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Short Communication

Broad-spectrum bactericidal activity of a new bioactive grafting material (F18) against clinically important bacterial strains

M.T. Souza [a,*], L.A. Campanini [b], C.R. Chinaglia [a], O. Peitl [a], E.D. Zanotto [a], C.W.O. Souza [b]

[a] CeRTEV–Center for Research, Technology and Education in Vitreous Materials, Vitreous Materials Laboratory (LaMaV), Department of Materials Engineering, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil
[b] Department of Morphology and Pathology, Federal University of São Carlos, São Carlos, SP, Brazil

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ABSTRACT

Infection is the most relevant surgical complication in implant or grafting procedures. Osteomyelitis and other chronic conditions pose a constant challenge in current medical practice. In this context, a grafting biomaterial that possesses antibacterial properties combined with bioactivity could have great clinical impact. Researchers at the Vitreous Materials Laboratory (LaMaV–UFSCar) recently developed a glass composition, named F18, that presents an improved workability range combined with high bioactivity. With F18, one can easily manufacture complex shapes, such as scaffolds, continuous fibres and coat implants. This biomaterial has proven to be a viable alternative for bone and skin regeneration in *in vivo* tests, however its antimicrobial properties have not been explored. Hence, the purpose of this study was to systematically investigate the antibacterial activity of F18 in powder and fibre forms according to the JIS Z 2801:2010 standard. Whether incorporation of silver into F18 glass could impact its antimicrobial activity was also evaluated. Four clinically relevant Gram-positive and Gram-negative pathogenic bacterial strains (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa*) were used in this study. In both powder and fibre forms, F18 presented extremely efficient bactericidal activity against all strains tested, eliminating virtually 100% of the bacterial cells after 24 h. Kinetic tests showed that silver doping further increased the bactericidal activity, leading to *S. aureus* eradication in only 30 min after incubation. Both doped and non-doped glasses demonstrated very high bactericidal activity, making F18 a promising infection-preventing alternative for bone and wound regeneration in clinical practice.

1. Introduction

Bioactive glasses (BAG) were developed by Hench and colleagues in the late 1960s and have since been comprehensively studied as a synthetic grafting material for hard and soft tissue regeneration [1]. In both scenarios, local bacterial infection prevention is certainly a relevant issue, especially when a rapid regeneration process is intended.

Indeed, infection and lack of tissue integration constitute the most significant surgical complications when using biomaterials and these problems are typically associated with conditions that can considerably affect the patients' life and the healthcare system owing to the resulting increase in morbidity and mortality. Severe infection cases such as osteomyelitis pose a constant challenge for medicine, not only due to the increasing number of hospitalised patients per year but also due to the increased expenses and lack of effective treatment alternatives [2]. The major causative agent of osteomyelitis is *Staphylococcus aureus*, followed by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*; the latter is normally linked to the use of metallic implants [2,3]. When established, osteomyelitis tends not to respond to antimicrobials, mainly due to poor penetrability of the drugs and prolonged courses of therapy at subtherapeutic concentrations that generally lead to bacterial antibiotic resistance [2].

Therefore, the development of effective alternatives for preventing infection during bone grafting and implant procedures is highly desirable and relevant to medical practice mainly for allowing bone fracture consolidation. The application of synthetic bone or skin grafts that present, besides bioactivity, antimicrobial properties could supply adequate antibiotic concentrations at the wound site and concomitantly accelerate the tissue regeneration and healing processes [2].

Recently, researchers at the Vitreous Materials Laboratory of the Federal University of São Carlos (São Carlos, Brazil) have developed a new highly reactive glass, named F18, which possesses a larger working range (i.e. does not crystallise during processing) while retaining very high bioactivity [4,5]. Previous studies have demonstrated the *in vitro* and *in vivo* biocompatibility of this new glass

* Corresponding author. CeRTEV–Center for Research, Technology and Education in Vitreous Materials, Vitreous Materials Laboratory (LaMaV), Department of Materials Engineering, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil.
E-mail address: marina.trevelin@gmail.com (M.T. Souza).

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composition and its ability to enhance new bone formation both in fibre and scaffold forms [4]. In addition, F18 fibres are highly bioactive, forming a hydroxycarbonate apatite (HCA) layer in <4 h in simulated body fluid (SBF) solution, and this phenomenon leads to a fast bone bonding mechanism [5,6]. However, its antimicrobial activity has still not been explored. Thus, the aim of this study was to evaluate the efficacy of F18 BAG against some clinically important Gram-positive and Gram-negative pathogenic bacteria using a standardised methodology, not only in particle form but also as fibre meshes. In addition, this study explored whether silver doping can impact the antibacterial activity of the F18 glass.

2. Materials and methods

2.1. Bioactive glass preparation

F18 BAG belongs to the SiO_2–Na_2O–K_2O–MgO–CaO–P_2O_5 system and has proven to be an efficient biomaterial for bone and skin regeneration [5]. Its fabrication and the manufacturing process of the fibres are described in detail elsewhere [4,5]. Briefly, the glass batch was well mixed and was melted in a platinum crucible at 1450 °C for 3 h and was then quenched by splat cooling. For the manufacture of the silver-doped composition (F18-Ag), 1% wt of $AgNO_3$ was added to the F18 original composition and the manufacturing procedure described above was repeated.

Powdered samples were prepared by grinding with a final mean particle size of 50 μm. F18 fibre meshes (5 × 5 cm fibres with 20 μm diameter) were obtained by a lab-scale downdrawing process (Supplementary Fig. S1).

2.2. Bioactive glass characterisation

To analyse whether incorporation of silver would affect F18 bioactivity, tests evaluating the doped glass apatite-forming ability with SBF solution and Fourier-transform infrared spectroscopy (FTIR) were carried out following the recommendations of ISO 23317:2007 [7]. Changes in pH over time were verified using phosphate-buffered saline solution with a weight/volume ratio of 50 mg/mL. Tests were performed in triplicate and the powdered quartz (mean diameter of 50 μm) was used as the control group.

2.3. Antimicrobial tests

Antibacterial activity tests were conducted using the methodology proposed by JIS (Japanese Industrial Standard) Z 2801:2010 [8] (Supplementary Fig. S2). Four classic pathogenic bacteria (*S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *Escherichia coli* ATCC 8739P and *P. aeruginosa* ATCC 27853), which are commonly found in infectious wounds, were used to assess the *in vitro* antimicrobial properties of F18. In this assay, the used powder concentration was ca. 50 mg/mL. Inert glass fibre meshes or inert glass plate samples were used as a control. Tests were performed in triplicate and the mean ± standard deviation (S.D.) of CFU for cell viability (initial inoculum), the control and the biomaterial (F18) group are presented.

2.4. Kinetic antibacterial activity

Kinetic tests were conducted with the aim of comparing the bactericidal action of F18 and F18-Ag at shorter periods of time than that indicated by the JIS 2801:2010 standard. The micro-organism used in this assay was *S. aureus* owing to its clinical relevance. The inoculum was prepared according to the methodology mentioned in the JIS standard and a concentration ratio of 50 mg/mL of F18 or F18-Ag was placed in Erlenmeyer flasks and was incubated with agitation (150 rpm) or without agitation (referred to as the dynamic and static tests, respectively) at 35 ± 1 °C. Aliquots of 100 μL were collected at 0, 0.5, 1, 3, 6 and 24 h. All plates were prepared by the spread plate method and were incubated for 24 h at 35 ± 1 °C before counting. Silica powder (mean diameter of 50 μm) was used as the control and tests were performed in triplicate.

3. Results

3.1. Glass bioactivity

The FTIR spectra for F18 and F18-Ag bulk samples (Supplementary Fig. S3) confirmed the formation of the HCA layer for all samples after 16 h of soaking in SBF solution, indicating that silver incorporation did not significantly affect the in vitro bioactivity of F18.

For F18, F18-Ag powder and F18 fibre samples, an increase in pH was detected, reaching a maximum value of ca. 10.8 at 24 h. For the SiO_2 powder samples (control group), no significant changes in pH values were observed during the incubation period (Supplementary Fig. S4).

3.2. Antibacterial activity

3.2.1. Powder samples

F18 particles exhibited a visible antibacterial effect against all four pathogenic bacteria (Table 1). Whilst the control group (inert glass) presented an increase in cell viability after 24 h, exposure to F18 reduced cell viability by up to 6.5 log, representing 99.9999% bacterial killing activity (Fig. 1). During counting, the F18 samples did not present any bacterial colonies. This result indicates that the obtained values were in the technique detection limit (1 CFU/mL) so the log of CFU is equal to zero (log 1 = 0).

3.2.2. Fibre meshes

The antibacterial activity of F18 fibre meshes exhibited a similar pattern to that of the glass powder form. For the fibre meshes, a reduction up to 7 log was observed and all four bacterial strains were killed after 24 h (Fig. 1). A S.D. of 0.6 was observed for *P. aeruginosa*, mainly due to the rapid growth rate of this particular bacterium (Table 1).

3.3. Kinetic antibacterial activity tests

The results obtained with the kinetic tests in static (without agitation) and dynamic (with agitation) conditions are presented in Fig. 2. In both methodologies, F18 and F18-Ag presented a similar

Table 1
Bactericidal activity of F18 in powder and fibre form after 24 h for all tested bacteria (results presented as mean ± standard deviation log CFU/mL).

Bacterium	Powder form			Mesh fibre		
	Cell viability[a]	Control	F18 powder	Cell viability[a]	Control	F18 mesh
Escherichia coli	5.8 ± 0.1	6.0 ± 0.1	0	5.8 ± 0.1	7.0 ± 0.2	0
Staphylococcus aureus	5.7 ± 0.2	6.1 ± 0.1	0	5.2 ± 0.1	6.2 ± 0.1	0
Staphylococcus epidermidis	5.2 ± 0.01	5.9 ± 0.4	0	5.1 ± 0.1	6.1 ± 0.2	0
Pseudomonas aeruginosa	5.8 ± 0.01	6.5 ± 0.1	0	5.8 ± 0.01	7.3 ± 0.2	0.8 ± 0.6

[a] Initial inoculum.

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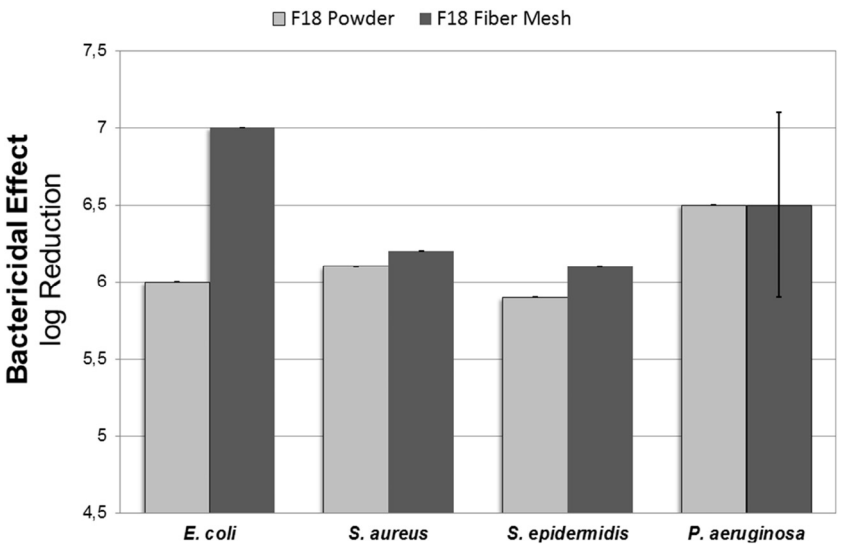

Fig. 1. Bactericidal activity (log reduction) of four different bacterial strains after 24 h in contact with F18 particles and fibre meshes.

trend even in the early stages of the experiment. After 30 min, F18 reduced the bacterial population by 1.5 log and F18-Ag reduced it by ca. 4.5 log, indicating very high bactericidal activity of this Ag-doped glass. After 3 h, the F18 glass yielded a 2 log reduction, which according to JIS 2810:2010 standard [8] is considered an efficient bactericidal agent. After 24 h of exposure, all tested groups presented a total reduction of the initial number of CFU except for the control group (powdered silica), as expected.

4. Discussion

The antibacterial mechanism triggered by BAG is not entirely clear and is still under discussion. Studies suggest that the bacteriostatic and/or bactericidal nature of BAG is linked to several factors, including high pH, increased osmotic pressure and 'needle-like' glass debris, which could potentially damage prokaryotic cell walls, thus inactivating bacteria [9–11]. The antibacterial action of BAG is highly influenced by its chemical composition and many other aspects, such as glass concentration in the medium, particle size distribution and the analysed bacterial strain [9,10,12]. Alkaline and alkaline-earth ions released from BAG into the medium can alter the pH of the bacterial cytoplasmic membrane, which plays an important role in the movement of nutrients into the cell. Consequently, the high pH can induce growth inhibition and toxic effects on the bacterial cell [11].

F18 presented a high bactericidal effect for all tested Gram-positive and Gram-negative bacteria strains both in powder and fibre forms. Although an increase in pH was observed for all F18 samples, its high biocidal activity may not be due only to pH elevation *per se*, but it could also involve other mechanisms, such as alterations in osmotic pressure and strong interaction with the bacterial cells owing to electrostatic forces. BAG are known to have a negative surface potential whilst the outer surface of the bacterial cell membrane is positively charged; this difference leads to microbial

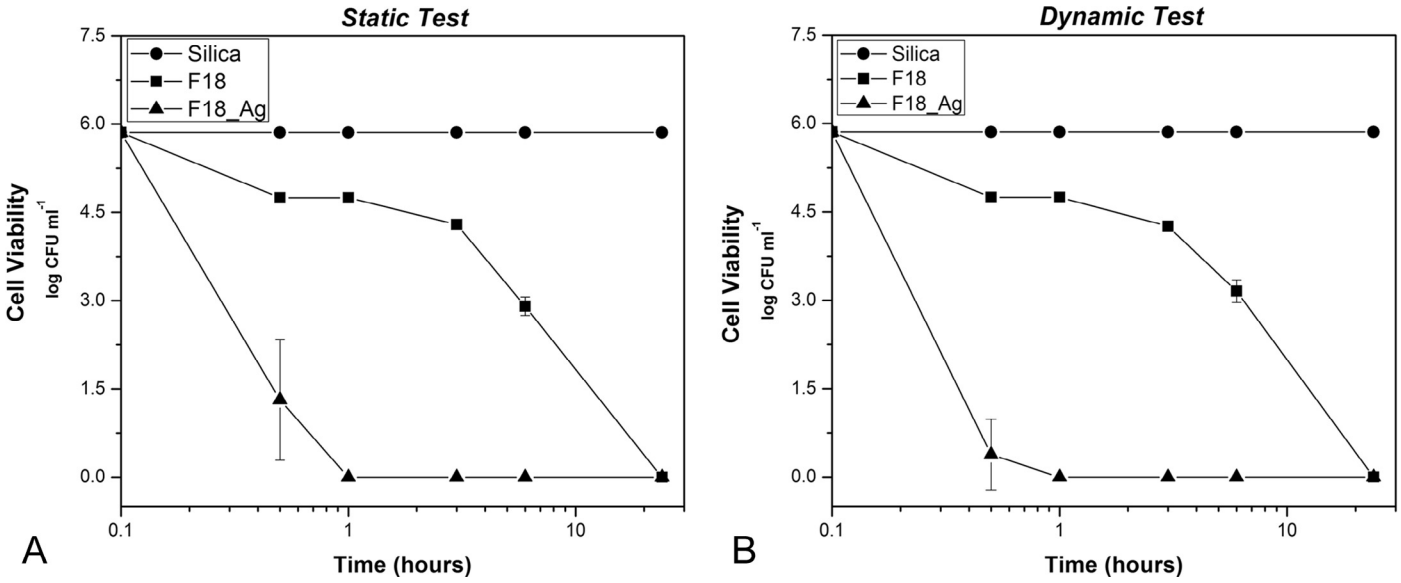

Fig. 2. (A) Static and (B) dynamic (agitation at 150 rpm) antimicrobial assay with F18 and the silver-doped F18 composition (F18-Ag) for different experimental times (0, 0.5, 1, 3, 6 and 24 h).

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adhesion on the glass particles, inducing a more effective bactericidal activity [13]. According to Cabal *et al* [14], the glass–cell membrane interface is crucial for the biocidal effect, since the adhesion results in a high calcium concentration release in the proximity of the plasma membrane, distorting its electrochemical potential gradient and thus causing rapid membrane depolarisation.

Considering the different experimental procedures used in previous studies, F18 presented a faster and more effective antibacterial activity compared with other BAG. As reported by Leppäranta *et al* [9], S53P4 BAG particles (≤45 μm) presented a killing effect on *S. epidermidis* when exposed to 100 mg/mL after 4 days, whilst 13–93 BAG presented only moderate antibacterial properties. Hu *et al* [15] reported that 45S5 BAG (≤50 μm) at a concentration of 50 mg/mL showed a bactericidal effect of up to 98% against *S. aureus*, *S. epidermidis* and *E. coli* after 24 h. Zhang *et al* [10] also reported a bactericidal effect on *S. epidermidis* after 48 h of exposure to S53P4 BAG particles (≤45 μm) in a 100 mg/mL ratio, whilst 18-04 BAG took 6 days to present the same effect.

The kinetic tests indicated that F18 particles significantly inhibited *S. aureus* growth both in static and dynamic conditions. Whilst the glass dissolution rate and other aspects of the suspension in these forms of testing are expected to diverge, no significant difference was detected between all analysed conditions (under Kruskal–Wallis and Dunn statistical analyses).

The results obtained with F18 and F18-Ag showed that the presence of Ag^+ decreases the time required for high bactericidal activity. In <1 h, a dramatic CFU log reduction was observed for F18-Ag-doped samples. Similar trends were also observed by Bellantone *et al* [16] by silver doping a sol–gel-derived glass. The marked antibacterial effect observed for F18-Ag is attributed to the leaching of ionic silver from the glass matrix into the medium. Although the efficacy of silver as an antimicrobial agent is well established in the literature, the mechanism by which silver ions exert their toxicity towards bacteria is not yet fully understood [16,17]. Studies indicate that the driving force is the complexation reaction involving the bacterial membrane, enzymes and other cellular molecular components such as amino and hydroxyl groups [16,18]. Silver ions exert their toxicity at various cellular sites, leading to disruption of the bacterial respiratory chain, phosphate uptake and storage systems, and cell wall synthesis [16].

Silver incorporation into F18 conferred a high antimicrobial property to the glass without compromising its bioactivity. This phenomenon has also been reported by previous studies with other glasses [17,19]. This feature opens the possibility to consider this material as a local Ag^+ delivery system where faster bacterial control is needed, such as skin wound healing applications. According to the current results, both F18 and F18-Ag glass compositions could be used to prevent implant-associated infections by significantly reducing the concentration of bacteria within a few hours after implantation.

5. Conclusion

F18 glass possesses broad-spectrum antibacterial activity and significant bacterial growth inhibition against Gram-positive and Gram-negative bacteria after 24 h under direct contact conditions. Incorporation of 1% wt of silver into the F18 glass positively impacted the kinetics of bacterial eradication. However, after 6 h of exposure both materials could be considered biocidal agents. The high antimicrobial activity combined with its elevated bioactivity makes F18 glass a very promising biomaterial for clinical applications in which microbial control is a relevant requisite, such as in bone defects and skin wounds.

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Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2017.08.015.

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