Supplementary Information for:

Breathing new life into old antibiotics: Overcoming antibacterial resistance by antibiotic-loaded nanogel carriers with cationic surface functionality

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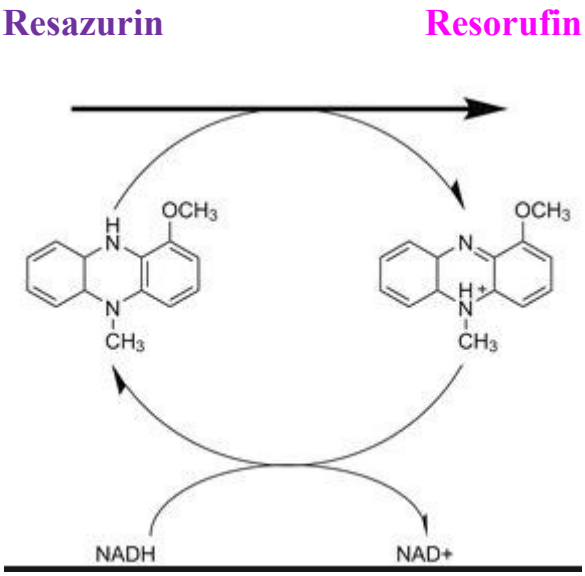

Figure S1. Resazurin reduction reaction. Resazurin in the presence of metabolically active cells is reduced to the Resorufin product, a blue colour indicates cells are inhibited and unable to reduce the Resazurin. Pink indicated the cells are metabolically active and are able to produce the pink Resorufin product.

Hydrodynamic diameter and zeta potential distributions of Carbopol Aqua SF1 nanoparticles

67 µL of the 30 wt% Carbopol Aqua SF1 solution was aliquoted and diluted to a final volume of 100 mL in deionized water yielding a 0.02 wt% Carbopol Aqua SF1 solution. Separate aliquots of the 0.02 wt% Carbopol Aqua SF1 solution was adjusted to a pH of 4-12 using droplets 0.25 M NaOH or 0.25 M HCl whilst been stirred on a magnetic plate. The particle size distribution was measured with a Mastersizer 2000 MS (1000 rpm, Malvern Instruments, UK) for each pH measurement. 1 mL of each aliquot was placed into a quartz cuvette prior to measurement. At pH 4-7 the refractive index was 1.450 and at pH 8-12 the refractive index of the swollen nanogel was 1.336, as determined by Al-Awady *et al*., J. Mater. Chem. B, 2017, 5, 7885-7897. The absorption was 1.000 and the temperature was 25^{o}C in both refractive index measurements. A zeta potential dipstick was added to the cuvette to measure the zeta potential distribution.

Zeta potential of wound bacterial species

Bacterial species were cultured as described in as described in the main article. The bacterial suspension was then centrifuged for 10 minutes at 5000 rpm and the supernatant discarded. The pellet was washed twice with deionized water and finally resuspended into 45 mL of deionized water. An1 mL aliquot of this suspension was then added to a quartz cuvette and the mean zeta potential distribution was measured using the Dipstick probe and a Malvern Mastersizer 2000 MS (1000 rpm, Malvern Instruments, UK). The refractive index was 1.384, the absorption 1.000, and the temperature was 25 ^{o}C for all measurements.

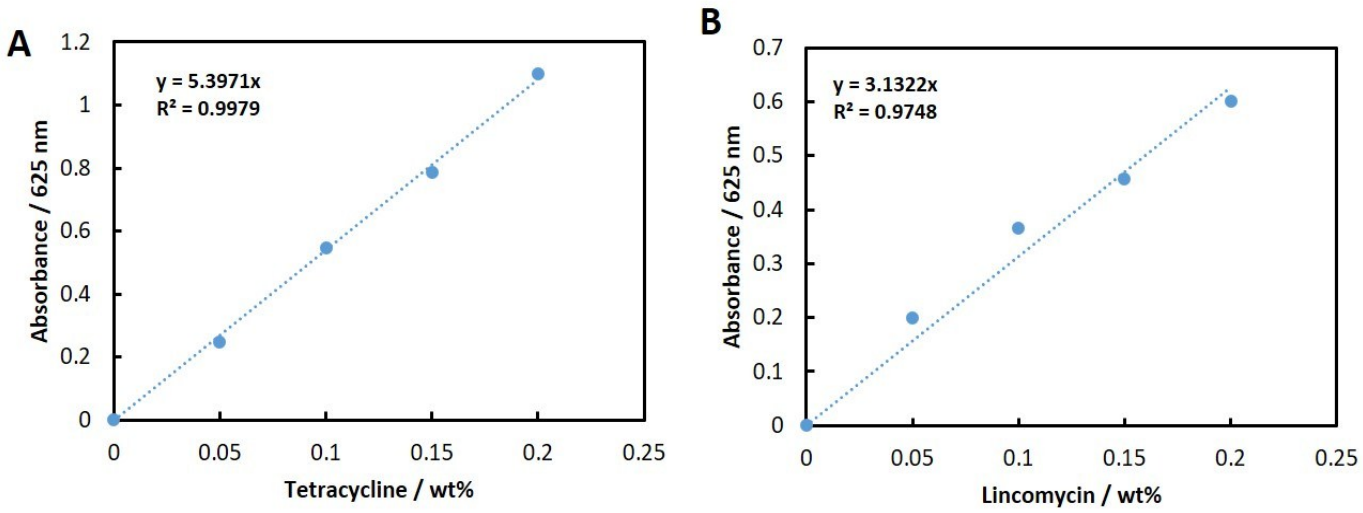

Figure S2. Standard calibration graphs of the absorption vs concentrations of (A) tetracycline and (B) lincomycin hydrochloride. Absorbance was measured at 625 nm. Aliquots were prepared by adding antibiotic hydrochlorides to deionized water at specific concentrations.

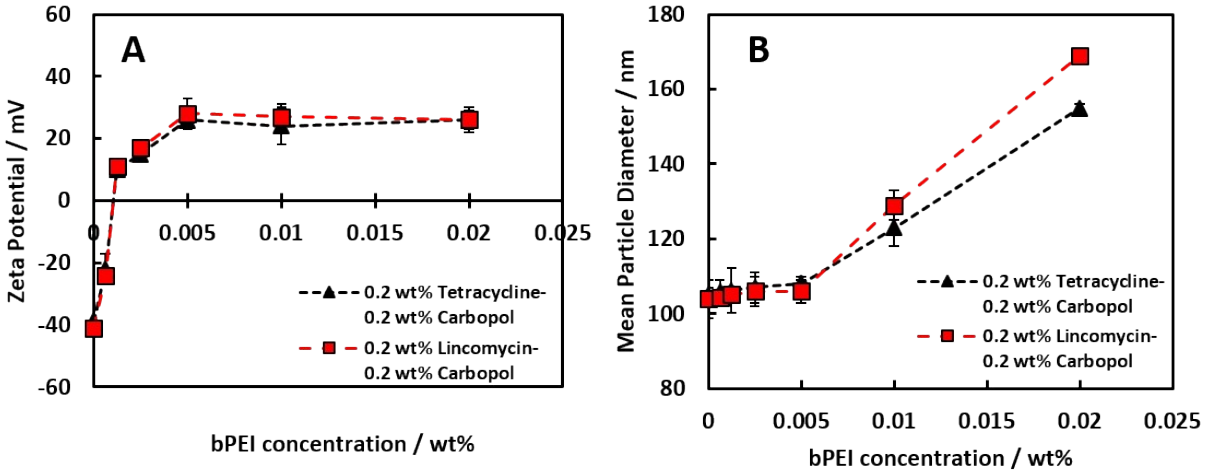

Figure S3. (A) The zeta potential of 0.2 wt% tetracycline-0.2 wt% Carbopol and 0.2 wt% lincomycin-0.2 wt% Carbopol NPs coated with various concentrations of bPEI and (B) the mean particle diameter of 0.2 wt% tetracycline-0.2 wt% Carbopol and 0.2 wt% lincomycin-0.2 wt% Carbopol NPs coated with various concentrations of bPEI. Each value represents a triple replicate with ± S.D. The lines are guides to the eye.

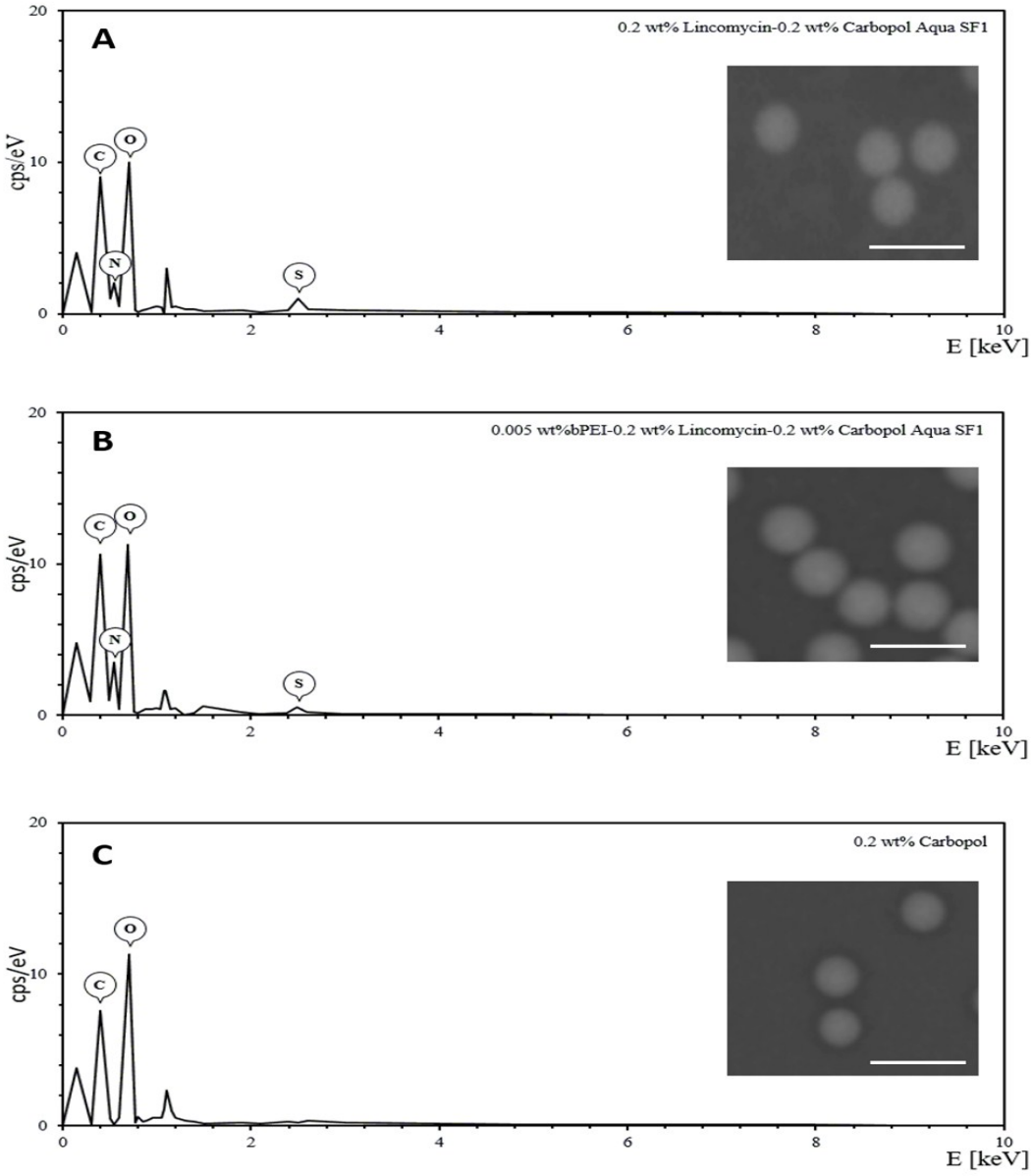

Figure S4. SEM images and EDX spectra of Carbopol Aqua SF-1 NPs. (A) shows spectra of 0.2 wt% lincomycin-0.2 wt% Carbopol Aqua SF1, (B) shows 0.005 wt% bPEI-0.2 wt% Lincomycin-0.2 wt%-0.2 wt% Carbopol Aqua SF1, and (C) 0.2 wt% empty Carbopol Aqua SF1. The measurement was taken in the centre of the NPs. Oxford Laboratories micF+ X-stream-2 EHX was used to take measurements, the results were analysed in Aztec One v.3.3. Images were acquired using a TM3030 Plus SEM. Scale bars represent 200 nm.

SEM images were acquired of the showing 0.2 wt% Carbopol loaded with 0.2 wt% lincomycin, 0.005 wt% bPEI-0.2 wt% lincomycin-0.2 wt% Carbopol, and 0.2 wt% Carbopol (empty nanocarrier). Lincomycin was chosen for the characterisation due to the presence of a sulphur atom in the molecular structure. EDX spectra shows the presence of sulphur in the lincomycin loaded Carbopol NPs (see Figure S4, ESI). This spectra peak is not present in the unloaded nanogel carrier. As the source of the sulphur is the loaded lincomycin, this additionally confirms that it has been successfully loaded into the nanocarrier, agreeing with the dynamic light scattering results (Figures 3A, 3C showing the nanocarrier size increase upon loading) and release curves (Figure 5) presented in the manuscript.

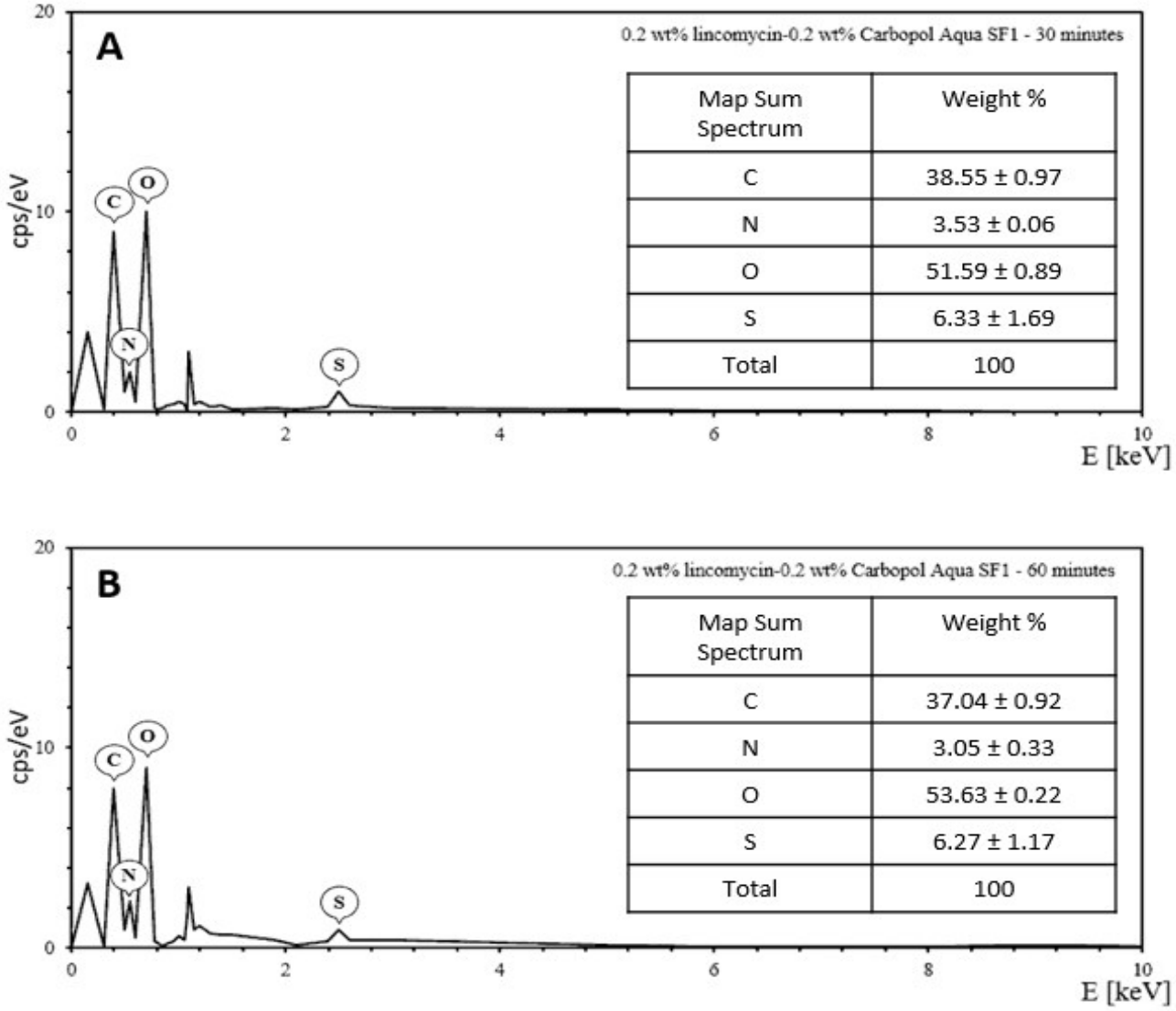

Figure S5. EDX spectra of Carbopol Aqua SF-1 NPs after 30 and 60 minutes incubation with lincomycin. (A) shows the EDX spectra of a sample of 0.2 wt% lincomycin-0.2 wt% Carbopol Aqua SF1 after 30 min loading time with the antibiotic at 37 ^{o}C, (B) shows 0.2 wt% Lincomycin-0.2 wt%-0.2 wt% Carbopol Aqua SF1 after 60 min loading time with the antibiotic at 37 ^{o}C. The measurement was taken in the centre of the nanocarrier NPs. Oxford Laboratories micF+ X-stream-2 EHX was used to take measurements, the results were analysed in Aztec One v.3.3.

Our experiments indicated that 30 min at 37 ^{o}C was the optimum time to incubate the antibiotic solution with the swollen Carbopol nanogel in order to gain the maximum encapsulation efficiency. Incubation times greater than this showed no increase in encapsulation efficiency. We demonstrate this using EDX data from lincomycin encapsulated Carbopol. Lincomycin was chosen as it contains a sulphur atom which can be detected on the EDX spectra. One can see that the sulphur-related peak in Figure S5B (60 min incubation) did not yield a significant increase compared with the sulphur-related peak on Figure S5A (30 incubation). Hence we can conclude that no further lincomycin was encapsulated into the nanocarrier after the 30 minutes incubation with lincomycin solution at identical conditions.

Table S1. The concentration of tetracycline encapsulated into 0.2 wt% Carbopol Aqua SF1 nanogel.

Carbopol-antibiotic mixture environment	Total tetracycline conc. attempted to encapsulate / wt%	Tetracycline in supernatant / wt%	Tetracycline encapsulated / wt%	Encapsulation efficiency / wt%
15 mins, 20 °C	0.2	0.042	0.158	78.9
15 mins, 37 °C	0.2	0.026	0.174	86.9
30 mins, 20 °C	0.2	0.032	0.168	84
30 mins, 37 °C	0.2	0.02	0.18	89.9

Table S2. The concentration of lincomycin encapsulated into 0.2 wt% Carbopol Aqua SF1 nanogel.

Carbopol-antibiotic mixture environment	Total lincomycin conc. attempted to encapsulate / wt%	Lincomycin in supernatant / wt%	Lincomycin encapsulated / wt%	Encapsulation efficiency / wt%
15 mins, 20 °C	0.2	0.095	0.105	52.4
15 mins, 37 °C	0.2	0.078	0.122	61
30 mins, 20 °C	0.2	0.081	0.119	59.5
30 mins, 37 °C	0.2	0.041	0.159	79.5

Table S3. Antimicrobial resistance tests of tetracycline and lincomycin by disk diffusion method for wound species.

Bacterial strains		Antibiotic disk	
Species	Origin	Tetracycline (30 µg) zone diameter (mm)	Lincomycin (15 µg) zone diameter (mm)
S. aureus	Ref 21213	**32** (S, 3/3)	**30** (S, 3/3)
S. epidermidis	Leg ulcer	**34** (S, 3/3)	**24** (S, 3/3)
MRSA	Leg ulcer	**32** (S, 3/3)	**26** (S, 3/3)
S. aureus	Cyst	**26** (S, 3/3)	**26** (S, 3/3)
P. aeruginosa	Leg ulcer	**12** (R, 3/3)	**10** (R, 3/3)
E. faecalis	Ulcer	**0** (R, 3/3)	**0** (R, 3/3)
S. pseudointermedius	Burn swab	**28** (S, 3/3)	**30** (S, 3/3)
P. aeruginosa	Ref 27853	**9** (R, 3/3)	**0** (R, 3/3)

Results of zone of inhibition repeats are in brackets i.e. S=susceptible, R=resistant, for zone diameter interpretation (EUCAST 8.1 (16 May 2018)) standards were used where available.

Table S4. Minimum inhibitory concentration (MIC) of free tetracycline and bPEI-coated nanogel loaded with the same concentration of tetracycline against several wound species clinical isolates. The lowest concentration of antimicrobial agent inhibiting growth was considered the MIC. > indicates that a concentration greater than 0.2 wt% is needed to inhibit growth.

	Tetracycline MIC (wt%)	**Encapsulated tetracycline MIC (wt%)**
***S. aureus* (ATCC - 21213)**	0.0125	0.00625
***S. aureus* (wound strain)**	0.025	0.0125
S. epidermidis	> 0.2	0.00625
MRSA	> 0.2	0.0125
S. psuedointermedius	0.0125	0.003125
***P. aeruginosa* (ATCC - 27853)**	> 0.2	0.025
***P. aeruginosa* (wound strain)**	> 0.2	0.025
E. faecalis	> 0.2	0.025

Table S5. Minimum inhibitory concentration (MIC) of free lincomycin and bPEI-coated nanogel loaded with the same concentration of lincomycin against several wound species clinical isolates. The lowest concentration of antimicrobial agent inhibiting growth was considered the MIC. > indicates that a concentration greater than 0.2 wt% is needed to inhibit growth.

	Tetracycline MIC (wt%)	**Encapsulated tetracycline MIC (wt%)**
***S. aureus* (ATCC - 21213)**	0.0125	0.00625
***S. aureus* (wound strain)**	0.05	0.0125
S. epidermidis	> 0.2	0.00625
MRSA	> 0.2	0.025
S. psuedointermedius	0.00625	0.003125
***P. aeruginosa* (ATCC - 27853)**	> 0.2	0.05
***P. aeruginosa* (wound strain)**	> 0.2	0.025
E. faecalis	> 0.2	0.025

Note that the cationic antibiotic alone can also adhere and potentially internalize through the bacterial cells. However, resistant bacteria often deploy efflux pumps to overcome the internalization of antibiotics. Unless the free antibiotic concentration is sufficiently high, these efflux pumps can easily cope with low-to-moderate antibiotic concentrations. Indeed, such type of antibiotic resistance can be overcome with conventional antibiotics, but at concentrations that far exceed the safe clinical dosage. Long term treatment and high dosages of antibiotics are known to lead to negative physiological conditions for patients, particularly hepatic and renal failures. We envisage that the reason our cationic nanogel-antibiotic formulation results in an increased antibacterial performance is due to the localised and sustained release of high concentration of the antibiotic from the nanogel carrier accumulated on the bacteria surface. Our methodology allows high concentrations of antibiotic to be delivered locally on the bacteria surface even at low nanocarrier concentration (as it concentrates on the bacteria), which can be effective even when the overall antibiotic concertation in the treatment formulation is low.

Table S6. Time-Kill assay statistical analysis between free tetracycline and encapsulated tetracycline at 6 and 24-hour time points. Data were expressed as average values ± standard deviations of the mean. *P*-values of less than 0.05 were considered significant. All One-Way ANOVAs and Tukey's post-test statistical analysis were performed in GraphPad v7.0.4.

Species	Multiple Comparison	P-value	Significance
***S. aureus* (ATCC - 21213)**	6 hour tetracycline vs 6 hour formulated tetracycline	0.02270416	*
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00401855	**
S. epidermidis	6 hour tetracycline vs 6 hour formulated tetracycline	0.03562452	*
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00145661	**
MRSA	6 hour tetracycline vs 6 hour formulated tetracycline	0.00238776	**
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00165489	**
***S. aureus* (wound strain)**	6 hour tetracycline vs 6 hour formulated tetracycline	0.00215412	**
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00545155	**
***P. aeruginosa* (wound strain)**	6 hour tetracycline vs 6 hour formulated tetracycline	0.00025125	***
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00014587	***
E. faecalis	6 hour tetracycline vs 6 hour formulated tetracycline	0.03454452	*
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00654552	**
S. pseudointermedius	6 hour tetracycline vs 6 hour formulated tetracycline	0.00245125	**
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00004412	**
***P. aeruginosa* (ATCC - 27853)**	6 hour tetracycline vs 6 hour formulated tetracycline	0.00021451	***
	24 hour tetracycline vs 24 hour formulated tetracycline	0.00019857	***

< 0.05 is considered significant. *P <0.05, **P <0.01, ***P <0.001

Table S7. Time-Kill assay statistical analysis between free lincomycin and encapsulated lincomycin at 6 and 24-hour time points. Data were expressed as average values ± standard deviations of the mean. *P*-values of less than 0.05 were considered significant. All One-Way ANOVAs and Tukey's post-test statistical analysis were performed in GraphPad v7.0.4.

Species	Multiple Comparison	P-value	Significance
***S. aureus* (ATCC - 21213)**	6 hour lincomycin vs 6 hour formulated lincomycin	0.00021545	***
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00025453	***
S. epidermidis	6 hour lincomycin vs 6 hour formulated lincomycin	0.00157455	**
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00068546	***
MRSA	6 hour lincomycin vs 6 hour formulated lincomycin	0.01589456	*
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00259874	**
***S. aureus* (wound strain)**	6 hour lincomycin vs 6 hour formulated lincomycin	0.00486549	**
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00845212	**
***P. aeruginosa* (wound strain)**	6 hour lincomycin vs 6 hour formulated lincomycin	0.00095455	***
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00026845	***
E. faecalis	6 hour lincomycin vs 6 hour formulated lincomycin	0.00985454	**
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00061795	***
S. pseudointermedius	6 hour lincomycin vs 6 hour formulated lincomycin	0.00245214	**
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00079878	***
***P. aeruginosa* (ATCC - 27853)**	6 hour lincomycin vs 6 hour formulated lincomycin	0.02458651	*
	24 hour tetracycline vs 24 hour formulated lincomycin	0.00751254	**

< 0.05 is considered significant. *P <0.05, **P <0.01, ***P <0.001.