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 REVIEW

Biomimetic and antibacterial composite for orthopedic implants

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Abstract. *The present paper shows how the development of synthetic nanostructured biomaterials, such as multisubstituted hydroxyapatite (msHAP) with Mg^{2+}, Zn^{2+} and Sr^{2+} ions is important and beneficial at the same time for the normal functioning of the body. Moreover, the paper discusses a broad topic of major importance in orthopedic and dental surgery, namely the incorporation of msHAP into the polymeric matrix of poly lactic acid (PLA). This composite is used in order to cover the surface of the titanium implant in order to obtain bone integration and heal bone fractures. The review also highlights the importance of improving silver nanoparticle (SNPs) coating in order to combat postoperative infections. Through such approaches, medicine has managed to evolve considerably, and the contributions brought by great personalities and young researchers in the field have increased its area of applicability.*

Keywords: multi-functional hydroxyapatites, orthopedic (medical) implants, silver nanoparticles

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1. Introduction

Materials science is a vast and intensely studied field as evidenced by the advances made in medicine. Composite materials have managed to bring surprising benefits in orthopedic and dental surgery by meeting the most important criteria of biocompatibility and osseointegration. These properties allow doctors to solve problems with implants [1].

Bone is a most complex and well-organized structure. It is a very well developed system, made up of bone components with the role of protection of organs and soft tissues, ensures mechanical resistance and allows the mobility of the whole body [2]. In terms of chemical composition, the major component is the inorganic phase containing the most stable form of calcium phosphate, hydroxyapatite. The organic phase such as collagen, water and other components are also present. Bone hardness is given by hydroxyapatite and the necessary flexibility is provided by collagen fibers. Bone contains essential minerals calcium and phosphorus that ensure bone reshaping when injuries are likely to allow it. In the case of severe bone diseases due to imbalances in the body or

serious injury, the natural healing capacity is insufficient. In this case healing can be achieved through surgery with metal implants coated with biomaterials or composite materials based on hydroxyapatite [3-7].

2. Types of implants

Metal implants can be made of: stainless steel, tantalum, magnesium-based alloys, cobalt-chromium-based alloys, titanium and its alloys. These metal implants are biocompatible and commonly used in orthopedic and dental surgery, and are accepted by the US Food and Drug Administration (FDA). The most common implant models are rods, plates, screws, and pins [8 - 14]. The main properties of the implants of interest are: high strength, low density, high resistance to corrosion and chemical attack of physiological fluids, complete inertia in the body, generation of non-inflammatory response of the host tissue, non-toxic, non-carcinogenic properties and not to cause allergic or immunological reactions, low modulus of elasticity [15-17]. The metal implant must be a material with a high strength and low modulus of elasticity, compared to the bone marrow.

The physico-chemical properties of an implant are important in order to assure that they are not rejected by the body. From a mechanical point of view, metal alloys must be resistant to compression and have a tensile strength to ensure the required stability. They also need resistance to the local force to which they are subjected to prevent rupture by implant fragility under cyclic loading. The modulus of elasticity of the bone varies from 4 to 30 GPa, depending on the bone.

Stainless steel and cobalt-chromium alloys have a much higher modulus of elasticity than bone, leading to bone resorption and implant weakening a few years after implantation. The modulus of elasticity of stainless steel is the largest, followed by cobalt-chromium alloys, tantalum, titanium alloys and magnesium alloys [15, 18-23]. Because metal biofunctionality is inadequate, metal implants need improvement. The major problem is related to the strength and adhesion between the metal substrate and the coating. The materials used as implants must be non-toxic and not cause allergic or inflammatory reactions in the human body. The success of the implant depends on the reaction of the foreign body, defined by biocompatibility. The selection of titanium-based materials for implantation is due to the combination of its special properties with its high ability to bind to the surrounding bone and tissues [15, 24-25].

The most commonly used implant now and in the 1960s is titanium. The current focus on it is due to its excellent compatibility, non-allergic nature, high mechanical properties, corrosion and wear resistance, chemical stability and very good bicombatibility [26]. Pure titanium and its alloys are advantageous materials compared to other metals that can be used as implants, as can be seen from their properties, but also from the fact that some devices such as wheelchairs, artificial limbs, etc. can be by using them [15-17, 27]. Titanium undergoes an allotropic transformation which means that it can change from one crystallographic form to another. At room temperature, it exists in a compact hexagonal structure and this is called the alpha (α) phase which at a high temperature, 883 ° C, changes into a centered cubic structure called the β phase. The temperature at which α or α + β changes in all β is called the β transus temperature [26, 28].

Pure commercial titanium (Ti) is among the most biocompatible metals due to its ability to form an inert and chemically stable oxide layer [29, 30].

The most important factors that characterize titanium and its alloys include a low level of electrical conductivity, high corrosion resistance, thermodynamic stability at physiological pH values, low tendency to form water-soluble ions and the isoelectric point of the oxide at pH between 5 and 6. Thus, the surface protected by the oxidized layer has only a slightly negatively charge at physiological pH. The dielectric constant of titanium is comparable to that of water, which makes the Coulomb interactions of charged particles similar to those in water [30].

Research has shown that phosphoric acid-activated titanium surfaces improve implant osseointegration by stimulating attachment to surfaces by stronger implant fixation [31].

The surface of the titanium samples was investigated by atomic force microscopy (AFM) using an JEOL JSPM 4210 Scanning Probe Microscope, Akishima, Tokyo, Japan equipment. The images were obtained in tapping mode, [32-34] using NSC15 Hard cantilevers with resonance frequency 325 kHz and force constant 40 N/m. The cantilevers were produced by MikroMasch company, Sofia, Bulgaria. All the AFM images was processing through the software: Jeol WinSPM 2.0, Tokyo, Japan. The surface roughness of Ti implants was expressed as the arithmetic mean, Ra and the root mean square, RMS, and was estimated using AFM, to have a roughness Ra 154 nm and Rq = 186 nm (Figure 1).

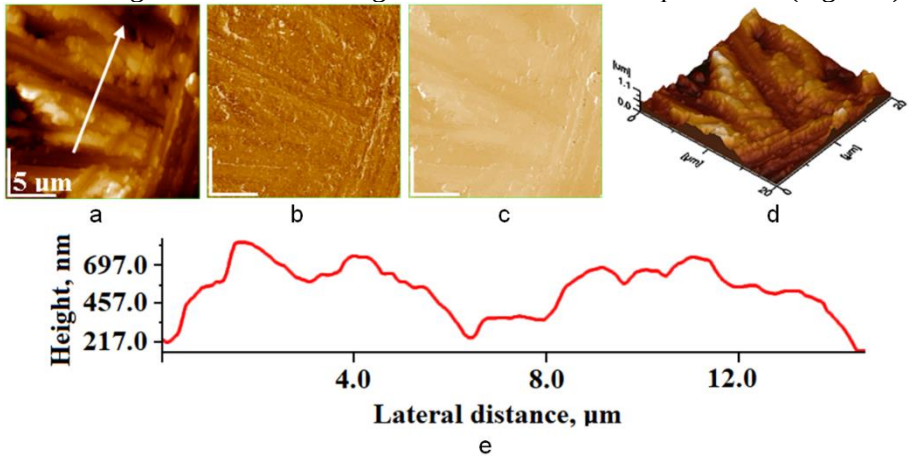

Figure 1. AFM images of Ti polished with P500: a) topographic image, b) phase image, c) amplitude image, d) 3D image and e) profile along the arrow in panel (a). Scanned area 20 µm x 20 µm; Ra = 154 nm; Rq = 186 nm

Table 1. Surface roughness of Ti implants before coating evaluated by AFM.

Ti	***Ti*** ***Cold pressed***		***Ti*** ***Grinding***		***Ti*** ***Grinding and acid etching***	
Fig.	Ra±SD	RMS±SD	Ra±SD	RMS±SD	Ra±SD	RMS±SD
-	240±25	305±30	-	-	-	-
1	-	-	154±14	186±19	-	-
-	-	-	-	-	196±18	244±20

3. Implants made of biocomposites

The biocompatibility of a material is determined by in vivo and in vitro tests, which involve the interaction of the material with biological fluids and cells [35]. Various methods of coating metal implants have been developed to improve the healing process. Hydroxyapatite coating is one of the most widely used surface modification techniques [17,36-39]. Hydroxyapatite has been shown to promote faster bone regeneration compared to uncovered titanium [40, 41].

As discussed in motivating the importance of choosing this research topic, HAP is extremely important for the health of the bone system but also for the development of modern treatments for the orthopedic field. That is why it is necessary to summarize the most important aspects starting from its structure and properties to the specific applications highlighted by the specialized literature.

Hydroxyapatite (HAP) is a phosphate class mineral with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. It occurs naturally in rocks rich in apatite and crystallizes in the hexagonal system. The direct implications of its complex and spatially ordered structure are evidenced in the X-ray diffraction spectra causing the appearance of many diffraction maxima with a slender appearance and relatively high intensities. X-ray diffraction data are very important for the crystallographic-mineralogical characterization of hydroxyapatite. Therefore, in Table 1.1. X-ray diffraction data for pure hydroxyapatite are shown as determined in JCPDS laboratories [42, 43].

Table 1.1. X-ray diffraction data for pure HAP

Nr. crt.	***2 theta, deg.***	***Lattice constant, d, Å***	***Relative intensity, a.u.***	***Miller Indices, h k l***
1	10.8406	8.1614	173	1 0 0
2	25.9048	3.4395	353	0 0 2
3	28.9456	3.0847	160	2 1 0
4	31.7927	2.8146	999	2 1 1
5	32.2226	2.7781	519	1 1 2
6	32.9245	2.7204	608	3 0 0
7	34.0921	2.6299	208	2 0 2
8	39.8249	2.2635	200	1 3 0
9	46.7353	1.9437	281	2 2 2
10	48.1236	1.8908	123	1 3 2
11	49.5336	1.8402	312	2 1 3
12	50.5200	1.8066	160	3 2 1
13	51.3002	1.7809	116	1 4 0
14	52.1216	1.7548	118	4 0 2
15	53.2673	1.7197	139	0 0 4

The dominant maximum (relative intensity taken as 1000) appears for the diffraction plane (2 1 1) with the following relevant maxima absolutely necessary for the identification of the HAP in a mixed composition being for the planes (3 0 0) and (1 1 2).

Data from the literature show that hydroxyl groups in the HAP structure can be replaced by other groups such as chloride or fluoride leading to the formation of

chloroapatite and fluorapatite, respectively. While these changes occur frequently in nature due to geological conditions in different mineral layers, synthetic hydroxyapatites can be obtained in the laboratory in which certain atoms can be replaced with doping elements [43].

4. Multisubstituted hydroxyapatites (msHAP)

For example, strontium is an atomic species used for doping hydroxyapatite for biomedical purposes [44 - 46]. Ca^{2+} ions in the HAP structure are replaced by Sr^{2+} ions.

Strontium doping enhances the bioactivity of hydroxyapatite by helping to increase the activity of osteoblasts [47] and decrease bone resorption by its action on osteoclasts [48], which recommends them for orthopedic applications [45, 46]. Table 1.2 compares the theoretical composition of Sr-substituted HAP with experimental ones.

Table 1.2. Atomic ratios (Ca + Sr) / P and Sr / (Ca + Sr) according to reference [44]

Content by Sr, % mol	***(Ca+Sr)/P***	***Sr/(Ca+Sr)***	***(Ca+Sr)/P***	***Sr/(Ca+Sr)***
	Nominal	***nominal***	***measured***	***measured***
0	1.67	-	1.68	-
1	1.67	0.010	1.67	0.011
5	1.67	0.050	1.67	0.058
10	1.67	0.100	1.69	0.112
15	1.67	0.150	1.70	0.169

Strontium atoms have an atomic radius of 215 pm, so bigger than calcium atoms that are only 176 pm, and will determine the elongation of the parameters of the crystalline lattice of the HAP. This increase found by the indexing of X-ray diffraction spectra is shown in specialized literature. Some values given by the literature are presented in table 1.3 reference [44].

Table 1.3. The variation of the crystallographic parameters of HAP-Sr depending on the amount of Sr

Crystallographic parameters, Å	***Strontium content, %***				
	1	***5***	***10***	***15***	***20***
a	9.4347	a	9.4347	a	9.4347
c	6.8893	c	6.8893	c	6.8893

Zinc hydroxyapatite doping is also very common in the literature due to the net improvement of certain bioactive properties. It contributes to the inhibition of osteoclast cells [49] and increases the response of osteoblast cells [50]. On the other hand, it intensifies the increase of collagen content and helps to obtain nano-scale biomedical materials [51, 52]. It should be noted that zinc has been reported to have anti-inflammatory [53] and antimicrobial [54] effects in the structure of HAP substituted with zinc.

From a structural point of view, it is very important to observe the variation of the crystallographic parameters depending on the zinc content, table 1.4.

Table 1.4. Variation of crystallographic parameters of HAP depending on the amount of Zn [55]

Crystallographic parameters, nm	***Zinc content, %***				
	0	***5***	***10***	***15***	***20***
a	0.9373	0.9422	0.9387	0.9397	0.9509
c	0.6887	0.6855	0.6829	0.6854	0.6836

Given the atomic radius of zinc, 134 pm, we would expect the values of the parameters of the crystal lattice of doped HAP to decrease with increasing Zn content. However, analyzes in the literature show an increase in the parameter a combined with a decrease in the value of the c-axis.

Advanced X-ray diffraction analyzes have shown that the contractions of the crystal lattice are due not so much to the replacement of Ca atoms with those of Zn but to the accumulation of structural defects [50]. Therefore, it is estimated that the limit of acceptability of Zn in HAP without leading to the segregation of new phases falls in the range of 5 - 15% Zn.

Magnesium is an important calcium ion substitute in hydroxyapatite, which is found in bones and teeth. It brings fundamental benefits through its involvement in bone growth and remodeling by activating osteoblast cells. Magnesium deficiency affects the transformations that take place in the bone matrix, therefore they become fragile and the risk of developing aosteoporosis is increased [56, 57].

Silicon present in the structure of hydroxyapatite intensifies the bioactivity of osteoblasts [57, 58] which means that bone regeneration takes place much faster. The involvement of silicon in the structure of HAP s also helps in the development of bone tissue and improved cell adhesion to pure hydroxyapatite [59].

4.1. Applications of HAP in biomedicine

Hydroxyapatite is the mineral component of bones so it is of particular medical interest. From a qualitative point of view, is strongly linked to the metabolism of calcium in the body which allows the living organism to extract it from the daily food and turn it into hydroxyapatite in the bones [61-63]. The process is natural and easy during the growth 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, ie manufactured in the laboratory, could be beneficial. Next we will point out the main directions of research in this field in the literature [60, 64-66].

Figure 2 schematically shows the biomedical fields in which hydroxyapatite has applications. These areas derive directly from the HAP requirement of the living body. The first category that requires a HAP contribution is the bone system for bone replacement in the form of implants or bone fill as a supplement to missing portions caused by trauma.

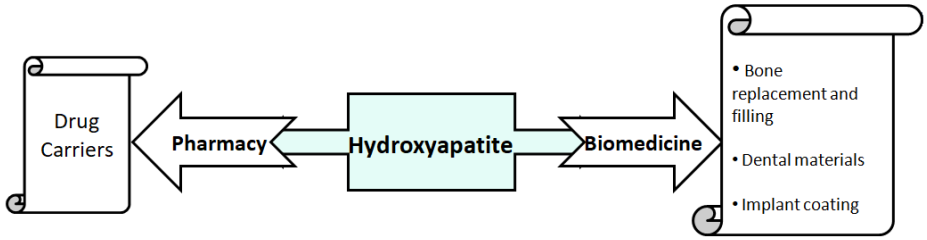

Figure 2. Fields of application of hydroxyapatites

On the other hand, the very high content of HAP s in dental enamel determines an important field, namely that dedicated to dental biomaterials. Last but not least, the functionalization of less environmentally friendly living surfaces creates a particularly important area for the deposition of biocompatible HAP -based coatings [67-69].

4.2. Ions release from hydroxyapatite

At the Babes-Bolyai University Physical Chemistry Center, the Faculty of Chemistry and Chemical Engineering has prepared a multi-substituted hydroxyapatite with the above-mentioned elements to demonstrate the release of ions in both water and SBF.

For the synthesis of hydroxyapatites 2 solutions were prepared. The first solution containing the cations Ca^{2+}, Mg^{2+}, Zn^{2+} and Sr^{2+} , with a total concentration of 0.25 M was prepared by dissolving nitrates in ultrapure water: $Ca(NO_3)_2 \cdot 4H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$ and $Sr(NO_3)_2$. The second solution contains the anions PO_4^{3-} and (for complex HAPs) SiO_4^{4-} with a total concentration of 0.15 M. The solution was obtained from diammonium hydrogen phosphate, $(NH_4)_2HPO_4$ and tetraethyl orthosilicate, TEOS, $Si(OC_2H_5)_4$ (98%) in an adequate ratio. The pH of the solutions was 11.5, fixed by adding a 25% ammonia solution. The equal volumes of solutions were mixed rapidly at room temperature, (22°C), using a peristaltic pump and a "Y" type impact reactor for the two liquid flows. The suspension was matured in two stages: at 22°C for 24 hours and at 70°C for another 24 hours, with intermittent stirring. The final precipitate was filtered and washed repeatedly with ultrapure water at room temperature until nitrates were removed. The drying process was performed by lyophilization followed by the calcination step at 300°C for one hour and the sample was disintegrated in a ball mill to obtain a fine powder [70-73].

FTIR and XRD spectroscopy established the single crystalline phase of HAP, and its chemical composition demonstrated by SEM-EDX.

The release of Ca, P, Si and Mg ions in water and in simulated body fluid (SBF) was monitored over time, from 1 to 90 days, using inductively coupled plasma optical emission spectrometry (ICP-OES). The amounts of Ca, P, Si and Mg in solutions were measured after immersion of 0.15 g of each sample in 15 ml of ultrapure water, respectively in simulated body fluid Kokubo (SBF) and incubation at 37°C in separate closed ampoules for each trial/day. After 1, 3, 7, 14, 21, 30, 60 and 90 days, the supernatant (after centrifugation) was filtered. For calibration, standard multi-element solutions were prepared by diluting Merck IV multi-element stock solutions to 1000 mg/L. The Zn content in the aqueous phase was below the detection limit for all samples.

A release of ions into water can be observed (Figure 3), for substituted hydroxyapatites, the amount of Ca released is higher than from pure HAP and very similar for all complex hydroxyapatites [71-81].

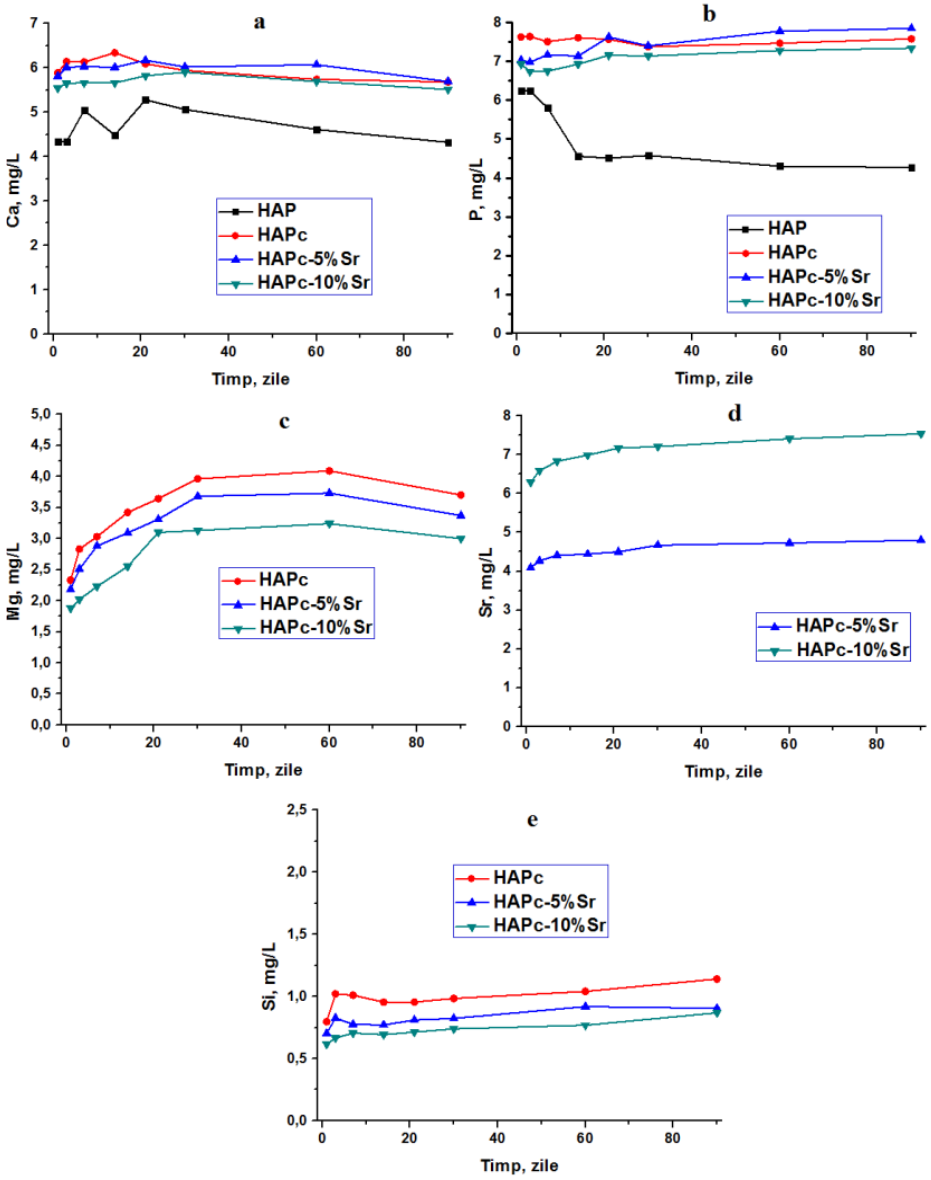

Figure 3. Release of ions in water after immersion of hydroxyapatite samples for 1 - 90 days: a) calcium, b) phosphorus, c) magnesium, d) strontium and e) silicon [71].

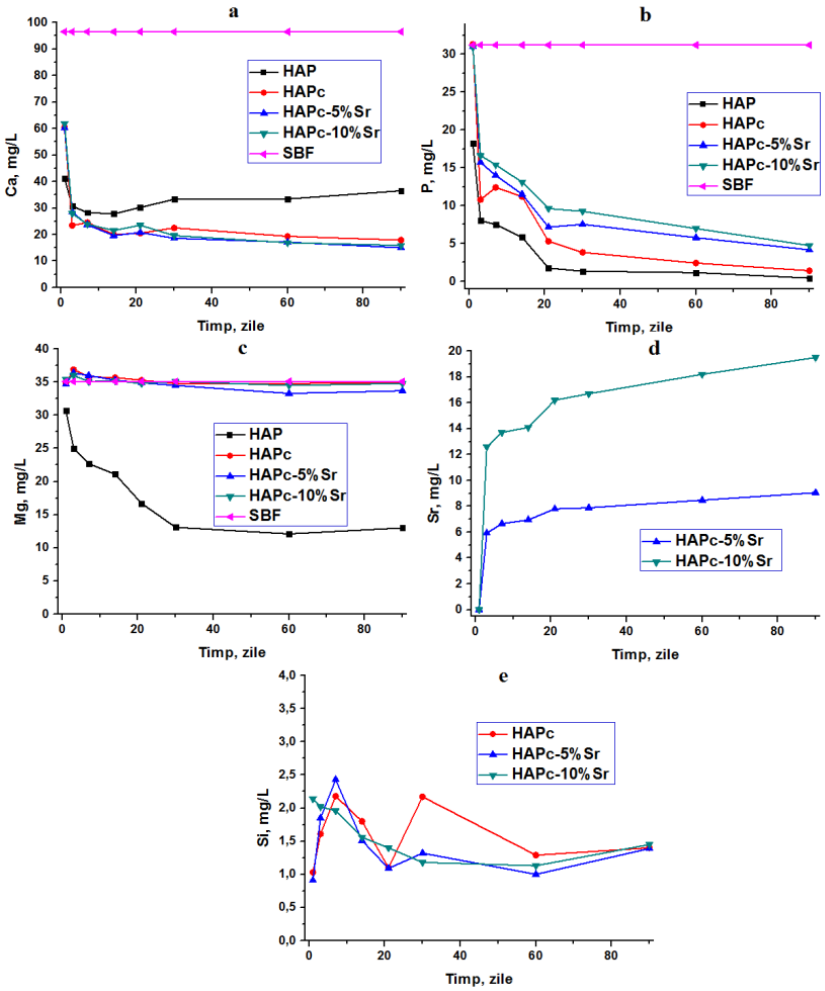

Figure 4. The ion content in SBF after immersion of hydroxyapatite samples for 1 day to 90 days: a) calcium, b) phosphorus, c) magnesium, d) strontium and e) silicon [71].

Multisubstituted hydroxyapatites with a long release of ions could be used as resorbable fillers for bone defects or even as coatings, as these particles could release ions for a longer period of time which can be beneficial for the formation of new bones. [82].

Implant coverage has many benefits, such as pain reduction and revision surgery, eliminating the need for a cemented implant with a shorter recovery period. Hydroxyapatite coatings have also been shown to increase implant life [83].

Coating the implant with multisubstituted hydroxyapatite improves protein adsorption, promotes cell proliferation and bone formation [84].

The combination of polymers with ceramics offers many opportunities to improve the mechanical properties of ceramics while maintaining its bioactivity. The addition of HAP nanoparticles to PLA has been shown to increase compressive and bending strengths [85].

4.3. Physical and chemical characterization of coating

Coating the implant with multisubstituted hydroxyapatite improves protein adsorption, promotes cell proliferation and bone formation [80, 84].

The combination of polymers with ceramics offers many opportunities to improve the mechanical properties of ceramics while maintaining its bioactivity. The addition of HAP nanoparticles to PLA has been shown to increase compressive and bending strengths [85].

In the present paper we have studied the nonostructured biomimetic composites that comprise three important contituents. These constituents which build the biomimetic composition are: HAPc nanoparticles functionalized with crown collagen - COL (core/shell nanoparticles), incorporated in polylactic acid, PLA, resulting in porous HAPc-COL@PLA coatings on Ti implants. Subsequently, these composites were coated with self-assembled COL fibers, resulting in HAPc-COL@PLA/COL biomimetic materials. The addition of collagen as a final layer to the porous HAPc-COL@ PLA coating material increased the mechanical strength of the composite, causing a reduction in its porosity. The reduction of the porosity and the increase of the mechanical resistance of the porous composite was achieved by adding the last layer of pure collagen. The titanium rods were coated with composite. Characterization of titanium-based composites was performed by X-ray diffraction and scanning electron microscopy (SEM) shown in Figure 5a, b [86, 87].

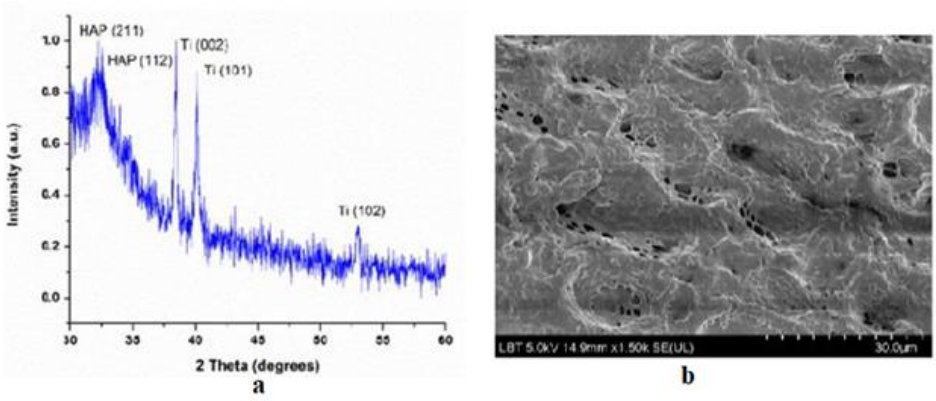

Figure 5. XRD pattern or HAPc composite (i.e., ms-HAP / COL @ PLA / COL coating) on Ti surface (a); SEM image (b) of the HAPc coating surface on Ti [87]

Multisubstituted hydroxyapatatite with collagen was prepared by the precipitation method described above. This type of HAP was chosen for in vivo experiments because it contains elements that are beneficial to the bones.

XRD analyzes 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. Data were collected in the range of 20–80°. A qualitative matching procedure was applied with the PDF card no. 74-0566 for the hydroxyapatite, and PDF card no. 89-4893 for pure Ti.

A thin layer of ms-HAP/COL@ PLA/COL composite was deposited on the titanium surface; from the XRD spectra in Figure 5a weaker maxima for ms-HAP / COL @ PLA composite and more intense maxima for titanium can be observed.

SEM analysis was performed with a Hitachi SU-8230 microscope (Hitachi Ltd., Tokyo, Japan) on ms-HAP / COL particles deposited on SEM grids. A very porous structure can be observed in the form of a lace in Figure 5b, in a higher magnification the collagen fibers can be observed more intensely and in some places the HAP particles.

5. Silver nanoparticles (SNPs)

Silver has been known since the time of the ancient Greeks (Herodotus, Hippocrates) and was used to disinfect water, treat ulcers and heal wounds long before the advent of antibiotics [88, 89]. Silver was appreciated due to its color and gloss, corrosion resistance, light processing and antibacterial properties [90]. Over time, this precious metal has been used in medicine by Dr. J. Marion Simm and in the treatment of epilepsy, its effect being discovered by mistake when an epileptic swallowed a silver coin [88].

The exploitation of silver and its compounds has led over the centuries, XIX-XX, to remedies for tetanus and rheumatism, colds and gonorrhea [91]. Due to its low toxicity to the human body, it has been introduced into a wide range of wound care products and biomedical applications [92].

Few studies have been published describing the use of silver in surgery, however the role and mechanism of action of silver ions in vivo continues to be mentioned in the surgical literature [93]. For silver to be biologically active it must be in a soluble form such as Ag^+ or Ag^0 clusters and the effectiveness of the silver coating is determined by the total silver available and not by the total silver contained in the dressing. Ag^0 is the uncharged metallic form of silver that is in a crystalline state including nanocrystalline structures. In the solution there are sub-crystalline forms with dimensions less than 8 atoms. Ag^+ is the common ionic form for silver that is found in silver nitrate, silver sulfadiazines, and other ionic compounds [94].

Because silver is toxic to bacteria by damaging the bacterial cell membrane, blocking DNA transcription, stopping the replication and distribution of DNA bonds [95-97] an alternative to combating bacterial resistance would be silver nanoparticles with antimicrobial properties. The synthesis, characterization and applications of nanoparticles are among the most important topics that have gained the interest of researchers. Among the physico-chemical properties of great importance is the size, as its decrease leads to an increase in the surface/volume ratio and plays a key role in the field where quantum effects predominate. As the specific surface area increases, the share of atoms on the surface of nanoparticles increases compared to those inside [98].

Silver nanoparticles are the new formula of old remedies with antibacterial properties and are exploited in a wide range of applications in medicine, cosmetics, renewable energy, environmental remedies and biomedical devices [99, 100]. They have attracted great interest due to their special physico-chemical and biological character compared to those of biomacromolecules, which are similar in size [100], the possibility of developing systems against pathogens and broad-spectrum bactericidal activity [101, 102].

Research into such metal nanoparticles aims to control the spread of antibiotic-resistant pathogens. Most research is devoted to silver nanoparticles both as such and functionalized with antibiotics. Although there are many studies, it should be noted that the mechanism of action of silver nanoparticles is not fully understood. Research in this area has found that obtaining silver nanoparticles as small as possible (1-10 nm) is essential in achieving enhanced antibacterial effects by facilitating a good interaction with the bacterial membrane.

The literature also highlights the importance of the shape of nanoparticles, for example silver nanoparticles with faces (111), in which there is the highest density of reactive silver atoms, capable of interacting with the bacterial cell wall, present a bactericidal effect [100-103].

The synthesis of silver nanoparticles (SNPs) can be done by methods such as: physical where the preparation is done by thermal methods using tubular or ceramic furnaces, laser ablation, gamma radiation; involving the use of non-toxic and harmless chemicals, such as from bacteria, plants, fungi. The most common method of obtaining it is the chemical method which uses various organic or inorganic reducing agents [104-106]. This method is based on the reduction of Ag+ in aqueous or organic solution. Various reducing agents have been proposed such as sodium citrate, sodium borohydride, hydrazine, glucose, hydroquinone, beta-cyclodextrin, ascorbic acid.

Trisodium citrate - TSC ($Na_3C_6H_5O_7$) is a non-toxic citric acid salt, produced by complete neutralization of citric acid with sodium hydroxide. It is used in food and in various applications as an emulsifying agent [107]. The most popular and simple chemical method of reducing silver nanoparticles is with trisodium citrate [105, 108, 109]. Trisodium citrate used with silver salt ($AgNO_3$) acts as both a reducing agent and a coating agent [110-113]. It influences the growth of silver nanoparticles in various forms (agglomerates) depending on the added concentration. It should be noted that a low concentration of trisodium citrate prevents the presence of excess citric acid in the dispersion of silver nanoparticles formed [38, 114]. Colloidal silver particles have a negative charge due to the adsorbed citrate ions, which produces repulsive forces between the particles, thus preventing their aggregation [109].

Co-reduction of silver nitrate with sodium citrate and tannic acid (TA) was achieved by mixing aqueous solutions of 0.017% silver nitrate with 0.1806% trisodium citrate and 0.34% tannic acid. These solutions were mixed on the heated magnetic stirrer. Finally a yello wish solution was obtained, containing 108% Ag mg / l. The molar ratio was Ag: TSC: TA 1: 7: 2 [115].

5.1. Characterization of silver naoparticles

These Np solutions were characterized by the UV-VIS absorption spectrum. The UV-VIS absorption spectrum was obtained by using the Jasco UV/Vis V650 spectrophotometer, in the wavelength range from 800 to 190 nm. Because the solution was too concentrated it had to be diluted with ultrapure water to be measurable.

Another characterization of these NPs was achieved with the Hitachi HD-2700 Electron Transmission Microscope (TEM), which operates at a maximum acceleration voltage of 200 kV, and the Hitachi SU-8230 Microscope. (Hitachi Ltd., Tokyo, Japan).

The UV-Vis spectrum of nanoparticles obtained by reduction with TSC and TA in molar ratio Ag: TSC: TA 1: 7: 2 is shown in Figure 6a, a maximum can be observed for SNPs at 428 nm and 418. The spectrum remains unchanged and after 5 days, which proves that these SNPs are stable over time.

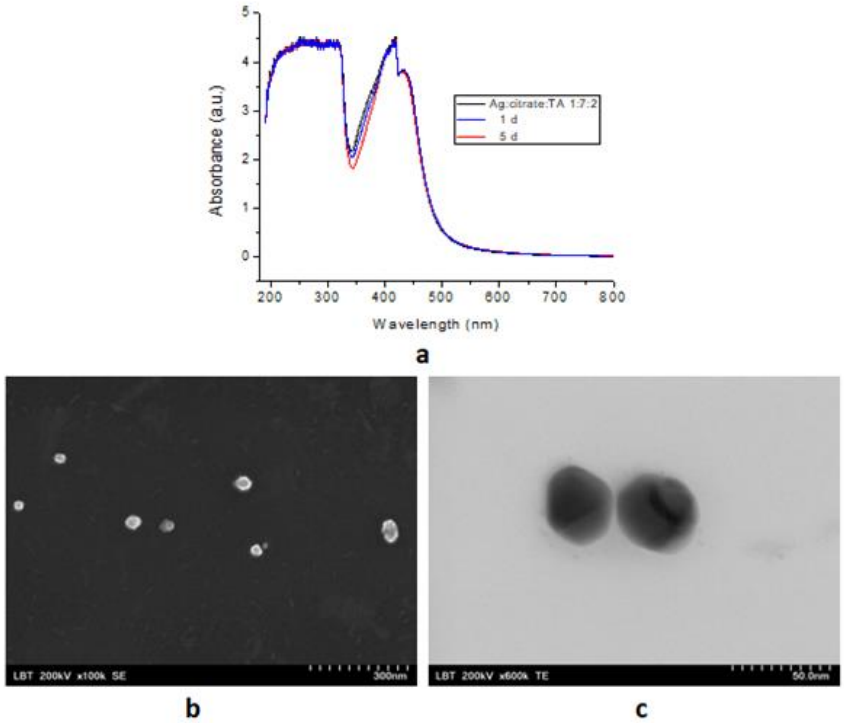

Figure 6. UV-VIS spectra evolution in time (a), SEM image (b) and TEM image (c) of silver colloidal solutions prepared from $AgNO_3$ with TSC and TA in molar ratio Ag: TSC: TA of 1: 7: 2 [115].

From the scanning electron microscopy (SEM) Figure 6b and the transmission electron microscopy (TEM) Figure 6c it can be seen that the SNPs are well dispersed in the mass of the solution and have a round shape. Their size is around 35 ± 5 nm. These nanoparticles have antimicrobial effects on Escherichia coli bacteria [115, 116].

The antimicrobial effect of SNPs on Gram-positive and Gram-negative bacteria is investigated, but the mechanism of growth inhibitors or bactericidal activity is not fully elucidated [114, 117-118].

It can be said that the activity of SNPs depends on the concentration, shape and size of SNPs and the type of bacteria to which it is exposed. Researchers have shown that gram-negative bacteria are found to be more sensitive to SNPs compared to gram-positive bacteria due to the thickness of the cell wall mainly from peptidoglycan [119-121].

The literature specifies various temporary mechanisms of interaction due to the physicochemical properties of SNPs such as the size and surface that allows it to pass or interact with the cell membrane which it affects [117,122]. The action mechanism of silver nanoparticles can be seen in Figure 7.

Silver nanoparticles can release continuously Ag ions, which kill bacteria. 3 ways of penetrating SNPs into the cell membrane are discussed. The first way consists in nanoparticles adhere to the surface of the bacteria and damaged the properties of the membrane. The second pathway is through nanoparticles that spear the bacterial cell, destroying DNA. The third way is that the dissolution of silver nanoparticles releases silver ions, which can interact with the cell wall and affect its functions [123]. SNPs infiltration can be done by anchoring the bacterial cell wall, making physical changes in bacterial membranes, e.g. damage to cytoplasmic membranes and disruption of the bacterial envelope.

It has been shown that there are electrostatic interactions between SNPs and cell membranes, for positively charged SNPs and bacterial membranes with negative charge. After adhering to the bacterial wall, SNPs can also penetrate the membranes, and then the bacteria. The effect depends on the size of the NP, smaller nanoparticles have a large surface area in contact with bacterial cells, and can reach the cytoplasm more easily than larger NPs. SNPs penetrates inside the cell and interacts with cellular structures and biomolecules, such as protein, lipids and DNA. These interactions lead to bacterial dysfunction and eventually to cell death.

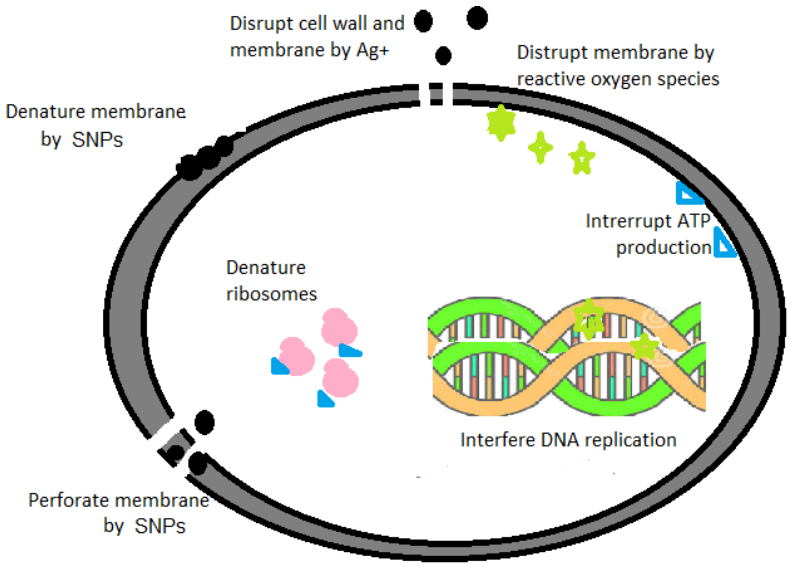

Figure. 7 The action mechanisms of silver nanoparticles.

The antibacterial mechanism of SNPs is also due to their ability to produce high levels of reactive oxygen species (ROS) and free reactive species such as hydrogen peroxide. Reactive oxygen species can destroy cell membranes and alter deoxyribonucleic acid (DNA). SNPs interacts with important components of DNA such as sulfur and phosphorus and causes problems in DNA replication [124, 125].

The SNPs self assembly is investigated in specialized literature by using Langmuir-Blodgett method [126]. This technique allow the preparation of ultrathin film of nanoparticles and their organization in lipid monolayer at air/water interface [127-138]

or oil/water interface [125, 126, 139, 140]. The manufacture of self-assembled structures at selected lateral pressures and the measurement of the surface potential of various biomolecules such as lipids, galactolipids, proteins, amino acids, fatty acids, carotenoids, is also possible and such research was conducted by a group of researchers in Romania [141-151].

Bacterial infections are one of the most serious problems in implant surgery, which can lead to severe injuries with more expensive additional surgical procedures or even death in some cases.

Interest in SNPs is increasing with the emergence and growth of drug-resistant bacteria. SNPs are more effective as mechanisms of action on the cell membrane. HAP-Ag are more effective because of their high bioactivity and osteoconductive stability. HAP-Ag can be applied as bone repair materials or as coatings for metal implants, preventing implant-related infections [152-155].

Silver-based composite implants in addition to the bactericidal effect may have a delayed release of silver from the material.

Looking to the future, hydroxyapatites doped with bacteriostatic ions such as Sr, Zn, Ce and Ag should be developed, which are not toxic to the body [156, 157].

In recent years, researchers have used nanostructured silver coatings and silver cations, which lead to changes in the polymer surface of the implant [158].

6. Antibacterial testing

Gram-negative bacteria patches, Escherichia coli ATCC 25922, were used for antimicrobial testing of SNPs on the agar plate (Merck KGaA, Germany), nutrient broth suspensions were prepared corresponding to a turbidity of 0.5 according to McFarland standards ($1.5x10^8$ CFU/mL). The Kirby-Bauer technique was used to verify the antimicrobial effect of the nanoparticles [106, 159]. 3 mm deep, 90 mm diameter wells were cut into the agar plates and 20 μL of SNPs dispersed were introduced. The plates were incubated for 24 hours at 37°C, after which the areas of inhibition were measured and photographed. They were monitored for 72 hours for any changes in the inhibition zone.

Figure 8 shows the inhibition zones of silver nanoparticles for Escherichia coli ATCC 25922 bacteria. The highest inhibition zone was found for sample 1 corresponding to the SNPs obtained by reduction with a TSC-TA mixture in the ratio: Ag: TSC: TA 1: 7: 2 and sample 2 corresponding at the molar ratio Ag: TSC: TA 1: 20: 0,1. Sample 6 is $AgNO_3$. Weaker areas of inhibition can be seen in sample 3 corresponding to the molar ratio Ag: TSC: TA 1: 7: 0.2, sample 4 corresponding to the molar ratio Ag: TSC: TA 1: 3: 0.2 and sample 5 which is citrate trisodic.

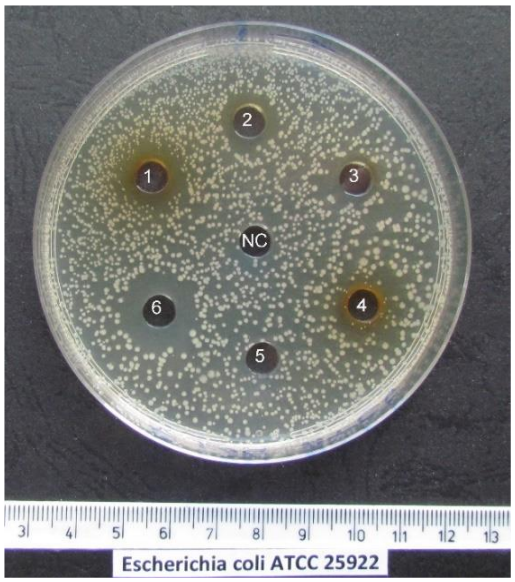

Figure 8. Areas of inhibition for Escherichia coli 25922 ATCC in the presence of SNPs dispersions (20 μl). Samples are numbered as follows: (1) - Ag (1 mM) - TSC-TA for 1: 7: 2 molar ratio; (2) - Ag (0.25 mM) - TSC-TA for the ratio 1: 20: 0.1; (3) - Ag (0.25 mM) - TSC-TA for the ratio 1: 7: 0.2; (4) - Ag (0.25 mM) - TSC-TA for 1: 3: 0.2 molar ratio; (5) - TSC (7 mM); (6) - $AgNO_3$ (1 mM); NC (negative control), ultrapure water sample. [115]

Antimicrobial activity is determined by the ratio of tannic acid (TA) to trisodium citrate (TSC) which determines the size and shape of SNPs. Smaller nanoparticles have a larger specific surface area and can penetrate the cell membrane more easily [160, 161].

7. Conclusions

The release of Sr from hydroxyapatites is better and longer than in the case of Mg. This effect is beneficial for orthopedic applications.

HAPc-coated titanium implants help heal fractures and increase its osseointegration.

Co-reduction of silver nitrate in aqueous solution with TSC and TA proved to be the best method for obtaining small Np sizes between 30-10 nm.

The antibacterial effect of SNPs was tested by measuring areas of inhibition on Escherichia coli cultures.

The effect was evident for all SNPs samples, but the smallest particle size dispersions proved to be the most active.

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Abbreviations

Hydroxyapatite – HAP, multisubstituted hydroxyapatite – msHAP, poly lactic acid – PLA, Food and Drug Administration - FDA, Silver nanoparticles – SNPs, atomic force microscopy – AFM, simulated body fluid – SBF, collagen – COL, Trisodium citrate – TSC, tannic acid – TA, reactive oxygen species – ROS, deoxyribonucleic acid – DNA.

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