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Research paper

A new multifunctionalized material against multi-drug resistant bacteria and abnormal osteoclast activity

Elisa Boanini[a,*], Paola Torricelli[b], Francesca Bonvicini[c,*], Maria Cristina Cassani[d], Milena Fini[b], Giovanna Angela Gentilomi[c], Adriana Bigi[a]

[a] Department of Chemistry "Giacomo Ciamician", University of Bologna, via Selmi 2, 40126 Bologna, Italy
[b] Laboratory of Preclinical and Surgical Studies, Codivilla-Putti Research Institute, Rizzoli Orthopaedic Institute, via di Barbiano 1/10, 40136 Bologna, Italy
[c] Department of Pharmacy and Biotechnology, University of Bologna, via Massarenti 9, 40138 Bologna, Italy
[d] Department of Industrial Chemistry "Toso Montanari", University of Bologna, viale del Risorgimento 4, 40136 Bologna, Italy

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ABSTRACT

The development of new biomaterials able to favor bone formation and to inhibit bone abnormal resorption is mandatory to face the increasing number of age-related musculo-skeletal disorders. Moreover, the increasing antibiotic resistance of clinically important bacteria, which is among the main causes of implant failure, requires new antimicrobial systems. In this study, we prepared multifunctional materials consisting of hydroxyapatite-zoledronate composite crystals decorated with Ag Nanoparticles (AgNPs). Zoledronate, a potent bisphosphonate widely applied for the treatment of pathologies associated to abnormal bone loss, was incorporated into hydroxyapatite up to about 8 wt%. Loading of poly(ethylenimine) – stabilized AgNPs onto the crystals was promoted by zoledronate functionalization and provoked a significant variation of the values of zeta potential. The results of in vitro tests demonstrate that the multifunctional materials combine the beneficial actions of zoledronate and AgNPs. In fact, they improve osteoblast differentiation and activity, whereas they inhibit osteoclastogenesis and osteoclast differentiation, and significantly hinder the growth of multi-drug resistant Gram positive and Gram negative bacteria. As a consequence, they can be exploited both as antiresorptive agents and as antimicrobial materials able to prevent the development of bone-associated infections.

1. Introduction

Thanks to its similarity to the inorganic phase of bone, hydroxyapatite (HA) is the most employed calcium phosphate for the preparation of biomaterials for the repair/substitution of the hard tissues of vertebrates. The good biocompatibility and bioactivity of HA can be enhanced and improved through functionalization with ions and molecules capable to promote bone growth and to inhibit bone abnormal resorption, as well as to prevent infections and/or adverse immune reactions [1–3]. In particular, functionalization with bisphosphonates (BPs) was shown to provide composite materials able to hinder abnormal osteoclast mediated resorption [4–6]. BPs are widely employed for the treatment of pathologies characterized by excessive bone turnover [7]. Moreover, nitrogen containing bisphosphonates (NBPs), which inhibit farnesyl pyrophosphate synthase (FPP) enzyme within the mevalonate pathway in osteoclasts, have both direct and indirect antitumor activities [8]. Zoledronate (ZOL), which has been reported to exhibit a high binding activity to synthetic HA [9], is one of the most potent antiresorptive NBPs [10]. On the other hand, several undesirable side effects, including osteonecrosis of the jaw and atypical subtrochanteric fractures, have been associated to the prolonged systemic administration of these drugs [11,12]. In order to avoid/reduce these drawbacks, a number of different materials, including calcium phosphates, have been proposed as delivery systems for N-BPs local administration at specific bone sites [13,14]. In particular, we have previously demonstrated that zoledronate functionalized HA (HAZOL) promotes in vitro osteoblast viability and activity, whereas it inhibits osteoclast proliferation and differentiation [5,15,16]. Herein we used HAZOL as support for silver nanoparticles (AgNPs) with the aim to get composite materials able to display both antiresorptive and antimicrobial properties.

Bacterial colonization of implants and medical devices is a big problem since clinically important bacteria exhibit increasing antibiotic resistance [3]. Silver nanoparticles display strong antimicrobial properties against a wide number of bacteria [17–20]. AgNPs are less toxic than silver ions, which can damage bacterial DNA, proteins, enzymes

* Corresponding authors.
E-mail addresses: elisa.boanini@unibo.it (E. Boanini), francesca.bonvicini4@unibo.it (F. Bonvicini).

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and cell walls [21–23]. Toxicity seems to depend on several nanoparticles properties, such as surface area, size and shape, surface charge, particle purity [24]. Anyhow, the concentrations for antimicrobial activity were reported to be much less than that capable to provoke cytotoxicity [25]. Clinical results on orthopedic applications of AgNPs, although still limited, are encouraging and supported by a number of in vitro and *in vivo* studies [26,27].

Silver doped/substituted HA has been the object of a number of studies [28–31], whereas a few investigations on the antibacterial activity of AgNPs supported on HA particles have been reported [32–36]. In this study we loaded different amounts of polyethylenimine (PEI) stabilized AgNPs onto HAZOL, as well as onto pure HA as control. To this purpose, we used low molecular weight PEI, in order to avoid the cytotoxicity problems that have been reported for the cationic polymer at high molecular weight [37]. Bone cells response was investigated using an osteoblast-osteoclast co-culture system. In particular, the study was performed using osteoclast (OC) derived from osteoporotic subjects in order to focus the attention on the action of ZOL on OC with abnormal activity. In fact osteoporotic OC show a different behavior when compared to normal ones, causing an impairment of the balance between bone resorption and formation [38–40]. The antibacterial properties of the composite materials were tested in vitro against a panel of Gram positive and Gram negative reference bacterial strains including pathogens frequently involved with implant-associated infections and against antibiotic-resistant clinical isolates recovered from patients with chronic bone or prosthetic joint infections.

2. Materials and methods

2.1. Synthesis and characterization of materials

The synthesis of hydroxyapatite (HA) was carried out in N_2 atmosphere using 50 mL of 1.08 M $Ca(NO_3)_2{\cdot}4\ H_2O$ at pH adjusted to 10 with NH_4OH. The solution was heated at 90 °C and 50 mL of 0.65 M $(NH_4)_2HPO_4$, pH 10 adjusted with NH_4OH, was added dropwise under stirring. After 5 h at 90 °C under stirring, precipitate was centrifuged at 10,000 rpm and repeatedly washed with CO_2-free distilled water. The product was dried at 37 °C overnight, then mildly ground in an agate mortar and sieved (d = 56 μm). Zoledronate-containing hydroxyapatites were obtained by following the same procedure, and by adding disodium zoledronate tetrahydrate (Chemos GmbH) to the phosphate solution [5]. Zoledronate concentrations calculated on final volume were 7 and 14 mM, thereby samples were labelled ZOL7 and ZOL14, respectively.

Bisphosphonate content was determined spectrophotometrically via complex formation with Fe(III) ions using a Varian Cary50Bio instrument (λ = 290 nm) [41].

PEI-stabilized Ag nanoparticles (AgNPs) were prepared by heating 100 mL of 10 mM $AgNO_3$. Once at the boiling point, 0.7 mL of 10% (w/w) polyethylenimine (MW ≈ 2000) solution were quickly added. Resulting boiling solution gradually turned brown and after 4 min it was allowed to cool at room temperature. Finally, to account for water evaporation, the volume was adjusted to 100 mL.

HA-AgNPs, ZOL7-AgNPs and ZOL14-AgNPs aggregates were prepared after incubation of 0.500 g of HA or ZOL7 or ZOL14 powders in freshly prepared AgNPs colloidal solution, just cooled at room temperature. The resulting suspension was stirred for 1 h. After filtration on a Buchner funnel, the solid was thoroughly washed with water and dried at 37 °C. Different volumes of AgNPs solution were tested, namely 5, 10, 20, 30, 40, and 50 mL. Accordingly, samples were labelled HA-5, HA-10, HA-20, etc.; ZOL7-5, ZOL7-10, ZOL7-20, etc.; and ZOL14-5, ZOL14-10, ZOL14-20, etc.

The same procedure was utilized to prepare some further samples containing PEI but no AgNPs, as control materials for cytotoxicity and antibacterial tests. In detail, the samples were prepared through incubation of 0.500 g of HA or ZOL14 in 5, 20 or 50 mL of a solution obtained by boiling 100 mL of an aqueous solution containing 0.7 mL of 10% (w/w) PEI for 4 min and then cooling at room temperature. The resulting suspensions were stirred for 1 h. After filtration on a Buchner funnel, the solids were thoroughly washed with water and dried at 37 °C. The samples were labeled HA-20P, HA-50P, ZOL14-5P and ZOL14-50P.

X-ray diffraction (XRD) analysis was carried out by means of a PANalytical X'Pert PRO powder diffractometer equipped with a fast X'Celerator detector. Ni-filtered CuKα radiation was used (40 mA, 40 kV). For phase identification the 2θ range was investigated from 10 to 60 2θ degrees with a step size of 0.1° and time/step of 100 s.

The amount of silver present on the different samples was determined with flame atomic absorption spectroscopy (AAS, Thermo Scientific) in air-acetylene flame with a wavelength of 328.1 nm and a spectral band-width of 0.5 nm. The analyses were conducted dissolving ca. 8 mg of previously grinded solid samples (weighted with a Mettler Toledo AT 21 Comparator balance), in 25 mL of a 0.5 M HNO_3 aqueous solution. The calibration line was made with 5 calibration standards (2, 4, 6, 8, 10 ppm), prepared by dilution to 50 mL of a 100 ppm silver standard for AAS in 0.5 M HNO_3 (Merck).

For Transmission Electron Microscopy (TEM) investigations, a drop of sonicated sample suspension in ethanol was transferred onto holey carbon foils supported on conventional copper microgrids. A Philips CM100 transmission electron microscope, operating at 80 kV was used.

Zeta potential was measured using Electrophoretic Light Scattering (ZetasizerNano; Malvern Instruments). 5 mg of powder sample was suspended in 50 mL of MilliQ water and sonicated for 2 min before zeta potential measurement. Each analysis was performed in triplicate.

The specific surface area was measured using a Carlo Erba Sorpty 1750 BET analyser using constant volume N_2 absorption with desorption at 80 °C.

For infrared (FT-IR) adsorption analysis, 1 mg of the powdered samples was carefully mixed with 200 mg of KBr (infrared grade) and pelletized under a pressure of 10 tons for 1 min. The pellets were analyzed using a Bruker ALPHA FT-IR spectrophotometer to collect 32 scans in the range 4000–400 cm^{-1} at a resolution of 4 cm^{-1}.

In vitro tests were performed on disk-shaped samples (∅ = 6.0 mm). Each disk was prepared by pressing 40 mg of powder into cylindrical moulds by using a standard evacuable pellet die (Hellma). Disk shaped samples for antibacterial activity, cytotoxicity and cell co-culture were sterilized using gamma rays.

Release of Silver from disk-shaped samples was measured in the medium used for cell culture differentiation (see Section 2.4). The supernatants were removed from the wells at increasing times up to 21 days and Ag content was analyzed using flame atomic absorption spectroscopy (AAS, Thermo Scientific) in air-acetylene flame with a wavelength of 328.1 nm and a spectral band-width of 0.5 nm. Results from this analysis represent the mean value of three different determinations.

2.2. Bacterial strains and Kirby-Bauer disk diffusion method

The in vitro antibacterial activity of the composite materials (HA-5, HA-20, HAZOL7-5, HAZOL7-20, HAZOL14-5, HAZOL14-20) was preliminary evaluated against a panel of Gram positive and Gram negative reference bacterial strains including *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 9591), *Pseudomonas aeruginosa* (ATCC 27853). Subsequently, having defined both the overall spectrum of antibacterial activity and the cytotoxic profile of the tested samples, HA-20 and HAZOL14-5, which contain almost the same amount of AgNPs (0.8–0.9 wt%), were assayed towards 10 clinical isolates recovered from patients with chronic bone or prosthetic joint infections. Strains included 5 *S. epidermidis* of which 3 methicillin-resistant (MRSE) and 5 *P. aeruginosa* of which 2 multi-drug resistant (MDR); they were isolated on BD