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ADVANCED NANOMATERIALS AND COATED SURFACES FOR ORTHOPEDIC IMPLANTS – A REVIEW

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Abstract. Critical-sized defects in bone induced by trauma have in numerous presented cases difficult challenges to the current treatment for bone repair. The main purpose of bone tissue engineered scaffolds is to use advanced materials to promote the natural healing process of bone which does not easily occur in critical-sized defects or on metallic implants. A synthetic bone scaffold and a coating on Ti implants must be biocompatible and biodegradable to allow the native tissue integration, and mimic the chemical composition and structure of native bone. In addition to being physically and chemically biomimetic, an ideal scaffold and the coating layers on metallic implants must be capable of releasing essential physiologic elements, like Mg, Zn, Sr and Si, and also containing bioactive molecules (e.g., collagen, COL) to accelerate extracellular matrix production and tissue integration. Also, these advanced materials might be doped with drugs (e.g., antibiotics, such as vancomycin) to prevent undesired biological response such as infections, especially with Staphylococcus aureus, *S. aureus*. Various biomaterials include hydroxyapatite (HAP) ceramics or multi-functional hydroxyapatite substituted with Mg, Zn, Sr and Si, mf-HAP, polymers, such as poly lactic acid (PLA, approved for medical applications by Food and Drug Administration, US FDA, and collagen, or their mixtures as biomimetic composites which have been investigated for their potential as bone scaffold materials and coatings on metallic implants. This article briefly reviews the physical and chemical characteristics of used advanced materials and describes the key-technologies in mimicking the physical and chemical environment of bone using synthetic materials, and provides an over view of local drug delivery as it pertains to bone tissue engineering.

Keywords: hydroxyapatite, multi-functional hydroxyapatites, biomimetic composites, orthopedic (medical) implants

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1. Introduction

Bone is a notable organ playing key roles in critical functions in human physiology including movement, protection and support of other critical organs, blood production, mineral storage and homeostasis, blood pH regulation and multiple progenitor cells (mesenchymal) location [1-4].

The importance of bone becomes very clear in the case of diseases such as osteoarthritis, osteomyelitis and osteoporosis in which bone does not function properly. These diseases along with traumatic injuries lead to or induce bone defects very difficult to treat through orthopedic surgeries (i.e., total joint arthroplasty, implant fixation and tumor resection). The clinical and economic impact of treatments of bone defects is staggering. All treatments mentioned above involve bone repair and regeneration or bone replacement. For a variety of reasons injured or diseased bone may not be capable of repairing itself by means of mechanical fixation alone which results in a nonunion. Each treatment has a different rate of incidence of nonunion [5-10].

Advantages to utilizing advanced synthetic bone nanomaterials might include fewer surgical procedures, a reduced risk of infection or immunogenicity, and the abundant availability of synthetic materials based on hydroxyapatites [11-13].

2. Design principles in bone tissue engineering

The main purpose of tissue engineering is to stimulate bone regeneration using porous synthetic materials. It is known that living organisms do not have the ability to heal in the event of major fractures, so biomaterials capable of replacing or regenerating diseased tissues have been developed [14]. Tissue engineering can be defined as the primary initiator through which the body can heal itself by delivering cells, biomaterials, and supporting structures to the right place [15-17]. The requirements for synthetic scaffoldings are as follows: porous structure to allow for vascularization and bone in-growth, their degradation products to be non-toxic, they can support and promote osteogenic differentiation within the non-osseous, synthetic scaffolds (i.e., they may support osteoconduction and osteoinduction), encourage bone cell migration into the scaffolds, provide temporary mechanical support to the affected area, act as a substrate for osteoid deposition, be capable of sterilization without loss of their bioactivity, not incite an active chronic inflammatory response, and degrade in a controlled manner to facilitate load transfer to developing bone.

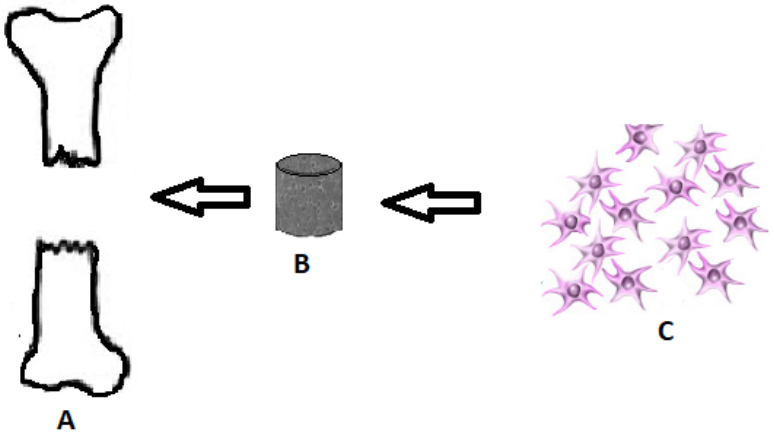

Figure 1. Defected bone (A) replacement using advanced materials (B) enriched in osteoblasts (C).

3. Advanced nanomaterials

Hydroxyapatite (HAP) is the most stable form of calcium phosphate found as an inorganic component in bones and teeth [18-21]. Rapid progress in science is due to its characteristics of biocompatibility, bioactivity, bioaffinity, osteoconductivity and osseointegration, which make it applicable in the biomedical field such as orthopedic and dental surgery. It is used as a filling material in bone substitution due to its resemblance to natural bone [22-26]. From a crystallographic point of view, hydroxyapatite belongs to the hexagonal crystalline system with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ [27].

HAP represents 65% of bone mass and provides most of its strength and rigidity. Improving the composition of hydroxyapatite with elements that are released in the natural developing bone, such as: Sr, Mg, Zn, Si can lead to beneficial effects on morphology, structure and mechanical properties. These ions help the development and proliferation of bone cells by ensuring osseointegration without side effects [28, 29].

Magnesium (Mg) is one of the elements that is found in a proportion of 60-65% in bones and teeth, the remaining 35-40% being spread in the nervous system, muscle tissues, body fluid and other tissues. It helps to maintain a stable and strong heart rate, ensures muscle contraction and expansion, reduces the risk of osteoporotic bone, and accelerates the process of natural bone regeneration [30-32]. Mg deficiency negatively affects all stages of skeletal metabolism causing the cessation of bone growth, decreased activity of osteoblasts and osteoclasts, as well as osteopenia and bone fragility [32-35].

Zinc (Zn) is known to be an element with stimulating effects on bone formation *in vivo* and *in vitro*. It inhibits bacterial growth in surgery and improves the healing process of wounds, facilitates bone formation by stimulating osteoblastic activity and reducing bone resorption of osteoclasts. It is known for its anti-inflammatory properties, activates osteoblast differentiation by enhancing alkaline phosphatase (ALP) activity and collagen control. It also prevents the differentiation of osteoclasts by suppressing the activation of the basal receptor of the NF-kß ligand with the nuclear factor (RANKL) which decreases the cellular absorption of RANKL. Zn deficiency leads to reduced bone density and ductility, which increases fracture rate [36 - 41].

Another important element is strontium (Sr) which can be useful in the treatment of fragile bone disorders, such as osteoporosis. It can also induce an antimicrobial response to help treat bone infections, such as osteomyelitis. An optimal concentration of Sr of around 3-7% improves osteoblastic activity and reduces osteoclast proliferation.

Strontium has been found to stimulate proliferation and differentiation from mesenchymal stem cells (MSCs) in osteoblasts by increasing the formation of new bone. It regulates ALP and type 1 collagen, which stimulates calcium deposition and bone nodule formation. Sr inhibits bone resorption by disrupting the actin-rich substance [38]. Sr facilitates cell attachment and proliferation [42, 43]. Another benefit of Sr is that it has dual effects of stimulating osteoblast differentiation and inhibiting osteoclast activity and bone resorption.

Silicon (Si) plays an important role in the early stages of bone formation. It is a cross linking agent in tissue connectivity and is important for vascular health. Bone metabolism is another important role where Si is involved. Soluble silicon has been reported to enhance osteoblast proliferation and cell differentiation, stimulates enzymes involved in type 1 collagen synthesis, and increases bone mineral density [44]. It also plays an important role in the health of the connective tissue, although its biochemical role remains unclear [45-48].

4. Biomimetic composites

Natural polymers are biologically active, unlike synthetic polymers, improve cell adhesion and growth, and are also biodegradable, allowing host cells to produce their own extracellular matrix by replacing the degraded backbone. The natural polymers used in tissue engineering are collagen, various polyglycans, arginate-based substrates and chitosan [49-52].

Collagen is a complex protein that has a repetitive sequence of amino acids: namely glycine, proline and hydroxyproline. More than 20 types of collagen have been identified, but type I and III collagen is the most abundant in nature. In the human body, collagen is found in the skin, tendons, cornea, cartilage and bones; its role is to ensure strength and structural stability [53-55].

Type I collagen is a major structural protein in bone, with beneficial properties in bone regeneration, such as biocompatibility, bone integration and cell proliferation [56]. It represents 89% of the organic matrix and 32% of the bone composition, with a significant potential for cell culture for bone production [53, 57].

It should be noted that the degradation process of synthetic and natural polymers did not alter the surface morphology and did not cause the formation of pores that could disturb the integrity of the metal [58]. Polymer coating can improve the biocompatibility of metal implants by reducing the amount of metal ions released from the metal substrate as well as improving the corrosion resistance [59].

The limitations of implants made from various materials presented above have led to the development of biomimetic composites, consisting of several phases. For example, different combinations of ceramic materials with natural or synthetic polymers were investigated, in order to improve their biological activity [53, 60]. Frequently, composite materials have better physical, chemical and mechanical properties than their constituent materials [61-64].

4.1. Hydroxiapatite, HAP, is used for bone tissue engineering

Hydroxyapatite is the mineral component of bones so it is of particular medical interest. This aspect is strongly linked to the metabolism of calcium in the body which allows the living organism to extract it from daily food and turn it into hydroxyapatite in the bones [65-68]. The process takes place naturally and easily during the growth period of the body, but once it reaches maturity it is more difficult, being even deficient in old age. From this point of view, the intake of hydroxyapatite of an exogenous nature to the human body, i.e. manufactured in the laboratory, could be beneficial [68-71].

Next, we will point out the main directions of research in this field in the literature. Figure 2 schematically shows the biomedical domains in which hydroxyapatite finds application. These areas derive directly from the HAP requirement of the living body. The first category that requires HAP intake is the bone system for bone replacement in the form of implants or bone filling as a supplement to missing portions caused by trauma. On the other hand, the very high content of HAP in dental enamel determines an important field, namely that dedicated to dental biomaterials. Last but not least, the functionalization of less body-friendly surfaces, e.g. of implants, creates a particularly important area for the deposition of biocompatible HAP-based coatings.

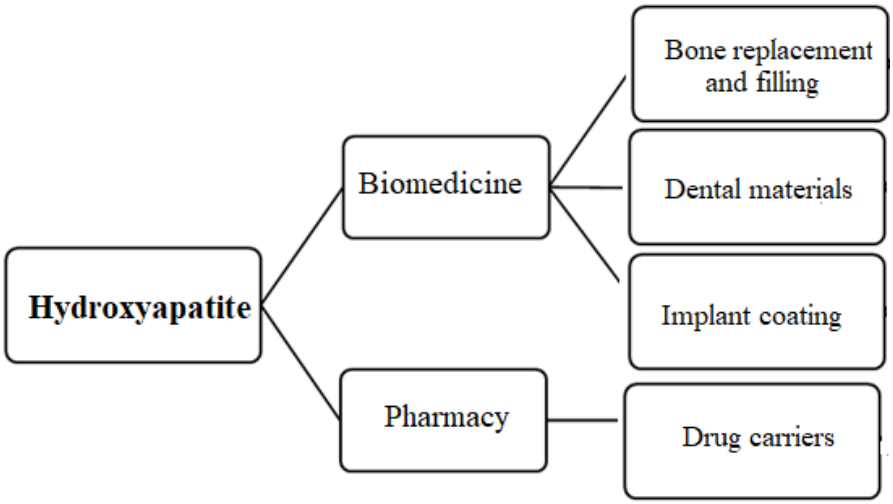

Figure 2. Applications of hydroxyapatites, HAPs, in biomedicine and pharmacy.

Hydroxyapatite multi-substituted with Mg, Zn, Si and Sr (ms-HAP = HAP-Mg-Zn-Si-Sr) were firstly synthesized in our laboratories by the wet precipitation method. Two solutions were prepared with different concentrations. The first solution had a concentration of cations Mg^{2+}, Zn^{2+} and Sr^{2+}. The second solution had a concentration of PO_4^{3-} and anions SiO_4^{4-} **[**72-74], resulting HAP-1,5%Mg-0,2%Zn-0,2%Si-5% Sr or *complex HAP* with *5% Sr*.

4.2. Physical and chemical characterization of ms-HAP

XRD analyses were performed using the XD8 ADVANCE X-ray diffractometer from Bruker AXS GmbH, Karlsruhe, Germany (Bragg-Brentano geometry) using Cu Kα radiation, wavelength 1.541874 Å, for step size of 0.02 at a scan speed of 2°/ min. The XRD spectrum recorded at angles 2θ between 10-95º (blue line) for the HAP-1,5%Mg-0,2%Zn-0,2%Si-5% Sr sample is compared with the powder diffraction file (PDF) 74-0566 corresponding to pure HAP hydroxyapatite (red lines) in Figure 3.

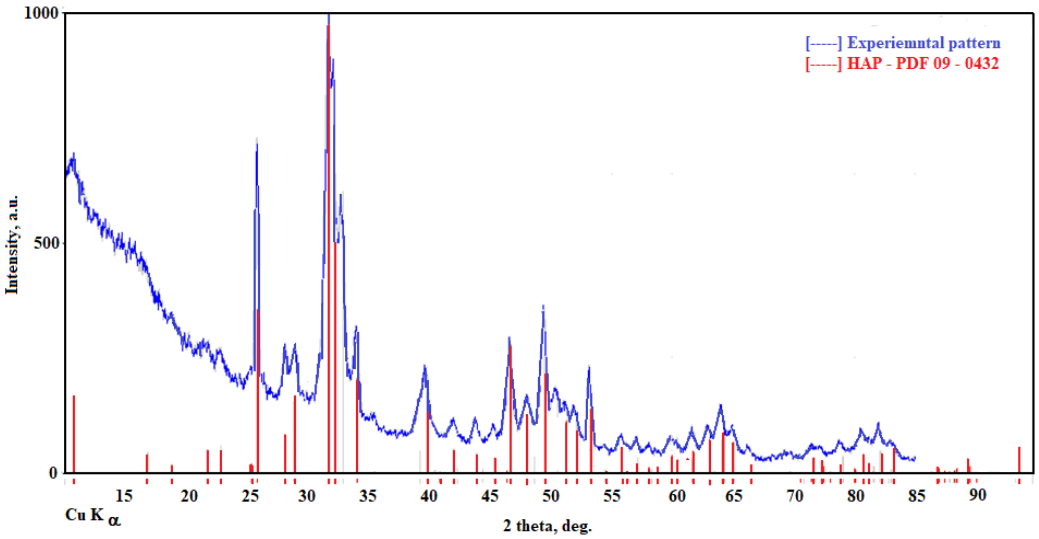

Figure 3. XRD spectrum for HAP-1,5%Mg-0,2%Zn-0,2%Si-5%Sr (blue line); pure hydroxyapatite, HAP, is presented with (red lines).

Bone replacement and filling requires the creation of a hydroxyapatite similar to that of bone as well as the formation of a composite material similar to bone, or ultimately biocompatible with native bone tissue. It consists mainly of the mineral component represented by HAP and the organic component represented by collagen [74-80].

The proportion of HAPs and collagen in bones differs depending on their specificity, influencing the fine microstructure and nanostructure of the bone [79]. Thurner et al. investigated with the help of atomic force microscopy (AFM) the structure of the cortical bone (massive bone) and of the trabecular bone (spongy bone), the trabeculae being the bone bridges that form the spongy structure.

The *in vitro* approach to the problem has led to bone generation on an inert biomedical surface. But bone filling and substitution requires the production of synthetic materials based on HAP and collagen that replicate as naturally as possible the natural bone so that they are successfully biointegrated into the living bone and ensuring osseoinduction and osseointegration. In general, this direction has been oriented towards the realization of ceramic implants based on hydroxyapatite [77, 78] which ensure a good mechanical resistance as well as a good osseointegration.

A special category of bone is the tooth. It has a complex structure with an outer layer of a very compact HAP nano-crystallite plate called enamel and the inside is made of a very robust and stress-resistant material made of HAP nanoparticles welded together by collagen fibers [79, 80].

Enamel is a very compact layer with an abrasion-resistant surface. If it is kept in good condition it is healthy and has a smooth and compact microstructure. If

the patient approaches poor oral hygiene, the acidic components of the food lead to enamel demineralization and the formation of depressions from which HAP nano-crystallites have been dislocated.

Dentin is a naturally occurring biocomposite made up of HAP nanoparticles strongly bonded together by collagen fibers [80 - 82]. It is traversed by a network of canals called dentinal tubules having a special role of increasing the mechanical resistance of the tooth to various mechanical shocks.

The dentin being located inside the tooth is relatively protected against the effect of acid erosion, but in the case of enamel lesions or in the case of dental caries, dentin appears exposed to demineralizing acid factors. Under the acidic action on HAP crystallites, they are dissolved in the structure of collagen fibers and are removed. This leads to the widening of the dentinal canals, with consequences that can be disastrous for the tooth. At first, dental sensitivity appears and then there may be acute pain that requires specialized medical treatment.

Therefore, HAP-based dental materials can be categorized into two large groups with different functional roles: - paste materials for the treatment of teeth with the role of cleaning and remineralization; - tooth enamel; - materials for endodontic implants and caries filling.

The prophylaxis of dental diseases is ensured by a correct dental hygiene which implies in the most rigorous case brushing with toothpaste after each meal, and in less rigorous cases 3 brushings daily are required: in the morning, in the afternoon and in the evening. If the subject performs less brushing or not at all, it will have poor oral hygiene and over time this will lead to chronic conditions related to acid erosion.

Here comes in the problem of the use of toothpaste for the treatment of demineralized portions. Specialist studies have shown that toothpastes with HAP nano dispersion are likely to remineralize tooth enamel [83 - 86].

In the case of major lesions on the enamel as well as dental caries, endodontic treatment is required. This is a very complex field in which a multitude of materials have been developed for filling the dental cavities. The data from the literature show that the use of HAP is an element of bio-activation of the materials utilized, making a more friendly interaction possible with the tooth tissue [87, 88].

Dental implant coating paves the way for the use of body-friendly materials for making implants, such as metal alloys: stainless steel or titanium alloys, coated with biomimetic materials to have superior mechanical and strength properties [89, 90]. Because of this, a top research direction is oriented towards the functionalization of these coating layers with layers containing HAPs and biologically active compounds that increase their biocompatibility, osseointegration and even promote local osteogeneration.

4.3. Development of biomimetic coatings for metallic implants based on HAPs and bioactive compounds

The literature has shown that biomimetic coatings contain hydroxyapatites substituted with zinc and strontium that help to produce bone tissue [91, 92]. The HAP and collagen composite has excellent biocompatibility and bioactivity, earlier bone remodeling around Ti rods, but it has a rather high fragility [93]. Therefore, the development of biomimetic coatings, for the activation of the surface of Ti (metallic) implants, is continued.

The 99.6% pure Ti, 20 mm long Ti rods were purchased from Goodfellow. To obtain the desired roughness, they were sanded with sandpaper. In order to clean these rods, a high-intensity ultrasonic processor Sonics Vibra-Cell, model VCX 750 (Sonics & Material Inc., Newtown, CT, USA) was used in deionized water at room temperature for 2 hours.

Atomic force microscopy determined the nano-roughness of the surface, ie the mean arithmetic value Ra 153±10 nm and calculated roughness as the root mean square, RMS 184±10 nm. The titanium rods were activated with 50% orthophosphoric acid then washed several times to remove the acid from the surface of the rod. The use of different concentrations of phosphoric acid for a set time and concentration of phosphoric acid increases the nano-roughness of the Ti surface in a controlled manner. Phosphate ions are formed on the Ti surface and can serve as an anchor for coating with the new biomimetic HAPc composite [94].

Figure 4 shows the scheme of coating on the titanium rods with the biomimetic composite. Separately we made a 4.8 percent PLA dispersion containing PLA and dichloromethane. From this dispersion we took a small amount and mixed it with acetone and with multi-substituted hydroxyapatite doped with collagen, resulting in the first dispersion which is rather diluted.

The chemically activated titanium rod, with orthophosphoric acid, was immersed in the first dispersion for three times successively with natural drying between the deposited layers using layer by layer (LbL) procedure resulting the first coating on Ti rods.

The second dispersion was obtained by mixing the PLA dispersion with multi-substituted hydroxyapatite doped with collagen (e.g., core-shell nanoparticles: ms-HAP/COL NPs). In this dispersion the Ti rods were immersed for three times with natural drying between the deposited layers, and the second coating, through layer by layer, on Ti implants was also obtained.

Finally, these composite-coated Ti rods were immersed in collagen solution, resulting in fibrous biocomposite-coated titanium rods, named also orthopedic (medical) implants.

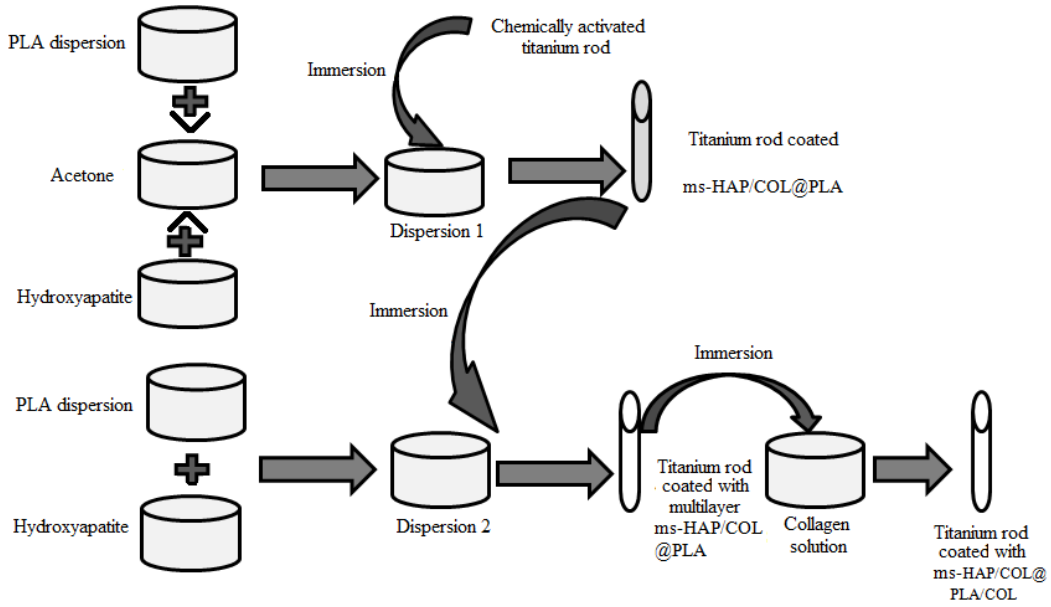

Figure 4. Scheme for the preparation of coated Ti implants with biomimetic material made of hydroxyapatite [e.g., ms-HAP/COL nanoparticles] embedded into PLA matrix and finally covered with COL fibers [i.e., ms-HAP/COL@PLA/COL].

4.4. Physical and chemical characterization of biomimetic coatings on Ti implants

The biomimetic coatings on Ti implants are made of HAPc composite comprising multi-substituted hydroxyapatite, ms-HAP: HAP-1.5% Mg-0.2% Zn-0.2% Si, collagen-coated nanoparticles (NPs), namely ms-HAP/6% COL (core/shell NPs), incorporated in matrix of poly lactic acid, PLA, (ms-HAP/COL @ PLA) and finally coated with a layer of COL (ms-HAP/COL @ PLA/COL). The biomimetic fibrous coating was deposited on the Ti surface by a self-assembly method, layer by layer (LbL) procedure, as demonstrated in Figure 4.

The surface of chemically activated titanium rods with orthophosphoric acid is examined by SEM image (Fig. 5A).

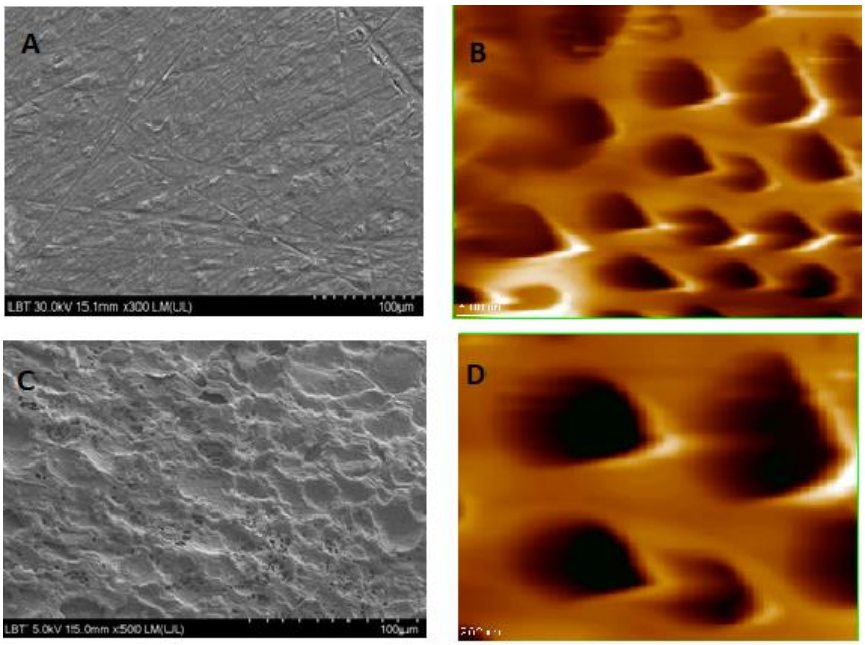

Figure 5. Ti implant surface, which is chemically activated (A, SEM image: scale bar of 100 μm), and coated with porous biomimetic composite (i.e., ms-HAP / COL @ PLA) as given in AFM image B: 2D-topography; scanned area: 20 μm x 20 μm; fibrous biomimetic composite (i.e., ms-HAP/COL@PLA/COL) is given in C: SEM image, scale bar of 100 μm; D represents AFM image: 2D topography, on the scanned area: 10 μm x 10 μm of porous coating, also given in AFM image, B.

The AFM characterization of the titanium surface coated with the porous biomimetic composite can be seen in Figure 5 (B and D) at different scanning areas, as shown in the legend of Figure 5. A network of pore structure evidenced in Figure 5 (B and D) is useful to facilitate the adhesion of osteoblasts to the coated surface of the Ti implant. The process of drying of the biocomposite by slow evaporation of dichloromethane (DCM) generates a mosaic of submicron and micron sized pores. Figure 5D shows a denser structure around the pores making a connection between the ms-HAP crystals with COL and PLA.

Figure 5C shows the surface of the biocomposite on titanium rod investigated by SEM. It can be seen a very rough structure in the form of a lace, which also contains collagen fibers. In some places, the compact structure of PLA, which binds nanoparticles to hydroxyapatite and collagen fibers, can also be seen.

The formation of the network of collagen fibers on the surface of the HAPc/6%COL@PLA/COL composite ensures the major transformation from a simple nano-composite material into a biomimetic structure, Figure 5C.

AFM exploring the surface of HAPc/6% COL@PLA/COL coating reveals self-assembled collagen fibers on the surface. This coating on the Ti implant is recently used in *in vitro* studies using a stem cell culture.

They adhere to the surface of the biomimetic composite, increasing the biocompatibility of the entire coating surface on the Ti implant. This method aims to achieve complex deposition layers that combine HAP nanoparticles with biocompatible binding components such as collagen [95-99].

We made coatings with ms-HAP embedded in the PLA polymer matrix which were analyzed by atomic force microscopy (AFM) using AFM JEOL 4210 equipment (JOEL GmbH, Freising, Germany), operated in tapping mode, [100-112] using standard cantilevers with silicon nitride peaks (resonant frequency in the range 200–300 kHz and elasticity constant 17.5 N / m).

Figure 5(B and D) and Figure 6 show a porous structure, which is conductive to the proliferation of osteoblast cells. The profile drawn through such a row of pores, selecting one pore of them, shows an approximately parabolic shape of the pore (Figure 6C) with an average depth of about 1000 nm. The surface roughness of porous HAPc/6%COL@PLA structure (Figure 5B) is relatively high [97].

The detail on a single pore can give the depth of the pore which is about 1 μm as presented in Figure 6.

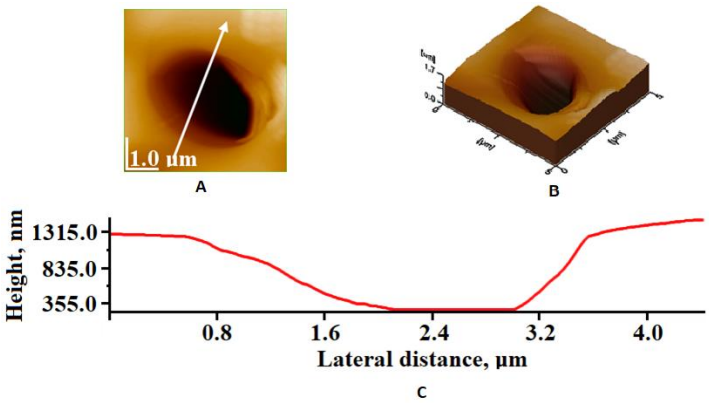

Figure 6. AFM images for Ti rod coated with HAPc/6% COL@PLA: A) topographic image, B) 3D image, and C) the profile along the white arrow in the image (A); scanned area: 5 μm x 5 μm.

The fibrous biomimetic composite HAPc/6%COL@PLA/COL surface characterized by SEM image (Figure 5C) is also visualized by AFM in Figure 7, at a scanned area of 2 μm x 2 μm. The surface 2D-topography (Figure 7A) shows a rather well developed COL fiber that is formed on the biomimetic composite surface, given in Figure 7(B, C, D). The collagen fiber is well developed with the tropocolagen rings very evident in the cross profile (Figure 7E). The COL fiber has a diameter of about 500 nm as shown in Figure 7F.

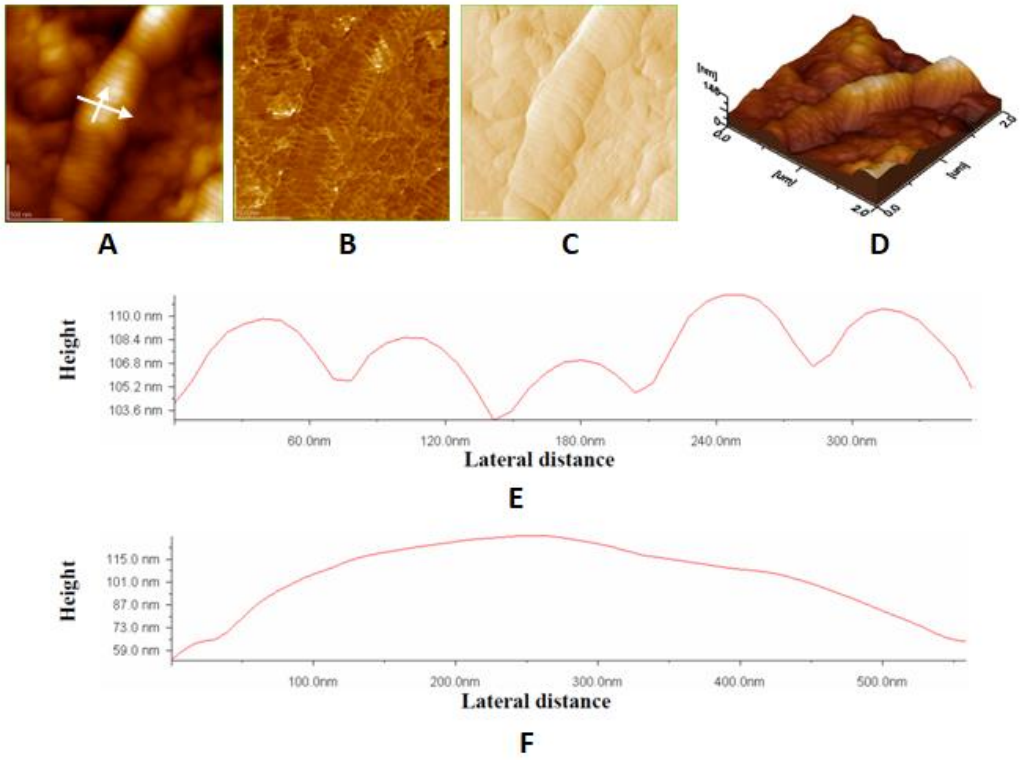

Figure 7. AFM images on the surface of Ti rod coated with fibrous biomimetic composite HAPc/6%COL@PLA/COL: A) topographic image; B) phase image; C) amplitude image; D) 3D image; cross profiles along the white arrows in image (A): on the long axis of COL fiber (E); and perpendicular on COL fiber axis (F); scanned area: 2 μm x 2 μm.

The phase image, Figure 7B, captures in more detail the structuring of the collagen fiber effectively highlighting the tropocollagen units that make up this fiber. The amplitude image in Figure 7C and the three-dimensional image in Figure 7D prove the outstanding quality of the scaffold surface.

The drawn profiles, Figure 7(E, F), confirm on the one hand the diameter of 500 nm of the COL fiber (Figure 7F) and on the other hand that the diameter of the tropocollagen rings (units) is about 65 nm (Figure 7E), which is a known value in accordance with the related data [112].

5. Studies of orthopedic (medical) implants *in vivo*

Biomimetic composites were studied *in vivo* to evaluate the biocompatibility and osseointegration [112-114]: Ti nails uncoated and coated with biomimetic composites in a rat model with a femoral fracture. Rat experiments with femoral fracture were chosen because most fractures occur in elderly patients with advanced osteoporosis of the femur [112].

With these experiments we want to make improvements in the osseointegration of Ti implants in the case of the at-risk population category. COL-based composites can increase cell adhesion, playing an important role in callus formation and fracture healing [115,116]. The role of HAPc compounds may be to increase the ability of cells to heal bone fractures in vivo. These composites can release essential ions from the ms-HAP nanoparticles embedded in the coating.

The most commonly used noninvasive methods for bone consolidation are pulsating electromagnetic field (PEMF) stimulators [117–119]. PEMF therapy presents various characteristics and ways to deliver energy, so that it can stimulate different pathways of bone formation and osteoblastogenesis [120, 121]. Osteogenic differentiation on murine precursor cells can be stimulated by high-frequency pulsed electromagnetic fields [122].

In vivo assessment of bone consolidation was performed on uncoated (Ti) and coated with biomimetic composite noted for simplicity as HAPc on Ti implants along with stimulation by high frequency pulsed electromagnetic short-waves (HF-PESW).

By making biomimetic coatings we pursued 2 objectives: 1) testing their quality and efficiency, and 2) *in vivo* testing of Ti implants coated with biomimetic HAPc composite versus Ti uncoated implants, both in the absence and in the presence of high-frequency pulsed electromagnetic short waves HF-PESW, which is biophysical stimulation.

In vivo experiments were performed on 24 albino Wistar rats divided into 2 groups, with 12 in each group. Surgical procedures resulted in a transverse fracture of the femoral shaft through a lateral approach to the thigh. Through the longitudinal incision at the knee, the implants were inserted into animals.

The rats were 2 months old and weighed about 226±13 g; they were general anesthetized using an intramuscular cocktail of xylazin 2% and ketamine 10%. All procedures were made by a team of two orthopedic surgeons. After realizing surgical asepsis, a transversal fracture of the femur diaphysis was produced by a lateral approach of the thigh. By a longitudinal incision at knee level Ti implants were introduced: uncoated (CG, N=12) and coated with biomimetic HAPc: ms-HAP/COL@PLA/COL (HAPc group, N=12) in the left femur.

Finally, the subcutaneous layer and the tegument were sutured. Postoperative, the animals were kept in cages under controlled environment at room temperature of 22°C with 12 hours day/night cycle, without food restrictions. The animals were euthanized using anesthetic over-dose, after 2 weeks (N = 12, N = 6/group) and after 8 weeks (N=12, N = 6/group); afterwards, the left femoral bone was carefully harvested, by the push-out method, for not to disrupt the bone callus, and then placed in 10% formaldehyde.

Markers of bone formation (Figure 8), osteocalcin (OCN) and alkaline phosphatase (ALP), were evaluated using the non-specific ELISA kit ER1205, Rat OC/BGP (Osteocalcin), commercially available (Wuhan Fine Biological Technology Co) and OSR6504 reagent (alkaline phosphatase), for use on the AU 680 (Beckman Coulter, USA).

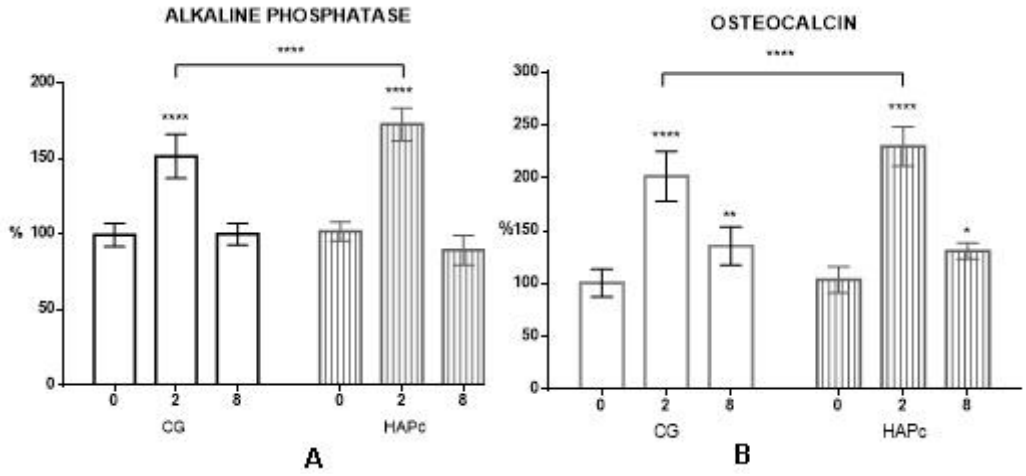

Figure 8. Bone markers, alkaline phosphatase (A) and osteocalcin (B), serum concentration at zero time (initially), two- and eight-weeks post-surgery; *statistically significant $p<0.05$; **statistically significant $p<0.01$; ***statistically significant $p<0.001$; ****statistically significant $p<0.0001$

An increase of alkaline phosphatase (ALP) (Figure 8A, Table 1) was observed in both groups at 2 weeks against the initial values ($p<0.0001$). The most significant increase was in the HAPc group (173%), as compared with CG (152%), $p<0,001$. After 8 weeks, ALP values decrease ($p<0.0001$), with concentrations in the bone serum equal or even lower against the initial values (0 weeks).

The osteocalcin values (OCN) (Figure 8B, Table 1) increased substantially in both groups against the initial values, with the highest increase, 230%, in the HAPc group.

Moreover, values in the control group, CG, after two weeks were significantly lower than for the HAPc grup ($p<0.0001$). Afterwards, at eight weeks, the OCN expression decreased significantly remaining with 31-36% over the initial value ($p <0.05$).

Table 1. Bone markers, alkaline phosphatase (ALP) and osteocalcin (OCN), serum concentration at initial (0 weeks), two- and eight-weeks post-operatively; *statistically significant $p<0.05$; **statistically significant $p<0.01$; ***statistically significant $p<0.001$; ****statistically significant $p<0.0001$; CG, control group, with uncoated Ti implants; HAPc: group with medical Ti implants coated with fibrous biomimetic composite.

Rat group		**CG**	**HAPc**
ALP (%)	0 weeks	100 ± 13	102 ±6
	2 weeks	152 ±14****	173 ± 10****
	8 weeks	100 ± 7	89 ± 8
OCN (%)	0 weeks	100 ± 15	104 ± 13
	2 weeks	202 ± 24****	230 ± 18****
	8 weeks	136 ± 18**	131 ± 8*

A multi-level threshold was applied to discriminate calcified bone and cartilage, dense cortical bone, and unmineralized tissue. At the time of slaughter, the left femoral bone with implants (N = 12/group) was scanned using the Bruker micro-CT SkyScan 1172 system (Kontich, Belgium).

The volume of interest (VOI) was set at 2 mm below the growth plate with a 1 mm in height and a 1.5 mm diameter ring around the implant, after which the percentage of bone volume (BV/TV) and the average trabecular number (Tb.N) in the volume of interest were determined (see, Table 2).

Table 2. Implant osseointegration assessed by micro-CT; bone volume per total tissue volume (BV/TV) and the mean trabecular number (Tb.N); *$p < 0.05$: HAPc group vs CG group; HAPc: group with orthopaedic (medical) Ti implants coated with fibrous biomimetic composite; CG: control group with uncoated Ti implants.

Rat group	CG	HAPc
BV/TV(%)	25.5±4.3	38.8±5.4*
Tb.N (1/mm)	154±18	180±18*

The biocompatibility of uncovered and coated Ti implants and their osseointegration at the fracture level were evaluated using light microscopy, on tissue samples stained with hematoxylin and eosin (H&E) from each of the two groups of animals studied, at eight-weeks postoperatively [98, 99, 112].

After eight weeks implantation the control group revealed fibrous tissue near the intramedullary Ti implant and residual cartilaginous tissue, indicating a transition from cartilaginous precursors to the incipient formation of bone trabeculae.

The group showed well-defined bone trabeculae around the HAPc Ti implants, compact bone with lamellar arrangement of the bone matrix and osteocytes around the Haversian canals, with osteoblasts covering their surface and a clear delimitation of the areola between the trabeculae and the areas of the compact lamellar bone.

6. Nanoparticles interaction with biological membranes and living system

Postoperative implant infections in surgery can lead to death. Bacteria can form biofilm on the surface of the implant by stopping antibiotics from penetrating and developing antimicrobial functions.

For this reason, advanced nanomaterials resistant to infections must be introduced. The antimicrobial effect of silver nanoparticles has been demonstrated since ancient times, and has been widely used in medicine. Recently, increased attention has been paid to modifying the orthopedic implant with silver nanoparticles [123-125].

Nanoparticles can mediate molecular processes and interact with living cells (e.g. membrane cell) [126]. Langmuir Blodgett method can best study the passage of NPs across the cell membrane.

The various self-assemblies can be described by Langmuir-Blodgett models, LBM, of molecules at air-water [127-140] and oil-water [141-147] interfaces, specifically fatty acids [138, 139, 148-152], galactolipids and carotenoids supramolecular structures [153-172], as well as gold nanoparticles functionalized with biomolecules [173, 174].

Conclusions

The developing of fibrous biomimetic coating onto the Ti surface proves to be a smart choice to enhance the osseointegration and ensure an optimal healing process, due to the creation of nanostructured biomaterials similar to those in native bone. Thus, we designed and prepared a composite coating (noted HAPc) based on multi-substituted hydroxyapatite (noted ms-HAP) nanoparticles, NPs, doped with essential elements: Mg, Zn and Si, functionalized with collagen type 1 (COL), embedded into poly lactic acid, PLA, matrix, and finally covered with COL layer to achieve biomimetic structures.

Thin layers of biomimetic composite were self-assembled onto Ti surface via dip-coating method. Both, initial and coated Ti implants were investigated by atomic force microscopy (AFM), which allows surface investigation at high resolution of nano-level.

The biocompatibility of uncovered Ti implants, and coated with innovative biocomposite was assessed on a rat model of femoral fracture. The biocomposite is based on multi-substituted hydroxyapatite, ms-HAP, containing Mg, Zn and Si, and is used as a coating material deposited on Ti implants, due to the excellent biocompatibility and osteoconductive property of ms-HAP.

The use of HAPc-coated Ti implants together with HF-PESW stimulation positively influenced the bone consolidation process, especially in its early phase.

This *in vivo* evaluation demonstrated that the association between HF-PESW stimulation and biomimetic HAPc coating on Ti implants promotes an accelerated healing process of bone fracture, enhancing bone consolidation in its early phase. Consequently, this combined method is potentially interesting and useful for clinical applications, providing a superior approach to the surface modification of biomedical implants.

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