Antibacterial potential of Zn- and Cu- substituted hydroxyapatite-based coatings deposited by RF-magnetron sputtering

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Abstract. The market of medical devices demanding new products for treatment of bone fractures. The demand is growing due to aging population and increased physical activity in senior people. We introduce newly developed implants – intramedullary fixators made from a set of biocompatible alloys including commercially pure titanium, Ti-6Al-7Nb and magnesium-based alloy. Surface of these implants is modified by Zn- or Cu-substituted hydroxyapatites coatings deposited by RF magnetron sputtering in order to diminish the risk of post-operative infection. The amorphous layers were deposited on the surface of biocompatible substrates for all deposition runs. Post-deposition annealing in air at the temperature of 700°C allowed us to crystallize coating in a way that the main hydroxyapatite peaks are well defined and lattice parameters calculated proving Zn\(^{2+}\) and Cu\(^{2+}\) substitution. The bacteriostatic effect of the coatings against the pathogenic strain 209P of Staphylococcus aureus was shown in vitro independent on the material of the metallic substrate

1. Introduction

In the last decades, a healthy lifestyle was vigorously promoted by a world health organization and mass media that led to increased interest in the physical activity among people of all ages. On the other hand, it is worth to mention that the increased physical activity is leading to a higher risk of traumatization leading to total hip contributing to the overall need for new approaches to bone fracture treatment [1]. A tremendous number of traumatic cases associated with the bone fractures fostered the market need for implants that can provide immobilization of bone fragments using minimally invasive surgical access and quicker recovery of the patient. In order to address this problem, we introduced newly developed implants the intramedullary fixators (IF). Those implants were previously made from commercially pure...
titanium (CP Ti) and already proved their effectiveness in cases where fixation of a proximal bone fracture of tubular bones is needed. However, in order to further improve the implantation success rate and make it possible to use these IF in various clinical cases. For this reason, we are moving from CP Ti to Ti-6Al-7Nb (Ti-Nb) due to the increased awareness of V toxicity and Nb higher osteogenetic potential. In cases where non load-bearing bone parts need to be treated, bioresorbable IF made from Mg-alloys can be used.

In order to accelerate bone healing calcium phosphate (CaP) coatings are deposited on the IF. In case of the IF made of Mg alloys, the CaP coating also serves a purpose of prolonging the bioresorption of the implant. Furthermore, the problem of postoperative infections remains the main challenge for modern health care. In case of severe post-implantation infection, revision surgery is usually needed as the treatment with antibiotics does not provide the desired outcome.

There are various methods that are used to deposit CaP coatings such as plasma spraying [2], micro-arc oxidation [3], sol-gel method [4] and RF magnetron sputtering [5]. An RF magnetron sputtering and other methods of physical vapor deposition stand out due to the high level of control over the coating’s morphology and structure and most importantly a high level of coating to substrate adhesion [6]. The adhesion and surface hardness were the most important parameters for us when choosing the method for deposition. It is because the surface of IF is undergoing significant mechanical stress during the implantation. For our implants, we should ensure the absence of delamination or formation of any debris from the coating during implantation. An RF magnetron sputtering was a method of choice to ensure reliable high-quality coatings for our purpose.

Therefore, our approach to this challenge lies in the surface modification of the IF by means of RF magnetron deposition of antibacterial CaP based coatings with the addition of Zn or Cu. The ions of Zn and Cu are known to have an antibacterial effect and their application is extensively researched in the biomedical engineering field.

In 2013 The International Consensus Meeting on Periprosthetic Joint Infection (Philadelphia, Pennsylvania, USA) concluded that surgical site infections and periprosthetic joint infection (PJI), alone and with its serious implications, continued to pose a challenge to the orthopedic community [7].

We aim to develop novel bioactive and antibacterial coatings consisted of CaP+Zn and CaP+Cu based materials on Ti-6Al-4V and Ti-6Al-7Nb alloys of medical applications. We aim for improvement of the immunocompatibility of the novel implants as well as their antibacterial properties. And finally, we are working towards a translation from model samples to coated implant prototypes for validation of the improved osseointegration, antimicrobial activity, and immunocompatibility in order to demonstrate the proof-of-concept of the developed surfaces.

2. Materials and methods

Custom made vacuum installation with an RF magnetron source operating at 13.56 MHz was used in order to deposit HA, HA-Cu and HA-Zn coatings. The powders of CaPs were prepared by mechanochemical synthesis. The precursor powders were put in the planetary ball mill with three drums, each having a volume of 1800 mL. The process of a synthesis took 12 minutes at room temperature. The phase composition of HA, HA-Cu and HA-Zn powder was confirmed by X-ray diffraction. Afterward, it was used as a precursor-powder to prepare a solid ceramic target for sputtering. The process of a target manufacturing from the calcium phosphate powder of HA-Zn is described in detail in our previous study. CP Ti (Ti-6Al-4V), and Ti-6Al-7Nb (TiNb) alloys were used as a substrate material. Samples were cut in the shape of disks with a diameter of 10 mm and the thickness of 1 mm in order to be used as the substrates for the deposition. The samples were polished in series using silicon-carbide paper up to 1200 grit. Prior to deposition, the samples were sonicated for 10 min consecutively in acetone, distilled deionized water, and ethanol. Samples were placed on the custom-made substrate holder. RF-power was ramped up to the working level of 250 W in a stepwise manner (50 W per 15 min) in order to ensure slow and homogeneous heat distribution along the surface of the target. The samples were moved in a position under the target erosion zone by the carousel mechanism in a vacuum chamber. The argon gas pressure (base pressure 10^-5 Pa, working pressure 0.1 Pa) and the distance between substrate and target...
(70 mm) were kept constant. The deposition time was set to 2.5 hours in all sputtering cases. The average thickness of the coatings deposited coatings was measured by a spectroscopic ellipsometry with an ELLIPS-1891 SAG setup (Rzhanov Institute of Semiconductors Physics of SB RAS, Novosibirsk, Russia) using Si (100) samples that were placed near the Ti and Ti-Nb substrates during every deposition run.

Investigation of the phase composition was done by X-ray diffraction (XRD) analysis (DRON-7, Burevestnik, Russia). The XRD experiments were carried out using the Bragg-Brentano geometry with angle 2θ = 15–60°, radiation source with CoKα radiation (λ = 1.7890 Å) and scanning by steps of 0.01°. Phases were identified with reference to the ICDD database (card number for HA is 9-432). Phase identification was performed with usage of the PDF-2 database, in the total profile analysis software POWDER CELL 2.4. To investigate the microstructure of the deposited films, transmission electron microscope (TEM) JEM 2100 (JEOL, Japan) with in-column energy dispersive X-ray (EDX) elemental composition analyzer INCA-Energy microanalyzer (Oxford Instruments, UK) was used. The distribution of Ca, P, O, Ti, Nb, Zn and Cu in the coatings was determined by EDX in three different regions across the lamella. An average content and Ca/P ratio were determined accordingly. For the cross-section sample preparation ion thinning equipment EM 09100IS ion slicer (JEOL, Japan) was utilized which is available at the Center for Collective Use of Scientific Equipment “Nanotekh” of ISPMS SB RAS. For the preparation of thin foils for investigation in TEM samples should be pre-thinned to 100 µm before to the processing in an ion slicer by conventional grinding and polishing techniques. A final thinning is performed in low-energy and low-angle Ar-ion beam mode.

For the comparative analysis of the antibacterial activity of the Zn- and Cu-containing HA coatings the experiments in vitro with the pathogenic strain 209P of Staphylococcus aureus (SA) (the collection of the Department of Microbiology of Siberian State Medical University, Tomsk) were carried out as described previously [8]. Extracts of the samples with the coating saturated by antibacterial ions were obtained using a 0.9% NaCl solution after 7 days incubating at 37°C (2 ml solvent per sample was used according to ISO 10993-5). The microbial suspension (500 microbial bodies) with an extract or a solvent (0.9% NaCl solution) was prepared in sterile conditions in 15 ml plastic tubes at the proportion of 1:1 (0.5 ml : 0.5 ml) and was incubated for 2 hours at 37°C.

Then 0.1 ml of the suspension from each group (100 microbial bodies) was placed on a nutrient agar medium in 90 mm plastic Petri dishes and was cultured for 24 hours at 37°C and 100% humidity. Three Petri dishes were taken for each group. The antibacterial activity was determined by the calculation of the area of colony-forming units (CFU) per Petri dish. Software measurements by Adobe Photoshop 10.0 (Adobe Inc., USA) was used.

Results were analyzed using Statistics 10.0 software. The data were shown as the median (Me), 25% quartile (Q1) and 75% quartile (Q3). To analyze the available data sets a normal distribution Kolmogorov-Smirnov test was used. Non-parametric Mann-Whitney’s U-test was performed, and the differences were considered significant at $p < 0.05$.

3. Results and discussion

The deposition parameters used in our work were kept constant for all targets for sputtering and types of substrates. These parameters, coating thickness estimated by ellipsometry from Si (100) substrates and elemental composition according to EDX obtained from coated Ti and Ti-Nb substrates are represented in table 1.

| Sputtered material | RF power (W) | Deposition time (hours) | Throw distance (mm) | Working gas pressure (Pa) | Thickness (nm) Ca/P |
|--------------------|-------------|------------------------|---------------------|---------------------------|---------------------|
| HA                 | 250         | 2.5                    | 70                  | 0.1                       | 1220±5% 1.96±10% |
| Cu-HA              | 250         | 2.5                    | 70                  | 0.1                       | 1251±5% 2.23±10% |
| Zn-HA              | 250         | 2.5                    | 70                  | 0.1                       | 1241±5% 2.2±10%  |
From the obtained data we can conclude that the coating thickness for HA, Cu-HA, and Zn-HA vary in a small range. The substituted HAs shown a slightly higher deposition ratio when compared to pure HA. The small differences between substituted HA and pure HA also noticeable in terms of elemental composition. The substituted HAs shown increased Ca/P ratio according to EDX. We connect these variations of elemental composition and thickness with a change of physico-chemical properties of substituted HAs and possible change in the melting point. According to K.Tõnsuadu et al [9] thermal stability is lowered the most by substitution of calcium and phosphate. The melting stability and ratio between the melting point and the substrate temperature strongly dictate the formation of the thin film according to the model proposed by J.A. Thornton [10]. The variation of the coatings’ elemental composition with relation to the substrate is negligible, the results represented in the table are averaged from coatings deposited on both types of substrates.

In figure 1 cross-section TEM of HA, Cu-HA and Zn-HA coatings deposited on Ti-Nb is presented. The coating is represented by a featureless homogeneous layer that repeats the topography of the initial Ti-Nb surface. From the TEM image, we can conclude that all CaP coatings appear to have the same amorphous type of structure. Selected area electron diffraction (SAED) gathered from the region of interest (ROI) represented in figure 1 is represented by the bright diffusive halo. It is confirming an amorphous state of the deposited coatings. The estimated coatings’ thickness from cross-section TEM is in good correlation with obtained results collected by Ellipsometry from Si samples. The number of scientific groups have reported the amorphous state of CaP coatings after deposition with RF magnetron sputtering. The amorphous layers are mainly formed when the substrate temperature is below 300°C.

![Figure 1](image-url)
The energy of the sputtered species is quickly dissipating after they arrive at the surface of the substrate prohibiting to rearrange in the crystalline state. The coatings’ crystallinity can be improved by depositing CaP on pre-heated substrates as was shown in our previous work [11].

Figure 2. The mapping of chemical elements in the Zn-HA coating: ROI used for EDX (a), distribution of Ti (b), Al (c), Nb (d), Ca (e), P (f), O₂ (g), Zn (h) in the coating.

In figure 3 GI XRD obtained from substituted HAs and Pure HA on Ti and Ti-Nb substrates are represented. The profiles from all six samples do not significantly different from each other. In each of the profiles, the lines of α-Ti phase are easily visible. It is because even under grazing incidence of the X-Ray the beam is interacting with the substrate producing the well-defined peak of α-Ti phase in diffractograms for both Ti and Ti-Nb substrates.
The discrete peaks related to HA lattice is not seen in the diffractograms. However, in the area of small angles (~30–40°), a halo is visible in all the cases. The halos are in the same angle range where the main peaks from HA lattice should be according to the databases. Taking into account the results obtained during TEM observations and the results produced by GI XRD we can confidently confirm the amorphous state of the CaP coatings. In order to crystallize the coatings post-deposition annealing was performed. The samples were annealed in the air in the furnace set to a stepwise increase in temperature at 15°C-min⁻¹ till the temperature of 300°C, 10°C-min⁻¹ till the temperature of 500°C and 10°C-min⁻¹ till the temperature of 700°C. The samples were annealed for 3 hours during the temperature 700°C and were cooled down to room temperature in a stepwise manner to reduce the risk of thermal stress.

In figure 4 GI XRD obtained from substituted HAs and Pure HA on Ti and Ti-Nb substrates after annealing is represented. Here we see the appearance of the main peaks in HA lattice for all CaP coatings after annealing. The annealing performed using above-mentioned strategy allowed us to crystallize coating in a way that the main HA peaks are well defined. Moreover, there was no obvious significant damage to the surface of the coatings after annealing.

The lattice parameters were calculated and compared to the reference parameters for pure HA. It was revealed that the lattice parameters for HA in our case and the ones that were found in the database were the same. However, a substitution of Ca²⁺ in the HA lattice for Zn²⁺ and Cu²⁺ led to the growth of volume in the primitive cell from 3 to 4 % when compared to pure HA. With these results, we can conclude that...
the ion substitution in the HA lattice occurred. It is of vital importance to obtain antibacterial elements in the form of ions and not agglomerates or nanoparticles as they could be potentially cytotoxic or produce an unwanted immune reaction. Here we ensure that we can produce the coatings with homogeneous distributed antibacterial ions that are going to be released during the dissolution in the body fluids.

Surgical and periprosthetic infectious complications are current problems of traumatology and orthopaedics. The results of in vitro antibacterial activity of Zn-HA and Cu-HA coatings against the pathogenic strain 209P of Staphylococcus aureus (SA) are shown in table 2. SA 209P grew well on PAS and occupied 1055 mm² (17%) per Petri dish surface. When the Zn or Cu was introduced into the coatings more than the 24-fold decrease in the area of the microbial growth occurred after contact with implant extracts.

### Table 2. Results of 24-hour growth of SA 209P in agar medium after preliminary 2-hour cultivation in the 7-day extracts of samples with rf-magnetron Zn-HA and Cu-HA coatings, Me(Q1-Q3).

| No | Groups investigated, n=3 | A type of metallic substrate | An area of bacterial CFU (mm² per Petri dish) |
|----|--------------------------|-----------------------------|--------------------------------------------|
| 1  | Growth control with a solvent | Ti6Al4V | 1055 (1005-1088) |
| 2  | HA coating               | Ti6Al7Nb                    | 1269 (1187–1395) | 1283 (983–1417) |
| 3  | Zn-HA coating            |                             | 227 (218–261)* | 334 (325–367)* |
|    |                          |                             | P1-2<0.05      | P1-2<0.05      |
| 4  | Cu-HA coating            |                             | 246 (193–361)* | 436 (114–642)* |
|    |                          |                             | P1-2<0.05      | P1-2<0.05      |

Note: n – number of Petri dishes studied in each group; * – statistical differences (P < 0.05) with the corresponded groups.

### 4. Conclusions

The novel bioactive and antibacterial RF coatings with enhanced osseointegration properties consisted of calcium phosphates with additions of Zn and Cu on Ti-6Al-4V and Ti-6Al-7Nb alloys of medical applications have been manufactured. As-deposited coatings are characterised by amorphous structure and Ca-enriched elemental composition. Annealing in air allows to crystallize amorphous CaP coatings. The extracts of Zn-HA or Cu-HA coating deposited by rf-magnetron sputtering have in vitro bacteriostatic effect against the pathogenic strain 209P of Staphylococcus aureus independent on the material of the metallic substrate. The RF CaP thin films doped with Zn or Cu ions and with enhanced osseointegration properties can be recommended as the bioactive and antibacterial coatings for IF made of Ti-6Al-4V and Ti-6Al-7Nb alloys.

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