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Silver-hydroxyapatite nanocomposites as bactericide and fungicide materials

Silver-hydroxyapatite nanocomposites containing 1 wt% of metallic silver have been obtained by a colloidal chemical route and subsequent chemical reduction process. The silver nanoparticles are supported onto the HA surface without a high degree of agglomeration. The bactericidal effect against common Gram-positive and Gram-negative bacteria, as well as antifungal activity against yeast have been investigated. The results indicated a high antimicrobial activity for *E. coli*, *M. Luteus* and *I. Orientalis*, so this material can be a promising antimicrobial biomaterial for implant and reconstructive surgery, among other applications.

Keywords: Hydroxyapatite, silver, nanocomposite, antimicrobial, antibacterial, antifungal.

1. Introduction

There is a necessity for replacing bone substance which has been lost due to traumatic or nontraumatic events [1]. The development of advanced materials for biomedical applications is among the most important problems facing modern materials engineering [2]. In the field of orthopaedic applications, metals have been widely used for major load-bearing, but there are, however, various problems related to metallic materials in the human body due to corrosion, wear and/or negative tissue reaction [1].

One alternative is the group of bioceramic materials, where the hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) stands out. This calcium phosphate is widely used as implants, drug carriers, and bone tissue engineering scaffolds, because its phase structure and chemical composition are similar to the mineral phase of hard tissue in the body [3]. Furthermore, HA has the ability to form chemical bond with bone tissue *in vivo* (i.e., bioactivity) and to lead bone tissue growth on the implants (i.e., osteoconductivity) [4]. So, when the implant based in HA is in direct contact with pre-existing bone is manifest the capacity to support bone ingrowth [5].

Although success rates of these kinds of implants are dependent on bone-implant osteointegration, the success and long-term survival of the implants are also dependent on the prevention of bacterial infection after implant placement [6]. The infection rates of total joint hip arthroplasties have been reported to range between 0.5 and 3.0% in primary total hip arthroplasty [7,8]. Infection is a devastating reason which severely affects patient outcome, reduces the lifespan of the prosthesis, and increases prosthesis failure rates. It accounts for an enormous medical costs, increased morbidity, and decreased patient satisfaction [9].

1 One common and accepted strategy to treat and prevent infections associated
2 with orthopedic implants is to deliver antibiotics in a controlled manner at the site of
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4 implantation in order to administer high local doses without exceeding the systemic
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6 toxicity of these drugs [10-13]. However, the capacity of certain bacterial strains to
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8 develop resistance against antibiotics has aroused an increasing interest for the
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10 controlled delivery of other antibacterial agents with a broader activity and low
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12 incidence of resistance. Such agents could be certain metals like silver or zinc, which
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14 are an alternative to avoid the formation of adhesive bacterial films [14,15].
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20 Silver, known as a disinfectant for many years, has a broad spectrum of
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22 antibacterial activity. The silver particles bind to the bacterial cell wall and cell
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24 membrane and inhibit the respiration process [16,17]. The nanoparticles release silver
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26 ions in the bacterial cells, which enhance their bactericidal activity [18,19]. The
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28 common bacterial strains involved in implant-associated infections are included in the
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30 pathogens that silver can eliminate. Likewise, it is well reported the lack of toxicity of
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32 low concentrations of silver for the mammalian cells[20,21].The silver nanoparticles
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34 show efficient antimicrobial properties compared to other silver salts due to their
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36 extremely large surface area and less solubility, which provides better contact with
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38 microorganisms. Silver nanoparticles in most studies are suggested to be non-toxic in
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40 low concentrations, but in high concentration could have toxic effects.
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48 In this work, a nano-nano composite of HA with a very low content of silver
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50 nanoparticles on its surface is presented (1 wt% Ag). The ceramic support avoids the
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52 metal agglomeration and keeps the nanoparticles attached. The antimicrobial benefits of
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54 the composite were evaluated against Gram-positive and Gram-negative bacteria, and
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56 the antifungal activity was evaluated against yeast.
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2. Experimental procedure

HA nanopowders with an average size (d_{50}) of 137 nm prepared *in situ* in the laboratory were used as raw material for the matrix of the composite. This powder was synthesised by a sol-gel route using triethylphosphite and calcium nitrate tetrahydrate as precursors. This process is described detailed in a previous work [22].

Suspensions containing 9 wt% solids with 1 wt% of an alkali-free organic polyelectrolyte as a surfactant (*Dolapix CE-64*) were prepared in distilled water as the liquid medium. The suspension was homogenised by milling with alumina balls in polyethylene containers for 24 hours. The pH was adjusted to 7 by adding drop-wise a 0.1 N NaOH aqueous solution under stirring. Then, AgNO_3 (*Riedel-de Haën*) in aqueous solution was added as a precursor to obtain a HA/nAg composite containing 1 wt% of metallic silver. The reduction of the cation Ag^+ and precipitation of Ag^0 was carried out employing sodium borohydride (NaBH_4 , *Sigma-Aldrich*). The stequiometric molar relation between the NaBH_4 and the Ag^+ is 1:8 according to the next reactions:

An excess of NaBH_4 was added to guarantee the total reduction of silver. This reducing agent was dropped quickly into the stirred mixture with the HA and the silver salt. Finally, the suspension was filtered and washed twice with distilled water. The resulting powder was dried at 60°C.

The particle size distribution was determined in a *Coulter LS 13320* particle size analyser equipped with a laser source ($\lambda=750$ nm). X-ray powder diffraction (XRD)

1 patterns were registered using a *Bruker D8 Advance* diffractometer with a
2 CuK_α radiation source at a scan speed of 0.5 degrees/minute and a step scan of 0.02°.
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4 Transmission electron microscopy (TEM) images were recorded on a *Jeol 2000 FXII*
5 instrument and a *JEM 2100F* using the HAADF-STEM technique, both operating at 200
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7 kV.
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13 The silver concentration in the final material was analyzed using an atomic
14 absorption spectrometer *Varian SpectrAA 220-FS*, with an air/acetylene flame 85/15
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16 and a light source of $\lambda = 328.1$ nm. The sample was previously dissolved in HNO_3 .
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22 Antibacterial tests were performed to investigate the effect of the HA/nAg
23 powder on three different microorganisms: *Escherichia coli JM 110* (Gram-negative
24 bacteria), *Micrococcus luteus* (Gram-positive bacteria) and *Issatchenkia orientalis*
25 (yeast). The three different microorganisms were incubated in a liquid media overnight
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27 at 37°C. After that, 10 μL from each culture were diluted to 5 mL, using the suitable
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29 media, and cultured at 37°C for 6 h. The media used were Luria Bertani (LB) for
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31 *Escherichia coli JM 110* and for *Micrococcus luteus*; the media was yeast extract
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33 dextrose (YEPD) for *Issatchenkia orientalis*. Then, 10 μL from the cultures were diluted
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35 in 1 mL of nutrient, according to the microorganism used. Subsequently, 150 μL of an
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37 aqueous suspension of HA/nAg composite (20 wt%) were added, obtaining a final
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39 concentration of silver in each culture of 0.026 wt%. A silver free media (a mixture of
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41 water containing the corresponding nutrient according to the microorganism tested: LB
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43 or YEPD) was used as control. The microorganisms were tested for viability after
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45 culture on appropriate dilution from the corresponding cultures. The inoculums were
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47 incubated at 30°C with horizontal shake until 72 h. The number of colonies was counted
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49 every 24 h.
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3. Results and discussion

According to the X-ray diffraction patterns recorded in Fig. 1, both the HA powder and the HA/nAg nanocomposite powder obtained present a good degree of crystallization. In the case of the nanocomposite, the Bragg's reflections corresponding to metallic silver are visible ($2\theta = 38.2^\circ$ and 44.4° , JCPDS 87-0720).

The parameters corresponding to the initial particle size distributions of the starting HA powders expressed in terms of d_{50} and d_{90} were 117 nm and 155 nm, respectively.

By comparing this granulometric analysis with the SEM image of the composite shown in Fig. 3, it is possible to infer that the powder matrix size continues in the same nanometric range after the metallic particles addition.

The silver concentration was analyzed in the final material to determine the efficiency of the deposition process. The efficiency was defined with respect to the theoretical concentration with the equation [1]:

$$\eta = \frac{C_s}{C_t} \times 100 \quad \text{Eq. [1]}$$

where η is the efficiency of the deposition process, C_s is the final concentration in the sample, and C_t is the theoretical concentration of Ag in the sample. As the value for C_s is 0.93%, the efficiency of the deposition was found to be $\eta=93\%$. This value indicates that only a small percentage of Ag was lost during the washing process of the sample. At that stage, the eliminated material was mainly formed by subproducts of the

precursors and dispersant, practically the total amount of silver is attached onto the HA surface.

TEM images show in Fig. 4 a size distribution of globular-shape silver particles that ranges between less than 10 nm and 50 nm, with hardly any agglomerates. This is in agreement with the particle size of 16 nm, diameter calculated by the Scherrer equation.

It is well reported that silver nanoparticles display a surface plasmon resonance around 400 nm. However, depending on the environment of the nanoparticles and their size and shape distribution, the precise position and shape of the band may vary [23,24]. As it can be observed in the UV-VIS spectrum in Fig. 5A, the light absorption maxima corresponding to the surface plasmon resonance of the HA/nAg powder is located at 426 nm. As a consequence, the powder shows a dark greenish grey colour (Fig. 5B). The colloidal silver solutions are yellow when the nanoparticles are well dispersed. However, if the particles are close enough, there is an overlap of the surface plasmon vibrations between neighboring particles and results in changes in the optical properties. This can occur due to the aggregations of the silver nanoparticles, leading to an effective decrease in the average interparticle distance. The absence of higher-wavelength features indicates the isotropy of the silver nanoparticles, in good agreement with Fig. 4. The position and shape of the surface plasmon absorption band is similar to those found by other authors [25-27], and indicate a low degree of agglomeration and polydispersity of the silver nanoparticles on the HA matrix surface, in agreement with the TEM images (Fig. 4). Therefore, the HA matrix acts as an effective scaffold, where silver nanoparticles are dispersed, avoiding agglomeration of the nanoparticles. This is an important feature, since the small size of silver particles ensures that a significantly

large surface area of the particles is in contact with the bacterial effluent. A larger contact surface is expected to increase the bactericidal activity.

To investigate the antibacterial and antifungal effect of the HA/nAg powders, a biocide test was performed inoculating $3.6 \cdot 10^{10}$ CFU/ml (CFU: colony forming units) of *Escherichia Coli* (gram-negative bacteria), $3.0 \cdot 10^9$ CFU/ml of *Micrococcus Luteus* (gram-positive bacteria) and $2.2 \cdot 10^{10}$ CFU/ml of *Issatchenkia Orientalis* (yeast). Table 1 shows the number of colonies (CFU) of *E. Coli*, *M. Luteus* and *I. Orientalis* corresponding to control and HA/nAg powder after 24 and 48 h.

After the exposition time of 24 h, the presence of HA/nAg caused a decrease larger than five orders of magnitude in the number of colonies of *E. coli*, *M. luteus* and *I. orientalis*. For the three microorganisms, the reduction percentage is above 99.99% only in the first 24 h. Finally, no antibacterial activity was found in the silver free media that was used as a control.

The logarithm reduction rate ($\log \eta$) has been used to characterized the effectivity of the biocide agent. It was calculated using the number of viable microorganisms in the inoculum control and substrate in the Equation 2:

$$\text{Log } \eta = \log A - \log B \quad \text{Ec. 2}$$

where A is the average number of microorganisms in the control, B is the average number of microorganism in the sample with HA/nAg. The results are shown in Fig. 6.

The antimicrobial activity tests have revealed that HA/nAg powder is an excellent antibacterial agent again Gram-positive and Gram-negative bacterias as well as yeast.

1 These results are similar to those reported by Esteban et al [28] in the case of glass-nAg
2 composite powder. In this regard a similar synergetic mechanism between the silver
3 nanoparticles and the Ca^{2+} lixiviated from the HA may operates. In Fig. 6, the
4 antimicrobial activity of the HA/nAg nanocomposite was compared with a commercial
5 broad-spectrum antibacterial/antimicrobial agent using the parameter of the logarithm
6 reduction [29]. The result confirm that the composite show an efficacy even higher than
7 that of commercial products. This activity of silver against microorganism combined
8 with the bioactivity of the matrix place the composite HA/nAg as potential candidate for
9 orthopedic and dental applications.
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26 4. Conclusions

27 Silver dispersed nanoparticles have been obtained into a HA matrix following
28 a simple low cost bottom-up route. The nanoparticles stay supported on a matrix,
29 avoiding agglomeration and health problems and consequently, allowing a better
30 manipulation and enhancing their biocide activity. The chemical reduction enables to
31 preserve the nanostructured character of the silver and HA phases, avoiding heat
32 treatments that can induce grain growth. This nanosized material is comparable to that
33 of the bone apatite.
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46 In vitro bactericidal activity of HA/nAg was observed against *E. coli* (Gram-
47 negative) and *M. luteus* (Gram-positive). It was achieved a logarithm reduction higher
48 than 20, which means a complete safe disinfection. In the case of the yeast (*I. orientalis*)
49 the logarithm reduction is comparable with commercial products, so its antifungal
50 activity has been proved. The present work is a contribution to a class of nanomaterials
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1 for orthopedic and dental applications combining the bioactivity of HA and the
2 additional advantage of silver antimicrobial effects.
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21 TEM study.
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Figure 1. X-ray diffraction patterns obtained from HA and HA/nAg nanocomposite powders. The Bragg’s reflections corresponding to metallic silver have been marked with an asterisk.

Figure 2. Particle size distribution of the starting HA powders.

Figure 3. HA/nAg composite SEM image

Figure 4. TEM micrographs showing the size and morphology of the spherical silver nanoparticles attached on the hydroxyapatite surface. Figure 4D was obtained using the HAADF technique.

Figure 5. According to the absorption spectrum of the HA/nAg composite powder (A), the light absorption maxima corresponding to the surface plasmon resonance of Ag nanoparticles is located at 426 nm. As a consequence, the obtained composite displays the dark greenish grey color showed in picture (B).

Figure 6. Logarithm reduction for *E. coli*, *M. luteus* and *I. orientalis* at 24 and 48 hours (— — — Commercial broad-spectrum antibacterial/antimicrobial).

Table 1. CFU ml⁻¹ of *E. coli*, *M. luteus* and *I. orientalis* corresponding to biocide agents at 0, 24 and 48 h.

Figure 1
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Figure 2
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Figure 3
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Figure 4A
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Figure 4D
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Figure 5A
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Figure 5B
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Figure 6
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Table

E. Coli			
	0h	24h	48h
Control (CFU/ml)	3,60E+10	1,40E+11	3,00E+11
HA/nAg (CFU/ml)	3,60E+10	1,00E-04	1,00E-04
M. Luteus			
	0h	24h	48h
Control (CFU/ml)	3,00E+09	3,00E+09	1,00E+09
HA/nAg (CFU/ml)	3,00E+09	1,00E-04	1,00E-04
I. Orientalis			
	0h	24h	48h
Control (CFU/ml)	2,20E+10	1,20E+11	4,80E+10
HA/nAg (CFU/ml)	2,20E+10	1,00E+04	1,00E+05