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Radiation mode and tissue thickness impact on singlet oxygen dosimetry methods for antimicrobial photodynamic therapy

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ABSTRACT

The target of the presented study is to evaluate the performances of illumination modes on Photodynamic therapy (PDT) for different tissue depths. For this purpose, radiation-based super pulse and pulse illumination modes were investigated for antimicrobial PDT (AmPDT). Singlet oxygen luminescence level was measured from two different points. The first one was to appraise the light penetration depth effect on singlet oxygen luminescence level for various radiation modes. The second one explored the singlet oxygen luminescence dosimetry (SOLD) method from deeper photosensitizer accumulated tissue levels. Two main experiments were performed in this study. The singlet oxygen concentration was calculated with singlet oxygen explicit dosimetry (SOED) and SOLD methods for various tissue depths in these experiments.

According to the results of the experiments, super pulse mode (SPM) provided relatively high Staphylococcus Aureus (S. aureus) cell death by 5–12%. The penetration depth was increased between 0.2 mm and 0.7 mm during the experiments. SOLD-based singlet oxygen detection system was utilized to detect singlet oxygen production levels from various tissue thicknesses to evaluate the system's usefulness for deeper infected tissues. It was observed that SPM was more effective than pulse mode radiation after a certain tissue depth ($\leq$ 2 mm).

1. Introduction

PDT is used for cancer and infection treatments. PDT requires three essential materials; photosensitizer (PS), light energy, and oxygen [1,2]. The main concept is to excite PS by an appropriate wavelength of light. The irradiation and PS produce singlet oxygen with the present oxygen molecules inside the target area [3–6]. The amount of the produced singlet oxygen can give information about the efficiency of the therapy. The light-tissue interaction is also an important aspect that depends on the depth of the targeted treatment area. PDT success is related, in part, to the concentration of PS, the fluence rate, light-tissue interaction, and the oxygenation of the treatment area [7–11].

There are many studies in the literature about light-tissue interactions [12–16]. The near-infrared light penetration depth is relatively higher due to visible light in tissue, and it generally takes values in the [1.0 mm, 3.0 mm] range [17]. This range of penetration ensures that it is possible to treat diseases in these layers by using phototherapy. The penetration depth can also affect the performance of PDT dosimetry techniques before detecting singlet oxygen luminescence. There exist several dosimetry techniques in the literature [10,11,18]. SOED is a mathematical calculation-based singlet oxygen determination method, and SOLD is a radiation measurement method commonly used for PDT treatment [20–22].

PDT can be used in various areas such as infection and cancer treatment. Infection can deeply spread throughout the tissue. So, infected tissue treatment can be challenging concerning the spread of infection inside the tissue. Under such conditions, radiation modes can improve the performance of the treatment choices. Temperature changes and optic power distribution can affect the tissue parameters, too [26]. Singlet oxygen measurement from the deeper part of the tissue is a crux because of the 1270 nm light-tissue interaction phenomena. Therefore, the singlet oxygen measurement systems need to be evaluated for different applications inside the various tissue depths.

The proposed study was conducted for singlet oxygen luminescence detection of methylene blue (MB) injected tissues from various depths. Singlet oxygen level was investigated with pulse and super pulse irradiation modes, and during the experiments, MB solution was prepared for different solution percentages. Singlet oxygen luminescence level from different tissue thicknesses was calculated with SOED and was measured with SOLD methods. Singlet oxygen luminescence level was

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investigated from two other points of view. The first one evaluates the light penetration depth effect on singlet oxygen luminescence level for various radiation modes. The second one is to estimate the photon measurement ability of the SOLD-based singlet oxygen detection system (SODS) acquired from deeper photosensitizer accumulated tissue levels. Different experiments were performed with an LED light source. Experiments were carried out with MB solution (1% and 0.5%) and LED light source (10 J/cm^2- 660 ± 10 nm). According to the results, the S. aureus mortality rate was relatively higher with SPM. Furthermore, about 5–12% higher singlet oxygen concentration was measured for the SPM case. It is observed that after a certain tissue depth (≤ 2 mm), SPM was more effective than PM.

The study is organized as follows. In Section 2, bacteria added MB solution placement in tissue, singlet oxygen generation, and experimental procedure were explained. The results, discussions, and conclusions were given in Sections 3–5, respectively.

2. Materials and methods

In this section, the experimental procedure, preparations, and singlet oxygen measurement process were discussed. Bacteria culture preparation and how to place it in tissue were explained. Mathematical equations used to calculate singlet oxygen amount were given, and then singlet oxygen measuring options under various tissue thicknesses were introduced. The dosimetry calculation function was improved to determine the singlet oxygen potential of the solution as made in references [19] and [21]. Finally, light source specifications and the experimental setup were mentioned in the order.

2.1. MB plus bacteria culture preparation and placement inside the tissue

S. aureus strain was used in the proposed study. Sheep blood agar was used as a cultural environment. Cells were incubated at 37 °C in 5% CO_2. S. aureus Petri plates were prepared as introduced in literature [9, 10]. After this inoculation, different percentages of the MB were inserted inside Petri dishes and 100 ul bacteria solution inserted inside Petri dishes. Before an experiment, the dye incubation time for a petri dish was 5 min. 0.05% and 0.1% (1 mg/ml) of MB was used.

The chicken breast tissue was used in this experiment because of its structure [22]. For this purpose, freshly slaughtered raw chicken breast tissues have been kept at room temperature for 20 min to make temperature-related tissue properties consistent. Then, fatty and skin parts have been removed to make them more homogeneous. Two different parts of chicken tissue were prepared. From the first part of the tissue, one scoop-shaped tissue was removed to place the MB solution. For the second part, 1–4 mm tissue parts have been prepared in various thicknesses (with 0.1 mm steps), and then each sample has been placed on the top of the Petri dish with S. aureus cultured MB solution.

2.2. Mathematical calculation and measurement of singlet oxygen

Singlet oxygen can arise from two different reactions (Type I, Type II). Type II reaction is more common than Type I reaction. Type II reactions produce singlet oxygen as the primary photocytotoxic agent that causes cell death [9,10]. Singlet oxygen can be measured and calculated with SOED and SOLD methods. In our previous works, SOED was used to validate SOLD measurements [18,19]. Calculation-based open dosimetry methods can be used during laboratory studies and applications. Researchers mainly focus on measuring phosphorescence and PS concentration. However, these measurements need to be evaluated from various points of view to make them useful in treatment plans.

Radiation parameters' effect on singlet oxygen generation was calculated according to references [23–25,30]. Macroscopic model-based singlet oxygen level was calculated with photophysical parameters. Time-dependent differential equations based function of singlet oxygen can be used to calculate singlet oxygen levels. These equations are mainly based on continuous illumination models. Furthermore, PM-based singlet oxygen determination models were introduced in the literature.

The equations were given with (1-4), and SOED-based model parameters were shown in Table 1.

$$\frac{d[S_0]}{dt} + \left(\xi\sigma\frac{I([S_0]+\delta[^3O_2])}{[^3O_2]+\beta}\right)[S_0] = 0 \quad (1)$$

$$\frac{d[^3O_2]}{dt} + \left(\xi\frac{I[S_0]}{[^3O_2]+\beta}\right)[^3O_2] - g\left(1-\frac{I[^3O_2]}{[^3O_2](t=0)}\right) = 0 \quad (2)$$

$$\frac{d[^1O_2]}{dt} - \left(\xi\frac{I[S_0]}{[^3O_2]+\beta}\right)[^3O_2] = 0 \quad (3)$$

$$[^1O_2]_{cumulative} = \int\left(\xi\frac{I[S_0]}{[^3O_2]+\beta}\right)[^3O_2]dt \quad (4)$$

The ξ, β, δ, σ, g parameters were obtained from references [27–30]. Instantaneous singlet oxygen luminescence level and overall singlet oxygen potential were calculated for experiments. The experiments were carried out to evaluate the light penetration depth and detect singlet oxygen from various tissue thicknesses.

A SOLD-based measurement model was made with SODS [19]. SODS system was used to detect 1270 nm irradiation emitted from singlet oxygen molecules during PDT. The system was able to see 2–5 fW optic power. The system can work at a DC to 20 MHz range appropriate for the small duration of the singlet oxygen occurrence time.

2.3. Light source specifications

The LED system can be used between 10 and 300 mW at 660 nm. Thus, the LED light source delivers the desired wavelength suitable for the PS. The optic power can be adjusted according to the application. LED system optic output was homogenous. The LED source was controlled between DC to 20 MHz. Powermeter and spectrometer were used to validate the output power. Singlet oxygen was measured with a SODS system that was capable of measuring 1270 nm in various frequencies. The light parameters were demonstrated in Table 2.

2.4. Experimental procedure

In the presented study, two main experiments were carried out to investigate the disinfection ability of the system in deeper infected tissues. Tissue structure and tissue depth effects on singlet oxygen luminescence level were acquired. The performed experiments were summarized in Fig. 1. The goal of the experiments was to answer the following questions: i) How deep infected tissues can be treated, and ii) Is it possible to monitor treatment efficiency with SOLD method through the radiation modes?

MB limitations and luminescence potential were also investigated by performing a group of experiments in this sense. In the experiments, MB was placed inside the scooped chicken breast tissue. The prepared

Table 1.
Super pulse and pulse mode photophysical parameters for calculating the MB solution singlet oxygen production capacity during the experiments.

Photophysical parameters	
[S0]	Zero state
I	Light fluence parameter
$[^3O_2]$	Higher state of oxygen(Triplet)
$[^1O_2]$	Excited-state of oxygen (Singlet)
ξ	O_2 usage speed
β	the triplet state decay rate
δ	Concentration correction coefficient (Low concentration)
σ	Photo-bleach coefficient
g	Bacteria oxygen consumption rate

Table 2
Light irradiation parameters.

Optic output specifications	
Wavelength (nm)	660 ± 10 nm
Mode	PM, SPM
Optic Power	100, 300 output power (mW)
Experiment duration (s)	200 s, 200 s, intervals
E (J/cm^2)	10 (J/cm^2)
Photosensitizer	MB
Beam profile	Uniform

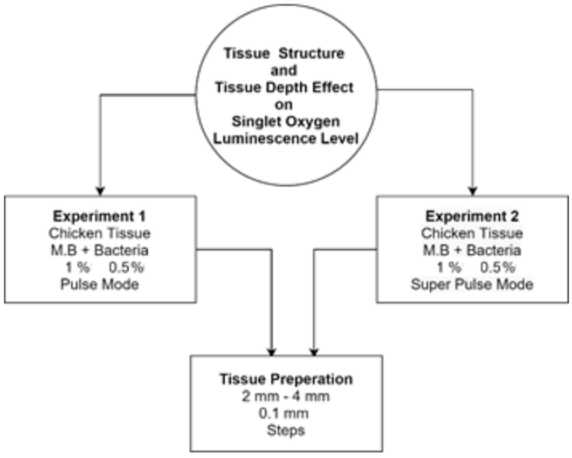

Fig. 1. The summary of the performed experiments.

chicken tissue layers were added up, and singlet oxygen measurement capacity was measured throughout every step. In this way, various tissue thicknesses were considered. The experimental setup was illustrated in Fig. 2.

As shown in Fig. 1, the performances of pulse and super pulse modes in the MB plus bacteria case were compared. One of the questions sought to be answered in the experiments was whether the super pulse mode increased the penetration depth. Thus the effect of SPM on penetration depth is studied during the experiments. Then it was investigated whether a singlet oxygen molecule can be detected from deeper infected tissues. A hole was scooped out of the base tissue part (4 cm diameter and 2 mm depth) in the experiments. The bacterial culture was filled the excavated section of the tissue. Due to the penetration level of infrared light wavelength corresponds to 2 mm, the tissue pieces were placed on this structure at 0.1 mm intervals starting from 2 mm. The standard penetration depth of 660 nm wavelength is between 2 and 3 mm.

In the proposed study, the SODS system was used to measure instantaneous and cumulative singlet oxygen luminescence. SOED and SOLD methods were compared. Measuring the photon counts per pulse can be achieved from the various depths with the same procedure via SOLD. The limit values are the critical aspect to choose the dosimetry method according to the application. SOED and SOLD methods' limits were examined for radiation modes and different tissue depths in the AmPDT application.

The survival rate of the bacteria was investigated with Petri film. The cell inoculation was made before and after the experiment for comparison. Analysis was made four times with the same procedure. The results were obtained by measuring the survival rate differences in percentages [31,32].

3. Results

In this section, the results of the performed experiments were given. Optical scattering and absorption coefficients were taken into account while placing MB solution inside the tissue. The PS concentration was evaluated according to oxygen [$^3O_2$] level throughout the time and PS concentration [S_0] under PM and SPM cases. The SOED and SOLD methods' limits were determined in our previous work [19]. Deeply infected tissue was investigated with different dosimetry techniques. SOED was used to calculate the total concentration of singlet oxygen and compared with the SOLD-based measurements.

MB photophysical specifications were taken from references [18,19], and MB solution singlet oxygen capacity was calculated as in the references [19,23].

3.1. Tissue thickness effect on singlet oxygen dosimetry methods

As discussed before, a singlet oxygen measurement task was performed for various tissue thicknesses. The SOED and SOLD methods

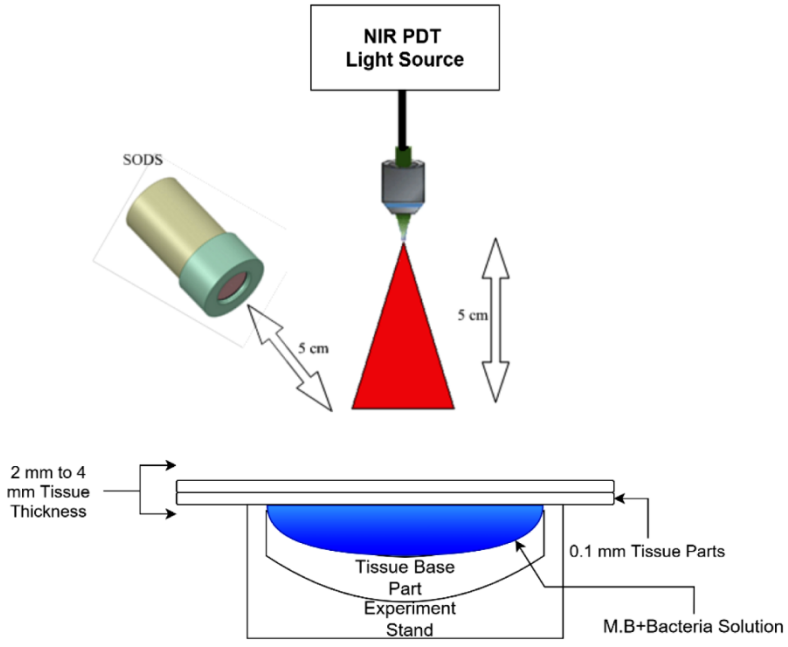

Fig. 2. The experimental setup for bacteria plus MB solution case.

were provided similar results from the thinnest tissue to the thickets. Even though the concentration was the same after a certain depth, performing measurement becomes much more difficult. For each pulse, the total amount of photons was relatively low when the tissue is thicker. The measurements were taken for every 0.1 mm tissue part from 2 mm to 4 mm. Singlet oxygen level achieved by SOLD method was demonstrated in Fig. 3. The SOLD and SOED methods were compared with each other as given in our previous studies [19,23].

Five different experiments were carried out to achieve cumulative singlet oxygen concentration. In this way, the results were validated. Due to the SPM nature, the survival rate of the bacteria was lower than that of PM mode. For 1% and 0.5% MB solution on AmPDT, SPM was shown relatively better results in different tissue thicknesses.

In clinical PDT, the tissue thickness is vital to excite the targeted area with the light source. During the experiments, the efficiency of the PM and SPM were analyzed for various tissue thicknesses. The first group of experiments was performed to investigate further PM and SPM on 1% and 0.5% MB solutions inside the tissue. The second group of experiments was carried out to inspect the PM and SPM effects on 1% and 0.5% bacteria plus MB solutions inside the different thicknesses of the tissue. The aim is to kill the bacteria without harming the tissue. Therefore, we wait 5 minutes with MB solution after bacteria injection. This is a suitable MB absorption time for bacteria but not for the tissue cells. The experimental results of 30 m, 1 h, and 6 h bacteria cultures were obtained. Survival bacteria percentages were shown in Table 3 [19]. According to the percentages given in the table, SPM can reach a relatively deeper part of the tissue. Thus, under the same fluence rate, higher singlet oxygen luminescence was measured. Cell viability analysis was made with a spectrophotometer. The cell death rates given in Table 3 were combined results, including necrosis and apoptosis.

As given in the reference [23], 400 s duration 10 J energy was used for S. aureus AmPDT application. Singlet oxygen levels obtained by SOED and SOLD methods were 3%± 0.5 different from each other. The calculation and measurement methods were provided almost the same information about the MB solution singlet oxygen capacity. In the deeper tissue part, the calculation results remain the same. However, performing measurement was much more difficult due to the 1270 nm light behavior inside the tissue. Different geometry and tissue thicknesses in deeper infected tissues, calculation-based dosimetry method can be used. On the other hand, the main drawback of the calculation-based dosimetry method was that it could not give real-time information about the treatment.

In summary, a higher level of singlet oxygen can be achieved by using SPM at relatively same tissue thicknesses. AmPDT light sources can be integrated with PM and SPM to achieve better results. However, SOLD-based instruments are pretty expensive, and SOED-based methods cannot give instantaneous information about the treatment. Therefore, the dosimetry model needs to be further improved by the researchers.

Table 3
S. aureus post-treatment percentages.

Medium/ Mode	Inactivated Bacteria (%)				
After 10 min.	1 mm	2 mm	2.5 mm	3 mm	4 mm
MB %0.5 PM	45.32 ± 2.58	39.32 ± 2.32	36.66 ± 2.51	20.12 ± 1.85	10.20 ± 2.55
MB %1 PM	52.44 ± 3.68	44.24 ± 3.36	41.78 ± 3.55	28.55 ± 2.32	15.35 ± 4.22
MB %0.5 SPM	47.55 ± 4.52	43.23 ± 4.66	39.55 ± 3.45	27.32 ± 2.12	17.31 ± 2.28
MB %1 SPM	53.35 ± 3.35	48.78 ± 3.89	43.66 ± 2.95	38.22 ± 3.05	22.33 ± 2.34
After 30 min.					
MB %0.5 PM	55.33 ± 3.88	46.57 ± 2.59	43.25 ± 2.53	24.88 ± 2.88	15.87 ± 2.55
MB %1 PM	62.58 ± 1.78	56.42 ± 2.56	52.15 ± 2.98	32.34 ± 2.55	19.65 ± 3.13
MB %0.5 SPM	58.22 ± 3.63	50.56 ± 2.53	47.15 ± 2.25	33.22 ± 3.55	20.65 ± 2.58
MB %1 SPM	65.35 ± 2.91	60.24 ± 3.11	57.37 ± 2.75	40.96 ± 1.90	25.65 ± 3.55
After 1 h.					
MB %0.5 PM	65.23 ± 2.83	57.63 ± 2.46	52.42 ± 3.26	30.30 ± 2.15	20.28 ± 2.22
MB %1 PM	68.36 ± 1.71	63.22 ± 2.51	58.51 ± 2.32	40.22 ± 1.90	23.55 ± 2.75
MB %0.5 SPM	67.65 ± 2.78	60.22 ± 1.99	54.66 ± 2.50	40.36 ± 1.90	24.68 ± 3.35
MB %1 SPM	72.33 ± 2.38	66.87 ± 3.69	62.55 ± 2.24	43.22 ± 3.12	29.31 ± 3.22
After 6 h.					
MB %0.5 PM	82.32 ± 3.65	75.13 ± 2.13	72.25 ± 3.55	50.12 ± 2.32	25.88 ± 1.69
MB %1 PM	91.73 ± 2.17	85.55 ± 3.05	80.32 ± 2.66	57.32 ± 1.66	29.25 ± 2.24
MB %0.5 SPM	88.25 ± 3.25	79.11 ± 2.99	77.32 ± 2.55	59.32 ± 2.78	31.66 ± 1.55
MB %1 SPM	93.35 ± 2.52	88.35 ± 3.12	83.15 ± 2.78	67.15 ± 1.88	39.22 ± 2.35

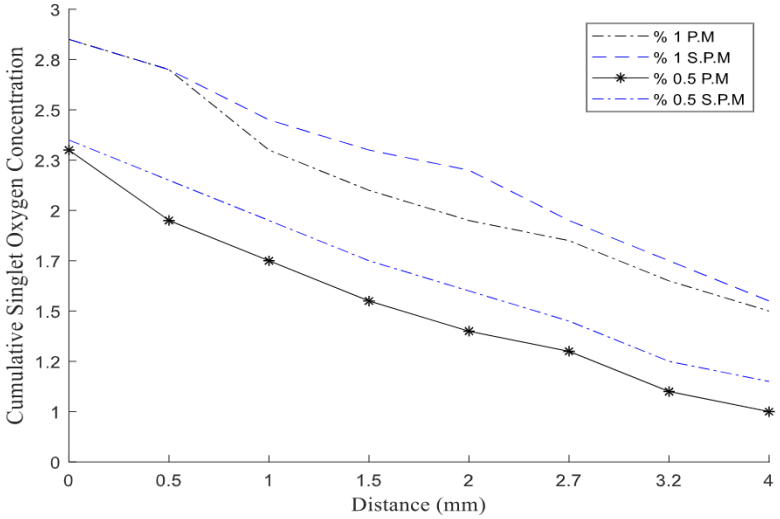

Fig. 3. Overall singlet oxygen concentration (mM) versus tissue thicknesses (mm).

4. Discussion

In a highly oxygenated environment, PDT can be more effective due to its high singlet oxygen-producing capacity. However, in a real-life biological environment, limited oxygen capacity is a challenging circumstance for PDT. Lower optic power administration can produce a solution, but the oxygen supply rate is directly related to the success of the treatment. Therefore, the radiation modes can be the solution for re-oxygenation and oxygen supply problems due to the light's pulse width modulation (PWM) period.

Tissue-related parameters like limited oxygen concentration, different geometry, and thickness may cause a reduction of treatment success [22,23]. The pulse-based radiation modes can also overcome tissue-related problems such as hypoxia and thermal damage [22,39,40]. PDT light source and dosimetric system optimization are crucial for experiments performed under different tissue thicknesses. The deeply infected tissue is a common problem for various cases, such as diabetic foot patients. As mentioned before, hypoxia and thermal damage can be more harmful to these patients. New generation light sources were developed with PM and SPM radiation modes to avoid undesired effects. These light sources can prevent hypoxia and thermal damage [19,23]. The optic properties of the tissue can be changed with temperature. The optical properties such as absorption coefficient, reflectance, and transmittance values need to be stabilized to make a more precise and stable treatment. Several studies analyzed the temperature effect on optical tissue properties and light tissue interaction [39,40]. Therefore, tissue temperature changes need to be minimalized with pulse-based light modes for more stable treatment during the radiation period. Also, the pulse-related method can provide an environment stress-free time for re-oxygenation. Thus, environment oxygen intake can occur between the pulses, and treatment time can be expanded with pulse modes. In a more extended period, as in metronome PDT, the significance of a hypoxic environment is somewhat reduced (but still very important). In many cases, irradiation, itchiness, and burned tissue can occur. During infection treatments, itchiness and burned tissue problem is deeply disturbing especially for diabetic foot patients. Pulse-based light sources become necessary in these situations.

In this paper, at first, the performances of SPM and PM were compared for various tissue thicknesses. Then the effectiveness of SOED and SOLD methods were investigated for different tissue thicknesses. As a first contribution, 5–12% more cell death was observed than PM by utilizing SPM. The SPM can penetrate the water-based solution slightly deeper; therefore, it can reach further inside the experimented environment. SPM mode can be used for both in vitro and in vivo environments. Another benefit of the SPM was the waiting period of the pulse, which is longer than other modes. Due to its nature, temperature changes can be minimized, and re-oxygenation can be maximized with the same treatment period.

The second contribution was to prove the success of SOED and SOLD models in the thinner and infected tissues. SOLD method-based singlet oxygen measurements were mildly ineffective for deeply infected tissues due to the 1270 nm light behavior inside the tissue. From the tissue point of view, the 1270 nm singlet oxygen luminescence can behave as a light source inside the tissue. In this case, the light level in the tissue can change. The detection system will detect a certain amount of 1270 nm radiation in tissue at 26 °C temperature but when the tissue temperature increased to 28 °C. However, the singlet oxygen production remains the same; the detected 1270 nm radiation amount would change.

On the other hand, the 1270 nm light source will act as if located at deeper tissues when SPM is used. Due to reflectance and transmittance coefficients variations, the SODS system's singlet oxygen detection ability will change. The information about the amount of singlet oxygen must be precise to evaluate the treatment success accurately. The specialist cannot distinguish whether the environmental parameters or the optical parameters have changed during the treatment. So, a stable environment is essential for singlet oxygen detection systems.

For each tissue thickness, oxygen level and PS singlet oxygen capacity were compared. SOED and SOLD methods provided similar results about singlet oxygen capacity. However, the SOLD model is much preferable when the infections were shallow. The SOED model can also be used for various situations, but the method cannot give instant information about treatment efficiency. For example, SOED-based calculations can be made to know about the PS effectiveness in previously simulated tissues. The calculations can be used as a decision support system to evaluate the success of the PDT in skin cancer and superficial infections. The SOLD plan can provide factual time information about the treatment and accurate results about the stability of the parameters during the treatment. Besides, hypoxia can be monitored in real-time with SOLD method. The singlet oxygen occurrence gives instantaneous information about the treatment outcome. When the singlet oxygen signal becomes week, the system can pause the treatment for re-oxygenation. This provides the tissue with more time for cooling and re-oxygenation. Singlet oxygen and timing can be used as a first-hand treatment outcome investigation tool for PDT. With pulse-based modes, singlet oxygen luminescence optimized SOLD, and SOED methods can be used in treatment plans. However, tissue thickness-related parameters need to be taken into account for more accurate treatments.

Pulse mode-related studies were presented for various research areas [33–38]. Tissue and light-related parameters need to be optimized to improve the performance of SOLD-based systems. Also, SPM can diminish the undesired effects of the treatment and reach the deeper parts of the tissue while reducing the treatment period. As far as we know, SPM was not used in the PDT field. Our previous studies have developed a singlet oxygen detection system, and we have investigated the super pulse effect on the tissue for PDT applications [18,19].

5. Conclusion

Cancer, infection, and AmPDT are the common execution areas of PDT. The activity of PDT can be increased by appropriate parameter optimization and dosimetric monitoring. The use of radiation mode and singlet oxygen measurement techniques is essential for real-time PDT monitoring. On the other hand, there are some drawbacks when the targeted area is deeper parts of the tissue. The penetration distance of the light in tissue is limited. Thus, re-oxygenation and light specifications become more critical to increase the accuracy of SOLD measurements. If the light source is not stable, the oxygen level can change. Indeed, the main reason for this change is not evident. The light source and radiation modes must be steady to achieve more reliable SOLD measurements. Tissue light interaction is an essential part of the PDT.

The MB can be treated as a light source at 1270 nm. The 1270 nm light behavior is essential when the infection influences the deeper parts of the tissue. The study demonstrated that the tissue depth effect of the radiation modes and the ability to measure singlet oxygen luminescence from the deeper parts of the tissue could be different. SOED and SOLD model was investigated with AmPDT application with varying thicknesses of tissue. The SPM and PM can be used in the AmPDT application to improve performance. However, most of the commercial systems are embedded with continuous light mode. The first step is the usage of pulse mode embedded stable light sources to use dosimetric methods. The second step is making regular measurements of 1270 nm luminescence with advanced technological systems. When these systems are combined, more accurate and more successful results can be acquired.

The pulse-based modes can be utilized to achieve more effective treatments. The real-time monitoring of the application can be a pretty challenging task. Therefore, more research needs to be done about tissue thicknesses, radiation modes, dosimetric methods, and these parameters' correlation. Also, dosimetric methods need to acquire light behavior characteristic coefficient as a function parameter in the SOED field. In future works, SOED and SOLD process-based hybrid models can be developed.

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