Research in Microbiology 169 (2018) 522–530

Contents lists available at ScienceDirect

Research in Microbiology

journal homepage: www.elsevier.com/locate/resmic

High precision microfluidic microencapsulation of bacteriophages for enteric delivery

Gurinder K. Vinner, Danish J. Malik*

Chemical Engineering Department, Loughborough University, Loughborough, LE11 3TU, United Kingdom

ARTICLE INFO

Article history:
Received 31 January 2018
Accepted 29 May 2018
Available online 7 June 2018

Keywords:
Microencapsulation
Bacteriophage
Solid dosage form
Enteric infections
Salmonella enterica
Felix 01

ABSTRACT

A *Salmonella* specific bacteriophage Felix O1 (Myoviridae) was microencapsulated in a pH responsive polymer formulation. The formulation incorporated a pH responsive methacrylic acid copolymer Eudragit® S100 (10% (w/v)) with the addition of the biopolymer sodium alginate, the composition of which was varied in the range (0.5% (w/v)–2% (w/v)). The microencapsulation process employed commercially available microfluidic droplet generation devices. We have used readily available low cost microfluidic chips instead of bespoke in-house fabricated glass capillary devices which are accessible only in specialist research facilities. We show that these co-flow microfluidic devices can easily be used to prepare phage encapsulated microparticles making them suitable for use by both the phage research community and industry in order to evaluate and optimise phage compatible formulations for microencapsulation. A novelty of the work reported here is that the size of the generated monodispersed droplets could be precisely controlled in the range 50 μm–200 μm by varying the flow rates of the dispersed and continuous phases. Consequently, alginate concentration and microparticle size were shown to influence the phage release profile and the degree of acid protection afforded to phages upon exposure to simulated gastric fluid (SGF). Bigger microparticles (~100 μm) showed better acid protection compared with smaller beads (~50 μm) made from the same formulation. Increasing the alginate composition resulted in improved acid protection of phages for similar particle sizes. The high viscosity formulations containing higher amounts of alginate (e.g. 2% (w/v)) negatively affected ease of droplet generation in the microfluidic device thereby posing a limitation in terms of process scale-up. Felix O1 encapsulated in the formulation containing 10% (w/v) ES100 and 1% (w/v) alginate showed excellent protection upon exposure of the gelled microparticles to SGF (pH 1 for 2 h) without the use of any antacids in the encapsulation matrix. Encapsulated phages previously exposed to SGF (pH 1 for 2 h) were released at elevated pH in simulated intestinal fluid (SIF) and were shown to arrest bacterial growth in the log growth phase. We have therefore demonstrated the microencapsulation of phages using readily available microfluidic chips to produce solid dosage microcapsule forms with a rapid pH triggered release profile suitable for targeted delivery and controlled release in the gastrointestinal tract.

1. Introduction

The emergence of antibiotic resistant bacteria is a serious global threat to human health. Common enteric bacterial pathogens have become progressively more resistant to standard antibiotics [1,2]. Government Health Departments are increasingly banning general antibiotic use in food animal production e.g. see EU directive on additives for use in animal nutrition [3]. In addition to treating infections in humans, a safe and low cost biocontrol strategy to reduce pathogen carriage in livestock and poultry is also needed. The development pipeline for new classes of novel antibiotics is not looking promising [4]. There are frequent calls from government health agencies around the world to explore alternative treatment options [5]. Lytic bacteriophages (phages) are viruses that infect and kill bacteria, and they represent a promising approach to targeting bacterial infections in a treatment known as phage therapy [6–9]. The specificity of bacteriophages and their potential role in maintaining healthy gut microbiota makes them an alternative to employing antibiotics [10]. The use of phage therapy is a

* Corresponding author.
E-mail address: d.j.malik@lboro.ac.uk (D.J. Malik).

https://doi.org/10.1016/j.resmic.2018.05.011

particularly promising alternative to broad spectrum antibiotic treatment for acute enteric infections because typically in such infections intestinal concentration of infecting bacteria is high, the causative agent and strain may be suitably diagnosed using rapid diagnostic tools, and application of phage therapy with a sufficiently high initial phage dose would promote rapid *in situ* phage multiplication and decrease in the host bacterial population [2,11–13]. Enteric infections worldwide are typically caused by pathogens such as *Clostridium difficile, Escherichia coli*, *Salmonella* spp, *Vibrio cholera* [14]. Any such pathogen is potentially a target for phage therapy however, there are major barriers to be overcome in terms of understanding the dynamics of the interaction between phage and bacteria in the gut environment, and in terms of the logistics of delivering a stable defined product to the infection site [15].

It is estimated that *Salmonella* alone accounts for 1.2 million foodborne illnesses in the United States, with 23,000 hospitalisations and 450 deaths costing an estimated 365 million dollars in medical costs each year [16]. Increasing centralisation and industrialisation of food supply enhances the probability of distribution of these hardy organisms. Antimicrobial resistance to 'first-line' drugs is increasingly common among *Salmonella* worldwide [17]. In animals, decolonisation of the gastrointestinal tract from *Salmonella* may be beneficial for biocontrol to reduce dissemination of harmful bacteria through the food chain e.g. lairage associated *Salmonella* transmission in pigs [18]. Ensuring delivery and subsequent release of a precise high dose of phages to the site of infection in the gastrointestinal tract remains an important challenge to ensure that phage therapy develops its full potential as a therapeutic or prophylactic antimicrobial agent [19]. Encapsulation may help protect phages from loss of activity during manufacturing and storage and during transit through the stomach *en route* further downstream in the gastrointestinal tract. Encapsulation in solid dosage forms e.g. microcapsules may also permit easy oral dose application allowing phage administration through feed instead of administration via oral gavage.

When free phages are delivered orally for phage therapy or for modulating microbiota, there is likely to be variable but significant losses of phage titre by the time phages reach the intended infection site [20]. Oral application of phages exposes them to stomach acidity, digestive tract contents (e.g. enzymes such as pepsin and pancreatin) which can inactivate phages [21]. A recent *in vivo* study in chickens (phage were added to animal feed) using alginate microencapsulated *Salmonella* Felix O1 phages showed a significant reduction in levels of phage titre (3 log reduction compared to the dose given) found in the gastrointestinal tract [22]. Potential reduction in phage titres due to inactivation attributed to stomach acidity may in part have been responsible for failure of a recent clinical trial treating acute bacterial diarrhoea symptoms in children using phage therapy [15]. Acidic conditions encountered in the stomach and the presence of bile and digestive enzymes and other proteases in the intestinal tract and stomach may inactivate unformulated phages [23–25]. Use of antacids may increase the risk of gastroenteritis and its use was not permitted in a recent clinical trial [15]. In a separate study, mice were given an oral dose (T4 coliphages in drinking water) of 10^9 PFU/g gut contents which resulted in a 1000-fold lower titre, indicating a sizable loss in phage activity [21]. Therefore, there are clear drivers to protect phages against adverse gastrointestinal conditions by encapsulating them and to control their targeted release at the site of infection e.g. in the lower gastrointestinal tract (GIT) i.e. ileum, cecum and colon for *Salmonella* and other enteric infections [24–26].

Previous phage encapsulation strategies have used basic homogeniser and extrusion techniques and have employed formulations that are gentle in terms of avoiding use of organic solvents that would inactivate phages [24,25,27–29]. These studies have shown modest protection of phages when the capsules were exposed to simulated gastric fluid at pH values in the range 2–2.5, for up to 2 h exposure duration. We recently showed for the first time the potential of microfluidic encapsulation for a *C. difficile* specific phage using an in-house developed microfluidic device [30]. In-house built microfluidic droplet generation devices are highly versatile however they require a high level of fabrication expertise including access to specialist micro-forging equipment, the devices are fragile, difficult to handle, prone at times to leakages and ingress of air bubbles causing contamination and production issues especially for processing of biological materials. The aim of this study was to use commercially available low cost glass microfluidic droplet generation systems to microencapsulate phage. Additionally, in our recent publication the phage titre for the encapsulated *C. difficile* specific phage was rather low (10^7 PFU ml^{-1}), the formulation was not optimised and acid protection was limited to pH 2. The objectives here were to precisely control microparticle size and hence investigate the effect of particle size and formulation parameters (alginate composition) in order to prepare pH responsive microparticles that were able to withstand significantly low gastric acidity (as low as pH 1 for 2 h) without appreciable loss in phage activity at high encapsulated phage titres.

2. Materials and methods

2.1. Chemical reagents

A methyl methacrylate co-methacrylic acid copolymer Eudragit® S100 was purchased from Evonik Germany. Miglyol 840, a propylene glycol diester of caprylic/capric acid, was purchased from Sasol Germany and used as carrier oil for the continuous phase. Polyglycerol polyricinoleate (PGPR) an oil soluble surfactant was purchased from Abitek USA. Para-toluenesulfonic acid and sodium chloride were purchased from Fisher Scientific, UK. Sorensen's buffer 0.2 M was used as a dissolution media for the microparticles and was prepared by mixing sodium phosphate (NaH_2PO_4) with sodium phosphate dibasic (Na_2HPO_4) (for pH 7) (Fisher Scientific UK). Alginate was purchased from Sigma Aldrich, UK.

2.2. Salmonella *strain and bacteriophage Felix O1*

Salmonella enterica ATCC19585 was purchased from LGC standards, EU. Phage Felix O1 was kindly donated by Dr Cath Rees, University of Nottingham, UK [31]. *S. enterica* strain was used to propagate and enumerate Felix O1. Brain heart infusion broth and agar (Oxoid UK.) were used for all bacterial work. A single colony from a streaked overnight culture on a BHI agar plate was used to inoculate fresh BHI broth and left shaking overnight at 37 °C. The culture was diluted and regrown to log phase at 0.2 O.D. (550 nm) for all phage work.

Felix O1 was propagated by infecting a log phase culture of *S. enterica* at MOI 0.01. Once the culture had cleared the lysate was centrifuged at 2000 g for 15 min at 4 °C and filtered using a 0.2 μm pore size filter (Millipore, USA). All phage stocks were stored at 4 °C until further use.

To titre the phage stocks, 10 μl of phage stock was serially diluted (10-fold) to 10^{-8} in 90 μl of BHI broth. This was spotted in triplicate on a double layer agar plate containing 10 μl of overnight culture of host, *S. enterica*. The plate was dried near a flame and then incubated overnight at 37 °C. The following day plaques were counted to determine the phage titre and expressed as plaque forming units (PFU) per ml.

2.3. Free phage sensitivity at different pH values and upon storage in formulations at different temperatures

Simulated gastric fluid (SGF) was used to test phage sensitivity to different pH values. SGF was prepared using the following formulation: 0.2 M NaCl with pepsin at 3.2 mg/ml for solutions of pH 1, 2, 2.5 and 3. For simulated intestinal fluid (SIF), pH solutions 4 to 7, 0.2 M Sorensen's buffer was used with the addition of 10 mg/ml pancreatin. Time points were taken at 0 min, 30 min, 1 h, 3 h, 6 h and 24 h. For pH 2 and 2.5 exposure time points were also taken every minute for the first 10 min. 10 μl was removed at each time point and serially diluted 10-fold to 10^{-8} as described above. The samples were spotted in triplicate on a lawn of the host bacteria introduced via the double layer agar method. The plates were incubated at 37 °C overnight and checked for PFU the following day.

Eudragit S100 (ES100) was dissolved in deionised water dH_2O by dripping 4 M NaOH until the solution was clear. The solution pH was adjusted to pH 7 using 0.1 M HCl. To this solution different concentrations (0.5% (w/v) designated D3, 1% (w/v) designated D2, 2% (w/v) designated D1) of alginate was added and dissolved by stirring at 60 °C overnight in 50 ml Duran bottles equipped with a magnetic stirrer to aid mixing (Table 1). The polymer solutions were left to cool before phage stock was added. Felix O1 stock was concentrated by centrifuging in Amicon tubes (Millipore, UK) with a 100 kDa filter for 15 min and 2000 g. This concentrated phage was added to the dispersed aqueous phase solution to make the final concentration of phage of ~10^8-10^{10} PFU ml^{-1}. Phage stability in the polymer solutions was measured at room temperature ~20 °C stored over five days and stored at 4 °C for up to a week.

2.4. Felix O1 encapsulation using microfluidic droplet generation system

The continuous oil phase was made of a 50:50 mixture of Miglyol 840 and Castor oil (Elf Foods, Loughborough, UK), with 5% (w/v) PGPR. The emulsion was collected in acidified oil, consisting of 0.05 M p-toluenesulfonic acid in Miglyol with 5% (w/v) PGPR.

Eudragit-alginate droplets were prepared by using a hydrophobic quartz droplet junction chip with an etch depth of 190 μm purchased from Dolomite, UK (Fig. 1). The dispersed aqueous phase (phages in polymer formulations D1-D3) was introduced through the inner/middle channel and the continuous phase from the two outer channels. The phases were pumped via 10 ml syringes (BD Plastipak, UK) mounted on syringe pumps (Harvard Apparatus, UK). The two phases were delivered and collected through medical delivery tubes (0.86 mm inner diameter and 1.52 mm outer diameter, Smiths Medical International Limited, UK). As the two phases met at the interface, the oil phase causes the aqueous phase to pinch and form droplets. The flow rates for all formulations were kept constant and maintained to keep the dripping regime (Table 1). The droplet formation was observed via a high-speed camera (Micro C110 Phantom Ametek, UK), connected to an inverted microscope (Nikon Eclipse E200) using x 4 magnification objective lens. The camera was connected via computer allowing recording of the droplet formation at around 3000 frames per second. The forming droplets were collected in acidified oil and allowed to crosslink for a minimum of 2 h.

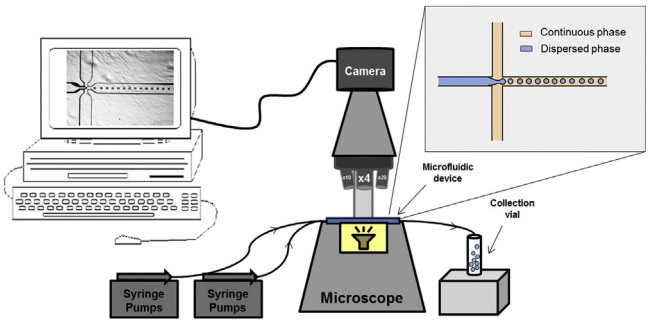

Fig. 1. Schematic of experimental setup used to produce water-in-oil (W/O) emulsions for phage microencapsulation. Microscope stage with mounted microfluidic chip connected to two high precision microfluidic syringe pumps suppling the aqueous dispersed phase (containing phages suspended in the polymer formulation) and the oil (continuous phase) respectively. High speed camera connected via computer to microscope for high frame rate visualisation of droplet generation and *in situ* control of droplet size through controlling of the flow parameters.

The acidified oil was removed by pipetting off the oil layer after gentle centrifugation at 300 g for 5 min to settle the acidified microparticles. An excess of 0.1 M $CaCl_2$ was added to the microparticles to crosslink the alginate for 1 h. Microparticles were collected by centrifuging at 2000 g for 5 min and removing the $CaCl_2$ solution using a pipette. The gelled particles were stored hydrated at 4 °C.

The drop and particle size were analysed using the Image J program (National Institute of Health, Washington, U.S.) by counting 10 drops or particles per sample. The particle sizes for gelled microcapsules were measured using Coulter LS series 130 (Beckman Coulter Inc.), employing a Fraunhofer optical model for data regression.

2.5. Scanning electron microscope (SEM)

Gelled particles were frozen on filter paper (0.2 μm pore size, Millipore Ltd. UK) at −20 °C overnight. The particles were freeze dried (VirTis Wizard 2.0) for 24 h at 50 Pa pressure and −20 °C. The particles were put on viewing stubs using double sided carbon tape, sputter coated with gold and viewed using a table top SEM (Hitachi TM3030 Microscope).

2.6. Encapsulated phage release in SGF and SIF

100 mg of Felix 01 encapsulated particles (approximate) were suspended in 10 ml prewarmed simulated intestinal fluid (SIF) containing 10 mg/ml pancreatin in 0.2 M Sorensen's buffer at pH 7 (Sigma Aldrich, UK). All dissolution experiments were carried out at 37 °C with shaking. For acid exposure to pH 1, 2, 2.5 and 3, 0.1 g microparticles were suspended in 10 ml prewarmed simulated gastric fluid (SGF) containing 3.2 mg/ml pepsin in 0.2 M NaCl at pH 2 (Sigma Aldrich, UK). After 2 h exposure to SGF at each pH, the particles were centrifuged at 2000 g for 10 min, SGF removed and

Table 1
Compositions of formulations and operating parameters for microfluidic device used to prepare emulsion droplets.

Formulation ID	D1	D2(s)	D2(b)	D3
Composition	10% (w/v) ES100 2% (w/v) Alginate ~10^9 PFU/ml	10% (w/v) ES100 1% (w/v) Alginate ~10^9 PFU/ml	10% (w/v) ES100 1% (w/v) Alginate ~10^9 PFU/ml	10% (w/v) ES100 0.5% (w/v) Alginate ~10^9 PFU/ml
Dispersed phase flow rate, Q_d/μl h^{-1}	50	25	100	50
Continuous phase flow rate, Q_c/μl h^{-1}	1000	1000	1000	500

microparticles re-suspended in SIF (10 ml) for dissolution and phage release. Phage release was monitored over 4 h, by taking samples to enumerate phage release kinetics at hourly time points. To do this, plaque assays were performed as described previously by taking 10 μl samples, serially diluting and spotting on a bacterial lawn using double overlay agar method. The plates were counted the following day for plaques.

2.7. *Encapsulated phage* Salmonella *killing*

Salmonella was cultured from a single colony with a starting O.D. (550 nm) of <0.001, shaking at 37 °C overnight. 60 ul of overnight culture was added to 60 ml of BHI. 2 ml of this starter culture was added to 24 well tissue culture plates. Approximately 0.2 g of microparticles were added to multiple wells (for hourly time points) to evaluate *in vitro* phage release, amplification and arresting of bacterial growth. Microparticles used were either virgin or previously exposed to pH 1 for an exposure period of 2 h. At hourly intervals 1 ml aliquots were withdrawn and the O.D. measured of the controls (bacteria only), pH 1 exposed particles and non-pH 1 exposed particles. An additional 1 ml aliquot per well was transferred to an Eppendorf tube for each sample and centrifuged at 2000 g for 3 min for plaque assays. 10 μl was removed from the supernatant to enumerate for phage using the double-layer agar method as described previously. The pellet was separated and re-suspended in fresh BHI broth, this was serially diluted and plated on BHI agar plate to enumerate residual bacteria concentration. The control was also serially diluted and plated to determine the bacteria concentration. The plates were incubated overnight at 37 °C and checked for plaques and colonies the following day. The results were presented as the mean of three repeats.

2.8. *Statistical analysis*

Statistical analysis was carried out using the statistical software Minitab 18. 2-Sample t tests were done to calculate 95% confidence intervals and to carry out hypothesis tests of the difference between two population means.

3. Results

3.1. *Production of phage encapsulated ES100-alginate microparticles*

Highly monodispersed droplets were produced for 0.5% (w/v) (formulation D3, viscosity, 25 cP) and 1% (w/v) (formulation D2, viscosity 64 cP) alginate compositions (all solutions contained 10% (w/v) ES100). Droplet generation was stable for formulations D2 and D3 over extended periods with drops collected for several days without interruption. Formulations with alginate compositions higher than 1% (w/v) were highly viscous, (2% (w/v) alginate solution having a measured viscosity of 574 cP) and this resulted in difficulties in maintaining uniformity of droplet generation for prolonged periods due to flow instabilities causing disruption in droplet generation and observation of jetting phenomena discussed elsewhere [30]. Stable production of uniform droplets required at-line optimisation of the hydrodynamic conditions. Microfluidic droplet generation devices can be controlled *in situ* by direct microscopic observation. Hydrodynamic conditions were optimised for each formulation in real-time by adjusting fluid flow rates during each experiment until uniform droplets were produced (Table 1). Thereafter only small changes to these values were needed to achieve droplets of similar sizes ensuring batch-to-batch uniformity of droplets (data not shown).

To enable better control of the droplet generation process and in order to reliably produce droplets with low polydispersity, the 'dripping regime' worked best. Here, the droplets were formed closer to the orifice of the collection capillary (Fig. 2a and b) because the interfacial tension forces dominated over the inertia of the dispersed phase and the viscous stress forces from the continuous phase. Droplet formation began as the dispersed phase entered into microfluidic flow focusing zone (Fig. 2a and b). The dispersed phase did not come into contact with the capillary wall due to repulsion from the hydrophobic surface of the collection capillary wall which was designed to resist wetting by the aqueous phase. The continuous phase forced the dispersed phase to elongate axially (Fig. 2b), collapsing the dispersed phase and producing

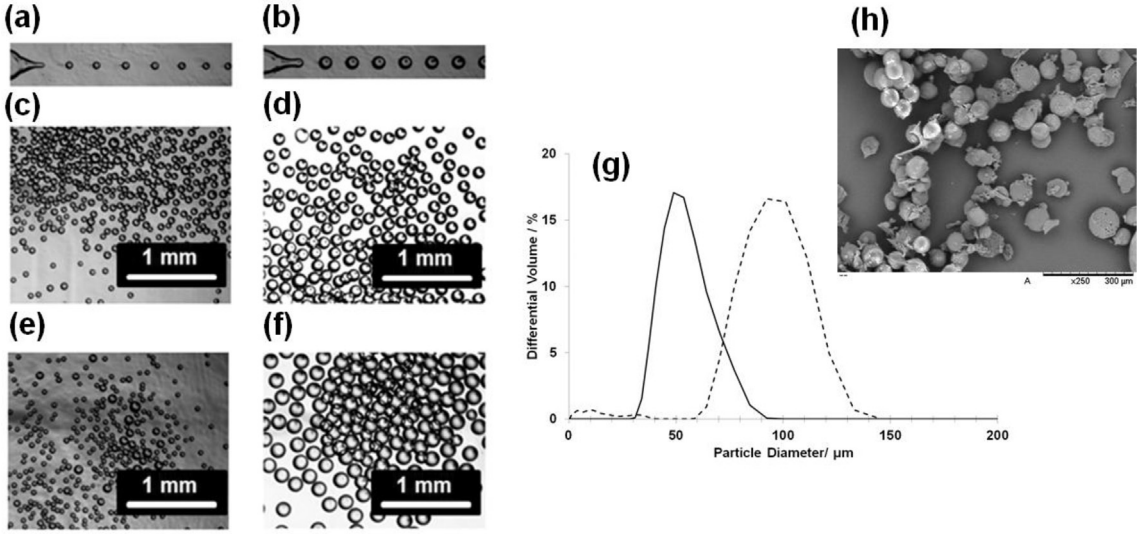

Fig. 2. Production of pH responsive microparticles using a microfluidic droplet generation system. At-line optimisation of hydrodynamic conditions allowed control over the droplet size. **(a)** *in situ* imaging showing generation of small droplets in the microfluidic chip using formulation D2(s) and **(b)** formation of big droplets using formulation D2(b); **(c)** collected small droplets with formulation D2(s) (mean drop size 73 μm ± 11 μm); **(d)** collected big droplets with formulation D2(b) (mean drop size 190 μm ± 5 μm); **(e)** collected small droplets with formulation D1 (mean drop size 64 μm ± 12 μm); **(f)** collected big droplets with formulation D3 (mean drop size 164 μm ± 19 μm); **(g)** particle size distributions of gelled microparticles (after TSA exposure and $CaCl_2$ crosslinking) using formulation D2(s) and D2(b); **(h)** scanning electron micrograph of freeze dried big microparticles prepared using formulation D2(b).

a neck behind the forming droplet. As the elongation progressed further, the neck thinned into a thread, eventually breaking the dispersed phase and releasing the droplet. Following retraction, the process restarted with the dispersed phase protruding at the orifice of the collection capillary. In this dripping regime, the formation of one droplet took ~95 ms, thus ~10 droplets were formed each second and subsequent droplets were produced at the same position as the previous droplets. The balance between the drag force and interfacial tension and negligible shear in the collection capillary after drop formation resulted in uniform droplets.

The ability to readily change the droplet size is an important feature of microfluidic droplet generation systems enabling control over the final microparticle size. Droplet generation in the dripping regime was optimised over a range of flow rates for alginate formulations of differing viscosities thereby enabling control of the droplet size whilst maintaining a reasonably high degree of the size uniformity (Fig. 2 c–f). This was done in real-time whilst observing (using the microscope camera) the change in the resulting droplet size. Increasing the flow rate of the inner phase relative to the outer phase resulted in an increased size of the droplets e.g. for formulation D2 (Fig. 2c and d, Table 1).

Droplet formation in the microfluidic droplet generation chip was followed by curing in acidified oil, removal of acidified miglyol and subsequent gelling of alginate in 0.1 M $CaCl_2$. This resulted in distinct gelled solid microspheres (Fig. 2h). The gelling process resulted in significant shrinkage in the size of the final microparticles measured using a Coulter particle size analyser (Fig. 2g) and through visualization of freeze dried particles using SEM imaging (Fig. 2h).

3.2. *Effect of acid exposure on Felix O1 viability and phage stability upon storage in formulations at different temperatures*

The effect of acidic pH on Felix O1 phage viability was tested. Phages were suspended in 0.2 M sodium chloride solutions with varying pH. The phages lost nearly all activity after 10 min of exposure to pH 2 and lost activity progressively upon exposure to pH 2.5 over a period of 6 h (Fig. 3a). Phage viability was significantly improved when the solution pH was above pH 3.

Phage Felix O1 was found to be stable and retain titre upon formulation in 10% (w/v) ES100 containing between 0.5% (w/v) to 2% (w/v) alginate (formulations D1-D3) upon storage under refrigerated conditions at 4 °C for up to 1 week (Fig. 3b) and over several days in syringes mounted in microfluidic pumps used during droplet generation at room temperature (data shown for formulation D2, Fig. 3c).

3.3. *Microencapsulated phage exposure to simulated gastric fluid (SGF) and phage release in simulated intestinal fluid (SIF)*

Having a controlled flow rate of polymer solution containing phages at a known phage titre allowed calculation of the theoretical yield of phages recovered in the final collected gelled microparticles. Near 100% (typically > 95%) encapsulation of phages was observed following the entire encapsulation process. Gelled microparticles prepared using formulations D2(s) and D2(b) were added to shaking solutions containing SGF with pH adjusted to pH 1, pH 2, pH 2.5 and pH 3 and microparticles were exposed for 2 h followed by centrifugation and separation of the microparticles from SGF. Microparticles were subsequently exposed to SIF and the kinetics of phage release was measured. Time point 0 h refers to phages released within the first 10 min upon exposure to SIF. Small 1% (w/v) alginate microbeads (D2(s), mean size 50 μm) were able to protect encapsulated Felix O1 phages to SGF with pH as low as pH 2.5 (Fig. 4a). However, lowering the pH further resulted in titre drop

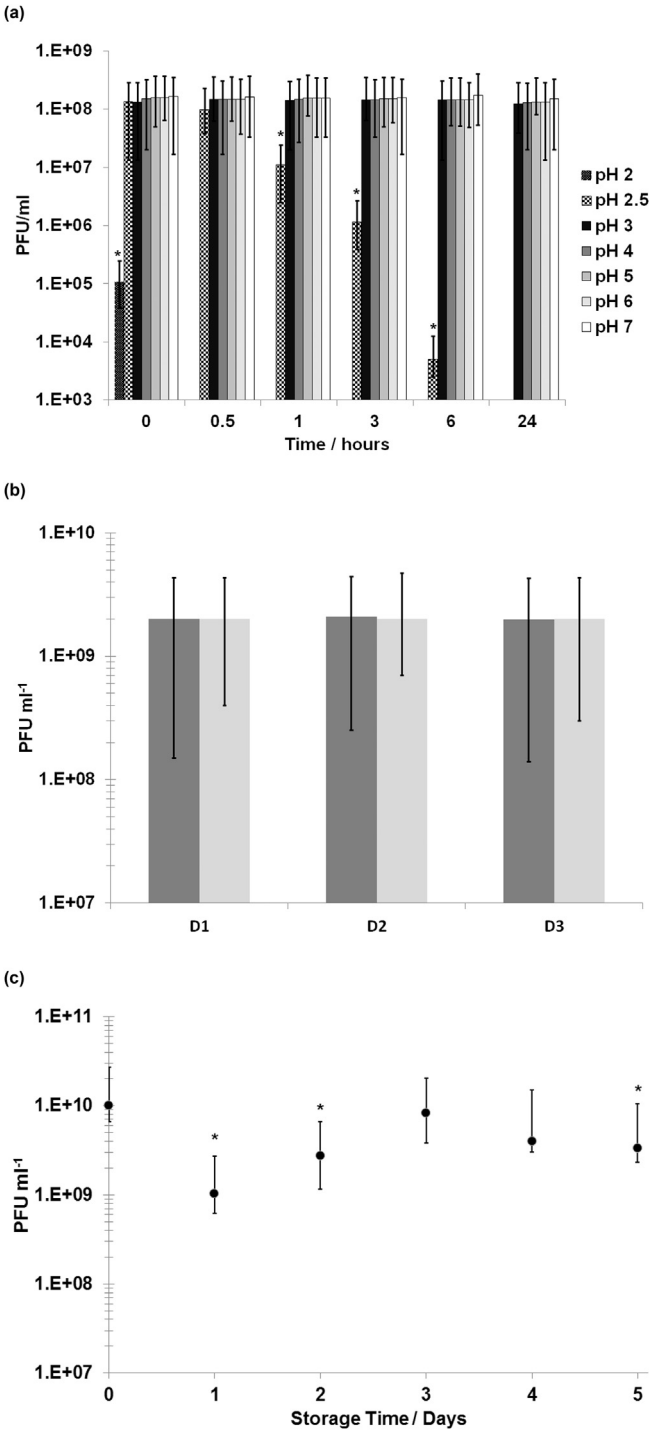

Fig. 3. Felix 01 stability upon exposure to solutions of different pH and in formulations D1-D3 upon storage. (a) Phage suspensions were exposed to 0.2 M NaCl with pH adjusted in the range pH 2 – pH 7. Phages were exposed for different time periods at each controlled pH. Time point 0 h refers to exposure for ~1 min. (b) Phages stored in formulations D1-D3 refrigerated at 4° C, dark grey bars (day 0), light grey bars (day 7). (c) Phages stored in formulation D2 stored at room temperature at ~20° C. Error bars are 95% confidence intervals (n = 3). * indicates significance ($p < 0.05$) using a 2-sample t-test as compared with sample at pH 7 exposed for the same time period in (a) and Day 0 in (c).

from 1×10^9 PFU ml^{-1} to 4×10^7 PFU ml^{-1} (at pH 2) and complete loss of phage titre upon exposure to pH 1. No phages were recovered for small microparticles prepared using formulation D2(s) exposed to SGF at pH 1. Using formulation D2(b) and increasing the

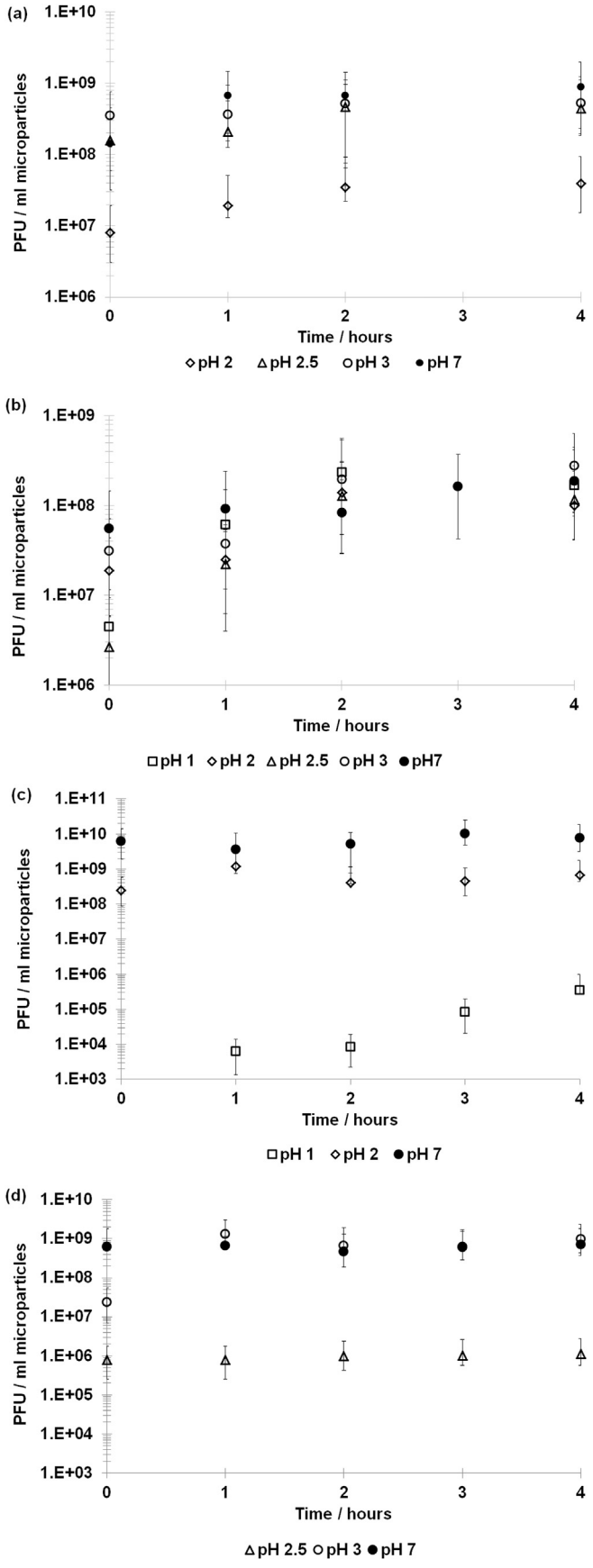

Fig. 4. Protection of encapsulated Felix O1 bacteriophage from SGF and release of phages upon exposure to SIF. (a) acid protection and phage release kinetics for small microparticles, formulation D2(s); (b) acid protection and phage release kinetics for big microparticles, formulation D2(b); (c) acid protection and phage release kinetics for small microparticles, formulation D1; (d) acid protection and phage release kinetics for big microparticles, formulation D3. Error bars are 95% confidence intervals (n = 3).

size of microparticles (mean size ~100 μm) resulted in significant improvement in phage viability with no measured loss in phage titre for microparticles exposed to pH 1 (Fig. 4b). The release profile also changed from burst release for small microbeads to slow release for the larger microbeads with around 20% of encapsulated phage dose released after 1 h and near complete release after 2 h of exposure to SIF. Small microbeads prepared using formulation D1 (containing 2% (w/v) alginate) significantly improved phage acid protection compared with particles prepared using formulation D2(s) with phages surviving exposure albeit with titre reduction upon exposure to SGF at pH 1 (Fig. 4c). For larger microbeads lowering the alginate composition (formulation D3) reduced acid protection with 0.5% (w/v) alginate samples showing acid protection down to pH 3 with significant drop in phage titres at pH 2.5 and no acid protection at pH values at or below pH 2 (Fig. 4d).

3.4. *Microencapsulated phage release and* Salmonella *killing*

Large 1% (w/v) alginate microbeads (D2(b)) pre-exposed to SGF (pH 1) rapidly released phage with the phage titre rising to ~1×10^6 PFU ml^{-1} shortly after addition (Fig. 5 c). Optical density measurements showed a clear deviation in *Salmonella* growth in cultures at time point 3 h post microparticle addition for small and large microparticles (particles had previously been exposed to SGF at pH 1 for 2 h, Fig. 5 a). For big particles (D2(b)) the CFU counts dropped sharply after 3 h with concomitant amplification of phage titres (Fig. 5 b, c). Exposure to SGF at pH 1 resulted in considerably lower phage titres in the small 1% (w/v) alginate microparticles (D2(s)), therefore phage release from the small microbeads resulted in much lower phage titre (1×10^3 PFU ml^{-1}). This resulted in a significant delay in both *Salmonella* CFU reduction (~4 h) and subsequent phage amplification (Fig. 5 b, c). 4 h post microparticle addition both sets of cultures had arrested *Salmonella* growth and considerable phage amplification was noted with phage titre rising to ~1×10^{10} PFU ml^{-1} however, the time taken to arrive at this level differed significantly for the two 1% (w/v) alginate samples (D2(s) and D2(b)) indicative of the effect of phage dose on phage-bacterium population dynamics.

3.5. *Storage stability of encapsulated phage*

Large microparticles (prepared using formulation D2(b)) stored over the course of 4 weeks under refrigerated conditions (at 4 °C) showed a modest drop in phage titre (Fig. 6). Phage titre dropped from ~4×10^8 PFU ml^{-1} microparticle at week 0–1×10^8 PFU ml^{-1} microparticles after 1 week of storage. Thereafter, phage titre remained stable up to week 4 (the extent for which measurements were taken).

4. Discussion

Motivation for research into solid dosage forms for oral application of phages is driven by the need to protect phages from the harsh gastrointestinal tract environment. Phage mediated reduction of bacterial levels *in vivo* requires high titres of viable phages delivered precisely at the site of infection that are able to target the host and arrest bacterial growth [19]. Previous efforts targeting *Salmonella* in the food chain have attributed poor phage stability in the gastrointestinal tract for insufficient *in vivo* efficacy [18]. *In vitro* phage stability experiments exposing phages to solutions of

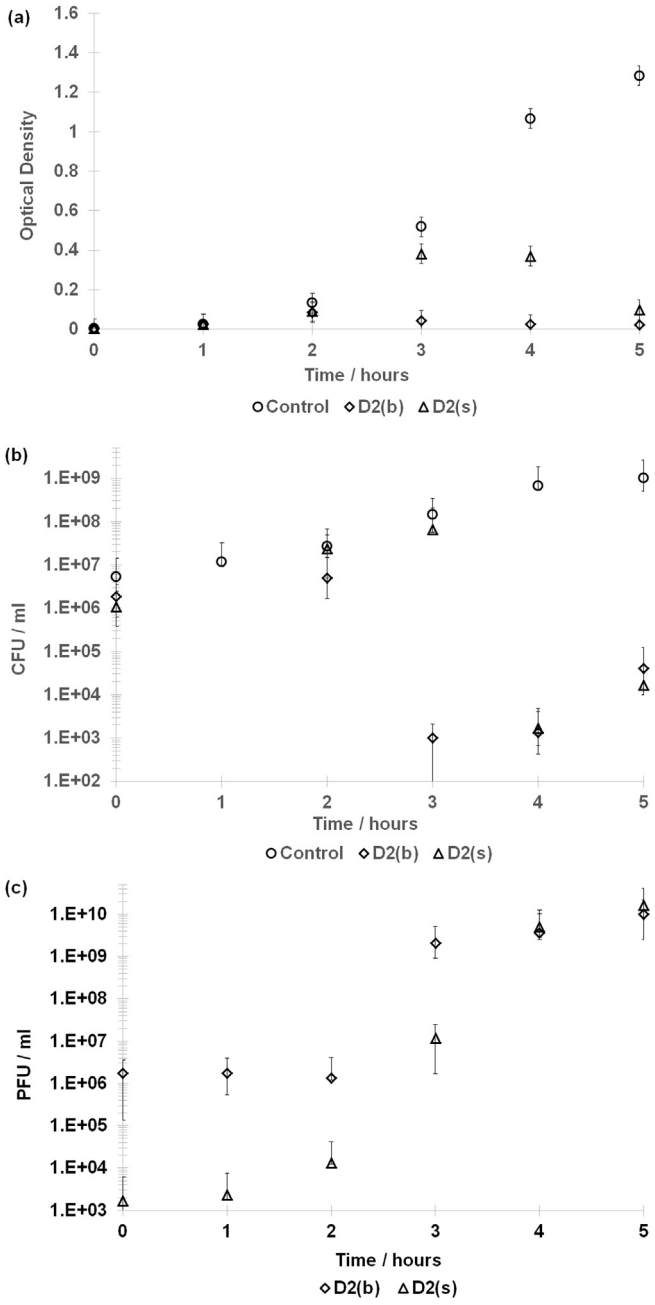

Fig. 5. Dynamics of phage killing of *S. enterica (serovar Typhimurium)* bacteria with microencapsulated Felix O1 bacteriophage. (a) Optical density curves showing bacterial growth without (controls) and with the addition of microparticles D2(s) and D2(b); (b) corresponding CFU data showing bacterial killing in the presence of added microencapsulated phages (D2(s) and D2(b)) pre-exposed to simulated gastric fluid (exposure period of 2 h at pH 1) prior to addition to *salmonella* bacterial cultures; (c) Phage titre amplification in *Salmonella* cultures following phage release from microencapsulated particles. Error bars are 95% confidence intervals (n = 3).

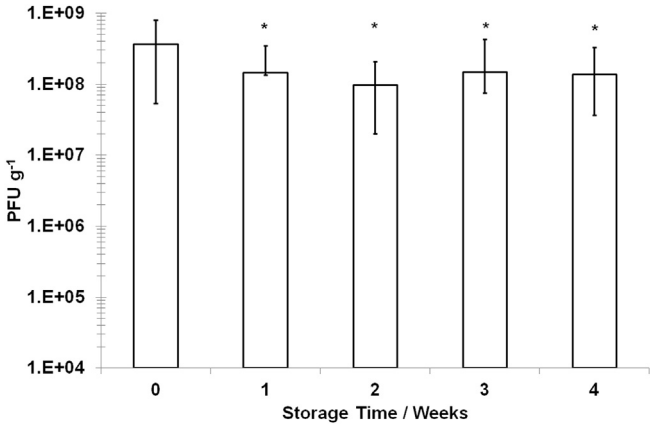

Fig. 6. Storage stability of Felix O1 phage encapsulated in microparticles prepared using formulation D2(b) (~100 μm beads) stored refrigerated at 4° C. Phage titre was evaluated by exposing microparticles to simulated intestinal fluid (pH 7). * indicates significance as compared with samples at day 0 (day of preparation) ($p < 0.05$) using a 2-sample t-test. Error bars are 95% confidence intervals (n = 3).

different acidic pH may be useful predictors of *in vivo* phage survival and a prerequisite during formulation development prior to testing in animal models [32]. *Salmonella* Felix O1 phages (belonging to the *Myoviridae* family) were shown here to be highly sensitive to acidic pH. Similar results have been reported previously for phage Felix O1, other *Salmonella* phages as well as *staphylococcus aureus* bacteriophage K (also a myovirus) and for *E. coli* specific phages [24,26,27,33,34]. This loss of phage activity highlights the need to protect phages from the harsh acidic environment of the stomach if controlled doses of phages are to be reliably delivered to treat *salmonellosis* in the infected gut. Phage Felix O1 titre was stable stored at 4 °C in formulations of dissolved ES100 10% (w/v) containing variable alginate amounts over the range 0.5% (w/v) – 2% (w/v). Microencapsulated Felix O1 formulated using 1% (w/v) alginate in large 100 μm microparticles was shown to survive 2 h exposure to SGF at pH values as low as pH 1 without loss of phage titre. Felix O1 microencapsulated in alginate beads has previously been shown to survive and amplify in the gastrointestinal tract of pigs [18]. *In vitro* results reported here suggest that high *Salmonella* phage titres could be delivered to the gastrointestinal tract, subsequently released there using a pH trigger and should remain viable thereafter. The pH of the colon can fall during bacterial infection to around or just below pH 7; in such a case, the released phage should remain viable and capable of lysing the infecting *Salmonella* bacteria present in the gut [35].

Alginate hydrogel pores tend to be in the 5–200 nm range depending on the degree of crosslinking [36]. The porosity of the alginate gel microparticles affects phage viability upon exposure to the acidic stomach environment. A number of previous studies have used alginate as the main encapsulating agent either on its own or in combination with whey protein or chitosan to improve acid stability [18,24–27,37–39]. Phages encapsulated in crosslinked pure alginate microparticles were found to be susceptible to acid damage following exposure to simulated gastric fluid [24,27,40,41]. We have shown that microparticle size and alginate composition were important in ensuring acid protection for encapsulated phage. The pH responsive character of Eudragit®S100 resulted in phages readily released upon exposure to pH 7. The combination of small microparticle size and pH responsive character of the microparticles resulted in rapid release of phage within the first 2 h upon exposure to SIF at pH 7. Previous studies on encapsulated phages in large microparticles (~1 mm) have reported slower sustained release kinetics for alginate encapsulated phages however, acid protection was reported to be poor [24,26]. Faster release from small alginate microparticles (mean size ~ 100 μm) containing $CaCO_3$ as antacid was recently reported [39]. However, exposure of these microparticles to simulated gastric fluid (pH 2.8 for 60 min) resulted in between 2 log and 3 log reduction in *Salmonella* phage titres suggesting that even with the addition of $CaCO_3$, phages were highly susceptible to SGF. Particle size was previously shown to be an important factor influencing phage protection from SGF for acid permeable beads [38]. The acid diffusion path length increases with particle size thereby affording

protection to phages located further away from the bead surface. We have shown that encapsulation of phages in composite Eudragit®/alginate microparticles with different mean sizes (50–100 μm) may permit control over the release rate. Small particles showed rapid burst release of phage cargo whilst larger beads showed an initial burst and then slower sustained release over 2 h. Hence control over the microparticle size may be used to manipulate the release rate of phages *in vivo*.

A number of *in vivo* animal studies have shown dose dependent phage therapy outcomes, with high doses of phages resulting in better clinical results [42–45]. Accurate control over the phage dose delivered at the site of infection and the timing of the delivery are important considerations [15,19,46]. Using *in vitro* experiments, we have shown that loss in phage titre upon acid exposure to SGF may result in a significant delay in phage amplification. This delay potentially could allow bacteria to attain much higher bacterial cell numbers in an *in vivo* setting with adverse treatment outcomes. Failure of a recently concluded phage therapy trial for the treatment of *E coli* diarrhoea in children was attributed to the lack of *in situ* phage amplification due to low host cell numbers which were nevertheless susceptible to phages in the phage cocktail administered [15]. Lack of phage formulation, no antacid administration and subsequent drop in phage titres due to phage exposure to stomach acidity may have been a factor in the consequent lack of *in situ* phage amplification [15]. These issues may be addressed through proper formulation development and microfluidic encapsulation of high phage titres for targeted delivery and controlled release of phages at the site of infection coupled with a better understanding of phage-bacterium population dynamics *in vivo* [13,47]. These aspects have heretofore received little attention in the published literature.

We have demonstrated here that even after exposure of phage encapsulated microparticles to simulated gastric fluid at pH 1 for 2 h, the released phage dose was unaffected and corresponded to ~1×10^8 PFU g^{-1}. The dose was sufficient to prevent rapid bacterial growth *in vitro* and resulted in rapid phage amplification from an initial titre of 1×10^6 PFU ml^{-1} to ~1×10^9 PFU ml^{-1} within 3 h of addition of microencapsulated phages. In small animal studies (e.g. mice) typical dose via oral gavage is around 100 μl which would allow dosing of around 0.1 g of microparticles containing around ~10^8 PFU or higher through phage stock concentration. Such a dose is indeed typical of many animal studies in literature [19,42,48–52]. Bacterial killing by phages is dependent on phage particles adsorbing to the target bacteria with killing rates affected by the *in situ* concentrations of both bacteria and phage [13,53,54]. It is known that in an *in vivo* situation, phage clearance mechanisms rapidly reduce the phage concentration in the absence of host bacteria [52]. We have shown here that a high phage dose is quickly able to arrest the rise of bacterial growth. Tanji et al. [55] showed similar results for *E. coli* using an *in vitro* chemostat experimental system. However, if low starting concentrations of bacteria are present, phage concentrations *in vivo* may decay significantly due to host clearance mechanisms and phages may be unable to amplify and achieve a sufficiently high concentration to eradicate the infecting bacteria until the bacteria have had time to grow bacterial numbers substantially [19]. If phages are administered prophylactically too early prior to infection or at the early onset of infection (when the bacteria concentration is low), clearance of phages by the host immune system or by other mechanisms (e.g. dilution or shortening of intestinal transit times during diarrhoea) may result in lowering of the *in situ* phage concentration resulting in poor phage therapy outcomes [19]. Under such conditions mucoadhesion of phages trapped in small microparticles and slow sustained phage release may be a good strategy. Encapsulation of phages and their slow controlled release may help in ensuring that the *in situ* phage concentration remains at a therapeutically effective level (over a realistic time period) allowing phages to amplify once the bacterial concentration increases to levels sufficient for *in situ* phage amplification [19]. The size of microparticles may play an important role in ensuring phage delivery is minimally affected by the conditions of the diseased state. Increased loss of fluid from the colon due to symptoms such as diarrhoea result in observed shortened mean transit times. Dilution of phages due to the high fluid environment may be a particular challenge. Larger particles (~mm size range) may be more prone to the influence of short transit times. Smaller particles predisposed to non-specific mucoadhesion which may aid in phage retention and sustained release over a significant time period [56,57]. A recent study showed better phage retention (for animals treated with alginate encapsulated phages versus those treated with free phages) in the caecum of chickens and a significant reduction in *Salmonella* colonisation [39]. This was attributed to the mucoadhesiveness of the small alginate microparticles (~100 μm) used for encapsulating phage.

The versatility of microfluidic encapsulation technologies readily allows screening and optimisation of formulations for phages. Microfluidic approaches may allow fabrication of complex microcapsules for co-encapsulation and simultaneous or sequential release of several different phages encapsulated in individually optimised formulations [19]. A high level of product innovation may be achieved through utilisation of a multitude of different trigger strategies including light, temperature, pH, enzymes etc [19]. Such strategies may allow phage biotechnology companies in the future to generate value and protectable intellectual property.

5. Conclusions

We have demonstrated the controlled production of highly uniform small composite microcapsules (using commercially available microfluidic droplet generation chips) with mean size 50–100 μm composed of Eudragit® S100 and with varying alginate amounts. Highly uniform microparticles were produced with low polydispersity enabling control over phage loading, acid stability upon exposure to SGF and their subsequent pH triggered release in SIF. Phage encapsulation and subsequent release kinetics revealed that microparticle size and alginate composition are factors affecting the pH stability and release profile of encapsulated phages. 100 μm beads prepared from 10% (w/v) ES100 and with 1% (w/v) alginate afforded phages excellent pH protection upon exposure to SGF (exposure for 2 h at pH 1) with no loss in phage titre. The microparticles released all their phage cargo within 2 h upon subsequent exposure to SIF making them suitable solid dosage forms for gastrointestinal delivery of phages. We have also demonstrated the storage stability of the encapsulated phages under refrigerated conditions over a 4 week storage period. Furthermore, we demonstrated the effect of acid stability and high phage dose delivery on the attenuation of *salmonella* bacterial growth upon release of encapsulated phages after exposure of the microbeads to SGF at pH 1. Future work will investigate the spatial and temporal delivery of encapsulated phages in a relevant small animal model and the suitability of these solid dosage forms to target *Salmonella* infections *in vivo*.

Conflict of interest

There is no conflict of interest.

Acknowledgements

The authors would like to acknowledge EPSRC support for this work (Grant no. EP/M027341/1) Tackling Antimicrobial Resistance: An Interdisciplinary Approach.

References

[1] Huttner A, Harbarth S, Carlet J, Cosgrove S, Goossens H, Holmes A. Antimicrobial Resistance : a Global View from the 2013 world healthcare-associated infections forum. 2013. p. 1–13.
[2] Merril CR, Scholl D, Adhya SL. The prospect for phage therapy in western medicine. Nat Rev Drug Discov 2003;2:489–97.
[3] The European Parliament and The Council of the European Union. Regulation (EC) No 1831/2003 of the European parliament and of the council of 22 September 2003 on Additives for Use in Animal Nutrition. 2003.
[4] Freire-moran L, Aronsson B, Manz C, Gyssens IC, So AD, Monnet DL, et al. Critical shortage of new antibiotics in development against multidrug-resistant bacteria — time to react is now. Drug Resist Updat 2011;14:118–24.
[5] Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, et al. Alternatives to antibiotics — a pipeline portfolio review. Lancet Infect Dis 2016;16:239–51.
[6] Alisky J, Iczkowski K, Rapoport A, Troitsky N. Bacteriophages show promise as antimicrobial agents. J Infect 1998;36:5–15.
[7] Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage 2011;1:66–85.
[8] Allen HK, Trachsel J, Looft T, Casey TA. Finding alternatives to antibiotics. Ann N Y Acad Sci 2014;1323:91–100.
[9] Abedon ST. Kinetics of phage-mediated biocontrol of bacteria. Foodborne Pathog Dis 2009;6:807–15.
[10] Barr JJ. A bacteriophages journey through the human body. Immunol Rev 2017;279:106–22.
[11] Barrow PA, Soothill JS. Bacteriophage therapy and prophylaxis : rediscovery and renewed assessment of potential. Trends Microbiol 1997;5:268–71.
[12] Galtier M, De Sordi L, Maura D, Arachchi H, Volant S, Dillies MA, et al. Bacteriophages to reduce gut carriage of antibiotic resistant uropathogens with low impact on microbiota composition. Environ Microbiol 2016;18:2237–45.
[13] Levin BR, Bull JJ. Population and evolutionary dynamics of phage therapy. Nat Rev Microbiol 2004;2:166–73.
[14] Viswanathan VK, Hodges K, Hecht G. Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea. Nat Rev Microbiol 2009;7:110–9.
[15] Sarker SA, Sultana S, Reuteler G, Moine D, Descombes P, Charton F, et al. Oral phage therapy of acute bacterial diarrhea with two coliphage preparations: a randomized trial in children from Bangladesh. EBioMedicine 2016;4:124–37.
[16] Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M, Roy SL, et al. Foodborne illness acquired in the United States — major pathogens. Emerg Infect Dis 2011;17:7–15.
[17] Hohmann EL. Nontyphoidal salmonellosis. Clin Infect Dis 2001;32:263–9.
[18] Wall SK, Zhang J, Rostagno MH, Ebner PD. Phage therapy to reduce preprocessing Salmonella infections in market-weight swine. Appl Environ Microbiol 2010;76:48–53.
[19] Malik DJ, Sokolov IJ, Vinner GK, Mancuso F, Cinquerrui S, Vladisavljevic GT, et al. Formulation, stabilisation and encapsulation of bacteriophage for phage therapy. Adv Colloid Interface Sci 2017;249:100–33.
[20] Young R, Gill JJ. Phage therapy redux — what is to be done? Science (80-) 2015;350:1163–4.
[21] Denou E, Bruttin A, Barretto C, Ngom-Bru C, Brüssow H, Zuber S. T4 phages against Escherichia coli diarrhea: potential and problems. Virology 2009;388:21–30.
[22] Ma Y, Islam GS, Wu Y, Sabour PM, Chambers JR, Wang Q, et al. Temporal distribution of encapsulated bacteriophages during passage through the chick gastrointestinal tract. Poultry Sci 2016;95:2911–20.
[23] Choinska-Pulit A, Mitula P, Sliwka P, Choi A, Wojciech Ł, Skaradzinska A. Bacteriophage encapsulation : trends and potential applications a. Trends Food Sci Technol 2015;45:212–21.
[24] Ma Y, Pacan JC, Wang Q, Xu Y, Huang X, Korenevsky A, et al. Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. Appl Environ Microbiol 2008;74:4799–805.
[25] Tang Z, Huang X, Baxi S, Chambers JR, Sabour PM, Wang Q. Whey protein improves survival and release characteristics of bacteriophage Felix O1 encapsulated in alginate microspheres. Food Res Int 2013;52:460–6.
[26] Kim S, Jo A, Ahn J. Application of chitosan-alginate microspheres for the sustained release of bacteriophage in simulated gastrointestinal conditions. Int J Food Sci Technol 2015;50:913–8.
[27] Ma Y, Pacan JC, Wang Q, Sabour PM, Huang X, Xu Y. Enhanced alginate microspheres as means of oral delivery of bacteriophage for reducing Staphylococcus aureus intestinal carriage. Food Hydrocolloids 2012;26:434–40.
[28] Shi LE, Zheng W, Zhang Y, Tang ZX. Milk-alginate microspheres: protection and delivery of Enterococcus faecalis HZNU P2. LWT – Food Sci Technol 2016;65:840–4.
[29] Puapermpoonsiri U, Spencer J, Walle CF, Van Der. A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. Eur J Pharm Biopharm 2009;72:26–33.
[30] Vinner GK, Vladisavljevi GT, Clokie MRJ, Malik DJ. Microencapsulation of Clostridium difficile specific bacteriophages using microfluidic glass capillary devices for colon delivery using pH triggered release. PLoS One 2017;12:1–27.
[31] de Siqueira RS, Dodd CER, Rees CED. Evaluation of the natural virucidal activity of teas for use in the phage amplification assay. Int J Food Microbiol 2006;111:259–62.
[32] Watanabe R, Matsumoto T, Sano G, Ishii Y, Tateda K, Sumiyama Y, et al. Efficacy of bacteriophage therapy against gut-derived sepsis caused by Pseudomonas aeruginosa in mice. Antimicrob Agents Chemother 2007;51:446–52.
[33] Albino LAA, Rostagno MH, Hungaro HM, Mendonca RCS. Isolation, characterization and application of bacteriophages for Salmonella spp. biocontrol in pigs. Foodborne Pathog Dis 2014;11:602–9.
[34] Saez AC, Zhang J, Rostagno MH, Ebner PD. Direct feeding of microencapsulated bacteriophages to reduce salmonella colonization in pigs. Foodborne Pathog Dis 2011;8:1269–74.
[35] McConnell EL, Fadda HM, Basit AW. Gut instincts: explorations in intestinal physiology and drug delivery. Int J Pharm 2008;364:213–26.
[36] George M, Abraham TE. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan - a review. J Contr Release 2006;114:1–14.
[37] Samtlebe M, Ergin F, Wagner N, Neve H, Küçükçetin A, Franz CMAP, et al. Carrier systems for bacteriophages to supplement food systems : encapsulation and controlled release to modulate the human gut microbiota. LWT – Food Sci Technol 2016;68:334–40.
[38] Tang Z, Huang X, Sabour PM, Chambers JR, Wang Q. Preparation and characterization of dry powder bacteriophage K for intestinal delivery through oral administration. LWT – Food Sci Technol 2015;60:263–70.
[39] Colom J, Cano-Sarabia M, Otero J, Aríñez-Soriano J, Cortés P, Maspoch D, et al. Microencapsulation with alginate/CaCO3: a strategy for improved phage therapy. Sci Rep 2017;7:41441.
[40] Dini C, Islan GA, de Urraza PJ, Castro GR. Novel biopolymer matrices for microencapsulation of Phages : enhanced protection against acidity and protease activity. Macromol Biosci 2012;12:1200–8.
[41] Soto MJ, Retamales J, Palza H, Bastias R. Encapsulation of specific Salmonella Enteritidis phage f3αSE on alginate-spheres as a method for protection and dosification. Electron J Biotechnol 2018;31:57–60.
[42] Smith HW, Huggins MB, Shaw KM. The control of experimental Escherichia coli diarrhoea in calves by means of bacteriophages. J Gen Microbiol 1987;133:1111–26.
[43] Wills QF, Kerrigan C, Soothill JS. Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model. Antimicrob Agents Chemother 2005;49:1220–1.
[44] Biswas B, Adhya S, Washart P, Paul B, Trostel AN, Powell B, et al. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. Infect Immun 2002;70:204–10.
[45] Cerveny KE, DePaola A, Duckworth DH, Gulig PA. Phage therapy of local and systemic disease caused by Vibrio vulnificus in iron-dextran-treated mice. Infect Immun 2002;70:6251–62.
[46] Atterbury RJ, Van Bergen MAP, Ortiz F, Lovell MA, Harris JA, De Boer A, et al. Bacteriophage therapy to reduce Salmonella colonization of broiler chickens. Appl Environ Microbiol 2007;73:4543–9.
[47] Leung CY (Joey), Weitz JS. Modelling the synergistic elimination of bacteria by phage and the innate immune system. J Theor Biol 2017;429:241–52.
[48] Nale JY, Spencer J, Hargreaves KR, Buckley AM, Trzepin P, Douce GR, et al. Bacteriophage combinations significantly reduce Clostridium difficile growth in vitro and proliferation in vivo. Antimicrob Agents Chemother 2016;60:968–81.
[49] Barrow P, Lovell M. Use of lytic bacteriophage for control of experimental Escherichia coli septicemia and meningitis in chickens and calves. Clin Diagn Lab Immunol 1998;5:294–8.
[50] Chibani-chennoufi S, Sidoti J, Bruttin A, Kutter E, Sarker S, Bru H. In vitro and in vivo bacteriolytic activities of Escherichia coli Phages : implications for phage therapy. Antimicrob Agents Chemother 2004;48:2558–69.
[51] Abdulamir AS, Jassim SAA, Abu Bakar F. Novel approach of using a cocktail of designed bacteriophages against gut pathogenic E. coli for bacterial load biocontrol. Ann Clin Microbiol Antimicrob 2014;13:1–11.
[52] Yen M, Cairns LS, Camilli A, Zuckerman JN, Rombo L, Fisch A, et al. A cocktail of three virulent bacteriophages prevents Vibrio cholerae infection in animal models. Nat Commun 2017;8:14187.
[53] Cairns BJ, Timms AR, Jansen VAA, Connerton IF, Payne RJH. Quantitative models of in vitro bacteriophage-host dynamics and their application to phage therapy. PLoS Pathog 2009;5:1–10.
[54] Payne RJH, Jansen V. Understanding bacteriophage therapy as a density-dependent kinetic process. J Theor Biol 2001;208:37–48.
[55] Tanji Y, Shimada T, Fukudomi H, Miyanaga K, Nakai Y, Unno H. Therapeutic use of phage cocktail for controlling Escherichia coli O157:H7 in gastrointestinal tract of mice. J Biosci Bioeng 2005;100:280–7.
[56] Alhnan MA, Cosi D, Murdan S, Basit AW. Inhibiting the gastric burst release of drugs from enteric microparticles: the influence of drug molecular mass and solubility. J Pharm Sci 2010;99:4215–27.
[57] Kietzmann D, Moulari B, Béduneau A, Pellequer Y, Lamprecht A. Colonic delivery of carboxyfluorescein by pH-sensitive microspheres in experimental colitis. Eur J Pharm Biopharm 2010;76:290–5.