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Comparing Two Antibacterial Treatments for Bioceramic Coatings at Short Culture Times

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Plasma-sprayed hydroxyapatite coatings were employed industrially for decades to improve osteointegration of articular implants, but many studies have warned about the problems inherent to this procedure (mechanical properties, harmful phases). Consequently, a combination of hydroxyapatite with TiO_2 sprayed by high velocity oxy-fuel spray was considered in this study. As infection after joint replacement surgery is one of the most critical concerns when considering implant performance, it is necessary to study possible ways to reduce or eliminate it. Two coating treatments were chosen for this study: addition of a percentage of ZnO and immersion in gentamicin for 24 h. Furthermore, three bacteria were considered: *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*. The evolution of bacteria viability in solution was measured at 0, 2, and 4 h; and plate assays were performed to study antibacterial effects by diffusion. The results show an important antibacterial effect of the as-sprayed coating, attributed to the presence of -OH radicals on the surface. The presence of ZnO did not have any additional influence on bacteria viability, but gentamicin-treated samples showed an improvement in antibacterial behavior for Gram-negative bacteria in solution, as well as a bactericidal effect in diffusion conditions.

Keywords antibacterial coatings, biomaterials, hydroxyapatite, HP/HVOF, TiO_2

1. Introduction

Infection in joint prostheses has been a major concern for years as it is the main cause of implant failure after short implantation times (Ref 1). Prosthesis implantation requires exceptional asepsis because of the large exposition area and the influence of a massive implant that lead to an important contamination risk (Ref 2). The patient's skin, airborne particles of the medical staff, or contaminated surgical material are the main sources of bacteria found in infected prostheses after removal (Ref 2, 3), which means that bacteria such as *S. aureus*, or different kinds of *Pseudomonas* or *Streptococcus* are some of the most common in these situations (Ref 3, 4).

As infection has devastating effects on the patient, research into possible solutions to this problem is a pressing field. In recent years, improvements in operational theater design and the use of systemic or local antibiotics and other antibacterial compounds directly present on the implant have decreased infection rates (Ref 3).

Among the use of these antibacterial compounds, the use of nanometric species is very well known and has many advantages. A small particle size, high surface area, and the capacity to produce oxygen species make nanometric species toxic, and this toxicity can be harnessed for bactericide purposes (Ref 5). ZnO nanoparticles are commonly used, due to the proven toxicity of the dissolved metallic ions, and the generation of oxyradical species in solution (this oxidative stress weakens and kills many kinds of bacteria). Furthermore, ZnO is itself an essential micro-nutrient for eukaryote organisms, and a ''safe'' product widely used in personal care (Ref 6).

Another possibility considered to prevent infection directly on the prosthesis is antibiotic loading. This approach is very common for bone cements (Ref 7) or biomimetic hydroxyapatite coatings (Ref 8), where it is easy to incorporate the antibiotic into the production of the cement or coating. Gentamicin sulfate, which belongs to the aminoglycoside antibiotic family, is extremely efficient against the majority of microorganisms responsible for bone infection, even when the infection is deep. It is, therefore, one of the most employed antibiotics, extensively used with success in orthopedics, since 1970s, in combination with bone cements (Ref 9).

Metallic materials have been employed for decades for large-bone or load-bearing replacements due to their mechanical properties (Ref 10). However, these materials are bioinert, and a fibrous capsule is formed between the implant and the surrounding bone that avoids the direct bonding between them and leads to an early failure of the

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device (consequence of micro-motion and an inadequate load distribution, corrosion problems, or the resultant damage of the surrounding tissues) (Ref 11-14). As a solution to this problem, the combination of the mechanical properties of a metal and the biological properties of a bioceramic coating was soon proposed (Ref 15), to ensure the bonding implant tissue. Hydroxyapatite is industrially the most employed bioceramic for this purpose, deposited by means of thermal spray technologies, specifically plasma-spraying (Ref 15).

Although in terms of effectiveness and productivity, the thermal spray techniques are the best choice, many studies widely showed the problems inherent to the plasma-sprayed hydroxyapatite coatings (Ref 16). The physiological behavior of the coated prosthesis is better than that of the uncoated, but the high temperatures inherent to the plasma process degrade the initial powders, leading to the formation of unwanted phases such as calcium oxide (harmful) or amorphous phases (easily dissolved) (Ref 16).

An approach to solve these problems is the addition of materials with better mechanical properties to the starting powder (e.g., carbon nanotubes to improve toughness and abrasion resistance (Ref 17, 18), silicium oxide to improve adherence and corrosion resistance (Ref 19, 20), ceramic interlayers (Ref 21), etc.). In this study, the addition of a low amount (20wt.%) of TiO_2 was considered, which previously proved to increase the mechanical properties of the coating without the loss of biological properties. The other approach considered was the substitution of plasma spraying by other thermal spray technique with lower beam temperatures, specifically high velocity oxy-fuel spray (HVOF). Previous studies showed the lower degradation of the starting material using this technique (Ref 22). Therefore, the starting material is a mixture of 80 wt.%HAp-20 wt.%TiO_2 sprayed using HVOF onto a substrate widely used in biomedical applications, TiAlV.

This work focuses then on a first comparison between the two antibacterial approaches cited in the first paragraphs applied to the starting material. The production process of the treated coatings, their characterization, as well as the bacterial tests performed with three bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) at different short times are reported in this paper.

Table 1 Spraying conditions

Parameters	Value
O_2 (L/min)	265
Propylene (L/min)	81
Compressed air (L/min)	264
Oxygen/propylene	3.96
Number of layers	5
Distance (mm)	200

2. Experimental Method

The initial powder was a combination that previously proved to have good biological and mechanical properties (Ref 23). It was a mechanical mixture of a Sulzer-Metco rutile powder (20 wt.%) and a hydroxyapatite powder (80 wt.%; Plasma-Biotal Ltd.), and its characterization is reported elsewhere (Ref 24). The substrate used was Ti6Al4V (5×5 mm^2 sheets), previously grit blasted in order to reach a surface roughness that ensured the optimum adhesion of the coating to the substrate. The thermal spray equipment was a Sulzer-Metco DJH 2600 HVOF system. The spraying conditions are listed in Table 1.

The ZnO was an American Elements agglomerated nanopowder. Its granulometric distribution was analyzed by laser light scattering (Beckman Coulter LS 13320), dispersing the powder in water without ultrasound, and the morphology was observed by scanning electron microscopy (SEM), with Jeol JSM-5310 equipment. The phases present were analyzed by x-ray diffraction. The powder was incorporated into the previous mixture in percentages of 2 and 5 wt.% (Ref 6, 25, 26), and the resulting mixture was sprayed using the same conditions as for the initial powder.

The sulfate gentamicin (Sigma Aldrich) treatment, after initial tests, was chosen to be 24 h immersion in Hank's solution with a concentration of 2 mg/mL, at 37 °C, followed by 60 min of drying at 55 °C (Ref 9, 27, 28).

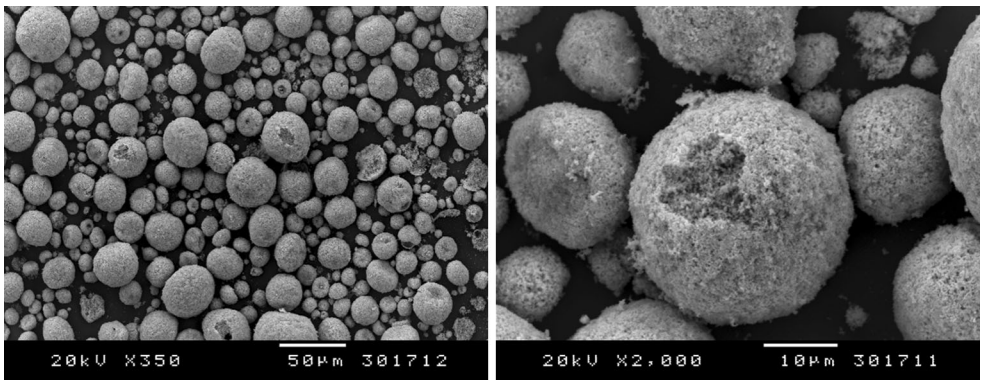

Fig. 1 ZnO particles. Morphology by SEM

The surface and the cross-section of the coatings were observed by SEM, in order to identify changes in the microstructure. XRD analysis were done to the base coating (non-treated HAp-TiO_2 coating) to identify and quantify the phases present. Additionally, energy-dispersive x-ray spectroscopy was used to analyze the three surfaces and to identify element distribution, and infrared spectroscopy was used to identify functional groups in the as-sprayed samples.

The antibacterial activity of ZnO-treated and gentamicin-treated samples was tested against the Gram-negative *E. coli* CECT405 and *P. aeruginosa* CECT116, and the Gram-positive *S. aureus* CECT 239, obtained from the Spanish Type Culture Collection. Overnight cultures of bacteria on LB medium at 37 °C with aeration were diluted into a fresh medium, and incubated until the culture reached an optical density of 0.6 OD at 600 nm (mid logarithmic phase of growth). The cells were harvested by

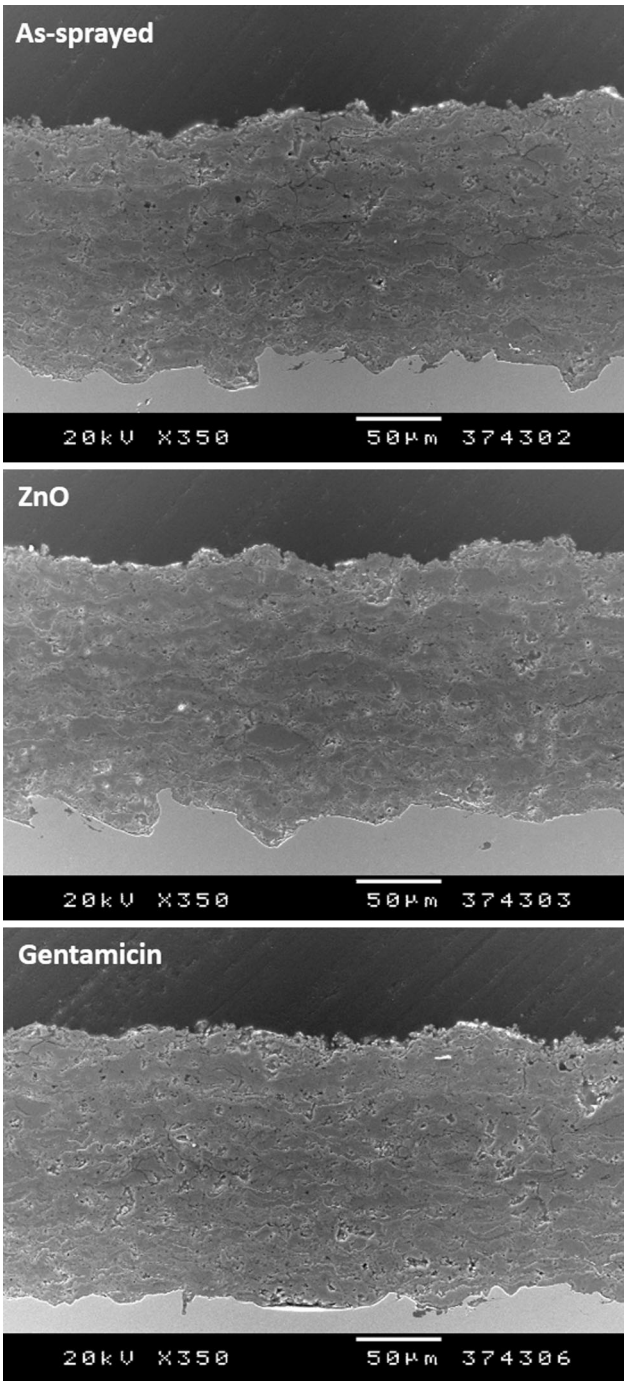

Fig. 2 Cross-section of the three samples

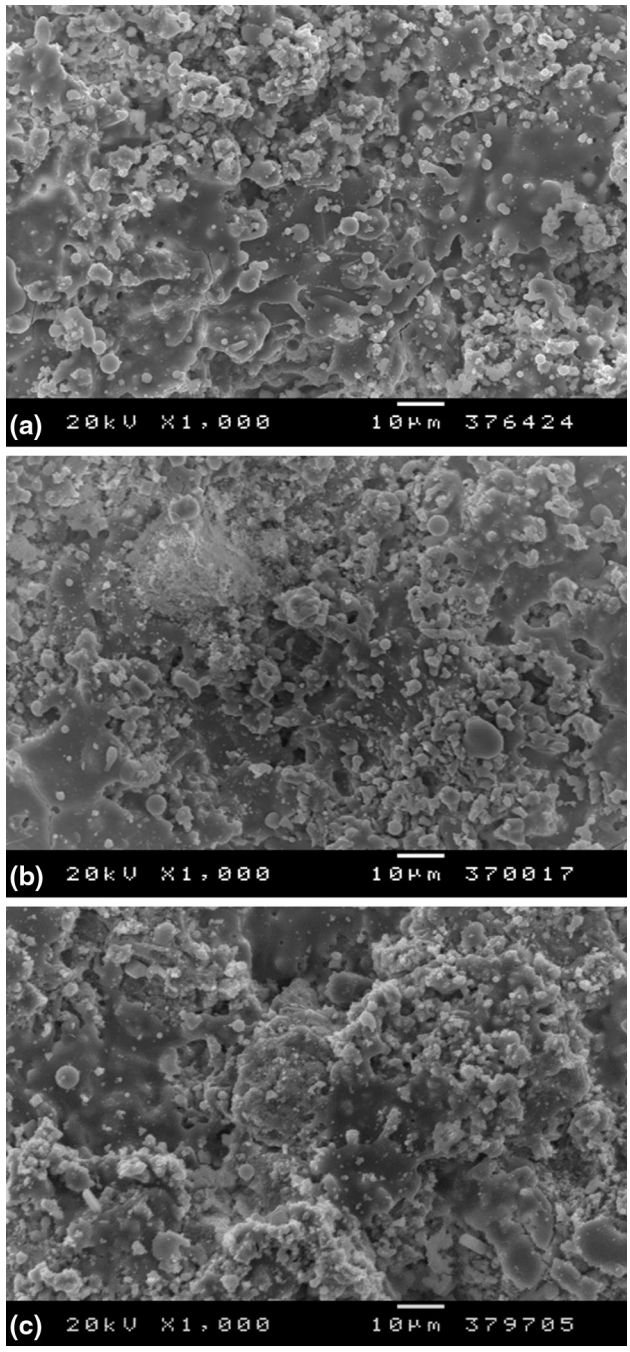

Fig. 3 Surfaces of the three samples: (a) as-sprayed, (b) with ZnO (5%), (c) gentamicin-treated

centrifugation (5 min; 3000×*g*), washed twice with Ringer ¼ solution, and adjusted to a final optical density of 0.6-0.7 (600 nm) with Ringer ¼ solution. All the samples were poured into a vial with 1 mL of a 1:10 dilution of the bacterial suspension in Ringer ¼ solution. The initial bacteria concentration in the vial was approximately 5×10^7 CFU/mL. To ensure that any decrease in the bacterial number was due to the exposure to different samples, a control was included with bacterial cells at the same initial concentration in Ringer ¼ solution without any sample. The bacterial suspensions were incubated for up to 4 h at 37 °C and 200 rpm. Samples were taken at the beginning (0 h) and after 2 and 4 h. After appropriate dilutions, the samples were plated in LB agar and incubated at 37 °C. The viable bacteria were monitored by counting the number of colony-forming units from the appropriate dilution. To assay the antibacterial effect by diffusion in plates, suspensions of the bacteria to be assayed were prepared and dispersed in solid medium (LB agar plates), and the different samples were dropped in different zones on the agar plate. After overnight incubation at 37 °C, the presence of inhibition growth zones was determined.

3. Results and Discussion

ZnO XRD characterization revealed that the powder was in a crystalline wurtzite phase. Its granulometric distribution, a Gaussian bell curve between 10 and 50 microns, was very similar to those of the other two powders. The morphology of the particles can be observed in Fig. 1. They had a spherical shape and porous structure resulting from the agglomeration of the nanopowder.

The analysis of the cross-sections of the coatings (Fig. 2) by SEM reveals compact microstructures, without imperfections (pores or cracks), either for non-treated or treated coatings, and the interface remains continuous. Mechanical analysis would be adequate to corroborate that the employed treatment do not affect the mechanical performance of the coatings.

The observation of the coating surfaces (Fig. 3a-c) showed that the surface of the as-sprayed sample is typical in this kind of spray process (Ref 29-31). Splats, unmelted particles, and splashes of the melted particles can be

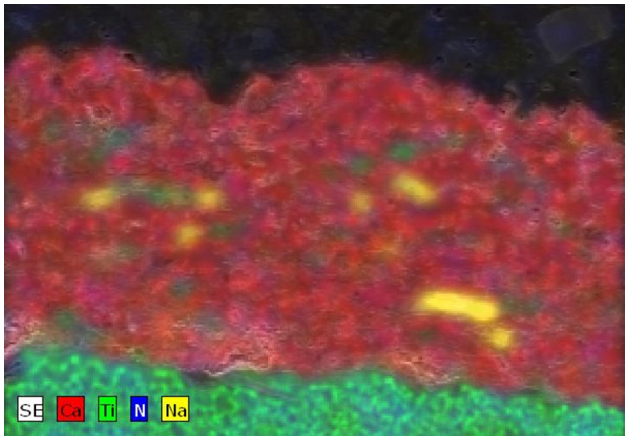

Fig. 5 EDX mapping of the cross-section of the gentamicin-treated sample

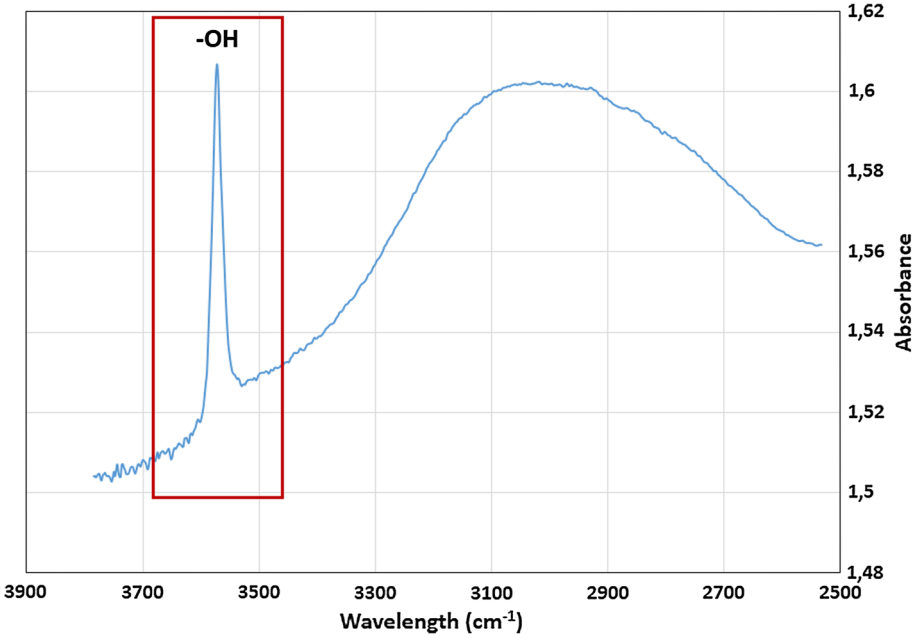

Fig. 4 IR spectra of the as-sprayed sample, -OH zone

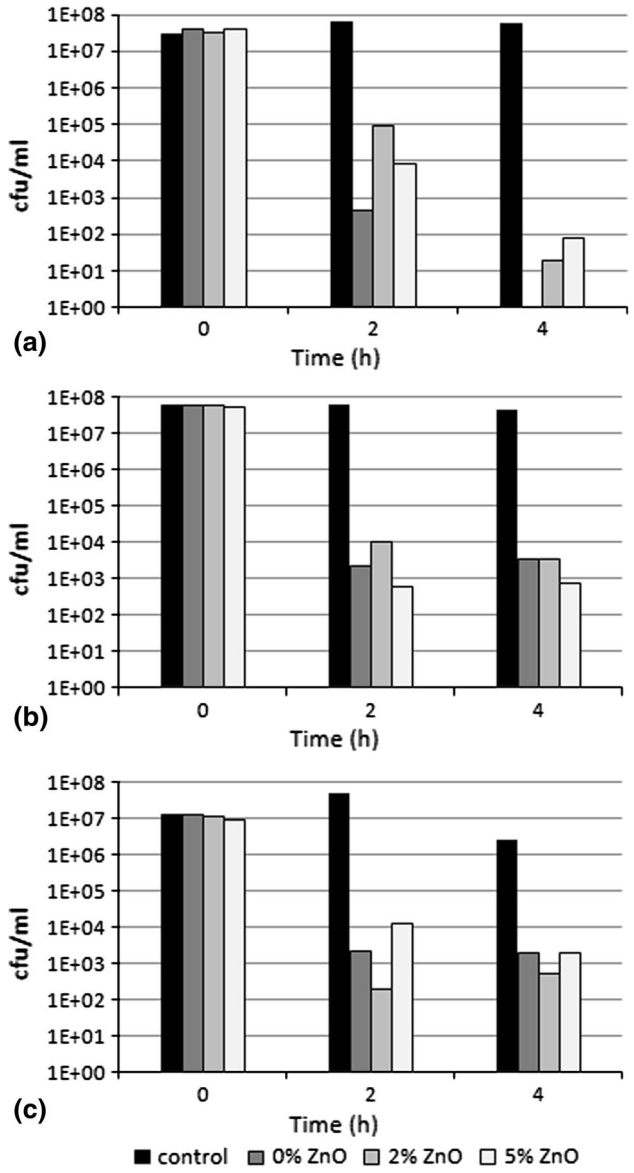

Fig. 6 Effect of the ZnO treatment on the evolution of bacteria survival in solution at 0, 2, and 4 h: (a) *E. coli*, (b) *P. aeruginosa*, (c) *S. aureus*

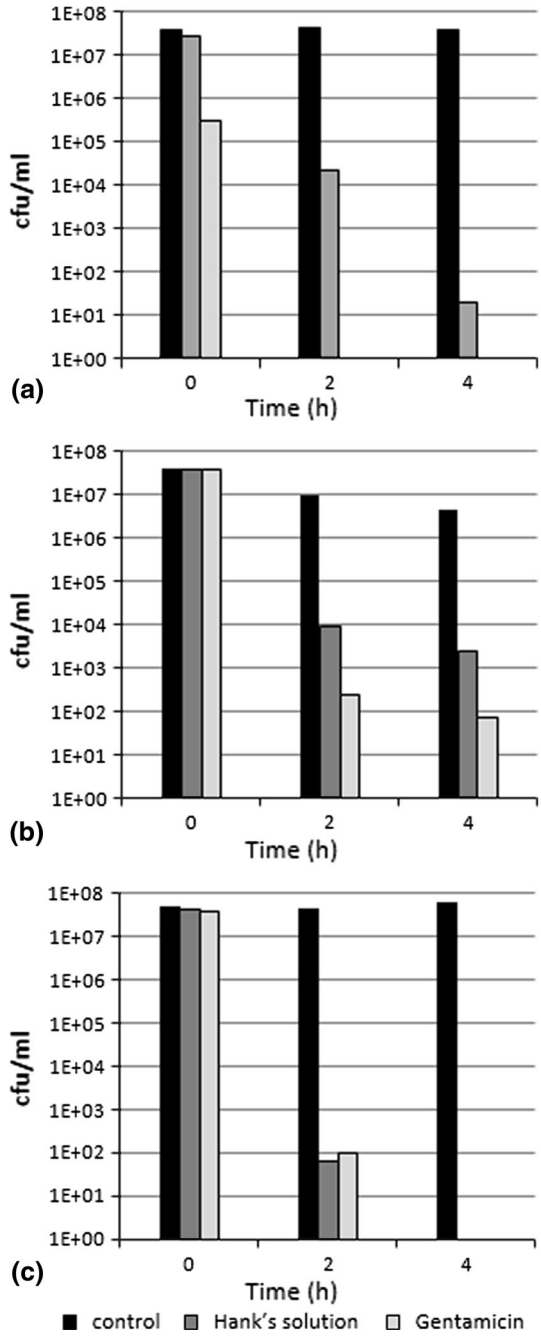

Fig. 7 Effect of gentamicin on the evolution of bacteria survival in solution at 0, 2, and 4 h: (a) E. *coli*, (b) *P. aeruginosa*, (c) *S. aureus*

identified in the micrographs. EDX analysis indicated, as expected, the elements present in the initial powder and the derived phases (tricalcium phosphates and anatase), Ca, P, Ti, O, and C. Infrared spectroscopy of these samples (Fig. 4) allowed the identification of a peak indicating the strong presence of -OH radicals on the surface.

The surface of the sample with ZnO (Fig. 3b) was very similar to that of the as-sprayed one. The presence of small amounts of ZnO did not influence the surface microstructure. EDX analysis showed the presence of the expected elements (the same than for the HAp-TiO_2 samples) and also a peak corresponding to zinc. Quantification indicated that the amount of zinc by weight was very close to the amount in the initial powder: 1.7 wt.% for the sample with 2 wt.% ZnO into the powder mixture and 4.4 wt.% for the sample with 5 wt.% ZnO into the powder mixture. The ZnO was, therefore, considered to be incorporated into the coating.

The surface of the gentamicin-treated sample (Fig. 3c) showed the typical microstructure of a bioceramic coating after a 24 h of immersion under physiological conditions. An apatite layer on the surface had started nucleation in different zones, in the form of globules. EDX analysis identified nitrogen (present in the gentamicin) on the

surface, but the limitations of the equipment made it extremely difficult to detect this element. Only the expected elements (present in the initial powder mixture), plus peaks corresponding to sodium and chlorine (present in Hank's solution) appeared. An EDX mapping of the cross-section (Fig. 5) revealed that sodium chloride penetrated to the layers near the substrate, by interconnected porosity derived from the partial dissolution of the coating after 24 h of immersion. The presence of a sodium chloride precipitation deep in the coating could be interpreted as an indicator of the presence of the gentamicin at the same levels. One consequence that could be expected is a slow liberation of the gentamicin over time, as this gentamicin must be released from such a deep level. The progressive coverage of the surface by an apatite layer is also expected to act as a stopper and helps the prevention of the complete liberation of the antibiotic in the initial stage of implantation.

XRD results showed that hydroxyapatite and rutile were the main phases (the same than in the starting powder), with small amounts of anatase and α-TCP. Broad bands due to the presence of amorphous phases derived from the thermal degradation of the initial powder were also detected. Rietveld calculations quantified an amorphous phase amount of 14%, in front of 18% obtained with HVOF-sprayed HAp, and 38% obtained with plasma-sprayed hydroxyapatite. These results are a witness of the good performance of these HAp-TiO_2 coatings.

The bacterial test results are shown in Fig. 6 and 7. The results with the samples with ZnO (Fig. 6) indicate that the addition of this compound did not have a significant effect on the behavior of any of the three bacteria. The non-treated sample showed an equivalent antibacterial effect as the samples with zinc oxide; or even higher. The infrared spectra of the non-treated sample showed an important peak attributed to the presence of -OH radicals. According to the literature (Ref 32, 33), these radicals are an important factor in the bactericide compounds, and cause an oxidative stress that has a strong effect on some kinds of bacteria. This could explain the antibacterial effect of the as-sprayed samples. The bactericidal effect was similar for the three bacteria, which could be an indicator of the effect of oxidative stress on Gram-positive and Gram-negative bacteria. The mere presence of the coatings reduces by approximately 4-5 orders of magnitude the presence of bacteria in the solution with regard to the controls, and consequently the presence of bacteria is reduced 100%. An interpretation of the slightly lower bactericide effect of the ZnO samples could be the lower presence of -OH radicals on the surface due to the partial coverage of the surface with ZnO. The fact that a well-known bactericide compound such as ZnO showed no effect in this case could be attributed to the bactericidal effect of zinc only for Zn^{2+} concentrations above 4-8 mM (the minimum inhibitory concentration) (Ref 6), and it is possible that this concentration was not reached in these cases. Further research is required to clarify the evolution of the concentration of zinc ions in solution at different culture times.

Concerning the tests with gentamicin, the results in Fig. 7 show that the blank coating (without gentamicin) also induced a reduction of bacteria viability, again a sign of the important antibacterial effect of the non-treated samples (the reduction of the viability was nearly 100%). Nevertheless, an improvement was detected for the treated samples. The addition of gentamicin clearly allowed an extra antibacterial effect against Gram-negative bacteria (which is especially important for resistant microorganisms such as *P. aeruginosa)*, whereas for *S. aureus* there was no marked difference with and without gentamicin.

Antibactericidal tests under diffusion conditions allowed the identification of differences between the samples. As an example, the *E. coli* assay can be observed in Fig. 8. Whereas for the ZnO and the non-treated samples, the antibactericidal effect by diffusion was not detectable; the gentamicin-treated samples clearly showed a growth inhibition zone around them. This capacity against bacteria by diffusion mechanisms makes the treatment with gentamicin

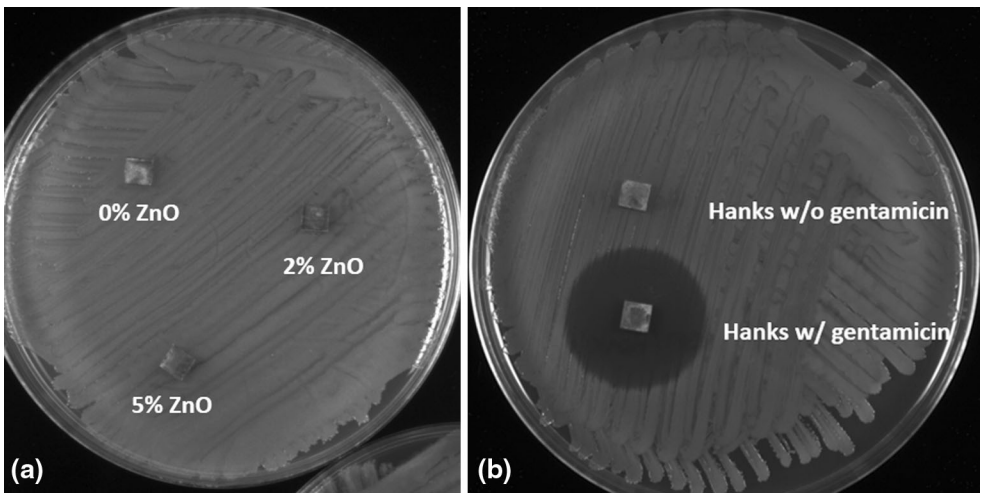

Fig. 8 Antibacterial test against *E. coli* of: (a) samples treated with ZnO and (b) samples treated with gentamicin

a good alternative, since it combines excellent antibacterial behavior in solution with an antibacterial effect by contact and diffusion, which makes it more appropriate for physiological conditions (both solution and diffusion conditions are important in such a situation).

4. Conclusion

Plasma-sprayed hydroxyapatite coatings, usually employed to improve tissue-implant bonding, show limited functionality. A combination HAp-TiO_2 sprayed by HVOF was chosen instead, to improve this functionality. Infection is also a major concern to these coatings, that is why research about antibacterial compounds is important. In this study, two antibacterial treatments were compared over short times in order to establish which one was the most appropriate for biomedical applications. The previous study of the coatings showed that the microstructure was maintained after the treatments, which validates their employment. Non-treated samples showed an important bactericidal effect in solution with the three bacteria analyzed, provided by the presence of -OH radicals. ZnO was found not to add any extra antibacterial activity to the samples, and the treated coatings showed comparable bactericide effects to the non-treated ones. Nevertheless, it was the gentamicin treatment that showed best behavior in solution as well as an important antibacterial effect by diffusion. Further research should be performed in order to study the antibacterial behavior over longer times, and the release kinetics of gentamicin and zinc ions. Possible alternatives include a two-stage spraying, before and after the gentamicin treatment, in order to block the immediate release with the superior layers; or search for a way to incorporate gentamicin in the initial mixture to avoid its degradation during the spray (inside non-conductor particles, for instance) and have more control over the release kinetics.

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