Sonodynamic Inactivation of Gram Positive and Gram Negative Bacteria using a Rose Bengal-Antimicrobial Peptide Conjugate

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Running title: Antimicrobial SDT.

Abstract

Combating antimicrobial resistance (AMR) is one of the most serious public health challenges facing society today. The development of new antibiotics or alternative techniques that can help combat AMR is a priority of many governments across the globe. Antimicrobial Photodynamic Therapy (APDT) is one such technique that has received considerable attention but is limited by the ability of light to penetrate deeply through human tissue reducing its effectiveness when used to treat deeply seated infections. The related technique sonodynamic therapy (SDT) has the potential to overcome this limitation given the ability of low intensity ultrasound to penetrate deeply through human tissue. In this manuscript, we have prepared a Rose Bengal-antimicrobial peptide conjugate for use in antimicrobial SDT (ASDT). We evaluate the ASDT efficacy of this conjugate upon irradiation with ultrasound in both *S. aureus* and *P. aeruginosa* bacterial strains. The ability of the conjugate to preferentially target bacteria over mammalian cells was also determined as was the ability of ultrasound to enhance the uptake of sensitisers through bacterial biofilms. Combined, the results from this study highlight ASDT as a targeted broad-spectrum modality with potential for the treatment of deeply-seated bacterial infections.

Keywords: Sonodynamic Therapy; antimicrobial; sensitiser; peptide

1. Introduction

Although the threat of antibiotic resistance has been prophesised for years, the issue has recently been described as an “apocalyptic scenario” by the UK’s chief medical officer representing “one of the most significant public health challenges facing society today”.[1] With 80% of gonorrhoeal infections now resistant to antibiotics and a reported 440,000 new cases of drug resistant tuberculosis per year, it has been suggested that we are fast approaching a post-antibiotic era.[2,3] This threat is not confined to systemic infections with the problem equally apparent in localised wound infection. Surgical wound infections account for 25% of nosocomial infections and result in a 2.5 times longer hospital stay with additional costs of ~£5,000 per patient.[4] Diabetic foot ulcers (DFU) and burns are equally problematic. In the US alone, 25 million people are estimated to have Diabetes Mellitus and 15-25% will develop DFU during their lifetime.[5] Over 50% of these ulcerations will become infected resulting in increased hospital admissions, amputation rates and mortality with an estimated one in six patients dying within 1 year of their infection.[6] The overall impact of this on both the patient and health service provider is significant and highlights an urgent need for alternative therapies.

Photodynamic therapy (PDT) is a clinical treatment that uses a combination of light, molecular oxygen and a photosensitising drug to generate a cytotoxic effect.[7] When the sensitiser absorbs light of an appropriate wavelength, the excited triplet state interacts with molecular oxygen by electron (Type I) or energy (Type II) transfer processes that result in the generation of cytotoxic singlet oxygen and other reactive oxygen species (ROS). Because of the high reactivity and short half-life (0.04 μs) of singlet oxygen, its diffusion radius is less than 20 nm meaning only cells close to the site of its generation are affected.[8] While predominantly used in the treatment of cancer, antimicrobial PDT (APDT) has also received considerable interest for the treatment of microbial infections.[9-11] The major attraction of APDT over conventional antibiotics is that multiple antibiotic resistant (AMR) strains are as easily killed as native strains and because it results in the production of

multiple forms of ROS, resistance to PDT is less likely to occur.[12] However, PDT is severely limited by the inability of light to penetrate to depth through mammalian tissue. This is due to endogenous pigments such as haem or melanin competing for light absorption with the sensitiser and is a particular problem in localised infection where the wound area may be severely discoloured due to bruising or inflammation.[13] Currently approved sensitisers absorb in the visible region of the electromagnetic spectrum limiting light penetration to only a few millimetres and reducing the ability of APDT to eradicate bacteria localised deeper within infected wounds.[14]

In recent years it has been demonstrated that many of the existing clinically-used photosensitisers can be 'activated' by ultrasound, although the precise mechanism(s) by which this occurs remain(s) unknown.[15-18] This approach has become known as Sonodynamic Therapy (SDT). Ultrasound can be tightly focused with penetration in soft tissue up to several tens of centimetres depending on the frequency used.[19] The efficacy of SDT as an anti-cancer treatment has been demonstrated in numerous pre-clinical and clinical studies.[20-23] Antimicrobial SDT (ASDT) has also emerged as an active area of research but reports to date have used clinically unsuitable ultrasound equipment / conditions and have not explored the potential damage of the treatment on host tissue.[24-26] As is the case for APDT, a major challenge for ASDT is specifically targeting the sensitiser to bacterial cells to reduce collateral damage to host tissue. A surgical site infection can be defined as a suppurating wound containing a variety of components such as host tissue (skin cells, muscle cells and extracellular matrix components), immune cells and bacterial cells (both live and dead).[27,28] The bacterial load can be as low as 10^5 bacteria (i.e. μg quantities) per gram of tissue meaning the majority of this complex environment is host tissue essential in the healing process.[29] As the cytotoxic agent(s) involved in APDT / ASDT are indiscriminate in their action on host or bacterial cells, it is imperative the sensitiser is preferentially directed to bacterial cells rather than host cells before activation with light or ultrasound. One method to achieve sensitiser selectivity is to exploit the differential binding

exhibited by cationic species to the cell wall of bacterial and mammalian cells. For example, it has been demonstrated that light irradiation of wounds in mice treated with a poly-L-lysine-chlorin(e6) conjugate exhibited a greater bacterial kill and less host tissue damage than the free sensitiser alone.[30] Similarly, when the antimicrobial peptide $(KLAKLAK)_2$ was conjugated to the sensitiser eosin, its antimicrobial photodynamic activity was enhanced with negligible photo-damage observed to normal cells.[31]

Inspired by these results, we have developed a Rose Bengal-$(KLAKLAK)_2$ conjugate for use in targeted ASDT. The potential of the conjugate to generate ROS during exposure to ultrasound was determined in cell-free solution and the antimicrobial efficacy was established using both *Staphylococcus aureus* and *Pseudomonas aeruginosa* as target microorganisms. The ability of the conjugate to preferentially target bacteria over healthy mammalian cells was also determined. Finally, the effectiveness of ultrasound to enhance the diffusion of sensitisers through bacterial biofilms was investigated.

2. Results and Discussion

The Rose Bengal-C(KLAKLAK)$_2$ conjugate was prepared by first synthesising the C(KLAKLAK)$_2$ peptide using Fmoc solid phase peptide synthesis on Rink Amide resin. In parallel, a carboxylic acid derivative of Rose Bengal was also prepared by reacting Rose Bengal with 1-bromooctanoic acid. This carboxylic acid derivative was added to the N-terminus of C(KLAKLAK)$_2$ while still on the resin using standard peptide coupling reagents (i.e. HOBt / TBTU).The Rose Bengal-C(KLAKLAK)$_2$ conjugate was then cleaved from the resin and purified using preparative reverse phase HPLC. Product formation was confirmed using MALDI-TOF and positive electrospray mass spectrometry (Fig S1).

The ability of the Rose Bengal-C(KLAKLAK)$_2$ conjugate to generate ROS upon exposure to low intensity ultrasound was determined using the chromogenic ROS probe 1,3-diphenylisobenzofuran (DPBF).[32] DPBF has an intense absorbance band centred at 410 nm in its native furan form but is readily bleached by ROS to the corresponding di-ketone. This conversion to the di-ketone is accompanied by a loss in absorbance at 410 nm that can be used to determine the amount of ROS produced. Solutions containing either Rose Bengal or Rose Bengal-C(KLAKLAK)$_2$ and DPBF were treated with ultrasound for 30 min and the DPBF absorbance at 410 nm measured every 5 min. The results are shown in figure 1 and show a significant reduction in DPBF absorbance for both Rose Bengal or Rose Bengal-C(KLAKLAK)$_2$ treated with ultrasound relative to the controls indicating efficient ROS production in the ultrasonic field. In addition, the almost identical profile observed for both Rose Bengal and Rose Bengal-C(KLAKLAK)$_2$ suggests the presence of the peptide does not inhibit ultrasound-induced ROS production by the sensitiser.

To determine the antimicrobial potential of this ROS generation, two candidate bacterial strains, Gram positive *S. aureus* and Gram negative *P. aeruginosa*, were subjected to ASDT treatment. In each case, suspensions containing 10^8 bacteria were added to the wells of a 96-well plate and incubated with 10 μM Rose Bengal or Rose Bengal-C(KLAKLAK)$_2$ for 30

145 min. The wells were then treated with ultrasound from the underside of the plate for either 10
146 min (*S. aureus*) or 6 min (*P. aeruginosa*). Following treatment, the number of viable bacteria
147 remaining was determined and expressed as CFU/mL. The results, shown in figure 2, reveal
148 that ultrasound treatment of *S. aureus* produces only a minor reduction (~0.5 log) in bacterial
149 number that was not statistically significant. Treatment of *S. aureus* with Rose Bengal-
150 C(KLAKLAK)$_2$ in the absence of ultrasound produced an ~1 log reduction in bacterial
151 number. This reduction was attributed to the antimicrobial effect from the AMP component of
152 the Rose Bengal-C(KLAKLAK)$_2$ conjugate as Rose Bengal alone in the absence of
153 ultrasound produced no change in bacterial number (data not shown). The magnitude of this
154 reduction is consistent with other literature where (KLAKLAK)$_2$ alone has been shown to
155 possess little activity against Gram positive bacteria.[31] However, when Rose Bengal-
156 C(KLAKLAK)$_2$ was combined with ultrasound treatment, a statistically significant 5 log
157 reduction in bacterial number was observed. This suggests that the ROS generated upon
158 interaction of ultrasound with the Rose Bengal component of Rose Bengal-C(KLAKLAK)$_2$
159 produces the desired antimicrobial effect. When this experiment was repeated using the
160 same concentration of Rose Bengal (i.e. without AMP attached) and the same ultrasound
161 conditions, the reduction in bacterial numbers was approximately one log less than for Rose
162 Bengal-C(KLAKLAK)$_2$ plus ultrasound. This difference, while not statistically significant,
163 suggests the slight antimicrobial effect observed for Rose Bengal-C(KLAKLAK)$_2$ alone (i.e.
164 no ultrasound) complements the ASDT effect of Rose Bengal.
165
166 It is generally considered that PDT is more toxic to Gram positive than Gram negative
167 bacteria and it has been suggested that this is due to structural differences in cell wall
168 composition.[33] Given that both the sensitisers used and the cytotoxic species generated (i.e.
169 ROS) are the same in PDT and SDT, one would expect that Gram negative bacteria would
170 also be more difficult to kill using SDT. Indeed, when *P. aeruginosa* was treated with Rose
171 Bengal and ultrasound, only a minor reduction in bacterial number was observed (~ 0.5 log)
172 which was considerably lower than for *S. aureus*. However, when *P. aeruginosa* was treated

with the Rose Bengal-C(KLAKLAK)$_2$ conjugate and ultrasound the results were even more dramatic than for *S. aureus*, with a 7 log reduction in CFU observed (Fig.2b). This large reduction in bacterial number cannot be explained by the antimicrobial nature of the peptide alone as treatment of *P. aeruginosa* with Rose Bengal-C(KLAKLAK)$_2$ in the absence of ultrasound produced a much lower 3.5 log reduction in bacterial number, suggesting the peptide positions the sensitiser close enough to the bacteria to exert its cytotoxic effect during ultrasound irradiation. To probe this interaction further, we incubated suspensions of both *S. aureus* and *P. aeruginosa* with different amounts of the Rose Bengal-C(KLAKLAK)$_2$ conjugate and measured the zeta potential before and after conjugate addition. Both bacterial strains showed strongly negative zeta potentials (-42.0 and -27.0 mV respectively) which are consistent with literature precedent.[34,35] Upon addition of increasing amounts of Rose Bengal-C(KLAKLAK)$_2$, the net charge of both bacteria increased but with significantly different magnitudes (Fig.3). For example, addition of 10 μM Rose Bengal-C(KLAKLAK)$_2$ to *P. aeruginosa* resulted in a 2.0 mV increase in zeta potential while for *S. aureus* an increase of 29.7 mV was observed. Indeed, only when 50 μM Rose Bengal-C(KLAKLAK)$_2$ was added to *P. aeruginosa* did the charge become positive while for S. aureus this occurred after only 10 μM. These results confirm a direct interaction between the positively charged peptide and negatively charged bacterial cell wall with *P. aeruginosa* requiring a significantly greater number of Rose Bengal-C(KLAKLAK)$_2$ molecules to bind in order to titrate the more negative surface charge.

Systemic delivery of sensitisers is not normally considered in APDT as damage to capillaries and host cells directly supplied by them is undesirable.[36] Therefore, while local administration is preferred, this form of delivery still requires the sensitiser to be targeted to bacteria so that collateral damage to host tissue crucial to the healing process can be minimised. To determine the ability of Rose Bengal-C(KLAKLAK)$_2$ to preferentially target bacteria over mammalian cells, solutions containing Rose Bengal or Rose Bengal-C(KLAKLAK)$_2$ were incubated with suspensions containing *S. aureus*, *P. aeruginosa* or

human fibroblast (HS27) cells for either 10, 20 or 30 min. Following incubation, the suspensions were centrifuged, the cells lysed and the Rose Bengal concentration determined using UV-Vis spectroscopy. The results are shown in Fig 4 and reveal a significantly enhanced uptake of the Rose Bengal-C$(KLAKLAK)_2$ in both bacteria compared to the Hs27 cells at the time points tested. Indeed, the uptake of Rose Bengal-C$(KLAKLAK)_2$ conjugate was also higher than Rose Bengal in both bacteria while it was generally lower in the Hs-27 cells which is ideal for bacterial targeting.

The presence of biofilms is a significant challenge associated with the local delivery of sensitiser drugs as it can act as a barrier between the applied sensitiser and bacteria. With as many as 80% of SSI's involving a microbial biofilm, strategies that can enhance dispersion of drugs through biofilms offer a significant advantage. It has been demonstrated that in addition to increasing the permeability of membranes through sonoporation, shear forces induced by ultrasound generates pores in the architecture of biofilms, enhancing the effectiveness of antibiotic treatment.[37] To test this hypothesis, we generated *P. aeruginosa* biofilms on the surface of trans-well inserts and tested the diffusion of Rose Bengal through the biofilm in the presence and absence of ultrasound (Fig 5a). Preliminary data (Fig 5b) show that pre-treatment of the biofilm with low intensity ultrasound for 5 min before addition of Rose Bengal produced a 2.6-fold increase in sensitiser diffusion through the biofilm compared to the untreated biofilm control. These results suggest that ultrasound can facilitate the dispersion of sensitisers through biofilms and potentially improve the efficacy of ASDT.

Having established the effectiveness of the SDT approach *in vitro* we were also interested if a similar effect would be observed *in vivo*. To determine this, wound abrasions (0.5 cm^2) were established in the dorsum of Balb/c mice and inoculated with a bioluminescent strain of *P. aeruginosa*. Once the infection had established, bioluminescent images were recorded using an IVIS whole body imaging system. The wound was then treated with a PBS solution

containing the Rose Bengal-C(KLAKLAK)$_2$ conjugate (4.5mg/kg) and 10 min later exposed to ultrasound. Bioluminescent images were then recorded 1 h and 24 h after ultrasound treatment. Control groups involving no treatment or treatment with Rose Bengal-C(KLAKLAK)$_2$ or ultrasound alone were also undertaken for comparative purposes. Representative images of the mice are shown in figure 6 and reveal substantial reductions in bioluminescent intensity for mice treated with the conjugate alone or SDT, with the SDT image being less intense, particularly after 24h. In contrast, the bioluminescent intensity of the untreated and ultrasound only groups were substantially more intense than the Rose Bengal-C(KLAKLAK)$_2$ or SDT treated animals. This pattern follows a similar trend to the results obtained for the *in vitro* experiments undertaken using *P. aeruginosa* where Rose Bengal-C(KLAKLAK)$_2$ alone produced a modest 3.5 log reduction while SDT treatment resulted in a much greater 7 log reduction. It was also apparent from the images presented in Figure 6 that the size of the wound 24 h following SDT treatment was much smaller when compared to 1 h following SDT treatment suggesting a degree of wound healing, a feature that was not apparent in any of the other groups. While there is an obvious limitation in the small sample size used in these experiments, the results do suggest that SDT using Rose Bengal-C(KLAKLAK)$_2$ is capable of substantially reducing bacterial burden in an *in vivo* model of localised infection. Interestingly the results also suggest that our approach does not elicit any collateral damage on host tissues. We are currently designing a larger animal study involving both *MRSA* and *P. aeruginosa* infection models and will report on this in due course.

In conclusion, a Rose Bengal-C(KLAKLAK)$_2$ conjugate has been prepared for use in targeted ASDT. A broad-spectrum ASDT effect was observed when the conjugate was used to treat *S. aureus* and *P. aeruginosa* in the presence of low intensity ultrasound. The conjugate also displayed improved uptake by these bacterial strains when compared to a mammalian cell line which promises to minimise damage to host tissue when considering *in vivo* ASDT applications. In addition, pre-treatment of a *P. aeruginosa* biofilm with low

intensity ultrasound before application of Rose Bengal enhanced diffusion of the sensitiser through the biofilm. A preliminary pilot *in vivo* experiment provided qualitative evidence of a substantial reduction in bacterial burden without collateral damage to host tissues when a *P. aeruginosa* infected wound was treated with SDT using the Rose Bengal-C(KLAKLAK)$_2$ conjugate. Combined, these results suggest that ASDT using Rose Bengal-C(KLAKLAK)$_2$ is an effective broad-spectrum antimicrobial technique with the potential to activate sensitisers at a much greater depth in human tissue than APDT enabling the treatment of more deep-seated infections.

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Declarations

Funding: None

Competing Interests: None

Ethical Approval: Not required

Supporting Information

Containing detailed materials & methods and characterisation of Rose Bengal-C(KLAKLAK)$_2$ conjugate.

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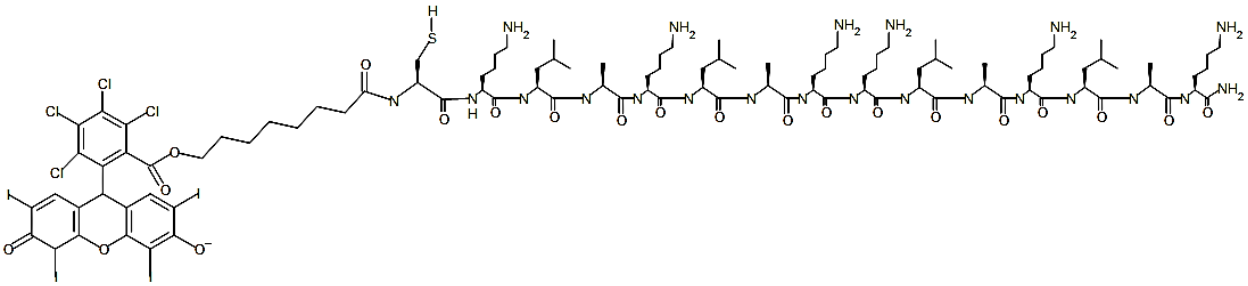
Figures and Diagrams



Scheme 1 Structure of Rose Bengal-C(KLAKLAK)$_2$.

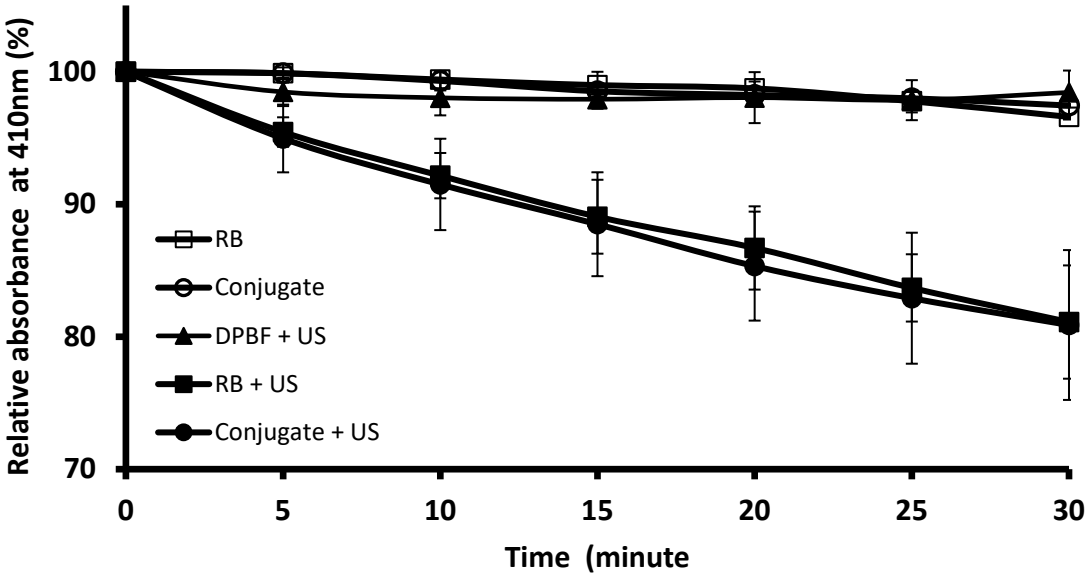


Figure 1 Plot of DPBF absorbance at 410 nm against time for solutions containing (i) Rose Bengal (ii) Rose Bengal-C(KLAKLAK)$_2$ conjugate (iii) DPBF alone plus ultrasound treatment (iv) Rose Bengal plus ultrasound treatment and (v) Rose Bengal-C(KLAKLAK)$_2$ conjugate plus ultrasound treatment.

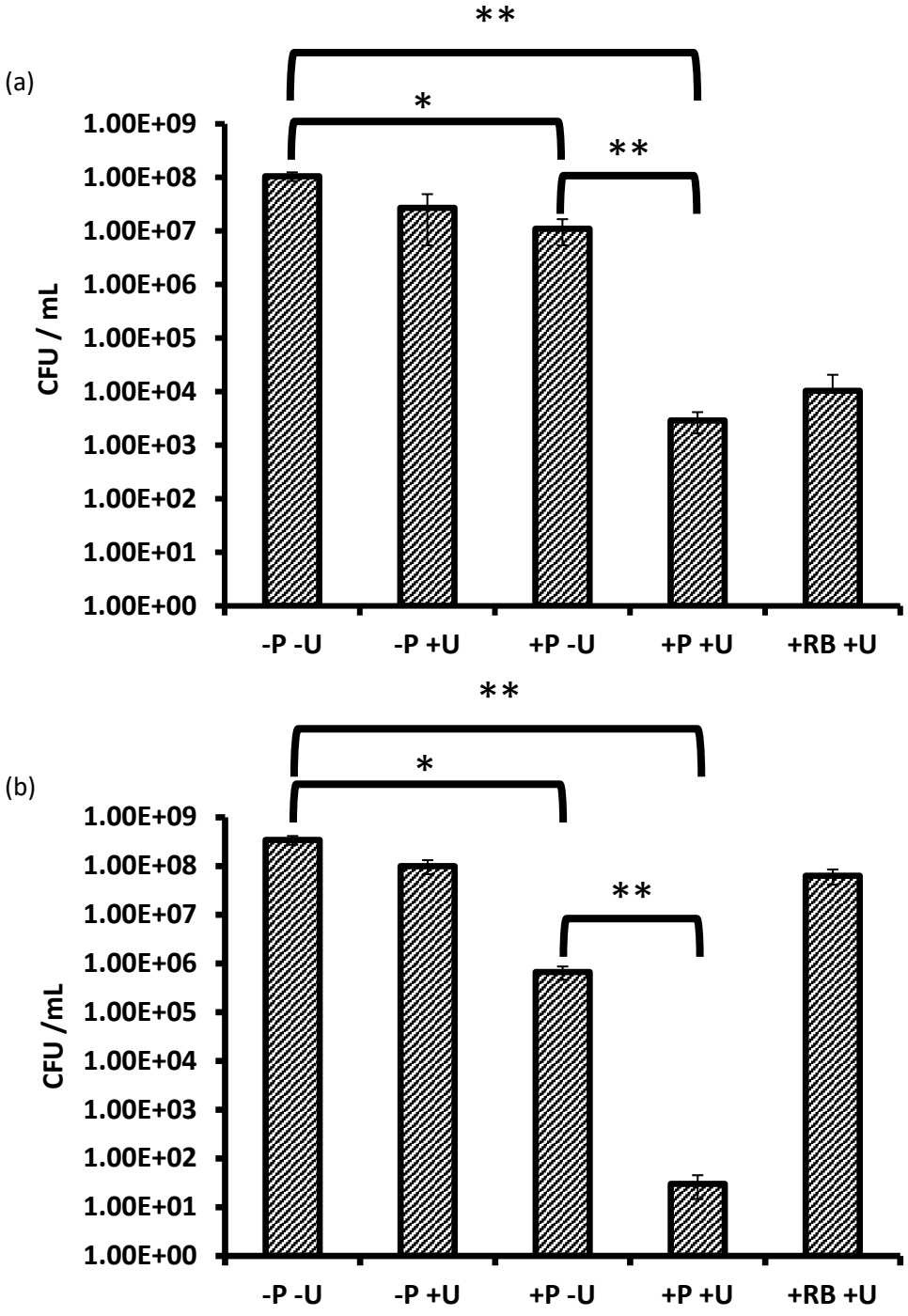
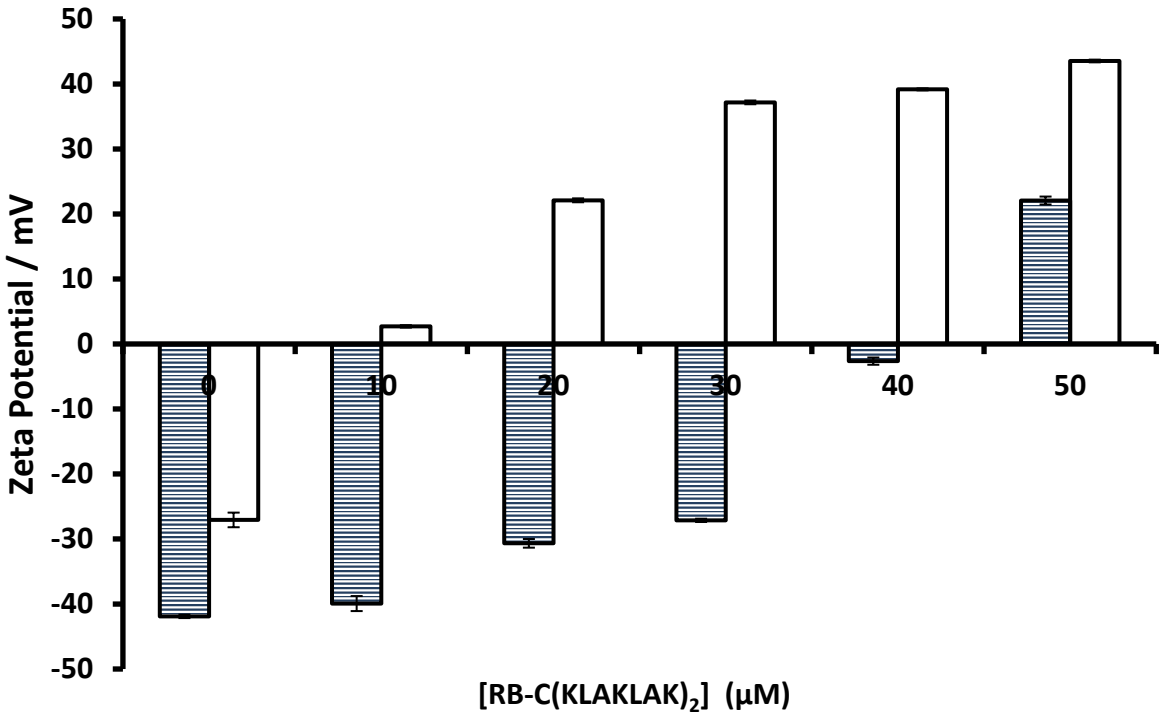


Figure 2 Plot of CFU/mL after treatment of (a) *S. aureus* and (b) *P. aeruginosa* with RB-C(KLAKLAK)$_2$ (P), Rose Bengal (RB) with / without ultrasound (+/- U). [RB-C(KLAKLAK)$_2$] = [RB] = 10 μM. Ultrasound conditions: 1 MHz, 3Wcm^{-2}, 10 min, 50 % duty cycle for *S. aureus* and 1 MHz, 3Wcm^{-2}, 6 min, 50 % duty cycle for *P. aeruginosa*. * represents $P \leq 0.05$, ** represents $P \leq 0.01$



443

444 **Figure 3** Plot of zeta potential for suspensions of *P. aeruginosa* (shaded columns) and *S.*

445 *aureus* (clear columns) recorded after addition of increasing amounts of RB-C(KLAKLAK)$_2$.

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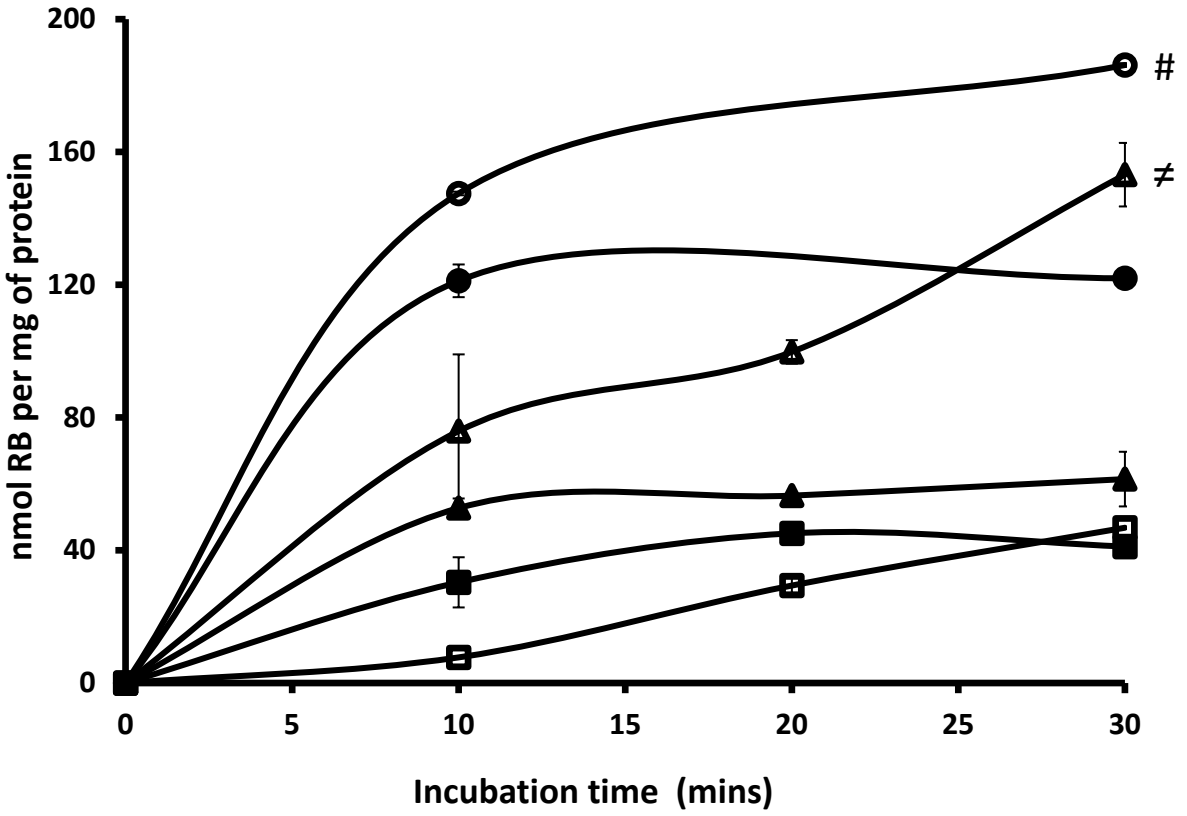

Figure 4 Plot of nmol of Rose Bengal per mg protein for suspensions of *S. aureus* (circles), *P. aeruginosa* (triangles) and HS27 RB cells (squares) incubated with RB (filled symbols) or RB-C(KLAKLAK)$_2$ (open symbols) for 10, 20 or 30 mins. (# represents P ≤ 0.001 with respect to uptake by RB alone and P ≤ 0.001 with respect to RB-C(KLAKLAK)$_2$ uptake in HS27 cells). (≠ represents P ≤ 0.01 with respect to uptake by RB alone and P ≤ 0.01 with respect to RB-C(KLAKLAK)$_2$ uptake in HS27 cells).

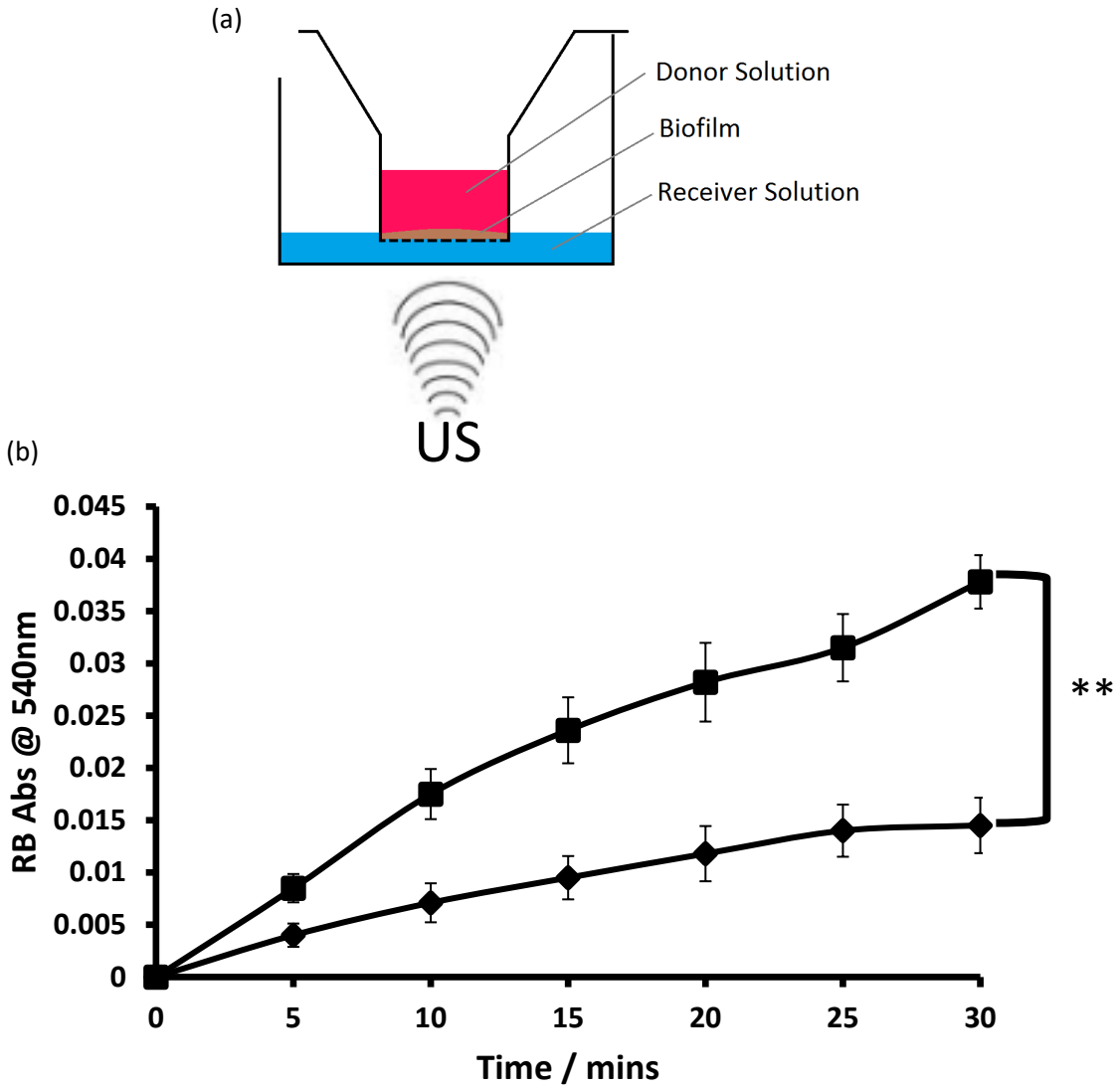

Figure 5 (a) Schematic representation of biofilm diffusion experiment. *P.aeruginosa* biofilms were generated on transwell inserts. The inserts were placed in wells containing PBS buffer and the base of each well irradiated (or not) with low intensity ultrasound. RB solution was added to the donor insert and the concentration of RB in the receiving PBS solution determined at various time points using UV-Vis spectroscopy (b) plot of RB absorbance against time for experiments performed in (a) ■ = wells pre-treated with US and ♦ = wells not pre- treated with US. ** represents $P \leq 0.01$

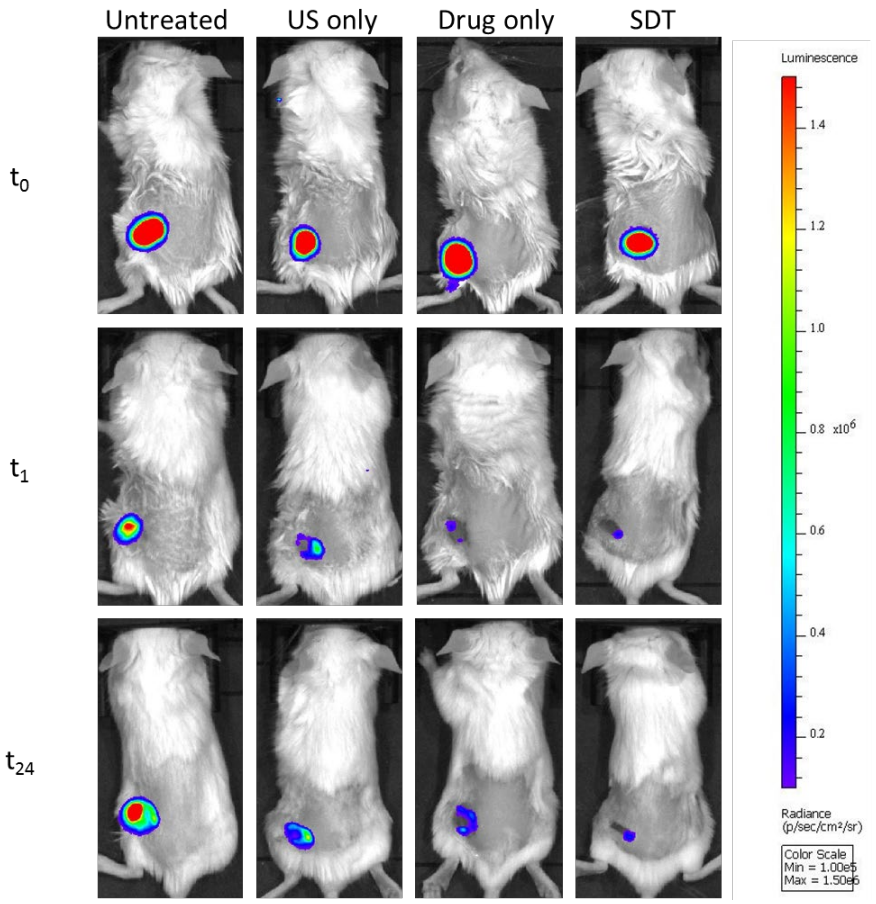

Figure 6 Whole body bioluminescent images of mice bearing 0.5 cm^2 wounds infected with *P.aeruginosa* and receiving (i) no treatment (ii) ultrasound only (iii) RB-C(KLAKLAK)$_2$ only or (iv) SDT, with images recorded immediately before, 1 h and 24 h after treatment.