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Calcium phosphate-based ceramic and composite materials for medicine

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Abstract. The topical problems in chemistry and technology of materials based on calcium phosphates aimed at both the replacement of damaged bone tissue and its regeneration are discussed. Specific features of the synthesis of nanocrystalline powders and the fabrication of ceramic implants are described. Advances in the development of porous scaffolds from resorbable and osteoconductive calcium phosphates and of hybrid composites that form the basis of bone tissue engineering are considered. The bibliography includes 131 references.

I. Introduction

According to the results of archaeological studies, even in antiquity the dental orthopaedy made use of implants, *i.e.*, means for replacing bone tissue defects that were fabricated from natural materials such as corals, ivory as well as human and animal bones.[1,2] The problem of substitutes for hard tissues goes beyond stomatology, because today, like earlier, a considerable part of population suffers from diseases of skeletal bone tissues caused by pathological degradation (osteoporosis), inflammatory processes and cancer. In the developed countries, the number of patients with such chronic diseases reaches $\sim 50\%$ of all population above 50. According to predictions, due to the overall increase in the life expectancy and the gain in population, the number of aged people will double to 2020.[3] Curing bone tissue diseases, especially malignant growths (osteosarcoma), was often associated with surgical operations that as a rule led to extended post-operative defects. The problem lies in recovery of lost functions of individual organs, parts of skeleton and the whole locomotor apparatus.[4]

Two fundamentally different approaches to this problem are feasible, namely, the mechanical replacement of a bone defect by an implant to develop an integral bioengineering structure and the regeneration of damaged bone tissues. Correspondingly, two kinds of materials that differ in their microstructures, properties and *in vivo* behaviour in the human body were elaborated.[5–8]

In this review, an attempt is undertaken to generalize and analyze the results of studies and developments in the field of chemistry and engineering of these materials. Insofar as the main qualitative criteria of materials used for implants include, besides their biocompatibility, the closeness of their mechanical properties to those of the bones under conditions of long-term reliability, the first part of this review provides (very briefly) information on the structure and properties of bone tissues. Next come the data on the structure and properties of certain calcium phosphates (CP), *i.e.*, the most promising candidates for the development of both implants and materials used in bone tissue regeneration, because their composition is close to that of the mineral component of bones. Further, this review discusses features of the chemical engineering aimed at the synthesis of CP materials with a high level of mechanical properties, which is, *inter alia*, due to their nanostructure nature. Then, the principles of a new biomedicinal technology are considered, namely, the *in situ* regeneration of bone tissues, the so-called bone tissue engineering, which requires materials with properties different from those of permanent implants. Actually, this technology is based on processes similar to those occurring in bone tissues of the human organism, namely, the processes of remodelling [resorption, *i.e.*, biodegradation and dissolution due to the interaction with cells (osteoclasts), and mineralization, *i.e.*, CP deposition on a collagen scaffold]. The requirements that should be met by various scaffolds for cellular cultures, namely, granules, porous ceramics and composite materials, are considered. The latter materials are the most promising, because their properties approach closer those of the bone tissue and also because they allow flexible manipulations during a surgical operation. A special place in the problem of development of CP materials for medicine is occupied by bone cements, *i.e.*, binding materials designed not only for plastic masking of bone defects, but for the preparation of porous scaffolds, too.

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II. Materials for bone tissue replacement

1. Bone tissue

The human bone tissue is a complex composite material with several levels of microstructure organization, which possesses unique mechanical properties.[9,10] The main components of the bone tissue are type I collagen (~20%), a mineral phase (~60%), water (~9%) and non-collagenous proteins (~3%), the remainder being polysaccharides and lipids. The mineral component, the so-called biological apatite $(Ca,Na,Mg)_{10}(PO_4,HPO_4,CO_3)_6(OH,F,Cl)_2$, represents a calcium-deficient hydroxyapatite (HA) with cationic (magnesium, sodium) and anionic (carbonate groups, chlorine and fluorine) substitutions.[8] The approximate content of ions in the human bones that are physiologically important for vital functions of the organism is as follows:

Ion	Mass %	Ion	Mass %
Ca^{2+}	36.51	K^+	0.03
PO_4^{3-}	15.2	CO_3^{2-}	7.4
Na^+	0.9	F^-	0.03
Mg^{2+}	0.72	Cl^-	0.13

The bone composition depends on many factors including the age of an individual. There is no agreement on the role of ionic substitution in the physiology of bones. It is known that magnesium destabilizes the HA structure. Carbonate groups play an important role in biochemical reactions between bone tissue and blood plasma, induce lattice deformations thus increasing the HA resorbability. The fluoride ions replace hydroxyl groups thus reducing the HA solubility at pH close to neutral due to their higher binding energy to calcium in HA.[11] Sodium is probably required to provide the optimal structure of the carbonate-containing HA.[12,13]

The organic component of the bone tissue is represented mainly by collagen fibres and proteins such as osteocalcin, osteonectin, fibronectin, *etc*.[14] Bioapatite is formed through the nucleation of crystals on collagen macromolecules and their subsequent growth due to the ionic transport and deposition from the body fluids.[15]

An important feature of bones is the presence of a system of interconnected pores with the sizes of 100–300 nm and 10–50 μm in the cortical bone tissue and 200–600 μm in the trabecular (spongy) bone tissue. The pore size and interconnection have an effect on biological flows, neovascularization processes and vital functions (proliferation) of cells.

The crystals of the mineral component look like plates ~40–60 nm long, ~20–30 nm wide and ~1.5–5 nm thick.[16] The crystals are oriented in such a way that their longitudinal axis is parallel to the axis of the collagen fibrils. The calcium-to-phosphorus ratio in the mineral phase of the bone tissue is lower than the stoichiometric value (1.67) for HA, *i.e*., biological apatite is always deficient in calcium.

The dense and compact cortical bone tissue constitutes the external part of all skeletal structures. The trabecular bone tissue is located inside long bones, especially in their end parts, in vertebra bodies, inside pelvis bones and other large flat bones. This tissue represents a network of thin bone elements, trabecules. Bones are very non-uniform as regards their microstructure and mechanical properties. The latter depend on the porosity (the pore content varies from 5% to 95%), the degree of mineralization and the orientation of collagen fibres. Table 1 shows the main parameters of mechanical properties of cortical and trabecular bone tissues.[8,17,18] These values can serve as the reference points in the development of materials for implants.

Table 1. Mechanical properties of cortical and trabecular bone tissues.

Property	Cortical bone tissue	Trabecular bone tissue
Compressive strength /MPa	100–230	2–12
Bending and tensile strength /MPa	50–150	10–20
Prefailure deformation (%)	1–3	5–7
Crack resistance /MPa $m^{1/2}$	2–12	–
Young's modulus /GPa	7–30	0.05–0.5

2. Requirements to materials for implantation

The medium of a human organism is highly corrosive. The main requirements imposed upon materials for implantation are their resistance to corrosive media and biomechanical compatibility. An implant should have the modulus of elasticity, the strength (static and fatigue) and the crack resistance close to those of bones. The modulus of elasticity of a material should be such that the bone tissues need not be protected from physiological loads that are required for its vital activity (in the absence of tensile loads, a bone degrades). The high strength and crack resistance are necessary for the reliable work of an implant in the body.

Initially, allo- and autografts, *i.e*., fragments of bones from either a donor or a patient himself, were used for displacing bone defects. However, this was associated with certain difficulties (possible immune reactions, the necessity of secondary operations). This is why for many years the efforts of scientists were aimed at the development of synthetic materials for these purposes. The evolution of developments in this field was marked by three generations of materials, namely, from bioinert (the first generation) to bioactive osteoconductive (the second generation) and to osteoinductive materials (the third generation) that stimulate the osteosynthesis.[4] The bioactivity of a material is its ability of interacting with the organism microenvironment, particularly, the formation of a bond between the material and the neighbouring bone tissue. Osteoconductivity of a material is its ability to sustain living functions of osteogenerating cells and the formation of a new tissue.[2,19] Osteoinductivity is the ability of a material to cause the ectopic (outside the bone) formation of bone tissue *de novo*. This property can be immanent, *i.e*., either manifest itself without the *ad hog* introduction of osteogenetic factors (bone morphogenetic proteins) or be induced by such factors.[2]

Bioinert materials (the first generation of biomaterials) do not affect the process of osteosynthesis; on the surface of implants manufactured therefrom, the formation of fibrous tissue occurs, which prevent the formation of a bond between the implant and the bone. There is risk of rejection of such materials by the organism, often by inflammatory reactions. The main problems to be solved in the design of implants based on bioinert materials include bringing the elastic characteristics of the implant closer to those of bones and suppressing the corrosion processes. The first-generation materials were widely used in orthopaedic facilities, for example, in endoprostheses of hip and knee joints.

Among all artificial materials that can, in principle, be used for implantation, the calcium phosphate-based

ceramics is the most promising material being similar in its chemical and phase composition to the mineral component of the bone tissue. In contrast to polymers and metals, the HA-based CP ceramics is biocompatible and bioactive, its brittleness being its drawback. The materials of choice for developing orthopaedic facilities for the locomotor apparatus that are subjected to mechanical loads are metals and alloys with CP coatings that ensure biocompatibility and biointegration of an implant with the bone tissue. An important problem is the long-term resorption resistance of the material; the stability depends not only on the adhesion strength, but also on the phase composition and the degree of crystallinity of the coating structure.[20–22] Resorption may result in the debris formation, the blood capillary occlusion and necroses. The introduction of a fluorine atom into the HA composition enhances the resistance against bioresorption, because the fluorohydroxyapatite has a substatially lower solubility product in aqueous salt solutions as compared with HA.[23]

HA-coatings were applied on metal implants using magnetron deposition, combined electron-beam evaporation of CaO and thermal evaporation of P_2O_5, ion-induced deposition, pulsed laser ablation, chemical vapour deposition, elecrophoretic deposition, sol–gel technology, biomimetic methods.[7,20] The main method of HA-coating formation that has found commercial application is the plasma spraying, despite all shortcomings of the resulting coatings such as their weak adhesion to the substrate and non-uniform morphology, crystallinity and phase composition.

3. Calcium orthophosphates

Table 2 provides information on the composition, density and solubility of calcium orthophosphates. Comprehensive survey of the data on the structure, synthesis and properties of calcium orthophosphates can be found in several publications.[24–28] It was suggested to divide all calcium orthophosphates into two groups. The first group includes compounds that can be synthesized under physiological conditions; the second group includes high-temperature compounds the synthesis of which involves heat treatment of intermediates.[29] The latter include tricalcium phosphate (TCP) in its α-modification and tetracalcium phosphate, which are the starting reagents in the synthesis of the majority of bone cements. This classification is convenient for the development of a technology of CP materials, being however a matter of convention. For example, HA that is assigned to high-temperature CP can be obtained in the crystalline form by hydrolysis of octacalcium phosphate (OCP). Calcium-deficient carbonate-containing HA is the main mineral of bone tissues, dentine and enamel in the human organism. Soft tissue calcificates were shown to contain amorphous calcium phosphate, $Ca_3(PO_4)_2 \cdot n\,H_2O$, and tricalcium phosphate in its β-modification; bladder stones were shown to contain OCP; pathological dental deposits contain OCP, magnesium-containing tricalcium phosphate and dicalcium phosphate dihydrate.[2] In the bone tissue, HA is present as nanosized crystals.

Table 2. Density and solubility at 25 °C of calcium orthophosphates.

Compound	Density /g cm^{-3}	$-\log K$ (see [a])
$Ca(H_2PO_4)_2 \cdot H_2O$	2.23	1.14
$CaHPO_4 \cdot 2\,H_2O$	2.32	6.59
$CaHPO_4$	2.89	6.90
$Ca_8H_2(PO_4)_6 \cdot 5\,H_2O$	2.61	96.6
α-$Ca_3(PO_4)_2$	2.86	25.5
β-$Ca_3(PO_4)_2$	3.07	28.9
$Ca_{10}(PO_4)_6(OH)_2$	3.16	116.8
$Ca_4(PO_4)_2O$	3.05	38–44

[a] K is the solubility product; concentrations are given in mol litre^{-1}.

4. Nanocrystalline hydroxyapatite and ceramics on its basis

Calcium phosphate nanocrystals possess two characteristics that are the most important for the bone tissue physiology, namely, they are in dynamic equilibrium with their biological environment within the remodelling cycle (resorption/mineralization) and exhibit high-level mechanical properties. Nanocrystalline HA (nano-HA) has the enhanced ability to adsorb proteins required for cell vital activity and the selectivity with respect to the functions of cells constituting bone and fibrous tissues.[30] The development of materials based on nanocrystalline calcium phosphates was carried out along the following two directions, *viz*., nanocrystalline ceramics and composite materials with biopolymeric scaffolds reinforced with phosphate nanoparticles.

The chronology of the development of methods for synthesizing nano-HA has been reviewed.[31] Several methods including the sol–gel technology, solid-phase reactions, the hydrothermal synthesis were developed.[32–35] The sol–gel method allows fairly easy preparation of HA with the stoichiometric composition, high purity and homogeneity.[31] However, the simplest method to obtain nano-HA is its precipitation from aqueous solutions. The effect of the reaction temperature and the precipitate ripening time on the size of HA particulates and their degree of crystallinity was studied.[36,37] The synthesis was carried out by reacting calcium chloride with ammonium hydrogenphosphate.[36] Both the crystallinity and the crystal size increased with the increase in the synthesis temperature. At $\sim$60 °C, a transition from mono- to polycrystalline HA particulates was observed. As the temperature of the precipitation of calcium nitrite and ammonium hydrogenphosphate from their solution with ammonia was reduced from 80 to 0 °C, the particle size of the synthesized product decreased from 37 to 12 nm (estimated by the Scherrer method). The size and shape of precipitated particles also depended on the cationic and anionic substitutions. Particularly, the anionic substitutions $CO_3 \rightarrow (PO_4, OH)$ and $SiO_4 \rightarrow PO_4$ in HA result in lattice deformations (due to the difference in the anion size) and decreased the size of HA crystals.[38]

The process of bone tissue formation in a human body involves collagen mineralization as a result of deposition of apatite crystals from the extracellular fluid. Collagen-containing carbonate-substituted HA is the product of the HA synthesis in a collagen solution.[39] Gelatine, the partial hydrolysis product of collagen, represents a mixture of tropocollagen chains and their fragments. It lacks the properties of animal collagen that are dangerous for medicinal application. Specific features of the preparation of nanocrystalline HA powders in gelatine solutions were studied.[40–44] Figure 1 shows the specific surface of powders as a function of the gelatine concentration; the synthesis was carried out through the precipitation from calcium nitrate and ammonium hydrogenphosphate solutions.[43] The maximum specific surface was $\sim$220 m^2 g^{-1} (which corresponded to the crystal size $<$15 nm). This was con-

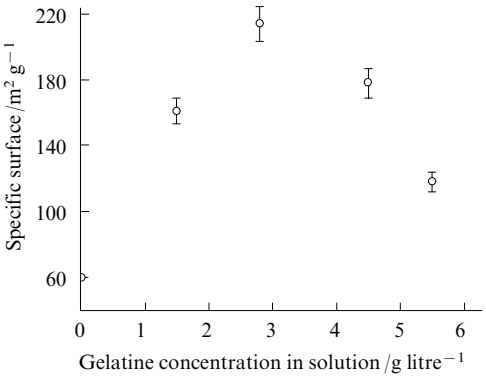


Figure 1. Dependence of the specific surface of HA powders on the gelatine concentration in solution.

firmed by a direct study using transmission electron microscopy (TEM) (Fig. 2). In the HA precipitation from a gelatine solution, the concentration of the latter was the decisive factor for the nucleation of HA crystals. The synthesis in an aqueous solution containing no gelatine occurred as homogeneous crystallization in the reaction between Ca^{2+} and PO_4^{3-} ions. In a gelatine solution, calcium ions react with the carboxyl groups of gelatine thus initiating the formation of crystals of a new phase. Phosphate ions attach to the growing calcium complexes to form the critical-size nuclei of a new phase to be later grown into HA nanocrystals. Apparently, the crystallization site density dependent on the gelatine concentration in solution is the decisive factor for the size of new phase particles. At high density of crystallization sites, the growing particles interact with one another, merge together and coarsen. According to certain data,[44] the IR spectra of the product of HA synthesis in gelatine solutions, in addition to bands typical of HA, contained stretching bands of the N—H groups at 3441–3425 cm^{-1}, C—H groups at 2925, 2855 and 1453 cm^{-1} and C═O groups at 1645 cm^{-1}, which are typical of gelatine. The presence of nitrogen and carbon atoms in the product was confirmed by the chemical analysis data. After calcination at 1000 °C and higher, the bands corresponding to gelatine disappeared from the spectra.

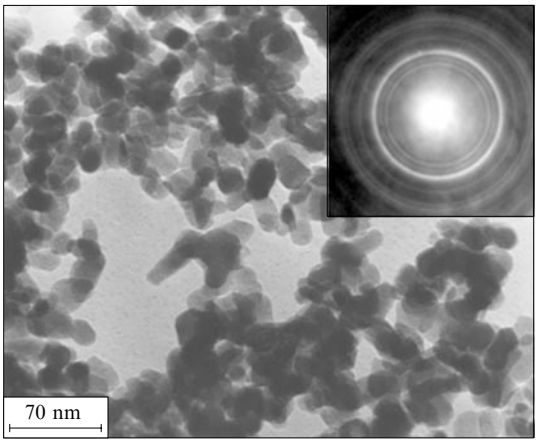


Figure 2. TEM Images of HA powder synthesized by precipitation in a gelatine solution.
Inset is an electron diffraction pattern.

This approach, *i.e.*, the *in situ* crystallization, can be used in the development of composite materials biopolymer–calcium phosphate nanoparticles. A drawback of gelatine that limits the prospects of using it in such composites consists of too high rate of its biological resorption in the organism, which is incompatible with the kinetics of osteosynthesis. This is why the possibilities of synthesizing CP in solutions of other polymers, particularly, non-protein polymers such as chitosan, were considered.[45]

Chitosan $(C_6H_{11}NO_4)_n$ is an aminopolysaccharide with the structure similar to that of cellulose. It is non-toxic and is biocompatible with the body tissues. The HA synthesis was studied in acetic acid solutions of chitosan, where $CaCl_2$ and $(NH_4)_2HPO_4$ were used as the starting components, ammonia was chosen as the precipitator and the pH of the medium varied from 8 to 10.6.[45] According to the data from chemical analysis, the products contained the nitrogen and oxygen atoms that are chitosan components. The IR spectra of products contained stretching bands of the N—H groups at 3426 cm^{-1} and of C—H groups at 2924, 2854 and 1446 cm^{-1}. In the course of air drying, the products transformed into solid uniform bioinorganic materials containing HA in the nanocrystalline state (according to XRD data, the size of the Cauchy blocks was 12.5–18.7 nm).

Retention at the nanolevel of the grain size of ceramics sintered from nanopowders is a problem that is difficult to solve, because nanograin powders are prone to considerable aggregative recrystallization during sintering. As a consequence, a ceramics sintered from nanopowders to the dense state is weaker than a ceramics prepared based on original powders of the micrometer size. Hence, it is important to reduce the sintering temperature. For this purpose, the sintering is usually carried out in the presence of a low-melting liquid phase formed in the melting of a relatively low-melting additive that has been introduced into the batch. This can be accompanied by secondary crystallization from the formed melt. Figure 3 shows examples of such microstructures. It is seen that the formation of secondary rod-like crystals from a melt formed in the presence of low-melting binary potassium–calcium carbonates gave rise to the 'self-reinforcing' effect. The bending strength of such materials sintered at 750 °C was 125 MPa, whereas for materials prepared from HA micropowders sintered at 1250 °C to the compact state, this parameter did not exceed 90 MPa. Rod-like crystals efficiently prevented the propagation of cracks, *i.e.*, led to the higher strength.

The grain size in a ceramics sintered from HA nanopowders could be retained by using high pressures in the pressing of raw samples.[46] Figure 4 shows TEM images for the original samples pressed from HA nanopowders prepared under high pressure (3 GPa) and for ceramic microstructures sintered at 670 °C. High pressure allowed reduction of the sintering temperature by ~550 °C as compared with samples pressed at 100 MPa, thus retaining the ceramic grain size at the level of 35–50 nm. The microhardness of this material reached 5.8 GPa, which is substantially (1.6-fold) higher than the microhardness of ceramics sintered from micropowders. The use of high pressures (~1 GPa in the centre of deformation) on rolling the HA nanopowders on titanium plates afforded compact ceramic films with the thickness of ~0.1 mm. After the thermal treatment at 690 °C, the size of HA crystals in the

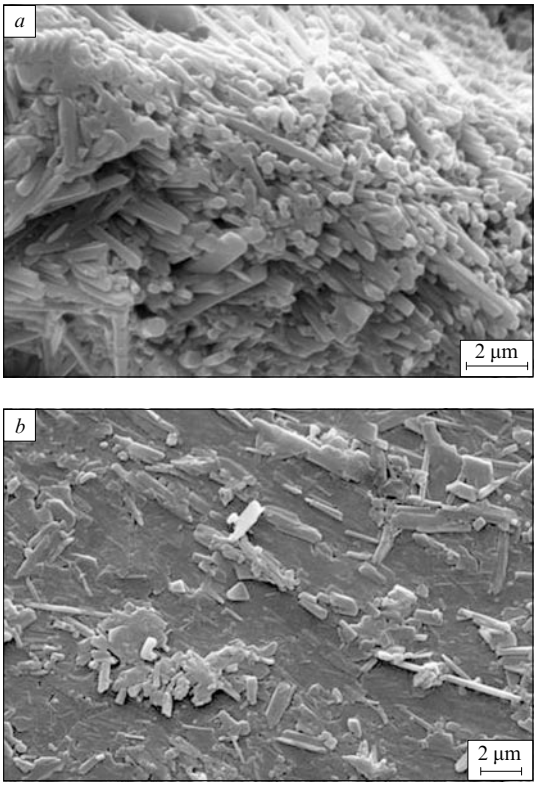


Figure 3. Microstructure of a ceramics sintered from HA nanopowders with carbonates added to the furnace mixture.
(*a*) is fracture surface; (*b*) is thin section.

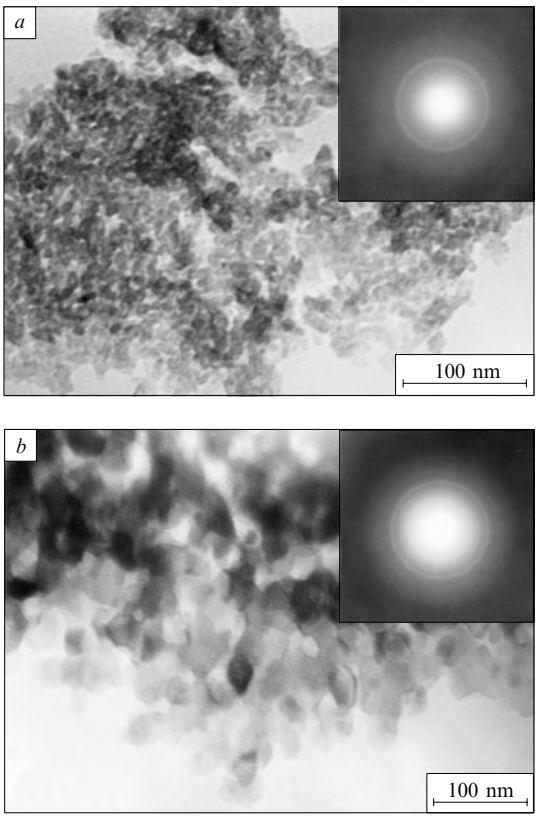


Figure 4. TEM Images of the original pressed sample (*a*) and sintered ceramics (*b*) of HA nanopowders.
Insets show electron diffraction patterns.

film sintered to the compact state was as small as 15–35 nm. Thus, the high-pressure activation allowed substantial reduction of the sintering temperature of HA nanoparticles. However, this technology was realized only for relatively thin objects. The necessity of further studies aimed at the preparation of bulk materials is evident.

Despite certain advantages achieved in the field of enhancing the mechanical properties of HA ceramics due to its nanostructuring, ceramic implants can be used only for displacing those bone regions that do not carry any substantial mechanical load. This is caused by the ceramics brittleness and its high sensitivity to stress corrosion in body fluids, which leads to the gradual destruction of the implant.[7, 28] The alternative lies in the concept of bone tissue regeneration.

III. Materials for bone tissue regeneration

As regards the combination of mechanical (elastic modulus, strength, fracture toughness) and biological (resorption kinetics, matrix ability for cell abstraction and protein adsorbtion) properties, the CP ceramics cannot compete with biological bone tissues and adequately replace the latter in the recovery of physiological functions.

The progress in the development and manufacturing of biomaterials of the second and third generations provided the necessary conditions for modifying the medicinal technologies of bone therapy, namely, the transition from the principle of displacement-reconstruction to the bone tissue engineering, *i.e.*, a cellular technology of self-regeneration of bones on osteoconductive scaffolds. This is a new trend developed and elaborated in the past decade; the bone tissue engineering is based on the results of studying biomineralization processes in the human body.

1. Biomineralization and bone tissue engineering

The human bone system is characterized by certain kinetics of remodelling, which consists of resorption of the mineral component and biomineralization (deposition).[14] The biomineralization process includes the heterogeneous nucleation of the mineral phase in pores of collagen fibrils catalyzed by the carboxyl groups, which is followed by crystal growth along the collagen fibrils.[47] The nature of the primary mineral phase was not identified unambiguously. It is believed that biological apatite is crystallized from calcium phosphate clusters, $Ca_9(PO_4)_6$, which form a random packing with interfacial water in the structure of the precursor, the so-called amorphous calcium phosphate. This is indirectly confirmed by the presence, in the mineral phase, of magnesium ions that stabilize the amorphous phase. According to another theory, it is dicalcium phosphate dihydrate and OCP phases that are the biological apatite precursors since their structures are similar to that of apatite.

The bone tissue engineering is based on the results of studies on the biomineralization processes; the main goal of these studies is the attainment of the maximum correspondence between the properties of a tissue formed *de novo* and the original tissue to be regenerated. It was assumed that the organism itself can heal the damage if a scaffold of a definite architecture (a carrier) fabricated of a material biologically compatible with the organism and containing cultured bone-forming cells (osteoblasts) were placed into the defective site and if there were stimuli for the vital activity of these cells, neovasculation (vascular invasion) and the biological fluxes to the scaffold. The following

major requirements to the scaffold materials were formulated:

— the material should correspond to the bone tissue in the mineral composition, mechanical characteristics, porosity and in the pore size and distribution;[28, 48, 49]

— the material should exhibit the high scaffold properties with respect to osteoclasts that maintain the resorption and osteoblasts that form collagen, thus sustaining the vital activity of cells;[14]

— the scaffold should exhibit the bioresorption kinetics compatible with that of the *de novo* bone tissue formation;[4, 50–53]

— the material should be osteoconductive and, in the ideal case, osteoinductive, *i.e.*, generate biological stimuli for osteogenesis, and also ensure compatibility with biologically active components that strengthen the system's regenerative potential such as morphogenetic proteins and steroids. Particularly, morphogenetic proteins stimulate the differentiation of osteoblasts and suppress that of muscular tissues, induce endochondrial formation of bone tissue.[2, 14] A scaffold should form a spatial structure for the bone tissue growth. Even a 3D packing formed of CP microspheres unconnected with one another can serve as a good scaffold.[14]

A version of the method of bone tissue engineering used mesenchymal stem cells capable of differentiation into osteoblasts. In this case, considerable success could be reached by using osteoinductive materials as the scaffolds.[2] Data are known according to which the implantation of certain CP into animals' soft tissues resulted in osteosynthesis, *i.e.*, these materials can exhibit immanent osteoinduction.[2] This opens up new prospects for the development of methods of regeneration therapy.

Among calcium phosphates that have controllable resorption kinetics and can be used as scaffolds, attention is drawn to the biphasic systems such as HA–tricalcium phosphate, carbonate-containing HA and OCP.

2. Biphasic systems hydroxyapatite – tricalcium phosphate

The concept of biphasic materials in the HA–TCP system (biphasic calcium phosphate, BCP) was developed based on the assumption that the resorption kinetics can be regulated by changing the ratio of the more and the less soluble phases (hydroxyapatite and tricalcium phosphate, respectively) in a single material.[54–56] The dissolution of the TCP component in body fluids favours the mineralization process, and the biological behaviour of biphasic materials depends on the HA : TCP ratio.[54]

Biphasic materials in the HA–TCP system can be prepared by both mechanical mixing of components and the thermal treatment of calcium-deficient apatite (Ca : P = 1.5–1.67) at a temperature >700 °C.[56–58] The latter method should be preferred because it provides higher uniformity of component distribution. Calcium-deficient apatite can be obtained by precipitation from solution or hydrolysis of dicalcium phosphate.

The effect of composition of calcium-deficient apatite and the thermal treatment temperature on the ratio of phases in composite materials synthesized by its thermal decomposition was studied.[59] Solutions of $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ were used as the starting components. The following reaction occurred:

$$Ca(NO_3)_2 + (NH_4)_2HPO_4 + NH_4OH \longrightarrow \quad (1)$$
$$\longrightarrow Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x} + NH_4NO_3 + H_2O.$$

The synthesis was performed at pH in the range from 7 to 8.5, which was important for the preparation of calcium-deficient apatite. The precipitate was dried in a microwave oven. X-Ray diffraction analysis has shown that the phase composition depended on both the Ca : P ratio in the product and the temperature of thermal treatment. For example, after the thermal treatment at 900 °C, a sample with the initial ratio Ca : P = 1.57 contained 62% β-TCP and 38% HA. As the temperature increased to 1000 °C, the HA content increased to 47% and the β-TCP content dropped to 53%. At 1100 °C, the main phase was HA (61%). After thermal treatment at a temperature >1200 °C, α-TCP appeared.

Thus, the method of precipitation of calcium-deficient apatite from solutions with the variable ratio of components (Ca : P varied from 1.54 to 1.64), followed by thermal treatment of the product at a temperature from 900 to 1300 °C enables manufacture of biphasic phosphates with the HA : β-TCP ratio in a wide range, namely, from 12 : 88 to 89 : 11, and triphasic phosphates with the HA : β-TCP : α-TCP ratio varying from 55 : 24 : 21 to 93 : 3 : 7.

Based on powders of biphasic materials, granules were prepared by the suspension methods using immiscible liquids.[60, 61] The powders were mixed with 10% gelatine solution; the resulting suspension was introduced into a dispersion medium (oil) and stirred. This induced the formation of beads of the water-in-oil suspension. These beads were hardened after cross-linking of the gelatine structure. After settling, the granules were subjected to thermal treatment at 1000 °C for sintering. The average granule size and the parameters of their size distribution in the range from 50 to 2000 μm depended on the suspension concentration, the dispersion liquid temperature and the stirring rate.

The solubility of granules of the fraction of 300–500 μm was measured in an isotonic 0.1 M NaCl solution for 28 days at a constant liquid phase volume (closed system); this method was used for quite justified reasons in studying biomaterials.[62] The sought value was the calcium concentration in the liquid phase. Figure 5 shows the results of studying the kinetics of dissolution of the HA and β-TCP granules and also of biphasic (HA–β-TCP) composite granules.

The regression analysis of kinetic curves has shown that the dissolution of granules is a multistaged process similar to the dissolution of Bioglass® granules.[63] In the initial stage (up to 1 day), the time dependence of the calcium concentration in solution [$c(t)$] could be approximated by a logarithmic or power function. Gradually, the dissolution decelerated and its kinetics obeyed the exponential dependence

$$c(t) = c_0 + c_m[1 - \exp(-bt)], \quad (2)$$

where c_0 is some starting concentration, c_m is the saturation concentration, b is the coefficient.

The exponential dependence corresponded to the first-order reaction kinetics, where the rate of variation of the number of 'active sites of dissolution' [$C(t)$] in the dissolved material was proportional to their number at a given moment

$$\frac{dC(t)}{dt} = -kC(t),$$

where coefficient k is independent of time.

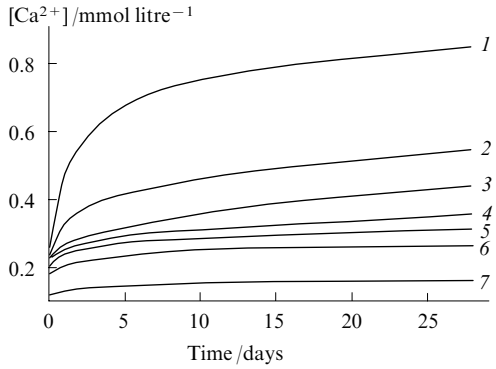


Figure 5. Changes in the Ca^{2+} ion concentration in an isotonic solution upon dissolution of granules.
(*1*) α-TCP, (*2*) β-TCP, (*3*) 80% β-TCP + 20% HA, (*4*) 60% β-TCP + 40% HA, (*5*) 40% β-TCP + 60% HA, (*6*) 20% β-TCP + 80% HA, (*7*) HA.

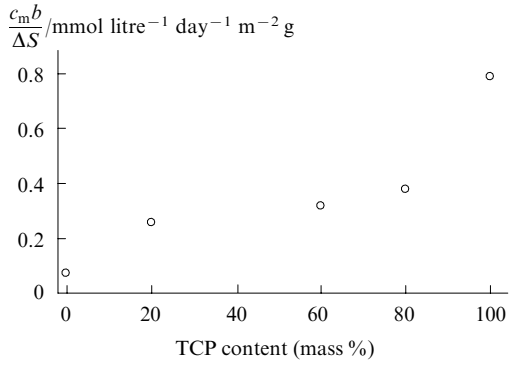


Figure 6. Relationship between the initial dissolution rate in the 'exponential' stage of dissolution normalized to the change in the specific surface and the β-TCP content in granules.

It is of interest to quantitatively assess the rate of calcium concentration variation in solution as a function of the HA : β-TCP ratio in granules. In the 'exponential' stage of dissolution, the rate of concentration variation decreased

$$\frac{dc(t)}{dt} = c_m b \exp(-bt);$$

hence, the initial rate of dissolution can be considered as the quantitative measure

$$\frac{dc(t)}{dt}(t=0) = c_m b.$$

Table 3 shows the results of quantitative assessment of kinetic parameters of this process. The initial rate of dissolution increased with the increase in the β-TCP content in granules. Granules of α-TCP exhibited the maximum solubility. It is commonly agreed to normalize the parameters of the kinetic equation of dissolution to the specific surface, which allows one to compare the solubilities of different materials. The initial rate of calcium deposition normalized to the change in the specific surface increased with the increase in the β-TCP content in granules (Fig. 6). Hence, the dissolution rate depends not only on the specific surface but also on the phase composition of the material. A substantial increase in the specific surface of granules after their exposure to an isotonic solution pointed to the selective dissolution of individual structural elements. According to the results of XRD analysis, the content of β-TCP in samples after the tests decreased. An increase in the specific surface of HA granules containing no β-TCP was possible as a result of dissolution of a part of HA.

Table 3.Parameters of the 'exponential' stage of dissolution, specific surface before (S_0) and after (S_k) solubility tests of granules as a function of their phase composition.

Content β-TCP (mass %)	c_0 (see [a])	c_m (see [a])	$c_m b$	b /day^{-1}	S_0 /m^2 g^{-1}	S_k /m^2 g^{-1}
0	0.123	0.019	0.119	6.28	0.639	2.248
16	0.184	0.039	0.208	5.33	1.391	2.181
36	0.225	0.030	0.165	5.50	—	—
53	0.236	0.043	0.230	5.34	0.402	1.113
77	0.250	0.074	0.392	5.30	0.325	1.367
100	0.28	0.104	0.517	4.97	0.027	0.680
α-TCP	0.407	0.199	0.953	4.79	—	0.898

[a] In mmol litre^{-1}.

The presence of magnesium is important for the bone tissue physiology. Synthesis of magnesium-containing biphasic calcium phosphates in the HA – TCP system was carried out by the precipitation from solutions of $Ca(NO_3)$, $Mg(NO_3)_2$ and $(NH_4)_2HPO_4$.[63]

The following reaction occurred:

$$Ca(NO_3)_2 + Mg(NO_3)_2 + (NH_4)_2HPO_4 + NH_4OH \longrightarrow \quad (3)$$
$$\longrightarrow Ca_{10-x}(MgHPO_4)_x(PO_4)_{6-x}(OH)_{2-x}\downarrow + NH_4NO_3 + H_2O.$$

The decomposition of calcium-deficient magnesium-containing apatite upon its thermal treatment proceeded by the following hypothetic reaction:

$$Ca_{10-x}(MgHPO_4)_x(PO_4)_{6-x}(OH)_{2-x} \longrightarrow \quad (4)$$
$$\longrightarrow (1-x)Ca_{10}(PO_4)_6(OH)_2 + 3x(CaMg_{1/9})_3(PO_4)_2 + x\,H_2O$$

to form HA and magnesium-containing β-TCP.

The added magnesium amounted from 1% to 6% of the ratio of magnesium to the total content of calcium and magnesium. An ammonium phosphate solution was added to a solution of calcium and magnesium nitrates, the resulting precipitate was ripened, filtered, dried and subjected to thermal treatment at 900 and 1100 °C. The powder particle size was 300 – 500 nm.

According to XRD data, all samples contained the following two phases: β-TCP and HA. The content of β-TCP increased with the increase in the magnesium content in calcium-deficient apatite. The substitution of magnesium for 1% of calcium ions resulted in the formation of 16% β-TCP, while for the 3% substitution, the HA : β-TCP phase ratio was 56 : 44. The introduction of > 6% magnesium led to the formation of virtually single-phase magnesium-containing β-TCP. The phase composition of synthesized powders changed insignificantly as the temperature increased from 900 to 1100 °C.

The position of diffraction peaks corresponding to β-TCP shifted to the long-wave range with the addition of magnesium. The unit cell parameters were determined using

the positions of reflection maxima (300), (021), (220) for β-TCP and (410), (213), (112), (211) for HA. The addition of magnesium to BCP had no effect on the HA unit cell parameters ($a = 9.432 \pm 0.002$ Å and $c = 6.883 \pm 0.002$ Å). However, with the increase in the content of magnesium cations in a BCP, the unit cell parameters of β-TCP decreased (Fig. 7), which was due to the difference in the ionic radii of calcium (1.14 Å) and magnesium (0.86 Å). Thus, it can be assumed that magnesium forms a substitutional solid solution in β-TCP and is not involved in the HA structure.[64]

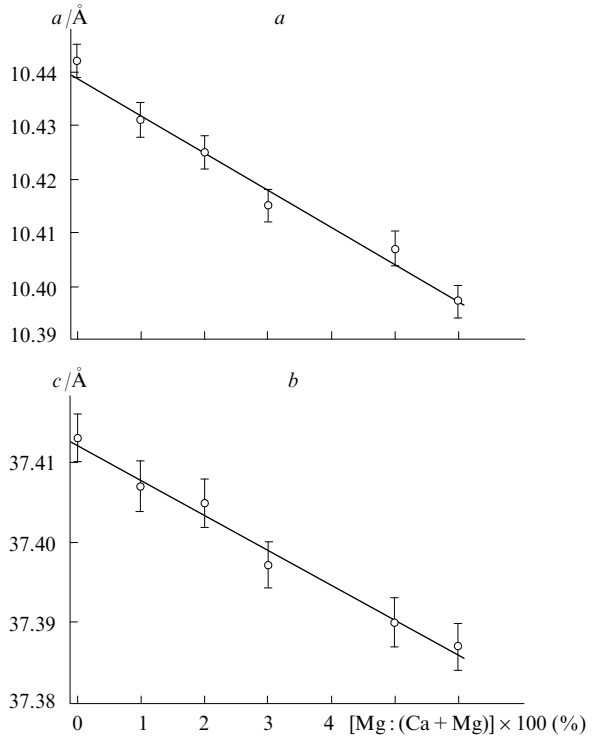


Figure 7. Unit cell parameters *a* (*a*) and *c* (*b*) for TCP as a function of the atomic ratio Mg : (Mg + Ca).

The *in vivo* studies on implanting BCP granules into a defect in the shin bone of Wistar rats have shown that the resorption of granules occurred non-uniformly, namely, the HA component remained 'immured' in the *de novo* bone tissue.[65] This is a substantial drawback of the BCP ceramics. It is assumed that a phase-uniform carbonate-containing HA would exhibit more favourable biological behaviour in the organism.

3. Carbonate-containing hydroxyapatite

Carbonate groups can occupy two positions in the HA structure, *i.e.*, substitute for either OH groups (A-type substitution) or PO_4 groups (B type).[66] The replacement of PO_4^{3-} anions by carbonate ions decreased both the grain size and the degree of crystallinity of HA. The replacement of OH groups led to the extension of the unit cell along the axis *a* and its slight compression along the axis *c*, which was accompanied by the change in the $P6_3/m$ space group to $P2_1/b$. In contrast, for carbonate-containing B-type HA, a decrease in parameter *a* and an increase in *c* were observed. Both the natural bone tissue and the synthetic carbonate-containing hydroxyapatite (CHA) prepared by the precipitation from solutions were of the mixed AB-type substitution with the A : B ratio depending on many factors. Particularly, in the chemical synthesis, this ratio could depend on the reaction conditions.

Carbonate-containing HA of the B or mixed AB types of substitution was prepared by the precipitation from aqueous solutions at high pH.[67–69] For this purpose, the reaction of solutions of calcium nitrate, diammonium phosphate and ammonium carbonate with ammonia can be used. For the B-type substitution, the problem consists of maintaining the charge balance. Hence, the synthesis of B-type carbonate-containing HA was usually carried out in the presence of sodium or ammonium ions that had partially substituted the calcium ions.[67] Sodium and calcium have close ionic radii. The synthesis temperature and the content of carbonate groups had a substantial effect on the morphology of crystallized particles (from spherical at high content to needle-like for low contents). The predominant type of substitution depended on the content of carbonate groups, namely, for the CO_3^{2-} concentration above 4 mass %, the latter preferentially occupied B positions in the structure.

Carbonate-containing HA was also obtained from calcium oxide, ammonium hydrogenphosphate and ammonium carbonate under the conditions of solid–liquid interaction.[70–72] The starting components in the solid state were mixed in a planetary ball mill, water was added to the mixture and the mixing was continued. Then, the mixture was placed into a microwave oven (microwave radiation is known to accelerate the CHA synthesis). The reaction product was subjected to the heat treatment in air for the HA crystallization.

Carbonate-containing HA is thermally unstable; hence, it is difficult to prepare ceramics on its basis. FTIR spectroscopic studies of the vapour phase condensate formed on heating the samples of carbonate-containing HA of different composition in a Knudsen cell have shown that the decomposition starts at as low as 300–400 °C and is accompanied by the evolution of CO and CO_2.[70–73] The thermal stability of carbonate-containing HA depended on both its composition and chemical prehistory (synthetic method and conditions). As the example, Fig. 8 shows the dependence of the peak areas of CO (2149 and 2138 cm^{-1}) and CO_2 (at 2345 and 2339 cm^{-1}) in the IR spectra of the vapour phase condensate on the temperature of evaporation of samples of carbonate-containing HA with 3.1 mass % and 6.3 mass % CO_3, respectively, and also 0.1 mass % Mg.[70] In the temperature range of 1200–1300 °C, carbonate-containing HA completely lost its carbonate groups. Carbonate-containing HA synthesized by heterophase interaction was more resistant to thermal decomposition, which is explained by its higher degree of crystallinity. According to the chemical analysis data, carbonate-containing HA lost >50% of carbonate groups after heat treatment at 1100 °C. In the IR spectrum, the absorption band of OH group disappeared and the carbonate group bands in the region of 1650–1300 cm^{-1} substantially changed. The analysis of ^1H NMR spectra has shown that after the heat treatment of carbonate-containing HA at 1100 °C, the proton content decreased threefold; this was accompanied by the HA restructuring into oxyapatite.[73]

The possibility of stabilizing carbonate-containing HA by compensating the charge disbalance appearing with the addition of CO_3^{2-} to the HA structure was studied.[12] As the compensation, a part of Ca^{2+} cations can be displaced in

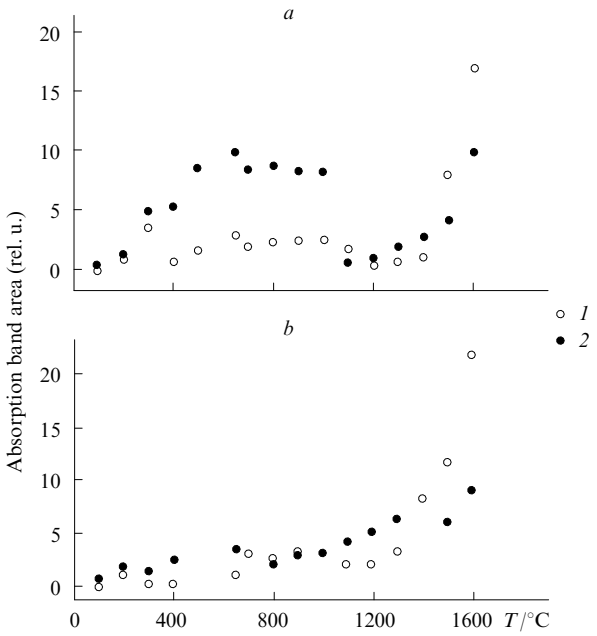


Figure 8. Temperature dependence of absorption band areas for CO (*1*) and CO_2 (*2*) in IR spectra of condensates of vapour phase formed in the CHA heating.
Samples containing 3 (*a*) and 6 mass % (*b*) CO_3.

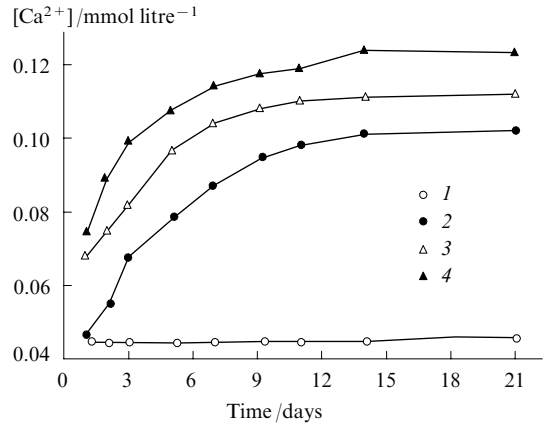


Figure 9. Time variations of the Ca^{2+} ion concentration in an isotonic solution at the dissolution of granules containing HA (*1*) and carbonate-containing HA with 0.6 (*2*), 6 (*3*) and 9 mass % (*4*) of carbonate groups.

due proportion by monovalent cations, preferentially Na^+ (due to their close ionic radii). It was found that sodium that occupies a position adjacent to the carbonate ion in the Ca1 sublattice favours the formation of B-type defects in the carbonate-containing HA that are thermodynamically less stable as compared with AB-type defects (the $2\,CO_3^{2-} \rightarrow PO_4^{3-},OH^-$ substitution).

Thus, in the synthesis of ceramics from carbonate-containing HA powders, their sintering temperature should be lowered by as least 450–500 °C as compared with the sintering temperature of HA ceramics (1250–1300 °C). With this purpose, experiments were carried out on the sintering of ceramics based on carbonate-containing HA at reduced temperature in the presence of the liquid phase. It was found that calcium and alkali metal carbonates are efficient liquid-phase-forming additives. The introduction of 8 mass % of such additive into the furnace mixture decreased the ceramics sintering temperature to 750–800 °C, which made it possible to avoid the substantial loss of carbonate groups. In the course of sintering, the secondary crystallization of the additive with the main-phase dissolution products took place in the melt, as confirmed by studies of the elemental composition of crystals using the method of local analysis. The crystallization of secondary rod-like crystals led to strengthening of the ceramics.

Porous ceramics based on carbonate-containing HA is a more promising scaffold material as compared with BCP ceramics. Figure 9 shows the kinetics of calcium ion concentration variations in an isotonic solution for granules with different degrees of substitution.[74] The solubility of granules increased with the increase in the carbonate group content. *In vivo* tests have shown that granules are osteocondutive; on their surface, the active formation of bone tissue was observed, the resorption rate of granules in a bone defect exceeded that observed for the β-TCP ceramics.[65,74] No information on the possible osteoinductive properties of carbonate-containing HA is available.

4. Octacalcium phosphate: possible osteoinduction

Octacalcium phosphate is a potential osteoinductive substance. Studies of OCP-based materials have begun only recently; hence, the literature data are quite limited. The stoichiometric composition of OCP corresponds to $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5\,H_2O$; the Ca : P atomic ratio is 1.33. The crystal structure of OCP pertains to triclinic system with the *P*1 space group.[75,76] The HA structure is to a certain extent similar to that of OCP and like the latter has layers with the apatite structure (A layers) separated by hydrated layers (B layers) containing water molecules.[76] Presumably, OCP represents a thermodynamically metastable polymorphous HA modification.[77] Layers B contain two types of the HPO_4^{2-} groups of which one is connected with layer A and the other is located between A layers in the $Ca-HPO_4Ca$ configuration.[78] This structure makes possible the intercalation of other anions into OCP by ion exchange with the HPO_4^{2-} groups. Intercalated OCP can be used for delivering drugs into the organism.[78] Partially hydrolyzed OCP may consist of OCP and HA domains.[79] The OCP structure is retained in a certain region of homogeneous composition so that generally the OCP composition corresponds to $Ca_{10}H_{4+x}(PO_4)_{12}(OH)_x \cdot (10-x)\,H_2O$.[80]

A unique feature of OCP is its ability to reversibly vary the content of HPO_4^{2-} anions in hydrated B layers. This content is varied as the medium changes from alkaline to neutral.[81] As to its morphology, OCP is crystallized in the form of planar crystals oriented in the {100} planes along the symmetry axis *a* of the triclinic structure.

Hydroxyapatite is the least soluble calcium orthophosphate at pH ~7, which matches the blood plasma pH; under these conditions, OCP dissolves much better.[82] The OCP synthesis was carried out by precipitation from aqueous solutions.[83] It often represents an intermediate product in the synthesis of more thermodynamically stable phases, for example HA in the range of neutral pH from solutions containing < 5 mmol litre^{-1} calcium ions and < 60 mmol litre^{-1} phosphate at ~ 40 °C.[83,84] In a recent work,[84] precipitation from solutions of $Ca(NO_3)_2$ (from 0.005 to

0.03 mol litre^{-1}) and $NH_4H_2PO_4$ was carried out by adding a solution of urea, which underwent hydrolysis with evolution of ammonia. In the course of reaction, the pH value changed from 4 to ~7. According to the results of XRD analysis of the precipitation product, OCP was the main phase in the presence of high (0.03 mol litre^{-1}) concentrations of calcium nitrate in solution. At lower concentrations, OCP was hydrolyzed to HA. Octacalcium phosphate particles possess morphology typical of lamellar crystals (Fig. 10). IR spectra contained bands at 873 and 1420 cm^{-1} corresponding to the vibration modes ν_2 and ν_3 of the carbonate groups originated from atmospheric CO_2.

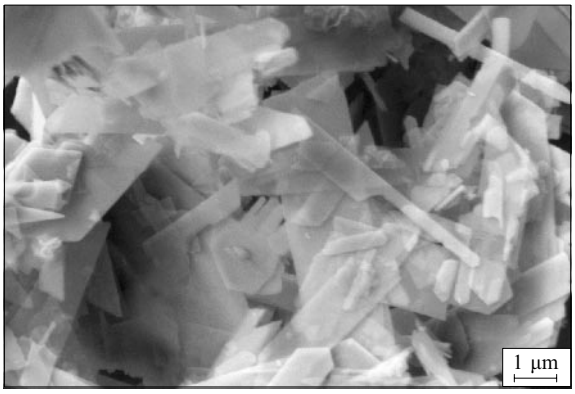


Figure 10. Morphology of octacalcium phosphate particles.

A version of the OCP synthesis by precipitation is based on the reaction of $Ca(NO_3)_2 \cdot 4\,H_2O$ with Na_2HPO_4 in a sodium acetate buffer. The reaction was carried out at 45–55 °C in the pH range of 6.5–7.2 for 48 h.[85] Its parameters such as temperature and pH were found to affect the size and morphology of the synthesized particles. They could be spherical or lamellar with the plate size varying from several tens to several hundreds of micrometres. Typical IR spectra of OCP contained an absorption band of the HPO_4 groups at 910 cm^{-1} the presence and the intensity of which made it possible to identify OCP. Another version of synthesis was described in detail in several studies.[79, 86, 87] A 0.04 M solution of $Ca(CH_3COO)_2$ (250 ml) was added to a solution containing Na_2HPO_4 and NaH_2PO_4 (5 mmol litre^{-1} each, 750 ml) with permanent stirring. The temperature was maintained at 60 °C at pH 5. After ripening for 10 min, the precipitate was washed with distilled water and dried at 37 °C. The resulting powder with a flaky structure represented pure OCP without traces of other phases.

Octacalcium phosphate was also synthesized by hydrolysis of other orthophosphates, namely, dicalcium phosphate dihydrate [brushite, $CaHPO_4 \cdot 2\,H_2O$ (see Ref. 88)] and α-tricalcium phosphate [α-TCP, α-$Ca_3(PO_4)_2$ (see Ref. 89)] according to the following reactions:

$$10\,CaHPO_4 \cdot 2\,H_2O \longrightarrow \quad (5)$$
$$\longrightarrow Ca_8(HPO_4)_2(PO_4)_4 \cdot 5\,H_2O + 2\,Ca^{2+} + 4\,H_2PO_4^- + 5\,H_2O,$$

$$8\,(\alpha\text{-}Ca_3(PO_4)_2) + 2\,H_3PO_4 + 15\,H_2O \longrightarrow \quad (6)$$
$$\longrightarrow 3\,Ca_8(HPO_4)_2(PO_4)_4 \cdot 5\,H_2O.$$

In both cases, under certain conditions, the final hydrolysis product could be calcium-deficient HA with OCP as the intermediate.[90]

Hydrolysis of α-TCP to OCP can be carried out, *e.g.*, as follows.[78] A powder of α-TCP (2.0 mmol) was suspended in 18 ml of sodium acetate buffer with the initial pH in the range of 3.9–4.7. The suspension was stirred at 50–70 °C for 3–24 h. The hydrolysis product was dried at 50 °C for 24 h.

The problem of OCP hydrolysis is important in the context of the biomineralization hypothesis according to which the formation of a phase stable in the organism, namely, biological apatite, involved an intermediate stage of formation of octacalcium phosphate as a metastable phase. The data in Table 4 demonstrate the increase in the Ca : P atomic ratio in the organism in accordance with the trend to gradual HA formation (Ca : P = 1.67).[91] The transformation of OCP into HA in the organism can occur as a result of either its permanent hydrolysis or by the mechanism of OCP dissolution and HA deposition from a supersaturated solution. In the course of hydrolysis, the crystallographic matching of phases at the OCP/HA interface remained unchanged.[92] The transformation of OCP into HA was spontaneous and irreversible.[81] The hydrolysis involved the addition of calcium ions from solution to the OCP molecule and partial transition of phosphate ions to solution according to reactions[93]

$$\frac{10}{8}\,Ca_8H_2(PO_4)_6 \cdot 5\,H_2O \longrightarrow \quad (7)$$
$$\longrightarrow Ca_{10}(PO_4)_6(OH)_2 + \frac{3}{2}\,H_3PO_4 + \frac{17}{4}\,H_2O,$$

$$Ca_8H_2(PO_4)_6 \cdot 5\,H_2O + 2\,Ca^{2+} \longrightarrow \quad (8)$$
$$\longrightarrow Ca_{10}(PO_4)_6(OH)_2 + 4\,H^+ + 3\,H_2O.$$

Table 4. Time variations on the calcium and phosphorus content in the chicken bone tissue.

Age	Ca (mass %)	P (mass %)	Ca : P	Bone tissue density /g cm^{-3}
Embryonal	17.8	9.6	1.43	1.85
10 weeks	24.6	12.5	1.52	2.11
1 year	26.2	12.5	1.61	2.14

It was observed experimentally that the hydrolysis of non-stoichiometric OCP with Ca : P = 1.26 afforded calcium-deficient HA (Ca : P = 1.49).[80]

Insofar as the biological bone tissue contains admixed ions, it was of interest to elucidate their effect on the hydrolysis process. It was found that magnesium has a pronounced inhibitory effect on the brushite hydrolysis to afford OCP and HA.[86] For high concentrations of magnesium ions (> 10^{-4} mol litre^{-1}), the hydrolysis could even produce β-TCP in place of OCP and HA.[93] The effect of ions of different metals (in amounts of 5 and 10 μmol litre^{-1}) on the hydrolysis of dicalcium phosphate dihydrate to OCP and HA was studied in a solution of orthophosphoric acid with addition of a metal nitrate.[94] The strongest inhibitory effects on the transformations was exerted by Zn^{2+} and Cu^{2+}. Hydrolysis in the presence of these ions was characterized by an induction period, a relatively extended stage of weak pH variations and a fast stage of the abrupt pH drop to its final value. The Pb^{2+} ions promoted the transformation of brushite into OCP; Mg^{2+} ions proved to be weak inhibitors of this process, while the

transition metal ions, namely, Cr^{3+}, Fe^{2+}, Fe^{3+}, were found to be an inhibitor, a weak inhibitor and a weak promoter of this conversion, respectively. Bivalent iron and zinc ions completely suppressed the conversion of brushite to HA. The mechanism of inhibition effect may be associated with the adsorption of ions impeding growth of new phase nuclei. Apparently, foreign ions can substantially affect the processes of bone tissue remodelling that occur in the human organism.

It is known that OCP exhibits high osteoconductive properties. According to experiments with Wistar rats, implanting of OCP granules into a bone defect accelerated the process of its regeneration.[95] Coating titanium implants with OCP favoured their biointegration into the neighbouring bone tissue.[96] It was found that OCP crystals are present in the human teeth dentine; moreover, OCP constitutes the central part of the dentine crystal phase covered with an apatite layer.[97] It was assumed that the HA crystallization occurs on its precursor, *i.e.*, OCP, and the latter plays an important role in the mineralization of bone tissues.

The OCP composition can deviate from stoichiometry due, for example, to the partial hydrolysis. These deviations can substantially affect the biological behaviour of a material. On a model of rat bone marrow, the effect of the OCP composition on osteoinductivity and immune response characteristics was studied.[98] Partially hydrolyzed, low-crystalline OCP with Ca : P = 1.37 was found to efficiently enhance the *de novo* bone tissue formation, suppress the activity of osteoclasts (cells resorbing the bone tissue), reduce inflammations and exhibit the higher osteoconductive properties as compared with both β-TCP and completely hydrolyzed OCP with Ca : P = 1.48.

It was demonstrated that titanium implants covered with OCP and morphogenetic protein rhBMP-2 exhibited osteoinductivity, whereas implants without such a coating lacked this property.[2] In contrast to HA and ceramics on its basis, OCP in a biological microenvironment exhibited immanent osteoinductivity, *i.e.*, the ability to initiate the *de novo* bone tissue formation, for example, at its hypodermic implantation.[2,99] The reasons for such biological behaviour of OCP are still unclear.[100] There are two viewpoints on the problem of its osteoinductivity.

It is believed[2] that the OCP composition is not the decisive factor of its osteoinductivity. Presumably, osteoinductivity of certain materials is due to their ability to concentrate morphogenetic proteins and bone-forming cells from the biological environment on their surfaces. The specific ability of OCP to adsorb proteins is explained by its microstructure, *i.e.*, the presence of micropores between OCP-constituting grains. Using a model of mouse calvarial defect, the effect of the OCP granule microstructure on the dynamics of bone tissue regeneration was studied.[101] Granules with diameters of 300–500 μm and two types (fine and coarse) of microstructures were implanted for up to 14 days. Histological studies revealed the active formation of bone tissue in the finer granules of plate-like OCP crystals. Presumably, the microstructure affects the adhesion of osteoblasts and the plate-like crystals favour adhesion. A hypothesis that the OCP microstructure is the main reason of its osteoinductivity was also indirectly confirmed by studying other CP materials.[2]

According to a different viewpoint, the *in vivo* hydrolysis of OCP to HA can itself be a factor that stimulates the differentiation of multipotent stem cells into osteoblasts.[99] The transformation of implanted OCP into HA in a rat defect was proved by X-ray microdiffraction studies.[81] Octacalcium phosphate acts not only as the source of calcium and phosphorus, but also as a template on which the bone tissue forms; it is gradually resolved in the body through its resorption by osteoclasts or multinucleated giant cells; moreover, the changes generated by such cells in the structures of OCP- and HA-based implants substantially differ. The rate of OCP resorption considerably exceeded the resorption rates of HA and TCP. After six weeks of being implanted into a rat calvarial defect, OCP virtually disappeared in contrast to TCP and, especially, HA. In the case of OCP implantation, the amount of bone tissue formed in the defect was the maximum.[81] A comparative study[101] of OCP and HA was carried out with the aim of elucidating whether the OCP osteoinductivity was determined by its special physicochemical properties. An *in vitro* study was carried out on a model of stromal cells of rat bone marrow. It was found that OCP induces the enhanced expression of osteogenic markers, whereas no effect was observed for HA. Hence, OCP induces the differentiation of stromal cells into osteoblasts, *i.e.*, cells that constitute the bone tissue.

Both hypotheses considered above have the right to exist; hence, the question on the mechanism of manifestation of osteoinductivity in OCP remains open so far.

No systematic studies were carried out on the directed synthesis of OCP particles of a given size and morphology. Nor were there any published data on the production process of bulky ceramic materials of OCP. Virtually all studies were carried out with powders, granules or coatings. Octacalcium phosphate is a thermally unstable compound, which decomposes at a temperature much lower than that required for sintering of ceramics. The loss of water in OCP begins at ~80 °C, the bonds between layers in the OCP structure are broken at 180 °C and transformations that accompany the pyrophosphate formation from OCP occur at 300 °C.[102] Thus, it is impossible to prepare an OCP-based ceramics by conventional methods. As an alternative to sintering, the method of chemical binding appears to be promising. In fact, this method is identical to the production of binding materials (cements). However, with the exception of an early study,[103] no research on the development of binding systems based on OCP is known,[104] which is apparently due to the fact that OCP transforms into another phase as a result of hydrolysis in the hardening liquid. The development of such systems would allow ceramics to be formed at physiological temperature without the sintering stage. The parameters of mechanical, chemical and biological properties of bulk materials based on OCP are unknown.

The synthesis of OCP as the setting product in a cementing system can be considered as an alternative version. The results of studies of a cementing system in which OCP is crystallized and its application for capping the dental pulp were decsribed.[105] The original system powder had the following composition:

System component	$Ca(H_2PO_4)\cdot H_2O$	$CaCO_3$	α-$Ca_3(PO_4)_2$
Content /mol litre^{-1}	3	5	9

A solution of sodium hydrogenphosphate with pH 7.4 was used as the hardening liquid. Octacalcium phosphate is the main crystal phase formed upon setting of this cement. The cement was found to favour the active formation of dentinal tubules in the pulp.

Recently, attention has been drawn to the development of scaffolds from materials based on biopolymers biologically compatible with the human organism. However, they lack biological activity and necessary mechanical properties, which proved to be crucial in the initial post-operative stage. This is why the active search for composite materials (CM) based on biopolymers is being carried out. Probably, the use of OCP as the filler would substantially improve the scaffold properties. Composite materials with a collagen scaffold reinforced with OCP particles were prepared.[106] These CM proved to have a very interesting structure in which lamellar OCP crystals are oriented along the collagen fibrils; moreover, the presence of a chemical bond between them was proved.

Taking into account the specific properties of ceramics, cements and CM with OCP, it can be assumed that the technological problems of their synthesis will be the subject of active studies in the field of biomaterials of the new generation.

5. Granules and porous ceramics for scaffolds

The results of *in vivo* studies have demonstrated the theoretical possibility of using three-dimensional packages of CP granules as the scaffolds for allogenic and autologous cell cultures.[107] Granules should be mainly spherical (to avoid the inflammatory reactions of the organism). To synthesize such granules, a suspension process based on the effect of spheroidizing in immiscible liquids was used.[108] This method is based of dispersing of a slurry of HA with a chitosan solution in liquid paraffin. Spherical drops of chitosan with HA are hardened with glutaric aldehyde. A drawback of this method is the use of molten paraffin.

A simpler and more flexible process was developed for the preparation of granules measuring from 50 to 2000 μm based on different CP.[60,61] A fine CP powder was mixed with an aqueous solution of a biopolymer to afford a suspension; the latter was vigorously stirred with a dispersion liquid, *e.g.*, vegetable oil. This technology made it possible to vary the granule size in wide limits.[7] Heat treatment of granules led to burning out of the biopolymer, formation of a system of open interconnected pores and shrinkage. The ~70% porosity was reached with small HA : biopolymer ratios and low heat treatment temperatures (~1100 °C). Pores with sizes of 0.1–5 μm dominated, their relative content in the total number of open pores reached 60%. The spherical geometry of granules is reflected in Fig. 11.

When a packing of granules was used as a scaffold, the granules were under risk of being carried away from the bone defect site by the fluxes of body fluids. The use of porous ceramics is more promising. The interconnected pores of such ceramics should measure no less than 100–135 μm in order to provide blood delivery to the surfaces in contact, neovascularization, bone tissue ingrowth and fixation. Smaller pores are also necessary, because they favour the adsorption of proteins. Thus, it is desirable that porous ceramics have a bimodal size distribution of pores (coarse and fine pores).

The technology of CP-based porous ceramics uses the procedures of burning out of additives, impregnation followed by burning out of organic (polyurethane) sponges and foaming, *e.g.*, by the addition of hydrogen peroxide. A method was developed for the synthesis of porous ceramics containing up to 70 vol.% of fine and coarse interconnected pores based on sintering of porous granules.[109] The burning out of biopolymers is accompanied by the formation of fine pores (intrapores) in granules, whereas the formation of intergranular interconnected pores (interpores) is determined by the packing of granules. Figure 12 shows the microstructure of such ceramics. The high porosity favours the ectopic formation of bone tissue due to the increased specific surface and higher solubility. The dissolution-induced increase in the concentration of calcium and phosphate ions in the extracellular fluid favours the differentiation of cells to osteogenic and also the deposition of an apatite-like phase on the scaffold.[47]

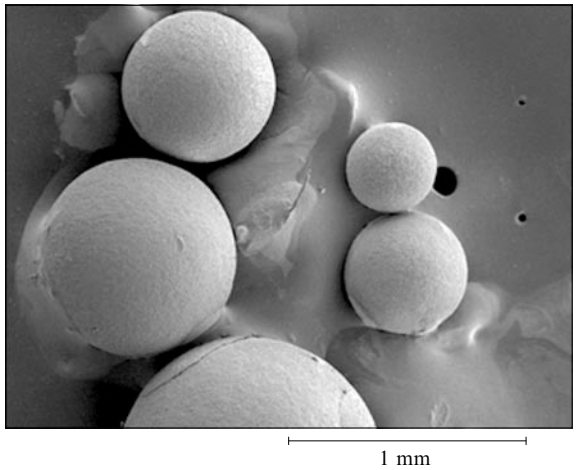


Figure 11. Microimages of CP granules synthesized by the suspension method.

Porous ceramic materials were successfully used as scaffolds in the bone tissue engineering. However, their serious drawback is the rigidity that prevents complete filling on the bone defect, especially if the latter has a complex configuration. Moreover, it is difficult to prepare ceramics by sintering powders of thermally unstable compounds such as CHA and avoid their sintering-induced chemical and phase changes that can affect the ceramics properties. As an alternative, a technology of binding systems (calcium phosphate cements) that settle and harden at physiological temperature can be used.

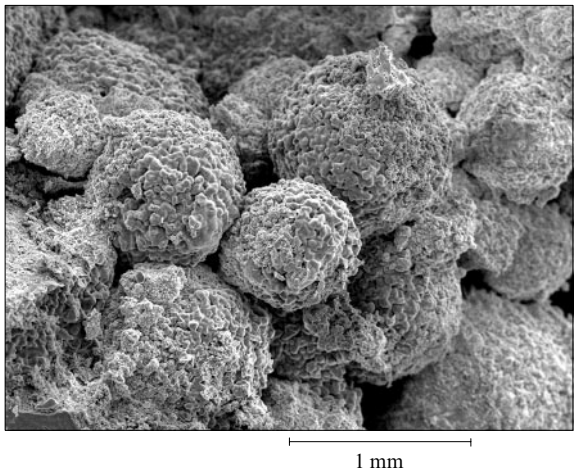


Figure 12. Microstructure of HA ceramics with bimodal (intra- and intergrain) porosity.

6. Porous scaffolds fabricated by the cement technology

The majority of binding CP systems are based on chemical reactions between the components with low and high Ca : P ratios in a hardening liquid.[110, 111] A typical example is cement with a composition including tetracalcium phosphate (Ca : P = 2.0) and dicalcium phosphate (Ca : P = 1.0) as well as water or orthophosphoric acid as the hardening liquid. The ratio of components is chosen depending on the required phase composition of the product. An alternative is a system that includes α-TCP, calcium carbonate and monocalcium phosphate monohydrate (Norian® cements with carbonate-containing HA as the crystallized phase). The so-called brushite cements (Ca : P = 1.0 in the crystallized phase) are obtained in the TCP – monocalcium phosphate monohydrate system. With a decrease in the Ca : P ratio in the final product from 1.67 to 1.5, the rate of *in vivo* resorption in an organism increased.[112] Brushite cements are characterized by the high solubility and resorbability.

The state of the art in the field of calcium phosphate cements that have found practical application in medicine was considered in a review.[113] The properties of such cements (kinetics of setting and hardening, porosity) depend not only on the chemical and phase composition of components, but also on their degree of dispersion, the solid : liquid ratio and the degree of precompacting.

Setting and hardening of cements is accompanied by the gradually decreasing liquid phase content due to the formation of a crystal hydrate in the crystallization of dicalcium phosphate dihydrate or the incorporation of hydroxyl groups into the apatite structure. The setting and hardening of calcium phosphate cements are usually accompanied by the formation of micropores with the sizes of about several micrometres. To provide vital activity of bone-forming cells, neovascularization and invasion of bone tissue into the porous space of scaffolds, the presence of interconnected pores measuring 100 – 500 μm is required. Such pores are formed in cement in the presence of soluble or foaming additives introduced into the binding system. NaCl was often used as the soluble additive, $(NH_4)_2CO_3$ was used as the foaming agent; this allowed manufacture of materials with porosity up to 80%.[114] Channels with the diameter of no less than 50 μm should be present between pores to provide permeability.[115]

A successful experiment on the preparation of cements of calcium-deficient HA with the ratio Ca : P = 1.5 and porosity up to 81% was described.[116] The starting components, namely, tetracalcium and dicalcium phosphates, were mixed in the 1 : 2 ratio; then water was added in the solid : liquid ratio of 3 : 1 to produce a paste. To the latter, NaCl crystals with the size of 400 – 500 μm were added as the porogen. The mixture was formed under a pressure of 2 MPa and then stored at 37 °C and 100% relative humidity for two full days. The salt crystals were washed out by immersing the samples into water; then, the samples were subjected to freeze drying, which was accompanied by the formation of a foamed structure. The prepared scaffolds were used for cultivating mesenchymal stem cells. *In vivo* tests have demonstrated the excellent scaffold properties of this biostructure that completely matched the requirements of bone tissue engineering.

The mechanical and rheological properties of calcium phosphate cements can be improved by the introduction of the corresponding gel-forming additives (for example, hydroxypropylmethylcellulose) and reinforcing fibres. In the system tetracalcium phosphate – dicalcium phosphate dihydrate, the injected materials with the porosity of up to 83% were synthesized by the introduction of a water-soluble porogen, namely, mannitol, with the particulate size of 300 – 500 μm. The cement porosity can be generated by the introduction of biopolymers, particularly, gelatine.[117] On the whole, the technologies based on the use of calcium phosphate cements for the preparation of porous scaffolds are new and insufficiently studied. Their main drawback is the inadequate mechanical properties apparently caused by the residual microporosity. The problem of decreasing this porosity with simultaneous retention of high macroporosity is yet unsolved.

7. Composite materials biopolymer – calcium phosphates

Hybrid composite materials with a polymeric component are more elastic than porous ceramics, which allows them to fill a bone defect of any form without leaving a gap between the bone and the scaffold. Hybrid composite materials with biopolymer scaffolds can correspond to the bone tissue as regards the structure and properties to a greater extent than sintered ceramics. The CP mineral phase imparts enhanced rigidity and the biological activity that is untypical of a polymer to the composite materials. Collagen and gelatine (the partial hydrolysis product of collagen), polysaccharides (chitin, chitosan, starch, hyaluronic acid) and the following synthetic polymers: polylactides and their co-polymers with polyglycolides, polycaprolactone and certain others, were used as the scaffolds.

The porosity in a polymer was achieved by the following methods. A solution of a polymer with dispersed porogenic additive was precipitated in a non-solvent and the additive was selectively leached.[118] A drawback of this method is that the complete removal of the solvent is difficult, which may cause cytotoxicity. Another method is freeze drying. The use of certain temperature conditions and compositions of the liquid phase to be frozen allowed large ice crystals of controllable geometry to be obtained.[119] Other methods such as phase separation, foaming and rapid prototyping were developed. Particularly, a microstructure similar to that of a trabecular bone could be obtained by thermally-induced phase separation.[120]

Composite materials can be prepared by different methods. Apparently, the simplest method is dispersing of CP particulates in a polymeric scaffold. Using this method, composite materials with the composition similar to natural bones were prepared in the system HA – collagen.[121, 122] These materials exhibited low tensile strength (up to 6.5 MPa). An advantage of this method is the possibility of preparing spatially reinforced materials.

A promising method is a process that simulates biomineralization, namely, the deposition of CP on the polymer surface in the cellular microstructure of a porous scaffold. The principles of this synthetic (biomimetic) mineralization were considered in detail in a review.[123] As a rule, CP were deposited from the liquids that simulate the body fluids and were oversaturated with calcium and phosphate ions. Their composition was brought close to that of blood plasma and included the following ions:[123]

Ion	Na^+	K^+	Mg^+	Ca^{2+}	Cl^-	HCO_3^-	$H_2PO_4^-$	SO_4^{2-}
Content /mmol litre^{-1}	142	5	1.5	2.5	103 – 148.8	4.2 – 27	1	0 – 0.5

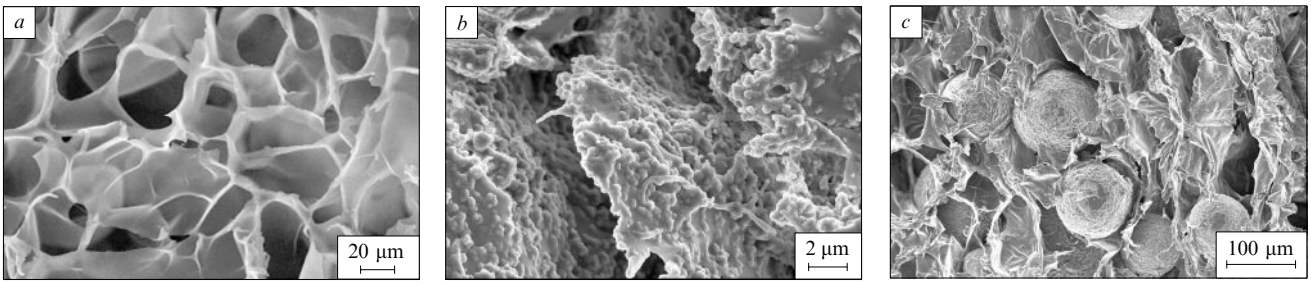


Figure 13. Porous chitosan/gelatine scaffold (*a*) with walls reinforced with ultrafine CP particulates (*b*) and CP granules (*c*).

Due to the risk of infection, the clinical use of collagen, a protein of the animal origin, was limited. Hence, attention was focused on the development of composite materials with scaffolds of gelatine, chitosan or their mixtures. Chitosan-based materials exhibited biocompatibility and bioresorbability. The addition of HA as the filler into a porous chitosan structure enhanced the adsorption of proteins and calcium ions and hence improved the adhesive properties and favoured better differentiation of mesenchymal stem cells.[124] Freeze drying was used as a rule to prepare porous chitosan sponges. Glutaraldehyde was used for cross-linking of the chitosan structure after the synthesis of a porous structure, its toxicity being the only drawback.

A simple method was developed for the preparation of porous chitosan and chitosan/gelatine composite materials containing bioactive calcium compounds.[125, 126] The procedure includes dissolution of biopolymers, addition of a filler and foaming, which is followed by the displacement of the solvent with a liquid that does not dissolve chitosan. A powder of chitosan (2 g) with the medium molecular weight (50 – 190 kDa) was dissolved in 50 ml of 8% acetic acid and equal amount of a filler and ammonium carbonate as the foaming agent were added to the solution. The resulting thick suspension was placed into a cylindrical polyethylene mould and frozen to produce crystals of ice at a temperature below -10 °C. The frozen solid sample was immersed in ethanol, so that gradual substitution of alcohol for acetic acid present in the sample occurred. Then the sample was dried at 60 °C up to complete removal of the liquid phase. As a result, composite materials with porosity $>90\%$, containing up to 50 mass % of the CP filler, with the specific surface approaching 120 $m^2\ g^{-1}$ were obtained. The composite materials had uniform microstructures, the content of coarse pores measuring 100 – 300 μm was $\sim 80\%$ of the total porosity. Figure 13 shows the microstructures of porous chitosan/gelatine scaffolds with walls reinforced with ultrafine CP particulates and CP granules.

Another method of introducing CP particulates is the impregnation of a polymeric framework with these particulates using a centrifugal filling technique.[127] First, a 10% solution of poly(caprolactone) containing certain amount of chitosan in acetic acid was treated with 40% NaOH and the precipitate was washed with water. Hydroxyapatite was sonicated in a 10% solution of poly(vinyl alcohol), the obtained slurry was mixed with the poly(caprolactone) – chitosan scaffold and centrifuged for 4 min at 4000 rpm. The reaction of Ca^{2+} ions of hydroxyapatite with poly(vinyl alcohol) provided the enhanced strength of the composite material. Materials with the porosity of up to 80% were prepared.

The phenomenon of phase separation was also used in the preparation of composite materials in the system nano-HA – poly-L-lactide (PLL).[123] The latter was dissolved in a suspension of HA particulates in a dioxane/water mixture and then frozen, which provided the conditions for phase separation. Then the product was subjected to freeze drying to remove the solvent. Materials with the porosity of up to 94% at the mass ratio PLL : HA = 70 : 30 were prepared.

In the HA – chitosan system, low-porosity composite materials with the microstructure close to that of bone tissue were prepared.[128] Low-molecular-weight chitosan (35 kDa) was dissolved in acetic acid, then ammonia was added and the solution pH was brought to 10 to afford a sol. In the sol, HA was synthesized by a reaction of

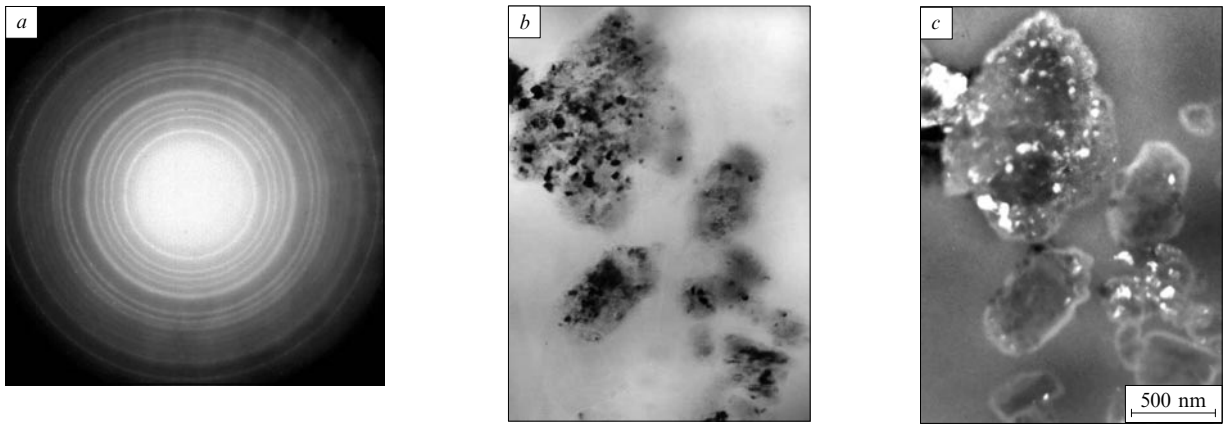


Figure 14. Electron-diffraction pattern (*a*), bright-field (*b*) and dark-field (*c*) TEM images of a chitosan scaffold with calcite nanoparticles.

dicalcium phosphate dihydrate with calcium nitrate at a calculated ratio HA : chitosan = 20 : 80. A suspension was prepared and then treated in a ball mill at room temperature. After being heated up to 25 – 60 °C, the precipitate was filtered, washed to the neutral pH and pressed under a pressure of 30 MPa. The structure of the thus synthesized composite material represented needle-like HA crystals on chitosan macromolecules (Fig. 14).

Setting and hardening of a binding system with a solution of chitosan as the hardening liquid might be an alternative process.[129] Due to the high rate of involved processes, the monitoring of the kinetics of phase formation could be carried out *in situ* by energy dispersive X-ray diffractometry.[130, 131]

The problems of control over the kinetics of resorption of composite materials with polymeric scaffolds aimed at their efficient application in the technology of bone tissue engineering are still unsolved. Yet another significant problem is modifying the polymer surface with the aim of governing the mineralization process. It is evident that further studies directed at the maximum approaching properties of composite materials to those of biological bone tissues should be carried out.

IV. Conclusion

The progress in chemistry of calcium phosphates has stimulated the studies in the field of development of materials destined for both the substitute of bone defects and the development of fundamentally new cell-based technologies of regeneration of damaged bone tissues. The most topical problems are associated with the development of nanostructured materials, especially, those with the structure and properties similar to those of bone tissues, including hybrid composite materials in the systems biopolymer – calcium phosphates, and also with the synthesis and design of materials based on the precursors of *in vivo* apatite mineralization such as octacalcium phosphate that are capable of exhibiting immanent osteoinductivity. The development of such materials and their introduction into the medical practice will cardinally improve the methods of therapy of bone tissue diseases especially oncological ones.

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