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ORIGINAL RESEARCH REPORT

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Hydroxyapatite sonosensitization of ultrasound-triggered, thermally responsive hydrogels: An on-demand delivery system for bone repair applications

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Abstract

While bones have the innate capability to physiologically regenerate, in certain cases regeneration is suboptimal, too slow, or does not occur. Biomaterials-based growth factor delivery systems have shown potential for the treatment of challenging bone defects, however, achieving controlled growth factor release remains a challenge. The objective of this study was to develop a thermally responsive hydrogel for bone regeneration capable of ultrasound-triggered on-demand delivery of therapeutic agents. Furthermore, it was hypothesized that incorporation of hydroxyapatite (HA) into the hydrogel could increase sonosensitization, augmenting ultrasound sensitivity to enable controlled therapeutic release to the target tissue. Alginate thermally responsive P(Alg-g-NIPAAm) hydrogels were fabricated and varying quantities of HA (1, 3, 5, and 7% wt./vol.) incorporated. All hydrogels were highly injectable (maximum injection force below 6.5 N) and rheological characterization demonstrated their ability to gel at body temperature. The study demonstrated the ultrasound-triggered release of sodium fluorescein (NaF), bovine serum albumin (BSA), and bone morphogenetic protein 2 (BMP-2) from the hydrogels. Release rates of BSA and BMP-2 were significantly enhanced in the HA containing hydrogels, confirming for the first time the role of HA as a son sensitizer. Together these results demonstrate the potential of these ultrasound-triggered thermally responsive hydrogels for on-demand delivery of therapeutic agents for bone regeneration.

KEYWORDS
bone, controlled delivery, hydrogel, thermally responsive, ultrasound

1 | INTRODUCTION

Bone has the ability to regenerate itself through complex physiologically processes. However, in the case of non-union fractures and certain pathological conditions regeneration is suboptimal, too slow, or does not occur. Autografting represents the current clinical "gold standard" treatment, however, this procedure has numerous associated limitations including risk of infection, chronic pain and donor site morbidity. As an alternative approach, recent research has focused on the development of biomaterial-based solutions. This approach incorporates therapeutic agents, such as the growth factors, for example, bone morphogenetic protein 2 (BMP-2) and bone morphogenetic protein 7 (BMP-7), into a scaffold or hydrogel that delivers the therapeutic agent to stimulate the bone repair processes while acting as a template for bone repair. Despite recent advances, the controlled release of growth factors from current systems has yet to be effectively achieved. Frequently there is insufficient local retention of these growth factors at the implantation site and typically a burst release of growth factors occurs within the first day of implantation.[1] As a result, large quantities of these growth factors are required to achieve a biological effect.[1] The harmful side effects that result from large doses of BMPs have been widely reported, including inflammation, undesirable ectopic bone formation and possibly an increased risk of cancer.[2,3] To overcome these challenges, new strategies for the controlled release of therapeutic agents are required.[2] Controlling the rate of BMP delivery would improve the safety of therapeutic delivery systems for bone repair while also enhancing the bone healing response, resulting in better quality bone tissue.[4]

Hydrogels have shown particular promise as carriers for therapeutic delivery for bone repair applications due to their potential for minimally-invasive delivery via injection.[1,5] An exciting approach in this area is the use of hydrogels for stimuli-responsive therapeutic delivery to achieve "on-demand", precisely-controlled release of therapeutic agents directly at the site of injury. Ultrasound-responsive systems have recently been developed, offering particular advantages in the context of on-demand therapeutic delivery due to their potential to be externally triggered.[6–8] Such systems have been widely explored for enhanced chemotherapy.[9–11] More recently the potential for applying ultrasound responsive hydrogel systems in minimally-invasive bone repair applications has been recognized, however, current systems are limited by the challenges relating to their delivery.[6,12] Dual stimuli systems offer potential to overcome these challenges, whereby the gelation of the hydrogel could be achieved once the hydrogel has been delivered to the site of injury and stimulation with ultrasound used to achieve controlled release of the therapeutic agents. Within this study, the ability to achieve ultrasound-triggered release from a thermally responsive Poly(Alginate-Graft-N-Isopropylacrylamide) (P(Alg-g-NIPAAm)) hydrogel is investigated. This hydrogel is synthesized via graft copolymerization of poly(N-isopropylacrylamide) onto alginate using a free-radical reaction and has been shown to be biocompatible and biodegradable.[13,14] Furthermore, it forms a gel at body temperature thus providing ideal properties for injectability.[13,14]

A further challenge in the development of ultrasound-triggered hydrogel systems relates to the low ultrasonic adsorption properties of many hydrogels, which limits their responsiveness to ultrasound. Recently 'solid-phase sonosensitization' has been reported, whereby solid-phase inclusions are incorporated into hydrogels to achieve a local increase in ultrasound sensitivity.[15] Osminkina *et al.* showed successful use of silicon nanoparticles as sonosensitizers for ultrasound assisted cancer therapy.[16] Inorganic compounds, such as calcium phosphates, have high ultrasonic absorption coefficients and thus the potential to act as solid-phase sonosensitizers within hydrogel systems. In addition, they have osteoconductive and osteoinductive properties, and therefore the potential to stimulate new bone formation, and have been widely used in bone repair applications.[17–20] Therefore the inclusion of hydroxyapatite (HA) into the hydrogel presents the potential to improve the efficacy of hydrogel therapeutic delivery systems for bone repair, enhancing both their ability to promote osteogenesis and their responsiveness to ultrasound stimulation.

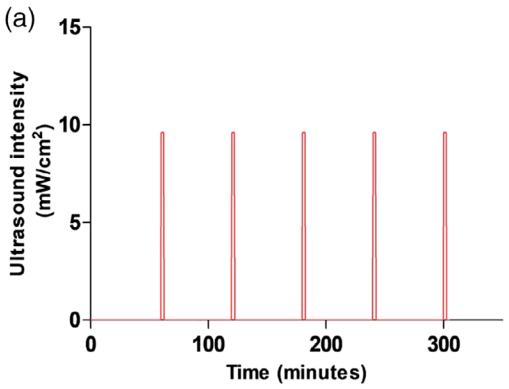
The overall objective of this work is to develop an ultrasound-triggered thermally responsive hydrogel as an on-demand system for the delivery of therapeutic agents for bone repair. This dual stimuli system would potentially allow thermally responsive hydrogels to be delivered to the site of injury and then the controlled release of therapeutic agents, such as growth factors, from the hydrogel using ultrasound. We hypothesized that the application of ultrasound could enhance the release of molecules from alginate-based thermally responsive hydrogels. Furthermore, we hypothesized that the incorporation of HA particles into an alginate-based thermally responsive hydrogel could augment the ultrasound sensitivity of the hydrogel, while maintaining the necessary thermal responsiveness, rheological and injectability requirements for minimally invasive application. Specifically, within this study, alginate thermally responsive hydrogels P(alg-g-NIPAAm) with incorporated HA particles were synthesized and the ultrasound-triggered release of small and large molecule drug mimics from these hydrogels was assessed prior to exploring the release of the therapeutic agent bone morphogenetic protein-2 (BMP-2).

2 | MATERIALS AND METHODS

2.1 | Preparation of alginate thermally responsive hydrogel P(Alg-g-NIPAAm)

Alginate thermally responsive hydrogel P(Alg-g-NIPAAm) (Pure TH) was prepared as previously described.[13] Briefly, grafted P(Alg-g-NIPAAm) was synthesized by dissolving 2.26 g of NIPAAm and 226 mg of sodium alginate (NaAlg) in 40 ml of PBS at pH 7.4 and stirring for 3 hr. Dry nitrogen was purged through the solution for 60 min to remove oxygen prior to adding 0.060 g of ammonium persulfate (APS) and 60 μl of N,N,N′,N′-Tetramethylethylenediamine (TEMED). Copolymerization was achieved within 24 hr at room temperature. The solution was then purified using dialysis in double distilled water (DDW) for 5 days. The solution was freeze-dried (VirTis AdVantage Plus, SP Industries Inc) at −40°C for 40 hr in order to remove water from the solution. The grafting efficiency of the copolymer hydrogel was determined by gravimetrical analysis using Equation 1 (n = 4).

(a)



(b)

Ultrasound Treatment Groups	Treatment Details
US Treatment 1	Ultrasound applied in 2.5 min pulses once per h for 5 h.
US Treatment 2	Ultrasound applied in 2.5 min pulses once per h for 5 h following an initial 24 h immersion period
US Treatment 3	Ultrasound applied in 2.5 min pulses once per hour for 5 h following an initial 72 h immersion period

FIGURE 1 Ultrasound treatment regimes. (a) Ultrasound with an intensity of 9.6 mW/cm^2 was applied in 2.5 min pulses, once per hour for 5 hr period.[9] For US Treatment 1, the ultrasound regime was applied at 0 hr, for US Treatment 2 the ultrasound regime was applied after incubation for 24 hr and for US Treatment 3 the ultrasound regime was applied after incubation for 72 hr

$$\text{Grafting Efficiency}\,(\%) = \frac{\text{Mass of graft copolymer}}{\text{mass of NIPAAm + mass of NaAlg}} \times 100. \quad \text{(Eqn.1)}$$

P(Alg-g-NIPAAm) was reconstituted at 5% wt./vol. and HA-loaded P(Alg-g-NIPAAm) hydrogels (TH + HA) fabricated by adding HA particles (Captal® R, Plasma Biotal Ltd., UK; particle size D_{50} of 4 μm) at 1, 3, 5 and 7% wt./vol. (TH + 1% HA, TH + 3% HA, TH + 5% HA and TH + 7% HA). An ultrasound sonicator (BRANSON Digital Sonifier, Model 450, U.S.A.) was used to apply 5 min pulses at an amplitude value of 10% to ensure that the HA particles were homogeneously distributed throughout the thermally responsive hydrogel suspension.

2.2 | Chemical characterization

All hydrogels were analyzed using Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer Spectrum Two FTIR, PerkinElmer Inc) to identify copolymer formation. Samples of PNIPAAm, NaAlg, HA the pure TH and TH + HA were prepared and scanned in the wavelength range of 400–4000 cm^{-1} at four scans with a resolution of 4 cm^{-1} with a strong apodisation and a scan speed of 0.2 mm/s (n = 6 per group).

2.3 | Rheological analysis

Rheological properties of the hydrogels were analyzed in oscillation mode with a parallel plate CP50-2 tool with a 49.97 mm diameter and a cone angle of 1.996° (Anton Paar MCR 301, Anton Paar GmbH, Austria). Temperature sweep experiments were carried out over a temperature range of 20–50°C with a ramp rate of 3°C min^{-1} with a normal force of 0.1 N. The experiments were performed in the linear viscoelastic region (between 0.1 and 10 Hz). Data points were collected every second. The testing time and temperature were measured to determine the gelation time and transition temperature to solid state. Additionally, storage modulus (G') and loss modulus (G") were analyzed and Tan δ was calculated as the quotient of G"/G'. The low critical solution temperature (LCST) was determined by measuring the G' and G" as a function of temperature.

2.4 | Injectability of thermally responsive alginate hydrogels

The injectability of the hydrogels was assessed using the Zwick mechanical testing machine (ZwickRoell, Zwick GmbH & Co., Germany), fitted with a 5 kN load cell. Syringes loaded with 10 ml of each hydrogel were mounted in a custom designed injectability rig during testing. Testing was conducted at room temperature over a displacement of 5 mm, at a crosshead speed of 30 mm/min, representative of the speed of manual delivery of a hydrogel from a syringe. The loading required to displace the plunger was measured as a function of plunger displacement and average maximum force values determined for each composition.

2.5 | Ultrasound release of sodium fluorescein (NaF) from alginate thermally responsive hydrogels

The ability to control particle release rates from the alginate hydrogels using ultrasound was firstly assessed using the water soluble fluorescent dye, sodium fluorescein (NaF) as a small molecule drug mimic. Two hydrogel groups were tested, Pure TH and TH + 7% HA. A 1 mg/ml NaF solution was prepared by adding 10 mg of NaF salt (Sigma Aldrich Products, Ireland) to 10 ml of double distilled water and then added to the hydrogels at a concentration of 100 μl/ml. For the TH + HA groups, NaF was added during hydrogel fabrication prior to the addition of the HA particles. For release studies, 1 ml hydrogel samples were immersed in 5 ml of buffer medium comprised of 0.01 M calcium and magnesium free Dulbecco's phosphate-buffered saline (DPBS) at pH 7.4 and stored

at 37°C for the duration of testing. Diffusion-based release of NaF, where no ultrasound treatment was applied, was assessed at timepoint 0, following 5 hr incubation, and following 24 hr incubation. Ultrasound (Vibra-Cell Ultrasonic Liquid Processor, Model CV18, Sonics & Materials Inc.) was then applied according to Ultrasound Treatment 1 (US Treatment 1) as detailed in Figure 1. This protocol involved application of ultrasound at timepoint 0 for 5 hr (US = 9.6 mW/cm^2, 25% amplitude, 2.5 min hr^{-1}, for 5 hr period at 37°C).[8,9] This protocol was based on previous work by Huebsch et al.[9] and Kearney et al.[8] that demonstrated effective ultrasound controlled release from alginate hydrogels while maintaining the temperature below 37 °C. NaF release following application of ultrasound was compared to the control at 5 hr where no ultrasound was applied. At the relevant timepoints, 1 ml of supernatant was collected and NaF concentration assessed using a spectrophotometer (Thermo Scientific Varioskan Flash, Thermo Fisher Scientific Inc) in the absorbance mode at 512 nm using a 1 s integration time.

2.6 | Ultrasound release of bovine serum albumin protein from alginate thermally responsive hydrogels

Ultrasound controlled release of bovine serum albumin (BSA) was then explored as a large molecule drug/protein mimic. For BSA release studies, two hydrogel compositions were tested, Pure TH and TH + 7% HA, prepared as described above. BSA solution was prepared by adding BSA (Sigma Aldrich Products, Ireland) to double distilled water at a concentration of 10 mg/ml and then added to the hydrogels to give a concentration of 1 mg/ml. Then 1 ml hydrogel sample were suspended in buffer medium comprising of 5 ml of 0.01 M calcium and magnesium free DPBS at pH 7.4 at a temperature of 37°C. Diffusion-based release of BSA, with no ultrasound applied, was assessed following incubation for 0, 5, 24, 29 and 72 hr. Ultrasound (Vibra-Cell Ultrasonic Liquid Processor, Model CV18, Sonics & Materials Inc.) was then applied as according to Ultrasound Treatment 1 (US Treatment 1), Ultrasound Treatment 2 (US Treatment 2) and Ultrasound Treatment 3 (US Treatment 3) as described in Figure 1, For US Treatment 1 ultrasound was applied at timepoint 0 for 5 hr (US = 9.6 mW/cm^2, 25% amplitude, 2.5 min hr^{-1}, for 5 hr period at 37°C).[8,9] For US Treatment 2 hydrogels were incubated for 24 hr at 37°C prior to applying the 5 h ultrasound treatment as per US Treatment 1.[8] For US Treatment 3 hydrogels were incubated for 3 days prior to applying the ultrasound treatment regime as per US Treatment 1. The results were compared to diffusion based release controls at the same timepoints with no ultrasound was applied. BSA quantification was carried out using a bicinchoninic acid (BCA) protein assay kit (Fisher Scientific Ireland Ltd, Dublin, Ireland) for the colorimetric detection and quantitation of total protein. The assay was carried out in accordance with the manufacturer's instructions with the absorbance of each sample was measured at 562 nm to determine BSA concentration.

2.7 | Ultrasound release of bone morphogenetic Protein-2 (BMP-2) from alginate thermally responsive hydrogels

To assess the potential of this system for therapeutic delivery, the ultrasound-triggered release of BMP-2 was explored. Recombinant Human/Mouse/Rat bone morphogenetic protein-2 (BMP-2) (Bio-Techne Ltd., UK) was reconstituted at a concentration of 100 μg/ml by adding 100 μl of 4 mM HCl and added to hydrogels at a concentration of 1 μg/ml. Two thermally responsive hydrogel groups were tested, pure TH, and TH + 7% HA. 1 ml hydrogel samples were then suspended in DPBS at pH 7.4 at a temperature of 37°C as before. Diffusion-based release of BMP-2 was assessed at 0, 5, 24, 29 and 72 hr. Ultrasound treatments (US Treatment 1, US Treatment 2 and US Treatment 3) were applied as detailed in Figure 1, and BMP-2 release compared to control groups at the same timepoints, as described for the BSA study. The supernatant (1 ml) was collected at each timepoint and analyzed using an ELISA kit (Human BMP-2 DuoSet ELISA kit, Bio-Techne Ltd., UK), to determine the relative values of BMP-2 released from the hydrogel. ELISA was conducted by coating the 96-well microplates overnight at room temperature with 100 μl per well at the working concentration of 1 μg/ml of diluted Capture Antibody. The wells were blocked for 1 hr at room temperature with 1% wt./vol. BSA in PBS before addition of BMP-2 samples. Bound BMP-2 was detected using detection reagents from BMP-2 DuoSet ELISA (biotin-conjugated anti-human BMP-2 polyclonal antibody). Streptavidin-conjugated horseradish peroxidase was then added to the plates. Enzyme substrate (tetramethylbenzidine and peroxide) was treated for 20 min, and the reaction was stopped by adding an acidic solution. Absorbance was measured at 450 nm using a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc.) and the amount of BMP-2 was calculated from a calibration curve based on known concentrations of BMP-2.

2.8 | Statistical analysis

Experiments were performed at least in triplicate unless otherwise indicated. Statistics were performed using GraphPad Prism software (GraphPad) and Microsoft Excel for Office 365. Statistical significance was determined using *t* tests and one-way analysis of variance (ANOVA).

3 | RESULTS

3.1 | Preparation of alginate thermally responsive hydrogel

P(Alg-g-NIPAAm) and P(Alg-g-NIPAAm) hydrogels containing 1, 3, 5 and 7% wt./vol. HA were successfully synthesized. The grafting efficiency of the P(Alg-g-NIPAAm) hydrogel was 80.01 ± 4.31%.

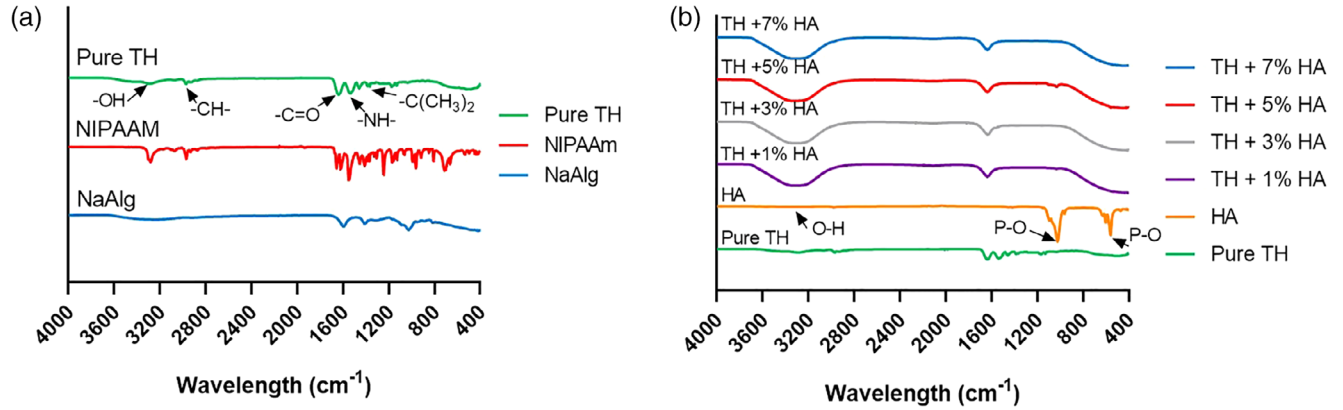


FIGURE 2 FTIR spectra for thermally responsive hydrogels. (a) shows the successful formation of the P(Alg-g-NIPAAm) co-polymer and (b) shows the incorporation of HA into the P(Alg-g-NIPAAm) co-polymer. FTIR, Fourier transform infrared spectroscopy

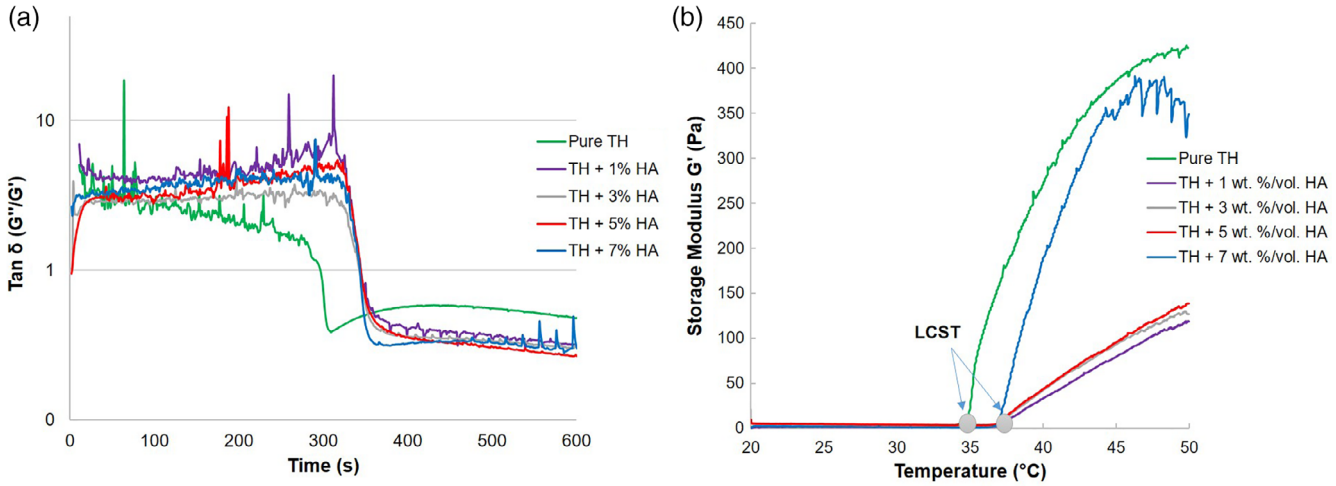


FIGURE 3 Rheological properties of pure TH, TH + 1% HA, TH + 3% HA, TH + 5% HA and TH + 7% HA thermally response hydrogels. (a) Typical plot showing Tan delta Tan (δ) as a function of hydroxyapatite concentration in the temperature range from 20–50°C. (b) Storage modulus as a function of hydroxyapatite concentration from 20–50°C indicating the LCST transition temperature points from liquid to solid-state. LCST, low critical solution temperature

3.2 | Chemical characterization

The characterization of the pure TH and TH + HA via FTIR confirmed the presence of P(NIPAAm) in the copolymers (Fig. 2). For the pure TH, the FTIR spectra showed that the important absorption bands regarding hydroxyl, ether and carboxylic functional groups were common between the homopolymer and copolymer (Fig. 2a). Stretching vibrations O-H bands of NaAlg appeared in the spectra ranging from 3000 to 3600 cm^{-1}. Stretching vibrations of the aliphatic C-H were observed at 2919–2932 cm^{-1}. At 1244 cm^{-1} a weak band appeared, which represents the C-O bond. Observed bands at 1639 and 1641 cm^{-1} corresponded to –C=O stretching. The characteristic bands between 1534 and 1539 cm^{-1} represented the –NH bending due to the presence of PNIPAAm within the copolymer. The bands appearing at 1459 cm^{-1} represented the asymmetric vibrations of the carboxylic groups COO- of the sodium alginate. Moreover, the bands between 1050 cm^{-1} and 1175 cm^{-1} corresponded to the symmetric and asymmetric vibrations of C-O-C bonds typical from alginate rings.

FTIR spectra of the P(Alg-g-NIPAAm) hydrogels containing 1, 3, 5 and 7% wt./vol. HA are shown in (Figure 2b). The FTIR spectra shows two strong peaks, one in the range between 3000 to 3700 cm^{-1} and the second peak at 1630 cm^{-1}. The first shoulder band ranging from 3000 to 3700 cm^{-1} corresponded to O-H stretching vibrations correlated to NaAlg as well as demonstrating the presence of HA within the copolymer. The second peak at 1630 cm^{-1} corresponds to amide bond and –C=O stretching due to presence of PNIPAAm within the copolymer.

3.3 | Rheological analysis

The thermally responsive properties of all hydrogels groups was confirmed using rheological assessment. The LCST of the pure TH was 34.5°C. The LCST increased following the addition of HA to the hydrogel with LCST values of 36.8°C, 36.7°C, 36.7°C and 36.2°C for the 1, 3, 5 and 7% wt./vol. HA hydrogels. Figure 3a shows Tan δ (G"/G') as a function of temperature. At temperatures below the LCST, Tan δ > 1,

indicating that the hydrogels behave like viscous liquids. At temperatures above the LCST, Tan δ < 1 and thus the sample behaves more like an elastic solid. For all hydrogels, at temperatures above the LCST an increase in G' was observed with increasing temperature (Figure 3b). The pure hydrogel demonstrated the highest G' ≈ 420 Pa at 50°C. The addition of HA to the pure TH led to a reduction in G', with the lowest G' recorded for the 1% HA hydrogel (≈110 Pa at 50°C). However, within the TH + HA groups it was observed that increasing the HA concentration in the hydrogel resulted in an increase in G'.

3.4 | Injectability of alginate thermally responsive hydrogels

Injectability testing was conducted to assess the suitability of fabricated hydrogels for use as injectable materials in a clinical setting (Figure 4). The TH + 1% HA group required the least force for injection, recorded at 4.32 ± 0.58 N. Injection force increased with increasing HA concentration for the TH + 3% HA (4.77 ± 0.54 N), TH + 5% HA (5.36 ± 0.30 N), and TH + 7% HA (5.69 ± 0.23 N) hydrogel groups. However, the pure hydrogel exhibited the highest injection force recorded at 6.46 ± 0.76 N. All values were below the maximum force that can be comfortably applied to a syringe by a surgeon's hand (22.6 N)[21] indicating that all hydrogels are suitable for use as injectable materials within a clinical setting.

3.5 | Ultrasound release of sodium fluorescein (NaF) from alginate thermally responsive hydrogels

The ultrasound-triggered release of NaF from two thermally responsive hydrogel groups, pure TH and TH + 7% HA was assessed. Analysis of the diffusion related release for each hydrogel group (Figure 5a) showed the gradual release of NaF over time, with up to 31% release observed to occur by 24 hr as a result of diffusion alone. The incorporation of HA into the thermally responsive hydrogels did not significantly influence the diffusion related release of NaF at 0 hr and 5 hr timepoints, however, a significantly lower (p value <.05) level of NaF release was observed from the TH + 7% HA groups compared to the pure TH group at the 24 hr timepoint indicating that the inclusion of HA into the hydrogels may reduce the diffusion related release of NaF. The application of ultrasound resulted in an increase in NaF release from both hydrogel groups. For the Pure TH group, US Treatment 1 resulted in 26.66% NaF release, compared to the control group at the same time point (5 hr) where a significantly (p value <.01) lower amount of NaF was released (13.7%) (Figure 5b)

3.6 | Ultrasound release of BSA protein from alginate thermally responsive hydrogels

The ultrasound-triggered release of BSA from the Pure TH and TH + 7% HA was then assessed. Analysis of the diffusion related release of BSA for each hydrogel group showed a low level of BSA release over time, with 5.98% and 6.53% BSA released from the Pure TH and TH + 7% HA groups respectively by the 72 hr timepoint (Figure 6a). No significant difference in diffusion related release was observed between the Pure TH and TH + 7% HA groups. The application of ultrasound resulted in an increase in BSA release in both the Pure TH and TH + 7% HA. For the Pure TH hydrogel 29.65% BSA release was recorded following US Treatment 1, which was significantly higher (p value <.0001) than the control at the same time point (5 hr) where just 4.45% BSA was released (Figure 6b). In comparison to US Treatment 1, reduced BSA levels were recorded for US Treatment 2 and US Treatment 3, possibly due to degradation of the BSA over time (Figure 8a). Notably, the incorporation of HA into the thermally responsive hydrogels led to an increase in BSA release in response to US Treatment 1, with 66.21% release recorded for the TH + 7% HA group compared to 29.65% for the Pure TH. Similar increases were observed for US Treatment 2 and US Treatment 3 (Figure 6c,d, Figure 8a).

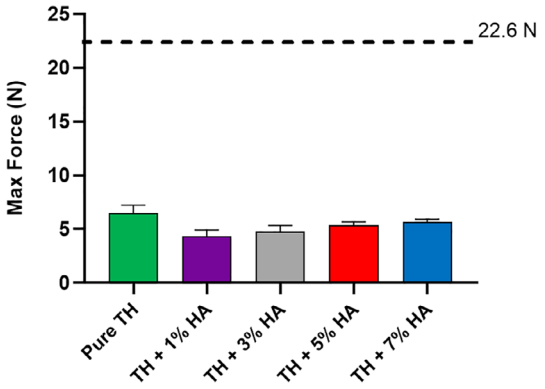


FIGURE 4 (a) Injectability of the pure thermally responsive hydrogel and thermally responsive hydrogels containing 1, 3, 5 and 7% wt./vol. hydroxyapatite. No significant difference was observed between groups. The forces recoded for all hydrogels was below the maximum force that can be comfortably applied to a syringe by a surgeon's hand is highlighted on the graph at 22.6 N. (n = 3 per group)

3.7 | Ultrasound release of bone morphogenetic Protein-2 (BMP-2) from alginate thermally responsive hydrogels

The ultrasound-triggered release of BMP-2 from the Pure TH and TH + 7% HA was then assessed. Analysis of the diffusion related release for each hydrogel group showed that BMP-2 was successfully maintained within the hydrogels, with only 2.71% and 2.91% released from the Pure TH and TH + 7% HA groups respectively by the 72 h timepoint (Figure 7a). No significant difference in diffusion rates was observed between the pure TH and TH + 7% HA groups. For the Pure TH, the application of ultrasound significantly increased BMP-2 release, compared to the control groups at the same timepoints. For US Treatment 1, 10.99% BMP-2 was released for the Pure TH

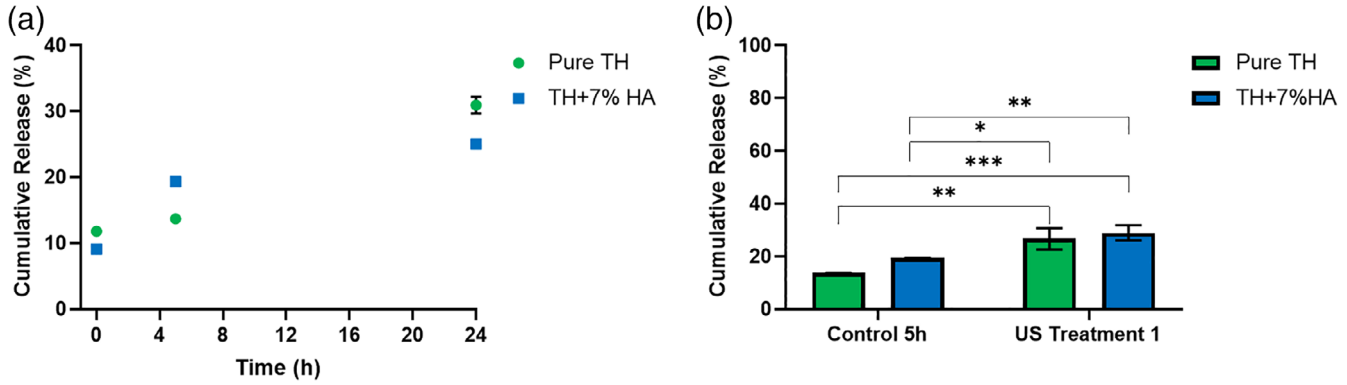


FIGURE 5 Cumulative release of NaF from NaF loaded thermally responsive hydrogels. (a) Diffusion related release of NaF from Pure TH and TH + 7% HA groups up to 24 h. (b) Ultrasound triggered release of NaF following US Treatment 1. $n = 3$. * indicates p value <.05, ** indicates p value <.01, *** indicates p value <.001

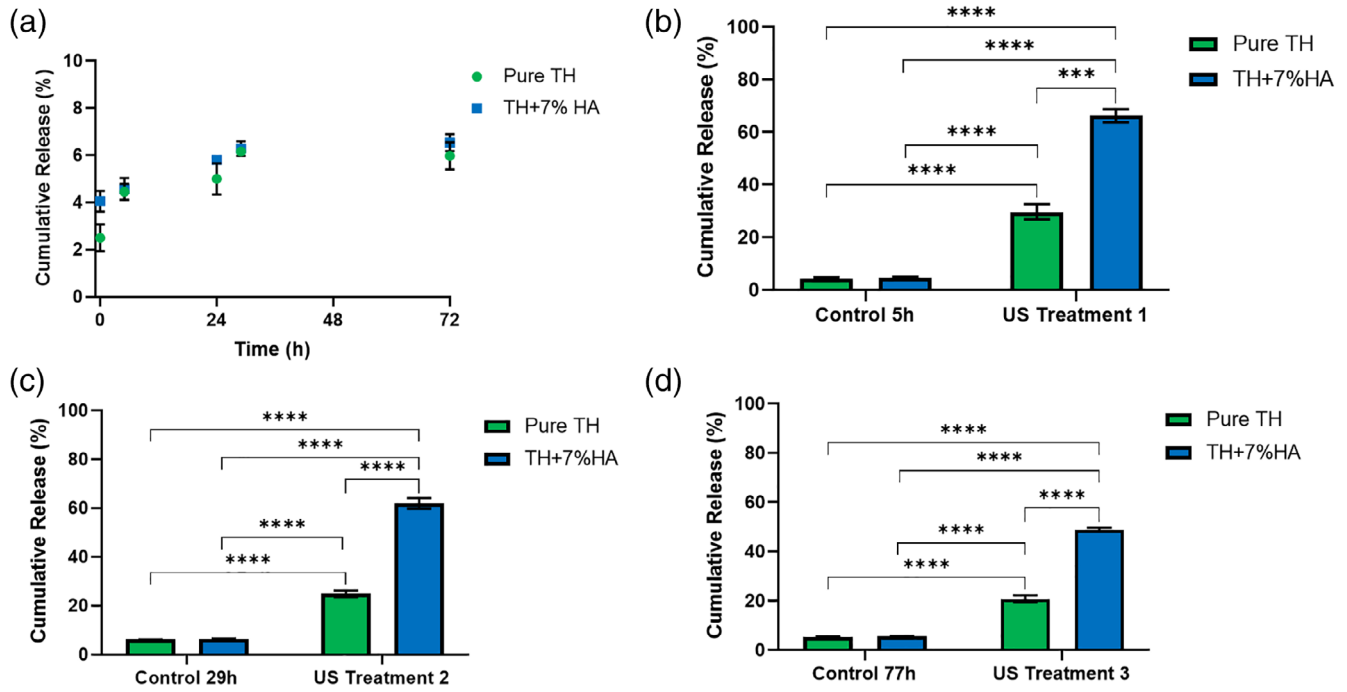


FIGURE 6 Cumulative release of BSA from BSA loaded thermally responsive hydrogels. (a) Diffusion related release of BSA from Pure TH, and TH + 7%HA up to 72 h. Ultrasound-triggered release of BSA is shown following a) US Treatment 1, (b) US Treatment 2 and (c) US Treatment 3. $n = 3$, *** indicates p value <.001, **** indicates p value <.0001

hydrogel compared to 2.06% for the control at the same timepoint (5 h) (Figure 7c). Similarly, US Treatment 2 resulted in 20.11% BMP-2 release compared to 2.76% released from the control at the same timepoint (29 h) (Figure 7c) and US Treatment 3 resulted in 26.65% released compared to 2.19% from the control at the same timepoint (77 hr) (Figure 7d). The addition of HA further increased BMP-2 release in response to ultrasound. While these increases were not significant for US Treatment 1 and US Treatment 2, significantly (p value <.05) higher BMP-2 released was observed for US Treatment 3 with 43.92% BMP-2 released compared to 26.65% for the Pure TH (Figure 8b).

4 | DISCUSSION

The development of minimally invasive system for precisely controlled therapeutic delivery for bone repair applications would present a significant advancement over current clinical approaches. To address this unmet clinical need, this study aimed to develop an alginate-based ultrasound-triggered, thermally responsive hydrogel as an on-demand delivery system for bone repair. This dual stimuli system would potentially allow for the minimally invasive delivery of a hydrogel that will gelate in-situ at body temperature, followed by the on-demand release of therapeutic agents, such as growth factors, using

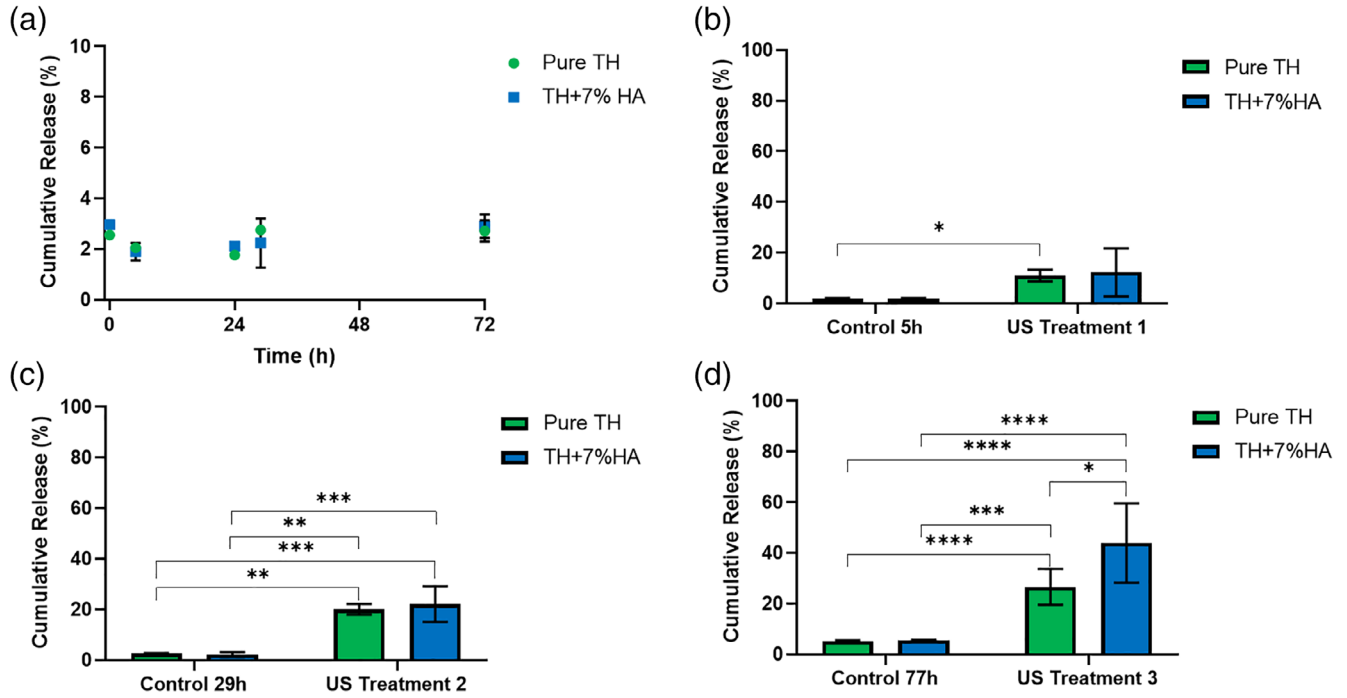


FIGURE 7 Cumulative release of BMP-2 from BMP-2 loaded thermally responsive hydrogels. (a) Diffusion related release of BMP-2 from the Pure TH, and TH + 7%HA groups up to 72 h. Ultrasound-triggered release of BMP-2 is shown following (b) US Treatment 1, (c) US Treatment 2 and (d) US Treatment 3. $n = 3$, * indicates p value <.05, ** indicates p value <.01, *** indicates p value <.001, **** indicates p value <.0001. BMP, bone morphogenetic protein 2

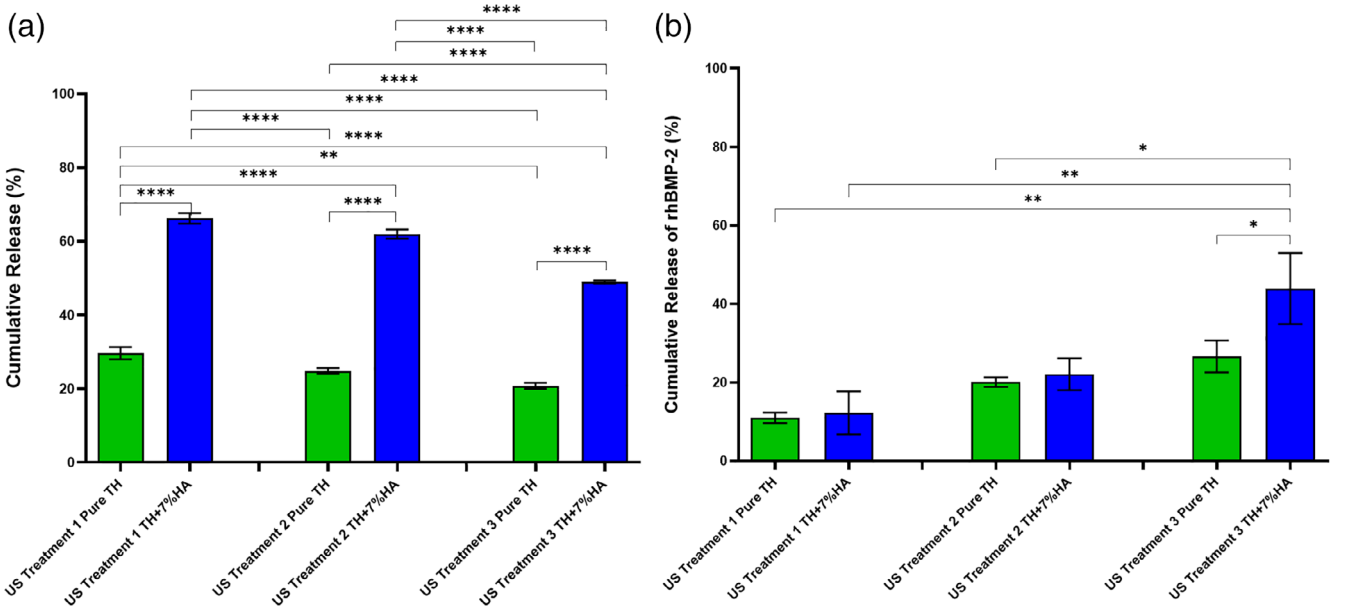


FIGURE 8 Comparison of cumulative release in response to US Treatment 1, US Treatment 2 and US Treatment 3 (a) Release of BSA from the Pure TH, and TH + 7% HA groups in response to US Treatment 1, US Treatment 2 and US Treatment 3. (b) Release of BMP-2 from the Pure TH, and TH + 7% HA groups in response to US Treatment 1, US Treatment 2 and US Treatment 3. $n = 3$, * indicates p value <.05, ** indicates p value <.01, **** indicates p value <.0001. BMP, bone morphogenetic protein 2

ultrasound. In this study, the ultrasound-triggered release of a small molecule mimic (NaF), a large molecule mimic (BSA) and a therapeutic agent (BMP-2) from thermally responsive pure TH and TH + HA was demonstrated. HA particles were incorporated into the thermally responsive P(Alg-g-NIPAAm) hydrogels at concentrations of 1, 3, 5 and 7% wt./vol. and their ability to act as sonosentising agents to enhance the rate of release of therapeutic agents in response to ultrasound was demonstrated.

Alginate is a natural biodegradable and biocompatible polymer that has shown promise in bone repair applications.[22] The addition of PNIPAAm to the alginate hydrogel has been shown to achieve a hydrogel with thermoresponsive properties.[13,14] Herein the successful co-polymerization of PNIPAAm on the alginate backbone structure properties was confirmed in all hydrogel groups using FTIR, demonstrating that the addition of HA into the P(Alg-g-NIPAAm) hydrogel did not interrupt the grafting process. Rheological characterization demonstrated that the incorporation of HA into the pure TH did not negatively impact the thermoresponsive properties of the hydrogel. The LCST is an important parameter as it indicates the temperature at which gelation of the hydrogel occurs. The LCST was found to be below 37 °C for all hydrogel groups, confirming that all groups are capable of forming a hydrogel at body temperature. Below the LCST the hydrogels were seen to behave like a viscous liquid whereas above the LCST a solid state is reached and they behaved like a stiff gel. Both storage (G') and loss modulus (G") were higher for the pure hydrogel than for the groups containing HA. For the HA containing hydrogels, the storage and loss modulus increased with increasing HA concentration. As the storage modulus is a measure of a material's elastic response, these results indicate that the capacity of the hydrogel to store elastic energy (i.e., stiffness) increases with increasing HA incorporation. The loss modulus, on the other hand, measures the viscous component of the material behavior and is proportional to the energy dissipated as heat. These results show that the Pure TH and TH + HA groups have the required properties for use as a thermoresponsive injectable hydrogel for use as a bone healing platform.

Injectability testing demonstrated that all hydrogel groups were suitable for use as injectable biomaterials. The maximum force required for injection was significantly below the 22.6 N force which is reportedly the maximum injection force that can be applied by hand.[21] The highest injection force was recorded for the Pure TH group (6.46 ± 0.76 N). The incorporation of HA into the P(Alg-g-NIPAAm) hydrogel led to a reduction in the force required for injection, however, a linear increase in injection force was observed as the amount of incorporated HA was increased from 1% wt./vol. (4.32 ± 0.58 N) to 7% wt./vol. (5.69 ± 0.23 N). This was due to the change in the viscous properties of the hydrogel with increasing HA content as observed from the rheological analysis. Taken together, these results demonstrate that the Pure TH and TH + HA have suitable injectability properties to enable minimally invasive delivery to the site of injury and the thermally responsive properties required to enable gelation once inside the body. As all TH + HA compositions demonstrate the required properties for an injectable hydrogel, the TH + 7% HA hydrogel was selected for the ultrasound-triggered release studies as it contains the highest percentage of osteoconductive HA particles.

The ultrasound-triggered release of loaded molecules from the thermally responsive alginate hydrogels P(Alg-g-NIPAAm), with and without HA, was then investigated. Firstly, the release of a small molecule drug mimic, NaF, from two thermally responsive hydrogel compositions (pure TH and TH + 7% wt./vol. HA) was investigated. The release profile of NaF from the thermally responsive alginates hydrogels shows a rapid release of NaF over the initial 24 hr time period. For the Pure TH group, approximately a third of the NaF was released through diffusion process by the 24 hr timepoint. A similar burst release of NaF from P(Alg-g-NIPAAm) hydrogels has been reported previously by Pentlavalli *et al*., with release of 65% of the loaded NaF observed by Day 28.[13] Diffusion related release was observed to be lower in the hydrogel groups that contain HA. These results indicate that NaF readily diffuses from the P(Alg-g-NIPAAm) hydrogels.

The application of ultrasound stimulation successfully triggered an increase in the rate of NaF release compared to the control groups for all hydrogel compositions. For the Pure TH group, application of ultrasound according to US Treatment 1 resulted in release of 26.67% of NaF, an almost two-fold increase over the control group at the same time point. No significant increase in the release of NaF in response to ultrasound was observed in the alginate-based hydrogel containing HA when compared with the Pure TH group. Thus while the ability to achieve ultrasound-triggered release of NaF has been demonstrated, the ability to induce a sonosensitization effect by incorporating HA into the hydrogel was could not be confirmed for these small molecule drug mimics. Further investigation into ultrasound-triggered release of NaF from these alginate hydrogels was therefore not pursued.

Following on from this, the ability to achieve ultrasound-triggered release of a large molecule drug mimic, BSA, was demonstrated. Two additional ultrasound treatment groups (US Treatment 2 and US Treatment 3) were included in this study, whereby samples were incubated at 37°C for 24 hr and 72 hr respectively prior to application of the ultrasound treatment. Notably here, the diffusion related release of BSA was much lower than observed for NaF with only 5.3% BSA release by the 77 hr timepoint in the Pure TH group. The lack of a burst release response indicates that the BSA is entrapped within the hydrogel and thus there is a greater ability to control BSA release using ultrasound stimulation than for the small molecule drug mimic. BSA is reported to have a hydrodynamic radius of 3.4–3.6 nm,[23] which is close to the pore size reported for alginate-based hydrogels, typically in the range of tens of nanometers.[8]

As was observed in the NaF study, the application of ultrasound to the Pure TH and TH + 7% hydrogel groups resulted in increased BSA release compared to the control groups. In the Pure TH group, the highest release was observed following US Treatment 1 with a six-fold increase over the control group. Furthermore, the sonosensitization effect achieved by inclusion of HA within the hydrogels was demonstrated with a ~ 2.five-fold increase in BSA release from the TH + 7% group compared to the pure TH for each US treatment. For both groups, higher levels of BSA release were observed following US Treatment 1 than for US Treatment 2 or US Treatment 3, perhaps indicating degradation of the BSA molecule. Previous studies investigating the delivery of BSA from polymer hydrogels have also reported structural changes to BSA over time. Estey et al. reported loss of BSA structure in PLGA hydrogels due to changes in pH within the hydrogel.[24] Hsieh *et al*. reported that the stability of BSA loaded within PNIPAM based thermoresponsive copolymers was influenced

by changes in hydrophilicity of the polymers.[25] Thus investigation into potential stabilization strategies may be important in the future development of successful controlled-release systems for some therapeutic proteins.

Following the successful demonstration of the ultrasound-triggered delivery of drug mimics from thermally responsive hydrogels, their ability to deliver the therapeutic agent BMP-2 was demonstrated. BMP-2 plays an important role in promoting osteogenesis and thus the ability to trigger its release at appropriate points during the bone healing process has the potential to significantly improve the safety and efficiency of BMP-2 use in bone repair applications. Furthermore, the ability to control the release rate of BMP-2 post-implantation represents a distinct advantage over existing systems which frequently demonstrate BMP release as a "burst release" immediately following implantation rather than a sustained release over time. This release profile differs greatly from the physiologic processes in normal bone healing and results in the formation of poor quality bone.[26] Slower, more sustained release kinetics are more biologically accurate and are reported to result in an enhanced bone healing response.[4,27–29] In one such study, Bez *et al*. report enhanced mineralization and an improvement in the average mechanical strength of the healed bone in rat cranial defect model following delayed injection of BMP-2 at 5 or 10 days after surgery compared to delivery intraoperatively.[4]

The results in this study demonstrated the ultrasound-triggered release of BMP-2 from the thermally response hydrogels, with a 5.five-fold, 7.five-fold and 13.five-fold increase in BMP-2 release compared to controls following US Treatment 1, US Treatment 2 and US Treatment 3 for the pure TH groups. Release of BMP-2 from the control groups was very low, indicating that it is bound or entrapped within the hydrogel structure. Binding between alginate and BMP-2 has been previously reported to occur through electrostatic interactions between the positively charged amino acid residues of BMP-2 and the negatively charged alginate carboxyl groups.[30] BMP-2 has also been previously shown to have a strong binding affinity for HA, reportedly interacting with HA through the –OH, -NH_2 and COO functional groups.[18,31] Here, no significant difference in the rate of BMP-2 diffusion was observed between the Pure TH and TH + 7% HA in the control groups, indicating that the HA present within the TH + 7% HA did not significantly influence BMP-2 binding. As a result of the ability of BMP-2 to bind within the alginate structure, these hydrogels show an improved controlled release profile compared to other materials such as collagen where BMP uptake and release is reported to occur purely by absorption and desorption processes.[30] The results here also demonstrate a further enhancement of BMP-2 release in response to ultrasound stimulation as a result of the addition of HA in the TH + 7% HA group compared to the Pure TH group. The greatest levels of BMP-2 release were observed in response to US Treatment 3 where a 1.7-fold increase in release was observed for the TH + 7% HA group compared to the Pure TH group. Comparing the TH + 7% HA group following US Treatment 3 to the control at the same timepoint where no ultrasound treatment was applied shows a 10-fold increase in BMP-2 release. The sonosentisation approach presented in this study shows greater levels of ultrasound-triggered BMP-2 release compared to other ultrasound-triggered hydrogel systems reported in the literature. For example, Kearney et al. achieved a six-fold increase in release of BMP-2 conjugated to gold nanoparticles from alginate hydrogels using a similar ultrasound protocol to that applied here compared to the control where no ultrasound was applied.[8]

Ultrasound, as a widely used technique clinically for non-invasive biomedical imaging, presents significant potential as a safe and translatable approach for externally controlled triggering therapeutic release. Furthermore, the application of ultrasound to BMP-2 has been previously shown not to influence the structural integrity of BMP-2 based on exposure within the diagnostic range for up to 30 min.[6] The maintained bioactivity of BMP following the exposure to a similar ultrasound protocol to that utilized here has also been reported.[8] HA has been widely demonstrated to have both osteoconductive and osteoinductive properties and is widely used in bone repair applications.[18,20,32] The successful synthesis of a HA-loaded thermally responsive hydrogels in this study presents an exciting material for use in minimally invasive bone repair applications. Furthermore, this study shows that HA can successfully achieve sonosensitization of hydrogels. The ability to achieve sonosensitization using HA has not previously been reported and thus this finding represents an important advancement in the development of ultrasound responsive materials for bone repair applications. While the mechanisms involved in ultrasound-triggered release of therapeutic agents from hydrogels have yet to be fully elucidated, studies report that both mechanical and thermal effects play a role.[11,15] The ultrasound protocol applied here has previously been shown to be effective at triggering release from alginate hydrogels while maintaining the temperature below 37°C.[9] Thus any change in molecular diffusion coefficients or hydrogel degradation due to heat could be ruled out of our analysis. It is hypothesized that rapid bubble formation and collapse in response to the applied ultrasound generates solvodynamic shear forces that alter the hydrogel mesh structure and interrupt the bonds formed between loaded drugs and the surrounding polymer network.[33] Huebsch *et al*. demonstrated that application of ultrasound resulted in a disruption of the calcium crosslinks in alginate hydrogels to accelerate drug release.[9] In addition to its ability to achieve triggered release of therapeutic agents, ultrasound also has the potential to promote bone repair in other ways. Osminkina *et al*. report that ultrasound increases the permeability of the cell membrane leading to an increase in the uptake of therapeutic agents which may further enhance bone healing.[16] This approach was also applied by Bez *et al*. to increase uptake of BMP-6 to enhance bone regeneration and fracture healing over 6 weeks in critical-sized tibial non-unions in mini-pigs.[4] Ultrasound has also demonstrated an ability to enhance bone formation due to microcrack formation.[34] In vivo and in vitro studies have demonstrated the positive role that ultrasound can play in the enhancement of fracture healing and in the reactivation of a failed bone healing process.[34]

The findings presented here show the development of a novel dual stimuli delivery system for the treatment of bone injuries. The results demonstrate the successful incorporation of HA in P(Alg-g-

NIPAAm) hydrogels to achieve thermally responsive alginate hydrogels suitable for minimally invasive delivery. Furthermore, the study demonstrates that the incorporation of HA particles into the P(Alg-g-NIPAAm) hydrogel, enhances its sensitivity to ultrasound stimulation resulting in an increase in rate of therapeutic factor release. Further studies will investigate the mechanisms involved in the ultrasound-triggered release of BMP-2 from these hydrogel systems and identify the optimal BMP-2 dose and timing of ultrasound application in order to achieve enhanced bone repair in vivo. Overall, the promising results reported for the dual stimuli system developed here present new possible applications for the successful treatment of otherwise hard to treat bone injuries in a relatively inexpensive and minimally invasive way.

5 | CONCLUSIONS

This study demonstrates the successful development of an ultrasound-triggered, thermally responsive hydrogel as an on-demand delivery system for bone repair. The results show that various concentrations of HA (1, 3, 5 and 7% wt./vol.) can be successfully integrated into thermally responsive P(Alg-g-NIPAAm) hydrogels, without compromising their overall structure. Furthermore, all hydrogels showed a gelation temperature of close to 37°C, to enable gelation upon delivery to the body, and the required injectability to enable minimally invasive delivery. The hydrogels demonstrated an ability to facilitate the sustained delivery of small (NaF) and large molecule (BSA) drug mimics and the therapeutic agent BMP-2 and an enhanced release as a result of the application of ultrasound was achieved compared to control groups in all cases. Importantly, hydrogel groups containing HA demonstrated an ability to achieve higher levels of release of both BSA and BMP-2 following ultrasound treatment compared to the pure hydrogel, thus demonstrating the ability of the HA particles to increase the sensitivity of the thermally responsive hydrogels to the ultrasound stimulation. Overall, the ultrasound-triggered thermally responsive hydrogels developed here hold tremendous promise for bone regeneration. Furthermore, these dual stimuli hydrogels have potential for delivery of a variety of therapeutic agents for other applications in clinical medicine.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Vo TN, Kasper FK, Mikos AG. Strategies for controlled delivery of growth factors and cells for bone regeneration. *Adv Drug Deliv Rev* 2012;*64*:1292–1309.
2. Wang Y, Newman MR, Benoit DSW. Development of controlled drug delivery systems for bone fracture-targeted therapeutic delivery: A review. *Eur J Pharm Biopharm* 2018;*127*:223–236.
3. Carragee EJ, Chu G, Rohatgi R, Hurwitz EL, Weiner BK, Yoon ST, Comer G, Kopjar B. Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. *J Bone Jt Surg - Ser A* 2013;*95*:1537–1545.
4. Bez M, Sheyn D, Tawackoli W, Avalos P, Shapiro G, Giaconi JC, Da X, Ben DS, Gavrity J, Awad HA, Bae HW, Ley EJ, Kremen TJ, Gazit Z, Ferrara KW, Pelled G, Gazit D. In situ bone tissue engineering via ultrasound-mediated gene delivery to endogenous progenitor cells in mini-pigs. *Sci Transl Med* 2017;*9*:390.
5. Liu M, Zeng X, Ma C, Yi H, Ali Z, Mou X, Li S, Deng Y, He N. Injectable hydrogels for cartilage and bone tissue engineering. *Bone Res* 2017;*5*.17014.
6. Crasto GJ, Kartner N, Reznik N, Spatafora MV, Chen H, Williams R, Burns PN, Clokie C, Manolson MF, Peel SAF. Controlled bone formation using ultrasound-triggered release of BMP-2 from liposomes. *J Control Release* 2016;*243*:99–108.
7. Zhou QL, Chen ZY, Wang YX, Yang F, Lin Y, Liao YY. Ultrasound-mediated local drug and gene delivery using nanocarriers. *Biomed Res Int* 2014;*2014*:1–13.
8. Kearney CJ, Skaat H, Kennedy SM, Hu J, Darnell M, Raimondo TM, Mooney DJ. Switchable release of entrapped nanoparticles from alginate hydrogels. *Adv Healthc Mater* 2015;*4*:1634–1639.
9. Huebsch N, Kearney CJ, Zhao X, Kim J, Cezar CA, Suo Z, Mooney DJ. Ultrasound-triggered disruption and self-healing of reversibly cross-linked hydrogels for drug delivery and enhanced chemotherapy. *Proc Natl Acad Sci U S A* 2014;*111*:9762–9767.
10. Zardad AZ, Choonara YE, du Toit LC, Kumar P, Mabrouk M, Demarco Kondiah PP, Pillay V. A review of thermo- and ultrasound-responsive polymeric systems for delivery of chemotherapeutic agents. *Polymers (Basel).* 2016;*8*:359.
11. Yang C, Li Y, Du M, Chen Z. Recent advances in ultrasound-triggered therapy. *J Drug Target* 2019;*27*:33–50.
12. Lavrador P, Gaspar VM, Mano JF. Stimuli-responsive nanocarriers for delivery of bone therapeutics – Barriers and progresses. *J Control Release* 2018;*273*:51–67.
13. Pentlavalli S, Chambers P, Sathy BN, O'Doherty M, Chalanqui M, Kelly DJ, Haut-Donahue T, McCarthy HO, Dunne NJ. Simple radical polymerization of poly(alginate-graft-N-Isopropylacrylamide) injectable Thermoresponsive hydrogel with the potential for localized and sustained delivery of stem cells and bioactive molecules. *Macromol Biosci* 2017;*17*(11).
14. Chalanqui MJ, Pentlavalli S, McCrudden C, Chambers P, Ziminska M, Dunne N, McCarthy HO. Influence of alginate backbone on efficacy of thermo-responsive alginate-g-P(NIPAAm) hydrogel as a vehicle for sustained and controlled gene delivery. *Mater Sci Eng C* 2019;*95*:409–421.
15. Nikolaev AL, Gopin AV, Bozhevol'nov VE, Treshchalina EM, Andronova NV, Melikhov IV. Use of solid-phase inhomogeneities to increase the efficiency of ultrasonic therapy of oncological diseases. *Acoust Phys* 2009;*55*:575–583.
16. Osminkina LA, Nikolaev AL, Sviridov AP, Andronova NV, Tamarov KP, Gongalsky MB, Kudryavtsev AA, Treshalina HM, Timoshenko VY. Porous silicon nanoparticles as efficient sensitizers for sonodynamic therapy of cancer. *Microporous Mesoporous Mater* 2015;*210*:169–175.
17. LeGeros RZ. Properties of osteoconductive biomaterials: Calcium phosphates. *Clin Orthop Relat Res* 2002;*395*:81–98.
18. Levingstone TJ, Herbaj S, Dunne NJ. Calcium phosphate nanoparticles for therapeutic applications in bone regeneration.

Nanomaterials [Internet] 2019;*9*:1570 https://www.mdpi.com/2079-4991/9/11/1570.

19. Samavedi S, Whittington AR, Goldstein AS. Calcium phosphate ceramics in bone tissue engineering: A review of properties and their influence on cell behavior. *Acta Biomater* 2013;*9*:8037–8045.
20. Levingstone TJ, Herbaj S, Redmond J, McCarthy HO, Dunne NJ. Calcium phosphate nanoparticles-based systems for RNAi delivery: Applications in bone tissue regeneration. *Nanomaterials* 2020;*10*:146.
21. Macdonald V, Wilson K, Sonne MWL, Keir PJ. Grip type alters maximal pinch forces in syringe use. *Hum Factors* 2017;*59*:1088–1095.
22. Venkatesan J, Bhatnagar I, Manivasagan P, Kang KH, Kim SK. Alginate composites for bone tissue engineering: A review. *Int J Biol Macromol* 2015;*72*:269–281.
23. Naddaf AA, Tsibranska I, Bart HJ. Kinetics of BSA release from poly(N-isopropylacrylamide) hydrogels. *Chem Eng Process Process Intensif* 2010;*49*:581–588.
24. Estey T, Kang J, Schwendeman SP, Carpenter JF. BSA degradation under acidic conditions: A model for protein instability during release from PLGA delivery systems. *J Pharm Sci* 2006;*95*:1626–1639.
25. Hsieh SR, Reddy PM, Chang CJ, Kumar A, Wu WC, Lin HY. Exploring the behavior of bovine serum albumin in response to changes in the chemical composition of responsive polymers: experimental and simulation studies. *Polymers (Basel)* 2016;*8*(6), 238.
26. Segredo-Morales E, García-García P, Évora C, Delgado A. BMP delivery systems for bone regeneration: Healthy vs osteoporotic population. Review. *J Drug Deliv Sci Technol* 2017;*42*:107–118.
27. Minardi S, Fernandez-Moure JS, Fan D, Murphy MB, Yazdi IK, Liu X, Weiner BK, Tasciotti E. Biocompatible PLGA-mesoporous silicon microspheres for the controlled release of bmp-2 for bone augmentation. *Pharmaceutics* 2020;*12*:118.
28. Brown KV, Li B, Guda T, Perrien DS, Guelcher SA, Wenke JC. Improving bone formation in a rat femur segmental defect by controlling bone morphogenetic protein-2 release. *Tissue Eng - Part A* 2011;*17*:1735–1746.
29. Jeon O, Song SJ, Yang HS, Bhang SH, Kang SW, Sung MA, Lee JH, Kim BS. Long-term delivery enhances in vivo osteogenic efficacy of bone morphogenetic protein-2 compared to short-term delivery. *Biochem Biophys Res Commun* 2008;*369*:774–780.
30. Lee YH, Lee BW, Jung YC, Yoon B Il, Woo HM, Kang BJ. Application of alginate microbeads as a carrier of bone morphogenetic protein-2 for bone regeneration. *J Biomed Mater Res - Part B Appl Biomater* 2019;*107*:286–294.
31. Dong X, Wang Q, Wu T, Pan H. Understanding adsorption-desorption dynamics of BMP-2 on hydroxyapatite (001) surface. *Biophys J Biophysical Society* 2007;*93*:750–759.
32. Habibovic P, Barralet JE. Bioinorganics and biomaterials: Bone repair. *Acta Biomater* 2011;*7*:3013–3026.
33. Sun W, Jiang H, Wu X, Xu Z, Yao C, Wang J, Qin M, Jiang Q, Wang W, Shi D, Cao Y. Strong dual-crosslinked hydrogels for ultrasound-triggered drug delivery. *Nano Res* 2019;*12*:115–119.
34. Padilla F, Puts R, Vico L, Raum K. Stimulation of bone repair with ultrasound: A review of the possible mechanic effects. *Ultrasonics* 2014;*54*:1125–1145.

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