Bactericidal activity and biocompatibility of TiO_2 coatings doped with bactericidal elements

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A Thesis for the Degree of PhD in Mechanical Engineering under the supervision of Prof. Dr. Paulo César Soares Jr.

Pontíficia Universidade Católica do Paraná

June 2018

Pontifícia Universidade Católica do Paraná
Escola Politécnica
Programa de Pós-Graduação em Engenharia Mecânica

PUCPR
GRUPO MARISTA

ATA DA SESSÃO PÚBLICA DE DEFESA DE TESE DE DOUTORADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA MECÂNICA DA PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ

DEFESA DE TESE Nº 27

Aos quatro dias do mês de junho de 2018, realizou-se a sessão pública de defesa da tese **"Bactericidal Activity and Biocompatibility of TiO2 Coatings Doped With Bactericidal Elements"** apresentada por **Luciane Sopchenski Santos,** como requisito parcial para a obtenção do título de Doutora em Engenharia Mecânica, perante uma Banca Examinadora composta pelos seguintes membros.

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Abstract

Metallic implants are susceptible to bacterial colonization even years after the implantation, impairing the osseointegration process. The treatment of a colonized implant is highly demanding and, in most cases, the implant's replacement is the only effective solution. To avoid the bacterial attachment and proliferation, bactericidal coatings are proposed as a long-term prevention tool. Those coatings must assure a bactericidal activity for a long period and cannot induce cytotoxic responses in eukaryotic cells. In this context, this research investigates TiO_2 coatings doped with three different bactericidal elements to be used in titanium-based implants. To achieve this goal, the investigation was divided in two parts. In the first study, coatings doped with different concentration of silver, a well know bactericidal agent used in biomaterials, are investigated to determine the optimum amount of silver to present bactericidal activity and bioactivity. In the second study, a silver doped coating was compared to coatings doped with zinc or boron, a new bactericidal coating. Coatings containing osteogenic elements (calcium and phosphorous) along with bactericidal elements (silver, zinc or boron) were obtained by plasma electrolytic oxidation (PEO) on commercially pure titanium grade 4 at 350 V for 60 s. Coatings were characterized by Scanning Electron Microscopy (SEM), X-ray diffraction (XRD) and X-ray Photoelectron Spectroscopy (XPS), while the ions dissolution were evaluated by Inductively Coupled Plasma (ICP). Coating's bactericidal activity were evaluated against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, while the biocompatibility and cytotoxicity were evaluated with adipose derived stem cells (ADSC). The characterization of the obtained coatings revealed that elements were successfully incorporated in the coatings structure, without changing the coating morphology and crystalline structure. It

was observed bactericidal action on the coatings with more than 0.6 %at Ag incorporated. Coatings with zinc or boron also presented bactericidal activity without inducing a cytotoxicity response against ADSC cells. Finally, this study revealed for the first time the bactericidal activity of a biocompatible boron doped coating, showing the possibility to use other bactericidal elements apart from silver as tools in the prevention of bacterial colonization in metallic implants.

List of publications

L. Sopchenski, S. Cogo, M. Dias-Netipanyj, S. Espósito, K. Popat, P. Soares, *Bioactive and antibacterial boron doped TiO_2 coating obtained by plasma electrolytic oxidation* Applied Surface Science 458 (2018), 49-58, ISSN 0169-4332 doi:10.1016/j.apsusc.2018.07.049.

L. Sopchenski, K. Popat, P. Soares, *Bactericidal activity and cytotoxicity of a zinc doped PEO titanium coating*, Thin Solid Films 660 (2018), 477-483, ISSN 0040-6090 doi: 10.1016/j.tsf.2018.05.055.

L.S. Santos, D. Francisco, E. Leite, S. Cogo, M. Dias-Netipanyj, S. Pinto, S. Espósito, K. Popat, P. Soares, *Bioactivity and Antibacterial Effects of Ag-Ca-P Doped PEO Titania Coatings*, J. Adv. Biotechnol. Bioeng. 6 (2018) 6–14. doi:10.12970/2311-1755.2018.06.02.

Patent

SOARES, PAULO; SANTOS, LUCIANE S.; Revestimento de implante de óxido de titânio dopado com boro biocompatível. 2017, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020170177289, Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial, Depósito: 18/08/2017 (*In Portuguese*)

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SANTOS, L. S.; Cogo S.; Espósito S.; Soares P. *Biocompatible calcium-phosphorous-boron doped titanium coating*. eCM Congress: Implant Infection, 2015. Davos, Switzerland. Abstract published on AO Research Institute, 2015. v. 30. p. 68-68.

Soares P.; SANTOS, L. S.; Cogo S.; Espósito S. *A Study on Properties of Zn Doped TiO2 Coatings Produced by Plasma Electrolytic Oxidation*. In: Materials Science & Technology 2015. Columbus, USA.

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Francisco, D. E. ; SANTOS, L. S. ; Pinto, S. C. S. ; Espósito S. ; Leite, E. L. ; Cogo S. ; Dias-netipanyj, M. F. ; Soares P. . *Comparative evaluation of different silver concentrations on PEO coatings*. 2016. 9° COLAOB - Congresso Latino-Americano de Orgãos Artificiais e Biomateriais. Foz do Iguaçu, Brazil.

SANTOS, L. S.; Laurindo, C. A. H. ; Espósito S. ; Gradowski, T. ; Soares P. . *Bioactivity and tribocorrosion of Sr containing PEO coating*. 2016. 9° COLAOB - Congresso Latino-Americano de Orgãos Artificiais e Biomateriais. Foz do Iguaçu, Brazil.

SANTOS, L. S.; Cogo S. ; Leite, E. L.; Dias-netipanyj, M. F.;Pinto, S. C. S.; Espósito S.; Soares P. *Bactericidal activity evaluation of tio2 coatings doped with silver, zinc and boron*. 24th ABCM International Congress of Mechanical Engineering. Curitiba, Brazil.

SANTOS, L. S. ; Francisco, D. E. ;; Cogo S. ; Dias-netipanyj, M. F. Espósito S. ; Pinto, S. C. S.; Popat, C. K.; Soares P. *Comparison of Ag, Zn and B Doped Titanium Coatings Bactericidal Activities*. 2017 4th Stevens Conference on Bacteria-Material Interaction. New Jersey, USA.

SANTOS, L. S.; Popat, C. K.; Soares P; *Bactericidal activity and cytotoxicity of a zinc doped PEO titanium coating.* 2018 45th International Conference on Metallurgical Coatings and Thin Films. San Diego, USA.

Acknowledgments

This research was supported by the Brazilian government agency CNPQ (Grants #309424/2012-7, #427025/2016-8, #305715/2016-0) and CAPES scholarship (PDSE-20161051632).

I would like to express my gratitude to my supervisor Prof. Paulo César Soares Jr. for the opportunity to develop this project and for all the guidance and support. I also would like to thank Prof. Ketul C. Popat for the guidance and for sharing his experience during my exchange at CSU. I also would like to thank Prof. Ricardo D. Torres and Prof. Carlos A. H. Laurindo, for their assistance and support at PUC-PR.

I am grateful for the assistance of Prof. Selene Espósito, Prof. Shelon Souza, Prof. James Self, PhD Geison Cambri and the students Marcela Dias-Netipanyj, Rachel Simon-Walker, Sheron Cogo, Yanyi Zang, Bella Neufeld, Irina Aimukanova and Dhanna E. Francisco. Thank you all the employees from PUC-PR and PPGEM that made this research possible, especially to Prof. Fred Lacerda Amorim and Jane Rocha.

My deepest gratitude also goes to my family and friends for their emotional support.

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1. Introduction

1.1. Motivation for the research

Endosseous metallic implants are used to reestablish motor and esthetic functions, in order to improve the patient's quality of life and decreasing the morbidity. Titanium and titanium alloys are the most used metals in implants fabrication, since they have excellent biocompatibility, adequate mechanical properties and high corrosion resistance (Khan, Williams, and Williams 1996; Long and Rack 1998).

The success of an implantation is directly related to the osseointegration quality (Albrektsson and Johansson 2001). Osseointegration is defined as "a direct, structural and functional connection between organized vital bone and the surface of a titanium implant, capable of bearing the functional load" (Adell et al. 1970). The osseointegration process occurs as a cascade of biochemical and biological processes, where the cell adhesion and proliferation are guided by the protein adsorption (Albrektsson and Johansson 2001; Yang, Cavin, and Ong 2003). The osseointegration depends simultaneously on the biological processes and the implants' surface; by this reason several researchers investigate superficial modifications on biomedical titanium, aiming to optimize the osseointegration process by altering the surface chemical composition, micro/nano topography, surface energy and roughness.

Despite the progress on the investigation of surface modification in implants, the osseointegration can be compromised by bacterial infections. The bacterial adhesion on the implant surface impair the adhesion and proliferation of eukaryotic cells, this process of competition was described for the first time in 1987 as a "race for the surface" (A. Gristina

1987). All endosseous implants are prone to bacterial infections, such as hip prosthesis, knee joints, dental implants and screws and plates for fracture fixation (Fig. 1).

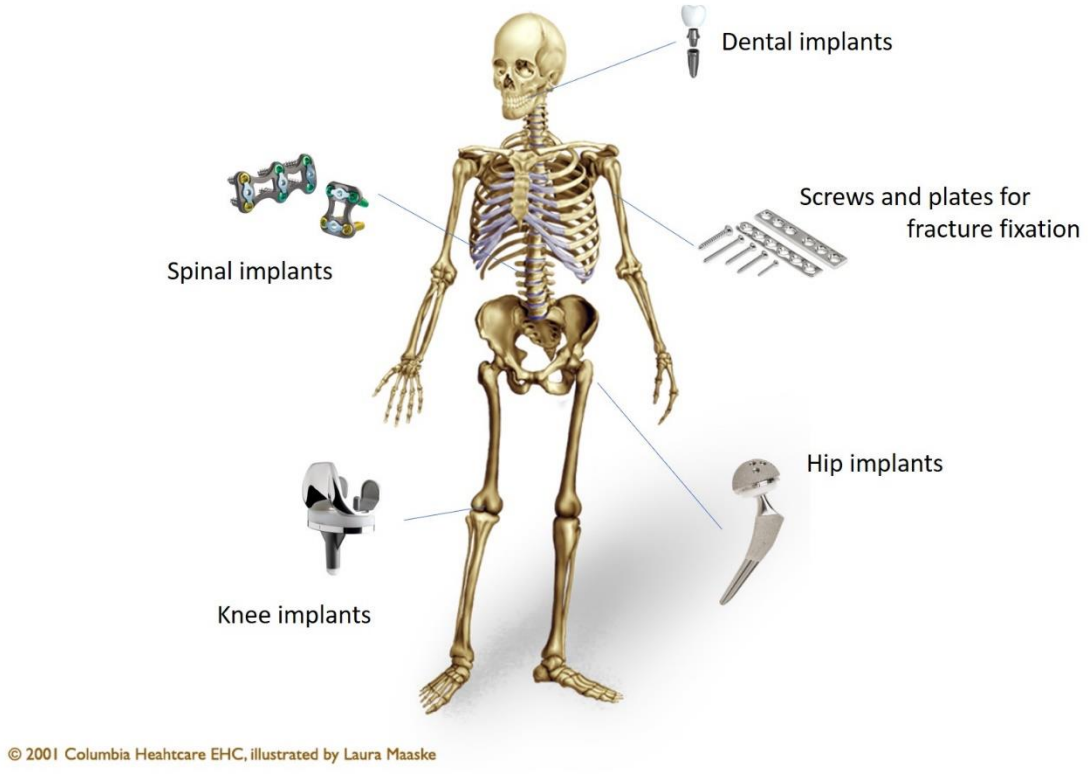

Figure 1: Metallic endosseous implants susceptible to bacterial infections.
Adapted from (Maaske 2017)

Bacterial infection in implants should be faced as a health care problem, since it increase the length of hospitalization, demands surgeon and hospital resources and multiple surgeries for the treatment, resulting in a projected expense of US$ 1.62 billion for 2020 for the health care system just in the USA (Kurtz et al. 2012). Unfortunately, there is no estimative of expenses or specific reports about implant infections in Brazil. The occurrence rate of bacterial infection in implants is variable among the implant function, reaching 14% of dental implants, 3 to 5% of orthopedic implants and more than 30% of

screws and plates used for fracture fixation (Darouiche 2001; Norowski and Bumgardner 2009; Young and Barrack 1994). Furthermore, bacterial colonization is one of the main reasons of implant failures, reaching expressive rates among dental implants and knee joints (25.2%) (Bozic et al. 2010).

The research and development of an antibacterial coating to be used in titanium-based implants is justified. The implant coating should prevent the bacterial proliferation at the same time that assure the osseointegration, looking for a reduction on the medical expenses and improving the quality of life of implanted patients.

1.2. Research objectives

General aim:

To develop and characterize bactericidal coatings on commercially pure titanium.

Specific aims:

1) To determine the optimum amount of silver on the coating assuring bactericidal activity and present biocompatibility.
2) To investigate the use of other bactericidal elements to assure bactericidal activity and present biocompatibility.
3) To evaluate the bactericidal properties of the coatings containing Ag, Zn and B on commercially pure titanium.
4) To evaluate the biocompatibility and cytotoxicity of the bactericidal coatings on Adipose derived stem cells (ADSC).

2. Literature review

2.1. Bacterial infections in implants

Endosseous implants are susceptible to bacterial colonization during the surgery, as a post-operative complication, as well years after the implantation, when endogenous bacteria may migrate to the prosthesis surface (Pulido et al. 2008). Factors related to the medical procedures influence the risk of develop a bacterial infection during the surgery. The operating room quality, contaminated air-conditioning and water systems, and transmission from the medical team to the patient are the major factors related to exogenous bacterial infections (Spagnolo et al. 2013; Loveday et al. 2014; Widmer et al. 2010; Uçkay et al. 2013b). Recently, Morgenstern et al. (2016) evaluated the nasal carriage of *staphylococci* on orthopedic, spine, head and neck surgeons and analyzed the correlation with their involvement on implant infection treatment. On that study, the nasal carriage of 1166 surgeons from 75 countries were analyzed and 85.8% were involved on the treatment of bone or implant infections, and just 4.7% presented no bacterial growth. Despite all the prophylactic measures before the surgery, the presence of bacteria on the surgeon's cohort may represent a risk for the patients.

Infections in implants may also be caused by endogenous bacterial strains, originated from colonies from the patient's own flora, located on skin, hair, digestive or respiratory systems (von Eiff et al. 2001; Pulido et al. 2008). Eighty percent of infections caused by *Staphylococcus aureus* are caused by strains identical to the ones found on

patient's nasal cavity, and patients colonized with *S. aureus* have increased from 2 to 9 times the risk of develop an implant infection (Wertheim et al. 2004).

Bacterial infection in implants may occur in all clinical settings, although obesity, diabetes, recurrent surgeries and smoking patients are factors that increase the risk of infection (Marmor and Kerroumi 2016; Crowe et al. 2015).

Implant centered infections may happen causing fever, edema, local pain and disturbance in the wound healing, as showed in Fig. 2. Late infections appear with sudden symptoms or without signs, being necessary the monitoring of inflammatory markers (Zimmerli 2006).

Figure 2: Fracture fixation plates with bacterial infection a) two weeks, b) six weeks and c) ten weeks after the firsts symptoms. Adapted from (Apivatthakakul et al. 2017).

2.2. Pathogens related to implant infections

More than 60% of implant centered infections are caused by *Staphylococcus aureus* or *Pseudomonas aeruginosa* (An and Friedman 1996; Arciola et al. 2012; Campoccia, Montanaro, and Arciola 2006; Mangram et al. 1999). *P. aeruginosa* is an opportunistic gram-negative bacterium, commonly associated to respiratory system and nosocomial infections, while *S. aureus* is a gram-positive bacterium and commensal inhabitant of

human body, present mainly on the skin and mucosae. *S. aureus* has a low virulence associated; however immunosuppressed patients may develop life risk infections by this bacterium. Other common strains related to nosocomial infections, as *Enterococcus faecalis* and *Escherichia coli*, are also related to implant infections, Fig 3.

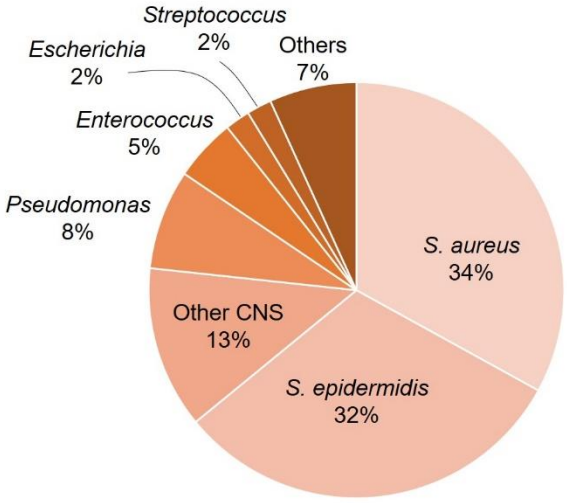

Figure 3: Frequency of main pathogenic species responsible for implant-associated infections. CNS: Coagulase-negative staphylococci. Adapted from (Campoccia, Montanaro, and Arciola 2006).

It is estimated that 60% of the population are intermittent carriers of *S. aureus* on the anterior nares of the nose (Kluytmans, van Belkum, and Verbrugh 1997). At least 80% of nosocomial infections caused by *S. aureus* are caused by the own *S. aureus* patient strain, previously present on the patient's skin or mucosae (Wertheim et al. 2005). Despite all the prophylactic measures, bacterial infection in implants still have an expressive mortality rate, Lora-Tamayo et al. showed 7% of deaths among patients infected just with *S. aureus* in total knee arthroplasty (Lora-Tamayo et al. 2013).

Other underestimated bacterial strains, as *Propionibacterium acnes*, related to the skin acne, can also be responsible for implant infections (Portillo et al. 2013). Hahn et al. showed the *P. acnes* was responsible for 7% of late infections in scoliosis surgery in a clinical study following one hundred and one patients. All the infected patients were submitted to antibiotic treatment and revision surgery for the implant removal and substitution (Hahn, Zbinden, and Min 2005).

2.3. Biofilms

The reason why bacteria with usual low virulence are capable to induce serious complication when in contact with implant surfaces is its ability to form biofilms. Biofilms are a structured cell community, enveloped by a self-produced polymeric matrix (J. Costerton, Stewart, and Greenberg 1999). The biofilm is formed when floating bacteria attach to a surface, mediated by physiochemical forces and multiply locally forming microcolonies. Those microcolonies are maturated and growth into microcolonies encased by an extracellular polymeric matrix. Inside the matrix, the bacteria behave differently from bacteria in planktonic state, through a biochemical communication (called *quorum sense*). Bacteria are capable to regulate gene expression and nutrient distribution in order to adjust population size, adapt the growth and spread the contamination, Fig. 4 (Resch et al. 2005; Hobley et al. 2015).

Biofilm compromises the soft tissue healing and bone apposition by inhibiting the attachment and growth of eukaryotic cells. As the bacteria inside the biofilm is protected

from the host defense and oral antibiotic therapies, in most cases the infection eradication is difficult and dispendious (Tande and Patel 2014; AG Gristina 1987). Nickel et al. showed 50 g/mL of Tobramycin for 8 h was capable to eradicate a *P. aeruginosa* planktonic culture, while 1000 g/mL for 12 h was not able to eradicate the same bacteria inside a biofilm (Nickel et al. 1985).

The use of bactericidal coatings on metallic implants represent a long-term alternative to prevent bacterial adhesion and further biofilm formation.

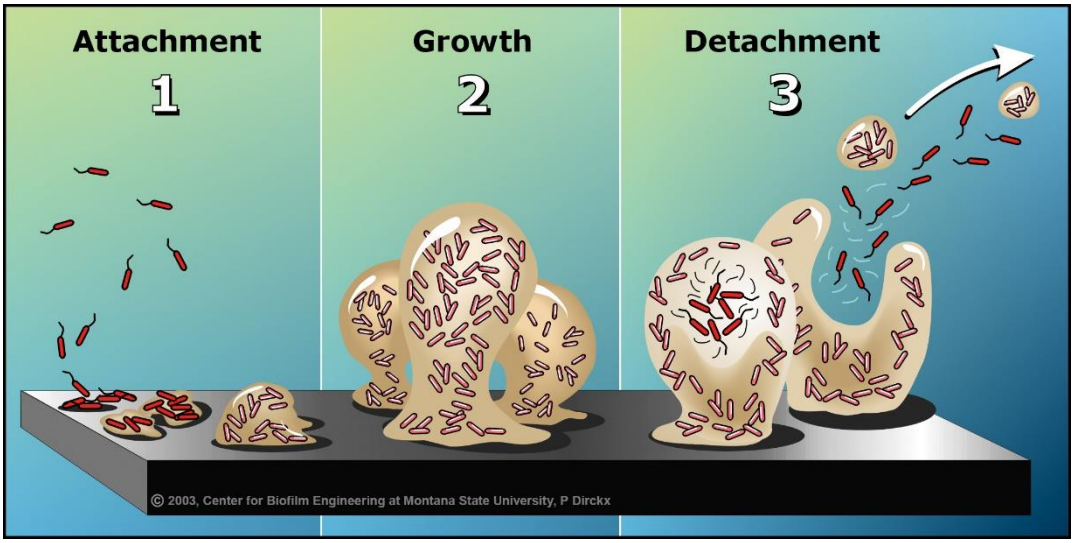

Figure 4: Growth cycle of a biofilm. Adapted from (P. Dirckx 2003)

2.4. Prevention and treatment

Prevention of infection in implants consists of standard surgery procedures such as the patient asepsis, but also encompass specific measures, like local and oral antibiotic perioperative prophylaxis (Uçkay et al. 2013a). The indiscriminate use of antibiotics can promote the spread of resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* (Bonomo and Szabo 2006; Enright et al.

2002). Those strains are usually stablished on hospital environments and are becoming increasingly difficult to eradicate, implying on high cost of treatment and suffering for the patient (Veltman et al. 2015; Vila et al. 2016). The main strategies to treat a bacterial implant infection consists in long term antibiotic administration, debridement without the prosthesis removal and resection of the prosthesis with or without the reimplantation, either at the time of the removal (one stage) or delayed from weeks to months (two-stages) (Tande and Patel 2014). Two-stage surgeries expose the patients to elevated risks and increase the costs in 4.8 times compared to the first surgery (Peel et al. 2013). Despite the high rate of infection control in a short term, the two-stage treatment still present high failure rate from reinfection and inability to perform the reimplantation (Berend et al. 2013).

2.5. Bactericidal coatings

Bactericidal surfaces in endosseous implants represent an effective tool in the prevention of bactericidal infections, since it prevents the biofilm formation from early stage after the surgery (Campoccia, Montanaro, and Arciola 2013; Cheng et al. 2007). Bactericidal surfaces can prevent bacteria attachment and proliferation based on antibiotic delivery or in the presence of some bactericidal agent (as nitric oxide, bactericidal elements, or nanoparticles) (Goudouri et al. 2014; Pelgrift and Friedman 2013). The prophylactic use of antibiotic could have some disadvantages, as the kinetic of elution, with a high release rate within the first hours, the possible ineffective concentration and the unnecessary exposure of health patients to the drug (Zhao et al. 2009; Campoccia et al. 2010; Tobin 2017; Goodman et al. 2013). Coatings with bactericidal activity based on

bactericidal elements have been researched to overcome those limitations. An effective bactericidal coating must assure a bactericidal activity for a long period without impair the bone healing by inducing cytotoxic responses in eukaryotic cells. For this reason, some bactericidal elements are not suitable to be used in biomaterials, like thallium, cadmium, chromium and mercury, for example.

Among all bactericidal elements this research choose tree of them, based on its distinct mechanisms of actions, described on the following section.

2.5.1. Silver

The bactericidal mechanism of silver is not fully understood yet, but it is well known that silver ions can interact with thiol groups (S-H) forming Ag-S bonds. Thiol groups are present in many proteins and their disruptions lead to loss of proteins shape and functionality (H. Li et al. 2016; Feng et al. 2000). Silver also kills bacteria by generating reactive oxygen species (ROS) which impairs enzymes from the respiratory chain and also prevents DNA replication, Fig. 5 (Park et al. 2009).

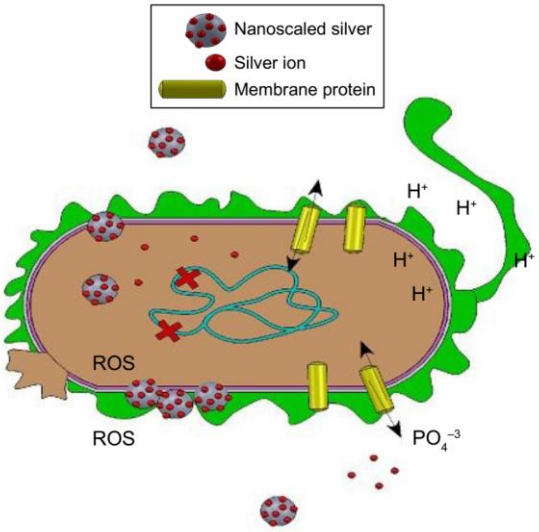

Figure 5: Bactericidal mechanisms of silver: Formation of ROS and disruption of membrane functionality. Adapted from (Vimbela et al. 2017).

Silver is used as a bactericidal agent in air disinfection systems, water filters and food package, but the use of silver in indwelling devices remain controversial as some cytotoxic reactions can be originated (Lin et al. 2015; Yoon et al. 2008; Miaśkiewicz-Peska and Łebkowska 2011; Lv et al. 2009; de Moura, Mattoso, and Zucolotto 2012; Paladini, Cooper, and Pollini 2014). Studies *in vitro* showed that silver nanoparticles decrease liver cells viability by ROS generation, inducing cells to apoptosis (Piao et al. 2011; Xue et al. 2016). A case study conducted by Trop et al., had also showed the increase of silver levels in blood and a higher production of liver enzymes when silver based wound dressings were used for burn healing (Trop et al. 2006). Furthermore, the indiscriminate use of silver can induce argyria, a cutaneous manifestation where silver precipitates are deposited on the skin, giving it a brownish-grey appearance (Jiravova et al. 2016; Sakai et al. 2007; Karakasli et al. 2014).

As the use of bactericidal coatings in endosseous implants must not damage eukaryotic cells around the implant, and silver has a dose-dependent cytotoxicity, an investigation of the proper amount of silver in implants is needed (Raphel et al. 2016; AshaRani et al. 2009).

2.5.2. Zinc

Zinc is an oligoelement present in abundance on the bone tissue (McBean et al. 1972). Zinc influences the bone growth and mineralization and its deprivation impairs the bone metabolism (Yamaguchi 1998). Moonga et al. showed that zinc is also involved on the control of bone resorption *in vitro*, by inhibiting the osteoclasts action (Moonga and Dempster 1995). Besides playing an important role on bone tissue, zinc is also essential for the function of more than 300 enzymes, proteins, regulate DNA synthesis, influences hormonal regulation and cell division (Prasad 2008; MacDonald 2000; Frassinetti et al. 2006).

Zinc has been extensively investigated as a broad spectrum bactericidal agent specially under the zinc oxide form (Zarrindokht Emami-Karvani 2012). Zinc oxide may act by the dissolution of zinc ions or the generation of reactive oxygen species (ROS), capable of disrupt the bacteria membrane (Mcquillan and Shaw 2014). Additionally, zinc oxide nanoparticles and structures may interact and attach to the cell wall, impairing the membrane function and possibility disrupting it (Ann et al. 2014). Zinc ions are believed to compete with di-valent binding sites on proteins, impairing the bacteria's metabolism,

as in the case of PsaA protein, where the binding zinc ion prevent the manganese absorption, resulting in the bacteria death (McDevitt et al. 2011).

In biomaterials, zinc has been widely studied as an antibacterial agent in hydroxyapatites, dental composites, bioglasses, and dopant on metallic materials due to its bactericidal activity against different pathogens (Sevinç and Hanley 2010; Hidalgo-Robatto et al. 2018; Huang et al. 2017; Atkinson et al. 2016). But most of the research are focused on zinc oxide, added on the surface of titanium implants by techniques as cathodic arc deposition (Tsai et al. 2013).

2.5.3. Boron

Boron is a trace element used for a long time as food preservative and recently its compounds has been investigated as a pharmaceutical agent (Leśnikowski 2016; Nielsen 1991). Boron compounds have bactericidal activity and antibiofilm properties against gram-positive and gram-negative bacteria, and antifungal activity (Baker, Tomsho, and Benkovic 2011; Sayin, Ucan, and Sakmanoglu 2016; Yilmaz 2012). The bactericidal mechanism of action of boron is not well stablished, although many studies show its presence and influence on the bacteria *quorum sense*, a molecular signaling system that coordinate gene expression (Dembitsky, Al Quntar, and Srebnik 2011; Chen et al. 2002; Coulthurst et al. 2002).

Boron is a trace element beneficial to human health by being involved in many biochemical processes (Nielsen and Meacham 2011; Nielsen 2014). Boron upregulates growth factors (VEGF and TGF-b) related to wound healing and has a suppressive effect

on the inflammatory response by down-regulating some specific enzymes involved on the inflammatory process (Hunt and Idso 1999; Dzondo-Gadet et al. 2002). Moreover, boron has an important role in bone formation and maintenance, and its presence on biomaterials is proved to enhance bone regeneration, and promote the osteogenic differentiation on bone marrow stromal cells (Hakki, Bozkurt, and Hakki 2010; Gorustovich et al. 2006; Brown et al. 2009; Ying et al. 2011).

The use of boron in biomaterials has been reported on polymers, glasses, and ceramics, but to the best of our knowledge, bactericidal activity and biocompatibility of boron on metallic implants coatings have never been reported (Wu et al. 2011; de Queiroz et al. 2006; Barheine et al. 2011).

2.6. Plasma electrolytic oxidation

Elements and nanoparticles can be incorporated on the surface of valve metals (titanium, niobium, tantalum, etc.) by electrochemical techniques, such as plasma electrolytic oxidation (PEO). PEO is largely used to obtain coatings on titanium-based implants since this technique promote the growth of a well adhered bioactive porous coating on the implant surface (Walsh et al. 2009). By selecting the appropriate oxidation parameters, it is possible to tune the coating properties to optimize the biological response.

During the PEO, the titanium piece is used as a work electrode in a two electrodes electrochemical cell, where the work sample (anode) and the counter electrode (cathode) are immersed in an aqueous electrolyte, Fig 6. By applying an external potential, the anode surface is oxidized while the cathode surface is reduced (Yerokhin et al. 1999). During the

first stage of oxidation, an oxide layer is formed on the titanium surface dictated by the following reactions:

a) In the cathode:

$$2H_2O \rightarrow 2O^{2-} + 4H^{+} \rightarrow H_2\,(gas) \quad (1)$$

b) In the vicinity of the work electrode:

$$2H_2O \rightarrow 2O^{2-} + 4H^{+} \quad (2)$$

$$2H_2O \rightarrow 2O^{2-} + 4H^{+} + 4e^{-} \quad (3)$$

c) In the work electrode:

$$Ti \rightarrow Ti^{2+} + 2e^{-} \quad (4)$$

$$Ti^{2+} + 2O^{2-} \rightarrow TiO_2 + 2e^{-} \quad (5)$$

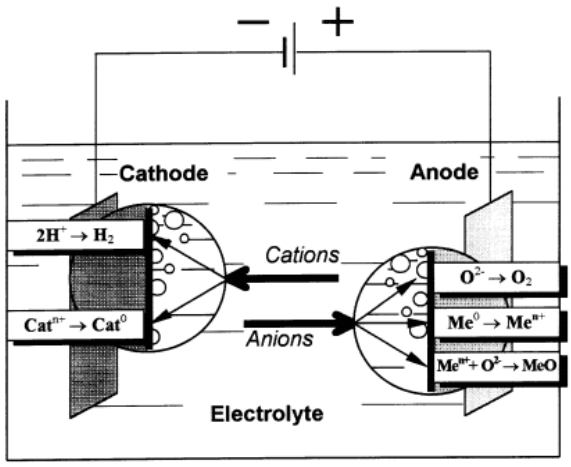

Figure 6: Electrolytic oxidation process in an aqueous solution.
Adapted from (Yerokhin et al. 1999).

The TiO_2 oxide layer acts a barrier layer, passivating the titanium substrate, although when the applied potential exceeds a critical value, the electrical breakdown of the oxide film take place and electrical discharges can be observed in the form of sparks of light. The electrical discharge give rise to short-lived plasma channels (μs) causing the local melting of the coating (reaching 3500 °C) (Dunleavy et al. 2009; Nominé et al. 2015a). The high temperature promote the formation of crystalline phases and during the resolidification, elements or nanoparticles present in the electrolyte are incorporated on the coating structure giving rise to doped TiO_2 coatings (Nominé et al. 2015b; Shokouhfar and Allahkaram 2016). The oxide properties such as roughness, thickness, porosity, crystallinity and chemical composition, are defined by the oxidation parameters, such as the potential/current applied, the oxidation time and the electrolyte composition (Q. Li, Liang, and Wang 2013; C.A.H. Laurindo et al. 2018).

PEO technique is widely used to obtain calcium/phosphorous doped titanium surfaces, as these elements turn the surface bioactive by promoting bone growth (Wang et al. 2015; Ribeiro et al. 2015). Ishizawa et al. showed for the first time the possibility to incorporate Ca and P on the TiO_2 structure by PEO, those coatings reduce the Ti^{4+} ion release, increase osteoblast adhesion and proliferation *in vitro*, and improve the osseointegration *in vivo* (Ishizawa and Ogino 1995; Mohedano et al. 2014; Xiaolong Zhu et al. 2004).

Multifunctional coatings that prevent the bacterial adhesion and improve the bone growth by the presence of osteoinductor elements can be obtained by adding bactericidal elements along with calcium and phosphorous sources in the electrolyte.

3. Experimental Procedure

This research was divided in two parts, on the first study a preliminary evaluation of the bactericidal activity of the samples containing silver was assessed in order to determine the proper amount of silver needed to assure bactericidal activity without inducing cytotoxic effects. On the second study this selected coating containing silver was compared to coatings containing zinc or boron.

The coatings were obtained and characterized on the Laboratory of biomaterials and surface engineering (LABES) at PUC-PR. The bactericidal activity and biocompatibility evaluation were done at Biomaterials Surface Micro/Nano-Engineering Laboratory at CSU during a six-month exchange.

3.1. Fabrication of doped TiO_2 coatings

3.1.1. Samples preparation

Commercially pure titanium grade 4 discs (6 mm diameter, 2 mm thickness, (Acnis do Brasil) were ground using #320 and #600 SiC abrasive papers, and cleaned ultrasonically in acetone, ethyl alcohol, and distilled water for 15 min, successively. After cleaning, samples were stored in 40 °C up to the coating obtainment.

3.1.2. Electrolyte preparation

Table 1 lists the different electrolyte compositions used during the plasma electrolytic oxidation. An aqueous solution containing 0.15 M calcium acetate ($Ca(C_2H_3O_2)_2$, Synth), 0.02 M calcium glycerophosphate ($C_3H_7O_6PCa$, Sigma-Aldrich) was used as a base electrolyte to obtain coatings doped only with calcium and phosphorous (named CaP group) to be used as a control group. The concentration of silver nitrate was varied from 0.02 mM to 0.64 mM to evaluate the minimum amount of silver needed to present bactericidal activity. Zinc or boron were added on 0.02 M of zinc acetate ($(CH_3CO_2)_2Zn$) or 0.02 M borax ($Na_2[B_4O_5(OH)_4]\cdot 8H_2O$), respectively.

Table 1: Electrolyte composition used for the plasma electrolytic oxidation process.

		Electrolyte composition				
					Base electrolyte	
Group	Sample name	Silver nitrate	Zinc acetate	Borax	Calcium acetate	Calcium glycerophosphate
Control	CaP	--	--	-	0.15 M	0.02 M
1	2Ag-CaP	0.2 mM	--	--		
2	4Ag-CaP	0.4 mM	--	--		
3	16Ag-CaP	0.16 mM	--	--		
4	64Ag-CaP	0.64 mM	--	--		
5	Zn-CaP	--	0.02 M	--		
6	B-CaP	--	--	0.02 M		

3.1.3. Plasma electrolytic oxidation

Samples were anodized by PEO under potentiostatic method at 350 V for 60 s at room temperature, using a DC power supply (62012P- 600-8/Chroma). The anodization parameters were chosen base on previous results from our group (Laurindo et al., 2014). The samples were oxidized using an acrylic container, where the sample was used as anode, while a titanium plate was used as cathode, Fig 7.

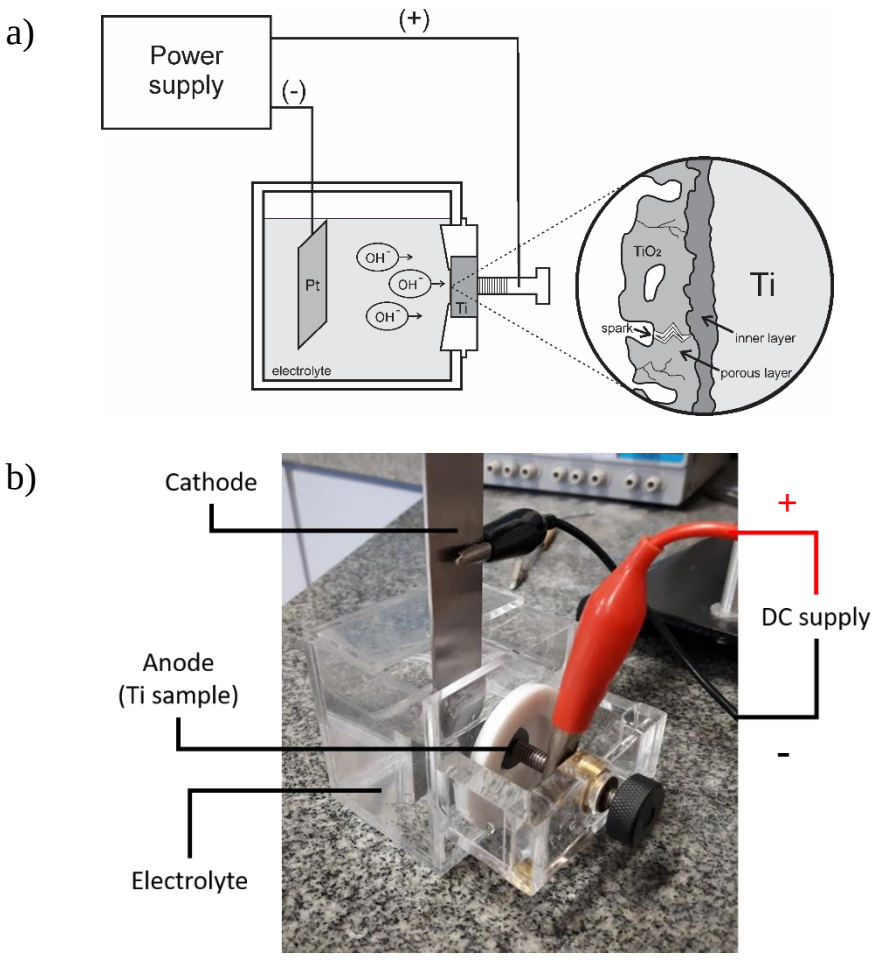

Figure 7: a) Scheme of the experimental setup used to obtain an oxide layer on the titanium samples and b) electrochemical cell used during for the PEO. Adapted from (Laurindo et al. 2018).

3.2. Samples characterization

The coating morphology were analyzed by scanning electron microscopy (SEM) using a Vega3/Tescan instrument. Chemical states on the coating surface was determined by X-ray photoelectron spectroscopy (XPS, ESCASystems X-ray Photoelectron Spectrometer 5800) using a monochromatic Al Kα X-ray source. XPS survey spectra were collected from 0 to 1100 eV with pass energy of 187.85 eV, and all spectra were referenced by setting the C 1s peak to 284.6 eV. High resolution spectra were recorded to assess the incorporation of the bactericidal elements. Data for percent elemental composition, elemental ratios and peak fit analysis were calculated using Multipack and XPSPeak 4.1 (Freeware) software.

Crystalline phases of oxide surfaces were analyzed by thin film X-ray diffraction (TF-XRD, XRD-7000, Shimadzu) using a Cu Kα (λ = 1.54 Å) radiation at 40 kV and 20 mA. A 5° incidence angle with a scan rate of 2 °/min from 20° to 80° in a step of 0.02° was used.

The bactericidal activity of the coating should endure for a long term and should not be quickly reduced by the contact with the body fluids, so the kinetic of ions dissolution from the coating was assessed by Inductively Coupled Plasma optical emission spectrometer (ICP – Optima 7000 DV, Perkin Elmer). Samples were immersed in deionized water at 37 °C for 1, 7, 14 and 28 days, after these time points 5 mL of the deionized water was analyzed by ICP to determine the concentration of Ca, P, Ag, Zn and B.

3.3. Biocompatibility evaluation

Adipose derived stem cells (ADSCs) were cultured in α-MEM medium, supplemented with 10% of Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin in a humidified incubator at 5% CO_2 and 37 °C. The coatings were cleaned, and sterilized in a sequence of acetone, alcohol and phosphate buffered saline (PBS), followed by UV radiation exposure for 30 min, prior to cell seeding. ADSCs were seeded on the coatings in a 48-well plate at a density of 10^4 cells per well.

3.3.1. Cell Viability

Adipose derived stem cells (ADSC) viability was measured after 1 and 7 days of culture using alamarBlue Assay Reagent (Promega). Adhered cells were incubated at 37 °C for 4 h in fresh α-MEM and 10% of alamarBlue Reagent. The alamarBlue Reagent is an oxidized form redox indicator that is blue in color. When incubated with viable cells, the reagent changes color from blue to red. After 4 h the optical density (OD) of the solution was measured at 570 nm and 600 nm using a spectrophotometer (FLUO-star Omega; BMG Labtech). The percentage of alamarBlue reduction was calculated according to manufacturer´s instructions.

3.3.2. Cell Adhesion and Proliferation

After 1 and 7 days of initial culture, the ADSCs adhesion and proliferation were investigated by fluorescence staining with Rhodamine Phalloidin (actin-cytoskeleton) at 70 nM, and 4' 6-diamidino-2-phenylindole DAPI (nucleus) at 300 nM. Samples were removed from the growing media, washed with PBS and fixed with 3.7% formaldehyde for 15 min at room temperature. To permeabilize the cells, samples were incubated with 1% of Triton-X100 for 3 min and then washed with PBS. Samples were then, incubated in Rhodamine-Phalloidin stain at a concentration of 70 nM for 30 min at room temperature, with addition of DAPI on the last 5 min. The solution was aspirated and the coatings were then washed with PBS and imaged using a Zeiss Imager-A2 fluorescence microscope.

3.3.3. Cell morphology

Morphology of ADSCs adhered on the coatings was evaluated using SEM after 1 and 7 days of culture. SEM was used to visualize how cells interacted with the coatings. The cells were fixed in a solution of 3% glutaraldehyde (Ted Pella), 0.1 M sodium cacodylate (Alfa Aesar), and 0.1 M sucrose (Fisher Scientific) for 45 min. Samples were then incubated in buffer solution of 0.1 M sodium cacodylate (Alfa Aesar) and 0.1 M sucrose (Fisher Scientific) for 10 min. After fixation, cells were dehydrated in increasing concentration of ethanol (35, 50, 70 and 100%) for 10 min each. Following, the surfaces were dehydrated by incubating in dexamethyldisilazane (HMDS, Sigma) for 10 min. The

surfaces were coated with 20 nm of Au and the SEM images were recorded with the samples tilted at 45º for a better visualization.

3.3.4. Cytotoxicity

The cytotoxicity of the coatings toward the ADSCs after 1 day of culture was evaluated by Lactate Dehydrogenase (LDH) assay kit (Cayman Chemical). Besides the cells cultured on the coatings, a set of 10 wells received just the cells in α-MEM, where 5 wells were used as negative control, and other 5 received 10% of Triton-X to be used as positive control. After 24 h of culture, 100 µL of the cell culture media was transferred from each well to a 96-well plate together with 100 µL of LDH solution. The 96-well was incubated for 30 min on an orbital shaker and then the absorbance of each well was measured at a wavelength of 490 nm using a plate reader (BMG Labtech).

3.4. Bactericidal activity evaluation

In the study I a preliminary evaluation of the bactericidal activity was assessed by the colony forming units (CFU) counting. *Staphylococcus aureus* (ATCC 25923) was cultivated in brain heart infusion (BHI) agar medium (Acumedia®, 107340A) for 24 h at 37 °C. A standard solution of the S. aureus was prepared in a density of 108 CFU/mL. A 1 mL aliquot of this solution was added into a test tube containing BHI broth medium and the coating samples; each tube with samples was tested independently. After 24-hour of culture, the coatings were gently rinsed with PBS and in order to detach adhering bacteria,

the test tubes were vigorously vortexed for 1 min. The solution containing the detached bacteria was diluted in PBS, in a 10-fold proportion, and re-cultivated in agar plates. The agar plate was incubated at 37 °C for 24 h and the number of CFU was counted using a plate counter.

In the second study, a detailed evaluation of the antibacterial activity of the coatings was assessed using a live/dead staining kit (LIVE/DEAD® BacLight, Thermo Fisher) against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (PA01). Stocks of *S. aureus* and *P. aeruginosa*, previously prepared in a glycerol solution and stocked at -80 °C, were thaw at room temperature and centrifuged at 4700 rpm for 10 min. The glycerol supernatant was discarded, and the pellet was resuspended in 50 mL of Lysogeny broth media (LBM) and allowed to grow for 24 h at 37 °C. The bacteria solution was adjusted to an OD600 nm ~ 0.35 and an aliquot of 500 µL of this solution was added over the coatings in a 48 well plate for 6 h and 24 h. After this period, samples were gently rinsed with the phosphate buffer solution (PBS) and fixed with formaldehyde for 15 min, rinsed with PBS and incubated for 25 min at 37 °C in the staining solution prepared following the manufacturer's instructions. The staining solution contains Propidium iodide (PI) to stain the dead bacteria and SYTO® 9 to stains the living bacteria, which together allow to determine the relative proportion of dead and live bacteria. Fluorescence images of the live and dead bacteria were recorded in a fluorescence microscope (Zeiss Imager-A2) and analyzed by ImageJ software to determine the percentage of live and dead bacteria on the coatings. *S. aureus* and *P. aeruginosa* morphologies on the coatings were analyzed by SEM. After the incubation, bacteria were fixed with 2.5% glutaraldehyde for 4 h,

dehydrated in ethanol series (60, 70, 80, 90, and 100%, each for 10 min) and coated with Au.

3.5. Statistical analysis

All the measurements were performed at an average of 3-10 replicates and are represented as mean ± standard deviation. The experimental data were subjected to a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test to determine the statistical difference between the groups. The significance was regarded at p value < 0.001.

4. Results and discussion

4.1. Study I: Preliminary study on the investigation and determination of the optimal Ag-CaP coating

4.1.1. Coating morphology

The coatings presented a volcano shape porous morphology, characteristic of coatings obtained by PEO process in calcium and phosphorous containing electrolyte(X Zhu, Kim, and Jeong 2001; Carlos A.H. Laurindo et al. 2014). No difference on the coating morphologies, as the porous distribution and dimensions were observed by the addition of silver nitrate on the electrolyte, as seen on the SEM images in Fig 8.

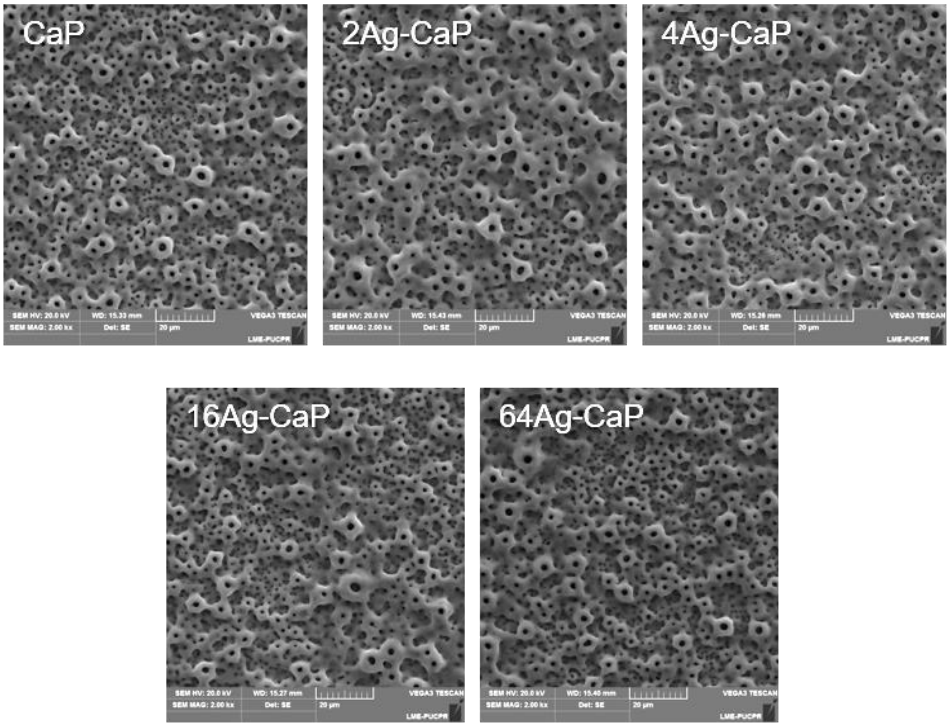

Figure 8: Scanning electron microscopy images of the coatings containing different amounts of Ag obtained by PEO.

4.1.2. Coating chemical composition

Elemental analysis performed by XPS showed the successful incorporation of Ca, P and Ag from the electrolyte (Table 2). Silver incorporation determined by XPS shows a non-linear relation of Ag concentration on the electrolyte (Fig. 9).

Table 2: Chemical composition of the samples containing Ag assessed by XPS.

	Chemical composition (% at)				
Sample name	**Ti**	**O**	**Ca**	**P**	**Ag**
CaP	6.3	67.4	12.7	13.6	--
2Ag-CaP	7.3	67.7	11.0	13.8	0.2
4Ag-CaP	4.1	68.7	13.8	12.8	0.6
16Ag-CaP	4.8	65.5	13.8	14.5	1.4
64Ag-CaP	3.7	64.8	13.8	15.2	2.4

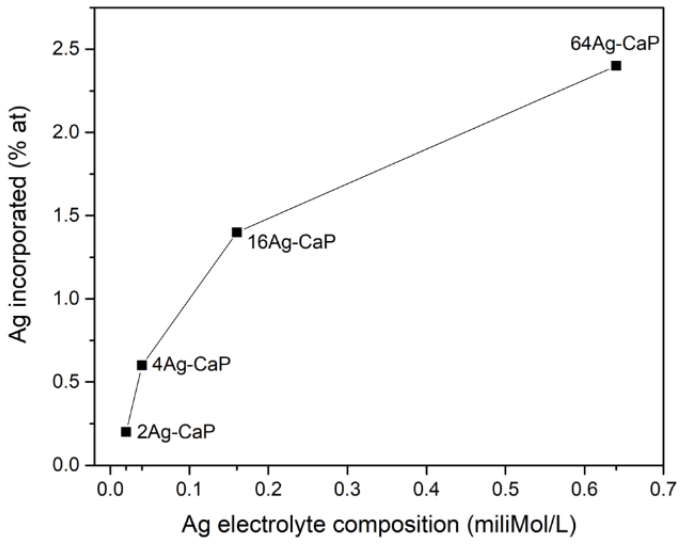

Figure 9: Ag incorporated on the coatings by the electrolyte's Ag concentration.

4.1.3. Crystalline structure

The XRD patterns on Figure 10 shows the formation of two titanium dioxide crystalline phases during the PEO process: Anatase and Rutile. Additionally, peaks from the titanium substrate can also be observed for all coatings, while peaks from the silver incorporation were not observed. No difference in the XRD pattern is observed among the coatings, showing the silver incorporation has not affected the crystalline composition.

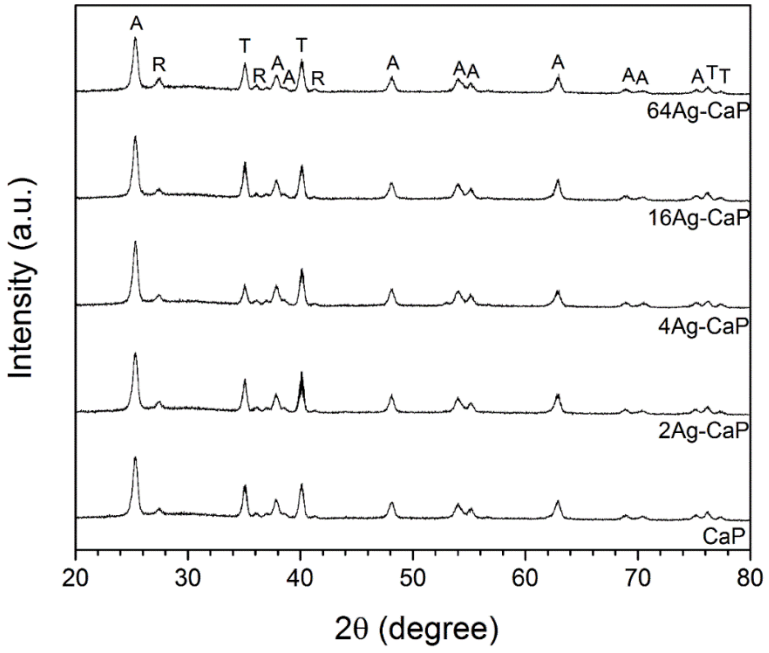

Figure 10: XRD diffraction pattern of the coatings containing different amounts of Ag. A, R and T stands for Anatase, Rutile and Titanium, indexed with the ICDD files #01-075-2547, 01-078-4187 and 01-071-4632, respectively.

4.1.4. Biocompatibility

ADSCs viability was not impaired by the silver incorporated coatings, as showed by the percentage of AlamarBlue reduced after 1 and 7 days of culture (Fig. 11). ADSCs

are viable on all coatings, with no significant difference among silver containing coatings and the reference group.

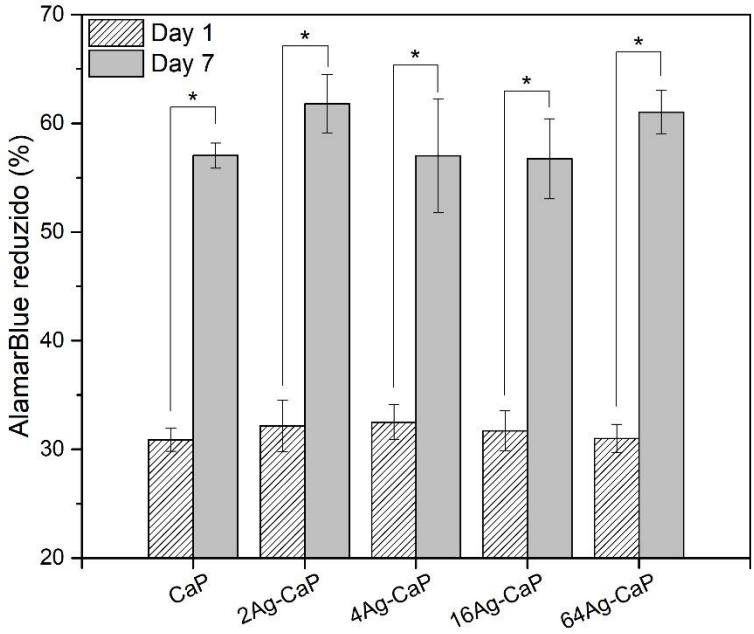

Figure 11: Cell viability on coatings doped with different amounts of Ag measured by the reduction percentage of AlamarBlue after 1 and 7 days of ADSCs culture. *p < 0.0001.

After one day of culture, the ADSCs morphology was dependent on the amount of silver in the coatings (Fig. 12). Coatings with higher amounts of silver presented less spread and thinner cells than samples with lower amounts of silver, where a spread star shape ADSCs can be observed. This difference in cell shape among the groups was not observed after 7 days of culture, since the cells had covered all samples surfaces, indicating the silver presence impacts only the early stage of cell proliferation. SEM images show ADSC flattened and adhered to the surface coating (Fig. 13). ADSCs presented good affinity with the porous coating, showing cellular extensions toward adjacent cells after just one day of culture. After 7 days of culture, cells have covered the entire surface. No difference in cell morphology was observed among the groups, showing that the amount of silver

incorporated does not induce deleterious effects in the ADSCs cell adhesion and proliferation.

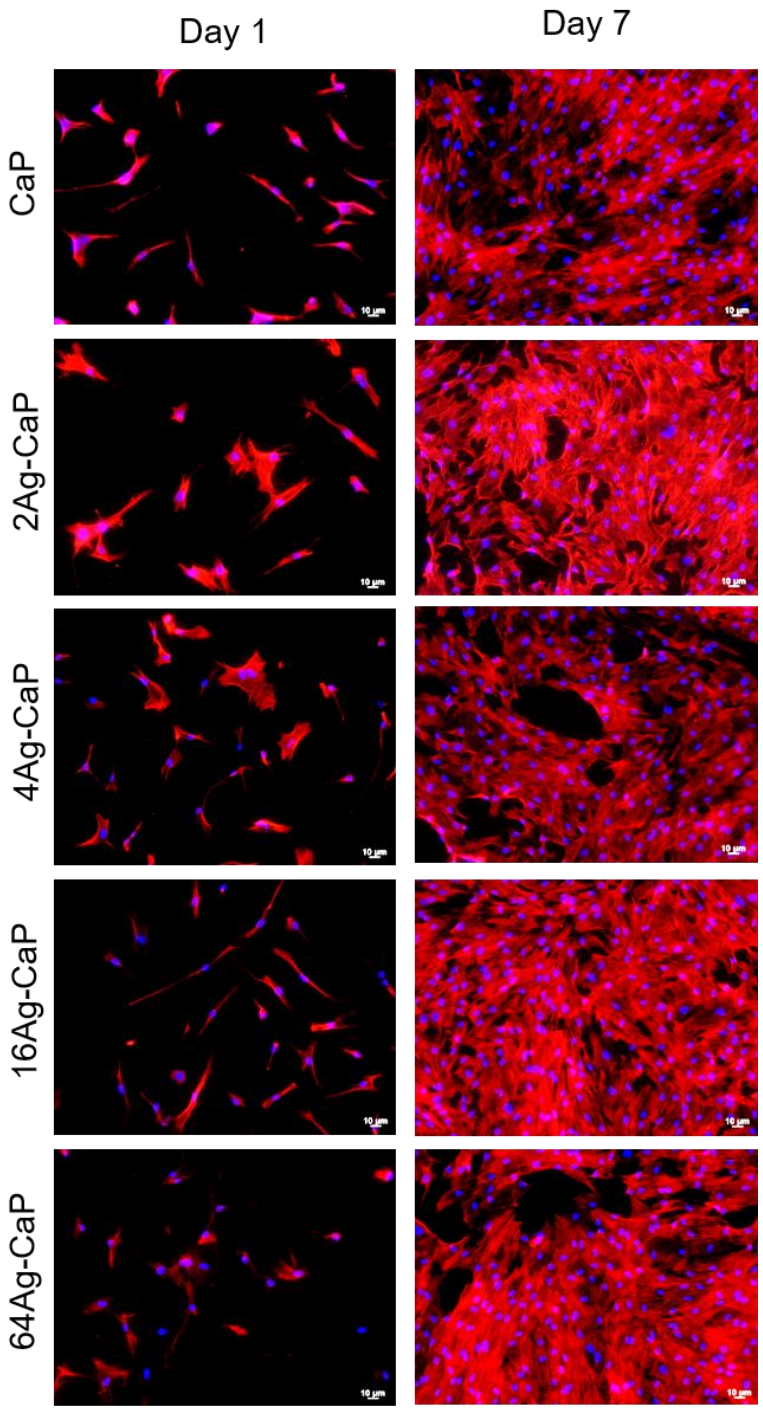

Figure 12: Fluorescence microscope images of ADSCs cultured for 1 and 7 days on different silver containing coatings.

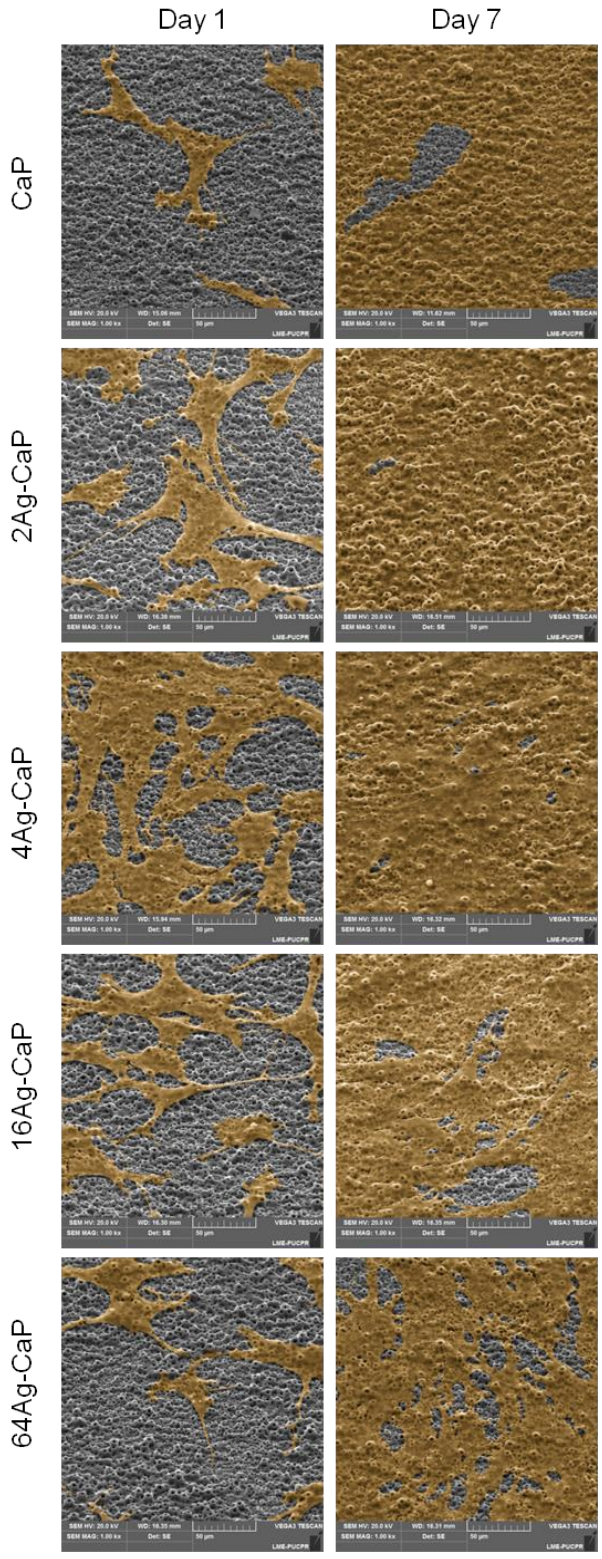

Figure 13: SEM images of ADSCs cultured for 1 and 7 days on different silver containing coatings. For better visualization, ADSCs are false-colored in orange.

4.1.5. Bactericidal activity

Results of colony forming units counting assay show a significant reduction of *S. aureus* CFU in groups 4, 16 and 64Ag-CaP after 24 h of incubation (Fig. 14). Surprisingly, the number of CFU has increased on the coating with the smaller amount of silver (group 2Ag-CaP, with 0.2 % Ag), but without significant difference from the reference group. A hormetic response was observed by increasing of *S. aureus* CFU in the coating containing a small amount of silver. A hormetic dose response occurs when lower doses of toxic agents promote a modest stimulatory response, resulting in an effect that is the opposite of what is expected, as a result of the disruption of the homeostasis (Calabrese 2004; Iavicoli et al. 2014). Biofilm formation induced by subminimal inhibitory concentration of antibiotics or nanoparticles have been extensively reported (Kaplan 2011; Davies, Spiegelman, and Yim 2006). Our results show that the 4Ag-CaP coating, containing 0.6% of Ag incorporated, achieves an 87 ± 20% of *S. aureus* reduction after 24h of culture. Coatings with higher amounts of silver presented similar rates of reduction, 72 ± 15 % and 88 ± 21 % for 16Ag-CaP and 64Ag-CaP, respectively. Among the tested coatings, the 4Ag-CaP group is selected as the most suitable to be used as a bactericidal coating in implants, since 0.6% Ag in the coating is sufficient to assure the bactericidal activity, and a higher early ADSC spread was observed. For this reason, the group 4Ag-CaP was selected for the next study.

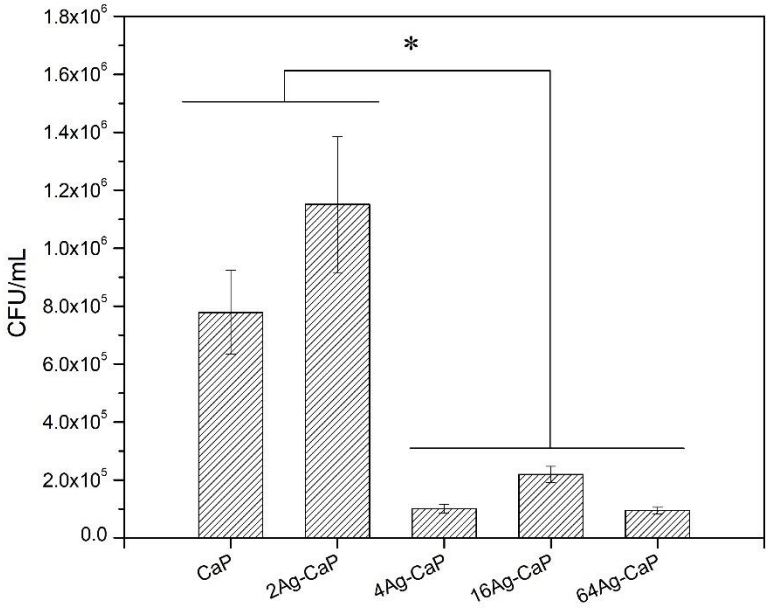

Figure 14: Colony forming units count assessing the antibacterial activity of different coatings against *Staphylococcus aureus* cultured for 24 h. *p < 0.0001.

4.2. Study II: Comparison of PEO coatings doped with Ag, Zn and B

4.2.1. Coating morphology

Similarly, to the observed on the study 1, the porous distribution and dimensions were not affected by the addition of zinc acetate or disodium tetraborate incorporation, as observed on the top-view SEM images Fig. 15.

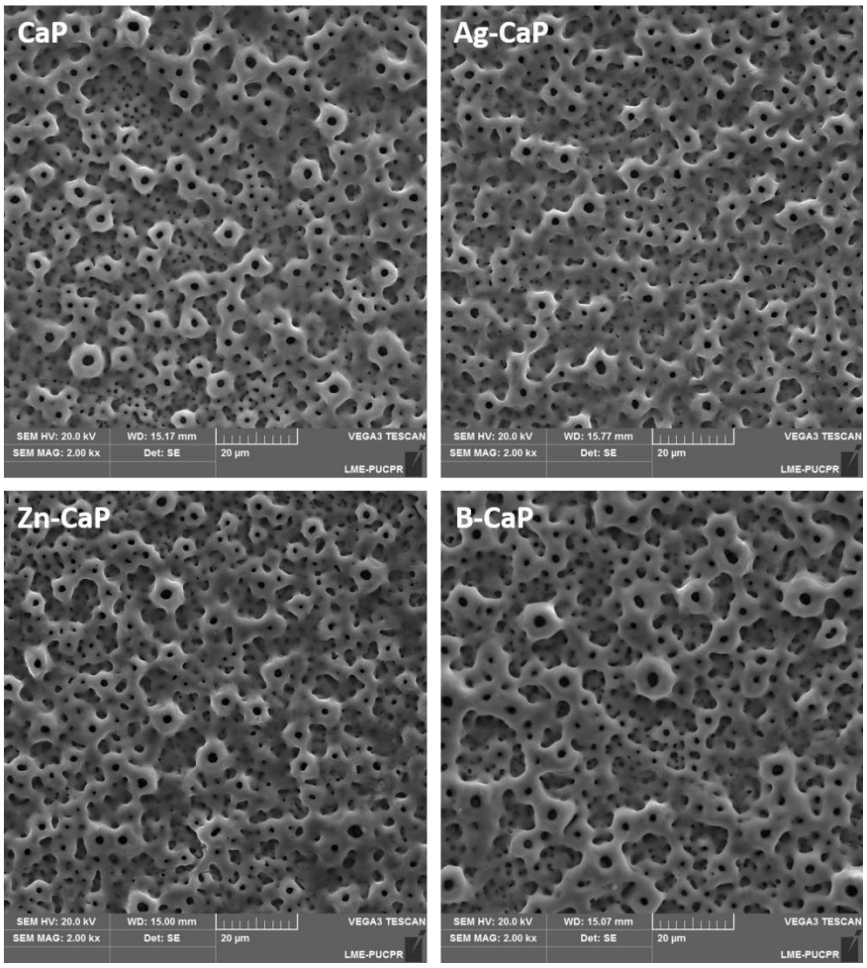

Figure 15: Scanning electron microscopy images of the coating without a bactericidal agent (CaP) and doped with Ag, Zn or B.

4.2.2. Coating crystalline structure

It is well stablished the coating crystallinity is directly dependent on the applied potential and the electrolyte composition (Xiaolong Zhu et al. 2002). The crystalline structure of the coatings was similar, despite the observation of a small amount of amorphous phase on the B-CaP group (Fig. 16). The presence of an amorphous phase is characteristic of the incorporation of extra elements, in this case boron, on the TiO_2 crystalline structure (Krupa et al. 2010; Rudnev et al. 2012). It is well established that crystalline TiO_2 can improve the osseointegration rate and quality, as their atomic structure promotes the apposition of apatite, the inorganic constituent of the bone (Uchida et al. 2003).

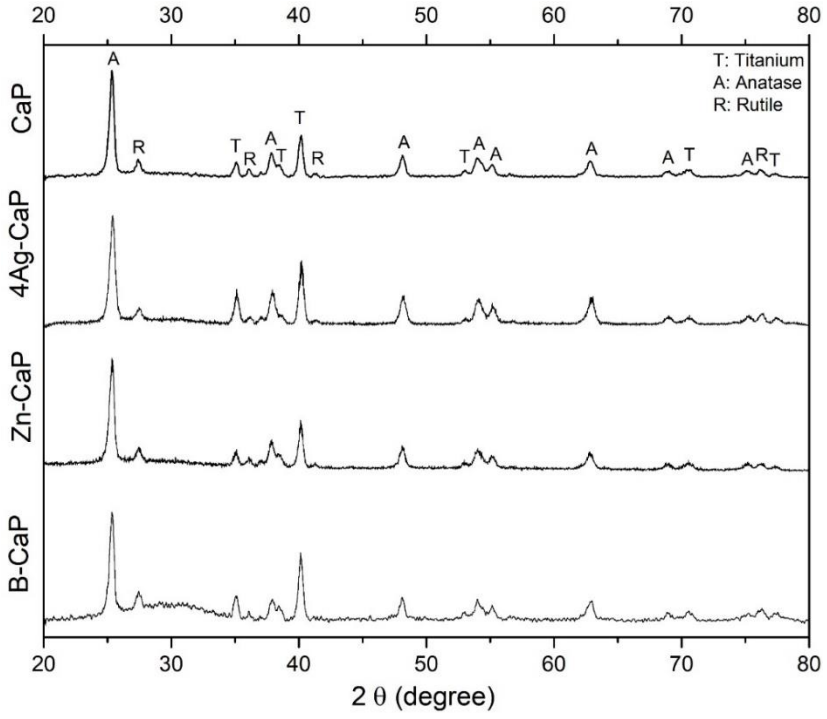

Figure 16: XRD diffraction pattern of the coatings. A, R and T stands for Anatase, Rutile and Titanium, indexed with the ICDD files #01-075-2547, 01-078-4187 and 01-071-4632, respectively.

4.2.3. Coating chemical composition

Calcium, phosphorous, silver, zinc and boron were successfully incorporated on the coatings as can be seen from the XPS survey spectra (Fig. 17). Ti 2p peaks at 458.4 eV and 464.4 eV correspond to the titanium doublet on TiO_2 structure (Bond and Flamerz 1989; Gonbeau et al. 1991). Ca 2p peaks at 347.1 eV and 350.6 eV are ascribed to calcium in $Ca_3(PO_4)_2$, in accordance with the P 2p peak at 133.1 eV, related to the P-O bond in phosphate ions (Chusuei 1999).

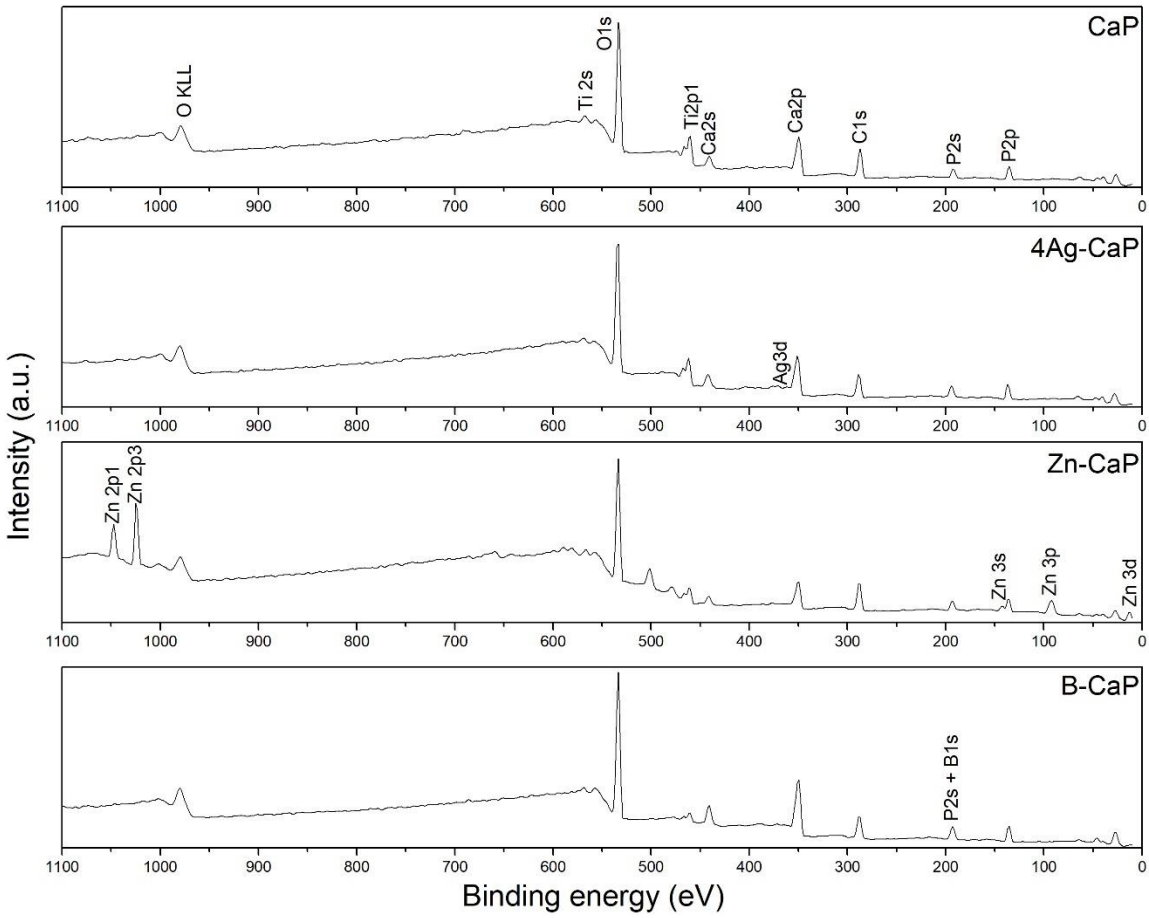

Figure 17: XPS survey spectra of the coatings.

Silver, Zinc and Boron incorporation on (4Ag-CaP, Zn-CaP and B-CaP, respectively) was confirmed by XPS high resolution analysis (Fig. 18). The high resolution Ag 3d spectrum shows two peaks at 367.6 eV (Ag $3d_{5/2}$) and 373.6 eV (Ag $3d_{3/2}$) which are ascribed to the presence of metallic silver (Agnihotri et al. 2015). The double peak on Zn 2p high resolution spectrum at 1021.6 eV and 1044.7 eV indicate the incorporation of Zn^{2+} (Fig 18b). The B 1s peak at 190.2 eV is ascribed to B-O bond in a BxOy boron suboxide, where $1.5 < x/y < 3$, although the B 1s and P 2s peaks are overlapped near ~ 191 eV, impairing the analysis (Yoshikawa et al. 2009; Moddeman et al. 1989). The incorporation of boron can be indirectly assessed by the comparation of P 2p/P 2s area ratio, by which it is possible to see the higher intensity of the peak around 191 eV in Figure 18c (Armelao et al. 2003).

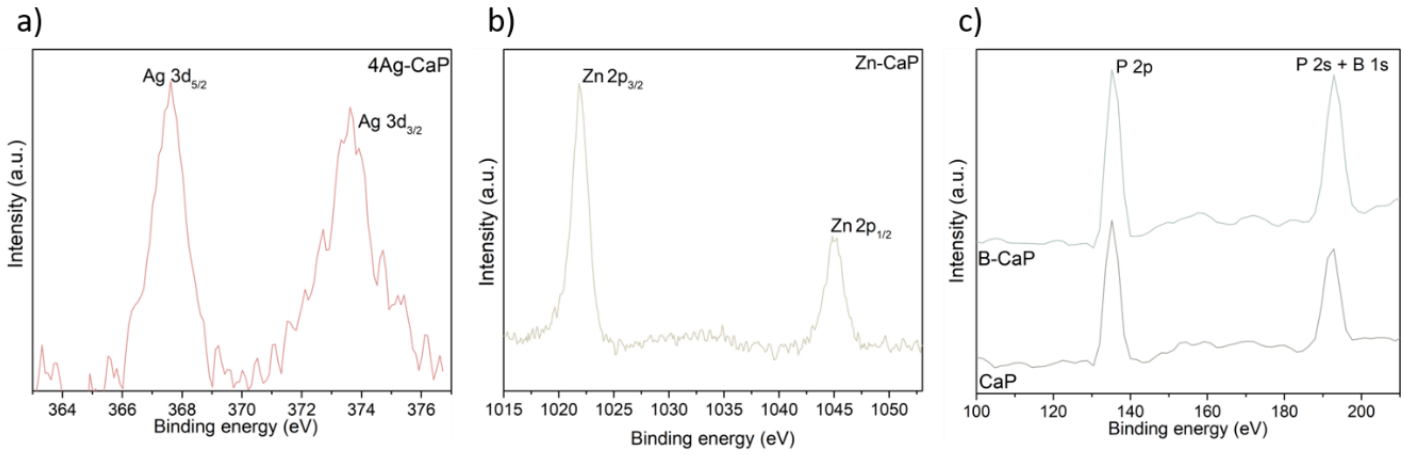

Figure 18: XPS high resolution spectra of the bactericidal elements on its respective coatings: a) Ag 3d on 4Ag-CaP coating, b) Zn 2p on Zn-CaP coating and c) B 1s on B-CaP coating.

The elemental composition of the samples is presented on the Table 3. During the oxidation the anions (PO_4^{3-}) are moved to the anode surface by electromigration, dictated by the external potential applied. Previous studies showed the Ca^{2+} anions are driven to the

anode surface by diffusion, and the same mechanism should be expected for the Ag^{+}, Zn^{2+} and B^{3+} ions (Qiao et al. 2016).

Table 3: Chemical composition of the samples containing Ag, Zn or B assessed by XPS

	Chemical composition (% at)						
Sample name	**Ti**	**O**	**Ca**	**P**	**Ag**	**Zn**	**B**
CaP	6.3	67.4	12.7	13.6	--		
4Ag-CaP	4.1	68.7	13.8	12.8	0.6		
Zn-CaP	3.9	66.7	8.4	11.3	--	9.7	--
B-CaP	3.1	54.7	12.8	10.0*	--	--	19.4*

4.2.4. Ion release

The release of calcium, phosphorus, silver, zinc and boron ions from the coatings were measured after 1, 7, 14 and 28 days of immersion in deionized water. Values under 10 ppm were found after 28 days of immersion, indicating the release of ions is minimal, as seen in the ion release profile in Fig. 19 and 20. PEO are broadly used to incorporate bactericidal elements on the coating structure, since it assures the long-term presence and availability of these elements as shown by the ion release results, unlike other techniques, as loaded surfaces, where the fast dissolution can be a drawback. Within the immersion time, the ions release increase gradually and tend to a stabilization state after 7 days of immersion. The slight difference of Ca^{2+} ion released on Zn-CaP coating is in accordance to the Zn-CaP coating elemental composition determined by XPS. After 28 days of

immersion the concentration of Ag^{+}, Zn^{2+} and B^{3+} ions in solution reached 0.0015, 0.8642 and 0.6551 ppm, respectively. Those concentrations are under the toxicity levels for those elements, 5, 13 and 300 ppm, for Ag, Zn and B, respectively (Garret 1998; Lansdown 2010; Song et al. 2010).

a) b)

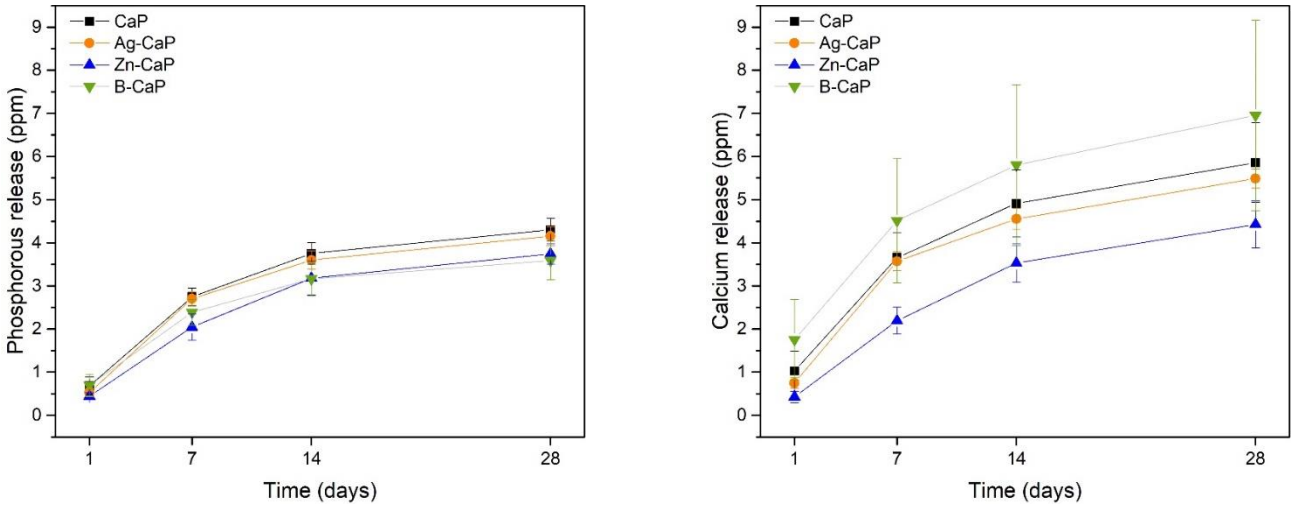

Figure 19: a) Phosphorous and b) calcium ion release profile from the coatings immersed in DI H_2O at 37 °C obtained by ICP.

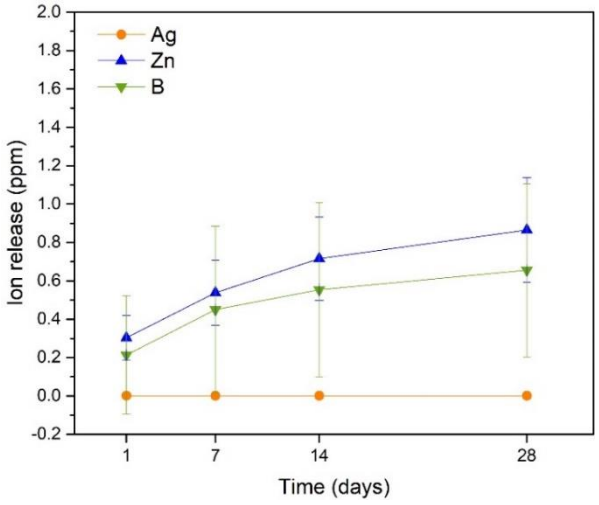

Figure 20: Silver, zinc and boron ion release profile from the coatings immersed in DI H_2O at 37 °C obtained by ICP

4.2.5. Biocompatibility

Cell viability assay shows that ADSCs remain viable and proliferate for up to 7 days of culture (Fig. 21). The percentage of AlamarBlue reduction has increased for all samples with no statistical difference between them, showing the presence of silver, boron and zinc had no influence on cell viability.

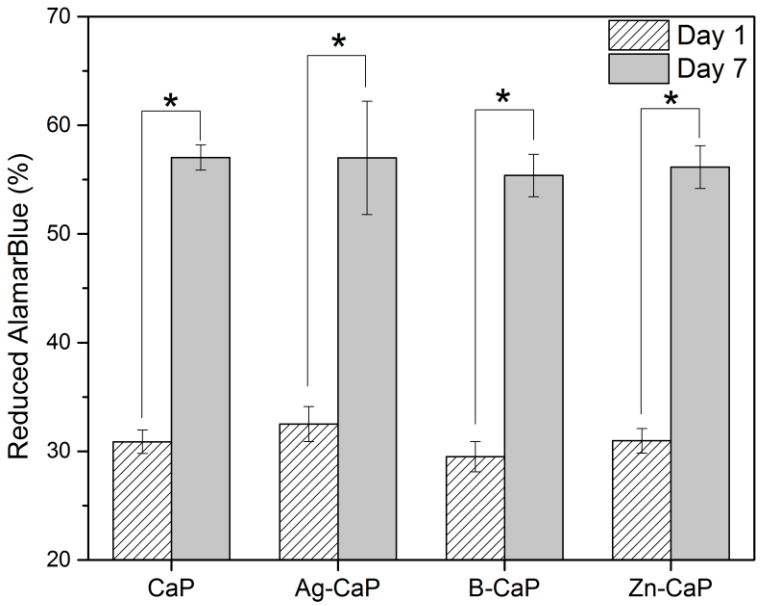

Figure 21: Cell viability on different coatings measured by the reduction percentage of AlamarBlue after 1 and 7 days of ADSCs culture. *$p < 0.0001$.

Fluorescence images shows ADSCs adhesion and proliferation on the coatings (Fig. 22). After 1 day of culture, cells on 4Ag-CaP and B-CaP coating are more spread than cells on CaP coating, presenting a larger shape. However, after 7 days of culture no difference can be observed since cells have covered the entire coating surface for all samples.

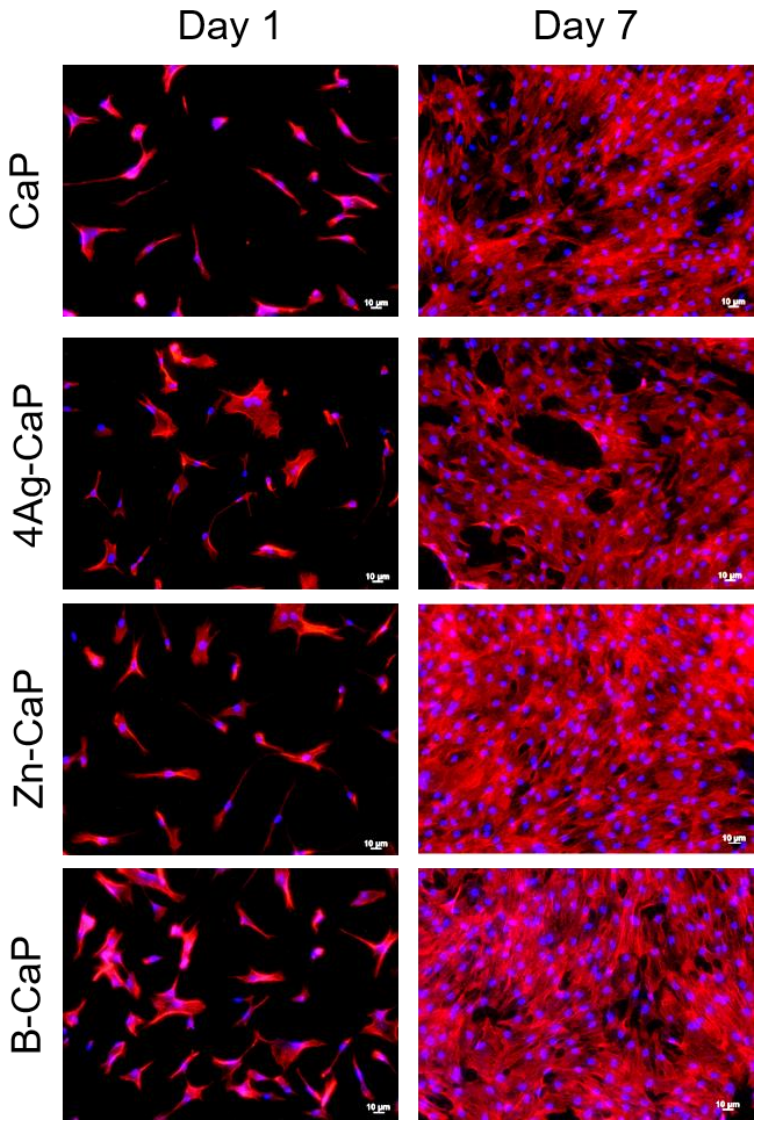

Figure 22: Fluorescence microscope images of ADSCs cultured on the coatings for 1 and 7 days.

The cell morphology after 1 and 7 days of ADSCs culture on the coatings are shown in Fig. 23. After 1 day of culture, cells start to spread and connect, being more spread on the silver containing coating, while after 7 days all the surface is covered in agreement with the observed in fluorescence images. Cells have adequate their shape following the coating

morphology and using the micropores as features for anchoring. The images indicate the silver, zinc and boron presence has not affected the cells morphology.

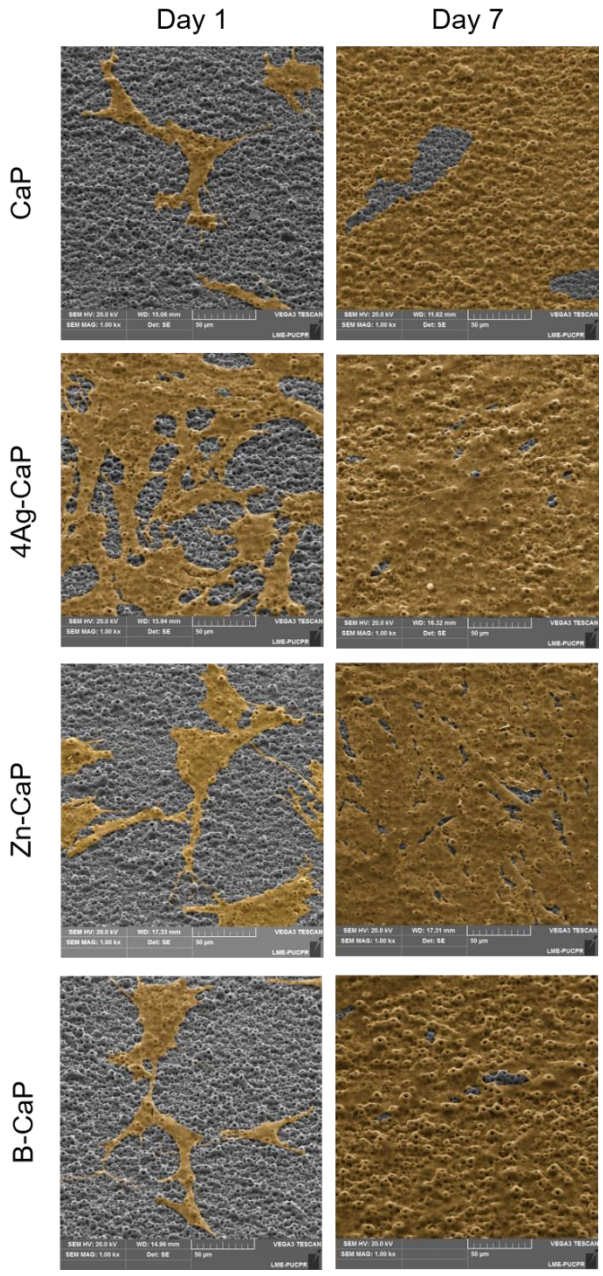

Figure 23: SEM images of ADSCs on coatings after 1 and 7 days of culture. For better visualization, ADSCs are false-colored in orange.

In order to determine if the bactericidal elements present on the coatings induce cell damage, the cytotoxicity was evaluated by the LDH assay. After 24 h of ADSCs cultivation on the coatings no obvious cytotoxicity was observed, the absorbance of the cells cultured on 4Ag-CaP, Zn-CaP and B-CaP coating was comparable to the ones cultured on the CaP coating and the negative control, corresponding to the cells cultured on α-MEM media (Fig. 24).

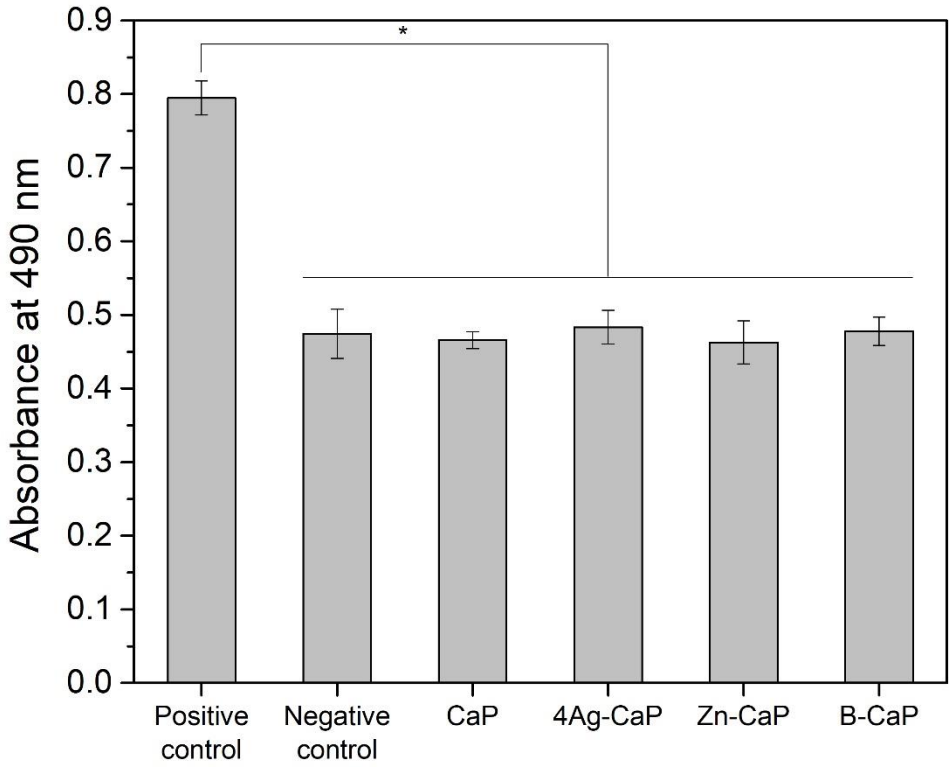

Figure 24: Cytotoxicity of the surfaces after 24 h of ADSC culture assessed by LDH assay. * $p < 0.001$

4.2.6. Bactericidal activity

Bacteria stablish a mature biofilm few hours after the microbial adhesion, so the bactericidal activity of the coatings were evaluated up to 24 h of culture (Romanò et al.

2015). Fluorescence images shows dead bacteria stained in red by the propidium iodide and the live bacteria stained in green by the SYTO9 dye of LIVE/DEAD® BacLight Kit. After 24 h of culture, it is possible to observe an increase on the dead bacteria on coatings containing Ag, Zn and B (Figs. 25 and 26). Despite the growth of *P. aeruginosa*, denoted by the bacterial clusters on Fig 26, it is possible to observe a significant reduction on the live bacteria on the coatings containing bactericidal elements.

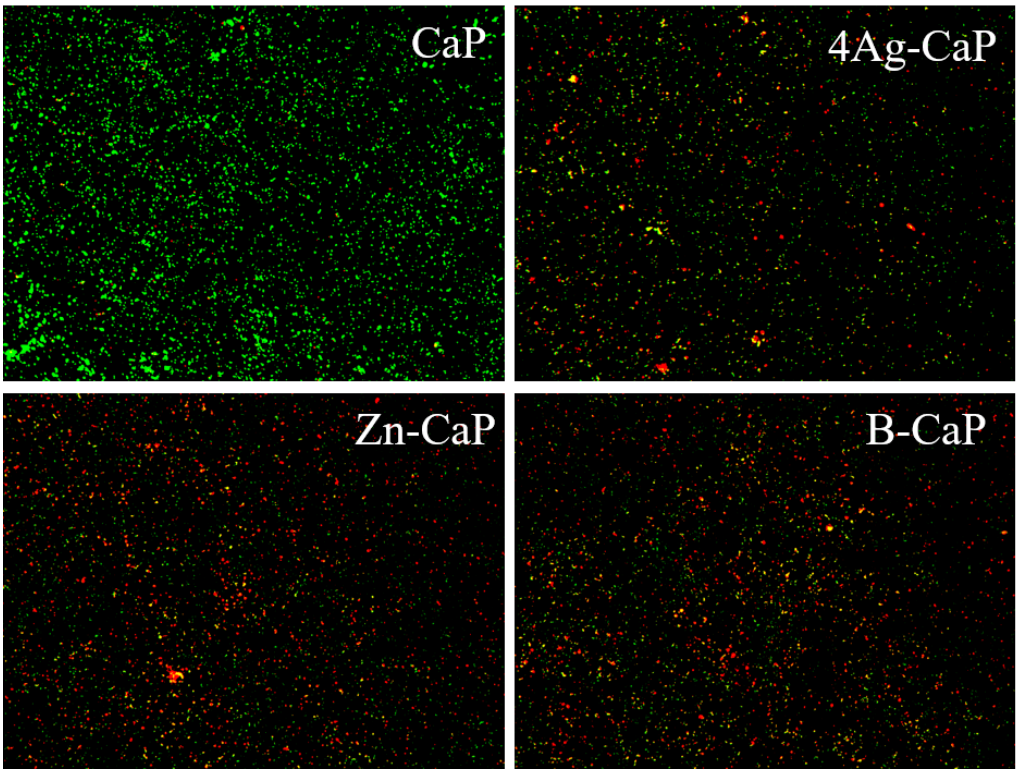

Figure 25: Live/dead fluorescence images of *Staphylococcus aureus* cultured for 24h on the coatings.

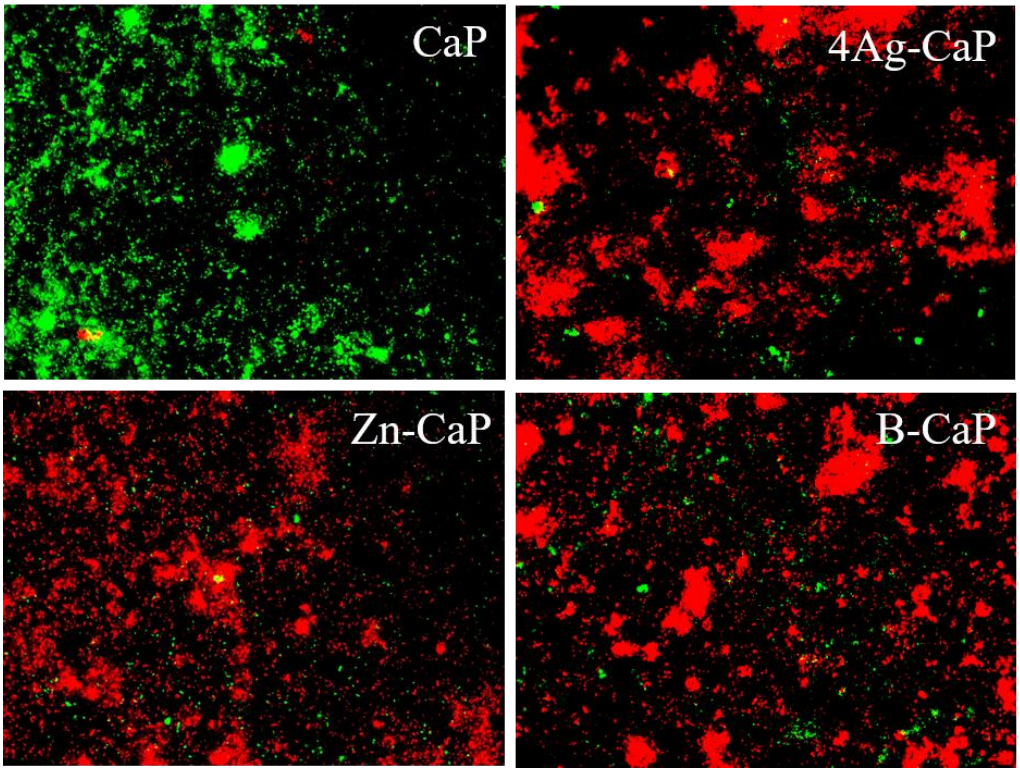

Figure 26: Live/dead fluorescence images of *Pseudomonas aeruginosa* cultured for 24h on the coatings.

The percentage of live and dead bacteria coverage obtained with ImageJ software allows to perform a quantitative analysis. After the culture of *S. aureus* on the coatings for 6 and 24 h it was possible to observe a significant decrease of the live bacteria coverage on the coating doped with boron (Fig 27). The coatings doped with silver and zinc presented a slight decrease on the live bacteria coverage after 24 h of culture. All the coatings increased the number of dead bacteria within 6 h of assay, sustaining the increasing after 24 h.

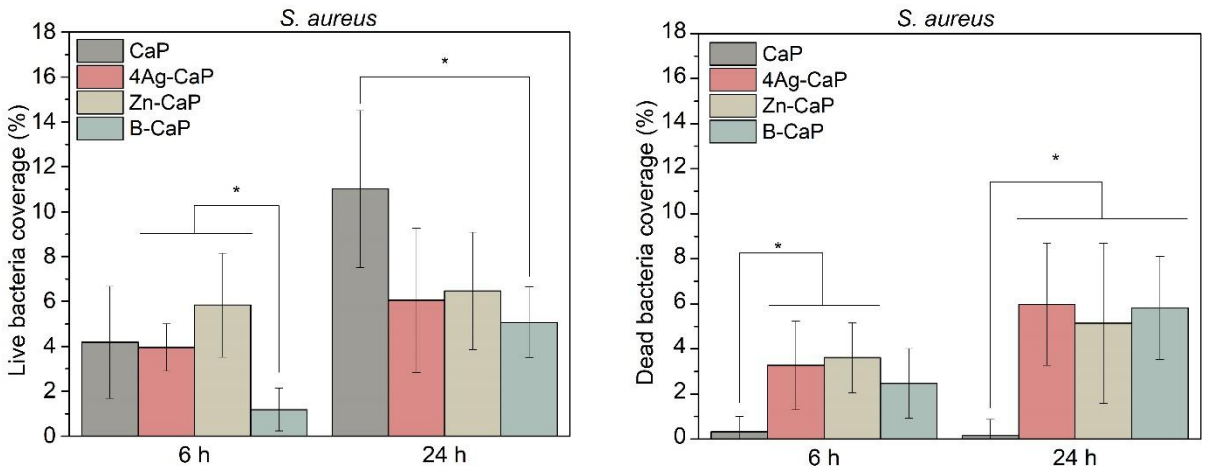

Figure 27: Percentage of live and dead *Staphylococcus aureus* after 6 h and 24 h of culture on the coatings. * p< 0.001.

After 6 h no significant difference on the live *P. aeruginosa* coverage was observed on the coatings, although after 24 h of culture all the coatings doped with bactericidal agents decreased the number of *P. aeruginosa* alive (Fig. 28). It is possible to see the increase of dead bacteria after 6 and 24 h in all coatings containing bactericidal elements, indicating the coatings' bactericidal activity.

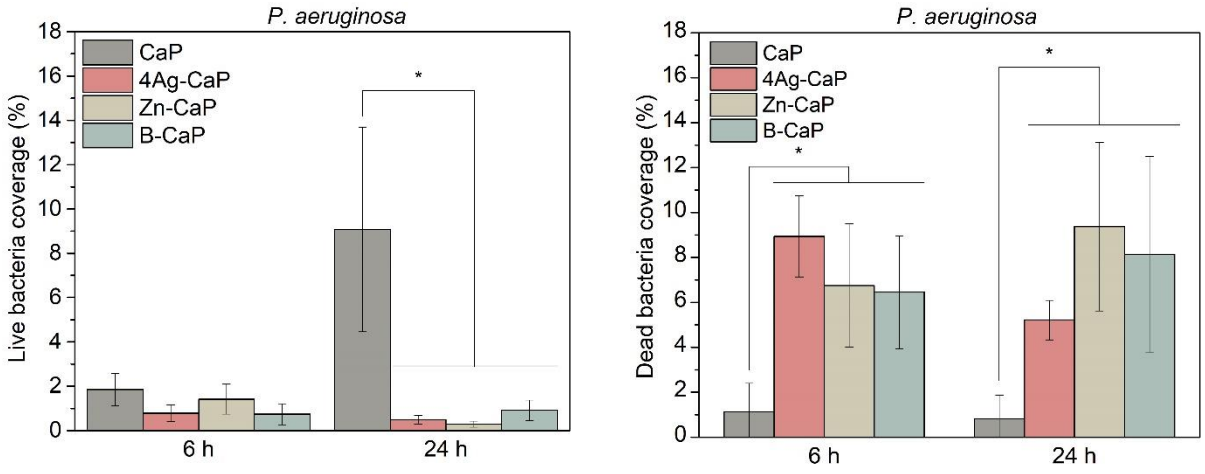

Figure 28: Percentage of live and dead *Pseudomonas aeruginosa* after 6 h and 24 h of culture on the coatings. * p< 0.001.

SEM images of bacteria corroborate with the Live/Dead assay results, as only few bacteria can be observed on 4Ag-CaP, Zn-CaP and B-CaP coatings, while bacteria clusters can be observed on CaP coatings (Figs. 29 and 30). The number of live bacteria increased from 6 h to 24 h of culture on the CaP coatings, showing the bacterial proliferation in absence of a bactericidal agent. Silver, zinc and boron inhibit the bacterial activity on the coating within the first 24 h of contact, preventing the bacterial proliferation and production of extracellular products, as observed on the CaP coating. The release of less than 1 ppm of bactericidal elements, within this 24 h period, was enough to prevent the bacterial proliferation. Subbiahdoss et al. showed that *S. aureus* and *P. aeruginosa* cause the death of adhering human osteoblasts after 24 hours of coculture (Subbiahdoss et al. 2011). This aggressive infection may be controlled by the presence of silver, zinc or boron in the coatings.

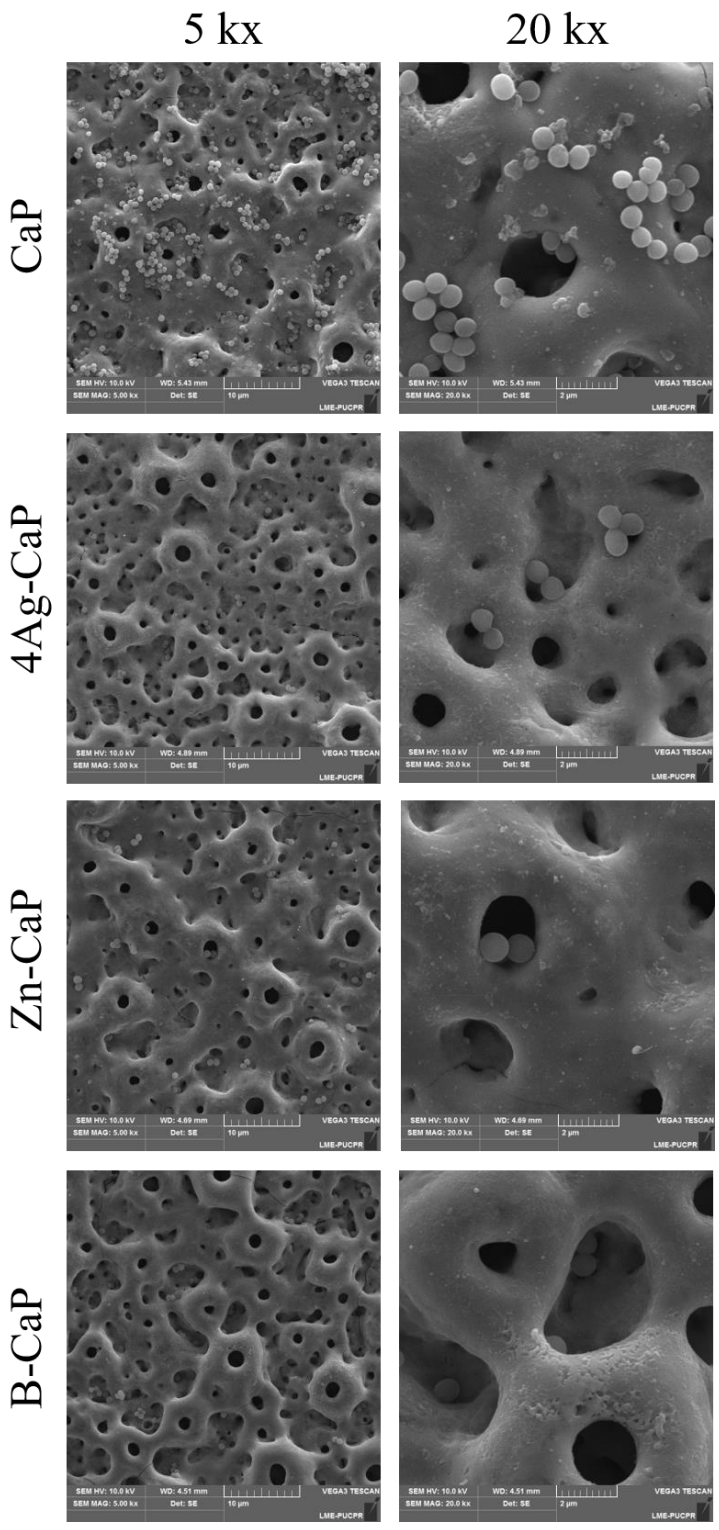

Figure 29: SEM adhesion of *Staphylococcus aureus* after 24 h of cultivation on the coatings.

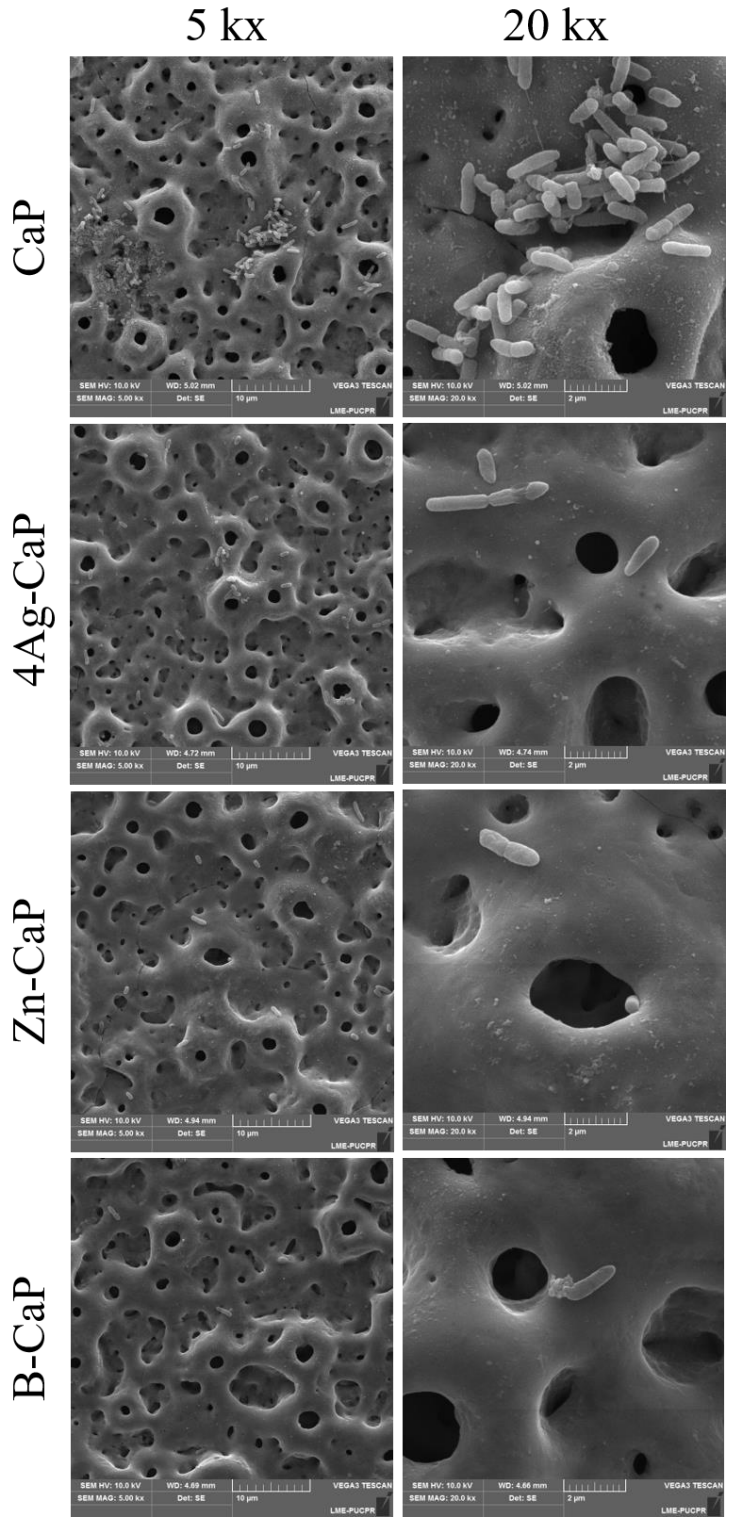

Figure 30: SEM adhesion of *Pseudomonas aeruginosa* after 24 h of cultivation on the coatings

The results show all the three coatings, which have bactericidal activity against *S. aureus* and *P. aeruginosa* after only 24 h of incubation, and represents a feasible approach to avoid the bacterial colonization in implants since *Staphylococcus* and *Pseudomonas* are the most common strains related to implant centered infections (Pulido et al. 2008).

Previous works showed the bactericidal properties of doped PEO coatings containing Ca, P, Zn and Ag-nanoparticles, were the bactericidal ability against *S. aureus* was credited to the combination Zn and Ag-nanoparticles (Zhang, Gao, and Han 2016). Our results show the incorporation of Ag nanoparticle is not mandatory, since Zn itself present bactericidal ability against *S. aureus* and *P. aeruginosa*. All three coatings presented similar bactericidal activity, biocompatibility and cytotoxicity after 24 h.

Taking in consideration the side effects of silver use in indwelling implants, our results show the importance to investigate other bactericidal ions, as zinc and boron. The use of bactericidal agents that inhibit the bacterial quorum-sensing, as boron does, is preferable over other bactericidal agents, since the bacterial resistance is less likely to occur (J. W. Costerton, Montanaro, and Arciola 2007). Boron presence on the coating has not affected the coating biocompatibility, in accordance with studies that evaluated the biocompatibility of other boron-containing biomaterials (Wu et al. 2011; Gümüşderelioğlu et al. 2015; Doğan et al. 2014).

5. Summary of conclusions

In this study, three different bactericidal coatings were developed and characterized. The bioactivity, cytotoxicity and bactericidal activity were evaluated against ADSC cells and two bacterial strains *S. aureus* and *P. aeruginosa*, respectively. Based on the results, it possible to conclude:

a) It's necessary the incorporation of at least 0.6 % at of silver to obtain a coating with bactericidal activity against S. aureus.

b) The incorporation of zinc or boron on the coatings grant bactericidal activity comparable to the coatings doped with silver, showing the possibility to use other bactericidal elements apart from silver.

c) The Ag, Zn or B incorporation grant the coating bactericidal ability against *S. aureus* and *P. aeruginosa* after 24 h of culture;

d) The bactericidal coatings are biocompatible and did not induce cytotoxic effects on ADSC cells.

In conclusion, it is possible to obtain a TiO_2 coating doped simultaneously with osteoconductor elements (Ca and P) and bactericidal elements (Ag, Zn or B), those doping elements are incorporated on the TiO_2 crystalline structure and present a minimum release up to 28 days of monitoring. The incorporation of bactericidal elements by PEO does not change the morphology and crystalline structure of Ca and P doped coatings, factors that are already proved to improve the osseointegration, showing the coatings obtained in this work can be potentially employed as a tool to prevent the bacterial colonization on endosseous implants.

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