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Multifunctional implant coatings providing possibilities for fast antibiotics loading with subsequent slow release

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Abstract The possibility to fast-load biomimetic hydroxyapatite coatings on surgical implant with the antibiotics Amoxicillin, Gentamicin sulfate, Tobramycin and Cephalothin has been investigated in order to develop a multifunctional implant device offering sustained local anti-bacterial treatment and giving the surgeon the possibility to choose which antibiotics to incorporate in the implant at the site of surgery. Physical vapor deposition was used to coat titanium surfaces with an adhesion enhancing gradient layer of titanium oxide having an amorphous oxygen poor composition at the interface and a crystalline bioactive anatase TiO_2 composition at the surface. Hydroxyapatite (HA) was biomimetically grown on the bioactive TiO_2 to serve as a combined bone in-growth promoter and drug delivery vehicle. The coating was characterized using scanning and transmission electron microscopy, X-ray diffraction and X-ray photoelectron spectroscopy. The antibiotics were loaded into the HA coatings via soaking and the subsequent release and antibacterial effect were analyzed using UV spectroscopy and examination of inhibition zones in a *Staphylococcus aureus* containing agar. It was found that a short drug loading time of 15 min ensured antibacterial effects after 24 h for all antibiotics under study. It was further found that the release processes of Cephalothin and Amoxicillin consisted of an initial rapid drug release that varied unpredictably in amount followed by a reproducible and sustained release process with a release rate independent of the drug loading times under study. Thus, implants that have been fast-loaded with drugs could be stored for ~10 min in a simulated body fluid after loading to ensure reproducibility in the subsequent release process. Calculated release rates and measurements of drug amounts remaining in the samples after 22 h of release indicated that a therapeutically relevant dose could be achieved close to the implant surface for about 2 days. Concluding, the present study provides an outline for the development of a fast-loading slow-release surgical implant kit where the implant and the drug are separated when delivered to the surgeon, thus constituting a flexible solution for the surgeon by offering the choice of quick addition of antibiotics to the implant coating based on the patient need.

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1 Introduction

In pace with an increased use of metallic implants, a growing interest in the development of mechanically and biologically stabile materials with nanostructured coatings is seen [1]. Examples of metallic implants include dental, cochlear, spinal, hip, arm and leg amputation implants, pedicle screws, as well as plates and nails [2]. The concept of biological stability in this connection focuses on three

different aspects, viz. inhibition of bacterial adhesion onto the implant, prevention of biofilm formation and direct extinction of bacteria [3]. At conferences and in research papers, orthopedic as well as dental surgeons have started to suggest more flexibility around the handling of implants, such as the possibility of fast-functionalization of implant surfaces at the site of surgery, in order to provide local treatment in vivo that is optimized for the patient's need. Citing Ginebra et al. "it would be of importance to propose a system that could be combined with many different drugs, in such way that the surgeon could choose the drug just before implantation" [4]. Ginebra et al. also says that "implant companies selling CPC (Calcium Phosphate Cement) do not have the know-how to deal with the drugs, whereas pharmaceutical companies do not have any know-how with CPC, and often do not have interest in working with such small markets". The market for implant materials is huge [1] and the development of such a complex object as a multifunctional implant might therefore be worth the effort of cross-disciplinary work. Several researchers have addressed this issue and used bioactive ceramics and ceramic coatings loaded with antibiotics for local delivery of antibacterial substances [4–8]. An other approach, proposed by Kälicke et al., is to use a polymer coating on titanium implant surfaces for incorporation and delivery of antibiotics [9].

Metallic implants made of titanium, titanium alloys, stainless steel and Co–Cr alloys are widely used as surgical implants owing to their mechanical strength, biocompatibility, chemical inertness and machinability. The interface properties between the material and tissue are crucial regarding fibrous tissue development and biofilm formation. Surface roughness and topology play an important role in cell attachment and proliferation [10–12]. Pure metal surfaces can be roughened through different techniques such as sandblasting and etching. For better incorporation of the implant to the bone without biofilm or fibrous tissue formation around the implant, a bioactive coating can be deposited on the metallic surface. Such coatings often comprise calcium phosphates [13] such as hydroxyapatite (HA). HA is a commonly used bioactive ceramic material and its use is predicated upon its excellent affinity to bone; HA is a calcium phosphate mineral ($Ca_{10}(PO_4)_6OH_2$) that constitutes the hard phase in bone and teeth. It can be synthesized and deposited on implants by several coating procedures such as plasma spraying [14], pulsed laser deposition [15], electron-beam deposition [16] and by biomimetic precipitation [17, 18]. Biomimetic HA coatings on metallic implants has been in focus of research for many years due to its excellent biocompatible and bioactive properties and also due to its formation via a low temperature deposition process that promotes good adhesion and step coverage [19].

It is well documented that native amorphous TiO_2 has very poor ability to form HA through biomimetic precipitation from a solution on its surface, whereas on the anatase and rutile phases HA spontaneously crystallizes when the TiO_2 is soaked in simulated body fluid [20, 21].

The new generation of HA coatings, presently under development, are not only bioactive but may also be functionalized to evoke a certain cell response or used as drug carriers as nano-porous HA can serve as a matrix for local administration of molecules [22–25].

Many attempts have been made to design an implant material with a functionalized surface coating for local drug administration [7, 25–29]. These inventions, though, are suffering from the problems pointed out by Ginebra et al. regarding the difficulties of dealing with both material and pharmaceutical in one device [4].

In the present work we investigate the possibility to fast-load biomimetic HA coatings with antibiotics in order to create the platform required for a multifunctional implant device where the implant and the drug are separated before implantation. The overall aim is thus to build knowledge for subsequent development of a device which (1) offers both sustained local anti-bacterial treatment at the site of the implant, thus, avoiding systemic administration of antibiotics, which (2) avoids problems with safe drug handling and storage, and which (3) lets the surgeon at the site of surgery choose which drug to incorporate in the implant based on the patient need.

2 Experimental

2.1 Ti oxide deposition and characterization

A coating of Ti oxide for which the oxygen content increased from the substrate towards the outermost surface was deposited on 10 × 20 mm plates of commercially pure grade 5 titanium by physical vapour deposition (PVD). The gradient coating, tailored to optimize the adhesion of the coating to the substrate [30], was prepared in a reactive dc magnetron-sputtering unit (Balzer UTT400). The working chamber was mounted with a turbo molecular pump TMU 521P. The chamber base pressure was $\sim 10^{-7}$ mbar after backing. Pure titanium (99.9%) was used as target. Pure argon (99.997%) and oxygen (99.997%) were used for the reactive sputtering. The substrate was kept at a constant temperature of 350°C during sputtering. The oxygen profile of the film was controlled by changing the oxygen flow: An increasing oxygen flow reduces the metallic content. A crystalline outermost TiO_2 surface was ensured by heat treatment of the sputtered film at 390°C for 1 h post-deposition. The targeted coating design parameters were as follows, described from the titanium substrate of grade 5;

50 nm of pure titanium, 50 nm gradually increasing oxygen content from Ti to TiO_2 and a top layer of 50 nm crystalline TiO_2.

The coatings were studied by Transmission Electron Microscopy (TEM, FEI Tecnai F30 ST microscopy equipped with a field emission gun operated at 300 kV) and profiles of the elemental composition in the gradient films were achieved using energy dispersive spectroscopy (EDS) in scanning TEM mode (STEM). Electron transparent samples for the TEM analysis were produced using focused ion beam (FIB) milling in a FEI Strata DB235. A thin ($\sim$1 μm) Pt coating was deposited on the Ti oxide surface and then cut out using the ion beam (Ga^{3+}). The sample was lifted out using an Omniprobe system and welded onto a TEM sample grid and further milled using the ion beam to electron transparency (thickness $<$ 100 nm). The thickness of the coatings was obtained from the EDS profiles and the order of crystallinity was examined using gracing incidence X-ray Diffraction (XRD, Siemens D5000 diffractometer).

2.2 Biomimetic coating of HA

A layer of HA was biomimetically precipitated [31] on the surfaces using PBS as ion source following the PVD deposition of the crystalline TiO_2. The term "biomimetic" refers to the spontaneous nucleation and crystal growth of HA on bioactive surfaces similar to the formation of apatite crystals in bone during bone formation and remodeling. The negative surface charge of the crystalline TiO_2 attracts positive calcium ions from the PBS solution and a calcium titanate layer is formed that initiates nucleation of HA on the surface [21]. After PVD deposition the plates were ultrasonically cleaned in acetone, ethanol and de-ionized water (5 min in each) and subsequently placed in plastic tubes with conical bottoms containing 40 ml PBS (Dulbecco's PBS with calcium chloride and magnesium chloride, Sigma–Aldrich Company Ltd.) preheated to 60°C and placed in an incubator at 60°C for 4 days. After the immersion, the plates were gently rinsed with de-ionized water and the precipitated HA coatings were examined using Scanning Electron Microscopy (SEM, Zeiss LEO 1550), X-ray Photoelectron Spectroscopy (XPS, Physical Systems Quantum 2000 spectrometer) and gracing incidence XRD.

2.3 Incorporation of antibiotics

The incorporation of antibiotics was preformed by soaking the HA coated plates in 10 ml PBS containing 50 μg of either Tobramycin, Cephalotin, Amoxicillin or Gentamicin sulfate (Sigma–Aldrich Company Ltd.). The drug loading was performed at room temperature in 50 ml conical test tubes for four different soaking times between 15 min and 24 h.

2.4 Antibacterial effect in bacteria agar

After incorporation of antibiotics, the plates were immediately transferred to new tubes filled with 40 ml of PBS preheated to 37°C in order to let the incorporated drug leak out for various time intervals before monitoring the antibacterial effect of the drug remaining in the coatings. After either 15 min, 60 min or 24 h the plates were removed and dried at room temperature for further analyze of the remaining antibacterial effect in the *Staphylococcus aureus* agar described below.

A Mueller–Hinton broth (Merck) containing 0.8% Agar was prepared by dissolving the powder in de-ionized water and sterilized by autoclaving at 121°C for 20 min. The broth was then tempered to 40°C in a water bath. *S. aureus* strain Cowan I (NCTC 8530, National Collection of Type Cultures, London, UK) in a PBS suspension was added directly to the broth giving it a final optical density at 600 nm of 0.005 (corresponding to approximately 5×10^6 cfu/ml). The mixture was mixed to homogenously distribute the bacteria in the substrate and then transferred to Petri dishes. The dishes were filled to a depth of 10 mm (50 ml) with the bacterial broth. As the Agar stiffened at room temperature the titanium plates were stuck down in the agar at a right angle to the surface, having both sides in contact with the bacterial substrate. The Petri dishes were then incubated at 37°C for 18 h. A HA coated reference plate without any antibiotics was also tested. The inhibition zones of bacterial growth formed around the plates were photographed by an imaging instrument (Geldoc 2000, Bio-Rad).

2.5 Antibiotic release studied by UV spectroscopy

The drug release rate was analyzed using UV spectroscopy (Shimadzu 1650PC, Shimadzu Corp.) by immersing plates that had been soaked in Amoxicillin and Cephalothin for 15 min, 1 h and 24 h in 8.2 ml of either water or PBS at room temperature. The release was measured (using a wavelength of 230 nm for Amoxicillin and 239 nm for Cephalothin) in water for 1 h to investigate the initial release and then in PBS for 22 h to investigate the slow release after the initial burst. The liquid was circulated through a cell in which the drug concentration was detected. After the release in PBS, the HA coatings soaked for 24 h in the antibiotic solutions were dissolved by lowering the pH of the PBS to 2 by addition of hydrochloric acid. This was done to release and measure the amount of remaining drugs still present in the coatings after 22 h of release. To relate the antibiotics concentration released to the antibacterial effect, antimicrobial susceptibility tests (E-test, AB Biodisk, Sweden) for Amoxicillin and Cephalothin were performed on strain *S. aureus* Cowan I and on

Table 1 Result from E-test for antimicrobial susceptibility testing, determining the minimum inhibitory concentration (MIC) of antimicrobial agents against microorganisms (inhibiting bacteria in 1 ml broth)

Antibiotic drug	Bacteria	
	Staphylococcus aureus, MIC (μg/ml)	*Staphylococcus epidermidis*, MIC (μg/ml)
Amoxicillin	0.064	0.19
Cephalothin	0.064	0.064

Staphylococcus epidermidis Kx293A1 (Department of Microbiology, Swedish University of Agricultural Sciences). Both these bacteria are major pathogens (*S. epidermidis* is an opportunistic pathogen) when it comes to infections and surgical implants and both are susceptible against the antibiotics (see Table 1). The chosen antibiotics are relevant for treatment of infections caused by these bacteria and as prophylactics after surgery. The *S. epidermidis* strain is from the normal human microbiota and not a clinical isolate. *S. aureus* Cowan I (serotype 1) is a well studied clinical isolate originally isolated (year 1935) from a patient with septic arthritis.

3 Results and discussion

3.1 Ti oxide

The PVD process resulted in a crystalline TiO_2 surface, mainly of anatase phase, see Fig. 1. Diffraction peaks originated from the crystalline titanium substrate is also seen in the figure, corresponding to the different crystallographic directions of alpha-Ti. The heat treatment after the coating deposition gave a more crystalline coating structure (more defined peaks in the thin-film XRD spectra) than the as-deposited coatings, see inset in Fig. 1.

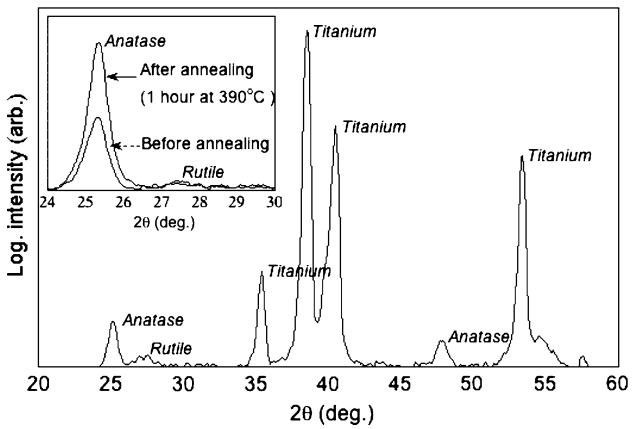

Fig. 1 XRD spectrum for coating surfaces after the PVD process. The location of peaks stemming from anatase and rutile structures as well as from pure titanium are indicated. The inset shows a comparison of XRD spectra from an as-deposited coating and a coating that has been heat treated for 1 h at 390°C

Figure 2a shows an STEM image of a sputtered coating in cross-section. The gradient can be seen as the change in contrast going from the Ti-bulk (dark grey) towards the TiO_2 surface (light grey). From this image, as well as from the titanium and oxygen EDS profiles displayed in Fig. 2b, it is obvious that the thickness of the gradient and the TiO_2 layer is $\sim$70 and $\sim$40 nm, respectively, and hence rather close to the targeted values.

3.2 Biomimetic coating of HA

Immersion of the titanium plates in PBS resulted in biomimetic precipitation of nano-porous and calcium deficient HA on the anatase TiO_2 surfaces. XPS analysis showed that the Ca/P ratio of the HA coatings was 1.36 compared to stoichiometric HA with a ratio of 1.67, see Fig. 3.

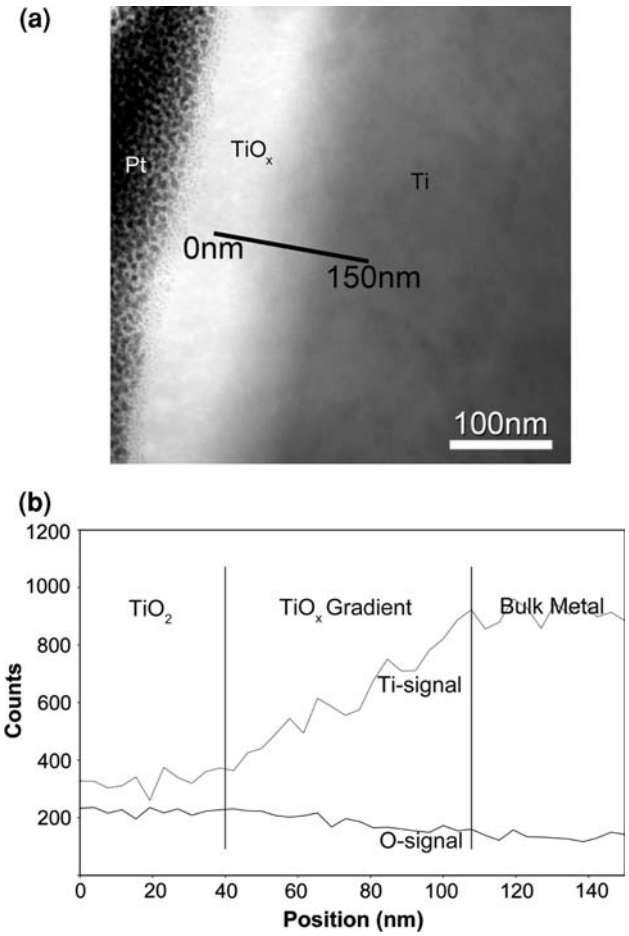

Fig. 2 **a** STEM image showing the titanium oxide layer in cross-section. The coating features are marked in the image and **b** the corresponding line profiles of Ti and oxygen over the marked line from 0 to 150 nm as obtained by EDS are displayed

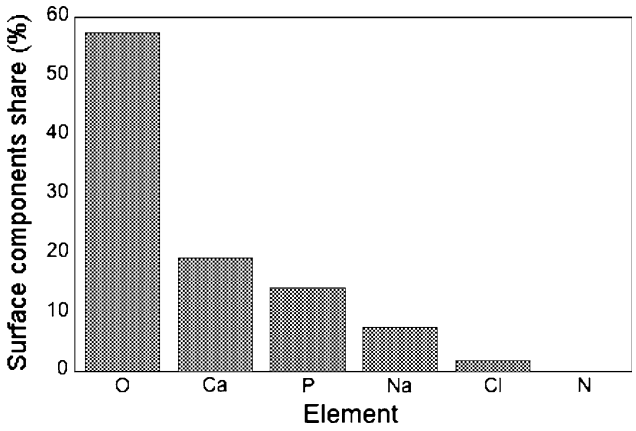

Fig. 3 XPS analysis of HA coatings displaying the elemental content of the coating surface. The data are average values after measurements made on 3 samples and the absolute deviation of these experiments were below 9% for all displayed elements

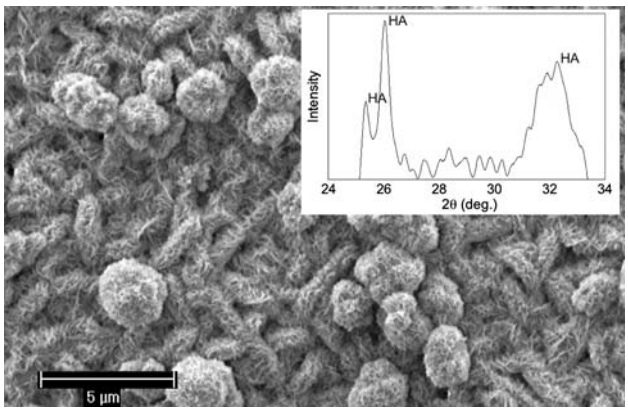

Fig. 4 SEM image of the HA coating biomimetically grown on anatase TiO_2 for 4 days at 60°C. An XRD spectrum of the surface is shown in the inset. Location of peaks stemming from crystalline HA are indicated

Further, the SEM and XRD analyses showed that a continuous layer of HA had formed all over the anatase surfaces, see Fig. 4. It has previously been shown that HA with a non-stoichiometric calcium deficient composition, like the one found here, is more favorable as a bone implant compared to stoichiometric HA because it dissolves faster in aqueous environments and induces a much more rapid bone colonization. As well, calcium deficient HA generally has a larger specific surface area than its stoichiometric counterpart, which has been found to facilitate the loading of therapeutic agents [32].

3.3 Antibacterial effect in bacteria agar

Figure 5 shows the inhibition zones of bacteria growth close to plates soaked in Tobramycin (panels a), Cephalothin (panels b) and Amoxicillin (panels c) at the indicated time periods; 15 and 30 min as well as 1 and 24 h for Tobramycin and 30 min for Amoxicillin and Cephalothin. Before immersion in the agar the plates were placed in PBS for 15 min, 1 h and 24 h, respectively, in order to investigate the effect of the antibiotics possibly remaining in the coatings after the various release times. For all experiments shown in the figure as well as those performed with loading times not shown here (15 min, 1 and 24 h for Amoxicillin and Cephalothin, 15 and 30 min as well as 1 and 24 h for Gentamicin) the agar solution closest to the plates remained clear showing that the antibiotic effect was preserved even for the most extreme values with 15 min drug loading followed by release for 24 h in PBS. The fact that the size of the clear zones closest to the plates did not show a clear dependence on loading and/or release time further indicated that the use of loading times longer than 15 min does not necessarily give higher drug release rates during the first 24 h after implantation. In all panels a–c it can be observed that the released antibiotics had an effect on both sides of the plates despite the fact that the HA coating containing the antibiotics only covered one side of the plates. This may be caused by a preferred diffusion direction along the sides of the plates rather than a spherical diffusion out into the agar. The turbid agar solution close to the unloaded HA surface indicating that this reference coating did not have any antibacterial effect is displayed in panel d. To our knowledge, the above presented results are the first of their kind experimentally showing on the possibility for a fast-loading slow-release procedure for surgical implant coatings, creating an opportunity for fast loading of implant surfaces by the surgeon in the operating room.

3.4 Antibiotics release studied by UV spectroscopy

Figure 6 shows examples on the initial drug release in water from HA coatings loaded with Cephalothin (panel a) and Amoxicillin (panel b) at three different loading times. As is apparent from this figure, an initial rapid drug release, hereafter referred to as the burst effect, varied in amount and did not show any clear dependence on drug loading time. In all experiments, the initial burst process lasted for less than 10 min after which a slow and reproducible release was detected. This slow release was found to be independent of drug loading time and somewhat faster for Amoxicillin than for Cephalothin.

In order to study the slow release process in more detail, similar experiments to those in Fig. 6 were carried out for 22 h in PBS, see Fig. 7. Also for these measurements the amount of drug released during the initial burst period was found to be erratic and not related to the drug loading time. The slow release process following the burst was, however, again found to be reproducible and rather similar for all

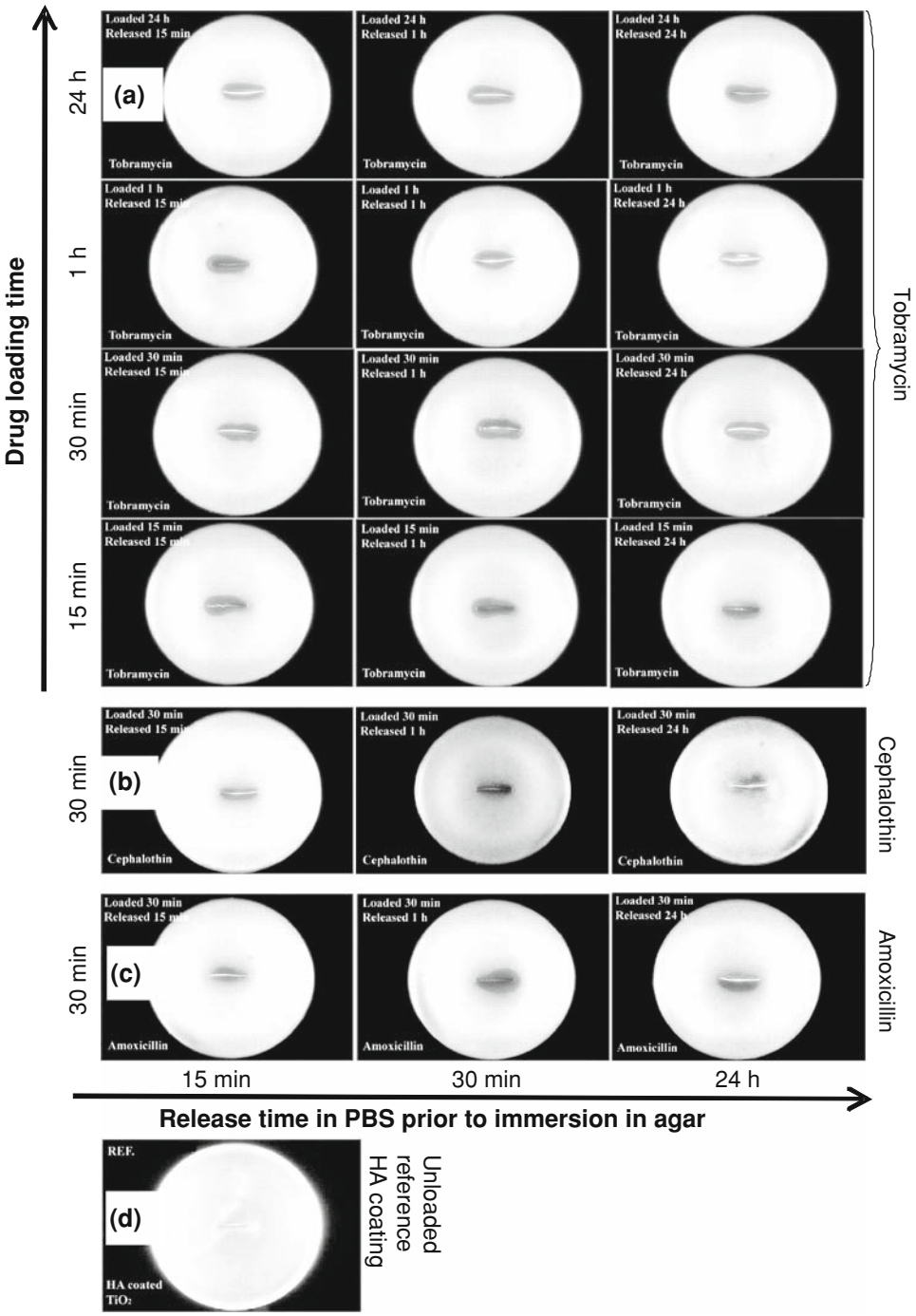

Fig. 5 Images showing inhibition zones of bacteria growth in *S. aureus* containing agar close to HA covered plates loaded with Tobramycin (**a**), Cephalothin (**b**) and Amoxicillin (**c**) at the indicated loading times (left vertical axis) and of the turbid agar solution close to the unloaded HA reference (**d**). Before immersion in bacterial agar the antibiotics loaded plates were stored in PBS for the indicated release times (horizontal axis)

drug loading times studied with the 15 min and 1 h loading process resulting in the most linear release processes. These findings lead to the suggestion that the implants preferably should be stored for about 10 min in PBS after drug loading to ensure reproducibility in the subsequent release process.

The drug release rate of Cephalothin was found to be 0.22 ± 0.06 μg/(h cm^2) while the corresponding release rate for Amoxicillin was found to be 0.42 ± 0.10 μg/(h cm^2) between 2 and 22 h after the experiment was started. The slower release rate for Cephalothin as compared to Amoxicillin is in accordance with earlier findings showing that the former molecule binds more strongly to HA [7].

Cephalothin and Amoxicillin have similar molecular structures. A possible explanation to a stronger binding of Cephalotin than Amoxicillin to HA may be found in the following: Cephalothin possesses one single carboxylic acid site, with a pK_a of 2.4 [33] that can interact and bind to calcium in the HA coating. Amoxicillin on the other hand

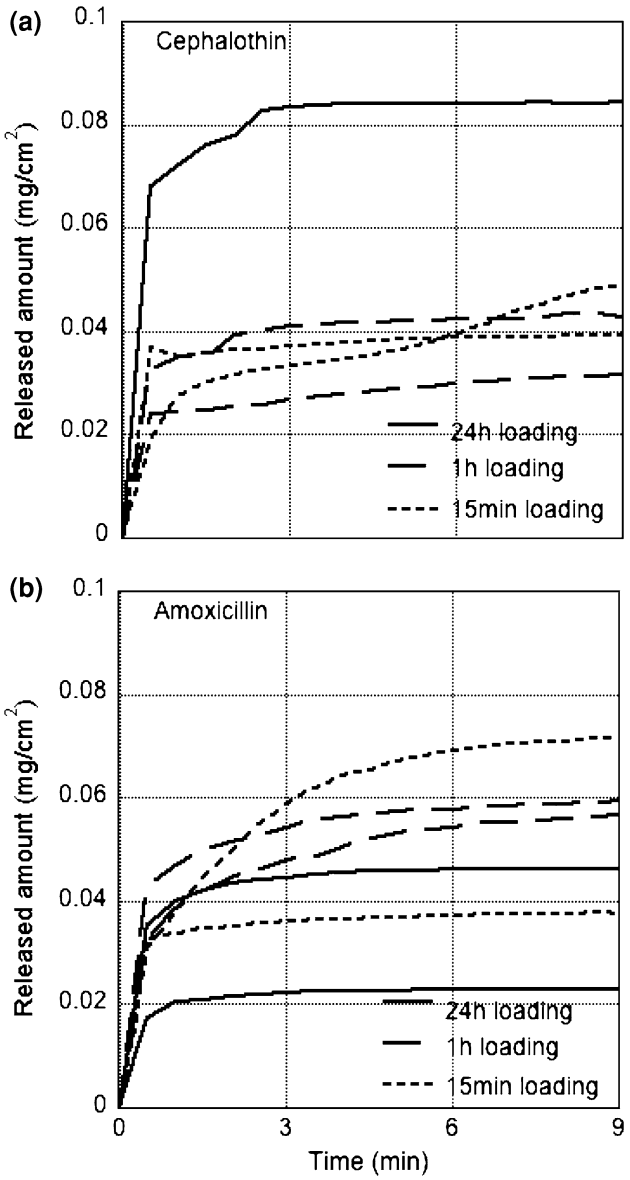

Fig. 6 Examples on release curves showing the amount of Cephalothin (**a**) and Amoxicillin (**b**) released in room tempered de-ionized water per surface area HA coated plates that had been soaking loaded with the antibiotics during the displayed loading times

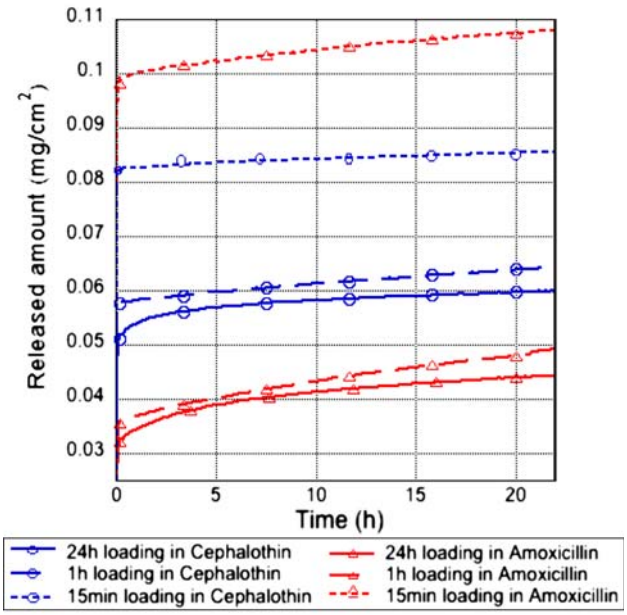

Fig. 7 Examples on release curves showing the amount of Cephalothin and Amoxicillin released in room tempered PBS per surface area HA coated plates that had been soaking loaded with the antibiotics during the displayed loading times

has an amine moiety with pK_a 7.4 [34] in addition to its carboxylic acid group. These amino groups will be partly protonated and thus negatively charged under the given conditions, and if these groups cannot interact and bind to a calcium ion, they will be repelled by the slightly negatively charged HA.

Table 1 presents the concentration of Amoxicillin and Cephalothin needed to ensure inhibition of *S. aureus* and *S. epidermidis*, as divulged from the antimicrobial susceptibility tests. Comparing the release rates of the slow release from the implant coatings with the doses necessary to accomplish bacteria inhibition according to the antimicrobial susceptibility tests, we find the following: During the slow release process from the implant coating, a sufficient dose to ensure inhibition of *S. aureus* in a radius of 1 cm from the implant is released every 17 min from the Cephalothin loaded coatings whereas the corresponding time is about 9 min from the Amoxicillin loaded ones. A sufficient dose to ensure inhibition of *S. epidermidis* in the corresponding region is released every 17 min from the Cephalothin loaded coatings and every 27 min from the Amoxicillin loaded ones. These numbers are calculated assuming that the transport of drugs from the region in vicinity of the implant is slow. The drug release rate from the implant surface being sufficient to maintain an antibacterial effect close to the implant in vivo, depends on the speed of the process transporting the antibiotics away from the region close to the implant as well as on how the drug release process is affected by other release conditions than those prevailing in our experiments.

After the release experiments in PBS two of the HA coatings that had been loaded for 24 h were dissolved in hydrochloric acid to determine the amount of antibiotics remaining after 22 h of release. We found that about 3.5 times more Cephalothin ($\sim$18 μg) was present in the coatings as compared to Amoxicillin ($\sim$5.0 μg). These amounts can be used to obtain a prediction of the expected total release time from the investigated coatings. Assuming that the constant release rate of 0.22 (Cephalothin) and 0.42 (Amoxicillin) μg/(h cm^2) found during the first day of release is upheld until the coatings are emptied, the amounts found to be remaining in the 2 cm^2 hydrochloric acid

treated coatings give an anticipated release time of between 27 and 63 h (including the 22 h of release before the hydrochloric acid treatment) for Amoxicillin and Cephalothin, respectively. These numbers are of course rough estimates, but they still give an indication on the state of the art from which the HA coating stoichiometry, porosity and thickness as well as the drug loading process should be optimized when longer release times are aimed for.

4 Summary and conclusion

The overall aim of the presented work was to investigate the possibility to fast-load biomimetic hydroxyapatite surgical implant coatings with antibiotics in order to create the platform required for a multifunctional implant device which offers sustained local anti-bacterial treatment, avoiding systemic drug administration, and which also avoids problems with safe drug handling and storage, as well as gives the surgeon the possibility to choose which antibiotics to incorporate in the implant at the site of surgery. For this purpose titanium surfaces were coated with an adhesion enhancing layer of Ti oxide, having a gradient in the oxygen content and resulting in bioactive crystalline anatase TiO_2 on the outermost surface, using physical vapour deposition. On this bioactive layer hydroxyapatite was biomimetically grown to serve as a combined bone ingrowth promoter and drug delivery vehicle. Four antibiotic drugs; Amoxicillin, Gentamicin sulfate, Tobramycin and Cephalothin were loaded into the implant coatings via soaking at different time periods between 15 min and 24 h. The subsequent release from Cephalothin and Amoxicillin loaded samples was studied using UV spectroscopy whereas the antibacterial effect of samples containing all 4 types of antibiotics was assessed by examination of inhibition zones around samples placed in a *S. aureus* containing agar after having been allowed to release their antibiotic content for various time periods in a PBS solution.

The antibacterial tests showed that even after as short drug loading times as 15 min, all 4 types of antibiotic loaded samples released enough drugs after 24 h to ensure bacterial inhibition in the vicinity of the samples. From the UV measurements it was found that the release processes of Cephalothin and Amoxicillin consisted of an initial rapid (<10 min) drug release which varied in amount followed by a sustained release process with a reproducible release rate found to be rather independent of the loading times used. The latter process was somewhat faster for Amoxicillin than for Cephalothin. These findings lead to the suggestion that implants that has been fast-loaded with drugs according to the process outlined in this work preferably should be stored for about 10 min in a simulated body fluid like PBS after loading to ensure reproducibility in the subsequent release process.

The calculated release rates and measurements of drug amounts remaining in the samples after 22 h of release indicated that a therapeutically relevant dose could be achieved close to the implant surface for about 2 days. In vivo studies are required to verify this estimate. In cases when a more prolonged release is aimed for, the porosity, stoichiometry and thickness of the HA coating could be optimized in several ways, e.g., by changing the solution temperature and deposition time of the biomimetic process as well as the Ca content of the PBS buffer used. As well, the drug loading process could be tuned by, e.g., changing the concentration of the drugs in the soaking solution.

Concluding, the present study provides an outline for the development of a fast-loading slow-release surgical implant kit where the implant and the drug are separated when delivered to the surgeon, thus avoiding some of the problems related to the lack of knowledge about drug handling at companies selling implants and also avoiding problems connected to sterilization of implants containing drugs prior to delivery. Most importantly, however, the suggested add-on feature could offer the choice of quick addition of antibiotics to the implant coating thus constituting a flexible solution for the surgeon.

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