SCAFFOLDURI FIBROASE COMPOZITE PROIECTATE PENTRU REGENERARE OSOASĂ
COMPOSITE FIBROUS SCAFFOLDS DESIGNED FOR BONE REGENERATION

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This work aims the obtaining of composite scaffolds based on polycaprolactone fibres loaded with inorganic powders, hydroxyapatite or/and barium titanate, through the electrospinning technique, as well as their characterization both from physicochemical point of view and biological side in order to establish the potential of such materials for integration in bone regeneration applications. The results confirmed the achievement of the designed materials, with a relative homogenous distribution of the mineral phases onto or inside the fibrous structures, together with a good response as respects the behaviour of fibroblast cells cultured in the presence of these multifunctional scaffolds with biocompatible, bioresorbable and bioactive features, combined with external stimulation capabilities (electric or/and magnetic).

Această lucrare are ca scop obținerea de scaffolduri compozite pe bază de fibre de policaprolactonă încărcate cu pulberi anorganice, hidroxiapatită sau/și titanat de bariu, prin tehnica electrofilării, precum și caracterizarea acestora atât din punct de vedere fizico-chimic, cât și din perspectivă biologică pentru a stabili potențialul unor astfel de materiale pentru integrare în aplicații de regenerare osoasă. Rezultatele au confirmat obținerea materialelor proiectate, cu o distribuție relativ omogenă a fazelor minerale pe sau în interiorul structurilor fibroase, împreună cu un răspuns favorabil în ceea ce privește comportamentul celulelor fibroblaste cultivate în prezența acestor scaffolduri multifuncționale cu trăsături de biocompatibilitate, bioresorbabilitate și bioactivitate, combinate cu capacități de stimulare externă (electrică sau/și magnetică).

Keywords: *Polycaprolactone; Hydroxyapatite; Barium titanate; Composite scaffolds; Electrospinning*

1. Introduction

According to the statistics, tens of millions of bone injuries occur in the world every year, which needs several millions of bone grafting procedures. Therefore, tissue engineering is one of the most important strategies for the treatment of bone injuries. Successful application of bone engineering can overcome the challenges related to other surgery opportunities, such as autografts or allografts [1]. Thus, tissue engineering is an interdisciplinary field in which engineering principles are applied for the development of biological functional substitutes inside the living bodies, that restore, maintain or improve the tissue functions [2].

One of the key components in tissue engineering for bone regeneration is the scaffold, which serves as a template for the cells interaction and formation of bone extracellular matrix to provide structural support to the newly formed tissue. In this context, the artificially designed scaffolds should display some of the following properties: three-dimensional appearance, high volume of open and interconnected pores, suitable mechanical properties, biocompatibility, biodegradability with controlled resorption rate, bioactivity and content of signalling molecules to induce new bone formation [3].

During recent years, the electrospinning method and the corresponding micro- or nanofibrous networks achieved remarkable interest, essentially due to the structural mimicking of the extracellular matrix and processing accessibility to a wide range of materials, as well as simple set-up and cost-effectiveness [1]. The electrospinning of biodegradable polymers, either with a synthetic or natural origin, was first reported to generate suitable bone cell matrices, largely due to their ease of processing. Furthermore, the flexibility and shape availability of polymeric materials provide them great potential in the bone regeneration field. Among all polymers, a group of poly(α-hydroxyl acids), such as poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers, has been the most extensively studied fibres system for the regeneration of tissues, including bone. PCL was first proposed as bioresorbable fibrous network for hard tissue healing, proving to supply a good support for the mesenchymal stem cells derived from the bone marrow of neonatal rats [4]. Newly, Jinga *et al.* [5] reported the obtaining of electrospun scaffolds based on PCL and zinc oxide, titanium dioxide or hydroxyapatite, their biodegradability and biocompatibility being highlighted.

As well, hydroxyapatite (HA) has been extensively investigated and employed in bone clinical applications for more than four decades. The increasing interest in HA is due to its similar chemical composition to that of the inorganic

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component of natural bone. Moreover, it displays favourable properties, such as biocompatibility, bioactivity, slow degradation, osteoinduction, osteoconduction and osseointegration. HA is commercially available either from natural sources or as synthetic derivatives. Lately, a variety of HA-polymer composites have been developed; while HA provides bioactivity, the addition of a polymeric matrix improves the mechanical properties, in particular brittleness, tensile strength and fracture toughness [3].

Electrical effects, such as piezoelectricity, pyroelectricity and ferroelectricity, play an important role in bone growth, remodelling and fracture healing. Recently, ferroelectric ceramics, including lithium niobate (LN) and barium titanate (BT), have been widely used as bone repair materials, with excellent biocompatibility and osseointegration due to their relatively high spontaneous polarization and inherent ability to sustain a charged surface [6].

Considering the aspects described above, as well as the advantages brought by each component separately, we report in this paper the fabrication and evaluation of the composites based on PCL fibres loaded with HA or/and BT powders. The novelty of this approach is sustained by the processing through electrospinning, as well as the integration of BT powder synthesized by a complex wet-chemistry method with the purpose of external stimulation of the physiological environment in order to yield beneficial conditions for the cellular metabolism.

2.Experimental

The composite fibrous scaffolds were obtained by the electrospinning technique, starting from three components:

- polymer, polycaprolactone (PCL, $(C_6H_{10}O_2)_n$, 80.000 Da) purchased from Sigma-Aldrich;
- inorganic powders, commercial hydroxyapatite (HA, $Ca_5(PO_4)_3(OH)$) provided by Sigma-Aldrich and laboratory synthesized barium titanate (BT, $BaTiO_3$); the last one was synthesized by using barium acetate and titanium isopropoxide as precursors and applying a combined method, sol-gel-hydrothermal (processing at 120 °C for 24 h, at the resulting vapor pressure), as previously reported in [7];
- solvent, chloroform (CF, $CHCl_3$) and dimethylformamide (DMF, C_3H_7NO), with a ratio CF/DMF of 4/1 (v/v).

The precursor solutions were achieved in two steps. First, a 5 w/v% suspension of inorganic powder, individual or combination, in the solvent mixture was prepared, with ultrasonication for 5 min. Second, the polymer was dissolved in the previously described suspension, at a concentration of 16 w/v%, with magnetic stirring for 24 h. The mineral phases were added as follows: HA, BT and HA+BT (weight ratio of 1:1), resulting in three materials, coded in this way: PCL-HA, PCL-BT and PCL-HA-BT.

The electrospinning process was carried out in the next operating conditions: stainless steel spinneret with inner diameter of 0.8 mm, 3 mL/h feeding rate, 15 kV applied high voltage, glass plates attached to a static collector placed at 25 cm from the spinneret, 28 °C ambient temperature and 56 % relative humidity.

The samples were characterized by: X-ray diffraction (XRD) with a Shimadzu XRD 6000 diffractometer or PANalytical Empyrean diffractometer, 2θ ranging between 20 and 70 °; Raman spectroscopy with a Horiba LabRAM HR Evolution spectrometer, the wavenumber ranging between 200 and 1000 cm^{-1}; scanning electron microscopy (SEM), coupled with energy-dispersive X-ray spectroscopy (EDX), with a FEI Quanta Inspect F electron microscope; Fourier-transform infrared spectroscopy (FTIR) with a Thermo Scientific Nicolet 6700 spectrophotometer, the wavenumber ranging between 400 and 2000 cm^{-1}.

The biological evaluation was performed from qualitative point of view by the test of cellular adhesion and proliferation after 7 days in culture, employing the LIVE/DEAD viability kit that allows fluorescence marking of living cells with calcein and dead cells nucleus with Ethidium Homodimer-1; a standard protocol was followed [8]. The fluorescence image was acquired with a Carl Zeiss LSM 880 confocal microscope.

3.Results and discussion

The inorganic powders were first characterized from compositional and structural point of view in order to establish if they are single-phase materials and their membership to a certain crystallization system. Thus, Fig. 1 displays the XRD patterns of HA and BT powders. The first one is composed of the hydroxyl end-member of the complex apatite group, with hexagonal symmetry and high degree of crystallinity, statement validated by the positioning and shape of the diffraction peaks, which identically copies the standard pattern from the database. Going to the second one, it is made of the targeted perovskite, with cubic symmetry and large crystallites size based on the narrow and tall diffraction peaks. However, taking into account the fact that BT powder was processed at high pressure, it is expected to discover domains with a distorted cubic structure, namely pseudo-tetragonal structure, within the material.

A proof in this sense is given by the Raman spectrum presented in Fig. 2. The corresponding curve includes several active modes of A1 (246, 518 and 713 cm^{-1}) or B1 (300 cm^{-1}) symmetry, in correlation with the

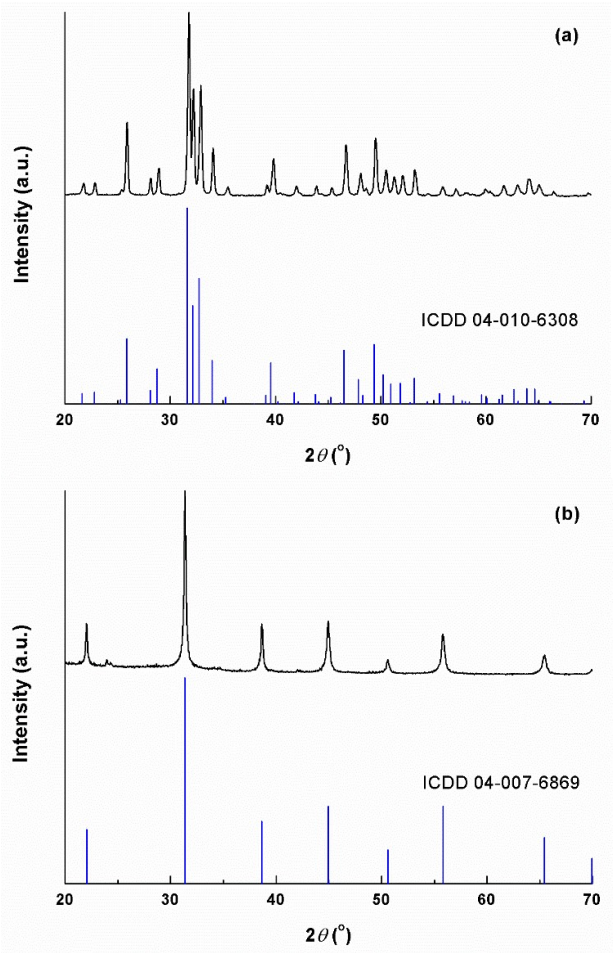

Fig. 1 - XRD patterns of: (a) HA powder and (b) BT powder, compared to the standard patterns from the database. *Analizele XRD ale: (a) pulberii HA și (b) pulberii BT, în comparație cu analizele standard din baza de date*.

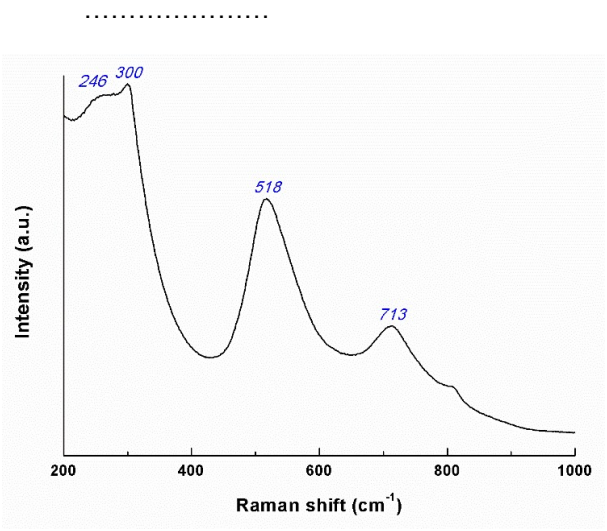

Fig. 2 - Raman spectrum of BT powder. *Spectrul Raman al pulberii BT.*

polycrystalline nature of BT powder and tetragonal phase presence, since the peak from 300 cm^{-1} is detectable and confirms the distortion of the cubic structure into a pseudo-cubic or slightly tetragonal one [9]. Such phenomenon can further generate an interesting behaviour in the field of magnetic properties, namely ferromagnetism due to the existence intrinsic point defects [10]. This can be subsequently exploited in the direction of local stimulation of the physiological environment of cell cultures [11, 12].

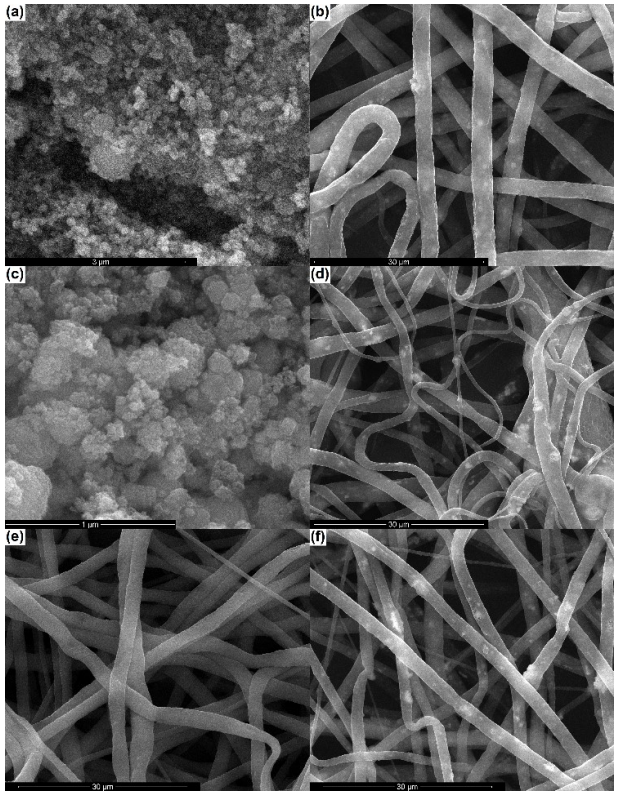

Fig. 3 - SEM images of: (a) HA powder, (b) PCL-HA scaffold, (c) BT powder, (d) PCL-BT scaffold, (e) PCL scaffold and (f) PCL-HA-BT scaffold./ *Imagini SEM ale: (a) pulberii HA, (b) scaffoldului PCL-HA, (c) pulberii BT, (d) scaffoldului PCL-BT, (e) scaffoldului PCL și (f) scaffoldului PCL-HA-BT.*

Fig. 3 shows the morphology of the individual phases and final composite scaffolds. The commercial HA powder (Fig. 3a) is made of quasi-spherical particles with a diameter around 100 nm for the most part of them, while the synthesized BT powder (Fig. 3c) consists of aggregates of nanometric particles, with spherical shape and sharp size distribution around 5 nm. Analysing the appearance of these two mineral phases, it is obvious that aggregates breaking and particles dispersion in the solvent mixture will be much easier in the case of HA compared to BT, which has larger specific surface area, higher surface energy and increased tendency of agglomeration. The simple polymeric fibres (Fig. 3e) are randomly arranged and form a uniform network of one-dimensional, continuous and constant in thickness structures, having diameters between 2 and 3 μm; only a few fibres with reduced diameter down to 700 nm can be identified. The addition of each powder separately or their mixture modifies the properties of the precursor solution, the stability of initial jet and subsequently the aspect of the final materials. Thus, in the case of HA (Fig. 3b), the fibres seem to be a little thicker, but with a tight size distribution, in the range 3-4 μm, and a quite good dispersion of the inorganic phase, predominantly inside their volume, at different depths. The loading of BT powder (Fig. 3d) generates significant changes, with the formation of curlier and more sticked fibres, with diameters in a wider interval, from 500 nm to 9 μm, the mineral aggregates having favourite areas, both at the surface and within the volume of the fibres. The combination of the two powders, HA and BT (Fig. 3f), leads to a mediation effect when it comes to the morphology of the resulting scaffold: a major proportion of fibres with 2-3 μm diameter and a minor proportion of fibres with 500-600 nm diameter. The homogeneity on large areas is affected to some extent because of the emergence of large agglomerations of particles from place to place. For all composites, beads-on-string structures can be observed at lower magnifications, especially for PCL-BT sample.

In order to demonstrate the fibres loading with particles of different compositions, the EDX and FTIR spectra were recorded and plotted centralized in Fig. 4. The elemental composition consists of C from PCL, Ca and P from HA, Ba and Ti from BT, the specific energy lines occurred in all three spectra demonstrating the achievement of the targeted materials. On another hand, the FTIR spectra contains vibrational bands specific to PCL (1722 cm^{-1} - C=O bonding, 1239 and 1165 cm^{-1} - C–O–C grouping) [13], HA (1088, 1043, 630, 601 and 571 cm^{-1} - P–O bonding) [14] and BT (528 and 438 cm^{-1} - Ti–O bonding) [15].

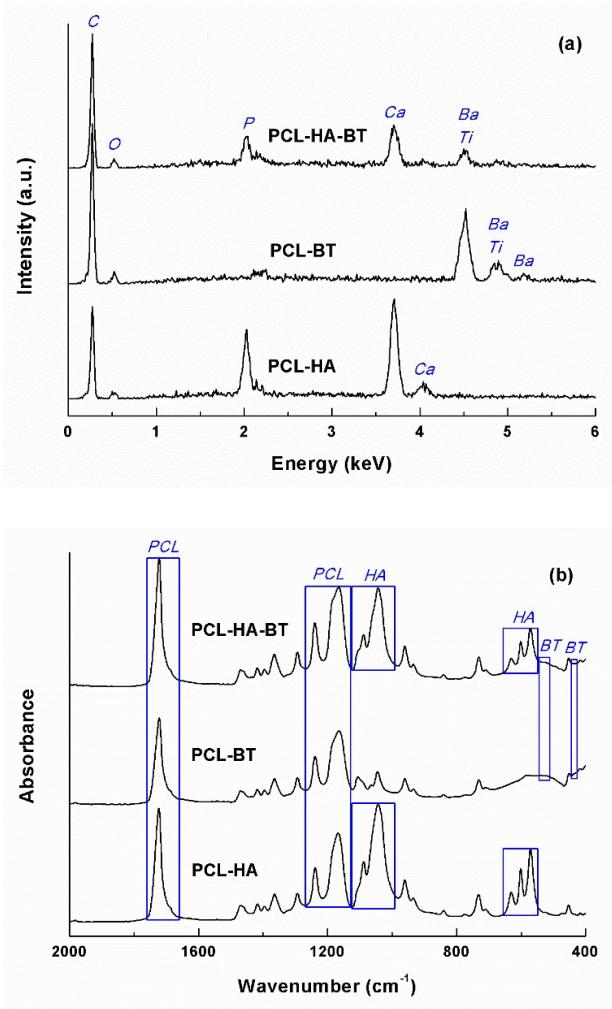

Fig. 4 - (a) EDX and (b) FTIR spectra of PCL-HA, PCL-BT and PCL-HA-BT scaffolds / *Spectrele: (a) EDX și (b) FTIR ale scaffoldurilor PCL-HA, PCL-BT și PCL-HA-BT.*

Since the material containing both HA and BT is the only one from which a synergistic contribution is expected, it was decided to conduct a preliminary biological investigation on PCL-HA-BT composite scaffold. Thus, the evaluation performed after 7 days in culture with fibroblast cells (3T3 line) through the LIVE/DEAD assay and fluorescence microscopy image (Fig. 5a) suggests that the cell culture adhered to the investigated hydrophilic support, the cells present an important proliferation, with a cellular morphology displaying extensions, building networks and promoting an arrangement in the material depth, based on the existing interconnected porosity and fibres directionality. Furthermore, the SEM image from Fig. 5b presents the aspect of PCL-HA-BT sample after dehydration in vacuum and gold coating by magnetron sputtering; it highlights the cells settlement on the three-dimensional porous network of fibres on favourite sites, especially at fibres crossing, at different depths, followed by their spreading in the radial direction, which

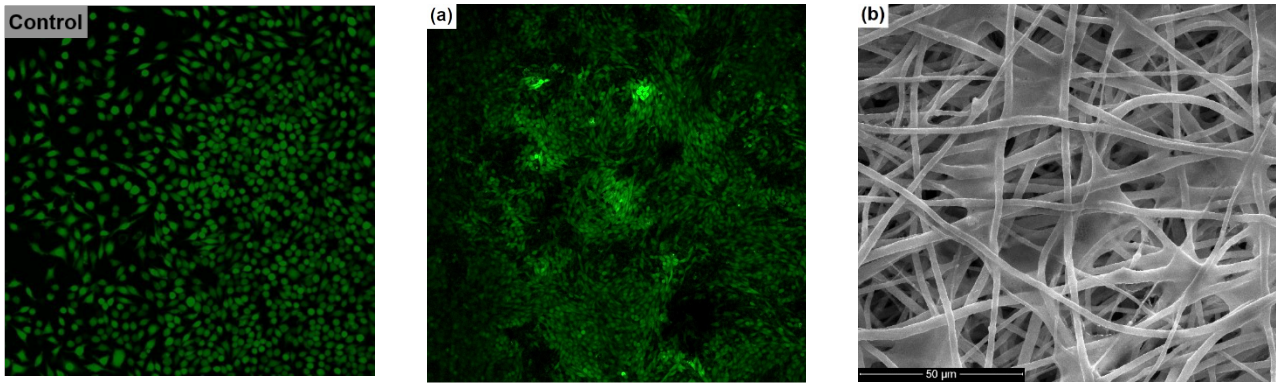

Fig. 5 - (a) Fluorescence microscopy and (b) SEM images recorded on PCL-HA-BT scaffold.
Imagini de: (a) microscopie de fluorescență și (b) SEM înregistrate pe scaffoldul PCL-HA-BT.

indicates that this multifunctional scaffold is well accepted and could be integrated in the future in hard tissue applications.

4.Conclusions

Composite fibrous scaffolds based on polycaprolactone, hydroxyapatite and barium titanate were successfully manufactured by the electrospinning technique. The employed inorganic powders were either commercially available or laboratory synthesized in order to control the final properties. The fibres diameter varied from less than 1 µm up to 4 µm, while the loaded mineral phases were distributed quite evenly as aggregates of different sizes both on the fibres surface and in their volume. The biological evaluation performed on fibroblast cells confirmed the biocompatibility of such materials. Further investigations will be carried out regarding composites bioresorbability, bioactivity, as well as bone healing through electric or/and magnetic stimulation.

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References

[1] Z. Rezvani, J.R. Venugopal, A.M. Urbanska, D.K. Mills, S. Ramakrishna and M. Mozafari, A bird's eye view on the use of electrospun nanofibrous scaffolds for bone tissue engineering: Current state-of-the-art, emerging directions and future trends, Nanomedicine: Nanotechnology, Biology and Medicine, 2016, **12**, 2181.

[2] D.P. Bhattarai, L.E. Aguilar, C.H. Park and C.S. Kim, A review on properties of natural and synthetic based electrospun fibrous materials for bone tissue engineering, Membranes, 2018, **8**, 62.

[3] V.S. Gshalaev and A.C. Demirchan, Hydroxyapatite: Synthesis, properties and applications, Nova Science Publishers, 2016.

[4] J.H. Jang, O. Castano and H.W. Kim, Electrospun materials as potential platforms for bone tissue engineering, Advanced Drug Delivery Reviews, 2009, **61**, 1065.

[5] S.I. Jinga, A.I. Zamfirescu, G. Voicu, M. Enculescu, A. Evanghelidis and C. Busuioc, PCL-ZnO/TiO_2/HAp electrospun composite fibres with applications in tissue engineering, Polymers, 2019, **11**, 1793.

[6] Y. Li, X. Dai, Y. Bai, Y. Liu, Y. Wang, O. Liu, F. Yan, Z. Tang, X. Zhang and X. Deng, Electroactive $BaTiO_3$ nanoparticle-functionalized fibrous scaffolds enhance osteogenic differentiation of mesenchymal stem cells, International Journal of Nanomedicine, 2017, **12**, 4007.

[7] A.V. Zanfir, G. Voicu, S.I. Jinga, E. Vasile and V. Ionita, Low-temperature synthesis of $BaTiO_3$ nanopowders, Ceramics International, 2016, **42**, 1672.

[8] LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells, Thermo Fisher Scientific, 2005.

[9] H. Hayashi, T. Nakamura and T. Ebina, In-situ Raman spectroscopy of BaTiO3 particles for tetragonal-cubic transformation, Journal of Physics and Chemistry of Solids, 2013, **74**, 957.

[10] S.G. Bahoosh, S. Trimper and J.M. Wesselinowa, Origin of ferromagnetism in $BaTiO_3$ nanoparticles, Physica Status Solidi, 2011, **5**, 382.

[11] A.V. Zanfir, G. Voicu, C. Busuioc, S.I. Jinga, M.G. Albu and F. Iordache, New Coll-HA/BT composite materials for hard tissue engineering, Materials Science and Engineering C, 2016, **62**, 795.

[12] C. Busuioc, G. Voicu, S.I. Jinga, V. Mitran and A. Cimpean, The influence of barium titanate on the biological properties of collagen-hydroxiapatite composite scaffolds, Materials Letters, 2019, **253**, 317.

[13] B Abderrahim, E. Abderrahman, A. Mohamed, T. Fatima, T. Abdesselam and O. Krim, Kinetic thermal degradation of cellulose, polybutylene succinate and a green composite: comparative study, Journal of Environmental Chemical Engineering, 2015, **3**, 95.

[14] S. Mondal, B. Mondal, A. Dey and S.S. Mukhopadhyay, Studies on processing and characterization of hydroxyapatite biomaterials from different bio wastes, Journal of Minerals and Materials Characterization and Engineering, 2012, **11**, 55.

[15] M.K. Trivedi, G. Nayak, S. Patil, R.M. Tallapragada, O. Latiyal and S. Jana, Impact of biofield treatment on atomic and structural characteristics of barium titanate powder, Journal of Industrial Engineering and Management, 2015, **4**, 166.

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