The effect of buffer sedimentation on the process of biometric deposition of calcium phosphates

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Abstract. In this work was considered the influence of the buffer solutions, modeling biogenic extracellular liquid, including Simulated Body Fluid and also physiological solution for comparison, on microstructure and properties of octacalcium phosphate granules. The research was conducted, according to Russian state standard specification № 10993-14-2001. The correlation of pH value and concentration of calcium and phosphorus ions in solutions on a degradation kinetics and recrystallization of the surface layer were analyzed.

1. Introduction

In the modern world more attention is drawn to the development of synthetic biocompatible materials for the replacement of the damaged tissues and organs, which will have the same composition and properties as biogenic, and will also be safe for transplantation and capable of accelerating the recovery of patients. Bone tissue engineering is one of the important area in this sphere.

Many types of materials are being studied to fill in bone defects. Recently considerable attention has been given to the materials based on calcium phosphates, which are characterized by biocompatibility, good connective activity with bone tissue and supporting de novo tissue formation. One of the most promising calcium phosphates is the precursor of biological hydroxyapatite (HA) - octacalcium phosphate (OCP), which is characterized by a resorption rate close to a new bone tissue formation process in the body. There is a need to study the behavior of this material in physiological environments.

The course of many reactions depends on the concentration of H⁺ ions in the solution. A change of concentration leads to the appearance of other products. To obtain successful synthesis results, it is necessary to control and maintain a certain pH during the reaction. In the biological processes of living organisms, many substances show biological activity in a specific, narrow range of pH values [1].

There are solutions that can keep the concentration of H⁺ ions constant being diluted and even when small amounts of strong acid or alkali are added to them. Such solutions (systems) are called buffered, and their capacity to keep the medium constant of pH when the ion concentration changes is called buffering.

The essence of buffer solutions can be visually examined on the example of titration curves. When titrating a weak acid with a strong base, with constant monitoring of the solution's pH, its value will gradually increase, and then, after neutralizing about a half of the initial acid, it will begin to go on a plateau, in the range of which the pH increase will be very slight.

The concentration of hydrogen ions H⁺ in cells and biological fluids is one of the important parameters for ensuring homeostasis/homeokinesis of the body. Although the H⁺ value in the extracellular fluid is relatively small (at pH 7.4 it is 40*10⁻⁹ mol/L), the concentration of the H⁺ ion significantly affects almost all the vital functions: the physicochemical and structural state of the...
membranes, the affinity of hemoglobin for oxygen, the sensitivity of receptors to biologically active substances, the intensity of the generation of reactive oxygen species, the kinetics of enzymatic reactions, the excitability and conductivity of nerve structures. Deviations of $\text{H}^+$ from the optimal range lead to disturbances in the metabolism, vital functions of cells (up to their death), tissues, organs and the whole body. A shift of $\text{pH}$ in the range of $\pm 0.1$ causes respiratory and circulatory disorders; $\pm 0.3$ - loss of consciousness, impaired hemodynamics and ventilation; in the range of $\pm 0.4$ and more - fraught with death of the body [2].

The buffer solution simulating blood plasma is a mixture of phosphate, bicarbonate and proteins. It was invented by Kokubo and is considered a classic buffer solution for studying the biological activity of materials (table 1.).

| Table 1. Ion plasma concentrations (mM). |
|-----------------------------------------|
| **Ions**  | **Blood plasma** |
| Na$^+$     | 142,0            |
| K$^+$      | 5.0              |
| Mg$^{2+}$  | 1,5              |
| Ca$^{2+}$  | 2.5              |
| Cl$^-$     | 103,0            |
| HCO$_3^-$  | 27,0             |
| HPO$_4^{2-}$ | 1,0          |
| SO$_4^{2-}$ | 0,5              |
| Buffer     | -                |

Known methods used to deposit a layer of calcium phosphates on the surface of the implants, such as electrochemical deposition, plasma deposition, sol-gel deposition, electrophoretic deposition, ion deposition, hot isostatic pressing and laser deposition, are associated with physical conditions that are far from physiological and, most importantly, are carried out at sufficiently high temperatures. The implant coverage by a calcium phosphate coating using the high-temperature method eliminates the possibility of introducing biological agents and can lead to thermal decomposition of the material.

However, materials that are placed in a biogenic environment need to improve their biological properties, minimize the risks of possible complications and the ability to create bioengineered structures with therapeutic drugs. Compared with the above-mentioned widely known methods of applying CP layers to various materials, the most promising method for the moment is the method of biomimetic deposition from buffer solutions. The main advantage of the method of biomimetic deposition of calcium phosphates is the usage of physiological temperatures, which may allow further incorporation of biological agents (proteins, growth factors, drugs) into the material, while maintaining their biological activity [4].

In this work, we studied the degradation kinetics of degradation of octacalcium phosphate granules in buffer solutions simulating the intercellular fluid of the body. The interconnected patterns of changes in the microstructure, composition and properties of the studied material depending on the concentration of buffer solutions, their ionic composition and exposure time are established.

Conclusions are made using modern research methods such as scanning electron microscopy (SEM), chemical analysis, x-ray phase analysis (XRD) and infrared spectroscopy (IR).

The main objective of the study was to study the relationship between the composition of buffer solutions to change the initial characteristics of octacalcium phosphate granules during the exposure of the material in a simulated physiological environment, to study the effect of the exposure time on the kinetics of degradation, phase composition and morphological characteristics of calcium phosphate phases, including the microstructure of the material, its properties and study of the possibility of controlling the characteristics of the material using buffer systems. Identification of the regularities of the influence of $\text{pH}$ of solutions on the solubility and reprecipitation of the surface layer of OCP granules.
2. Materials and methods

2.1. Starting materials
OCP granules were synthesized and buffer solutions were prepared (table 2.): SBF and physiological saline NaCl 0.9% [3].

| Ions  | Concentration, mM |
|-------|-------------------|
| Na⁺   | 284.0             |
| K⁺    | 10.0              |
| Mg²⁺  | 3.0               |
| Ca²⁺  | 5.0               |
| Cl⁻   | 295.6             |
| HPO₄²⁻| 2.0               |
| SO₄²⁻ | 1.0               |
| HCO₃⁻ | 8.4               |
| H₂PO₄⁻| -                 |
| pH    | 7.4               |

2.2. Research Methods
X-ray phase analysis (XRD) is one of the most important methods for studying the substance structure, which allows a comprehensive study of x-ray diffraction by bodies of various structures and compositions.

The diffraction patterns were recorded on a 2Θ – Θ SHIMADZU XRD-600 X-ray diffractometer in monochromatized copper CuKα radiation (wavelength = 1.54183 Å) at the temperature of 20 °C and normal humidity of 40-60%. The crystalline phases were identified using the 2004 ICDD powder diffractogram database. Generator operation parameters: accelerating voltage 40 kV, tube current 200 mA. The shooting was carried out in aluminum cuvettes without averaging rotation. No solvents were used to fix the powder samples. Shooting parameters: angle interval 2Θ = 10–60°, step 2Θ = 0.02°, spectra registration speed 2° / min. For spectrum correction, silicon powder (ASTM file cabinet) was used as an internal standard. The analysis of the obtained X-ray diffraction patterns was carried out using the WinXPOW program with the JCPDS database.

The IR spectra of the samples were obtained by a Fourier transform IR spectrometer Avatar Nikolet (USA) for synchronous thermogravimetric and calorimetric analysis in the range of 400 - 4000 cm⁻¹ with a scan step of 4 cm⁻¹. The analysis of the spectra was carried out on the basis of published and existing reference data. Vibrational IR absorption spectra are widely used to identify substances by their molecular composition.

Samples were prepared for the experimental study by pressing in the form of tablets with a diameter of 13 mm with potassium bromide (pressing force of 6 metric tons) at the rate of 1 mg of powder per 50 mg of ultrapure potassium bromide.

Chemical analysis was performed by inductively coupled plasma atomic emission spectroscopy (ICP) in an ULTIMA 2 instrument (France). Weighed portions of the studied materials were dissolved in 10% hydrochloric acid (grade OSCH). The relative error (error) of the determination of elements is given in the table (table 3).
Electron microscopic studies of the samples were carried out using scanning electron microscopes - Tescan VEGA II (Czech Republic), with the usage of a SE sensor, current strength 10–20 kW. To study the morphology of the particles, the samples were glued onto a copper substrate using conductive carbon glue. Since calcium phosphate samples are good dielectrics, when being irradiated with an electron beam, their surface does not only heat up, but it also accumulates an electric charge, which makes it difficult and sometimes excludes the possibility of obtaining high-quality images of the surface morphology during SEM studies, therefore, before studying OCF in SEM on the surface of the samples by ion-plasma spraying (Q150R Quorum Technologies vacuum spraying machine, England) a thin continuous film of a conductive coating was sprayed I - gold [Au] (thickness 10–20 nm), which, on the one hand, has good electrical conductivity and ensures the draining of the induced electrostatic charge, on the other hand, a high secondary emission coefficient significantly increases the brightness and contrast of the SEM image.

The assessment of the degradation kinetics was carried out in fluids simulating the extracellular fluid of the body, according to ISO 10993-14-2001 “Medical devices. Assessment of the biological effects of medical devices. Part 14. Identification and quantification of ceramic degradation products.”

To determine the hydrogen index (pH), we used the potentiometric pH-metric method, which involves measuring the hydrogen index using stationary (laboratory) instruments - a pH meter - EXPERT (accuracy up to ± 0.05 pH units). The potentiometric method is based on measuring the EMF of an electrode system consisting of an indicator electrode and a reference electrode. To measure the pH, the glass electrode was immersed in the test solution (without introducing any foreign substances into it).

The accuracy of the analysis was controlled by testing specially prepared control buffer solutions having pH values close to the given samples on a control scale.

For buffer solutions the pH value plays a decisive role since it is the main stable and characteristic property of such systems. Accurate control of the pH changes was carried out in the preparation of solutions and then its stability over time was evaluated, therefore:

- Buffer solutions were selected with a pH value close to the desired = 7 ± 1;
- Used buffer systems with a concentration of from 1 to 2 M;
- The selected buffer solutions do not form insoluble complexes with cations or anions of the studied samples;
- Selected buffers are non-toxic and safe.

Possible errors in determining the pH of the device include: unplanned changes in solution temperature; calibration errors and improper electrode care; impurities in the reagents used and in the air of the laboratory; insufficient time to establish equilibrium between the electrode and the analyzed solution.

The DTA method consists in measuring the temperature difference between a sample and a standard when heating or cooling a sample and a standard at a constant speed. For inorganic samples, alumina (Al₂O₃) or silicon carbide (SiC) is usually used as standards, since the standard should not undergo any changes. This analysis makes it possible to determine the temperature lifetimes of low-weight material
samples and its phase transformations due to the increased sensitivity of the method. Almost all modern termoanalytical instruments have a differential thermal analysis unit.

For thermal analysis, samples weighing from 50–300 mg to 10–12 g are used. Analyzed substances, if possible, should be close in size and the packing density in the crucible uniform.

3. The experimental part

3.1. Obtaining granules OCP

The initial α-TCP powder was obtained by precipitation from aqueous solutions of salts of calcium nitrate Ca(NO₃)₂·4H₂O (0.1 M solution), ammonium hydrogen phosphate (NH₄)₂HPO₄ (0.6 M solution) in the ratio Ca/P=1/5 and 25% solution of ammonium NH₄OH (to maintain pH ≈ 7). Reaction (3.1) proceeds at room temperature (T = 20 °C) and normal pressure (pH = 1 atm.) In a neutral medium (pH = 7):

\[ 3\text{Ca(NO}_3\text{)}_2\cdot4\text{H}_2\text{O}+2(\text{NH}_4\text{)}_2\text{HPO}_4+2\text{NH}_4\text{OH} = \text{Ca}_3(\text{PO}_4)\text{O}_2\downarrow+6\text{NH}_4\text{NO}_3+6\text{H}_2\text{O} \]  

(3.1)

To obtain TCP granules, the method of immiscible liquids was used. Then special sieves for the selection of granules of the required size were used.

OCP granules were obtained by chemical transformation in a solution of TCP granules ranging in size from 1000 to 2000 μm. This method was used to transform α-TCP particles into OCP granules at physiological temperatures (not exceeding 40 °C), which is very important, since OCP is a thermally unstable compound. The first stage (4.2) was the chemical modification of α-TCP in DCPD, treatment with solutions of salts of phosphoric acid, according to the reaction:

\[ \text{Ca}_3(\text{PO}_4)\text{O}_2 + \text{H}_3\text{PO}_4 \leftrightarrow \text{CaHPO}_4\cdot2\text{H}_2\text{O} \]  

(3.2)

Hydrolysis of DCPD in OCP was carried out in a 1.5 M aqueous solution of sodium acetate (CH₃COONa • 3H₂O). The pH value of the medium was maintained by the addition of ammonium bicarbonate (NH₄HCO₃) to neutralize the acids formed during hydrolysis. Reactions (4.3, 4.4, 4.5) describe the processes occurring in the solution:

\[ \text{CaHPO}_4 \cdot 2\text{H}_2\text{O} \leftrightarrow \text{Ca}^{2+} + \text{HPO}_4^{2-} + 2\text{H}_2\text{O} \]  

(3.3)

\[ \text{HPO}_4^{2-} \leftrightarrow \text{PO}_4^{3-} + \text{H}^+ \]  

(3.4)

\[ 8\text{Ca}^++2\text{HPO}_4^{2-}+4\text{PO}_4^{3-}+5\text{H}_2\text{O} \leftrightarrow \text{Ca}_8(\text{PO}_4)\text{O}_2(\text{PO}_4)\text{O}_4 \cdot 5\text{H}_2\text{O} \]  

(3.5)

Then, the obtained ceramic material was washed in distilled water to a pH value of 7.4 ± 0.2. Dried in an oven for 24 hours. The phase composition of the obtained material was established using the XRD method.

As a result of the chemical transformation, ceramic materials from OKF were obtained, the size and microstructure of which corresponded to the starting materials from α-TCP, that is, granules of OCP were obtained with sizes of 1000 - 2000 μm.

3.2. Preparation of simulated body flu (SBF) buffer solutions and 0.9% NaCl.

The following were used as buffer solutions: simulated body fluid (2 M SBF) and 0.9% physiological saline (NaCl), which did not contain calcium and phosphorus ions, as a control solution.

A physiological solution of NaCl (0.9%) per 1 liter was prepared by adding 9 g of sodium chloride (Scharlau brand) with M = 58.44 with a purity of 99.5% in distilled water.

The preparation of a 2 M buffer solution of simulated body fluid (SBF) in a volume of 1 liter was carried out basing on the original Kokumbo composition, in terms of a bipolar solution. The temperature was maintained in the range of 36.5 ± 1.5 °C.
Table 4. Ionic composition and pH of the initial buffer solutions.

|       | pH [un. pH] | Ca\(^{2+}\) [mg/l] | PO\(_4^3-\) [mg/l] | Cl\(^-\) [mg/l] | CO\(_3^{2-}\) [mg/l] |
|-------|-------------|---------------------|---------------------|-----------------|---------------------|
| NaCl  | 5,9         | 0                   | 0                   | 10,86           | 1,49                |
| SBF   | 7,7         | 80,76               | 27,75               | 13,22           | 21,93               |

The study was carried out in closed systems; for each time point, 3 samples were needed in each solution. OCP granules were placed in solutions and placed in a thermostat while maintaining a constant physiological temperature of 37 °C for the required period.

On days 1, 3, 7, 14, 28, and 44, the containers were removed from the thermostat, a sample of the buffer solution was taken by the dispenser for research, and placed in epindorfs for chemical analysis. OCP granules were washed with distilled water, and then left to dry in a thermostat for 24 hours, after what they were weighed to compare the mass loss with an error of 1% and samples were prepared for analysis.

4. Discussion of the results

The kinetic studies of OCP granules degradation were carried out in buffer solutions simulating extracellular body fluids. Dependence of the samples mass change (in wt.%) on the exposure time in buffer solutions are determined.

![Figure 1](image1.png)

Figure 1. Changes in the concentration of calcium and phosphorus ions during the exposure of OKF granules in buffer solutions

![Figure 2](image2.png)

Figure 2. The microstructure of the surface of ceramic granules a - source material, b - after exposure to NaCl and c - after exposure to SBF for 28 days.
The greatest value of mass loss can be observed in the NaCl standard solution, since there are no calcium and phosphorus ions in it, and its pH is approximately 5–6; therefore, the surface layer degrades rapidly and, as the solution is saturated with ions, it reaches a plateau, which can also be seen by chemical analysis of the solution (Fig. 1).

Microstructure photographs of the granules after 44 days of exposure in physiological buffer is presented in Fig. 2. The sharp edges of the petals of the OCP were dissolved, the structure looks more ordered and resembles a honeycomb in shape.

5. Conclusions
The direction development of the biomaterials creating is currently going forward so-called regenerative approach, in which the emphasis is not on the replacing of a defect with an implant with suitable mechanical characteristics, but on a rapid biodegradation of the material and replacing it with bone tissue formed by the body itself. Thus, in the first place for such materials go their biological and physico-chemical properties, which substantially depend on the method of their preparation.

There is a strong reason to believe that the chemical and morphological correspondence of biomaterial and bone mineral is one of the basic principles that underlie the construction of new materials for biomedical use. Deviations in the composition and size of the crystals of the artificial material from the natural analogue can be used for the directed synthesis of biomaterials with a given biological activity.

The study was carried out to observe the changes in the microstructure and properties of octacalcium phosphate granules when exposed in buffer solutions of various ionic composition simulating the extracellular fluid of the body.

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