Society For Biomaterials

New method for the fabrication of highly osteoconductive β-1,3-glucan/HA scaffold for bone tissue engineering: Structural, mechanical, and biological characterization

Katarzyna Klimek,[1] Agata Przekora,[1] Krzysztof Pałka,[2] Grażyna Ginalska[1]

[1]Department of Biochemistry and Biotechnology, Medical University of Lublin, Chodzki 1, 20-093, Lublin, Poland
[2]Department of Materials Engineering, Lublin University of Technology, Nadbystrzycka 36, 20-618, Lublin, Poland

Received 9 April 2016; revised 20 May 2016; accepted 26 May 2016
Published online 8 June 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.35798

Abstract: Recent studies have shown that thermal method for β-1,3-glucan (curdlan) gelation performed at temperature above 80°C enables fabrication of biocompatible bone scaffolds. The aim of this study was to establish new method for fabrication of β-1,3-glucan/hydroxyapatite (glu/HA) scaffold using ion-exchanging dialysis for curdlan gelation that allows for the modifications of the glu/HA material with thermo-sensitive agents like growth factors or adhesive proteins. Obtained results reveal that fabricated scaffold appears to be highly osteoconductive as it is nontoxic, promotes osteoblast growth and proliferation as well as increases bone alkaline phosphatase level thereby enhancing cell differentiation. It was demonstrated that developed new method for the glu/HA scaffold fabrication allows to obtain material that not only can be modified with thermo-sensitive agents at the stage of production process but also is a promising candidate for bone tissue engineering applications to act as a framework for osteoblasts to spread and form new bone. It should be noted that dialysis method for curdlan gelation has never been used before to fabricate bone scaffold. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 2528–2536, 2016.

Key Words: curdlan, osteogenic differentiation, compressive strength, Young's modulus

How to cite this article: Klimek K, Przekora A, Pałka K, Ginalska G. 2016. New method for the fabrication of highly osteoconductive β-1,3-glucan/HA scaffold for bone tissue engineering: Structural, mechanical, and biological characterization. J Biomed Mater Res Part A 2016:104A:2528–2536.

INTRODUCTION

Fabrication of available implantable materials is a fundamental element of tissue reconstruction.[1] Two types of biomaterials for bone regeneration may be distinguished within biomedical materials field. First type includes bone substitutes that are often used to fill large bone defects and thus may be exposed to high mechanical stress. Therefore, this type of biomaterials should have mechanical properties, such as compressive/tensile strength or Young's modulus values, close to human bone. The second type involves bone scaffolds, whose pivotal role is to improve the regeneration process in the place of bone defects. For this reason, high biocompatibility of these materials is often favored over their mechanical properties and thus their implantation is limited to the areas that are not exposed to high mechanical loads.[2,3] Considering bone regeneration process, the scaffolds should be primarily osteoconductive which is defined as promotion of osteoblast adhesion, proliferation, and differentiation.[4]

Hydroxyapatite (HA) and tricalcium phosphates (α-TCP and β-TCP) are considered to be major implantation ceramic materials used in bone tissue engineering. HA, an essential mineral component of bone, was shown to be completely biocompatible, osteoconductive, and bioresorbable.[5,6] Nevertheless, calcium phosphate bioceramics reveal relatively long degradation time in human organism, possess unsatisfactory mechanical properties, and due to the their friability do not exhibit good surgical handiness. Fabrication of two- or three-component composite biomaterials composed of bioceramics and natural or synthetic polymers exhibiting high bioactivity and *in vivo* gradual biodegradability allows to connect advantages of both types of materials.[7]

Curdlan (β-1,3-glucan) is a bacterial linear D-glucose homopolymer which possesses various biological properties such as anti-cancer, anti-septic, anti-inflammatory, or anticoagulant activities.[8] Furthermore, it has unique ability to form firm but flexible gel. Curdlan gelation may be induced either by heating its aqueous suspension to above 55°C[8] or by neutralization its alkaline solution in acids as well as dialysis against alkali metal salts at room temperature.[9,10] Recent studies performed by our research team have shown that curdlan gel obtained by heating to above 80°C is a suitable HA binder for bioactive bone substitutes.[11,12] Moreover, thermally obtained curdlan gel has also been successfully

Correspondence to: A. Przekora; e-mail: agata.przekora@umlub.pl
Contract grant sponsor: Ministry of Science and Higher Education (MNmb1 and DS2/15 project of Medical University of Lublin, Poland)

used as a constituent of highly biocompatible tricomponent chitosan/β-1,3-glucan/bioceramic scaffolds.[4,13]

The aim of this study was to establish new method for fabrication of β-1,3-glucan/hydroxyapatite (glu/HA) scaffold for bone tissue engineering applications by dialysis of the mixture of the alkaline β-1,3-glucan solution and HA granules into aqueous calcium chloride at room temperature. The goal of the study was also to demonstrate that applied method, which may yield benefits associated with modifications of the material with thermo-sensitive agents (e.g., growth factors, adhesive proteins) at the stage of production process, allows to fabricate the biomaterial characterized by high biocompatibility and osteoconductivity. It should be noted that dialysis method for curdlan gelation has never been used before in the biomedical materials field to fabricate biomaterials for bone tissue engineering.

MATERIALS AND METHODS

HA granules preparation and characterization

HA granules were produced according to the common procedure described elsewhere.[14,15] Briefly, 1.67*M* H_3PO_4 (Avantor Performance Materials, Poland) was added to 1 *M* aqueous suspension of $Ca(OH)_2$ (Avantor Performance Materials, Poland) and pH was adjusted with 2*M* NaOH (Avantor Performance Materials, Poland) to approximately 11. Received precipitate was dried at 90°C for 24 h, crumbled into smaller particles, sintered at 1150°C for 2 h, and then sieved to collect 0.3–0.6 mm in diameter granules. The phase of prepared HA granules was identified by X-ray diffraction (XRD). XRD analysis was performed using the CuKα radiation generated at 40 kV and 20 mA (conventional sealed 1500 watt X-ray tube was applied as the X ray source) and a scan range between 20° and 50° 2θ at a step size of 0.01° (2θ). XRD spectrum was recorded in Bragg–Brentano focusing mode with the diffractometer type HZG-4 (Zeiss) and scintillation detector. Identification of the HA phase was performed using the PDF-4 database (ICDD).

β-1,3-Glucan/hydroxyapatite scaffold fabrication

Beta-1,3-glucan/hydroxyapatite (glu/HA) scaffold was produced in accordance with procedure described in Patent pending.[16] The appropriate amount of β-1,3-glucan powder (Wako pure Chemicals Industries, Japan) was dissolved in NaOH solution of pH 12.7 (Avantor Performance Materials, Poland) and mixed to make homogeneous gel. Then, the desired quantity of HA granules was gradually added. After thorough spread of HA granules in curdlan solution using the planetary centrifugal mixer, the prepared uniform mass was put into dialysis tubing and dialyzed against $CaCl_2$ solution (Avantor Performance Materials, Poland) at room temperature to gel the β-1,3-glucan component of the material. Afterwards, the prepared two-component scaffold was rinsed three times for 30 min. with deionized water to remove residues of $CaCl_2$ from the surface of the material. Fabricated glu/HA material, containing 8 wt % of curdlan and 80 wt % of HA granules, was cut into suitable sizes and left to air dry for 24 hours. Prepared biomaterial in a wet and dry state is presented in Figure 1.

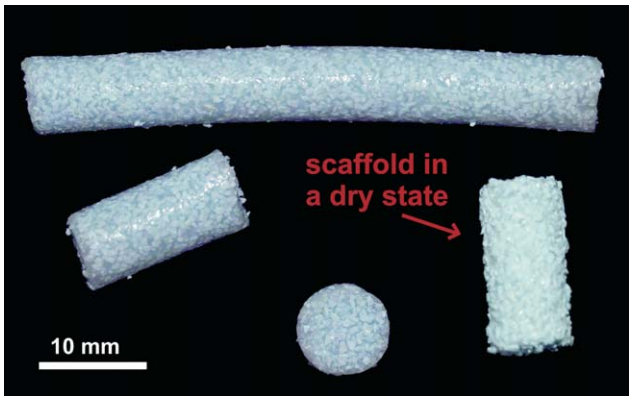

FIGURE 1. Photograph of the glu/HA scaffold in a wet state just after fabrication process and in a dry state after air drying (red arrow).

Scaffold microstructure visualization

The cylinder-shaped samples (8 mm in diameter, 15 mm in length) were used to evaluate the structure of the composite. Surface of the glu/HA scaffold was visualized using scanning electron microscopy (SEM, Nova NanoSEM 450, FEI). The microstructure of the scaffold was determined by the computed microtomography (SkyScan 1272, Bruker Micro-CT) using samples in a wet state (after soaking in normal saline solution—0.9% NaCl). Obtained set of images (a total of 2000 pieces) was reconstructed into cross-sections using NRecon software (Bruker Micro-CT, Belgium) and further analyzed. The isotropic voxel size obtained was 5 μm in each axis. Moreover, to quantitatively evaluate the total, open, closed, and potential porosity (after β-1,3-glucan degradation), the CTAn software (Bruker Micro-CT, Belgium) was applied.

Compression test

The cylinder-shaped scaffold samples (the same like in the case of computed microtomography analysis) were allocated to compression test after soaking in phosphate buffered saline (Sigma-Aldrich Chemicals, Poland) as biomaterials occur in a wet state within organism. Then, wetted samples were subjected to compression testing using Zwick Roell Z2.5 testing machine (preload value of 1 *N*, crosshead moving speed 10 mm/min followed by basic load rate 0.5 mm/min). Based on obtained data, Young's modulus (*E*) and compressive strength values were determined as described earlier.[3] The maximum strain value of 30% was applied. Compression test was conducted using four separate scaffolds ($n = 4$). The results were expressed as mean values ± standard deviation (SD).

Water uptake determination

The scaffold samples measuring 8 mm in diameter and 15 mm in length were used to determine water uptake behavior. Water absorption capacity of the glu/HA scaffold was evaluated by measuring the weight increase with time after placing the samples into normal saline solution (0.9% NaCl). The normal saline solution was used so as to keep the actual procedure that is applied during pre-operative treatment (before the implantation surgery, biomaterials are often

soaked in normal saline solution) and to assess the potential of the tested scaffold as a drug carrier—antibiotic solutions are often prepared in 0.9% NaCl. The weighting was performed until no further increase in weight of the sample was observed (60 min.). The water uptake ability was expressed as weight increase (W_i) using the equation: $W_i = (W_t\text{-}W_0)/W_0 \times 100$, where W_0–weight of the dry sample at time 0; W_t–weight of the soaked sample at time t.[3,11] The experiment was performed using four separate samples of the glu/HA scaffold ($n = 4$). The results were expressed as mean values ± SD.

Cell culture experiments

For cell culture experiments the cylinder-shaped material (8 mm diameter) was cut into 2 mm thick discs and sterilized via ethylene oxide. Before setting up the tests, the discs placed in wells of a 48-well plate were incubated in a complete cell culture medium for 24 h at 34°C or 37°C (depending on the kind of cell line used).

Cell lines. Two osteoblastic cell lines were used in this study: hFOB 1.19 (normal human fetal osteoblasts) and MC3T3-E1 Subclone 4 (normal mouse calvarial preosteoblasts) obtained from ATCC (American Type Culture Collection). The hFOB 1.19 cells were cultured in a DMEM/Ham F12 medium (Sigma-Aldrich Chemicals, Poland) with addition of 300 μg/mL G418 (Sigma-Aldrich Chemicals, Poland), whereas MC3T3-E1 cells were cultured in MEM Alpha (Gibco, Poland). Both culture media were supplemented with 10% fetal bovine serum (Pan-Biotech, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich Chemicals, Poland). The osteoblasts were grown at 34°C (hFOB 1.19) or 37°C (MC3T3-E1) in a humidified incubator with 5% CO_2. Cell differentiation was initiated via addition of osteogenic supplements as it was described earlier.[13]

Cytotoxicity evaluation. The hFOB 1.19 cells (at concentration of 1×10^5 cells/mL) and MC3T3-E1 cells (at concentration of 2×10^5 cells/mL) were seeded directly on the glu/HA discs in 500 μL of complete growth medium. After 24-h incubation at recommended for each cell line temperature, cell viability on the scaffold was assessed by fluorescent staining using *Live/Dead Double Staining Kit* (Sigma-Aldrich Chemicals, Poland) according to the manufacturer instruction. Live cells emitted green fluorescence, while dead cells emitted red fluorescence of nuclei. The cells were observed under confocal microscope (Olympus Fluoview equipped with FV1000).

Cell growth assessment. To evaluate the growth of osteoblast cells on the scaffold surface, the hFOB 1.19 (at concentration of 5×10^4 cells/mL) and MC3T3-E1 cells (at concentration of 1×10^5 cells/mL) were seeded on the glu/HA discs in 500 μL of complete growth medium. The culture media were replaced every third day. On the second, fifth, and eighth day of culture, F-actin filaments and nuclei were stained with AlexaFluor635-phalloidin (Invitrogen, Poland) and Hoechst33342 fluorescent dyes (Sigma-Aldrich Chemicals, Poland), respectively. The detailed procedure of cell staining was described previously.[4] Stained osteoblasts were observed under confocal microscope.

Cell differentiation evaluation. The hFOB 1.19 and MC3T3-E1 cells were seeded as described in *Cytotoxicity evaluation* section. Control culture was conducted directly on polystyrene and analyzed in parallel. After 24-h incubation, the growth media were replaced by osteogenic ones. The medium replacement was performed every third day throughout the duration of the experiment. Markers of the osteogenic differentiation were determined on the 4th, 12th, and 18th day of culture either in cell culture supernatants (osteocalcin [OC]) or in cell lysates (bone-specific alkaline phosphatase and runt-related transcription factor 2). Supernatants were collected via centrifugation of the plate at $250 \times g$ for 4 min. (4°C). Afterwards, cell lysates were obtained via two frozen/thawed cycles and sonication as described earlier.[13] The OC concentration was determined in supernatants using enzyme-linked immunosorbent assays (ELISAs) specific for mouse (muOC ELISA kit, Immunotopics) and human (huOC ELISA kit, Quidel®). Bone-specific alkaline phosphatase (bALP) and runt-related transcription factor 2 (RUNX2) levels were evaluated in cell lysates using appropriate ELISA kits specific for mouse (muALPL ELISA kit and muRUNX2 ELISA kit, Uscn Life Science) and human (huBAP ELISA kit, Quidel® and huRUNX2 ELISA kit, Uscn Life Science). Four separate control and glu/HA samples were analyzed ($n = 4$). The results were expressed as mean values ± SD. Obtained data were analyzed using unpaired t test to assess statistical differences between scaffold and control samples. Differences were considered as statistically significant with $p < 0.05$ (GraphPad Prism 5, Version 5.04 Software).

RESULTS

Phase analysis of HA granules

The XRD pattern of fabricated HA granules (Fig. 2) obtained in the range between 20° and 50° 2θ were consistent with the ICDD data indicating that prepared HA granules were pure-phase, well-crystallized, and without any presence of other calcium phosphates. Crystallite size of HA granules (calculated using Scherrer equation) was equal 22.97 nm.

Microstructure analysis of the scaffold

Porosity of the glu/HA scaffold was determined by computed microtomography (μCT). Total porosity was composed of closed (12.61 ± 1.31%) and open (0.54 ± 0.25%) porosity and had relatively low value of 13.08 ± 1.34%. Potential porosity, defined as a space created after potential degradation of β-1,3-glucan gel in organism environment [purple color in Fig. 3(a)], was much higher and reached the value of 64%. The μCT analysis showed uniform distribution of HA granules [yellow–green color in Fig. 3(a)] in whole volume of fabricated composite without any clusters of the HA particles. Air voids (pores) were visible in the form of black areas in the interparticulate spaces. Moreover, SEM image demonstrated that HA granules, with an irregular shape and sharp edges, were uniformly covered by β-1,3-glucan gel layer [Fig. 3(b)].

Mechanical behavior of the scaffold

The stress-strain curve obtained with compression test is presented in Figure 4. The glu/HA scaffold revealed poor mechanical properties with the average Young's modulus value of

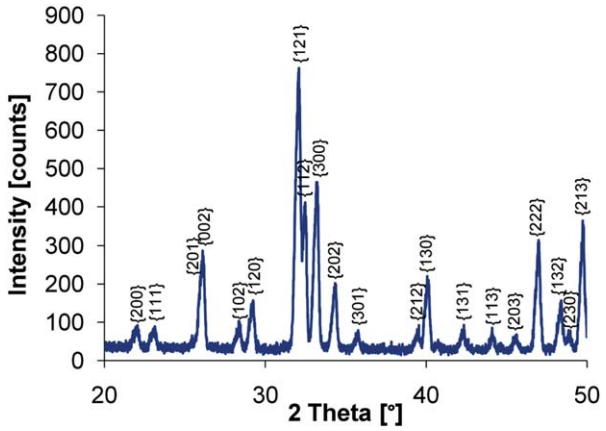

FIGURE 2. XRD pattern of fabricated hydroxyapatite (HA) granules including Miller's indices.

0.17 ± 0.05 MPa and the compressive strength value of 0.057 ± 0.006 MPa. The strain value to 30% did not cause decohesion process and complete destruction of the material, only some cracks within the sample were observed.

Water uptake determination

The glu/HA scaffold exhibited meaningful liquid uptake ability after placing the dry sample in normal saline solution (Fig. 5). Already after 2 min. soaking, the W_i value reached $34.71 \pm 7.45\%$. However, the sorption tendency of the glu/HA sample still increased with time and after 20 min. the W_i value was equal $53.02 \pm 7.86\%$. At this point the glu/HA scaffold reached sorption equilibrium and further weight increase was not considerable.

Cytotoxicity evaluation

The live/dead staining revealed group of viable osteoblast cells (green fluorescence) and only single dead cells (red fluorescence) on the glu/HA scaffold surface (Fig. 6). The osteoblasts were mostly well flattened, especially in the case of MC3T3-E1 cell line, indicating their good adhesion to the biomaterial surface.

Osteoblast proliferation assessment

The osteoblast growth on the glu/HA scaffold was assessed by confocal microscopy observation on cell cytoskeleton and nucleus staining (Fig. 7). Confocal microscope observation revealed clusters of well spread osteoblasts on the material surface already after 2-day culture. Moreover, both hFOB 1.19 and MC3T3-E1 cells had extensive system of cytoskeletal filaments, many filopodia, and large nuclei indicating their good condition and proliferation on the scaffold. More importantly, the considerable increase in cell number was observed with time, so on the eighth day of the experiment the surface was covered by almost multilayer of osteoblasts.

Osteogenic differentiation evaluation

In the case of both cell lines, hFOB 1.19 and MC3T3-E1, the levels of RUNX2 determined in the control and glu/HA samples were almost the same at each time interval [Fig. 8(a,b)].

In turn, the glu/HA scaffold showed tendency to increase bALP concentrations in hFOB 1.19 and MC3T3-E1 cultures compared to the control cells [Fig. 8(c,d)]. More importantly, hFOB 1.19 cells cultured on the glu/HA exhibited significantly ($p < 0.05$) higher bALP levels at each time point compared to the control cells [Fig. 8(c)]. On the 4th, 12th, and 18th day, the bALP concentrations in control samples were as follows: 1.19 ± 0.16 μg/mL, 4.08 ± 0.78 μg/mL, and 8.84 ± 0.66 μg/mL, respectively, whereas the following bALP levels were detected in the glu/HA samples: 2.8 ± 0.61 μg/mL ($p = 0.0021$), 6.23 ± 0.77 μg/mL ($p = 0.0079$), and 11.23 ± 0.61 μg/mL ($p = 0.044$), respectively. In the case of MC3T3-E1 cells, significantly higher ($p = 0.0005$) amount of bALP in the glu/HA sample compared to the control cells was detected only on the 18th day of the osteogenic differentiation [Fig. 8(d)]. It should be noted that similarly to hFOB 1.19 cells, MC3T3-E1 cells cultured on the glu/HA scaffold generated higher bALP levels compared to the control throughout duration of the experiment, but differences observed

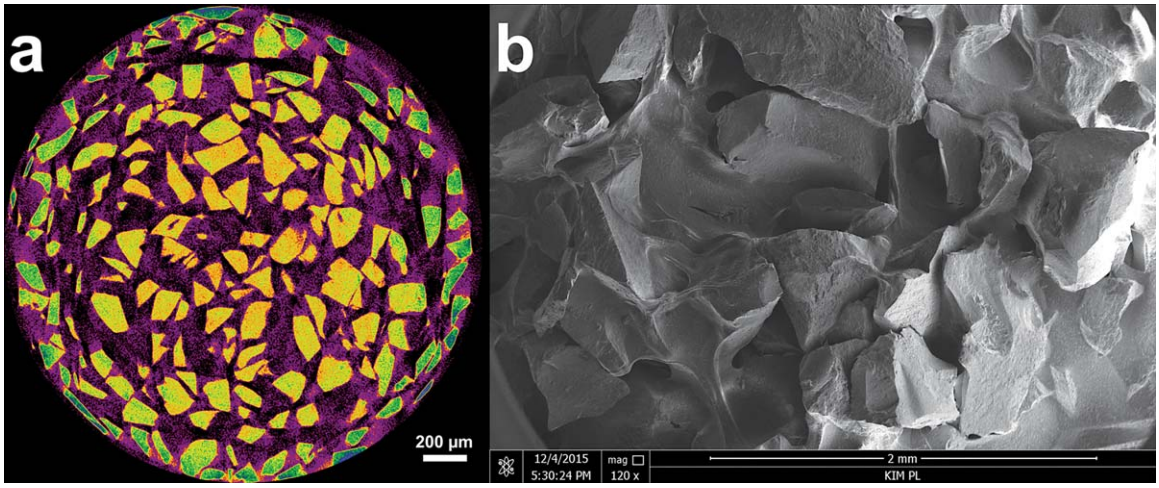

FIGURE 3. Microstructure of the glu/HA scaffold visualized by (a) μCT (HA—yellow–green color, β-1,3-glucan gel-purple color, air voids-black color) and (b) SEM, magn. 120×.

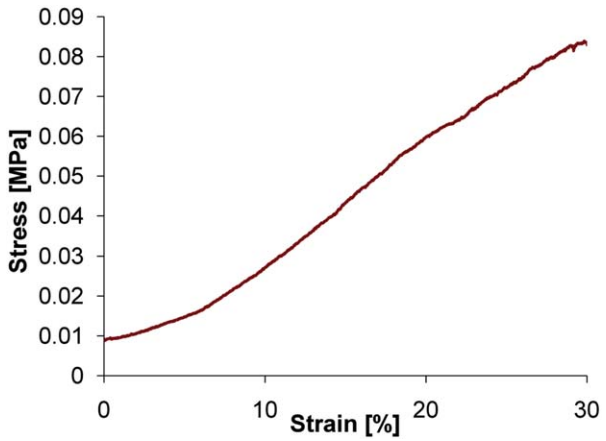

FIGURE 4. The stress-strain curve obtained with compression test for the glu/HA scaffold.

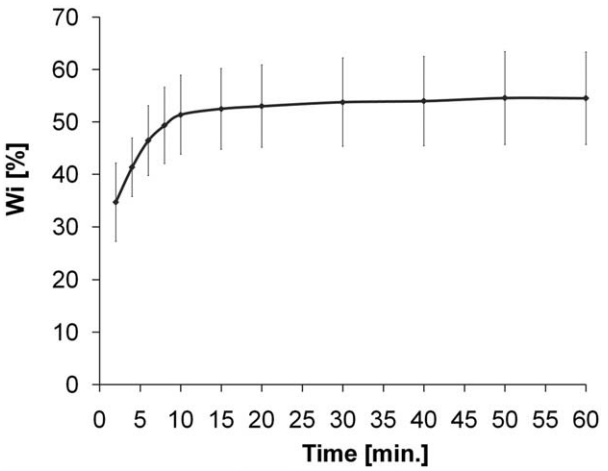

FIGURE 5. Water uptake ability of the glu/HA scaffold expressed as percent of weight increase (W_i) with time after soaking in normal saline solution (mean ± SD, $n = 4$).

on the 4th and 12th day were not statistically significant. Surprisingly, although significantly augmented bALP production by hFOB 1.19 cells cultured on the glu/HA scaffold was observed throughout duration of the experiment, the OC synthesis was undetectable using ELISA test even on the 18th day. In the case of control hFOB 1.19 cells, the OC production was detected at comparable levels (~3 ng/mL) at each time point [Fig. 8(e)]. Unlike hFOB 1.19 osteoblasts, the MC3T3-E1 cells started synthesis of OC protein on the 12th day of the experiment [Fig. 8(f)], but OC concentration in the scaffold sample (1.84 ± 0.44 ng/mL) was significantly lower ($p = 0.0001$) compared to the control cells (13.67 ± 0.52 ng/mL). The OC level in the glu/HA sample increased with time, but on the 18th day it was still significantly lower (8.69 ± 1.96 ng/mL, $p = 0.0003$) than the OC concentration detected in the control sample (20.32 ± 3.14 ng/mL).

DISCUSSION

Natural bone grafts possess osteoinductive and osteoconductive properties and are considered to be a "gold standard" for regenerative medicine applications. However, their use is limited due to some risk they might carry like infections[17] leading to increased morbidity and transplant rejection.[18] Taken these drawbacks into consideration, there is a constant need to fabricate the synthetic biomaterials as substitutes for natural grafts. In this study, new method based on dialysis of alkaline curdlan solution into calcium chloride was successfully applied to fabricate biomaterial composed of bacterial β-1,3-glucan (curdlan) and HA.

The μCT analysis [Fig. 3(a)] showed low total porosity (~13%) of the fabricated glu/HA scaffold. However, its high potential porosity (64%)—defined as a space filled by the

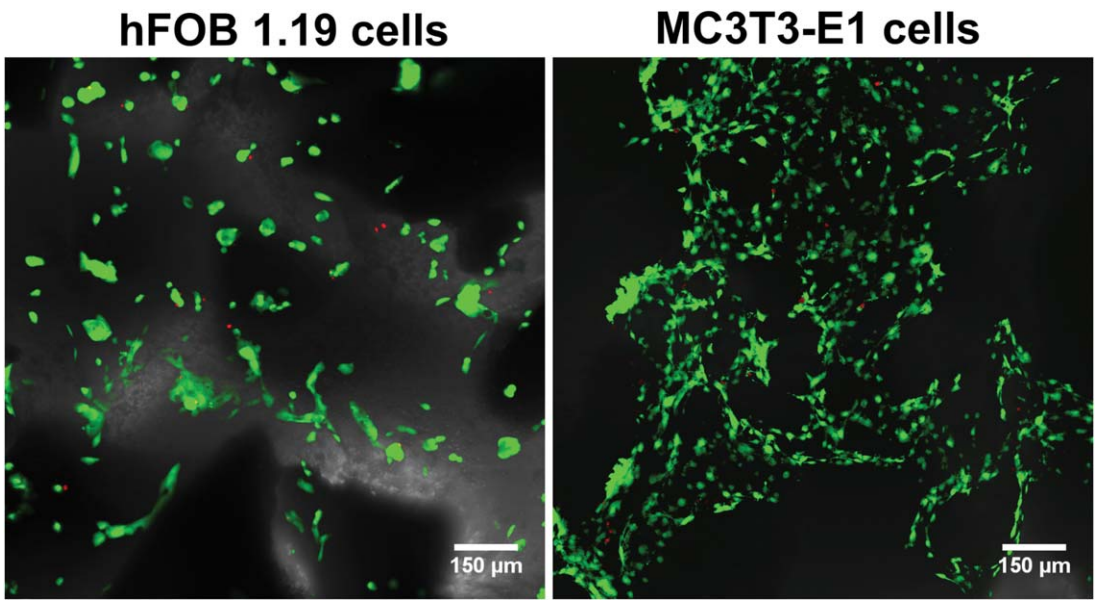

FIGURE 6. Confocal microscope images presenting viability of osteoblast cells on the glu/HA scaffold after 24-h culture (viable cells-green fluorescence, dead cells-red fluorescence), magn. 100×; Nomarski contrast was also used for visualization of the glu/HA scaffold structure.

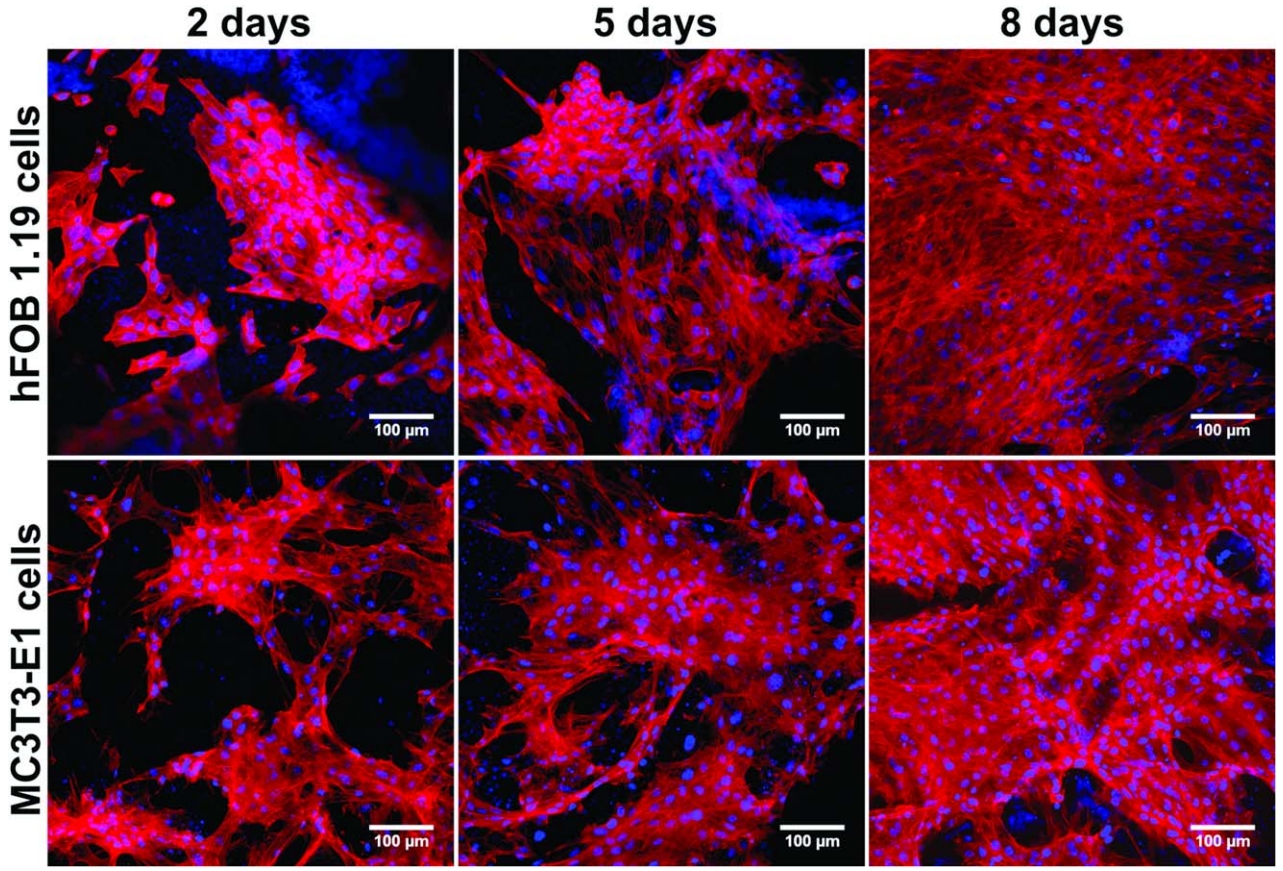

FIGURE 7. Confocal microscope images showing proliferation of osteoblast cells on the glu/HA scaffold after 2-, 5-, and 8-day culture (cytoskeletal filaments-red fluorescence, cell nuclei-blue fluorescence), magn. 200×.

β-1,3-glucan hydrogel matrix—may indicate that this biomaterial would allow for the easy transport of oxygen, nutrients, and waste metabolic products *in vivo* across its structure. Note that appropriate microenvironment is essential for cell proliferation, differentiation, and new bone growth. Moreover, β-1,3-glucan gel obtained by dialysis method was proved to undergo acidic hydrolysis and consequently degradation.[19] Therefore, it may be assumed that glu/HA scaffold after polysaccharide matrix degradation *in vivo* would provide optimal space for osteoblast ingrowth as well as for angiogenesis process and formation of blood vessels.

The compression test demonstrated that glu/HA scaffold is characterized by low Young's modulus (0.17 ± 0.05 MPa) and compression strength (0.057 ± 0.006 MPa) in comparison to human bone. It was shown that elastic moduli for cortical and cancellous bone are equal 7–30 GPa and 0.02–0.05 GPa, respectively, whereas compressive strength values are 130–230 MPa and 2–12 MPa, respectively.[20] Inferior values of Young's modulus and compressive strength estimated for the glu/HA may be a result of its specific composition. In contrast to the HA granules, the β-1,3-glucan matrix is a very flexible component of the glu/HA material and presumably it is especially susceptible to the strain. Moreover, the compression test was performed using material in a wet state what was justified by the implantation procedure and conditions in a living organism. Dry glu/HA sample is characterized by huge hardness and in contrary to wet scaffold is not flexible at all. Thus, dry glu/HA material would certainly reveal higher stiffness (Young's modulus) and compressive strength value. Obtained results indicate that application of glu/HA scaffold is limited to the implantation areas that are not exposed to high mechanical loads. Nevertheless, due to low Young's modulus value, it also reveals high flexibility indicating its good surgical handiness in wet state and ability to fit into the bone defect area.

Water uptake behavior is an essential feature of absorbent, porous materials (like glu/HA scaffold) that should be assessed. Water uptake test provides information about the time required for the complete absorption of drugs or physiological solution by the sample. It is especially important in regards to drug delivery applications and pre-operative procedure.[21,22] The glu/HA scaffold revealed huge capacity to absorb liquid (Fig. 5) in relatively short time of soaking (~20 min.). Thus, it may be suggested that glu/HA scaffold is a promising candidate as drug delivery carrier and its surgical applications are not limited due to too long time needed for complete saturation.

Moreover, bone scaffold for regenerative medicine applications should be osteoconductive, which is characterized

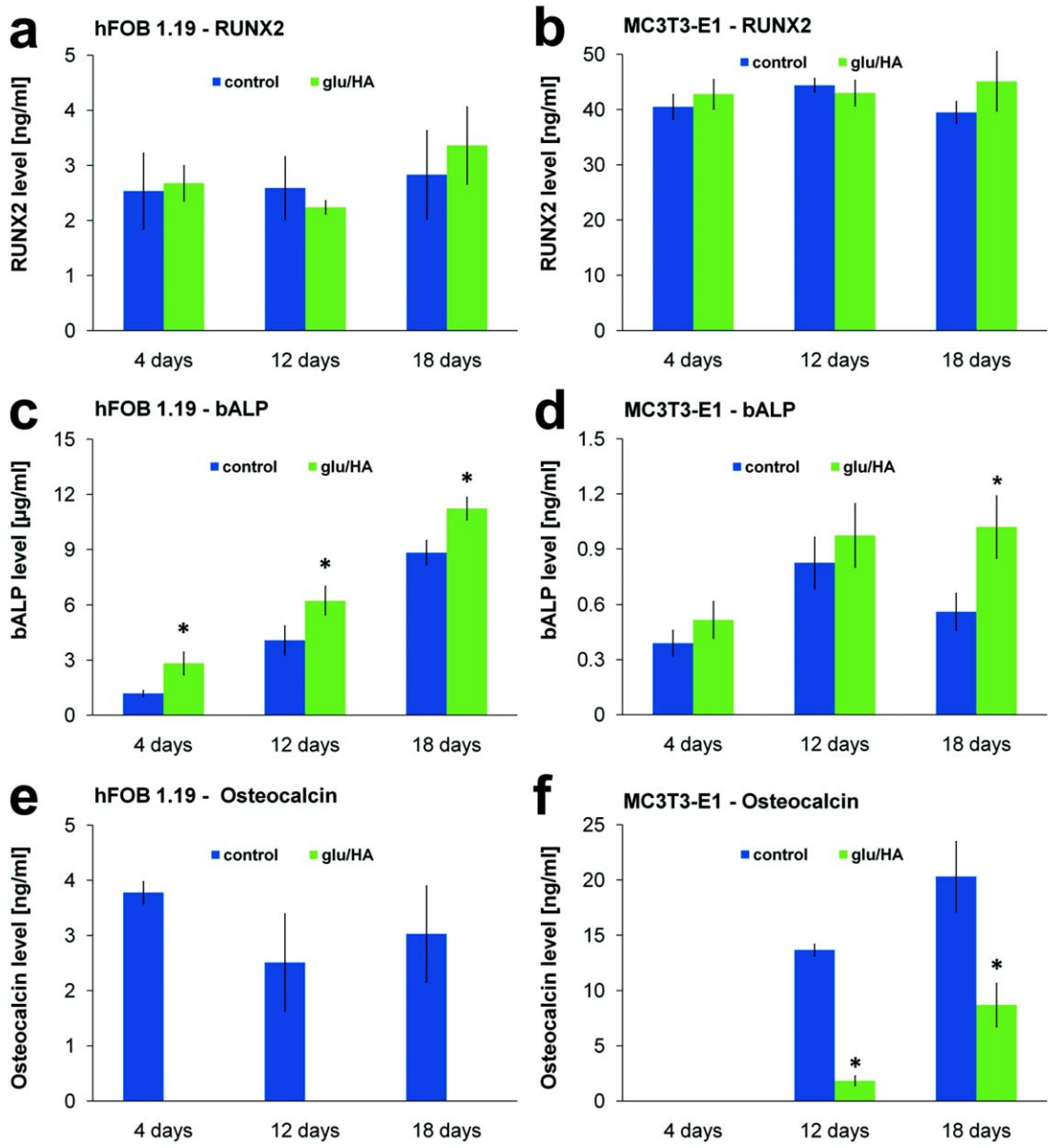

FIGURE 8. Determination of RUNX2 (a,b), bALP (c,d), and osteocalcin (e,f) production during differentiation process of hFOB 1.19 (a,c,e) and MC3T3-E1 (b,d,f) osteoblasts. The differences between the results obtained with the cells cultured on the glu/HA scaffold and control cells cultured on polystyrene were considered as statistically significant based on unpaired *t* test ($*p < 0.05$, $n = 4$).

by biological features like promotion of osteoblast adhesion, spreading, proliferation, and differentiation followed by bone extracellular matrix (ECM) formation and mineralization. In the first step of the *in vitro* culture experiment, the cytotoxicity of the glu/HA scaffold was assessed. The microscopic observation after live/dead double staining (Fig. 6) demonstrated that both hFOB 1.19 and MC3T3-E1 cells cultured on the glu/HA scaffold were mostly viable and well flattened indicating their good adhesion to its surface. Moreover, confocal microscope observation performed at 3 intervals during long-term culture of osteoblasts on the glu/HA material revealed good cell spreading and proliferation on the scaffold (Fig. 7). The amount of hFOB 1.19 and MC3T3-E1 cells noticeably increased with the time and 8 days after cell inoculation almost entire surface of the glu/HA scaffold was covered by multilayer of osteoblast cells suggesting that glu/HA scaffold surface is beneficial to cell growth and proliferation.

Osteoconductivity of the material is primarily determined by good osteoblast differentiation on its surface and effective new bone formation. Osteoblast differentiation is a complex process related to synthesis and release of specific proteins (e.g., type I collagen, OC) and enzymes (bALP) that are considered as osteogenic markers.[13] Moreover, the differentiation process is regulated by some transcription factors such as osterix or runt-related transcription factor 2 (RUNX2).[23] RUNX2 is considered to be a key regulator of osteoblast differentiation enhancing expression of fibronectin, type I collagen,

osteopontin, and OC. It has also been shown to be essential for maintenance of osteoblast biological function.[24]

In this study, RUNX2, bALP, and OC levels were evaluated as osteogenic markers during osteoblast differentiation process *in vitro*. At each time interval, the amounts of RUNX2 in hFOB 1.19 and MC3T3-E1 cultures grown on the glu/HA and in the corresponding control samples were comparable [Fig 8(a,b)] suggesting that osteoblast cells retained their functions and the differentiation process run correctly. Moreover, the meaningful increase in bALP level with the time was observed in the case of both tested osteoblast cell lines [Fig. 8(c,d)]. More importantly, cells cultured on the glu/HA scaffold released significantly higher amounts of bALP in comparison to control cells cultured on polystyrene what may suggest that glu/HA enhances osteogenic differentiation. The OC ELISA test showed the lack of OC synthesis (marker of the ECM mineralization) in hFOB 1.19 osteoblasts cultured on the glu/HA scaffold at each time point during the experiment [Fig. 8(e)] indicating that on the 18th day of the incubation, the hFOB 1.19 cells were still in the phase of bone ECM synthesis/maturation and did not begin ECM mineralization phase. In the case of MC3T3-E1 cells cultured on the glu/HA, OC ELISA test revealed initiation of OC synthesis on the 12th day [Fig. 8(f)]. Interestingly, control osteoblasts of both cell lines grown on polystyrene produced great amounts of OC protein [Fig. 8(e,f)]. Control hFOB 1.19 osteoblasts synthesized significant levels of OC at each time interval during the experiment, whereas control MC3T3-E1 cells, similarly to MC3T3-E1 osteoblasts cultured on the glu/HA, produced OC beginning from the 12th day—however, the amounts of OC protein released by control MC3T3-E1 cells were significantly higher than those produced by cells grown on the scaffold [Fig. 8(f)]. As glu/HA scaffold significantly increased bALP synthesis and because the OC production (mineralization phase) was still undetectable or at low level in osteoblasts cultured on the material on the 18th day, it may be implied that glu/HA scaffold enhances osteogenic differentiation mainly via promotion and prolongation of the ECM synthesis/maturation phase, which is characterized by the highest bALP synthesis and activity during differentiation process. However, it should be noted that prolonged time of the experiment would probably result in the initiation of the ECM mineralization and OC would be detected also in osteoblast cultures grown on the glu/HA scaffold. Thus, to fully understand the effect of fabricated scaffold on the osteogenic differentiation, it is planned to repeat the experiment and performed it for longer period of time to detect the last stage of differentiation as well.

Based on the results obtained with *in vitro* culture experiments, it may be inferred that application of dialysis method for glu/HA scaffold fabrication allows to obtain non-toxic material that possesses ability to support osteoblast growth and proliferation and also to enhance osteogenic differentiation. It should be noted that recent studies performed by our research team demonstrated that addition of β-1,3-glucan (curdlan) to chitosan/HA scaffold results in promotion of osteoblast adhesion, growth, and proliferation.[3,4,25] Moreover, chitosan/β-1,3-glucan/HA scaffold was proved to enhance osteogenic differentiation[13] and not to induce osteoblasts-, monocytes-, and macrophages-mediated inflammatory response *in vitro*[26] indicating its high biocompatible properties. Nevertheless, fabrication process of the chitosan/β-1,3-glucan/HA scaffold requires the gelation of the β-1,3-glucan component at 90°C what limits its modification by addition of thermo-sensitive biomolecules such as adhesive proteins/growth factors or antibiotics at the stage of scaffold production.[25] In contrary to described in the literature thermal method for curdlan gelation and biomaterial fabrication, presented in this study dialysis method for the glu/HA scaffold production allows for the β-1,3-glucan gelation at room temperature and thus makes the glu/HA scaffold an excellent base to thermo-sensitive biomolecules modifications as described in Patent pending.[16] Scaffold modifications with adhesive proteins/growth factors are of great interest in biomedical materials field as they enable to facilitate osteoblast adhesion, bone ECM formation followed by mineralization and consequently bone tissue regeneration at the site of implantation. These kind of the material modifications will be the subject of our further research on the glu/HA scaffold.

CONCLUSIONS

This study clearly indicates that new method for the β-1,3-glucan/hydroxyaptite scaffold fabrication allows to obtain non-toxic, and supportive to osteoblast growth, proliferation, and differentiation material. Fabricated via dialysis method glu/HA material exhibits osteopromotive and highly osteoconductive properties *in vitro*. However, fabricated scaffold has relatively poor mechanical properties limiting its application to the implantation areas that are not exposed to high mechanical loads. The glu/HA material possesses also meaningful ability to aqueous solution uptake and retention extending its potential application as drug delivery carrier. Based on obtained results it may be implied that produced using dialysis method glu/HA material has high biomedical potential as bone scaffold and is a promising candidate for regenerative medicine applications.

ACKNOWLEDGMENTS

The authors have no conflict of interest to declare. The article was developed using the equipment purchased within agreement No. POPW.01.03.00–06-010/09–00 Operational Program Development of Eastern Poland 2007–2013, Priority Axis I, Modern Economy, Operations 1.3. Innovations Promotion. The authors would like to thank Michal Wojcik from the Department of Biochemistry and Biotechnology, Medical University of Lublin, Poland for the help with confocal microscopy analysis.

REFERENCES

1. Bose S, Roy M, Bandyopadhyay A. Recent advances in bone tissue engineering scaffold. Trends Biotechnol 2012;30:546–554.
2. O'Brien FJ. Biomaterials & scaffolds for tissue engineering. Mater Today 2011;14:88–95.
3. Przekora A, Palka K, Ginalska G. Biomedical potential of chitosan/HA and chitosan/β-1,3-glucan/HA biomaterials as scaffold for bone regeneration—A comparative study. Mater Sci Eng C Mater Biol Appl 2016;58:891–899.

4. Przekora A, Palka K, Ginalska G. Chitosan/β-1,3-glucan/calcium phosphate ceramics composites—Novel cell scaffolds for bone tissue engineering application. J Biotechnol 2014;182–183:46–53.
5. Knowles JC. Phosphate based glasses for biomedical applications. J Mater Chem 2003;13:2395–2401.
6. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials 2005;26:5474–5491.
7. Gloria A, De Santis R, Ambrosio L. Polymer-based composite scaffolds for tissue engineering. J Appl Biomater Biomech 2010;8: 57–67.
8. Zhang R, Edgar KJ. Properties, chemistry, and applications of the bioactive polysaccharide curdlan. Biomacromolecules 2014;15: 1079–1096.
9. Kanzawa Y, Harada T, Koreeda A, Harada A. Curdlan gel formed by neutralizing its alkaline solution. Agric Biol Chem 1987;51: 1839–1843.
10. Sato M, Nobe M, Dobashi T, Yamamoto T, Konno A. Multifold curdlan gel formation by dialysis into aqueous solutions of metal salts. Colloid Polym Sci 2005;284:293–300.
11. Belcarz A, Ginalska G, Pycka T, Zima T, Ślósarczyk A, Polkowska I, Paszkiewicz Z, Piekarczyk W. Application of β-1,3-glucan in production of ceramics-based elastic composite for bone repair. Cent Eur J Biol 2013;8:534–548.
12. Borkowski L, Pawłowska M, Radzki RP, Bieńko M, Polkowska I, Belcarz A, Karpiński M, Słowik T, Matuszewski Ł, Ślósarczyk A, Ginalska G. Effect of a carbonated HAP/β-glucan composite bone substitute on healing of drilled bone voids in the proximal tibial metaphysis of rabbits. Mater Sci Eng C Mater Biol Appl 2015;53: 60–67.
13. Przekora A, Ginalska G. Enhanced differentiation of osteoblastic cells on novel chitosan/β-1,3-glucan/bioceramic scaffolds for bone tissue regeneration. Biomed Mater 2015;10:13
14. Farzadi A, Solati-Hashjin M, Bakhsi F, Aminian A. Synthesis and characterization of hydroxyapatite/β-tricalcium phosphate nanocomposites using microwave irradiation. Ceram Int 2011;37:65–71.
15. Belcarz A, Zalewska J, Pałka K, Hajnos M, Ginalska G. Do Ca^{2+}—Adsorbing ceramics reduce the release of calcium ions from gypsum-based biomaterials? Mater Sci Eng C Mater Biol Appl 2015;47:256–265.
16. Klimek K, Przekora A, Ginalska G. Rusztowanie kostne na bazie β-1,3-glukanu (kurdlanu) i bioceramiki oraz sposób jego wytwarzania/Bone scaffold based on β-1,3-glucan (curdlan) and bioceramics as well as the method for its fabrication. Polish Patent pending nr. P.415936, filed to Polish Patent Office. Forthcoming.
17. Khan SN, Cammisa FP Jr, Sandhu HS, Diwan AD, Girardi FP, Lane JM. The biology of bone grafting. J Am Acad Orthop Surg 2005;13:77–86.
18. O'Malley MJ, Sayres SC, Saleem O, Levine D, Roberts M, Deland JT, Ellis S. Morbidity and complications following percutaneous calcaneal autograft bone harvest. Food Ankle Int 2014;35:30–37.
19. Kanzawa Y, Harada T. Difference of molecular association in two types of curdlan gel. Carbohydr Polym 1989;10:299–313.
20. Velasco MA, Narváez-Tovar CA, Garzón-Alvarado DA. Design, materials, and mechanobiology of biodegradable scaffold for bone tissue engineering. Biomed Res Int 2015;2015:729076 doi: 10.1155/2015/729076.
21. Boesel LF, Reis RL. The effect of water uptake on the behaviour of hydrophilic cements in confined environments. Biomaterials 2006; 27:5627–5633.
22. Prabaharan M, Rodriguez-Perez MA, de Saja JA, Mano JF. Preparation and characterization of poly(L-lactic acid)-chitosan hybrid scaffolds with drug release capability. J Biomed Mater Res B Appl Biomater 2007;81:427–434.
23. Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem 2006;99:1233–1239.
24. Ducy P. Cbfa1: A molecular switch in osteoblast biology. Dev Dyn 2000;219:461–471.
25. Przekora A, Ginalska G. Addition of 1,3-β-D-glucan to chitosan-based composites enhances osteoblast adhesion, growth, and proliferation. Int J Biol Macromol 2014;70:474–481.
26. Przekora A, Ginalska G. *In vitro* evaluation of the risk of inflammatory response after chitosan/HA and chitosan/β-1,3-glucan/HA bone scaffold implantation. Mater Sci Eng C Mater Biol Appl 2016;61:355–361.