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Local injection of a hexametaphosphate formulation reduces heterotopic ossification in vivo

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1. Introduction

Heterotopic ossification (HO), the pathological formation of ectopic bone, is a debilitating condition which can cause chronic pain, limit joint movement, and prevent prosthetic limb fitting. The prevalence of this condition has risen in the military population, due to increased survivorship following blast injuries. Current prophylaxes, which aim to target the complex upstream biological pathways, are inconsistently effective and have a range of side-effects that make them unsuitable for combat-injured personnel. As such, many patients must undergo further surgery to remove the formed ectopic bone. In this study, a non-toxic, U.S. Food and Drug Administration (FDA) -approved calcium chelator, hexametaphosphate (HMP), is explored as a novel treatment paradigm for this condition, which targets the chemical, rather than biological, bone formation pathways. This approach allows not only prevention of pathological bone formation but also uniquely facilitates reversal, which current drugs cannot achieve. Targeted, minimally invasive delivery is achieved by loading HMP into an injectable colloidal alginate. These formulations significantly reduce the length of the ectopic bone formed in a rodent model of HO, with no effect on the adjacent skeletal bone. This study demonstrates the potential of localized dissolution as a new treatment and an alternative to surgery for pathological ossification and calcification conditions.

Increased military occurrence has also highlighted the prevalence of HO in civilian patients, after total hip arthroplasty (24–28% [5]), burn injuries (5.6% [6]), and traumatic brain and spinal cord injuries (4% and 11%, respectively [7]).

Notably, there is a lower prevalence (23%) of HO in civilians after amputation [2]. The large discrepancy in prevalence between military and civilian amputees, as well as the diversity of causes, highlights the complex etiology of HO. There is a general consensus that HO is formed via a combination of systemic factors, such as upregulation of cytokines, and local conditions, including hypoxia, inflammation, and hematoma formation [8]. Kaplan et al. [9] described four factors necessary for the formation of HO: (1) an inciting event, usually traumatic, which may cause a hematoma; (2) cellular signaling from the injured cells or the inflammatory cells that move to the site; (3) a supply of undifferentiated mesenchymal stem cells, which will differentiate into chondroblasts and...
Heterotopic Ossification
Non-Specific Anti-Inflammatory Drugs
Radiotherapy
NSAIDs
Targetted Demineralisation

HMP

\[(PO_4)^{3-} + 3Ca^{2+} \rightarrow Ca_6(PO_4)_6\] (1)

The early work by Fleisch and Neuman [34] and Fleisch et al. [35] showed that HMP is able to prevent precipitation of calcium phosphate, even in supersaturated solution. HMP has further been shown capable of dissolving solid hydroxyapatite (HA), the main mineral constituent of bone [36,37]. Eisenstein et al. [38] have recently shown that HMP can demineralize biological bone, without affecting the adjacent collagen, and that it is hydrolyzed by alkaline phosphatase, an enzyme present

The most common prophylaxes currently used to prevent HO are non-steroidal anti-inflammatory drugs (NSAIDs) and radiotherapy. NSAIDs, typically orally administered indomethacin, aim to prevent HO by suppressing proliferation and inducing apoptosis of osteoblasts and chondrocytes [15]. NSAIDs may reduce the incidence of HO but can cause gastrointestinal side-effects, acute renal failure, and bleeding, and increase the risk of fracture non-union [16–18]. This can result in discontinuation, even in relatively healthy civilian patients, as their deleterious side-effects become worse than the initial condition. Perioperative radiotherapy is administered to suppress HO by inhibiting mesenchymal stem cell proliferation or inducing terminal differentiation [19]. This prevention has been poorly studied, with the exception of HO following hip arthroplasty, with inconclusive timing and dosage guidelines [20, 21]. Radiotherapy may also give rise to non-union and contracture of soft tissues and delay wound healing [22,23]. Both NSAIDs and radiotherapy are non-specific, aiming to reduce HO by decreasing inflammation, and neither are entirely effective. They reduce the probability of developing HO, though effectiveness varies between studies [23]. Furthermore, the vast majority of these studies examine the development of HO after elective joint surgeries in controlled medical environments, where the side-effects may be tolerable. This is magnified for combat-injured patients with complex injuries, in whom delayed wound healing and fracture non-union are unacceptable.

Current research on new prophylactics for HO aims to target the specific biological pathways by which the ectopic bone forms. Strategies such as antibiotic administration, remote adenosine triphosphate hydrolysis, and agonism of retinoic acid receptor gamma have been investigated [24–27]. Despite their potential, the complexity of the biological pathways of HO, which are not completely understood, limits understanding of how these interventions prevent the disease and may not prevent all etiologies of HO. Furthermore, therapies which target these upstream biological processes of bone formation, including NSAIDs and radiotherapy, will never be able to treat HO once formed, leaving excision surgery as the only option for many.

The biological mechanisms leading to HO are complex and may have multiple pathways. As such, no specific biological prevention are used clinically, and currently used prophylaxes are non-specific, not entirely effective, and have a range of side-effects. However, all forms of HO share a common final step; formation of solid calcium phosphate mineral in bone. A prevention that targets this chemical pathway would not only be able to treat all forms of this disease but would be uniquely able to dissolve formed mineral. This would offer not only a specific prophylactic for HO but also a medical alternative to surgery.

Hexametaphosphate (HMP), commonly supplied as a sodium salt, is an inorganic, multivalent polyphosphate. It is used in several industries including food, minerals, and ceramics, as well as in medical and dental applications [28–33]. HMP may be used as a deflocculant, by altering the charge of particles, but is more commonly used as a sequestant because it forms strong complexes with metal cations, particularly calcium (Equation (1)).

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ubiquitously in vivo. This is important as it will temporarily limit the action of HMP, preventing continuous demineralization. These properties of HMP advocate its use as a therapeutic to target the chemical formation pathway of HO to prevent and reverse the mineralization of bone. However, the dissolution mechanism of HMP is not specific to pathological, rather than skeletal, bone. It must therefore be loaded into a delivery vehicle for targeted release to the HO site. This study proposes the use of colloidal alginate as a delivery vehicle for HMP. The resulting injectable formulation could be used as a prophylactic or minimally invasive alternative to surgery to improve outcomes and quality of life for sufferers of HO. In this manuscript, we report on the formulation of an injectable delivery system and examine its efficacy in vitro, ex vivo, and in an in vivo model of the disease.

2. Methods and materials

2.1. Study design

The objective of this study was to formulate, optimize, and characterize an injectable HMP-alginate formulation, which may be used as a novel prophylactic and treatment for HO. For in vitro evaluation, a standard n = 3 was used for most studies and for in vivo, n = 6. In vivo work was batch randomized, with 3 animals per batch, and blinded so that the injector did not know which animals were in the active or control group. As a de novo study, time points for injection were based on the stipulations of the project license, and the endpoint was determined by assurance of HO development from initial model validation studies. A moderate level of discomfort at any point was also an endpoint, though this was not enacted in this study. No data were excluded.

2.2. Material characterization

2.2.1. Formulation

Alginate-HMP systems were prepared by dissolving HMP powder (sodium hexametaphosphate, general purpose grade, Fisher Scientific) in deionized water and then codissolving alginate powder (alginic acid sodium salt from brown algae, BioReagent grade, MW 100–200 kDa [39], cn 71238, Sigma-Aldrich), by stirring at 400 rpm for 30 min at 20 °C. Sodium hexametaphosphate (sodium hexametaphosphate, general purpose grade, Fisher Scientific) was dried and ground to give a powder for precipitate, which was then dried and ground to give a powder for analysis.

2.2.2. Rheology

Rheological characterization of formulations was carried out on an AR-G2 rheometer (TA Instruments), using 40 mm diameter sandblasted parallel plates, to minimize wall slip for highly viscous samples, with a 1 mm gap. Shear ramps were carried out by continuously increasing the shear rate from 1 to 1000 s⁻¹, over 10 min, and measuring the viscosity instantaneously. Recovery behavior was examined by performing a series of peak holds, at a shear rate of 1 s⁻¹ and 1000 s⁻¹, respectively, for 1 min. All measurements were taken at 20 °C.

2.2.3. Zeta potential

Zeta potential of the polymers in solution was determined via electrophoretic light scattering, using a Zetasizer Nano ZS (Malvern), which calculated the zeta potential after measuring the electrophoretic mobility via laser Doppler velocimetry, at a wavelength of 633 nm. For each measurement, 10–100 runs were taken at 25 °C.

2.2.4. Fourier-transform infrared spectroscopy

Scans were performed in transmission mode, with attenuated total reflectance, on a Nicolet 380 Fourier transform infrared (FTIR) spectroscope (Thermo Electron Corporation). For each sample, spectra were averaged from 128 repeats. Samples of HMP and alginate were analyzed directly in their powder form. To prepare the mixed system, a formulation of 1 w/v% alginate and 1 M HMP was filtered to collect the precipitate, which was then dried and ground to give a powder for analysis.

2.2.5. Injectability

Injectability studies were carried out on a Z030 universal mechanical tester (Zwick Roell), as shown in Fig. 4A. A displacement-controlled compression test was applied, using a 100 N load cell, on the plunger of a 1 mL Luer-Lok syringe (Beckton Dickinson), suspended by clamps. The attached needle was either a 19-gauge (690 μm internal diameter, 50 mm length) or 30-gauge (160 μm internal diameter, 12 mm length) needle and was initially full. Displacement was carried out over 45 mm, and the maximum allowable force was set at 50 N, as the limit for a reasonable injection force by hand is 38 N [40].

2.3. Bioactivity

2.3.1. HMP release

Release from the formulations was studied via the regular dialysis method [41], and conductivity was used to measure concentration. Dialysis tubing, with a nominal molecular weight cutoff of 2000 Da, was loaded with 5 mL of formulation and tied at each end to create a sealed parcel. Each sample was placed into 50 mL of deionized water at 37 °C, and the conductivity of the release medium was measured up to two weeks. The HMP concentration in the medium was calculated from the conductivity using a standard curve and corrected to remove the contribution to conductivity from alginate, using an alginate only control. The data are presented as a percentage of the total release, with 100% taken as the point at which the HMP concentration is the same both inside and outside the dialysis tubing barrier.

2.3.2. In vitro dissolution of HA

HA was synthesized by a sol-gel precipitation method [42]. Discs (12 mm diameter, 1 mm thickness) were formed in a pellet press and were then sintered at 700 °C for 4 h. Pellets were then embedded in EpoFix (Struers) resin and were polished with silicon carbide discs (Struers) of decreasing grain size, down to 5 μm.

Interferometry was carried out on a MicroXAM2 (Omniscan), using green light. Scans on day 0 were carried out over a depth of 20 μm, while scans on day 7 were carried out over a depth of 30 μm. All scans had a noise reduction of 0.05. Scanning electron microscopy (SEM) images were taken using secondary electron detection, with a TM3030 Plus (Hitachi) at 15 kV. Embedded pellets were attached to a steel mount with carbon tape, sputter coated with 15 nm of gold, and connected to the mounting with copper tape to improve conductivity. Pellets were analyzed via both modalities, and then, 40 μL of 2 w/v% alginate with or without 0.2 M HMP was applied. The pellets were stored in a closed container with water to create a humid environment and reduce evaporation. The formulation was removed with deionized water, the pellets were dried with absorbent paper, taking care not to scratch the surface, and formulation was reapplied in the same location. This was repeated daily, for 5 days, before reanalysis of the pellet surfaces.

2.3.3. Ex vivo demineralization of the bone

Femurs were harvested immediately postmortem from male Sprague Dawley rats (Charles River) and frozen at –20 °C until further use. The distal end, with an approximate volume of 30 mm³, was removed and placed in 2 w/v% alginate solution, with or without 0.2 M HMP, adjusted to pH 7. Microcomputed tomography (micro-CT) scans were taken at 0, 7, and 14 days with a SkyScan 1172 (Bruker), using the following settings: 0.5 mm aluminium filter, current 100 mA, voltage 75 kV, exposure time 950 ms, pixel size 5.4 μm, camera resolution 2000 × 1332 pixels, rotation step 0.3°, frame averaging 10. Scans were reconstructed using NRecon (version 1.6.10.2, Bruker), and 3D models were produced in CTVox (version 3.0.0, Bruker). The same scanning, reconstruction, and postreconstruction parameters were used for all scans.
2.3.4. In vivo prevention of HO

Ethical approval for this work was given by the University of Birmingham's Animal Welfare and Ethical Review Board, and experiments were licensed by the UK Home Office. All work was carried out in strict accordance to the guidelines of the UK Animal (Scientific Procedures) Act 1986 and the Revised European Directive 1010/63/EU and conformed to the guidelines and recommendation of the use of animals by the Federation of the European Laboratory Animal Science Associations.

Unilateral Achilles tenotomy was performed on the right hind limb of adult male Sprague Dawley rats (Charles River) to induce HO [43]. Surgery was performed under general anaesthesia, induced and maintained by 5–2% isoflurane, and the incision was closed with two interrupted sutures and sealed with surgical glue. Opioid analgesia, typically buprenorphine, was given as required; NSAIDs were not used at any point in this study.

Surgery and injection of the formulations were performed under sterile conditions, and all equipment used was either purchased sterile or autoclaved before use. Formulations were sterilized by passing them through a 0.2 μm filter and aspirating them into syringes, which were kept sterile until use.

This study was performed in two batches, with 6 rats in each batch: 3 in the treatment group and 3 in the control group. At 2, 4, 6, and 8 weeks after the operation, each animal was given an injection into their right hind limb, directly adjacent to the site of HO formation. The control group was given 200 μL of 1 w/v% alginate, and the treatment group was given 200 μL of 1 w/v% alginate with 0.2 M HMP, via a 0.6 mm diameter needle. Animals were sacrificed at 10 weeks, by exposure to CO2 in rising concentration, and their hind limbs were immediately harvested and frozen at –80 °C until scanning.

The tissue was defrosted, and micro-CT scans were taken using a SkyScan 1172 (Bruker). The scan settings used were as follows: 0.5 mm aluminium filter, current 100 mA, voltage 75 kV, exposure 400 ms, pixel size 21.5 μm, camera resolution 1000 × 666 pixels, rotation step 0.6°, frame averaging 2. Scans were reconstructed using NRecon (version 1.6.10.2, Bruker), and quantitative analysis was performed in CTAn (version 1.15.4.0, Bruker). For length measurement, the data were first orientated in DataViewer (version 1.5.1.2, Bruker) so that the longest axis of the HO was orthogonal to the x-y plane. The data were then loaded in CTAn, and the length was calculated from the height of the z-stack. The cortical bone tissue mineral density (TMD) of the tibia directly adjacent to the HO site (Fig. 6B blue square) was calculated following the method supplied by the manufacturer, by calibrating with phantoms of a known HA density [44]. 3D models were produced in CTVox (version 3.0.0, Bruker). The same scanning, reconstruction, and post reconstruction parameters were used for all scans.

2.4. Statistical analysis

Normality was determined using a Shapiro-Wilk test. All data sets were then analyzed using two-tailed unpaired t-tests, with <0.05 defined as significant in all tests (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001). Standard deviation (SD) was used as the measure of uncertainty. Statistical analysis was performed in Prism (version 7, GraphPad).

3. Results

3.1. Shear-thinning formulations with recoverable post-shear viscosity

Therapeutic formulations were prepared by dispersing alginate into an aqueous solution of the HMP (Fig. 2A). This created viscous, fluid formulations which could be injected through a needle and subsequently recohere (Fig. 2B). Formulations containing HMP at a concentration of up to 0.2 M exhibited a similar yellow-brown color and translucency to alginate alone, however, above this concentration, a precipitate formed and phase separation was observed (Fig. 2C).

Rotational rheology was performed to characterize the shear-dependent viscosity of the formulations. The viscosity of all samples was found to decrease with applied shear, that is, they were shear-thinning (Fig. 2D). HMP concentration was also found to influence the nature of this behavior, demonstrated by the change in concavity of the shear response curves. Furthermore, HMP concentration altered the instantaneous viscosity of the formulations at all tested shear rates. Alginate solution viscosity was increased by raising the HMP concentration, up to a critical value of approximately 0.2 M (Fig. 2E). However, further increasing HMP content above this concentration decreased the viscosity. This is concurrent with the observation of precipitate formation at HMP concentrations greater than 0.2 M. The time it took for the formulations to recover their standing viscosity following the application of high shear (1000 s⁻¹) was also assessed. The 0.2 M HMP system, which did not precipitate, recovered its viscosity on the removal of shear almost instantaneously (Fig. 2F). The precipitated samples with 1 M HMP, however, displayed thixotropy. There was time dependence to the shear-thinning behavior, with the formulation taking around 30 s to recover.

3.2. Interaction between the demineralizing agent and polymer network

The strong effect of HMP concentration on the rheological properties of the formulations (Fig. 2) suggested that there was a molecular interaction between the alginate and active component. This behavior was found to be concentration dependent, and up to 0.2 M HMP, the network density increased, as evidenced by increased viscosity. Above this concentration, precipitation occurred. To investigate the electrostatic forces, the zeta potential of the formulations was measured. Alginate alone in solution is strongly anionic, having a zeta potential more negative than −30 mV, indicating that the electrostatic repulsion between polymer chains is enough to prevent aggregation [45]. However, the addition of HMP, at any concentration tested (0.2, 0.4, and 1 M), reduced the zeta potential to less than −30 mV (Fig. 3A). This suggested that it decreased the electrostatic repulsion and allowed the polymer chains to come into closer contact, which above 0.2 M HMP resulted in flocculation and precipitation. The pH appeared to have little effect on the zeta potential of this system.

To investigate the chemical mechanisms of this interaction FTIR was conducted on the individual components, alginate and HMP, and the formed precipitate. No new peaks were seen in the spectra for the precipitate that were not present in the raw material spectra, discounting covalent bonding between species. For some peaks, however, the wavenumber was observed to shift, suggesting ionic interactions between the alginate and HMP (Fig. 3B). In particular, there was a large shift in the peak at 1589 cm⁻¹ (circled), which corresponded to the COO⁻ group on the alginate [46], and shifts in the peaks at 1234 and 1065 cm⁻¹, which corresponded to the P=O and P–O groups, respectively, on the HMP (Fig. 3C) [29]. This implied that these groups were interacting ionically, and thus a mechanism can be proposed, whereby the resonating groups on both species stabilize around one or more sodium ions, of which there are an abundance in these formulations (Fig. 3D). This may explain the effects of HMP on the bulk rheological properties. At concentrations up to 0.2 M, each HMP ion binds multiple polymer chains, increasing the network density, but without causing phase separation. At higher HMP concentrations, the increased number of interactions causes the polymer chains to come much closer together, which forces the water phase out (Fig. 3E). This polymer-rich phase, having a lower density, then creams out, leaving a polymer-poor phase below (Fig. 2C). The precipitate removes the polymer available to form the network, reducing the viscosity of the system.

3.3. Injectable formulations for targeted release

Rheological experiments provided an important insight into the shear
rate response of the formulations; however, the tests performed did not entirely represent the intended delivery device. To provide a more fidelitous assessment of the in situ behavior during injection, a displacement controlled compression test was used to obtain a direct quantitative measure. The plunger was compressed at a set rate, and the force required to push the formulation through the needle was measured (Fig. 4A).

Injectability was determined as the ability of the formulation to entirely pass through the needle with a reasonable amount of force. The value at the plateau was taken as the injection force (Fig. 4B, black arrow). Uninjectable systems were defined as those which required a force above 38 N to be injected [40]. Formulations in which a precipitate had formed and where the solid material did not pass through the needle, had an initial force-displacement trace similar to injectable samples. However, at a certain point (Fig. 4B, light gray arrow), the injection force rapidly increased due to a build-up of precipitate in the syringe, resulting in filter pressing.

A range of parameters, known from the Hagen-Poiseuille equation to determine injectability, were investigated: alginate concentration (corresponding to formulation viscosity), needle diameter, and injection speed [40]. While previous experiments indicated that 0.2 M may be the maximum HMP concentration which does not cause phase separation, these additional data may help to identify an optimum alginate concentration in the formulations. Two needle gauges were investigated: the smaller, which is suitable to test the treatment in a rodent model, and the larger which is more appropriate for clinical use. A range of injection speeds were tested; 1 mm s\(^{-1}\) is representative of manual injection [40], however a larger range was tested to examine the effects of shear-thinning.

The required force for injection was found to increase with increasing polymer concentration (Fig. 4C). This was because increasing the alginate concentration increases the formulation viscosity. Doubling the polymer concentration increased the standing viscosity by approximately a factor of 10 (Fig. 2E); however, a more complex relationship was seen for injection force. This is likely due to the shear-thinning behavior of the formulations, which decreased their viscosity as they experienced high shear in the narrow gap of the needle, and the composite geometry in a needle-syringe system.

Decreasing the needle diameter greatly increased the force required for injection (Fig. 4D). This may be attributed to an increase in average flow speed through the smaller diameter needles. Increasing the flow speed increased the injection force (Fig. 4E) because of the higher wall shear stress. It should also be noted that different gauge needles are different lengths, which is another parameter included in the Hagen-Poiseuille equation that is known to affect injection force.

Fig. 2. Formulations are shear-thinning and recover their viscosity following shear. (A) Schematic of the formulation process; HMP and alginate were cos dissolved in deionized water, creating a formulation which can then be loaded into a syringe. (B) Photograph of a formulation (containing 5 w/v% alginate and 0.2 M HMP) being injected, with insert demonstrating viscosity recovery after delivery through a 160-μm diameter needle. (C) Photographs of 2 w/v% alginate with varying HMP concentrations showing phase separation above 0.2 M. (D) Shear rate ramps revealing the shear-thinning behavior of 2 w/v% alginate and varying concentrations of HMP. (E) Viscosity of formulations at a shear rate of 2 s\(^{-1}\), as a function of HMP concentration for various alginate concentrations. (F) Recovery of viscosity as a function of time, when 2 w/v% alginate was exposed to high shear (1000 s\(^{-1}\), shaded sections) to mimic injection and then low shear (1 s\(^{-1}\), white sections) to assess the ability of the formulation to remain localized. (C), (E), and (F) show mean ± SD (n = 3). SD, standard deviation; HMP, hexametaphosphate.
The release of the active component from the formulation over time was assessed in vitro through a regular dialysis method. Around 40% of HMP was released in the first 6 h, with apparent zero-order kinetics and a further 30% released over the following two weeks (Fig. 5A). From the first release region (Fig. 5B), a higher polymer concentration was found to slow release.

The force required to inject formulations (0.2 M HMP, 690 μm needle diameter, 1 mm s⁻¹ injection speed) at varying alginate concentrations. (C) shows mean ± SD (n = 5) and (B) shows the mean of 128 scans. SD, standard deviation; FTIR, Fourier-transform infrared spectroscopy; HMP, hexametaphosphate.

The force required to inject formulations (2 w/v% alginate, 0.2 M HMP, 690 μm needle diameter) at varying injection speeds. (C), (D), and (E) show mean ± SD (n = 3), with p values determined by two-tailed unpaired t-tests, not significant (ns) when p > 0.05. SD, standard deviation; HMP, hexametaphosphate.
Specifically, 2 and 5 w/v% alginate formulations released the HMP 21% and 34% slower, respectively, than the 1 w/v% sample over 6 h. This was due to the higher polymer content increasing the path length and retard ing solute release.

The demineralizing capacity of the formulations was first tested in vitro using sintered HA pellets. Interferometric analysis revealed that applying 0.2 M HMP containing formulations to HA increased the surface roughness more than six-fold (Fig. 5C). This was likely because the formulation dissolved the surface of the HA, but the free ions then precipitated out as they cannot flow away in this static in vitro system. The observed surface morphology supported this mechanism; the formation of discreet spheres suggested nucleation and growth of solid mineral precipitating from solution (Fig. 5D). A small increase in surface roughness (24%) was also seen for the formulation without HMP. This may be due to some HA dissolution in the aqueous phase, perhaps enhanced by alginate’s affinity for calcium, or simply caused by water ingress into the ceramic. In contrast to the active sample, reprecipitation was not observed for the control without HMP (Fig. 5E), and this is likely because supersaturation was not achieved.

The targeted delivery of the formulations is demonstrated by Fig. 5F, where a distinct boundary between regions treated with and without HMP can be seen. Notably, the treated region was lighter in the SEM image than the untreated one; this indicates that this area had steeper surfaces. This agrees with the morphology observed at higher magnification (Fig. 5D).

### 3.4. Effect of HMP formulations on biological bone

Having shown efficacy of the formulations in dissolving HA in vitro, the capacity to demineralize bone ex vivo was studied. These experiments provided further evidence of the potent efficacy of these formulations; mineral volume was reduced by 75% in two weeks (Fig. 6A). The total tissue volume changed little, however, as the collagen matrix of the bone remained.

The promising demineralization efficacy of HMP in vitro and ex vivo substantiated progression to testing the formulation in an in vivo model of HO. Ectopic bone formation was induced by Achilles tenotomy surgery in hind limbs of rats, and the formulations were used to treat the HO formed. Initial model validation studies showed consistency and reliability; 100% of the animals developed HO in the operated (right) limb after 10 weeks (Fig. 6B). No HO was seen in the control (left) limb, and no adverse effects were seen during or after surgery.

The effect of the formulations was then examined, by injecting them into the site of HO formation. An active formulation containing 1 w/v% alginate and 0.2 M HMP or a control formulation containing 1 w/v% alginate only were administered fortnightly. After treatment, the length of HO which formed in the hind limb of the animals was determined via micro-CT. Animals which received the active prophylactic developed, on average, 2.8 mm less HO than controls, a reduction of 19% (Fig. 6C, p < 0.01). This shows that the formulations are active in a biological system and able to significantly reduce the effects of HO in living animals. The TMD of the tibia, adjacent to the site of injection, was also determined to assess the effect of the treatment on the surrounding skeletal bone. No significant difference in TMD was observed, which suggested that the active formulation did not demineralize the orthotopic bone (Fig. 6D). This demonstrated that the viscosity recovery (Fig. 2E) and localized targeting (Fig. 5F) seen in vitro are maintained in vivo.

### 4. Discussion

#### 4.1. Shear-thinning formulations with recoverable post-shear viscosity

Current preventions for HO are inconsistent in their efficacy, with a range of undesirable side-effects, and surgical excision has a host of risks. This necessitates the need for the development of a new, minimally invasive prophylactic and treatment to improve quality of life for HO sufferers in both military and civilian populations. In this study, we have presented such a treatment, in the form of an injectable formulation. This allows it to be used in a civilian outpatient clinic and also in any level of military medical facility; no specialist equipment is required. Furthermore, this treatment is distinct from other proposed prophylaxes, which aim to target the upstream biological pathways of HO. By focusing on the chemical level, this treatment may also be used to dissolve the formed bone, offering a minimally invasive alternative to surgery. This therapy is also favorable from a translational perspective; it is a simple two-component system, and both components are recognized as safe by the United States Food and Drug Administration (FDA) [47,48]. Furthermore, both alginate and HMP are relatively cheap, processing is a single filtration. These factors lend themselves to a scalable and cost-effective treatment.

The formulations were found to be shear-thinning, as their viscosities decreased with increasing shear rate. This behavior is typical of
concentrated polymer solutions, where the chains are entangled. At low shear rates, the chains form new entanglements at the same rate as they are broken by shear, and their viscosity remains constant. At a critical point, the rate of entanglement lags and the polymer network breaks down, resulting in a reduction in viscosity \[49,50\]. The formulations with up to 0.2 M HMP are examples of this behavior. Flocculated particles in suspension also display shear-thinning behavior, as the shear forces break apart the flocs, reducing the viscosity. However, these curves have a different shape, with a much steeper shoulder after the Newtonian plateau \[51\], similar to those seen for the phase-separated samples with \(>0.2\) M HMP. Shear-thinning formulations are favorable for injectable systems; their viscosity decreases when they experience the high shear force in the needle during injection and thus will take less force to inject than a Newtonian fluid of the same standing viscosity. Shear-thinning would not be possible were the polymers covalently linked or linked by strong ionic bonds, as in a calcium alginate gel, such that the bonds between polymers could not be easily broken during injection and reformed in vivo \[52\].

The time taken for the formulations to recover their viscosity, following the high shear force experienced during injection, is also important to this application as short recovery times prevent delocalization. Formulations with 0.2 M HMP recovered their viscosity on the removal of shear almost instantaneously. This is expected, as alginate solutions are pseudoplastic materials, whose viscosity is independent of time \[53,54\]. The formulations with \(>0.2\) M HMP, which formed a precipitate, displayed thixotropy. There is time dependence to their shear-thinning behavior, taking around 30 s to recover their initial viscosity. This is typical of weakly flocculated particle suspensions; the flocs take a finite amount of time to recoagulate, after the removal of shear \[55\].

HMP concentration had a profound effect on the rheological properties of the formulations. Low HMP concentrations increased the viscosity of alginate in solution, by increasing interaction density in the entangled polymer network. This is advantageous because it makes the treatment more site-specific, without adding more polymer which the body would be required to eliminate. The peak value of viscosity appeared at around 0.2 M HMP, for all alginate concentrations. Further increasing the HMP concentration decreased the viscosity, concurrent with the formation of a precipitate and phase separation. This decrease in viscosity is due to the precipitate layer removing polymer from solution.
and thus the formulation moves from an entangled polymer network to a
suspended floc system. This interaction behavior is thus the limiting
factor for drug loading. In addition to the decrease in viscosity at con-
centrations exceeding 0.2 M, the formation of the precipitate removes the
possibility of filter sterilization, which is one of the key advantages of this
system. Henceforth, 0.2 M HMP systems were investigated for inject-
ability and bioactivity, in vitro and in vivo.

4.2. Interaction between the demineralizing agent and polymer network

The significant effect of HMP concentration on the rheological
properties of the formulations suggested there was an interaction, on the
molecular level, between the HMP and the polymer. Investigating this via
zeta potential measurements suggested that the HMP reduced the elec-
 trostatic repulsion between alginate chains, allowing them to come closer
together and interact. Increasing salt concentration has previously been
shown to increase the zeta potential of alginate solutions [56]. This is
because the greater ionic strength reduces the size of the surrounding
electrical double layer, allowing for a greater degree of interaction [57].
This effect also increases with increasing ionic valence [58]. The pro-
found effect of HMP on zeta potential may thus be explained by high
sodium and hexavalent polyphosphate ion concentrations. The low pKa
values of alginate (3.38–3.65, depending on polymer chain composition)
and HMP (2.96) may explain why pH has a negligible effect on zeta
potential [59,60]. Owing to their low pKa values, neither species was
substantially protonated in the tested pH range (3.9–10.0), thus the
surface charge and by extension zeta potential did not vary considerably.

The peak shifts in the FTIR spectra suggested that it is the resonating
groups on the alginate and HMP which were interacting, and it is pro-
posed that these stabilize around positively charged sodium. There is a
high concentration of sodium in these formulations, as both alginate and
HMP are sodium salts. Each molecule may form several interactions, as
HMP anions have six resonating groups and alginate has one carboxyl
group per uronic acid moiety. This interaction increases the viscosity of
the formulations, up to 0.2 M HMP, which is beneficial for this applica-
tion; the formulations will have a lower propensity to disperse, without
the need for additional polymer. Beyond this limit, the interaction binds
the polymers strongly and causes phase separation. This is undesirable
from a translational standpoint, as it prevents filter sterilization and may
reduce reproducibility of treatment.

4.3. Injectable formulations for targeted release

To obtain a more pragmatic understanding of clinical application,
quantitative injection tests were performed to measure the force required
to inject the formulations through a hypodermic needle. The majority of
tests were performed on formulations with 0.2 M HMP, as the rheological
characterization suggested that this is the optimum drug loading. Tests
were also performed with higher HMP concentrations to understand the
effect of precipitation on injectability. Injectable samples passed through
the needle smoothly in a single stream, which is desirable for clinical
application. For uninjectable samples, the force required to extrude the
formulation at a clinically relevant

The force which can sensibly be applied by hand will vary between
clinicians; however, a conservative view should be taken so that a given
formulation can be delivered by any healthcare professional if required.
This limitation will place a threshold on these parameters, particularly
the alginate concentration in the formulation, and also the selected
needle size.

The study of the in vitro release of the HMP from the formulations
revealed two phases. In the initial 6 h, 40% of the drug was released, and a
further 30% was released over the following two weeks. This initial fast
release may be due to the system swelling via osmosis; the dialysis tubing
swelled to its maximum volume when immersed in the release medium.
Once fully swollen, the HMP diffused out of the system, with the polymer
chains acting as obstructions to mass transport [63]. This may explain the
two distinct release regions. The positive control with no alginate released
faster initially but the release rate slows earlier than for the other formu-
lations. This may be due to a faster swelling rate, as there is no polymeric
hindrance to water ingress. The release in vivo, however, may have a
different profile. The swelling, while bounded somewhat by the pressure
of the surrounding tissue, will be less finely constricted. Furthermore, the
rate of swelling may decrease, as the osmotic pressure in body fluids will
be less than that of deionized water, used as the release medium in vitro.
The profile in vivo may therefore be purely swelling controlled but over a
longer time period. The release of the HMP may be retarded by increasing
the alginate concentration because the greater network density increases
the path length of the HMP. The alginate concentration can therefore be
altered to tailor the release profile of HMP; however, this will also affect
formulation viscosity and injectability.

The demineralization capacity of the formulation was studied in vitro
on a pellet of HA. The HMP-containing formulation greatly increased the
surface roughness of the pellet, due to dissolution and reprecipitation.
This demonstrates the ability of the formulation to dissolve HA, the main
mineral constituent of bone; however, in vivo reprecipitation is not ex-
pected to occur, as the ions can flow away from the site. The formulations
also exhibit targeting, with a clear distinction between the treated and
untreated area of the pellet. This is promising for in vivo studies, as it
suggests the formulation will be able to demineralize the HO where it is
injected, without damaging the nearby orthotopic bone.

4.4. Effect of HMP formulations on biological bone

Having demonstrated that the formulations were injectable and dis-
solved HA in vitro, the effect of the formulations on biological bone was
then studied. This was important in order to understand whether the
organic components of bone, such as collagen, prevented demineraliza-
tion in some way. Femoral condyles were used, as they consist largely of
trabecular bone, which has a macroscopic structure more similar to HO
than cortical bone. The HMP-containing formulation reduced the mineral
volume by over 75% in two weeks but did not dissolve the collagen
matrix. This is expected, as HMP demineralizes the bone by sequestering
calcium, to dissolve the calcium phosphate phase, but does not affect the
organic phase [38]. This suggests the formulation will not have dele-
tious effects on the soft tissue surrounding the HO in vivo. Although this
treatment only removes one component of the HO, it is this mineral
component which causes most, if not all, of the complications arising
from HO. Demineralizing the ectopic tissue could improve the range of
joint motion, prevent skin ulceration, reduce pain, and allow amputees to
more easily wear their prostheses.

Having shown efficacy ex vivo, the formulations were studied in an
animal model of HO. An Achilles tenotomy model was chosen for this
study because it induces HO reliably from a non-pharmacological event.
As the biology of HO formation was not under investigation, blast and
injury models, which are more representative but also more harmful and
less controlled, were not necessary. Implantation, injection, or genetic
modification models, the majority of which induce artificially high bone
morphogenetic protein levels, are less representative and are no less
complex. Initial model validation demonstrated that the animals
recovered quickly and that all animals developed HO in the operated limb after 10 weeks. The formulations were then studied using this model, with fortnightly injections following surgery. It should be noted that only one animal in the active group died immediately after injection at six weeks. Veterinary review concluded that this was due to unintended intravascular injection, causing an embolism. As a result of this event, future treatments included an aspiration step prior to injection, and no further events were observed.

The length of the developed HO was chosen as the marker of efficacy because it is the length of the spurs of bone that is responsible for the chronic pain, skin ulceration, and ankylosis which may be experienced by patients. These spurs may thus be a promising initial target for this type of treatment to alleviate these symptoms. The length of HO was found to be significantly reduced by the active formulation, which suggested that the proposed therapeutic may help to relieve these symptoms and be an efficacious prophylactic or treatment for formed HO.

There was no significant change in the TMD of the tibia, adjacent to the injection site. In addition to targeted delivery to the HO site, this may also be because HMP is more effective in low pH conditions, which exist in the local inflammatory site of HO [38]. Furthermore, as treatment took place during HO formation, HMP may be more effective at preventing mineralization than demineralizing the existing skeletal bone. Formed HO has a floral appearance and thus has a much larger specific surface area than the skeletal bone for dissolution and reaction of the mineral phase. Moreover, mature skeletal bone is covered by the periosteum, which may offer some protection against HMP. These factors may all contribute to the preferential demineralization of HO over skeletal bone.

This study demonstrates that targeted demineralization may be a viable strategy to prevent or treat HO. However, this preliminary study is limited to the examination of one delivery vehicle and treatment strategy in vitro. While colloidal alginate is a suitable delivery vehicle, other systems may provide improved rheological or release properties and sustain HMP release over a longer period. Further in vitro and ex vivo experiments in physiologically representative environments, followed by in vivo studies, to determine the material and release properties of the formulations over time are also warranted. In addition, this study examines a single dosing regimen. HMP concentration, dosing timing, and frequency are all factors that can be optimized, which may give increased demineralization in vitro. The effect of applying HMP to HO once completely formed, and on the HO once treatment has ceased, are also important considerations. Other effects of injected HMP, such as temporal changes to local and systemic calcium concentration, may also help to elucidate the precise mechanism of HO demineralization in vivo.

5. Conclusion

This study has shown that HMP, a potent calcium chelator, can be incorporated into a vehicle with optimal material properties for injection and targeted delivery. This formulation is effective at dissolving HA, demineralizing biological bone, and can curb the growth of HO in an animal model, without affecting the adjacent skeletal bone. These data suggest that demineralization may be a viable strategy for HO management and, unlike biological prophylaxes, could act as an alternative to surgery. This preliminary study warrants further exploration into this area to provide new therapies and improve the quality of life for sufferers of HO and other pathological ossification and calcification diseases.

Declaration of competing interest

The authors have no competing interests to declare.

CRediT authorship contribution statement

T.E. Robinson: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. N.M. Eisenstein: Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Supervision. R.J.A. Moakes: Conceptualization, Writing - review & editing. A.M. Thompson: Investigation. Z. Ahmed: Methodology, Investigation, Writing - review & editing. E.A.B. Hughes: Methodology, Writing - review & editing. L.J. Hill: Methodology, Writing - review & editing. S.A. Stapley: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. L.M. Grover: Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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