DEVELOPMENT AND CHARACTERIZATION OF NOVEL POROUS COLLAGEN BASED BIOCOMPOSITE FOR BONE TISSUE REGENERATION

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Biomaterialele compozite ce au ca bază colagenul pot fi folosite ca biomateriale pentru regenerarea defectelor osoase. Materialele compozite au fost caracterizate prin spectroscopie în infraroşu cu transformată Fourie, microscopie electronică de baleiaj şi degradare enzimatică în colagenză. Compozitele au fost obţinute prin liofilizarea gelurilor de colagen în care au fost adăugate pulberi ceramice de fosfat tricalcic, formă β (β-TCP) şi un amestec de pulbere ceramică şi metalică (Mg:β-TCP). Rezultatele au evidenţiat o structură poroasă cu particule aderente la matricea de colagen. În urma analizelor FTIR şi de degradare enzimatică cele mai bune rezultate au fost obţinute de compozitele în care a fost adăugată pulbere metalică de Mg, amestecată cu β-TCP. Materialele compozite obţinute pot fi utilizate ca potenţiale biomateriale pentru regenerarea ţesutului osos.

Biocomposite materials based on collagen matrix could be used as biomaterials for bone defects repair. The spongious composites were characterized by Fourier transform infra-red (FT-IR) spectroscopy, scanning electron microscopy (SEM) and enzymatic degradation by collagenase. The composites were obtained by lyophilization of the collagen hydrogel in which have been added β tricalcium phosphate ceramic powder (β-TCP) and a mixed metallic and ceramic powder (Mg:β-TCP). The results showed spongious composites with interconected pores and embedded particles. As the revealed data from FT-IR analysis and collagenase degradation, the best results were obtained for the composite with magnesium powder mixed with ceramic β-TCP powder. Due to their properties, the obtained composites could be used as potential biomaterials in bone tissue engineering.

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1. Introduction

The fracture of bones due to various traumas or natural aging is a typical type of tissue failure. A fast aging of the population and serious drawbacks of natural bone grafts make the situation even worse. Therefore, there is a high clinical demand for bone substitutes [1]. Bone defects are very challenging in orthopedic practice. They can result from a high-energy traumatic event, from large bone resection for different pathologies such as tumor or infection, or from the treatment of complex non-unions (en-bloc resection) [2].

Historically, autogenous bone grafts, allograft, and a variety of biomaterials have been used for the repair of osseous defects and the augmentation of compromised bone. The ideal bone-graft substitute is biocompatible, bioresorbabile, osteoconductive, osteoinductive, having a similar structure to bone. In this way, it's easy to use, cost-effective [3] and strong enough to support load bearing functions **[**4,5**].** Synthetic bone graft substitute materials are mineral structures similar to the mineral content of human bone. In the Table 1 is being presented the chemical composition of human bone.

Table 1

The composition of human bone [6, 7]

Mineral phase	***wt%***	***Organic phase***	***wt%***
Hydroxyapatite	~ 60	Collagen	~20
Carbonate	~ 4	Water	~9
Citrate	~ 0.9	Non-collagenous proteins (osteocalcin, osteonectin, ostepontin,	~3
Na^{2+}	~ 0.7	BMP)	
Mg^{2+}	~ 0.5		
Other: Cl^-, F^-, K^+, Sr^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}	Traces	Other: polysaccharides, lipids, cytokines	Traces

Due to the fact that hydroxyapatite and collagen are the main components of the human bone, by using a combination of this type of materials can be revealed a promising biomaterial in regeneration of bone tissue. Composite materials which are made of bioactive calcium phosphates and polymers can lead to the improvement of the final material properties.

The main calcium phosphates which have been used in the development of the bone substitute composites are calcium phosphates like hydroxyapatite (HAp), β tricalcium phosphate (β-TCP) and amorphous calcium phosphate (ACP) [8].

In the present study our attention was focused on β-TCP due to its remarkable properties of biodegradation. It can be completely absorbed within a few months at the implantation site via the simultaneous formation of new bone

[9]. In the same time it has good biological compatibility and bone conductivity [10,11].

Magnesium (Mg) has been recently recognized as a biodegradable metal which can be used in bone substitute applications [12]. Magnesium is one of the main substitutes for calcium in biological apatite [13], and is associated with mineralization of calcified tissue and influences indirectly the mineral metabolism [14]. Taking in consideration cationic substitutes, magnesium is quantitatively one of the most important bivalent ions associated with biological apatite.

Some studies have shown that the small amount of magnesium which can be found in enamel, dentin and bone is about 0.44, 1.23, respectively 0.72 wt% [15]. It was also showed that the maximal amount of Mg substitution is between 0.3÷1.0 wt % in apatite [17,18], while other ones have reported a higher upper limit of Mg substitution between 1.5÷7.6 wt% [19,20].

Calcium phosphate with magnesium substitution has been an extensive subject of research, due to its potential for developing artificial bone substitutes or being used in other medical applications [21-23].

There are many techniques to process biomaterials into various porous composite. These include conventional techniques such as impregnation and sintering ceramic scaffolds processing, solvent casting and particulate leaching, gas foaming, emulsion freeze drying for manufacturing scaffold and lots of other applications [24].

Lyophilization is one of the most efficient methods to produce a porous structure and works by freezing the material. In this way it is reducing the surrounding pressure to allow the frozen water in the material and sublimates directly from the solid to the gas phase. The pores are created by the ice crystals, sublimating subsequently and leaving gaps or pores in their place. This phenomena is especially important when it comes to bone substitutes, drug delivery systems or pharmaceutical uses. In order to meet the requirements for the medical applications, a porous structure which can be obtained by lyophilization is necessary.

The aim of this work is to develop a new type of composite materials by adding different ratio collagen/ceramic powder or collagen/ceramic powder/metallic powder. All samples were obtained by lyophilization. The morphology of the composite was evaluated by SEM technique, the chemical structure was analyzed by FTIR spectroscopy in the near IR spectrum, and the weight loss was monitored by collagenase enzymatic degradation.

2. Experimental part

2.1. Materials and preparation of the developed composites

In order to obtain a porous structure, a special attention should be given to the manufacturing method and process parameters. Collagen gel was obtained from the Collagen Department of the Romanian National Research & Development Institute for Textiles and Leather. The magnesium and β -tricalcium phosphate powders were purchased from STREM Chemicals, Inc. USA and Plasma Biotal Limited UK, respectively. Collagenase of Clostridium histolyticum was from Sigma-Aldrich (USA) and glutaraldehyde (GA) from Merck (Germany). Sodium hydroxide and phosphate buffer solution (PBS), pH 7.4 were of analytical grade.

One of the most used techniques that have been used in the development of synthetic composites is freeze-drying (also known as lyophilization or cryodesiccation). The starting bases of the experimental composites are presented in Table 2. Type I collagen which has been extracted from bovine calf hide by a technology which is describes somewhere else [25]. The experimental composite materials were obtained by adding different weight percentages (wt %) of β-TCP and combined powders of Mg:10% β-TCP into the type I collagen, with respect to the samples with 75% β-TCP. The experimental samples have been codified as following: Collagen: β-TCP in different weight percentages as T1, T2 T3, and T4 as the composite materials in which has also been added metallic magnesium powder. The control sample has been coded with C (pure collagen 100%). In the Table 2 we summarize the chemical composition of the tested composites.

Table 2

The chemical composition of the composites materials

Codification	*Composition*	*Ratio*
C	***Collagen 100%***	
T1	***75 wt% Collagen: 25% wt β-TCP***	***3:1***
T2	***50 wt% Collagen: 50% wt β-TCP***	***1:1***
T3	***25 wt% Collagen: 75% wt β-TCP***	***1:3***
T4	***Collagen:3% (Mg:10%wt β-TCP)***	

The pH value was adjusted to physiological environment, 7.4-7.6. All gels were cross-linked with 0.25% glutaraldehyde (GA), reported to the weight of dry collagen. Then it was casted in polystyrene dishes and kept for 24 hours at 4°C.

In order to obtain porous structures, the composites were freeze-dried for 48 hours as follows: cooling to -40°C (4 hours), keeping up for 8 hours, then freeze-dried at -40°C and 0.1 mbar for 10 hours, then heating to +20°C for 18 hours at 0.1 mbar, then heating (6 hours) to +30°C at and finally freeze-dried at -

35°C at 0.01 mbar for 6 hours, using the Christ Model Delta 2–24 LSC freeze-dryer (Germany) (Fig. 1). In Fig. 1 we present the lyophilization program.

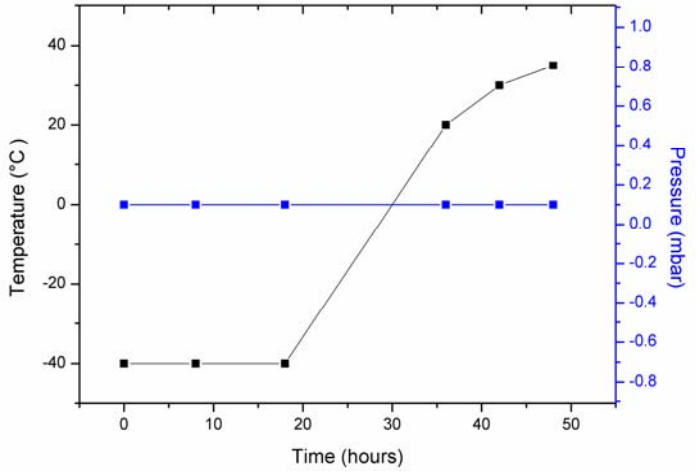

Fig. 1. The lyophilisation program after the 4 hours in which the apparatus was brought to the -40°C temperature

The freezing rate and the collagen/powder content are the most important factors in controlling pore size. The dimensions of the pores and also their interconnectivity are important factors for our biocomposite materials. This morphology details will determine the mechanical properties of the composite, favors cell adhesion, so the new tissue formation. A collagen matrix was used as control sample in all experiments.

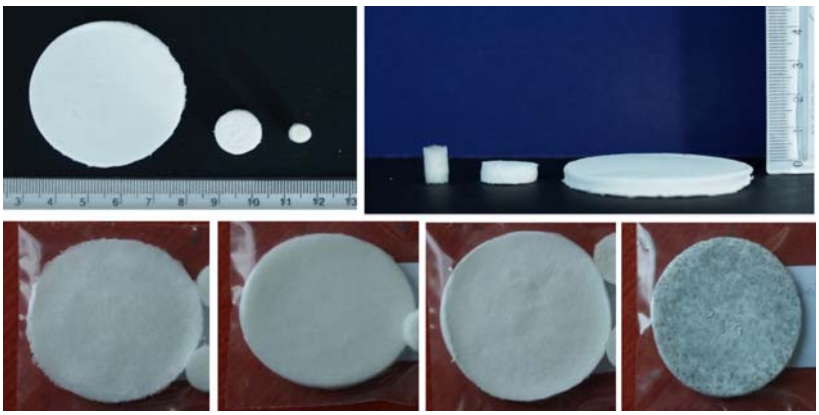

Fig. 2. Porous composite sponge made from collagen, β-TCP and magnesium (macroporous view, the scale shows centimeters)

2.2. Investigation and testing methods

The morphological aspects of the samples surface, the adhesion of β-TCP and Mg with β-TCP particles into the collagen matrix and the pore interconnectivity were determined by Scanning Electron Microscopy, using a Philips ESEM XL 30 microscope.

The chemical reaction between the functional groups of collagen and the filler was evaluated by infrared spectroscopy using a JASCO 6200 FTIR Spectrometer. All spectra were recorded in absorption mode at in near infrared spectrum interval and 160 scans.

In order to simulate the behavior of matrices *in vivo* conditions and to evaluate the biological stability, we performed *in vitro* experiment regarding to its degradation by collagenase. Among all enzymes, only collagenase is able to cleave the collagen completely. The matrices were tested by collagenase degradation according a specific protocol. Enzymatic degradation of collagen hydrogels was investigated by monitoring the weight loss depending on exposure time to collagenase solution. Involved working protocol is the following: 3 g of collagen hydrogels were accurately weighted, placed in PBS solution and collagenase (1 μg/mL) and incubated at 37°C. At regular intervals, the swollen composites were removed from the degradation solution, blotted dry and weighted again. The percent of hydrogel degradation was determined by the following relation:

$$\%\, weight\ loss = \frac{W_i - W_i}{W_i} \cdot 100 \qquad (1),$$

where W_i is the initial weight and W_t is the weight after time t.

3. Results and discussions

The SEM investigation offer information about the morphology and distribution of the particle to/at the collagen matrix. In Fig. 3 is presented the control collagen sample, having a porous structure with interconnected micro- and macropores, similar to the spongious bone. The pure collagen has a smooth surface which began to modify with the incorporation of ceramic fillers (β-TCP and Mg with β-TCP). This fact will induce a gradually rough surface**.** The content of ceramic fillers increased, doesn't disturb the porous structure, but significant modification on the morphology is produced.

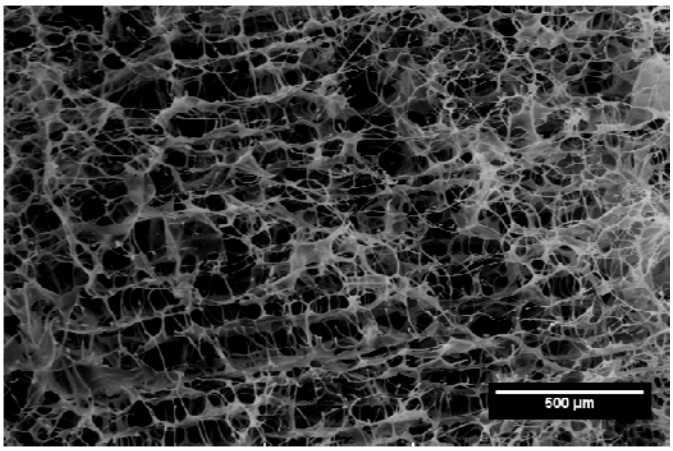

Fig. 3. SEM image of the collagen matrix – control sample C (pure collagen 100%)

In Fig. 4 (a-d), the SEM images of the samples (T1, T2, T3, T4) are presented.

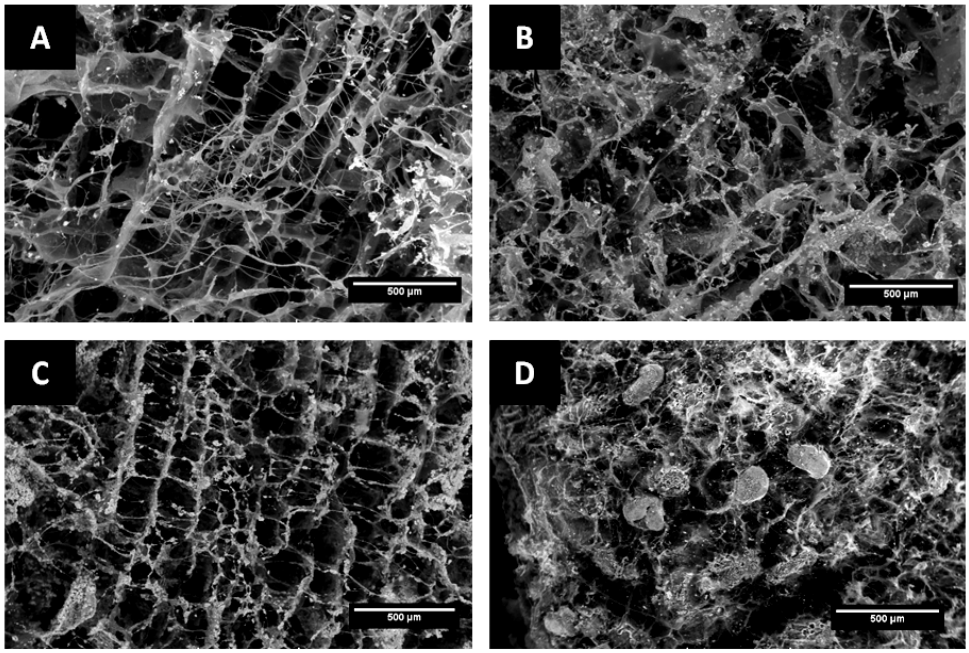

Fig. 4. SEM images of the general morphology for A) T1, B) T2, C) T3 and D) T4 composites

The morphological results by SEM analysis revealed a differentiated homogeneity and pore interconnectivity in our biocomposites. Also the distribution and adhesion of the β-TCP and Mg particles on the collagen fibrils is visible. Thus, the pore dimension decreases with increasing fillers quantity into the collagen matrix. The walls of the collagen network appear to be thicker for the composite T2 and T3, while the particle are beginning to agglomerate and form clusters on the collagen fibrils.

The FT-IR spectra for collagen reference composite (C) showed the characteristic peaks as following: 3302 cm^{-1} (amide A), 3078 cm^{-1} (amide B), 1636 cm^{-1} (amide I), 1547 cm^{-1} (amide II) and 1239 cm^{-1} (amide III). The phosphate bands are located between 900 and 1200 cm^{-1} [26].

The FT-IR measurements presented in Fig. 5, showed that there are not any alterations of triple helix of collagen, because there are no changes in amide II, III and pyrrolidine ring of collagen. The shift of amide I from 1636 cm^{-1} to 1644 cm^{-1} is due to new chelate bonds between Ca^{+2} and C=O bond [27]. Our measurements showed peak for β-TCP at 1013 and 1108 cm^{-1} associated to with the asymmetric vibration of the P-O bond from phosphoric group. The shifts from amide A towards higher wave numbers when β-TCP was added showed strong bonds between β-TCP and collagen.

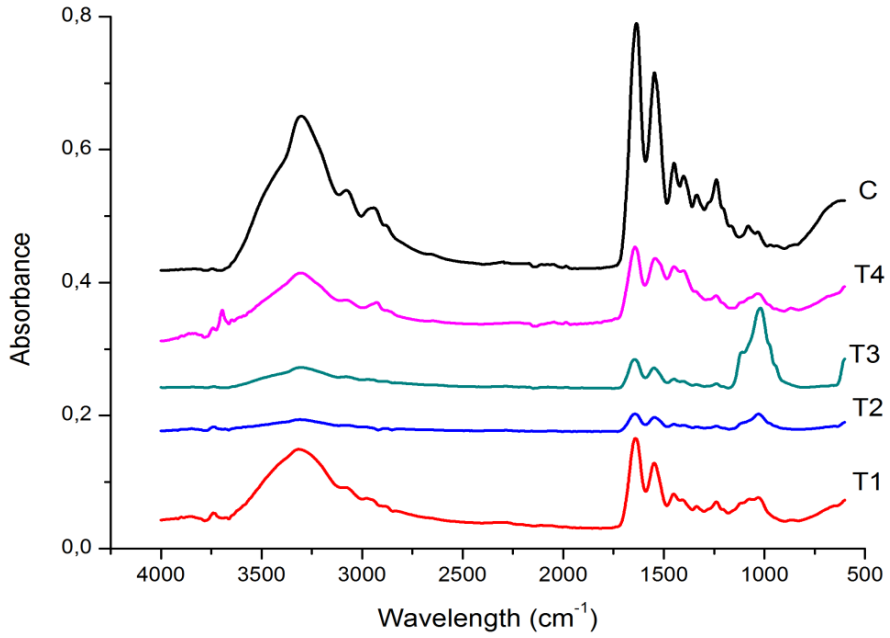

Fig. 5. FTIR spectra of the composites samples

Table 3

Assignments of the observed vibrational frequencies of C, T1, T2, T3, T4 composites

Assignments	Observed vibrational frequencies wave number (cm^{-1})					
	C	T1	T2	T3	T4	**β-TCP**
structural OH	-	3743	3742	3741	-	3741
N-H (amide A)	3302	3316	3309	3305	3305	-
C-H (Amide B)	3079	3090	3071	3077	3082	-
$C-H_3$	2943	2979	2984	2962	2927	-
C=O (amide I)	1636	1641	1641	1644	1643	-
N-H amide II), C-N	1548	1549	1549	1550	1543	-
pyrolidine rings	1450	1450	1451	1453	1449	-
C-H,	1402	1407	1401	1406	1403	-
$-COO^-$	1336	1337	1335	1338	1345	-
C-N (amide III), N-H	1239	1239	1238	1238	1240	-
PO_4^{-3} bend ν_3	-	1075	-	1111	1033	1109
PO_4^{-3} bend ν_3	-	1032	1030	1020	-	1013
PO_4 bend ν_1	-	-	-	-	868	-

In the Table 3, it can be observed the appearance of a new chemical reaction between carboxylate ion ($-COO^-$) and Mg^{2+} and Ca^{2+} of the mixed filler (Mg: β-TCP), existing in the T4 composite.

The enzymatic degradation, evaluated according to described protocol is shown in the histogram presented in Fig. 6.

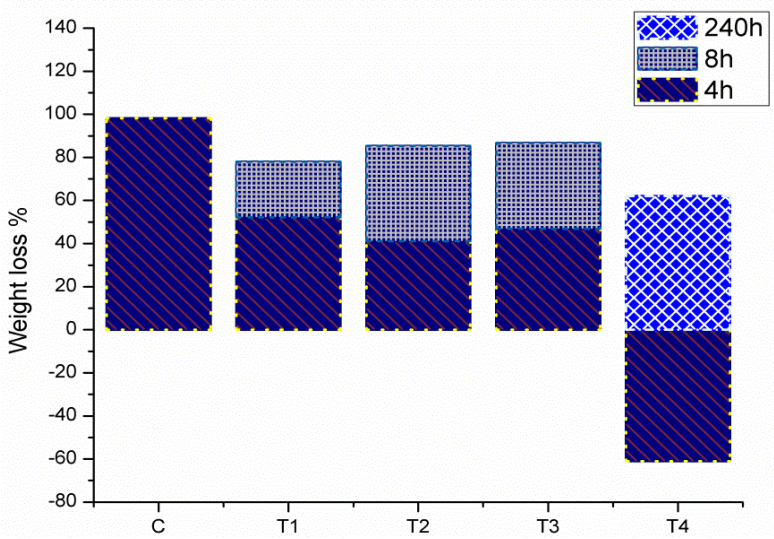

Fig. 6. *In vitro* enzymatic degradation of the experimental composites

Collagen composite (C) was completely degraded during less than 4 hours, while the composites containing β-TCP were digested more slowly comparing

with C sample (pure collagen 100%). As well the composites with mineral phase were digested at different periods, as shown in Fig. 6.

Considering Fig. 6, the resistance to collagenase increases with increasing the percentage of the added powder. The samples with β-TCP powder are digested by collagenase in about 8 hours. The higher resistance is manifested by the samples containing mixed Mg powder with β-TCP powder.

In the development composites, the Mg powder mixed with the ceramic powder is being added into the collagen hydrogels and could lead to the appearance of a hydroxide layer on the surface of the particles. This layer of oxide has influenced the behaviour of the final composite T4 by improving the rate to colagenase digestion.

4. Conclusions

Resorbable biocomposites based collagen with β-TCP (T1, T2, and T3) and the mixed Mg with β-TCP powder (T4) obtained experimentally, appear to be suitable for use as biocomposites for bone regeneration. The particles which had been added into the collagen, can act as fillers to stiffen and to improve the degradation rate, improving the properties of the organic matrix.

The SEM images of the composite revealed a high porous structure, common in all samples. The homogeneity of the structure and the interconnectivity of the pores are also an important advantage of our experimental biocomposites.

The FTIR analysis revealed the chemical interaction between the collagen and the ceramic and metallic filler. The FTIR spectra showed that the interactions between the T4 components of the composite are not only interacting at a physical level but also in a chemical manner.

The enzymatic degradation have shown a slower degradation rate for the T4 composite, proving the fact, that the composite in which have was added the mixed metallic powder and ceramic powder, presents a better behaviour to the collagenase digestion.

The obtained results are close related to the clinical applications regarding the rate of new bone formation in conjunction with the biocompatibility requirement. These work provide a framework for generating synthetic composites with organic/inorganic interface similar to natural bone.

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REFERENCES

[1] *S. Ramakrishna, J. Mayer, E. Wintenmarntel, K.W. Leong* – Biomedical applications of polymer-composite materials: a review, Composites Science and Technology **61** (2001) 1189-1224

[2] *G.M. Calori, , E. Mazza, M. Colombo, C. Ripamonti* - The use of bone-graft substitutes in large bone defects: Any specific needs?, Injury, Int. J. Care Injured **42** (2011) S56–S63

[3] *A Kolk, Handschel J, Drescher W, Rothamel D, Kloss F, Blessmann M, Heiland M, Wolff KD, Smeets R* Current trends and future perspectives of bone substitute materials e From space holders to innovative biomaterials - J Craniomaxillofac Surg. **30** (2012)

[4] *S.V Dorozhkin* - Calcium orthophosphates. J Mater Sci. **42** (2007) 1061-1095

[5] *M. Bohner, Y. Loosli, G. Baroud, D. Lacroix* - Commentary: Deciphering the link between architecture and biological response of a bone graft substitute Acta Biomaterialia **7 (2)** (2011) 478-484

[6] *R.Z. Legeros*, Biological and synthetic apatites, In: Brown PW,. Constantz B, editors. Hydroxyapatite and related materials. Boca. Raton: CRC Press **(**1994)

[7] *R Murugan, S. Ramakrishna* - Nanostructured biomaterials. In: Nalwa HS, editor. Encyclopedia of nanoscience and nanotechnology **7** California: American Scientific Publishers (2004) 595–613.

[8] *D.A. Wahl, J.T. Czernuszka* Collagen-Hydroxyapatite composites for hard tissue repair –, European Cells and Materials **1** (2006) 43-56

[9] *W.F. Neuman, M.W. Neuman* - The chemical dynamics of bone mineral. Chicago: University of Chicago Press; 1958

[10] *H.H. Horch, R. Sader, C. Pautke, A. Neff, H. Deppe, A. Kolk*: Synthetic, pure-phase betatricalcium phosphate ceramic granules (Cerasorb) for bone regeneration in the reconstructive surgery of the jaws. Int J Oral Maxillofac Surg **35** (2006) 708-713

[12] *W.R. Walsh, F. Vizesi, D. Michael, J. Auld, A. Langdown, R. Oliver, Y. Yu, H. Irie, W. Bruc*e Beta-TCP bone graft substitutes in a bilateral rabbit tibial defect model Biomaterials **29** (2008) 266-271

[12] *H. Zhuang, y. Han, A. Feng* – Preparation, mechanical properties and in vitro biodegradation of porous magnesium scaffolds - Materials Science and Engineering C **28** (2008) 1462 – 1466

[13] *R.Z. LeGeros* - Calcium phosphates in oral biology and medicine, Monographs in Oral Sciences. **15** Myers H (ed). Basel: Karger (1991)

[14] *J. Althoff, P. Quint, E.R. Krefting, H.J. Hohling*. Morphological studies on the epiphyseal growth plate combined with biochemical and X-ray microprobe analysis. Histochemistry **74** (1982) 541–552

[15] *J.T. McCarthy R. Kumar* – The Schrier Atlas of Diseases of the Kidney Volume I, Chapter 4 Divalent Cation Metabolism: Magnesium, Disorders of Water, Electrolytes, and Acid-Base (**1999**)

[16] *Wojciech L. Suchanek, K. Byrappa, P. Shuk, R.E. Riman, V.F. Janas, K.S. TenHuisen*- Preparation of magnesium-substituted hydroxyapatite powders by the mechanochemical–hydrothermal method, Biomaterials **25** (2004) 4647-4657

[17] *E. Bertoni, A. Bigi, G. Cojazzi, M. Gandolfi, S. Panzavolta, N. Roveri* - Nanocrystals of magnesium and fluoride substituted hydroxyapatite. J Inorg Biochem **72 (**1998);:29-35

[18] *R.Z. LeGeros* - Incorporation of magnesium in synthetic and in biological apatites. In: Tooth Enamel IV, Fearnhead RW, Suga S, editors. Amsterdam: Elsevier, (1984) 32-36

[19] *I. Mayer, R. Schlam, J.D.B. Featherstone* - Magnesium-containing carbonate apatites, J Inorg Biochem **6** (1997) 1-6

[20] *A. Bigi, G. Falini, E. Foresti, A. Ripamonti, M. Gazzano, N. Roveri* - Magnesium influence on hydroxyapatite crystallization. J Inorg Biochem **49** (1993) 69-78
[21] *F. Ren, Y. Leng, R. Xin, X. Ge* - Synthesis, characterization and ab initio simulation of magnesium-substituted hydroxyapatite - Acta Biomaterialia **6** (2010), 2787-2796
[22] *I.V. Fadeev, L.I. Shvorneva, S.M. Barinov, V.P. Orlovskii* - Synthesis and structure of magnesium-substituted hydroxyapatite. Inorg Mater **39** (2003) 947-950.
[23] *S.R. Kim, J.H. Lee, Y.T. Kim, D.H. Riu, S.J. Jung, Y.J. Lee* - Synthesis of Si, Mg substituted hydroxyapatites and their sintering behaviors. Biomaterials **24** (2003) 1389-1398
[24] *C. Liu, Z. Xia, J.T. Czernuszka* - Design and Development of Three-Dimensional Scaffolds for Tissue Engineering" - Chemical Engineering Research and Design, **85**, (2007) 1051-1064,
[25] *M.G. Albu* - Collagen gels and matrices for biomedical applications, LAP LAMBERT Academic Publishing, Saarbrücken, Germany (2011**)** 23-24
[26] *A. Ficai, E. Andronescu, G. Voicu, C.Ghiţulică, B.S. Vasile, D. Ficai, V. Trandafîr* – Self-assembled collagen/hydroxyapatite composite materials, Chemical Engineering Journal **160** (2010) 794-800
[27] *M. Kikuchi, S. Itoh, S. Ichinose, K. Shinomiya, J. Tanaka* – Self-organization mechanism in a bone-like hydroxyapatite/collagen nanocomposite synthesized in vitro and its biological reaction in vivo, Biomaterials **22** (2001) 1705-1711