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Abstract

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Rose bengal uptake by *E. faecalis* and *F. nucleatum* and light-mediated antibacterial activity measured by flow cytometry

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A R T I C L E   I N F O

Article history:
Received 14 March 2016
Received in revised form 20 June 2016
Accepted 22 June 2016
Available online 23 June 2016

Keywords:
Antibacterial photodynamic therapy
Bacteria
Rose bengal
Photosensitizer uptake
LIVE/DEAD BacLight
Flow cytometry

A B S T R A C T

Antibacterial photodynamic therapy (aPDT) using rose bengal (RB) and blue-light kills bacteria through the production of reactive oxygen derivates. However, the interaction mechanism of RB with bacterial cells remains unclear. This study investigated the uptake efficiency and the antibacterial activity of blue light-activated RB against *Enterococcus faecalis* and *Fusobacterium nucleatum*. Spectrophotometry and epifluorescence microscopy were used to evaluate binding of RB to bacteria. The antibacterial activity of RB after various irradiation times was assessed by flow cytometry in combination with cell sorting.

Uptake of RB increased in a concentration dependent manner in both strains although *E. faecalis* displayed higher uptake values. RB appeared to bind specific sites located at the cellular poles of *E. faecalis* and at regular intervals along *F. nucleatum*. Blue-light irradiation of samples incubated with RB significantly reduced bacterial viability. After incubation with 10 μM RB and 240 s irradiation, only 0.01% (±0.01%) of *E. faecalis* cells and 0.03% (± 0.03%) of *F. nucleatum* survived after treatment. This study indicated that RB can bind to *E. faecalis* and *F. nucleatum* in a sufficient amount to elicit effective aPDT. Epifluorescence microscopy showed a yet-unreported property of RB binding to bacterial membranes. Flow cytometry allowed the detection of bacteria with damaged membranes that were unable to form colonies on agars after cell sorting.

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1. Introduction

During antibacterial photodynamic therapy (aPDT), reactive oxygen derivates are produced by the interaction of visible light with a photosensitizing chemical called a photosensitizer (PS) [1]. Photons excite the PS to the highly unstable singlet state, which then shifts to the long-lived triplet state before transferring electrons to organic molecules or energy to molecular oxygen, respectively producing free radicals (type I mechanism) or singlet oxygen (1O2 – type II mechanism) [2–4]. These highly reactive species are antibacterial because they irreversibly damage proteins, membrane lipids and DNA [1].

Rose bengal (RB, 4,5,6,7-tetrachloro-2,4,5,7-tetra-iodo-fluorescein disodium salt) is an anionic, hydrophilic xanthene dye characterized by light absorption at wavelengths of 450–600 nm. This photosensitizer was previously shown to specifically generate singlet oxygen (1O2) upon light irradiation [5].

Rose bengal has several applications in medicine that include the diagnosis of ocular damage, the detection of oral pre-cancerous lesions and the use as a dental plaque disclosing agent [6,7]. The combinational use of rose bengal and blue-light has recently gained interest to inactivate several oral pathogens causing dental and periodontal diseases [8,9,10]. The use of blue light-mediated aPDT has stemmed from the widespread availability of dental blue-light sources having a broad emission spectrum (400–500 nm) and a high energy per photon [11]. Blue light-activated rose bengal was shown to inactivate *S. mutans*, *A. actinomycescomitans* and *C. albicans* in several research reports that also confirmed the influence of the amount of light energy delivered on bacterial killing [10,12,13].

However, the exact interaction between RB and bacteria remains a subject of debate. For Gram-positive bacteria, Demidova et al. [14] showed that RB was able to inactivate *S. aureus* after entering the cell, as evidenced by the antibacterial effect produced after removing the PS from the culture medium. George et al. [15] suggested that RB is a substrate of *E. faecalis* efflux-pumps, thereby supporting the intracellular incorporation of the dye. More recently, Ishiyama et al. [16] investigated the incorporation and the 1O2 production of phloxine, erythrosine and RB in *S. mutans* suspensions. Despite a lower 1O2 production, RB
displayed the highest incorporation rate and antibacterial activity among the three photosensitizers tested [16]. The relative porous layer of peptidoglycan surrounding the cytoplasmic membrane of Gram-positive bacteria was shown to allow the penetration of neutral or anionic PS’s [17].

For Gram-negative bacteria, the penetration of RB having both anionic and hydrophobic properties, is less likely. Gram-negative bacteria are known to be less permeable to hydrophobic compounds and the presence of a negatively charged outer membrane also known to restrict the penetration of the PS [18,19]. Studies on the interaction mechanism of RB with Salmonella typhimurium suggested that the dye locates at the outer membrane rather than inside the cell [20]. Demidova et al. [14] investigated RB uptake in E. coli and concluded that the lack of light-mediated killing after washing RB was testimony of a superficial subcellular location of the dye.

Finally it must be pointed-out that most published reports on the antibacterial properties of RB used a standard plating technique (CFU/mL) to estimate bacterial survival after treatment. Although this technique is still considered as the gold-standard for bacterial viability assessment, it fails to identify subpopulations of viable but not cultivable (VBNC) bacteria whereas antibacterial activity after various irradiation times was assessed by flow cytometry. The proliferation capacities of the fluorescent populations observed by flow cytometry were assessed using cell sorting and plating.

2. Materials and Methods

2.1. Bacterial Cultures

A Gram-positive bacterium (Enterococcus faecalis 135737, culture collection of the University Hospitals of Geneva, Geneva, CH) and a Gram-negative bacterium (Fusobacterium nucleatum OMZ 598, Orale Mikrobiologie Zürich culture collection, Zurich, CH) have been used in this study. E. faecalis and F. nucleatum were cultured from frozen stocks onto Columbia and Schaedler agar plates respectively (Oxoid AG, Pratteln, CH). Bacteria retrieved from agar cultures were transferred into 8 mL of liquid media (BHI for E. faecalis and Schadler broth for F. nucleatum, Oxoid AG, Pratteln, CH) and incubated at 37 °C, 5% CO₂ until the stationary phase of growth was reached. Cultures of F. nucleatum were maintained under anaerobic conditions using packs of OMZ 598, Orale (excitation: 500/20, emission: 535/30) photomicrographs were acquired using a camera connected to a Zeiss Axioskop 40 microscope. To confirm the spectrophotometric findings on RB uptake, E. faecalis and F. nucleatum were observed under an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss, Gottingen, DE). Bacteria were incubated with 10 μM RB or 0.9% NaCl and followed the same washing procedures as described for uptake experiments. After the last centrifugation, bacteria were re-suspended in 500 μL of 0.9% NaCl and sonicated for 20 s (Sonorex) to disperse aggregates. Aliquots of 40 μL were placed on microscope slides and covered with a coverslip (Menzel-Glasser, Braunschweig, DE). Differential interference contrast (DIC) and fluorescence (excitation: 500/20, emission: 535/30) photomicrographs were acquired using VisiView software 5.1 (Visirion System, Puchheim, DE) and analyzed with ImageJ 1.60.

2.4. Epifluorescence Microscopy

To confirm the spectrophotometric findings on RB uptake, E. faecalis and F. nucleatum were observed under an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss, Gottingen, DE). Bacteria were incubated with 10 μM RB or 0.9% NaCl and followed the same washing procedures as described for uptake experiments. After the last centrifugation, bacteria were re-suspended in 500 μL of 0.9% NaCl and sonicated for 20 s (Sonorex) to disperse aggregates. Aliquots of 40 μL were placed on microscope slides and covered with a coverslip (Menzel-Glasser, Braunschweig, DE). Differential interference contrast (DIC) and fluorescence (excitation: 500/20, emission: 535/30) photomicrographs were acquired using VisiView software 5.1 (Visirion System, Puchheim, DE) and analyzed with ImageJ 1.60.

2.5. Antibacterial Photodynamic Therapy – aPDT

For aPDT experiments, bacteria were incubated with the same concentrations of RB and followed the same washing procedures described for uptake experiments. After the third centrifugation, the bacterial pellet was re-suspended in 1 mL 0.9% NaCl and transferred into black opaque 24 well culture dishes (Greiner BioOne, Millian, CH) for light-irradiation. To ensure a complete irradiation of the surface of each well (16 mm diameter), the light guide of the QTH lamp was placed 10 mm above the samples. For each concentration of RB to be tested, samples were irradiated 15 s, 60 s or 240 s. Each sample has been irradiated separately. Control cultures received blue-light alone or photosensitizer alone. Experiments were performed in triplicate and repeated 3 times.
2.6. Samples Staining

After irradiation, samples were diluted $\times 10^3$ and sonicated 20 s at 35 kHz (Sonorex) to generate single cell suspensions. Cells were labeled with two nucleic acid intercalating agents SYTO 9 (excitation/emission: 485/500) and propidium iodide (PI; excitation/emission: 535/617) for monitoring viability as a function of membrane integrity. SYTO 9 is a membrane permeable green fluorescing dye that enters all cells, whereas PI only penetrates membrane-damaged cells. When propidium enters a cell, it displaces the SYTO 9 from the DNA due to its higher association constant with nucleic acids, therefore shifting the fluorescence of the cell from green to red[25].

A SYTO 9/PI solution has been prepared by mixing 6 $\mu$M of each dye in 2 mL 0.9% NaCl. Two hundred microliters of bacterial suspension were mixed to an equal volume of the SYTO 9/PI solution to a working concentration of 6 $\mu$M. Labeled samples were incubated with the FACSAria cytometer (Becton Dickinson, New-Jersey, USA) equipped with a solid-state blue laser (488 nm) and a 100 kHz (Sonorex) to generate single cell suspensions. Cells were labeled with two nucleic acid intercalating agents SYTO 9 (excitation/emission: 485/500) and propidium iodide (PI; excitation/emission: 535/617) for monitoring viability as a function of membrane integrity. SYTO 9 is a membrane permeable green fluorescing dye that enters all cells, whereas PI only penetrates membrane-damaged cells. When propidium enters a cell, it displaces the SYTO 9 from the DNA due to its higher association constant with nucleic acids, therefore shifting the fluorescence of the cell from green to red[25].

A SYTO 9/PI solution has been prepared by mixing 6 $\mu$M of each dye in 2 mL 0.9% NaCl. Two hundred microliters of bacterial suspension were mixed to an equal volume of the SYTO 9/PI solution to a working concentration of 6 $\mu$M and 30 $\mu$M respectively. Labeled samples were incubated for 15 min at room temperature in the dark.

2.7. Flow Cytometry Analysis

Samples were acquired with an Accuri C6 flow cytometer and analyzed with the Accuri C6 Software (BD Accuri Cytometers, Ann Arbor, USA). This cytometer is equipped with a solid state blue laser 488 nm (20 mW) used for excitation of both SYTO 9 and PI. SYTO 9 signal has been collected in the FL1 channel (BP 533/30) and PI in the FL3 channel (LP > 670). Bacterial density and flow rate (14 $\mu$L/min) were set to keep event rate below 800 events/s during acquisition. Bacteria have been discriminated from debris and gated on a FL1-height/Forward Scatter-height (FSC-H) plot. Thresholds were set on the FSC and FL1 channels to exclude debris from analysis. All parameters were acquired as log-arithmetic signals. Twenty thousands events were collected in the bacterial gate for each sample. Microbial subpopulations were identified on the basis of fluorescence detected on a FL1-A/FL3-A plot. Gates applied for population discrimination were set manually based on control samples. Untreated bacteria were used as live controls (SYTO 9 – FL1) and heat-treated cells as dead controls (60 °C for 20 min) (PI – FL3).

Previous studies that used FCM analysis of SYTO/PI labeled bacteria showed that not only intact (SYTO 9 positive) and damaged cells (PI positive) are observed, but that double-stained cells (SYTO 9/PI) are also detected [26–29]. For results, SYTO 9 positive cells were referred to as “live”, PI positive cells as “dead”, and cells displaying a double positive signal (SYTO 9/PI) were designated “injured”.

2.8. SYTO 9/Propidium Iodide Staining Characterization

Fluorescent-activated cell sorting (FACS) was used to separate the three populations observed; SYTO 9 positive, PI positive and double-stained bacteria. Each population was then plated on agar plates to determine its proliferation capacity, and more importantly the growth potential of the double-stained cells. Appropriate aPDT doses were selected to maximize the ratio of cells under investigation to be sorted. SYTO 9 positive and PI positive cells were respectively sorted from untreated controls and from samples incubated with 10 $\mu$M RB irradiated 240 s. Samples of E. faecalis incubated with 10 $\mu$M RB irradiated 15 s, and samples of F. nucleatum incubated with 1 $\mu$M RB irradiated 60 s were used to sort double-stained cells. Sorting has been performed with the FACSAria cytometer (Becton Dickinson, New-Jersey, USA) equipped with a solid-state blue laser (488 nm) and a 100 $\mu$m nozzle (air pressure 20 psi). Sheath fluid was IsosFlow Sheath Fluid (Beckman Coulter, Nyon, CH) diluted hundred times in phosphate buffer saline. A threshold was set on the Side Scatter-height (SSC-H) channel at 200. SYTO 9 has been collected in the FITC channel (BP 530/30) and PI in the TexasRed channel (BP 610/20). All parameters were acquired as log-arithmetic signals. Debris have been excluded from analysis in a Fitzgerald/FSC-H plot and bacterial fluorescent populations gated in a FITC-A/ Texas Red-A plot. PI positive events were sorted first followed by double stained and SYTO 9 positive events to avoid false positive colonies. Fifty thousand cells of each population were sorted in 1 mL 0.9% NaCl. After sorting, cells were sonicated 20 s at 35 kHz (Sonorex) to disaggregate potential clusters and immediately plated. Culture media and conditions were the same as described in “bacterial cultures”. CFU (colony forming units) from both strains were counted after 72 h. Results are expressed as follows: (number of colonies counted on agars / number of cells plated) $\times$ 100. Experiments were performed in triplicate and repeated 3 times.

2.9. Statistics

Results were statistically analyzed using one-way analysis of variance (ANOVA) and Tukey multiple comparison intervals ($\alpha = 0.05$).
3. Results

3.1. Rose Bengal Uptake

Fig. 2 shows that both strains have retained the photosensitizer after three washing steps and that rose bengal uptake significantly increased in a concentration dependent manner ($p < 0.05$). After 30 min incubation with $1 \mu M$ RB, *E. faecalis* incorporated approximately $0.5 \pm 0.1 \mu M$ of photosensitizer. At higher concentrations, $5 \mu M$ and $10 \mu M$ RB, bacterial uptakes respectively increased to $2.1 \pm 0.2 \mu M$ and $3.8 \pm 0.3 \mu M$.

For *F. nucleatum*, $0.3 \pm 0.2 \mu M$ of RB were retained after incubation in presence of $1 \mu M$, whereas $1.7 \pm 0.2 \mu M$ and $2.6 \pm 0.3 \mu M$ were incorporated by the bacterium after incubation with $5 \mu M$ and $10 \mu M$ of RB. For all concentrations except $1 \mu M$, the Gram-positive *E. faecalis* exhibited a significantly higher uptake of RB than the Gram-negative *F. nucleatum*.

3.2. Epifluorescence Microscopy

Samples of both strains that were incubated without RB displayed no detectable fluorescent signal under epifluorescence microscopy observation (data not shown). *E. faecalis* and *F. nucleatum* acquired a fluorescent signal after incubation with $10 \mu M$ rose bengal (Fig. 3B and D). For *E. faecalis* this signal was concentrated at the bacterial poles, whereas *F. nucleatum* exhibited several fluorescent spots along its filamentous structure.

3.3. Antibacterial Photodynamic Therapy – aPDT

*E. faecalis* cultures exposed to light irradiation only (240 s) or rose bengal only had similar populations to control cultures (no light/no rose bengal) with approximately 97% of live cells, 1% injured and 2% dead (Fig. 4). When *E. faecalis* cultures incubated in presence of $1 \mu M$ of RB were light-irradiated, the percentage of live cells decreased in a light-dose dependent manner. A 15 s exposure to blue light reduced bacterial viability by almost 50% and increased the percentage of injured cells to $43.8\% \pm 6.7\%$. After 60 s of light irradiation, $82\% \pm 4.5\%$ of the bacterial population was injured and $14.6\% \pm 4.4\%$ was dead. Only $2.9\% \pm 1.3\%$ of cells remained live. A longer light-irradiation (240 s) killed $96\% \pm 1.6\%$ of the bacterial culture whereas $3.8\% \pm 1.9\%$ remained injured. Exposure of *E. faecalis* cultures to $5 \mu M$ or $10 \mu M$ of light-activated RB had similar effect on bacterial viability except a higher percentage of injured cells immediately after 15 s of light irradiation (Fig. 4). Increasing irradiation times progressively increased bacterial death. Fig. 6A illustrates representative samples of *E. faecalis* exposed to increasing irradiation times after incubation with $5 \mu M$ RB. After incubation with $10 \mu M$ RB and 240 s of light irradiation, $99.6\% \pm 0.4\%$ of *E. faecalis* cultures were dead whereas $<0.4\% \pm 0.4\%$ remained injured and $0.01\% \pm 0.01\%$ live.
Control cultures of *F. nucleatum* (no light/no rose bengal) exhibited 95.2% (±2.4%) of live cells, 1.3% (±0.7%) of injured cells and 3.5% (±1.8%) of dead cells as measured by FCM. The viability of *F. nucleatum* cultures was slightly reduced (86.1% ± 6.4%) after blue-light irradiation (240 s) and concentrations of RB higher than 5 \( \mu \text{M} \) induced a dark toxicity to bacterial cultures \( (p < 0.05) \) (Fig. 5). At 1 \( \mu \text{M} \), irradiation for 15 s only reduced viability to 80% (± 3.8%), but longer irradiation time resulted in further viability reduction; 21.2% (± 3.7%) remained live after 60 s of light irradiation whereas only 0.25% (± 0.15%) survived after 240 s. The bacterial suspensions incubated with 5 \( \mu \text{M} \) RB and light-irradiated 15 s showed 17.4% (±10%) of live cells, 25.1% (±8.7%) of injured cells and 57.5% (±13%) of dead cells (Fig. 5). When suspensions were irradiated 60 s, 95.3% (±3.5%) of the population was dead, 4% (±2.5%) was injured and 0.8% (±0.8%) was recorded live. After 240 s irradiation, 99.7% (±0.2%) of the population died, 0.3% (±0.1%) of cells were injured and only 0.05% (±0.04%) of cells remained live (Fig. 5). Fig. 6B illustrates representative samples of *F. nucleatum* exposed to increasing irradiation times after incubation with 5 \( \mu \text{M} \) RB. Increasing RB concentration to 10 \( \mu \text{M} \) did not induce any additional effect to RB mediated aPDT against *F. nucleatum*.

### 3.4. Sorting the Different Bacterial Populations

For *E. faecalis*, 82.4% (±5%) of the SYTO 9 positive cells formed colonies on agars after sorting and plating, whereas only 0.02% (±0.01%) of the injured population was able to grow. No growth was observed among the PI positive cells. For *F. nucleatum*, none of the sorted populations were able to form colonies after sorting and plating.

### 4. Discussion

It is generally accepted that, upon light activation, rose bengal produces singlet oxygen \( (\text{O}_2^*) \) in the vicinity of target cells that subsequently die from oxidative stress \( [30–32] \). Because singlet oxygen is effective in the nanometer range (20 nm), photosensitizers that tightly bind or penetrate bacterial cells may elicit more antibacterial activity than those producing oxygen derivates nearby cells \([14,16,33]\).

The results of the current study indicate that RB uptake by *E. faecalis* and *F. nucleatum* reached sufficient concentrations to kill bacteria upon light-irradiation. Uptake values were measured spectrophotometrically as the concentration of RB in the whole bacterial pellet after three washing steps. Thus, only RB retained by cells was considered for calculations. To keep optical readings above the detection limit of the spectrophotometer, a high bacterial concentration (1.5 × 10^9 cells/mL) had to be used; this definitely influences the concentration of RB measured in the lysed pellet.

To overcome this limitation, uptake values can be expressed as the number of RB molecules per cell. As suggested by Demidova et al. \([14]\), these values can be obtained by dividing the number of \( \mu \text{mol} \) of RB in the lysed pellet by the number of bacteria in the sample, and the number of RB molecules per cell calculated by using Avogadro’s number. Using such calculations, uptake values at 1 \( \mu \text{M} \), 5 \( \mu \text{M} \) and 10 \( \mu \text{M} \) RB were respectively 0.3, 1.5 and 2.7 \( (\times 10^6) \) molecules per cell of *E. faecalis* and 0.2, 1.19 and 1.8 \( (\times 10^6) \) molecules per cell of *F. nucleatum*. The
lower uptake of \textit{F. nucleatum} confirms the resistance of Gram-negative bacteria to the penetration of the dye across the LPS layer. Nevertheless, the uptake value of $1.19 \times 10^6$ molecules per cell (RB: 5 μM) fairly correlates with another report on Gram-negative bacteria showing that approximately $0.9 \times 10^6$ molecules of RB can bind per cell of \textit{E. coli} after 20 min incubation \cite{14}. However, it must be noted that the concentration of RB retained by cells did not increase proportionally with applied concentrations.

Fig. 6. Effect of rose bengal-mediated aPDT on bacterial subpopulations: flow cytometric dot plots of \textit{Enterococcus faecalis} (left side) and \textit{Fusobacterium nucleatum} (right side) stained with SYTO 9 and PI after 30 min incubation with 5 μM RB and blue-light irradiated for various times (0 s to 240 s). SYTO 9 fluorescence (530/33 nm) on the X-axis is plotted against PI fluorescence (λ670 nm) on the Y-axis. Numbers in each gate represent the percentage of events.
(Fig. 2). This suggests a maximum cellular uptake capacity after which further addition of dye will remain in solution by saturation of the binding sites [34]. This assumption is somewhat supported by epifluorescence microscopy showing RB binding to specific sites located at the cellular poles or at regular intervals along F. nucleatum (Fig. 3). This binding pattern was consistently observed for all samples incubated with RB and fairly correlates with previously published reports on 10-nonyl-acridine-orange (NAO). NAO, which shares similar physico-chemical properties with RB (anionic, hydrophobic), was shown to label anionic phospholipid rich domains (phosphatidylglycerol and diphosphatidyl-glycerol) of the bacterial membranes, precisely located at the cellular poles or at regular intervals along cells [35–37].

The antibacterial properties of blue light-activated RB were demonstrated despite the spectra of emission and absorption of the light source and the PS respectively do not perfectly coincide (Fig. 1A). It is demonstrated despite the spectra of emission and absorption of the light source and the PS respectively do not perfectly coincide (Fig. 1A). It is also shown that increased membrane damage occurred, allowing more PI to enter the cytoplasm. None of these PI positive bacteria were able to recover and grow on appropriate media after cell sorting, which agrees with others [22,43,44]. PI was therefore considered testimony of dead cells. All together, this indicates that bacteria exposed to increasing aPDT doses progressively lose their membrane integrity, which ultimately results in cell death.

Unfortunately, the proliferation capacity of F. nucleatum could not be tested, since the bacterium was probably too sensitive to the aerobic conditions applied during sorting experiments [45]. It has also been shown that stressed cells may die immediately after plating on agars due to the oxidative burst induced by the excess of nutrients [46].

Finally, it must be noted that the antibacterial activity of light-activated RB was assessed on planktonic cultures of bacteria frequently retrieved from infected dental roots [47,48]. However, the relevance to clinical conditions would be further improved by using bacterial biofilms as usually encountered in infected teeth.

Within the limitations of the current study, results indicate that RB binds to E. faecalis and F. nucleatum and kill bacteria upon light irradiation. Epifluorescence microscopy showed a yet-unreported property of RB binding to specific phospholipid rich domains of the bacterial membranes. Flow cytometry followed by fluorescent cell sorting confirmed that SYTO 9/PI double-stained bacteria are losing their proliferation capacity.

Acknowledgments

This study was supported by Grant #31003A-149962 of the Swiss National Science Foundation.

Manufacturers of the light sources used in this study (KerrHawe SA, Bioggio, CH) are acknowledged for their support. No competing financial ties exist.

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