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Paper

Enzymatically regulated demineralisation of pathological bone using sodium hexametaphosphate

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The pathological formation of bone in soft tissue can result in significant disability, prevent prosthetic limbs from fitting, and limit joint movement. A range of conditions exist, which are characterised by this local tissue ossification. The awareness of one such condition, heterotopic ossification, has increased recently due to the extraordinarily high incidence of the condition in military amputees (64.6%). Although the process of formation is biologically mediated through a massive inflammatory response, there is currently no adequate treatment or prophylaxis for the condition. This study investigates the use of hexametaphosphate (HMP) as a demineralising agent for the treatment of pathological ossification. Other demineralising agents exist but their application is limited due to unwanted effects on biological processes such as blood clotting and an inability to control their activity. This study demonstrates, for the first time, that the demineralising effect of HMP can be modified by local pH and is controlled through the activity of alkaline phosphatase, an enzyme that is found throughout the body. HMP was shown, using micro computed tomography, to cause large scale demineralisation of samples of pathological bone and was able to inhibit hydroxyapatite precipitation in a supersaturated solution. Stiffness and maximum force to failure of rat tibiae incubated in HMP were 49% ($p = 0.001$) and 41% ($p = 0.03$) lower, respectively, than controls. In contrast, no significant difference was observed in yield force, demonstrating specificity of action of HMP against hydroxyapatite, with no unwanted effect on collagen. Contrary to established understanding of the mechanism of its dissolution of calcium phosphate salts, micro X-ray fluorescence measurements of the hydroxyapatite surfaces suggested that the demineralising effect was mediated in the solution rather than surface binding of HMP. These findings suggest that HMP is effective at dissolving hydroxyapatite and, as such, is a promising a candidate for the treatment of a range of pathological ossifications.

1. Introduction

Heterotopic ossification (HO) is the pathological formation of lamellar bone in soft tissues. It causes disabling symptoms of pain, ankylosis, ulceration of the skin, peripheral nerve injury, and prosthetic limb fitting problems.^{1, 2} Due to the large numbers of combat injured patients from the conflicts in Iraq and Afghanistan, the incidence of HO is increasing. Radiological evidence of HO is found in 64.6% of combat injured patients.³ In a series of military casualties who required limb amputation, the incidence was 79.6% if the amputation was performed through the zone of injury.⁴ In the civilian setting, the incidence of HO after amputation may be lower, with 22.8% of patients reporting symptomatic HO.² The incidence of radiological evidence of HO secondary to other causes has been reported as follows: traumatic brain injury 37%, spinal cord injury 5 to 60%, hip replacement surgery 5%, fractures of

the distal humerus 8.6%, and burn injuries 0.15%.⁵⁻⁹

Bisphosphonates, non-steroidal anti-inflammatory drugs (NSAIDs), and radiotherapy are the main preventative therapies currently available. NSAIDs are favoured due to their availability and ease of administration but have many side effects that may make them unsuitable for traumatically injured patients. These include blood clotting dysregulation, gastrointestinal ulceration, renal injury, and an increased risk of non-union.^{10, 11} Radiotherapy has been used to prevent HO but there is significant controversy surrounding the timing and dose of treatment.¹² A Cochrane review of bisphosphonate use for HO prophylaxis found no evidence of efficacy. Despite this, they are still used widely in clinical practice.¹³

Non-surgical options for the alleviation of symptoms related to HO include rest, analgesia, nerve blocks, and nerve ablations.¹⁴ However, these treatments are only partially effective and many patients require surgery to alleviate their symptoms. In a series of civilian patients who underwent amputation for trauma, 11% required surgical HO excision.² This figure is 19% in combat-related trauma patients.⁴ Surgery carries risks of pain, infection, anaesthetic complications, and delay in patient rehabilitation (a mean interval between injury and excision of 8.2 months).⁴ Therefore, there is a need to

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develop new methods for the treatment of this debilitating condition.

Polyphosphates are a family of chemical compounds containing chains of phosphorous atoms covalently linked by phosphoester bonds. They can be linear or cyclical (Fig. 1) and the chain length can vary from one (orthophosphate) to many thousands.

Polyphosphates have been shown to be potent regulators of biomineralisation. The addition of minute concentrations (10^{-7} M) of HMP to a saturated calcium phosphate solution has been shown to inhibit crystallisation.^{15, 16} Further, incubation of chick femurs in the presence of polyphosphates can inhibit mineralisation.¹⁷ Detailed mechanistic work to attempt to understand the interaction between hydroxyapatite (HA) ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and a range of polyphosphates showed that HMP could dissolve HA in an inorganic system.¹⁸ The proposed mechanism of dissolution was the substitution of the polyphosphate's terminal phosphate group into the HA crystal lattice.

In vivo, the activity of condensed phosphates is regulated by phosphatase enzymes, such as inorganic pyrophosphatase and alkaline phosphatase (ALP), which function to mediate hydrolysis of the phosphoester bridges between the orthophosphate moieties. ALP, is of particular interest in the regulation of condensed phosphates *in vivo* since it is present in five isoforms throughout the body. The ubiquity of this family of enzymes means that ALP may enable the regulation of HMP following administration, such that its demineralising activity can be localised and is unlikely to have any deleterious systemic effects.

Many researchers are currently working on HO prophylaxis but all of these approaches focus on the upstream biological processes that lead, eventually, to ectopic bone formation. Examples include selective agonism of the retinoic acid receptor gamma pathway, remote ATP hydrolysis, and administration of echinomycin.¹⁹⁻²¹ However, upstream inhibition can never treat HO once it has formed. The application of the results presented in this paper will be the development of a clinically relevant formulation of HMP. This would be used to treat patients at high risk of developing HO as well as those in which the pathological bone had already formed.

In this study, the demineralising affects of HMP on hydroxyapatite monoliths and pathological bone samples were determined. The mechanism of this process was investigated using micro X-ray fluorescence (micro-XRF), which allowed us to determine whether the process was surface mediated as previously reported in the literature. Finally, the potential for the regulation of mineralising activity was evaluated by controlling local pH value and through the exposure of HMP to ALP.

2. Experimental

2.1 Materials and Methods

2.1.1 Hydroxyapatite Pellet Dissolution. Disc-shaped pellets of HA were formed by isostatically pressing 400 mg of HA powder (Sigma-Aldrich, Gillingham, UK) with 100 μL deionised water (dH_2O) at 2 kN for 5 seconds. These were sintered at 600 $^\circ\text{C}$ for 1 hour. After cooling in ambient conditions, pellets were weighed and incubated at room temperature (20 $^\circ\text{C}$) in 50 ml of a 0.1 M solution of each of the following reagents: sodium pyrophosphate decahydrate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$), sodium tripolyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$), trisodium trimetaphosphate ($\text{Na}_3\text{P}_3\text{O}_9$) (all Sigma-Aldrich), and sodium HMP ($\text{Na}_6\text{P}_6\text{O}_{18}$) (Fisher, Loughborough, UK). Ethylenediaminetetraacetic acid (EDTA) ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$) (Sigma-Aldrich) was used as a positive control. Before incubation, each reagent was adjusted to pH 7.4 with 0.1 M NaOH or 0.1 M HCl. There were triplicate repeats of each condition. The pH-adjusted media were replaced every 3.5 days. The pellets were removed from solution every 3.5 days, surface fluid removed with absorbent paper, and weighed. After 21 days the pellets were recovered (except for the EDTA group, which had dissolved completely), dried overnight at 80 $^\circ\text{C}$, and were then analysed by micro XRF.

2.1.2 Micro X-Ray Fluorescence. Elemental maps of the recovered and dried HA pellets were generated using a Tornado M4 micro-XRF system (Bruker Nano GmbH, Berlin, Germany) fitted with a Rhodium micro focus X-Ray tube and a polycapillary lens. The X-Ray spot size from the polycapillary lens was 25 μm . All samples were analysed under ambient conditions with an X-Ray tube voltage of 50 kV and a tube current of 400 μA . The X-rays were rastered over the sample surface with a step size of 100 μm and an XRF spectrum was collected at each point with an acquisition time per pixel of 2.5 ms. Elemental maps were then generated in real time by gating around the Phosphorous $\text{K}\alpha_1$ (2.0137 keV) and the Calcium $\text{K}\alpha_1$ (3.692 keV) X-Ray fluorescence emission peaks in the XRF spectra, creating an image where pixel intensity represented detected X-Ray counts per second per eV from each measurement point on the sample. Pixel intensity increased monotonically with X-Ray counts with maximum pixel intensity normalised to the highest count rate per eV for a given element across the whole sample. The instrument was set to map samples three times to generate a single averaged map for each element.

2.1.3 Hydroxyapatite Sol Dissolution. HA sol was synthesised according to the method of Afshar *et al.*²² After aging overnight, the sol was added to serial dilutions of HMP in a 96-well plate. Each concentration was conducted in triplicate. dH_2O was added to each well to give a constant final well volume of 245 μL . A Glomax 9301-010 plate reading spectrophotometer (Promega, Wisconsin, USA) was used to measure light transmission through the sample at 650 nm as a quantitative determination of the degree of dissolution. Results were normalised to a dH_2O blank. This experimental method was repeated with adjustment of the pH to 6.2, 7.4,

and 9.6 using 0.1 M HCl or 0.1 M NaOH as required. See supplementary figure 1. for calibration data.

2.1.4 Alkaline Phosphatase Control Of HMP. 3.6 mL of undiluted HA sol was dissolved completely in 25 mL 0.1 M HMP (adjusted to pH 7.4 using 0.1 M NaOH). The resulting solution was optically clear on macroscopic examination. 2 mL aliquots of this stock solution were incubated at 37 °C for 9 days with addition of either 400 units (in 200 µL) of bovine ALP (Sigma-Aldrich) for the experimental samples or 200 µL diH₂O for the control samples. The optical density of the solution was measured at day zero and day nine using a plate-reading spectrophotometer (see above). Each condition was conducted in triplicate and the results compared using a two-tailed Student's t-test.

2.1.5 Rat Tibia Mechanical Testing. Three pairs of tibiae were harvested immediately post mortem from male Lister hooded rats of 275-325 g bodyweight. Baseline non-destructive 4-point bend testing was performed on all of the samples using a Bose Electroforce 5500 mechanical tester (Bose / TA Instruments, Minnesota, USA). Testing parameters were as follows: upper points 4.0 mm apart, lower points 10.4 mm apart, displacement ramp 0.02 mm/s, non-destructive displacement limit 0.2 mm, destructive displacement limit 2.0 mm (see supplementary figure 2). The left-sided tibiae were incubated in 50 mL 1% w/v gellan (Gelzan CM, Kelco, Atlanta, USA) loaded with 0.1 M HMP. Right-sided tibiae were incubated in 50 mL 1% w/v gellan only. The gellan was used as an inert delivery vehicle in this experiment. All samples were incubated at room temperature (20 °C) for 6 days. Destructive 4-point bending was undertaken after this time. Data from the mechanical testing were analysed using WinTest 7 software (Bose). Stiffness was calculated as the gradient of the elastic region of the force-displacement curves, yield force was identified as the force corresponding to the upper limit of the elastic region of the force-displacement curves, maximum force was taken as the maximum recorded force during destructive testing. A two-tailed paired t-test was used to compare the stiffness, yield force, and maximum load to failure for each pair of tibiae.

2.1.6 Human HO Sample Dissolution. Samples of human HO excised from a single patient who had suffered civilian poly-trauma were retrieved from the Human Biomaterials Resource Centre at the University of Birmingham. These were incubated in 5 mL of either 0.1 M HMP (adjusted to pH 7.4 with 0.1 M NaOH) or diH₂O as a control for 7 days. Before and after incubation, these samples were wrapped in parafilm and placed inside a polystyrene sample tube. These were scanned using a Bruker SkyScan 1172 X-ray micro computed tomography (microCT) scanner (Bruker, Coventry, UK) with the following settings: no filter, camera position near, pixel size 4.96 µm, rotation step 0.2°, camera resolution 4000 x 2664 pixels, exposure time 400 ms, frame averaging 6, current 70 µA, voltage 70 kV. The scans were reconstructed using NRecon software (version 1.6.10.2, Bruker microCT) and analysed using CTAn (version 1.15.4.0, Bruker microCT). Three-dimensional models were generated using a double time cubes algorithm. Models were visualised with CTvol (version 2.3.1.0, Bruker

microCT). The same scanning, reconstruction, and post reconstruction processing settings were used for the samples before and after incubation.

2.1.7 Inhibition of HA formation. HA synthesis (as above)²² was undertaken in the presence of 10⁻³ M of each of the potential inhibiting reagents: pyrophosphate, linear tripolyphosphate, cyclic trimetaphosphate, EDTA, and HMP. The reaction products were aged for 1 hour then centrifuged (4000 rpm for 10 minutes) and washed with diH₂O three times. The product was dried overnight at 80 °C and finely ground. X-ray diffraction patterns were generated using a Bruker D8 diffractometer (2θ 5-80°) and analysed using Bruker EVA *diffraction.suite* software (version 3.1). The raw diffraction data was baseline corrected but not smoothed. Reference data for HA (PDF 00-009-0432) and calcium hydroxide (Ca(OH)₂) (PDF 00-044-1481) were obtained using the software program PDF-4+ with database version 4.4103 (International Centre for Diffraction Data).

3. Results and Discussion

3.1 HMP Dissolves HA *in vitro* and *ex vivo*

Incubation in HMP reduced the mean mass of HA pellets by 30.2% over 504 hours (Fig. 2). The EDTA positive control reduced the mass of pellets by 95.0% in the same time period. diH₂O water control, cyclical trimetaphosphate, linear tripolyphosphate, and pyrophosphate had negligible effect on HA pellet mass.

Incubation of rat tibiae in gellan loaded with HMP caused a reduction in stiffness and maximum load to failure. Stiffness was 49% lower in the HMP/gellan group compared to gellan-only controls ($p = 0.001$). Maximum load to failure was 41% lower in the HMP/gellan group compared to gellan-only controls ($p = 0.03$) (Fig. 3). There was no significant difference in the yield force between the two conditions. The reduction in stiffness of the bone is consistent with the dissolution of hydroxyapatite from the bone matrix.^{23, 24} Interestingly, the yield force was unaffected by incubation in HMP. As the yield force is determined by the collagenous component of bone, this finding suggests that HMP is able to dissolve HA from within the bone composite structure without affecting the collagen component.^{25, 26}

By using the same scanning, reconstruction, and processing protocols for the samples before and after incubation, micro-CT imaging and volume analysis demonstrated that HMP caused the demineralisation of samples of human HO at physiological pH. The bone volume of the HO sample incubated in 0.1 M HMP at pH 7.4 for 7 days decreased from 11.6 mm³ to 3.43 mm³, a reduction of 70.4%. The bone volume of the control sample changed from 9.34 mm³ to 8.76 mm³, a reduction of 6.2%. Three-dimensional models of the mineralised component of the HO samples are shown in Fig. 4. The sample incubated in HMP appears grossly attenuated. In contrast, the sample incubated in diH₂O appears largely unchanged compared with its pre-incubation state.

3.2 Evidence for Solution-Mediation Dissolution

Elemental maps illustrate the resulting changes in surface chemistry as a result of incubation in the polyphosphates for 21 days (Fig. 5). diH_2O , HMP, linear tripolyphosphate, and cyclical trimetaphosphate all showed little change in Ca:P ratio over the period of the study. The pellets incubated in pyrophosphate, however, showed an increased phosphorus signal distributed heterogeneously across the surface of the pellet, suggest the adsorption of pyrophosphate ions to the surface of the samples. There are no images for the pellets incubated in EDTA as these were dissolved completely.

The finding that the HMP group did not cause any change in the Ca:P ratio compared with deionised water controls is significant because, if the mechanism of dissolution was initial incorporation of the terminal phosphate group of HMP into the HA matrix, an increase in phosphorus signal relative to calcium signal would be expected.¹⁸ The lack of change in Ca:P, coupled with the monotonic rate of dissolution was more consistent with a solution- rather than surface-mediated mechanism. Certainly, HMP has a very strong affinity for Ca^{2+} ions, forming a 1:1 complex with a high thermodynamic stability constant.²⁷ Thus it is possible that HMP is complexing any available Ca^{2+} ions from the hydroxyapatite matrix and forming a highly-stable Ca-HMP complex in solution. Indeed, HMP has such a high affinity for cations that it has been demonstrated to dissolve kaolin and kaolinite by complexing the aluminium cations in these minerals.²⁸

The finding that incubation of HA pellets in pyrophosphate caused an increase in the surface phosphorus signal on micro-XRF suggests adsorption to the surface of the pellet, which is consistent with previous reports in the literature.²⁹

3.3 HMP Activity is Regulated by pH and Alkaline Phosphatase

At every concentration of HMP, higher pH values reduced the amount of HA sol dissolved (Fig. 6). For a HMP concentration of 0.1125 M, the available HA in sol form in the samples at pH 6.2, 7.4, and 9.6 was 0.15, 13.3, and 23.4 g/L, respectively (note: control samples of sol had 26.43 g of HA per L). See supplementary figure 3 for additional data derived using HA residual mass to demonstrate the HA-dissolving ability of HMP. This pH-controlled effect was demonstrated across a range of concentrations of HMP spanning four orders of magnitude. This may prove to be a key mechanism by which the activity of HMP can be targeted towards the sites of pathological bone formation in HO. This is because hypoxia is thought to be a key step in the biological pathway for HO formation, and hypoxia leads to local low pH in tissues through switching toward anaerobic respiration and the generation of lactate.^{30, 31} Therefore, in sites where pH is low and HO is most likely to develop, HMP will also be most potent at dissolving HA. While these data provide compelling evidence that pH controls the HA-dissolving ability of HMP, they do not provide any mechanistic explanation for the relationship.

Addition of ALP to a solution of HA sol dissolved in HMP caused HA to precipitate out of solution as demonstrated by a change from clear to turbid over 9 days. The mean HA

concentration in the ALP group increased from 1.08 to 11.8 g/L ($p = 0.00$) whereas that of the control group fell from 1.13 to 0.94 g/L ($p = 0.007$) (Fig. 7). See supplementary figure 4 for additional data derived using HA residual mass to demonstrate the effect of ALP on HA sol concentration.

ALP is an exophosphatase found in many tissues that can cleave the terminal phosphate group from polyphosphates. As demonstrated in Fig. 2, shorter chain length polyphosphates have little or no HA-dissolving ability. Therefore, we interpret the increased turbidity in the presence of ALP to be due to HA precipitating out of solution as the solvent (HMP) is cleaved. The importance of this is that it demonstrates that ALP-mediated degradation is a feasible mechanism for the inactivation of HMP over time in the body. It would not be desirable to deliver a therapeutic agent with HA-dissolving activity into the body without the possibility of limiting its duration of action. It is noted that, in contrast to the ALP group, the HA concentration of the control group decreased. This may represent further dissolution of any undissolved HA sol over the incubation period.

3.4 HMP Inhibits HA Synthesis

All of the experiments and results discussed above relate, in various ways, to the dissolution of HA by HMP. In addition to dissolving HA, HMP is also able to inhibit its synthesis. Polyphosphates (10^{-3} M) were added to the reaction mixture when synthesising HA using the Afshar method.²² X-ray diffraction (XRD) patterns generated from unsintered reaction products are shown in Fig. 8. The patterns generated in the control sample and in the presence of cyclic trimetaphosphate, EDTA, linear tripolyphosphate, and pyrophosphate match the reference standard of HA with no detectable secondary phases. In contrast, the pattern generated by the sample synthesised in the presence of HMP has additional peaks that match the reference standard for $\text{Ca}(\text{OH})_2$, one of the reaction precursors. This suggested that the HMP inhibited the formation of HA as previously reported elsewhere.^{17, 18, 32} Given that HMP was able to reduce the reaction yield of HA precipitated in a super-saturated solution, it is possible that *in vivo*, it could inhibit the formation of HA in ectopic bone.³³

3.5 Application and Future Developments

The next stage in the development of HMP as a therapeutic agent will be to test it upon an *in vivo* model of heterotopic ossification. Selection of the most appropriate model for testing will be crucial for allowing researchers to apply any findings to clinical development.^{34, 35}

4. Conclusions

This paper demonstrates that HMP can dissolve hydroxyapatite in inorganic and biological settings, that this effect is controllable through alteration in pH and through the action of ALP, that *ex vivo* human HO is attenuated by its action, and that HA formation can be inhibited in the presence of small concentrations of this simple polyphosphate. Existing

clinical prophylaxis and current innovations in this field focus on the upstream biological pathways. However, chemical means of dissolving the final product, hydroxyapatite, could be an effective way of addressing this problem and the data presented here show this to be a possibility. If efficacy is proven in the condition of heterotopic ossification, this opens the possibility of broadening the application to other diseases of extra-skeletal mineral deposition.

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Ethical Approval

Ethical approval for the use of HO tissue for analysis and publication has been given by the Human Biomaterials Resource Centre (HBRC) via the North West 5 Research Ethics Committee, Haydock Park; Ref 09/H1010/75. The patient has given full consent for their tissue to be used for analysis and for dissemination of the results.

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Fig. 1 Schematic chemical structures of ionic phosphate and polyphosphates: orthophosphate (Pi), pyrophosphate (PPI), linear triphosphate (LTPP), cyclic trimetaphosphate (CTMP), and hexametaphosphate (HMP).
42x22mm (600 x 600 DPI)

Fig. 2 Mass loss of hydroxyapatite pellets over time incubated in various potential dissolving agents. This demonstrates the potent dissolving ability of hexametaphosphate. Ethylenediaminetetraacetic acid (EDTA) was used as a positive control. Deionised water (diH₂O), cyclic trimetaphosphate (CTMP), linear tripolyphosphate (LTPP), pyrophosphate (PPI), hexametaphosphate (HMP). Error bars are +/- SEM. 82x82mm (300 x 300 DPI)

Fig. 3 Results of 4-point mechanical testing of rat tibiae after incubation in either 1% gellan alone or 1% gellan loaded with 0.1 M HMP for 6 days. Incubation in HMP caused a significant reduction in the stiffness and maximum load to failure compared to matched controls group. Error bars are +/- SEM. * = $p < 0.05$.
82x82mm (300 x 300 DPI)

Fig. 4 Three-dimensional surface-rendered models of the mineralised volumes of samples of heterotopic ossification. Before (A) and after (B) incubation in 0.1 M HMP at pH 7.4 for 7 days. Before (C) and after (D) incubation in diH₂O at pH 7.4 for 7 days. The sample incubated in HMP is clearly attenuated after incubation whereas the control sample shows little change. Scale bar = 1 mm.

Fig. 5 Micro X-ray fluorescence mapping of calcium and phosphorus on the surface of pellets of hydroxyapatite after 21 days of incubation in the following potential dissolving agents: deionised water (diH₂O), cyclic trimetaphosphate (CTMP), linear tripolyphosphate (LTPP), pyrophosphate (PPi), hexametaphosphate (HMP). Note that for each dissolving agent, this figure shows the micro-XRF data for different elements on the surface of a single pellet. There were, in fact, triplicate repeats under each condition and the changes shown here were typical for all repeats.
82x102mm (300 x 300 DPI)

Fig. 6 Demonstration of the effect of varying the pH and concentration of HMP on the amount of available HA in sol form. Lower amounts (in g / L) of available HA sol indicates higher amounts dissolved by the HMP. Dissolving ability is increased with increasing concentration and decreasing pH. Error bars are +/- SEM. 82x82mm (300 x 300 DPI)

Fig. 7 Change amount (in g / L) of available HA in sol form in a nearly-saturated solution of HA dissolved in 0.1 M hexametaphosphate after incubation with either alkaline phosphatase (ALP) or control (diH₂O) for 9 days. Error bars are +/- SEM. * = $p < 0.05$.
82x82mm (300 x 300 DPI)

Fig. 8 X-ray diffraction patterns of the products of hydroxyapatite synthesis. In the presence of hexametaphosphate, there is an increased amount of unreacted calcium hydroxide (Ca(OH)_2) precursor in the product suggesting inhibition of hydroxyapatite crystallisation. The other reagents had no measurable effect compared to control. Pyrophosphate (PPi), linear tripolyphosphate (LTPP), ethylenediaminetetraacetic acid (EDTA), cyclic trimetaphosphate (CTMP), hexametaphosphate (HMP).

82x40mm (300 x 300 DPI)

A

B

Human heterotopic ossification before (A) and after (B) incubation in hexametaphosphate