Materials Science and Engineering C 62 (2016) 260–267

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

The comparison study of bioactivity between composites containing synthetic non-substituted and carbonate-substituted hydroxyapatite

Leszek Borkowski [a,*], Anna Sroka-Bartnicka [b], Piotr Drączkowski [c], Agnieszka Ptak [a], Emil Zięba [d], Anna Ślósarczyk [e], Grażyna Ginalska [a]

[a] Chair and Department of Biochemistry and Biotechnology, Medical University of Lublin, Chodźki 1, 20-093 Lublin, Poland
[b] Department of Biopharmacy, Medical University of Lublin, Chodźki 4a, 20-093 Lublin, Poland
[c] Department of Synthesis and Chemical Technology of Pharmaceutical Substances, Medical University of Lublin, Chodźki 4a, 20-093 Lublin, Poland
[d] SEM Laboratory, Department of Zoology and Ecology, John Paul II Catholic University of Lublin, Al. Kraśnicka 102, 20-718 Lublin, Poland
[e] Faculty of Materials Science and Ceramics, AGH-University of Science and Technology, Mickiewicza 30, 30-059 Krakow, Poland

ARTICLE INFO

Article history:
Received 28 July 2015
Received in revised form 12 January 2016
Accepted 23 January 2016
Available online 26 January 2016

Keywords:
Apatite
Bioactivity
Bone
Composite
Raman spectroscopy

ABSTRACT

Apatite forming ability of hydroxyapatite (HAP) and carbonate hydroxyapatite (CHAP) containing composites was compared. Two composite materials, intended for filling bone defects, were made of polysaccharide polymer and one of two types of hydroxyapatite. The bioactivity of the composites was evaluated *in vitro* by soaking in a simulated body fluid (SBF), and the formation of the apatite layer was determined by scanning electron microscopy with energy-dispersive spectrometer and Raman spectroscopy. The results showed that both the composites induced the formation of apatite layer on their surface after soaking in SBF. In addition, the sample weight changes and the ion concentration of the SBF were scrutinized. The results showed the weight increase for both materials after SBF treatment, higher weight gain and higher uptake of calcium ions by HAP containing scaffolds. SBF solution analysis indicated loss of calcium and phosphorus ions during experiment. All these results indicate apatite forming ability of both biomaterials and suggest comparable bioactive properties of composite containing pure hydroxyapatite and carbonate-substituted one.

1. Introduction

Hydroxyapatite (HAP) is the most frequently used representative of calcium phosphates in commercially available ceramic-based bone substitute materials. It has been widely used in medicine and dentistry for more than 40 years, because of its good biocompatibility with hard human tissues and osteoconductive properties [1]. Mostly it is used as pure, non-substituted HAP in the form of ceramic (such products as: Osteograf/D®, Osteogen®, Osprovit®, Endobon®, OSTIM®), ceramic composites (eg. 4BONE®, Camceram®, BoneSave®, ProOsteon®, HT Biocer®, NanoBone®) or ceramic-polymer composites (Healos®). However, biological apatite present in bone is a carbonate-substituted one of a low crystallinity. Carbonate ions (present in bone and dentine tissues at the level of 2.3–8 wt.% [2] were claimed to increase the solubility and decrease the crystallinity of ceramics [3,4]. Therefore, there is growing interest in the potential application of carbonate-substituted hydroxyapatite (CHAP, also called as *biomimetic*) in bone tissue engineering. Up to now, CHAP found an application as a coating on metals, mostly titanium [5,6,7,8,9] and polymers [10,11,12]. Numerous studies were performed on CHAP containing scaffolds either in the form of ceramic [13,14,15,16,17,18] or ceramic-based composites [19,20,21,22]. The results of *in vitro* studies on ceramics showed the enhanced attachment and proliferation of rat osteoblasts as well as the formation of mineralization nodules in comparison with non-substituted HAP. On the other hand, some investigators report that carbonate substitution can either delay or accelerate the proliferation of osteoblastic-like cells and constrain the osteoclastic differentiation. The studies on CHAP-based composites lead to the general conclusion that addition of CHAP affects the bioactivity of the composites, increases adhesion and differentiation of osteoblast cells, collagen and osteocalcin expression and mineral deposition. Similarly, *in vivo* studies do not provide explicit conclusions concerning the biological impact of carbonate-substituted hydroxyapatite [23,24,25,26,27]. On the basis of literature analysis, it seems that there is still a high need to explore the difference in properties between HAP and CHAP.

Bioactivity of biomaterials is described as its ability to bond with host bone tissue [1]. The mechanism of bone bonding is through the formation of a surface layer of apatite, which is similar to the mineral component of bone. Currently, two common methods have been used for testing the *in vitro* bioactivity of biomedical materials. One method (also called the biomineralization test) is to evaluate the apatite-formation ability of biomaterials in the simulated body fluids (SBFs) [28]. It was firstly established by Kokubo and colleagues in 1990 to

* Corresponding author.
E-mail address: leszek.borkowski@umlub.pl (L. Borkowski).

http://dx.doi.org/10.1016/j.msec.2016.01.056
0928-4931/

assess bioactive potential of ceramics [29,30]. The second method is to investigate the effect of material on osteogenic differentiation using cell experiments [13,31,32,33]. Selection of methods for the evaluation of *in vitro* bioactivity of biomaterials depends on the composition of materials and the mechanism of their bone formation [34].

In this study we compared apatite forming ability of hydroxyapatite and carbonate hydroxyapatite using two recently developed ceramic-polymer composite materials [35,36]. Two types of scaffolds contained HAP or CHAP granules and the same polysaccharide (β-1,3-glucan) playing a role of joining and elasticity increasing agent. The polymer used in these composites is a natural, relatively cheap, non-animal and nontoxic glucan. It has already been commercialized as a formulation aid, processing aid, stabilizer and thickener or texturizer in foods [37]. The bioactive properties of HAP and CHAP were assessed by detailed examination of precipitated hydroxycarbonate layer on the surface of polymer. The investigation was conducted in SBF under semi-dynamic conditions for 1 month. *In vitro* composite mineralization was analysed qualitatively and quantitatively, using such tools as scanning electron microscopy (SEM), an energy dispersive X-ray detector (EDS) and Raman mapping.

2. Materials and methods

2.1. Composite preparation

12 CHAP-glucan composite and 12 HAP-glucan composite scaffolds were prepared according to the procedure described in european patent [38]. Briefly, samples were fabricated by mixing 3 g of CHAP or HAP granules with β-1,3-glucan aqueous suspension (0.625 g of glucan + 5 ml of distilled water), thus obtaining wt.% proportion granules to β-1,3-glucan 83:17. The mixture of ceramic/glucan solution was put into a special glass mould and baked at 90 °C for 15 min. Finally, the fabricated material was cut with scalpel into cubes with the side length of 10 mm (±1), dried for 4 days at 37 °C, subjected to exsiccation for the next 3 days and sterilized.

CHAP and HAP granules were synthesized at the AGH-University of Science, according to patented procedures [39,40]. Physicochemical data of the HAP/CHAP granules and the HAP/glucan and CHAP/glucan composite samples were presented in Tables 1 and 2.

β-1,3-Glucan (curdlan) from *Alcaligenes faecalis* (DP 450) was supplied by Wako Chemicals, Japan.

2.2. Soaking in a semi-dynamic SBF conditions

Prior to the experiment, all composite samples were weigh on an analytical balance with accuracy 0.0000 g. Laboratory glassware was washed, soaked for 24 h in HCl, rinsed with distilled water and autoclaved. The SBF was prepared by dissolving reagent grade NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and Na_2SO_4 into ultra-pure water, $(CH_2OH)_3CNH_3$ was added and the solution was buffered at pH 7.25 with HCl (according to the method proposed by Kokubo et al., [29]). Afterwards, the fluid was sterilized by mechanical filtration, using the Stericup filter (500 ml, 0.22 μm; Millipore Corporation) under vacuum.

During experiment the composite samples were named as follows:

- CHAP Composite — treated in SBF (n = 9)
- HAP Composite — treated in SBF (n = 9)
- CHAP Control — treated in water (n = 3)
- HAP Control — treated in water (n = 3)

CHAP and HAP Composite samples were soaked in 100-ml bottles (Simax, Czech Republic) with 80 ml of SBF (one bottle contained 3 samples). The SBF solution was replaced with new every 2–3 days to ensure sufficient ion concentrations for mineral growth. Control samples were kept in 80 ml of ultra-pure water. Incubation was conducted at 37 °C for various periods: 10, 20 and 30 days. After a given period, the specimens were dried for 4 days at 37 °C, subjected to exsiccation for the next 3 days and weighed.

Quantitative analysis (weight measurements) was performed for samples treated with SBF for 10, 20 and 30 days. Structural analysis of the scaffolds (aimed at showing the presence of apatite crystals on the surface of composites) was performed on the samples after 30-day SBF-treatment because the differences between CHAP and HAP Composites were the highest.

2.3. Structural analysis of the samples

2.3.1. SEM–EDS

The polymeric surface of specimens before and after soaking in SBF wasanalysed using scanning electron microscopy (FE-SEM; Zeiss ULTRA plus) with an energy dispersive X-ray detector (EDS; Bruker). The regions of interest (polymer phase of composites) for EDS analysis were defined using magnified SEM micrographs. The chemical composition (presented in wt.%) was calculated automatically by software on the basis of EDS spectra.

2.3.2. Raman spectroscopy and mapping

2.3.2.1. Spatial distribution. The Raman experiments were performed using a DXR confocal Raman Microscope equipped with the Omnic™ 8 software from Thermo Fisher Scientific (Madison, WI, USA) and a X–Y motorized sample stage. The excitation laser wavelength was 780 nm. Filters of 780 nm and 400 lines/mm grating were used. A Peltier-cooled CCD detector registered dispersed light with a wavenumber range between 150 and 2500 cm^{-1}. The mapping measurements were carried out using a long working distance ×50 objective due to uneven surface of the samples, the autofocus at each point of the map was used in case of non-flat samples.

All area maps (sampling point spread along X and Y axes) were carried with an exposure time of 4 s with laser power set to 20 mW, and 5 exposures per point using an operating spectral resolution of 4 cm^{-1} of

Table 1
Chemical compositions and physical parameters of the hydroxyapatites used for the fabrication of composites.

Characteristics	Granules	
	HAP	CHAP
Ca/P ratio	1.67	1.69
Fraction size (mm)	0.2–0.6	0.2–0.6
Open porosity (%)	68	66
Surface area (m^2/g)	24	74
Carbonate content (wt.%)[a]	0	5.2

[a] Confirmed by FT-IR and TGA analysis.

Table 2
Chemical compositions and physical parameters of the composite samples used in the study.

Characteristics	Composites	
	HAP/glucan	CHAP/glucan
(HAP or CHAP)/glucan (wt.% ratio)	83:17	83:17
Carbonate content (wt.%)	0	4.3
Compressive strength (MPa)[a]	5.9	6.1
Young's modulus (GPa)[a]	0.78	0.64
Sorption index (%)	121.7	119

[a] Measured for dry composite samples.