Alginate chitosan microbeads and thermos-responsive hyaluronic acid hydrogel for phage delivery

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ABSTRACT

Bacteriophages are potential alternatives to conventional antibiotics in the treatment of multidrug resistant infections. However, optimal treatment administration protocols are not established. The direct application of bacteriophages in local biomaterials-based carriers offers solutions to many of the limitations of current modalities. In this study, thermo-responsive hyaluronic acid-poly(N-isopropylacrylamide) (HA-pNIPAM) hydrogels and alginate-chitosan microbeads have been developed as biodegradable local bacteriophage delivery systems designed to enable rapid and delayed phage release, respectively. The long-tailed Staphylococcus aureus phage ISP and the short-tailed Pseudomonas aeruginosa phage LUZ19 were selected as structurally diverse phages to load within both biomaterials. HA-pNIPAM hydrogels were synthesized and their thermo-responsive crosslinking was confirmed after rheological assessment. Alginate microbeads were prepared by ionic-gelation and chitosan surface deposition was confirmed by amine sensitive assays. After embedding phages, their antimicrobial potential was retained when stored at 4 °C. Release of both phages from HA-pNIPAM hydrogels was gradual and consistent over 21 days, whereby, continuous release of the short-tailed LUZ19 was observed for 21 days from the microbeads, while release of the long-tailed ISP portrayed burst-like release during early timepoints, with gradually reducing phage release thereafter. These findings suggest that the release kinetics of phage from biomaterials are impacted by both the biomaterial and the phages themselves. These results highlight the potential of HA-pNIPAM hydrogel and alginate-chitosan microbeads for local delivery of ISP and LUZ19 bacteriophages for both rapid burst release and controlled, extended release.

1. Introduction

Bacteriophages (phages) are naturally occurring viruses that selectively infect and lyse bacteria in a rapid manner. They are emerging as an adjunct therapy for antibiotics in difficult-to-treat infections [1]. While the discovery of bacteriophages predates the development of antibiotics, research on bacteriophages was mostly abandoned in Western countries after antibiotics were successfully produced on an industrial scale. A main factor in the renewal of interest in phage therapy is the growing problem of antibiotic resistant pathogens [2].

A key benefit of phage therapy over antibiotic therapy is the specific nature of the phage, with limited off-target effects expected compared with conventional antibiotics, particularly broad-spectrum antibiotics. Phages are self-replicating in the presence of host bacteria, and once the host bacteria are all lysed, the phage titer will slowly decrease. In phage therapy, where phages are used to treat infection in vivo, the formation of new bacteriophages is countered by bacteriophage inactivation resulting in a declining bacteriophage presence over time [3]. Since the
dynamics of these competing processes are highly variable and difficult to predict, precise dosing regimens have not established for phage therapy to the same extent as they have for antibiotic therapy. Antibiotic dosing regimens are calculated based on body weight, knowledge of pharmacokinetics and pharmacodynamics, and antibiotic susceptibility data. In contrast, phage therapy regimens still lack an evidence-based approach for optimal outcomes. Current practice in orthopedic device-related infection involves either intravenous administration or local instillation through surgical drains for up to 10 days, with good outcomes [4,5]. However, the instillation of phage suspensions through a percutaneous tube creates a risk of a further hospital-acquired infection and requires extended hospitalization for the patient [6,7]. Bacteriophage therapy for orthopedic infections would benefit greatly from bioresorbable delivery systems or carrier materials that could be placed within the infected site, allow wound closure and provide a sustained release of bacteriophages locally at the site of infection in one single application [8,9]. The concept is well established for local antibiotic delivery [10], but less so for phage delivery at the present time [9].

Hydrogels, either derived from natural or synthetic polymers, are highly suitable to embed and subsequently release bacteriophages. Our group previously reported on the synthesis of a semi-natural thermo-responsive hyaluronic acid-poly(N-isopropylacrylamide) (HA-pNIPAM) hydrogel that is suitable as a carrier for human mesenchymal stem cells (hMSCs) [11] or as a drug delivery system for water soluble antibiotics [12-15]. A hydrogels porosity and net charge are two parameters that influence phage retention and release kinetics [16]. Phages loaded in loosely crosslinked, porous hydrogel networks are prone to show a more rapid release compared to phages embedded in densely crosslinked matrices. Secondly, as phage tails and phage capsids can carry a net positive or negative charge at physiological pH [16], electrostatic interactions between phage and hydrogel plays an integral part in phage stability and release. As charged moieties with unique architectures, every type of phage potentially shows a specific level of release or retention in a hydrogel environment. Therefore, a combination of biomaterials may be required to provide adequate release profile for a wide variety of phages or, in future, even phage cocktails. HA-pNIPAM at body temperature can be considered as a dense network with physically crosslinked and hydrophobic pNIPAM regions. Therefore, the addition of hydrophilic biopolymer microbeads to the HA-pNIPAM could result in a composite environment from which a wider variety of phage could be loaded and released. In this work, microbeads with an alginate core (anionic) and chitosan shell (cationic) were studied separately and in combination with HA-pNIPAM hydrogels. The microbeads contain a high water content and are, therefore, considered very compatible with biological payloads such as cells or bacteriophages. Phage-loaded alginate/chitosan materials have been previously investigated for potential applications in food processing [17], animal husbandry [18] and gastrointestinal drug delivery [19], although studies investigating phage delivery to sites other than the gastro-intestinal tract have not been described to date.

The aim of this study was to investigate the release of phages from thermo-responsive HA-pNIPAM hydrogels, alginate-chitosan microbeads and combinations of these two phage-loaded biomaterials. The hypothesis is that the alginate-chitosan microbeads would not only offer additional protection to the phages, but also further delay delivery to extend the total phage release profile of the final HA-pNIPAM + Alginate-Chitosan composite material. The phages selected for testing included Staphylococcus aureus phage ISP and Pseudomonas aeruginosa phage LUZ19. These established therapeutic phages show specific activity against two bacterial species commonly identified in orthopedic infections, and with relatively high antibiotic resistance rates [20]. In addition, there are also structural differences between the long-tailed phage ISP (myovirus morphology, ~175 nm rigid tail-length) [21] and the short-tailed phage LUZ19 (podovirus morphology with typical tail-length < 10 nm) that might alter phage diffusion rates out of carrier materials [9] and so testing both should be indicative of the role of phage structure on interaction and release from biomaterials.

2. Materials and methods

2.1. Materials

HA sodium salt was acquired from Contipro Biotech (Czech Republic), number average molecular weight (Mn) = 170.6 kDa and polydispersity (Mw/Mn) = 1.73. N-isopropylacrylamide, dimethylformamide (DMF), cysteamine hydrochloride, diethylether (DEE), dimethyl sulfoxide (DMSO) sodium bromide, calcium chloride (CaCl2), Chitosan, anhydrous acetic acid, sodium chloride, anhydrous magnesium sulfate, sodium bicarbonate, Dowex Resin M – 31 and Carbonyl dimidazole were purchased from Sigma Aldrich (St. Louis, MO, USA). Phosphate buffer saline (PBS) tablets were bought from Sigma Aldrich, Buchs, Switzerland. Tetrabutylammonium (TBA), methanesulfonic acid, 2,2'-azobis (2-methylpropionitrile (AIBN) and Tris-(hydroxymethyl)-aminomethane hydrochloride (TRIS-HCl) was bought from Fluka (Buchs, Switzerland). Alginate (LVM, high G content) was purchased from Nova Matrix (Sandvika, Norway). Sodium citrate was acquired from Karl Roth (Karlsruhe, Germany). Bacteriological agar, Tryptic soy agar (TSA) plates and Tryptic soy broth (TSB) were purchased from Oxoid AG (Basel, Switzerland). Boric acid was purchased from Sigma Aldrich (Buchs, Switzerland). O-phthalaldehyde, sodium hydroxide, 2-mercaptoethanol and methanol was purchased from Carl Roth (Karlsruhe, Germany).

2.2. Methods

2.2.1. pNIPAM-NH2 and HA-pNIPAM synthesis

The synthesis of amine terminated poly(N-isopropylacrylamide) (pNIPAM-NH2) was performed according to ter Boo et al. [12]. Briefly, 10 g (88.4 mmol) of NIPAM monomer was dissolved in 20 mL of N2 degassed DMF. The NIPAM solution was brought under an inert atmosphere by further N2 degassing. Next, 100 mg of Cysteamine hydrochloride and 15 mg AIBN were added to the NIPAM solution. The solution was heated to 70°C and NIPAM polymerization occurred for 7 h under continued N2 bubbling. The pNIPAM-NH2 solution was cooled down to RT and was precipitated in DEE. The precipitate was vacuum-filtered and washed with DEE, followed by three days of desiccation under vacuum.

The subsequent synthesis of HA-pNIPAM was adapted from D’Este et al. [22]. Briefly, HA sodium salts were converted to TBA salts by ion-exchange of Na+ and TBA+ ions using Dowex Resin M-31. HA-TBA was lyophilized and 2.0 g of dried HA-TBA was dissolved in 160 mL DMSO. Separately, 3.5 g of pNIPAM-NH2 was dissolved in 40 mL of DMSO. Eighty µL of Methanesulfonic acid and 540 mg Carbonyl dimidazole were added to the dissolved HA-TBA and stirred at 42°C in an oil bath for 1 h. The pNIPAM-NH2 solution was added to the HA-TBA solution, and the mixture was stirred at room temperature for 48 h. Then, 20 mL of sodium bromide solution (930 g/L) was added dropwise to the reaction mixture and was stirred for 2 h. The solution was dialyzed using dialysis membranes with a 3.5 kDa molecular weight cut off, against cold tap water, and was changed daily. After five days, the HA-pNIPAM was purified using the Mestreneova software.

2.2.2. Preparation of buffers and bacteriological agar

Bacteriophages were maintained and diluted in a buffer containing sodium and magnesium ions and is hereafter described as SM-buffer. SM-buffer was prepared by adding 5.8 g sodium chloride, 1 g anhydrous magnesium sulfate and 50 mL of 1 M Tris-HCl solution at pH 7.4–950 mL ddH2O. To break alginate or alginate-chitosan microbeads, 200 mL ‘bead-breaker solution’ was prepared containing 50 mM sodium ion exchange of Na+ and TBA+ ions using Dowex Resin M-31. HA-TBA was lyophilized and 2.0 g of dried HA-TBA was dissolved in 160 mL DMSO. Separately, 3.5 g of pNIPAM-NH2 was dissolved in 40 mL of DMSO. Eighty µL of Methanesulfonic acid and 540 mg Carbonyl dimidazole were added to the dissolved HA-TBA and stirred at 42°C in an oil bath for 1 h. The pNIPAM-NH2 solution was added to the HA-TBA solution, and the mixture was stirred at room temperature for 48 h. Then, 20 mL of sodium bromide solution (930 g/L) was added dropwise to the reaction mixture and was stirred for 2 h. The solution was dialyzed using dialysis membranes with a 3.5 kDa molecular weight cut off, against cold tap water, and was changed daily. After five days, the HA-pNIPAM was purified using the Mestreneova software.
citrate, 0.2 M sodium bicarbonate, and 50 mM Tris-HCl solution at pH 7.5. Bacteriological soft agar 0.6% (w/v) and 0.8% (w/v), was prepared by adding 1.2 g (for ISP) or 1.6 g (for LUZ19) agar, respectively, and 6 g TSB to 200 mL ddH₂O. All buffers and agar solutions were autoclaved at 121 °C prior to use.

2.2.3. Bacteriophages and bacterial strains

The bacteriophages used in this study were S. aureus phage ISP (obtained from M. Merabishvili, University of Ghent (Belgium) [23] and P. aeruginosa phage LUZ19 (obtained from Rob Lavigne, University of Leuven (Belgium) [24]. S. aureus (JAR060131) [25], isolated from a patient with an infected hip prosthesis, was used for to propagate S. aureus phage ISP. This strain is available at the Swiss Culture Collection, with accession number CCOS 890. P. aeruginosa strain PA01 was used for the propagation of P. aeruginosa phage LUZ19 [24].

2.2.4. Bacteriophage quantification

In all methods and experiments described below bacteriophage titers were quantified by a modified agar overlay method [26]. Serial dilutions (from log(0) to log(-10)) of the bacteriophage suspension were prepared. One hundred µl of each dilution was mixed with 3 mL of molten (45 °C) 0.6% or 0.8% TSB top agar and 100 µl of the host bacterial suspension and plated onto TSA plates (Oxoid, Switzerland). Plates were incubated for 18–24 h at 37 °C. Plates with 100–1000 plaques were manually counted. The phage titre (PFU/ml) was calculated for all below described experiments by using the following equation: PFU/ml = counted plaques* dilution factor*10.

2.2.5. Preparation of alginate and alginate-chitosan microbeads

A solution of alginate was made by dissolution of 60 mg in 3.6 mL of MilliQ H₂O. After complete dissolution of the alginate, 0.4 mL of ISP or LUZ19-phage stock (10^10-10^11 plaque-forming units (PFU)/ml) was added bringing the total alginate concentration to 1.5% w/v. The Alginate solution containing phage was electrosprayed using a commercial instrument (Spraybase, Kildare, Ireland) with a pressure of 1 bar, emitter voltage of 10 kV, spray height of 10 cm and a 27-gauge needle. The solution was sprayed in a collection bath containing 30 mL of 100 mM CaCl₂. After the complete volume of the alginate/phage solution was sprayed, the solidified alginate microbeads were filtered using vacuum-assisted filtration on filter paper with mesh size grade 3 (6 µm) and washed with MilliQ H₂O to remove excess CaCl₂.

To fabricate alginate-chitosan microbeads, a Chitosan solution containing 4 mg/mL Chitosan in 2% Acetic acid was made and its pH was adjusted to 7.5. The deposition of Chitosan on the surface of the alginate beads was confirmed by O-phthalaldehyde (OPA) assay [27]. Briefly, Alginate-Chitosan beads were dissolved by submerging them in ‘bead-breaker solution’ (50 mM sodium citrate, 0.2 M sodium bicarbonate, and 50 mM Tris-HCl solution at pH 7.5). The OPA assay solutions were prepared by solving 1.25 g boric acid in 48 mL dH₂O and buffering the solution to a pH of 10.4 through addition of concentrated sodium hydrosilate solution (1 M). Next, 100 µL of 2-mercaptoethanol was added to the boric acid solution. 100 µg of OPA was dissolved in 2 mL methanol under vortex and added to the previously prepared boric acid/2-mercaptoethanol solution. The total volume of the OPA reagent was approximately 50 mL. The samples were prepared by adding 50 µL of dissolved Alginate-Chitosan beads to 100 µL OPA reagent and 50 µL methanol. The samples were incubated for 45 min at room temperature under mild rocking motion and protected from light. Multiple samples could be analyzed by loading the samples in self-packed wells made from light absorption assays and measuring UV absorption at 332 nm using a spectrophotometer (MultiskanGo, Thermo Scientific).

The collected phage loaded alginate microbeads were dispersed in 20 mL of chitosan solution and dispersed by vortex. The alginate microbeads were stirred in the Chitosan solution for 10 min, after which the microbeads were filtered and washed as mentioned previously. The alginate-chitosan microbeads were kept in airtight containers at 4 °C until further use. Imaging of alginate and alginate-chitosan microbeads was done by light microscopy (AxioVert A1, Zeiss, Oberkochen, Germany) using Axiovision4 software. The alginate or alginate-chitosan microbeads were used within 24 h of the electrospray procedure.

2.2.6. Rheological properties of phage loaded HA-pNIPAM hydrogels

To investigate changes in gelation kinetics and network properties caused by the presence of bacteriophages, hydrogels with and without ISP or LUZ19 phage load were tested. Rheological changes of the HA-pNIPAM hydrogel over a temperature range of 20–40 °C were assessed on an MCR302 Rheometer instrument (Anton Paar) equipped with a Peltier device. The hydrogel was applied on the rheometer bottom plate and a cone plate with diameter of d = 25 mm (CP25S) was used as a mobile top plate. The gap between the two surfaces during measurement was 50 µm. To assess the thermos-responsive mechanical properties of HA-pNIPAM, the temperature increased with approximately 1 °C/min and a strain within the hydrogels linear viscoelastic range (0.5% strain) was applied at a frequency of 1 Hz.

2.2.7. Bacteriophage stability in HA-pNIPAM hydrogels

HA-pNIPAM hydrogels were made by preparing a 10% w/v solution with SM-buffer containing approximately 10^10-10^11 PFU/mL. In quintuplicate (n = 5), 200 µL of the phage loaded hydrogel was pipetted into glass vials which were stored at either 4 or 37 °C. After 1, 3, 7, 11, and 14 days, a single vial was taken and the phage titer inside the hydrogel was determined to allow phase transition of the HA-pNIPAM gel. Control groups included phages kept in SM buffer. In addition, to assess potential adverse effects of HA-pNIPAM gelation on phage activity, phage loaded hydrogels were incubated at 37 °C and cooled at 4 °C in intervals of 5 min. Phage titers were assessed after three gelation cycles.

2.2.8. Bacteriophage release studies

2.2.8.1. Bacteriophage release from HA-pNIPAM. Lyophilized HA-pNIPAM was reconstituted at a concentration of 10% w/v with SM-buffer containing approximately 10^10-10^11 PFU/mL. Reconstitution occurred overnight at 4 °C under mild rotation. Aliquots of 800 µL of phage loaded HA-pNIPAM were made in glass vials and the hydrogel could solidify at 37 °C for 30 min, after which 3.2 mL of SM buffer was added on top of the gels. The complete release buffer was aspirated and refreshed every day for 21 days. After 21 days, the hydrogels were cooled in the fridge and phage remaining in the gels was quantified. Phage quantification of the release media and endpoint hydrogels was performed by plaque assay as previously described.

2.2.8.2. Cumulative bacteriophage release from combinations of HA-pNIPAM and alginate-chitosan microbeads. Alginate and alginate-chitosan microbeads were weighed and dispersed in 5 mL SM buffer at a concentration of 10 mg/mL and 30 mg/mL, respectively. Samples were incubated at 37 °C and after 1, 2, 3 and 4 days, without disturbing the sedimented beads, 100 µL was withdrawn and further diluted for phage quantification by plaque assay. After four days, 5 mL of bead-breaker solution was added to the samples, bringing the total volume up to approximately 10 mL. The alginate or alginate-chitosan microbeads were allowed to dissolve, and remaining phage was quantified by plaque assay.

2.2.8.3. Bacteriophage release from combinations of HA-pNIPAM and alginate-chitosan microbeads. Phage loaded alginate and alginate-chitosan microbeads were weighed (50 mg and 150 mg, respectively) in glass vials. Next, 800 µL of phage loaded 10% w/v HA-pNIPAM containing 10^8-10^11 PFU/mL was added to the vials with the microbeads. The microbeads and hydrogel were gently stirred by pipette tip and by mild vortex. Samples were incubated at 37 °C for 30 min to allow...
gellation of the HA-pNIPAM and 3.2 mL of SM-buffer was subsequently added to the vials. The complete release buffer was aspirated and refreshed every day for 21 days. After 21 days, the hydrogels were cooled in the fridge and 5 mL of bead-breaker solution was added. Phage quantification of the release media and endpoint hydrogels was performed by plaque assay.

2.2.9. Statistics
Statistical analysis was done in GraphPad Prism, Version 9.3.1. An independent t-test was performed to evaluate significant differences between reported averaged mean values and p-values were reported in Fig. 4. Error-bars in figures correspond to standard deviations to the reported average values.

3. Results

3.1. 1H NMR analysis and rheological properties of HA-pNIPAM hydrogels

Analysis of the molecular structure of HA-pNIPAM copolymers can be seen in Fig. 1A. Protons from the grafted pNIPAM polymers are labeled a at 1.14 ppm, b at 3.82 ppm, c at 1.96 ppm, and d at 1.3–1.7 ppm. The broad distribution of d protons was previously attributed to limited rotation of the c-d carbon atoms due to steric hindrance [22]. Protons on the HA backbone of the copolymer are labeled e at 3.2–3.6 ppm, h at 4.3–4.5 ppm, f at 1.96 ppm and g at 3.65 ppm. The degree of substitution (DS%) of pNIPAM grafting was calculated by integrating the peaks from the 8 HA protons labeled e and the peaks from pNIPAM labeled a. The DS% of pNIPAM grafted to the HA backbone was calculated to be 6.5 mol/mol%. Fig. 1B shows the gelation properties of HA-pNIPAM hydrogels in absence or presence of phage load. All hydrogels, regardless of presence of ISP or LUZ19 phage, showed an equal storage modulus after complete gelation at physiological temperatures (HA-pNIPAM: 2255 ± 648 Pa; HA-pNIPAM + ISP-phage: 1982 ± 119 Pa and HA-pNIPAM + LUZ19: 1667 ± 110 Pa). At lower temperatures, the presence of phages slightly increased the viscosity of the hydrogels resulting in higher storage modulus measurements. The temperature range in which gelation took place (±28–31 °C), as well as gelation kinetics, were not affected by phage loads. This rheological assessment ensured that phages did not alter the thermo-responsive behavior of the HA-pNIPAM hydrogels. Practical limitations prevented rheological assessment of HA-pNIPAM with alginate-chitosan microbeads, due to the large diameter of the microbeads (characterized below).

3.2. Stability of phages in HA-pNIPAM hydrogels

Since embedding phages in biomaterials can be associated with mechanical stresses that could damage the structural integrity of the phage, the compatibility of two phages was assessed with HA-pNIPAM. Phages with long rigid tails may be especially susceptible [28]. Active phage titers present inside HA-pNIPAM hydrogel and buffer were monitored over time (Fig. 2). Phage activity was not altered during the hydrogel hydration phase, which occurred in 4 °C under slow continuous vial inversion (Fig. 2A). This shows that mechanical stresses applied during this step did not inhibit the activity of phage ISP. Phage in SM-buffer at 4 °C was found to be a suitable storage condition over 14 days, while there was a gradual reduction in SM buffer at 37 °C or in the HA-pNIPAM hydrogel at 4 °C (Fig. 2A).

Titers of ISP (solid line) and LUZ19 (dotted line) phages in hydrogels kept at 37 °C are seen in Fig. 2B. Over a period of two weeks, the titer of both embedded phages steadily decreases. In this experimental set-up, small volumes of 10 w:v% HA-pNIPAM hydrogels were kept in glass vials and condensation could be observed on the inside of the vials. The decreasing water content in the hydrogel might have contributed to the reduction in phage activity, as hydrated environment has been recognized as an important factor in maintaining phage activity [29]. Because a sharp reduction of 2log10 was observed for ISP phage activity within 24 h, physical crosslinking of the HA-pNIPAM copolymers during gelation could have exerted mechanical stresses on the larger and tailed ISP phages. In Fig. 2C the gelation of ISP loaded HA-pNIPAM hydrogels was repeated three times. As no decrease in phage activity can be observed in Fig. 2C, it was ruled out that the thermally driven physical crosslinking of HA-pNIPAM gels reduced active ISP phage titers.

3.3. ISP and LUZ19 release from HA-pNIPAM hydrogels

Fig. 3 shows the release of ISP (A.) and LUZ19 phages (B.) from HA-pNIPAM hydrogels at 37 °C. The dashed lines represent the percentage of cumulative phage release and are plotted on the right Y-axis. The initial phage load in the HA-pNIPAM hydrogel samples differed slightly between the two experiments (3.1·10^10 PFU/gel for ISP and 9.8·10^9 PFU/gel for LUZ19). The cumulative release of ISP was higher (42% of initial phage load by day 21) compared to LUZ19 (28% of initial phage load by day 21). However, most of the ISP release occurred within 48 h, with released reducing to approximately 10^8 PFU/gel at day 12 while LUZ19 phage release remained more constant over the 21-day experiment.

After following the phage release for 21 days, the remaining PFU in the HA-pNIPAM hydrogel was 1.3·10^8 PFU/gel for ISP and 2.2·10^7 PFU/
3.4. Characterization of alginate and alginate-chitosan microbeads

Alginate microbeads and alginate-chitosan microbeads were prepared to provide extended release in comparison to the rapid release from the hydrogel. Beads were imaged immediately after fabrication and images are shown in Fig. 4A and B. During the chitosan deposition on the microbeads, the size average increases, as can be observed from the microscopic images and from Fig. 4C, which shows the average diameter of the two groups of microbeads (n > 100). Because chitosan deposition occurs in wet, pH-neutral conditions and alginate is known to swell in non-acidic environments, it was important to make sure that the increased bead diameter was not only the result of bead swelling, but also of chitosan deposition. In Fig. 4D we observed a significant (p = 0.0084) increase in absorption of the amine labeling agent o-phthaldehydraldehyde, indicating an increased presence of amine groups associated to the microbeads once alginate-chitosan microbeads were formed.

3.5. ISP and LUZ19 release from alginate and alginate-chitosan microbeads

Release of ISP and LUZ19 phages from alginate and alginate-chitosan microbeads can be seen in Fig. 5. Short-term release studies were performed as the in vitro experimental set-up involved immersing beads in SM buffer. Alginate and alginate-chitosan microbeads were expected to swell when freely dispersed in SM-buffer, due to the presence of sodium ions that can transform the crosslinked calcium-alginate beads into water-soluble sodium-alginate polymers. Alginate-chitosan microbeads released ISP and LUZ19 phages across the four-day experimental period (Fig. 5A&B). Release of LUZ19 from alginate microbeads increased sharply after 48 h, which contrasted to ISP release from alginate microbeads which decreased over this time period. While release of ISP from alginate-chitosan microbeads does not completely plateau after 24 h, release of LUZ19 increases at later time points.
3.6. ISP and LUZ19 release from combined HA-pNIPAM hydrogel and alginate-chitosan microbeads

Fig. 6 shows the release profiles of ISP and LUZ19 phages from phage-loaded HA-pNIPAM hydrogels with or without addition of phage-loaded alginate or alginate-chitosan microbeads. The release profile of ISP in Fig. 6A reveals that the addition of either ISP-loaded alginate or ISP-loaded alginate-chitosan microbeads does not affect the ISP release kinetics over time. In contrast, Fig. 6B shows that prolonged release of high titers of LUZ19 phage is established after addition of LUZ19-loaded alginate-chitosan microbeads. The cumulative release profiles of ISP and LUZ19 phages can be seen in Fig. 6C and D, respectively. ISP phage release from HA-pNIPAM with alginate or alginate-chitosan microbeads was more complete compared to only HA-pNIPAM hydrogel, plateauing at approximately 35% phage released in 21 days. However, the ISP release profile was not affected as most phage was released during the initial burst release in the first five days. Interestingly, LUZ19 release from HA-pNIPAM gels with alginate-chitosan microbeads increased after day 7, with both alginate and alginate-chitosan bead enriched HA-pNIPAM showing a slight increase in phage release after day 14 and 8, respectively (Fig. 6D). This mirrors the result when the microbeads were incubated in buffer, albeit much earlier at 48 h (Fig. 5). This increased
release of phage load shows the benefits of addition of alginate materials, with alginate-chitosan yielding the most sustained and highest in vitro LUZ19 release.

4. Discussion

Phage therapy protocols for treating orthopedic infection require further evidence and trials to identify optimal protocols. Both intravenous administration and direct instillation are cumbersome due to the need for repeated administration [1]. Such draining systems can be considered a burden for the patient and pose substantial risk for bacterial contamination, potentially leading to an additional hospital acquired infection. In this paper we investigated the potential of a thermo-responsive HA-pNIPAM hydrogel containing alginate chitosan microbeads to provide a sustained release of bacteriophages that may be applied in a single application. A single case report exists of a phage loaded hydrogel in the treatment of musculoskeletal infection [30]. Customized hydrogels, protecting and delivering phage in a more controlled manner may enable improved treatment outcome in these difficult to treat infections.

Embedding bacteriophages in biomaterials comes with the risk of phage inactivation, for example if the proteinaceous phage particle is altered due to exposure to heat, mechanical stresses, or chemical biocides [31]. Since the thermo-responsive HA-pNIPAM undergoes physical crosslinking at physiological temperatures, there is a risk of physical damage. As long-tailed phages such as ISP are reported to be susceptible to structural damage due to mechanical stresses [32], it was a positive outcome to see that the mechanical stresses involved in gelation and hydrogel preparation did not cause significant phage inactivation. The thermo-responsive feature enables injection of the fluid-like gel, and then jellification at body temperature, filling the wound space with phage-loaded hydrogel.

The stability of the phages in the HA-pNIPAM hydrogel over 21 days suggests the chemical environment is also suitable for phage delivery. However, the rapid release of phage may not provide the prolonged release of phage to match current treatment targets. Alginate and chitosan are established biomaterials for bacteriophage delivery [17,19,33–35]. The release of phage from alginate materials is diffusion driven and expedited by swelling and subsequent disintegration of the carrier material. When contained within the HA hydrogel, this should occur slowly, and further delay/control phage release. It is a challenge to replicate this in vitro. Alginate microbeads are kept together in aqueous media by interactions of Ca\(^{2+}\) ions with guluronic segments of alginate, but structural integrity is affected by ion exchange of Ca\(^{2+}\) with other monovalent ions present such as Na\(^{+}\) [36]. It has been shown that the swelling and degradation behavior of alginate is affected by NaCl concentration with increased swelling rates observed at higher NaCl concentrations, while alginate microbeads did not show swelling in distilled water [37]. Bajpai et al. (2004) showed that alginate microbeads (with an unknown diameter) took approximately 4–5 h to disintegrate in PBS [37]. In our experimental setup, SM-buffer was used to ensure optimal phage viability upon release from the microbeads. SM-buffer contains 100 mM NaCl (5.8 g/L), similar to the NaCl concentration in PBS (8 g/L, 137 mM). Additionally, SM-buffer contained 8.3 mM MgSO\(_4\) (1.0 g/L). Mg\(^{2+}\) ions have been identified as ions that induce alginate gelation, however a 5–10 times higher Mg\(^{2+}\) concentration was necessary to yield similar results compared to Ca\(^{2+}\) gelation [38]. As the alginate and alginate-chitosan beads in our study were already gelled in CaCl\(_2\) solution prior to exposure to SM-buffer, we cannot exclude that the Mg\(^{2+}\) ions in SM-buffer contributes to the reduced microbead degradation. Nevertheless, this may be a largely in vitro phenomenon, or at least accelerated in vitro relative to in vivo degradation within the hydrogel.

As most bacteriophages have an isoelectric point between 3 and 7 [39], the cationic character of chitosan was expected to further slow bacteriophage release from alginate-chitosan microbeads in pH neutral buffers due to electrostatic interactions. The effects on phage release of coating the microbeads with chitosan was most profound for LUZ19 phages in combined HA-pNIPAM + alginate-chitosan materials, where a reduced release was observed over the first nine days of the release experiment. This finding is supported by literature on short-term release of T4 phages from alginate-chitosan materials [35]. However, LUZ19 release from HA-pNIPAM + alginate-chitosan materials increased sharply compared to HA-pNIPAM and HA-pNIPAM + alginate materials after nine days (Fig. 6B & D). Considering that the alginate and alginate-chitosan microbeads lose their structural integrity after approximately nine days in SM-buffer, the higher release of LUZ19 from alginate-chitosan microbeads could be due to higher maintenance of phage activity in alginate-chitosan microbeads. The protective properties of chitosan coatings for embedded bacteriophages has been described previously [33–35].

Striking differences were found between ISP phage and LUZ19 phage release from alginate and alginate-chitosan microbeads, which were also
observed when the microbeads were embedded in HA-pNIPAM hydrogels. Bacteriophage interaction with the polymer network, and, consequently, mobility inside the hydrogel, is affected by charge and the hydrophobicity of the bacteriophage outer surfaces [40]. Vandersteegen et al. (2011) showed that the ISP phage proteome consisted of 22 structural proteins [41]. Upon consulting the NCBI gene databank it was found that 39% of amino acids from these proteins consisted of hydrophobic amino acids (Gly, Ala, Val, Leu, Ile, Pro, Phe, Met or Trp) [42]. The LUZ19 genome was published by Lammens et al. (2009) [43] and the hydrophobic amino acid content in the most abundant proteins was 59%. The affinity between the relatively hydrophobic LUZ19 and hydrophobic pNIPAM regions in gelled HA-pNIPAM could explain the differences in ISP and LUZ19 release kinetics, as well as the higher LUZ19 retention in HA-pNIPAM gels after 21 days. Even though the porosity of the hydrogel was not studied in this work, according to the literature, pore size of calcium alginate gels is approximately 6–16 nm [44–45]. As both phages are larger than this, it is expected that phage release is expedted by the swelling/degradation of the hydrogel structure. The fact that we see faster release of the larger ISP phage, leads us to believe that there are other factors contributing to phage release kinetics aside from pore size and swelling/degradation. The amino acid composition of LUZ19 has been shown to have a higher prevalence of hydrophobic amino acids structural peptides than ISP. Another possibility is that the tagged phages are more susceptible to structural damage, yielding them undetectable by the plaque assay method. Due to the wide variety of phage potentially useful in phage therapy, and the wide range of surface properties and biomaterials, further work is required to fully comprehend any phage-loaded hydrogel for both release properties and phage-material interactions.

5. Conclusion

Thermo-responsive HA-pNIPAM hydrogels and alginate-chitosan microbeads were investigated as bioerodable phage delivery materials. HA-pNIPAM hydrogels show sustained release of ISP and LUZ19 phages for 21 days. Although being a phage delivery material with easy handling and efficient phage incorporation, HA-pNIPAM hydrogels display rapid release, and a slight reduction in ISP and LUZ19 phage activity over prolonged incubation times. Therefore, coformulations with alginate materials were developed to improve release kinetics and phage activity over time. Alginate-chitosan microbeads in combination with HA-pNIPAM hydrogels showed the most consistent and sustained release of LUZ19 bacteriophages over a period of 21 days. Our findings also suggests that release kinetics can be phage dependent and further impacted by charges and hydrophobicity of the hydrogel and should therefore not be generalized for any future phage-loaded material. The alginate chitosan beads within the hydrogel show potential for sustained bacteriophage release over the course of multiple days. Further in vitro and in vivo experiments should investigate the efficacy of these phage delivery systems in preventing and treating infection.

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Declaration of competing interest

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Data availability

Data will be made available on request.

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