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Effect of calcination parameters on behavior of bone hydroxyapatite in artificial saliva and its biosafety

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HIGHLIGHTS

- Evaluation of physicochemical properties of natural origin HAp is proposed.
- Bioactivity tests confirm the stability during incubation in simulated body fluids.
- Tests conducted exhibit potential application in medicine and dentistry.

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ABSTRACT

Being the main inorganic constitution of hard tissues (bone and teeth), calcium phosphates have been attractive in medical and dental applications in hard tissue repair. The evaluation of predicted osteointegration of implant material with bone most often begins with *in vitro* tests simulating living organism environment. The present study aims to present the behaviour in artificial saliva of hydroxyapatite (HAp) obtained from pork bone sludge using pioneer method and its features related to biosafety. Novel, waste-free HAp natural origin preparation method has been developed. Evaluation of diverse parameters, including physicochemical properties by various characterization techniques (eg. X-ray diffraction (XRD), FTIR, SEM and EDS methods) and biological behaviour (immersion in artificial saliva, NRU and MTT *in vitro* cytotoxicity) of natural origin bioceramic material intended to dentistry applications was the main goal of the research. On the basis of bioactivity tests in *in vitro* conditions, it was found that hydroxyapatite of natural origin exhibits the composition stability during incubation in artificial saliva. Performed cytotoxicity tests revealed no cytotoxic effects.

1. Introduction

Hydroxyapatite bioceramic (HAp) has the highest biocompatibility of all synthetic inorganic implant materials. Because of the similarity to chemical composition of natural apatite in bone, hydroxyapatite does not irritate the surrounding tissue, does not cause acute or chronic inflammation, instead it stimulates bone repair processes, which enable the creation of chemical bond at the implant-bone interface [1–3]. Discussing hydroxyapatite it is important to consider its behavior in the living body environment. Typical reaction of living organism to a foreign body is its destruction by resorption. Resorption in a living organism is a complex process and involves chemical and biological disintegration stimulated by osteoclasts adsorbed on both bone and implant materials [4–7]. Biodegradation of some implanted materials is associated with dissolution of intergranular necks, separation of single particles from bulk materials and their absorption by phagocytes [5,8]. The above processes (especially biodegradation) have adverse effects such as mechanical weakness of implant or even its complete destruction [1,6]. HAp exhibits high resistance to resorption and biodegradation tests in contrast to another calcium phosphate - tricalcium phosphate (TCP) with much higher tendency to degrade in the human body [8,9]. The tendency of apatitic calcium phosphate to resorption decreases with increasing Ca/P molar ratio. The greatest degree of resorption is observed for a hydroxyapatite with Ca/P molar ratio in the range of 1.3–1.4, while at a ratio in the range of 1.8–2.0 this phenomenon is almost unnoticeable [10,11]. Porous materials are more prone to resorption

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and biodegradation processes compared to dense materials [12]. Also, heat treatment of hydroxyapatite materials has significant impact on their stability in body environment - HAp bioceramics exposed to higher temperatures exhibit a higher stability in body fluids [13,14]. Hydroxyapatite bioceramic material can be assigned to nontoxic and biologically active materials because it is characterized by a unique bioactivity – the surface layer of hydroxyapatite reacts with a physiological medium and creates chemical bonds between the implanted material and the bone [2,6]. Hydroxyapatite stimulates intense bone ingrowth into bone pores and provides the biological stability of the reconstructed tissues by osteoconductive action. Hydroxyapatite by mimicking the natural bone microstructure acts as a porous scaffold that facilitates tissue ingrowth into the implant [1,4,6,12]. HAp in the form of powder, granules, dense materials, porous sinters, layer on various bases or composites component has been applied in orthopedics, dentistry, maxillofacial surgery, ophthalmology, laryngology and traumatology [1,4,6,15–19]. Powders and granules are used as components of dental cements in the dental pulp, dentin hypersensivity, hypoplastic enamel defects, pathological dental abrasion and after extraction defects treatment. They are also used in sealing of root canals, root perforations and to preparations of dentifrices in conservative dentistry [1,11,20,21]. In some cases to increase the capacity to restore and stimulate the healing of bone tissue, hydroxyapatite powder is used in combination with diverse materials, i.e. freeze-dried bone, bone marrow, fibrin and tissue or fibrin glues [1]. HAp granules have been used for the treatment of periodontal bone defects and maxillofacial surgery as postoperative filling in the maxilla and mandible [1,4,21]. Porous HAp fittings are currently used as fillings of post-extraction alveolus to delay the decline of alveolar bone. Moreover, they serve as drug carriers, and scaffolds to cell culture [1,2,22]. Hydroxyapatite layers are applied as coatings on long-term metal implants such as hip replacement and dental implants [6,20]. There are also implants called PD, used as parts of complex apparatus for dialysis or other therapies [4,5,10,12].

Several *in vitro* methods have been established as standards for preclinical studies of biosafety and are recommended by the International Organization for Standardization [23,24]. The evaluation of predicted osteointegration of implant material with bone most often begins with *in vitro* tests simulating living organism environment. In the environment of the living body, behavior of hydroxyapatite bioceramics depends on the chemical and phase composition, shape and size of crystallite, crystal lattice defects, as well as biological conditions around the implant. Among many available fluids simulating internal environment of living body, the most frequently used is SBF (Simulated Body Fluid) with composition similar to plasma fluid, Ringer's solution – a fluid isotonic with the blood and artificial saliva as an environment of mouth cavity [25–31]. The bioactivity *in vitro* tests in simulated body fluids provide interesting yet limited information about the behavior of the materials in the real environment of the living organism in comparison with *in vivo* tests on cell lines or model animals. An important advantage of immersion the samples in artificial body fluids is the ease of conducting an experiment without a necessity of involving animals in the early stages of research [7,27].

The present study aims to present the behaviour in artificial saliva of hydroxyapatite (HAp) obtained from pork bone sludge using pioneer method and its features related to biosafety. Novel, waste-free HAp natural origin preparation method (mentioned previously in paper [32]) has been developed. Evaluation of diverse parameters, including physicochemical properties and biological behaviour, of natural origin bioceramic material intended to dentistry applications was the main goal of the research. The overriding conclusion is expected stability of resultant biomaterials upon contact with artificial saliva during long-term *in vitro* incubation tests. Moreover, performed cytotoxicity tests revealed no toxic effects.

2. Experimental

2.1. Hydroxyapatite preparation

An animal origin hydroxyapatite was applied. Detailed preparation method was presented in previous paper [32]. In brief, a raw bone material was preliminary defatted and deproteinized by hydrolysis in lactic acid. Bone sludge obtained was calcined in two-step process in a chamber oven or in a rotary kiln. Preliminary calcination was carried out at a temperature of 650 °C in a chamber oven for 3 h and in a rotary kiln for 40 min. In the second stage of the process material was re-calcined in a chamber oven at temperatures 750, 850 and 950 °C in air atmosphere for different times (2.0, 2.5, 3.0 h) or in a rotary kiln at three temperatures: 750, 850 and 950 °C. Hydroxyapatite used in the experiments have been obtained with a novel method using pig bones, which allows a significant cost reduction in obtaining this type of material in comparison with conventional methods. It eliminates aggressive hydrolyzing agents, which generate significant quantities of hazardous waste that need to disposed of in a special way.

2.2. Characterization techniques

The pH measurements were determined with an Elmetron CX-741 device equipped with a pH electrode. The molar ratio of Ca/P was determined by chemical analyses of HAp samples. Calcium was analysed by complexometry titration (EDTA in the presence of calcein and thymolphthalein as indicators), while phosphorus as a P–Mo–V complex by colorimetry (Marcel Media UV-Vis spectrophotometer). The phase composition of the samples was analysed with the use of X-ray diffraction with Philips X'Pert diffractometer equipped with a graphite monochromator PW 1752/00, Cu Kα 1.54 nm, Ni filter (40 kV, 30 mA). FT–IR infrared analyses, within the basic infrared range of 400–4000 cm^{-1}, were conducted with the use of Scimitar Series FTS 2000 spectrophotometer produced by the Digilab Co. The microstructure of samples was examined using scanning electron microscope S-4700 Hitachi supported by chemical analysis carried out using energy dispersive X-ray spectroscope (EDS) at 20.0 kV and 15.0 mA.

2.3. Bioactivity in vitro tests

For assessment of bioactivity in *in vitro* conditions 1.1 g of all calcined hydroxyapatite powders obtained in two-step process in varied kiln and temperatures were formed in cylindrical disks with 13 mm diameter by the uniaxial compaction in a steel mould at a pressure of 74 MPa using hydraulic press. The compacts were sintered at 950 °C in air for 3 h.

The assessment of *in vitro* bioactivity was carried out in artificial saliva prepared according to [33]. The immersion study was carried out at 37 °C by soaking the discs placed vertically in a plastic container with 40 ml of fluid. The time of soaking was 62 days. To evaluate the behavior of hydroxyapatite in stimulated oral environment, pH measurement around hydroxyapatite sinters were performed at 25 °C every day for the first week of immersion and subsequently once a week. Starting on the 13th day the weight changes of sinters were investigated once a week. After immersion time the discs were removed from the solution, washed with distilled water, dried at 105 °C and analyzed.