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Controlled release of chlorhexidine antiseptic from microporous amorphous silica applied in open porosity of an implant surface

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ABSTRACT

Amorphous microporous silica (AMS) serving as a reservoir for controlled release of a bioactive agent was applied in the open porosity of a titanium coating on a Ti–6Al–4V metal substrate. The pores of the AMS emptied by calcination were loaded with chlorhexidine diacetate (CHX) via incipient wetness impregnation with CHX solution, followed by solvent evaporation. Using this CHX loaded AMS system on titanium substrate sustained release of CHX into physiological medium was obtained over a 10 day-period. CHX released from the AMS coating was demonstrated to be effective in killing planktonic cultures of the human pathogens *Candida albicans* and *Staphylococcus epidermidis*. This surface modification of titanium bodies with AMS controlled release functionality for a bioactive compound potentially can be applied on dental and orthopaedic implants to abate implant-associated microbial infection.

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1. Introduction

The development of multifunctional materials for medical implants is an active research area. Key issues of implant materials are the fixation by osseointegration and the prevention of infections around the implant. Implant fixation can be facilitated by increasing the roughness of the surface, but rough surfaces present the drawback of enhanced microbial adhesion and biofilm formation (Teughels et al., 2006). Biofilms consist of dense layers of microorganisms that are surrounded by a self-produced extracellular polymer matrix, in which they show a dramatically reduced sensitivity to most of the currently used antibiotics. Medical devices and especially implants present the risk of biofilm development. The most recent generation of implants with open porosity enable fast osseointegration, but also present an increased risk of microbial biofilm-associated infection. One approach of preventing implant loss due to microbial biofilm formation is by preventing adhesion of microorganisms on the implant surface. Surface modifications include changes in hydrophobicity, surface electric potential, roughness, or chemical composition. In addition to surface modification to prevent bacterial adhesion, drug-releasing devices which affect bacterial growth through a controlled release of bioactive substances can be foreseen on the implant. Drug releasing organic polymer coatings have already been applied (Jones and Medlicott, 1995; Medlicott et al., 1996, 1999; Riggs et al., 2000; Zilberman, 2005). Among the inorganic controlled release systems, porous silica or titania coatings have also been reported (Ayon et al., 2006; Radin and Ducheyne, 2007; Anglin et al., 2008). Popular antibacterial agents are the anti-inflammatory dexamethasone (Zilberman, 2005; Ayon et al., 2006) and the antibiotic vancomycin (Radin and Ducheyne, 2007) as well as the antiseptic chlorhexidine (CHX), the latter is specifically used on dental implants (Jones and Medlicott, 1995; Medlicott et al., 1996, 1999; Riggs et al., 2000). CHX has some advantages over an antibiotic due to the limited occurrence of resistance and its broad-spectrum activity (McBain et al., 2003). It is widely used as active component in mouth rinses, but as implant infections are in general located in the transgingival region, rinsing the oral cavity is not sufficient to prevent infections around dental implants. This inspired to the idea of loading the CHX in the implant itself and to release it during the critical healing time. The development of a porous silica-based controlled release system was recently reported (Aerts et al., 2007, 2010; Verraedt et al., 2010).

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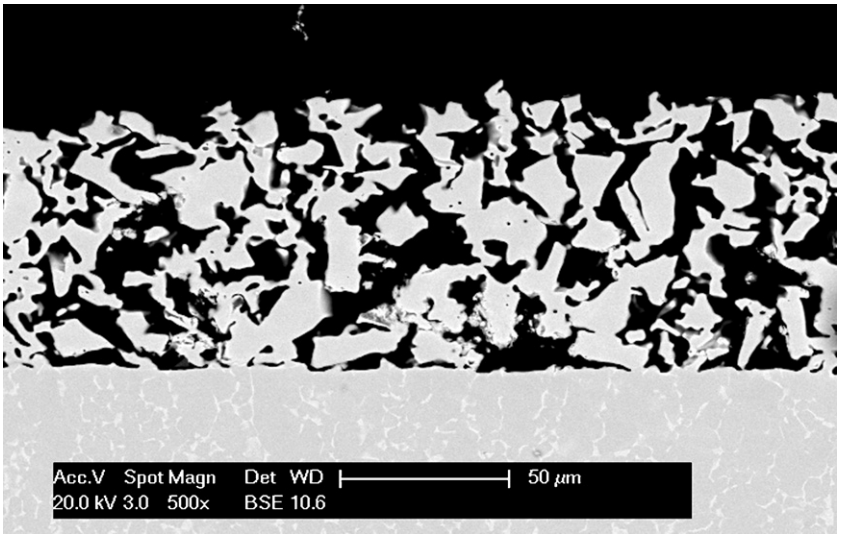

Fig. 1. Scanning electron microscope (SEM) micrograph of a cross section of a porous Ti coating obtained by electrophoretic deposition of TiH_2 powder followed by vacuum sintering.

Amorphous microporous silica (AMS) is a highly porous silica material, the pore size of which can be tuned in the range 0.4–0.6 nm range depending on synthesis conditions. Therapeutic compounds can be introduced into the pores via adsorption or impregnation. The release of therapeutic compounds from AMS was demonstrated to occur via a pore diffusion process and to be dependent on the pore diameter and diffusion path length. AMS was shown to be appropriate for CHX controlled release from powders and grains, as well as from thin film deposited on a silicon wafer (Verraedt et al., 2010). The release of CHX from an AMS film with 500 nm thickness covered a 4 h time period only, which for a dental implant would be much too short a time to prevent biofilm formation during healing. In the present study, large AMS particles were synthesized and fixed inside the open pores of a macroporous titanium surface structure in order to extend the drug release period. Controlled release as well as the antimicrobial activity of the released CHX against a bacterial and fungal cell culture were assessed. To this end, the bacterium *Staphylococcus epidermidis* and the human pathogenic yeast *Candida albicans* were used. Both pathogens have been found in association with the oral cavity (Niimi et al., 2010; McNicol and Israels, 2010).

2. Materials and methods

2.1. Substrate

The substrate material was Ti–6Al–4V (grade 5, LIMA Lto) machined disk of 15.5 mm diameter with a thickness of 2 mm. An open macroporous Ti coating was applied by means of electrophoretic deposition (EPD) of TiH_2 powders (Grade P, Chemetall GmbH) followed by dehydrogenation and vacuum sintering. The coatings have an average pore width of $51.2 \pm 3.9\%$ and an interconnective pore size of 2–8 μm (Braem et al., 2011). A representative cross section of the macroporous titanium layer is shown in Fig. 1.

2.2. Synthesis of AMS inside open macroporous Ti surface layer

Preparation of AMS coatings departed from an AMS sol prepared according to Maier et al. (1993). Under magnetic stirring, tetraethoxysilane (TEOS, 98%, Acros) and technical EtOH were dropwise combined with HCl (37%, Chem-Lab) respecting a TEOS:EtOH:HCl:H_2O molar ratio of 1:3:1.74:6. This sol was allowed to age for 3 h, and applied using a glass pasteur pipette on top of the porous Ti structure. Penetration of the silica sol into the titanium porous network was improved by treating the silica loaded Ti support structure under vacuum during ca. 20 h. Subsequently the samples were calcined in air to a final temperature of 300 °C at a heating rate of 0.1 °C min^{-1}. After 5 h at the maximum temperature, the samples were cooled to ambient temperature.

Metallographic cross sections were prepared. First, the samples were embedded in an epoxy resin (EPON 812, Fluka). Vacuum was applied to ensure a good penetration of the resin in the open pore structure. Afterwards the resin was cured for 2 h at 70 °C. Cross sections were prepared using a Secotom-10 sawing machine (Struers) with 10S20 saw blade (Struers). Finally, the cross sections were ground using 1200 and 4000 grit SiC papers (Hermes), followed by polishing with 3 μm diamond particles (Diapat S, VEM Metallurgie). The polished cross sections were examined using scanning electron microscopy (SEM, Philips XL 30 FEG, FEI) with associated energy dispersive spectroscopy (SEM-EDS, EDAX).

2.3. Glassware pretreatment

To avoid adsorption effects of CHX on the glassware, all glassware was subjected to a silanization procedure. The procedure was adopted from earlier work by Tsutsumi et al. (2003). The glass was treated during 1 h with a 10% solution of dichlorodimethylsilane (98%, Acros) in toluene (Chem-Lab), followed by rinsing with toluene and methanol (BDH prolabo).

2.4. CHX loading

CHX (chlorhexidine diacetate hydrate, Sigma–Aldrich) loading was performed using an incipient wetness impregnation method. The macroscopic pore volume was estimated at 5.12 μL cm^{-2}. The micropore volume of the silica was unknown but was estimated from the silica loading and assuming a porosity of 18–20%. A CHX loading of 200–250 μg per disk was targeted. An appropriate amount of CHX dissolved in ethanol was applied on top of the evacuated coating, where after the ethanol was allowed to evaporate at ambient temperature.

2.5. CHX release experiments

CHX release was monitored over an extended time period using a pharmaceutical dissolution test procedure. The coated

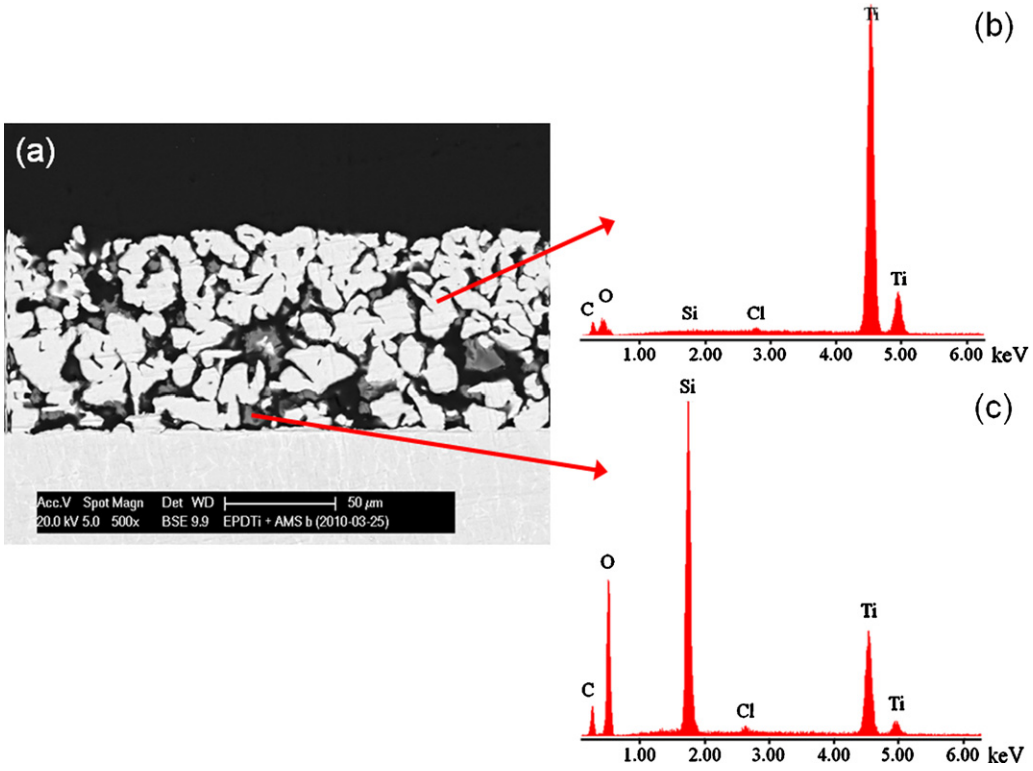

Fig. 2. Backscattered electron (BSE) micrograph of a cross-sectioned AMS containing porous titanium layer on titanium metal (a), and EDX point-analysis of the bright (b) and grey (c) BSE contrast spots indicated by arrows.

disks were immersed in 40 mL, pH 6.8, phosphate buffer (0.01 N, KH_2PO_4 and K_2HPO_4 both >99%, Merck) in 50 mL Falcon tubes (Greiner). A temperature of 37 °C was maintained, and the tubes were swirled at 100 rpm. At certain time points, 1 mL samples were withdrawn from the medium and replaced with 1 mL fresh medium to maintain the volume of the release medium constant. Samples were transferred to silanized vials for HPLC analysis.

An HPLC-UV setup was used to determine CHX concentrations. The setup consisted of a Lachrom L-7100 pump, a L-7200 autosampler, an L-7420 UV detector set at 260 nm and a D-7000 interface. Peaks were integrated using the D-7000 HSM software (Merck). A LiChrospher 60 RP-select B (125 × 4 mm, 5 μm, Merck) column was used. The mobile phase in the HPLC analysis consisted of a 1% triethylamine solution (>99% Sigma–Aldrich), adjusted to pH 3.0 using acetic acid (99–100% Chem-Lab), and acetonitrile (HPLC grade, >99.7%, Biosolve LTD) in a 70:30 (v/v) ratio. The flow was set at 1 mL min^{-1}. 70 μL was used as injection volume. Note that the analysis protocol did not allow determination of acetate. Therefore, in the presentation of the experimental data, CHX was quantified assuming the presence of diacetate salt.

2.6. In vitro antimicrobial testing

The activity of CHX loaded coatings was verified *in vitro* against *C. albicans* SC5314 and *S. epidermidis* LMG10474 cell cultures. To this end, overnight cultures of *C. albicans* and *S. epidermidis* in YPD (1% yeast extract, 2% peptone, 2% glucose) and TSB (30 g/L BBL Trypticase Soy Broth, BD, Sparks, MD), respectively, were diluted 1/100 and 1/1000 in PBS, respectively, resulting in a cell density of approx. 10^6 cells/mL. Two milliliters of these cultures were added to Falcon tubes containing the CHX-containing Ti disk and incubated at 30 °C. At different incubation times, 20 μL samples of these cultures were analyzed for viability of the culture by colony counting on YPD or TSB plates. A CHX single dose of 100 μg/mL was used as positive control.

3. Results and discussion

3.1. Preparation and characterization of controlled release coating

The successful incorporation of AMS in the macroporous Ti surface structure of the Ti–6Al–4V disks was verified by SEM using backscattered electron, i.e. atomic number contrast, imaging combined with X-ray analysis (EDX). The Si-containing AMS appeared as grey contrast regions in a bright Ti network structure (Fig. 2a). Part of the original porosity remained open, as reflected by the black contrast regions. EDX point analysis confirmed the assignment of the dark grey zones to silica and the AMS (Fig. 2c) and bright areas to Ti (Fig. 2b). The presence of Ti in the spectrum of the AMS was due to the low spatial resolution of the EDX analysis, partially incorporating some of the surrounding Ti into the interaction volume. AMS was observed throughout the entire porous network (Fig. 2a). Pores of the AMS material itself, however, because of their very small diameter of less than 1 nm cannot be observed at this magnification. The through EDX detected C and Cl chemical elements originated from the polymer embedding resin. These SEM/EDX data indicated the AMS coatings were spread over the whole porous titanium structure without losing the original open porosity. The introduction of the AMS did not deteriorate the porosity of the titanium surface being beneficial for implant integration (Teughels et al., 2006).

3.2. Cell adhesion and proliferation

The behavior of AMS-coated titanium surface was compared to the bare commercial pure Titanium (cpTi) surface with respect to

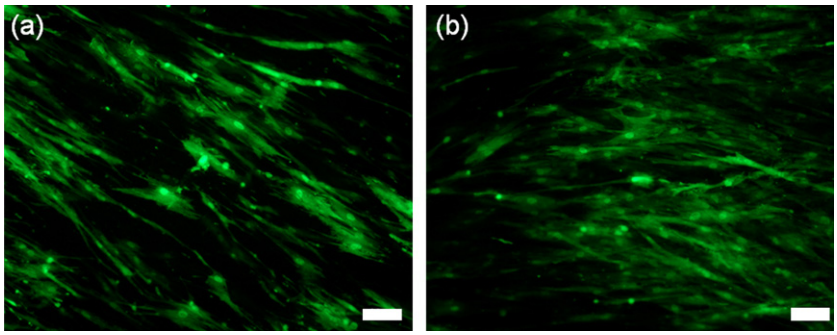

Fig. 3. Fluorescence images of GFP-labeled hMSCs after 21 days proliferation on cpTi (a) and AMS coated (b) surfaces. Scale bars represent 0.1 mm.

cell adhesion and proliferation. Green Fluorescence Protein (GFP)-labeled human mesenchymal stem cells (hMSCs, pool of 5 donors) were seeded on the surfaces at cell density of 3×10^3 cm^{-2} (experiment in triplicate). Cell behavior and proliferation was qualitatively tracked by fluorescence microscopy. The fluorescence images of hMSCs grown on cpTi (Fig. 3a) and AMS (Fig. 3b) surfaces for 21 days illustrated no adverse effect of AMS coating on the cell adhesion and proliferation. This indicated the cell behavior was not influenced by the presence of AMS, which is important regarding the potential application and implant integration (Albrektsson and Johansson, 2001). This behavior of AMS corresponded to earlier observations for similar silica materials displaying good biocompatibility (Quintanar-Guerrero et al., 2009; Falaize et al., 1999).

3.3. In vitro CHX release

CHX release from the disks was investigated over a 10 day period, in triplicate (Fig. 4). The CHX release consisted of an initial burst release (data point taken after 1 day), followed by a slow additional release that was almost complete within the investigated period. A total amount of ca. 220 μg CHX was released per disk, which corresponded with the original content. The observed initial burst release of about 70% of the loading was attributed to the presence of CHX outside of the AMS pores, as previously demonstrated for CHX loaded on AMS powder (Verraedt et al., 2010). The remaining 30% CHX was released from the AMS during an extended release period. The slow release obeyed a diffusion controlled behavior, similar to earlier observations with AMS powders and films (Verraedt et al., 2010). The plot of the percentage release against square root of time was linear as expected according to a Fickian diffusion model (Fig. 5).

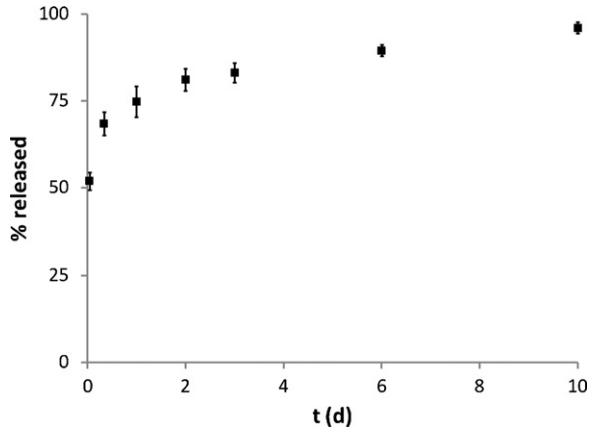

Fig. 4. CHX release from Ti disk with AMS on porous Ti surface layer ($n = 3$).

3.4. Anti-microbial activity

As the CHX-releasing coatings were designed to prevent biofilm formation, the killing kinetics of CHX released from the disks were assessed against a bacterial (*S. epidermidis*, Fig. 6a) and yeast (*C. albicans*, Fig. 6b) culture, $n = 3$. The CHX as released from the disks was compared to the effect of a single dose CHX of 100 μg/mL on the suspended microbial cultures. This CHX dose represented the total amount of CHX loaded in the coatings. Fig. 6 represents the viability of the *S. epidermidis* (Fig. 6a) and *C. albicans* (Fig. 6b) cultures in the presence of the CHX-containing disks. It is clear that a single dose of CHX results in immediate microbial death, whereas the same killing potential of CHX released from the disks was only reached after 3–4 h of release. In conclusion, both *C. albicans* as *S. epidermidis* planktonic cell cultures are effectively killed by the CHX released from the AMS coatings after 3–4 h of controlled release. In this regard, the burst CHX release followed by a sustained release over a 10-days period could provide an improved strategy in infection prevention.

3.5. Discussion

The development of implants with both excellent integration characteristics and limited infection risks is a difficult task. Increased implant roughness results in enhanced implant fixation, but as a consequence increased infection rates have been observed (Teughels et al., 2006). Here we present a combined silica–titanium device which potentially offers both the desired increased surface roughness and an integrated biocide delivery system integrated at the implant surface to prevent infections. The surface roughness is

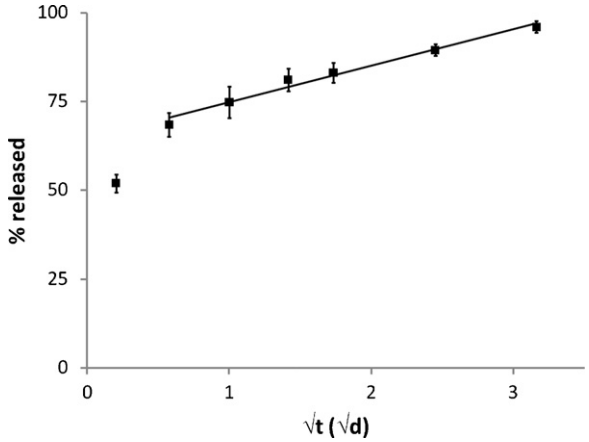

Fig. 5. Chlorhexidine release from Ti disk with AMS on porous Ti surface layer plotted against square root of time (data from Fig. 4 replotted).

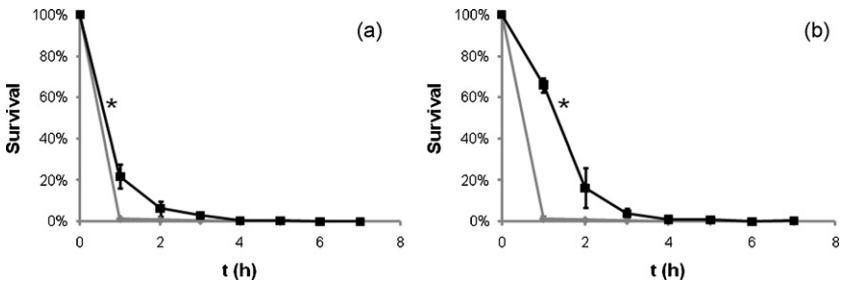

Fig. 6. Survival of *S. epidermidis* (a) and *C. albicans* (b) in the presence of CHX loaded Ti/AMS coating (curves marked with *) and in presence of a CHX single dose (100 μg/mL).

provided by a macroporous titanium coating, applied on top of a Ti–6Al–4V substrate (Braem et al., 2011). The data of Section 3.1 show the AMS coating was introduced successfully throughout the whole porous Ti structure without blocking pores. Thus the AMS is not expected to interfere with the fixation of an implant having such surface.

The main advantage of silica materials over polymer-based systems is their safety and biocompatibility (Quintanar-Guerrero et al., 2009; Falaize et al., 1999). Cell adhesion and proliferation data for AMS (Section 3.2) were in line with the advantageous behavior of silica.

The incorporation of CHX in the AMS coating of the titania surface was conceived as a means to counter-act the increased infection risk associated with the implant roughness. CHX as active agent has some advantages over antibiotics due to its broad-spectrum activity without occurrence of resistance (McBain et al., 2003) and the absence of toxicity for human tissue (Darouiche, 2007). CHX release from the AMS coated Ti lasted 10 days (Section 3.3). The use of a CHX-releasing coating associated with the implant delivers the biocide molecules at the implant site itself which is more difficult to achieve via conventional drug administration or rinses. The observed initial burst release will take care of the potential pathogens present after implanting, while the subsequent period of sustained release will ensure an extended infection-free healing period. The released CHX molecules retained their activity as both *C. albicans* as *S. epidermidis* were efficiently killed, as discussed in Section 3.4. Controlled release properties of AMS materials for other active molecules have been demonstrated before (Aerts et al., 2007, 2010; Verraedt et al., 2010). The AMS system could be extended to sustained release of antibiotics and anti-inflammatory agents from implant surfaces.

4. Conclusions

Amorphous microporous silica (AMS) was successfully incorporated into an open macroporous Ti surface layer on Ti–6Al–4V substrate. Chlorhexidine diacetate was successfully incorporated into the AMS. In physiological medium a sustained release of CHX from this type of coating lasted 10 days. The released chlorhexidine was effective to kill planktonic cultures of *S. epidermidis* and *C. albicans*. Given the controlled release properties of AMS demonstrated with other bioactive molecules (Aerts et al., 2007, 2010; Verraedt et al., 2010), the results are promising for the development of dental implants with dedicated controlled release function. Beside antiseptics, the AMS/Ti porous composite surface layer might be useful for sustained release of all kinds of anti-inflammatory and antibiotic molecules and their combinations.

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