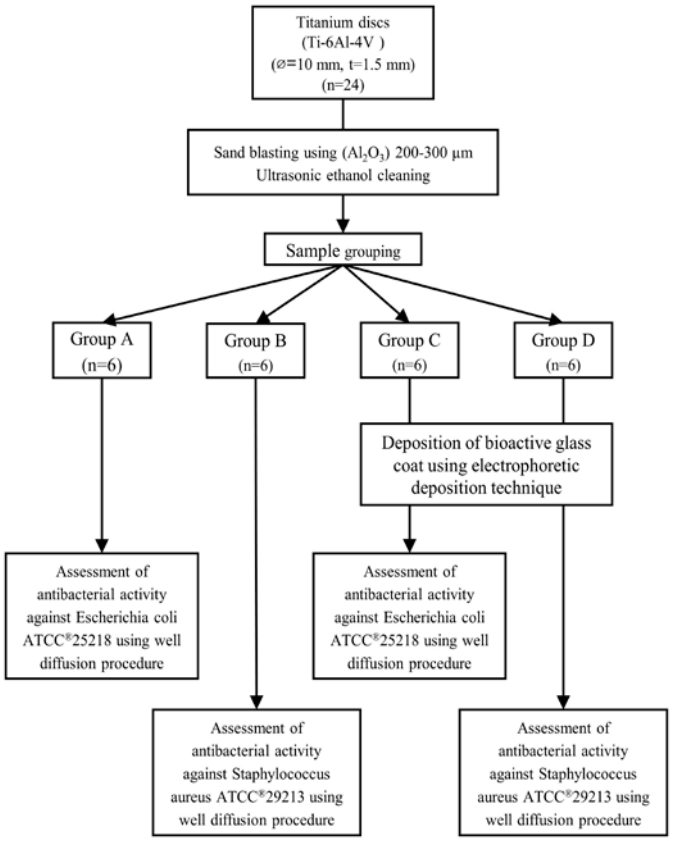

Figure 2: Sample groping of the prepared titanium substrates.

Electrophoretic deposition of bioactive glass nanoparticles on titanium surface (15):
The prepared BG suspension was used for surface coating process of the titanium substrates (group C and group D) via EPD using a DC power supply with controlled voltage as seen in (Fig. 3). Following the deposition, the resulting coatings were exposed to heat processing at 700°C for 2 h.

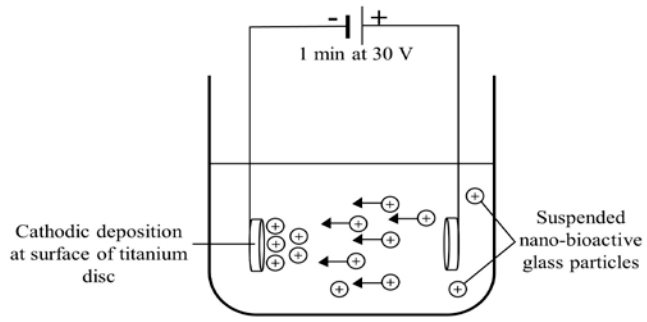

Figure 3: Electrophoretic deposition cell used for BG nanoparticles coating on the titanium substrate.

Antibacterial study:
The experiment was performed as per the protocol of disc diffusion susceptibility test by Kirby-Bauer (16) and following the guidelines of European Committee on Antimicrobial Susceptibility Testing (17) and Clinical and Laboratory Standards Institute (18).
Staphylococcus aureus - ATCC 29213 (S. aureus) and Escherichia coli - ATCC 25218 (E. coli) bacteria picked up from LGC Standards ***(GmbH – Germany)*** were used in the examination of the antibacterial action of titanium substrates with and without BG nanoparticles coating via well diffusion method.
For inoculum suspension preparation, S. aureus and E. coli were adjusted using saline to 0.5 McFarland standard of turbidity (Fig. 4A). Six holes were created in previously prepared Mueller-Hinton Agar (MHA) ***(CM0337-Oxoid Ltd, UK)*** plates using a sterile customized punch of the same diameter of the titanium discs. A template was used to guide the holes placement in order to maintain spaces between discs (not closer than 24 mm from center to center) as recommended to avoid zones overlapping that may prevent reliable zone diameters measuring (Fig. 4B-C).
Then the bacterial suspension was inoculated in the MHA plate using cotton swab that was streaked evenly over the entire dry MHA surface three times; by rotating the plate about 60° each time to allow an equal spread of the inoculum (Fig. 4D). Titanium discs with and without the coating were placed in the holes using sterile disposable forceps then gentle pressure was applied over the discs to ensure intimate contact with the agar surface (Fig. 4E-F). Plates were then located in an incubator ***(ICT 52 FALC Co., ITALY)*** for 24 h at 35°C. Following the incubation period, the plates were examined for the formation of inhibition zones around the discs.

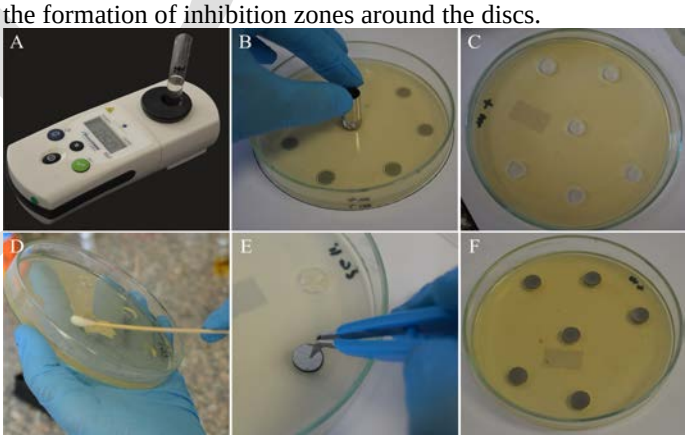

Figure 4: Preparatory steps for the antibacterial susceptibility test
A- Turbidity adjustment of the inoculum suspension using turbidity meter device.
B- Customized punch used to create holes for the placement of titanium discs guided with previously prepared template.
C- Agar plate with six holes kept apart from each other to avoid inhibition zones overlapping.
D- Inoculation of the Muller Hinton Agar plates with bacterial suspension.
E- Confirming intimate contact of the disc with agar surface.
F- Titanium discs seated in the places.

RESULTS

Characterization of the prepared BG nanoparticles:

TEM image of the prepared powder (Fig. 5A) is showing that the BG powder was agglomerated with nano sized particles (<30 nm). The infrared absorption spectra of the prepared nanoparticles (Fig. 5B) are showing the characteristic peaks of SiO_2. The peaks observed at 1074 cm^{-1} assigned to Si–O–Si asymmetric stretching vibration and at 795 cm^{-1} assigned to the Si–O–Si symmetric stretching vibration. The peak at 468 cm^{-1} assigned to Si–O–Si bending mode. The peak at 1636 cm^{-1} and the broad band at 3451 cm^{-1} correlates to the physically adsorbed water bending and stretching modes respectively (19). These findings show that condensation reaction between Si–OR groups is successful.

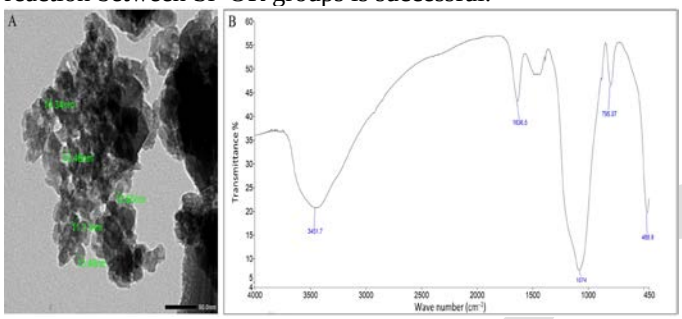

Figure 5: Characterization of the synthesized bioactive glass nanoparticles

(A) TEM image of prepared powder and (B) its corresponding FTIR spectra peaks.

Antibacterial study:

Following the incubation period of 24 h at 35°C, both control (uncoated) and test (coated) groups were showing normal bacterial growth with no observed inhibition zones for both S. aureus and E. coli bacteria, indicating the absence of any antibacterial activity (Fig. 6).

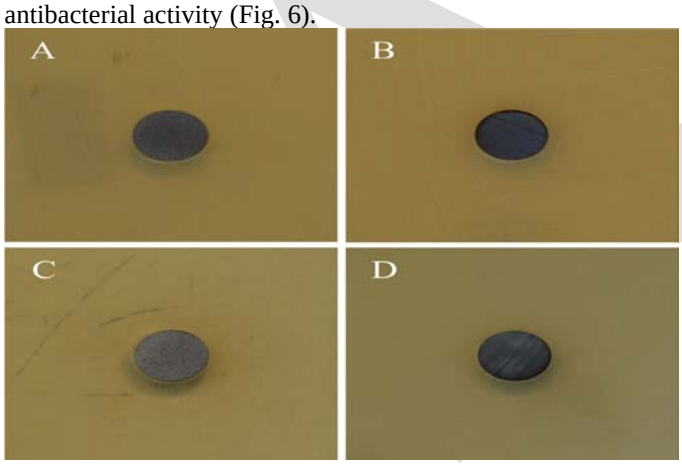

Figure 6: Disk diffusion assay reporting the results correlated to the antibacterial activity following 24 h incubation at 35°C against S. aureus for (A) uncoated, (B) coated titanium substrates and E. coli for (C) uncoated, (D) coated titanium substrates.

DISCUSSION

Owing to the serious medical issues of patients resulting from infections, protection against bacterial infection is an important necessity in orthopedic/dental surgery. This bacterial colonization or infections could result in failure of implants. These failures can be resolved by supplying the patient with systemic antibiotics. However, allergic reactions, reduction of microbial flora or bacterial resistance may result from this solution. Although the percentage of postoperative infections has been dramatically reduced by meticulous hygienic measures and protective prophylactic antibiotics, several types of bacteria have acquired selective resistance to antibiotics that can trigger infections. These infections are difficult to recover from and typically result in subsequent operations. An other alternative to the use of systemic antibiotics is the use of implants or prostheses made using engineered materials with inherent antibacterial activity (20). For this reason, many research efforts into the possible antibacterial properties of BGs has been conducted.

In the present study to evaluate the antibacterial action of the obtained BG nanoparticles coating, well diffusion susceptibility test was used as it is one of the oldest approaches and remains among the most widely used methods of antimicrobial susceptibility testing in routine clinical laboratories (18).

In fact, many parameters have been suggested to be the basis for the antibacterial activity of BGs including the elevated pH level and the osmotic effect induced by the concentration of the dissolved glass ions. It is also affected by the chemical composition and the environmental conditions for its degradation (21).

Certain compositions of BG were found to have antibacterial activity on a number of clinically significant microorganisms. Khanmohammadi and Ilkhchi (22) showed that, 55% SiO_2, 40% CaO and 5% P_2O_5 BG coating deposited over pure titanium substrates has antibacterial effect against E. coli and S. aureus bacteria using well diffusion susceptibility test. This inhibitory effect was discussed to be linked to the elevated pH level related to BG dissolution.

Particulate Bioglass® (45S5 BG) was found to have antibacterial action on some oral bacteria related to periodontal diseases (23). In addition, particle size reduction has been shown to improve the antibacterial activity of BG 45S5 (24). The use of BG nanoparticles was found to increase their solubility and ions release in the medium, thus raising the level of pH.

Stoor et al. (25) showed the silica-based S53P4 BGs (53%SiO_2, 23%Na_2O, 20%CaO and 4%P_2O_5) paste to provide antimicrobial action against supra- and sub-gingival plaque containing microorganisms owing to the elevated pH, ionic concentration, and osmotic pressure produced.

In the present study, the obtained sol-gel derived 70S30Ca BG nanoparticles coatings are shown not to possess antibacterial properties against both S. aureus and E. coli. This finding is in agreement with several previous studies in which high silica content BGs have shown no inhibitory action against bacterial growth.

Mortazavi et al. (26) evaluated the antibacterial function of BG nanopowders (20-90 nm) with different silica compositions (58S, 63S, and 72S) related to the SiO_2-CaO-P_2O_5 system and prepared using the sol-gel method. It was concluded that, the 72S BG nanopowder showed no antibacterial effect against E. coli and S. aureus, while 58S and 63S BG nanopowders had an antibacterial activity against

these bacteria. It was discussed that, the 72S with high content of SiO_2 and increased release of silica in the broth resulted in reducing pH level and increasing its dissolution rate. The 63S displayed the same actions, however, its reduced content of silica contributed to a higher pH level. The 58S was found to be a good agent for antibacterial use owing to the synergistic effects of increased calcium and alkaline pH levels.

To overcome the limitations to the antibacterial property, modifying the biomaterials with antibacterial agents (e.g., Ag, Cu, and Zn) is being intensively investigated (27-32). The incorporation of these bioactive ions with antibacterial activities has the ability to enable localized antibacterial treatments that are beneficial relative to systemic delivery of antibiotics.

Catauro et al. (19) evaluated the antibacterial properties of silver-containing 70% SiO_2 - 30% CaO BG. It was shown that the antibacterial activity against the S. aureus was increased with increasing the silver content, however, the biocompatibility and bioactivity of the coatings were reduced.

Balamurugan et al. (33) demonstrated the BG with molar composition of SiO_2 64%, CaO 26%, P_2O_5 10% and 100 - 700 µm particle size not to have antibacterial effect on E. coli. However, the incorporation of silver into this composition inhibited the bacterial growth.

CONCLUSION

The obtained modified sol-gel derived 70S30Ca BG nanoparticles coating, deposited on titanium alloy substrate using EPD under 30 volt for 1 min, has no antibacterial activity against S. aureus and E. coli bacteria.

CONFLICT OF INTEREST:

The authors declare that they have no conflicts of interest. Also, there is no granting or funding to be declared.

REFERENCES

1. de Viteri VS, Fuentes E. Titanium and Titanium Alloys as Biomaterials. In: Gegner J, ed. Tribology-Fundamentals and Advancements. United Kingdom: IntechOpen; 2013:155-81.
2. Amin Yavari S, van der Stok J, Chai YC, Wauthle R, Tahmasebi Birgani Z, Habibovic P, et al. Bone regeneration performance of surface-treated porous titanium. Biomaterials. 2014;35:6172-81.
3. Esposito M, Hirsch J-M, Lekholm U, Thomsen P. Biological factors contributing to failures of osseointegrated oral implants, (II). Etiopathogenesis. Eur J Oral Sci. 1998;106:721-64.
4. Liu X, Chu PK, Ding C. Surface modification of titanium, titanium alloys, and related materials for biomedical applications. Mater Sci Eng R Rep. 2004;47:49-121.
5. Mandracci P, Mussano F, Rivolo P, Carossa S. Surface treatments and functional coatings for biocompatibility improvement and bacterial adhesion reduction in dental implantology. Coatings. 2016;6:7.
6. Jones JR. Reprint of: Review of bioactive glass: From Hench to hybrids. Acta biomaterialia. 2015;23:S53-S82.
7. El Shazley N, Hamdy A, El-Eneen H, El Backly R, Saad M, Essam W, et al. Bioglass in alveolar bone regeneration in orthodontic patients: randomized controlled clinical trial. JDR Clinical & Translational Research. 2016;1:244-55.
8. Falk MM, Kowal TJ, Almeida RM, Saad M, Marei MK, Thamma U, et al. Sol–Gel Glass and Nano–Macro Porous Bioscaffolds. In: Boccaccini AR, Brauer DS, Hupa L, eds. Bioactive Glasses: Fundamentals, Technology Applications. United Kingdom:

RSC Publishing; 2016:105-35.
9. Saravanapavan P, Hench LL. Low-temperature synthesis, structure, and bioactivity of gel-derived glasses in the binary CaO-SiO2 system. J Biomed Mater Res. 2001;54:608-18.
10. Liu S, Gong W, Dong Y, Hu Q, Chen X, Gao X. The effect of submicron bioactive glass particles on in vitro osteogenesis. RSC Adv. 2015;5:38830-6.
11. Nguyen AT, Sathe SR, Yim EK. From nano to micro: topographical scale and its impact on cell adhesion, morphology and contact guidance. Journal of Physics: Condensed Matter. 2016;28:183001.
12. Ferraris S, Venturello A, Miola M, Cochis A, Rimondini L, Spriano S. Antibacterial and bioactive nanostructured titanium surfaces for bone integration. Applied Surface Science. 2014;311:279-91.
13. Chen Q, Li W, Goudouri O-M, Ding Y, Cabanas-Polo S, Boccaccini AR. Electrophoretic deposition of antibiotic loaded PHBV microsphere-alginate composite coating with controlled delivery potential. Colloids and Surfaces B: Biointerfaces. 2015;130:199-206.
14. Wang S, Falk M, Rashad A, Saad M, Marques A, Almeida R, et al. Evaluation of 3D nano–macro porous bioactive glass scaffold for hard tissue engineering. J Mater Sci Mater Med. 2011;22:1195.
15. Stojanovic D, Jokic B, Veljovic D, Petrovic R, Uskokovic PS, Janackovic D. Bioactive glass–apatite composite coating for titanium implant synthesized by electrophoretic deposition. J Eur Ceram Soc. 2007;27:1595-9.
16. Hudzicki J. Kirby-Bauer disk diffusion susceptibility test protocol. 2009.
17. Disk Diffusion Method for Antimicrobial Susceptibility Testing. Version 8.0. European Committee on Antimicrobial Susceptibility Testing; 2020.
18. Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
19. Catauro M, Bollino F, Papale F, Ciprioti SV. Investigation on bioactivity, biocompatibility, thermal behavior and antibacterial properties of calcium silicate glass coatings containing Ag. Journal of Non-Crystalline Solids. 2015;422:16-22.
20. Campoccia D, Montanaro L, Arciola CR. The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials. 2006;27:2331-9.

21. Leppäranta O, Vaahtio M, Peltola T, Zhang D, Hupa L, Hupa M, et al. Antibacterial effect of bioactive glasses on clinically important anaerobic bacteria in vitro. Journal of Materials Science: Materials in Medicine. 2008;19:547-51.
22. Khanmohammadi S, Ilkhchi MO. Effect of suspension medium on the characteristics of electrophoretically deposited bioactive glass coatings on titanium substrate. Journal of Non-Crystalline Solids. 2019;503:232-42.
23. Allan I, Newman H, Wilson M. Antibacterial activity of particulate Bioglass® against supra-and subgingival bacteria. Biomaterials. 2001;22:1683-7.
24. Waltimo T, Brunner T, Vollenweider M, Stark WJ, Zehnder M. Antimicrobial effect of nanometric bioactive glass 45S5. J Dent Res. 2007;86:754-7.
25. Stoor P, Söderling E, Salonen JI. Antibacterial effects of a bioactive glass paste on oral microorganisms. Acta Odontol Scand. 1998;56:161-5.
26. Mortazavi V, Nahrkhalaji MM, Fathi M, Mousavi S, Esfahani BN. Antibacterial effects of sol-gel-derived bioactive glass nanoparticle on aerobic bacteria. J Biomed Mater Res Part A. 2010;94:160-8.
27. Esteban-Tejeda L, Díaz LA, Prado C, Cabal B, Torrecillas R, Moya JS. Calcium and zinc containing bactericidal glass coatings for biomedical metallic substrates. International journal of molecular sciences. 2014;15:13030-44.
28. Chen X, Brauer D, Karpukhina N, Waite R, Barry M, McKay I, et al. 'Smart'acid-degradable zinc-releasing silicate glasses. Materials letters. 2014;126:278-80.
29. Bellantone M, Coleman NJ, Hench L. Bacteriostatic action of a novel four-component bioactive glass. J Biomed Mater Res. 2000;51:484-90.
30. Bellantone M, Williams HD, Hench LL. Broad-spectrum bactericidal activity of Ag2O-doped bioactive glass. Antimicrob Agents Chemother. 2002;46:1940-5.
31. Hoppe A, Güldal NS, Boccaccini AR. A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics. Biomaterials. 2011;32:2757-74.
32. Kaya S, Cresswell M, Boccaccini AR. Mesoporous silica-based bioactive glasses for antibiotic-free antibacterial applications. Mater Sci Eng C. 2018;83:99-107.
33. Balamurugan A, Balossier G, Laurent-Maquin D, Pina S, Rebelo A, Faure J, et al. An in vitro biological and anti-bacterial study on a sol–gel derived silver-incorporated bioglass system. dental materials. 2008;24:1343-51.