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Structure and *in vivo* anticalcification properties of a polymeric calcium–sodium–phosphocitrate organic–inorganic hybrid ☆

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Abstract

This paper describes the synthesis, characterization and crystal structure of an organic–inorganic polymeric hybrid composed of Ca, Na, and phosphocitrate (CaNaPC). CaNaPC is synthesized by reaction of $CaCl_2 \cdot 2H_2O$ and $Na_4(HPC) \cdot 3H_2O$ in water, at pH 2. Its structure is polymeric with $Ca(PC)_2(H_2O)$ "monomers" connected through Na^+ bridges. The 9-coordinate Ca occupies the center of an irregular polyhedron defined by four phosphate, four carbonyl, and one H_2O oxygens. Ca—O(=C) distances are in the 2.446(2)–2.586(2) Å range. There is a short distance of 2.477(1) Å between Ca and the ester *O* from C–*O*–PO_3H_2. All –COOH groups are protonated. There are three dissociated protons per two PC molecules, all coming from $–PO_3H_2$. Na ions are six-coordinate surrounded by –COOH's. The anticalcification properties of CaNaPC on plaque growth were studied *in vivo* using rats as model systems. Na–phosphocitrate is an effective inhibitor, but its effectiveness diminishes when a lower dose is used (9.7 mg as H_5PC), resulting in only 30% plaque reduction. Superior inhibition activity becomes evident by following treatment with CaNaPC, at an equal dose (9.6 mg as H_5PC) giving nearly quantitative (95%) plaque inhibition.

Keywords: Phosphocitrate; Biomineralization; Calcium phosphonates; Anticalcification; Crystal growth

1. Introduction

Biological systems utilize organic molecules, such as proteins and polysaccharides, as natural additives that function variously as nucleators, cooperative modifiers, matrices or molds for minerals. They exert precise control over complex biomineralization processes, resulting in unique inorganic–organic composites (e.g., bone, teeth, seashells, diatomea and many others) with their structure and morphology regulated to optimally fit their functions as biomaterials [1].

There is great interest in these processes from the biomedical community because disruption of normal biomineralization processes may lead to pathological conditions, such as in atherosclerotic plaque formation, encrustation of biomaterials (such as urinary catheters and heart valve calcification), kidney stone build-up, dental calculus formation, or bone and tooth demineralization. Research related to those processes has revealed that phosphorylated carboxylic acids are powerful inhibitors of pathological biomineralization as it relates to calcium-containing crystal deposition diseases [2]. Phosphocitrate (PC, Fig. 1), a phosphorylated analog of citrate, has been the subject of interest for several years. It has been shown to inhibit several biomineralization-related processes, such as the transformation of calcium phosphate to hydroxyapatite ($Ca_5(PO_4)_3(OH)$) [3], the crystallization of calcium oxalates (various hydrated forms), [4] octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) [5], calcium pyrophosphate ($Ca_2P_2O_7 \cdot 2H_2O$) [6], and struvite ($Mg(NH_4)(PO_4) \cdot 6H_2O$) [7].

In this paper we describe the preparation and crystal and molecular structure of a polymeric mixed salt of PC,

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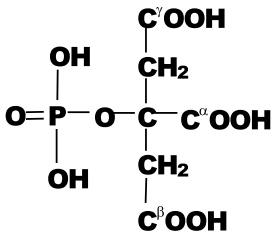

Fig. 1. Schematic representation of phosphocitric acid (H_5PC).

$[CaNa(PC)_2(H_2O)]_n$ (CaNaPC). Also, its anticalcification properties in inhibiting induced plaque growth in rats are described. Herein, the abbreviation "PC" is used with no specific reference to proton content, unless otherwise noted.

2. Experimental section

2.1. Synthesis

Synthesis and characterization of NaPC, as the *tetrakis*-deprotonated $Na_4(HPC)\cdot 3H_2O$, has been described [8]. NaPC (3.24 g, 7.83 mmol) was dissolved in 200 ml distilled water. $CaCl_2\cdot 2H_2O$ (2.86 g, 19.64 mmol; this amount of Ca^{2+} gave the highest yield) was added slowly as a solid under stirring. The pH started decreasing and some turbidity appeared. Final pH was adjusted to ~2 with dilute HCl. The solution was then taken to dryness using a Rotovap®. Excess Ca^{2+} and Na^+ were removed by washing briefly with small amounts of distilled water. Yield 2.5 g (70%). Product purity was verified by ICP of a solution of the material in water. FT-IR spectrum (KBr pellets): $\nu_{C=O}$ 1717, 1636 cm^{-1}, ν_{O-H} 3573, 3496 cm^{-1}, $\nu_{P=O}$ (asym) 1260, 1230 cm^{-1}, and $\nu_{P=O}$ (sym) 1090, 1075 cm^{-1}. Rapid salt precipitation has prevented preparation of crystalline CaPC salts at higher pH regions, yielding amorphous solids.

2.2. Crystallography

Single crystals of CaNaPC (Fig. 2) were grown by slow evaporation of a concentrated aqueous solution of the salt prepared as above. They are colorless, monoclinic (space group C_2/c), with $a = 22.331(3)$ Å, $b = 7.966(1)$ Å, $c = 13.233(2)$ Å, $\beta = 107.877(2)°$, $V = 2240.2(5)$ Å^3, $Z = 8$, $FW = 311.13$, and $d_{calc} = 1.845$ g/cm^3. Intensity data were collected on a CCD SMART diffractometer with Mo-Kα radiation. A total of 4751 reflections were collected (1612 unique), 1478 with $I > 2\sigma(I)$ used in the structure refinement by full-matrix least-squares techniques (173 parameters). Final R indices $R_1 = 0.0277$, $wR_2 = 0.0753$, and GoF = 1.072 (for all reflections $R_1 = 0.0303$ and $wR_2 = 0.0770$).

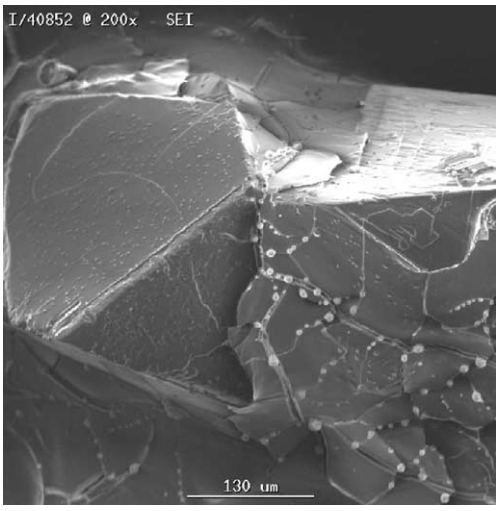

Fig. 2. Morphology of CaNaPC single crystals (bar length = 130 μm). The film that covers part of the crystal is $CaCl_2$ from drying.

2.3. Animal experiments

In the present study, male Hooded Wistar rats (200 g) were randomly divided into three treatment groups of 5 as A = controls; B = sodium phosphocitrate (NaPC, administered as $Na_4(HPC)\cdot 3H_2O$); C = calcium phosphocitrate (CaNaPC, administered as polymeric $\{CaNa(H_3PC)(H_4PC)(H_2O)\}_n$; *vide infra* for explanation of the structure). Rats had access to water and chow ad libitum at all times.

Plaque formation was chemically induced on day 1 by subcutaneous injection of a 0.1% $KMnO_4$ solution (200 μl dose) in two positions on either side of the interscapular region. Respective salts of PC were dissolved in 0.1 M Tris–HCl (pH 7.2) and administered 6 h after plaque induction treatment was commenced. Group A were injected intraperitoneally with 300 μl buffer alone, Group B received 300 μl NaPC solution (9.7 mg as H_5PC) in buffer, and Group C was treated with 300 μl CaNaPC solution (9.6 mg as H_5PC). Thereafter, therapy with the above treatments was given on alternate days. Plaque growth proceeded over 10 days, at which time the experiment was terminated and plaque material was excised and weighed.

3. Results and discussion

Reaction of NaPC and $CaCl_2$ at pH ~2 gives CaNaPC according to the Eq. (1) (proton content on PC also shown):

$$CaCl_2\cdot 2H_2O + 2Na_4(HPC)\cdot 3H_2O + 5HCl \rightarrow CaNa(H_3PC)(H_4PC)(H_2O) + 7NaCl + 4H_2O \quad (1)$$

The structure of CaNaPC (Fig. 3) is polymeric with $CaNa(H_3PC)(H_4PC)(H_2O)$ "monomers" connected

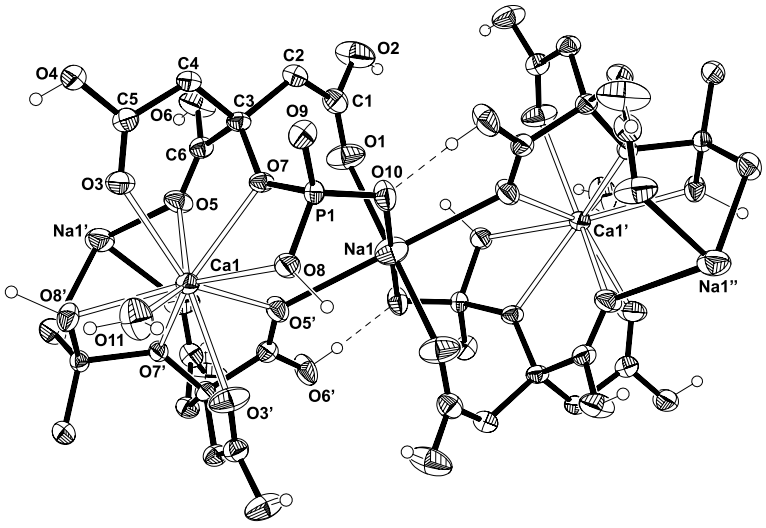

Fig. 3. Partial ORTEP diagram of the CaNaPC polymeric structure (50% probability ellipsoids). O-attached protons and two H-bonds (dashed lines) are shown. Relevant bond lengths and distances (Å): Ca···Ca 8.794(1), Ca···Na 4.3972(5), Ca(1)–O(11) 2.388(2), Ca(1)–O(3) 2.446(2), Ca(1)–O(7) 2.477(1), Ca(1)–O(8) 2.527(2), Ca(1)–O(5) 2.586(2).

through Na^+ bridges into chains running parallel to the *c*-axis. The chains are interconnected by hydrogen bonds into layers parallel to the (1 0 0) plane. The Ca cation occupies the center of an irregular polyhedron defined by four phosphate, four carbonyl, and one water O-atoms. Coordination number 9 for Ca is rather rare [9]. In that regard, the unexpected presence of a coordinated H_2O (albeit a common ligand for Ca^{2+}) is the result of the strain imposed by the PC ligand on the coordination geometry, making a wide site available to H_2O. Two examples of 9-coordinate, biologically relevant Ca are in the structures of β-calcium-pyrophosphate [10] and hydroxyapatite minerals [11].

An interesting structural feature is the short distance of 2.477(1) Å between Ca and the ester *O* from C–*O*–PO_3H_2. For comparison, the Ca–O (pyrophosphate ester) distance in β-$Ca_2(P_2O_7)$ is 2.855 Å. Interestingly, this is consistent with the apparent resistance of the P–O–C moiety to hydrolysis in an acidic environment, suggesting that strong calcium coordination exerts a "protective" effect on the overall molecule. Ca—O(=C) distances are in the 2.446(2)–2.586(2) Å range, much shorter than those in Ca hydrogen citrate trihydrate (2.37–2.49 Å) [12]. Similarly, the Ca–O(PO_2H) distance is 2.527(2) Å, much longer than Ca–O distances in related complexes (2.3–2.4 Å) [13]. As coordination number increases Ca–O distances become elongated. Ca–O distances in CaNaPC are consistent with these observations. All –COOH groups are protonated. There are three dissociated protons *per two PC molecules* [14], all coming from –PO_3H_2. The second proton from –PO_3 is dissociated before that from α-COOH and is involved in a short hydrogen bond (2.453(3) Å) connecting two phosphate moieties of adjacent polymeric "ribbons". A carbonyl oxygen (O(5)) from –COOH acts as a bridge between Ca^{2+} and Na^+. Na ions are six-coordinate, a feature commonly found in Na-carboxylate salts [15]. Other structural features of CaNaPC compare well with those of NaPC [16].

Although PC's biological excretion is rapid, it is an effective inhibitor of pathological biomineralization *in vivo* [17]. An alternative form of PC that exhibits slower, more sustained release could offer substantial therapeutic benefits. Solubility of Ca^{2+} salts is typically much lower than that of the analogous Na^+ salts. This prompted the present study as a comparison between the efficiencies of the Ca and Na salts of PC to inhibit hardening of an induced plaque in rats [18]. This model has been used before to demonstrate anticalcification potency of PC [19]. Results from the animal study are presented in Table 1.

NaPC is an effective plaque inhibitor but at higher and more frequently administered doses than those described herein [20]. However, as shown in results from Group B, its effectiveness is greatly diminished when a lower dose is used (9.7 mg as H_5PC), resulting in only 30% plaque reduction. Superior inhibition activity becomes evident by following treatment with CaNaPC (Group C), at an equal dose (9.6 mg as H_5PC) giving nearly quantitative (95%) plaque inhibition.

Possible explanations for the improved anticalcification efficiency of CaNaPC compared to that of NaPC could be relevant to: (a) the slower and more sustained release of "active PC", thus ensuring its bioavailability at all times by limiting the excreted amount; (b) the more effective stereospecific interaction between CaNaPC and crystal face(s) of hydroxyapatite. This latter probability could be resolved through molecular modeling. Such studies are underway [21], following similar

Table 1
Inhibition of plaque growth using NaPC and CaNaPC as inhibitors[a]

Treatment groups	Treatment dosage (as mg) H_5PC)	Plaque weight (mg)[b]	Plaque weight reduction (%)
A (control)	0	211 ± 9.244	0
B (NaPC)	9.7	147 ± 8.825	30
C (CaNaPC)	9.6	11 ± 4.444	95

[a] Data were processed to establish one way analysis of variance with significance determined as pair-wise comparison (Student–Newman–Keuls method).
[b] Results are expressed as means ± SEM for 10 plaques. Statistical significance was determined at the level of $P < 0.001$ for single groups and pair-wise group comparisons.

ones on interactions of NaPC with other calcium minerals [22].

4. Conclusions

The present results reveal a novel organic–inorganic hybrid polymeric material that can be synthesized under mild conditions. The polymeric structure of CaNaPC combines interesting features that include a 9-coordinate Ca center, a Ca–O (phosphate ester) linkage, and Ca—O═C bonding (from –COOH) [23]. CaNaPC is a potent inhibitor of plaque growth *in vivo*, as demonstrated by calcification inhibition experiments on rats. Delineation of the inhibition mechanisms of NaPC, CaNaPC, and other phosphonates [24], both *in vitro* and *in vivo* is currently underway in our laboratories.

Supplementary material

Details on the structure (crystal data, bond lengths and bond angles, packing diagrams), SEM images and an EDS spectrum of the CaNaPC single crystals (10 pages) are deposited as Supplementary material.

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