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Is there a relationship between solubility and resorbability of different calcium phosphate phases *in vitro*?

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ABSTRACT

Background: Does chemistry govern biology or it is the other way around - that is a broad connotation of the question that this study attempted to answer.
Method: Comparison was made between the solubility and osteoclastic resorbability of four fundamentally different monophasic calcium phosphate (CP) powders with monodisperse particle size distributions: alkaline hydroxyapatite (HAP), acidic monetite (DCP), β-calcium pyrophosphate (CPP), and amorphous CP (ACP). Results With the exception of CPP, the difference in solubility between different CP phases became neither mitigated nor reversed, but augmented in the resorptive osteoclastic milieu. Thus, DCP, a phase with the highest solubility, was also resorbed more intensely than any other CP phase, whereas HAP, a phase with the lowest solubility, was resorbed least. CPP becomes retained inside the cells for the longest period of time, indicating hindered digestion of only this particular type of CP. Osteoclastogenesis was mildly hindered in the presence of HAP, ACP and DCP, but not in the presence of CPP. The most viable CP powder with respect to the mitochondrial succinic dehydrogenase activity was the one present in natural biological bone tissues: HAP.
Conclusion: Chemistry in this case does have a direct effect on biology. Biology neither overrides nor reverses the chemical propensities of inorganics with which it interacts, but rather augments and takes a direct advantage of them.
Significance: These findings set the fundamental basis for designing the chemical makeup of CP and other biosoluble components of tissue engineering constructs for their most optimal resorption and tissue regeneration response.

1. Introduction

The field of hard tissue engineering has been preoccupied for a long time by the problem of designing bone replacement materials with the most optimal bioresorption rates. It has been established over time that, ideally, the degradation rate of the bone implant is to match the new bone tissue in growth rate. Hydroxyapatite (HAP), the chemically pure version of the mineral component of bone, however, is typified by impractically low resorption rates [1–3]. Reduction of the particle size [4], an increase in porosity [5] and introduction of ionic substitutions, such as magnesium [6], sodium [7], fluorine [8] and/or carbonate [9], so as to mimic the composition and microstructure of biological apatite, have presented approaches to resolving this issue, though with limited success. Namely, even the least sparingly soluble calcium-deficient apatites usually resorb slower than the new bone tissue formation rate [10], whereas HAP with high porosity is weak even to compression and not suitable for load-bearing applications [11]. An alternative approach has comprised admixing more resorbable calcium phosphates, typically tricalcium [12] or dicalcium [13] phosphates, or other calcium compounds, such as sulfates [14] or carbonates [15], to pure HAP.

Calcium phosphates (CPs), in fact, exist in a variety of phase compositions, whose solubility ranges from extremely soluble monocalcium phosphates (MCPs) to moderately soluble dicalcium phosphates (DCPs) to relatively insoluble tricalcium phosphates (TCPs) to sparingly soluble octacalcium phosphate and HAP. The main problem that the use of more resorbable CP phases as bone implants has faced is little control over bioresorption in spite of the known solubility profiles. For example, even though DCP dissolved three times faster than HAP in a solution whose inorganic composition matched that of blood serum [16], implantation of DCP often does not guarantee any faster resorption than implantation of HAP [17–19]. This phenomenon supposedly stems from the highly mobile surface layer of charged species common to all CP phases [20]. Constant dissolution and reprecipitation of this layer are expected to take place in solution and in body fluids, leading to an incessant reorganization of the surface in search of the most stable and chemically equilibrated phase composition for the given chemical conditions of its microenvironment. Thus, for example, after the first burst of dissolution, as a temporary equilibrium is established between the solid surface and the solution, DCP in contact with the body fluid is expected to form a layer of HAP on its surface, which would protect it from further dissolution

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[21–25]. Being the most stable CP phase at pH > 6.5 [26,27], HAP is supposed to partially cede place to DCP as an *in vivo* precipitate only under pathological conditions, such as cariogenesis [28], dental calculus formation [29], corneal calcification [30], atherosclerosis [31], calcific arthritis [32], renal and urinary lithiasis [33], osteomyelitis [34], or aggressive osteoporosis [35], or in the zones of excessive osteoclastic activity [36]. Such circumstances are not uncommon, which explains why uncertainties regarding the fate of CP implants in the body are still tied to their use. This is not even to mention the more complex biological processes, ranging from (a) phagocytic to (b) enzymatic degradation to (c) local variations in acidity to (d) more complete wetting of hydrophobic surfaces in biological environments than in aqueous solutions to (e) osteoblastic formation of new bone at the tissue/implant interface as a barrier to osteoclastic access and resorption [37], which could all, individually and in synergy, have an equal say in terms of contributing to deviations of the resorption rates *in vitro* and *in vivo* from the degradation profiles observed in simple solutions [38]. Some CP phases, moreover, do not reach a plateau in the concentration of dissolution units in the supernatant even after 30 days of immersion time [39], suggesting the unusual complexity of the dissolution process. Implant geometry, location of the surgical placement and the identity and composition of cells, proteins and ionic solutes at the implant/tissue interface are all involved in defining the resorption rate of CPs in the body [23]. As a result, the structural transformations conditioning the kinetics of resorption of CP are expected to be complexly dependent on an array of factors other than the simple dissolution propensities of the individual phases in question.

The purpose of this study was to assess the correlation between *in vitro* resorption profiles and solubilities of four different CP phases, ranging from 0.3 to 48 mg/l. Osteoclasts, multinuclear cells whose *in vitro* model, RAW264.7, was used in this study, dissolve the bony tissues by creating a low pH (3–4) environment [40] and phagocytosing the mineral particles. They work in precise orchestration with their antagonists, osteoblasts, cells that internally precipitate mineral nanoparticles and deposit them extracellularly, building new bone thereby. As ever, when it comes to proposing correlations between *in vitro* observations and the corresponding patterns present *in vivo*, caution is to be exercised, primarily because osteoclasts are not the only factors that govern the resorption of CPs in the body. Phagocytosis by macrophages is an additional effect that contributes to resorption of bioceramics [38,41]. The more rapid body fluid flow, reducing the concentration of dissolution units in the near vicinity of the implant, while simultaneously buffering it at a fairly constant level elsewhere, is another factor that greatly affects the resorption rate and is not so readily mimicked under *in vitro* conditions. Coupled to attrition to mimic cyclic mechanical loading, frequent solvent replenishments indeed promoted dissolution of DCP in buffered saline, perhaps by interfering with the protective HAP layer formation at physiological pH [42]. These results have implicitly suggested that different chemical environments and different mechanical loads greatly affect the degradation profile of CP implants and reiterated the unusual complexity of their fate in the body. Given that their degradation determines the outcome of the bone regeneration and healing process, a broader knowledge of these processes is needed to ensure more reliable, patient- and case-specific orthopedic and dental treatments, which is the niche that the purpose of this work falls in. Although the complexity of degradation pathways present in the body is far more complex than that of the *in vitro* model used in this study, the latter can still offer insights that deepen our knowledge of the degradation of CPs and help us better understand and clinically control its flipside: the regeneration process.

2. Materials and methods

2.1. Synthesis and characterization of different CP nanopowders

The synthesis of different CP powders followed the previously established protocols [43] and involved precipitation from either alkaline or pH-neutral aqueous solutions followed by bringing the precipitate alongside its parent solution to boiling in case of DCP and HAP, as well as annealing at 800 °C for 2 h in air for CPP. The degrees of saturation (DS) were calculated using an algorithm based on Debye-Hückel equation [14]:

$$DS = pK_{sp} - pQ \quad (1)$$

$$Q = \left\{Ca^{2+}\right\}^{x}\left\{PO_4^{3-}\right\}^{y}\left\{H^{+}\right\}^{z}\left\{OH^{-}\right\}^{w} \quad (2)$$

Q is the ionic activity product of the solution, and pK_{sp} is the negative logarithm of the solubility product of a given CP phase (Table 1). Activity coefficients were calculated through $\log\gamma = -Az_i^2 I^{1/2}$, where z_i is the charge number of ion species i, I is the ionic strength of the solution, and A is the-temperature dependent constant equal to 0.5115 at 25 °C. B and a_i are, like A, constants depending on temperature, dielectric constant of the solution and Debye screening length; $a_i = 6 \times 10^{-8}$ for Ca^{2+}; 9×10^{-8} for H^+; and 4×10^{-8} for $H_xPO_4^{x-3}/CaH_{2x}PO_4^{2x-1}$. Appropriate dissociation constants for H_2O and $H_xPO_4^{x-3}$ and association constants for $CaH_xPO_4^{x-1}$, $CaH_xCO_3^{x}$ and Ca—OH were taken into account as functions of pH [44].

X-ray diffraction (XRD) studies were carried out on a *Siemens* D500 diffractometer using $Cu_{K\alpha} = 1.5418$ Å as the wavelength of the radiation source. The average size of the crystallites was determined using a structural refinement approach (Topas 2.0) based on the previously identified crystal structure (PCPDFWIN & Eva) and taking the instrumental broadening into account. The morphology of the powders was analyzed on a *Hitachi* S-4300SE/N scanning electron microscope (SEM) at the electron beam energy of 15 kV.

2.2. Cell culture

Mouse monocyte macrophage RAW264.7 cells were obtained from *ATCC* and cultured in Dulbecco's modified Eagle medium (DMEM) with 10 vol% fetal bovine serum and 5 vol% amphotericin B (streptomycin/penicillin/fungisone) as the antibiotic/antimycotic. To differentiate them into an osteoclast-like phenotype, they were seeded at 1.5×10^5 cells/well in 24 well plates and incubated for 7–9 days in the medium additionally containing 35 ng/ml RANKL/TRANCE (*R&D system*). The medium was replaced every other day and differentiated, large multinucleated cells could be seen in the wells by day 5. On day 7, the cells were stained for Tartrate-resistant acid phosphatase (TRAP) using a commercial acid phosphatase leucocyte kit (*Sigma*). Images were obtained using an Olympus BX51 microscope (UIC core imaging facility).

2.3. Real time qPCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen) and cDNA synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems) from 100 ng of total RNA. cDNA was quantified using custom TaqMan probes for cathepsin K (CTSK) Mm00484039_m1, Acp5 (TRAP) Mm00475698_m1, Tnfsf11 (RANKL) Mm00441906_m1 and Polymerase (RNA) II (Polr2a) Mm00839502_m1

Table 1
CP phases synthesized as a part of this study and arranged in the order of their solubility: dicalcium phosphate anhydrous (DCP), β-calcium pyrophosphate (CPP), amorphous calcium phosphate (ACP) and hydroxyapatite (HAP). Solubility products and solubility values in pure water (pH 7.0) at 25 °C are empirical in origin and obtained from Refs. [51] (DCP), [52] (CPP), [53] (ACP), and [54] (HAP).

Phase	Chemical formula	Space group	pK_{sp}	Solubility (g/dm^3)
DCP	$CaHPO_4$	Triclinic $P^{\bar{1}}$	7.0	4.8×10^{-2}
CPP	$Ca_2P_2O_7$	Tetragonal $P4_1$	18.5	1.5×10^{-2}
ACP	$Ca_3(PO_4)_2 \cdot nH_2O$	/	25	2.5×10^{-3}
HAP	$Ca_5(PO_4)_3OH$	Hexagonal $P6_3/m$	58.5	3×10^{-4}