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Structural transformation of synthetic hydroxyapatite under simulated *in vivo* conditions studied with ATR-FTIR spectroscopic imaging

Anna Sroka-Bartnicka [a,*], Leszek Borkowski [b], Grazyna Ginalska [b], Anna Ślósarczyk [c], Sergei G. Kazarian [a,*]

[a] *Department of Chemical Engineering, Imperial College London, South Kensington Campus, SW7 2AZ London, United Kingdom*
[b] *Chair and Department of Biochemistry and Biotechnology, Medical University of Lublin, Chodźki 1, 20-093 Lublin, Poland*
[c] *Faculty of Materials Science and Ceramics, AGH-University of Science and Technology, Mickiewicza 30, 30-059 Krakow, Poland*

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ABSTRACT

Hydroxyapatite and carbonate-substituted hydroxyapatite are widely used in bone tissue engineering and regenerative medicine. Both apatite materials were embedded into recently developed ceramic/polymer composites, subjected to Simulated Body Fluid (SBF) for 30 days and characterized using ATR-FTIR spectroscopic imaging to assess their behaviour and structures. The specific aim was to detect the transition phases between both types of hydroxyapatite during the test and to analyze the surface modification caused by SBF. ATR-FTIR spectroscopic imaging was successfully applied to characterise changes in the hydroxyapatite lattice due to the elastic properties of the scaffolds. It was observed that SBF treatment caused a replacement of phosphates in the lattice of non-substituted hydroxyapatite by carbonate ions. A detailed study excluded the formation of pure A type carbonate apatite. In turn, CO_3^{2-} content in synthetic carbonate-substituted hydroxyapatite decreased. The usefulness of ATR-FTIR spectroscopic imaging studies in the evaluation of elastic and porous β-glucan hydroxyapatite composites has been demonstrated.

1. Introduction

For several decades, many efforts have been put to improve the effectiveness of bone substitutes. The most significant characteristics of biomaterials are their bioresorbtion and bioactivity. Bioactivity shows an ability of an implant to create a chemical bond with bone tissue [1]. Significant efforts have been made designing modern hydroxyapatite materials [HAP, $Ca_{10}(PO)_4(OH)_2$] that may be substituted by numerous ions such as cations Na^+, Mg^{2+}, Mn^{2+}, Zn^{2+} and anions CO_3^{2-}, Cl^-, F^-. HAP have a specific type of substitution by anions either for phosphate groups PO_4^{3-} in B sites of the apatitic structure (B-type substitution) or for hydroxide groups OH^- in the A sites (A-type substitution). The first substitution mechanism (A-site substitution) involves the replacement of channel site hydroxyl ions with carbonate. The second mechanism (B-site substitution) corresponds to the carbonate replacing a phosphate group [2,3]. The third mechanism is known as a AB substitution when both substitutions are allowed in the carbonated hydroxyapatite [4]. The structural roles of these substitution types of apatite have been studied extensively by X-ray and neutron diffraction, chemical analyses, infrared (IR) and Raman spectroscopy, proton and ^{13}C NMR spectroscopy, and computer modeling [5–8]. (See Tables 1 and 2.)

The FT-IR assay has been most frequently used for the assignment of the substitution types of ions in biominerals [9] or synthetic apatite products and finally bone tissue regeneration process [10]. Applications of ATR-FTIR spectroscopic imaging to biological systems has been proposed for studying multiple protein solutions [11–13] drugs disproportionations [14,15]. The use of ATR-FTIR spectroscopic imaging for high-throughput analysis of many samples was introduced for studies of polymer/drug formulations [16,17]. The increase of the spatial resolution in FTIR imaging using Attenuated Total Reflection (ATR) mode with Ge crystal in a microscope objective is possible, however, the imaged area in such case is small, ca. 50 × 50 mm^2 [18,19].

The *in vitro* bioactivity test in Simulated Body Fuid (SBF) applied for the first time by Kokubo et al. [20] has been widely used for the determination of *in vivo* bone bioactivity and observation of apatite formation on the surfaces of different types of biomaterials. It is known that bioactive material soaked in a SBF solution participate in the ion-exchange process. As a result the hydroxyapatite layers can form on the surface of materials, which is the common characteristic of a their bioactivity. The ion exchange in SBF fluid was discussed in the context of influencing changes in the crystallinity of sample. In Park et al. paper [21] where the bioactivity of calcium phosphate coatings with the electrodeposition in modified SBF containing CO_3^{2-} ions was revealed as the most bioactive showing transformation into carbonate apatite similar to bone apatite. The bone like hydroxyapatite precipitation was recently studied using 10 SBF-like solutions and microwave irradiation [22]. The modified

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* Corresponding authors.
E-mail addresses: annasroka@tlen.pl (A. Sroka-Bartnicka), s.kazarian@imperial.ac.uk (S.G. Kazarian).

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Table 1
Chemical compositions and physical parameters of the composite samples used in the study.

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

[a] Measured for dry composite samples.

SBF was also used for production HA samples with the sol-gel method [23].

Recently, two novel ceramic-polymer composites were designed for bone tissue engineering. The composites HAP/glucan and CHAP/glucan contained HAP or carbonate hydroxyapatite (CHAP), respectively and the same polysaccharide polymer. Synthetic HAP has been widely used in medicine and dentistry since 1970s, because of its excellent biocompatibility, osteoconductivity, lack of toxicity and immunogenicity, and bone affinity [24]. CHAP is a biomimetic variation of HAP because it is more similar to biological apatite of bone and dentine tissues that contains carbonate ions at the level of 2.3–8 wt% [25,26]. According to the previous reports, CHAP stimulates adhesion and proliferation of rat osteoblasts and *in vitro* formation of mineralized nodules more than HAP [27,28]. The second phase of the developed composite was β-1,3-glucan that is a natural, relatively cheap, non-animal and nontoxic polysaccharide. The polymeric component played the role of a joining and elasticity increasing agent due to its specific gelling properties. Therefore, HAP/glucan and CHAP/glucan composites combine the benefits of calcium phosphate ceramics with a high level of elasticity convenient for surgeons. Our *in vivo* studies demonstrated their effective osseointegration, penetration of bony tissue into scaffolds and increasing mineralization leading to the repair of bone defects [15,29,30]. However, previous studies were insufficient to determine chemical and structural alterations within the lattice of those hydroxyapatites (such as substitutions, phase transitions, *etc.*) that occur after implantation. Better understanding of these changes would help to evaluate the role of carbonate ions on the biological behaviour of HAP and its influence on the bone regeneration process.

HAP/glucan and CHAP/glucan composites exhibit a high soaking capacity, as reported before [31]. Sorption index (S_w) significantly varied within the range of 102.6 to 195.8% depending on the ceramic/polymer ratio. In general, the higher the amount of glucan, the higher ability for fluid absorption. For example, estimated S_w for biomaterial containing 83.3 wt% HAP and 16.7 wt% glucan was 121.7%. Such capability to uptake and preserve liquids within material structure is an important feature because it may be used as antibiotic-loaded carrier for local drug delivery. Moreover, the soaking capacity of a material is closely related to its elasticity and plasticity. HAP/glucan and CHAP/glucan composites exhibit considerable flexibility, can be compressed or bent, thus may easily adapt to the shape/size of a bone defect.

The aim of this work was to specify band assignments and spectral alterations in hydroxyapatite (HAP) and carbonate-substituted hydroxyapatite (CHAP) using two new elastic ceramic-polymer composites with ATR-FTIR spectroscopic imaging. This approach was applied to detect changes *i.e.* ion exchange, carbonate incorporation and crystallinity, influenced by the SBF. Furthermore, the apatite forming ability and type of substitution in HAP and CHAP containing composites were compared.

Table 2
Assignment of IR spectral bands of carbonate groups in calcium phosphates of A, B, AB apatite type [38].

v_2	v_3	Phosphates apatite type
880; 878	1450,1545; 1540,1500,1465	A-type
873	1465,1415	B-type
871	1470,1420; 1460,1420 (CO_3,OH)	B-type (CO_3^{2-})
866	–	CO_3^{2-} absorbed
–	1452, 1470, 1500, 1545, 1568	A or B type

2. Materials and methods

2.1. Sample preparation

β-1,3-Glucan (curdlan) from *Alcaligenes faecalis* (DP 450) was supplied by Wako Chemicals, Japan. Apatite granules were synthesized at the AGH-University of Science, according to patented procedures [32,33]. Pure granules were used as a standard for spectroscopic analysis. Their chemical compositions and physical parameters were as follows:

- HAP - 0.2–0.6 mm; Ca/P ratio 1.67; open porosity 68%; surface area 25 m^2/g
- CHAP - 0.2–0.6 mm; Ca/P ratio 1.69; open porosity 66%; surface area 74 m^2/g; carbonate content 5.2 wt.%

CHAP-glucan ($n = 12$) and HAP-glucan ($n = 12$) composite scaffolds were prepared by mixing 17 wt% β-1,3-glucan (suspended earlier in distilled water) with 83 wt% of CHAP or HAP ceramic granules according to the patented procedure [34]. The mixture of ceramic/glucan solution was put into a special mould and baked at 92 °C for 15 min. Finally, the fabricated material was cut into cubes with the side length of 10 mm (±1), dried for 4 days at 37 °C and sterilized. Physicochemical data of the composite samples were presented in Table 1.

2.2. Soaking in a semi-dynamic SBF conditions

Laboratory glassware was washed, incubated for 24 h in HCl, rinsed with distilled water and autoclaved. The SBF solution was prepared according to method proposed by Kokubo et al. [35]. Briefly, reagent grade NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and Na_2SO_4 were dissolved into ultra-pure water, $(CH_2OH)_3CNH_3$ was added and the solution was buffered at pH 7.25 with HCl. Afterwards, mechanical filtration of the fluid was performed using the Stericup filter (500 ml, 0.22 μm; Millipore Corporation) under vacuum.

CHAP-glucan or HAP-glucan composite samples were soaked in 100-ml bottles (Simax, Czech Republic) filled with 80 ml of SBF. The SBF solution was replaced with new every 2–3 days to ensure sufficient ion concentrations for mineral growth. Control samples were incubated in 80 ml of ultra-pure water. Incubation was conducted at 37 °C for 30 days according to the recommendation for *in vitro* bioactivity test of biomedical implant materials (ISO 23317) [36]. Finally, the specimens were removed from the solution and dried for 4 days at 37 °C.

During experiment the composite samples were named as follows:

- CHAP SBF - treated in SBF ($n = 9$)
- CHAP Control - treated in distilled water ($n = 3$)
- HAP SBF - treated in SBF ($n = 9$)
- HAP Control - treated in distilled water ($n = 3$)

2.3. FTIR spectroscopy

FTIR spectra of the pure composites components were measured Using an Alpha-P spectrometer (Bruker, UK) in ATR mode. A spectral resolution of 4 cm^{-1} with 64 co-added scans was used for collection of reference spectra over a spectral range of 4000–600 cm^{-1}.