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Biomimetic fabrication of antibacterial calcium phosphates mediated by polydopamine

Lucia Forte[a], Paola Torricelli[b], Francesca Bonvicini[c], Elisa Boanini[a,*], Giovanna Angela Gentilomi[c], Gigliola Lusvardi[d], Elena Della Bella[b], Milena Fini[b], Edoardo Vecchio Nepita[e], 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 Chemical and Geological Sciences, University of Modena and Reggio Emilia, via Campi 103, 41125 Modena, Italy
[e] Microbiology Operative Unit-Bacteriology Section, S. Orsola-Malpighi Hospital, via Massarenti 9, 40138 Bologna, Italy

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ABSTRACT

In this work we developed new antibacterial composite materials using polydopamine (PDA) to trigger the deposition of silver nanoparticles (AgNPs) onto calcium phosphates, namely octacalcium phosphate (OCP) and α-tricalcium phosphate (αTCP). Functionalization of OCP and αTCP with a self-polymerized polydopamine layer was obtained by soaking the calcium phosphates in dopamine solution. The PDA surface of functionalized calcium phosphates (OCPd and αTCPd) promoted the deposition of AgNPs by reducing silver ions when soaked in a silver nitrate solution. The amount of deposited AgNPs can be modulated by varying the concentration of silver nitrate solution and the type of substrate. The results of in vitro tests carried out with osteoblast-like MG63 cells indicate that the combination of AgNPs with OCP provides more biocompatible materials than those obtained using αTCP as substrate. In particular, the study of osteoblast activity and differentiation was focused on the samples OCPdAg5 (silver content = 8.2 wt%) and αTCPdAg5 (silver content = 4.7 wt%), which did not show any cytotoxicity, and compared with those obtained on pure OCP and αTCP. The results demonstrate that the AgNPs loaded materials support osteoblast viability and differentiation, whereas they significantly inhibit the growth of relevant antibiotic-resistant pathogenic bacteria.

1. Introduction

Calcium phosphates (CaPs) are widely employed for the preparation of biomaterials for hard tissues substitution and repair, because of their similarity to the inorganic phase of the mineralized tissues of vertebrates. In particular, octacalcium phosphate, $Ca_8H_2(PO_4)_6{\cdot}5H_2O$ (OCP), which is considered the precursor phase of biological apatites, exhibits osteoconductive properties and converts into hydroxyapatite (HA) when implanted in bone defects [1]. Enhancement of bone formation has been demonstrated both when OCP granules are used to repair bone defects and when OCP is applied as a coating on metallic surfaces [2–6]. OCP can hydrolyze into HA in aqueous solution [7,8] However, the process is relatively slow at physiological pH and temperature, whereas it is accelerated at higher temperatures and in the presence of small amount of fluoride [9–12]. Also other CaPs, in particular α-tricalcium phosphate (αTCP), hydrolyze into HA [13]. At variance with OCP, αTCP is considered unsuitable for surgical implants [14]; however, it is widely employed for the preparation of calcium phosphate bone cements. αTCP has a relatively higher solubility than its polymorph, βTCP, and the hardening reaction of αTCP containing cements implies its conversion into HA [15,16]. One of the main problems of medical devices, including CaPs based biomaterials, is the relatively high risk of infections associated to their use. Silver nanoparticles (AgNPs) display antimicrobial properties against a broad spectrum of pathogens and are currently applied in a number of biomedical applications, including bone cements and implant coatings [17]. We have previously developed a fast and low cost procedure to support AgNPs on HA crystals, and shown that the composite material displays significant, long-standing antibacterial activity towards both *S. aureus* and *E. coli* [18]. In this study we propose to utilize OCP and αTCP, which are more soluble and resorbable than HA, as supports for AgNPs, using polydopamine (PDA) as functionalizing and reducing agent. Polydopamine is a polymer

* Corresponding author at: Department of Chemistry "G. Ciamician", University of Bologna, Italy.
E-mail address: elisa.boanini@unibo.it (E. Boanini).

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formed under slightly basic conditions by the oxidative polymerization of dopamine, which creates a stable layer that is adherent to the surface of materials [19]. Stable PDA films with controlled thickness can be deposited on virtually any substrate. Moreover, PDA is biocompatible and exhibits several functional groups, such as catechol, amine and imine groups, which are able to react with a wide range of molecules [20]. In particular, the catechol group can oxidize into the corresponding quinone group and trigger reduction processes of metallic cations [21]. The peculiar characteristics of PDA prompted a number of studies that yielded an increasing number of new applications of PDA based materials, spanning in different fields, from energy to environment, to biomedical science [19]. Herein we optimized the experimental conditions to prepare PDA functionalized OCP and αTCP at increasing AgNPs contents, and we investigated the influence of the presence of AgNPs on the antibacterial properties of the composite materials against Gram positive and Gram negative reference bacterial strains, as well as against antibiotic-resistant clinical isolates recovered from patients with bone or prosthetic joint infections. Moreover, we tested the ability of the materials to support *in vitro* growth and differentiation of osteoblast-like cells MG63. MG63 activity and differentiation were investigated using the most common markers of osteoblast metabolism, which were tested both on the supernatants of cell culture and by means of quantitative Polymerase Chain Reaction (qPCR).

2. Materials and methods

2.1. Materials synthesis and characterization

The synthesis of OCP was carried out as previously reported [22]. Briefly, 250 ml of 0.04M $Ca(CH_3COO)_2{\cdot}H_2O$ were added dropwise into 750 ml of a phosphate solution containing 5 mmol of $Na_2HPO_4{\cdot}12H_2O$ and 5 mmol of $NaH_2PO_4{\cdot}H_2O$ previously adjusted to pH 5 with CH_3COOH. The reaction was carried out at 60 °C under mechanical stirring. After 15 min the precipitate was filtered, repeatedly washed with distilled water and dried at 37 °C.

αTCP was obtained by solid-state reaction of a mixture of $CaCO_3$ and $CaHPO_4{\cdot}2H_2O$ in the molar ratio of 1:2 at 1300 °C for 5 h [23]. The solid product was carefully ground and sieved (< 40 μm) before being submitted to further treatment.

Functionalization with polydopamine was obtained through immersion of 100 mg of OCP or αTCP into 50 ml of a solution of dopamine hydrochloride (2 mg/ml) at pH 8.5 (TRIS buffer), under stirring [19], for different periods of time (1, 2 and 18 h) at room temperature. Then, the samples were filtered, repeatedly washed with distilled water and dried at 37 °C overnight. In the following, the samples functionalized with PDA are indicated as OCPd and αTCPd, respectively.

Deposition of silver nanoparticles was obtained by simultaneous addition of 20 ml of $AgNO_3$ solution, at different concentrations (1, 5 and 10 mM), and 20 ml of a 13 mM sodium citrate solution, to the PDA functionalized phosphates (100 mg) at room temperature under stirring. After 1 h, the solid samples were filtered, repeatedly washed with distilled water and dried at 37 °C overnight. The two series of samples were labeled respectively OCPdAgX and αTCPdAgX, where X indicates the concentration (mM) of $AgNO_3$ solution.

X-ray diffraction analysis was carried out by means of a PANalytical X'Pert PRO powder diffractometer equipped with a X'Celerator detector (40 mA, 40 kV). For phase identification the 2θ range was investigated from 3 to 60 2θ ° with a step size of 0.1 and time/step of 100 s.

Thermogravimetric analysis was carried out using a Perkin- Elmer TGA-7. Heating was performed in a platinum crucible in air flow (20 cm^3/min) at a rate of 10 °C/min up to 700 °C. The samples weights were in the range 5–10 mg. Results from this analysis represent the mean value of determinations for three different samples of each composition.

Morphological investigations of crystals were performed using a Phenom ProX desktop-scanning electron microscope at beam acceleration voltage of 10 kV. The samples were observed as prepared and not sputter coated before examination.

The quantification of Ag was performed using a quadrupole mass spectrometer with plasma source ICP MS XSeries Thermo Fisher Scientific. The samples were prepared by dissolving 10 mg of powders in water/methanol solution 1/1 V/V. 5 ml were taken and added in 50 ml of 5% nitric acid solution.

Static contact angle measurements were performed on disk shaped samples (Ø = 13.0 mm). Each disk was prepared by pressing 100 mg of powder into cylindrical moulds by using a standard evacuable pellet die (Hellma). A KSV CAM101 instrument was used under ambient conditions by recording the side profiles of deionized water drops for image analysis. The shape of the drop was recorded in a time range of 0–60 s, by collecting an image every 0.033 s. At least six drops were observed for each sample.

In vitro cell and antibacterial tests were performed on disk-shaped samples (Ø = 6.0 mm). Each disk was prepared by pressing 40 mg of powder into cylindrical moulds by using a standard evacuable pellet die (Hellma), and sterilized using gamma rays (Cobalt-60) at a dose of 25 kGy.

AFM analysis of the disk-shaped samples was performed using a Veeco Nanoscope 3D instrument. The samples were analyzed in tapping mode using an E scanner (maximum scan size 15 μm) and phosphorus (n) doped silicon probes (spring constant 20–80 N/m; resonance frequency 250–290 kHz; nominal tip radius < 10 nm). Roughness parameters, namely arithmetic mean roughness (Ra), root-square roughness (Rq), and the vertical distance between the highest and lowest points within the evaluation length (Rt), were recorded.

Silver release was measured in the medium used for cell culture differentiation (see Section 2.2.2.). The surnatants were removed from the wells at 1, 2 and 7 days and Ag content was analyzed using a quadrupole mass spectrometer with plasma source ICP MS XSeries Thermo Fisher Scientific.

2.2. Cellular tests

2.2.1. Cytotoxicity tests

Human osteoblast-like cells MG63 (OB, Istituto Zooprofilattico Sperimentale IZSBS, Brescia, Italy), were cultured in DMEM medium (Dulbecco's Modified Eagle's Medium, Sigma, UK) supplemented with 10% FCS, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Cells were detached from culture flasks by trypsinization, and cell number and viability were checked by Erythosine B dye exclusion test. OB cells were plated at a density of 5×10^4 cells/ml in 24-well plates containing sterile disk-shaped samples (Ø = 6.0 mm) (of the following biomaterial: OCPdAg1, OCPdAg5, OCPdAg10, αTCPdAg1, αTCPdAg5, αTCPdAg10. Wells for negative (CTR −, DMEM only) and positive (CTR +, DMEM + 0.05% phenol solution) controls were also prepared. Plates were cultured in standard conditions, at 37 ± 0.5 °C with 95% humidity and 5% ± 0.2 CO_2 up to 72 h.

The quantitative evaluation of cytotoxicity was performed by measuring cell viability, lactate dehydrogenase enzyme (LDH) release, Interleukin-6 (IL-6) and Caspase 3 activity. Cell proliferation and viability at 24 and 72 h was assessed by WST1 (WST1, Roche Diagnostics GmbH, Manheim, Germany) colorimetric reagent test. The assay is based on the reduction of tetrazolium salt into a soluble formazan salt by a reductase of the mitochondrial respiratory chain, active only in viable cells. 100 μl of WST1 solution and 900 μl of medium (final dilution: 1:10) were added to the cell monolayer, and the multi-well plates were incubated at 37 °C for a further 4 h. Supernatants were quantified spectrophotometrically at 450 nm with a reference wavelength of 625nm. Results of WST1 are reported as optical density (OD) and directly correlate with the cell number. Proliferation percent relative to CTR − are also reported. At the end of experimental times the supernatant was collected from all wells and centrifuged to remove