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A new polyphosphate calcium material with morphogenetic activity

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ABSTRACT

Polyphosphate [polyP] has been proven to elicit morphogenetic activity on bone cells. By applying mild reaction conditions, a Ca-polyP material that displays a hardness of ≈1.3 GPa has been fabricated. The Ca-polyP granules are prone to hydrolytic degradation during in vitro incubation of the cells, suggesting that this property is associated with the observed bioactivity.

1. Introduction

The need for suitable biofabricated bone graft materials, due to the shortage and risks of autogeneic and allogeneic implants, drives the development of new biosynthetic bone substitutes [1]. Besides of cell-based bone graft substitutes, ceramic-based materials have been proven to be useful bone scaffolds for implants. Among the calcium phosphate-based ceramics, hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and bioactive glass gained upmost relevance [2–4].

Since all of those mineralic implant materials are fabricated at temperatures higher than 800 °C and in turn are not, or only at a limited degree, osteoinductive like the bioglass, we tried to develop a new potential biomaterial perhaps likewise suitable to be used as bone implant. This new material is based on polyphosphate [polyP]. Previously, polyP has been used, after calcination, as a potential scaffold for bone implants [5]. The polymer was integrated into different inorganic matrices which had been sintered at 550–1000 °C, a process during which the amorphous material was transformed into a crystalline one [6]. Those sintered polyP samples displayed a highly edged morphology; the cubes were of large sizes with >100 μm [7,8]. The material displayed both biocompatibility and degradability [9] but elicit no osteoinductive activity. PolyP is a natural polymer that has been found in bone-forming cells [10] as well as in platelets [11]. We described that polyP has an inductive effect on osteoblasts [12], mainly as an anabolic polymer that stimulates differentiation of bone cells and mineralization [13,14] (reviewed in: [15,16]). Interesting, polyP induces the enzyme alkaline phosphatase [ALP] [13], an enzyme that degrades polyP [17]. In the present study we describe an amorphous, polyP-based biomaterial that is fabricated at ambient temperature.

2. Experimental section

Preparation of calcium polyphosphate nanospheres: Na-polyphosphate (Na-polyP of an average chain of 40 phosphate units) was obtained from Chemische Fabrik Budenheim (Budenheim; Germany). Na-polyP (10 g) was dissolved in 500 ml of distilled water and the pH was adjusted to 10 with a 1 M NaOH aqueous solution. A solution of 14 g of calcium chloride dihydrate (C3306 Sigma) or of 28 g of $CaCl_2$ in 250 ml was added dropwise (1 ml/min) to the Na-polyP solution, adjusting steadily the pH to 10 at room temperature. The suspension formed was stirred for 4 h. The particles formed were collected by washing twice with ethanol. Then the particles were dried at 60 °C. The Ca-polyP material obtained by addition of 14 and 28 g of $CaCl_2$ were named as "Ca-polyP1" and "Ca-polyP2", respectively

The Ca-polyP material obtained by addition of 14 g of $CaCl_2$ was named "Ca-polyP1", and the one with 28 g of $CaCl_2$ was termed "Ca-polyP2".

Chemical characterization by FTIR: Fourier transformed infrared (FTIR) spectroscopy was measured by using a Varian 660-IR

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spectrometer with Golden Gate ATR auxiliary (Agilent, Darmstadt; Germany).

Scanning electron microscopy: For the scanning electron microscopic (SEM) analyses a HITACHI SU 8000 electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) was employed.

XRD analysis: X-ray diffraction (XRD) of the dried powder samples was conducted on a Philips PW 1820 diffractometer with monochromatic Cu-K_α radiation (λ=1.5418 Å, 40 kV, 30 mA, 5 s, $\Delta\theta$=0.02) [18].

Cells and cell culture conditions: Human osteogenic sarcoma cells, SaOS-2 cells, were used for the experiments and cultivated in McCoy's medium with fetal calf serum [FCS] [19]. Cultivation of the cells was performed in 24-well plates; 3×10^4 cells were seeded per well. After an initial incubation period of 3 d, the cultures were supplemented with a solution of 10 μg/mL of Na-polyP, supplemented with $CaCl_2$ in a 2:1 stoichiometric ratio, in order to compensate for the chelating activity of polyP as described [13], or with a suspension of 10 μg/mL of the two polyP samples, prepared here, with Ca-polyP1, Ca-polyP2, nano-hydroxyapatite (HA; 677418 Sigma-Aldrich) or β-TCP (13204 Sigma-Aldrich); in the controls none of those materials were added. Then the cells were continued to be incubated in the presence of the osteogenic cocktail [OC]. After a 7 d incubation period the cells were either stained with Alizarin Red S to assess the extent of mineralization [20] or subjected to qRT-PCR [quantitative real-time RT-PCR] analysis [19].

Determination of the hardness: The hardness of the polyphosphate material Ca-polyP2 was determined by a ferruled optical fiber-based nanoindenter [21].

Polyp degradation in vitro: SaOS-2 cells were incubated with 50 μg/mL of solid Ca-polyP2 and incubated in the standard assay for 1 or 7 d. In parallel assays 50 μg/mL of solid Ca-polyP2 had been incubated for 1 or 7 d in PBS [phosphate buffered saline] in a final volume of 1.5 ml. Then samples (50 μl) were taken and assayed for the chain length of polyP [17].

Statistical analysis: The results were statistically evaluated using the paired Student's *t*-test [22].

3. Results and discussion

FTIR analyses: The two phosphate materials, Ca-polyP1 and Ca-polyP2, were characterized by FTIR and compared with the spectrum obtained for Na-polyP (Fig. 1). The complete spectra between the wavenumbers 4000 and 600 cm^{-1} are shown in Fig. 1A, while segments between 1400 and 600 cm^{-1} are given in Fig. 1B. However, the spectra of Ca-polyP showed the same characteristic features as those of the described polyP samples [23]. However, the peaks are shifted in the Ca-polyP1 and Ca-polyP2 samples if compared the Na-polyP spectrum. This shifting is also seen for other bands in the Ca-polyP spectra; they even increase by increasing the Ca^{2+} content in the polyP.

The XRD patterns for Na-polyP, Ca-PolyP1 and Ca-Polyp2, shown in Fig. 1C, indicate a clear amorphous phase, with a broad peak from 20° to 40° for Na-polyP and centered around 30° for Ca-polyP.

Morphology of polyP samples: The three samples, Na-polyP, Ca-polyP1 and Ca-polyP2, were analyzed by SEM (Fig. 2). The Na-polyP particles, of a non-regular shapes, often show a tapered morphology (Fig. 2A and B). The sizes of the particles vary between 1 and 300 μm with an average size of ≈ 100 μm. Likewise non-regular shapes show the Ca-polyP1 particles (Fig. 2C and D). They are smaller than the Na-polyP particles with an average

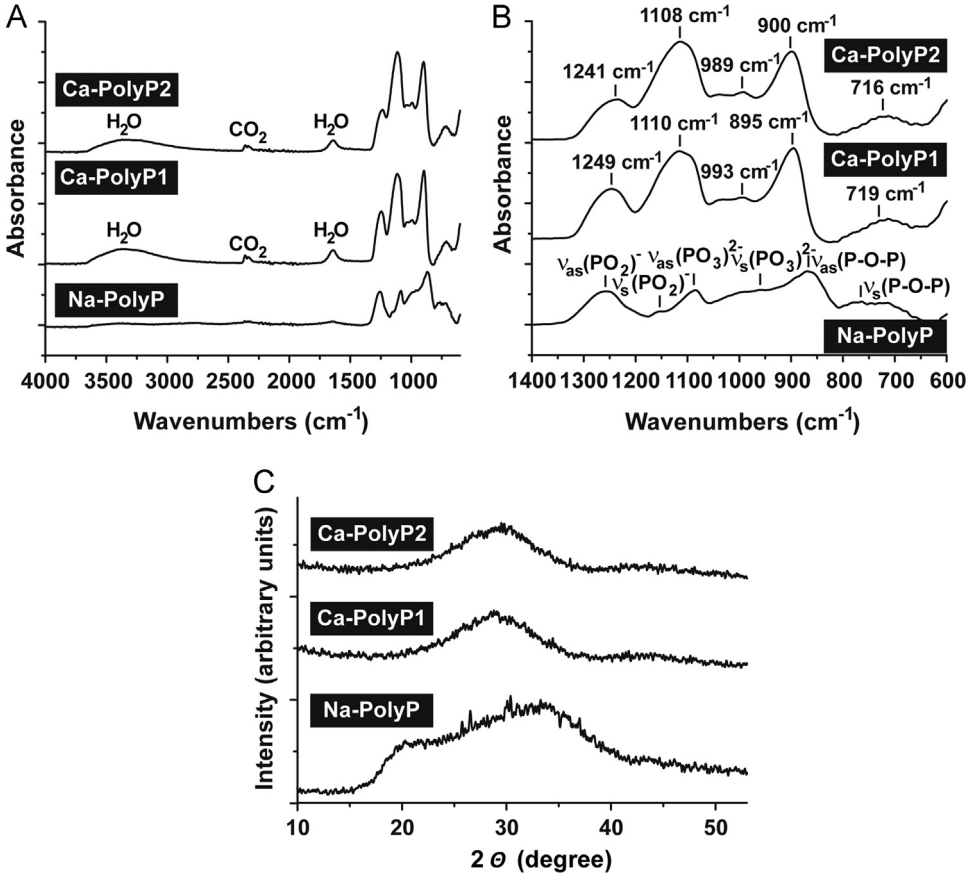

Fig. 1. FTIR spectra and XRD pattern of Na-polyP, Ca-polyP1 and Ca-polyP2; (A) wavenumbers 4000–600 cm^{-1} and (B) wavenumbers 1400–600 cm^{-1}. (C) XRD analysis.