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Synthesis of fluorine substituted hydroxyapatite nanopowders and application of the central composite design for determination of its antimicrobial effects

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ABSTRACT

Synthetic biomaterials based on fluorine substituted hydroxyapatite are potentially attractive for orthopedic and dental implant applications. The new synthesis of fluorine substituted hydroxyapatite samples were done by neutralization, which consists of adding the solution of HF and H_3PO_4 in suspension of $Ca(OH)_2$. Characterization studies from XRD, SEM and FTIR spectra showed that crystals are obtained with apatite structure and those particles of all samples are nano size, with an average length of 80 nm and about 15–25 nm in diameter. The central composite design was used in order to determine the optimal conditions for the antimicrobial activity of the synthesized samples. In order to evaluate the influence of operating parameters on the percent of viable cell reduction of *Streptococcus mutans*, three independent variables were chosen: exposure time, pH of saline and floride concentration in apatite samples. The experimental and predicted antimicrobial activities were in close agreement. Antimicrobial activity of the samples increases with the increase of fluoride concentration and the decreased pH of saline. The maximum antimicrobial activity was achieved at the initial pH of 4.

1. Introduction

Biomaterials based on calcium orthophosphate are especially attractive for use in medicine, for bone and teeth implants due to their biological properties, such as biocompatibility, bioactivity and biodegradability [1,2]. Among them, hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$) is used particularly because of its similarities to the inorganic component of bone. Fluorapatite (FAP; $Ca_{10}(PO_4)_6F_2$) was recognized as a possible biomaterial for bone repair due to its biocompatibility and potential antibacterial activity [3–9]. Fluoride ions are not natural constituents of bones but in vivo it is mainly associated with calcified tissue, bone and teeth, replacing the hydroxyl groups in hydroxyapatite phase producing its partial conversion into fluoroapatite [10,11]. Compared to pure hydroxyapatie, fluoroapatite has much higher physic-chemical stability, such as an increased resistance to dissolution by acid [12]. Dental caries is one of the most widespread bone diseases. Acidogenic bacteria are the main cause of dental caries, when fermentation sugars and starches in food accumulate on the surface of teeth, it leads to the formation of organic acids, which then cause demineralization, and can lead to complete destruction of teeth [13]. The ability of fluorine ions to stabilize the apatitic structure against demineralization by acid is a useful way in preventing tooth degradation. Upon dissolution of fluorapatite in an acidic environment, leads to the release of fluorine ions, which can act as an antimicrobial agent [14]. Yamagishi et al. [15] has reported that it is possible to use the fluoridated-apatite as biomaterial to repair an early stage of caries lesion in a lower premolar tooth. The nature of the mechanisms of antibacterial actions of fluoride ions is still not entirely clear. It is assumed that fluoride acts in multiple ways to affect the metabolism of bacteria. Primarily, fluoride ions in bacterial cells inhibit enolase, a glycolytic enzyme, resulting in a decrease in acid production from glycolysis and subsequently decrease the bacterial population [16,17]. Low concentrations of fluoride ions are not toxic to humans, but high concentrations are toxic and can lead to enamel fluorosis [18]. Currently, fluoride is one of the most common anticaries agents, present as primary components in toothpaste and mouthwash [19,20].

Various methods such as precipitation, sol–gel processing, mechanochemical synthesis, microwave decomposition and flame spray pyrolysis have been used to prepare fluorapatite [21–26]. The main problem with these methods is that the starting chemicals,

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contain ions which are the basic ingredients of fluorapatite. These ions such as ammonium, can be incorporated into the fluorapatite structure and thus affect its chemical and biological properties.

In this work, fluorine substituted hydroxyapatite ($Ca_{10}(PO_4)_6Fx(OH)_{2-x}$, $0 \le x \le 2$) samples with different fluoride concentrations were synthesized by the neutralization method, starting from calcium oxide, phosphoric acid and hydrofluoricacid. The main reason for using the neutralization method to produce fluorine substituted hydroxyapatite biomaterials in this study is the possibility of preparing pure products from relatively inexpensive chemicals, and the suitability for an industrial production. The antimicrobial activity of synthesized samples was tested against the Gram-positive bacterium *Streptococcus mutans*, one of the main causative agents of dental caries.

2. Materials and methods

2.1. Preparation of the materials

Pure and fluorine substituted hydroxyapatite powders were prepared by a neutralization method. Starting chemicals used for the synthesis of powders were calcium carbonate ($CaCO_3$; min. 98.5%), hydrofluoric acid (HF; min. 40%) and phosphoric acid (H_3PO_4; min. 85%) (all analytical grade, Merck). Calcium oxide (CaO) was obtained by calcinations of $CaCO_3$ for 24 h at 1000 °C. Experiments were performed with inert atmosphere (N_2). Double distilled water was used throughout all the experiments. The $Ca(OH)_2$ suspension was prepared by stirring (at a rate of 300 rpm) a required amount of CaO into 500 mL water, heated to 95 °C. A required amount of HF was dissolved in 300 mL of 0.5 M H_3PO_4. Then the solution was added dropwise at a rate of about 3 mL min^{-1} to a suspension of $Ca(OH)_2$. The titration was stopped at pH 7.0. After the titration, stirring (at a rate of 200 rpm) in the suspension at 20 °C was continued for a further 24 h. The obtained precipitate was filtered in a Buchner funnel, flushed with distilled water, dried at 105 °C and pulverized into powder.

Three precipitates were produced with starting Ca/P molar ratios at 1.67. These compositions correspond to a 0%, 50% and 100% fluoride substitution for OH^- groups and can be further referred to as HAP, FHAP and FAP in this paper.

2.2. Characterization of synthesized apatite samples

The X-ray diffraction (XRD) characterization of the samples was performed using a Philips (PW1710, Almelo, Netherlands) diffractometer. The diffracted X-rays were collected over 2θ range 20–80° using a step width of 0.02° and measuring for 1 s per step. Lattice parameters a, b and c of the apatite hexagonal unit cell and average crystallite size were determined using the Powder Cell 2.4 program.

According to Landi et al. [27], the fraction of crystalline phase (Xc) of the samples can be approximately determined by the following Eq. (1):

$$Xc \approx 1 - \left(\frac{V_{112/300}}{I_{300}}\right); \quad (1)$$

where I_{300} is the intensity of (3 0 0) reflection and $V_{112/300}$ is the intensity of the hollow between (1 1 2) and (3 0 0) reflections, which completely disappears in non-crystalline samples.

The FTIR spectra of synthesized apatite samples, were recorded by a Nicolet 6700 FTIR spectrophotometer (Thermo Scientific) using the ATR technique, in the frequency interval of 4000–400 cm^{-1}.

The morphology of the obtained powders was studied by scanning electron microscopy (SEM) TESCAN Mira3 XMU at 20 kV.

The semi-quantitative chemical analysis of the samples was analyzed using an energy-dispersive spectrometer, EDS, with a SiLi X-ray detector (Oxford Instruments, UK) connected to a scanning electron microscope and a computer multi-channel Analyzer. The measurements were performed to detect Ca, P and F. The quantitative analysis of Ca and P were determined by inductively coupled plasma (ICP) spectrometry, the Spectro-flame model of Spectro-Analytical Instruments.

Table 1
Experimental ranges and levels of the independent variables in the experimental design.

Factors	Range and level		
	−1	0	+1
A: exposure time (min)	30	105	180
B:F concentration (%)[a]	0	50	100
C: initial solution pH	4	6	8

[a] Fluoride concentration in samples: 0 (HAP); 50 (FHAP) and 100 (FAP).

2.3. In vitro antimicrobial activity

The antimicrobial activity of samples was tested against bacterial strains *S. mutans* OMZ-70. Decimal dilution of fresh overnight broth culture (Tripton soy broth with 0.6% yeast extract–TSYB, Torlak, Belgrade) was prepared in saline to obtain the initial number of cells of ca. 10^5 cfu/mL in samples (adjusted using McFarland turbidity standard).

A quantitative test was performed according to Stanić et al. [28], with some modifications. The 0.1 g of an appropriate FAP sample (according to design) was challenged to 1 mL saline solution (with a defined pH), inoculated with an indicator of a microorganism prepared to obtain ca. 10^5 cfu/mL. After the designed time of incubation at 37 °C, 9 mL of saline was added and the suspension was vigorously vortexed 3 min to separate the cells since they tend to adsorb to the particles [28,29]. 1 mL of an aliquots suspensions were taken as sample for viable cell determination. The viable cells were determined after 48 h of incubation of a Petri dish with TSY agar at 37 °C. The percentage of viable cell reduction (*R*%) was calculated using Eq. (2).

$$R(\%) = \left[\frac{C_0 - C}{C_0}\right] \times 100 \quad (2)$$

where C_0, is the initial number of microorganisms (inoculum) and *C* is the number of microorganism colonies of the samples. The average of two replicated values of each run is taken as dependent variables or response.

2.4. Experimental design and statistic

A central composite design (CCD) of a response to surface methodology (RSM) was used for the experimental design (Design Expert Version 8.0.7.1, Stat-Ease, Inc., Minneapolis, United States). The RSM is equipped with statistical tools to determine the significance of a factor over a response. The RSM focused on the construction of geometrical models that can predict the behavior of the factors in the area of evaluation. In addition, the RSM enables the independent evaluation of each factor and their interaction within the proposed model. The central composite design is an experimental design used to allocate the operation variables into a range of evaluation [30].

In order to evaluate the influence of operating parameters on the percent of viable cell reduction (response, Y), three independent variables were chosen: exposure time (A), F concentration (B; fluoride concentration in the samples) and initial pH of saline (adjusted by 0.1 N HCl and 0.1 N NaOH). The value of F concentration for: HAP = 0, FHAP = 50 and FAP = 100. Table 1 shows the highest and lowest limits of the independent variables. A total of