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Anti-sessile bacterial and cytocompatibility properties of CHX-loaded nanohydroxyapatite

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ABSTRACT

Nanohydroxyapatite possesses exceptional biocompatibility and bioactivity regarding bone cells and tissues, justifying its use as a coating material or as a bone substitute. Unfortunately, this feature may also encourage bacterial adhesion and biofilm formation. Surface functionalization with antimicrobials is a promising strategy to reduce the likelihood of bacterial infestation and colonization on medical devices. Chlorhexidine digluconate is a common and effective antimicrobial agent used for a wide range of medical applications. The purpose of this work was the development of a nanoHA biomaterial loaded with CHX to prevent surface bacterial accumulation and, simultaneously, with good cytocompatibility, for application in the medical field. CHX (5–1500 mg/L) was loaded onto nanoHA discs and the materials were evaluated for CHX adsorption and release profile, physic-chemical features, antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, and cytocompatibility toward L929 fibroblasts. Results showed that the adsorption of CHX on nanoHA surface occurred by electrostatic interactions between the cationic group of CHX and the phosphate group of nanoHA. The release of CHX from CHX-loaded nanoHA showed a fast initial rate followed by a slower kinetics release, due to constraints caused by dilution and diffusion-limiting processes. NanoHA.50 to nanoHA.1500 showed strong anti-sessile activity, inhibiting bacterial adhesion and the biofilm formation. CHX-nanoHA caused a dose- and time-dependent inhibitory effect on the proliferation of fibroblasts for nanoHA.100 to nanoHA.1500. Cellular behavior on nanoHA.5 and nanoHA.50 was similar to control. Therefore, CHX-loaded nanoHA surfaces appear as a promising alternative to prevention of devices-related infections.

1. Introduction

Devices-related infections (DRIs), from bacteria attaching and proliferating on surfaces of biomedical devices/implants, are a significant issue in implant surgery and short-term biomedical devices [1]. These infections have a huge impact in terms of morbidity, mortality and medical costs. Different microorganisms have been implicated in DRIs; the most prevalent are the Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* and the Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* [2]. Difficulty of antibiotherapy on the eradication of DRIs is known to be related to a significant decrease in the susceptibility of biofilms against antimicrobial agents, compared with cultures grown in free-floating suspension [2,3]. The high cell density and the extra-cellular polymer substances of microbial biofilms contribute to their higher antimicrobial resistance. Mechanical management to remove the biofilm in the peri-implant vicinity is almost impossible since the roughness and composition of the implant surface, which modulate osteoblast attachment and proliferation, cannot be altered [3].

Therefore, the most commonly used preventive approach is surface modification of the device with antimicrobial agents in order to

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inhibit bacterial development in the biofilm and, hopefully, to prevent device-associated infections. The immobilization of bioactive molecules on biomaterial surfaces has been subjected of considerable research in the search for antimicrobial surfaces [1]. There is a broad choice of active molecules such as anticoagulants, anti-inflammatory drugs, antibiotics and extracellular matrix proteins [2]. Several authors [4–8] reported that the adsorption of antibiotics (e.g., gentamicin, ciprofloxacin, amoxicillin, and erythromycin) on medical devices has a huge potential in the medical field. However, there is a consensus in the medical area on the rational use of antibiotics in order to avoid microbial resistance.

Chlorhexidine digluconate (CHX) is an antimicrobial organic substance, with low risk of associated drug resistance, and it has a large broad-spectrum, acting against Gram-negative and Gram-positive bacteria as well as bacterial spores, lipophilic virus, yeast, and dermatophytes [9,10]. CHX has been used for a wide range of medical applications such as mouthwash, topical antimicrobial, surgical scrub and vascular catheters [9]. The mechanism of action is based on the cationic nature of dissociated chlorhexidine salts which bind to negatively charged regions in the cytoplasmic membrane of microorganisms, through electrostatic attraction, causing interference in metabolic processes and loss of osmotic control (bacteriostatic action), and subsequently leakage of intracellular components (bactericide action) [9]. The CHX effect is concentration-dependent, being bacteriostatic at low concentrations and bactericidal at high concentrations [10,11]. CHX has been loaded to different materials like titanium, polymethyl methacrylate-based resin cement, dentin slabs, glass ionomer cements, PLGA microspheres, titanium/polybenzyl acrylate, anatase/rutile titanium dioxide, cellulosic fibers, hydroxyapatite, and polyurethane nanocomposites [3,9,12–20]. Most of these studies focused on the development of controlled CHX release systems aiming to prevent implant-associated infections.

Hydroxyapatite, an inorganic calcium phosphate material, has long been preferred as the material used in hard tissue repair over autografts and allografts, drug carrier and coatings due to the chemical similarities with bone and teeth mineral enamel [21–23]. However, hydroxyapatite has some disadvantages such as brittleness, low tensile strength and fracture toughness [24]. Nanohydroxyapatite (nanoHA) appears able of rectifying the problems of standard hydroxyapatite. NanoHA possesses a significantly higher surface area, porosity and densification, which may improve its mechanical properties under load, shows solubility in vivo and the capacity to penetrate cell membranes [25]. Also, nanoscale topography have a positive effect on osteoblastic proliferation and differentiation, and the nano-crystals allow a constant regeneration of bone, resulting in improved biocompatibility and osteointegration [26]. Some authors have addressed the capacity of blending nanoHA with antimicrobial agents (e.g., amoxicillin, erythromycin, minocycline, zinc oxid, cobalt) [8,27–29] or materials (e.g., hydroxide, chitosan) [30,31] for the treatment of infections related to biomedical devices and implants. However, the possibility to incorporate CHX into nanoHA substrates was not addressed so far.

In this work, it was hypothesized that the unique surface properties of nanoHA substrates compared to its bulk-phase counterpart, namely the significantly higher surface area, would favor an adsorption profile that will endow the material with antibacterial adhesive properties. This approach is of upmost relevance as bacterial adhesion in the first step to the formation of biofilms. Therefore, the purpose of this work was the development of a nanoHA biomaterial loaded with an antimicrobial agent (chlorhexidine digluconate–CHX) effective in preventing the bacterial accumulation on its surface and with a good cytocompatibility, for application in the medical field.

2. Materials and methods

2.1. Adsorption of CHX on nanoHA discs

NanoHA discs, 10 mm diameter and 1 mm height, were prepared as previously described [32]. Briefly, nanoHA discs were produced using 150 mg of dry power under uniaxial compression stress of 2 MPa. Then, the discs were sintered at 830 °C with a 15 min plateau and applying a heating rate of 20 °C/min. The sintering cycle was completed with a natural cooling process inside the furnace. The discs were sterilized by dry heat (180 °C, 2 h).

Then, different solutions of CHX were prepared by diluting an aqueous stock solution of 20% chlorhexidine digluconate (VWR—USA) in sterile deionized water in order to obtain the following concentrations: 5, 50, 100, 500, and 1500 mg/L. The nanoHA discs were incubated with 5 mL of the previously prepared solutions of CHX for 24 h at 37 °C with moderate shaken in a batch system. The nanoHA discs incubated with sterile deionized water were used as control (nanoHA.0). The CHX-containing materials will be further mentioned as nanoHA.5, nanoHA.50, nanoHA.100, nanoHA.500, nanoHA.1500 and nanoHA.0, according to the concentration of the CHX solution used to load the nanoHA discs. After the contact period, the supernatant was removed and analyzed by UV spectrophotometry at 254 nm. The amount of the CHX adsorbed on nanoHA discs at equilibrium was estimated from the difference between the initial and final concentration of CHX in solution. These assays were carried out in triplicate. The CHX mass adsorbed per unit area of nanoHA discs (Q_e, mg/m^2) versus final equilibrium concentration of CHX in solution (C_e, mg/L) was plotted, and Langmuir and Freundlich adsorption isotherms were fitted to the experimental data. The specific surface area of nanoHA discs (4.9 m^2/g) was previously obtained by Barros et al. [32].

2.1.1. Materials characterization

Materials chemical characterization was performed using Attenuated total reflectance—Fourier transformed infrared spectroscopy (ATR—FTIR), with a Perkin–Elmer 2000 FTIR spectrometer. For that purpose, all materials were analyzed at a spectral resolution of 2 cm^{-1} and 100 scans were accumulated per sample.

The morphologic characterization of CHX-loaded nanoHA discs was obtained by scanning electron microscopy (SEM), using a FEI Quanta 400FEG/ESEM microscope (FEI, USA). Before analysis, all materials were sputter-coated with a thin gold/palladium film, using a sputter coater (SPI-Module) in an argon atmosphere. Five fields for each sample were randomly chosen, under a 5000× magnification.

Zeta potential (ZP) was measured to evaluate the negative net charge of CHX-loaded nanoHA discs using an electrokinetic analyzer (EKA), applying the “automatic” mode method. Measurements were performed at pH 6 in 1 mM KCl.

2.2. Release kinetics of CHX from CHX-loaded nanoHA discs

Two protocols were used to quantify the release kinetics profile of CHX. The nanoHA discs were immersed in 0.9% NaCl (1 mL/disc) and were incubated at 37 °C for 14 days. During this period, at days 1, 4, 7 and 14, the total or half of the medium (0.9% NaCl) was withdrawn and replaced. The amount of CHX released was determined by UV spectrophotometry at 254 nm. All experiments were performed in triplicate. Results were expressed as the concentration of CHX released at each time-point.

2.3. Minimum inhibitory concentration of CHX

The minimum inhibitory concentration (MIC) of CHX was determined for *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and