

**Exploitation of the bilosome platform technology to formulate antibiotics and enhance efficacy of Melioidosis treatments**

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**Abstract**

*Burkholderia pseudomallei* is a Gram-negative intracellular bacterium which is recalcitrant to antibiotic therapy. There also is currently no licenced vaccine for this potentially fatal pathogen, further highlighting the requirement for better therapeutics to treat the disease melioidosis. Here we use an oral delivery platform, the bilosome to entrap already- licenced antibiotics. Bilosome-entrapped antibiotics were used to treat mice infected via the aerosol route with *B. pseudomallei*. When treatment was started by the oral route at 6h post-infection and continued for 7 days, bilosome levofloxacin and bilosome doxycycline formulations were significantly more efficacious than free antibiotics in terms of survival rates. Additionally, bilosome formulated levofloxacin protected mice from antibiotic and infection induced weight loss following *B. pseudomallei* infection. The microbiomes of mice treated with levofloxacin were depleted of all phyla with the exception of Firmicutes, but doxycycline treatment had minimal effect on the microbiome. Encapsulation of either drug in bilosomes had no deleterious or clear advantageous effect on microbiome. This indicates that the ability of bilosomes to ameliorate antibiotic induced weight loss is not due to microbiome effects. The bilosome platform not only has potential to reduce adverse effects of orally delivered antimicrobials, but has potential for other therapeutics which may cause detrimental side-effects or require enhanced delivery.

## Introduction

*Burkholderia pseudomallei* is an environmental pathogen and the causative agent of the human condition of melioidosis [1, 2]. The bacteria survive in wet conditions (e.g. paddy fields) [3] with most common human exposure occurring through scratches or other lesions of the skin. It can cause an acute febrile illness or go unnoticed, depending on the inoculum size and host. The bacteria can also infect by the inhalational route, causing a more acute pneumonic infection. There is currently no licensed vaccine for melioidosis, meaning there is a heavy reliance on antibiotics to treat overt infection [4]. As an intracellular pathogen, *B. pseudomallei* has evolved multiple secretory and immunomodulatory mechanisms to evade innate host defences and to survive in granulomatous lesions for long periods. Consequently, this insidious pathogen is extremely difficult to access and treat effectively, limiting the use of antibiotics to clear the infection [5-7].

A comparison of the minimum inhibitory concentrations (MICs) determined *in vitro* for *B. pseudomallei* indicates that all the classes of antibiotics (apart from the aminoglycosides e.g. streptomycin/gentamicin) have some inhibitory effect on bacterial growth, with the combination drug co-trimoxazole (sulfamethazole plus trimethoprim) looking most consistent, while ceftazidime, ciprofloxacin and piperacillin have been shown to be partially effective therapies against *B. pseudomallei* [8, 9]. A potential explanation for the relative resistance of *B. pseudomallei* to multiple antibiotics is its ability to actively export antibiotics by efflux pumps [10]. This has a direct impact on human melioidosis where treatment is required initially to prevent overwhelming sepsis and mortality, with a subsequent prolonged oral eradication phase to kill residual bacteria. The success of this strategy requires a selection of antibiotics with, for example, ceftazidime and trimethoprim-sulphamethoxazole [11]. There is a significant risk of side effects arising from a lot of these combinations. Doxycycline has been used in the past for treatment of localised melioidosis (and still used in some parts of the world) and combining doxycycline with another bactericidal drug provides a broad spectrum therapy for systemic disease [12, 13]. More recently however, the use of the newer fluoroquinolones such as levofloxacin has been expanded to include the treatment of other pathogens such as pneumonic plague and anthrax. Building on this, another member of this class of antibiotic, finafloxacin, which has a modified structure able to

tolerate and retain activity in a low pH environment such as in a eukaryotic cell, has been reported to be efficacious in the treatment of experimental melioidosis [14].

Here, we have taken the approach of encapsulating each of levofloxacin and doxycycline in synthetic microvesicles which have been modified for oral delivery, to determine if this enhances intracellular delivery/uptake and therapeutic efficacy in experimental melioidosis. The synthetic microvesicles used are comprised of monopalmitoyl glycerol, cholesterol and the surfactant dicetyl phosphate, and thus are termed non-ionic surfactant vesicles (NISVs) or niosomes [15]. Furthermore, by the simple incorporation of bile salts, these microvesicles are converted to bilosomes, which are completely biocompatible and biodegradable and provide a platform technology for the oral delivery of a range of small molecules including hormones, proteins, DNA and vitamins [16] and now, antibiotics. As well as enhancing cellular uptake [17], the physical properties of bilosomes confer many other advantages including ease of modification to achieve tissue-targeted drug delivery [18], increased bioavailability [19], sustained release [20], extreme stability in air and at temperatures up to 130°C, and are inexpensive and simple to synthesize, and amenable to lyophilisation [21].

Here we report for the first time the entrapment of doxycycline and levofloxacin in bilosomes and the efficacy of these oral formulations in a murine post-exposure therapeutic model of inhalational melioidosis. We demonstrate that such formulations confer a statistically significant survival benefit, together with an unexpected and highly significant benefit in protection against infection and antibiotic-induced weight loss.

## Materials and Methods

### Antibiotics

The antibiotics, levofloxacin  $\geq 98.0\%$  (HPLC) and doxycycline hyclate  $\geq 98.0\%$  (HPLC) (Sigma-Aldrich) were obtained in dry powder form and used in all studies. For making the bilosome formulations and dosing as unformulated drugs, they were suspended in PBS at pre-determined concentrations.

### Bacteria

The clinical isolate *Burkholderia pseudomallei* K96423 was used for *in vitro* and *in vivo* studies. *B. pseudomallei* is a Containment Level 3 (Biosafety Level 3) pathogen that requires specialist facilities, laboratories, cabinets and isolators to carry out the work safely. The Defence Science and Technology Laboratory contains suites a number of ACDP containment level 3 (equivalent to BSL3) experimental laboratories with primary containment of the pathogen in double-HEPA filtered class III closed cabinets and ACDP CL3 animal rooms containing rigid-walled half-suit isolators for housing experimentally infected animals. Bacteria were grown in Luria broth at  $37^{\circ}\text{C}$  on a rotary platform for aerosol challenges and enumerated on L-Agar plates.

### Preparation of bilosomes

Bilosomes were made by the melt method. Briefly, 1-monopalmitoyl glycerol, cholesterol, and dicetyl phosphate in a 5:4:1 molar ratio were combined and heated to  $130^{\circ}\text{C}$ . Following the addition of sodium deoxycholate in 0.025 M carbonate buffer and the relevant antibiotic, preparations were vortexed vigorously for 2 min. Antibiotics were entrapped as vesicles were formed. Non-entrapped antibiotic was removed through centrifugation and the pelleted vesicles re-suspended in the appropriate buffer containing the relevant antibiotic at a concentration equivalent to that entrapped within the vesicles to give a 50/50 entrapped: free preparation.

### Biophysical characterisation of bilosomes

Large batches of bilosome preparations were formulated and aliquoted for lyophilisation and storage. Aliquots were recovered as required and resuspended in reverse osmosis water, followed by vigorous agitation. Vesicle size and zeta-potentials were determined using a Malvern Zeta-sizer (Zetasizer 3000HS, Malvern

Instruments Ltd., UK). Vesicles were pelleted by centrifugation and HPLC used to determine entrapped antibiotic content.

### HPLC Methodologies

HPLC analysis was carried out on an Agilent1290 Infinity Series HPLC, using a C18 column (150mm x 4.6mm, 5 $\mu$ ) maintained at 50°C. The mobile phase for both antibiotics (doxycycline and levofloxacin) consisted of 0.02M Na<sub>2</sub>HPO<sub>4</sub>, pH2 with H<sub>3</sub>PO<sub>4</sub> and either acetonitrile or methanol.

### Dissolution Studies

Drug release studies were carried out using a CE7smart USP-4 system (SOTAX AG, Switzerland) in a closed loop system with buffers being circulated at 20ml/min at 37°C ( $\pm$  1°C). Synthetic gastric fluid (SGF; 0.1M HCL, 39nM sodium taurocholate) and synthetic intestinal fluid (SIF; 50mM PBS, 2.171 $\mu$ M sodium deoxycholate pH8.5) was used to simulate oral delivery of the formulations. 1ml of 5mg/ml of either free drug or drug encapsulated in bilosomes were sealed in 300kDa dialysis membrane and placed in the USP-4 system. Formulations were incubated for 2 hours in SGF with 1ml samples being taken at 0.25, 0.5, 1 and 2 hours. The buffer was exchanged to SIF and 1ml samples taken at 0.25, 0.5, 1, 2, 4 and 6 hours. After sampling at each time point 1ml of the appropriate fresh buffer was added back into the system to maintain the sink volume. Sample drug concentrations were then assessed using HPLC (described above) and drug release expressed as cumulative milligrams over time elapsed.

### Minimum inhibitory concentrations

MICs for antibiotic formulations were determined for *B. pseudomallei* strain K96243 using the broth micro dilution method in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines. Assays were performed in 96 well micro-titre plates with antibiotic concentrations in the range of 64 mg/L to 0.03 mg/L, and bacteria at a final concentration of approximately 5 x 10<sup>5</sup> CFU/mL. Following incubation at 37°C for 24 h the optical densities (OD) of the plates were read in an automated plate reader at a wavelength of 590nm. MICs were determined as the

concentration that inhibited >80 % of bacterial growth via OD and confirmation by eye.

### **Minimum bactericidal concentrations**

Minimum bactericidal concentrations (MBCs) for antibiotic formulations were determined by plating 100  $\mu$ L aliquots of the MIC dilutions showing no visible growth onto L-agar plates in triplicate and incubating at 37°C for 48 hours. The MBC was recorded as the lowest concentration of antibiotic that killed 99.9 % of the bacteria in the original inoculum.

### **Microbiome and Toxicology studies :**

BALB/c mice were allocated into groups of twelve mice, and treated via the oral route (0.1ml/day at 10mg/ml concentration) with PBS, Empty bilosomes, levofloxacin, bilosome levofloxacin, doxycycline or bilosome doxycycline on days 1 to 7. Fecal samples were collected three days prior to treatment, 24 h. after the final dose (day 8) and 28 days after the cessation of treatment (day 36). Animals' weights and condition were recorded daily. On day 8, half the mice in each group were sacrificed and serum, small intestine and large intestine were collected. Serum samples were stored frozen at -80°C, the large and small intestines were cleaned of contents, rolled into pinwheels and fixed in 10% formalin. The remaining mice were weighed and observed for an additional 28 days before they were sacrificed and serum and tissues harvested as described for the mice sacrificed on day 8.

### **DNA Extraction and bTEFAP®:**

Genomic DNA was isolated from fecal samples using the PowerSoil® DNA Isolation Kit (Qiagen) following the manufacturer's instructions. As an alternative to the recommended 250mg of soil, approximately 200mg of fecal sample was added to the PowerBeads tube to undergo cell lysis. Purified DNA was eluted from the spin filter using 50 $\mu$ L of solution C6 and stored at -20°C until PCR amplification.

The 16S universal Eubacterial primers 515F GTGCCAGCMGCCGCGGTAA and 806R GGACTACHVGGGTWTCTAAT were utilized to evaluate the microbial ecology of each sample on the HiSeq 2500 with methods via the bTEFAP® DNA analysis

service. Each sample underwent a single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute; after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing the Illumina HiSeq chemistry following manufacturer's protocols.

The Q25 sequence data derived from the sequencing was processed using a proprietary analysis pipeline ([www.mrdnalab.com](http://www.mrdnalab.com), MR DNA, Shallowater, TX). Sequences were depleted of barcodes and primers then short sequences < 200bp were removed, sequences with ambiguous base calls removed, and sequences with homopolymer runs exceeding 6bp removed. Sequences were then de-noised and chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity). OTUs were then taxonomically classified using BLASTn against a curated GreenGenes/RDP/NCBI derived database and compiled into each taxonomic level into both "counts" and "percentage" files. Counts files contain the actual number of sequences while the percent files contain the relative (proportion) percentage of sequences within each sample that map to the designated taxonomic classification.

Statistical analysis was performed using a variety of computer packages including XLstat, NCSS 2007, "R" and NCSS 2010. Alpha and beta diversity analysis was conducted as described previously using Qiime ([www.qiime.org](http://www.qiime.org)).

### **Animal Infection Studies:**

Six to eight week old female BALB/c mice (Charles River, UK) were transferred to a high containment Class III rigid isolator, where they were given unlimited access to food and water and allowed to acclimatise for at least 5 days. Mice were allocated to treatment groups (15 per group) and housed in cages of 5. Mice were challenged with 50-100 CFU (10MLD) of *B. pseudomallei* K96243 via the aerosol route in a nose-only exposure system using a computerised delivery platform (Biaera



Technologies). A sub-optimal therapy study design was used, in which antibiotic administration was started at 6h post-infection and administered orally once daily for only 7 days, to test therapeutic efficacy. The treatment groups comprised bilosome-formulated antibiotics or unformulated antibiotics. The antibiotics levofloxacin, doxycycline, bilosome encapsulated levofloxacin and bilosome encapsulated doxycycline were delivered daily by the oral route at 50mg/kg and treatment was continued for 7 days. A subgroup of 5 mice per treatment group was culled at day 3 p.i. to determine bacterial loads in lung, spleen and liver. All mice were checked twice daily and scored for clinical signs and mice were weighed daily. Mice reaching a humane end-point, based on a pre-determined set of objective clinical signs, were promptly culled. Survival times were recorded for some mice and others were culled for analysis of tissues at different time points. All procedures and housing complied with the UK Animal (Scientific Procedures) Act (1986).

### **Statistical Analysis:**

Drug release profiles between free and encapsulated drug were compared using the  $f_2$  similarity test in accordance to the FDA guidelines (Appendix II of the "Note for Guidance on the investigation of bioavailability and bioequivalence". If the  $f_2$  score is less than 50 then the formulation being tested is considered to be dissimilar to the reference control [22]. A variety of statistical analyses have been performed using the program SPSS V21.0 (IBM) or Graphpad PRISM V6.0. Graphs have been constructed using Graphpad PRISM V6.0. Survival data were compared using log rank tests. Continuous data were analysed by parametric analysis (ANOVA, T tests, GLM) when conditions were met (QQ plots to assess Gaussian distribution and Levene's/Bartlett tests for unequal variation) or non-parametric tests (Kruskal-Wallis, Mann-Whitney, Moods) where these criteria were not met. In some cases it was possible for parametric criteria to be attained in the use of transformations such as logarithmic transformation. Contingency tables were used for binary data. Multiple testing corrections for familywise error were performed on individual comparisons with analyses. These included Bonferroni's and Dunn's corrections.

## Results

### Entrapment of antibiotics into bilosomes.

The bilosome formulation technology was adapted to successfully entrap the fluoroquinolone levofloxacin and the tetracycline doxycycline. The total antibiotic delivered by each formulation in mg/ml, together with percentage of this which was entrapped, is recorded in Table 1. For each formulation, more than 50% of the total antibiotic was entrapped. The mean size of bilosomes with antibiotic cargo was in the range 2700 -3400 nm with zeta potentials in the range of -30 to -23, where negative values for zeta potential indicate formulation stability.

### Assessment of formulation stability.

The stability (determined via Zeta potential) of the bilosome formulations was tested as freeze-dried preparations at a range of temperatures (room temperature, +4°C and -20°C) and at a range of time post- manufacture (1 week, 1 month and 3 months) (Table 2). The bilosome zeta potential remained relatively consistent across all time points and all temperatures. There was slight variability in the bilosome doxycycline formulation if kept at room temperature, however all formulations used in these studies were stored at -20 °C and used within a month of manufacture.

### Dissolution studies

Release profiles of encapsulated drug were studied in a system to simulate oral delivery and to compare the dissolution of bilosome encapsulated and free drug controls. Bilosome encapsulation of levofloxacin modestly reduced dissolution rate over the first 2 hours compared with free drug when incubated in synthetic gastric fluid (SGF). However, the vast majority of both levofloxacin formulations had been released by 2 hours. Following buffer exchange to SIF the remaining levofloxacin was released by the end of the 8 hour study. Free levofloxacin and bilosome levofloxacin release profiles were found to be dissimilar SGF using the  $f_2$  similarity test gave (score: 48.8) (Figure 1). For doxycycline formulations the free drug displayed a rapid dissolution profile over the 2 hours in SGF medium with all of the 5mg being released. In comparison bilosome encapsulated doxycycline had a dissimilar, slower release profile (score: 15.56) with only 2.2mg of the doxycycline

being released into the SGF. Doxycycline continued to be released from the bilosome formulation in the SIF over the remaining 6 hours resulting in a cumulative total of 2.9mg being released (score 14.89 for the cumulative release in SGF and SIF) (Figure 1).

### ***In vitro* assessment of bilosome formulations for minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC).**

To rule out the possibility that the formulation of antibiotics in bilosomes would adversely affect antimicrobial function, the MICs and MBCs of the formulations were tested *in vitro* against *B. pseudomallei*. In each of these assays, bilosome-formulated antibiotics exerted as much antimicrobial activity as unformulated antibiotics, indicating that the process of formulation had not adversely affected the antibiotic cargo (Table 3).

### **Safety/ toxicology studies on bilosome formulations**

Since the bilosome antibiotic formulations are novel, a repeat- dose murine safety/toxicology study was also carried out to evaluate the tolerability of the formulations.

In these specific toxicology studies, the effects of treatment with two different antibiotics, formulated in either saline or bilosomes, were evaluated on weight gain, microbiome composition, small intestine histology and serum serotonin levels (therapeutic efficacy is discussed later). Levofloxacin or doxycycline were given orally for seven days, and half the mice were sacrificed on day 8 to evaluate the immediate impact of treatment. Faecal pellets were collected from mice sacrificed at day 8. The remaining mice were monitored for 28 days after the cessation of treatment, with faecal pellets collected at day 22 and terminal samples collected on day 36. No mortality was noted, small intestine histology was normal in all animals, and there were no statistically significant differences between groups in serum serotonin levels (data not shown).

However statistically significant differences in weight gain were noted between treatment groups (Figure 2). In particular the bilosome levofloxacin group lost

significantly less weight than mice treated with unformulated levofloxacin at days 14 and 36 post treatment ( $p < 0.05$ ). Interestingly therapy ended at d7 indicating that the bilosome formulation increases the recovery phase. No significant difference was seen between bilosome doxycycline and free doxycycline treated groups.

### Microbiome analysis

To investigate the ability of bilosomes to ameliorate antibiotic-induced weight loss, the gut microbiome of mice was monitored prior to antibiotic treatment and 8 days following treatment and analysed by 16s RNA sequencing. A total of fifty-nine (59) genera were defined as high frequency (present in at least 75% of specimens). There was some variation in the presence of these 59 genera in the baseline samples, but at least 57 genera were present in all groups. A total of eighty-one (81) genera were defined as low frequency (present in less than 25% of specimens). No more than 15 of these 81 genera were present in any one group at any time point, with most genera being absent from most specimens. In total, these genera accounted for very little of the total microbial complement. The results demonstrate that bilosomes had no adverse effects on the microbiome (Figure 3).

Formulation of levofloxacin with bilosomes did not protect the vast majority of the microbiome in treated mice. All phyla of bilosome formulated levofloxacin and free levofloxacin treated mice, with the exception of the *Firmicutes* were reduced to minimal levels following treatment as observed on day 8 (Figure 3). However, within the *Firmicutes*, only subtle differences were noted in the genera maintained in the bilosome levofloxacin mice, compared with the free levofloxacin treated mice (Figure 3).

Doxycycline treatment had less severe effects on host microbiome, irrespective of formulation as determined on day 8. Mice treated with free doxycycline or bilosome doxycycline all had similar levels of *Firmicutes* and *Bacteroidetes* as observed in mice pre-treatment, although mice given doxycycline in bilosomes had an increased ratio of Firmicutes to Bacteroidetes. No significant differences were noted between animals treated with PBS over the course of the study. The ratio of Firmicutes to

*Bacteroidetes* was increased in mice treated with empty bilosomes compared with the same animals before commencing treatment (Figure 3). Minor phyla with the exception of Actinobacteria were greatly diminished in groups of animals at day 8 post infection (Figure 3).

By day 36 of the experiment (4 weeks post treatment), bacteroidetes levels had recovered in levofloxacin and levofloxacin in bilosome treated animals to pre-treatment levels. All groups of animals including control PBS treated had some Verrucomicrobia present at this timepoint. Levels of minor phyla including Spirocaetae, Tenericutes, Actinobacteria and Proteobacteria had also recovered to pre-treatment levels at this time point (Figure 3).

### ***In vivo* Assessment of bilosome formulations to treat *B. pseudomallei* infection**

The efficacy of bilosome formulations of levofloxacin and doxycycline was tested in an aerosol model of melioidosis, using a sub-optimal therapy study design, in which antibiotic administration was started at 6h post-infection and administered once daily for only 7 days, to test therapeutic efficacy. The treatment groups comprised bilosome-delivered antibiotics or unformulated antibiotics, with control groups receiving PBS or empty bilosomes. This study was repeated twice with exactly the same design, conditions and identical treatment groups, the data were stratified and combined for analysis, to give an overall significant survival advantage for bilosome-encapsulated versus free levofloxacin ( $p=0.014$ ) with an average survival rate of 90% vs 55% and a median survival for both the PBS and empty bilosome controls of d4 p.i (Median survival for levofloxacin and bilosome levofloxacin is undefined) (Figure 4). A significant survival advantage for bilosome-encapsulated versus free doxycycline was also seen ( $p<0.001$ ). An average survival rate of 40% vs 0% was recorded with median survival for both the PBS and empty bilosome controls of d4 p.i, doxycycline was d6 p.i and bilosome doxycycline d10 p.i (Figure 5).

In addition to the survival advantage conferred by delivering levofloxacin in bilosomes, over free levofloxacin, bilosome levofloxacin- treated groups lost

significantly less body weight compared to levofloxacin (Figure 6). In the levofloxacin treatment groups, the protection against antibiotic-induced weight loss in mice receiving the bilosome formulations, was very marked, such that the combined data from the bilosome–levofloxacin treated groups gave a highly significant difference in weight loss ( $p < 0.001$ , Figure 6). No difference in weight loss between bilosome doxycycline and free doxycycline groups was noted, but this was due to the majority of free doxycycline mice succumbing to infection before antibiotic dosing was completed (Figure 7).

Bacteriological, immunological and blood chemistry analyses show supporting but non-significant changes between bilosome and free antibiotic- treated groups. At d.3 p.i., cytokine analysis of lung, spleen and liver tissue samples showed a decrease in pro-inflammatory cytokines in bilosome levofloxacin treated groups and this correlated with a reduction in ALT and GGT, enzymes associated with liver damage (data not shown).

## Discussion

The emergence of antimicrobial resistance in a number of clinically important bacteria along with the natural ability of microbes to evade treatment is a continuing concern [23, 24]. It is well documented that there has been a lack of investment in new antimicrobial therapies and thus repurposing or re-formulation of already licensed drugs is a potentially attractive solution [25]. Encapsulation of therapies to improve delivery and access to intracellular niches is a developing area both academically and industrially [26]. For these reasons, we reformulated two existing antibiotics, levofloxacin and doxycycline and utilised a mouse model of melioidosis to determine their efficacy. Treatment options for *B. pseudomallei* are not always completely effective and this intransigent pathogen has been documented in one extreme case to persist in a person and emerge 60 years after the original exposure [27]. Current suggested clinical treatment of melioidosis consists of two phases. Phase 1 is administration of Ceftazidime or Meropenem via the intravenous route followed by an elongated Phase 2 where Trimethoprim-sulfamethoxazole or Amoxicillin/clavulanic acid (co-amoxiclav) are administered orally for several months [28-30]. Other antibiotic combinations have been explored clinically, including the use of doxycycline and ciprofloxacin [31, 32]. Therefore the optimisation of oral drug delivery is of importance for melioidosis treatment and many other pathogens (e.g. biothreat agent) where treatment is difficult or inappropriate.

Liposome encapsulation of various drugs including antibiotics has been explored for decades and has shown promise [33]. Indeed encapsulation of ciprofloxacin has been demonstrated to reduce bacterial load and increase survival of mice infected with *Francisella tularensis*, *Coxiella burnetti* or *Yersinia pestis* [34-37]. However, less literature is available regarding NISVs, despite their many perceived advantages over liposomes, including their enhanced stability, simpler formulation and cheaper synthesis [16, 21, 38]. However, recent reports demonstrate the successful encapsulation of ciprofloxacin in NISV (comprising a range of nonionic surfactants) and demonstrate the drug maintains *in vitro* efficacy against the Gram-positive *Staphylococcal aureus* (40.41).

In these studies, we demonstrate that NISVs stabilised with bile salts for oral delivery as bilosomes are suitable platforms for two antibiotics, a tetracycline and a fluoroquinolone. Formulated drugs had improved dissolution profiles with both

levofloxacin and doxycycline release being prolonged in synthetic gastric fluid. This was particularly striking in the doxycycline formulation. The formulations generated are stable and retain antimicrobial activity *in vitro*. *In vivo* data suggests that bilosome vesicular formulations of both levofloxacin and doxycycline have increased efficacy in a murine model against *B. pseudomallei*. Thus mice receiving these formulations had significantly increased survival rates and time, compared to those receiving non-encapsulated drugs.

The data presented here show that the freeze-dried formulations generated are stable and that the encapsulation process has not impaired the antimicrobial activity of the cargo when tested *in vitro*. Both bilosome formulations had the same MIC and MBC values against *B. pseudomallei* and *F. tularensis* (Francisella data not shown) as free antibiotic. This was expected as the concentration of the antibiotic acting on the bacterial pathogen is the same irrespective of how it was formulated. The advantages of encapsulation as envisaged in an *in vivo* infection model, such as slower release and targeting, would not be seen in the *in vitro* MIC assay employed.

Stability of adapted and/or novel therapeutic formulations is of increasing interest. This is especially important for distribution to low/middle income countries where cold storage may not be readily available. Previous work in our laboratory has shown that both NISV and bilosome formulations can be freeze dried and maintain entrapment efficiency for 6 months at room temperature. Other measures of stability such as, size and charge of vesicles were also shown to be relatively consistent over time in our studies and are similar to previous reports where NISVs were stored for 90 days [39, 40]. Further interesting studies have demonstrated the ability of NISVs to enhance the photo-stability of compounds entrapped [41]. This again would be an added advantage for NISVs and bilosomes as delivery platforms.

Despite the obvious benefits of antibiotics, side effects can occur, especially associated with repeated dosing over a long duration. Indeed it is well documented that antibiotics from the fluoroquinolone family cause significant side effects including weight loss [42]. This is most likely due to the antibiotic disturbing the gut microbiota and altering the digestive tract [43]. Herein we demonstrated that entrapment of levofloxacin in bilosomes ameliorates antibiotic-induced weight loss in mice. Further, we have found that the formulation of ciprofloxacin in NISVs or bilosomes



protected against antibiotic-induced weight loss (data not shown). Bilosome doxycycline showed no change in weight loss compared to controls. This may be attributed to the different properties of this tetracycline compound, compared to the fluoroquinolones, including its bacteriostatic, rather than bactericidal effects.

Further, when the antibiotic dosing regimen was used to treat *B. pseudomallei* infection, the protection against weight loss was pronounced in the bilosome levofloxacin treated groups. Indeed it has previously been reported that NISV can protect from weight loss induced by FK-565, an experimental anti-tumour drug and also during an infection setting, where NISVs have been shown to protect against *Toxoplasma gondii* induced weight loss during vaccination studies (Patent No. CA2228298A1).

Other perceived advantages of vesicular delivery include altering PK/PD of therapeutic compounds, such as cancer agents [44]. To date, we have observed only minor changes in PK/PD with these antibiotics, but since levofloxacin and doxycycline are in any case highly bioavailable drugs, there is less potential benefit of encapsulation on PK/PD.

To determine whether there was a therapeutic advantage of encapsulation of antibiotics in bilosomes it was necessary to utilise a sub-optimal antibiotic model where we know that free levofloxacin or doxycycline would not totally clear *B. pseudomallei* infection. Despite starting our therapy early (6 hours post-infection) the concentration of levofloxacin and doxycycline used were below those previously reported to clear infection [45, 46].

The survival rate and time of *Burkholderia*-infected mice were significantly increased when treated with bilosome formulated levofloxacin or doxycycline rather than free drug. The increases in protection seen from the bilosome formulations were not directly related to pathogen clearance at early time points as determination of bacterial loads showed no significant difference between treatment groups. The lack of bactericidal differences between vesicle formulated antibiotic and free antibiotic seen in our studies may be due to the route of administration and/or the fact that we are using different antibiotics. Identification of the full mechanism of protection associated with the bilosome platform is underway. It was initially hypothesised that the mechanism of protection could be related to difference in the effects of each drug

formulation on the microbiome. It is well documented that changes in the microbiome can alter systemic immunity [47, 48]. Indeed a recent paper it has demonstrated that disrupting the gut microbiome with a broad cocktail of antibiotics prior to infection with *Burkholderia* increased growth and dissemination of the bacteria systemically, including changes in the lung [49]. In our studies we saw only minor differences in the microbiomes of mice treated with entrapped and free antibiotic formulations. Additional in- depth analyses needs to be carried out, but preliminary data would suggest that the advantages seen with the bilosome formulation are not solely related to changes in the microbiome.

There are many methods of NISV preparation which can alter the characteristics of the vesicles and therefore make them highly adaptable for numerous uses [50]. The method used for these studies was the melt method [51], where the 3 components are heated to high temperature then mixed with PBS containing antibiotic. Other methods such as ether injection, Ph gradient and micro- fluidisation have all been used and shown to be suitable to generate NISVs [52-54] and we have also automated and scaled-up production to achieve controlled batch consistency. NISVs have also been shown to have a variety of applications including vaccine platforms, delivery of anticancer or anti-parasitic drugs [51, 55-57]. Further adaptations of the vesicles make them suitable for crossing the blood brain barrier [58, 59].

Overall, the data reported here support bilosomes as a broad platform technology for the delivery of antibiotics. This technology not only provides a significant survival advantage in the infection models examined, but also reduces the serious side effect of weight loss associated with repeated antibiotic dosing and infection. Therefore there is potential that this drug delivery system would have utility in a biothreat scenario where drugs would most likely be delivered orally as a post exposure prophylaxis [60]. The precise mechanism responsible for this protection will need further investigation.

**Table 1: Total loading, percentage entrapment and zeta potential of Bilosomes formulations**

The values presented in the table are generated from making bilosomes via the melt method with entrapped concentrations determined via HPLC following removal of free antibiotic. Size and zeta potential measurements were determined by a Malvern Zeta-sizer and data are presented as raw values for 3 independent samples. Means and SEM are highlighted.

Antibiotics Formulations	Total antibiotic in formulation (mg/ml)	Antibiotic entrapped in formulation (mg/ml)	Percentage entrapped (%)	Size	Zeta Potential (mV)
<b>Bilosome Levofloxacin</b>	19.4	11.4	58.9	2846.0 ±124.42	-29.667 ±0.31
<b>Bilosomes Doxycycline</b>	17.2	9.2	53.5	3329.33 ±85.62	-23.33 ±0.29

**Table 2: Stability of bilosomes with time and storage conditions, as measured by zeta potential**

Bilosome antibiotic formulations were made as previously described and subsequently freeze dried (FD). Formulations were left at a variety of different storage temperature (37°C, Room Temperature, 4°C or -20°C) and assayed at multiple time points post manufacture (1 week, 1 month and 3 months) to determine the zeta potential of the vesicles. FD formulations were rehydrated at each time point for the assay. Measurements were determined by a Malvern Zeta-sizer and data are presented as raw values (zeta potential) for 3 independent samples per time point and per storage conditions. Means and SEM are highlighted in bold.

Storage Time	Bilosome Formulation					
	Levofloxacin			Doxycycline		
	RT	4°C	-20°C	RT	4°C	-20°C
<b>Start</b>	-28.10			-19.80		
	-30.20			-22.60		
	-32.70			-27.80		
<b>Mean</b>	-30.33	N/A	N/A	-23.40	N/A	N/A
<b>SEM</b>	0.42			0.74		
<b>1 Week</b>	-24.40	-28.90	-17.90	-24.00	-28.30	-28.10
	26.10	-31.90	-18.00	-30.00	-27.90	-25.90
	25.90	-31.50	-19.00	-27.30	-26.50	-28.50
<b>Mean</b>	-25.47	-30.77	-18.30	-27.10	-27.57	-27.50
<b>SEM</b>	0.17	0.30	0.11	0.55	0.17	0.26
<b>1 Month</b>	-24.20	-26.5	-29.80	-16.50	-26.70	-22.20
	26.30	-30.00	-30.20	-18.40	-28.2	-22.80
	-28.00	-31.00	-32.70	-18.70	-30.00	-26.50
<b>Mean</b>	-26.17	-29.17	-30.90	-17.87	-28.30	-23.83
<b>SEM</b>	0.35	0.43	0.29	0.22	0.30	0.43
<b>3 Month</b>	-28.90	-29.90	-26.00	-35.3	-23.40	-33.6
	29.40	-30.9	-28.50	-36.4	28.10	-36.40
	29.20	-32.60	-27.90	-37.00	29.00	-35.00
<b>Mean</b>	-29.17	-31.13	-27.47	-36.23	-26.83	-35.00
<b>SEM</b>	0.05	0.25	0.24	0.16	0.55	0.26

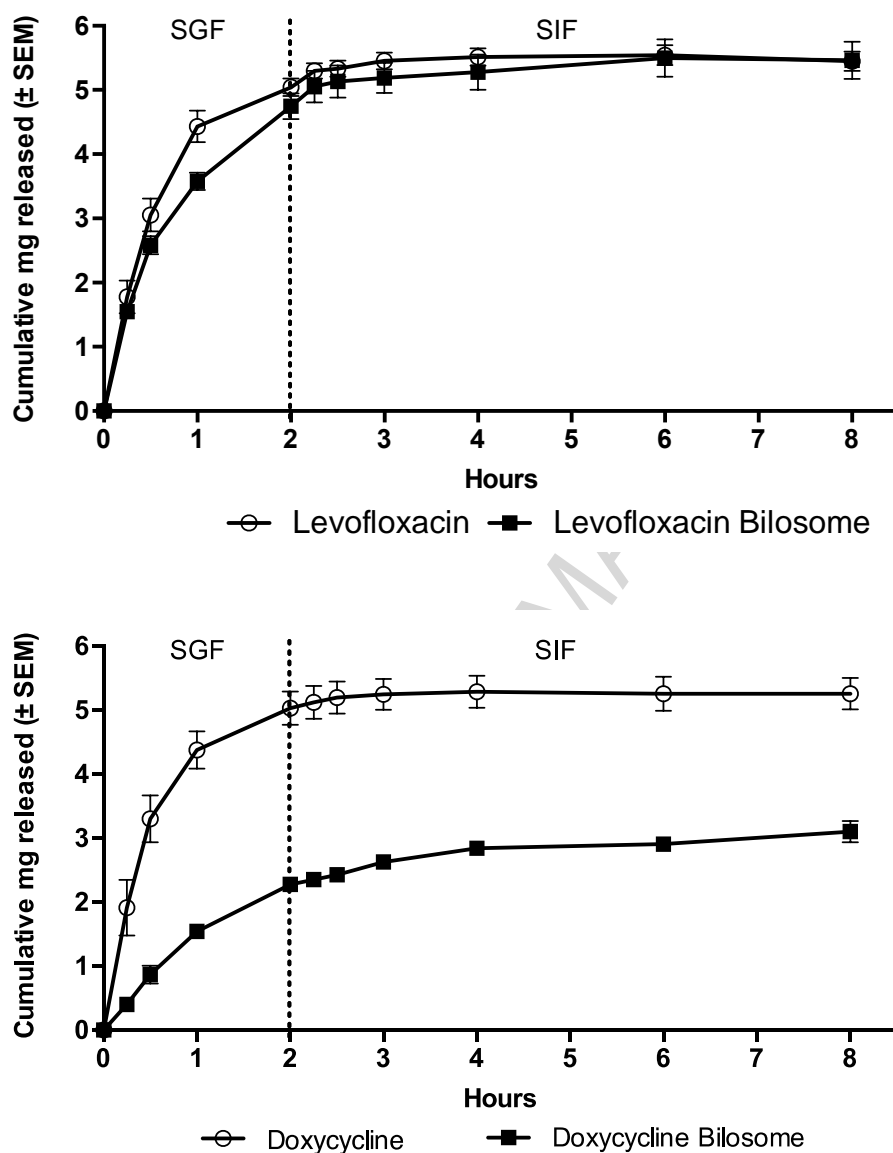
**Table 3 *In vitro* assessment of antimicrobial properties of formulated antibiotics by determination of Minimum Inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC)**

Values represented on the table are the median value generated from 3 independent experiments with 3 technical repeats in each experiment. MIC is the lowest concentration that inhibits growth i.e. Value is recorded when growth is less than 10% of positive control. Measured by OD. MBC is the lowest concentration that prevents 99.9% of positive control growth i.e. No bacterial colonies present from 10 $\mu$ l drops on agar plates.

Antibiotic Formulation	<i>Burkholderia pseudomallei</i> K96243	
	MIC ( $\mu$ g/ml)	MBC ( $\mu$ g/ml)
Free Levofloxacin	2	32
Bilosome Levofloxacin	4	8
Free Doxycycline	1	16
Bilosome Doxycycline	1	16

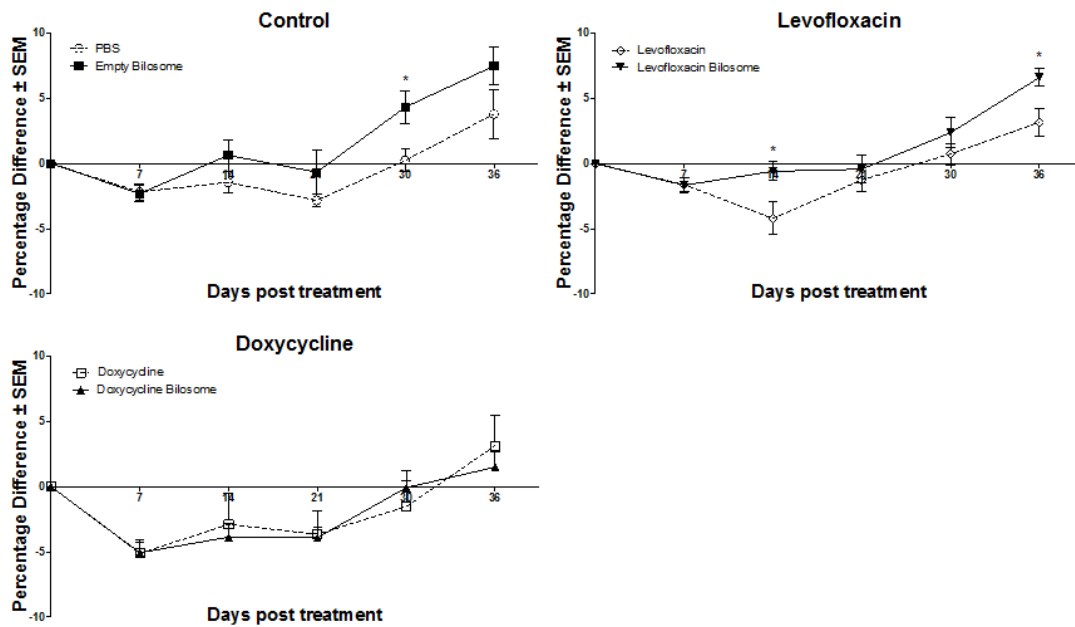
## Figures and Figure Legends

Figure 1



**Figure 1: Cumulative drug release profiles using USP-4 for free and bilosome encapsulated Levofloxacin or Doxycycline.** SGF buffer was used for the first 2 hours and exchanged to SIF for the remaining 6 hours of the study. Buffers were circulated at 20ml/min at 37°C ( $\pm 1^\circ\text{C}$ ). Drug concentrations were determined by HPLC. Results are from an  $N=3 \pm \text{SEM}$  and plotted as cumulative mg of drug released.

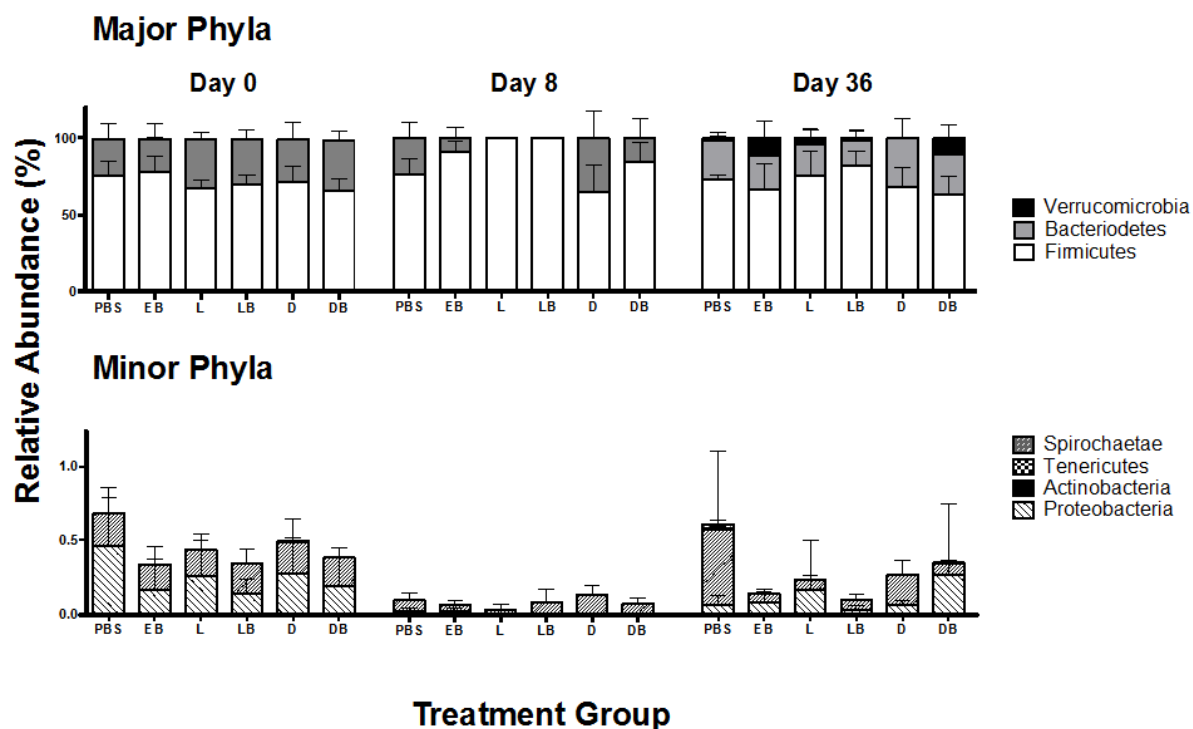
Figure 2



**Figure 2: Weight change following daily dosing with antibiotic formulations.**

Groups of 10 mice were dosed daily with 0.1 ml (at a concentration of 1mg per mouse) of each antibiotic. Bilosome formulations were administered by the oral route. Suitable controls (free antibiotic in PBS) were included for all groups. Mice were weighed daily and data recorded as percentage change from starting weight. Control panel compares PBS treated to empty bilosome treated. The levofloxacin and Doxycycline panels compare free drug to bilosome formulated. (\*  $p < 0.05$ )

Figure 3

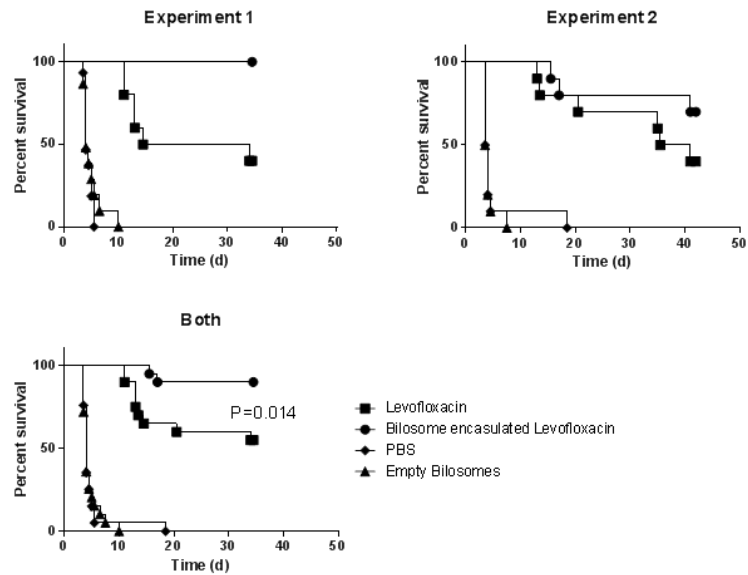


**Figure 3: Broad Microbiome analysis following treatment with antibiotic formulations**

Genus data was categorised into specific phyla to which they belong and percentage abundance for each treatment group was calculated. Data was graphed on area charts/stacked bar charts + SE. Major Phyla (Verrucomicrobia, Bacterioidetes and Firmicutes) presented on top panel with minor phyla (Spirochaetae, Tenericutes, Actinobacteria and Proteobacteria) presented at the bottom. PBS = Phosphate buffered saline, EB = Empty Bilosomes, L = Levofloxacin, LB = Levofloxacin Bilosomes, D = Doxycycline and DB = Doxycycline Bilosomes.

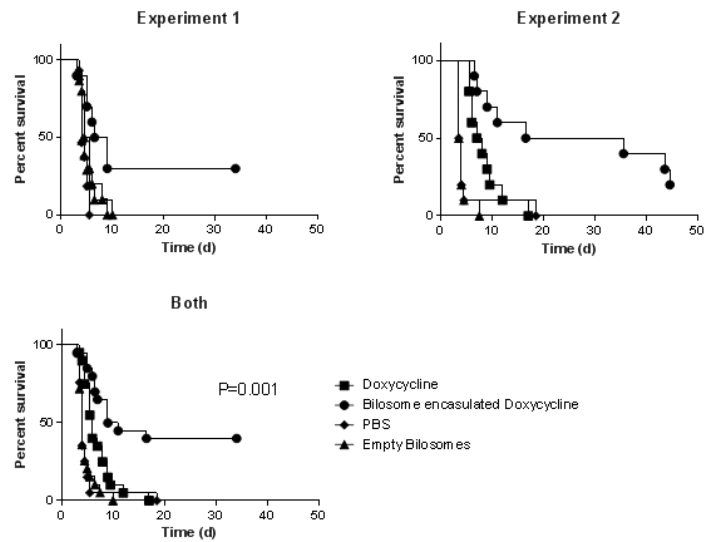


Figure 4



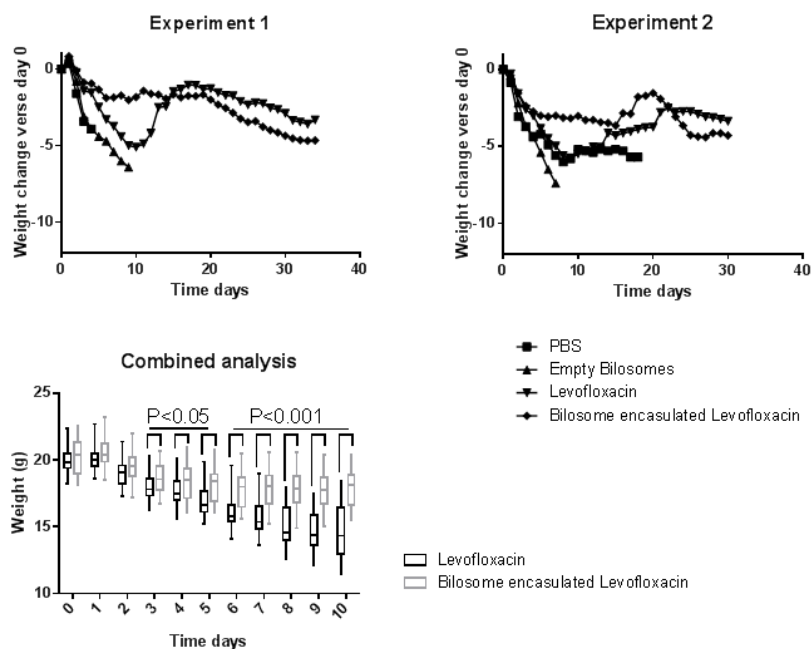
**Figure 4: Bilosome levofloxacin comparison to free levofloxacin in an aerosol model of *B. pseudomallei*.** Data represented on the graphs are Kaplan Meier plots, with 10 mice per group. Experiment 1 (top left panel), Experiment 2 (top right panel) and combined stratified data (bottom panel). PBS and Empty Bilosome treatment groups were included as controls.

Figure 5



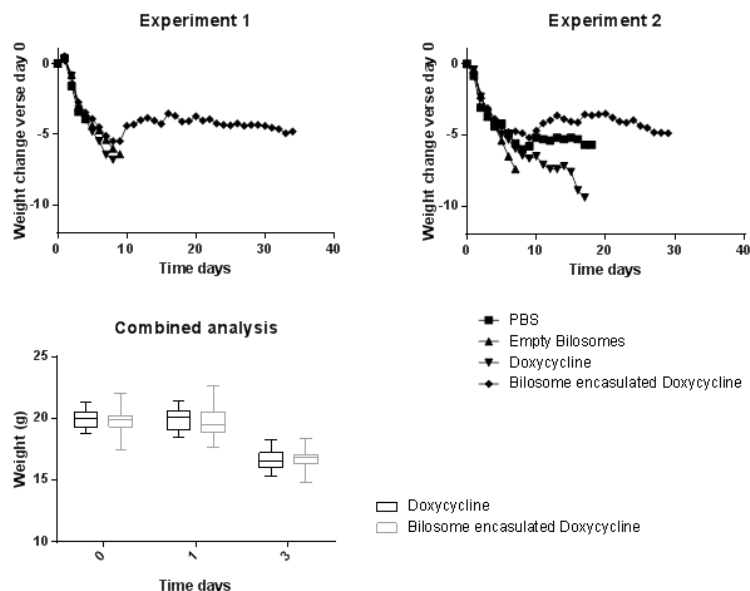
**Figure 5: Bilosome doxycycline comparison to free doxycycline in an aerosol model of *B. pseudomallei*.** Data represented on the graphs are Kaplan Meier plots, with 10 mice per group. Experiment 1 (top left panel), Experiment 2 (top right panel) and combined stratified data (bottom panel). PBS and Empty Bilosome treatment groups were included as controls.

Figure 6



**Figure 6: Animal weight data following exposure to an aerosol challenge of *B. pseudomallei* and treatment with levofloxacin or Bilosome levofloxacin. Data represented are plots for individual mice. Experiment 1 (top left panel), Experiment 2 (top right panel). Combined data comparing levofloxacin and Bilosome levofloxacin uses box and whisker plots with Inter Quartile Ranges (bottom panel). PBS and Empty Bilosome treatment groups were included as controls but omitted in the combined analysis.**

Figure 7



**Figure 7: Animal weight data following exposure to an aerosol challenge of *B. pseudomallei* and treatment with doxycycline or Bilosome doxycycline.** Data represented are plots for individual mice. Experiment 1 (top left panel), Experiment 2 (top right panel). Combined data comparing levofloxacin and Bilosome doxycycline uses box and whisker plots with Inter Quartile Ranges (bottom panel). PBS and Empty Bilosome treatment groups were included as controls but omitted in the combined analysis.

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**Graphical abstract****Highlights**

- Bilosomes are Non-Ionic Surfactant Vesicles suitable for oral delivery
- Bilosomes have been shown to entrap the antibiotics ciprofloxacin and doxycycline
- Bilosomes increase antibiotic efficacy in a Burkholderia model of infection
- Bilosomes can protect against antibiotic and infection induced weight loss

ACCEPTED MANUSCRIPT