Enhancement of the Antimicrobial Properties of Bacteriophage-K via Stabilisation in Oil-in-Water Nano-emulsions

Short title: Bacteriophage K in o/w Nano-emulsions

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ABSTRACT

Bacteriophage therapy is a promising new treatment that may help overcome the threat posed by antibiotic-resistant pathogenic bacteria, which are increasingly identified in hospitalised patients. The development of biocompatible and sustainable vehicles for incorporation of viable bacterial viruses into a wound dressing is a promising alternative. This paper evaluates the anti-microbial efficacy of Bacteriophage K against *Staphylococcus aureus* over time, when stabilised and delivered via an oil-in-water nano-emulsion. Nano-emulsions were formulated via thermal phase inversion emulsification, and then bacterial growth was challenged with either native emulsion, or emulsion combined with Bacteriophage K. Bacteriophage infectivity, and the influence of storage time of the preparation, were assessed by turbidity measurements of bacterial samples. Newly prepared Bacteriophage K / nano-emulsion formulations have greater anti-microbial activity than freely suspended bacteriophage. The phage loaded emulsions caused rapid and complete bacterial death of three different strains of *Staphylococcus aureus*. The same effect was observed for preparations that were either stored at room temperature (18 - 20°C), or chilled at 4°C, for up to 10 days of storage. A response surface design of experiments was used to gain insight on the relative effects of the emulsion formulation on bacterial growth and phage lytic activity. More diluted emulsions had a less significant effect on bacterial growth, and diluted bacteriophage-emulsion preparations yielded greater antibacterial activity. The enhancement of bacteriophage activity when delivered via nano-emulsions has yet to be reported. This prompts further investigation into the use of these formulations for the development of novel anti-microbial wound management strategies.

Keywords: *S. aureus* infections, Phage therapy, oil-in-water nano-emulsion, burn wound related infections, responsive wound dressings.

INTRODUCTION

The rise in antibiotic resistant bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) is widely reported[1], but the rate of development of new antibiotics to treat these emerging 'superbugs' is low[2]. Only Linezolid has been approved for the treatment of acute skin infections since 2000[3], although Tedizolid is currently being developed[4]. Skin is the primary defence mechanism against infection[5]; hence injuries or burns constitute significant pathways for bacterial infection. Burns are especially susceptible to bacterial colonization with an estimated 10% of all burn injuries becoming infected[6]. Infection consequences include increased patient morbidity and mortality, and increased cost of treatment[7,8] due to prolonged hospitalisation. *Staphylococcus aureus* (MRSA) is the most frequently detected antibiotic-resistant pathogen in hospitals worldwide[9,10]. The lack of effective new antibiotics has led to interest in alternative therapies[11], including antimicrobial peptides[12], microbial therapy, and the use of viruses that kill bacteria[13,14]. Such bacterial viruses, called bacteriophages or phages, attach to bacterial cell surfaces, use them as a host for their own replication, and eventually produce bacterial lysis. Bacteriophages therefore have potential for control of microbial infections[15].

Therapeutic use of bacteriophages dates from the 1930s and they are now being reconsidered as alternatives to antibiotics. The advantages of bacteriophage therapy include their capacity to infect bacterial cells, their abundance and ecological 'friendliness'. They can be used as a 'phage-cocktail', they multiply exponentially, and they do not affect human microflora and hence do not generate unwanted side-effects[16]. However, their broad diversity presents a challenge to our detailed understanding of their potential[17]. Bacteriophages have been used against skin and wound infections, with success rates up to 90% against *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*[14]. Phage cocktails have been shown to increase survival significantly in mice with burn wound injuries[18], and used in ear

infections of dogs[19]. Once a safe phage or 'phage-cocktail' has been identified, the aim is to deliver it to the point of infection without losing efficacy, either during delivery or storage.

Surface burns are normally treated via cleaning to remove dead tissue, and covering the burn in a dressing to promote re-epithelisation and reduce or prevent infection. Various anti-microbial wound dressings are available, including Acticote^{TM}, a silver-containing dressing. However, recent analysis of anti-microbial wound dressings has queried their effectiveness compared to passive alternatives[20].

Nano-emulsions provide enhanced biocompatibility compared to emulsions as they require less surfactant, which may affect the skin at high concentration[21]. Additionally the small droplet size allows greater penetration and distribution through the skin compared to emulsions[22,23]. A benefit of nano-emulsions as carriers of bacteriophage is prevention of aggregation[24] which can result in precipitation and loss of biological activity. Nano-emulsions can also provide stability in long-term phage storage without a significant decrease in infectivity[25]. Oil-in-water emulsions enhance transdermal penetration, and depending on the composition and the use of thickeners, numerous formulations can be achieved. However screening the wide variety of potential emulsion formulations is a challenge when looking for biocompatibility so careful design of experiments is essential. The efficacy of encapsulation for delivery and storage of bacteriophage has been demonstrated[26,27]. Nevertheless, phage infectivity and survival may be influenced by formulation composition[28–35], which can damage phage structural components. Phage may be exposed to abnormal environmental conditions such as the emulsion components themselves. Furthermore, the compounds present in the nano-emulsion may influence bacterial growth itself, and thus mask the specific effect of the phage.

This work evaluates the *in vitro* anti-microbial efficacy of Bacteriophage K when stabilised in an oil-in-water nano-emulsion, compared to simple delivery as an aqueous dispersion.

Bacteriophage K is lytic for a wide range of *S. aureus* strains as well as other staphylococci – three strains are selected here. The emulsion / phage formulation will form the basis for development of a wound dressing or topical cream, containing stabilised phage.

MATERIALS AND METHODS

Chemicals

Brij® O10 (Polyoxyethylene (10) oleyl ether), soybean oil, Tryptic soy broth (TSB), tryptic soy agar (TSA), NaCl, $MgSO_4 \cdot 7H_2O$, Tris-Cl, and gelatine were purchased from Sigma-Aldrich (Dorset, UK). Reverse Osmosis water was produced in the laboratory.

Bacterial and bacteriophage strains

Staphylococcus aureus strains H560, H325, and Btn766 (Bacteriophage K sensitive strains), and Bacteriophage K, were obtained from AmpliPhi Biosciences (Bedfordshire, UK).

Emulsification method

Thermal phase inversion emulsification was used[36,37] to produce formulations containing 5% (w/w) soybean oil as the organic phase, 15% (w/w) Brij O10® as surfactant, and 80% (w/w) SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-Cl, 0.002% (w/v) gelatin, pH 7.5) as the aqueous phase. The relatively high concentration of emulsifier allowed the more pronounced effects on bacterial growth and bacteriophage infectivity to be detected during a shorter storage time. The presence of SM buffer as the aqueous phase resulted in smaller emulsion droplets (via the salting out effect) and stabilised pH to an appropriate value for bacteriophage storage. The emulsion components were initially sterilised by filtration, and formulation took place under aseptic conditions. Brij O10® was added to the corresponding amount of SM buffer under mixing, and the required amount of soybean oil was added. Stirring continued for at least one hour until a homogenous mixture was achieved (complete dissolution of emulsifier). The coarse emulsion was transferred into a round-bottom flask with a condenser and heated with a hot water bath until the Phase Inversion Temperature

(PIT, approximately 80°C) was reached, as indicated by the appearance of a clear or bluish colour. Finally the emulsion was cooled rapidly by immersion in an ice bath, while stirring, to avoid coalescence.

Bacteriophage propagation and purification

S. aureus H560 was used as a host for the propagation of Bacteriophage K. It was grown in 10 ml TSB overnight with orbital agitation. 100 µl of actively growing culture was mixed with 40 µl of Bacteriophage K stock solution, i.e. suspended in SM buffer, and incubated for 5 minutes at room temperature to allow bacteriophages to adsorb. 3 ml of top tryptic soy agar (TSB containing 1.5 % (w/w) of bacteriological agar) was added to the bacteria-bacteriophage mixture and poured onto a TSA plate, which was incubated overnight. 3 ml of SM buffer were added to the resulting confluent lysis plate. This plate was incubated with gentle shaking for approximately 4 hours at room temperature (18-20°C). The phage-containing liquid was removed, centrifuged and passed through a 0.22 µm filter to eliminate possible bacterial debris. The titre of the phage lysate was determined by plaque assay.

Incorporation of bacteriophage into emulsions

The introduction of Bacteriophage K into the emulsion required dilution of the phage lysate to a concentration that lead to a noticeable decrease in bacterial growth, as indicated by a reduction in optical absorbance. Dilutions were made using emulsion as both diluent and storage media. The final concentration of bacteriophage used for infectivity tests corresponded to a multiplicity of infection (MOI) of about 0.1. Bacteriophage-emulsion preparations were kept either at room temperature (18-20°C) or cold temperature (4°C) over ten days and their ability to infect bacteria was tested regularly.

Plaque assays and colony counting

Standard colony counting was used to quantify bacterial concentration (colony forming units, CFU ml^{-1}) and standard plaque assays were used to measure bacteriophage concentration

(plaque forming units, PFU ml^{-1}) using overnight cultures of the tested strain with an initial optical density (O.D) of 1 – 1.5 absorbance units (a.u) at 600 nm, or about 10^8 CFU ml^{-1}. Plaque assays could only be used for the quantification of bacteriophage infectivity in buffer, as the effect of the emulsion on the growth of the bacterial lawn was not measurable via this technique. Therefore, a fair comparison between free phage suspension and phage-emulsion formulations was not possible via standard PFU counting.

Measurement of turbidity of bacterial samples

Visual PFU counting may lead to errors and lack of reproducibility, hence optical density measurement was used as an alternative, as the turbidity of bacteria cultures in TSB should be reduced by the lytic action of bacteriophage. Variation of optical density with time was measured in triplicate via 96 multi-well plates, using a micro-plate reader (SPECTROstar Omega, BMG LABTECH). Measurements took place overnight for approximately 15 hours at 37°C and 600 nm, as a simulation of bacterial growth and phage infection in the incubator.

Measurement of infectivity of bacteriophage preparations

Bacteriophage infectivity was followed by the decrease in optical density of bacterial suspensions. Bacterial growth was challenged with either aqueous suspensions of bacteriophage in SM buffer, or bacteriophage-emulsion preparation, all in triplicate. The initial concentration of bacteriophage was the same in each case and equivalent to a MOI of 0.1. The initial bacterial concentration was equivalent to 1 a.u., corresponding to about 10^8 CFU ml^{-1}. As samples comprised 100 μl of the appropriately diluted overnight bacterial culture, and 100 μl of the corresponding bacteriophage formulation, the MOI was preserved. The optical density of samples was measured at 600 nm for approximately 15 hours at 37°C. The bacteriophage-emulsion formulation was stored for a 10 day period, and infectivity was measured over time by taking samples and running growth curves and measuring the final bacterial concentration after the 15 hour treatment for each sample.

Characterisation of emulsions and stability

Droplet size can determine the stability of emulsions as it influences the possibility of coalescence or creaming. This was measured using a ZETASIZER Nano Series (Malvern Instruments), based on the principle of dynamic light scattering (DLS) and Brownian motion of particles. Samples needed to be suitably diluted in water or buffer before being subjected to measurement. The stability of emulsions was also tracked via measurement of optical density over time[38] using the micro-plate reader.

Surface response experimental design to evaluate the influence of emulsion formulation on bacterial growth and bacteriophage infectivity

A mixture containing emulsion droplets, bacteriophage, and bacteria could result in complex interactions between any pair of components, or all three. To understand the relative influence of these three components, experiments were designed using Response Surface Methodology (RSM). The main objective was to evaluate the effect of emulsion droplet concentration on bacterial growth under different growth conditions, and to detect variations in bacteriophage infectivity when emulsion was present. RSM was selected as the relationship between these variables was initially unknown. Experiments were designed using Minitab16® (Minitab Statistical Software), and experiments were randomised to meet the statistical assumptions and reduce the effects of other factors. Initial concentration of bacteria and the bacteria : emulsion droplet ratio were the factors used to study bacterial growth in emulsion for each of the bacterial strains and the different levels selected for each factor are presented in Table 1. Growth rate and carrying capacity were the response variables characterised in relation to bacterial growth. Values for these parameters were estimated from the Verhulst (logistic) model of population growth (Equation 1).

$$\frac{dN(t)}{dt} = aN(t)\left(1 - \frac{N(t)}{C}\right) \qquad [1]$$

where $N(t)$ is the concentration of bacteria (CFU ml^{-1}), t is time (h), a is the growth rate of the

bacterial population (h^{-1}), and *C* is the carrying capacity or maximum population size (CFU ml^{-1}). The logistic growth curve was preferred as it gives a reasonable fit despite its simplicity[39]. Bacterial growth was measured by turbidity assays (600 nm) at 37°C with orbital agitation for 20 hours, under the conditions specified by the experimental design, using six replicates. Optical density growth curves were used to estimate the parameters in the integrated form of the Vershulst model (Equation 2) using Origin 8 software (OriginLab, Northampton, MA)[40].

$$OD(t) = \frac{C}{1 + Be^{-at}} \qquad [2]$$

Here *OD(t)* is optical density of bacteria at time t (a.u.) – equivalent to *N(t)* in Equation 1, *t* is time (h), *a* is the growth rate of the bacterial population (h^{-1}), *B* is an integration constant, and *C* is the carrying capacity or maximum population size (a.u.) as before. A non-linear least squares error minimisation algorithm was used to estimate the parameter values.

Three parameters were considered for the factorial study of bacteriophage infectivity: the initial concentration of bacteria, the bacteria : emulsion droplet ratio, and the initial concentration of bacteriophage for each of the bacterial strains. The factor levels are listed in Table 1. The response variable was the final concentration of bacteria after contact with the bacteriophage-emulsion preparation (20 hours).

RESULTS AND DISCUSSION

Characterisation of emulsions

Nano-emulsions prepared using the PIT method were transparent, with a droplet size of about 20 nm, as previously reported for this formulation (5% (w/w) Soybean oil, 15% (w/w) Brij O10® surfactant, 80% (w/w) SM buffer)[41]. Nano-emulsions remained clear and transparent to the naked eye during the 10 day testing period, and optical density was also constant. No clouding, phase separation or coalescence was observed when stored at room or cold

temperature. Nano-emulsion stability is not only caused by the small droplet size, but also by the narrow droplet size distribution[36].

Influence of emulsion on bacterial growth

The measurements of bacterial OD were all performed in triplicate, with average values being plotted in Figures 1 to 3. One in five of the error bars (representing standard deviation of the replicates) are presented for clarity. Nano-emulsion and TSB are virtually transparent at 600 nm; however, their baseline OD was subtracted from the raw data and appropriate propagation of errors was performed.

S aureus H560 (Figure 1) grew at an initial high rate in both TSB and in 1: 10 diluted nano-emulsion. When *S aureus* H560 was grown in more concentrated emulsion (1:1 dilution) the growth rate is slower, with a lag period in the first two or three hours. No noticeable difference in final bacterial concentration was observed for *S. aureus* H560 growing in TSB and 1:10 diluted nano-emulsion, but there was a decrease of about 25% in the final OD at the stationary phase when *S. aureus* H560 was grown in emulsion diluted 1:1. Figure 2 shows that growth of *S. aureus* H325 in both 1:1 and 1:10 diluted emulsion results in similar growth rates and final bacterial concentration. The OD in the stationary phase was reduced by 46% when emulsion was present, if compared with normal growth in TSB. Figure 3 shows that *S. aureus* Btn766 growth differed from H560 and H325 mainly in the 1:1 diluted emulsion, with lag period of almost 5 hours, a concentration peak at about 9 hours, and a slight decline before the stationary phase. When Btn766 was grown in 1:1 or 1:10 emulsion dilutions the final bacterial concentration was about the same, at almost 33% less than in TSB.

To summarise, strains H560, H325, and Btn766 experienced the slowest growth when they were grown in 1:1 diluted emulsion. The maximum concentration of bacteria achieved was also affected, mainly being reduced for strains H325 and Btn766 growing in 1:10 and 1:1 nano-emulsion dilutions (Figures 2 and 3). The slight decrease in bacterial concentration at

the end of the measurement period was attributed to the gravitational deposition of bacteria on the wells. The growth was generally slower in the presence of emulsion, but there was no apparent overall growth inhibition or antimicrobial effect, which is supported by the literature[42–44]. The distinctive growth patterns for the three bacterial strains suggest that variations in growth might be related to the specific metabolism of each strain. Bacteria tend to grow in the aqueous phase of food-related emulsion formulations, and the presence and concentration of emulsion droplets constitutes an important factor that can influence bacterial growth[45,46]. The observed decelerating effect of emulsions on bacterial growth is supported by published work, where *E. coli* is shown to grow slower when higher concentrations of emulsion were present in the growth medium[47]. These authors summarise possible explanations for this phenomenon as being impeded diffusion of nutrients through the organic phase, spatial limitations between bacteria and emulsion droplets, and facilitated accumulation of waste products, leading to growth inhibition. Interactions between emulsion droplets and bacteria have been shown to take place mainly due to electrostatic interactions[48]. This paper shows that emulsions formulated with non-ionic surfactants did not show changes in droplet size, and concludes that interactions bacteria and emulsion droplets did not affect emulsion stability. In our work, a non-ionic surfactant was used and the emulsion droplet size was in the range of nanometres. This explains the lack of variation in emulsion transparency when bacteria were present. However, it is clear that appropriate controls are required when bacteriophage and bacteriophage-emulsion formulations are tested against *S. aureus*, if they are to be effectively compared. When bacteriophage infectivity was tested, bacterial growth in TSB was selected as the control, whilst bacterial growth in emulsion was chosen as the control for bacteriophage-emulsion formulation testing. In this way the killing effects of phage, measured via reduction in bacterial OD, may be effectively compared. The influence of nano-emulsions on bacterial growth also prevented the use of standard plaque forming unit

assays for the quantification of bacteriophage infectivity. This restricted the CFU and PFU counting methods to the determination of initial concentrations of bacteria and bacteriophage.

Efficacy of bacteriophage preparation

Measurements were performed in triplicate, and one in five of the error bars, representing the standard deviation of triplicates, for the data are shown for clarity. When *S. aureus* H560, H325 and Btn766 were challenged with bacteriophage-emulsion preparations different responses were observed. Figures 4 to 6 show that for all strains, bacteriophage stored in SM buffer had no significant killing effect on bacteria after nine days of storage. The growth curves of *S. aureus* in TSB (●) and in the presence of bacteriophage (◇) show almost identical shapes, and they reach the same final concentration after 15 hours. For *S. aureus* H560 and Btn766 (Figures 4 and 6), bacterial concentration was dramatically reduced after the first five hours of treatment with bacteriophage-emulsion formulation (+), when compared with bacterial growth in emulsion (▼). From hour 7 the concentration is close to zero, indicating almost complete lysis of bacterial cells. In Figure 5, *S. aureus* H325 continues growing to about 8 hours, but the subsequent decrease in concentration shows a significant killing effect by 15 hours. Enhanced lytic activity of bacteriophage has been demonstrated when stored in nano-emulsions for nine days. No similar effect has been reported in the literature, except for mention of a higher bacteriophage titre achieved within an emulsion[49]. Bacteriophages may be protected against inactivation due to electrostatic interactions between bacteriophage surface proteins and nano-emulsion droplets[50]. This shielding mechanism, which could preserve lytic activity, and combined with more favourable contact between bacteriophage and bacteria in the presence of emulsion, could result in an enhanced killing effect. The literature suggests that certain surfactants (e.g. emulsan) do not interfere with phage binding to bacterial surfaces; moreover the binding occurs at the emulsion interface[51].

Surface response experimental design to evaluate the influence of emulsions on bacterial growth and bacteriophage infectivity

Enhancement of infectivity of Bacteriophage K against *S. aureus* by nano-emulsion has been demonstrated (Figures 4 – 6), but the mechanisms of enhancement are not specifically understood. Factorial experimental design followed by RSM analysis was used to identify the most influential variables in the system on bacterial growth and bacteriophage infectivity.

Normal probability plots show a normal distribution of the residuals between RSM predictions and experimental data for the three responses: bacterial growth rate, bacterial carrying capacity, and final concentration of bacteria in the presence of bacteriophage-emulsion formulations. The residuals align to a straight line with $R^2 = 0.99$. The random distribution of the residuals demonstrates agreement with the desired normal distribution, although some outliers were found. This statistical analysis gives an idea of the effectiveness of RSM for the evaluation of the responses as a function of the input factors (Table 1).

Bacterial growth may be influenced by attachment of emulsion droplets to the outer cell membrane, causing a switch in metabolism, for instance inducing anaerobic behaviour due to the lack of available oxygen, or depriving bacteria of nutrients present in the medium. Emulsion droplets may also contribute to more effective bacteria-bacteriophage interaction, creating a framework that reduces adsorption distances. These effects may combine to influence both bacterial growth and phage-bacterial binding mechanisms. The significance of the experimental factors was determined by the Fisher test, which compares the p-values from the statistical software with a fixed value, α, at the desired confidence level. In this case 95% confidence was selected ($\alpha = 0.05$), and the influence of any factor was considered significant if its corresponding p-value was < 0.05.

The most influential factors on bacterial growth rate were the initial concentration of bacteria (p-value=0.000) and the dilution factor of emulsion (p-value=0.000). The physical meaning

of these is understandable, and the relationships are confirmed in Figure 7.

For bacterial carrying capacity, the most influential factors were emulsion concentration (p-value=0.000), and the interaction between the initial concentration of bacteria and the emulsion concentration, i.e. bacteria : emulsion droplet ratio (p=0.001). A different growth pattern was observed for H560, H325 and Btn766 bacteria - see Figures 1, 2 and 3. Carrying capacity was not influenced by initial concentration (p-value=0.057), as time was sufficient for consumption of all nutrient resources - Figures 1 to 3 indicate the stationary phase.

When final bacterial concentration was tested against varying bacteriophage-emulsion preparations, the most important factor was initial bacterial concentration (p-value=0000). The interaction of these factors is shown in Figures 4, 5 and 6 via different patterns of growth. Interaction between initial bacteria concentration and emulsion dilution factor was also significant (p-value=0.001), suggesting that bacterial growth is inhibited by emulsion.

The results for the growth rate and carrying capacity response variables are shown in Figure 7. The R^2 values are closer to 1 for strains H560 and H325 than for Btn766. This means that Btn766 growth pattern differs from the logistic curve, see Figure 3. Maximum bacterial growth rates for strain H560 in Figure 7A are observed for lower concentrations of emulsions and for smaller initial concentrations of bacteria. Competition for resources is less acute when a reduced number of cells coexist during their initial growth phase. The maximum growth rate for H560 was 1.46 – 1.73 h^{-1} in 1:10 diluted emulsion. The most effective emulsion concentration (dilution factor of 1:10) was the same for all strains, see Figures 7C and 7E for strains H325 and Btn766. For the more concentrated emulsion (1:1 dilution) the large number of droplets could inhibit nutrient uptake by the bacteria. However, the optimum growth rate varies from strain to strain depending on the initial bacterial concentration. For all initial concentrations of bacteria, growth in more dilute emulsions yielded higher growth rates. The dilution factor of emulsions had a slight effect on bacterial

carrying capacity, supporting a greater capacity for the most diluted emulsion. In Figure 7B, 7D and 7F, H560 achieved the maximum number of bacteria with the higher dilution factor (1:100) leading to a higher carrying capacity. A less obvious effect is seen with Btn766.

To summarise, emulsion formulations with least effect on bacterial growth were those that were diluted, supporting the hypothesis that droplets might inhibit the uptake of oxygen or nutrients by bacterial cells. If the killing effect of bacteriophage in dilute emulsions is high compared to the enhanced lytic activity seen with more concentrated emulsion (1:1 dilution), then dilute formulations would be selected as they affect bacterial growth less.

Figure 8 shows the minimum final bacterial concentration (OD) values, corresponding to a maximum killing effect. For H560 (0.5 a.u.), Figure 8A shows minima at a bacteriophage concentration of about 1×10^{-3} PFU ml^{-1} for 1:100 diluted emulsion, and about 1×10^{-4} PFU ml^{-1} for 1:10 diluted emulsion and undiluted emulsion. For H325 (0.5 a.u.), Figure 8D shows a minimal final bacterial concentration at a bacteriophage concentration of 1×10^{-3} PFU ml^{-1} for all dilution factors of emulsion. For Btn766 (0.5 a.u.), Figure 8G, the minima were found at the most diluted bacteriophage preparation in undiluted emulsion. For all diluted emulsions a concentration of 1×10^{-5} PFU ml^{-1} seems optimal. Similar patterns were observed for all initial concentrations of the three strains, although the figures are not shown.

Figures 8B, 8E and 8H show similar patterns for the initial concentration of bacteriophage for H560, H325 and Btn766 respectively. For all initial concentrations of bacteria, highest initial concentrations of bacteriophage (1×10^{-3} PFU ml^{-1}) seem to have the highest killing effect, except for Btn766 (Figure 8H) where the optimum can be seen between 1×10^{-4} and 1×10^{-5} PFU ml^{-1}. The same patterns were observed for all dilution factors of emulsion, although this data is not shown. For smaller initial concentrations of bacteria, in Figures 8D and 8E, there is a regrowth effect indicates a threshold below which active therapy of bacteriophage is not possible [52–54].

Figures 8C and 8I show that for an initial bacteriophage concentration of 1×10^{-4} PFU ml^{-1}, diluted emulsions give greater killing effect for medium to high initial concentrations of H560, and medium initial concentrations of Btn766. The killing effect of H325 at medium to high initial concentrations of bacteria is not affected by the emulsion dilution factor (Figure 8F). The highest and medium initial concentrations of bacteria gave the lowest final bacteria concentration, so more concentrated emulsions generally give the highest killing effect.

Shelf-life of bacteriophage – emulsion preparations

A relative killing effect was defined, Equation 3, to compare the lytic activity of bacteriophage in buffer suspension with bacteriophage-emulsion formulations.

$$Relative\ killing\ effect = \frac{OD_{20h}(control) - OD_{20h}(within\ phage\ preparation)}{OD_{20h}(control)} \qquad [3]$$

Optical density (OD) is equivalent to bacterial concentration. Hence, the relative killing effect is the difference in OD between normal bacterial growth (control) and bacterial growth in the presence of bacteriophage, normalised to 20 hours of treatment. The controls were different in the normal growth medium and in the emulsion environment. The relative killing effect will have values between 0 and 1, where 0 corresponds to no killing at all (for bacteria in the presence of bacteriophage or bacteriophage-emulsion preparation with or without bacteriophage), and a value of 1 means a total kill, irrespective of the treatment.

Bacteriophage-emulsion formulations show enhanced antibacterial activity (relative killing effects close to 1) against the three strains of *S. aureus* when stored at room and cold temperatures. It has been shown that emulsion influenced bacterial growth, but this effect is eliminated by the use of bacterial growth in undiluted emulsion as an appropriate control. Figures 9A, B and C show no significant variations of bacteriophage-emulsion activity over a 10 day period, while bacteriophage lytic activity shows oscillations, giving different results for every day of treatment, and for each temperature of storage.

CONCLUSIONS

A novel approach for the efficient storage and delivery of Bacteriophage K for the treatment of *Staphylococcus aureus* infections is demonstrated. The nano-emulsion-bacteriophage preparations show enhanced antimicrobial activity, with reduced fluctuations in infectivity over time when compared to simple aqueous suspensions of bacteriophage. The nano-emulsion-bacteriophage formulations were more stable and effective over time. The effects of emulsion and emulsion preparations on bacterial growth were used to indicate both more optimal formulations insight into key interactions. This work provides the basis for a viable product and prompts further research into the biological mechanisms within the system, and into formulations with enhanced biocompatibility and low cost.

RSM analysis confirmed the influence of emulsion concentration on the growth patterns of *S. aureus*, and identified important system interactions. The interactions between emulsion droplets and bacteria require a better model than the logistic growth curve. RSM analysis of the final concentration of bacteria in the presence of bacteriophage-emulsion preparations shows the potential to optimise the concentration rations of the components to reach a balance between desired killing effect and stable emulsion formulation.

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NOMENCLATURE

A	Bacterial growth rate (h^{-1})
B	Integration constant
C	Carrying capacity (CFU ml^{-1} or a.u.)
CFU ml^{-1}	Colony forming units per millilitre
DLS	Dynamic Light Scattering

MOI	Multiplicity of infection
MRSA	Methicillin Resistant *Staphylococcus aureus*
N(t)	Concentration of bacteria (CFU ml^{-1})
OD	Optical Density (absorbance units, a.u.)
PIT	Phase Inversion Temperature (°C)
PFU ml^{-1}	Plaque forming units per millilitre
RO	Reverse Osmosis
RSM	Response Surface Methodology
T	Time
TSA	Tryptic soy agar
TSB	Tryptic soy broth

REFERENCES

(1) Alanis, A. J. Resistance to antibiotics: are we in the post-antibiotic era? *Archives of Medical Research* **2005**, *36*, 697–705.

(2) Freire-Moran, L.; Aronsson, B.; Manz, C.; Gyssens, I. C.; So, A. D.; Monnet, D. L.; Cars, O. Critical shortage of new antibiotics in development against multidrug-resistant bacteria-Time to react is now. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy* **2011**, *14*, 118–24.

(3) Fung, H.; Kirschenbaum, H.; Ojofeitimi, B. Linezolid : An Oxazolidinone Antimicrobial Agent. *Clinical Therapeutics* **2001**, *23*.

(4) Prokocimer, P.; De Anda, C.; Fang, E.; Mehra, P.; Das, A. Tedizolid Phosphate vs Linezolid for Treatment of Acute Bacterial Skin and Skin Structure Infections. *Journal of American Medical Association* **2013**, *309*, 559–569.

(5) Church, D.; Elsayed, S.; Reid, O. Burn wound infections. *Clinical Microbiology Reviews* **2006**, *19*, 403–434.

(6) Fitzwater, J.; Purdue, G. F.; Hunt, J. L.; O'Keefe, G. E. The risk factors and time course of sepsis and organ dysfunction after burn trauma. *The Journal of Trauma* **2003**, *54*, 959–66.

(7) Griffiths, H. R.; Thornton, K. L.; Clements, C. M.; Burge, T. S.; Kay, A. R.; Young, A. E. R. The cost of a hot drink scald. *Burns : Journal of the International Society for Burn Injuries* **2006**, *32*, 372–4.

(8) Wolf, S. E.; Rose, J. K.; Desai, M. H.; Mileski, J. P.; Barrow, R. E.; Herndon, D. N. Mortality determinants in massive pediatric burns. An analysis of 103 children with >

or = 80% TBSA burns (> or = 70% full-thickness). *Annals of Surgery* **1997**, *225*, 554–65; discussion 565–9.

(9) Rashid, A.; Solomon, L. K.; Lewis, H. G.; Khan, K. Outbreak of epidemic methicillin-resistant *Staphylococcus aureus* in a regional burns unit: management and implications. *Burns : Journal of the International Society for Burn Injuries* **2006**, *32*, 452–7.

(10) Murray, C. K.; Holmes, R. L.; Ellis, M. W.; Mende, K.; Wolf, S. E.; McDougal, L. K.; Guymon, C. H.; Hospenthal, D. R. Twenty-five year epidemiology of invasive methicillin-resistant *Staphylococcus aureus* (MRSA) isolates recovered at a burn center. *Burns : Journal of the International Society for Burn Injuries* **2009**, *35*, 1112–7.

(11) Taylor, P. W.; Stapleton, P. D.; Paul Luzio, J. New ways to treat bacterial infections. *Drug Discovery Today* **2002**, *7*, 1086–91.

(12) Nizet, V.; Ohtake, T.; Lauth, X.; Trowbridge, J.; Rudisill, J.; Dorschner, R. A; Pestonjamasp, V.; Piraino, J.; Huttner, K.; Gallo, R. L. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* **2001**, *414*, 454–7.

(13) Kutateladze, M.; Adamia, R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends in Biotechnology* **2010**, *28*, 591–5.

(14) Ahmad, S. I. Treatment of post-burns bacterial infections by bacteriophages, specifically ubiquitous *Pseudomonas* spp. notoriously resistant to antibiotics. *Medical Hypotheses* **2002**, *58*, 327–31.

(15) Adhya, S.; Merril, C. The road to phage therapy. *Nature* **2006**, *443*, 754–5.

(16) Hanlon, G. W. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International Journal of Antimicrobial Agents* **2007**, *30*, 118–28.

(17) Ackermann, H.W. 5500 Phages examined in the electron microscope. *Archives of Virology* **2007**, *152*, 227–43.

(18) McVay, C. S.; Velásquez, M.; Fralick, J. A. Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. *Antimicrobial Agents and Chemotherapy* **2007**, *51*, 1934–8.

(19) Harper, D. R.; Enright, M. C. Bacteriophages for the treatment of *Pseudomonas aeruginosa* infections. *Journal of Applied Microbiology* **2011**, *111*, 1–7.

(20) Carter, M. J.; Tingley-Kelley, K.; Warriner, R. A. Silver treatments and silver-impregnated dressings for the healing of leg wounds and ulcers: a systematic review and meta-analysis. *Journal of the American Academy of Dermatology* **2010**, *63*, 668–79.

(21) Bouchemal, K.; Briançon, S.; Perrier, E.; Fessi, H. Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. *International Journal of Pharmaceutics* **2004**, *280*, 241–51.

(22) Prakash, U. R. T.; Thiagarajan, P. Nanoemulsions for drug delivery through different routes. *Research in Biotecnhology* **2011**, *2*, 01–13.

(23) Azeem, A.; Khan, Z. I.; Aqil, M.; Ahmad, F. J.; Khar, R. K.; Talegaonkar, S. Microemulsions as a surrogate carrier for dermal drug delivery. *Drug Development and Industrial Pharmacy* **2009**, *35*, 525–47.

(24) Bondoc Jr, L.; Fitzpatrick, S. Size distribution analysis of recombinant adenovirus using disc centrifugation. *Journal of Industrial Microbiology & Biotechnology* **1998**, 317–322.

(25) Schramm, L. L. *Emulsions, Foams, and Suspensions: Fundamentals and Applications*; WILEY-VCH Verlag GmbH & Co.KGaA, Ed.; Weinheim, 2005; pp. 226–325.

(26) Puapermpoonsiri, U.; Spencer, J.; Van der Walle, C. F. A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. *European Journal of Pharmaceutics and Biopharmaceutics* **2009**, *72*, 26–33.

(27) Kumari, S.; Harjai, K.; Chhibber, S. Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by *Klebsiella pneumoniae* B5055. *Journal of Medical Microbiology* **2011**, *60*, 205–10.

(28) Capra, M. L.; Patrignani, F.; Quiberoni, A. D. L.; Reinheimer, J. A.; Lanciotti, R.; Guerzoni, M. E. Effect of high pressure homogenization on lactic acid bacteria phages and probiotic bacteria phages. *International Dairy Journal* **2009**, *19*, 336–341.

(29) Avsaroglu, M. D.; Buzrul, S.; Alpas, H.; Akcelik, M.; Bozoglu, F. Use of the Weibull model for lactococcal bacteriophage inactivation by high hydrostatic pressure. *International Journal of Food Microbiology* **2006**, *108*, 78–83.

(30) Olson, M. R.; Axler, R. P.; Hicks, R. E. Effects of freezing and storage temperature on MS2 viability. *Journal of Virological Methods* **2004**, *122*, 147–52.

(31) Jończyk, E.; Kłak, M.; Międzybrodzki, R.; Górski, A. The influence of external factors on bacteriophages-review. *Folia Microbiologica* **2011**, *56*, 191–200.

(32) Iriarte, F. B.; Balogh, B.; Momol, M. T.; Smith, L. M.; Wilson, M.; Jones, J. B. Factors affecting survival of bacteriophage on tomato leaf surfaces. *Applied and Environmental Microbiology* **2007**, *73*, 1704–11.

(33) Mylon, S. E.; Rinciog, C. I.; Schmidt, N.; Gutierrez, L.; Wong, G. C. L.; Nguyen, T. H. *Langmuir : The ACS Journal of Surfaces and Colloids* **2010**, *26*, 1035–42.

(34) Matsubara, T.; Emoto, W.; Kawashiro, K. A simple two-transition model for loss of infectivity of phages on exposure to organic solvent. *Biomolecular Engineering* **2007**, *24*, 269–71.

(35) Branston, S.; Stanley, E.; Ward, J.; Keshavarz-Moore, E. Study of robustness of filamentous bacteriophages for industrial applications. *Biotechnology and Bioengineering* **2011**, *108*, 1468–72.

(36) Tadros, T.; Izquierdo, P.; Esquena, J. Formation and stability of nano-emulsions. *Advances in Colloid and Interface Science* **2004**, 303–318.

(37) Fernandez, P.; André, V.; Rieger, J.; Kühnle, A. Nano-emulsion formation by emulsion phase inversion.*Colloids and Surfaces: Physicochemical and Engineering Aspects* **2004**, *251*, 53–58.

(38) Mason, T. G.; Wilking, J. N.; Meleson, K.; Chang, C. B.; Graves, S. M. Nanoemulsions: formation, structure, and physical properties.*Journal of Physics: Condensed Matter* **2006**, *18*, R635–R666.

(39) Zwietering, M. H.; Jongenburger, I.; Rombouts, F. M.; Van 't Riet, K. Modeling of the bacterial growth curve. *Applied and Environmental Microbiology* **1990**, *56*, 1875–81.

(40) Lindqvist, R. Estimation of *Staphylococcus aureus* growth parameters from turbidity data: characterization of strain variation and comparison of methods. *Applied and Environmental Microbiology* **2006**, *72*, 4862–70.

(41) Marino, H. Phase Inversion Temperature Emulsification: From Batch to Continuous Process, University of Bath, 2010, p. 142.

(42) Langevin, P. B.; Gravenstein, N.; Doyle, T. J.; Roberts, S. A.; Skinner, S.; Langevin, S. O.; Gulig, P. A. Growth of *Staphylococcus aureus* in Diprivan and Intralipid: implications on the pathogenesis of infections. *Anesthesiology* **1999**, *91*, 1394–400.

(43) Kim, C.; Lewis, D.; Kumar, A. Bacterial and fungal growth in intravenous fat emulsions. *American Journal of Health Systems and Pharmacy*. **1983**, *40*, 2159–2161.

(44) Tessler, M.; Dascal, A.; Gioseffini, S.; Miller, M.; Mendelson, J. Growth curves of *Staphylococcus aureus*, *Candida albicans*, and *Moraxella osloensis* in propofol and other media. *Canadian Journal of Anaesthesia* **1992**, *39*, 509–11.

(45) Parker, M. L.; Brocklehurst, T. F.; Gunning, P. A.; Coleman, H. P.; Robins, M. M. Growth of food-borne pathogenic bacteria in oil-in-water emulsions: I - Methods for investigating the form of growth. *The Journal of Applied Bacteriology* **1995**, *78*, 601–8.

(46) Brocklehurst, T. F.; Parker, M. L.; Gunning, P. A.; Coleman, H. P.; Robins, M. M. Growth of food-borne pathogenic bacteria in oil-in-water emulsions: II - Effect of emulsion structure on growth parameters and form of growth. *The Journal of Applied Bacteriology* **1995**, *78*, 609–15.

(47) Prachaiyo, P.; McLandsborough, L. Oil-in-Water Emulsion as a Model System to Study the Growth of *Escherichia coli* O157: H7 in a Heterogeneous Food System. *Journal of Food Science* **2003**, *68*.

(48) Li, J.; McClements, D. J.; McLandsborough, L. A. Interaction between emulsion droplets and *Escherichia coli* cells. *Journal of Food Microbiology and Safety* **2001**, *66*, 570–574.

(49) Kiss, M. M.; Babineau, E. G.; Bonatsakis, M.; Buhr, D. L.; Maksymiuk, G. M.; Wang, D.; Alderman, D.; Gelperin, D. M.; Weiner, M. P. Phage ESCape: an emulsion-based approach for the selection of recombinant phage display antibodies. *Journal of Immunological Methods* **2011**, *367*, 17–26.

(50) Fujito, B.; Lytle, C. Elution of Viruses by Ionic and Nonionic Surfactants.*Applied and Environmental Microbiology* **1996**, *62*.

(51) Pines, O.; Gutnick, D. Specific Binding of a Bacteriophage at a Hydrocarbon-Water Interface. *Journal of Bacteriology* **1984**, *157*, 179–183.

(52) Payne, R. J. H.; Jansen, V. A. A. Pharmacokinetic principles of bacteriophage therapy. *Clinical Pharmacokinetics* **2003**, *42*, 315–25.

(53) Payne, R. J. H.; Jansen, V. A. A. Evidence for a Phage Proliferation Threshold ? Letter to the Editor. *Journal of Virology* **2002**, *76*, 13123–13125.

(54) Payne, R. J. H.; Phil, D.; Jansen, V. A. A. Phage therapy: the peculiar kinetics of self-replicating pharmaceuticals. *Clinical Pharmacology and Therapeutics* **2000**, *68*, 225–30.

Table 1: Factors, Levels and Response for the RSM

Factor	Levels	Response
Initial bacterial concentration (Initial OD)	1 High – 1 a.u. 0 Medium – 0.5 a.u. -1 Low – 0.1 a.u.	Bacterial growth rate, a (h^{-1}) Carrying capacity, C (a.u.)
Dilution factor of raw emulsion	1 High – 1:1 0 Medium – 1:10 -1 Low – 1:100	
Initial bacterial concentration (Initial OD)	1 High – 1 a.u. 0 Medium – 0.5 a.u. -1 Low – 0.1 a.u.	Final concentration of bacteria after 20 h, expressed as OD(t) (a.u.)
Dilution factor of raw emulsion	1 High – 1:1 0 Medium – 1:10 -1 Low – 1:100	
Dilution from bacteriophage stock	1 High – $1:10^3$ 0 Medium – $1:10^4$ -1 Low – $1:10^5$	

Figure Titles

Figure 1: Concentration of *S. aureus* H560 over time, expressed as Optical Density at 600 nm, at 37°C in TSB (▼), in undiluted emulsion (×), and in diluted emulsion (⊕). Data points are the mean value of triplicates, after discounting the OD of TSB and emulsion. Error bars are the standard deviation of experimental values; one in five error bars are shown.

Figure 2: Concentration of *S. aureus* H325 over time, expressed as Optical Density at 600 nm, at 37°C in TSB (▼), in undiluted emulsion (×), and in diluted emulsion (⊕).Data points are the mean value of triplicates, after discounting the OD of TSB and emulsion. Error bars are the standard deviation of experimental values; one in five error bars are shown.

Figure 3: Concentration of *S. aureus* Btn766 over time, expressed as Optical Density at 600 nm, at 37°C in TSB (▼), in undiluted emulsion (×), and in diluted emulsion (⊕). Data points are the mean value of triplicates, after discounting the OD of TSB and emulsion. Error bars are the standard deviation of experimental values; one in five error bars are shown.

Figure 4: Concentration of *S. aureus* H560 over time, expressed as Optical Density at 600 nm, in TSB (○), in undiluted emulsion (▼), in the presence of bacteriophage K (◇), and in the presence of Bacteriophage K – emulsion preparation (+). All preparations were stored at 4°C and tested after 9 days of storage. Data points represent the mean value of triplicates, after discounting the OD of TSB and emulsion or SM buffer. Error bars are the standard deviation of experimental values; one in five error bars are shown.

Figure 5: Concentration of *S. aureus* H325 over time, expressed as Optical Density at 600 nm, in TSB (○), in undiluted emulsion (▼), in the presence of bacteriophage K (◇), and in

the presence of bacteriophage K – emulsion preparation (+). All preparations were stored at 4°C and tested after 9 days of storage. Data points represent the mean value of triplicates, after discounting the OD of TSB and emulsion or SM buffer. Error bars are the standard deviation of experimental values; one in five error bars are shown.

Figure 6: Concentration of *S. aureus* Btn766 over time, expressed as Optical Density at 600 nm, in TSB (●), in undiluted emulsion (▼), in the presence of bacteriophage K (◇), and in the presence of bacteriophage K – emulsion preparation (+). All preparations were stored at cold temperature and tested after 9 days of storage. Data points represent the mean value of triplicates, after discounting the OD of TSB and emulsion or SM buffer. Error bars are the standard deviation of experimental values; one in five error bars are shown.

Figure 7: RSM Contour Plots for bacterial growth rate and carrying capacity.
Bacterial growth rate vs. dilution factor of emulsion and initial concentration of H560 (Panel A), H325 (Panel C) and Btn766 (Panel E). Carrying capacity vs. dilution factor of emulsion and initial concentration of H560 (Panel B), H325 (Panel D) and Btn766 (Panel F). Six replicates for each of the design combinations of factors were performed.

Figure 8: RSM Contour Plots for final concentration of bacteria in the presence of bacteriophage-emulsion preparations. Final concentration of bacteria vs: Panel A: dilution factor of emulsion and bacteriophage for an initial concentration of H560 of 0.5 a.u.; Panel B: dilution factor of bacteriophage and initial concentration of H560 at a dilution factor of emulsion of 1:10; Panel C: dilution factor of emulsion and initial concentration of H560 at a dilution factor of bacteriophage of $1:10^4$; Panel D: dilution factor of emulsion and bacteriophage for an initial concentration of H325 of 0.5 a.u.; Panel E: dilution factor of

bacteriophage and initial concentration of H325 at a dilution factor of emulsion of 1:10; Panel F dilution factor of emulsion and initial concentration of H325 at a dilution factor of bacteriophage of $1:10^4$; Panel G: dilution factor of emulsion and bacteriophage for an initial concentration of Btn766 of 0.5 a.u.; Panel H: dilution factor of bacteriophage and initial concentration of Btn766 at a dilution factor of emulsion of 1:10; and Panel I: dilution factor of emulsion and initial concentration of Btn766 at a dilution factor of bacteriophage of $1:10^4$. Six replicates were performed for each of the factor combinations.

Figure 9: Bacteriophage K infectivity against *S. aureus* over a period of ten days, expressed as Relative Killing Effect, when stored in SM buffer at 18-20°C (▭), in undiluted emulsion at 18-20°C (▨), in SM buffer at 4°C (▬), and in undiluted emulsion at 4°C (▩). Panels A to C correspond to H560, H325 and Btn766 respectively. The bar height is the mean value of triplicates; error bars are the standard deviation of replicates.

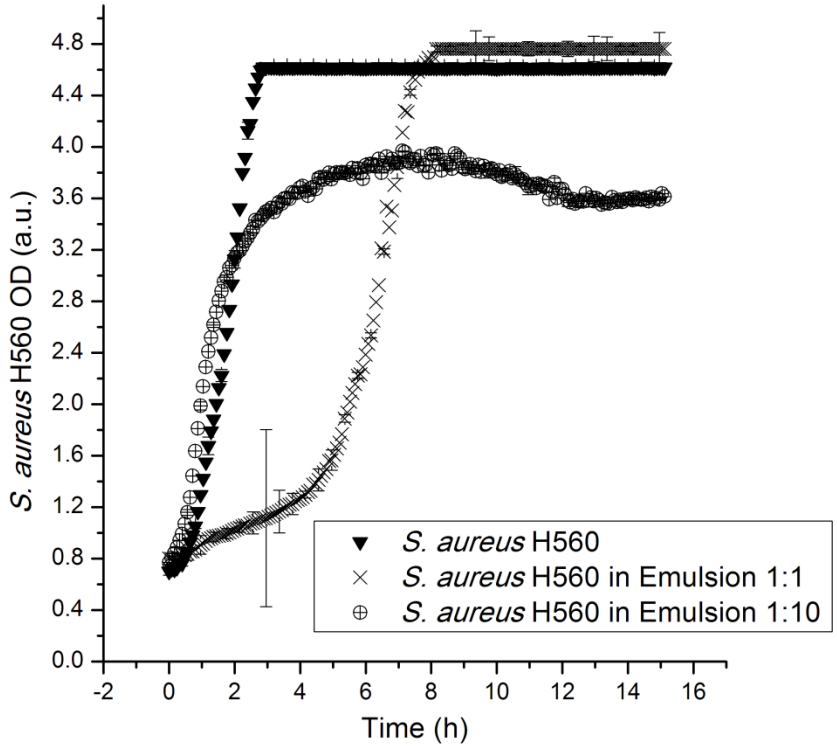

Figure 1

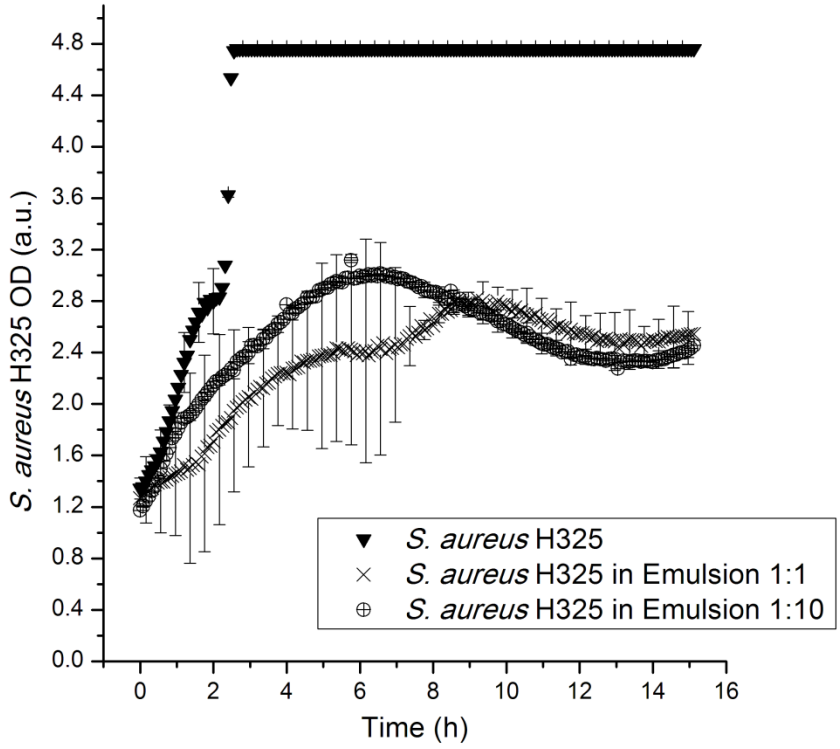

Figure 2

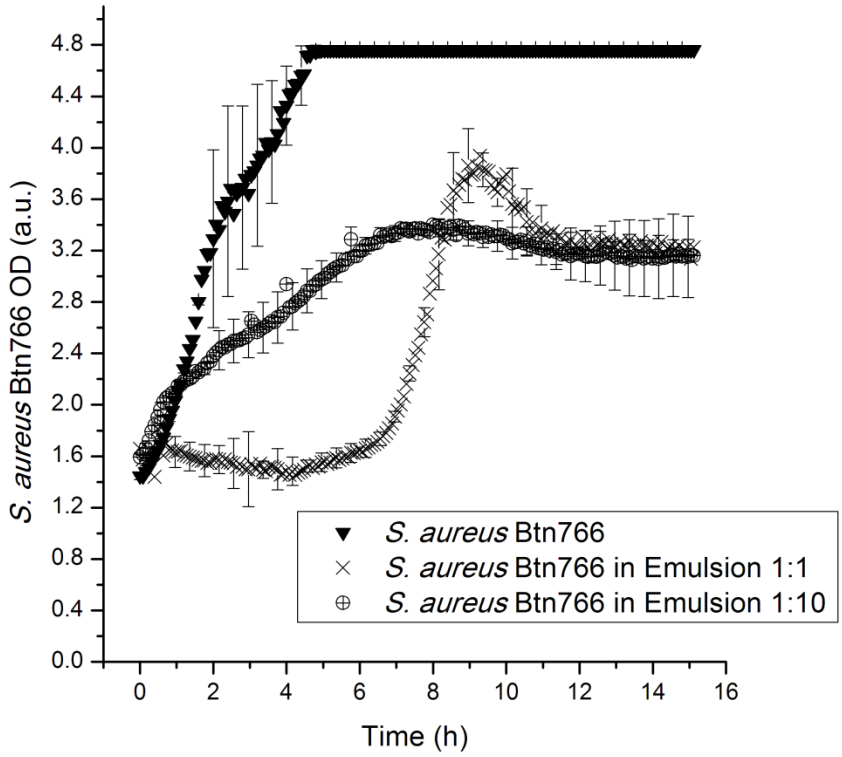

Figure 3

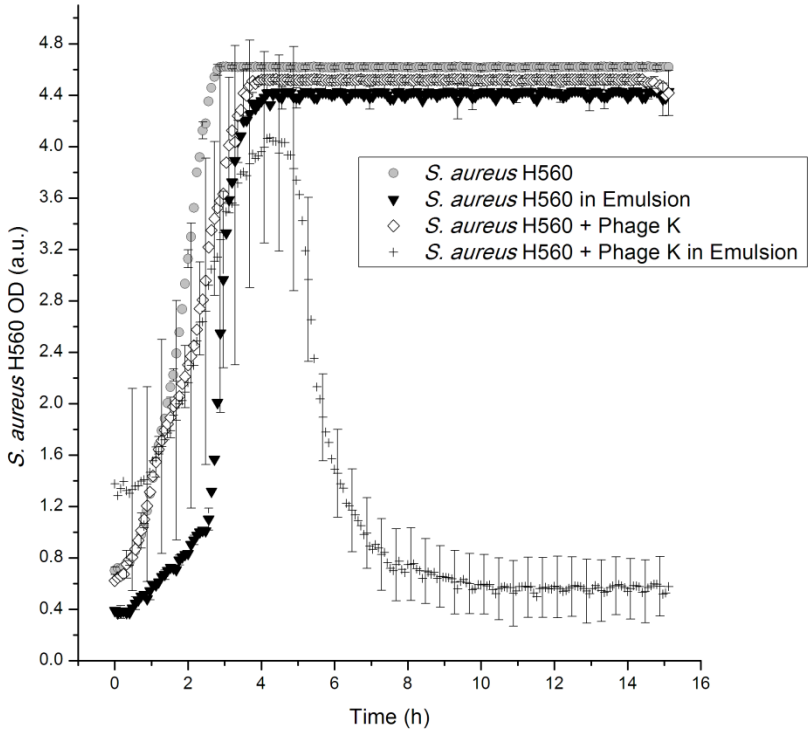

Figure 4

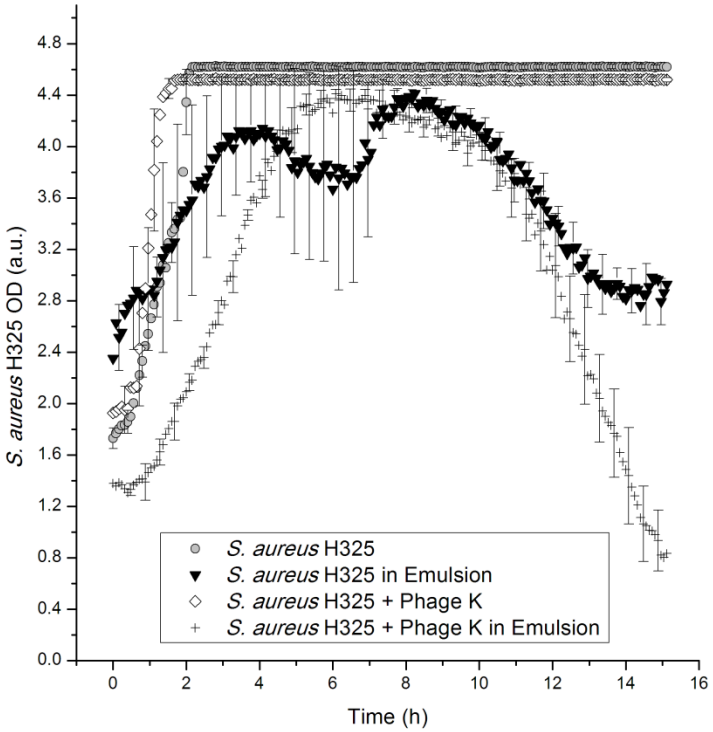

Figure 5

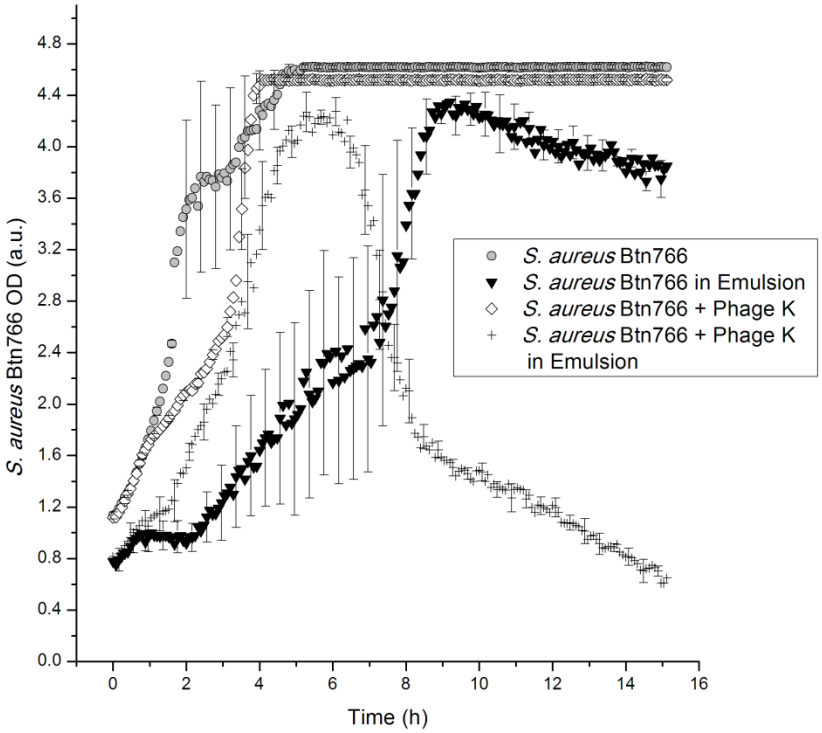

Figure 6