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Metallurgical Gallium Additions to Titanium Alloys Demonstrate a Strong Time-Increasing Antibacterial Activity without any Cellular Toxicity

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ABSTRACT: Orthopedic metallic devices are often related with devasting complications due to acute prosthetic joint infections. Gallium (Ga) antibacterial activity has been demonstrated by the evidence that Ga in solution ionizes in a Ga³⁺ trivalent form that replace Fe³⁺ thus arresting metabolism. However, it is not clear whether such effect is restricted only to Ga³⁺ release laps. Accordingly, here we investigated Ga addition into titanium alloys using metallurgical methods, thus realizing intermetallides of a very high stability that contain Ga in the range of 1, 2, 20, and 23% wt. ICP-OES analysis confirmed that Ga ions were not released from the specimens regardless of the Ga amount. These alloys ensured long-lasting Ga effect toward multidrug resistant Staphylococcus aureus, whose metabolic activity was reduced of >80% in comparison with controls. Finally, specimens cytocompatibility was confirmed by direct and indirect contact evaluations with mature osteoblasts and preosteoblasts progenitor cells.



KEYWORDS: biofilm, gallium, titanium alloy, antibacterial, orthopedic, cytocompatibility

■ INTRODUCTION

Orthopedic medical devices are aimed as a treatment of a wide range of skeletal diseases and disabilities.^{1–3} As for any surgery, complications might arise after implantation, and those of main concern are usually associated with prosthetic joint infections (PJI). PJIs are known to be a devastating hurdle linked with substantial patient morbidity.⁴ In general, quality of life of the patients is radically compromised due to PJI, causing immobility and excess pain. A revision to correct the situation commonly requires a multistage additional operation with extra costs, time, and bone/soft tissue loss.

Multiresistant pathogens (bacteria) present in the patient or brought externally into the wound greatly increase risk of secondary complications (over 25%), even leading to death.^{5,6} Most of the pathogenic microorganisms cannot be easily avoided because of their presence in the host flora and in the hospitals operating rooms.^{7,8} The alarming issue in PJI is fast growing due to resistance of these bacterial strains against all known antibiotics and antibacterial drugs.

Bacteria colonize on the surfaces of medical devices (implants) leading to diverse populations of bacteria/fungi into the autogenous extracellular matrix, forming biofilms. These biofilms may also comprise different multivalent cations, inorganic particles, colloidal, and other dissolved compounds.

Both Gram-negative (such as P. aeruginosa, P. fluorescens, E. coli) and Gram-positive (S. epidermidis, S. aureus, enterococci) bacteria are responsible for such infections, where Staphylococci are usually associated with metallic and polymer biomaterials in orthopedic implants.⁸ Biofilm offers a good protection for bacteria from the environment and the patients immune system response as well as from antibiotics (thousands times more than planktonic bacteria).⁹ Intercellular biofilm communications ("quorum sensing") stimulate the gene expression and enable temporal adaptation, substantially improving pathogens ability to survive in "harsh" conditions.^{3,8,10} For example, a 100 000-fold decrease of minimal infecting dose of S. aureus causing permanent abscess just due to the presence of the implant has been reported.¹⁰

Most of metallic biomaterials for orthopedic applications are nowadays based on titanium alloys thanks for their optimal combination of critical properties. $^{11-13}$ Biofilm formation on

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Figure 1. Microstructures of the 1% (a,d), 2% (b,e), and 20% (c,d) wt. Ga alloys observed by SEM at different magnifications (upper panel = 400×, lower panel = $2.5K\times$). The formation of intermetallic phases with hexagonal symmetry as well as primary crystals following eutectic-like phase are clearly seen for 20% Ga alloy. (g–i) Main average phase compositions (% wt., balance Ti) as identified by SEM microanalysis. The approximate intermetallics grain composition (darker than matrix, some examples indicated by the red arrows) is (Ti,Zr)₃(Si,Al,Ga) for 1% and 2% Ga alloy and (Ti,Zr)₅(Si,Al,Ga)₃ for 20% Ga.

titanium alloys is highly dependent on the biomaterial surface state, treatment, roughness, and composition of the alloy; accordingly, these material parameters should be targeted against bacteria without decreasing the affinity with the native tissue that is fundamental for a successful implant integration.^{14,15}

The biofilm eradication from contaminated surfaces is still very challenging (or simply not possible), and disappointing results in antibiotics therapies often lead to device replacement. Therefore, intense research is focused on the devices' surface modification to prevent bacteria adhesion.¹⁶

Because biofilm is able to proliferate the infection to the surrounding tissues, an ideal device design must forecast to prevent the biofilm formation and thus to protect nearby tissues. The general approaches are thus (i) incorporation or coating of potential bacteria-killing agents such as antibiotics, silver, and so forth or (ii) suppression or prevention of biofilm matrix development by dispersing agents, quorum sensing silencing, or other combined cues. For titanium biomaterials, one of the options is doping or alloying with other metals (like Ag, Cu, Zn) capable of reduction of biofilm formation. Especially, silver is well recognized as a powerful bacteria killer agent, despite some concerns for Ag ions on cell toxicity.^{17–19}

Gallium (Ga) is a novel attractive player in the large category of inorganic antimicrobial agents potentially due to its ability to replace Fe^{3+} in bacterial metabolism.^{20,21} The similarity of Ga^{3+} and Fe^{3+} in terms of charge, ionic radius, mass, electronic configuration, and coordination number simplifies iron substitution with gallium into siderophore-dependent biological systems.²² The key difference, however, is that Ga^{3+} cannot be reduced to lower oxidation states in the same manner as Fe^{3+} to Fe^{2+} , so the vital redox processes become irreversible in impairing related bacterial biological

functions to cease.^{20–22} Gallium owns also unique antibacterial properties that are very different from other ions: as an example, authors have recently demonstrated that negative effects of silver could be overridden with coatings containing gallium ions.²⁰ Gallium was reported also to have anti-resorptive effects on bone and bone fragments blocking osteoclast resorption after being absorbed onto the bone surface, without visible cytotoxic effects.²¹ Moreover, it was sown that Ga³⁺ can directly stimulate bone formation; the formulation with gallium nitrate is now used in clinical practice to counteract bone defects due to hypercalcemia.²³

Despite the evidence of such large positive effects for infection and bone treatment, it is not yet clear whether such highly positive effects are typical to Ga^{3+} release, and how this would affect longer term performance.

According to these premises, in this study we evaluate the hypothesis that addition of gallium is also possible to titanium alloys using metallurgical methods, similarly to how aluminum is being alloyed. As gallium and aluminum are analogous, some presence of aluminum in the alloy might be beneficial during melting.²⁴ On the other hand, despite its low melting point gallium has a very high boiling point and it forms intermetallides with titanium of a very high stability.²⁵ Some presence of silicon in titanium is beneficial to be an effective strengthening agent and for improvement of creep, oxidation, and corrosion resistance,^{26,27} as also observed for zirconium and niobium additions.^{11,28}

EXPERIMENTAL SECTION

Materials. Titanium alloys with different compositions were prepared using melting under argon atmosphere using pure (>99.0% wt.) granulated elements (Goodfellows, U.K.) and die cast into uncooled steel mold to obtain rods of ~15 mm diameter. The rods



Figure 2. Cytocompatibility analysis of experimental alloys. (a,b) The indirect assay confirmed that no toxic compounds were released from the specimens as no significant differences (p > 0.05) were detected by comparing test alloys and polystyrene controls at each time point. (c,d) Similarly, when cells were directly seeded onto the alloys' surface, they showed a comparable metabolism in comparison with cells cultivated onto polystyrene (p > 0.05). Values are representative for means and standard deviations.

were cut into disks and rectangular pieces of a few millimeters in thickness, mechanically ground with a SiC paper (grit size from 400 to 1200), then polished to achieve a surface roughness $R_a < 0.2 \ \mu$ m. The compositions produced have different gallium content as the addition of 1% and 2% Ga (targeted to α -phase structure), 2% and 20% Ga (targeted to $\alpha + \alpha_2$ phase) to the base alloy Ti, 8% Al, 3% Zr, 3% Si (here and later % values for alloy compositions shown are by weight, unless stated otherwise). Also, Ti-23% Ga without other additions was prepared in the same way. These compositions were optimized by calculation of the phase equilibria to ensure that no brittle phases (high-gallium intermetallics) or unfavorable microstructure would form during solidification. Low-gallium concentrations are expected to localize gallium within the titanium-rich α -matrix, whereas higher concentration would likely to spread it into the intermetallic compounds. However, as seen from the microstructure (SEM) and microanalysis (Figure 1), gallium is rather uniformly present in all the phases despite increase of its overall concentration. No significant differences were observed between 1 and 2% Ga alloy, but for 20% Ga changes in the intermetallic phase, overall microstructure, and phase composition were evident (Figure 1). The Al, Si, and Zr-free alloy with 23% Ga did not exhibit intermetallics, showing a uniform very fine $\alpha + \alpha_2$ phase microstructure (data not shown), typical for binary Ti-Al alloys of similar composition.

These specimens were examined using TGA-DSC (STA449C "Jupiter", Netzsch Gerätebau GmbH, Germany) with repeatable heating and cooling until 1480 °C in argon to assess possible phase transformation temperatures and to align these with known phase equilibria, where available (data not shown). Specimens of Al-free TNZ alloy with added silicon (Ti 11%, Nb 11%, Zr 2.2% wt. Si) were used as an alloy control for estimation of gallium effect (TNZ-Si). All specimens were sterilized in autoclave (121 °C for 20 min at 1 bar) prior to use for biological experiments.

Gallium lons Release. The stability of the alloys was intended also as the ability to retain Ga ions in contact with solutions. Gallium ions release was quantified from Dulbecco's modified Phosphate buffered saline solution (D-PBS, Sigma-Aldrich, Milan, Italy) soaked materials 1 Ga and 23 Ga. These alloys were selected as representative for the lowest and higher Ga amount insider the test specimens' group. Tests were performed in triplicate for two time points, 3 and 7 days. Samples, whose surface average was 3 cm², were placed in Falcon tubes filled with 6 mL of D-PBS and maintained in static condition at 37 $^\circ\text{C}.$ At day 3, the solutions were totally removed and stored at 5 °C until analysis, and Falcon tubes were refilled with 6 mL of fresh D-PBS. At day 7, the solutions were totally collected and stored at 5 °C until analysis before analysis. Inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis was made on all collected solutions using an Optima 8300 (PerkinElmer, Waltham, U.S.A.). ICP-OES analysis failed to reveal any trace of Ga ions into the collected solutions (data not shown; all results were negative), confirming that no ions were released into the liquid (or at least that no volume >20 μ g/L that represents the instrument limit of detection were released).

Cells. Cells were purchased from the American Type Culture Collection (ATCC, Manassas, U.S.A.). Human fetal progenitor hFOB (ATCC CRL-11372) and human osteosarcoma U2OS cells (ATCC HTB-96) were selected for cytocompatibility experiments. hFOB were cultivated in MEM/F12 mixture (50:50), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.3 mg/mL G418 salt at 34 $^{\circ}$ C, 5% CO₂, and U2OS were cultivated in DMEM 10% FBS, 1% penicillin/streptomycin (all Sigma-Aldrich, Milan, Italy) at 37 $^{\circ}$ C and 5% CO₂ (all cells until 80–90% confluence), then detached with trypsin–EDTA solution and used for experiments.

Nondirect Cytotoxicity Evaluation. Specimens were individually placed into the wells of a 12-multiwell plate (Nunc, Thermo Fisher Scientific, U.S.A.), submerged with fresh medium (2 mL/ specimen) for 1 week and stored at 37 °C and 5% CO₂. Afterward, supernatants were collected and used to seed cells (both U2OS and hFOB) in a defined number (1×10^4 cells/well, 1 mL/well) into the wells of a 24-multiwell plate (Nunc). Cells viability was evaluated after 24, 48, and 72 h by the colorimetric alamarBlue assay (alamarBlue,



Figure 3. Antibacterial effect of the experimental alloys. (a,b) *S. aureus* metabolic activity in function of single time-points (a) and time (b); (c,d) *S. aureus* viable colonies count in function of single time-points (c) and time (d). Ga-doped specimens were always significant in comparison with polystyrene control (a-d, p < 0.05, indicated by asterisk) but a concentration-dependent trend was also observed as both 20Ga and 23Ga resulted as being significant toward other Ga-doped specimens (a-d, p < 0.05, indicated by # and § marks, respectively). Values are representative for means and standard deviations.

Thermo Fisher Scientific, U.S.A.). At each time-point, supernatants were removed from each well containing cells and replaced with alamarBlue (10% v/v in fresh medium plus 10% FBS). Plates were incubated in the dark for 4 h and then 100 μ L was removed, spotted into a new 96-wells plate with fluorescence signals evaluated at 590 nm by spectrophotometer (Victor, PerkinElmer). Cells cultivated with fresh medium were used as null control; all experiments were performed in triplicate.

Direct Cytotoxicity Evaluation. Specimens were individually placed into the wells of a 12-multiwell plate (Nunc, Thermo Fisher Scientific, U.S.A.); cells were seeded directly dropwise (100 mL) onto the specimens' surfaces in a defined number (1×10^5 cells/specimen). After 2 h of adhesion onto the specimens' surface, cells were rinsed with 1 mL of fresh medium and cultivated for 24, 48, and 72 h. At each time point, specimens were moved to a new multiwell plate and the cells viability was evaluated by the colorimetric metabolic alamarBlue assay as previously detailed in the indirect assay.

Bacteria Strain. A strong biofilm-former, multidrug resistant (MDR) *S. aureus* strain was provided by the Microbiology and Virology Unit of the "Maggiore" University-Hospital in Novara, Italy, from a clinical specimen. All procedures were performed with patients informed consent and in full compliance with the Declaration of Helsinki. Bacteria were cultivated onto selective Mannitol Salt Agar (MSA) plates until single round yellow colonies were formed; then before any experiment, single colonies (3–4) were collected and incubated into 30 mL of fresh Luria–Bertani broth (LB, Sigma) for 16 h in agitation (200 rpm) to obtain exponentially growing bacteria cultures.

Biofilm Formation. To obtain *S. aureus* biofilm in vitro, bacteria broth cultures, obtained as above, were diluted into fresh LB medium until the final concentration of 1×10^5 cells/ml, evidenced by the spectrophotometer lecture (OD = 0.001 at 600 nm). Then each specimen was submerged in the solution for 90 min in circular agitation (120 rpm) to force cell adhesion (adhesion phase). Afterward, supernatants containing planktonic cells were removed and specimens were rinsed with 2 mL of fresh LB medium to select

only the attached cells (separation phase). Biofilm was grown for 24, 48, and 72 h.

Viable Biomass Quantification. At each time-point, specimens were moved to a new 12-wells plate and rinsed with 2 mL of alamar blue solution as described for cytocompatibility assay. Fluorescence signals were spectrophotometrically evaluated (Victor, PerkinElmer) at 590 nm. Biofilm cells were detached from specimens' surfaces by sonication and vortexed (30 s each, 3 times) and collected into 1 mL of sterile PBS. Afterward, detached cell suspension was used to perform six 10-fold scalar dilutions by mixing 20 μ L of bacteria solution with 180 μ L of PBS. From each dilution, 20 μ L were collected and spotted into new LB agar plates and incubated at 37 °C until single round colonies were observed. The colonies forming unit number (CFU) were calculated as

$$CFU = [(number of colonies \times dilution factor)^{(serial dilution)}]$$

where "number of colonies" means countable round single colonies, "dilution factor" means a dilution from the initial 1 mL solution, "serial dilution" comprises 1-6 10-fold dilution area where colonies were counted. Biofilm cells cultivated onto polystyrene dishes were used as null control and those of gallium-free alloy (TNZ with 2.2% Si) as an alloy control.

Statistical Analysis of Data. All these experiments were performed in triplicate. Data were analyzed using SPSS software (v. 25, IBM, NY, U.S.A.) by means of one-way ANOVA followed by the Tukey's test as post hoc analysis. Significance level was set at p < 0.05.

RESULTS AND DISCUSSION

Alloys were first analyzed on their cytocompatibility, and the results are shown in Figure 2. The indirect (Figure 2a,b) assay confirmed that no toxic elements were released into the culture media from the specimens' surface within the 7 days of direct contact. Both hFOB (Figure 2a) and U2OS (Figure 2b) cells reported similar RFU values in comparison with controls. These data are in accordance to those obtained from ICP-OES

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analysis that excluded the presence of ions in the physiological solution applied for the soaking.

When cells were directly cultivated onto alloys' surface in the direct assay (Figure 2c,d), no significant differences were noticed by comparing the metabolic activity obtained by the cells on the samples' surface and the control ones in polystyrene wells (a-d, p > 0.05). These results were evaluated at each time-point (24, 48, and 72 h) when the cells viability was measured, thus demonstrating that cytocompatibility is not related or limited by the exposure time. Moreover, cells proliferated rather well as seen by the constant increase in fluorescence signals due to cells number increase.

Specimens' in vitro direct antibacterial activity evaluation results are reported in Figure 3. As seen, all specimens with gallium have displayed an effective activity in reducing S. aureus biofilm viability in comparison with polystyrene controls (a-d, data with p < 0.05 are indicated by asterisk). The bacteria killing activity seems to be related to Ga amount and time. Already at 24 h, results of bacteria viability was reduced by about 70-80% (Figure 3a) and CFU number by about 3-4 logs (Figure 3c) for 20 and 23% Ga specimens. Alloys with 1 and 2% Ga decreased S. aureus biofilm viability of <50% and <3 logs, which is considered the minimal effective ratio to define as "antibacterial" for a novel treatment. So, 20 and 23% Ga alloys were significant in respect to other Ga-doped specimens (Figure 3a-d, p < 0.05, indicated by # and §, respectively). However, looking at the 48 and 72 h (Figure 3b,d) time-points, the effect of these alloys was substantially increased, showing a time-dependent activity.

After 72 h a similar reduction of about 85–95% in viability was observed for all Ga-alloyed specimens. A 5-log decrease in CFU number was detected for alloys with 2% Ga and more after 72 h. Both alamarBlue and CFU count assays displayed a continuous reduction in biofilm viability and number that is due to gallium progressive entry in bacteria metabolism. As expected, no effect was noticed for alloy control specimens lacking gallium (TNZ-Si, p > 0.05).

As earlier debated in the Introduction, gallium antibacterial activity is strongly related to the inhibition of Fe-dependent pathways due to its ability to compete for the iron-binding sites uptake. However, ICP-OES analyses have failed to detect Ga³⁺ ions into the medium thus allowing the suggestion that ion release of these alloys is not effective (gallium is more tightly bound with intermetallic phases than in a $Ga(NO_3)_3$ -like compound. Iron has a key role in the Fenton chemistry in biological objects involving creation of free radicals in vivo, based on Fe³⁺/Fe²⁺ redox process. It has been suggested that patients with active infections should thus not be administrated with excess iron.²⁹ Therefore, an alternative or complementary explanation of the observed antimicrobial effect comes from Lemire et al.³⁰ who classified Ga as one of the Fenton-inactive metals (together with Ag and Hg) generating ROS. Once bacteria attach to the Ga-contained alloys, the surface-exposed gallium gets in touch with the bacteria solvent-exposed [4Fe-4S] clusters of proteins, which represents the cellular donor ligands that coordinate iron exchange.³¹ The direct impairment of the [4Fe-4S] clusters due to gallium presence results in the toxic release of additional Fenton-active Fe into the cytoplasm, thus leading to deadly ROS amount formation. In either case of the potential pathways of gallium, the effect observed has potential clinical relevance in suppressing bacterial biofilm formation on these alloys without use of antibiotics.

CONCLUSIONS

The study has confirmed that metallurgical addition of gallium even in small amounts (1-2% wt.) to titanium alloys have highly efficient antibacterial function without any visible cytotoxic effect. The presence of gallium within the metal matrix might ensure that antibacterial effect will persist for a long time, which might not be possible for gallium embedded in thin degradable coatings. Since gallium is metallurgically analogous to aluminum in titanium alloys, it might be easily used without affecting other alloy properties, also in the case when it has other elements like zirconium or silicon.

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L.R. and M.G. co-shared authorship. M.G. was responsible for alloys preparation, microstructure and chemical-physical analysis; A.C., B.A., R.C, and L.R. were responsible for biological assays.

Notes

The authors declare no competing financial interest.

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