Incorporation of Tobramycin Biomimetic in Hydroxyapatite Coating on CoCrMo Alloy and its Antimicrobial Acitivity

MIHAELA GRECU, GABRIELA NOVAC, DANIELA IONITA*, CAMELIA UNGUREANU
University Politehnica of Bucharest, 1 Polizu Str., 011061, Bucharest, Romania

In this study, we have investigated the incorporation of tobramycin into biomimetic HA coatings applied on CoCrMo implants. Tobramycin can prevent growth of Escherichia coli, a gram-negative bacillus frequently responsible for post-surgical infections in orthopaedic surgery and such antimicrobial activity was demonstrated on biomimetic HA coating. Such bioactive HA coatings on metallic implants have been shown to enhance early bone apposition and fixation of prostheses. Our results demonstrated the efficiency of the biomimetic coatings combined with tobramycin, to prevent local post-surgical infections. This study demonstrated an affinity of tobramycin for HA surfaces as well.

Keywords: tobramycin, hydroxyapatite coating, CoCrMo implants

Surfaces containing calcium (Ca) and phosphorous (P) are known to become bioactive and to cause osteoconduction of new bones. Hydroxyapatite (HA) coating has been extensively used as an implant material due to its similarity with human bone composition and thereby its ability to form a strong bond to human hard tissue [1]. Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is the main mineral component of bone, and its synthetic form is one of the most widely used biomaterials for reconstruction of the skeleton due to the lack of local or systemic toxicity together with its osteoconductive properties [2].

It is used as an implant material both in its bulk mainly porous form, for filling in or reconstructing bone defects, and as a thin coating on metals, titanium and CoCrMo alloys, for hip, knee, and dental prostheses. They are the choice for orthopedic application. The many advantages of these materials include biocompatibility, good resistance to corrosion, and excellent mechanical properties [3]. However, CoCrMo based materials are unfortunately not bioactive materials and generally encapsulated after implantation into the living body by fibrous tissue that isolates them from the surrounding bone. Therefore, coating CoCrMo with HA and other bioactive ceramic materials including surface modification becomes a popular method for providing them with a bone-bonding ability. Recently a biomimetic method for coating medical devices with carbonated HA and others Ca-P phases was developed [4]. This coating technology involves the immersion of metal implants into supersaturated solutions of calcium phosphate, which resemble physiological fluid, at ambient temperature [5]. Biologically active agents can be added to the supersaturated solutions and gradually co-precipitated with the calcium phosphate crystals, forming a layer on the metal implants [6, 7]. This creates the possibility to incorporate an antibiotic uniformly within the biomimetic coating and release it at a controlled rate, thus preventing or stopping local post-operation infection [8].

Although successful rates of these kinds of implants are dependent on bone-implant osteointegration, the successful and long-term survival of the implants is also dependent on the prevention of bacterial infection after implant placement. A good approach to the local treatment of implant-associated infections is the use of HA coatings for antibiotics delivery, exploiting the osteoconductive properties of this material [9, 10].

The purpose of this study was to obtain hydroxyapatite coating on CoCrMo alloy, characterize, incorporate in this coating the tobramycin antibiotic and then to evaluate the antibacterial effect of this surface. Tobramycin was used to confer microbial resilience on biomaterials. Local release of antibiotic may eliminate bacteria introduced at the site of implantation. It could also prevent invasion by micro-organism through the wound, when external fixation pins are used.

Among antibiotics, tobramycin was chosen, because of its broad spectrum against most gram-negative bacteria and some gram-positive bacteria [11]. The inhibition test was conducted with *Escherichia coli* bacteria.

Tobramycin can prevent growth of *Escherichia coli*, a gram-negative bacillus frequently responsible for post-surgical infections in orthopaedic surgery [12].

The amino glycoside tobramycin presents several hydrophilic groups, which could interact with ions present in the solution coating (i.e. Ca^{+2},HPO_4^{2-}). Tobramycin also has an isoelectric point of approximately 8.2, meaning that it is positively charged at neutral pH when biomimetic coating is deposited [13].

Tobramycin was incorporated in the biomimetic HA coating did inhibit the growth of *Escherichia coli* bacteria *in vitro*. Various concentrations of tobramycin in the coating solution were tested (n=2).

Experimental part

Samples preparation

Samples of CoCrMo alloys with dimensions of 10 mm in diameter and 2 mm in height were obtained. The composition of CoCrMo alloys was: 28 % Cr, 5.2 % Mo, 1 % Si, 1 % Mn, 1 % Ni, 0.75 % Fe, 0.35 % C, and 62.7 % Co. These samples were ground with silicon carbide papers ranging from 80 to 1200 grit. Finally, the samples were washed with deionised water and ethanol, dried in air and stored in desiccators before testing.

Alkaline treatment

The CoCrMo alloy discs were soaked in NaOH solution at a concentration of 5 M for 24 h at 60°C. After the

* email: md_ionita@yahoo.com

Ion	Plasma (mmol/L)	SBF (mmol/L)
Na^+	142	142
K^+	5	5
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	103	147.8
HCO_3^-	27	4.2
HPO_4^{2-}	1	1
SO^{2-}_4	0.5	0.5

Table 1
COMPOSITION OF SBF COMPARED WITH THE HUMAN BLOOD PLASMA

treatment, the specimens were slowly washed with distilled water and dried at room temperature.

Thermal treatment

The Co-Cr alloy discs were alkaline- treated with 5 M NaOH at 60 °C for 1 day, after which they were washed and dried as described in the previous section. The specimens were dried at 220 °C for 8 h.

Preparation of simulated body fluid, SBF

The SBF solution was prepared by dissolving reagent-grade NaCl, KCl, $NaHCO_3$, $MgCl_2 .6H_2O$, $CaCl_2$ and KH_2PO4 into distilled water and buffered at *p*H = 7. 25 with tris-hydroxymethyl aminomethane (TRIS) and 36.5 g/L HCl at 37°C. Its composition is given in table 1 and is compared with the human blood plasma. The discs were then soaked in an SBF and a solution with ion concentration 1.5 time of SBF (1.5 SBF) solutions at 37°C for 21 days.

Co-precipitation of tobramycin

Following the application of the coating, CoCrMo samples were then subsequently coated with a thick, crystalline biomimetic hydroxyapatite (HA) layer containing the antibiotic. The HA-coated CoCrMo samples were immersed in another calcium phosphate supersaturated (CPS) solution containing NaCl (146 mM), $CaCl_2 . 2H_2O$ (4 mM), $MgCl_2 .2H_2O$ (0.05 mM), $Na_2HPO_4 . 2H_2O$ (2 mM) and $NaHCO_3$ (1 mM). This solution was buffered at *p*H 7.4. In this solution were dissolved 300 mg/L or 600 mg/L tobramyicin. The CoCrMo samples covered with HA were immersed in CPS solution containing tobramycin for 48 h at room temperature.

Surface characterization

The morphological investigations of the specimens before and after alkaline and heat treatment and soaking in SBF were performed by using an Environmental Scanning Electron Microscope FEI/Philips XL30 ESEM (SEM).

Surface hydrophilicity/hydrophobicity

The hydrophilic/hydrophobicy balance vas evaluated using a contact angle meter - KSV Instruments CAM 100. The surface hydrophilicity of CoCrMo was studied by measuring the static contact angle with a sessile drop of distilled water deposited on the sample surface. Wetting was evaluated by measuring the contact angle formed between the liquid drop and the solid surface. The volume of the liquid was kept constant (10 μL). Each contact angle value is the average of minimum 10 measurements. All measurements were performed at ambient temperature. The water adhesion tension (τ) was calculated by:

$\tau = \gamma \cos\theta$; where θ is the measured water contact angle and γ =72.8 dyn/cm for water.

Surface roughness evaluation

The surface topography of all surfaces was examined with atomic force microscopy (AFM). The surface roughness was evaluated on each 3D surface image to obtain the average roughness value (Ra) with standard deviation for each specimen. The used equipment was an atomic force microscope from APE Research, Italia.

Evaluation of antibacterial activity

For antibacterial activity evaluation three sterile samples were used as following: one native passive CoCrMo sample, a sample of CoCrMo coated with HA and one coated with tobramycin/HA composite.

Escherichia coli (K 12-MG1655) was aerobically cultured in a tube containing Luria Bertani medium at 37°C. The initial concentration of bacteria was adjusted to 10^{-9} colony forming units (CFU)/mL by dilution with sterile water. Four microliters of bacterial solution was pipetted onto each specimen. A sterile glass was placed over the specimen and the bacterial solution. After 3h, the specimen and sterile glass were transferred to a 10 mL of sterile water, and then fully vibrated (Disruptor Genie vortex) in order to harvest the cells. 1 mL of each rinse was placed onto Bacto-Agar plates. The plates were incubated for 48 h at 37°C to determine the number of viable *E. coli* in terms of CFU.

Results and discussions

The SEM photograph (fig 1) revealed that the control specimen, the polished CoCrMo alloy has a smooth surface texture with abrasive marks.

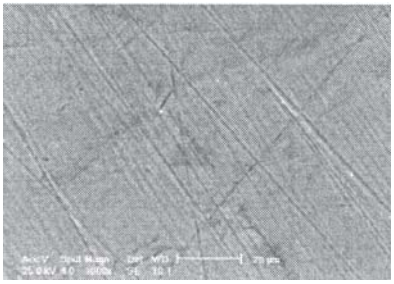

Fig. 1. SEM image of polished CoCrMo alloys

Figure 2 and 3 show the SEM image of CoCrMo after various surface treatments. After alkali treatment, the alkaline-treated surface SEM image of CoCrMo relieved a porous surface as can be seen in figure 2.

EDS analysis detects on the surface of CoCrMo a compound containing Na, such as chromates (fig. 2), which may be stabilized during the thermal treatment.

After alkaline and thermal treatment the specimens are immersed 7 days in SBF solution at 37°C. A thin amorphous calcium phosphate layer was first deposited on the surface of the CoCrMo alloy. This served as a seed surface to induce the precipitation and growth of the second and more crystalline layer. The secondary layer was obtained by soaking the coated CoCrMo specimens other 14 days in 1.5 SBF solution at 37°C. The EDAX analysis of coating relieved the presence of hydroxyapatite on the coating composition. In some cases, early precipitation in the supersaturated calcium phosphate solution was observed.

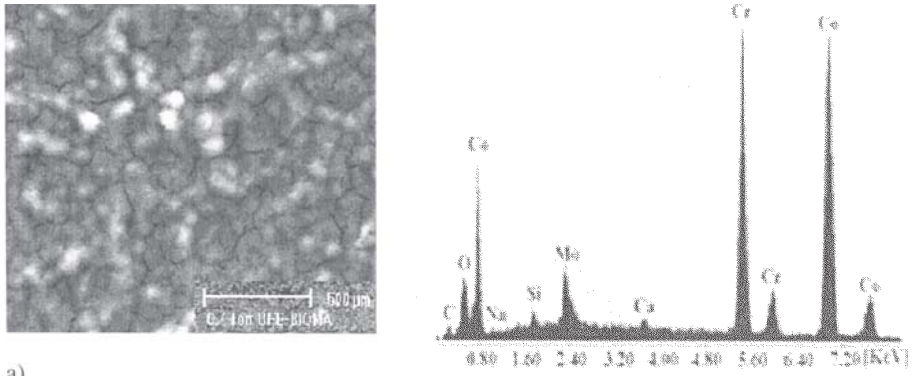

Fig. 2. SEM image (a) and EDS spectrum (b) of CoCrMo alloy alkali treated

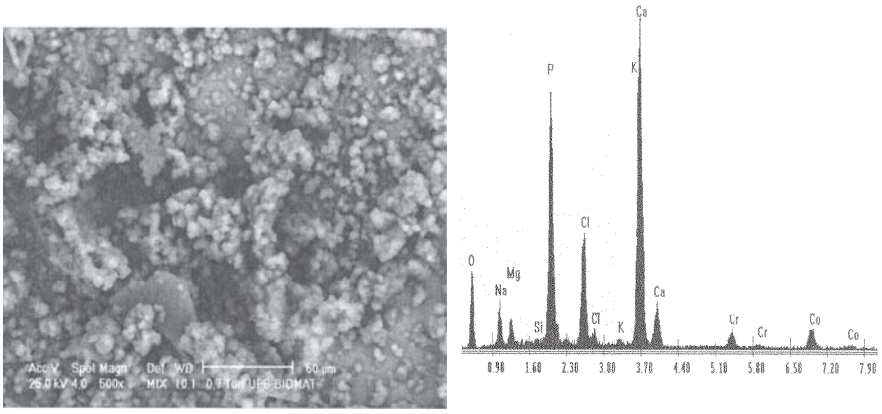

Fig. 3. SEM image (a) and EDS spectrum (b) of CoCrMo covered with HA

The AFM images of surface of CoCrMo alloy are presented in figure 4-6.

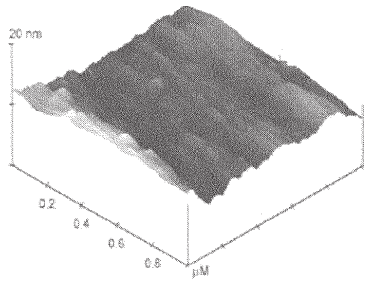

Fig. 4. The polished CoCrMo

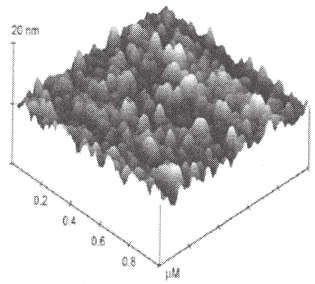

Fig. 5. CoCrMo covered with HA

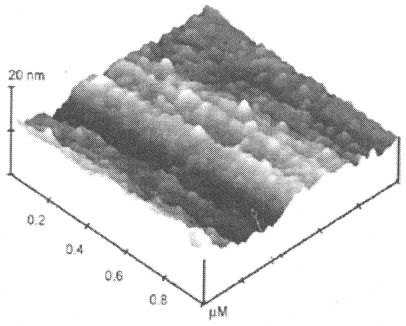

Fig. 6. CoCrMo covered with HA with tobramycin

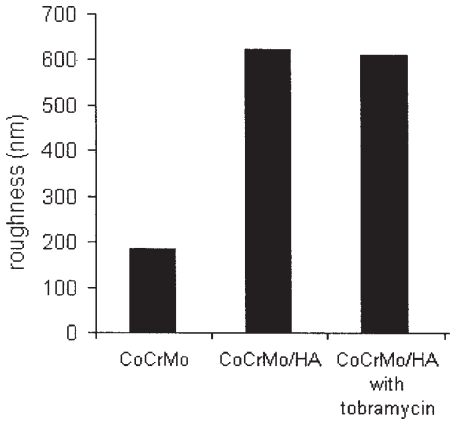

Fig. 7. The average roughness values for the CoCrMo alloy

It can be seen, in figure 4, that the surface of the polished implant presents scratches produced by the mechanical machining process. This surface has a typical average roughness of 0.186 μm ± 0.10 mm, and a surface area of 10.190 mm^2. The surface properties of the CoCrMo substrates changed significantly after the deposition of HA (fig. 5). The layer presented a porous structure with pore sizes up to 5 mm and surface roughness about three times larger (0.624 mm) than polished CoCrMo samples. The results on pore size, phase composition and surface roughness are in line with previous studies on deposition of HA on CoCrMo alloy using the same electrolyte [14].

Figure 7 show the average roughness values for the CoCrMo alloy. Hydroxyapatite deposition is a process that increases the surface roughness of the CoCrMo. When incorporating tobramycin into the porous coatings, the average roughness was slightly lower compared to the CoCrMo with HA coating specimens, but not significantly. Published data from the literature [15, 16] show that good integrated implants present Ra between 0.5 and 1.0 μm.

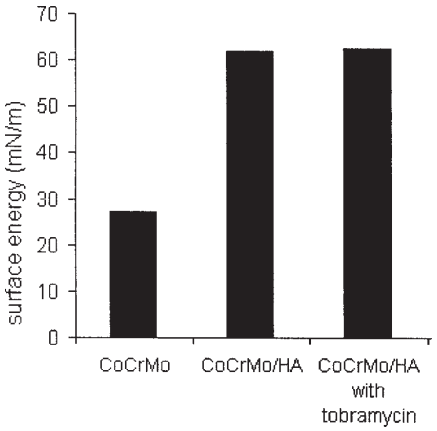

Fig. 8. The surface energy values for the CoCrMo alloy

In the present work, the Ra values were between these values.

Regarding contact angle values, the experimental data indicated a hydrophilic behaviour. For polished CoCrMo alloy the contact angle was 68°. After depositing HA, the corresponding surfaces had remarkably reduced contact angles at 32°. The decrease of contact angles by HA deposition is ascribed to high surface energy of nano HA particles. Ions Ca^{2+} and PO_4^{3-}, as hydrophilic solutes in the surface oxides, promote surface hydration resulting in a decrease of contact angles. The presence of tobramycin on the coating did not determine a change in wettability (contact angle was 32°). Some researchers observed that proliferation of the cells increases with surface wettability; the fibroblasts have greater adhesion on hydrophilic surfaces than on hydrophobic surfaces [17]. Surface energy calculations from contact angle data indicated that increasing surface roughness, surface energy increased. Surface alloys with HA and HA and tobramycin had a surface energy significantly higher than that of unmodified CoCrMo surfaces (fig. 8). In addition, the alloys with modified surfaces had a lower contact angle.

It has been demonstrated that there is a linear relationship between nano-roughness, surface energy, and protein adsorption [18, 19].

The last year researches have shown that increased protein adsorption, such as fibronectin, resulted in decreased bacteria attachment [20, 21].

As shown in figure 9, tobramycin was released from the HA coating and inhibition of bacterial growth in vitro was demonstrated.

These bacteriological experiments performed in vitro demonstrated efficacy of tobramycin incorporated in the biomimetic HA coated implants against growth of *Escherichia coli* bacteria.

The biomimetic HA coating produced in the absence of tobramycin showed no antibacterial effect.

On the plate corresponding to the sample incorporating tobramycin, *E. coli* culture was inhibited confirming the antibacterial effect of this surface, (0 CFU/mL of *E. coli*).

In the samples with HA, a more pronounced growth of *E. coli* was noticed ($648 \cdot 10^3$ UFC/mL of *E. coli* for CoCrMoHA sample versus $77 \cdot 10^3$ CFU/mL for CoCrMo sample); this is explained by the nutrient content of HA.

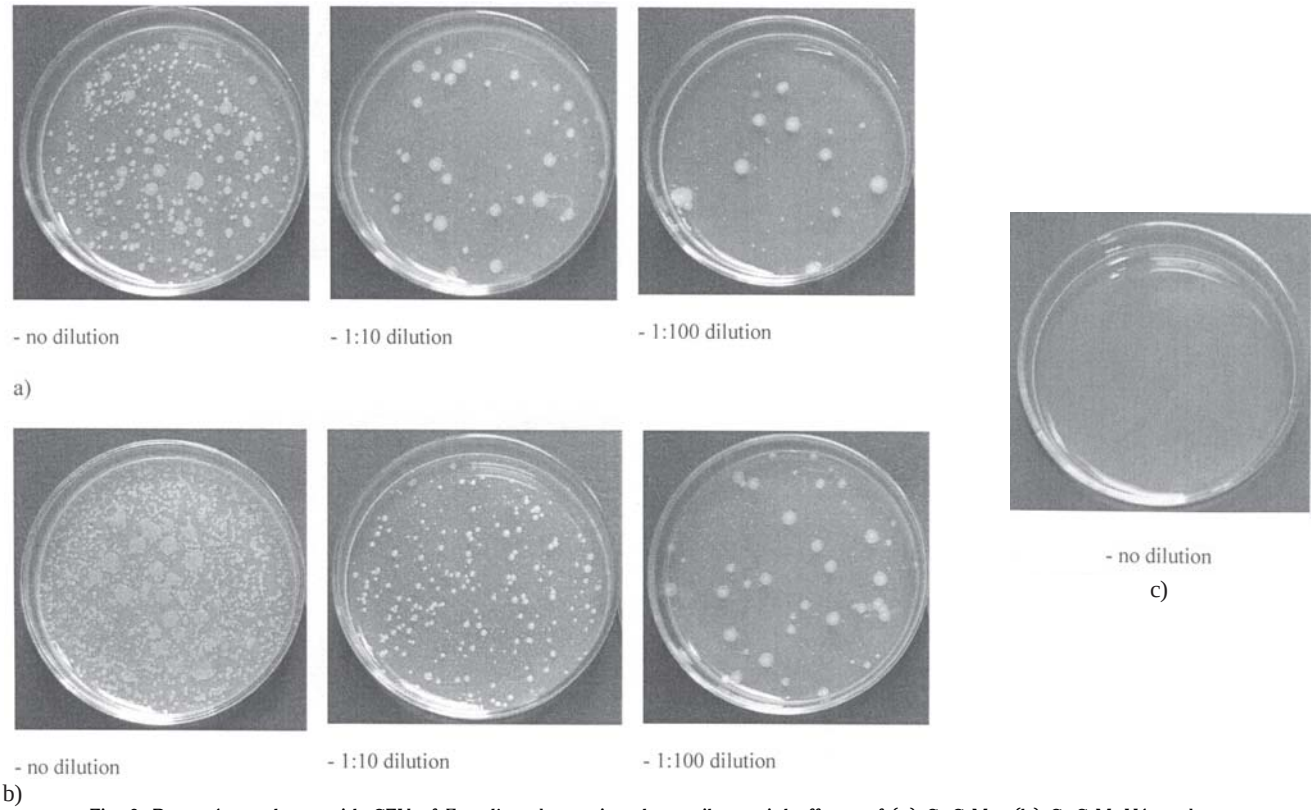

Fig. 9. Bacto-Agar plates with CFU of *E. coli* to determine the antibacterial effects of (a) CoCrMo, (b) CoCrMoHA and (c) CoCrMoHA/ tobramycin

Conclusions

Bioactive HA with tobramycin coatings on CoCrMo implants were obtained using incorporation of tobramycin in biomimetic hydroxyapatite coating.

These results demonstrated the efficiency of the biomimetic coatings combined with tobramycin, to prevent local post-surgical infections, and an affinity of tobramycin for HA surfaces as well.

This method represents an efficient approach for producing a biomimetic therapeutic coating that could reduce post-surgical infections, while increasing implant stability.

References

1. OONISHI, H, Biomaterials, **12,** 1991, p. 171
2. TIRRELL, M., KOKKOLI, E., BIESALSKI, M., Surface Science, **500**, no. 1-3, 2002, p. 61
3. MARTI, A., Injury, 31, 2000, p. 18
4. BARRERE F., VAN BLITTERSWIJK, C.A., DE GROOT, K., LAYROLLE P., Biomaterials, **23**, no. 9, 2002, p. 1921
5. AIONICESEI, E., IONITA, D., MICULESCU, F., CIMPEAN, A., DEMETRESCU, I., Rev. Chim. (Bucharest), **57**, no. 8, 2006, p. 813
6. IONITA, D., MAN, I., DEMETRESCU, I., Key Engineering Materials, **330**, 2007, p. 545
7. COVALIU, C. I., GEORGESCU, G., JITARU, I., NEAMTU, J., MALAERU, T., OPREA, O., PATRO, E., Rev. Chim. (Bucharest), **60,** no.12, 2009, p. 1254
8. STIGTER, M., BEZEMER, J., DE GROOT, K., LAYROLLE, P., Journal of controlled release, **99**, 2004, p. 127
9. HETRICK, E. M., SCHOENFISCH, M. H., Chemical Society Reviews, **35**, no. 9, 2006, p. 780
10. HASEGAWA, S.M., FUKUDA, A., UCHIDA, A., The Journal of Arthroplasty, **23**, no. 1, 2008, p 145
11. KANG, M.K., MOON, S.K., LEE, S.B., KIM, K.M., LEE, Y.K., KIM, K.N., Thin Solid Films, **517**, 2009, p. 5390
12. STIGTER, M., DE GROOT, K., LAYROLLE, P., Biomaterials, **23**, 2002, p. 4143
13. LIU, Y., LAYROLLE, P., VAN BLITTERSWIJK, CA., DE GROOT, K., J. Biomed Mater. Res., **57**, 2001, p. 327
14. WARDA, B. C., WEBSTER, T. J., Biomaterials, **27,** 2006, p. 3064
15. ALBREKTSSON, T., WENNERBERG, A., Int. J. Prosthodont., **17**, 2004, p. 536
16. GEISGERSTORFER, J., LINDMANN, W., SCHEIDER, L., A presented at the 10th Int Friadent Symposium, 2003 Mannheim, Germany
17. BOYNA, B.D., DEAN, D.D, LOHMANN, C.H., Titanium in Medicine, 2001, SpringerVerlag, Berlin
18. WOO, K.M., CHEN, V.J., MA, P.X., J. Biomed. Mater. Res. A, **67,** 2003, p. 531 19. KHANG, D., KIM, S.Y., LIU-SNYDER, P., PALMORE, G.T., DURBIN, S.M., WEBSTER, T.J.., Biomaterials, **28**, 2007, p. 4756
20. ANAGNOSTOU, F., DEBET, A, PAVON-DJAVID, G, GOUDABY, Z, HELARY, G, MIGONNEY, V., Biomaterials, **27**, 2006, p. 3912
21. KINNARI. T.J., PELTONEN, L.I., KUUSELA, P., KIVILAHTI, J., KONONEN, M., JERO, J., Otol. Neurotol., **26**, 2005, p. 380

Manuscript received: 23.07.2010