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Metaphosphates as packing materials for biochromatography

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Abstract

The chromatographic properties of metaphosphates of Mg, Ca, Sr, Mn, Fe, Al and Y prepared as crystalline particles of 4–10 μm were studied. Each metaphosphate (MPi) column except the $Mn_2[P_4O_{12}]$ column showed similar retentions and elution orders of all the basic proteins tested and retention of only a few acidic proteins such as γ-globulins and some iron-binding proteins. The $Mn_2[P_4O_{12}]$ column retained most acidic and all the basic proteins used. The difference in the conformation between the iron-complexed and iron-poor proteins was discriminated in transferrin chromatography using the MPi columns, except for the $Mn_2[P_4O_{12}]$ column, and in conalbumin using the β-$Ca(PO_3)_2$ and β-$Sr(PO_3)_2$ columns. The $Mg_2[P_4O_{12}]$ and $Mn_2[P_4O_{12}]$ columns retained DNA, whereas the other MPi columns could not retain all the nucleotides and nucleic acids tested. The MPi columns studied had excellent mechanical strength and thermal and chemical stability. The zeta potentials of all the MPi materials studied were negative over a wide pH range of 0.01 *M* sodium phosphate solution.

1. Introduction

In a previous study [1], pyrophosphate (PPi), one of the condensed phosphates, was found to have interesting chromatographic properties: the retention properties of basic proteins of various PPi materials with different crystal systems and metals were similar to each other and to those of hydroxyapatite (HAP), but those for acidic proteins, nucleotides and nucleic acids were different, depending on the kind of PPi column. PPi materials and orthophosphates such as HAP can be used as packing materials for high-performance liquid chromatography.

The condensed phosphates form a very large group of pentavalent phosphorus compounds. A metaphosphate (MPi) is also a form of a typical condensed phosphate. Although the type of phosphate group mainly dominates the physical and chemical properties of the condensed phosphates, the component metal ion may also be important [2,3]. Various metaphosphates of alkaline earth metals, transition metals and other polyvalent metals display excellent resistance to dissolution in acidic and alkaline solutions [4]. Moreover, they are inorganic salts, so that it is possible to work in a wide temperature range. Taking into account these properties, MPis are expected to be promising packing materials for chromatographic applications. This paper reports the chromatographic properties of various MPi materials including long-chain polyphosphates which approximate the MPi composition, which were all prepared as crystalline particles.

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2. Experimental

2.1. Materials

All proteins, nucleotides and nucleic acids used in this study, purchased from Sigma (St. Louis, MO, USA), were the same as those used previously [1].

2.2. Preparation of metaphosphates

The seven metaphosphates listed in Table 1 were synthesized by a solid-state reaction as follows. $Mg_2[P_4O_{12}]$ (MgMP), $Mn_2[P_4O_{12}]$ (MnMP), $Fe(PO_3)_3$(C) [FeMP(C)] and $Y(PO_3)_3$ (YMP) were prepared by mixing the corresponding metal oxides and ammonium dihydrogenphosphate (molar ratio of divalent metal to phosphorus = 1:2, trivalent metal to phosphorus = 1:3, respectively), heating at 600°C in air, grinding and reheating at 800°C. $Al_4[P_4O_{12}]_3$(A) [AlMP(A)], β-$Ca(PO_3)_2$ (β-CaMP) and β-$Sr(PO_3)_2$ (β-SrMP) were prepared by the same method, except that the corresponding metal hydroxides instead of oxides were used as raw materials.

These products were classified using an air classifier, and particles of 4–10 μm were used as packing materials.

Table 1
Metaphosphates prepared in this study

Metaphosphate	Abbreviation	Crystal system	Linkage of PO_4 tetrahedra
$Mg_2[P_4O_{12}]$	MgMP	Monoclinic	Ring
β-$Ca(PO_3)_2$	β-CaMP	Monoclinic	Long chain
β-$Sr(PO_3)_2$	α-SrMP	Monoclinic	Long chain
$Mn_2[P_4O_{12}]$	MnMP	Monoclinic	Ring
$Fe(PO_3)_3$(C[a])	FeMP(C)	Monoclinic	Long chain[b]
$Al_4[P_4O_{12}]_3$(A[a])	AlMP(A)	Cubic	Ring
$Y(PO_3)_3$	YMP	Monoclinic	Long chain

[a] According to the notation of d'Yvoir [5] and Remy and Bulle [6].
[b] According to Ref. [7].

2.3. Analysis of products

The crystalline phases, morphology, pore-size distribution and specific surface area of the MPi products were measured by the methods described previously [1]. The zeta potentials of the MPi products were measured by a streaming potential technique [8] using a 0.01 *M* solution of sodium phosphate with a ZP-10B streaming potential analyser (Shimadzu, Kyoto, Japan). The zeta potential and also the pH of the supernatant solution after allowing the MPi products to settle were measured periodically until an apparent equilibrium was attained.

2.4. Chromatographic procedure

The MPi products (4–10 μm) were packed in a stainless-steel tube (100 mm × 8 mm I.D.) under a 300 kg/cm^2 pressure using the slurry packing method. The chromatographic procedures were the same used previously [1], and the experimental conditions will be only briefly summarized here: eluent, sodium phosphate buffer (pH 6.8); gradient, $5.00 \cdot 10^{-3}$ *M*/min from 0.001 to 0.3 *M* for proteins and from 0.001 to 0.8 *M* for nucleotides and nucleic acids; flow-rate, 1 ml/min; detection, UV at 280 nm for proteins and 260 nm for nucleotides and nucleic acids.

3. Results and discussion

3.1. Properties of metaphosphates

MgMP, MnMP and AlMP(A) have a tetrametaphosphate ring structure and β-CaMP, β-SrMP, FeMP(C) and YMP have a long-chain structure of PO_4 tetrahedra. Although the latter should be polyphosphates, they can be interpreted as being approximately represented by a metaphosphate composition because of the chain being extremely large. Hence these polyphosphates were included in the MPi class in this study.

All MPi products were virtually non-porous

materials: pores with diameters larger than 60 Å, which affect the diffusion of large-sized samples such as biomolecules in a column, were not recognized by mercury porosimetry, and specific surface areas less than 1 m^2/g were measured by the BET method.

The zeta potential of the studied MPi materials in 0.01 *M* sodium phosphate solution is given as a function of pH in Fig. 1a–g. For comparison, the zeta potential of the HAP measured under the same solution conditions, using the HAP powder prepared in the previous study [1], is given in Fig. 1h. The data were obtained after equilibration for 1 week. All the MPi materials studied showed negative zeta potentials in a solution of pH > 5. During the measurements, it was also found that addition of the MPi samples to 0.01 *M* sodium phosphate solution of pH in the range 6–8 caused no net change in the pH of the supernatant solution. Moreover, regarding MnMP and FeMP(C), even in the experiments involving an extended pH range of the solution from 5 to 9, only a small pH change was observed. Hence the MPi materials are considered to be chemically stable under the chromatographic conditions of this study.

YMP was also similar to the other MPi materials concerning the above-described properties. However, an unusual rise in the back-pressure of the YMP column occurred during the chromatographic experiments, the reason for which was not clear. Hence the data on the elution of proteins obtained using the YMP column are not given in Table 2. However, the column showed some interesting chromatographic properties such as a very high retention ability for basic proteins (even higher than that of the HAP column), elution of γ-globulins over a very wide molarity range and an elution behaviour of transferrin similar to that obtained using the other MPi columns.

All these MPi materials had great mechanical strength and a sufficiently high thermal stability to withstand heating above 600°C.

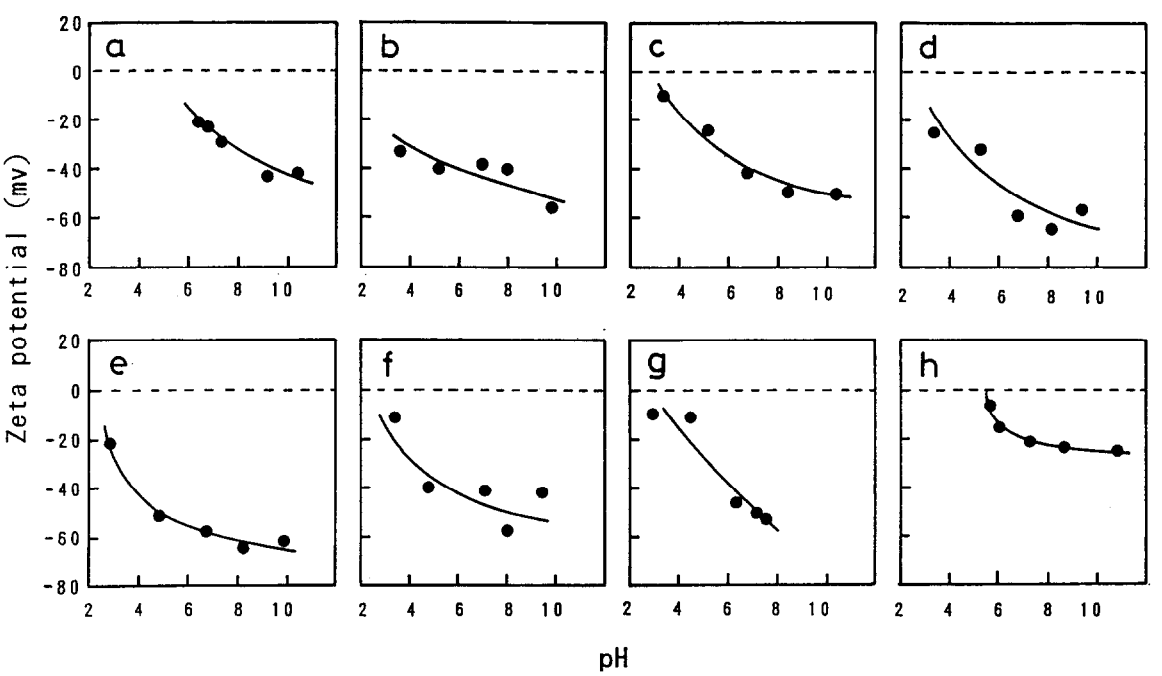

Fig. 1. Zeta potential of metaphosphates as a function of pH after equilibration for 1 week in 0.01 *M* sodium phosphate solution, obtained using (a) MgMP, (b) β-CaMP, (c) β-SrMP, (d) MnMP, (e) FeMP(C), (f) AlMP(A), (g) YMP and (h) HAP particles.

3.2. Chromatography of proteins

Elution of basic proteins

As Table 2 reveals, all the MPi columns retained the basic proteins with values of the isoelectric point (p*I*) larger than 7.0. The elution order of these basic proteins obtained using each MPi column was similar to that obtained using the HAP and PPi columns [1]. The difference in the elution molarity of a basic protein among the MPi columns except for the YMP column was very small. For the elution of cytochrome *c*, the chromatographic resolution of two peaks based on the reduced and oxidized forms obtained using the β-CaMP and β-SrMP columns was superior to that obtained using the other MPi columns. FeMP(C) and AlMP(A) showed chromatographic similarity, despite the fact that their properties in the crystal system and linkage of PO_4 tetrahedra are different.

Elution of acidic proteins

The MPi columns except for the MnMP column showed weak retention for acidic proteins with p*I* values of 5.0–7.0 and no retention for the most acidic proteins with p$I < 5.0$. Although MnMP and MgMP are crystalline isomorphs [9], the MnMP column showed a higher retention ability than the MgMP column for usual acidic proteins.

Phosphoproteins and β-lactoglobulins are considered to be special acidic proteins that interact strongly with HAP [10,11] and with most PPi materials [1]. In contrast, the β-CaMP, β-SrMP and AlMP(A) columns showed no retention of the phosphoproteins tested (i.e., α- and β-casein

Table 2
Elution of proteins from metaphosphates

Protein	p*I*	Elution molarity					
		MgMP monoclinic	β-CaMP monoclinic	β-SrMP monoclinic	MnMP monoclinic	FeMP(C) monoclinic	AlMP(A) cubic
Trypsin inhibitor	4.3–4.6	N.r.[a]	N.r.	N.r.	N.r.	N.r.	N.r.
α-Lactalbumin	4.5	N.r.	N.r.	N.r.	0.02	N.r.	N.r.
Ovalbumin	4.6	N.r.	N.r.	N.r.	N.r.	N.r.	N.r.
β-Casein		N.r.	N.r.	N.r.	>0.3	>0.3	N.r.
α-Casein		N.r.	N.r.	N.r.	>0.3	N.r.	N.r.
Phosvitin	4.8	N.r.	N.r.	N.r.	>0.3	N.r.	N.r.
Albumin	4.7–4.9	N.r.	0.01	0.01	0.02	N.r.	N.r.
β-Lactoglobulin A	5.1	0.08	N.r.	N.r.	0.12	0.04	N.r.
β-Lactoglobulin B	5.2	0.1	N.r.	N.r.	0.13	0.06	N.r.
holo-Transferrin (human)	5.2	N.r.	N.r.	N.r.	0.02	N.r.	N.r.
apo-Transferrin (human)	5.5	0.01	0.02	0.01	0.02	0.01	0.01
holo-Transferrin (bovine)		N.r.	N.r.	N.r.	0.02	N.r.	N.r.
apo-Transferrin (bovine)		0.01	0.04	0.04	0.02	0.01	0.02
Catalase	5.5	N.r.	N.r.	N.r.	0.04	N.r.	N.r.
Conalbumin (iron-complexed)	6.0–6.8	0.05	0.01	0.02	0.06	0.04	0.02
Conalbumin (iron-poor)	6.0–6.8	0.04	0.06	0.06	0.05	0.04	0.03
Haemoglobin	6.8–7.0	0.03	0.01	0.01	0.04	0.02	0.01
Myoglobin	7.2	0.02	0.01	0.01	0.02	0.02	0.01
α-Chymotrypsin	8.1–8.6	0.08	0.07	0.07	0.06	0.07	0.07
Papain	8.8–9.5	0.04	0.04	0.04	0.05	0.04	0.03
α-Chymotrypsinogen A	9.5	0.07	0.08	0.07	0.07	0.08	0.07
Ribonuclease A	9.5–9.6	0.07	0.10	0.10	0.06	0.07	0.07
Cytochrome *c* (reduced)	10.1	0.13	0.12	0.12	0.12	0.16	0.14
Cytochrome *c* (oxidized)		0.14	0.14	0.14	0.13	0.16	0.15
Lysozyme	11.0–11.4	0.09	0.11	0.11	0.07	0.08	0.08

For details of measurements, see Experimental section.
[a] N.r. = No retention under the experimental conditions.

and phosvitin) and β-lactoglobulin A and B. The MgMP column showed no retention of phosphoproteins and retention of β-lactoglobulins. The FeMP(C) column eluted only α-casein and phosvitin without retention in the column. On the other and, the MnMP column strongly retained both phosphoproteins and β-lactoglobulins, similarly to the cases in HAP and PPi chromatography.

γ-Globulin was retained on all the MPi columns. This may be because γ-globulin, in general, is a mixture of a large number of immunoglobulin species and has a wide range of p*I* values even involving the basic range.

Elution of iron-binding proteins

It was found that the MPi columns except for the MnMP column discriminated the difference in conformation between Fe-bound and Fe-unbound transferrins: holo-transferrins (both human and bovine) were not retained on the MPi columns, and the apo-transferrins were retained to and eluted from the column at phosphate concentrations higher than 0.01 *M*. In contrast, in MnMP chromatography, transferrins with different conformations were eluted at the same molarities.

The retained apo-transferrin showed one peak in MPi chromatography except for the β-CaMP and β-SrMP columns. However, three peaks of apo-transferrin (bovine) were obtained when using the β-CaMP column, as shown in Fig. 2a. Rechromatography of each of the three fractions showed that they appeared again at the same three different positions as in the first experiment, although the first peak became very small and the other two peaks became large. Because transferrin is reported to be capable of binding various metal ions [12], the three peaks may be based on the uptake of calcium ions in apo-transferrin from the β-CaMP crystal, followed by the formation of different forms of the protein; the first peak is probably a form with actually no metal ion in both the N- and C-sites in transferrin, and the protein of this form may have a chance to take up metal ions in rechromatography. This may be the reason why the first peak becomes small in rechromatography. On the other hand, the other two peaks are probably forms in which metal ions in part occupied the N-site and/or C-site. In addition to rechromatography, the uptake of calcium ion in transferrin was confirmed by inductively coupled plasma emission spectrometry. Calcium ion was detected in each of the three fractions, but detection in the first fraction was weaker than that in the other fractions.

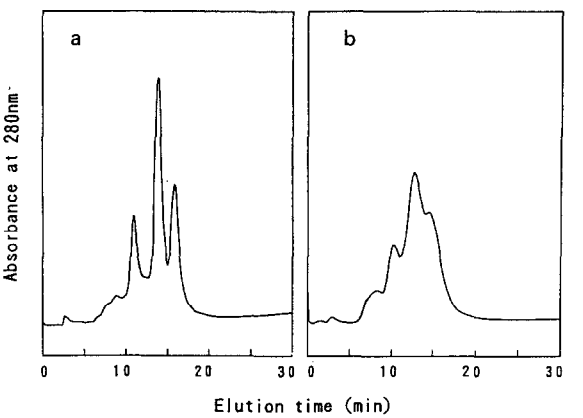

Fig. 2. Chromatograms of apo-transferrin (bovine) obtained using (a) β-CaMP and (b) β-SrMP columns. Elution conditions: gradient, $5.00 \cdot 10^{-3}$ *M*/min from 0.001 to 0.3 *M* sodium phosphate buffer (pH 6.8); flow-rate, 1 ml/min.

A chromatogram similar to Fig. 2a was obtained in β-SrMP chromatography, as shown in Fig. 2b, although the resolution was inferior to that with the β-CaMP column.

With regard to conalbumin also being an iron-binding protein, elution similar to that of transferrin was obtained using the β-CaMP and β-SrMP columns; holo-type conalbumin was eluted at a molarity lower than that of apo-type conalbumin. The reverse result, however, was obtained when using the MgMP and MnMP columns: the elution molarity of holo-type conalbumin was slightly higher than that of apo-type conalbumin. In AlMP(A) chromatography, the dependence of elution on the iron-binding state was hardly recognized.

Fig. 3a and b illustrate chromatograms of holo- and apo-type conalbumins obtained using the MnMP column. In the chromatogram of apo-type conalbumin two peaks were observed,

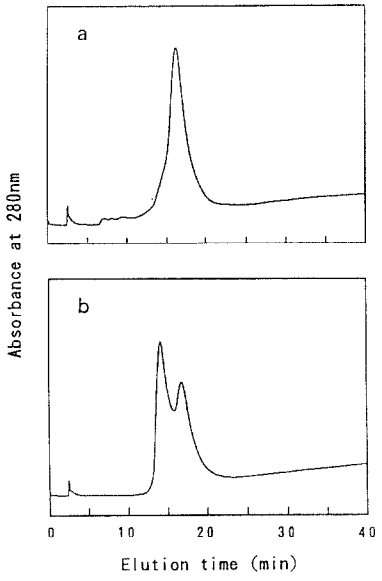

Fig. 3. Chromatograms of (a) iron-complexed conalbumin and (b) iron-poor conalbumin, obtained using the MnMP column. Elution conditions as in Fig. 2.

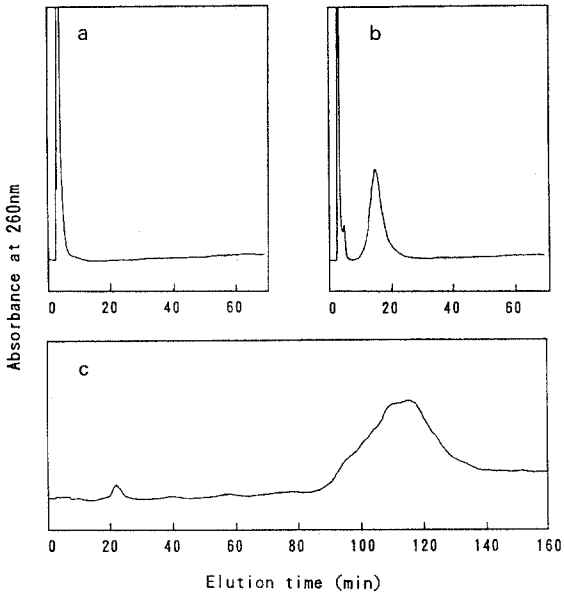

Fig. 4. Chromatograms of (a) ADP, (b) tRNA and (c) DNA, obtained using the MnMP column. Elution conditions as in Fig. 2, except that buffer concentration was from 0.001 to 0.8 *M*.

in which the latter peak was eluted at a molarity that agreed with that of holo-type conalbumin. Hence this peak might be based on the uptake of manganese ions in conalbumin from the MnMP crystal.

From the above-mentioned results for the chromatography of transferrin and conalbumin, the uptake of metal ions in some metalloproteins was shown to be influenced by the kind of metal ion composing the MPi crystal.

3.3. Chromatography of nucleotides and nucleic acids

The chromatographic properties of MPi for nucleotides and nucleic acids were also studied. Based on the experiment, the MPi columns studied except for the MgMP and MnMP columns were found not to retain all tested nucleotides and nucleic acids. The MgMP and MnMP columns could retain DNA. However, even when using these two columns, the retention of RNA was incomplete and nucleotides were not retained at all. Fig. 4a–c illustrate typical chromatograms for ADP, tRNA and DNA obtained using the MnMP column.

3.4. Chromatographic properties of the MPi column

In β-CaMP, β-SrMP and MnMP, their metal ions can form pure single-cation HAP, leading to the possibility of alternation in surface composition due to hydrolysis of the MPi in solution and formation of HAP on the MPi crystal. If that is the case, then the chromatographic properties would be expected to be similar among these MPi columns. However, as described above, the retention properties of the MnMP column for acidic proteins were different from those for the other two columns. Hence the metal arrangement in the crystal surface, which depends on both the type of anion (i.e., ortho-, pyro- or metaphosphate ion) and the linkage of PO_4 tetrahedra (i.e., ring or chain structure and its configuration), may influence the adsorption of acidic proteins. On the other hand, in MgMP, FeMP(C) and AlMP(A), their metal ions cannot

form pure single-cation HAP, so their surfaces probably maintain their intrinsic crystal structures or change, if at all, to compounds other than HAP. Nevertheless, similar elution behaviour for basic proteins was found among all studied MPi columns. Hence it is considered that the difference in the component metal and crystal structure of MPi does not greatly influence the adsorption of basic proteins on the MPi column.

As with the HAP crystal, there may be two types of adsorbing sites on the MPi crystal surface: one is the negatively charged sites formed by phosphorus ions and oxygen ions, and the other is positively charged sites related to the metal ions of the MPi crystal. The adsorption of biomolecules on the MPi crystal is considered to take place fundamentally because of the electrostatic interaction between the adsorption groups of these molecules and the adsorbing sites of the MPi crystal. However, the chemical properties of the MPi surface in a solution are dependent not only on the MPi's crystallographic properties but also on the solution conditions such as pH and ions present in the system as a result of the various hydrolytic reactions. From the fact that the MPi's zeta potential shows a negative values near neutral pH (Fig. 1), the positively charged sites which are available to adsorb acidic molecules (acidic proteins, nucleic acids and nucleotides) are probably partly or nearly saturated with the phosphate ions of the buffer under the chromatographic conditions in this study. To be adsorbed on the HAP surface an acidic molecule requires a contact area of fixed adsorbing sites that are free of adsorbed phosphate ions [13]. Hence, if the contact area contains less than fixed free sites, then no adsorption will take place. This may be one of the reasons why the MPi columns show weak or no retention of most acidic molecules. This interpretation is not inconsistent with the findings that the HAP with affinity for most acidic proteins has a p*I* of about pH 6 in 0.01 *M* potassium dihydrogenphosphate solution [14]. Almost the same result for the p*I* of HAP was also obtained under the present solution conditions (i.e., 0.01 *M* sodium phosphate solution), as shown in Fig. 1h.

Transferrin is known to have two conformations [15,16]: one is the conformation of apotransferrin in which the iron-binding site is in an open cleft near the protein surface and is exposed to the surrounding solution, and the other is the conformation of holo-transferrin in which the iron-binding site is in a closed cleft and is buried under the protein surface so that it cannot approach interacting agents. Hence, on the basis of the chromatographic results obtained in this study and the published findings on the conformation of proteins, it is suggested that the elution of iron-binding proteins may be the result of the main interaction between the metal-binding site or other negative adsorption groups of the protein and the metal ion of the MPi crystal. Moreover, the difference in chromatographic resolution of iron-binding proteins among MPi columns may be the result of the different metal uptake in a protein, which depends on both the protein and the metal of the MPi crystal.

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