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New method for HA/glucan bone scaffold preparation reduces cytotoxic effect of highly reactive bioceramics

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ABSTRACT

Highly reactive bioceramics deprive culture medium of Ca^{2+} ions which is lethal to the cells *in vitro*. The aim of this work was to demonstrate that new method for HA/glucan scaffold fabrication, involving calcium as a cross-linker for β-1,3-glucan gel, may support cell survival *in vitro* when highly reactive bioceramics are the component of the biomaterial. Two composites made of highly reactive hydroxyapatite (HA) and β-1,3-glucan were prepared basing on 2 different methods. Then, ion reactivity of the materials and cell behaviour on their surfaces were compared. Obtained results proved that new method for HA/β-1,3-glucan fabrication protects against Ca^{2+} uptake by reactive HA supporting osteoblast growth *in vitro*.

1. Introduction

Calcium phosphates (CaPs) are widely used as components of biomaterials for bone tissue engineering applications. CaPs may be prepared using various sintering temperatures resulting in their different structural and mechanical properties [1] as well as ion reactivity – especially with respect to Ca^{2+} and HPO_4^{2-} [2]. According to the available literature, unsintered and sintered at low temperature CaPs (<900 °C) reveal high ion reactivity in aqueous environment, which is often well correlated with increased bioactivity *in vitro* and *in vivo* [2,3]. Whereas sintered at high temperature CaPs (>900 °C) show poor ion reactivity and do not critically change the ionic composition of the surrounding environment [2].

Many CaP-based biomaterials cause changes in the ion concentrations of culture medium *in vitro* either via ion release resulted from simply dissolution of bioceramics or via ion uptake often associated with apatite formation [2,4,5]. While slight release of divalent cations (Ca^{2+}, Mg^{2+}) may be supportive for osteoblast survival [6,7], decrease in ion concentration nearly always leads to significant cell viability reduction *in vitro* [2–5]. Thus, there is huge need to develop method for biomaterial fabrication allowing for reduction of cytotoxic effect of highly reactive CaPs.

The aim of this work was to compare osteoblast behaviour on biomaterials comprising highly reactive hydroxyapatite and prepared basing on 2 different methods for β-1,3-glucan (curdlan) gelation in order to demonstrate that recently developed method involving calcium as a cross-linker for β-1,3-glucan gel is better than thermal one since it introduces Ca^{2+} into the culture medium and eliminates cytotoxic effect of reactive CaPs *in vitro*.

2. Materials and methods

Composites made of hydroxyapatite granules (60 wt.%) sintered at low temperature of 800 °C (HA-LT) and β-1,3-glucan (8 wt.%) (Wako pure Chemicals Industries) were prepared basing on 2 different methods: 1) well described in the literature method requiring heating at 90 °C (sample marked as HA-LT/glu H) [8,9] and 2) recently developed requiring dialysis against $CaCl_2$ (HA-LT/glu D) [10], which is described in details in Polish Patent pending [11]. The surface of the scaffolds was characterized using SEM (Nova NanoSem 450, FEI) equipped with Octane Pro EDS detector (EDAX).

For ion reactivity tests, scaffolds were immersed in alpha MEM medium (Gibco) for 48 h (short-term test) and 15 days (long-term test). The experiments were conducted so as to simulate actual procedures that are applied during cell culture tests. Thus, after 24 h incubation at 37 °C, the culture medium was renewed to simulate cell seeding procedure. The Ca^{2+} and HPO_4^{2-} concentrations in the medium were assessed during first 24 h and 24 h after medium

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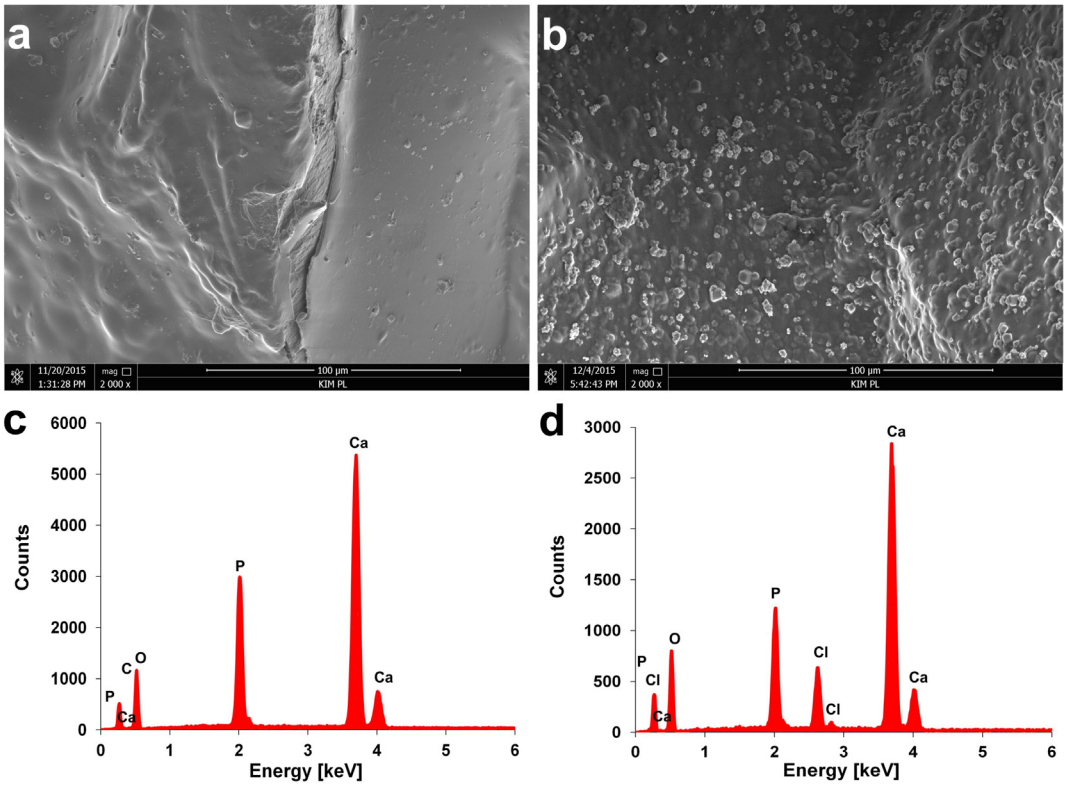

Fig. 1. SEM images of HA-LT/glu H (a) and HA-LT/glu D (b); EDS analysis of the HA-LT/glu H (c) and HA-LT/glu D (d).

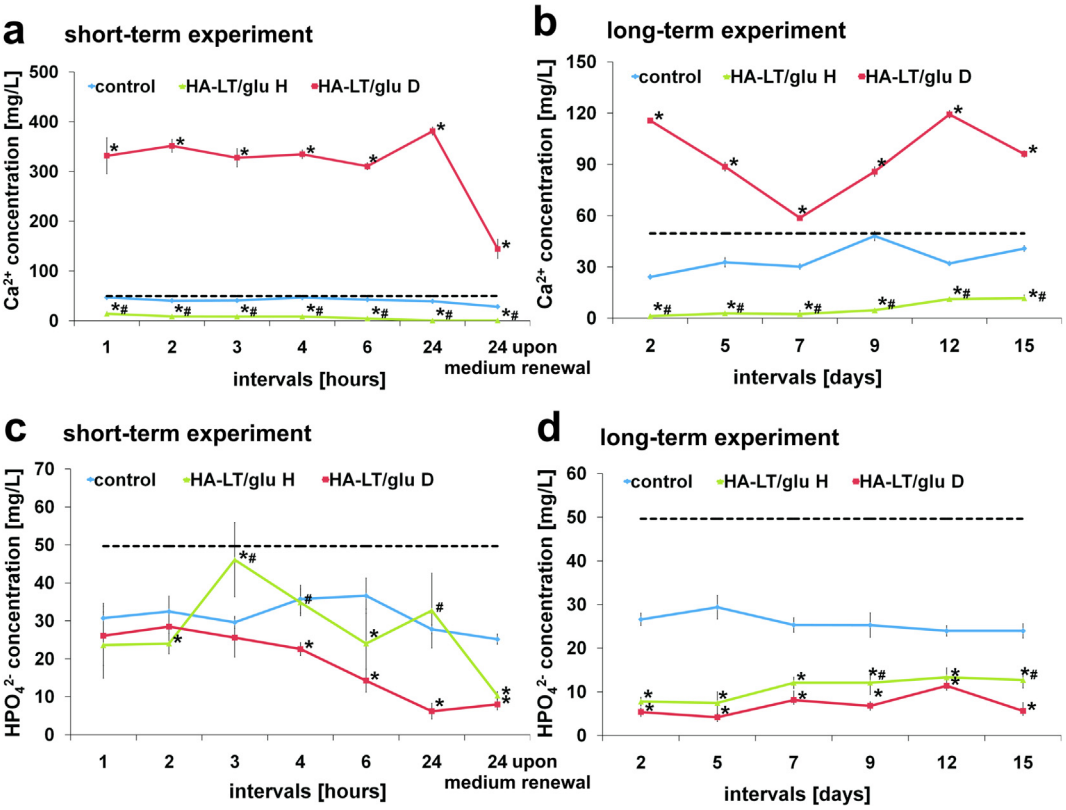

Fig. 2. Ion reactivity tests with respect to Ca^{2+} (a,b) and HPO_4^{2-} (c,d); dotted line indicates ion concentrations in fresh medium; *statistically significant results compared to the control and #compared to the HA-LT/glu D.

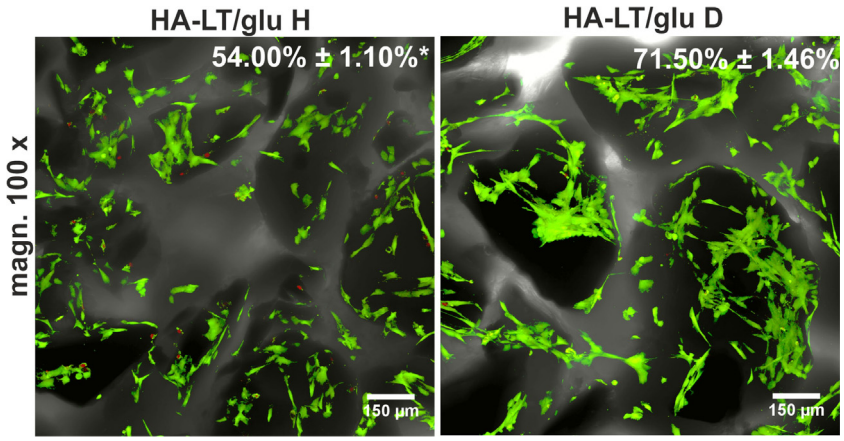

Fig. 3. Live/dead staining of osteoblasts; green – viable cells /calcein-AM, red – dead cells/propidium iodide, *significantly lower cell viability (WST-8 test) compared to the HA-LT/glu D. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exchange using calcium and phosphorus detection kits (BioMaxima). In the case of long-term test, the medium renewal and ion concentration measurements were repeated every 2–3 day. Medium incubated without materials served as a control. The experiments were repeated 4 times. Statistical significance (considered at $P < 0.05$) among samples was assessed using one-way ANOVA test followed by Tukey's test.

Cell culture experiments were conducted using mouse calvarial preosteoblasts (MC3T3-E1 Subclone 4, ATCC) maintained in alpha MEM with 10% foetal bovine serum (Pan-Biotech). The cells were seeded on the scaffold discs at a concentration of 1×10^5 cells/sample and incubated at 37 °C for 24 h to evaluate cytotoxicity and for 48 h to assess differences in cell number/spreading. Cytotoxicity was evaluated by Live/Dead Double Staining Kit and WST-8 test (Sigma–Aldrich Chemicals), which was repeated 3 times. Statistical significance (considered at $P < 0.05$) between HA-LT/glu H and HA-LT/glu D was assessed using unpaired *t*-test.

Cell number/spreading was determined upon fluorescent staining of F-actin filaments with AlexaFluor635phalloidin (Invitrogen) and nuclei with Hoechst33342 (Sigma–Aldrich Chemicals) as described previously [12]. Images obtained with confocal

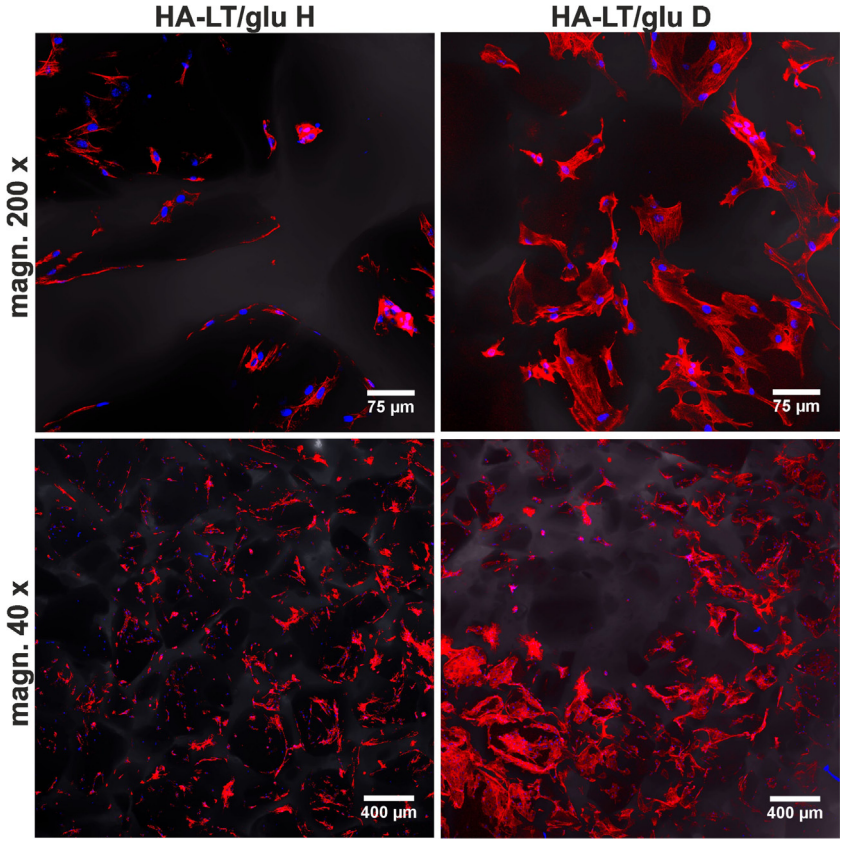

Fig. 4. Fluorescent staining of osteoblast cytoskeleton (red) and nuclei (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microscope (Olympus Fluoview equipped with FV1000) and analyzed using ImageJ software.

3. Results and discussion

Unlike HA-LT/glu H material, the matrix of HA-LT/glu D was covered by precipitates as indicated by SEM analysis (Fig. 1a,b). It was probably the result of the production process requiring dialysis step against $CaCl_2$. EDS analysis confirmed that observed precipitates were in fact made of Ca^{2+} and Cl^- ions (Fig. 1d).

Although HA-LT/glu D comprised highly reactive sintered at low temperature HA (known to uptake Ca^{2+} from the medium), ion reactivity tests showed that this material released great amounts of Ca^{2+} through duration of the experiments and the highest Ca^{2+} release (310–350 mg/l) was observed during first 24 h (Fig. 2a,b). It was probably due to the gradual dissolution of $CaCl_2$ precipitates. More importantly, HA-LT/glu H material, which was composed of the same constitutes as HA-LT/glu D but was fabricated based on the heat treatment method, caused critical sorption of the Ca^{2+} ions. After 24 h incubation, culture medium was completely devoid of Ca^{2+} (0.4–0.6 mg/l).

According to the available literature, calcium-deprived culture medium is lethal to the cells [2,3]. Whereas increased concentration of Ca^{2+} in medium positively affects osteoblast proliferation and differentiation [6]. Obtained results are in good agreement with abovementioned statements. There were significantly more viable cells on the HA-LT/glu D scaffold – which released Ca^{2+} into the medium – compared to the HA-LT/glu H, which reduced the Ca^{2+} concentration (Fig. 3).

Moreover, there were almost 2-fold more osteoblasts on the HA-LT/glu D sample ($3.2 \times 10^4/cm^2$) than on the HA-LT/glu H material ($1.8 \times 10^4/cm^2$). Osteoblasts cultured on the HA-LT/glu D were also noticeably better spread (spreading area of approx. 1900 μm^2) compared to the cells on the HA-LT/glu H material (approx. 446 μm^2) indicating that HA-LT/glu D scaffold reduced cytotoxic effect of highly reactive HA and was more favourable to osteoblast growth (Fig. 4).

Considering *in vivo* applications, highly reactive bioceramics have some desired features like good bioresorbability, high microporosity, and biomineralization ability [3]. However, if the material is designed for *in vitro* applications, e.g. to generate living bone graft, highly reactive bioceramics would deprive the culture medium of crucial for cell survival ions causing cell death. Obtained results clearly showed that the use of calcium ions instead of heat treatment for β-1,3-glucan gelation during fabrication process of HA/β-1,3-glucan biomaterial significantly reduces cytotoxic effect of highly reactive CaPs caused by the Ca^{2+} uptake from the medium. Thus, new method for HA/β-1,3-glucan fabrication may be considered as effective way to support cell survival *in vitro* when highly reactive CaPs are used for biomaterial fabrication.

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References

[1] J. Vivanco, J. Slane, R. Nay, A. Simpson, H.L. Ploeg, The effect of sintering temperature on the microstructure and mechanical properties of a bioceramic bone scaffold, J. Mech. Behav. Biomed. Mater. 4 (8) (2011) 2150–2160.
[2] B.P. Malafaya, L.R. Reis, Bilayered chitosan-based scaffolds for osteochondral tissue engineering: Influence of hydroxyapatite on in vitro cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor, Acta Biomat. 5 (2009) 644–660.
[3] K. Klimek, A. Belcarz, R. Pazik, P. Sobierajska, T. Han, R.J. Wiglusz, G. Ginalska, "False" cytotoxicity of ions-adsorbing hydroxyapatite - Corrected method of cytotoxicity evaluation for ceramics of high specific surface area, Mater. Sci. Eng. C Mater. Biol. Appl. 65 (2016) 70–79.
[4] J. Gustavsson, P.M. Ginebra, E. Engel, J. Planell, Ion reactivity of calcium deficient hydroxyapatite in standard cell culture media, Acta Biomat. 7 (2011) 4242–4252.
[5] A. Przekora, J. Czechowska, D. Pijocha, A. Slosarczyk, G. Ginalska, Do novel cement-type biomaterials reveal ion reactivity that affects cell viability in vitro?, Cent Eur. J. Biol. 9 (2014) 277–289.
[6] S. An, Y. Gao, J. Ling, X. Wei, Y. Xiao, Calcium ions promote osteogenic differentiation and mineralization of human dental pulp cells: implications for pulp capping materials, J. Mater. Sci. Mater. Med. 23 (2012) 789–795.
[7] N. Cao, B.X. Chen, J.D. Schreyer, Influence of calcium ions on cell survival and proliferation in the context of an alginate hydrogel, ISRN Chem. Eng. (2012), http://dx.doi.org/10.5402/2012/516461 516461.
[8] A. Belcarz, G. Ginalska, T. Pycka, A. Zima, A. Ślósarczyk, I. Polkowska, Z. Paszkiewicz, W. Piekarczyk, Application of β-1,3-glucan in production of ceramics-based elastic composite for bone repair, Cent. Eur. J. Biol. 8 (2013) 534–548.
[9] L. Borkowski, M. Pawłowska, R.P. Radzki, M. Bieńko, I. Polkowska, A. Belcarz, M. Karpiński, T. Słowik, Ł. Matuszewski, A. Ślósarczyk, G. Ginalska, 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. 53 (2015) 60–67.
[10] K. Klimek, A. Przekora, K. Pałka, G. Ginalska, 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. A. (2016), http://dx.doi.org/10.1002/jbm.a.35798.
[11] K. Klimek, A. Przekora, G. Ginalska, 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 no. P.415936, filed to Polish Patent Office. Forthcoming.
[12] A. Przekora, K. Palka, G. Ginalska, Chitosan/β-1,3-glucan/calcium phosphate ceramics composites – novel cell scaffolds for bone tissue engineering application, J. Biotechnol. 182–183 (2014) 46–53.