**UNIVERSIDADE DE SÃO PAULO
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**Study of the principles involved in the activation of
indocyanine green by infrared radiation in the
photodynamic inactivation of pneumonia**

São Carlos

2020

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indocyanine green by infrared radiation in the
photodynamic inactivation of pneumonia**

Dissertation presented to the Graduate Program in Physics at the Instituto de Física de São Carlos, Universidade de São Paulo to obtain the degree of Master of Science.

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Advisor: Profa. Dra. Cristina Kurachi

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2020

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*“No puedo concebir a un auténtico
hombre de ciencia sin una profunda fe.”
Albert Einstein*

ABSTRACT

DIAZ TOVAR, J. S. **Study of the principles involved in the activation of indocyanine green by infrared radiation in the photodynamic inactivation of pneumonia.** 2020. 87p. Dissertation (Master of Science) - Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2020.

Pneumonia is one the main causes of death worldwide, and it is mainly due to the increase of antibiotic microbiological resistance. Photodynamic therapy (PDT), which uses the combination of light and a photosensitizer (PS) drug to cause damages in biological target, has emerged as a non-invasive clinical approach for different kind of treatment to which development of resistance is reported to be unlikely. Our research group has demonstrated the efficient of photodynamic inactivation (PDI) of *Streptococcus pneumoniae* *in vitro* and *in vivo* using Indocyanine green as a PS. In this work was investigated the efficient of generation of reactive oxygen species (ROS) of Indocyanine green (ICG) by comparing two wavelengths, 780 and 808 nm. As well as the efficient of 808 nm wavelength to pass through structures with similar optical properties of skin and activate ICG by extracorporeal illumination to generate PDI in *Streptococcus pneumoniae*. For the first part of the work, photobleaching experiments were performed at 780 and 808 nm, different oxygen concentrations and solvents. Sensitizer bleaching was recorded by absorption spectra and then analysed by using the PDT bleaching macroscopic model to extract important parameters of ICG. It was found higher photobleaching rates when degradating with 808 nm than 780 nm wavelength, and deactivation of ICG molecule was observed to be due to type I and type II mechanisms of PDT. For second part, the PDI efficiency was validated when incident light is attenuated by phantom barriers. Characterization of the panel of 200 laser prototype was performed by emission wavelength, irradiance statibility and temperature increase. The optical transmission attenuated light was detected as phantom barriers increased. On the other hand, Monte Carlo simulation were performed in a computerized model phantom of the thoracic cage to . Finally, for the PDI experiments it was found that even with a barrier thickness of 37.10 mm a energy dose of 197.96 J/cm² at surface of the laser panel/phantom interface is needed to achieve a total reduction of the bacterial burden. In conclusion, ICG in combination with extracorporeal illumination at 808 nm wavelength demonstrate a high efficient for treatment of lung infections as pneumonia.

Keywords: Pneumonia. Photodynamic inactivation. Indocyanine green. Phantom. Monte Carlo.

RESUMO

DIAZ TOVAR, J. S. **Estudo dos princípios envolvidos na ativação da indocianina verde pela radiação infravermelha na inativação fotodinâmica da pneumonia.** 2020. 87p. Dissertação (Mestrado em Ciências) - Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2020.

A pneumonia é uma das principais causas de morte em todo o mundo, principalmente devido ao aumento da resistência microbológica a antibióticos. A terapia fotodinâmica (TFD), que usa a combinação de luz e um medicamento fotossensibilizador (FS) para causar danos no alvo biológico, emergiu como uma abordagem clínica não invasiva para diferentes tipos de tratamento aos quais é relatado que o desenvolvimento de resistência é improvável. Nosso grupo de pesquisa demonstrou a eficiência da inativação fotodinâmica (IFD) de *Streptococcus pneumoniae* *in vitro* e *in vivo* usando verde de indocianina como FS. Neste trabalho, investigou-se a eficiência da geração de espécies reativas de oxigênio (ERO) da indocianina verde (ICV), comparando-se dois comprimentos de onda, 780 e 808 nm. Assim como a eficiência do comprimento de onda de 808 nm para atravessar estruturas com propriedades ópticas semelhantes da pele e ativar o ICV por iluminação extracorpórea para gerar IFC em *Streptococcus pneumoniae*. Para a primeira parte do trabalho, foram realizadas medidas de fotodegradação a 780 e 808 nm, em diferentes concentrações de oxigênio e solventes. A fotodegradação do sensibilizador foi registrado por espectros de absorção e, em seguida, analisado usando o modelo macroscópico de branqueamento TFD para extrair parâmetros importantes do ICV. Verificou-se maiores taxas de fotodegradação ao se degradar com comprimento de onda de 808 nm a 780 nm, e a desativação da molécula de ICG foi devida aos mecanismos de PDT tipo I e tipo II. Para a segunda parte, a eficiência do IFD foi validada quando a luz incidente é atenuada por barreiras fantasmas. A caracterização do protótipo de iluminação de 200 lasers foi realizada pelo comprimento de onda de emissão, estabilidade de irradiância e aumento de temperatura. A luz atenuada da transmissão óptica foi detectada à medida que as barreiras fantasmas aumentavam. Por outro lado, a simulação de Monte Carlo foi realizada em um modelo de phantom computadorizado da caixa torácica. Finalmente, para as experiências IFD, verificou-se que, mesmo com uma espessura de barreira de 37,10 mm, é necessária uma dose de energia de 197,96 J/cm² na superfície da interface painel laser/phantom para obter uma redução total da carga bacteriana. Em conclusão, o ICV em combinação com a iluminação extracorpórea no comprimento de onda de 808 nm demonstram alta eficiência no tratamento de infecções pulmonares como pneumonia.

Palavras-chave: Pneumonia. Inativação fotodinâmica. Indocianina verde. Phantom. Monte Carlo.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMR	Antibiotic microbiological resistance
PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PS	Photosensitizer
IC	Internal conversion
ISC	Intersystem crossing
ROS	Reactive oxygen species
NIR	Near infrared
ICG	Indocyanine green
FDA	Food Drug Administration
TQY	Triplet quantum yield
IR	Infrared
PBS	Phosphate buffered saline
SD	Standard deviation
CW	Continuous wave
FWHM	Full width at half maximum
MC	Monte Carlo
MCX	Monte Carlo eXtreme
GPU	Graphics Processing Units
CFU	Colony-forming unit
BHI	Brain heart infusion
LD	Lethal dose
MPE	Maximum permissible exposure

LIST OF SYMBOLS

1O_2	Singlet oxygen
dH ₂ O	Distilled water
N ₂	Molecular nitrogen
O ₂	Molecular oxygen
λ	Wavelength
h	Planck constant
c	speed of light
μ_a	Absorption coefficient
μ_s	Scattering coefficient
η	Refraction index
g	Anisotropy coefficient

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1 INTRODUCTION

Pneumonia is responsible for high mortality and is a common illness that continues to be the major killing cause of young children and the fourth leading cause of death in elderly people around the world.¹ Pneumonia usually begins as a colonization of the mucosa of the nasopharynx followed by spread into the lower respiratory tract. Several pathogens are known to cause pneumonia: bacteria, viruses, atypical organisms, and fungi.^{1,2} However, the predominant pathogen in pneumonia is *Streptococcus pneumoniae*. A study made by *Ranganathan* and *Sonnappa* showed that the bacterial cause was major responsible in 60% of cases of pneumonia, with *S. pneumoniae* causing 73% of the cases in which a bacterial cause was identified.³

Patient should receive antibiotic treatment when pneumonia is diagnosed, even though when the evidence is by viral infection.¹ The main antibiotics employed for treatment are macrolides, β -lactams and fluoroquinolones.⁴ However, antibiotic microbiological resistance (AMR) has been increasing, resulting in a relevant public health concern. The United Nations recently reported an alarming prediction of 10 million annual deaths caused by AMR in 2050 with no effective measurements are taken. The urgent development of treatment alternative to antibiotics is pointed out as one of these must-have actions.^{3,5} A promising alternative or auxiliary treatment for pneumonia is photodynamic inactivation (PDI), a non-invasive treatment, which is the microbiological application of photodynamic therapy (PDT).

PDT was first time reported more than a hundred years ago by Oscar Raab.⁶ It is a treatment method that involves the administration of a nontoxic dye (photosensitizer, PS) and subsequently, exposure to light to excite the PS leading to cell death. The cell death can be by necrosis or apoptosis and PDT is a highly selective process since only cells that have absorbed PS and only the irradiated site are affected.^{7,8} This treatment has been applied for tumors, localized infection, and other non-malignant conditions.^{9,10} Furthermore, it is been reported that the development of tolerance or resistance to PDI by microorganisms must be considered highly unlikely.¹¹

Figure 1 illustrates the process that occurs when a PDT dye absorbs a photon. Initially, electrons are at ground state of the PS, then a photon is absorbed and a promotion of the electron to an excited singlet state, usually S_1 , happens. The excited molecule may dissipate this energy by radiative (fluorescence) and non-radiative (internal conversion (IC), intersystem crossing (ISC)) processes. ISC is a spin-forbidden transition of S_1-T_1 interaction, and radiative transition known as phosphorescence can bring the electrons back to the ground state. The lifetime of the excited triplet state is typically 10^{-6} - 1 s.¹² For PDT is accepted that long lifetime of the triplet state allows sufficient time for the

Figure 1 – Simplified Jablonski diagram showing the possible fates of a PDT dye molecule following photon excitation. $h\nu$ is the photon energy, 1PS is the singlet ground state, $^1PS^*$ is the first excited state, and 3PS is the triplet state of the PS. 3O_2 is the ground state triplet oxygen and 1O_2 is the excited state singlet oxygen.

Source: By the author.

PS interacts with molecules in the neighbor microenvironment (usually oxygen), and then produced cytotoxic species. This process is know as energy transfer and results in the generation of singlet oxygen.¹³ There are two mechanisms recognized in PDT, type I and type II reactions.

In type I reaction, the PS interacts with the solvent or biological substrate by electron/hydrogen transfer to produce highly reactive free radicals and other reactive species, such as: anion/cation species of the PS, superoxide radical, hydrogen peroxide, hydroxyl radical.¹⁴ The second photochemical reaction, type II, produces an electronically excited state of oxygen known as the singlet oxygen (1O_2) through energy transfer. In an enviroment with high oxygen concentration, and dependending on the PS used, is widely accepted that the phototherapeutic effect of PDT is a result of the generation of 1O_2 .¹³ However, both type I and II processes contribute to the production of reactive oxygen species (ROS) and their contribution depends on the PS used, PS concentration and oxygen concentration.¹⁵

Even though there are several PS types that can be excited by light at the UV-visible region, sometimes a PS absorbing at the near infrared (NIR) spectrum is preferred, due to a higher light penetration depth at biological tissues.¹⁷ The use of PS with NIR absorption for PDT applications may represent a higher volumetric tumor response or the targeting of an internal infected organ. Indocyanine green (ICG), a tricarbocyanine dye (figure 2a) that is able to penetrate living cells without inducing immediate degenerative changes, and it is excited at the near infrared spectrum, has been used in medicine since 1956 and proposed as a possible PS.^{17,18} ICG is approved by Food Drug Administration (FDA) for medical applications such as the diagnostic of liver function, cardiac output,

Figure 2 – (a) Chemical structure of indocyanine green, ICG. (b) Normalized absorbance spectrum of 5 μ M ICG dissolved in distilled water.

Source: (a) GIRAUDEAU *et al.*¹⁶ (b) By the author.

and blood volume.^{19–22} ICG shows a highest amplitude of absorption at 780 nm in water (figure 2b) and 805 nm in human plasma.²³ At the spectral region of 600 - 1300 nm, most biological tissues are relatively transparent (figure 3) thus presenting highest optical penetration depth of light.²⁴

Aggregation of dye molecules in aqueous solution is very common in cyanine and other organic dyes.²⁵ ICG instability in various media and under different conditions has been studied, and it was found that for certain concentrations and solvents, ICG do not follow Lambert-Beer's law^{25,26} because it forms large aggregates in water and aqueous salts. ICG is reported to have two predominant peaks, 780 and 715 nm, the first at 780 nm is associated with a monomeric species, and the one at 715 nm is associated with dimeric species due to H-aggregates forms.^{25–27} Also, it is known that transition of fundamental singlet state to the first excited singlet state occurs at the peak of 780 nm. Furthermore, for ICG at room temperature a red-shifted J-aggregate absorption band is formed within about two weeks in aqueous solution, and the J-aggregation is accelerated by heat treatment and salt addition.²⁸ All this behaviors influence its degradation, and the concentration of the ICG solution tends to be more unstable, for that reason, lower concentrations are preferred to have better stability of the molecule.

PDT action strongly depends on the efficient PS excitation and production of reactive oxygen species and free radicals to induce cell death. The overall PDT effectiveness will only occur when the reaction is achieved over a threshold limit to produce enough damage to the target cells. In this sense, to assure enough light energy and PS concentration is essential. PS is a critical element in PDT, and an ideal one should have the following requirements³⁰:

- Selectively to accumulate in biological targets.

Figure 3 – Relative absorbance of the main absorbing biomolecules present in tissues at the 400-2000 nm spectral window.

Source: HUANG *et al.*²⁹

- Minimum dark toxicity and be cytotoxic in the presence of light.
- Absorb significantly in the tissue optical window.
- Have a high quantum yield and long lifetime o triplet state.
- Be rapidly excreted from the body.

ICG meets most of the requirements, however the triplet quantum yield (TQY) of ICG has been reported to be extremely low,^{23,31} furthermore, direct detection of the singlet oxygen luminescence at 1270 nm for ICG was unsuccessfully measured.³² Then, since the mechanism of PDT is based on the generation of ROS, ICG is not believed to be a good PS.

In somehow, different studies base on the use of ICG as a treatment agent in PDT have been reported, and are summarized in Table 2 of the article published by *Giraudeau et. al.*¹⁶ There, has been identified authors who observed generation of ROS.^{20,33–37} Some of them suggested that the basis of the inhibition effect on biological target killing involves the singlet oxygen.^{20,35,36} There have been any other reports of the cytotoxicity of ICG in the presence of light,^{17,20,34,38–40} even though the exact mechanism of cytotoxicity of ICG remains unclear.

Most of PDT studies are made at the UV-Vis spectrum because of the absorption of the used PSs.⁷ As mentioned before, at this region a higher absorption is presented, and to avoid this behavior from biological tissue, PSs with long-wavelength are required, this is where ICG presents itself as a possible candidate. Even with the low efficient of TQY of ICG, it is chosen due to its infrared (IR)-NIR absorbance and also because cytotoxicity has been reported. Then the possibility of using extracorporeal illumination in the IR region together with ICG for the treatment of pneumonia arises.

The use of ICG as a PS activated by extracorporeal IR light has been studied by our research group since 2013. In 2017 was demonstrated the photodynamic inactivation of *Streptococcus pneumoniae* in an *in-vivo* study.³⁸ After a single PDI session using ICG at 100 μ M and 120 J/cm² of light at 780 nm, no bacteria was recovered in 80% of the animals.³⁸ Also, small differences were observed in infected mice treated just with light and just ICG as compared to untreated controls.

Furthermore, a study published in same year was made to proof the safe of PDT treatment in RAW 264.7 macrophages.⁴⁰ It was found low cytotoxic impact on the macrophages cultures when using concentration of ICG at 5 μ M + 780 nm laser illumination or 10 μ M + 850 nm LED device. Therefore, safe and efficient *in-vitro* protocol for the ICG-PDI inactivation of *Streptococcus pneumoniae* was successfully presented.⁴⁰

In the race to turn previous studies into a clinical protocol for humans, ways to deliver the PS have been studied. Previously, *Geralde et. al*³⁸ delivered ICG using instillation, which is uncomfortable and not well accepted in clinical practice.⁴¹ Then, in 2019 a study was published by *Kasaab et. al* proposing nebulization as a tool to deliver the PS to the respiratory tract.⁴² Their conclusions suggest jet nebulization as an adequate method for delivering PSs with the optimal size and keeping stable the compounds through process to the lungs. Specifically, it was shown conditions in which ICG is deposited in the respiratory tract and activated with extracorporeal light and not cause acute lung or liver damage.⁴²

Moreover, all these studies show that ICG has a great potential to be a good PS due to its strong absorption band in the region of deeper penetration in biological tissue. Also, since it does not have a high TQY and therefore it is not likely to go through the reaction of type II, it is believed that ICG produces highly reactive free radicals, involved in the type I reaction.

On the other hand, an undergraduate student of our group has performed an experiment of the photodynamic inactivation efficiency comparing two wavelengths, 780 and 808 nm. In table 1 is presented the results at different incubation times of *Streptococcus pneumoniae* (0 and 4 h), as well as the PDI treatment, light treatment and control for each condition. It is clearly observed that immediately after PDI treatment, 808 nm wavelength is capable to reduce the total bacterial burden. This behavior is unexpected since absorption at 780 nm is approximately two times greater than 808 nm. These unconventional results show us that the mechanism of ROS generation by ICG is not well clear.

Additionally, it is known that ICG is unstable in aqueous solution, since these solutions undergo transformation as degradation changes. Different factors affect this degradation kinetics such as concentration, pH, light exposure, and temperature.^{23,25} These parameters are relevant in the ROS production process of ICG.

Table 1 – Effect of laser and PDI applications at two different wavelengths and incubation times of *Streptococcus pneumoniae* (Unpublished data).

Condition in $\log_{10}(\text{CFU/mL})$	Incubation time	
	0 h	4 h
Control	6.222 ± 1.107	5.226 ± 0.831
Light 780	5.671 ± 0.352	5.048 ± 0.841
PDI 780	4.496 ± 0.482	0
Light 808	5.706 ± 0.633	5.108 ± 1.386
PDI 808	0	0

Source: Samara S. da Silva and co-workers (not published).

Following this introduction, the photobleaching kinetics of ICG is presented in Chapter 2. The degradation behavior at two different irradiation wavelengths (780 and 808 nm) and solvents are discussed; and an analysis by the use of the PDT bleaching macroscopic model is presented.

In Chapter 3, the effect of PDI is presented when barriers of phantoms attenuated the initial radiation. The discussion includes Monte Carlo simulation, characterization of the illumination plate, attenuation of initial radiation by phantom thickness increases and finally, PDI response.

Finally, conclusions are presented in Chapter 4.

1.1 Objectives

1.1.1 General objective

The main aim of our present study is to evaluate the photodynamic inactivation efficiency when using ICG and different conditions, mostly under 780 and 808 nm irradiation.

1.1.2 Specific objectives

- To analyse the behaviour of degradation in aqueous solutions of ICG and understand the population in the triplet and singlet oxygen state induced by continuous wave (CW) laser excitation at 780 and 808 nm.
- To perform Monte Carlo simulations for understanding the photon migration through biological tissues.
- To evaluate the photodynamic inactivation efficiency using phantom barriers mimicking optical attenuation for lung application.

2 PHOTBLEACHING OF ICG

To assess changes in ICG following laser irradiation and variations in the concentration of dissolved oxygen, spectrophotometric measurements of ICG were prepared. The main objective of this chapter was to understand how ICG photodegradation is affected by NIR illumination and presence of oxygen.

2.1 Methodology

2.1.1 Materials

ICG was purchased from Ophthalmos S.A., Brazil. ICG has a molecular weight of 774.96 g/mol, and it was used without further purification. The solvents used were distilled water and phosphate buffered saline (PBS). PBS was prepared at pH of 7.4. Aliquots of 1 mg were stored at room temperature (around 26°C), protected from light until the time of its use. Fresh aliquots were diluted in 1 mL of distilled water (dH₂O) and kept in the dark; this was considered the stock solution. At the time of each experiment, the samples were prepared at 5 μ M since this concentration falls into a linear range of Beer-Lambert law for ICG, when using absorbance spectroscopy. The samples were diluted either in dH₂O, or in a mixture of 50% dH₂O and 50% PBS, this last to present more similar biological conditions.

ICG in relative lack of dissolved oxygen was investigated just in dH₂O. To remove dissolved oxygen in the samples, N₂ was bubbled into the solution. All the experiments were performed at room temperature, new stocks were prepared each day, and freshly prepared ICG solutions were used for each experiment.

2.1.2 Spectrophotometric measurements

The absorbance measurements were carried out using a Cary 50-Varian Bio UV-Vis spectrophotometer. All samples were placed in four sides clear quartz cuvettes of 1 cm path length and absorbance spectra were recorded at room temperature in the 300 - 900 nm spectral range.

2.1.3 Dissolved oxygen measurements

The dissolved oxygen quantification was performed using the NEOFOX-KIT Oxygen Sensing System (Ocean Optics, USA), which was calibrated for our specific experiment. The calibration was performed using a two user entered O₂ data points, first point was the saturated oxygen in ICG diluted in dH₂O, and the second one was the zero oxygen point, it was reached bubbling N₂ gas to the sample. The oxygen sensor used was the FOSPOR probe, which is suitable for gas and liquid samples, with high-sensitivity for low levels

of O₂ (0.001%). The freshly prepared ICG samples in dH₂O were bubbled with N₂ gas, and the experiments were performed at two relative concentrations of dissolved oxygen, at approximately 4% and 50% (this was the minimum dissolved oxygen concentration reached), finally, the cuvette was sealed.

2.1.4 Photobleaching measurements

The irradiation experiments were performed using two different customized continuous wave (CW) laser cluster devices, one emitting at 780 nm and the other at 808 nm. Scheme of the experimental set up is presented in figure 4, which is a laser cluster used in previous studies in our research group. It is made up of 18 lasers with centralized illumination in a specific position, as presented by the laser arrows. The delivered irradiances at cuvette wall were of $I_{780} = 79 \text{ mW/cm}^2$ and $I_{808} = 116 \text{ mW/cm}^2$, the irradiances were measured using a spectroradiometer (calibrated USB 4000, Ocean Optics, USA). In order to calculate the irradiation time, a normalization with respect to the absorbed photons by ICG molecule was performed in the following way:

- The emitted photons were calculated for each device using, $ph_E = \frac{I_{ex}(0,\lambda)\lambda}{hc} \left[\frac{\text{photon}}{\text{cm}^2\text{s}} \right]$. Where $I_{ex}(0, \lambda)$ is the irradiance, λ wavelength, h Planck constant and c speed of light.
- Then, using the absorption coefficient at the respective wavelength, the absorbed photons were calculated, $ph_A = \mu_a(\lambda)ph_E \left[\frac{\text{photon}}{\text{cm}^3\text{s}} \right]$. Where μ_a is the absorption coefficient.
- The dose of absorbed photons was used as the fixed parameter, $1 \times 10^{19} \text{ photon/cm}^3$, which is a threshold energy dose described in the literature for a PDT effect.⁴³ After that, $t_{illumination} = \frac{1 \times 10^{19}}{ph_A} [s]$.

Subsequently, the samples were placed in front of the devices and the absorbance spectra were measured at different irradiation times, as a function of absorbed photons. As a non-irradiated control sample, the absorbance spectra of the self-aggregating sample were recorded at the same investigated times. For each condition, three samples were measured, and the mean and standard deviation (SD) were calculated.

2.1.5 PDT bleaching macroscopic model

The simplified Jablonski diagram (Figure 5) shows transitions of interest in PDT given by the type I and II mechanisms for any PS. Briefly, the PS in the ground state absorbs a photon and it generates transitions of electrons to an excited singlet state upon light irradiation at a suitable wavelength. The electrons in the excited singlet state can go

Figure 4 – Illumination set up used for the photobleaching degradation.

Source: By the author.

to the triplet state of the PS by a change of the spin of electrons known as intersystem crossing. $[S_1]$ and $[T]$ can interact with surrounding molecules and produce ROS through two different pathways.⁴⁴ Type I reaction is known as the transfer of free radical formation or electron transfer reaction starting from excited singlet or triplet states with cellular targets or acceptor.²³ Type II reaction involves the energy transfer between triplet state and the molecular oxygen in the ground state $[^3O_2]$, and subsequently the formation of a highly reactive state of the oxygen known as singlet oxygen $[^1O_2]$.⁴⁴

Terms in brackets represent the concentrations measured in units of molar (M), typical concentrations and transition constants are all described in Table 2. From Figure 5 a complete set of coupled differential equations describing the physical-chemical process during PDT:

Figure 5 – Simplified Jabloski diagram of the main transition that occurs for a PS in PDT process.

Source: By the author.

$$\frac{d[S_0]}{dt} = -I_a[S_0] + k_f[S_1] + k_p[T] + k_{ot}[T][^3O_2] - k_{os}[S_0][^1O_2], \quad (2.1)$$

Table 2 – Definitions and units of variables used in the kinetic analysis.

Symbol	Definition	Units
$[S_0]$	Ground-state sensitizer.	M
$[S_1]$	Excited-state sensitizer.	M
$[T]$	Triplet-state sensitizer.	M
$[^3O_2]$	Ground-state triplet oxygen.	M
$[^1O_2]$	Excited-state singlet oxygen.	M
$[A]$	Cellular targets or acceptor.	M
I_a	Rate of photons absorbed by PS per second ($\frac{\sigma_{so}F_{ex}}{h\nu}$).	s ⁻¹
σ_{so}	Absorption cross section of PS.	cm ²
F_{ex}	Irradiance of incident light.	mW cm ⁻²
S_Δ	Fraction of triplet PS that produces $[^1O_2]$.	-
ϕ_T	Sensitizer triplet-state quantum yield ($\frac{k_{isc}}{k_{isc}+k_f}$).	-
k_f	Fluorescence constant of $S_1 \rightarrow S_0$	s ⁻¹
k_p	Phosphorescence constant of $T \rightarrow S_0$	s ⁻¹
k_{isc}	Intersystem crossing of $S_1 \rightarrow T$	s ⁻¹
k_d	Phosphorescence constant of $^1O_2 \rightarrow ^3O_2$	s ⁻¹
k_{ot}	BRCQ ^a of T_1 by 3O_2	M ⁻¹ s ⁻¹
k_{os}	BRCQ ^a of 1O_2 by S_0	M ⁻¹ s ⁻¹
k_{oa}	BRCQ ^a of 1O_2 by A	M ⁻¹ s ⁻¹
k_{sa}	BRCQ ^a of S_1 by A	M ⁻¹ s ⁻¹
k_{ta}	BRCQ ^a of T_1 by A	M ⁻¹ s ⁻¹

^aBRCQ: Bimolecular rate constant for quenching.

Source: By the author.

$$\frac{d[S_1]}{dt} = I_a[S_0] - k_f[S_1] - k_{isc}[S_1] - k_{sa}[A][S_1], \quad (2.2)$$

$$\frac{d[T]}{dt} = k_{isc}[S_1] - k_p[T] - k_{ot}[T][^3O_2] - k_{ta}[T][A], \quad (2.3)$$

$$\frac{d[^3O_2]}{dt} = -S_\Delta k_{ot}[T][^3O_2] + k_d[^1O_2], \quad (2.4)$$

$$\frac{d[^1O_2]}{dt} = S_\Delta k_{ot}[T][^3O_2] - k_d[^1O_2] - k_{oa}[A][^1O_2] - k_{os}[S_0][^1O_2]. \quad (2.5)$$

It is common to use this set of coupled differential equations in PDT, because it describes the transition of PS when absorbs a photon and interact with molecules around, such oxygen. Terms with positive or negative sign indicate the increase or decrease of the rate of concentration of a specific state by process of energy absorption or transfer to other states. Equation 2.1 is normalized with respect to the rate of absorbed photons by the term I_a , which involves absorption cross section of PS (σ_{so}), Irradiance of incident

light (F_{ex}) and energy of incident photon ($h\nu$). Fraction of PS triplets by produced singlet oxygen (S_Δ) is considered in equations 2.4 and 2.5, observe that oxygen supply term is not considered in our model.

This model has been presented and discussed before by several authors.⁴⁵⁻⁴⁷ However, to the best of our knowledge, it has not been applied to study ICG kinetics. Generally speaking, since ICG aggregates and degrades by different factors, other paths of PS degradation may occur, such as interaction of the PS excited singlet state with the acceptor, interaction of the ground-state PS with some other photoexcited acceptor (apart from molecular oxygen), photoisomerisation, etc. Here, we will limit our discussion to the phenomena taking place during triplet interaction with the acceptor and singlet oxygen mediated PDT without additional complexity. Also, due to its rapid decay (typically S_1 lifetimes for ICG are in the order of 0.16 ns in 75% water - 25% methanol mixture⁴⁸ and 0.111 ns in water⁴⁹) the reactions between $[S_1]$ and acceptor are unlikely to occur. Therefore, the $k_{sa}[A][S_1]$ term in equation 2.2 can be neglected.

A solution to the set of coupled differential equations (2.1-2.5) may be obtained in the quasi-steady state, since the lifetime of the excited states $[S_1]$, $[T]$ and $[^1O_2]$ are shorter compared to the inherent time of its ground states.⁴⁷ In order to find the instantaneous equilibrium concentrations of these species, we set their time derivatives to zero.⁴⁵ For equations 2.2, 2.3 and 2.5 we obtained:

$$[S_1] = \frac{I_a[S_0]}{k_f + k_{isc}}, \quad (2.6)$$

$$[T] = \frac{k_{isc}[S_1]}{k_p + k_{ot}[^3O_2] + k_{ta}[A]}, \quad (2.7)$$

$$[^1O_2] = \frac{S_\Delta k_{ot}[T][^3O_2]}{k_d + k_{oa}[A] + k_{os}[S_0]}. \quad (2.8)$$

Finally, after some algebraic manipulation and combining equations 2.6, 2.7 and 2.8 with equations 2.1 and 2.4, we obtain:

$$\frac{d[S_0]}{dt} = -\frac{\phi_T I_a}{(k_p + k_{ot}[^3O_2] + k_{ta}[A])} \left[k_{ta}[A][S_0] + \frac{S_\Delta k_{ot} k_{os} [^3O_2][S_0]^2}{(k_d + k_{oa}[A] + k_{os}[S_0])} \right], \quad (2.9)$$

$$\frac{d[^3O_2]}{dt} = -\frac{S_\Delta k_{ot} \phi_T I_a [^3O_2][S_0]}{(k_p + k_{ot}[^3O_2] + k_{ta}[A])} \left[1 - \frac{k_d}{(k_d + k_{oa}[A] + k_{os}[S_0])} \right], \quad (2.10)$$

where it is defined $\phi_T = \frac{k_{isc}}{k_{isc} + k_f}$ as the quantum yield of the triplet state. Using substitutions:

$$\begin{aligned}
C_1 &= \phi_T I_a, & C_5 &= S_\Delta k_{ot}, \\
C_2 &= k_{ta}[A], & C_6 &= k_{os}, \\
C_3 &= k_p, & C_7 &= k_d + k_{oa}[A], \\
C_4 &= k_{ot}, & C_8 &= k_d,
\end{aligned}$$

the equilibrium concentration of $[^1O_2]$ (equation 2.7) and $[T]$ (equation 2.8) can be written as:

$$[T] = \frac{C_1[S_0]}{C_2 + C_3 + C_4[^3O_2]}, \quad (2.11)$$

$$[^1O_2] = \frac{C_1 C_5[^3O_2][S_0]}{(C_7 + C_6[S_0])(C_2 + C_3 + C_4[^3O_2])}. \quad (2.12)$$

Finally, the set of differential equations to be solved are:

$$\frac{d[S_0]}{dt} = -\frac{C_1}{C_2 + C_3 + C_4[^3O_2]} \left(C_2[S_0] + \frac{C_5 C_6[^3O_2][S_0]^2}{C_7 + C_6[S_0]} \right), \quad (2.13)$$

$$\frac{d[^3O_2]}{dt} = -\frac{C_1 C_5[^3O_2][S_0]}{C_2 + C_3 + C_4[^3O_2]} \left(1 - \frac{C_8}{C_7 + C_6[S_0]} \right). \quad (2.14)$$

The concentration decrease in ICG under photobleaching is an irreversible process that results in the formation of new chemical species called photoproducts. Assuming that photoproducts are formed as a result of reactions between S_0 and T ; and S_0 and 1O_2 , the respective photoproduct concentration as a function of time is given by

$$[P_I]_t = \int_0^t S_{Ip} k_{ta} [T]_{(t')} [S_0]_{(t')} dt' = C_1 C_2 S_{Ip} \int_0^t \frac{[S_0]_{(t')}}{C_2 + C_3 + C_4[^3O_2]_{(t')}} dt', \quad (2.15)$$

$$[P_{II}]_t = \int_0^t S_{IIp} k_{os} [^1O_2]_{(t')} [S_0]_{(t')} dt' = C_1 C_5 C_6 S_{IIp} \int_0^t \frac{[^3O_2]_{(t')} [S_0]_{(t')}^2}{(C_7 + C_6[S_0]_{(t')})(C_2 + C_3 + C_4[^3O_2]_{(t')})} dt', \quad (2.16)$$

where S_{Ip} and S_{IIp} are the fraction of S_0 and T ; and S_0 and 1O_2 reactions which produce photoproducts.

2.2 Results and Discussion

Figures 6 and 7 show the absorption spectra of ICG going through self-aggregation over time in dH₂O and 50% of PBS, respectively. Both spectra have a major absorbance peak at approximately 780 nm, a shoulder peak at approximately 715 nm and a lower peak at approximately 393 nm. The main broad band is mostly reported to be the result of the superposition of two bands,²³ the absorption peak at 780 nm is associated to a monomeric species (which is the form that ICG primarily exists at low concentrations), and is reported to be the molecules that undergo through the transition of electrons from ground state (S_0) to the first excited singlet state (S_1). The second absorption peak at 715 nm is due to vibronic side band coupling⁵⁰ and it is also associated to a dimeric band. The peak at approximately 393 nm is assigned to be the transition of electron from (S_0) to the second excited singlet state (S_2).^{50,51}

Figure 6 – Optical absorbance spectra of ICG showing its self-aggregation in aqueous solution for dH₂O.

Source: By the author.

To obtain more information from the absorption peaks in the spectrum, a multiple peak fitting deconvolution analysis was performed. ICG in distilled water showed 6 absorption peaks centered at around: 781, 719, 682, 446, 386, 310 nm with R^2 value of 0.9997. As it was discussed before, the peaks at 781 and 719 nm are correlated to monomer and dimer species, and the existence of a peak at 682 nm suggests the appearance of H-aggregate, also due to side-to-side arrangement of these two molecules. Although the dominance of dimers is above around 80 μ M,^{23,25} the relatively low presence is enough to generate some H-aggregates. Furthermore, the peaks obtained at 446 and 310 nm can be associated to vibronic coupling of the main peak at 386 nm.

On the other hand, the spectral fitting for ICG samples diluted in 50% of PBS show a best fit with 7 peaks at around: 835, 782, 719, 700, 448, 389, 311 nm with R^2 value of 0.9999. All the peaks discussed before for ICG in distilled water are observed here, with one additional peak in the NIR region (835 nm). The presence of this peak suggests that J-aggregates are being formed. Additionally, it is observed that the amplitude for the peak at 719 nm decreases substantially, and the peak at around 700 nm increases for ICG in 50% of PBS; all of these behaviors can be attributed to the presence of ions in the 50% PBS, leading to a further aggregation of ICG.

Figure 7 – Optical absorbance spectra of ICG showing its self-aggregation in aqueous solution for 50% PBS in water.

Source: By the author.

A mathematical equation to express the ICG absorbance in those solutions can be defined as a function of wavelength and correlated molecule concentration,

$$A_{dH_2O}(\lambda, C) = A_M(\lambda, C) + A_D(\lambda, C) + A_{H-agg}(\lambda, C) + A_{S_0 \rightarrow S_2}(\lambda, C) + A_{S_0 \rightarrow S_2, \nu_1}(\lambda, C) + A_{S_0 \rightarrow S_2, \nu_2}(\lambda, C). \quad (2.17)$$

Where every single term at the right of equation 2.17 are the partial absorption of monomeric, dimeric, H-aggregates, $S_0 \rightarrow S_2$ electronic transition, vibrational mode 1 of $S_0 \rightarrow S_2$ and vibrational mode 2 of $S_0 \rightarrow S_2$ respectively, in the solution at concentration C . This mathematical expression fits the behaviour for ICG in distilled water. In the case of ICG in 50% of PBS, the mathematical expression is the same as for distilled water, but

Figure 8 – Optical absorbance spectra of ICG showing its photobleaching in air-saturated solutions: (a) in distilled water using 780 nm, (b) in distilled water using 808 nm, (c) in 50% PBS using 780 nm, and (d) in 50% PBS using 808 nm.

Source: By the author.

with an additional term:

$$A_{50\%PBS}(\lambda, C) = A_{dH_2O}(\lambda, C) + A_{J-agg}(\lambda, C). \quad (2.18)$$

Where $A_{J-agg}(\lambda, C)$ is the partial absorption of J-aggregates.

2.2.1 ICG air-saturated samples

The photobleaching measurements of ICG in air-saturated samples using 780 and 808 nm illumination are presented in figure 8 for both solvents. When ICG is diluted in distilled water, the degradation kinetics for the two wavelengths seems to be similar and no shift is observed in the peaks. However, for ICG in 50% of PBS, we can observe a faster degradation for 808 nm than 780 nm irradiation. It is also noticeable that for both

clusters, the monomeric species of ICG in 50% of PBS tends to a equilibrium with dimeric species, losing the spectral shape of the original ICG molecule.

Figure 9 – Normalized absorbance at 780 nm vs dose of absorbed photons for ICG: (a) in distilled water, and (b) in 50% of PBS. Oxygen concentration vs dose of absorbed photons for ICG solutions in: (c) distilled water, and (d) 50% PBS.

Source: By the author.

The maximum amplitude (780 nm) was used to monitor the absorbance decay caused by the bleaching. Prior to averaging the triplicates, the absorbance for each measurement was normalized in respect to the amplitude at $t=0$. Then, to solve the set of differential equations 2.13 and 2.14, we developed a computational algorithm in MATLAB[®] with which we could solve the model using as input the initial conditions of $[S_0]$ and $[^3\text{O}_2]$, and the experimental data of $[S_0]$ through time. The algorithm solves the differential equations using *ode15s*, and then, performs iteratively a least-squares fit to obtain the corresponding best-fit values of C_1 to C_8 . Unfortunately, the required constants are not reported in the literature for ICG, so for a initial guess, values of others PSs were used,⁵² and upper and lower bounds were taken into account. Finally, once the best fit of $[S_0](t)$

and $[^3O_2](t)$ were obtained, the integrals for the photoproducts (equations 2.15 and 2.16) were solved and adjusted with least-squares fit to achieve the best value of S_{Ip} and S_{IIp} .

Table 3 – Table of fitted constants for the photodynamic bleaching of ICG in a macroscopic model.

Constants	dH ₂ O		50% of PBS		dH ₂ O - 4% of O ₂		dH ₂ O - 50% of O ₂	
	780nm Cluster	808nm Cluster	780nm Cluster	808nm Cluster	780nm Cluster	808nm Cluster	780nm Cluster	808nm Cluster
$C_1 = \phi_T I_a$	0.00805	0.00649	0.00902	0.0304	0.0014	8.739×10^{-4}	0.09926	0.30734
$C_2 = k_{ta}[A]$	1.987×10^7	4.927×10^7	1.136×10^8	5.774×10^7	7.263×10^6	2.204×10^5	5.354×10^7	2.411×10^7
$C_3 = k_p$	1.921×10^3	4.499×10^4	3.436×10^3	7.179×10^3	9.957×10^3	2.533×10^3	9.990×10^3	9.638×10^3
$C_4 = k_{ot}$	8.446×10^7	1.2839×10^8	9.815×10^7	1.592×10^8	4.140×10^6	3.420×10^6	3.804×10^7	1.107×10^8
$C_5 = S_\Delta k_{ot}$	2.833×10^7	4.553×10^7	2.620×10^7	3.941×10^7	9.999×10^7	9.310×10^7	9.123×10^7	8.665×10^7
$C_6 = k_{os}$	9.848×10^8	8.448×10^8	6.777×10^8	1.008×10^8	4.493×10^6	1.933×10^6	1.246×10^6	1.571×10^5
$C_7 = k_d + k_{oa}[A]$	1.001×10^7	1.006×10^7	1.010×10^7	1.274×10^8	6.902×10^8	1.447×10^8	8.235×10^8	3.322×10^7
$C_8 = k_d$	8.769×10^4	3.992×10^5	3.196×10^4	2.619×10^4	1.759×10^4	2.067×10^3	1.661×10^4	3.633×10^4
ϕ_T	2.359×10^{-4}	1.208×10^{-4}	2.643×10^{-4}	6.331×10^{-4}	5.401×10^{-5}	1.627×10^{-5}	2.908×10^{-3}	5.723×10^{-3}
S_Δ	0.3354	0.3546	0.267	0.2476	–	–	–	–
R ²	0.98324	0.99812	0.99564	0.99219	0.99974	0.99832	0.99972	0.99451
S_{Ip}	–	–	–	–	0.158	0.150	0.149	0.1197
S_{IIp}	0.139	0.1175	0.128	0.1025	–	0.125	–	0.114
R _p ²	0.98662	0.99382	0.98751	0.97592	0.98554	0.99317	0.99713	0.9882

Source: By the author.

Figure 9 shows the result of the discussed process. It includes the best fit of $[S_0](t)$ and $[^3O_2](t)$ for 780 and 808 nm irradiation clusters for: distilled water, figure 9 (a) and (c), and 50% of PBS figure 9 (b) and (d). All the fitted constants are presented in table 3. In both cases we can observe that a faster degradation is occurring for 808 nm than 780 nm. ICG in 50% of PBS shows a steeper decay with 808 nm irradiation. Moreover, the quantum yield of the triplet state was calculated using the fit constant C_1 , the absorption cross section of ICG used for the calculation was the one reported in water by Gratz *et al.*, $\sigma_{so} = 1.1 \times 10^{-16} \text{ cm}^2$.

As mentioned earlier, a low TQY state of ICG is expected and different values have been reported previously.^{23,31,53} Reindl *et al.* were the first to report the quantum yield of ICG, obtaining values of 0.14 and 0.16 in water and methanol, respectively.⁵³ However, in 1999 the same group reported different values of 1.7×10^{-6} (in water) and 2.5×10^{-5} (in methanol), concluding that ICG was not efficient for PDT applications.²³ In 2006, Sudeep *et al.* suggested the absence of long-lived species.³¹ Further studies of intersystem crossing and triplet spectroscopy of ICG have not been found in the literature. Our fitted quantum yield values are rather low, corroborating with the hypothesis that ICG has a relatively low efficiency for intersystem crossing related to the shorter lifetime values of fluorescence.^{48,49} Besides, the other fitted constants have a smaller value when compared with the constants of other PSs.⁵² C_8 , for example, in the air-saturated experiment has a order of magnitude between $10^4 - 10^5$, while for many PSs it has an order of magnitude between $10^6 - 10^9$.

This corroborates with the fact reported by Engel *et al.* that the direct proof of singlet oxygen by the detection of its luminescence at 1270 nm failed in ICG solution.³²

No photobleaching differences related to the oxygen concentration were expected between 780 nm and 808 nm since for both cases the same dose of absorbed photons were delivered. However, relative differences were observed between the oxygen consumption during photobleaching when the laser clusters were compared. Using a photochemical parameter defined by Wang *et al.*, we can better understand the variations. $\xi(\lambda)$ represents the photochemical oxygen consumption rate per light fluence rate and PS concentration under the condition of infinite $[^3O_2]$ supply⁴⁶:

$$\xi(\lambda) = S_{\Delta}\phi_T \frac{k_{oa}[A]}{k_d + k_{oa}[A]} \frac{\sigma_{so}\lambda}{hc} \quad (2.19)$$

Equation 2.19 was calculated based on our fitted constants for the experiments performed at air-saturated and it was found to have values: 3.383×10^{-5} and 1.841×10^{-5} $\text{cm}^2\text{mW}^{-1}\text{s}^{-1}$ for ICG in distilled water using 780 nm and 808 nm respectively; and 3.039×10^{-5} and 7.014×10^{-5} $\text{cm}^2\text{mW}^{-1}\text{s}^{-1}$ for ICG in 50% of PBS using 780 nm and 808 nm, respectively. These results are smaller than the ones presented for other PSs in the literature (order of magnitude between 10^{-3} - 10^{-2}).⁴⁶ Faster consumption is observed for ICG in distilled water when it is irradiated at 780 nm, while for 50% PBS the opposite occurs. This behaviour was not expected since it was normalized in respect to the dose of absorbed photons, but it seems that the presence of J-aggregates for ICG in 50% of PBS stimulates the rapid oxygen consumption in the photobleaching kinetics, or the presence of charges induces a higher bleaching through a non-oxygen dependent process. Finally, it can be concluded that for air-saturated samples the analysis of the model can be performed by a solution under conditions of constant oxygen concentration ($\frac{d[^3O_2]}{dt} = 0$), as it is explained by Finlay *et al.*,⁴⁵ where they simplified the analysis considering the local oxygen concentration is held constant, and then, the resulting expression is just for the time-dependent sensitizer concentration.⁴⁵

2.2.2 ICG in relative lack of dissolved oxygen

When using lower concentrations of dissolved oxygen, longer times of irradiation exposure were needed to achieve the threshold of normalized absorbance (which is approximately 0.2). Figure 10 shows the best fit of $[S_0](t)$ and $[^3O_2](t)$ for 4% of dissolved oxygen, (a) and (c); and for 50% of dissolved oxygen, (b) and (d). It is observed that for figure 10 (a) shows a shoulder at around $7 \times 10^{19} \gamma/\text{cm}^3$, and after this inflection point the exponential decay is observed. This behaviour may be better understood by looking the oxygen consumption in figure 10 (c), where it is observed that at this point all of the remaining oxygen has been consumed. Consequently, $7 \times 10^{19} \gamma/\text{cm}^3$ could be considered

Figure 10 – Normalized absorbance at 780 nm vs dose of absorbed photons of ICG in distilled water for: (a) 4% of $^3\text{O}_2$, and (b) 50% of $^3\text{O}_2$. Oxygen concentration vs dose of absorbed photons of ICG in distilled water for: (c) 4% of $^3\text{O}_2$, and (d) 50% of $^3\text{O}_2$.

Source: By the author.

the threshold dose of absorbed photons necessary to finish the remaining oxygen under this condition.

Furthermore, the TQY state is found to be somewhat shorter for 4% of oxygen. The order of magnitude of the rest of the constants decreases and the factor S_Δ could not be calculated because it is over the upper limit value $[0,1]$. This inconsistency can be explained by analyzing at the experiment performed at 50% of dissolved oxygen. The ϕ_T value is one order of magnitude greater than in the case of air-saturated samples. Also, oxygen is being consumed during the bleaching in figure 10 (d), however our exposure times did not result in complete consumption of the present oxygen. Eventually, in longer times the oxygen will be consumed since the cuvette is sealed. Finally, it suggests that an additional transition is occurring. This transition could be associated to indirect interaction of singlet oxygen with triplet-state PS. This has been previously reported by Kilger *et al.*

and it describes the energy back transfer from singlet oxygen state to the ground-state PS which will be excited and populate the triplet-state.^{52,54} So, higher ϕ_T values are observed at 50% of dissolved oxygen and lower values are observed at 4% of dissolved oxygen since the $[^3O_2]$ is rapidly consumed and there is no time for the back transfer. Once again, the S_Δ value could not be calculated for these conditions, this may be due to the limitations of our model at lower oxygen concentrations, and therefore a redefinition of C_5 need to be included. In this new definition, the energy back transfer could be taken into account when solving the model at lower oxygen concentrations.

2.2.3 Photoproduct formation

The amplitude of photoproduct accumulation predicted by ICG's photodynamic bleaching macroscopic model is plotted in figures 11 and 12. The values of S_{Ip} and S_{IIp} are presented in table 3 together with the R^2 value. The light induced decomposition products for ICG were studied by Engel *et al.* They reported that the photoproducts of ICG were independent of the light source used, and showed that these photoproducts can affect cellular integrity.³²

Figure 11 – Amplitude of photoproduct absorbance at 389 nm vs dose of absorbed photons for: (a) ICG in distilled water, and (b) ICG in 50% of PBS.

Source: By the author.

We observed that the photoproduct formation in the air-saturated experiments, for both solvents, are just due to the reaction of ICG singlet ground state with the singlet oxygen state, figure 11. No contribution of the reaction with triplet state was observed at this condition. Even taking 1 for S_{Ip} , which is the maximum value that it can reach, the theoretical behaviour does not match with the experimental values. The photoproduct generation may be dependent on the solvent, and this condition seems to be observed in figure 11 (a) and (b). However, the fit value of S_{IIp} does not show great variation from one to the other. This suggests that the fraction of S_0 and 1O_2 reactions which

produce photoproducts are almost the same for both irradiation sources and solvents in air-saturated samples.

Figure 12 – Amplitude of photoproduct absorbance at 389 nm vs dose of absorbed photons of ICG in distilled water for: (a) 4% of $^3\text{O}_2$, and (b) 50% of $^3\text{O}_2$.

Source: By the author.

The photoproducts formed at relative lack of dissolved oxygen are presented in figure 12. At this condition, less photoproducts are expected since the samples are not well-oxygenated. However, the opposite is observed. When using 780 nm, the type I reaction is the dominant one, and type II reaction is not observed to occur. On the other hand, when the samples were irradiated with 808 nm, the best fit to understand the photoproduct formation was obtained when both reactions are combined, type I and II. Interestingly, this only happens for 808 nm, and the fact to present type II reaction in relative lack of oxygen justifies in some way that energy back transfer is occurring.

A possible explanation for this behavior could be that an extra pathway of deactivation of PS is being missed when 808 nm excitation is used. In somehow, 808 nm wavelength may show the ability to induce more heat than 780 nm, and this may translate into the fact that more vibrational states are being populated due to internal conversion by an increase in temperature, consequently, intersystem crossing is favourable to happen^{55,56} and then more energy could be transferred to oxygen and subsequently generates singlet oxygen. It has been shown by *Soper and Mattingly*⁴⁸ that the nonradiative pathways for ICG increase at higher temperature for the ground and first excited singlet state, so higher rate of internal conversion was observed.⁴⁸

In somehow, all these results show that greater efficient is being observed for excitation at 808 nm than 780 nm. In this behavior found between the PDT action, observed through the bleaching of the dye itself, with excitation at 808 nm, when compared to 780 nm, we must rely on the mechanisms that occur in the energy transfer process. As we know, and already discussed earlier in the text, the transfer of energy reaching the formation of

the singlet oxygen, has several stages. The process starts with the excitation of the singlet state (S_n) that has a large band, due to the vibration states. Then there is an internal conversion, with respect to the vibrational states. In this process, energy from this decay is transferred out of the molecule. Then, an intersystem crossing occurs, populating the triplet state of the dye. Another internal conversion can occur, with the final non-radioactive process of transferring energy to oxygen. Excitations of the excited state by the first photon, can excite π electrons preferentially, being able, as more energy is the excitation photon, to excite also other types of electrons. It happens that fluorescence is more likely when electrons $\pi-\pi^*$ (promotion of a π electron from bonding, π , to an antibonding, π^* , orbital). Alternatively, intersystem crossing is more favourable when we excite the so-called electrons $n-\pi^*$ (promotion of a nonbonding electron, n , to an antibond orbital). Depending on the photon energy of excitation, the fluorescence can be more intense, and therefore the energy is returned in the form of light, or to favor the intersystem crossing, with greater production of singlet oxygen. For excitations involving 780 nm, fluorescence seem to be favored, while for excitations with 808 nm, system intercrossing is favored. This may explain the greater efficiency of bleaching, and therefore of photodynamic action with longer wavelength excitations, and outside the peak of absorption. The hypothesis is that the system treated here, due to the gap between the states, create a competition between fluorescence and intersystem crossing, resulting in a dependence on the overall efficiency with the excitation photon energy.

2.3 Partial Conclusions

In conclusion, the photobleaching of ICG in aqueous solvents was investigated under two different excitation wavelengths (780 and 808 nm). The measurements were monitored spectrophotometrically and results were interpreted using the PDT bleaching macroscopic model. For case at air-saturated samples, faster degradation for 808 nm irradiation was observed when compared to 780 nm. Steeper decay was observed for ICG in 50% of PBS when was irradiated under 808 nm wavelength. The triplet-state quantum yield obtained by our model is an order of magnitude greater than the one reported by H. Gratz *et al.*, this difference is because of the experimental set-up (laser beam chopped, $\lambda_{exc} = 812.5$ nm and $I_{exc} = 390 \text{ Wcm}^{-2}$). However, these values are consistent with what was found in the literature.^{23,31,53} The oxygen concentration through the photobleaching was relatively constant. Moreover, small differences were observed, specially for the case of ICG in 50% of PBS irradiated with 808 nm wavelength, which showed the faster oxygen consumption.

For ICG in low concentrations of dissolved oxygen, longer irradiation times, meaning higher energy doses, were needed to achieve the threshold of normalized absorbance (~ 0.2). Also, it was observed for 4% of dissolved oxygen that delivering up to $7 \times 10^{19} \gamma/\text{cm}^3$ is necessary to consume the remaining oxygen in the samples. Shorter triplet-state quantum

yield were obtained for this condition. However, in the case of 50% of dissolved oxygen, the obtained ϕ_T was obtained greater, even greater than the one from the air-saturated samples suggesting the evidence of energy back transfer process.

For the case of air-saturated samples, the photoproduct formation is attributed to the type II reaction. Because of the fit value S_{IIp} the photoproduct generation are almost the same for both irradiation sources and solvents. In relative lack of dissolved oxygen photoproducts were obtained. The generation is mainly due to the type I reactions. However, for 808 nm irradiation was obtained the best fit when both reaction are combined.

Nonradiative pathways for ICG when excited with 808 nm seems to take place through reaction, then intersystem crossing results in a dependence on the overall efficiency with the excitation photon energy.

Finally, 808 nm wavelength has faster photobleaching than 780 nm, suggesting a more efficient photodynamic effect.

3 PHOTODYNAMIC INACTIVATION WITH PHANTOM BARRIERS

Many diagnosis and therapeutic applications of light require the knowledge of the optical properties of biological tissues, such media are called turbid media, total attenuation (μ_t), absorption (μ_a) and scattering (μ_s) coefficients, refraction index (η) and phase function ($p(\cos(\theta))$).⁵⁷ Together, they will determine the total transmission through the tissue at a certain wavelength. This information is extremely relevant when using PDT, especially for in depth applications and bulky target tissues. The aim of this chapter is to evaluate the total transmission of 808 nm wavelength in optical phantoms with features similar to human skin and simulate *in-vivo* condition of a PDI treatment.

3.1 Preparation and characteristics of the phantom

For this study, an optical phantom with properties of skin was used. Optical phantom is a material capable to mimic optical properties at specific or over a broad range of wavelengths. The phantoms were prepared based on a publication made by Lualdi *et. al.*⁵⁸ The basic medium consists of a high purity silicone polymer, curable at room temperature (SQ 8000/3,5M, São Paulo, Brasil). The silicon and the catalyst were mixed at the ratio 5mL/200mL and were cured for 48 hours at room temperature before handling. To mimic absorption property of skin a cosmetic base was used (Koloss Cosméticos Ltda, São Paulo, Brasil); for the scattering properties, Al_2O_3 particles were used (Sigma Aldrich Brasil Ltda, São Paulo, Brasil). Five phantoms were made for the purpose of our experiment with dimension of (205, 115, 7.42) mm, figure 13.

Figure 13 – Geometry of the homemade phantom.

Source: By the author.

Phantoms with 10 mm thick sections were characterized by a double-integrating sphere geometry for the measurements of absorption coefficients. Those measurements were carried out at Photobiophysics group, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo. Because of the resolution equipment, wavelengths greater than 800 nm could not be achieved, also, the magnitude of absorption coefficient is not the same as the reference paper due to at concentrations presented in the paper of

cosmetic base and Al_2O_3 were not able to be detected by the equipment. However, the shape of the curve was really similar (figure 14), then, the final phantoms were made based on the concentrations suggested by *Lualdi et. al.*,⁵⁸ 2.4% of cosmetic base and 8.8% of Al_2O_3 .

Figure 14 – Absorption coefficients as function of wavelength of the homemade phantom.

Source: By the author.

3.2 Characterization of lasers panel

The characteristics of laser radiation also influence the light/biological tissue interaction, due to the optical coupling when irradiating the tissue surface, and the further photon traveling behavior. Parameters such as wavelength, exposure time, applied energy, focal spot size, energy density, and power density need to be taken into account as they can trigger unwanted effects.⁵⁷ In our case, the illumination system is a panel of 200 lasers prototype (figure 15) each laser has a power of 100 mW, emitting at 808 nm wavelength. Emission wavelength, irradiance stability and temperature increase are important parameters to be known.

3.2.1 Emission wavelength

In order to measure the emission wavelength a spectral irradiance measurement was made using a spectroradiometer (calibrated USB 4000, Ocean Optics, USA). The spectroradiometer was calibrated using a National Institute of Standards Technology (NIST) traceable light source (DH-3plus-BALCAL, Ocean Optics, USA). Figure 16 shows

Figure 15 – Prototype of the laser panel for PDI with extracorporeal illumination.

Source: By the author.

power density at a specific wavelength. A Gaussian model was used to make a fitting to the laser profile, it was found a full width at half maximum (FWHM) of 2.29 nm and a center wavelength at 808.39 nm.

Figure 16 – Normalized spectral irradiance of 808 nm laser.

Source: By the author.

3.2.2 Irradiance stability

In order to characterize the illumination uniformity of the laser panel, power measurements were made for each laser using a Laser Power and Energy Meter (LabMax-

TOP, Coherent, USA). Figure 17 presents the irradiance map through the laser panel, irradiance variations are observed at some points, however, a large part of the panel presents the desired working value and is within the limits of more or less 15% of the working irradiance ($I_0 = 79.4 \pm 8.8 \text{ mW/cm}^2$).

Figure 17 – Irradiance map of the 808nm laser panel.

Source: By the author.

Moreover, since longer times will require for the PDI treatment, irradiance stability is an important parameter to be maintained through the experiment. Figure 18 shows the irradiance stability for a continuous period of 50 min, it is observed that over this time the initial value is well maintained with a mean of 79.50 and SD of 0.39 mW/cm^2 .

Figure 18 – Irradiance stability of the 808nm laser panel for 50 min.

Source: By the author.

3.2.3 Temperature

Temperature measurements were performed using thermal-graphic camera FLK-Ti400 (Fluke®, Everett, EUA). Temperature was monitored during 27.5 minutes and the hottest point was plotted individually, figure 19b, as can be observed, after 10 minutes, temperature is well stable at around 38°C, this value was under the operating temperature of the laser according to the data-sheet (40°C). Center part of the laser panel was the hottest because of the set up of refrigeration, where two coolers were located at the ends and hot air came out through the middle of the panel. As this equipment is a prototype, upgrades have to be made to improve the cooling of lasers. For the PDI experiments, the 96 well plate was positioned near from the coolers, avoiding the center region. As it is presented by the black square in figure 19a. At this region, temperature increases were of 4°C, which is not considerable enough to affect the bacteria.

Figure 19 – (a) Temperature map of the 808nm laser panel. (b) Temperature increase of the 808 nm laser panel during 27.5 min at the center position.

Source: By the author.

3.3 Optical attenuation through the phantoms

Optical transmission attenuated light was detected using a wide field imaging system in the IR region developed by Govone.⁵⁹ Figure 20 presents qualitative information about the IR images as well as the irradiance value of the pixel through a choose specific line path (dash line). The images were captured for each increment in phantoms and the acquisition values were normalized with respect to the values of the initial image (figure 20a). It is observed that as the thickness of the phantoms increases, the illumination becomes more uniform.

In figure 21 is presented direct measurement of the optical and percentage of transmitted light, it was made measuring the power at different points using the power

Figure 20 – Optical transmission through the homemade phantoms for: (a) 7.44 mm, (b) 15.02 mm, (c) 23.37 mm, (d) 29.72 mm and (e) 37.10 mm.

Source: By the author.

Figure 21 – Optical and percentage of transmitted light of the initial irradiance for the different thickness of phantoms.

Source: By the author.

meter. The percentage of transmitted light is less as thickness phantom increases.

At the first phantom attenuating the initial irradiance can be observed the the illumination is not homogeneous, however, when measuring with the power meter small differences were observed in the power. This is mainly due that the power that is reaching the detector is diffuse light, it means that it is the mean value of all scattering and absorption events that occur as photons migrate through the turbid medium. As the other phantom thickness are added, the distribution of irradiance turns more homogeneous, as well as decrease the transmitted light.

3.4 Monte Carlo simulation

Photon transport in such turbid media as biological tissues is fairly well described by an integro-differential equation of radiation-transport theory.⁶⁰ However, it is not trivial to solve it analytically without introducing a number of approximations and simplifications. For that reason, one such method is the Monte Carlo (MC) method, which is widely used to numerically model complex systems in many areas of science.^{57,60}

The present study uses an optimized method, Monte Carlo eXtreme (MCX), an open source software that was proposed by *Qianqian Fang* and *David A. Boas*,⁶¹ which has been widely used in recent years because of the uses of a massive number of parallel threads of a Graphics Processing Units (GPU). MCX is a free access software and for the simulation one photon is initialized for each sub-process and its trajectory is calculated considering the optical properties of the medium (μ_a , μ_s , g and η).⁶¹

For the simulation was created a heterogeneous 5-layer optical computerized phantom with parallelepiped structure and dimensions of (360, 230, 100)mm (figure 22). The resolution voxel was $0.5 \times 0.5 \times 0.5$ mm³.

Figure 22 – Geometry and structure of the optical computerized phantom (Laser array, skin, subcutaneous fat, muscle, rib bone and lung tissue).

Source: By the author.

The structure consists of skin, subcutaneous fat, muscle, rib bone and lung tissue. The dark gray rectangle with the red arrow indicates the illumination and direction source, in this case, it was used 200 disk sources with aperture of 3 mm, with the first one located at position (65, 60, 1) mm with equal separation distance of 12 mm at x and y . Values of the optical properties at 808 nm of each tissue layer are listed in Table 4, These values were obtained from different authors.^{24,62-64} Value of g that are presented in bold were not found in the literature, so they were set as 0.95, in order to indicate forward-direction of scattering as it is major present in biological tissues.⁶⁰

Table 4 – Values for the absorption coefficient, scattering coefficient and anisotropy coefficient for each biological tissue at 808 nm.

$\lambda = 808 \text{ nm}$	$\mu_a \text{ (mm}^{-1}\text{)}$	$\mu_s \text{ (mm}^{-1}\text{)}$	g	thickness (mm)
Skin	0.042 ⁶²	23.04 ²⁴	0.92 ²⁴	2
Fat	0.105 ⁶²	22.22 ²⁴	0.95	20
Muscle	0.011 ⁶³	5.06 ²⁴	0.95 ²⁴	14
Bone	0.028 ⁶⁴	37.92 ²⁴	0.95	8
Lung	0.034 ²⁴	11.77 ²⁴	0.957 ²⁴	60

Source: By the author.

Simulation was performed using a total amount of 1×10^9 photons, each source delivering 5×10^6 photons. Also, it was considered reflection at boundaries and refraction index mismatch. Figure 23 shows the flux of photons in any position within the optical computerized phantom.

Figure 23 – Flux profiles inside the optical computerized phantom for (a) cross section. (b) interface muscle-rib/lung.

Source: By the author.

Figure 23a represents a cross section of the optical computerized phantom, dash

lines represents the different tissue layers. The flux has units of $1/(\text{mm}^2\text{s})$, and the logarithmic scale is used because of greater values are at the neighborhood of the sources than in deeper penetrations. Visually, most of the photons are being absorbed in the skin and subcutaneous fat, it was expected since absorption coefficients are greater for these two layers. Furthermore, photon that survives to the absorption of the two layers above can migrate and reach other tissue such as muscle and rib bone. In figure 23b is presented the interface between muscle-rib/lung, it is well observed how a good amount of photon are being absorbed by the rib bone, since it has a greater absorption coefficient. To better understand the distribution of the flux, different positions were plotted to observe homogeneity. Figure 24a presents the flux at position $(180, 115, z)$; $(100, 115, z)$ and $(250, 115, z)$ mm; and figure 24b presents the flux at position $(180, y, 39)$ and $(180, y, 60)$ mm. The flux was equal when taken positions under the illuminated area, also, in the near field region (close of the source) is observed an relative increase of the flux, this is mainly because scattering coefficient is greater than absorption coefficient in all biological tissues here presented, and then the prevalence of multiple scattering is likely to occur. However, photons that can continuous its trajectory can suffer absorption by the next tissue, and then the nature of the statistical model used by Monte Carlo is affected in deeper penetrations. This can be observed after 40 mm, where the signal to noise ratio seems to increase due to low quantity of photon available to run the simulation. At the interface muscle-rib/lung is well observed a good amount of photons, remembering that the simulation are normalized to 1, then, by the flux obtained it can be concluded that for every 1×10^9 photons launched at the surface, 1×10^6 photons are reaching the interface muscle-rib/lung, in a deeper position $(180, y, 60)$ mm, around 1×10^3 photons arrive.

Figure 24 – (a) Flux profiles versus depth in tissue. (b) Flux profiles at the interface muscle-rib/lung.

Source: By the author.

3.5 Photodynamic inactivation

3.5.1 Bacterial strain

For the PDI experiments, a strain of *Streptococcus pneumoniae* was used. The samples were stored in a biofreezer at -80°C in cryotubes containing 10^8 colony-forming units (CFUs). To perform the assays, a pre-inoculum was prepared as follows: $100\text{ }\mu\text{L}$ of the stored sample was diluted in 9.9 mL of brain heart infusion (BHI) broth, also, $10\text{ }\mu\text{L}$ of the stored sample was plated on a blood agar plate, as well as 5 mL of BHI broth in a separated falcon tube. Subsequently, the prepared samples were grown aerobically at 37°C during 16 hours. After that, 1 mL of the pre-inoculum was diluted in 9 mL of BHI broth and one colony grown on agar was mixed in 10 mL BHI broth and grown in a microaerobiosis jar at 37°C for 8 hours more. After the grown period, the absorbance measurement was done at a wavelength of 600 nm to confirm the concentration of bacteria present in the sample. Reached a value between 0.3 , corresponding to $3\times 10^8\text{ CFU/mL}$, the sample proceeded to be centrifuged at 3000 rpm for 15 minutes. The supernatant was removed and the pellet re-suspended in PBS. Finally, inoculum was adjusted by dilutions to the concentration of 10^8 CFU/mL for every $125\text{ }\mu\text{L}$, volume used in each well.

3.5.2 Photosensitizer

ICG (Ophthamos, Brazil) was used as PS, aliquots of 1 mg were stored at room temperature (around 26°C), protected from light until the moment of use. Fresh stock solutions were prepared in PBS (pH of 7.4) at $10\text{ }\mu\text{M}$ before each experiment and kept in dark.

3.5.3 Study design

To verify the photodynamic inactivation efficiency using ICG and 808 nm irradiation, the irradiation was performed using the laser panel at the surface of the homemade phantoms and the culture of the viable bacteria of *Streptococcus pneumoniae* was counted. The following groups were tested:

- ICG (Bacteria + PBS + PS).
- Light (Bacteria + PBS + Light).
- PDI (Bacteria + PBS + PS + Light).

Bacterial suspension ($125\text{ }\mu\text{L}$) with 10^7 CFU/mL was poured into the specific well presented in figure 25a of the 96-well plate. For PDI and ICG group, $125\text{ }\mu\text{L}$ of ICG at $10\text{ }\mu\text{M}$ was added to specific well and mixed with bacteria. After addition of ICG, the 96-well

Figure 25 – Position of the light and PDI group in the 96-well plate.

Source: By the author.

plate was incubated in dark for 35 min. For light group equal volume of PBS was added and mixed. The final experimental set up is presented in figure 25b.

The bacterial suspension in the PDI and light group was irradiated for the times presented in table 5 and the experiment was repeated for each thickness of the homemade phantoms.

Following all this procedure, all groups were diluted according to the serial dilution method; five-fold serial dilutions in sterile PBS were prepared and spread in blood agar to finally incubate for 18 to 24 h aerobically. The number of CFU were counted, multiplied by dilution factor and divided by the volume of the drop to determinate the concentration (CFU/mL) after each experiment. All experiments were repeated at least three times, and all conditions were plated in triplicate.

Table 5 – Irradiation times for each homemade phantom thickness.

Barriers thickness (mm)	Time (min)
0	0, 3.88, 8, 15
7.44	0, 3.88, 8, 15, 23
15.02	0, 3.88, 8, 15, 23, 33.3
23.37	0, 3.88, 8, 15, 23, 33.3
29.72	0, 3.88, 8, 15, 23, 33.3, 40, 48
37.10	0, 3.88, 8, 15, 23, 33.3, 40, 48

Source: By the author.

3.5.4 Data processing

For better understand the energy dose required at surface to achieve a 100% bacteria reduction, a logistic model was used to model the bacterial death. Logistic model

suggests that the rate population increase maybe limited.⁶⁵ The dynamics population can be described as:

$$\frac{dN(t)}{dt} = aN(t) \left(1 - \frac{N(t)}{b} \right), \quad (3.1)$$

where $N(t)$ is the population size at t , a is the intrinsic growth and $b > 0$ is the carrying capacity of the population.⁶⁵ Logistic model provide a good mathematical description for many biological population of microorganisms,^{66,67} animals, and plants.⁶⁵ As observed in figure 26 for PDI experiments, they represent population mortality, then, logistic model can be applied in conditions in which the value of a is in a steady state condition, a state in which the bacteria is alive but slowly growing because of the PBS solvent, then, the factor a/b is greater due to accelerated death from treatment. Then, the fitting for the experimental curves were fitted using the Multiple Peak Fit tool from Origin[®] software, as well as the derived lethal dose(LD) 99 parameter, which is the lethal dose to kill 99% of the bacteria.

3.6 Results and Discussion

Figure 26 shows the result of bacterial strain of every condition after application of their corresponding energy dose. It is observed that ICG and light group compared with the PDI groups did not cause significant death, then ICG and light cytotoxicity was not observed. As increasing the phantom thickness, more energy dose was necessary to achieve a total reduction of the bacterial strain.

PDT studies are usually made at the UV-visible spectrum. However NIR spectrum gives a higher penetration into biological tissue. Our study shows the feasibility of ICG inactivate *Streptococcus pneumoniae*, even when illumination is attenuated by phantom barriers with optical properties of skin. ICG has been widely used in many PDT/PDI applications, and has shown good properties as a PS.

Several bacteria inactivation has been studied by the use of ICG, *Omar et al.* reported the reduction up to $6\log_{10}$ of *Staphylococcus aureus* and *Streptococcus pyogenes* by the use of near-infrared light.³³ *Topaloglu et al.* investigated optimal parameters of ICG concentration and energy dose with 809 nm laser to kill *Pseudomonas aeruginosa* *in-vitro*.⁶⁸ PDI using ICG and NIR against *Streptococcus pneumoniae* has been studied by our research group^{38,40} *in-vivo* and *in-vitro*. These findings hold great promise for the treatment of diseases where bacteria strains develop resistance very quickly.

Figure 26 – Effect of ICG, light and PDI experiments on *Streptococcus pneumoniae*.

Source: By the author.

Figure 27 – PDI experiments on *Streptococcus pneumoniae* fitted with logistic model.

Source: By the author.

However, those studies are made in the use of PDI/PDT for the elimination of microorganisms by direct illumination. In our case, the interesting thing about expanding the use of PDI by extracorporeal illumination in cases of pneumonia, led us to the construction of phantoms with optical properties similar to skin at NIR spectrum to simulate scenario (as a relative approximation) in which illumination is attenuated by the

biological tissues found in the thoracic cage region. Optical transmitted light through the phantoms was successfully measured by qualitative and quantitative analysis, now the PDI efficient at this conditions must be demonstrated.

Results with logistic fit are presented in figure 27. As the thickness increases, the energy dose delivered onto the surface must be greater to completely inactivate the bacteria. Table 6 shows the value obtained as derivative parameter from logistic fitting, this result reflects the necessary energy dose on the surface to inactivate the 99% of bacteria, being, for the 37.10 mm thickness, 197.96 J/cm² necessary to kill the bacteria. Base on the transmitted light presented in figure 21, the energy dose that arrives to the bacteria can be calculated, being 13.48, 15.32, 6.33, 6.53 and 6.92 J/cm² for 7.44, 15.02, 23.37, 29.72 and 37.10 mm, respectively. The decrease in the energy dose that arrives directly to the bacteria as the thickness increases, may be due to another pathways of cytotoxicity of ICG. The generation of photoproducts of ICG while is degrading, as it was shown in Chapter 2, can be the responsible of the decrease in that energy dose. *Engel et al.* suggest that those photoproduct generated can be toxic compounds that decrease the cell viability.³²

These parameters represent a safe procedure as it is stablish by the safety requirements specified by the ANSI standard Z136.1 (2014).⁶⁹ According to this standard, the maximum permissible exposure (MPE) of the light source is organ and wavelength dependent. For skin exposure, the MPE at 850 nm is about 400 mW/cm².

Table 6 – Value of the LD99 obtained as a derived parameter from fitting of logistic model.

Barriers thickness (mm)	LD99 (J/cm ²)	R ²
0	21.40	1
7.44	49.37	1
15.02	101.50	0.99956
23.37	85.323	0.99956
29.72	167.44	0.99894
37.10	197.96	0.99456

Source: By the author.

On the other hand, longer exposure to illumination can carry out a series of effects such as increase in temperature in biological tissues and photodynamic effect of ICG by temperature. However, it is know that IR and NIR light can present therapeutic benefits,⁷⁰⁻⁷³ then damage from the wavelength used is not expected. Also, higher temperatures accelerate the degradation of ICG in aqueous solution (thermal degradation),⁷⁴ and photothermal effects of ICG has been reported by *Chen et al.* using 808 nm diode laser with power between 5-10 W on murine mammary tumours.⁷⁵ Moreover, *Topaloglu et al.* conclude that photothermal effect is not caused on bacteria by the application of PDT

in-vitro when using a diode laser at 809 nm with 1 W of power and delivering energy dose of 84, 168 and 252 J/cm².

In our experimental conditions, temperature increase was not observed in the bacterial strain, then the cytotoxic effect is completely due to PDI action. These results led us to consider the use of 808 nm wavelength for extracorporeal illumination with ICG in PDI to kill *Streptococcus pneumoniae*.

3.7 Partial Conclusions

In conclusion, it was observed the optical penetration of 808 nm wavelength through the home made phantoms by experimental measurements, obtained a transmitted light of 3.5% for a thickness of 37.10 mm.

By Monte Carlo simulation was observed the deeper penetration of 808 nm wavelength at the different biological tissue localized in the thoracic cage. Photons are found to reach the lung, making this wavelength suitable for extracorporeal illumination.

It was shown that PDT using ICG and 808 nm irradiation resulted in a bacterial inactivation through optical phantoms. Also, the activation of ICG at 10 μ M was even observed when just 3.5% of the incident light is transmitted and led to PDI effects that completely kill *Streptococcus pneumoniae*. The results of the present study suggest that ICG is a promising PS, and use combined with extracorporeal illumination and NIR light demonstrate effectiveness for treatment of lung infections as pneumonia.

4 CONCLUSIONS

The findings of this study showed improved photobleaching of ICG when irradiated with 808 nm than 780 nm wavelength. This result is due to nonradiative pathways that take place when excited with 808 nm, and then long lifetime species are generated and likely to interact with molecules around (oxygen or biological targets). Generation of photoproducts were observed even in cases of relative lack of oxygen.

It was possible to verify the optical penetration of 808 nm wavelength through phantom with optical properties of skin at the NIR region, as well as validate the potential of ICG as PS. Even though knowing that the efficiency is not comparable with other PSs found in the literature, it was observed the complete inactivation of *Streptococcus pneumoniae* by extracorporeal illumination when just 3.5% of the initial irradiance pass through the phantoms.

Finally, we are confident that the use of ICG + 808 nm wavelength is promising for infections at deeper no-surface tissues, such as pneumonia, by an extracorporeal illumination.

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Appendix

APPENDIX A – PHOTBLEACHING SIMULATION FOR NON-CONSTANT OXYGEN CONCENTRATION

A.0.1 Function script

```

1 function S = ICGKineticsSvsO(C,t,~)
2 % Comentarios
3 c0 = [1.0; 170.63]; % initial conditions for S0 and 3O2.
4 [T,Sv] = ode15s(@DifEq,t,c0);
5     function dS = DifEq(t,x)
6         dxdt = zeros(2,1);
7         dxdt(1) = -(C(1)./(C(2) + C(3) + C(4).*x(2))) .* (C(2).*
            x(1) + (C(5).*C(6).*x(2).*(x(1).^2))./(C(7) + C(6).*x
            (1)));
8         dxdt(2) = -((C(1).*C(5).*x(2).*x(1))./(C(2) + C(3) + C
            (4).*x(2))) .* (1 - C(8)./(C(7) + C(6).*x(1)));
9         dS=dxdt;
10     end
11 if nargin == 2
12     S = Sv(:,1);
13 elseif nargin == 3
14     S = Sv;
15 end
16 %whos
17 end

```

A.0.2 Solver script

```

1 data = xlsread('ICV-5uM-dH2O-808nmC-5,46ppm-O2.xlsx');
2 Time = data(:,4);
3 Sdata = data(:,5);
4 %O2 = 344.38
5
6 %Sdata = Sdata';
7 % 780 nm dH2O y PBS, So-dH2O = 0.9810 y So-PBS = 0.9923:
8 % C0 = [1e-4, 1e08, 1e03, 1e08, 1e07, 1e05, 1e08, 1e04];
9 % lb = [1e-6, 1e06, 1e02, 1e07, 1e06, 1e04, 1e07, 1e01];
10 % ub = [1e-2, 1e10, 1e04, 1e09, 1e08, 1e09, 1e09, 1e05];
11
12 % 808 nm PBS, So-PBS = 0.9969:

```

```
13 % C0 = [1e-2, 1e08, 1e03, 1e08, 1e07, 1e08, 1e08, 1e04];
14 % lb = [1e-6, 1e06, 1e02, 1e07, 1e06, 1e06, 1e07, 1e01];
15 % ub = [1e-1, 1e09, 1e04, 1e09, 1e08, 1e10, 1e09, 1e05];
16
17 % 808 nm dH2O, So-dH2O = 0.9644:
18 % C0 = [1e-5, 1e08, 1e04, 1e08, 1e07, 1e08, 1e08, 1e05];
19 % lb = [1e-6, 1e07, 1e02, 1e07, 1e06, 1e07, 1e07, 1e04];
20 % ub = [1e-2, 1e10, 1e05, 1e09, 1e08, 1e09, 1e09, 1e06];
21
22 %-----
23
24 % 780 nm dH2O 3.9% de O2, So = 1.0 y O2 = 13.44:
25 % C0 = [1e-4, 1e08, 1e03, 1e08, 1e06, 1e05, 1e08, 1e04];
26 % lb = [1e-6, 1e06, 1e02, 1e06, 1e05, 1e04, 1e07, 1e01];
27 % ub = [1e-2, 1e10, 1e04, 1e09, 1e08, 1e07, 1e09, 1e05];
28
29 % 808 nm dH2O 3.9% de O2, So = 1.0 y O2 = 13.44:
30 % C0 = [1e-4, 1e07, 1e03, 1e06, 1e06, 1e05, 1e08, 1e04];
31 % lb = [1e-6, 1e05, 1e02, 1e05, 1e05, 1e04, 1e07, 1e01];
32 % ub = [1e-2, 1e09, 1e04, 1e08, 1e08, 1e07, 1e09, 1e05];
33
34 % 780 nm dH2O 49.54% de O2, So = 1.0 y O2 = 170.63:
35 % C0 = [1e-2, 1e08, 1e03, 1e08, 1e06, 1e05, 1e08, 1e04];
36 % lb = [1e-6, 1e06, 1e02, 1e06, 1e05, 1e04, 1e07, 1e01];
37 % ub = [1e-1, 1e10, 1e04, 1e09, 1e08, 1e07, 1e09, 1e05];
38
39 % 808 nm dH2O 49.54% de O2, So = 1.0 y O2 = 170.63:
40 C0 = [1e-1, 1e08, 1e03, 1e08, 1e07, 1e05, 1e08, 1e04];
41 lb = [1e-6, 1e06, 1e02, 1e06, 1e06, 1e04, 1e07, 1e01];
42 ub = [ 1e0, 1e09, 1e04, 1e09, 1e08, 1e07, 1e09, 1e05];
43
44 %-----
45 opt = odeset('reltol',1e-12);
46 tic;
47 [C,Rsdnrm,Rsd,ExFlg,OptmInfo,Lmda,Jmat] = lsqcurvefit(
    @ICGKineticsSvsO, C0, Time, Sdata, lb, ub, opt); %,
    MonodKin1Opts
48 toc;
49 FitData = ICGKineticsSvsO(C,Time,1);
```

```

50 S = FitData(:,1);
51 X = FitData(:,2);
52
53 obj = sum((Sdata - S).^2)./sum((Sdata - mean(Sdata)).^2);
54 R2 = 1 - obj;
55
56 expTime = data(:,1);
57 expData = data(:,2);
58 err = data(:,3);
59
60 figure(1);
61 errorbar(expTime, expData, err, 'ro', 'MarkerSize',6);
62 hold on;
63 plot(Time,S, 'k', 'LineWidth',2);
64 legend('Exp. Data', '[S_{0}] curve fit')
65 xlabel('Irradiation Time (s)', 'FontSize', 18)
66 ylabel('Normalized Concentration (M)', 'FontSize', 18)
67 grid on;
68
69 figure(2);
70 plot(Time,X, 'k', 'LineWidth',2);
71 legend('[O_{2}] concentration', 18)
72 xlabel('Irradiation Time (s)', 'FontSize', 18)
73 ylabel('Oxygen Concentration (\mu M)', 'FontSize', 19)
74 grid on;
75 disp(['R^2 = ' num2str(R2)]);
76
77 % Integration of photoproducts
78
79 FotoPro = data(:,6);
80 fotoProextr = data(:,8);
81 ErroFotoPro = data(:,7);
82
83 % Photoproducts due to singlet oxygen
84 funO2 = (X.*(S.^2))./((C(7) + C(6).*S).*(C(2) + C(3) + C(4).*X))
85 ;
86 intO2 = 0.2.*C(1).*C(5).*C(6).*cumtrapz(Time, funO2);
87
88 % Photoproducts due to triplet interaction

```

```
88 funTri = (S)./(C(2) + C(3) + C(4).*X);
89 intTri = 0.21.*C(1).*C(2).*cumtrapz(Time,funTri);
90
91 % Photoproducts due to both reactions
92 Sum = 0.57*(intO2 + intTri);
93
94 %


---


95 % Photoproducts Error
96 objOxy = sum((fotoProextr - intO2).^2)./sum((fotoProextr - mean(
    fotoProextr)).^2);
97 R2oxy = 1 - objOxy;
98 disp(['R^2-oxy = ' num2str(R2oxy)]);
99
100 objTri = sum((fotoProextr - intTri).^2)./sum((fotoProextr - mean(
    fotoProextr)).^2);
101 R2tri = 1 - objTri;
102 disp(['R^2-tri = ' num2str(R2tri)]);
103
104 objSum = sum((fotoProextr - Sum).^2)./sum((fotoProextr - mean(
    fotoProextr)).^2);
105 R2sum = 1 - objSum;
106 disp(['R^2-sum = ' num2str(R2sum)]);
107 %


---


108
109 figure(3)
110 errorbar(expTime, FotoPro, ErroFotoPro, 'ro', 'MarkerSize',6);
111 hold on;
112 plot(Time,intTri, 'k—', 'LineWidth',2);
113 hold on;
114 plot(Time,intO2, 'b', 'LineWidth',2);
115 hold on;
116 plot(Time,Sum, ':', 'LineWidth',2)
117 legend('Exp. Data', 'Photoproduct type I', 'Photoproduct type II',
    'Photoproduct type I and II'); %,
118 xlabel('Irradiation Time (s)', 'FontSize', 18)
```



```
119 ylabel( 'Photoproduct amplitude' , 'FontSize' , 19)
120 grid on;
```


APPENDIX B – MONTE CARLO SIMULATION FOR THE THORACIC CAGE REGION AT 808 NM WAVELENGTH

```

1 clear cfg
2
3 cfg.seed = hex2dec('623F9A9E');
4 cfg.nphoton=1e9;
5 cfg.issrcfrom0=1; % First voxel is [0 0 0]
6
7 fesc=0.5; % Resolution of voxel00
8 cfg.unitinmm=fesc;
9 fa=1/fesc; % factor para poder escribir todas las medidas
10 cfg.unitinmm=fesc;
11 % define a 4 layer structure
12 x=1:360*fa;
13 y=1:230*fa;
14 z=1:100*fa;
15 x_esc=x.*fesc;
16 y_esc=y.*fesc;
17 z_esc=z.*fesc;
18 cfg.vol=ones(360*fa,230*fa,100*fa); % skin 0.5 – 5.2
19 cfg.vol(:, :, 1)=0; % pad a layer of 0s to get diffuse reflectance
20 cfg.vol(:, :, 4*fa:24*fa)=2; % fat 5.2 – 81.2
21 cfg.vol(:, :, 25*fa:39*fa)=3; % muscle 81.3 – 131.6, including
    bone 136
22 cfg.vol(1:30*fa, :, 31*fa:39*fa)=4; % bone 131.6 – 136
23 cfg.vol(61*fa:90*fa, :, 31*fa:39*fa)=4;
24 cfg.vol(121*fa:150*fa, :, 31*fa:39*fa)=4;
25 cfg.vol(181*fa:210*fa, :, 31*fa:39*fa)=4;
26 cfg.vol(241*fa:270*fa, :, 31*fa:39*fa)=4;
27 cfg.vol(301*fa:330*fa, :, 31*fa:39*fa)=4;
28 cfg.vol(:, :, 40*fa:100*fa)=5; % Lung with optical properties
    of air as a first aproximation
29 cfg.vol=uint8(cfg.vol);
30
31
32 cfg.gpuid=1;
33 cfg.autopilot=1;

```

```
34
35 % format: [mua(1/mm) mus(1/mm) g n]
36 cfg.prop=[0 0 1 1          % medium 0: the environment
37     0.042  23.04   0.92  1.55   % medium 1: skin
38     0.105  22.22   0.95  1.45   % medium 2: fat
39     0.028   5.06   0.95  1.41   % medium 3: muscle
40     0.011  37.92   0.95  1.50   % medium 4: bone
41     0.034  11.77   0.957 1.45  ]; % medium 5: Lung
42
43 % time-domain simulation parameters
44 cfg.tstart=0;
45 cfg.tend=5e-9;
46 cfg.tstep=5e-9;
47
48 cfg.issaveref=1; % save diffuse reflectance/transmittance in the
    non-zero voxels net to a boundary voxel.
49 cfg.isnormalized = [1]; %normalize the output fluence to unitary
    source
50
51 cfg.issaveref=1; %save the dissufe reflectance/transmittance in
    the non-zero voxels next to a boundary voxel.
52 cfg.isreflect=1; % enable reflection at exterior boundary /
    consider refractive index mismatch
53 cfg.isrefint=1; % enable reflection at interior boundary too
54
55 cfg.srcpos=[65*fa,60*fa,1];
56 cfg.srkdir=[0 0 1];
57
58 tic
59 [flux, vol]=mcxlab(cfg);
60 toc
61 fcw      =flux.data; %.*cfg.tstep
62 drefcw   =flux.dref; %.*cfg.tstep
63
64 % Running 200 disk beam sources at different locations and
    average
65 fcw=zeros(size(fcw));
66 drefcw=zeros(size(drefcw));
67 cfg.srctype='disk';
```

```

68  cfg.srcparam1=[2*fa 0 0 0];
69  cfg.nphoton=cfg.nphoton/200;
70
71  tic
72  for i=0:19
73      for j=0:9
74          cfg.srcpos=[65*fa+i*12*fa, 60*fa+j*12*fa, 1];
75          [flux,vol]=mcxlab(cfg);
76          fcw=fcw+flux.data;
77          drefcw=drefcw+flux.dref;
78      end
79  end
80  fcw=fcw.*(1/200);
81  drefcw=drefcw.*(1/200);
82  toc
83
84  center = squeeze(fcw(180*fa,115*fa,:));
85  pos100 = squeeze(fcw(100*fa,115*fa,:));
86  pos250 = squeeze(fcw(250*fa,115*fa,:));
87  rib     = squeeze(fcw(180*fa, :, 39*fa));
88  pul     = squeeze(fcw(180*fa, :, 60*fa));
89
90  figure(1)
91  mcxpreview(cfg);
92  set(gca, 'Zdir', 'reverse');
93  title('Domain preview');
94  xlabel('x (mm)', 'FontSize',16); ylabel('y (mm)', 'FontSize',16);
95  zlabel('z (mm)', 'FontSize',16);
96
97  figure(2)
98  imagesc(x_esc, y_esc, log10(abs(squeeze(drefcw(:, :, 1))))');
99  axis equal; colormap bone; colorbar; caxis([-25 0]);
100 xlabel('x (mm)', 'FontSize',16); ylabel('y (mm)', 'FontSize',16);
101
102 figure(3)
103 imagesc(x_esc, z_esc, log10(abs(squeeze(fcw(:, 115*fa, :))))');
104 hold on
105 plot([0 360],[4 4], 'k—', [0 360],[25 25], 'k—', [0 360],[40 40], '
      k—', ...

```

```

106     [0 30],[35 35], 'k—',[30 30],[35 40], 'k—',[61 90],[35 35], '
        k—', ...
107     [61 61],[35 40], 'k—',[90 90],[35 40], 'k—',[121 150],[35
        35], 'k—', ...
108     [121 121],[35 40], 'k—',[150 150],[35 40], 'k—',[181
        210],[35 35], 'k—', ...
109     [181 181],[35 40], 'k—',[210 210],[35 40], 'k—',[241
        270],[35 35], 'k—', ...
110     [241 241],[35 40], 'k—',[270 270],[35 40], 'k—',[301
        330],[35 35], 'k—', ...
111     [301 301],[35 40], 'k—',[330 330],[35 40], 'k—');
112 axis equal; colormap bone; colorbar; caxis([-25 0]);
113 xlabel('x (mm)', 'FontSize',16); ylabel('z (mm)', 'FontSize',16);
114
115 figure(4)
116 imagesc(x_esc,y_esc,log10(abs(squeeze(fcw(:,:),40*fa))))')
117 axis equal; colormap bone; colorbar; caxis([-20 -5]);
118 xlabel('x (mm)', 'FontSize',16); ylabel('y (mm)', 'FontSize',16);
119
120 figure(5)
121 semilogy(z_esc,center, '.k', 'MarkerSize',8);
122 hold on;
123 semilogy(z_esc,pos100, '.r', 'MarkerSize',8);
124 hold on;
125 semilogy(z_esc,pos250, '.b', 'MarkerSize',8);
126 grid on;
127 title('Flux decay along z', 'FontSize',14);
128 xlabel('Depth in tissue (mm)', 'FontSize',16);
129 ylabel('Flux (1/mm^2s)', 'FontSize',16);
130 legend('(180,115,z)', '(100,115,z)', '(250,115,z)')
131
132 figure(6)
133 semilogy(y_esc,rib, '.', 'MarkerSize',8);
134 hold on;
135 semilogy(y_esc,pul, '*r', 'MarkerSize',8);
136 grid on;
137 title('Flux along ribs and muscle', 'FontSize',14);
138 xlabel('y (mm)', 'FontSize',16);
139 ylabel('Flux (1/mm^2s)', 'FontSize',16);

```

```
140 legend( '(180,y,39)', '(180,y,60)' )
141
142 %mcxplotvol(log10(fcw)) — mcxplotvol(log10(abs(squeeze(fcw))));
    colormap jet
```