Physiological and Pathological Mineralization: Some problems and possible solutions

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In the first part of this review similarities and differences between physiological and pathological biomineralization (bone and tooth formation, osteoarticular and tooth pathologies, urolithiasis) are discussed. Both processes may be seen as the deposition of ionic crystals from high ionic strength solutions upon / within a preformed organic matrix and the presence or deficiency of crystallization promoters or inhibitors plays a decisive role. However, while physiological mineralization is strictly biologically controlled, urolithiasis is more likely driven by physicochemical factors, such as urinary supersaturation and lack of inhibitors of crystallization and aggregation.

In the second part of the review physicochemical research, performed to facilitate understanding of biomineralization processes is described. It comprises precipitation diagrams for calcium and magnesium phosphates, calcium oxalates, uric acid, sodium and calcium urates, precipitating from high ionic strength solutions at biologically relevant conditions. Here we present a general precipitation diagram of the calcium phosphate system, in which the precipitation boundary and the boundary between the regions of direct crystallization and crystallization via precursor phases are defined by the mean ion activity products and the pH. Kinetic diagrams of spontaneously precipitating systems are represented, which may be interpreted in terms of subsequent or parallel precipitation processes, such as nucleation, crystal growth and aggregation. The dual role of macromolecules, poly-L-glutamate and poly-L-lysine, as crystallization promoters and/or inhibitors is demonstrated by a kinetic study of the phase transformation of amorphous calcium phosphate, ACP, into octacalcium phosphate and/or apatite. It is shown that the polyelectrolytes at low concentrations induce and at higher concentrations inhibit secondary precipitation of the crystalline phase.

In the third part of the review applied interdisciplinary research is described. Three chemical methods for testing the inhibitory potential of whole urine were developed. The methods are simple and can be used in a clinical laboratory for discrimination between urines of calcium stone formers and healthy persons. Furthermore a new class of biomimetic organic-inorganic composites, as coatings for bioinert metal implants was recently developed. The coating procedure involves “in situ” growth of calcium phosphate crystals within an organic - inorganic matrix, the inorganic part being ACP. The coatings are tightly anchored to the substrate and exhibit excellent biological properties as verified by in vitro testing and animal experiments.

Key words: Aggregation; Amorphous calcium phosphate - chemistry; Biomineralization; Crystallization; Inhibitors; Interdisciplinary research; Organic – inorganic composite coatings; Phase transformation; Precipitation diagrams; Precipitation kinetics; Promoters; Urolithiasis – pathology, prevention & control, urine;

Introduction

Osteoarticular pathologies such as traumas, child growth defects, osteoarthritis, osteoporosis and related failures are, at all ages, a serious cause of handicap and concern in public healthcare. Bone diseases and induced defects are also of prime importance in maxillo-facial surgery and odontology especially for aging populations.

Other related serious health problems that need to be addressed are calcifications occurring in soft tissues, such as urinary and kidney stones, some forms of arteriosclerosis and related diseases. Urolithiasis, i.e. the formation of urinary and kidney stones presents a particularly important medical and social problem because of its debilitating effects and high rate of recurrence among working age populations.

This review is focused on interdisciplinary research carried out with the purpose of (a) designing substitute materials for bone and/or cartilage regeneration and (b) prevention of soft tissue mineralization, specifically urinary and kidney stone formation. Similarities and differences between the

TABLE 1.
The main components of bone, dentin and tooth enamel as organic-inorganic composites.
TABLICA 1.
Glavni sastojci kosti, dentina i zubne cakline kao organsko-anorganskih kompozita.

bone, dentin kosti, dentin	organic matrix primary structure primarna struktura organske matrice	tooth enamel zubna caklina
collagen - kolagen	+ proteoglycans + + acidic proteins + + proteoglikani + + kiseli proteini +	amelogenin
carbonate apatite karbonat apatit	Mineral	carbonate apatite karbonat apatit
osteoblast, odontoblast	cells - stanice	osteoblast, odontoblast

two problems are discussed and it is shown that progress can only be made by close collaboration between medical doctors, materials scientists, cell and molecular biologists.

1. The mechanisms of physiological and pathological mineralization:

The first step in any research on mineralized tissues should be the understanding of the basic mechanisms of physiological and pathological mineralization.

1.1 Physiological mineralization: Bone and teeth are precisely organized organic – inorganic nanocomposites, the main components of which are listed in Table 1. Bone and dentin are characteristically composed of type I collagen fibrils, which comprise about 85 -90% of the organic matrix and are intimately associated with precisely oriented apatite nanocrystals (1, 2). Additional minor components include an array of noncollageneous macromolecules (proteoglycans, acidic and/or phosphorylated glycoproteins) which have been found to have a decisive role in the mineralization process. In fact it has been assumed that collagen fiber networks provide the organizational framework and spatial constraint for crystal deposition, whereas noncollageneous, acidic and/or phosphorylated matrix macromolecules are involved in the control of nucleation and growth of the mineral phase (1, 3, 4).

The first step in controlled mineralization is the secretion of the organic matrix by specialized cells (i.e. osteoblast and odontoblast for bone and dentin respectively). The subsequent process of mineralization of the organic matrix is still being considered with several possible mechanisms being discussed.

The source of ions for mineralization is the blood serum, which is a well defined, high ionic strength (I = 0.15) aqueous solution just slightly supersaturated and metastable with regard to calcium phosphate crystallization (Table 2, column A). It is therefore inconceivable that blood serum itself would induce homogeneous nucleation of hydroxyapatite crystals. It may be assumed that multiple mechanisms initiating mineralization are possible, which at times may act synergistically to boost extracellular matrix mineralization when necessary (5). Mineralization may be induced either locally by heterogeneous nucleation at specific sites, mediated by acidic or phosphorylated matrix macromolecules (3, 4) or by homogeneous nucleation within matrix vesicles, which can actively concentrate calcium and phosphate ions by enzymatic activity and thus acquire mineral in the form of amorphous calcium phosphate, ACP or poorly crystalline apatite. (6). The involvement of matrix vesicles (7) and/or amorphous calcium phosphate precursors (8) in certain forms of biomineralization has been demonstrated.

1.2 Osteoarticular pathologies and tooth defects: Whether induced by trauma or disease these pathologies can cause small or large defects in the skeleton. In the case of small defects it may be possible to fill the voids with suitable composite materials, which, if based on reverse thermoreversible gels may be injectible (M. Dutour Sikirić, A. Sosnik, D. Cohn and H. Fűredi-Milhofer, unpublished results). However, in the case of large defects the need arises for surgical replacement and/or repair of mineralized tissues with load bearing applications (such as hip replacement, tooth implants). This need led to extensive research with the aim to design substitute materials that mimic mineralized tissue's mechanical, chemical, biological and functional properties. An attractive solution is to use metals with the required biomechanical properties, such as titanium, titanium alloys, zirconium and/or its alloys, etc.

TABLE 2.
Ionic concentrations in blood serum and in 24h urine (mean values obtained from 20 male patients, after ref. (12).
TABLICA 2.
Ionske koncentracije u krvnom serumu i 24 satnom urinu (srednje vrijednosti dobivene na uzorku od 20 muških pacijenata, prema ref. (12).

ion - molecule ion - molekula	blood serum krvni serum (mmol/ml)	urine mokraća (mmol/24h)
K^+	5.0	45.9 ±13.0
Mg^{2+}	1.5	
Ca^{2+}	2.5	7.3 ±2.7
HCO_3^-	27.0	
HPO_4^{2-}	1.0	30.7 ±8.8
SO_4^{2-}	0.5	
pH	7.5	5.9 ±0.8
NaCl	150.0	202.1 ±65.9
$C_2O_4^{2-}$		0.28 ±0.20
citrate / citrat		1.0 ± 0.8
urate / urat		3.8 ±1.2
creatinin / kreatinin		15.2±2.9
urinary volume / volumen urina		1434.6 ±503.3

In order to successfully integrate such materials into the body; they may be covered with biocompatible and/or preferably bioactive coatings. (9).

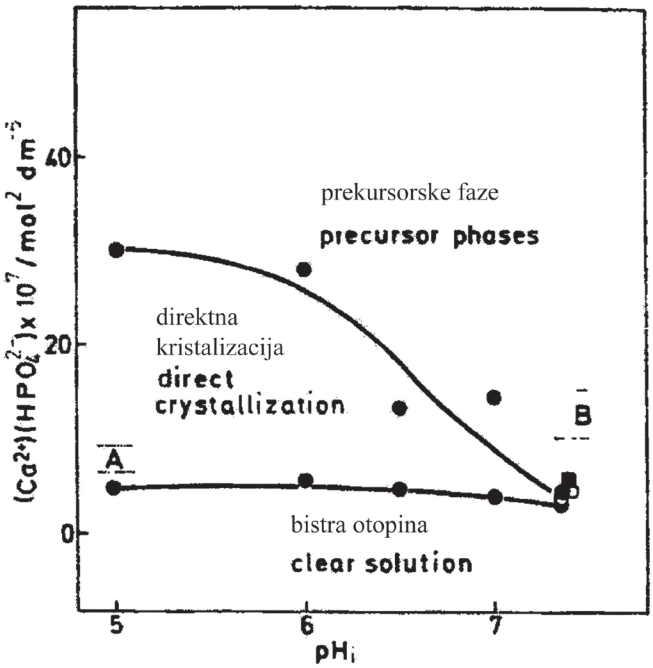
1.3 Soft tissue mineralization - urolithiasis: In contrast to the mineralization of bone and teeth, which is due to strictly controlled biological mechanisms, in urolithiasis the first step is the formation of crystals within the urine, which depends on physicochemical factors, such as urinary supersaturation, deficiency of inhibitors, etc (10). The urine is a complex system with variable flow rate, pH (5 – 7), high ionic strength (0.33 mol/l (11)) and variable composition. It is usually supersaturated with regard to one or more of the urinary salts. As an example in Table 2 column B are listed the mean values and standard deviations of the main urinary parameters in 24h urine samples from 20 male stone formers from the Osijek region (12). An analysis of the frequency of occurrence of abnormal metabolic parameters in this group showed that 35 % of patients had hypercalciuria, 15% had hyperoxaluria, 36% hyperuricosuria, 15% hyperphosphaturia and 25% had hypocitraturia.

It seems that urinary supersaturation is a necessary, but not sufficient condition for urolithiasis to occur. Even though it almost always results in the voiding of crystals (crystaluria) (13), this does not necessarily always lead to renal stone growth. Other properties of the urine and the urinary tract, such as the presence or absence of inhibitors of crystal growth and/or aggregation and the attachment of crystals or crystal aggregates to the renal walls must also be taken into account. It has been suggested (11) that a protective layer consisting of glycosaminoglycans covers the internal renal walls in healthy kidneys and that crystal attachment with subsequent stone growth is restricted to areas with a defective layer.

2. Physicochemical background:

2.1. The conditions of precipitation of biomineral from high ionic strength aqueous solutions. Precipitation diagrams and kinetics. Both physiological and pathological mineralization involves crystallization of slightly soluble salts from high ionic strength solutions (I = 0.15 for blood serum and 0.33 for urine). The relevant salts, calcium and magnesium phosphates,

FIGURE 1.
After ref. (14).General 24h precipitation diagram of the calcium phosphate system, showing the solubility boundary and the regions of direct crystal growth (A) and crystallization via precursor phases (B). In region B the initial precursor phase was amorphous calcium phosphate (ACP). The data, expressed in terms of the mean activity product vs. pH have been recalculated from precipitation diagrams of the system: $CaCl_2$ – HPO_4 – 0.15 M NaCl obtained at 37^0C and initial pH 7.4, 7.0, 6.5, 6.0 and 5.0 (15, 16).
SLIKA 1.
Prema ref. (14). Opći taložni dijagram kalcijevih fosfata dobiven nakon 24 sata. Naznačene su granica topljivosti te granica između područja direktnog kristalnog rasta (A) i područja kristalizacije preko prekursorske faze (B). U području B početna prekursorska faza bila je amorfni kalcijev fosfat (ACP). Podaci, izraženi kao prosječni produkti aktiviteta u ovisnosti o pH preračunati su iz taložnih dijagrama sustava : $CaCl_2$ – HPO_4 – 0.15 M NaCl dobivenih pri 37^0C i početnim pH 7.4, 7.0, 6.5, 6.0 i 5.0 (15, 16).



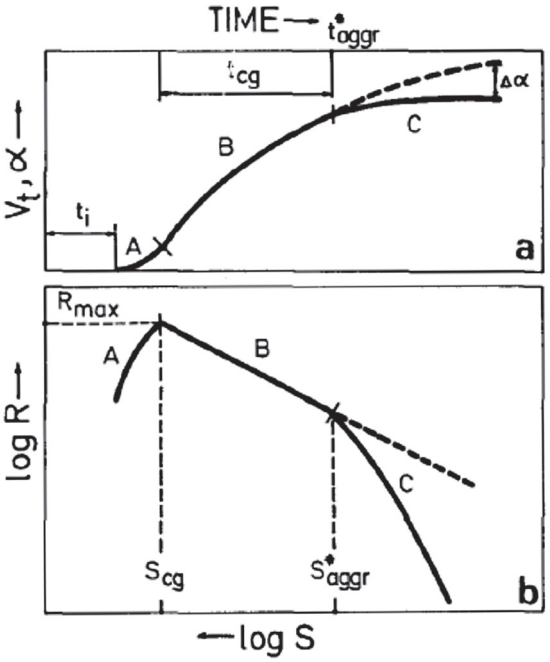
calcium oxalates, uric acid and urates appear as different crystal hydrates, which are likely to undergo phase transformation and/or aggregation processes. The thermodynamics and kinetics of crystallization processes depend on the experimental conditions (reactant concentrations, pH, ionic strength, temperature, time of aging, additives). Thus the first approach might be to construct adequate precipitation diagrams to map the properties of precipitates formed at specified times as functions of the experimental conditions, while in the next step one should learn about the kinetics of crystallization in specified systems. In the Laboratory for Precipitation Processes at the Rudjer Boskovic Institute precipitation diagrams were used to study the properties of biomineral and urinary salts precipitating from solutions at biologically relevant pH, temperature and ionic strength. The following systems were investigated: calcium phosphates (14-16, 18), (calcium oxalates (17, 18), magnesium phosphates (struvite and newberyite, (19, 20)), uric acid, sodium and calcium urates (21, 22).

A general precipitation diagram of the calcium phosphate system, in which the precipitation boundaries are defined by the mean ion activity products and the pH is shown in Fig. 1 (after ref. (14)). It shows that the mechanism of crystallization (and consequently the composition and structures of the emerging solid phases) are pH dependent, the critical transition region lies between pH 6 and 7.

Two precipitation regions are defined: A region of direct precipitation (region A) and, at higher ionic products and higher pH values, a region of crystallization via precursor phases (region B). At pH $\geq$ 7.5 region B is prevailing in the whole concentration range. The data used in Fig. 1 are from precipitation diagrams constructed at different constant initial pH values (15, 16), which show that in region A the prevailing crystal phase is calcium hydrogenphosphate dihydrate, DCPD, presumably formed by heterogeneous nucleation upon solution impurities. At higher activity products and pH > 6 (region B) the first precipitate formed was amorphous calcium phosphate (ACP) (16), which later transformed into octacalcium phosphate (OCP) and/or calcium deficient apatite, DA. These findings are in accordance with kinetic data (23- 25). The number of particles recorded in region B is in the range found for precipitates formed by homogeneous nucleation (ref. (14), for details about heterogeneous and homogeneous nucleation see also ref. (26)).

FIGURE 2.
Schematic presentation of a kinetic precipitation experiment. Upper diagram represents experimental data plotted in terms of ά vs time or V_t vs. time curves from which the rate vs supersaturation curve (lower diagram) is recalculated according to equ. 1. Sections A, B and C correspond in time and indicate time periods in which nucleation (A), crystal growth (B) and crystal growth inhibited by aggregation (C) are rate controlling. After ref. (28).

SLIKA 2.
Shematski prikaz kinetičkog taložnog eksperimenta. Na gornjem dijagramu prikazani su eksperimentalni podaci kao krivulje ά ili V_t u ovisnosti o vremenu iz kojih je krivulja brzina u ovisnosti o presićenju (donji dijagram) preračunata prema jednadžbi 1. Područja A, B, C označavaju vremena u kojima su nukleacija (A), kristalni rast (B) i kristalni rast inhibiran agrgacijom (C) stupnjevi koji kontroliraju brzinu. Prema ref. (28).



Further efforts have been concentrated to study the kinetics of precipitation and phase transformation in some of the most relevant systems in physiological mineralization (i.e. the calcium phosphate system, refs (24, 25, 27)) and in urolithiasis (i.e. calcium oxalates, refs (27 – 29) and magnesium phosphates (30)). It was possible to construct kinetic diagrams of spontaneous precipitation systems, by which subsequent precipitation processes (nucleation, crystal growth, growth and aggregation) could be resolved in time (27, 28). A schematic presentation of the procedure is shown in Fig. 2 (after ref. (28)). From experimental kinetic data (upper diagram) the rate of precipitation R has been calculated according to equation (1) and plotted as a function of the supersaturation to a given salt (lower diagram in Fig 2).

$$R = (d\acute{\alpha}/dt)\ \acute{\alpha}^{-2/3} = kN^{1/3}S^p \qquad (1)$$

where S is the supersaturation, N is the number of particles per cm^3, k is a proportionality constant and p is the order of the reaction.

The degree of the reaction, ά is defined in terms of the reactant concentrations, c or the precipitate volume V as

$$\acute{\alpha} = (c_t - c_s)/(c_o - c_s) = V_t / V_{max} \qquad (2)$$

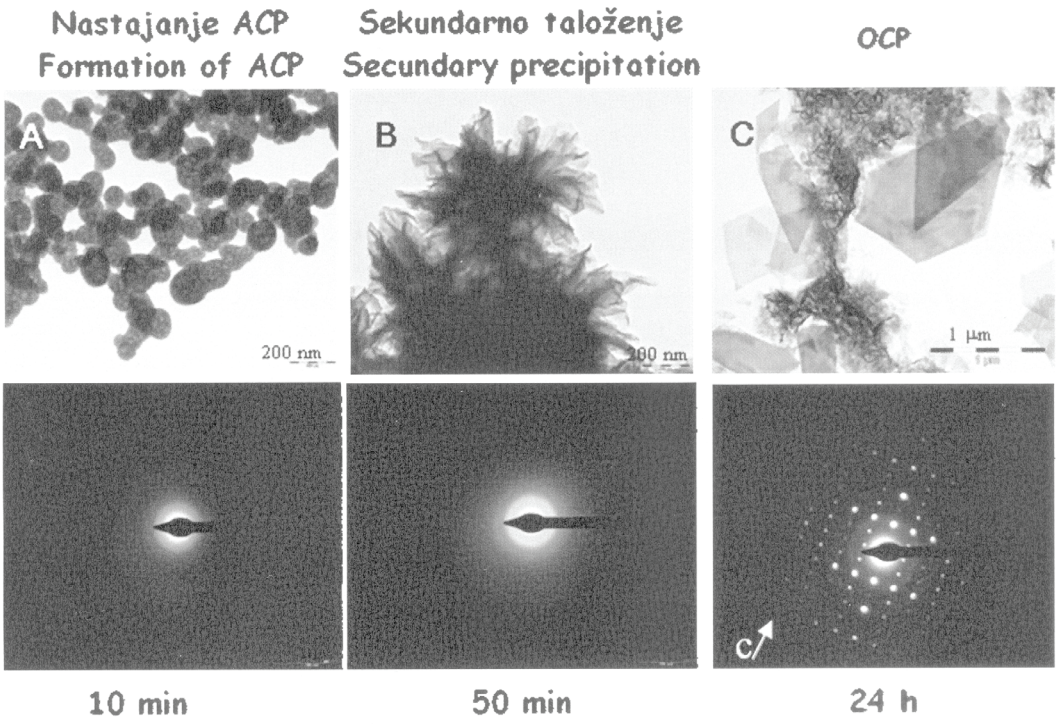
In equation (2) c_o , c_s and c_t are solute concentrations at zero time, time t and at equilibrium, respectively, while V_t and V_{max} are the precipitate volume at time t and at equilibrium.

In such diagrams sections corresponding to the respective rate controlling processes could easily be distinguished (see Fig.2 and ref. (28)). The method has been applied to studies of precipitation of calcium oxalates from high ionic strength solutions and the influence of additives thereon (27 - 29).

The kinetics of ACP to crystalline phase transformation is shown in Figs. 3 and 4 (after ref. (24, 25, 40)). In Figure 3 the process is visualized by electron micrographs, while the corresponding kinetic curve in Fig 4 shows regions of ACP metastability (A) followed by fast secondary crystal growth at the surfaces of ACP particles (B) and a region of ripening and phase transformation on expense of the remaining amorphous material (C). Under the specific conditions of precipitation ACP converted into octacalcium phosphate within 24h (Fig. 3). A kinetic parameter, the induction time, t_t i.e. the time needed for the onset of secondary precipitation, could be defined as shown in Fig. 4.

2.2. Promoters and inhibitors - the multiple roles of additives in crystallization. For the materials scientist two important conclusions emerge from section 1:

FIGURE 3.
Electron micrographs and corresponding electron diffraction patterns of amorphous calcium phosphate (ACP) particles, initial crystallization upon an ACP particle and aggregates of octacalcium phosphate (OCP) crystals observed 24h after sample preparation. After (24, 45).
SLIKA 3.
Elektronske mikrografije i odgovarajuće difrakcijske slike elektrona čestica amorfnog kalcijevog fosfata (ACP), početne kristalizacije na česticama ACP i agregata kristala oktakalcijevog fosfata (OCP) dobivenih nakon 24 sata od pripreme sustava. Prema (24, 45).



- Both physiological and pathological mineralization may be seen as crystallization upon and/or within an organic matrix.
- In both processes the presence or deficiency of promoters and/or inhibitors of crystallization is critical.

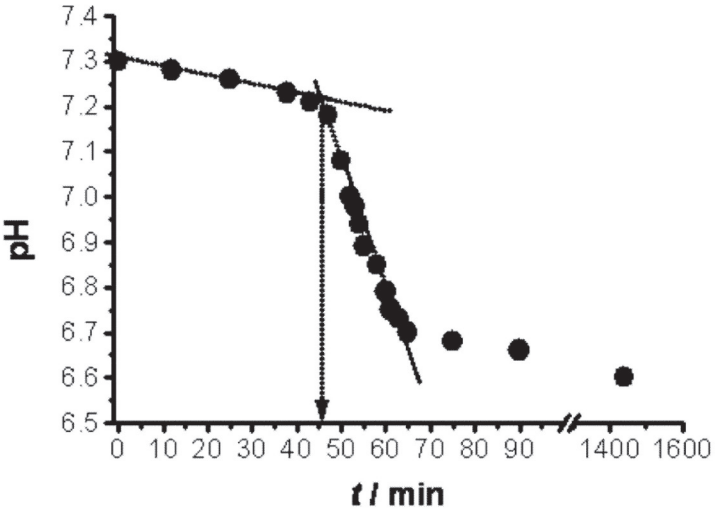
In physiological mineralization (i. e. bone and tooth formation) the collagen and/or amelogenin matrices provide a framework for mineralization while non-collageneous acidic and/or phosphorylated matrix macromolecules are involved in the control of nucleation, crystal growth and phase transformation of the mineral phase (1 – 4). In some forms of pathological mineralization, such as kidney stone formation, crystals are originally generated in the body fluid, then attach to the "organic matrix" (i.e. to defective parts of the kidney walls) and proceed growing and aggregating into an urolith (11 – 13). As in physiological mineralization, at all stages of urolith formation the presence or absence of small and macromolecular inhibitors / promoters in the matrix and/or the body fluid (the urine) is a controlling factor.

It has been shown by numerous examples, including examples relevant to biomineralization and urolitiasis (31, 32) (see also M. Dutour Sikirić, V. Babić-Ivančić and N. Filipović-Vinceković in this volume) that small and macromolecular inhibitors/promoters can influence every step of the crystallization process, i.e. they can promote or inhibit heterogeneous nucleation, retard crystal growth and/or induce habit modification, stabilize metastable phases and inhibit or promote aggregation of crystals or amorphous precursor phases. Of special interest for this treatise, because of their relevance to physiological and pathological mineralization, are organic – inorganic interactions in the calcium oxalate (33 – 38) and calcium phosphate (39 - 45) crystallization systems. Here we discuss but one example, the influence of polyelectrolytes on the phase transformation of ACP in high ionic strength solutions (45).

The role of ACP as precursor in calcium phosphate crystallization has been discussed in section 2.1 and its involvement in some forms of biomineralization has been demonstrated by other authors (8). When ACP transforms into crystalline phases (octacalcium phosphate, calcium deficient hydroxyapatite) the induction time, t_i and the structure of the ensuing solid phase depend on the experimental conditions (pH, reactant concentrations, ionic strength, concentrations of additives). Using the kinetic parameter, t_i (i.e. the time elapsed before the onset of secondary precipitation) as a criterion (Figs. 4, 5) it was possible to explore the influence of additives on ACP – apatite phase transformation (45).

As an example the influence of increasing concentrations of high and low molecular weight poly-L-glutamate (PGA)

FIGURE 4.
pH vs time curve representing the formation and phase transformation of amorphous calcium phosphate (ACP). Sections A, B and C correspond to the respective electron micrographs in Fig. 3. A – metastable region of ACP particles, B – secondary precipitation. New crystals form at the surfaces of the ACP particles. C – region of solution mediated crystal growth on account of the amorphous material. The kinetic parameter, t_i (time of metastability of ACP or induction time elapsed before the onset of secondary precipitation) is marked with an arrow. After ref. (45).
SLIKA 4.
Krivulje pH u ovisnosti o vremenu nastajanja i fazne transformacije amorfnog kalcijevog fosfata (ACP). Odgovarajuće elektronske mikrografije taloga nastalog u područjima A, B, C prikazane su na slici 3. A- metastabilno područje čestica ACP, B – sekundarno taloženje. Novi kristali nastaju na površini čestica ACP. C – područje kristalnog rasta putem otopine na račun amorfnog materijala. Kinetički parametar, t_i (vrijeme metastabilnosti ACP ili vrijeme indukcije proteklo prije početka sekundarnog taloženja) označen je strelicom. Prema referenci (45).



and poly-L-lysine (PLL) on t_i is shown in Fig. 5 (after ref. (45)). When the values of t_i are plotted as a function of the polymer concentration the dual effect of the molecules becomes apparent – at low concentrations the polymers act as promoters, while at higher concentrations they act as inhibitors of phase transformation of ACP.

A schematic presentation of likely mechanisms of these effects is shown in Fig. 6 (after ref. (45)). Induction and inhibition of crystal nucleation at the surfaces of ACP particles may be explained as follows: At low concentrations polyelectrolyte molecules adsorb in random conformation and the adsorption process is reversible. Since the adsorbed molecules can overcharge the ACP particles, and also inhibit aggregation, a large number of small, highly charged particles is created, (see also ref. (41)), which concentrate oppositely charged Ca^{2+} and/or HPO_4^{2-} ions and thus provide effective sites for secondary nucleation and crystal growth (Fig. 6A). On the other hand, at high concentrations polymer molecules spread until they completely envelop the amorphous particles (Fig. 6B) thus hindering the transport of ions to the template surface, consequently delaying or completely hindering nucleation of the secondary precipitate. This effect was especially strongly expressed in the presence of poly-L-glutamate (45).

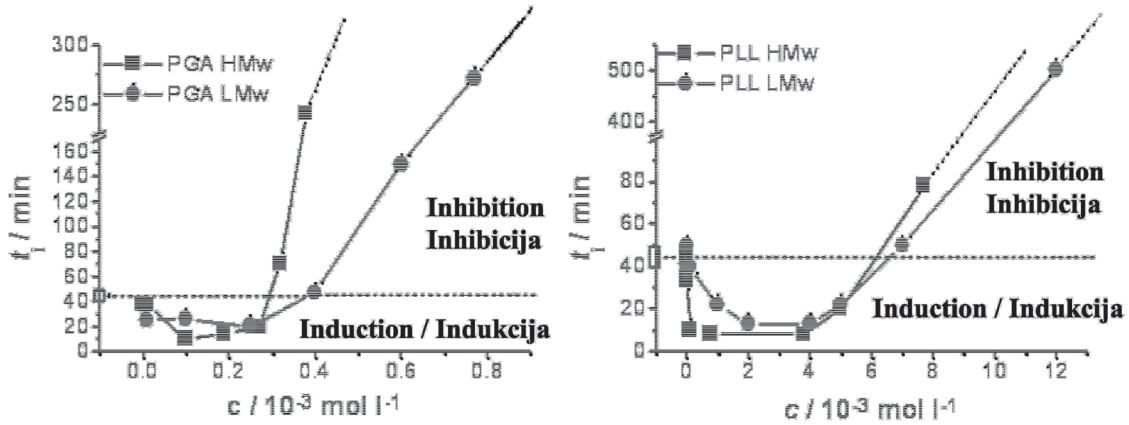
The findings are consistent with other studies of the behavior of polyelectrolytes in inorganic precipitation systems (31). Another interesting example is the study of the effect of polyglutamic acid, polylysine and heparin on the crystallization of calcium oxalate (46). In this experiment the authors show that by far the most efficient retardation is achieved with polyglutamate, but the addition of a specific low concentration of the polymer greatly enhanced the nucleation rate in the same crystallization system. It might be possible to envisage a mechanism similar to the one outlined above (Fig. 6.) in this case as well.

3. Applied interdisciplinary research

The results described in this section have been obtained by interdisciplinary teams, involving MD's, physical, analytical and clinical chemists, cell biologists and their laboratories. Because of the complexity of the systems involved, the research could not have been accomplished without a strictly interdisciplinary approach.

3.1. Methods for discriminating between calcium stone formers and healthy individuals. The formation of kidney stone is an extremely complex process, some aspects of which have been discussed above (Sections 1.3 and 2.2.). In the eastern continental region of Croatia (Osijek and surroundings) calcium oxalate monohydrate, (COM) stones, apatite stones and their mixtures were most frequently found (12). From metabolic analysis the following risk factors for calcium stone formation have been identified (12, 47): (a) Hypercalciuria, hyperoxaluria and high pH, which determine

FIGURE 5.
Plots of t_i as a function of the polyelectrolyte concentration for amorphous calcium phosphate (ACP) phase transformation in the presence of A – high and low Mw poly-L-glutamate and B – high and low Mw poly-L-lysine. The horizontal dotted lines correspond to t_i in the control system without polyelectrolyte. Values of $t_i < t_i$(control) indicate induction and $t_i > t_i$(control) show inhibition of secondary precipitation (marked on the diagrams). After ref. (45).
SLIKA 5.
Grafovi ovisnosti t_i o koncentraciji polielektrolita za faznu transformaciju amorfnog kalcijevog fosfata (ACP) u prisutnosti A – poli-L-glutamata visoke i niske molekulske težine i B – poli-L-lizina visoke i niske molekulske težine. Vrijednosti $t_i < t_i$(kontrola) ukazuju na indukciju, a $t_i > t_i$(control) pokazuju inhibiciju sekundarnog taloženja (označeno na grafovima). Prema (45).



A B

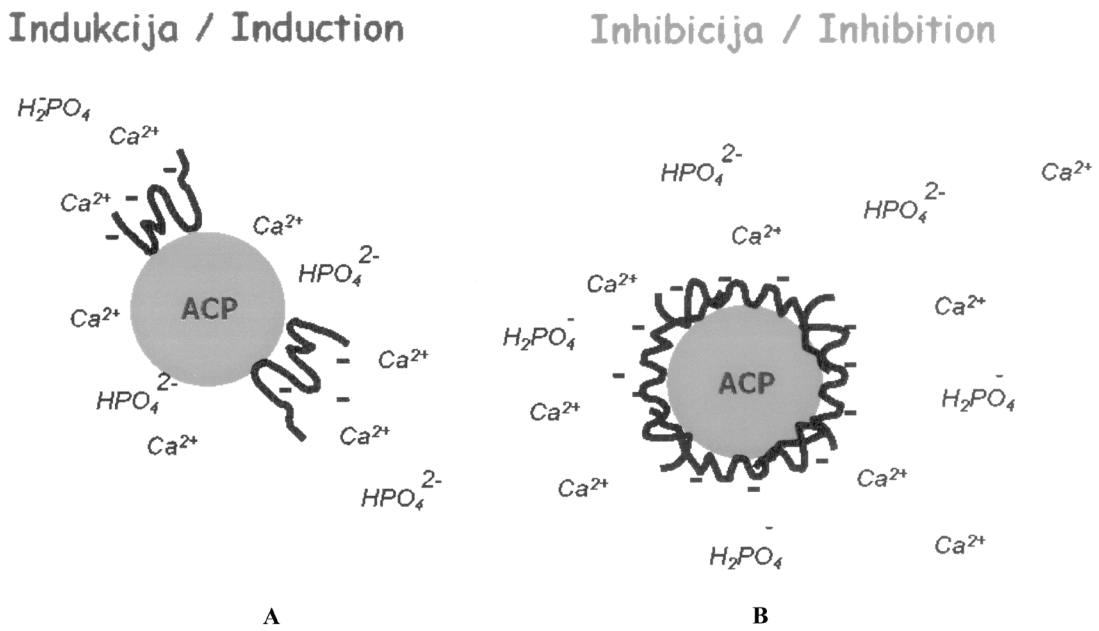
the supersaturation of the urine to the critical urinary salts and (b) a lack of inhibitors, i.e. a high Ca/citrate ratio (a consequence of hypocitrateuria, (12)) and low concentrations of acid mucopolysaccharides (AMPS (47)). Complicating diagnoses based on metabolic factors is also the dual role of organic molecules as promoters and/or inhibitors of crystallization (see Section 2.2). A practical way to circumvent these difficulties and to enable relatively quick analysis in a clinical Laboratory is the determination of the risk of stone formation in whole urine. Three simple chemical methods to distinguish between stone formers and healthy individuals have been developed (48, 49). Two of the methods are based on overnight precipitation from the test urines (48). In the first method (method A) precipitation was initiated by the addition of COM seed crystals, while in the second method (method B) the concentrations of calcium and oxalate in unseeded urine were adjusted to equal, predetermined values. After overnight incubation the differences in calcium concentrations between unseeded and seeded urine (A) and of total and residual soluble calcium concentrations (B) were analytically determined. The analysis was carried out for urines of stone formers and healthy individuals and impressive differences were obtained, showing that stone formers lack inhibitors of precipitation that are present in urines of non-formers. The third method (49) is based on the determination of the calcium binding capacity (CBC) of whole urine by titration of early morning urine with a calcium chloride solution. For this purpose a calcium selectrode with a disposable membrane, which can be prepared and replaced in the laboratory, was used. Plots of the calcium ion concentration versus the concentration of total (added) calcium were linear up to a point, where precipitation of calcium salts commenced. The slopes of such titration lines are inversely proportional to the CBC and are therefore indicative of the amount of calcium complexing molecules in the urine. Thus synthetic urine-like solutions, if devoid of calcium binding matter, should give titration curves with the slope of 1. In the experiments described in (49) titration curves of healthy subjects had slopes of 0.2 – 0.3 in contrast to stone forming urines, with slopes of titration curves of 0.5 – 0.8. Statistical evaluation gave mean values of 0.31 for healthy urines (13 samples) and 0.64 for stone formers (26 samples). There was good correlation with the clinical situation as well.

3.2. Materials for the replacement and/or repair of bone and teeth:

To alleviate the problem of osteointegration of metals, differently prepared surface coatings consisting of calcium phosphates have been applied. Coating methods involving the immersion of pretreated material into "simulated body fluid" (i.e. a solution containing the inorganic constituents of blood serum, (see Table 2.) have attracted attention (9, 50).

In the course of international collaborations between materials scientists, cell biologists, dentists and medical doctors, a new class of biomimetic organic-inorganic composite coatings has been designed in our laboratories (refs. (51, 52) see also M. Dutour Sikirić, V. Babić-Ivančić and N. Filipović-Vinceković in this volume). In the design we tried to mimic the basic strategy by which mineral is deposited in some forms of biomineralization, i.e. to first lay down an organic matrix, which is then mineralized in stages through the deposition of

FIGURE 6.
Schematic presentation of amorphous calcium phosphate (ACP) particles formed in the presence of negatively charged polyelectrolyte. A – induction of secondary precipitation at low polyelectrolyte concentration, B – inhibition of secondary precipitation at high concentration of polyelectrolyte. After ref. (45).
SLIKA 6.
Shematski prikaz čestica amorfnog kalcijevog fosfata (ACP) nastalih u prisutnosti negativno nabijenog polielektrolita. A – indukcija sekundarnog taloženja pri niskim koncentracijama polielektrolita, B – inhibicija sekundarnog taloženja pri visokim. koncentracijama polielektrolita. Prema ref. (45).



poorly crystallized mineral and subsequent maturation (see Section 1.1 and ref. (8)).

In these experiments the organic matrix consisted of PLL/PGA multilayers (for polyelectrolyte multilayer design see ref (52)) into which layers of ACP particles were incorporated. The assembly was then immersed into a metastable calcifying solution, thus enabling ACP phase transformation and crystal growth "in situ" within the organic matrix. The rough crystal surfaces obtained were then smoothened by adding a final $(PLL/PGA)_3$ multilayer, thus enabling good attachment and proliferation of bone forming cells. The coatings have been tested by extensive cell culture tests and by a validated "in vivo" pull-out model in rabbit tibia with uncoated chemically etched titanium plates serving as controls (51). The results of both "in vitro" and "in vivo" experiments show excellent biological properties of the coated titanium plates. Thus, when tested in the animal experiment, the coating significantly enhanced the attachment of titanium implants to the bone as compared to uncoated titanium plates, with more than twice the force needed to detach coated implants.
The above described organic-inorganic composite coatings have several advantages:

- The deposition of polyelectrolyte multilayers is largely independent of the type and topology of the substrate. It is thus possible to apply the coatings to a wide variety of bioinert implant materials of different shapes and sizes.
- Bioactive molecules can be co-adsorbed into the organic matrix or coprecipitated with the inorganic phase without loosing their bioactivity.
- Because of "in situ" crystal growth of the inorganic phase the coating is tightly anchored to the substrate exhibiting satisfactory mechanical stability.
- The coating procedure is simple, energy saving and environmentally friendly and could be easily up scaled for factory production.

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REFERENCES

1. Loewenstam HA, Weiner S. On Biomineralization. New York, Oxford: Oxford University Press; 1989.
2. Weiner S, Traub W. Bone structure: from angstroms to microns. FASEB J 1992;882:879-85.
3. Addadi L, Weiner S. Interactions between acidic proteins and crystals - stereochemical requirements in biomineralization. Proc Natl Acad Sci (USA) 1985;82:4110-14.
4. Veis A, Perry A. The Phosphoprotein of the Dentin Matrix. Biochemistry 1967;6:2409 -16.
5. Murshed M, Mc Kee MD. Molecular determinants of extracellular matrix mineralization in bone and blood vessels. Cur Opin Nephrol Hypertens 2010;19:359-65.
6. Ali SY, Sajdera SW, Anderson HC. Isolation and Characterization of Calcifying Matrix Vesicles from Epiphyseal Cartilage. Proc Natl Acad Sc. 1970;67:1513-20.
7. Landis WJ. A study of calcification in the leg tendons from the domestic Turkey. J Ultrastr Mol Struct Res 1986 ;94 :217-38
8. Loewenstam HA, Weiner S. Transformation of Amorphous Calcium Phosphate to Crystalline Dahillite in the Radular Teeth of Chitons. Science 1985;227:51-53.
9. Kokubo T. Apatite formation on surfaces of ceramics, metals and polymers in body environment. Acta Mater 1998;46:2519-27.
10. Achilles W, Dekanić D, Burk M, Schalk Ch, Tucak A, Karner I. Crystal growth of Calcium oxalate in urine of stone-formers and normal controls. Urol Res 1991;19: 159-64.
11. Söhnel O, Grases F. Calcium oxalate monohydrate renal calculi. Formation and development mechanism. Adv Coll Interf Sci. 1995;59:1-17.
12. Tucak A, Šerić V, Kozmar D, Sikirić M, Zorić I, Babić-Ivančić V. Correlation of urine metabolic factors and urinary stones composition. Period Biol 1999; 101: 35 - 44.
13. Wernes PG, Bergert JH, Smith LH. Crystalluria. J Crystal Growth 1981; 53: 166-181.
14. Füredi-Milhofer H, Brečević Lj, Purgarić B. Crystal growth and phase transformation in the precipitation of calcium phosphates. Faraday Disc Chem Soc 1976;6:184-193.
15. Füredi-Milhofer H, Purgarić B, Brečević Lj, Pavković N. Precipitation of Calcium Phosphates from Electrolyte Solutions. I. A Study of Precipitates in the Physiological pH Region. Calc Tissue Res 1971;8:142-53.
16. Füredi-Milhofer H, Oljica-Žabčić E, Purgarić B, Pavković N. H. Precipitation of calcium phosphates from electrolyte-solutions. IV. Precipitation diagrams of the system calcium chloride-sodium phosphate-0.15 M sodium chloride. J Inorg Nucl Chem 1975;37:2047-51.
17. Babić-Ivančić V, Füredi-Milhofer, Purgarić B, Brničević N , Despotović Z. Precipitation of Calcium Oxalates from High Ionic Strength Solutions. III. The Influence of Reactant Concentrations on the Properties of the Precipitates. J Crystal Growth 1985;71: 655-63.
18. Füredi-Milhofer H, Marković M, Uzelac M. Precipitation and Solubility of Calcium Phosphates and Oxalates in the System $Ca(OH)_2$ - H_3PO_4 - $H_2C_2O_4$ - NaCl - H_2O, J Crystal Growth 1987;80:60-8.
19. Babić-Ivančić V, Kontrec J, Kralj D, Brečević Lj. Precipitation Diagrams of Struvite and Dissolution Kinetics of Different Struvite Morphologies. Croat Chem Acta 2002; 75: 89-106.
20. Babić-Ivančić V, Kontrec J, Brečević Lj. Formation and transformation of struvite and newberyite in aqueous solutions under conditions similar to physiological. Urol Res, 2004;32:350-56.
21. Füredi-Milhofer H, Babić-Ivančić V, Milat O, Brown WE, Gregory TM. Precipitation of Sodium Acid Urate from Electrolyte Solutions. J Crystal Growth 1987; 83 572-80.
22. Babić-Ivančić V, Füredi-Milhofer H, Brničević N, Marković M. Precipitation and Solubility of Calcium Hydrogenurate Hexahydrate. J Res Natl Inst Stand Technol 1992;97:365-72.
23. Eanes ED, Gillesen IH, Posner AS. Intermediate states in the precipitation of hydroxyapatite. Nature 1965;208:365-7.
24. Brečević Lj, Füredi-Milhofer H. Precipitation of calcium phosphates from electrolyte solutions .2. Formation and transformation of precipitates. Calcif Tissue Res 1972;10:82-90.
25. Despotović R, Filipović-Vinceković N, Füredi-Milhofer H. Precipitation of calcium phosphates from electrolyte solutions. 3. Radiometric studies of kinetics of precipitation and aging of calcium phosphates. Calcif Tissue Res 1975;18:13-26.
26. Füredi-Milhofer H, Walton AG. Principles of precipitation of fine particles. U: Parfitt GD, ur. Dispersion of powders in liquids. 3rd ed. New Jersey: Appl. Sci. Publ. London; 1981. str. 203-272.
27. Marković M, Füredi-Milhofer H. Precipitation of Calcium Oxalates from High Ionic Strength Solutions. Part 6. Kinetics of Precipitation from Solutions Supersaturated in Calcium Oxalates and Phosphates. J Chem Soc Faraday Trans 1988;1:1301-10.
28. Škrtić D, Marković M, Füredi-Milhofer H. Precipitation of calcium oxalates from high ionic strength solutions IV. Testing of kinetic models. J Cryst Growth 79 (1986) 791-6.
29. Füredi-Milhofer, H.; Škrtić, D.; Marković M, Komunjer Lj. Kinetic Analysis of the Influence of Additives on Spontaneous Precipitation from Electrolytic Solutions, Prog Colloid Polymer Sci 1988; 77:195-207.
30. Babić-Ivančić V, Kontrec J, Brečević Lj, Kralj D. Kinetics of struvite to newberyite transformation in the precipitation system $MgCl_2$–$NH_4H_2PO_4$–NaOH–H_2O. Water Res 2006;40:3447-55.
31. Füredi-Milhofer H, Sarig S. Interactions Between Polyelectrolytes and Sparingly Soluble salts. Prog Cryst Growth Charact Mater 1996; 32: 45-74.
32. Dutour Sikirić M, Füredi-Milhofer H.The influence of surface active molecules on the crystallization of biominerals in solution. Adv Colloid Interface Sci 2006;128:135-58.
33. Gardner GL, Doremus RH. Crystal growth inhibitors in human urine. Effect on calcium oxalate kinetics. Invest Urol 1978;15:478-85.
34. Eidelman NR, Azoury R, Sarig S. Reversal of trends in impurity effects on crystallization parameters. J Cryst Growth 1986;74:1-9.
35. Škrtić D, Filipović-Vinceković N. Inhibition of calcium oxalate crystallization by sodium dodecyl sulphate. J Crystal Growth 1988;88:313-20.
36. Brečević Lj, Kralj D. The influence of some amino acids on calcium oxalate dihydrate transformation. J Crystal Growth 1986;79:178-84.
37. Füredi-Milhofer H, Tunik L, Filipović-Vinceković N, Škrtić D, Babić-Ivančić V, Garti N. Crystallization of Calcium Oxalate Dihydrate in Micellar Solutions of Anionic Surfactants. Scan Microscopy 1995;9:1061-70.
38. Füredi-Milhofer H, Bloch R, Škrtić D, Filipović-Vinceković N, Garti N. Induction of Crystallization of Specific Calcium Oxalate Hydrates in Micellar Solutions of Surfactants. J Disp Sci Techn 1993;14: 355-71.
39. Hlady V, Füredi-Milhofer H. Adsorption of human serum albumin on precipitated hydroxyapatite. J Colloid Interface Sci 1979;69:460-8.
40. Brečević Lj, Sendijarević A, Füredi-Milhofer H. Precipitation of Calcium Phosphates from Elactrolyte Solutions. VII. The Influence of Di- and Tricarboxylic Acids. Colloids and Surfaces 1984;11:55-68.
41. Brečević Lj, Hlady V, Füredi-Milhofer H. Influence of gelatin on the precipitation of Amorphous Calcium Phosphate. Colloids and Surfaces 1987;28:301-13.
42. Füredi-Milhofer H, Babić-Ivančić V, Brečević Lj, Filipović-Vinceković N, Kralj D, Komunjer Lj, Marković M, Škrtić D. Factors Influencing Nucleation from Solutions Supersaturated to Different Crystal Hydrates. Colloids and Surfaces 1990;48:219-30.
43. Füredi-Milhofer H, Moradian-Oldak J, Weiner S, Veis A, Mintz KP, Addadi L. Interactions of matrix proteins from mineralized tissues with octacalcium phosphate. Connect Tissue Res 1994;30:251-264.
44. Sikirić M, Babić-Ivančić V, Milat O, Füredi-Milhofer H. Factor Influencing Additive Interactions with Calcium Hydrogenphosphate Dihydrate Crystals. Langmuir 2000;16:9261-66.

45. Bar Yosef Ofir P, Govrin-Lippman R, Garti N, Füredi-Milhofer H. The influence of polyelectrolytes on the formation and phase transformation of amorphous calcium phosphate. Cryst Growth Des 2004;4:177-83.
46. Drach GW, Sarig S, Randolph AD, Thorson S. The paradox of inhibition and enhancement of the formation of urinary stones. Urol Res 1982;10:165–8.
47. Robertson WG, Peacock M, Heyburn PJ, Marshall DH, Clark PB. Risk Factors in Calcium Stone Disease of the Urinary Tract. British J Urol 1978;50:449-54.
48. Marković M, Vicković Đ. Methods for testing urines for the precipitation of calcium salts. U: Excerpta Medica: Proceedings of the 1st European Symposium on Urolithiasis. Amsterdam, Hong Kong, Princenton, Sydney, Tokio: 1990. p. 114-6.
49. Füredi-Milhofer H, Kiss K, Kahana F , Sarig S. New Method for Discriminating between Calcium Stone Formers and Healthy Individuals. Brit J Urol 1993; 71: 137–42.
50. Wen HB, de Wijn JR, Cui FZ, de Groot K. Preparation of calcium phosphate coatings on titanium implant materials by simple chemistry. J Biomed Mater Res 1998;41:227-36.
51. Dutour Sikirić M, Gergely C, Elkaim R, Wachtel E, Cuisinier FJG, Füredi-Milhofer H. Biomimetic organic-inorganic nanocomposite coatings for titanium implants J Biomed Materi Res Part A 2009;89A:759-71.
52. Schade R, Dutour Sikirić M, Lamolle S, Ronold HJ, Lyngstadass SP, Liefeith K, Cuisinier F, Füredi-Milhofer H. Biomimetic organic-inorganic nanocomposite coatings for titanium implants. In vitro and In vivo Biological Testing. J Biomed Materi Res Part A, 2010;95A:691-700.
53. Decher G. Fuzzy nanoassemblies: Toward layered polymeric multicomposites. Science 1997;277:1232-7.

FIZIOLOŠKA I PATOLOŠKA MINERALIZACIJA: NEKI PROBLEMI I MOGUĆA RJEŠENJA

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Pregledni rad

SAŽETAK

U prvom dijelu ovog pregleda prikazali smo sličnosti i razlike između fiziološke i patološke biomineralizacije, tj. nastajanja kostiju i zubi, osteoartikularne i zubne patologije, te urolitijaze. Pojednostavljeno možemo ove procese smatrati taloženjem ionskih kristala iz otopina visoke ionske jakosti na i/ili unutar organske matrice. Prisutnost ili manjak promotora ili inhibitora kristalizacije u svim su slučajevima od izuzetne važnosti. Međutim, dok je fiziološka mineralizacija strogo biološki kontrolirana, urolitijaza je uglavnom kontrolirana fizičko-kemijskim faktorima kao sto su prezasićenost urina, pomanjkanje inhibitora kristalizacije i agregacije, itd.

U drugom dijelu preglednog rada opisana su fizičko-kemijska istraživanja, izvedena zbog boljeg razumijevanja procesa biomineralizacije. Konstruirani su taložni dijagrami za sustave kalcij i magnezij fosfata, kalcij oksalata, mokraćne kiseline, te natrij i kalcij urata za slučaj taloženja iz otopina visoke ionske jakosti u biološki relevantnim uvjetima. Prikazan je generalni taložni dijagram kalcij fosfata sa granicom taloženja i granicom između područja, gdje kristali nastaju direktnim rastom i onim gdje nastaju putem transformacije prekusorske faze. Istraživanja kinetike taloženja prikazana su dijagramima, koje možemo interpretirati u smislu sukcesivnih ili paralelnih taložnih procesa, kao što su nukleacija, kristalni rast i agregacija. Kinetičkom studijom fazne transformacije amorfnog kalcij fosfata (ACP) u oktakalcij fosfat i/odn. apatit pokazali smo, da makromolekule, kao što su poli-L-glutamat i poli-L-lisin mogu imati dvostruko djelovanje, tj kada su prisutne u niskoj koncentraciji djeluju kao promotori, a u visokim koncentracijama kao inhibitori sekundarnog taloženja kristalne faze na površini amorfnih čestica.

U trećem dijelu pregleda opisana su primijenjena interdisciplinarna istraživanja. Razrađene su tri kemijske metode za testiranje inhibitorskog potencijala nerazrijeđenog urina. Metode su jednostavne i pogodne su za primjenu u kliničkim laboratorijima za razlikovanje urina pacijanata s urolitijazom od urina zdravih osoba.

U posljednje vrijeme pripremljene su nove vrste organsko – anorganskih kompozita. kao presvlake za bioinertne metalne implante. Postupak pripreme uključuje „in situ" rast kristala kalcij fosfata unutar organsko - anorganske matrice (anorganska komponenta je ACP), koja je ranije adsorbirana na metalnu površinu. Tako pripremljene presvlake čvrsto prianjaju uz površinu supstrata i imaju izvrsna biološka svojstva, što smo provjerili „in vitro" i „in vivo" biološkim testovima.

Ključne riječi: Agregacija; Amorfni kalcijev fosfat – kemija; Biomineralizacija; Fazna transformacija; Inhibitori; Interdisciplinarna istraživanja; Kinetike taloženja; Kristalizacija; Organsko-anorganske kompozitne presvlake; Promotori; Taložni dijagrami; Urolitijaza – mokraća, patologija, prevencija & kontrola