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Characterization and cytocompatibility of an antibiotic/chitosan/cyclodextrins nanocoating on titanium implants

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ABSTRACT

A novel ciprofloxacin loaded chitosan nanoparticle-based coating onto titanium substrates has been developed and characterized to obtain an orthopaedic implant surface able to in situ release the antibiotic for the prevention of post-operative infections. Ciprofloxacin loaded chitosan nanoparticles were obtained using the combination of sulfobutyl ether-beta-cyclodextrin and gamma-cyclodextrin. The resulting nanoparticulate system was characterized by TEM, HPLC and XPS. Particle size was in the range 426–552 nm and zeta potential values were around +30 mV. This antibacterial coating was able to in vitro inhibit two nosocomial *Staphylococcus aureus* strains growth, with a reduction of about 20 times compared to controls. No impairment in MG63 osteoblast-like cells viability, adhesion and gene expression were detected at 48 h, 7 and 14 days of culture. Overall, the investigated coating represents a promising candidate for the development of a new antibiotic carrier for titanium implants.

1. Introduction

Infections caused by nosocomial bacteria still remain a major challenge in orthopaedic surgery. Data reported by the Nosocomial Infection National Surveillance Scheme (NINNS) indicated that in hip replacement surgery over 50% of superficial and deep wound infections detected during the in-patient stay were due to *Staphylococcus aureus* (Cooke et al., 2000), which are also capable to produce a biofilm protecting the microbial populations from host immune defence. Today, in particular for orthopaedic implant associated infections, different strategies for the local delivery of antibiotics have been developed: thin biodegradable polymer coatings (Buchholz & Engelbrecht, 1970; Gollwitzer et al., 2003; Lucke et al., 2003), biodegradable polypeptide multilayer nanofilms (Li, Ogle, Jiang, Hagar & Li, 2010); biomimetic hydroxyapatite coatings (Brohede et al., 2009); modification of titanium implant surfaces by covalently bonded antibiotics (Jose, Antoci, Zeiger, Wickstrom, & Hickok, 2005) or linkage through a self-assembled monolayer (Noreen, Hickok, & Shapiro, 2012), etc. Limited investigation has been, indeed, devoted to the use of hydrogels carrying antimicrobial agents as possible coatings of metal implants (Giammona et al., 2010; Romano, Giammona, Giardino, & Meani, 2011). Certainly, hydrogel networks, due to their specific ability to entrap high water amounts, represent an ideal candidate for a sustained antibiotic delivery system. We already reported the electrosynthesis of hydrogel coatings onto titanium substrates (De Giglio, Cometa, Satriano, Sabbatini, & Zambonin, 2009; De Giglio et al., 2010) that, due to their swelling capabilities, were able to entrap and release antibacterial agents, demonstrating an interesting activity against microorganisms (Cometa, Iatta, Ricci, Ferretti, & De Giglio, 2013; De Giglio, Cometa, et al., 2011; De Giglio et al., 2013).

Biodegradable polymeric nanoparticles (NPs) could be also used as alternative delivery systems to achieve antibacterial coatings for titanium implants. In this context, chitosan, a cationic polysaccharide obtained from the alkaline deacetylation of chitin, is particularly attractive for pharmaceutics applications (Agnihotri,

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Mallikarjuna, & Aminabhavi, 2004; Paul & Sharma, 2000). Several reasons induce to adopt chitosan as a coating layer for titanium implants. When chitosan is protonated in acid solutions, its positive charges can promote cell adhesion (Bumgardner et al., 2003), and several studies already reported its ability to improve specific cell line growth, such as osteoblasts (Amaral, Sampaio, & Barbosa, 2006; Heinemann, Heinemann, Bernhardt, Worch, & Hanke, 2008; Lahiji, Sohrabi, Hungerford, & Frondoza, 2000). Because of its excellent biocompatibility, biodegradability, nontoxicity and adsorption properties, it has been widely used in biomedical area, such as haemostatic agent (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013; Thatte, Zagarins, Khuri, & Fischer, 2004) or as drug vehicle (Park, Saravanakumar, Kim, & Kwon, 2010). Promising results have also been obtained when chitosan has been used to formulate NPs that showed high loading and good delivery ability of various compounds like dopamine (De Giglio, Trapani, Cafagna, Sabbatini, & Cometa, 2011; Trapani et al., 2011) or antibiotics (Motwani et al., 2008; Ventura et al., 2008, 2011). In this respect, we have previously demonstrated that chitosan nanoparticles, obtained using sulfobutyl ether-β-cyclodextrin, were able to vehicle ciprofloxacin, thus providing an effective inhibition of *S. aureus* and *P. aeruginosa* growth (De Giglio et al., 2012).

In this work, we developed a novel chitosan nanoparticle based coating on titanium as ciprofloxacin (CIP) delivery system, exploiting for the first time the joint action of sulfobutyl ether-β-cyclodextrin (SBE_{7m}-β-CD) and γ-cyclodextrin (γ-CD). In literature, the interaction between the antibiotic CIP and CDs has been widely explored as reported for the natural β-CD (Chandran, Shirwaikar, Sarala, & Devi Kiron, 2010) and the derivative methyl-β-CD (Blanchemain et al., 2011). CIP was chosen for its broad antimicrobial spectrum associated with no genotoxic effects (Herbold, Brendler-Schwaab, & Ahr, 2001) and its common use in orthopaedic local delivery systems (De Giglio, Cometa, et al., 2011; Mattioli-Belmonte et al., 2010; Sansone et al., 2009). Chemico-physical characterization, antibiotic release ability, in vitro antibacterial activity against different nosocomial strains as well as the MG63 osteoblast-like cells proliferation, cytoskeletal organization and gene expression were extensively investigated in this study.

2. Materials and methods

2.1. Materials

Chemicals, listed below, were obtained from commercial sources and used without further modification. Chitosan hydrochloride (UP CL 113, *Mw* 110 kDa, deacetylation degree 75–90% according to manufacturer instructions) was obtained from Pronova Biopolymer (Norway). Titanium foils (thickness 0.25 mm) and ciprofloxacin hydrochloride (*Mw* 385.82 Da) were purchased from Sigma–Aldrich (Italy). Sulfobutyl ether-β-cyclodextrin sodium salt (SBE_{7m}-β-CD, *Mw* 2163 Da, average substitution degree = 6.40) and γ-cyclodextrin (γ-CD, *Mw* 1320 Da) were purchased from CyDex, Inc. (USA) and Wacker-Chemie GmbH (Italy), respectively. CDs were kept in a desiccator until use. Ultrapure water, by Carlo Erba (Italy) was used throughout the study. All other chemicals were reagent grade.

2.2. Preparation of chitosan nanoparticles-based coatings onto titanium substrates

A modified ionic gelation method was adopted to formulate chitosan nanoparticles (CSNPs) in the presence and in the absence of CIP. Briefly, for the preparation of chitosan nanoparticles loaded with CIP (hereafter mentioned as "CIP-loaded CSNPs"), two separate inclusion complexes (i.e., SBE-β-CD/CIP and γ-CD/CIP) were prepared. In order to maintain the molar ratio stoichiometry 1:1 (CD:CIP), for the SBE-β-CD/CIP one, the concentrations were equal to 4.50 mg/mL and 0.81 mg/mL, respectively, whereas for γ-CD and CIP, the concentrations were set at 4.50 mg/mL and 1.30 mg/mL, respectively. An aliquot of 0.2 mL of the complex γ-CD/CIP was firstly mixed with 1.5 mL of chitosan and, in the following step, the use of 0.7 mL of the complex SBE-β-CD/CIP allowed the formation of NPs via a non-covalent crosslinking. Chitosan nanoparticles without CIP (i.e., "unloaded CSNPs") were formulated following the same protocol in the absence of the antibiotic. To promote the adsorption of an additional amount of antibiotic, a further aliquot of the complex γ-CD/CIP was incubated with the final CIP-loaded CSNPs suspension for 3 h at room temperature without any stirring, obtaining the so-called "CIP-enriched CSNPs". The CIP-enriched CSNPs coatings were deposited onto titanium sheets by casting on each sample 1.4 mg of CSNPs, which had previously adsorbed 1.5×10^{-2} mL of the γ-CD/CIP complex. On the other hand, the unloaded CSNPs coatings were obtained by casting 1.4 mg of unloaded CSNPs, which had previously adsorbed 1.5×10^{-2} mL of the aqueous solution of γ-CD (4.5 mg/mL). The resulting coatings were taken as control during biological experiments. Titanium sheets were coated on both sides and were oven-dried at 40 °C.

2.3. Chemico-physical characterization of nanoparticles

Particle size and polydispersity index (PI) of all tested NPs were determined in ultrapure water by Photon Correlation Spectroscopy (PCS) using a Zetasizer NanoZS (ZEN 3600, Malvern, UK). The determination of the ζ-potential was performed using laser Doppler anemometry with 1 mM KCl as carrier.

TEM nanoparticles characterization. Morphological evaluation of unloaded CSNPs and CIP-loaded CSNPs was performed with transmission electron microscope (TEM). 5 μl of each sample solution was dropped on carbon/formvar grids and air dried. Samples were counterstained with 2% (w/v) phosphotungstic acid, air dried and analysed by a TEM Philips CM10 (FEI, Eindhoven, the Netherlands). Images were recorded using a Megaview III digital camera (FEI).

2.4. X-ray photoelectron spectroscopy (XPS) analysis

XPS has been exploited for the surface characterization of all the investigated nanoparticle systems. XPS spectra were obtained with a Thermo VG Thetaprobe spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a microspot monochromatized Al Kα source.

Survey (binding energy range 0–1200 eV, pass energy 150 eV) and high resolution scans (pass energy 50 eV) were recorded in the FAT analyser mode.

Nonlinear least-squares curve fitting of the detailed spectra was performed by using the Avantage software package. Quantification was obtained by peak areas normalized by acquisition parameters and sensitivity factors provided by the manufacturer, whereas the binding energy (BE) scale was set by fixing the C1s component due to adventitious hydrocarbon at 285 eV (De Giglio, Cafagna, et al., 2011).

Before XPS analysis, all coatings were prepared by casting following the procedure reported above, while CIP powder was pressed onto a small square of double-stick carbon tape.

2.5. Ciprofloxacin determination by HPLC

High-performance liquid chromatography (HPLC) analyses of CIP were performed with an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA), equipped with a MW detector, 20 μL