IN VITRO BEHAVIOR OF COMPOSITE MATERIALS BASED ON Zn-MODIFIED CALCIUM PHOSPHATE POWDERS

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Abstract

Composite scaffolds were prepared using two calcium phosphate powders simultaneously modified by Zn and SBF-ions and hydrogels of different natural polymers as binding components. Two biomimetic precipitation procedures (quick mixing and continuous precipitation) with subsequent sintering up to 1000^{o}C were applied and the ion-modified calcium phosphate powders were obtained. The ratio $Zn^{2+}/(Ca^{2+}+Zn^{2+}+Mg^{2+})$ was 0.13 and the ratio $(Ca^{2+}+Zn^{2+}+Mg^{2+}+Na^{+}+K^{+})/P$ was 1.4 and 1.45. The biocompatibility of these composite scaffolds was tested with two cell lines, rat bone marrow cells and murine BALB/c 3T3 fibroblasts. The studies revealed that the studied materials had different effects on the survival and proliferative activity of the two cell lines. This result we explain by the different cellular targets that displayed specific cell responses, as well as by the different physico-chemical characteristics of the two ion-modified calcium phosphate powders resulting from the different precipitation procedures.

Key words: *Zn-modified calcium phosphates, composite scaffolds, biomimetic precipitation, in vitro testing*

1. INTRODUCTION

Calcium ortho-phosphates are suitable compounds for the preparation of ceramics, cements, composites, etc. for orthopedic, reconstructive and maxillofacial surgery, because of their chemical and structural similarity with the inorganic part of the hard tissues. Their bioactivity depends on the chemical and phase compositions and on the morphologies which are highly affected by the method of precursor preparation and its subsequent treatment. The possibility of incorporating ionic modifiers into the calcium phosphates allows them to look like the mineral component of bone tissues and to strengthen some specific biologically important behaviors (Koroleva 2010, Shepherd et al. 2012). Zinc is one of the essential elements for the human body due to its significance for natural biological growth and development of the skeleton system (Yamaguchi 1998). Moonga and Dempster (1995) found that zinc inhibits osteoclast differentiation and promotes osteoblast activity. Zn-doped hydroxyapatite (Zn-HA), like hydroxyapatite (HA), possesses very low solubility and does not actively participate in the process of bone remodeling (Sogo et al. 2004). The solubility of Zn-doped β-tricalcium phosphate (Zn-β-TCP) is lower than that of pure β-TCP (Xue et al. 2008). This feature favors the biological behavior of materials containing Zn-β-TCP (Dorozhkin 2009, Xue et al. 2008) by reducing the high resorbability of β-TCP (Tampieri et al 2005, Dorozhkin 2009).

The preparation of composite materials on the base of ion-modified calcium phosphates and polymer matrices is a subject of extensive studies (Dorozhkin 2011, Ilieva et al. 2013, Rabadjieva et al. 2013). These composites mimic the hierarchical mineral-organic structure of bones (Dorozhkin 2011) and provide adequate long-term porosity of the materials. Polymers are a special class of organic materials that are compatible with the major functional components of the biological environment. There is a variety of biocompatible polymers, such as polyacrylates (Furuichi et al. 2006, Shen 2007), polyurethanes (de Groot 1996, Resiak & Rokicki 2000), polycarbonates (Agrawal & Ray 2001), etc., which are suitable for biomedical applications. Natural polymers like gelatin (Eiden-Assmann et al. 2002, Goebel et al. 2004), chitosan (Khor & Lim 2003, Di Martino et al. 2005) or their derivatives,

have been used as an alternative to synthetic polymers because of their biodegradability and biological activity.

In vitro tests are usually performed to prove the new materials applicability. The studies on cell response of calcium phosphate composite materials by *in vitro* tests include different cell lines, such as human mesenchymal stem cells (MSCs) (Li et al. 2012, Reddy et al. 2013), permanent cell line Lep-3 obtained from 3-month human embryos; osteoblastic cells derived from newborn rat calvaria and isolated by sequential collagenase digestion (Nezafati et al. 2013), 7F2 cells corresponding to adult mouse osteoblasts (Wagner et al. 2013), C2C12 preosteoblastic cells (Okuda et al. 2008)). The procedures usually include culturing of the cells in DMEM or α-MEM solution, interaction of the cultured cells with the materials and cell proliferation measurements.

The aim of this study was to investigate the *in vitro* behavior (survival and proliferation activity of cultured cells) of composite scaffolds based on Zn-modified calcium phosphate powders and hydrogel polymers. For this purpose two biomimetic precipitation procedures (quick mixing and continuous precipitation) were applied and ion-modified calcium phosphate powders with a $Zn^{2+}/(Ca^{2+}+Zn^{2+}+Mg^{2+})$ ratio of 0.13 and $(Ca^{2+}+Zn^{2+}+Mg^{2+}+Na^{+}+K^{+})/P$ ratios of 1.4 and 1.45 were obtained. Natural polysaccharide xanthan gum, carrageenan and gelatin were the binding components in the scaffolds. The *in vitro* testing was performed with two cell lines, rat bone marrow cells and murine BALB/c 3T3 fibroblasts.

2. EXPERIMENTAL PART

2.1. Materials – Simulated body fluids

The popular conventional simulated body fluid (SBFc) (Kokubo 1990) was used to provide an electrolyte medium for the precursor preparations. Modified calcium-free conventional simulated body fluid (SBFc-*Cam*) was used as a solvent for K_2HPO_4 and modified phosphorus-free conventional simulated body fluid (SBFc-*Pm*) was used as a solvent for $CaCl_2$ and $ZnCl_2$, respectively, thus preliminary precipitation being avoided (Rabadjieva et al. 2011). All simulated body fluids used in the experiments (Table 1) were prepared by successive mixing of previously prepared solutions of KCl, NaCl, $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, $ZnCl_2.1.5H_2O$, $NaHCO_3$, Na_2SO_4, and K_2HPO_4 or Na_2HPO_4 salts in distilled water. The pH of the solutions was adjusted to 8.2 – 8.4 using 0.1M HCl or 0.05M Tris (hydroxymethyl) aminomethane. Analytical grade reagents were used.

Table 1. Inorganic composition of Simulated Body Fluids (SBF) ($mmol.dm^{-3}$)

Composition	***SBFc*** [Kokubo, 1990]	***SBFc-Cam*** (Solution 1)	***SBFc-Pm*** (Solution 2)	***SBFc-Pm*** (Solution 3)
Na^+	142.0	141.9	141.9	141.9
K^+	5.0	506.4	3.0	3.0
Mg^{2+}	1.5	1.5	1.5	1.5
Ca^{2+}	2.5	-	364.78	2.5
Zn^{2+}	-	-		54.1
Cl^-	147.8	142.8	867.3	256.1
SO_4^{2-}	0.5	0.5	0.5	0.5
HCO_3^-	4.2	4.2	4.2	4.2
HPO_4^{2-}	1.0	251.7	-	-

2.2. Preparation of ion modified calcium phosphate powders

Calcium phosphate precursors simultaneously modified by Zn and SBF-ions were biomimetically synthesized using two precipitation procedures. The $Zn^{2+}/(Ca^{2+}+Zn^{2+}+Mg^{2+})$ ratio was 0.13 and the initial $(Ca^{2+}+Zn^{2+})/P$ ratio was 1.67.

Procedure 1. The method of quick mixing of the initial solutions was applied. Solution 2 and Solution 3 (Table 1) were rapidly poured into Solution 1 (Table 1) at room temperature under intense stirring, keeping pH 10-11 by 25% NH_4OH. The precipitate was filtered, immediately washed with water and with acetone (solid-to-liquid ratio of 1:1) and freeze-dried for a week at a temperature of -18ºC in the presence of silica gel. The dried precursor was sintered at 1000^{o}C and atmospheric pressure in a high-temperature furnace, type VP 04/17 of LAC Ltd Company. The working regime was: heating with a rate of 10^{o}C/min up to the desired temperature and keeping it constant for 2 h. This powder is further denoted as Zn-β-TCP(1).

Procedure 2. The method of continuous co-precipitation was applied. All reagents (Solutions 1-3, (Table 1)) were added to a glycine buffer medium with a rate of 3 ml/min assuring continuous precipitation at a constant $(Ca^{2+}+Zn^{2+})/P$ ratio of 1.67, keeping pH 8 by 1 M KOH. The precipitate was matured in the mother liquid for 24 h at room temperature and then the thick suspension was subjected to gelling with xanthan gum, lyophilization at -56ºC, and baking at 300ºC. After washing (solid-to-water ratio 1:100) the precipitate was lyophilized again. The dried precursor was sintered gradually at 200, 400, 600, 800 and 1000ºC and atmospheric pressure in a high-temperature furnace. The working regime was: heating with a rate of 3ºC/min up to the desired temperature and keeping it constant for 1 h. This powder is further denoted as Zn-β-TCP(2).

2.3. Preparation of composite scaffolds

Hydrogels of gelatin (20 mass %), xanthan gum (1 mass %) and carrageenan (1 mass %) were previously prepared. Appropriate amounts of these gels were mixed with fine ceramic powders of both Zn-β-TCP powders. Additionally, saccharose was added as a porosifier. Thus, two samples of composite scaffolds with the composition given in Table 2 were prepared. Then, the dense pastes were moulded in the shape of cylinders (2 mm diameter) and lyophilized. The moulds were impregnated with water for 24 h and hardened in 1% glutaraldehyde.

Table 2 Composition (mass %) of the composite scaffolds

Zn-β-TCP (powder)	Xanthan gum	Carageenan	Gelatin	Saccharose	Water
73.89	0.12	0.12	2.46	1.23	22.1

2.4. Physico-chemical characterizations

Chemical analyses - The sum of Ca^{2+}, Mg^{2+} and Zn^{2+} ions in the solid samples was determined complexometrically with EDTA at pH 10. The concentrations of Zn^{2+}, Mg^{2+}, K^+ and Na^+ ions were determined by atomic absorption spectrometry on a THERMO SOLAAR M5 instrument. The concentrations of $P\text{-}PO_4^{3-}$ and Cl^- ions were determined spectrophotometrically on a NOVA 60 equipment using Merck and Spectroquant® test kits.

X-ray diffraction analysis - The phase composition of the calcium phosphate samples was determined on a Bruker D8 Advance XRD apparatus operating at 40 kV and 40 mA with CuK□ radiation and a SolX detector within the 2θ range 10-90^o 2θ, using a step of 0.04^o 2θ and counting time of 1 s/step.

Morphology studies – SEM images - The powder samples were sputter-coated with gold. Their morphology and microstructure were observed using scanning electron microscope JEOL JSM-5510 equipment operating at a voltage of 10 kV.

2.5. In vitro testing

Cell cultures – Two cell lines were used in this study: rat bone marrow cells isolated by us (Andonova-Lilova et al. 2012) and a permanent cell line BALB/c 3T3 clone 31 (mouse embryo fibroblasts) (from the Cell Culture Collection of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences).

Testing procedure: In the case of rat bone marrow cells, the cells were seeded in 6-hole plates at a concentration of 1.5×10^6 cells per hole and were cultivated in the course of 3, 6 and 9 days in presence of the composites. The cell number and survival were determined by the test of trypan blue incorporation using an electronic cell counter of the firma Invitrogen *** - $p < 0.001$. In the case of BALB/c 3T3 cell line, the cells were cultivated for 72 h in a modified nutrient medium, and incubated in the course of 3, 6, 8 and 10 days in the presence of the composites. The study was carried out by a MIT test.

Statistical analysis: The data are presented as mean ± standard error of the mean. Statistical differences between control and treated groups were assessed using one-way analysis of variance ANOVA followed by a Dunnett post-hoc test.

3. RESULTS AND DISCUSSIONS

3.1 Zn and SBF-ions modified calcium phosphate powders

XRD-amorphous calcium-deficient phosphates simultaneously modified by Zn and SBF-ions were biomimetically precipitated. Their $Zn^{2+}/(Ca^{2+}+Zn^{2+}+Mg^{2+})$ ratio was 0.13 and the $(Ca^{2+}+Zn^{2+}+Mg^{2+}+Na^{+}+K^{+})/P$ ratio was 1.4 and 1.45 (Table 3). The level of Zn incorporation was initially set The modification with Mg^{2+}, Na^{+}, K^{+}, and Cl^{-} ions was due to the SBFs and their levels were close to those of natural enamel, dentin and bone (Dorozhkin, 2009). The higher content of Mg^{2+}, Na^{+}, K^{+}, and Cl^{-} ions in the precursor obtained according to *Procedure 1* is due to the applied technique that allows incomplete washing of the mother liquid.

Table 3. Composition of the ion-modified precursors and natural enamel, dentin, cementum and bone

$Zn^{2+}/(Ca^{2+}+Zn^{2+}+Mg^{2+})$	$(Ca^{2+}+Zn^{2+}+Mg^{2+}+Na^{+}+K^{+})/P$	Mg mmol/g	Na mmol/g	K mmol/g	Cl mmol/g
Zn-β-TCP(1) precursor (Quick mixing)					
0.13	1.45	0.13	0.20	0.45	0.03
Zn-β-TCP(2) precursor (Continuous co-precipitation)					
0.13	1.4	0.04	0.08	0.02	<0.05
Enamel, Dentin, Cementum, Bone (Dorozhkin, 2009)					
	1.61 -1.77	0.02 - 0.29	0.22 - 0.39	2.10^{-4} - 0.02	0.03 – 0.1

Zn-β-TCP powders (Fig. 1) were obtained after sintering the two precipitates at 1000°C. The results showed that only the temperature and not the duration nor the way of implementation of the sintering process influence the calcium phosphates phase composition.

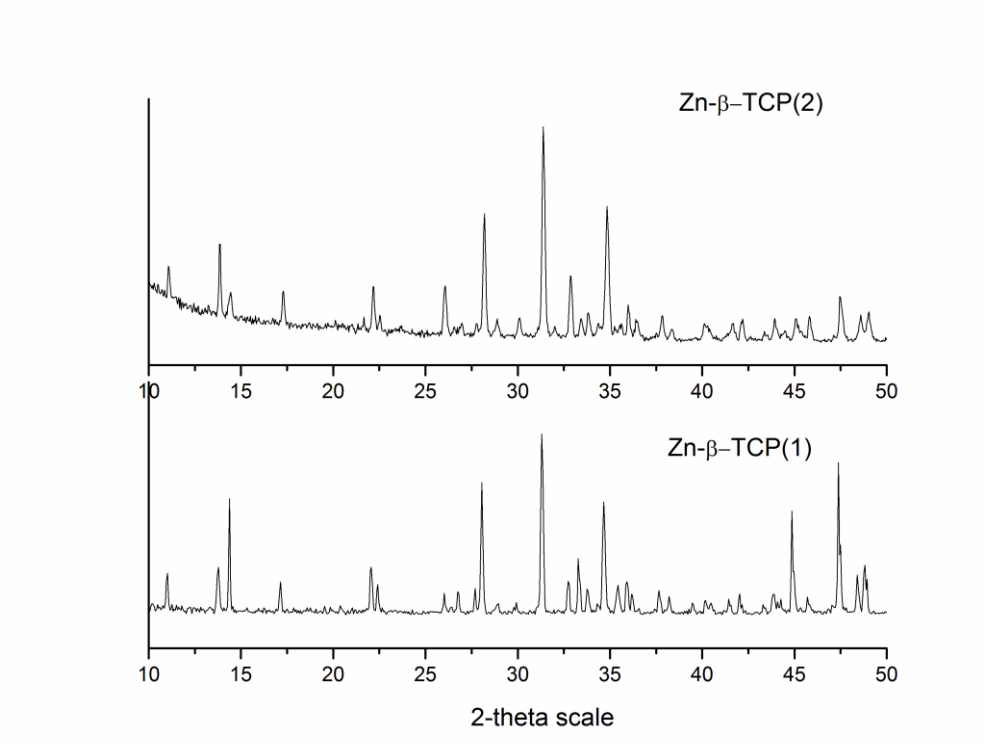
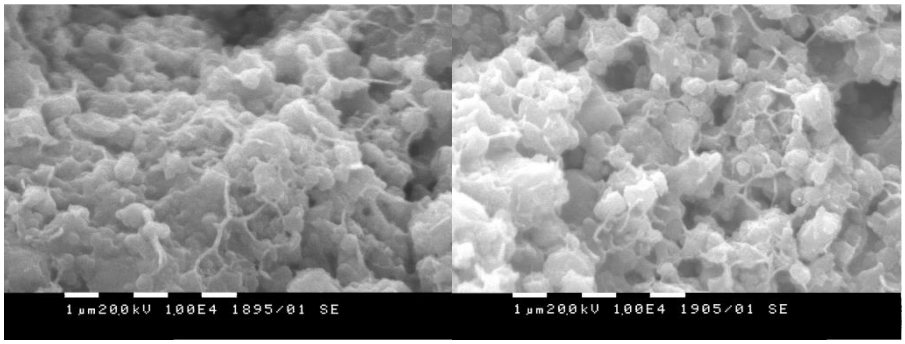


Fig. 1 XRD powder patterns of Zn-β-TCP(1) and Zn-β-TCP(2) after sintering at 1000^{o}C

3.2. Composite scaffolds

The two powders synthesized (Zn-β-TCP(1) and Zn-β-TCP(2)) were used for preparation of two samples of composite materials differing only in the quality of the solid phase. Natural bioresorbable polysacharides xanthan gum and carrageenan, as well as gelatin were used for composite scaffold preparation. Owing to their different solubilities, these polymers provide the necessary elasticity of the scaffold and the step-wise porosity formation. The preliminary porosity of the samples was imparted by saccharose during washing (Fig.2b). The SEM images (Fig.2) show the good cross-linking of the powder particles by the polymers.



a b

Fig. 2. SEM images of composite scaffolds: a) before and b) after saccharose dissolution.

3.3. In vitro testing

The effect of the examined Zn-β-TCP(1) composite scaffolds on cell viability and proliferation of murine BALB/c 3T3 fibroblasts was studied by MTT test assays and Mosmann method (Mosmann 1983) after 72 h of incubation (Fig. 3). NIH 3T3 murine fibroblasts have been successfully applied in various studies on bone regeneration. Moreover, there are data that after genetic manipulation, these cells can become suitable tools for cell therapy of bone defects. The increasing interest in such non-osteogenic fibroblasts could also be explained by some of their advantages: easy harvesting from autologous donors, expansion, and *in vitro* genetical engineering (Phillips et al. 2007, Lattanzi et al. 2008, Lee et al. 2011).

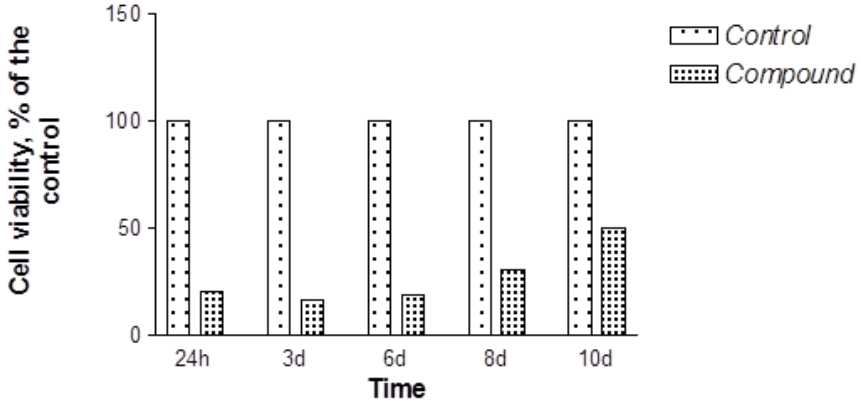


Fig. 3 Effect of Zn-β-TCP(1) composite scaffolds on the viability and proliferation of murine BALB/c 3T3 fibroblasts

The results revealed high mortality of the cells just after the 1st day that increased after the 2nd day. Then the mortality decreased; an increase in the cell number was observed after the 6th day, due to the start of a reproductive process.

For *in vitro* testing of the Zn-β-TCP(2) composite scaffolds we chose rat bone marrow cells, because in contrast to permanent cell lines, primary cultures better resemble the biological characteristics of the cells in the original tissue. The results are shown on Figs. 4 and 5.

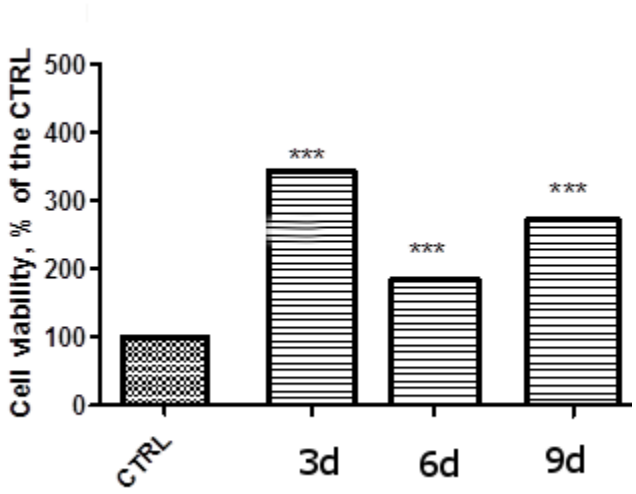
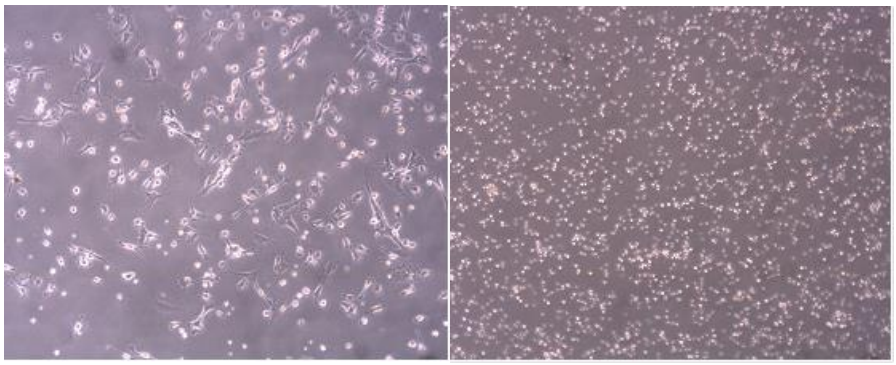


Fig. 4 Effect of Zn-β-TCP(2) composite scaffolds on the viability and proliferation of rat bone marrow cells



a b

Fig. 5 Rat bone marrow cells (a) control and (b) cultivated for 6 days in the presence of Zn-β-TCP(2) composite scaffolds

The results show a high survival degree and reproductive ability of the rat bone marrow cells in the presence of Zn-β-TCP(2) composite scaffolds.

The different behavior of the studied calcium phosphate scaffolds towards the two cell lines could be explained first by the different cellular targets that express specific cell response and second, by the different physico-chemical characteristics of the ion-modified calcium phosphate powders resulting from the different precipitation procedures. Ion-modified calcium phosphates are homogeneous mixedcrystals, in which the substituting ions are incorporated in the calcium phosphate structure during the precipitation process. This phenomenon is an equilibrium process. It is rather probable to reach equilibrium at quick precipitation. It is more probable a part of the ion inclusions determined by us to be due not only to the incorporation into the crystal structure, but also to the mother liquid. In the case of zinc, co-crystallization of zinc hydroxide is possible at pH 10-11. It cannot be registered by the method of XRD analysis because of its amorphous form and its small quantity. During the sintering process the salts undergo different transformations. Their higher concentrations (Table 2) and easier dissolution determine their easier transition into the surrounding environment. This resulted in a higher toxicity of the Zn-β-TCP(1) scaffolds in comparison with Zn-β-TCP(2) scaffolds.

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