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Biological properties of novel chitosan-based composites for medical application as bone substitute

Research Article

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Abstract: Hydroxyapatite is the main inorganic component of bones and teeth. In order to improve mechanical properties and surgical handiness of bioceramics, a plasticizing agent *e.g.* polysaccharide can be added. Chitosan is a polysaccharide with biological properties that make it an ideal component of bioceramics-based composites for medical application as bone substitute. In this study, biocompatibility of two types of novel krill chitosan-based composites was evaluated. *In vitro* experiments were carried out using human foetal osteoblast cell line. Cytotoxicity, cell adhesion, and bone ALP activity tests were performed to assess biocompatibility of the composites. Osteoblast growth on composites was observed using confocal microscope. Our results demonstrated that fabricated novel composites are non-toxic, are favorable to cell adhesion and growth, and provoke increase in b-ALP activity with time, thus inducing osteoblast differentiation. Based on this data composites have promising clinical potential as a bone defect filler in regenerative medicine. It is worth emphasizing that our work resulted in fabrication of flexible and surgical handy, bone substitutes that possess absolute biocompatibility with structural and mechanical properties similar to trabecular bone.

Keywords: *Chitosan • Hydroxyapatite • Composites • Biocompatibility • Cell culture • Regenerative medicine*

1. Introduction

Hydroxyapatite (HAp) is the main inorganic component of bones and teeth. Calcium phosphate ceramics in the form of granules and porous scaffolds are widely used as bone substitutes in dentistry and orthopedics [1-3]. In order to improve mechanical properties and surgical handiness of bioceramics, a plasticizing agent *e.g.* polysaccharide can be added [4,5]. Biological properties of chitosan make this polysaccharide an ideal component of calcium phosphate-based composites. Chitosan is a partially N-deacetylated derivative of chitin that naturally occurs in the exoskeleton of crustaceans [6,7]. This polysaccharide is often applied in tissue engineering because it biodegrades rapidly, is non-toxic, is prone to chemical and enzymatic modification, has similar structure to glycosaminoglycans (GAG) of bone extracellular matrix (ECM), and it stimulates cell adhesion, proliferation and osteoinduction [8-10]. In regenerative medicine, chitosan-based composites are often applied in the form of hydrogels, implants or scaffolds capable of delivering drugs, growth factors or cells into the implantation area [10].

Scaffolds with good biological properties should also possess appropriate structure and act as bone ECM providing physical support for the surrounding cells, inducing cell proliferation and regulating cell differentiation. Thus, the goal of tissue engineering is to create a porous, three-dimensional scaffold that is capable of supporting, reinforcing and allowing for regeneration of the surrounding tissues [11,12]. Referring to the mechanical properties, scaffolds should be of optimum porosity and elasticity, adjusted to the place of implantation (*e.g.* cortical or trabecular bone) [13,14]. Scaffolds that are structurally and mechanically similar to the bone tissue will facilitate the process of tissue ingrowth into the implanted material, improving fatigue strength of the implant and ensuring a good implant-tissue connection [15].

The aim of this work was to assess biocompatibility of novel krill chitosan- based composites and evaluate their potential clinical application as a bone defect filler in regenerative medicine.

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2. Experimental Procedures

2.1 Biomaterials fabrication

Two types of biomaterials were fabricated using high molecular weight krill chitosan (1174 kDa and 73% deacetylated) and calcium phosphate bioceramics according to procedure described in our previous report [16]. We applied two types of manufactured bioceramics (Chema Elektromet Rzeszow, Poland):

— HA BIOCER bioceramics (HAp granules, Ø 0.5-1.6 mm) – composite marked as chitosan/HA BIOCER

— HT BIOCER bioceramics (mix of HAp/β-TCP granules, Ø 0.5-1.6 mm) – composite marked as chitosan/HT BIOCER.

We used high molecular weight chitosan derived from krill shells (*Euphausia superba*) obtained according to technology developed by Anna Wojtasz-Pajak from Sea Fisheries Research Institute in Gdynia, Poland [17,18]. Production of chitosan/HA BIOCER and chitosan/HT BIOCER composites was as described earlier [16]: krill chitosan was dissolved in 1% acetic acid solution, then HA BIOCER or HT BIOCER granules were added into chitosan solution (80% wt.). The resulting homogenous paste was moulded into cylinder-shaped samples, which were then gelled in 1% NaOH solution, washed in distilled water and left to air dry for 48 hours. Pictures of the fabricated composites are presented in Figure 1 a, b, and c.

2.2 Cell culture *in vitro* experiments

2.2.1 Materials and reagents for in vitro tests

The following materials were used for *in vitro* experiments: DMEM/Ham F12 culture medium without phenol red, G418 disulfate salt solution, penicillin-streptomycin solution, 0.25% trypsin-EDTA solution, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), cell counting WST-8 kit, lactate dehydrogenase (LDH) cytotoxicity kit, sodium dodecyl sulfate (SDS), β-glycerophosphate, L-ascorbic acid, dexamethasone, p-nitrophenyl phosphate (pNPP), p-nitrophenol (pNP), and Hoechst 33342 dye were purchased from Sigma-Aldrich Chemicals. Foetal bovine serum (FBS) was supplied by PAA Laboratories and phosphate buffered saline (PBS) was from BIOMED Serum and Vaccine Production Plant (Lublin, Poland). Hydrochloric acid (HCl), sodium hydroxide (NaOH) were purchased from POCH (Gliwice, Poland). Draq5 fluorescent stain was supplied by Cell Signaling Technology. Calcium, magnesium and phosphorus detection kits were acquired from BioMaxima (Lublin, Poland).

2.2.2 Evaluation of cytotoxicity

All *in vitro* experiments were carried out using normal human foetal osteoblast cell line (hFOB 1.19) obtained from ATCC (American Type Culture Collection, England, UK). The cells were cultured in accordance with ATCC recommendation: in a 1:1 mixture of DMEM/Ham F12 medium without phenol red supplemented with 10% FBS, 300 μg mL^{-1} G418, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and maintained at 34ºC in a humidified atmosphere of 5% CO_2 and 95% air.

Cytotoxicity of the biomaterials was evaluated indirectly by means of fluid extracts obtained by immersing the test materials in a complete culture medium supplemented with 2% FBS under standard conditions: 24 h, at 37ºC in a humidified atmosphere of 5% carbon dioxide and 95% air. The ratio between sample weight and the volume of the extraction vehicle was 0.1 g mL^{-1} beyond the absorptive capacity of the biomaterial. Culture medium incubated at 37ºC without test material served as a negative control of cytotoxicity and 0.1% phenol solution served as a positive control of cytotoxicity. Before the extraction, biomaterials were cut into cylindrical discs 1 mm thick and 15 mm in diameter in order to enhance submersion in the medium. After 24 hours of incubation at 37ºC, the extracts were collected

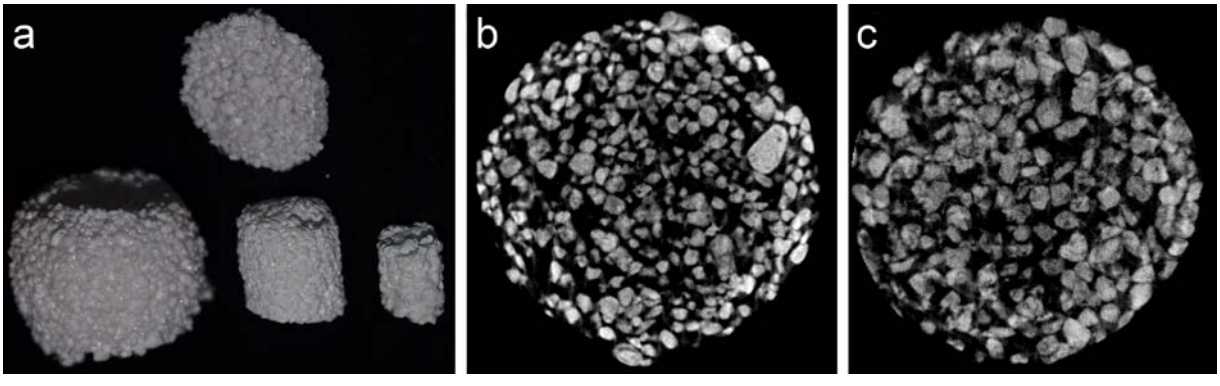

Figure 1. Images of fabricated composites: macrophotography of composites (a), cross section image of chitosan/HA BIOCER (b) and chitosan/HT BIOCER (c) obtained with computed microtomography scanning.

and crucial ion concentration (magnesium, calcium, and phosphate) was assessed. The ionic composition of the samples was measured using appropriate colorimetric detection kits. The measurements were repeated three times. Extracts were prepared according to ISO 10993-5 standards [19] and subjected to *in vitro* cytotoxicity tests. To assess cytotoxicity, hFOB 1.19 cells were seeded in flat bottom 96-multiwell plates in 100 µL complete culture medium at a concentration of 1.5 x 10^4 cells $well^{-1}$. After 24-hour incubation at 34ºC the growth medium was replaced with 100 µL of the appropriate extracts or 100 µL of 0.1% phenol solution. After 24 and 48 hours of incubation, MTT and LDH total tests were performed to evaluate cell viability. MTT test (evaluation of cell metabolic activity via measurement of mitochondrial dehydrogenases activity) was carried out as described earlier [20]. LDH total test allows for cell number evaluation via total cytoplasmic LDH activity measurement after cell lysis. LDH total test was conducted using *LDH cytotoxicity kit* in accordance with the manufacturer's recommended procedure. The results of MTT and LDH total tests were expressed as the percentage of OD values obtained with control cells. The cytotoxicity indirect tests were repeated in three separate experiments.

2.2.3 Cell adhesion assessment

WST-8 (measurement of metabolic activity of living cells) and LDH total tests were performed to evaluate cell adhesion into tested composite surface. WST-8 test differs from the MTT assay by producing orange water-soluble formazan dye, thus WST-8 assay possesses better sensitivity than MTT. Before the experiment, composites were cut into discs 1 mm thick and 8 mm in diameter then preincubated overnight in 24-multiwell plates in a complete culture medium at 34ºC. Culture medium was then removed and hFOB 1.19 cells were seeded directly on chitosan/HA BIOCER and chitosan/HT BIOCER surfaces in 25 µL of the complete culture medium at a concentration of 3 x 10^4 cells per composite. Plates were returned to the incubator for 2 hours and cells were allowed to attach to the composite surface, then 500 µL of fresh medium per well was carefully added. After 24-hour incubation at 34ºC, WST-8 and LDH total tests were conducted in accordance with manufacturer's (Sigma-Aldrich) recommended procedures. WST-8 and LDH total assays were repeated in 2 separate experiments in quadruplicate.

2.2.4 Cell growth evaluation

Cell growth on the surface of chitosan/HA BIOCER and chitosan/HT BIOCER composites was evaluated using confocal microscopy after staining with fluorescent dyes. Both Hoechst 33342 and Draq5 are cell permeable nucleic acids stains. Hoechst gives blue fluorescence when bound to DNA whereas Draq5 emits red fluorescence. Composite discs 1 mm thick and 8 mm in diameter were pre-incubated overnight in 24-multiwell plates in a complete culture medium at 34ºC. A solution of 25 µL of hFOB 1.19 cell suspension (3 x 10^4 cells per composite) was applied to each sample and cells were allowed to attach for 2 hours, then 500 µL of fresh medium per well was added. After 16 days of culture at 34ºC, composites were gently rinsed with PBS then stained with 500 µL of Hoechst 33342 solution (1 µg mL^{-1} prepared in PBS buffer) in each well for 15 minutes. After staining, the Hoechst solution was removed and composites were rinsed with PBS. For comparison parallel staining with Draq5 was applied. Composites were rinsed with PBS and 500 µL per well of Draq5 solution (5 µmol L^{-1} prepared in PBS buffer) was added. Cells were stained for 5 minutes, Draq5 solution was removed and composites were rinsed with PBS. Stained cells growing on the surface of chitosan/HA BIOCER and chitosan/HT BIOCER composites were then observed using a confocal microscope (Olympus Fluoview equipped with FV1000).

2.2.5 Bone alkaline phosphatase activity assessment – pilot experiment

Osteoblast differentiation was evaluated *via* measurement of bone alkaline phosphatase activity (b-ALP). Composite discs 1 mm thick and 5 mm in diameter were placed in 96-multiwell plate and pre-incubated overnight in a complete culture medium at 34ºC. Culture medium was then removed and hFOB 1.19 cells were seeded directly on chitosan/HA BIOCER and chitosan/HT BIOCER surfaces in 10 µL of complete culture medium at a concentration of 1.5 x 10^4 cells per composite. Cells were allowed to attach for 2 hours, then 150 µL of fresh medium was added to each well. After a 24-hour incubation at 34ºC, the culture medium was gently removed and 150 µL per well of osteogenic medium supplemented with 0.05 mg mL^{-1} ascorbic acid, 10 mmol L^{-1} β-glycerophosphate, and 10^{-7} mol L^{-1} dexamethasone was added. As a control, hFOB 1.19 cells were cultured directly on polystyrene plate. After 4, 8, 12, 16, and 20 days of culture in osteogenic medium, b-ALP activity was evaluated on the transformation of p-nitrophenylphosphate (pNPP) into p-nitrophenol (pNP) at 37ºC and pH~10. To assess b-ALP activity, 200 µL of appropriate glycin-NaOH buffer containing 7 mmol L^{-1} pNPP, 12 mmol L^{-1} $MgCl_2$, and 0.1 M glycin was added to each well of 96-multiwell plate then plate was incubated at 37ºC for 60 minutes. The reaction was

inhibited by addition of 50 µL of 2 mol L^{-1} NaOH to each well. Absorbance was then measured at 405 nm using microplate reader (Biotek ELx50) and the concentration of pNP was calculated from the calibration curve. ALP activity test was conducted in 1 experiment (pilot test) in quadruplicate.

2.3 Statistical analysis

The results were presented as mean values ± standard deviation (SD) for each group of samples. The Kolmogorov-Smirnov test was applied to demonstrate normal distribution of variables, then unpaired t-test was performed to assess statistical differences among groups by two population comparison. Statistical significance was considered at a probability $P < 0.05$ (GraphPad Prism 5, Version 5.03 Software).

3. Results

3.1 Ionic composition of the extracts and their cytotoxicity

Appropriate level of crucial ions (Mg^{2+}, Ca^{2+}, and HPO_4^{2-}) in the medium is necessary to maintain cell viability and proliferation. Neither chitosan/HA BIOCER nor chitosan/HT BIOCER composite affect Ca^{2+}, Mg^{2+}, and HPO_4^{2-} ions concentration in the medium after incubation at 37ºC for 24 hours (Table 1). It is in agreement with the cytotoxicity tests results that clearly showed no toxic effect of chitosan/HA BIOCER and chitosan/HT BIOCER composites extracts on hFOB cells (Figure 2 and 3). Both cytotoxicity tests revealed that composite extracts did not induce cytotoxic effect and hFOB cell viability was near 100% compared to the control throughout the full length of the experiment (48 hours).

3.2 Cell adhesion assessment

The LDH total and WST-8 tests showed good cell adhesion to the composite surfaces. However, LDH total test revealed that chitosan/HA BIOCER surface is slightly (but statistically significant) more favorable to cell adhesion than chitosan/HT BIOCER surface (Figure 4).

3.3 Cell growth evaluation

Confocal microscope observation showed good osteoblast growth on chitosan/HA BIOCER and chitosan/HT BIOCER composite surfaces. Furthermore, both Hoechst 33342 and Draq5 staining suggest that better cell proliferation is on the chitosan/HA BIOCER surface than on the chitosan/HT BIOCER surface (Figure 5).

3.4 Bone alkaline phosphatase activity assessment

Osteoblast differentiation assessment demonstrated that the chitosan/HA BIOCER and chitosan/HT BIOCER composites provoke increase in b-ALP activity with time (Figure 6). Osteoblasts cultured on the surface of chitosan/HA BIOCER composite revealed slightly higher b-ALP activity compared to the control cells

	control medium	chitosan/HA BIOCER extract	chitosan/HT BIOCER extract
Mg^{2+}	15.2 ± 0.9	15.8 ±1.0	16.2 ± 0.6
Ca^{2+}	36.9 ± 1.3	38.5 ± 0.6	40.1 ± 1.6
HPO_4^{2-}	33.5 ± 0.7	33.3 ± 1.1	30.5 ± 1.7

Table 1. Crucial ions (magnesium, calcium and phosphate) composition of extracts (mg L^{-1}) ± SD.

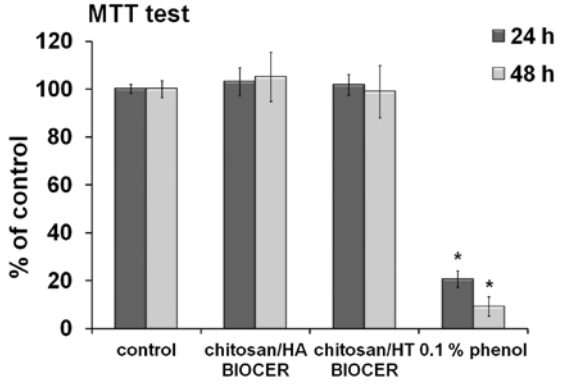

Figure 2. Cytotoxicity evaluation of composite extracts using hFOB 1.19 cells by means of MTT test. The results were expressed as the percentage of OD values obtained with the control cells and reported as the mean ± SD. The values for the control cells and the extract-treated cells were significantly different according to unpaired t-test (*$P < 0.05$).

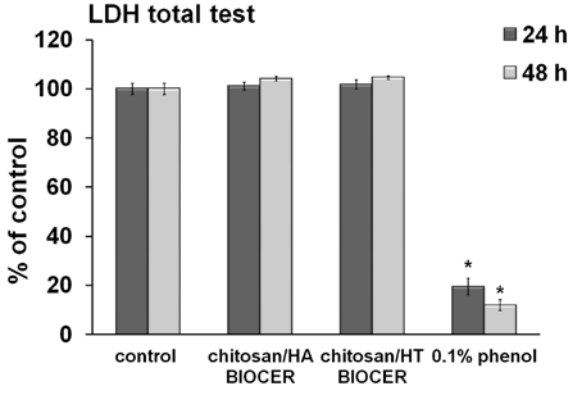

Figure 3. Cytotoxicity evaluation of composite extracts using hFOB 1.19 cells by means of LDH total test. The results were expressed as the percentage of OD values obtained with the control cells and reported as the mean ± SD. The values for the control cells and the extract-treated cells were significantly different according to unpaired t-test (*$P < 0.05$).

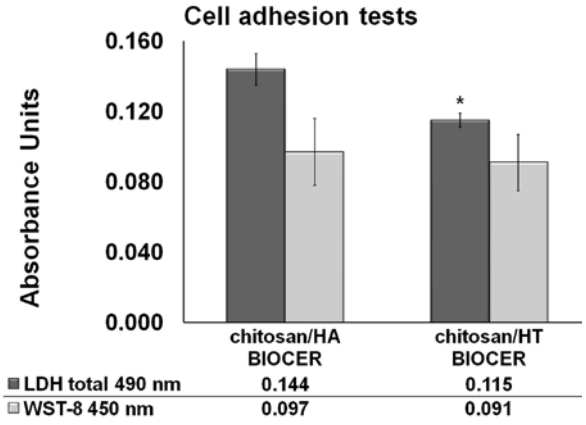

Figure 4. Cell adhesion evaluation 24 hours after cell inoculation by means of LDH total and WST-8 tests. The results were reported as the mean value of obtained absorbance ± SD. The values for the cells growing on the chitosan/HA BIOCER surface and cells growing on the chitosan/HT BIOCER surface were significantly different according to unpaired t-test (*$P < 0.05$).

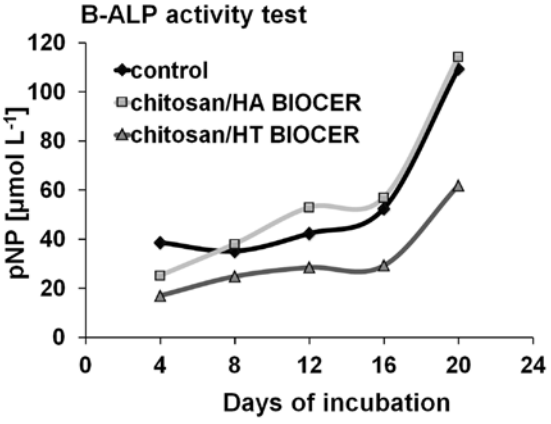

Figure 6. B-ALP activity assessment via transformation of pNPP into pNP (μmol L^{-1}).

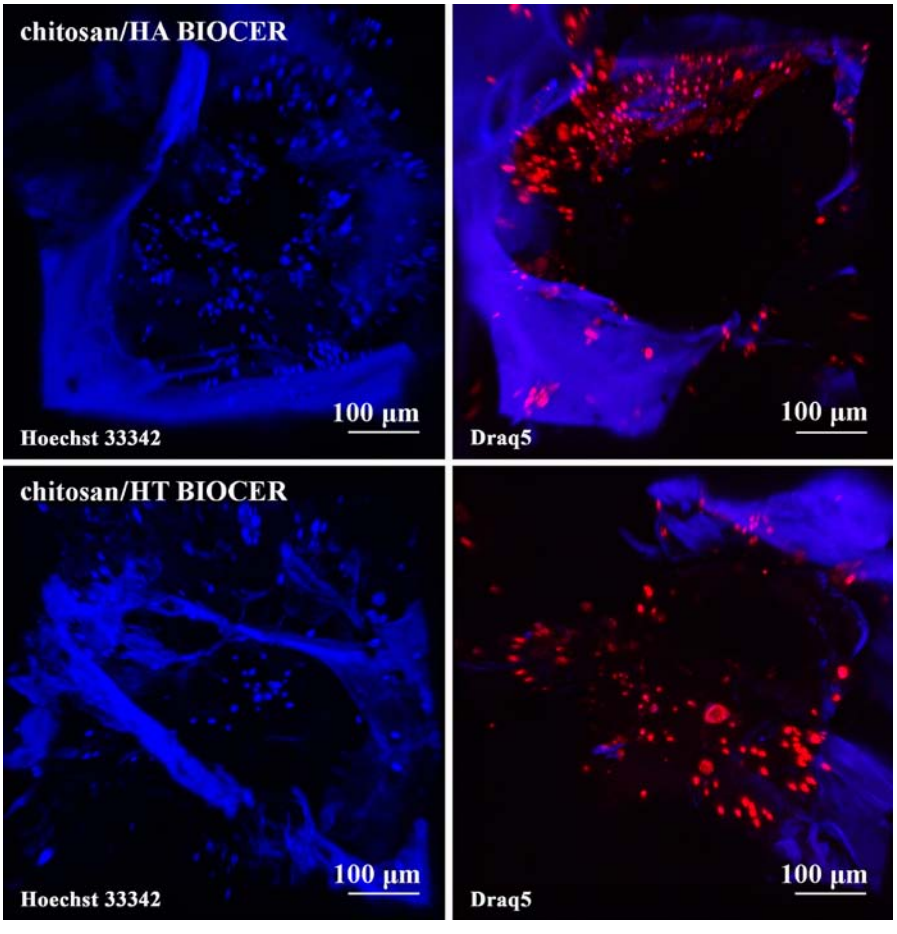

Figure 5. Confocal microscope images of cells growing on the surface of chitosan/HA BIOCER and chitosan/HT BIOCER composites, magn. 200x. On the left cells stained with Hoechst 33342 (blue fluorescence), on the right cells stained with Draq5 (red fluorescence); there is also visible blue autofluorescence of chitosan.

cultured on polystyrene plate. It may indicate that novel chitosan/HA BIOCER composite has osteoinductive properties. In contrast, cells cultured on the chitosan/HT BIOCER composite showed lower b-ALP activity compared to the control. After 20 days of culture, pNP production was approximately 114.3 μmol L^{-1} for cells cultured on chitosan/HA BIOCER, 61.8 μmol L^{-1} for cells cultured on chitosan/HT BIOCER and 109.4 μmol L^{-1} for control cells on polystyrene plate.

4. Discussion

Chitosan possesses several biological properties such as non-toxicity, rapid biodegradation, promotion of cell proliferation or osteoinduction, that make it a great candidate for bone tissue engineering applications [10]. Furthermore, chitosan possesses structural similarity to GAG of bone ECM [8,10] and has natural antimicrobial activity against Gram-positive and Gram-negative bacteria [21].

In our previous report [16] we found very good mechanical and structural properties of novel chitosan/HA BIOCER and chitosan/HT BIOCER composites, similar to the human trabecular bone. Presented earlier [16] images and virtual model of the composite scaffolds obtained with SEM and computer microtomography revealed individual grains, clusters and networks of open pores with irregular geometry and various sizes. Porosity of both materials was about 30% with a predominance of closed pores. Approximately 5-10% of the pores were open with average pore diameters of 426 mm for chitosan/HA BIOCER and 446 mm for chitosan/HT BIOCER. It is worth noting that composites porosity and their Young's modulus values, were similar to values for human trabecular bone (porosity in the range 30-90%, E in the range 0.18-0.33 GPa). Young's modulus values of composites were as follows:

— Chitosan/HA BIOCER: E = 1.38 GPa ± 0.06 GPa

— Chitosan/HT BIOCER: E = 1.22 GPa ± 0.15 GPa [16].

These valuable structural and mechanical properties of novel composites were achieved thanks to specific high molecular weight krill chitosan (1174 kDa) that was applied as a biopolymer component of each composite. We used that particular type of chitosan because of its characteristic physical properties such as very high viscosity of chitosan solution and ability to form flexible, solid, and stable gel-type material after neutralization in NaOH solution. We were not able to achieve the same results with other types of chitosan. In pilot experiments, we applied three different chitosan samples to fabricate novel composites: a medium molecular weight krill chitosan (218 kDa and 91% deacetylated), a high molecular weight krill chitosan (1174 kDa and 73% deacetylated) obtained according to technology developed by Anna Wojtasz-Pajak [17,18], and a manufactured low molecular weight chitosan (app. 100 kDa and 75-85% deacetylated, obtained from Sigma-Aldrich Chemicals). However, neutralization in NaOH solution only resulted in a solid and stable material when high molecular weight krill chitosan (1174 kDa and 73% deacetylated) was used. Thus, it should be noted that although there are many chitosan-based materials, their physical and mechanical properties may differ significantly depending on what type of chitosan was used.

Our previous work [16] was focused on mechanical and structural properties assessment of chitosan/HA BIOCER and chitosan/HT BIOCER composites. In the present work we put emphasis on biological properties of novel composites in order to evaluate their clinical potential as a bone defect filler in regenerative medicine.

We assessed biocompatibility *in vitro* of chitosan/HA BIOCER and chitosan/HT BIOCER composites using hFOB 1.19 cell line. It is widely known that surface-reactive biomaterials can cause changes in the crucial ion concentration in the surrounding environment, thereby affecting cell metabolism and viability [9,20,22]. Biomaterial composition and microstructure may have influence on the ion interactions between biomaterials and surrounding ionic environment, causing ion release or uptake. Appropriate level of crucial ions (Mg^{2+}, Ca^{2+}, and HPO_4^{2-}) in the medium plays an important role in cell adhesion, viability, proliferation, and differentiation [9,20,23,24]. We measured crucial ion concentration in the extracts in order to assess potential ion reactivity of chitosan/HA BIOCER and chitosan/HT BIOCER composites and we revealed that both composites did not significantly change ion concentration in the culture medium (Table 1). Furthermore, both MTT and LDH total cytotoxicity assays showed that novel composites are non-toxic and do not affect cell metabolism and viability (Figure 2 and 3). This is in agreement with the data presented by other authors. Malafaya and Reis revealed that extracts from the polymeric chitosan scaffolds and chitosan/sintered HAp scaffolds do not affect L929 fibroblast viability [9]. Similarly, Kucharska *et al* proved the lack of toxicity of chitosan microspheres agglomerated scaffolds against human osteoblast using XTT assay [25]. We evaluated also cell adhesion to the composite surfaces 24 hours after cell inoculation. Both, WST-8 and LDH total tests revealed good osteoblast adhesion to the novel composite surfaces (Figure 4). Moreover, confocal microscope observation showed well proliferated cells

on the composite surfaces (Figure 5). According to the available literature, chitosan stimulates cell attachment and proliferation [8,10,11]. Chun *et al.* used WST-8 assay to demonstrate very good human dermal fibroblasts (HDFs) initial attachment to the surface of chitosan scaffold [11]. Whereas Kim *et al.* based on the *in vitro* studies conducted using Chinese hamster ovary cell line (CHO-K1) implied that the collagen/chitosan sponge has a potential as a scaffold for cell adhesion and growth in tissue engineering [8].

Chitosan also promotes growth and mineral-rich matrix deposition by osteoblasts in culture [10]. The results of our pilot experiment evaluating osteoblast differentiation via measurement of b-ALP activity clearly indicate that novel composites provoke increase in b-ALP activity with time, thus inducing osteoblast differentiation (Figure 6). However, it should be noted that it was only a pilot experiment that should be repeated in the future using sensitive ELISA test to confirm these results.

Obtained promising results have encouraged us to continue this research in order to improve structural properties of the fabricated composites and modify them with growth factors *e.g.* BMP-2 to enhance osteoinductive properties, with antimicrobial agents to prevent post-surgery infections or with glycoproteins of ECM *e.g.* fibronectin to induce better cell adhesion and growth.

5. Conclusions

In summary, based on these results, it can be concluded that novel chitosan/HA BIOCER and chitosan/HT BIOCER composites are non-toxic, are favorable to cell adhesion and growth, and provoke increase in b-ALP activity with time, thus inducing osteoblast differentiation. The chitosan/HA BIOCER composite shows slightly greater biocompatibility than the chitosan/HT BIOCER composite and is, therefore, more appropriate for medical application as a bone substitute. It is worth emphasizing that our work resulted in fabrication of flexible and surgically handy bone substitutes that possess structural and mechanical properties similar to the trabecular bone [16] with absolute biocompatibility. Considering structural and mechanical properties tests results reported earlier [16] and satisfactory biological tests results reported here, we infer that fabricated novel composites have promising clinical potential as a bone defect filler in regenerative medicine or as a cell scaffold in tissue engineering applications.

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Conflict of interest

The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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