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3D printing of hybrid biomaterials for bone tissue engineering: Calcium-polyphosphate microparticles encapsulated by polycaprolactone

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ABSTRACT

Here we describe the formulation of a morphogenetically active bio-ink consisting of amorphous microparticles (MP) prepared from Ca^{2+} and the physiological inorganic polymer, polyphosphate (polyP). Those MP had been fortified by mixing with poly-ε-caprolactone (PCL) to allow 3D-bioprinting. The resulting granular PCL/Ca-polyP-MP hybrid material, liquefied by short-time heating to 100 °C, was used for the 3D-printing of tissue-like scaffolds formed by strands with a thickness of 400 μm and a stacked architecture leaving ≈0.5 mm^2-sized open holes enabling cell migration. The printed composite scaffold turned out to combine suitable biomechanical properties (Young's modulus of 1.60 ± 0.1 GPa; Martens hardness of 153 ± 28 MPa), matching those of cortical and trabecular bone, with morphogenetic activity. This scaffold was capable of attracting and promoting the growth of human bone-related SaOS-2 cells as demonstrated by staining for cell viability (Calcein AM), cell density (DRAQ5) and SEM studies. Furthermore, the hybrid material was demonstrated to upregulate the steady-state-expression of the cell migration-inducing chemokine SDF-1α. EDX analysis and FTIR measurements revealed the presence of hydroxyapatite in the mineral deposits formed on the scaffold surface. Based on the results we conclude that granular PCL/Ca-polyP-MP hybrid material is suitable for the fabrication of bioprintable scaffold which comprises not only biomechanical stability but also morphogenetic potential.

Statement of Significance

In present-day regenerative engineering efforts, biomaterial- and cell-based strategies are proposed that meet the required functional and spatial characteristics and variations, especially in the transition regions between soft (cartilage, tendon or ligament) and hard (bone) tissues.

Statement of Significance: In a biomimetic approach we succeeded to fabricate amorphous Ca-polyP nanoparticles/microparticles which are highly biocompatible. Together with polycaprolactone (PCL), polyP can be bio-printed. This hybrid material attracts the cells, as documented optically as well as by a gene-expression studies. Since PCL is already a FDA-approved organic and inert polymer and polyP a physiological biologically active component this new bio-hybrid material has the potential to restore physiological functions, including bone remodelling and regeneration if used as implant.

1. Introduction

In order to avoid possible adverse effects of synthetic inorganic- or organic-based implants the use of biohybrid materials fabricated from biomolecules and other (inert) components e.g. ceramics, metals or biodegradable polymers, can be effective alternatives. Focusing on bone, as a hybrid formed of an inorganic mineral and organic template, a suitable biomimetic substitution material should include an inorganic scaffold, giving mechanical stability to the implant material and a biopolymer that provides the scaffold with functional properties, such as biocompatibility, proliferation-inducing and/or regeneration-promoting activity.

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Since those biohybrid materials are usually expensive and since implants should be designed and prepared for individualized applications, additive manufacturing techniques like the 3-dimensional (3D) printing technology are appropriate. In addition, during this process only little waste accumulates. The products can be quickly fabricated and controlled by computer-aided 3D design data. This technology is expected to dominate the manufacturing of orthopedic implants in the nearest future, especially for the fabrication of patient-specific implants made of regeneratively active materials (reviewed in: [1]).

In present-day, regenerative engineering efforts biomaterial- and cell-based strategies are proposed that meet the required functional and spatial characteristics and variations, especially in the transition regions between soft (cartilage, tendon or ligament) and hard (bone) tissues. To reach this goal scaffolds have been engineered, comprising stratified and gradient properties [2]. Until now the successful application of the 3D printing technology for the fabrication of cell-based scaffolds is hampered by the fact that a suitable bio-ink has not been developed. Therefore, efforts have been undertaken to enrich bio-inks with organic growth factors, like vascular endothelial growth factor or platelet-derived growth factor, to accelerate cell growth and differentiation [3]. We succeeded to identify a physiological inorganic polymer, polyphosphate (polyP) that acts as a morphogenetically active and growth promoting polymer (reviewed in: [4,5]). polyP is found in almost any metazoan cell type, and in high concentrations especially in blood platelets and serum, in an amorphous state like nanoparticles [6,7]. Ca-polyP accumulates at sites of new bone formation [8]. This polymer does not only elicit anabolic signals but also fuels, due to its energy-rich phosphate anhydrides linkages, metabolic processes within cells as well as in the extracellular space [9]. If bone-like SaOS-2 cells are exposed to polyP those cells respond with an increased intracellular ATP synthesis and also an accumulation of ATP in the extracellular space. This effect is linked to the process of enzymatic hydrolysis of the polyP phosphoanhydride linkages by the alkaline phosphatase (ALP). The Gibbs free energy released by this process becomes biochemically and metabolically available after transfer to adenine nucleotides.

It is plausible to take as a rule that in a living system, especially in multicellular organisms, any crystalline structure formed originates from an amorphous precursor(s) [10]. With respect to polyP, this polymer occurs both in an amorphous and in a crystalline state; the latter phase is formed only at higher temperature (>650 °C; [11]). The second, amorphous phase is stabilized in the presence of a molar excess of Ca^{2+} at room temperature [12].

In a biomimetic approach we succeeded to fabricate amorphous Ca-polyP nanoparticles/microparticles [12], similar to those found in cells as acidocalcisomes [6]. In this amorphous phase Ca-polyP is highly bioavailable. Having this material, polyP and Ca-polyP, in hand it was also possible to elucidate the biosynthetic route of bone formation. This process starts from amorphous Ca-carbonate and is enzymatically driven by the carbonic anhydrase [13]. The amorphous phase of Ca-carbonate can be stabilized by low concentrations of the polyanion polyP [14]. Learning from nature, the subsequent transition processes from the amorphous to the crystalline $CaCO_3$ phases can be blocked and controlled by negatively charged D/E peptides, e.g. isolated from the spicules of the siliceous sponge *S. domuncula* [15]. Based upon Gibbs free energy calculations it has been proposed that after initial supersaturation of the solution for Ca^{2+} (aq) and HCO_3^- (aq) a polymer-induced liquid-precursor (PILP) process runs prior to formation of the amorphous Ca-carbonate (s) phase [16]. After this amorphous Ca-carbonate bio-seed formation [17] a non-enzymatic exchange of carbonate by phosphate proceeds which is exergonic [18]. Again, an amorphous Ca-phosphate is formed. It could be established that *in vitro* Ca-polyP, if added together with $CaCl_2$ and ammonium phosphate, blocks the transition of the amorphous to the crystalline Ca-phosphate phases [19]. Subsequently, the crystalline Ca-phosphate [hydroxyapatite (HA)], with the intermediate octacalcium phosphate, develops [20].

polyP can be printed alone or with other negatively charged polymers, like alginate or carboxymethylated chitosan, to individualized implants [21,22]. Embedded in this scaffold the cells readily proliferate and differentiate; hence polyP can be ascribed as morphogenetically active bio-ink. However, such 3D printed tissue-like specimens are not strong enough to match with the biomechanical properties of bone. Therefore, we developed in the present study a 3D printed scaffold which is sufficiently resistant and appears to be suitable as bone implant. As solid scaffold we used the FDA-approved polymer polycaprolactone (PCL) [23] together with polyP and demonstrate that this hybrid material is suitable for 3D printing. PCL is relatively hydrophobic and requires modifications to improve its biocompatibility. This deficiency was eliminated by co-application of PCL with polyP. The formed hybrid material comprises suitable biomechanical properties (due to PCL) and retains the morphogenetic activity of polyP.

The biocompatibility as well as the morphogenetic activity of the PCL/polyP hybrid material was demonstrated by the intriguing activity of this material to allow the attachment and growth of cells, we used the human bone-related SaOS-2 cells [24], as well as the strong inducible function to activate metabolic pathway (s). In addition, evidence is presented that polyP within this hybrid material attracts the cells, as documented optically as well as by a gene-expression study which revealed that the steady-state-expression level of the stromal cell-derived factor-1α (SDF-1α) is substantially upregulated after incubation of the cells with the material. This factor stimulates the migration of cells [25,26].

2. Materials and methods

2.1. Materials

Powdered poly-ε-caprolactone (PCL) with a relative molecular mass of 50,000 was purchased from Polysciences Europe (Hirschberg; Germany). According to the instruction of the supplier, this raw PCL material has a particle size of <600 μm (98%). The melting point is denoted as 58 °C–60 °C. Sodium polyphosphate (Na-polyP) with an average chain length of approximately 40 phosphate units was obtained from Chemische Fabrik Budenheim (Budenheim; Germany).

2.2. Formulation of amorphous Ca-polyP microparticles

Calcium-polyphosphate microparticles (Ca-polyP-MP) were prepared basically as described [12,27]. In brief, the 100 nm–400 nm large amorphous Ca-polyP-MP were obtained by adjusting the $Ca[CaCl_2]$:P[polyP] weight ratio to >2. In the present study 2.8 g of $CaCl_2 \cdot 2H_2O$ (Sigma, Taufkirchen; Germany) were dissolved in 50 mL ethanol and added drop-wise to 1 g of Na-polyP, dissolved in 50 mL distilled water at room temperature. The suspension formed was kept at pH 10 (using 1 N NaOH) and stirred for 5 h. The microparticles formed were collected by filtration through a Nalgene filter unit with 0.45 μm pore size (Cole-Parmer, Wertheim; Germany). After filtration the particles were washed three times with ethanol and subsequently dried in an oven at 60 °C overnight.

2.3. Preparation of the PCL/Ca-polyP-MP composite material

The material used for the 3D-printing of the scaffolds was prepared by mixing the PCL particles with Ca-polyP-MP. In order to