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Amorphous polyphosphate–hydroxyapatite: A morphogenetically active substrate for bone-related SaOS-2 cells in vitro

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ABSTRACT

There is increasing evidence that inorganic calcium-polyphosphates (polyP) are involved in human bone hydroxyapatite (HA) formation. Here we investigated the morphology of the particles, containing calcium phosphate (CaP) with different concentrations of various Na–polyP concentrations, as well as their effects in cell culture. We used both SaOS-2 cells and human mesenchymal stem cells. The polymeric phosphate readily binds calcium ions under formation of insoluble precipitates. We found that addition of low concentrations of polyP (<10 wt.%, referred to the CaP deposits) results in an increased size of the HA crystals. Surprisingly, at higher polyP concentrations (>10 wt.%) the formation of crystalline HA is prevented and amorphous polyP/HA hybrid particles with a size of ≈50 nm are formed, most likely consisting of polyP molecules linked *via* Ca^{2+} bridges to the surface of the CaP deposits. Further studies revealed that the polyP–CaP particles cause a strong upregulation of the expression of the genes encoding for two marker proteins of bone formation, *collagen type I* and *alkaline phosphatase*. Based on their morphogenetic activity the amorphous polyP–CaP particles offer a promising material for the development of bone implants, formed from physiological inorganic precursors/polymers.

Statement of significance

Hydroxyapatite (HA) is a naturally occurring mineral of vertebrate bone. Natural HA, a bio-ceramic material which is crystalline to different scale, has been used as a biomaterial to fabricate scaffolds for *in situ* bone regeneration and other tissue engineering purposes. In contrast to natural HA, synthetic apatite is much less effective. In general, while HA is bioactive, its interaction and biocompatibility with existing bone tissue is low. These properties have been attributed to a minimal degradability in the physiological environment. In the present study we introduce a new Ca–phosphate (CaP) fabrication technology, starting from calcium chloride and dibasic ammonium phosphate with the HA characteristic Ca/P molar ratio of 10:6 and report that after addition >10% (by weight) of polyphosphate (polyP) amorphous CaP/HA samples were obtained. Those samples elicits strong morphogenetic activity let us to conclude that polyP/HA-based material might be beneficial for application as bone substitute implant.

1. Introduction

Two main stages of bone formation (ossification) are distinguished [1]. *First*, during the primary phase, the endochondral ossification, the epiphysial cartilage, a combination of ground substance and loosely interconnected fibril bundles of collagen, interspersed with a large number of matrix vesicles, allows a relatively rapid mineralization process (reviewed in: [2]). The mineralized, "woven" bone microstructures comprise collagen type-I, which exists in rat cartilage to ≈17%. The crystals formed are not closely associated with collagen; the clusters of hydroxyapatite (HA) crystals are deposited into the collagen-surrounding proteoglycan matrix [3]. While during primary ossification phase collagen does not markedly direct the mineralization deposits, during the subsequent *secondary* phase of bone formation the primary woven bone is remodeled into an organized tissue comprising concentric

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lamellae that make up the osteons [4]. The nanoscopic HA platelets become oriented and aligned within the self-assembled collagen fibrils and are arranged concentrically around blood vessels [5]. The cells that form the mineral deposits within bone are the osteoblasts. In a tightly controlled process, the collagen fibrils undergo a progressive mineralization starting with poorly crystalline carbonated apatite (see: [6]).

Three main mechanisms have been proposed to explain the initial bone mineral deposition. *First:* a cell-independent process, whereby charged non-collagenous proteins facilitate mineral nucleation from ions in solution [7]. *Second:* a cell-controlled mechanism during which vesicles are budding off from the cellular plasma membrane. In these matrix vesicles, and supported by phosphatases including the alkaline phosphatase (ALP), as well as by Ca-binding molecules, initial calcium phosphate (CaP) mineral crystals are formed. Subsequently the matrix vesicles break down at the cell membrane, releasing the mineral deposits into the extracellular space [8]. *Third:* more recently an alternative route has been proposed in which amorphous mineral precursors are transiently produced. They are deposited within the collagen fibrils where they transform into crystalline apatite platelets [9]. However, the origin of the proposed amorphous CaP remained still to be elucidated. Experimental evidence has been presented that revealed that osteoblasts contain intracellularly localized CaP-containing vesicles which are potential candidates for the extracellular mineralization process [10]. Element analyses suggested that the intracellular mineral granules consist of a highly metastable phase and, in addition, a potential precursor stage of carbonated HA. Moreover, experimental evidence indicates that initially, on the surface of osteoblasts, Ca-carbonate deposits are synthesized in concert with the carbonic anhydrase that act as bio-seeds for the biomineralization process [11,12]. It is reasonable to assume that those Ca-carbonate deposits are amorphous, since this material is unstable and readily processes to other states of Ca-carbonate [13,14]. This proposition is supported by the finding that Ca-carbonate is converted to CaP by exposure to external orthophosphate [15]. In addition, during the evolution of the animals first Ca-carbonate-based skeletons, e.g. in sponges or corals, developed while later CaP skeletons are dominating [16]. The analytical demonstration that Ca-carbonate exists in vertebrate bone goes back to Pellegrino and Biltz [17]; and, to mention, that in the vestibular labyrinth of the vertebrate ear the otoliths predominantly consist of Ca-carbonate [18].

Bone, being a composite material, forms the inorganic deposits around the collagen fibrils. Evidence has been presented indicating that the uniaxially oriented apatite crystals are formed within the periodic 67 nm cross-striated pattern of the collagen fibril at the less dense 40-nm long gap zone [19]. It is proposed that the positive net charge at the *C*-termini of the collagen molecules initiates and promotes the infiltration of the fibrils with amorphous CaP (ACP). The latter discovery was supported by the finding that during bone and teeth formation the development of the apatite crystals starts from an ACP precursor phase [20,21] at the prenucleation clusters. Those amorphous clusters have also been demonstrated for amorphous $CaCO_3$ (ACC) [22,23]. The transition from ACP to crystalline HA in those clusters is a fast process that is decelerated when a size is reached at which the increasing surface energy is counter-balanced by the reduction of bulk energy, originating from the process of crystal lattice formation. The resulting primary critical-sized crystal nuclei grow further, driven by the associated reduction of the Gibbs free energy. The basic building blocks, as in tooth crowns, are dense arrays of needle-shaped carbonated apatite crystals (≈50 nm in diameter and tens of μm long), with crystalline *c*-axes, arranged along the rods [24]. Interesting is the finding that *in vitro* collagen-mediated mineralization is decelerated by substituting the spacing of non-collagenous proteins either with polyaspartic acid or with fetuin; both of them are inhibitors of HA crystallization [2,25,26]. Those additional components added during *in vitro* HA synthesis are considered as structure-forming elements during the intrafibrillar formation of oriented HA crystals.

HA is a naturally occurring mineral with the formula $Ca_5(PO_4)_3(OH)$ [or: $Ca_{10}(PO_4)_6(OH)_2$ to signify that the HA crystal unit cell comprises two entities]. Natural bone HA elicits biological activity, at least bone conducting properties ([27]). In turn, natural HA, a bio-ceramic which is crystalline to different scale has been used as a biomaterial to fabricate scaffolds for *in situ* bone regeneration and other tissue engineering purposes (reviewed in: [28–30]). While natural HA shows pronounced mineralization-promoting activity *in vivo*, synthetic apatite is much less effective. In general, although HA is bioactive, its interaction and biocompatibility with existing bone tissue is low. These properties have been attributed to a minimal degradability in the physiological environment as observable by the lack of resorption via the surrounding intact tissue [31]. Recent advances in nanotechnology allowed the synthesis of nano-hydroxyapatite of various morphologies with a reasonable biocompatibility [32]. Moreover, it has been demonstrated that ACP has a better osteoconductivity and biodegradability than tricalcium phosphate and HA *in vivo* [33].

In the present study we introduce a new CaP fabrication technology, starting from calcium chloride and dibasic ammonium phosphate [34]. Besides of the preparation of HA, with the characteristic Ca/P molar ratio of 10:6, we prepared CaP mixed with various amounts of polyphosphate (polyP). While the CaP/HA samples were found to consist of a crystalline phase, those CaP preparations that contained >10% by weight of polyP (with respect to the modified HA deposits) are amorphous. All those polyP-supplemented CaP samples were found to support the growth of bone cell-related SaOS-2 cells [35] as well as human mesenchymal stem cells (hMSC). Surprisingly, only the CaP preparation, containing 10 weight percent (wt.%) of polyP, elicits strong morphogenetic activity, as measured by gene expression analysis and using the marker genes *alkaline phosphatase* (*ALP*) and *collagen type I* (*COL-I*) for differentiation of bone and bone-related cells [36]. These results let us to conclude that polyP/HA-based material might be beneficial for application as bone substitute implant.

2. Material and methods

2.1. Materials

Na^+–polyphosphate (Na–polyP) with an average chain length of ≈40 phosphate units was obtained from Chemische Fabrik Budenheim (Budenheim, Germany).

2.2. Synthesis of HA and polyphosphate–hydroxyapatite

Hydroxyapatite (HA) nanoparticles were synthesized by a wet chemical precipitation method from calcium chloride ($CaCl_2$; Sigma–Aldrich, Taufkirchen; Germany) as Ca^{2+} source, and ammonium phosphate dibasic ($(NH_4)_2HPO_4$; Sigma–Aldrich 215996) as phosphate source [34]. To precipitate stoichiometric HA ($Ca_{10}(PO_4)_6(OH)_2$; Ca/P ratio of 1.667), 100 mL of 0.3 M aqueous solution of $(NH_4)_2HPO_4$ was dropwise added to 100 mL 0.5 M aqueous solution of $CaCl_2$. The amount of reagents was calculated in order to obtain the Ca/P molar ratio for HA of 10:6. The pH of the reaction was maintained at 10 with the addition of sodium hydroxide solution.

In order to prepare polyP-substituted HA nanoparticles of various polyP content, the starting components ($CaCl_2$ and $(NH_4)_2HPO_4$) were additionally supplemented with 2.5, 5 or

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