Inorganic Pyrophosphatase–Nanodiamond Conjugates Hydrolyze Pyrophosphate in Human Synovial Fluid

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ABSTRACT: The present work is focused on testing enzyme-based agents for the partial dissolution of calcium pyrophosphate (CaPPi) deposits in the cartilages and synovial fluid of patients with pyrophosphate arthropathy (CPPD disease). Previously, we suggested that inorganic pyrophosphatases (PPases) immobilized on nanodiamonds of detonation synthesis (NDs) could be appropriate for this purpose. We synthesized and characterized conjugates of NDs and PPases from Escherichia coli and Mycobacterium tuberculosis. The conjugates showed high enzymatic activity and resistance to inhibition by calcium and fluoride. Here, we tested the effectiveness of pyrophosphate (PPi) hydrolysis by the conjugates in an in vitro model system simulating the ionic composition of the synovial fluid and in the samples of synovial fluid of patients with CPPD via NMR spectroscopy. The conjugates of both PPases efficiently hydrolyzed triclinic crystalline calcium pyrophosphate (t-CPPD) in the model system. We evaluated the number of phosphorus-containing compounds in the synovial fluid, showed the possibility of PPi detection in it, and estimated the hydrolytic activity of the PPase conjugates. The soluble and immobilized PPases were able to hydrolyze a significant amount of PPi (1 mM) in the synovial fluid in short periods of time (24 h). The maximum activity was demonstrated for Mt-PPase immobilized on ND–NH–(CH2)6–NH2 (2.24 U mg⁻¹).

1. INTRODUCTION

Calcium pyrophosphate deposition (CPPD) disease, alternatively known as pseudogout, is a type of arthropathy caused by the deposition of microcrystals of calcium pyrophosphate (CaPPi) in joint tissues, particularly in fibrocartilage and hyaline cartilage. The possible manifestations of pyrophosphate arthropathy vary in severity from asymptomatic chondrocalcinosis to acute inflammation attacks and degenerative changes typical of arthritis. The pathogenesis of CPPD disease is caused by defects in the metabolism of inorganic pyrophosphate (PPi) produced from ATP in the extracellular matrix of the cartilage. Normally, the equilibrium between the production and degradation of extracellular PPi is maintained due to its regulated transport to and from the fibroblasts, where it is degraded by intracellular inorganic pyrophosphatase (PPase). The known cases of familial CPPD disease include mutations in some of the PPi, homeostasis system, such as the transmembrane transport protein ANK or ectonucleotide pyrophosphatase/phosphodiesterase 1 (eNPP1), leading to the increased efflux of PPi and its accumulation in the extracellular matrix, where it forms deposits of crystalline CaPPi. Age-related alterations in PPi homeostasis, physical damage to chondrocytes, magnesium disorders, and alkaline phosphatase deficiency are other common causes of CPPD.

To date, there is no specific treatment for CPPD disease. Conventional therapy is focused on anti-inflammatory and immunosuppressive treatment for the prevention of acute symptoms. In an animal model, it was shown that phosphocitrate or probenecid inhibits the local mineralization of CaPPi, and, therefore, they may be used as anticrystal agents. We hope that other factors affecting PPi, homeostasis and concentration and/or the morphology of deposited CaPPi crystals will be developed in the future for specific treatment of CPPD disease. Yeast inorganic pyrophosphatase has been shown to dissolve CaPPi crystals in vitro. Rate-limiting factors for crystal dissolution were the accessibility of the crystal surface for PPase and Ca⁺⁺ concentration in the solution. In our previous work, we suggested the use of bacterial PPases immobilized on the detonation synthesis nanodiamond (ND) as potential therapeutic agents for the treatment of CPPD disease. When injected into the joint, the enzymatic component of this hybrid material should hydrolyze the PPi in the synovial fluid and extracellular matrix of the cartilage, while the carrier ND would facilitate penetration of the cartilage and enhance the enzyme’s access to the crystals. Protein–nanoparticle conjugates are believed to be more resistant than soluble proteins to proteolytic degradation and other possible destructive factors in the physiological environment. Our preliminary data suggested that the PPase:ND
conjugates retained significant hydrolytic activity under a wide range of conditions, including the presence of 2 mM Ca\(^{2+}\), a known inhibitor of Family I PPases [unpublished data]. Other conjugates of PPases have been fabricated, which also exhibited higher resistance to various inhibiting factors. PPase conjugated with gold nanoparticles together with poly(N-isopropylacrylamide) (pNIPAM)\(^8\) or poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA)\(^7\) was shown to be more tolerant to protease digestion than the soluble form. The conjugate of PPase with poly(2-methacrylamido glucopyranose) (PMAG) retained hydrolytic activity at extreme pH and high salt concentrations, and showed increased tolerance to protease and glycosidase degradation.\(^10\)

In this work, we tested the possibility of dissolution of the specially synthesized triclinic calcium pyrophosphate dihydrate (t-CPPD) crystals by PPases from Escherichia coli (Ec-PPase) and Mycobacterium tuberculosis (Mt-PPase) conjugated with aminated NDs [ND–NH\(_2\) and ND–NH–(CH\(_2\))\(_6\)–NH\(_2\)] in a model in vitro system. We evaluated the PPase activity of the immobilized PPases in the synovial fluid of patients with pyrophosphate arthropathy.

The synovial fluid of patients with pyrophosphate arthropathy typically has a moderate soluble PP concentration (4.6–34.2 \(\mu\)M) and high soluble Pi concentration (up to 3.5 mM).\(^11,12\) It contains CPPD crystals that are 0.42–17.9 \(\mu\)m in size.\(^13\) Canonical PPase assays do not work in the raw synovial fluid due to heterogeneity, viscosity, and complex chemical composition of this fluid, including high levels of endogenous Pi (up to 3.76 \(\mu\)M L\(^{-1}\)).\(^14\) Therefore, in this work, we assayed the PPase activity by monitoring the reduction in the substrate (PP\(_i\)) concentration when adding fixed portions of endogenous PP\(_i\) to the samples of the synovial fluid.\(^13\)\(^15\) P NMR spectroscopy was performed to determine the PP\(_i\) concentration. NMR spectroscopy is a simple and nondestructive method for the qualitative and quantitative analyses of miscellaneous compounds in various samples, in particular, biological samples.\(^15\)\(^16\)\(^17\) NMR methods of metabolomics for the analysis of biological samples, including human tissues, are especially developed.\(^18\)\(^19\)\(^20\)\(^21\)\(^22\)\(^23\)\(^24\)\(^25\)\(^26\)\(^27\) For instance,\(^13\)\(^15\) P NMR spectroscopy was performed to characterize the phospholipid composition of the synovial fluid and blood serum.\(^28\)\(^29\) The ratio of phosphatidylcholine to lysophosphatidylcholine determined by this method was used by Fuchs and colleagues to detect the progression of rheumatoid arthritis.\(^2\)

In this work, we showed that the addition of the conjugates to the model in vitro system containing Mg\(^{2+}\), Ca\(^{2+}\), and exogenous crystalline CPPD results in the time-dependent accumulation of inorganic phosphate in the solution. This indicates that the conjugates shift the equilibrium to CaPP\(_i\)(cryst.) \(\leftrightarrow\) Ca\(_{4}(sol.)^{2+}\) + PP\(_i\)(sol.) thus facilitating the dissolution of crystals. We determined the presence of Pi and PP\(_i\) in the synovial fluid and estimated the ratio of their concentrations. We evaluated the hydrolytic activity of the PPase conjugates in the synovial fluid samples of patients with pyrophosphate arthropathy.

2. RESULTS AND DISCUSSION

2.1. Characterization and Hydrolysis of the Synthesized t-CPPD. Cartilage samples from patients with CPPD disease contain a large amount of calcium pyrophosphate in various forms.\(^29\) The major forms are triclinic or monoclinic calcium pyrophosphate dihydrate (t-CPPD or m-CPPD).\(^24\) According to the reference literature, CaPP\(_i\) is mostly insoluble in water.\(^25\) However, in a special study, it was shown that some crystalline CPPD was slowly dissolved in water-based buffers. The maximum solubility was about 60 \(\mu\)M PP\(_i\) (or Ca\(^{2+}\)), and it strongly depended on the pH, ionic strength, and the addition of protein.\(^26\) It also depended on the factors that affect the concentrations of PP\(_i\) in solution. Exogenous PP\(_i\) decreased the solubility of CPPD, while yeast PPase hydrolyzing PP\(_i\) provided the partial dissolution of CPPD crystals.\(^26\)

We suggested further exploration of this possibility and estimated the time-course parameters of phosphate release from crystalline t-CPPD in the presence of PPase-based conjugates. For the in vitro enzymatic hydrolysis tests, we synthesized t-CPPD crystals. Their characteristics, obtained by Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) analyses (Figure S1), are fully consistent with the literature.\(^27\)

To study the hydrolysis of synthesized t-CPPD, the mixture containing PPases (soluble or immobilized) and excess t-CPPD was incubated on a shaker for about 2 weeks in a buffer solution in the presence of a cofactor (Mg\(^{2+}\)). The hydrolysis of PP\(_i\) was monitored by the release of Pi. To estimate the possible effect of Ca\(^{2+}\) ions present in the synovial fluid, the hydrolysis of t-CPPD was also studied at different ratios of Mg\(^{2+}\) and Ca\(^{2+}\). As a control, the nonenzymatic hydrolysis of PP\(_i\) was studied under the same conditions. The data obtained are presented in Figure 1 for 2 mM Ca\(^{2+}\) and 10 mM Mg\(^{2+}\), and Figure S2 (Supporting Information) is for 2 mM Ca\(^{2+}\) and 2 mM Mg\(^{2+}\).

![Figure 1](https://dx.doi.org/10.1021/acsomega.9b04429)
concentrations. The most notable effect was observed with 2 mM of Ca$^{2+}$ and 10 mM of Mg$^{2+}$ (Figure 1). In this case, the concentration of released Pi in the presence of PPases showed saturation at a level of approximately $60 \pm 80 \mu M$, which is significantly higher than the control values. At a lower Mg$^{2+}$ concentration, the effect of the PPases becomes negligible, partly due to their inhibition by Ca$^{2+}$ and partly due to the high amount of nonenzymatically hydrolyzed CaPP$_i$ (about 40 μM at 2 mM Mg$^{2+}$ versus 20 μM at 10 mM Mg$^{2+}$).

The levels of phosphate observed in these experiments were approximately 0.5–1% of the maximum possible level expected if all added t-CPPD was hydrolyzed.

The calculated parameters of the time dependencies, rate constants ($k$), and the maximum levels of the product ($[P_i]_{max}$), are presented in Tables 1 and S1 (Supporting Information). The nonenzymatic hydrolysis of PP$_i$ occurs with a rate constant of 0.01–0.02 h$^{-1}$. The presence of PPases does not increase this rate to values that are typical of the hydrolysis of a soluble pyrophosphate: 10–15 ME mg$^{-1}$ (15–20 × 10$^3$ h$^{-1}$) for Ec-PPase and 30–40 ME mg$^{-1}$ (40–50 × 10$^3$ h$^{-1}$) for Mt-PPase. This demonstrates that the rate-limiting factor of the observed Pi release is crystal dissolution rather than the hydrolysis of PP$_i$ itself. Mt-PPase-based samples show significantly higher facilitation of Pi release than Ec-PPase-

Table 1. Parameters of the Phosphate Release from the Synthesized Calcium Pyrophosphate by PPase Samples$^a$

| samples                  | $[P_i]_{max}$ [μM] | $k$ [h$^{-1}$] |
|--------------------------|--------------------|----------------|
| control                  | 20 ± 2             | 0.02 ± 0.01    |
| Ec-PPase                 | 76 ± 2             | 0.04 ± 0.01    |
| immobilized on ND–NH$_2$ | 62 ± 2             | 0.04 ± 0.01    |
| immobilized on ND–NH–(CH$_2$)$_4$–NH$_2$ | 66 ± 2 | 0.03 ± 0.01 |
| Mt-PPase                 | 58 ± 2             | 0.08 ± 0.01    |
| soluble                  | 62 ± 2             | 0.09 ± 0.02    |
| immobilized on ND–NH$_2$ | 74 ± 2             | 0.04 ± 0.01    |
| immobilized on ND–NH–(CH$_2$)$_4$–NH$_2$ | 74 ± 2 | 0.04 ± 0.01 |

$^a$The conditions included the following: 50 mM Tris-HCl pH of 7.5, 10 mg mL$^{-1}$ Ca$_2$P$_2$O$_7$·2H$_2$O, 10 mM MgCl$_2$, 2 mM CaCl$_2$, and 0.01 μg mL$^{-1}$ PPase.

Figure 2. $^{31}$P NMR spectra of the synovial fluid before the addition of Pi (Ia) and PP$_i$ (IIa) and after the addition of Pi (Ib) and PP$_i$ (IIb).
based samples, especially at low Mg\(^{2+}\) concentrations (Table S1). This can be attributed to the fact that Mt-PPase is less sensitive to inhibition by Ca\(^{2+}\) at the concentrations used in this experiment. This is consistent with the fact that the smallest effect on the rate of t-CPPD hydrolysis was observed for conjugates with ND\(^{-}\)NH\(^{-}\)(CH\(_2\))\(_6\)NH\(_2\), which, according to our data, have the highest affinity for Ca\(^{2+}\) of the tested PPases [unpublished data].

The results of this experiment confirm that different PPases in various forms may be applied for the dissolution of t-CPPD in an ionic context similar to that of the synovial fluid. To maximize the efficiency of these catalysts in a Ca\(^{2+}\)-rich biological environment, Mg\(^{2+}\) should be added to PPase-based samples to a concentration of 5–10 mM.

### 2.2. Assignment of Pi and PPi Signals in the \(^{31}\)P NMR Spectrum of the Synovial Fluid.

To evaluate the composition of phosphorus compounds in the synovial fluid, \(^{31}\)P NMR spectra of the fluid were recorded. To identify Pi and PPi peaks, exogenous sodium pyrophosphate and orthophosphate were added to the synovial fluid samples up to a 1 mM concentration, and \(^{31}\)P NMR spectra were recorded. The spectra are presented in Figure 2.

In the reference, the \(^{31}\)P NMR spectrum of the synovial fluid without exogenous additives, three intense peaks were observed (Figure 2, red curves). This indicates the presence of three soluble, magnetically nonequivalent forms of phosphorus in the synovial fluid in the millimolar concentration range. Peaks 2 and 3, with chemical shifts of −0.2 and −0.8 ppm, respectively, can correspond to phospholipids, such as phosphatidylcholine and lysophosphatidylcholine.

When orthophosphate was added to the synovial fluid, one of the NMR peaks (peak 1, \(\delta = 2.7\) ppm) increased significantly, while the other two peaks remained unchanged (Figure 2, top panel). This indicates that peak 1 in the spectrum corresponds to endogenous inorganic phosphate in the synovial fluid.

When pyrophosphate was added to the synovial fluid, none of the peaks of the initial spectrum increased. However, a new peak (peak 4) appeared in the NMR spectrum (Figure 2, bottom panel) (\(\delta = −5.2\) ppm). This means that pyrophosphate was absent in the collected synovial fluid or its concentration was below the detection threshold of NMR. Thus, the new peak (peak 4) in the spectrum corresponds to the exogenous pyrophosphate. A moderate increase in peak 1 (orthophosphate) may have been caused by the nonenzymatic hydrolysis of the added pyrophosphate under the conditions of the NMR experiment.

The nondetectable level of endogenous PPi rendered it impossible to assay the PPase activity in the raw synovial fluid. However, it was possible to assay the PPase activity using the exogenous PPi, quantified by the area under the peak, with \(\delta = −5.2\) ppm.

### 2.3. PPi Hydrolysis in the Synovial Fluid.

A series of experiments were performed to evaluate the PPase activity in the synovial fluid. Soluble or immobilized PPases were added to the samples of the synovial fluid at a protein concentration of 0.8 \(\mu\)g mL\(^{−1}\), and 1 mM sodium pyrophosphate was used as

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**Figure 3.** Time course of pyrophosphate hydrolysis in the synovial fluid without the addition of PPases (a) and with the addition of Ec-PPase (b) and Mt-PPase (c) in the soluble form (1) or immobilized on ND\(^{-}\)NH\(_2\) (2) or ND\(^{-}\)NH\(_2\)-(CH\(_2\))\(_6\)-NH\(_2\) (3). The lines are the best fit to the first-order reaction kinetics equation.
an exogenous substrate. $^{31}$P NMR spectra were recorded at various time intervals, ranging from 2.5 to 4 h, and the fraction of unhydrolyzed PP$_i$, was determined for each kinetic series as the ratio of the PP$_i$ peak ($\delta = -5.2$ ppm) to its initial value. Figure 3 shows the time course of PP$_i$ hydrolysis for different PPases. The rate constants for each kinetic series, calculated using first-order reaction kinetics, are presented in Table 2.

The nonenzymatic hydrolysis of exogenous PP$_i$ in the synovial fluid was moderate or not observed at all (Figure 3a). The addition of the samples of Ec-PPase (Figure 3b) has only a moderate, if any, effect on the rate of hydrolysis, with the residual PP$_i$ levels above 80%. However, the addition of Mt-PPase samples (Figure 3c) facilitates hydrolysis. In two cases [soluble or immobilized on ND−NH$_2$−(CH$_2$)$_6$−NH$_2$ Mt-PPase], PP$_i$ was almost completely hydrolyzed within 20 h.

The comparison of the rate constants determined from the obtained data (Table 2) shows that though all Ec-PPase-based samples have much poorer performance than Mt-PPase-based samples, Ec-PPase is most active in the soluble form, while Mt-PPase is most active when immobilized on ND−NH$_2$−(CH$_2$)$_6$−NH$_2$. This pattern is in good agreement with our previous findings. Among the samples of Ec-PPase, the soluble form had the greatest specific activity, and its conjugate with ND−NH$_2$ had the lowest specific activity. Under the conditions of the model system simulating the ionic composition of the synovial fluid, the most active were the Mt-PPase samples, especially the soluble form and the conjugates with ND−NH$_2$−(CH$_2$)$_6$−NH$_2$.

All samples of Ec-PPase had significantly lower activity in the synovial fluid than our preliminary tests in the model in vitro system [unpublished data] showed. An inhibitory effect of some components of the synovial fluid was expected, though the samples of Ec-PPase were active in the model system in the presence of up to 2 mM of Ca$^{2+}$. Mt-PPase-based conjugates, as expected, were less sensitive to this inhibition. This expectation was based on the unique combination of the structural and functional properties of Mt-PPase, which stands alone among known homologues, Family I PPases. First, while the active site residues of Family I PPases demonstrate the remarkable evolutionary conservation, Mt-PPase represents the only group of mycobacterial PPases that contain histidine residues at the active site (His21 and His86). Second, its polypeptide chain lacks 12 residues at the N-terminus compared to homologous PPases, which manifests in the significant differences in the intersubunit interfaces, organization of a hexamer, and allosteric properties. Functionally, Mt-PPase exhibits some unusual properties not observed in its homologues, including low affinity to the mechanism-based inhibitors Ca$^{2+}$ and F$^-$, and activation by substrate analogues. Most authors agree that the catalytic mechanism of PP$_i$ hydrolysis by Mt-PPase may differ from other family I PPases.

To summarize, Mt-PPase immobilized on aminated NDs, and to some extent, Ec-PPase retains its hydrolytic activity in the synovial fluid. Maximum activity was found with Mt-PPase immobilized on ND−NH$_2$−(CH$_2$)$_6$−NH$_2$ (2.2 U mg$^{-1}$). The hydrolytic activity in the synovial fluid for all studied enzymes was only 1–2% of hydrolytic activity under optimal in vitro conditions. Nevertheless, the absolute values of the activity are comparable to the values typical for some classes of hydrolases. This allows us to consider the suggested PPase-based materials, especially Mt-PPase-based conjugates, as promising agents for the hydrolysis of pyrophosphate in joint tissues in vivo.

### Table 2. PP$_i$ Hydrolysis in the Synovial Fluid by Soluble or Immobilized PPases

| samples | $k$ (h$^{-1}$) | $A$ (U mg$^{-1}$) |
|---------|--------------|------------------|
| control |              |                  |
| ND−NH$_2$ | 0.010 ± 0.001 | 0                |
| ND−NH−(CH$_2$)$_6$−NH$_2$ | 0.13 ± 0.002 | 0                |
| soluble | 0.025 ± 0.003 | 0.33 ± 0.04      |
| immobilized on ND−NH$_2$ | 0 | 0 |
| immobilized on ND−NH−(CH$_2$)$_6$−NH$_2$ | 0.017 ± 0.003 | 0.22 ± 0.04      |
| Mt-PPase |              |                  |
| soluble | 0.10 ± 0.01 | 1.3 ± 0.1        |
| immobilized on ND−NH$_2$ | 0.040 ± 0.004 | 0.53 ± 0.05 |
| immobilized on ND−NH−(CH$_2$)$_6$−NH$_2$ | 0.17 ± 0.02 | 2.2 ± 0.3      |

3. CONCLUSIONS

In this work, we tested pyrophosphatase−ND conjugates in an in vitro model system and applied $^{31}$P NMR spectroscopy to estimate the quantity of P$_i$ and PP$_i$ in the synovial fluids of patients with pyrophosphate arthropathy (calcium pyrophosphate deposition disease or CPPD disease). We demonstrated that in both these systems, immobilized PPases were capable of PP$_i$ hydrolysis. PPase activity varied in different samples, though in both systems, it was much lower than the activity measured under standard conditions. The major factors determining PPase activity in the synovial fluid were the type of PPase and the type of ND. The performance of Mt-PPase-based samples in the synovial fluid was significantly better than that of Ec-PPase-based samples, and the activities of both PPases were higher when they were conjugated with ND via the hexamethylene diamine linker. This factor presumably affects PPase activity by changing the density of PPase molecules packed at the surface layer since the addition of the linker increases the effective diameter and surface area of the nanoparticle. In turn, the protein packing density may determine two features of a conjugate crucial for PPase activity: the conformational freedom of protein and the diffusional logistics of substrate/product delivery to or from the active sites. Previously, we observed that the PPase activity of conjugates depended on the protein:ND ratio that support this idea. Liu et al. reported similar effects on conjugates of gold nanoparticles (AuNPs) with PPase and poly(N-isopropylacrylamide) (pNIPAM); too high coverage of polymer on the nanoparticle surface tended to sterically interfere with the interaction of PPase with the substrate and decreased the activity of conjugates. PPase conjugated with AuNP and pNIPAM with randomly bound protein molecules exhibited low activity (ca. 15% of the soluble form), while the proper orientation increased PPase activity up to 50% at 25 °C. In this work, we used the system with
nonoriented conjugation of PPase. We specifically protected the active sites from modification, provided the flexible enough linker, found the optimal protein density avoiding the undesired crowding effects on the protein conformation and activity. As a result, the conjugation was not exactly random, and the conjugates obtained by this protocol exhibited high PPase activity under standard conditions (up to 95% at 20 °C).

The conjugates, in the presence of Mg2+, facilitated the partial dissolution of t-CPPD, which is the cause of CPPD deposition disease. However, even partial dissolution of t-CPPD would be beneficial for patients with CPPD deposition disease. The altered crystal size and morphology can decrease the damage to the cartilage tissue and quench the inflammatory response. Also, smaller crystals of t-CPPD tend to loosen from the mold of the cartilage matrix to the joint cavity, and they can eventually be evacuated, i.e., surgically. The conjugates of bacterial PPases with denotation ND retained their enzymatic activity in the hydrolysis of exogenous PPi in the human synovial fluid. These results provide the basis for further tests of PPase-based conjugates in animal models in vivo.

4. MATERIALS AND METHODS

4.1. Reagents. The modified ND samples, ND–NH2 and ND–NH–(CH2)6–NH2, were synthesized and characterized as previously described. The soluble inorganic PPases from E. coli (Ec-PPase) and M. tuberculosis (Mt-PPase) were expressed, purified, and characterized as previously described. The catalytic activities of soluble enzymes under standard conditions ranged from 100 to 150 U mg–1. The enzymes were immobilized on the NDs via a previously developed protocol. In brief, the PPase:metal cofactor (Mg2+):substrate (MgPPi) complexes for both PPases were stabilized using the reversible inhibitor (F−) to ensure the protection of active site lysine residues from modification. The enzymes in protected form were cross-linked to the aminated ND with glutaraldehyde using the ratios protein:ND, protein:glutaraldehyde, concentrations of protecting agents, incubation, and washing protocols optimized previously to yield the highest PPase activity. The protein load of the final samples (mg PPase per mg of ND) was 0.6, 0.9, 0.5, and 0.4 for Ec-PPase:ND–NH2, Ec-PPase:ND–NH–(CH2)6–NH2, Mt-PPase:ND–NH2, and Mt-PPase:ND–NH–(CH2)6–NH2, respectively. The catalytic activities of Ec-PPase:ND–NH2, Ec-PPase:ND–NH–(CH2)6–NH2, Mt-PPase:ND–NH2, and Mt-PPase:ND–NH–(CH2)6–NH2 measured under standard conditions were 64, 95, 82, and 95% of their native forms, respectively.

All used chemicals were purchased from Sigma (USA), Fluka (Switzerland), Merck (Germany), and Pharmacia Fine Chemicals (Sweden) and used without further purification. The deionized water was purified by a MilliQ plus system (Millipore) to an impedance of at least 18 MΩ cm–1.

4.2. Synthesis of Triclinic Calcium Pyrophosphate (t-CPPD). Triclinic calcium pyrophosphate dihydrate was obtained using a previously described method. To this end, 200 mL of aqueous solutions of Ca(NO3)2·4H2O (7.16 g, 3.03 × 10–2 mol) and K2P2O7 (5.00 g, 1.51 × 10–2 mol) were gradually added in 45 min to 400 mL of acetate buffer heated to 70 °C with constant stirring (400 mL of distilled water, 12 mL of glacial acetic acid, aqueous ammonia solution to adjust pH to 3.5) - so that, the molar concentration ratio for Ca2+ and P2O74– was 2:1. After the addition of the solutions, the precipitate was incubated in the mother liquor for 10 min at a temperature of 70 °C to maximize precipitation. The resulting precipitate was filtered via a Buchner funnel with a paper filter, washed three times with deionized water, and left in an oven at 37 °C overnight.

4.3. X-ray Phase Analysis of t-CPPD. Characterization of the obtained t-CPPD was performed using a Rigaku D/Max-2500 X-ray diffractometer with a rotating anode (Japan). The assay was carried out in reflection mode (Bragg-Brentano geometry) in quartz cells, without averaging rotation, using Cu Kα radiation (wavelength: 1.54183 Å). The assay parameters included a 2θ interval of 2–60°, 2θ step of 0.02°, and a recording speed of 5° min–1. The generator parameters included an accelerating voltage of 50 kV and a tube current of 250 mA. Qualitative analysis of the X-ray images was performed using the WinXPOW program and the ICCDD PDB-2 database.

4.4. Hydrolysis of t-CPPD. To study the hydrolysis of the synthesized t-CPPD, PPase solutions (Ec-PPase and Mt-PPase) or hydrosols of covalently immobilized PPase were added to 50 mM of Tris (pH 7.5) containing 10 mg mL–1 of crystalline t-CPPD, 2 mM of CaCl2, and 2–10 mM of Mg2+ to a final protein concentration of 0.01 mg mL–1 (0.55 nM), and they were incubated at room temperature with continuous shaking using a Vortex. Over the course of the reaction, aliquots of the solution or suspension were taken and centrifuged (14 000 rpm, 10 min), and the concentration of the phosphate ion was measured in the supernatant, as described previously. The principle of phosphate determination is based on the formation of the phosphomolybdate complex in the acidic medium. The obtained complex can be visualized by the malachite green dye and stabilized by sodium citrate. The optical density of the solution can be measured at 620 nm. The amount of phosphate can be determined by the calibration curve. The data were processed using eq 1 of the first-order reaction kinetics, as follows

\[ \frac{[P_i]}{[P_i]_{\text{max}}} = (1 - \exp^{-kt}) \]  

where \([P_i]\) is the measured concentration of the product, \([P_i]_{\text{max}}\) is the maximum concentration of the product, \(k\) is the rate constant, and \(t\) is the incubation time.

4.5. Synovial Fluid Collection and Characterization. Synovial fluid sampling was performed in patients with clinical signs of CPPD (acute or chronic arthritis in combination with chondrocalcinosis signs according to X-ray analysis and/or sonography of knee joints). In advance, written consents were taken from all patients for carrying out an arthrocentesis and synovial fluid analyses. The knee joints of all patients were punctured. Five minutes after aspiration, the fluid was examined using light and polarization microscopes with a compensator (Olympus CX31PF, ×400) to confirm the presence of CaPP1 crystals. Extracellular or intracellular rhomboid or parallelepiped crystals ranging in size from 1 μm to 20 μm and showing weak positive birefringence were regarded as CaPP1 crystals. For this study, we used only samples that contained calcium pyrophosphate crystals. If CaPP1 crystals were detected, the synovial fluid was frozen and stored at −70 °C.

4.6. Hydrolysis of PPi in Synovial Fluid. 4.6.1. Sample Preparation for NMR Spectroscopy. The synovial fluid was thawed at room temperature. For identification of PPi, and P2O74–, NMR peaks, sodium orthophosphate or sodium pyrophosphate was added to the samples up to 1 mM concentration. One NMR spectra were recorded.

https://dx.doi.org/10.1021/acsomega.9b04429
ACS Omega 2020, 5, 8578–8586
To estimate PPi stability in the synovial fluid, sodium pyrophosphate and pure ND hydrosols of both types were added to the samples up to 1 mM and 0.016 mg mL\(^{-1}\) concentrations, respectively. Five NMR spectra were recorded sequentially.

To monitor PPi hydrolysis in the synovial fluid, sodium pyrophosphate and soluble or immobilized Ec- or Mt-PPases were added to the samples up to 1 mM and 0.8 μg mL\(^{-1}\) concentrations, respectively. Thus, for immobilized PPases, conjugates were taken at a concentration of 1.6 μg mL\(^{-1}\). This corresponded to a protein concentration of 0.8 μg mL\(^{-1}\) due to the protein–conjugate mass ratio of 1:2 mg mg\(^{-1}\). Five to six NMR spectra were recorded sequentially.

4.6.2. \(^{31}\text{P}\) NMR Experiments. Before NMR experiments, deuterium oxide was added to all samples for the 2H frequency lock up to 5% v/v concentration. All samples (\(V = 350 \mu L\)) were transferred to 5 mm Shigemi tubes.

NMR measurements were performed using a Bruker AVANCE 600 spectrometer operating at 242.9 MHz for \(^{31}\text{P}\). The spectra were obtained by the collection of 15,000–27,000 scans at 25 °C. A 90° pulse angle (22.7 μs) was used, the delay time between each scan was 0.1 s, the acquisition time was 0.5 s, and the spectral width was 60.6 kHz (250 ppm).

The phosphorus spectra were proton decoupled. Spectra were obtained without sample spinning. The chemical shifts were indirectly referenced to external 85% H\(_3\)PO\(_4\) (at \(\delta = 0.0\) ppm) via the signal lock.

4.6.3. Calculation of the PPi Fraction and the Rate of PPi Hydrolysis. The phosphate and pyrophosphate signals were assigned by the addition of Na\(_3\)PO\(_4\) or Na\(_4\)P\(_2\)O\(_7\) to the synovial fluid samples. The quantities of the various forms of phosphorus were calculated from the peak area ratios. The peaks were numbered from 1 to 4 according to their chemical shifts, and peak areas were determined by integration using the Bruker TopSpin-3.6.1 program. For the initial spectra of each kinetic series, the area of peak 4 (\(\delta = -5.2\) ppm), assigned to pyrophosphate, was normalized to the area of reference peak 3 (\(\delta = -0.8\) ppm). The fraction of unhydrolyzed PPi, in each kinetic series was determined as the ratio of the PPi peak area at various times to the initial value. The data were approximated using SigmaPlot for Windows, version 10.0 (Systat Software Inc.). For each kinetic series, eq 2 of the first-order reaction kinetics was used for the approximation, as follows

\[
\frac{\varphi_{\text{PPi}}}{\varphi_{\text{PPi,0}}} = \exp^{-kt}
\]

where \(\varphi_{\text{PPi}}\) is the fraction of unhydrolyzed PPi, \(\varphi_{\text{PPi,0}}\) is the initial PPi signal, and \(k\) is the hydrolysis rate constant.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at this link.

FTIR and XRD spectra of the synthesized t-CPPD; data and calculated parameters for the hydrolysis of synthesized calcium pyrophosphate by the enzymes in the presence of 2 mM MgCl\(_2\) and 2 mM CaCl\(_2\) (PDF)

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ACKNOWLEDGMENTS

This work was supported by a grant from the Russian Science Foundation (No. 19-14-00115). We appreciate the Laboratory of Magnetic Tomography and Spectroscopy of the Department of Fundamental Medicine of the M.V. Lomonosov Moscow State University for NMR spectroscopy. We express our gratitude to the employees of the Nasonova Scientific Research Institute of Rheumatology: E.V. Ilinykh, M.N. Chikina, and O.V. Zhelyabina for the collection and preparation of synovial fluid samples. We thank Dr. N.N. Vorobyeva and Dr. S.A. Kuraliova (Department of Chemistry and Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University) for the enzymes that they provided. We thank Dr. V.I. Putlyaev and D.S. Larionov (Faculty of Material Science, Lomonosov Moscow State University) for their assistance in the synthesis of t-CPPD.

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