ANTIMICROBIAL ACTIVITY OF EUROPIUM DOPED HYDROXYAPATITE POWDERS AFTER IMMERSION IN SBF SOLUTION

Ecaterina ANDRONESCU[1], Alina SURUGIU[2], Monica Luminita BADEA[3], Carmen Steluta CIOBANU[4*], Alin IOSIF[5]

The europium doped hydroxypatite samples were obtained by an adapted coprecipitation method. After the synthesis, the samples were immersed in simulated body fluid (SBF) solution for 6 and 24 h respectively. After the immersion in the SBF solution, the morphology and antimicrobial activity of the powders were studied. Our antimicrobial studies revealed that all the samples exhibit antimicrobial activity against gram positive and gram negative strains.

Keywords: antimicrobial activity, SBF solution, europium, hydroxyapatite

1. Introduction

One of the most interesting bioceramic material with application in the biomedical field is hydroxyapatite (HAp). This biomaterial is well-known for its similarity to the inorganic part of bone, its non-toxicity, excellent bioactivity and biocompatibility [1-3]. Due to its affinity, the HAp structure allows an important number of substitution for Ca^{2+}, PO_4^{3-} and OH^- [4-6]. These substitutions improve the physico - chemical and biological properties. As reported before, HAp possess luminescent properties. However, in order to enhance this property, hydroxyapatite is doped with lanthanide (Ce, Eu, Sm, Tb, etc) ions [7-8].

Fluorescent labeling can realize the continuous and non-destructive observations, which are helpful for monitoring the implanted subject [9], the delivering progression of drug carriers [10] and the bioimaging probes etc.

[1] Prof., Department of Science and Engineering of Oxide Materials and Nanomaterials, University POLITEHNICA of Bucharest, and *Academia Oamenilor de Știință din România*, Romania, e-mail: ec_andronescu@yahoo.com

[2] Physician, Hospital of obstetrics and gynecology, Ilfov, Romania, e-mail**:** surugiualina@yahoo.com

[3] PhD, National Institute of Materials Physics, and University of Agronomic Sciences and Veterinary Medicine, Bucharest, Romania e-mail: monalisa588@yahoo.com

[4]* PhD, National Institute of Materials Physics, and Department of Science and Engineering of Oxide Materials and Nanomaterials, University POLITEHNICA of Bucharest, Romania, e-mail: ciobanucs@gmail.com

[5] Manager, S.C.Biosintex SRL, Snagov, Ilfov ,Romania, e-mail: alin.iosif@biosintex.com

In this study, we chose to dope hydroxyapatite nanoparticles with europium in order to improve their luminescence. It is well known that europium ions (Eu^{3+}) possess a remarkable and stable red luminescence under UV radiation. Also, europium is characterized by a long-life luminescence which can be recorded by time-resolved fluorescence measurement. These properties could lead to the use of europium doped hydroxyapatite in *in vivo* imaging [11-12].
Previous studies reported in literature [13] have shown that the immersion of hydroxypatite in a simulated body fluid is an efficient method to evaluate the bioactivity of HAp in *in vitro* conditions [14]. Moreover, recent studies reported by S. L. Iconaru et. al [15] revealed that europium doped hydroxyapatite nanoparticles exhibit antibacterial activity against Gram-negative and Gram-positive bacteria as well as against fungi.

In this paper we report the synthesis, characterizations and antimicrobial activity of europium doped hydroxyapatite (HAp:Eu) powders after immersion in a SBF solution for 6 and 24h respectively. The HAp:Eu samples were prepared by an adapted coprecipitation method. The morphology and optical properties of the obtained powders was studied by scanning electron microscopy (SEM) and FT-Raman spectroscopy. Also, the antimicrobial activity of HAp:Eu powders after immersion in the SBF solution against *Enterococcus faecalis* and *Pseudomonas aeruginos*a was studied.

2. Samples preparation

The HAp:Eu ($Ca_{10-x}Eu_x(PO_4)_6(OH)_2$, x_{Eu} = 0.1) nanopowder was synthesized by an adapted coprecipitation method. In order to obtain the HAp:Eu nanopowder, calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$; Sigma Aldrich], ammonium hydrogen phosphate ($(NH_4)_2HPO_4$; Alpha Aesar) and europium nitrate pentahydrate ($Eu(NO_3)_3\ 5H_2O$; Sigma Aldrich) were used. Appropriate quantities of ammonium hydrogen phosphate and europium nitrate were dissolved in ethanol. After adding distilled water, the solution was kept under vigorous stirring for 8 h at 80 °C. In a distinct recipient, a specific amount of calcium nitrate was dissolved in ethanol under vigorous stirring for 8 h at 80°C. The calcium containing solution was added drop by drop in to the phosphorus containing solution. The atomic ratio used in order to obtain HAp:Eu sample was [Ca+Eu]/P = 1.67 [15-16]. The obtained powders were dried at 80 °C for 12 hours.

On the other hand, the HAp:Eu SBF samples were obtained by immersing HAp:Eu samples into SBF solution. These mixtures were maintained at body temperature and incubated for 6 (HAp:Eu SBF 6h) and 24 h (HAp:Eu SBF 24h) respectively**.** After incubation, the powders were removed from the SBF solution, dried and milled.

3. Samples characterizations

Scanning electron microscopy studies were performed with a QUANTA INSPECT F microscope operating at 30 kV accelerating voltage. For the elemental analysis, the electron microscope was equipped with an energy dispersive X-ray attachment (EDAX/2001 device).

Optical properties of the powders were investigated by **FT-Raman spectroscopy**. The studies were performed with a RFS 100 FT-Raman Bruker spectrophotometer (λ=1064 nm).

***In vitro* antibacterial studies.** The antibacterial activity of the HAp:Eu SBF powders was evaluated on clinical and reference ATCC bacterial strains of gram positive (*Enterococcus faecalis* ATCC 29212) and gram-negative bacteria (*Pseudomonas aeruginos*a 1397), using twofold serial dilutions performed in 96-multi-well plates [17]. For the quantitative antibacterial screening, the inoculums were prepared using a broth culture of each bacterial strains equivalent to a 0.5 McFarland standard. Then, twofold serial dilutions of the samples were prepared in DMSO (dimethyl sulfoxide) with a starting stock solution of 1000 μg/mL concentration. After that, the plates were incubated for 24 h at 37 °C and the bacterial growth was assessed by measuring the absorbance of the obtained culture at 620 nm. The experiments were done in triplicate.

4. Results and discussions

In Figs. 1 and 2, the SEM images obtained at different magnifications for HAp:Eu SBF samples are presented, after the immersion of HAp:Eu in SBF for 6 and 24 hours, respectively.

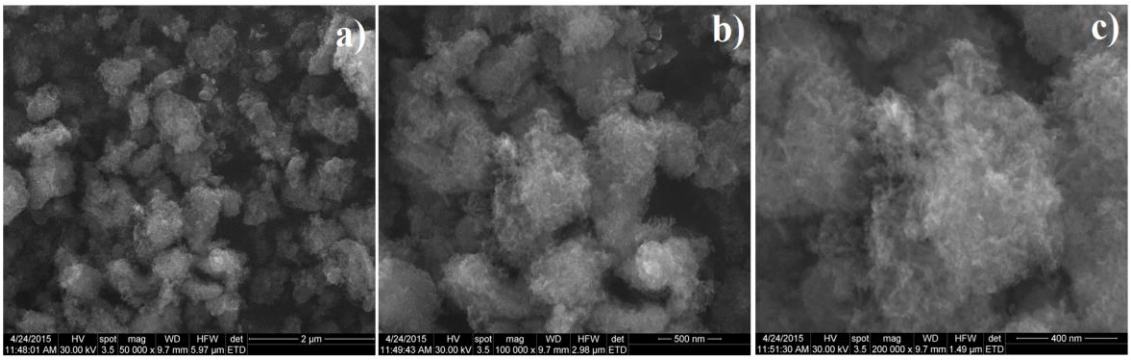

Fig. 1. SEM images of HAp:Eu samples after immersion in SBF solution for 6h at various magnifications: (a) 50000x (b) 100000x, (c) 200000x.

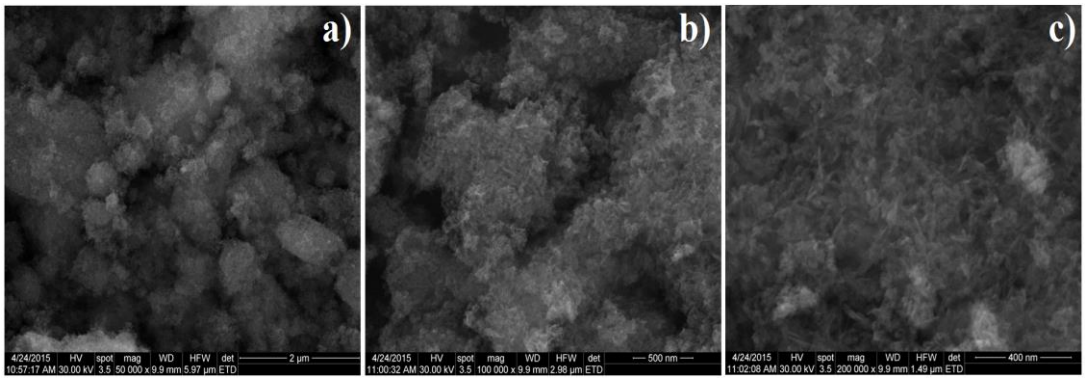

Fig. 2. SEM images of HAp:Eu samples after immersion in SBF solution for 24h at various magnifications.(a) 50000x (b) 100000x, (c) 200000x.

It must be noted that the porosity of the HAp:Eu samples increased after immersion in SBF. The increase of the porosity of HAp:Eu SBF could lead to new applications in bone tissue engineering [18]. Moreover, it was observed that the morphology of the nanoparticles is slightly influenced by the immersion time. Also the nanometric dimensions and elipsoidal shape of the particles were confirmed by SEM studies. The morphology of HAp:Eu samples before immersion in SBF have been reported in our previous work [8].

The EDAX results and mapping analysis obtained for the HAp:Eu SBF samples after immersion in SBF for 24 hours are shown in Fig. 3. The EDAX findings confirmed the presence of Ca, P, Eu, O, Na, Cl and Mg. The major peaks attributed to Ca, P, and O certified the hydroxyapatite composition, as shown in Fig. 3. On the other hand, the mapping analysis confirmed a uniform and continuous distribution of all the constituents of the HAp:Eu SBF sample.

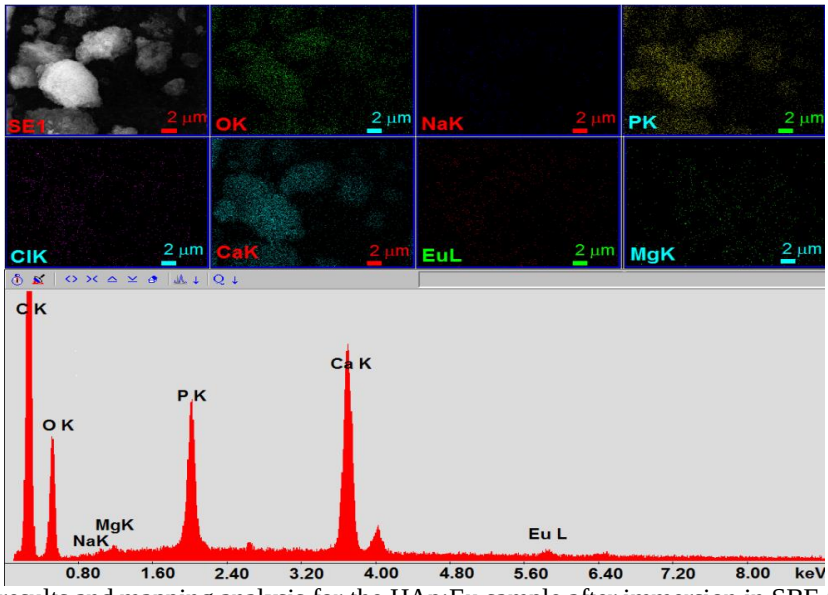

Fig. 3. EDAX results and mapping analysis for the HAp:Eu sample after immersion in SBF for 24 hours.

In Fig. 4, the Raman spectra recorded for the studied samples are presented (HAp:Eu SBF 6h and HAp:Eu SBF 24h). It was noticed, in both spectra, the presence of the bands characteristic to the ν_1 (960 cm^{-1}), ν_2 (429 cm^{-1}), ν_4 (590 cm^{-1}) and ν_3 (1045 cm^{-1}) vibrational modes associated to the phosphate group from the structure of hydroxyapatite [4-8]. Moreover, a decrease of the vibrational bands intensity could be observed. This behavior could indicate a decrease of the powders' degree of crystallization. None of the two spectra presented in Fig. 4 exhibited any additional bands. This suggests that the samples kept the apatitic structure even after they were immersed in the SBF solution. Moreover, the behavior of HAp:Eu samples before immersion in SBF have been reported in our previous work [19].

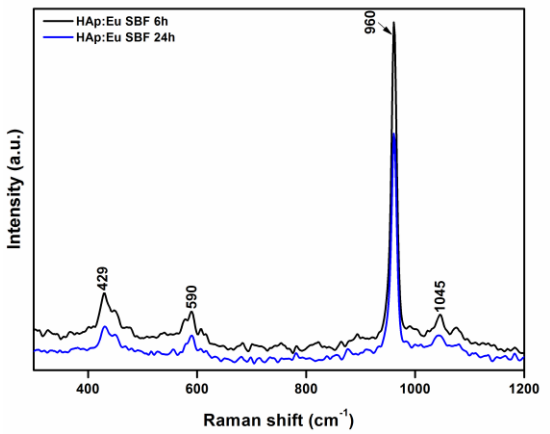

Fig. 4. Raman spectra of HAp:Eu SBF 6h and HAp:Eu SBF 24h powders.

Hydroxyapatite is a widely investigated material for potential biomedical applications due to its remarkable biocompatibility. In the last years, a special attention was directed towards the development and characterization of europium-doped hydroxyapatite for potential use as a biological probe [20]. Furthermore, recent studies regarding the antimicrobial activity of europium-doped hydroxyapatite have been reported [15]. In order to better understand the *in vitro* behavior of these types of materials, most often they are put into contact with a simulated body fluid (SBF) solution.

The aim of this study was to evaluate the antibacterial properties of europium doped hydroxyapatite powders previously immersed in SBF for 6 and 24 hours. The antibacterial activity of HAp:Eu SBF was tested using two of the most common bacterial strains, *Pseudomonas aeruginosa* 1397 (Gram-negative) and *Enterococcus faecalis* ATCC 29212 (Gram-positive).

The bactericidal effect of the HAp:Eu SBF 6 h and HAp:Eu SBF 24 h assessed against gram negative bacterial strain *Pseudomonas aeruginosa* 1397 is presented in Fig. 5. The results obtained indicated that the HAp:Eu SBF 6 h and

HAp:Eu SBF 24 h powders exhibit antibacterial activity against *Ps. aeruginosa*. The bactericidal effect on the *Ps. Aeruginosa* microbial suspensions was observed from 1mg/L to 0.125 mg/L.

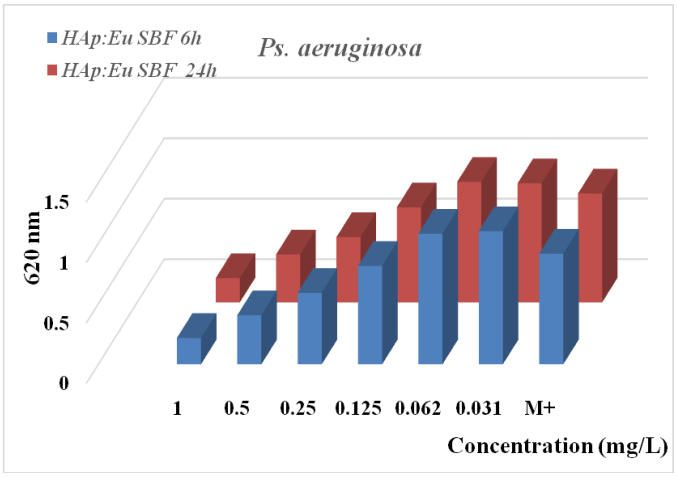

Fig. 5. Antibacterial activity of HAp:Eu SBF 6h and HAp:Eu SBF 24h against gram-negative *Pseudomonas aeruginosa* 1397.

Although HAp:Eu SBF 6 h and HAp:Eu SBF 24 h had a similar effect against *Ps. aeruginosa*, there was observed a slight difference for the absorbance at 620 nm. The values of the absorbance measured in the case of HAp:Eu SBF 24 h were smaller than the ones obtained for the HAp:Eu SBF 6 h.

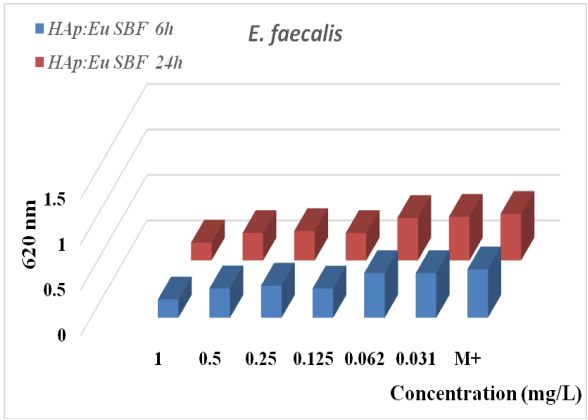

Fig. 6. Antibacterial activity of HAp:Eu SBF 6h and HAp:Eu SBF 24h against gram-positive *Enterococcus faecalis* ATCC 29212.

Thus, the results revealed that the period of exposure to SBF influenced the antibacterial activity of the europium doped hydroxyapatite powders. The antibacterial effect of the HAp:Eu SBF 6 h and HAp:Eu SBF 24 h was also tested

against a gram-positive bacterial strain. Fig. 6 presents the antibacterial activity of HAp:Eu SBF 6 h and HAp:Eu SBF 24 h against gram-positive *Enterococcus faecalis* ATCC 29212.

The results indicated that HAp:Eu SBF 6 h and HAp:Eu SBF 24 h powders have a good antibacterial activity against *E. faecalis* (Fig. 6) for all the studied concentrations (from 1mg/L to 0.031 mg/L). In the case of the HAp:Eu SBF 24 h sample, the inhibition of *E. faecalis* ATCC 29212 was more evident than in the case of HAp:Eu SBF 6 h sample, demonstrating that contact time of europium-doped hydroxyapatite nanoparticles with SBF influenced their antibacterial activity. In addition, the authors reported in their previous work the antimicrobial activity of HAp:Eu samples before immersion in SBF [15].

5. Conclusions

In this study we have reported the preparation of HAp:Eu powders by co-precipitation method and the evaluation of the morphology, optical properties and antimicrobial activity after immersion in a SBF solution for 6 and 24 h respectively. The SEM study revealed that the samples morphology is slightly influenced by the immersion time. Moreover, the elemental composition studies confirmed the presence of the chemical elements from the structure of HAp:Eu and SBF solution. In conclusion, this study on the antibacterial activity of HAp:Eu SBF 6 h and HAp:Eu SBF 24 h nanoparticles describes a novel approach of the potential usage of luminescent Eu^{3+} doped hydroxyapatite in biomedical applications. Also, the study describes the effect of simulated body fluid on the antibacterial activity of europium doped hydroxyapatite powders. These results suggest that this kind of materials possess great potential in being used as biomaterials with antibacterial properties.

Acknowledgements

This work was financially supported by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Ministry of European Funds through the Financial Agreement POSDRU/159/1.5/S/134398 and PN II 259/2014.

R E F E R E N C E S

[1]. *O. Kaygili, S. Keser, T. Ates, A.A. Al-Ghamdi and F. Yakuphanoglu,* "Controlling of dielectrical and optical properties of hydroxyapatite based bioceramics by Cd content", in Powder Technol., **vol. 245**, sept. 2013, pp. 1–6.

[2]. *I. Sopyan, A. Fadli and M. Mel,*" Porous alumina-hydroxyapatite composites through protein foaming-consolidation method", in J. Mech. Behav. Biomed. Mater., vol. 8, apr. 2012, pp. 86–98.

[3]. *G. Bezzi, G. Celotti, E. Landi, T.M.G. La Toretta, I. Sopyan and A. Tampieri,* " A novel sol–gel technique for hydroxyapatite preparation ", in Mater. Chem. Phys., **vol. 78**, feb. 2003, pp. 816–824.

[4]. *C. L. Popa, C. S. Ciobanu, S. L. Iconaru, M. Stan, A. Dinischiotu, C.C. Negrila, M. Motelica-Heino, R. Guegan and D. Predoi,* "Systematic investigation and *in vitro* biocompatibility studies on mesoporous europium doped hydroxyapatite", in Cent. Eur. J. Chem., **vol. 12**, Iss. 10, apr. 2014, pp. 1032-1046.

[5]. *C. S. Ciobanu, F. Massuyeau, L. V. Constantin and D. Predoi,* "Structural and physical properties of antibacterial Ag-doped nano-hydroxyapatite synthesized at 100°C", in Nanoscale Research Letters, **vol. 6**, dec. 2011, pp. 613.

[6]. *C. S. Ciobanu, C. L. Popa and D. Predoi*, "Sm:HAp nanopowders present antibacterial activity against *Enterococcus faecalis*", in J. Nanomat., **vol. 2014**, apr.2014, pp. 1-9.

[7]. *C. S. Ciobanu, E. Andronescu, B. S. Vasile, R. Trusca and D. Predoi*, "Preliminary studies on hydroxyapatite doped with europium", in U.P.B. Sci. Bull., Series B, **Vol. 73**, Iss. 4, dec. 2011, pp. 63-70.

[8]. *C. S. Ciobanu, S. L. Iconaru, F. Massuyeau, L. V. Constantin, A. Costescu and D. Predoi*, "Synthesis, Structure and Luminescent Properties of Europium-Doped Hydroxyapatite Nanocrystalline Powders", in J. Nanomat., **vol. 2012**, feb. 2012, pp. 1-9.

[9]. *F. Chen, P. Huang, Y. Zhu, J. Wu, C. Zhang and D. Cui*, "The photoluminescence, drug delivery and imaging properties of multifunctional Eu^{3+}/Gd^{3+} dual-doped hydroxyapatite nanorods", in Biomaterials, **vol. 32**, Iss. 34, dec. 2011, pp. 9031-9039.

[10]. *P. Yang, Z. Quan, C. Li, X. Kang, H. Lian and J. Lin,*" Bioactive, luminescent and mesoporous europium-doped hydroxyapatite as a drug carrier", in Biomaterials, **vol. 29**, iss 32, nov. 2008, pp. 4341-4347.

[11]. *X. Wang, H. Chang, J. Xie, B. Zhao, B. Liu, S. Xu, W. Pei, N. Ren, L. Huang and W. Huang*, "Recent developments in lanthanide-based luminescent probes", in Coord. Chem. Rev., **vol. 273–274**, aug. 2014, pp. 201–212.

[12]. *S. Y. Lee, S. I. Jeon, S. Jung, I. J. Chung and C.-H. Ahn*, "Targeted multimodal imaging modalities", in Adv. Drug Deliv. Rev., **vol. 76**, sept. 2014, pp. 60–78.

[13]. *A. Oyane, K. Onuma, A. Ito, H.M. Kim, T. Kokubo and T. Nakamura*, "Formation and growth of clusters in conventional and new kinds of simulated body fluids", in J. Biomed. Mater. Res., **vol. 64A**, feb. 2003, pp. 39–48.

[14]. *T. Kokubo and H.Takadama*, "How useful is SBF in predicting *in vivo* bone bioactivity?", in Biomaterials, **vol. 27**, iss. 15, may 2006, pp. 2907–2915.

[15]. *S. L. Iconaru, M. Motelica-Heino and D. Predoi*, "Study on Europium-Doped Hydroxyapatite Nanoparticles by Fourier Transform Infrared Spectroscopy and Their Antimicrobial Properties", in J. Spectroscopy**, vol. 2013**, aug. 2013, pp. 1-10.

[16]. *C. S. Ciobanu, E. Andronescu, B. S. Vasile, C. M. Valsangiacom, R.V. Ghita and D. Predoi*, "Looking for new synthesis of hydroxyapatite doped with europium", in Optoelectron. Adv. Mat., **vol. 4**, oct. 2010, pp. 1515 – 1519.

[17]. *J. H. Jorgensen and J. D. Turnidge*, "Susceptibility test methods: dilution and disk diffusion methods. Manual of Clinical Microbiology", 8th ed., American Society for Microbiology, Washington, DC, 2003.

[18]. *N. Ninan, M. Muthiah, I.K. Park, A. Elain, T.W. Wong, S. Thomas and Y. Grohens*, "Faujasites incorporated tissue engineering scaffolds for wound healing: *in vitro* and *in vivo* analysis", in ACS Appl Mater Interfaces, **vol. 5**, 2013; pp. 11194–11206.

[19]. *E. Andronescu, F. M. Iordache, C. S. Ciobanu, M. L. Badea, A. Costescu, A. M. Prodan*, "Optical properties of bioactive europium doped hydroxyapatite (HAp:Eu^{3+})", Optoelectron. Adv. Mat. **Vol. 9**, No. 9-10, September – October 2015, pp. 1155 – 1159

[20]. *T. Kushida*, "Site-selective fluorescence spectroscopy of Eu^{3+} and Sm^{2+} ions in glass", in J. Lumin., **vol. 100**, no. 1–4, dec. 2002, pp. 73–88.