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Antibacterial Effect and Cytotoxic Evaluation of Mg alloy Functionalised by Sol-Gel Magnesium Hydroxide and Copper doped.

A. De Luca[1], L. Raimondi[1], R. Alduina[2], A. Presentato[2], R. Chiesa[3], A. D'Agostino[4], A. Piccininni[4], D. Bellavia[1], G. Giavaresi[1], V. Costa[1].

[1]*Surgical Sciences and Technologies, IRCCS Istituto Ortopedico Rizzoli, Bologna, Italy*
[2]*Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze, Bd. 16, 90128 Palermo, Italy.*
[3] *Department of Chemistry, Materials and Chemical Engineering 'G. Natta', Politecnico di Milano, 20135 Milan, Italy.*
[4] *Department of Engineering and Applied Sciences, University of Bergamo, Viale Marconi 5, 24044 Dalmine, BG, Italy*
[5]*Department of Mechanics, Mathematics and Management, Polytechnic University of Bari, Via Orabona 4, 70125 Bari, Italy.*

***Abstract* —** *Introduction:* Biodegradable alloys are promising candidates for implants that offer stability during healing and controlled degradation after healing, eliminating the need for second surgery and reducing the risk of chronic inflammation or allergic reactions. In particular, Mg alloys have a high capacity for bone regeneration and mechanical properties similar to natural bone, making them very promising for biomedical applications. However, there is a growing need to find new treatments with antimicrobial properties while slowing and controlling the dissolution kinetics of Mg alloys. This study aims to evaluate the antibacterial properties of a coating doped with magnesium hydroxide ($Mg(OH)_2$).
Materials and Methods: MgAZ31 magnesium alloy discs produced by the superplastic forming (SPF) process were coated by sol-gel technique with $Mg(OH)_2$ doped with copper (Cu) ions. Microbiological testing assessed the antibacterial properties of the material through growth inhibition (viable count) and biofilm formation (SEM analysis) using *Staphylococcus aureus* strains (ATCC 25923). The WST-1 viability assay is performed on fibroblast and pre-osteoblast mouse cell lines to assess the toxicity of the Sol Gel -Cu doped coating.
Results: The antibacterial data reported on MgAZ31 SPF-Sol-Gel-Cu doped alloys show that the doping reduces bacterial growth of *S.aureus* strain and also inhibits bacterial biofilm formation. The effect on cell viability was different in the two lines analysed. No change in viability was observed in the L929 cell line, whereas viability decreased in the more differentiated pre-osteoblast MC3T3.
Conclusion: Despite inconsistent data on cell viability, pre-osteoblasts and osteoblastic cells, encouraging antibacterial data reported on MgAZ31-Sol-Gel-Cu doped alloys show that doping reduces bacterial growth of two *S.aureus* strains and also inhibits bacterial biofilm formation. These promising data should encourage research to evaluate the effects of doped coatings more broadly, assessing immunotoxic effects and osteointegration capabilities.

***Keywords*—Mg alloy, Magnesium Hydroxide Sol-Gel, Copper doping, antibacterial and cytotoxic properties.**

I. INTRODUCTION

Magnesium alloys are a promising material for temporary orthopaedic implants. Magnesium mechanical properties, which are very similar to those of natural bone, biocompatibility, bioresorbability and antibacterial properties make it attractive to researchers and engineers to manufacture of prostheses[1]. The growing demand for safe biomaterials for various orthopedic surgeries and the increasing incidence of recurrent surgical infections are driving industrial research to find biomaterial coating solutions that both slow down the degradation of magnesium alloys and provide enhanced antibacterial properties [2]. The sol-gel coating technique is commonly used to add antimicrobial agents to alloy surfaces [3]. Various antimicrobial compounds are used in the preparation of sol-gel coatings and fall into two categories: chemical or natural compounds [4],[5]. These sol-gel coatings can be applied by various techniques: dipping, spraying or spinning before gelling. The antimicrobial compounds added to the sol-gel layer act by releasing molecules or ions with antibacterial activity over time, preventing bacterial adhesion, inhibiting microbial growth and biofilm formation [6]. Several articles have highlighted the antimicrobial activity of copper ions, but also their economic advantage over silver ions, as they are a less expensive and more readily available chemical resource with a similar level of inhibitory activity [7],[8]. Many properties attributed to the copper ion drive the use of this element as a dopant for the sol-gel layer. Copper is an essential element and can be rapidly excreted from the body, and different states can have different biological activities, including antibacterial activity [9]. In this work we have investigated new copper-doped magnesium hydroxide thin films deposited on Mg alloy AZ31 for temporary implants to provide antimicrobial activity.

II. MATERIALS AND METHODS

- The discs of Mg ally AZ31 were manifactured as reported in De Luca A. et al. article [10]. The sol-gel-Cu doped specimens were obtained by dipping untreated AZ31 disks into a solution containing: the $Mg(OH)_2$ solution, the (3-glycidaxypropyl)-trimethoxysilane (GLYMO) and $Cu(NO_3)_2$. The $Mg(OH)_2$ solution was prepared by adding drop by drop a NH4OH solution (2.2 M) to a $MgCl_2$ solution (0.4 M). The silane solution was prepared by mixing GLYMO, 2-propanol, and distilled water with a volume ratio of 8:8:1. The $Cu(NO_3)_2$ was dissolved in the silane solution with a concentration of 0.04 M. Then, the $Mg(OH)_2$ solution and the GLYMO one were mixed and kept under magnetic stirring for 2 hours. Dip-coated samples were thermally treated in the oven at 160°C for 2 hours [11]. The discs were individually placed in PA/PE double pouches, thermo-sealed and then gamma-sterilized at 25.0 kGy.

In vitro evaluation of antibacterical effects of MgAZ31-Sol-Gel-Cu doped alloys.

- Bacterial Growth Inhibition Test: MgAZ31 SPF and MgAZ31 SPF-Sol-Gel-Cu doped were placed in direct contact with the bacterial strain *S. aureus* (ATCC® 25923™) at a concentration of $1x10^8$ colony forming units (CFUs) per milliliter of culture. All samples were incubated at 37°C for 24 hours, kept under constant mechanical agitation, and then

seeded onto LB agar plates for CFU counting. To quantify the actual number of CFUs in the culture broth, aliquots of bacterial cultures were harvested, serially diluted, and plated on LB agar plates.

- Biofilm inhibition: *S. aureus* (ATCC® 25923™) strain was incubated overnight at an initial microbial titer of 10^8 CFU/mL in LB medium in the presence of MgAZ31 SPF and MgAZ31 SPF Sol-Gel-Cu doped samples. At the end of the incubation, each scaffold was washed 3 times with sterile saline solution. The Mg alloy samples were transferred in tubes containing 2 mL of saline solution and vortexed for 10 seconds. Finally, they were sonicated for 5 minutes (frequency: 35 kHz) and vortexed again for 10 seconds, allowing bacterial detachment from the scaffold. The sonication product was serially diluted, and the microbial titer (CFU/mL in the logarithmic scale) was estimated after the recovery of bacterial cells on LB agar plates.

- Biofilm inhibition evaluation by vital CFU/mL: *S. aureus* (ATCC® 25923™) strain was incubated overnight at a concentration of 10^8 CFU/mL in LB medium in the presence of MgAZ31 SPF and MgAZ31 SPF-Sol-Gel-Cu doped samples. At the end of the incubation, each scaffold was washed 3 times with saline solution. The Mg alloy samples were transferred in tube containing 2 mL saline solution and subjected to 10" vortex shaking. Finally, they were sonicated for 5 minutes (frequency: 35 kHz) and vortexed again for 10". The sonicated solution was serially diluted from 10^{-1} to 10^{-7} concentration and CFU/mL were counted the next day.

- Biofilm inhibition evaluation by SEM analysis: The MgAZ31 SPF and MgAZ31 SPF Sol-Gel-Cu doped scaffolds, colonized by S. aureus biofilms, were subjected to three successive washes with 5 mL of saline solution under continuous agitation to dislodge loosely adherent cells. Subsequently, the bacterial cells were fixed using a 2.5% glutaraldehyde solution prepared in 0.1 M phosphate buffer for 1.5 hours at room temperature. Following fixation, residual fixative was removed by rinsing the samples with 0.1 M phosphate buffer. The specimens were then progressively dehydrated through a graded ethanol series (30%, 50%, 70%, and 95%) for 20 minutes at each step. Finally, they were immersed in absolute ethanol for one hour before undergoing further dehydration with hexamethyldisilazane (Sigma-Aldrich) in two successive five-minute treatments. The metallisation was carried out with gold using an agar sputter coater B7340 and observed with a Zeiss SEM EVO HD15 at different magnifications (500x; 15Kx; 5Kx) under high vacuum conditions, Iprobe current of 250 pA, NTS-BSD detector, WD of 8 mm and voltage (EHT) of 10 kV

In vitro effect of MgAZ31-Sol-Gel-Cu doped alloy on cell viability.

- Mg extract prepared according to the method described by De Luca *et al.*[10] to treat cell lines.

- Quantification of Mg^{2+} ions: Magnesium Assay Kit (Sigma-Aldrich) was used to measure the amount of Mg2+ ions in both Mg extracts. The colour intensity, measured at 500 nm using the ChroMate 4300 ELISA microplate reader (Awareness Technology INC), is directly proportional to the magnesium concentration in the sample.

- WST-1 viability assay: L929 and MC3T3 cell lines were seeded at a density of 5×10^3 per 96-well plate and exposed to extract MgAZ31 SPF and MgAZ31 SPF-Sol-Gel-Cu doped for 24, 48 and 72 hours. The pH value at each time point was measured and reported in Table I. The WST-1 assay was performed by adding 1/10 volume of WST-1 reagent to each well and incubating the cells at 37°C in a 5% CO2 humidified atmosphere for 3 hours. The absorbance of the samples was measured against the background (culture medium plus WST-1 reagent without cells) at 450 nm using a BioTek 800 TS microplate reader (BioTek Instruments, Vermont, USA). Absorbance values obtained from the WST-1 assay are proportional to the total number of viable cells.

III. Results

The MgAZ31 magnesium alloys (96% Mg, 3% Al, 1% Zn) produced by the SPF process have undergone chemical pickling in sulphuric, nitric and hydrofluoric acid baths at room temperature for times appropriate to the amount of residue. The sol-gel technique has been used to produce a coating of magnesium hydroxide favouring also slowing down and controlling the dissolution kinetics of the alloy. Dati not shown report that the sol-gel technique extends the degradation time by showing good corrosion resistance after 21 days in SBF, no great change in mass, and no morphological change by SEM or chemical carried out by XRD or EDS elemental spectroscopic analysis.

The antibacterial evaluation performed *in vitro* showed a reduction in the growth of the *S. aureus* strain in contact with MgAZ31 SPF-Sol-Gel-Cu doped (Figure 1) and an inhibition of biofilm growth (Figure 2), quantified as a reduction in the number of CFUs during 24 hours treatment in contact with MgAZ31 SPF-Sol-Gel-Cu doped, compared to the Mg alloy control.

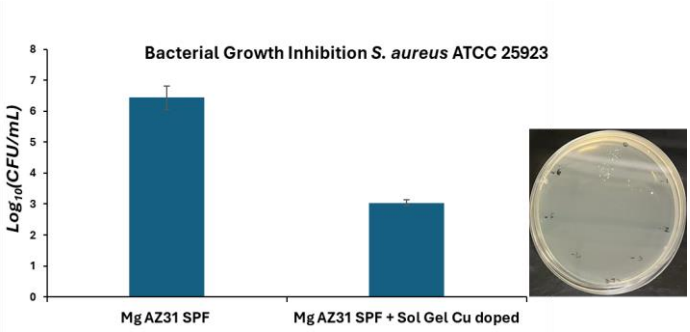

Fig. 1: Bacterial growth inhibition evaluated by vible cell count of *S.aureus* ATCC 25923 after 24 hours. The results showed a reduction of bacterial growth in MgAZ31 SPF-Sol Gel-Cu doped.

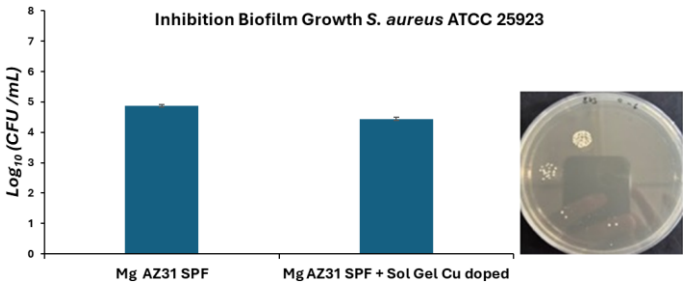

Fig. 2: *S. aureus* ATCC 25923 Biofilm growth inhibition after. The results showed a reduction the bacterial biomass grown as a biofilm onto MgAZ31 SPF-Sol-Gel-Cu doped.

SEM micrographs at high magnification (5Kx) showed the strong inhibition of biofilm formation (Figure 3) in the Mg Cu-doped sample as compared to the Mg control sample. These data align with Tatullo *et al.* [11], which reported that the Mg alloy coated with a thin film of $Mg(OH)_2$ applied by the sol-gel tecnique has the antibacterical effects. Our data further showcase the relevance of this coating and the opportunity to implement the antibacterial effect by adding Cu doping. Effects evidenced not only as inhibition of bacterial growth but also in the inhibition of biofilm formation.

To perform the cytotoxicity tests, extracts of both samples were prepared and placed in contact with the two cell lines to assess their effects on viability. The pH at each point of the experiment was also measured, which, as shown in Table I, was slightly lower in the cell supernatants in contact with the Mg Sol-Gel-Cu doped. This could be due to the increased degradation of the sample causing changes in pH values and consequently on cell viability of *in vitro* systems [12].

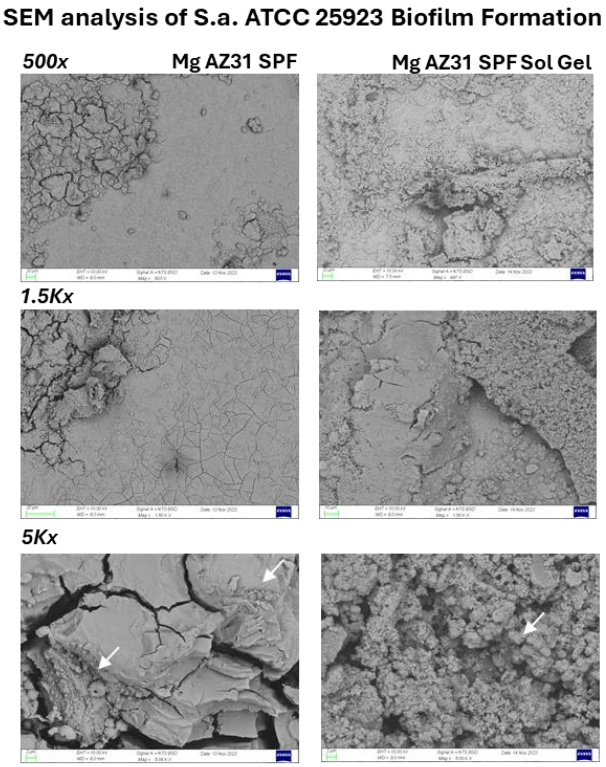

Fig. 3: SEM micrographs of S. aureus ATCC 25923 biofilms. The micrograph showed a reduction of the bacterial biofilm formation on MgAZ31 SPF Sol-Gel-Cu doped.

The results of the cytotoxicity analysis of the Cu-doped Mg alloy showed that no reduction in viability was recorded on the more plastic cell line L929 (Figure 4). When using a more differentiated cell line, such as the pre-osteoblastic murine MC3T3 line, the effect of the Cu-doped Mg alloy is much more toxic, reducing cell viability. Probably, the viability in MC3T3 is significantly reduced in favour of the differentiation process of pre-osteoblasts. The scientific literature suggests that the degradation process of Mg alloys, in particular the amount of released Mg ions and hydroxyl ions could determine the rise of the pH, influencing the viability and differentiation of pre-steoblasts [13]. As shown in Table II, the Mg extracts were quantified before contact with the cell lines. The results show that the amount of Mg ions in the Cu-doped Mg alloy is higher than in the undoped Mg alloy. This could explain the different behaviour of the two cell lines based on existing data in the literature.

TABLE I. pH values of Mg extracts at different time points.

pH values	24h	48h	72h
Mg AZ31 SPF	8.673	8.884	8.973
Mg AZ31 SPF+ Sol Gel Cu doped	9.001	8.979	8.987

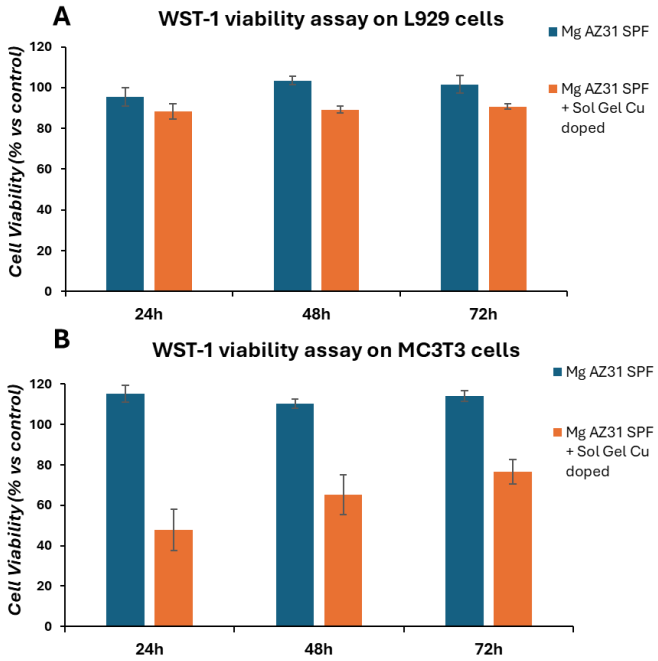

Fig. 4: WST-1 viability assay. During the different time points, the L929 and MC3T3 showed a different proliferation rate. The graphs reported how the growth of L929 did not change after contact with the extract deriving from the MgAZ31 SPF Sol-Gel-Cu doped alloy, while the viability of MC3T3 is reduced compared to Mg alloy not doped.

TABLE II. Mg ions quantifiction in the Mg exract.

Mg assay quantification	mg/dl
MgAZ31 SPF	47,5
Mg AZ31 SPF+ Sol Gel Cu doped	60,5

IV. Conclusion

The promising results of the antibacterial effect of Mg alloy coated with Mg $(OH)_2$ and Cu-doped using the sol-gel technique encourage further research into biodegradable materials and further refinement of coating techniques. The inhibitory effect on bacterial growth and the formation of biofilms is helping research into solutions that can increasingly promote the reduction of device resorption over time. This will allow the sustained release of Cu ions, which have been shown to have a strong antibacterial effect. On the other hand, the

cytotoxicity data obtained require further investigation of the effects to confirm whether the reduction in viability observed on the pre-osteoblastic cell line correlates with real differentiation. Therefore, further studies on markers of differentiation in the osteoblastic direction and further evaluation of the immunotoxic effects of the coating subject of this study are required to establish the level of safety of the coating produced for this Mg alloy.

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