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Chemiluminescence as a PDT light source for microbial control

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ABSTRACT

The photodynamic therapy (PDT) is a combination of using a photosensitizer agent, light and oxygen that can cause oxidative cellular damage. This technique is applied in several cases, including for microbial control. The most extensively studied light sources for this purpose are lasers and LED-based systems. Few studies treat alternative light sources based PDT. Sources which present flexibility, portability and economic advantages are of great interest. In this study, we evaluated the *in vitro* feasibility for the use of chemiluminescence as a PDT light source to induce *Staphylococcus aureus* reduction. The Photogem[®] concentration varied from 0 to 75 µg/ml and the illumination time varied from 60 min to 240 min. The long exposure time was necessary due to the low irradiance achieved with chemiluminescence reaction at µW/cm² level. The results demonstrated an effective microbial reduction of around 98% for the highest photosensitizer concentration and light dose. These data suggest the potential use of chemiluminescence as a light source for PDT microbial control, with advantages in terms of flexibility, when compared with conventional sources.

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1. Introduction

Photodynamic therapy (PDT) is a technique which, besides its application in cancer treatment, has also shown promising results for *in situ* treatment of infected lesions [1]. The PDT indication for such application has been demonstrated by several authors [2–4], employing different combinations of microorganisms and photosensitizers. The photodynamic reaction is induced after an interaction of a photosensitizer (PS) and light in the presence of oxygen. The essential point of photodynamic action is the illumination of the target tissue, previously photosensitized, resulting in PS excitation, and in a sequence of events, including singlet oxygen production, which induces a local toxicity [5,6].

The appearance of bacteria species which are resistant to antibiotics drugs, raised the need to establish and improve other systems of microbial control. PDT is one of the alternative procedures and based on its mechanisms, it is not likely that microorganisms develop resistance [7,8].

As for tumor treatment, PDT for microbial control also relies on induced cell killing. In the case of bacteria photosensitization, the

cellular targets of PDT sensitizers have been cellular membranes and nucleic acids, depending on PS physico-chemical properties [2].

For most PDT applications the appropriate light source constitutes the main limitation. Illumination parameters as wavelength, irradiance and fluence are essential for the appropriate PS excitation and cellular killing. The matching of the excitation spectrum with, at least, one of the absorption bands of the PS is need for the photodynamic response. For the treatment of infected lesions, it is also important to establish a PDT protocol that results in microorganism killing but is safe to human cells.

Several types of light sources have been employed in PDT protocols, including lasers, lamps and LED-based systems. Those light sources are distinct when considering certain parameters, i.e. spectral emission, collimation/divergence and optical coupling. These light sources are electricity-dependent systems and therefore may not be fully portable. Laser systems are widely used due to their spectral characteristics, light energy concentration, and the best optical fiber coupling, when compared to the other sources. Laser sources are so far the first option for the PDT treatment of internal organs as in esophagus, lung and bladder cancers. There are already some portable light sources based on battery, powered systems for PDT treatments [9,10].

Unfortunately, even though the cost of a PDT laser equipment has been decreasing, it remains a prohibitive factor for a large scale acquisition, especially in developing countries. LED-based systems,

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which are less expensive compared to lasers, have been presented as an attractive light source option for the treatment of skin and oral lesions where the access of the illumination tip is direct.

Both light sources, lasers and LEDs, show advantages and disadvantages when considering their applications, but there is still room for instrumentation of non-electrical light source. Some countryside regions, located far from metropolitan areas, are not provided with well-equipped clinical facilities, and in several cases there is not even electricity. Homecare treatment is also an example where a non-wired PDT system can be used and is actually required, especially when patient's convenience is taken into consideration. The possibility for more practical light sources may even make the introduction of new PDT procedures possible.

Chemiluminescence (CL) stands for a chemical reaction which results in light emission [11–14]. Depending on the reagents of the chemical reaction, the emission wavelength can be tuned. Although chemiluminescence shows a very low intensity emission when compared to the available PDT light sources, its emission may be sufficient for microbial control in infected lesions. The use of chemiluminescence combined to PDT action is a topic not yet widely explored and its main principles still remain to be demonstrated.

Chemiluminescence is a chemical reaction that results in light emission, completely independent on electricity. There are distinct CL reactions as the oxidation of luminal or luciferin and the peroxyoxalate system. The reactants used in the reaction determine the optical characteristics of the emitted light, including spectrum, intensity and lifetime. The mechanism of the CL reaction was proposed by Rauhut in 1967 describing the reaction of the peroxyoxalate system [11–14].

The mixture is performed with an oxalate reactant of bis(2,4,6-trichlorophenyl) (TCPO) in the presence of hydrogen peroxide (H_2O_2) and an activator (ACT), in this case, 9,10-bis(phenylethynyl)anthracene (BPEA). This reaction occurs in a solution of imidazole (IMI-H) and ethyl acetate (EtOAc).

The TCPO reacts with the H_2O_2 producing a cyclic peroxide, a highly energetic molecule that later reacts with the BPEA. A sequence of events, called Chemically Initiated Electron Exchange Luminescence mechanism – CIEEL mechanism – is induced. According to Rauhut, there is a highly energetic metastable intermediate that reacts with the activation and constitutes one of the main components for the CIEEL mechanism.

Current PDT studies have demonstrated the positive aspects of the use of low irradiances during long-time illumination. This could be one attempt to avoid the effect on the oxygenation system in the illuminated area, due to a lower vasculature collapse and thus getting improved results in photodynamic response. This kind of analysis has been carried out by Seshadri, Bisland and Wilson, where low doses (2 and 5 J/cm²), at an intensity of 10 mW/cm² were able to reduce some types of bacteria using long exposure time [15–22]. To the best of our knowledge, no studies have been performed for the investigation of the PDT efficacy when operating on an irradiance level of $\mu W/cm^2$.

Another factor which must be considered when low irradiances are used, is that long illumination times are necessary. This fact may turn into a drawback for an in-office application, because of an unfeasible long working time, but it may not be a problem for a home treatment, if a suitable illumination device is developed.

Also, the possibility of a flexible and portable non-wired device with a spectral emission tunability regarding the chemical reagents, makes chemiluminescence a potential light source for antimicrobial PDT.

The microbial control and bacterial reduction studies became objects of great interest to public health, especially when these microorganisms have been related to hospital infection, as the *Staphylococcus aureus* [3,7,23–30]. Therefore, the development of

techniques for microbial control that are not antibiotic dependent became very important [31,32] as shown by Meisel, Zeina, O'riodan and Hasan where they verified that an infection control using different types of photosensitizers (methylene blue, rose bengal, chlorophyll, phthalocyanines and others) were effective on both Gram negative and Gram positive microorganisms, e.g. *Candida albicans*, *Porphyromonas gingivalis*, *Staphylococcus epidermidis* and *S. aureus*. In general, the development of techniques that do not induce bacterial resistance is of great relevance [7,30].

In this sense, the aim of this study was to evaluate *in vitro* the feasibility of chemiluminescence as a light source for PDT on *Staphylococcus aureus* growth reduction.

2. Materials and methods

2.1. Bacterial culture

S. aureus (American Type Culture Collection – ATCC 25923) was chosen as a first microbial target, since it is the most common species of hospital infections. The bacterial culture was aerobically grown overnight at 37 °C in 5 ml of Tryptic Soy Broth (TSB) (Acumedia Manufactures Inc., Lansing, USA). The culture was harvested after centrifugation at 2000 rpm for 10 min, washed twice with sterile distilled water and resuspended in sterile saline to a turbidity of 10^6 cells ml⁻¹, determined by a spectrophotometer BioPhotometer (Eppendorf AG, Hamburg, Germany) set at 600 nm.

2.2. Photosensitizing agent (PS)

The PS used in this study was Photogem[®] (Photogem[®], Moscow, Russia) a first generation PDT sensitizer, a mixture of monomeric, dimers and oligomers of porphyrin units, with similar properties compared to Photofrin[®] [1,2]. Photogem[®] was chosen as the PS due to the previously presented results on bacteria growing control [28]. Fig. 1 presents the chemical structure of dimeric form of the Photogem[®]. Solutions were prepared by diluting Photogem[®] powder in sterile saline and protecting from light exposure. Four sensitizer concentrations were evaluated 6; 25; 50 and 75 $\mu g/ml$. These concentrations were determined after a previous experiment, where a high dark toxicity was observed for PS concentration of 150 $\mu g/ml$ (results not shown).

2.3. Light source

A volume of 30 ml of chemiluminescent solution were used for each one hour of PDT illumination. The chemical reagents for CL were obtained from 30 luminescent light sticks (Light Stick Ltd., Pequim, China) which present the following reagents: ethyl acetate, 9,10-bis(phenylethynyl) anthracene; oxalate, bis(2,4,6-trichlorophenyl) imidazole and hydrogen peroxide. The solution was removed from the sticks and placed in a Petri dish with 5.0 cm diameter and 1.5 cm height, with the solution filling its whole volume. This setup provided uniform illumination over the area of 12 wells in a 96-well culture cell test plate. The CL solution was replaced every 60 min, which corresponds to 2x of the half life of the CL reaction, for a more uniform delivered light intensity during all illumination time, for the groups with 120 and 240 min.

2.4. In vitro procedure

Aliquots of 100 μl of *S. aureus* suspension were individually transferred to 12 wells of a 96-well test plate. An equal volume of PS solutions was added to each well to achieve final concentrations of 6; 25; 50; and 75 $\mu g/ml$. The 2 min of dark incubation was chosen based on another experiment [28] as where this time

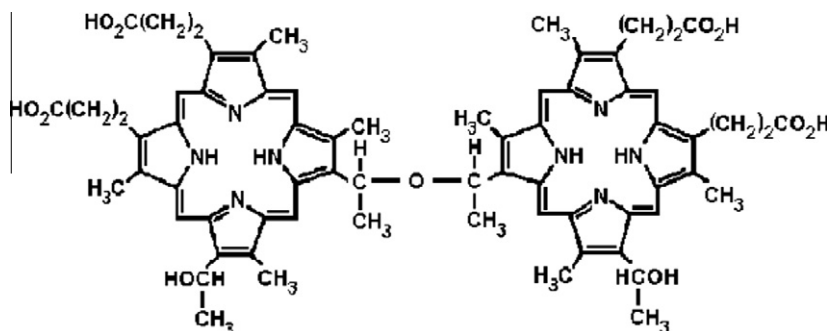


Fig. 1. Chemical structure of dimeric form of Photogem®.

showed already good results. The 96-well plate was positioned over a Petri dish containing 30 ml of the chemiluminescence solution. The CL solution was 40 mm distant from the bottom of the cell culture plate. Illumination was performed for 60, 120 and 240 min, resulting in a total fluence of 0.2, 0.4 and 0.8 J/cm², respectively.

To determine the dark toxicity of the PS itself, for each concentration, the pre-photosensitized bacteria suspensions were protected from ambient and chemiluminescent illumination for all tested times. The cytotoxicity effect of the chemiluminescence for 60, 120 and 240 min was also investigated in bacteria suspensions without PS exposure. The bacteria suspensions with neither photosensitization, nor illumination, constituted the non-treated control group.

Table 1 presents the experimental groups: (I) PS–L–(no photosensitizer, no light); (II) PS–L+(treated only with light); (III) PS + L–(treated only with photosensitizer), and (IV) PS + L+(PDT treated).

After illumination, the bacteria survival rate was determined by counting the number of bacterial *colony forming units* (c.f.u.). Serially diluted aliquots (10⁻¹–10⁻³) of treated and untreated bacteria were plated on Mannitol Salt Agar (Acumedia manufactures, Inc., Lansing, USA) using 25 µl aliquots. The number of c.f.u. was counted after 48 h incubation at 37 °C. Each serial dilution for each group was performed in triplicate.

3. Results

Initial attention should be give to the optical properties of CL and PS. The optical spectrum of CL emission was obtained using a spectrophotometer (Spectral-Fluorescence System “Spectr-Cluster” v. 2.05 m, Cluster Ltd., Moscow, Russia). This signal is shown in Fig. 2 and reveals significant intensity emitted between 450 nm and 600 nm with a peak at 505 nm and 34 nm FWHM. The total optical intensity emitted by CL solution (30 ml) was still monitored during 60 min using an optical power meter Field Master (Coherent Inc., San Jose, USA) coupled to an optical sensor for low level visible radiation (up to 50 mW). Inset of Fig. 2 shows this

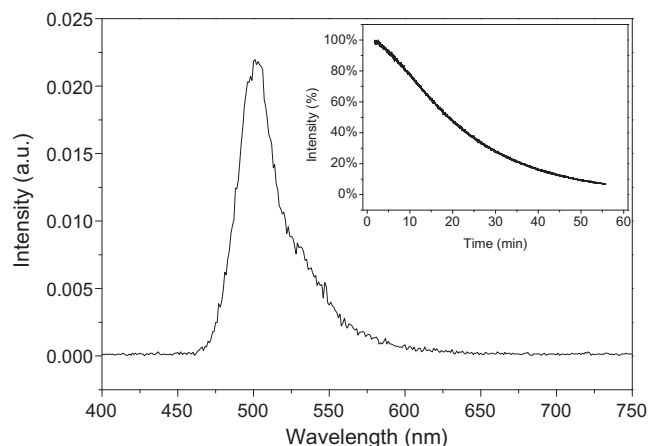


Fig. 2. Chemiluminescence emission spectrum and its decay time (inset).

result and depicts the temporal profile of CL emission as a like-exponential decay with close to 20 min decay time. At ($t = 0$), the chemiluminescent reaction emits $100 \pm 7 \mu\text{W}/\text{cm}^2$; with time the temporal profile of CL emission drives the behavior of light dose delivered to cells-targets. In a PDT cycle (60 min) CL yielded round to 0.2 J/cm², which was sufficient to cause a photodynamic action.

The absorption spectrum of the photosensitizer was further examined in order to check the spectral region where the CL emission is a strongly absorbed by the PS solution. In fact, it is easy to conclude that the efficacy of the PDT treatment depends on the overlapping between these spectra. Based on this concept, the absorption spectrum of PS as well the emission spectrum of CL was plotted, as shown in Fig. 3. Both spectra were normalized by maximum peak intensity in order to focus on predominant spectral information and this graph shows a strong overlap between CL emission and PS absorption.

A correlation parameter (η) was defined to represent the matching between PS absorption and CL emission spectra (Eq. (1)).

$$\eta = \frac{\sum_{i=475}^{600} E_i A_i}{\sum_{i=475}^{600} A_i A_i} \quad (1)$$

where E_i is CL emission spectra and A_i is PS absorption spectra.

Since the correlation (η) between CL emission and PS absorption can be used as a quantitative tool of overlapping, η was calculated by performing the product of both spectra intensity at the same wavelength and revealed the value of η chemiluminescence (η_{ch}) is $\eta_{\text{ch}} \approx 0.5$. We used two LED-devices LUXEON III (green LED, LXHL-PM09; red LED, LXHL-PD09, Philips Lumileds Lighting Co., San Jose, USA) to compare the CL to other light sources. The emission spectra of both LED-devices are presented in Fig. 3 normalized

Table 1

Scheme of the organization per experimental group. Different photosensitizer concentrations (6;25;50 and 75 µg/ml) were investigated in the experiments with the presence of photosensitizer.

		Group I	Group II	Group III	Group IV
60 min	Light	No	Yes	No	Yes
	PS	No	No	Yes	Yes
120 min	Light	No	Yes	No	Yes
	PS	No	No	Yes	Yes
240 min	Light	No	Yes	No	Yes
	PS	No	No	Yes	Yes

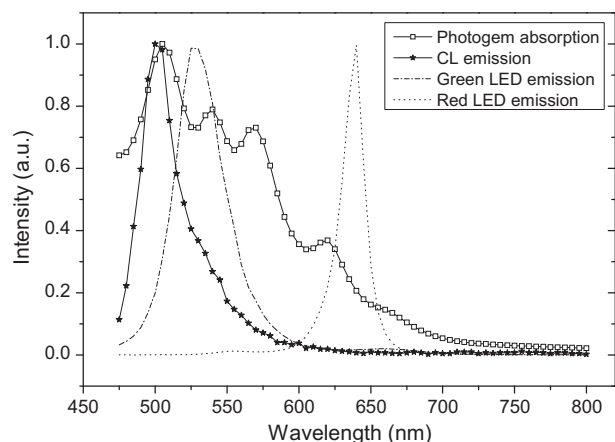


Fig. 3. Light sources (CL, green LED and red LED) emission and photosensitizer absorption at visible spectral range showing the spectra overlap.

by the highest intensity. In a visual analysis it is easy to see the CL emission to have a stronger overlap with PS absorption than for other LED-devices. Despite this analysis, the correlation of LED emission and Photogem[®] absorption were performed and the results present values of $\eta_g \approx 0.5$ for green LED and $\eta_r \approx 0.1$ for red LED. These values demonstrate that CL and green LED have similar absorption efficiency, leading to conclude that chemiluminescence could be an as effective light source as a commercial green LED to induce the photoactivity of Photogem[®] in a PDT treatment.

The microorganism survival after 60, 120 and 240 min without Photogem[®] sensitization, nor illumination, was evaluated for group I (PS–L–) in order to assure that the treatment times were not influencing bacterial growth. No bacterial death was observed up to 240 min, showing that *S. aureus* resisted at room temperature, at experimental conditions which no lack of nutrients was found. Similar results were obtained for group II (PS–L+) when cells were illuminated without the presence of PS. In this case, the chemiluminescence has not shown any significant effect on bacterial growth for all investigated exposure times.

The dark toxicity of Photogem[®] (group III; PS + L–) was analyzed for different PS concentrations (6, 25, 50 and 75 $\mu\text{g/ml}$). The results due to the *in vitro* incubation at 240 min are presented in Fig. 4. While the PS concentrations up to 25 $\mu\text{g/ml}$ did not induce microorganism reduction, higher concentrations caused the mentioned toxicity effects. The maximum reduction rate was 22.6% (log reduction < 0.1) at 240 min incubation and 75 $\mu\text{g/ml}$ PS concentration. This reduction is not significant when considering microbial growth control. Even though these toxicity effects could be tolerable, it indicates that higher PS concentrations must be carefully managed in order to avoid undesirable effects on healthy tissues. Nevertheless, these are concerns for future investigation.

The combined effect involving PS and light, the PDT effect was evaluated with group IV (PS + L+) and it presented a significant bacterial reduction compared to group III. While the maximum reduction rate induced by dark toxicity was 22.6%, with this same 240 min of illumination, the reduction rate induced by photodynamic effect was near 60% for 6 $\mu\text{g/ml}$ and 98% for 75 $\mu\text{g/ml}$ Photogem[®] concentration. These reduction rates indicate that even with use of lowest photosensitizer concentration, the PDT treatment is three times more efficient for microbial control than PS dark toxicity.

In order to show details of group IV results, Fig. 5 depicts the microbial reduction rate for all PDT subgroups. Fig. 5a presents the percentage of c.f.u counting as a function of the light dose and Fig. 5b presents the microbial population at logarithm scale

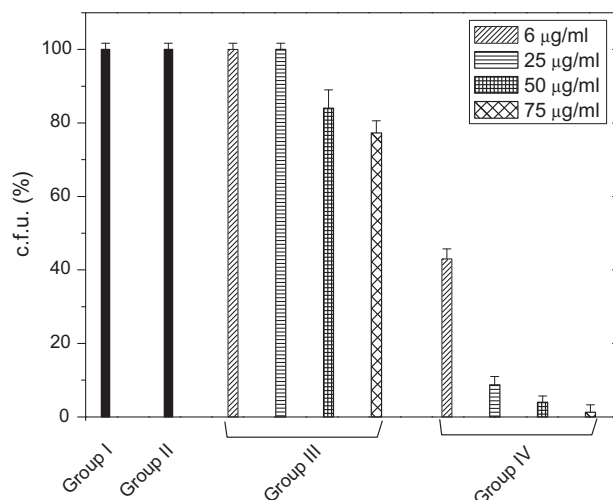


Fig. 4. Percent values of c.f.u. counting for all experimental groups using 240 min of illumination time. Black bars represent the standard deviation. Group I (PS–L–), group II (PS–L+), group III (PS + L–), group IV (PS + L+).

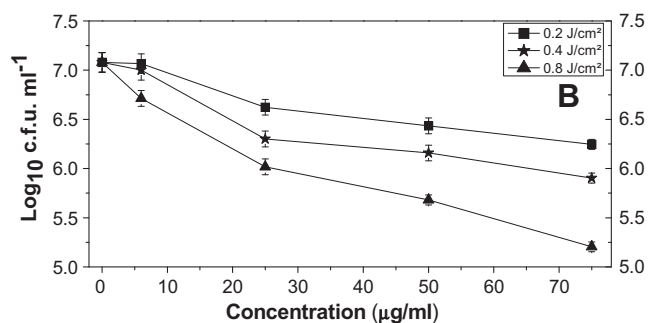
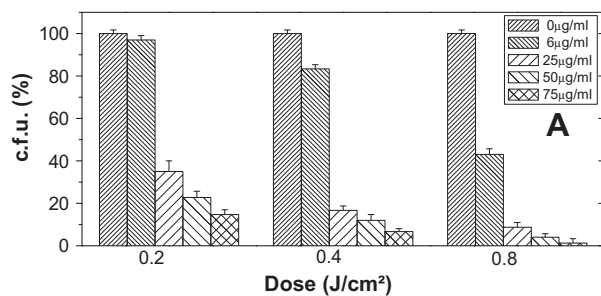


Fig. 5. Detailed results of the experimental group IV. (A) Percent values of c.f.u. counting for all PS concentrations as a function of light doses. (B) Logarithm decrease in the microbial population as a function of PS concentration. Black bars represent the standard deviation.

as a function of PS concentration. The results reveal that for a light dose of 0.2 J/cm^2 the bacterial reduction achieved was 3% (log reduction < 0.01) using 6 $\mu\text{g/ml}$ PS concentration and 84% (log reduction < 0.8) using 75 $\mu\text{g/ml}$ PS concentration. Increasing the light dose to 0.8 J/cm^2 the bacterial reduction achieved was 54% (log reduction < 0.4) using 6 $\mu\text{g/ml}$ PS concentration and 98% (log reduction < 2) using 75 $\mu\text{g/ml}$ PS concentration.

4. Discussion

It is possible to determine PDT protocols in accordance with the PS concentration and the light dose. The bacterial reduction obtained with 75 $\mu\text{g/ml}$ concentration and 0.2 J/cm^2 light dose is sim-

ilar to the one obtained with 25 $\mu\text{g/ml}$ and 0.4 J/cm^2 light dose. This indicates that the PDT protocol using CL as a light source is flexible and the choice of parameters could be adapted to the patient's condition. The same standard is observed between the protocols with 75 $\mu\text{g/ml}$ and 0.4 J/cm^2 and with 25 $\mu\text{g/ml}$ and 0.8 J/cm^2 . There is no direct correlation between the here tested PS concentrations and the potential concentration to be used at the clinic. In an infected lesion the microorganisms are present in a biofilm, resulting in distinct PS and oxygen distributions. Based on our experience, and also on several studies from other groups, we expect that both PS concentration and light dose needed for PDT response in an infected lesion will be higher than the experimental values determined in planktonic culture.

Besides, it should be pointed out that the microbial reduction over 95% (log reduction ≈ 2) obtained using the PS concentration of 50 or 75 $\mu\text{g/ml}$ and 0.8 J/cm^2 light dose are in accordance with the results obtained with other conventional microbial reduction techniques [8,25,28]. It is clear that a higher microorganism reduction can be obtained with the much higher light intensities achieved with laser or LED sources, as presented by studies where the log decrease is of 4–6 units [2,29,33]. We believe that this log reduction range is difficult to obtain using the chemiluminescence as a light source. In order to achieve an antimicrobial effect, the PDT protocol can be modified balancing the PS concentration and light dose. In our study, to achieve higher log reduction units, the PS concentration would have to increase over 75 $\mu\text{g/ml}$, but in this case, the PS dark toxicity begins to be relevant. Even though the microbial reduction was about 2 log units, the possibility of the application of multiple PDT sessions may overcome this limitation. Based on the concept of a CL-PDT for a home treatment this can be feasible. A home treatment for infectious lesions is attractive for health policies of country communities and small towns without hospital facilities.

The goal of this *in vitro* study was to demonstrate the efficacy of the light generated by a chemiluminescent reaction as a light source for photodynamic therapy applied to microbial control. The idea of a portable, flexible and low cost light source based on chemiluminescence shows a potential clinical use.

The results presented in this paper demonstrated the feasibility of applying this technique for *in vivo* studies, even though the PS and light parameters may change for tissue application. For all investigated protocols, the chemiluminescence effectiveness for PDT can be observed (group IV). The other groups (I, II and III) showed that there is no relevant bacterial reduction in the absence of either photosensitizer or light.

Under the presented experimental conditions, two important behaviors were verified. Firstly, the higher is the PS concentration, the smaller is the importance of illumination time. Secondly, the longer the illumination, the smaller the importance of the photosensitizer concentration in presented experimental conditions. Chemiluminescence showed itself as being a potential light source for microbial control; however, further investigations are necessary and will be the topic of future studies.

The light intensity decreases as a function of time, a behavior that is related to the CL reaction. We chose to replace the CL solution every 60 min for the groups with 1 and 2 h of illumination. The decrease in intensity affects the overall PDT, effect since it modifies the delivered energy. In this first study we aimed to determine the CL efficiency as PDT light source, in further experiments it may be possible to assemble a device where the CL solution is continuously replaced to overcome this intensity decrease issue.

The light intensity provided by the CL reaction decreases continuously as a function of time. The initial value for our setup is of 100 $\mu\text{W/cm}^2$ and the observed decay is ruled by an exponential equation. In this way, after 20 min of reaction, the intensity is around 50 $\mu\text{W/cm}^2$. After 60 min of reaction, the solution was re-

placed by a new one. This non-uniform intensity during the whole experiment has influence on the dosimetry and also on the final PDT response. Even though this is not ideal for the PDT response, we still wanted to test its overall efficacy on the microbial reduction.

The inactivation efficacy would be indeed improved if the illumination time for each CL solution was of 10 min, but in this experiment we aimed to evaluate the efficacy of the total reaction. Improvements can be done if we use a 10 min time of CL replacement.

We believe that a CL source for clinical PDT could be a transparent and flexible bag filled with the CL reactants. The CL reaction would be initiated just before its placement on the target tissue after the breaking of small glass tubes containing the reactants. The treatment time for each bag must be previously determined based on the half lifetime of the CL reaction. If needed, the CL bag can be replaced for new ones until the total light dose is delivered to the target tissue.

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