### Transparent Photochemistry: Infrared photosensitizers and singlet oxygen microscopy

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#### Abstract

There is a wide interest to the use of porphyrin related dyes in medical applications. Their photophysical and photochemical properties, and high quantum yield of generation of reactive oxygen species and absorption in the red part of the spectrum where tissues are most transparent, has made this group of molecules a target of interest to use as sensitizers in photodynamic therapy (PDT).

We had available a family of halogenated tetraphenyl macrocycles that were designed to achieve properties of an ideal PDT photosensitizer. It was our interest to explore the photochemical and photophysical properties of these dyes in order to establish correlations that provide a greater understanding of the oxygen photosensitization mechanism for porphyrins, chlorins and bacteriochlorins in solution and in cells.

The deactivation of the triplet excited states of these photosensitizers by interaction with oxygen was found to follow distinct pathways. The progressive macrocycle reduction introduces an increase in the quenching rate constant and a decrease of oxidation potential. Porphyrins and chlorins generate mostly singlet oxygen through a mechanism of energy transfer to molecular oxygen. Bacteriochlorins on the other hand show evidence for charge transfer, with generation of superoxide ion and consecutively hydroxyl radical.

A direct consequence of having a system where light and oxygen are combined with a sensitizer is the irreversible photodamage of the sensitizer leading to its photodegradation. The photobleaching kinetics was evaluated in solution. Bacteriochlorins photodegradation kinetics changes according to their peripheral substituents reaching levels of stability comparable to porphyrins.

Subcellular localization and light induced-changes on the intracellular fluorescence of the photosensitizers were followed by microscopy in mammalian in cells and the primary sites of PDT action were identified.

Intracellular detection of singlet oxygen at 1275 nm was observed upon irradiation of a photosensitizer in time-resolved experiments in single cells and in cell suspensions. Single cell results are consistent with a model in which long lived singlet oxygen can readily cross barriers between phase-separated domains.

In light of bleaching-dependent problems and with the desire to achieve spatial and temporal control in detecting singlet oxygen at the single cell level, we set out to identify sensitizers which show photostability properties that enable the collection of meaningful data.

#### Resumo

Há um vasto interesse no uso de compostos porfírinicos para aplicações médicas. As suas propriedades fotofísicas e fotoquímicas, como elevado rendimento quântico de formação de espécies reactivas de oxigénio e absorção no infravermelho onde os tecidos são mais transparentes, fez com que este grupo de moléculas fosse alvo de interesse para serem usadas como sensibilizadores em terapia fotodinâmica.

Tinhamos à nossa disposição uma familia de macrociclos tetrapirrólicos halogenados que foram desenhados para alcançar as propriedades de um fotossensibilizador ideal. Era do nosso intereste explorar as propriedades fotoquímicas e fotofísicas deste grupo de compostos de forma a estabelecer correlações que possam fornecer um melhor entendimento do mecanismo de fotosensibilização do oxigénio em porfirinas, clorinas e bacterioclorinas em sistemas em solução e em células.

Verificamos que a desactivação dos estados excitado tripleto destes fotossensibilizadores por interação com o oxigénio segue por diferentes caminhos. A redução progressiva do macrociclo introduz um aumento na constante de velocidade de supressão e uma dimunição no potencial de oxidação. Porfirinas e clorinas geram predominantemente oxigénio singuleto através de um mecanismo de transferência de energia para o oxigénio molecular. Por ouro lado, as bacterioclorinas mostram evidencias de um processo de transferências de carga para gerar ião superóxido e radical hidroxilo.

Uma consequência directa da combinação de luz, oxygénio e fotossensibilizador num sistema, é a degradação irreversível do sensibilizador. A cinética de fotodegradação foi avaliada em solução e para as bacterioclorinas varia de acordo com os substituintes periféricos atingindo níveis de estabilidade comparáveis aos das porfirinas.

A localização subcellular e a variação na fluorescência induzidas por irradiação dos fotossensibilizadores foi seguida por microscopia em células. Os locais iniciais de acção da PDT foram identificados.

A detecção intracelular de oxigénio singuleto resolvida no tempo foi observada a 1275 nm após a irradiação de um fotossensibilizador ao nível unicelular e em suspensão de células. Os resultados para experiências a nivel unicelular são consistentes com um modelo onde o oxigénio singuleto gerado rapidamente atravessa as barreiras entre separação de fases.

Tendo em conta os problemas de fotodegradação associados à perda de controlo espacial e temporal em experiências a nível unicelular na detecção do oxigénio singuleto, procuramos identifiar de um grupo de sensitibilizadores aqueles que reunem condições de estabilidade essenciais para a obtenção de resultados com significado.

#### List of papers

#### Papers relevant for the dissertation work:

"Photodynamic Therapy Efficacy Enhanced by Dynamics: Charge-Transfer and Photostability Favor a Fluorinated Bacteriochlorin" L. G. Arnaut, M. M. Pereira, J. M. Dąbrowski, E. F. F. Silva, F. A. Schaberle, A. R. Abreu, C. J. P. Monteiro, N. P. F. Gonçalves, G. N. Costa, A. V. C. Simões, L. C. Gomes-da-Silva, L. B. Rocha, M. M. Barsan, L. Cortes, M. Krzykawska-Serda, M. Elas, K. Urbańska, G. Stochel, S. Simões, C. M. A. Brett, S. Formosinho, submitted to Chem. Eur. J., **2013**.

"The challenging combination of intense fluorescence and high singlet oxygen quantum yield in photostable chlorins – a contribution to theranostics" E. F. F. Silva, F. A. Schaberle, C. J. P. Monteiro, J. M. Dabrowski, L. G. Arnaut, Photochem. Photobiol, Sci., 12, 1187-1192, **2013**.

"Irradiation- and Sensitizer-Dependent Changes in the Lifetime of Intracellular Singlet Oxygen Produced in a Photosensitized Process" E. F. F. Silva, B. W. Pedersen, T. Breitenbach, R. Toftegaard, M. K. Kuimova, L. G. Arnaut, P. R. Ogilby, Journal of Physical Chemistry B, 116, 445-461, **2012**.

"Mechanisms of Singlet-Oxygen and Superoxide-Ion Generation by Porphyrins and Bacteriochlorins and their Implications in Photodynamic Therapy" E. F. F. Silva, C. Serpa, J. M. Dabrowski, C. J. P. Monteiro, S. J. Formosinho, G. Stochel, K. Urbanska, S. Simoes, M. M. Pereira, L. G. Arnaut, Chemistrya european journal, 16, 9273-9286, **2010**.

"Synthesis and photophysical characterization of a library of photostable halogenated bacteriochlorins: An access to near-infrared chemistry" M. M. Pereira, C. J. P. Monteiro, A. V. C. Simões, S. M. A. Pinto, A. R. Abreu, G. F. F. Sá, E. F. F. Silva, L. B. Rocha, J. M. Dabrowski, S. J. Formosinho, S. Simões, L. G. Arnaut, Tetrahedron, 66, 9545-9551, **2010**.

"Synthesis and photophysical properties of amphiphilic halogenated bacteriochlorins: New opportunities for photodynamic therapy of cancer" M. M. Pereira, C. J. P. Monteiro, S. M. A. Pinto, A. V. Simões, G. F. F. Sá, E. F. F. Silva, L. G. Arnaut, S. J. Formosinho, S. Simões, L. Rocha, Journal of Porphyrins and Phthlacyonines, 13, 567-573, **2009**.

#### Other papers not discussed in this dissertation:

"Antioxidant β-Carotene Does Not Quench Singlet Oxygen in Mammalian Cells"; G. N. Bosio, T.Breitenbach, J. Parisi, M. Reigosa, F. H. Blaikie, B. W. Pedersen, E. F. F. Silva, D. O. Martire, P. R. Ogilby, J. Am. Chem. Soc., 135, 272-279, **2013**.

"Photoacoustic spectroscopy of a weakly absorbing media using nanosecond laser pulses"; L. A. Reis, F. A. Schaberle, E. F. F. Silva, L. G. Arnaut, Proc. SPIE, 8427 **2012**.

"Infrared absorbing dyes tailored for detection and therapy of solid tumors"; F. A. Schaberle, L. G. Arnaut, C. Serpa, E. F. F. Silva, M. M. Pereira, A. R. Abreu, S. Simões, Proc. SPIE, 7376, 73760X, **2010**.

#### Structures

The following structures correspond to the photosensitizers studied or referred in this dissertation. Respective scientific names are presented at the bottom of the table.

Structure	Substituents		Name
$\begin{array}{c} & & \\$	$\begin{array}{c} X_1{=}X_2{=}F\\ X_1{=}X_2{=}Cl\\ X_1{=}F, X_2{=}H\\ X_1{=}X_2{=}F\\ X_1{=}X_2{=}F\\ X_1{=}X_2{=}Cl\\ X_1{=}X_2{=}Cl\\ X_1{=}X_2{=}Cl\\ X_1{=}X_2{=}Cl\\ X_1{=}X_2{=}Cl \end{array}$	$\begin{array}{l} R{=}H \\ R{=}H \\ R{=}SO_2NHCH_3 \\ R{=}SO_3H \\ R{=}SO_2NHCH_3 \\ R{=}SO_2NHCH_3 \\ R{=}SO_2NHCH_3 \\ R{=}SO_2NHCH_2CH_3 \\ R{=}SO_2NHCH_2(CH_2)_5CH_3 \end{array}$	F <sub>2</sub> P Cl <sub>2</sub> P FPMet F <sub>2</sub> POH Cl <sub>2</sub> POH Cl <sub>2</sub> POH Cl <sub>2</sub> PMet Cl <sub>2</sub> PEt Cl <sub>2</sub> PHep
$\begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$	$\begin{array}{l} X_1 \!\!=\!\! X_2 \!\!=\!\! F \\ X_1 \!\!=\!\! X_2 \!\!=\!\! C l \\ X_1 \!\!=\!\! F, X_2 \!\!=\!\! H \\ X_1 \!\!=\!\! X_2 \!\!=\!\! F \\ X_1 \!\!=\!\! X_2 \!\!=\!\! C l \end{array}$	R=H R=H R=SO <sub>2</sub> NHCH <sub>3</sub> R=SO <sub>2</sub> NHCH <sub>3</sub> R=SO <sub>2</sub> NHCH <sub>2</sub> CH <sub>3</sub>	F2C Cl2C FCMet F2CMet ClCEt
$\begin{array}{c} \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$	$\begin{array}{c} X_1{=}X_2{=}F\\ X_1{=}X_2{=}Cl\\ X_1{=}X_2{=}F\\ X_1{=}F, X_2{=}H\\ X_1{=}F, X_2{=}H\\ X_1{=}K, X_2{=}H\\ X_1{=}Cl, X_2{=}H\\ X_1{=}Cl, X_2{=}H\\ X_1{=}Cl, X_2{=}Cl\\ X_1{=}X_2{=}Cl\\ \end{array}$	$\begin{array}{l} R{=}H \\ R{=}H \\ R{=}SO_3H \\ R{=}SO_2NHCH_3 \\ R{=}SONH (CH_3)_2 \\ R{=}SO_2NHCH_3 \\ R{=}SO_3H \\ R{=}SO_2NHCH_2CH_3 \\ R{=}SO_3H \\ R{=}SO_2NHCH_2CH_3 \\ R{=}SO_2NHCH_2CH_3 \\ R{=}SO_2NHCH_2(CH_2)_5CH_3 \end{array}$	F2B Cl2B F2BOH FBMet FBMet2 F2BMet ClBOH ClBEt Cl2BOH Cl2BEt Cl2BHep
$\begin{array}{c} \begin{array}{c} R \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \begin{array}{c} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \\ \\ \\ \end{array} \\ $	R=H R=OH		TPP m-THPP
	R=H R=OH		TPC m-THPC



F <sub>2</sub> P	5,10,15,20-Tetrakis(2,6-difluorophenyl)porphyrin
Cl <sub>2</sub> P	5,10,15,20-Tetrakis(2,6-dichlorophenyl)porphyrin
FPMet	5,10,15,20-Tetrakis(2-fluoro-5-N-methylsulfamoylphenyl)porphyrin
F <sub>2</sub> POH	5,10,15,20-Tetrakis(2,6-difluoro-3-sulfophenyl)porphyrin
F <sub>2</sub> PMet	5, 10, 15, 20 - Tetrakis (2, 6 - difluoro - 3 - N - methyl sulfamoyl phenyl) por phyrin
Cl <sub>2</sub> POH	5,10,15,20-Tetrakis(2,6-dichloro-3-sulfophenyl)porphyrin
Cl <sub>2</sub> PMet	5,10,15,20-Tetrakis(2,6-dichloro-3-N-methylsulfamoylphenyl)porphyrin
Cl <sub>2</sub> PEt	5,10,15,20-Tetrakis(2,6-dichloro-3-N-ethylsulfamoylphenyl)porphyrin
Cl <sub>2</sub> PHep	5, 10, 15, 20 - Tetrakis (2, 6 - dichloro - 3 - N - heptyl sulfamoyl phenyl) por phyrin
F <sub>2</sub> C	5,10,15,20-Tetrakis(2,6-difluorophenyl)chlorin
Cl <sub>2</sub> C	5,10,15,20-Tetrakis(2,6-dichlorophenyl)chlorin
FCMet	5,10,15,20-Tetrakis(2-fluoro-5-N-methylsulfamoylphenyl)chlorin
F <sub>2</sub> Cmet	5, 10, 15, 20 - Tetrakis (2, 6 - diffuoro - 3 - N - methyl sulfamoyl phenyl) chlorin
CICEt	5,10,15,20-Tetrakis(2-chloro-5-N-ethylsulfamoylphenyl)chlorin
$\mathbf{F}_2\mathbf{B}$	5,10,15,20-Tetrakis(2,6-difluorophenyl)bacteriochlorin
Cl <sub>2</sub> B	5,10,15,20-Tetrakis(2,6-dichlorophenyl)bacteriochlorin
F <sub>2</sub> BOH	5,10,15,20-Tetrakis(2,6-difluoro-3-sulfophenyl)bacteriochlorin
FBMet	5, 10, 15, 20 - Tetrakis (2 - fluoro - 5 - N - methyl sulfamoyl phenyl) bacterio chlorin
FBMet <sub>2</sub>	5, 10, 15, 20 - Tetrakis (2 - fluoro - 5 - N - dimethyl sulfamoyl phenyl) bacterio chlorin
F <sub>2</sub> BMet	5, 10, 15, 20 - Tetrakis (2, 6 - diffuoro - 3 - N - methyl sulfamoyl phenyl) bacterio chlorin
CIBOH	5,10,15,20-Tetrakis(2-chloro-5-sulfophenyl)bacteriochlorin
ClBEt	5, 10, 15, 20 - Tetrakis (2-chloro-5-N-ethyl sulfamoyl phenyl) bacterio chlorin
Cl <sub>2</sub> BOH	5,10,15,20-Tetrakis(2,6-dichloro-3-sulfophenyl)bacteriochlorin
Cl <sub>2</sub> BEt	5, 10, 15, 20 - Tetrakis (2, 6 - dichloro - 3 - N - ethyl sulfamoyl phenyl) bacterio chlorin
Cl <sub>2</sub> Bhep	5, 10, 15, 20 - Tetrakis (2, 6 - dichloro - 3 - N - heptyl sulfamoyl phenyl) bacterio chlorin and the second state of the second sta
TPP	5,10,15,20-Tetrakisphenylporphyrin
m-THPP	5,10,15,20-Tetrakis(m-hydroxyphenyl)porphyrin
ТРС	5,10,15,20-Tetrakisphenylchlorin
m-THPC	5,10,15,20-Tetrakis(m-hydroxyphenyl)chlorin
ТРВ	5,10,15,20-Tetrakisphenylbacteriochlorin
m-THPB	5,10,15,20-Tetrakis(m-hydroxyphenyl)bacteriochlorin
ТМРуР	5,10,15,20-Tetrakis(N-methyl-4-pyridyl)-21H,23H-porphine
PPa	Pyropheophorbide-a
BP	benzo[cd]pyrene-5-one
DMP-60	N,N-dimethylfulleropyrrolidiniuim iodide
BBB	1,4-Bis[4-(N,N-diphenylamino)phenylethynyl]-2,5-dibromobenzene

### **1** Introduction

- 1.1 Tetrapyrrolic photosensitizers: porphyrins, chlorins and bacteriochlorins
- 1.2 Photodynamic therapy
- 1.3 Photochemistry and photophysics of PDT
- 1.4 Reactive oxygen species
  - 1.4.1 Molecular oxygen: Ground state and excited state
  - 1.4.2 Quenching of triplet states by molecular oxygen
- 1.5 Cell death
- 1.6 Phototransformation of sensitizers
- 1.7 References

## **1.1 Tetrapyrrolic photosensitizers: porphyrins, chlorins and bacteriochlorins**

Porphyrins, whose name comes from the Greek word *porphura* (purple),<sup>1</sup> embrace an important class of intensely colored molecules that serve nature in a variety of ways.<sup>2</sup> The different forms of porphyrins are involved in a number of biologically important roles, from oxygen transport and storage (hemoglobin and myoglobin) to electron transfer (cytochrome c, cytochrome oxidase) and energy conversion (photosynthesis). Their extremely versatile synthesis provides applications also for a variety of materials particularly in optoelectronics.<sup>3</sup>



**Figure 1.1** Comparative structures of porphyrin (I), chlorin (II) and bacteriochlorin (III). Numbering of porphyrin is shown, with *beta* ( $\beta$ ) positions at bold and *meso*-positions underlined.

Porphyrins are tetrapyrrolic molecules that possess in the heart of its skeleton a heterocyclic macrocycle, known as porphine (Figure 1.1). The fundamental porphine framework consists of four pyrrolic sub-units on opposing sides ( $\alpha$ -positions, numbered 1, 4, 6, 9, 11, 14, 16, and 19) through four methine (CH) bridges (5, 10, 15 and 20) known as the *meso-* carbon atoms/positions, as shown in Figure 1.1. The resulting conjugated planar macrocycle can be turned into a porphyrin macrocycle when substituted at the *meso-* and/or  $\beta$  - positions by non-hydrogen atoms or groups. The insertion of four phenolic groups in the macrocycle skeleton to tetraphenylporphyrins, causes amphiphilic modification and gave rise to a series of potent tumor photosensitizers.<sup>4</sup>

By progressive reducing the porphyrinic macrocycle leading to chlorins and bacteriochlorins, profound changes in chemical and physical properties are found. The reduction alters the symmetry of the molecule, though each macrocycle still maintains an 18  $\pi$ -electron conjugated system as required for aromaticity. A notable change upon reduction is the striking increase in absorption in the red or near-IR region of the spectrum.

In chlorins the loss of one double bond destabilizes the  $\pi$  system, the highest occupied molecular orbital (HOMO) rises in energy, and the molecule becomes easier to oxidize. Only a small effect is observed on the lowest unoccupied molecular orbital (LUMO), and a reduction on the energy gap HOMO-LUMO is observed, and consequently, a red shift is occurs to the lowest energy absorption band. The energy gap between HOMO and LUMO is reduced in the order: porphyrin>chlorin>bacteriochlorin. While the energy required for oxidation follows the opposite order.

Their different physical properties are exploited in biological systems where the chlorin macrocycle provides the basis for chlorophyll *a* and *b* in plant photosynthesis while the bacteriochlorins are present as a photosynthetic pigment (bacteriochlorophyll *a*) in bacteria.<sup>5</sup> The strong near-infrared (NIR) absorption, resultant from the macrocycle reduction, of chlorins and mainly bacteriochlorins, makes them well suited for a wide variety of applications in medicine and materials chemistry.<sup>6-8</sup> However, the pronounced tendency of bacteriochlorins to undergo oxidation and generate photoproducts as the corresponding chlorin has been the main handling obstacle to their use with implications to the shelf-life.<sup>9</sup> Surprisingly, until recently only a few methods described the preparation of bacteriochlorins despite the importance of this class of compounds. Researchers have now become actively involved in developing new synthetic routes to yield more stable and more efficient photosensitizers.<sup>10-16</sup>

#### **1.2 Photodynamic therapy**

Light has been employed in the treatment of disease since the antiquity. The first uses of photomedicine date back thousand years ago in ancient Egypt, India and China, for the treatment of skin diseases, although it was only until relatively recently that it has been used to any significant degree in medicine and surgery.<sup>17,18</sup>Niels Finsen was awarded with the Nobel Prize in Medicine in 1903 for the treatment of cutaneous tuberculosis by ultraviolet radiation. Early in the 20<sup>th</sup> century light and a photosensitizer were combined in medicine and originated the field of photodynamic therapy. In 1900 Raab<sup>19</sup> showed that acrydine dyes would be lethal to paramecia when light exposed. Later in 1925, Policard<sup>20</sup> showed the phototoxicity of porphyrins, including hematoporphyrin. With the synthesis of hematoporphyrin derivative, HpD,<sup>21</sup> a step forward was achieved when Lipson et al. observed preferential accumulation in the cancerous tissue rather than in the surrounding tissue<sup>22</sup> and Dougherty et al.<sup>23</sup> initiated pioneering studies on basic science and clinical applications. In 1983, a purified form of HpD, now commercially known as Photofrin® was developed. Photofrin®<sup>24,25</sup> was the first photosensitizer to receive regulatory approval for treatment of various cancer types in more than 40 countries throughout the world, including the United States. Since their work, a variety of

compounds has been tested *in vitro* and *in vivo*, but only tetrapyrrole macrocycles, with the exception of methylene blue, have been clinically approved and are the majority of photosensitizers used on PDT.

Photodynamic therapy has been approved as a treatment modality against some forms of cancer, precancerous lesions and age-related macular degeneration (AMD). The most relevant clinical photosensitizers are listed in Table 1.1.

Trade name	Approval	Indications
Photofrin	Worldwide	Advanced and early lung cancer, Oesophageal adenocarcinoma, cervical cancer and bladder cancer.
Foscan	Europe	Palliative head and neck cancer
Levulan	Approved in 2000 in USA	Actinic keratosis
Metvix	Europe	Actinic keratosis and basal cell carcinoma
Visudyne	Approved in 65 countries	Age-related macular degeneration

**Table 1.1** Clinically approved photodynamic therapy photosensitizers. Table information obtained from.26,27

HpD and Photofrin® are part of the first generation of photosensitizers. Although they have shown to be efficient in the treatment of many cancer types, they also have exhibited some disadvantages such as: prolonged patient photosensitivity (poor clearance), suboptimal tumor selectivity, low wavelength absorption and poor light penetration into the tumor tissue. Additionally, Photofrin® is a complex mixture of uncertain structures. Therefore, in order to overcome these drawbacks and also improve treatment efficacy, several strategies have been developed to find more tumor-selective agents with reduced side effects, especially skin phototoxicity. The synthesis of many second generation photosensitizers, most of them modified tetrapyrrolic compounds, improved various properties over the first generation of photosensitizers, and some of them were approved for clinical use or are under clinical trials (Table 1.1).

The third generation of photosensitizers is usually meant to designate second generation photosensitizers coupled to carriers such as cholesterol, antibodies or liposomes, for selective tumor accumulation and targeting tumor tissue.



Figure 1.2 Structures of approved photosensitizers Photofrin (I), Foscan (II) and Visudyne (III).

For the purpose of PDT, the following characteristics have been proposed for the ideal photosensitizer<sup>28,29</sup>: i) chemically pure and of known specific composition, ii) stable at room temperature and with a straightforward synthesis, iii) minimal dark toxicity and only cytotoxic in the presence of light of a defined wavelength, iv) should have preferential retention by target tissue, v) should be rapidly excreted from the body and have a low systemic toxicity, vi) should have strong absorption with high absorption coefficient in the near-infrared (between 700 and 800 nm) where light penetration in tissue is maximum (Figure 1.3), vii) should have long triplet state lifetimes and effectively produce singlet oxygen and other reactive oxygen species (ROS), viii) be affordable and readily available in order to promote extensive use, ix) be easy to dissolve in biocompatible formulations, x) and have a subcellular localization that promotes a strong generation of ROS, preferentially the endoplasmic reticulum (ER), where oxidative stress has shown to induce immunogenic cancer cell death.<sup>30-32</sup>



**Figure 1.3** Depth of penetration into the skin at different wavelengths. The percentages indicate the incident radiation reaching a given skin layer. The maximum light penetration depth in tissue is found for wavelengths in the range 650 - 800 nm, the therapeutic window. In this region light is poorly absorbed by most of the biological tissues and has lower scattering than in the visible region. Source: ILO - Encyclopedia of Occupational Health and Safety at: <u>http://www.ilo.org/oshenc/part-vi/radiation-non-ionizing/item/654-infrared-radiation</u>.

#### **1.3 Photochemistry and photophysics of PDT**

Light, oxygen and a photosensitizer are the three essential elements involved in photodynamic therapy. After administration and delivery of the photosensitizer to the target tissue, light irradiation of a specific wavelength matching the absorption of the photosensitizer is used and the undesired tissue is destroyed.

Upon irradiation and absorption of a photon, the photosensitizer is excited from the ground state  $({}^{1}S_{0})$  to the first excited singlet state  $({}^{1}S_{1})$ , from which several physical pathways, represented in the Jablonski diagram in Figure 1.4, leading to deactivation can be followed. The excited singlet state  ${}^{1}S_{1}$  can rapidly return to the ground state level  ${}^{1}S_{0}$  by a radiative process, called fluorescence or by a nonradiative process of internal conversion (IC), where the excess of energy is released as heat that dissipates to the surrounding medium (solvent or tissue). In addition, the excited singlet state can generate the triplet state  $({}^{3}S_{n})$ , by a fast spin inversion, in a process called intersystem crossing (ISC). The long-lived triplet state enables the interaction of the excited photosensitizer with the surrounding molecules increasing the number of paths by which it can be deactivated. The excited triplet state of a PDT photosensitizer can react by two mechanisms, normally defined as Type I and Type II.<sup>33</sup> The Type I mechanism involves the hydrogen/electron abstraction between the sensitizer and either a biological substrate, a solvent

or another sensitizer, resulting in the formation of free radicals or radical ions. If molecular oxygen is present, these free radical species can interact and generate superoxide  $(O_2^{\bullet})$  a direct electron transfer from the triplet state of the photosentitizer to oxygen may occur. Dismutation or one electron reduction of  $O_2^{\bullet}$  gives  $H_2O_2$  (equation 1.1), which on one electron reduction can generate hydroxyl radical (OH<sup>•</sup>). In biological systems where ferrous ion is present, it is generally assumed that OH<sup>•</sup> is generated from  $H_2O_2$  by the Fenton reaction (equation 1.2). <sup>34</sup>

$$2O_2^{\bullet} + 2H^+ \rightarrow O_2 + H_2O_2 \tag{1.1}$$

$$\operatorname{Fe}^{2+}$$
 +  $\operatorname{H}_2\operatorname{O}_2 \rightarrow \operatorname{Fe}^{3+}$  +  $\operatorname{OH}^{\bullet}$  +  $\operatorname{OH}^{-}$  (2.1)

In a Type II mechanism, energy transfer takes place from the excited triplet state of the sensitizer to ground state molecular oxygen, generating singlet oxygen. These oxygen species that result from a Type I and Type II mechanisms are highly reactive and can interact with a large number of biological molecules. In particular, hydroxyl radical reacts unspecifically with biomolecules such as proteins, polysaccharides and nucleic acids located less than a few nanometers from the generation site. Singlet oxygen, generated in a Type II process, has a wider action radius which can reach hundreds of nm, according to its estimated lifetime in cells of ~3  $\mu$ s.<sup>35,36</sup>



Figure 1.4 Jablonski diagram representing the excitation and relaxation of a photosensitizer (S).

#### **1.4 Reactive oxygen species**

#### 1.4.1 Molecular oxygen: Ground state and excited state

The properties of oxygen are a reflection of its unique electronic structure. Unlike many other molecules, oxygen's electronic ground state is a spin triplet.

Mullikan<sup>37</sup> predicted that the electronic configuration of molecular oxygen would give rise to three energetically close lying states and specified them as a  ${}^{3}\Sigma_{g}^{-}$  triplet ground state, and  ${}^{1}\Sigma_{g}^{+}$ and  ${}^{1}\Delta_{g}$  excited singlet states. Three different optical transitions are possible between the ground state  ${}^{3}\Sigma_{g}^{-}$  and the two excited states ( ${}^{1}\Sigma_{g}^{+} = 31.5$  kcal mol<sup>-1</sup>;  ${}^{1}\Delta_{g} = 22.5$  kcal mol<sup>-1</sup>) of molecular oxygen, and all of them are forbidden. The transition  ${}^{3}\Sigma_{g}^{-} \rightarrow {}^{1}\Delta_{g}$  was first observed by Ellis and Kneser<sup>38</sup> in absorption experiments with liquid O<sub>2</sub> at ~1261 nm, and identified O<sub>2</sub>( ${}^{1}\Delta_{g}$ ) as the metastable O<sub>2</sub> specie, commonly known as singlet oxygen.<sup>38</sup> The higher energy state  ${}^{1}\Sigma_{g}^{+}$  is not known to react with other molecules, at least in solution phase systems, because of its short lifetime due to the spin-allowed transition to the  ${}^{1}\Delta_{g}$  state. The  ${}^{1}\Delta_{g}$  lifetime is relatively long and this state has a rich chemistry that is distinctly different from that of the ground triplet state.<sup>39</sup>

#### 1.4.2 Quenching of sensitizer triplet states by molecular oxygen

Singlet oxygen can be produced with varying efficiency as a consequence of quenching of both excited singlet and triplet states of organic molecules. Despite the major contributions of many authors to elucidate the mechanism of quenching of electronically excited states by molecular oxygen many aspects remain unclear.<sup>39</sup>

Molecular oxygen can quench both excited singlet and triplet states. In the first case,  $O_2$  quenching of the singlet excited states gives five spin allowed and two spin-forbidden possible processes.

The quenching of triplet states by molecular oxygen can be described by Figure 1.5, proposed by Wilkinson et al.<sup>40</sup>

$${}^{1}_{k_{A}}$$

$${}^{1}_{k_{A}}$$

$${}^{1}_{k_{diff}} = {}^{1}({}^{3}S \dots O_{2}({}^{3}\Sigma^{-}g)) \xrightarrow{{}^{1}_{k_{et}}} {}^{1}(S^{\delta^{+}} \dots O_{2}^{\delta^{-}}) \xrightarrow{{}^{1}_{k_{ic}}} {}^{1}({}^{1}S \dots O_{2}({}^{1}\Delta g)) \xrightarrow{{}^{1}_{k_{diff}}} {}^{1}S + O_{2}({}^{1}\Delta g)$$

$${}^{3}S + O_{2}({}^{3}\Sigma^{-}g) \xrightarrow{{}^{3}_{k_{et}}} {}^{3}({}^{3}S \dots O_{2}({}^{3}\Sigma^{-}g)) \xrightarrow{{}^{3}_{k_{et}}} {}^{3}(S^{\delta^{+}} \dots O_{2}^{\delta^{-}}) \xrightarrow{{}^{3}_{k_{ic}}} {}^{3}({}^{1}S \dots O_{2}({}^{3}\Sigma^{-}g)) \xrightarrow{{}^{k_{diff}}} {}^{1}S + O_{2}({}^{3}\Sigma^{-}g)$$

$${}^{3}S + O_{2}({}^{3}\Sigma^{-}g) \xrightarrow{{}^{3}_{k_{et}}} {}^{3}({}^{3}S \dots O_{2}({}^{3}\Sigma^{-}g)) \xrightarrow{{}^{3}_{k_{et}}} {}^{3}(S^{\delta^{+}} \dots O_{2}^{\delta^{-}}) \xrightarrow{{}^{3}_{k_{ic}}} {}^{3}({}^{1}S \dots O_{2}({}^{3}\Sigma^{-}g)) \xrightarrow{{}^{k_{diff}}} {}^{1}S + O_{2}({}^{3}\Sigma^{-}g)$$

$${}^{5}({}^{3}S \dots O_{2}({}^{3}\Sigma^{-}g)) \xrightarrow{{}^{3}_{k_{et}}} {}^{5}({}^{3}S \dots O_{2}({}^{3}\Sigma^{-}g))$$

Figure 1.5 Quenching of triplet excited states of a photosensitizer by molecular oxygen.

According to this mechanism, molecular oxygen in a collision dependent process can quench triplet excited states of a photosensitizer (<sup>3</sup>S) leading to the reversible formation of encounter complexes  ${}^{1,3,5}({}^{3}S - O_{2}({}^{3}\Sigma_{g}^{-}))$ . One out of nine of the collisions results in a singlet state complex which can decay by energy transfer to generate singlet oxygen and ground state photosensitizer at a rate  $k_{\Delta}$ .

Because there is no spin or energy allowed product state for the quintet complex, quenching of  ${}^{3}S^{*}$  can only occur via the singlet and triplet collision complexes. Which means that only 4/9 of all triplet collisions will be responsible for quenching. If only the singlet channel contributes to the deactivation of the triplet state of the sensitizer by molecular oxygen then  $k_{T}^{Q} = 1/9k_{diff}$  and singlet oxygen generation efficiency (S<sub> $\Delta$ </sub>) should be unit. However, if the triplet channel also participates in the quenching, then  $k_{T}^{Q} = 4/9k_{diff}$  and singlet oxygen generation efficiency drops to 0.25.

The mechanism of quenching the triplet states by molecular oxygen as presented on Figure 1.5 is based on the mechanism first proposed by Gijzeman, Kaufman and Porter,<sup>41</sup> which considered that for a group of aromatic hydrocarbons the energy transfer channel was the only possible quenching path since their quenching rates were in good agreement with 1/9  $k_{diff}$ . Although Wilkinson et al.<sup>42</sup> observed the same behavior for anthracene derivatives in cyclohexane, this behavior is rarely observed. In order to account for values higher than 1/9  $k_{diff}$ , and even higher

than  $4/9 \ k_{diff}$ , the involvement of charge transfer complexes in the mechanism of quenching by molecular oxygen and the possibility of intersystem crossing between channels has been considered, and the mechanism initially proposed was modified to the version presented on Figure 1.5.<sup>43-46</sup>

Quantitative correlations between physical properties of a sensitizer and its triplet state quenching rate constants by molecular oxygen, and the overall efficiency on singlet oxygen generation have been explored by a number of authors.<sup>41,47-49</sup> Systematic studies by Wilkinson and Schmidt <sup>39,43,46,50-53</sup> have contributed significantly to the present knowledge, revealing meaningful tendencies.

It has been shown that the fraction of triplet states quenched by oxygen which yield singlet oxygen depends on several factors including the excited state energy, the nature of the excited state, the redox potential of the excited state and the nature of the solvent.<sup>40,43,46,50,52</sup>

Wilkinson et al. have demonstrated for a group of biphenyl derivatives that the rate constant for quenching of the triplet excited states  $(k_T^Q)$  by molecular oxygen and the efficiencies with which singlet oxygen is produced  $(S_\Delta)$  are inversely correlated, and both show pronounced sensitivity to oxidation. Furthermore these and other authors observed that the charge transfer and non-charge transfer  $({}^1k_\Delta)$  pathways compete in the quenching of triplet states by  $O_2$  and both yield  ${}^1O_2$  with different efficiencies.<sup>40,43,46,52-55</sup> Schmidt and co-workers after studying a series of organic compounds in CCl<sub>4</sub>  ${}^{51,56-59}$  have proposed some modifications to the mechanism in Figure 1.5, by including the formation of ground state oxygen from the encounter complexes and excluding intersystem crossing in charge transfer complexes  $({}^{1.3}({}^3S^{\delta^+}...O_2^{\delta^-}))$ .

In addition to other pathways to generate singlet oxygen Tsubomura and Mulliken <sup>60</sup> have first proposed the formation of a ground state complex between the organic molecule S and  $O_2(^{3}\Sigma_{g})$  that after light absorption can populate to a charge-transfer state. This charge-transfer state has been shown by Scurlock and Ogilby to be a precursor of singlet oxygen  $(O_2^{1}\Delta_g)$ .<sup>61,62</sup>

Since the first excited singlet state of most photosensitizers rapidly undergoes intersystem crossing into a triplet state, quenching of singlet excited states by molecular oxygen has only a very short time to occur and so it will not be explored further in the discussion of the work in this thesis.

#### 1.5 Cell death

The generation of reactive oxygen species after light induced excitation of an intracellularly localized photosensitizer may lead to cell death mainly by apoptosis and/or necrosis. <sup>63,64</sup> In the case of a mammalian tissue being the target, necrosis and apoptosis can cause vascular damage with ischemia of the target tissue and/or an immune systemic response. <sup>63</sup> More interesting, the activation of the immune system increases the priming of T-lymphocytes that recognize tumor antigens, leading to the development of an immune memory which can fight the recurrence of cancer. <sup>65,66</sup>

Apoptosis is a very complex, multi-step, multi-pathway cell-death program controlled by intracellular and extracellular signals. It can be initiated either through the activation of death receptors or the mitochondrial release of cytochrome c. Both events eventually lead to activation of caspase cascades in a process tightly controlled by various proteins which leads to cell dismantling into apoptotic bodies without leakage of intracellular material to the immediate envirnoment.<sup>67,68</sup> Necrotic cell death normally occurs when high light fluence doses or high photosensitizer concentrations are used with cells. <sup>69</sup> A quick and violent degeneration characterized by cytoplasm swelling, destruction of organelles and disruption of the plasma membrane, leading to release of material into the extracellular compartment is normally observed.

When the PDT damaged cells try to contain and remove damaged proteins, an autophagy mechanism of initial rescue is activated. Only when the PDT damage is sufficiently robust and the cells are damaged beyond repair, apoptosis occurs.<sup>70</sup> PDT, at its highest dose may also lead to necrosis, as the proteins that participate in both autophagy and apoptosis may be immediately destroyed and the cellular integrity may be broken. The initial site of PDT-related damage may determine which cell pathway is initially activated. The extent of PDT related damage may also regulate how the PDT treated cells respond.<sup>70</sup>

An important factor determining the outcome of PDT is how the photosensitizer interacts with cells within the target tissue. The key characteristic of this interaction is the subcellular localization of the photosensitizer, since photosensitizers can localize within many different cellular organelles such as mitochondria, lysosomes, endoplasmic reticulum and/or Golgi apparatus.

#### **1.6 Phototransformation of sensitizers**

The same source of photons which is used to trigger a photosensitizer to destroy undesired tissue is also the cause of its photobleaching. The loss of sensitizer absorption or emission intensity by light is called photobleaching. The photobleaching of a photosensitizer has been a major concern of scientists from various fields. In materials science, the issue of producing stable electroluminescent polymers, for example, has been an important limiting factor in the evolution of this particular technological innovation.<sup>71,72</sup> In biology, where photoinduced bleaching is likewise a general phenomenon, common photo-functional molecules include i) fluorescent probes used to assess cell structure and/or activity, and ii) sensitizers used to generate reactive oxygen species that, in turn, elicit cell death. The photobleaching of photosensitizers used in PDT is a field of interest since it can influence the success of the treatment in several ways.



Figure 1.6 Schematic illustration of photobleaching mechanism after generation of triplet state.

The mechanistic processes involved in the photobleaching of a sensitizer are generally oxidative and can be caused by singlet oxygen generated in a Type II process, and/or by free radicals in a Type I process.<sup>73</sup> Light-induced molecular rearrangements, bond making/breaking reactions, and functional group modifications all contribute to the photobleaching phenomenon. Two types of irreversible photobleaching leading to chemical change in a chromophore are normally considered: i) photomodification, where loss of absorbance or fluorescence occurs at some wavelengths, but the chromophore retains its modified form and ii) true photobleaching, which involves fragmentation of the sensitizer in species that no longer absorb in the visible region.

The photobleaching quantum yield varies significantly with the reaction path involved (Type I or Type II) and specific features of the sensitizer itself (e.g., the oxidation potential, lipophilicity etc.).

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## 2

# Photochemistry and photophysics of porphyrins and bacteriochlorins

2.1 Introduction

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## **2.1 Introduction**

This chapter introduces a group of synthetic porphyrins, chlorins and bacteriochlorins prepared by a synthesis group in Coimbra in a joint collaboration with our group. The structures of these macrocycles were strategically modified by the presence of phenyl groups with different substituents at the *meso* positions to modulate the hydrophobic/hydrophilic character of these new photosensitizers.<sup>1-4</sup> Reduction of the porphyrinic macrocycle along this series causes a number of changes. A most remarkable change is the increase in absorption in the red or near-IR region of the spectrum, allowing deeper light penetration through tissue, and making bacteriochlorins interesting for biomedial applications. The introduction of halogen atoms in the *ortho* positions of the phenyl ring of the sensitizer should accelerate the intersystem crossing to the triplet excited state and maximize the triplet quantum yield ( $\Phi_T$ ). Additionally, the steric interaction between the halogen atoms and hydrogen atoms in  $\beta$  positions, increases the angle between the macrocycle and the phenyl ring, and diminishes the tendency of porphyrin derivatives to aggregate.<sup>2.5</sup>

Porphyrins are part of the most widely studied photosensitizers due to their photosensitizing ability in PDT applications. Under illumination with light of an appropriate wavelength, porphyrins generate singlet oxygen with quantum yields considerably high, typically ~0.5 or higher. However, these photosensitizers are limited by their low light absorption in the near infrared region, where the effective depth of light penetration is higher.

Bacteriochlorins efficacy in PDT has been associated to their ability to transfer a large part of their triplet energy to molecular oxygen, with consequent production of singlet oxygen (O<sub>2</sub>,  ${}^{1}\Delta_{g}$ ), but also to their ability to transfer an electron to molecular oxygen to generate superoxide ion (O<sub>2</sub><sup>-</sup>). Because singlet oxygen and superoxide radical have active roles in the production of oxidative stress in cancer cells, it is important to establish the mechanism of energy transfer and electron transfer from the photosensitizer to molecular oxygen.

Taking into account the photophysical and photochemical properties of these dyes, their potential application as photosensitizers for photodynamic therapy and to generate singlet oxygen in cells will be discussed.

# 2.2 Results and discussion

## 2.2.1 Absorption and fluorescence properties

Figure 2.1 shows the absorption spectra of the porphyrin  $Cl_2PEt$ , chlorin  $F_2CMet$  and bacteriochlorin  $Cl_2BMet$ .



Figure 2.1 Electronic absorption spectra in ethanol at room temperature of porphyrin  $Cl_2PEt$ , chlorin  $F_2CMet$  and bacteriochlorin  $Cl_2BEt$ .

Cl<sub>2</sub>PEt has a typical free base porphyrin absorption spectrum with an intense band around 400 nm and four other less intense bands at lower energies. A simple model to interpret the electronic spectra of porphyrins has been proposed by Gouterman.<sup>6,7</sup> According to this model the absorption bands in porphyrin systems arise from transitions between the two highest occupied molecular orbitals (HOMOs) and the two lowest unoccupied molecular orbitals (LUMOs), and the identities of the substituents of the ring can affect the relative energies of the transitions. A representation of the energy levels for the two HOMOs ( $a_{1u}$ ;  $a_{2u}$ ) and the two LUMOs ( $e_{gx}$ ;  $e_{gy}$ ) for free base porphyrin, chlorin and bacteriochlorin are shown in Figure 2.2.

In porphyrins, the two HOMO orbitals and two LUMO orbitals lie close enough in energy and as result the optical transitions have nearly the same energy. By interaction and splitting of the electronic states a pair of low energy and low intensity transitions gives rise to the absorption bands  $Q_x$  and  $Q_y$ , and a pair of high energy transitions lead to the generation of  $B_x$  and  $B_y$  bands. Most of the intensity of the transition is carried by the B bands as a result of the addition of the transition dipoles.  $B_x$  and  $B_y$ , are observed as a single intense band called the Soret band.

Whereas the Q bands come from the near cancelation of the transition dipoles and are observed as low intensity absorption bands.<sup>2</sup>



Figure 2.2 Schematic representation of HOMO and LUMO orbitals for tetraphenyl porphyrins, chlorins and bacteriochlorins.<sup>8,9</sup>

Within the series, porphyrin, chlorin and bacteriochlorin the lowest LUMO's are nearly isoenergetic, and so the energy does not change as the number of the  $\pi$  electrons is reduced. On the other hand, the macrocycle distortion causes destabilization of the HOMO, which rises along the series. As a result the HOMO-LUMO gaps get progressively smaller and the Q<sub>y</sub> bands shift to the red,<sup>2</sup> explaining the red absorption of the chlorin around ~650 nm and the infrared absorption of the bacteriochlorins at ~745 nm. A less intense band around 519-529 nm, normally labeled as Q<sub>x</sub> is observed along the series and the Soret band is observed at 400-420 nm for porphyrins and chlorins. For bacteriochlorins the Soret band splits into two independent bands with absorption peaks for wavelengths lower than 380 nm. The peak of higher energy (350-360 nm) is made up mainly of the  $a_{2u} \rightarrow e_{gy}$  configuration while the absorption band around 370-380 nm is mainly  $a_{1u} \rightarrow e_{gy}$ . The strong absorption of chlorins and the even higher of bacteriochlorins in the near infrared, where the tissues are most transparent, confer to this group of photosensitizers a spectroscopic advantage for PDT.<sup>10</sup> The exact position and intensity of the absorption peaks can also be dependent on both the concentration and the nature of the solvent.

The absorption spectra of  $Cl_2PEt$ ,  $F_2CMet$  and  $Cl_2BEt$ , presented in Figure 2.1, and of all the other related sensitizers analyzed in this study, represent the main optical characteristics of typical tetraphenyl porphyrins, chlorins and bacteriochlorins, respectively.<sup>2</sup> The variation on the peripheral substituents of the phenyl ring causes only relatively small changes on intensity and shifts on the absorption bands (Table 2.1).



Figure 2.3 Normalized fluorescence spectra of Cl<sub>2</sub>PEt, F<sub>2</sub>CMet and Cl<sub>2</sub>BEt in ethanol.

Figure 2.3 shows the fluorescence spectra of  $Cl_2PEt$ ,  $F_2CMet$  and  $Cl_2BEt$ , typical for tetraphenylporphyrins, chlorins and bacteriochlorins. The fluorescence spectra are a good mirror image of the absorption spectra and a small Stokes shift (~5 nm) is observed in ethanol solutions. Fluorescence emission maximum and fluorescence quantum yields are presented in Table 2.1.

Comparing the fluorescence quantum yield of porphyrins and bacteriochlorins, a tendency is found according to the halogen atom substituent of the phenyl rings.  $\Phi_F$  organizes as following  $Cl_2 < Cl < F_2 \le F$ . The presence of halogenated atoms in the *ortho* position of the phenyl ring in the porphyrins or bacteriochlorins structure increases the rate of intersystem crossing to the triplet state and further reduces the fluorescence intensity, a process normally explained by the internal heavy atom effect.<sup>5,11</sup> Chlorine atoms, Cl, are heavier than fluorine, F, and so the process is enhanced because the spin-orbit coupling constants are increased. The fluorescence quantum yield determined for halogenated porphyrins are half of that of TPP,  $\Phi_F = 0.10 \pm 0.01$ .<sup>12</sup> Similarly, bacteriochlorins also show low fluorescence quantum yields.

Chlorin  $F_2$ CMet shows the highest value of fluorescence quantum yield of 0.36. A similar value was obtained by Monteiro et al.<sup>13</sup> for a related chlorin FCMet (0.396).<sup>14</sup>

From the crossing of the normalized absorption Q(0,0) and fluorescence bands we have estimated the singlet state energy (E<sub>s</sub>) in ethanol. Values of 44 kcal mol<sup>-1</sup> were obtained for porphyrins. Bacteriochlorins have singlet states about ~6 kcal mol<sup>-1</sup> lower in energy. The values are registered in Table 2.1.

		$\lambda_{Qx(0,0)}$	$\epsilon_{\rm max}/10^3$	$\lambda_{ems}$	Es	$\Phi_{ m F}$
		/nm	/M <sup>-1</sup> cm <sup>-1</sup>	/nm	/kcal mol <sup>-1</sup>	
	<b>TPP</b> <sup>a</sup>	650	9.6	652/719	44.0	$0.100\pm0.010$
	mTHPP <sup>b</sup>	644	3.4	649/715	44.2	0.120
	FPMet <sup>f</sup>	639	$0.79\pm0.03$	644/709		$0.096 \pm 0.021$
	$\mathbf{F}_{2}\mathbf{P}^{c}$	655	5.3	657/713	43.6	$0.069\pm0.015$
yrins	F <sub>2</sub> POH	637	$1.0\pm0.16$			
rphy	F <sub>2</sub> PMet <sup>e</sup>	639	$0.68\pm0.03$	654/720	44.8	$0.049 \pm 0.022$
Pc	Cl <sub>2</sub> P <sup>c</sup>	660	2.1	661/706	43.3	$0.005\pm0.002$
	Cl <sub>2</sub> POH <sup>e</sup>			654/720	43.8	
	Cl <sub>2</sub> PEt	652	$5.2\pm0.2$	655/720	43.8	$0.017\pm0.003$
	Cl <sub>2</sub> PHep <sup>e</sup>	653	$3.9\pm0.2$	658/724	43.6	$0.017\pm0.004$
	mTHPC <sup>b</sup>	650	29.3	653		0.089
	FCMet <sup>e</sup>	652	$34 \pm 3$	657	43.6	$0.396\pm0.05$
ins	$F_2C^c$	655	40	658	43.6	$0.124\pm0.026$
Chlor	F <sub>2</sub> CMet <sup>e,h</sup>	655	$50 \pm 4$	657	43.6	$0.360\pm0.04$
	Cl <sub>2</sub> C <sup>c</sup>	660	2.1	661	43.4	$0.005\pm0.022$
	Cl <sub>2</sub> CEt <sup>f</sup>	658	29	662		0.028
	mTHPB <sup>b</sup>	735	91	746		0.110
	FBMet <sup>e</sup>	743	62	746		$0.060\pm0.01$
	$\mathbf{F}_{2}\mathbf{B}^{d}$	744	140	745	38.4	$0.068 \pm 0.07$
s	F <sub>2</sub> BOH <sup>e</sup>	745	56	745		$0.023\pm0.05$
orin	F <sub>2</sub> BMet <sup>e</sup>	743	$140\pm3$ <sup>g</sup>	746	38.1	$0.138\pm0.01$
iochl	<b>CIBOH</b> <sup>e</sup>	742	61	745		$0.040\pm0.08$
lcteri	<b>ClBEt</b> <sup>e</sup>	743	76	746		$0.038 \pm 0.07$
Ba	$\mathbf{Cl}_{2}\mathbf{B}^{d}$	747	126	748	38.3	$0.012\pm0.002$
	Cl <sub>2</sub> BOH <sup>e</sup>	745	61	748	38.3	$0.006 \pm 0.001$
	Cl <sub>2</sub> BEt <sup>e</sup>	745	97	747	38.3	$0.008 \pm 0.002$
	Cl <sub>2</sub> BHep <sup>e</sup>	746	76	749	38.2	$0.008 \pm 0.002$

**Table 2.1** Absorption and emission properties of tetraphenyl porphyrins, chlorins and bacteriochlorins in ethanol and of other relevant macrocycles.

a) from ref. <sup>12</sup>, b) in methanol from ref.<sup>15</sup>, c) from ref.<sup>16</sup>, d) from ref. <sup>15</sup>, e) published at ref. <sup>3,4,17-19</sup>, f) published at ref.<sup>13</sup> g) a chromatographic evaluation of the sample content was performed for this photosensitizer. The absorption coefficient determination was corrected based on 80 % of sample purity, h) corrected value for 12% of impurity content. The chromatographic evaluation of the other bacteriochlorins was not determined. Errors obtained from the standard error associated to the linear fit ( $\varepsilon_{max}$  determination) or from the average of at least two independent experiments, whichever was higher.

### 2.2.2 Generation of the triplet state

The generation of the triplet state is a requisite in order to proceed in the photosensitization process by a Type I and/or Type II reaction, and produce reactive oxygen species.

We made use of the internal heavy atom effect to accelerate the intersystem-crossing  $S_1 \rightarrow T_1$  rate and consecutively maximize the triplet quantum yield ( $\Phi_T$ ) of our photosensitizers. Arnaut et al.<sup>5</sup> predicted how spin-orbit coupling would affect the photophysical properties of tetraphenyl porphyrins when substituted in the *ortho* positions of the phenyl rings, with halogenated atoms such as F, Cl or Br. The predictions were verified by introducing heavy atoms in the *ortho* phenyl position of porphyrins. The observed increase in the triplet quantum yield was not accompanied by a significant decrease in the triplet lifetime. The triplet quantum yields were measured for some of the difluorinated and dichlorinated photosensitizers, and the values are present on Table 2.2.

The determination of the triplet quantum yield was accessed using the singlet depletion method<sup>20</sup>. A more complete description is given in the material and methods section. The general equation is given as:

$$\Phi_{\rm T}^{\rm S} = \frac{\varepsilon_{\rm T}^{\rm ref}}{\varepsilon_{\rm T}^{\rm S}} \frac{\Delta 0 D^{\rm s}}{\Delta 0 D^{\rm ref}} \frac{(1-10^{\rm -A(\rm ref)})}{(1-10^{\rm -A(\rm s)})} \Phi_{\rm T}^{\rm ref}$$
(2.1)

Where, the superscripts *s* and *ref* denotes sample and reference,  $\varepsilon$  is the molar absorption coefficient, *A* the absorption and  $\Phi_{\rm T}$  the triplet quantum yield ( $\Phi_{\rm T}^{\rm ref} = 1.0$ ).  $\Delta OD$  values correspond to the slopes obtained from the linear dependence of the pre-exponential parameters, calculated by fitting the triplet decays at different laser energies (figure 2.4).



**Figure 2.4** Changes in intensity of triplet-triplet absorption decays extrapolated to t=0 as a function of the laser relative energy, for  $F_2PMet$  (445 nm),  $F_2CMet$  (450 nm),  $Cl_2BOH$  (400 nm) and benzophenone, BP, (545 nm).

A triplet quantum yield close to unity is observed for Cl<sub>2</sub>PMet, while for fluorinated dyes the fraction of singlet states that undergo intersystem crossing is much less. The intersystem crossing is favored for the macrocycles with chlorine atoms since their spin-orbit coupling is higher, than for fluorinated ones. The combination  $\Phi_F + \Phi_T$  gives 0.94 for F<sub>2</sub>CMet showing that virtually all the photons absorbed lead either to fluorescence or to intersystem crossing. For F<sub>2</sub>PMet and F<sub>2</sub>BMet the same combination gives 0.75 and 0.74, respectively. Internal conversion is considered to have a substantial contribution for the singlet state decay in porphyrins and bacteriochlorins.

We observed saturation in the triplet absorption curve at high light intensities for the porphyrin, chlorin and mainly for bacteriochlorins but not for benzophenone (figure 2.4).Different systems exhibit different degrees of non-linearity with the laser energy.<sup>21,22</sup> The relationship between the triplet transient absorption with the laser intensity was explored by Lachish et al.<sup>23</sup> which emphasized that in a monophotonic process a linear dependence would be expected at low light intensities although saturation is expected for higher intensities. Bacteriochlorin's capacity to absorb light is very high, ( $\varepsilon$ >60000 M<sup>-1</sup> cm<sup>-1</sup>) and at the laser intensities that we use in flash photolysis experiments, we easily achieve an unbalance between the number of photons generated at 355nm and the number of molecules presented in solution (absorbance ~0.2).



Similar saturation behaviors have been observed and documented for phtalocyanines<sup>24</sup> and other bacteriochlorins <sup>25</sup>, which similarly have high absorption coefficients.

**Figure 2.5** Triplet state detection in ethanol for Cl<sub>2</sub>PEt (A), FCMet (B), and Cl<sub>2</sub>BEt (C). Left side - timeresolved transient absorption spectra and right side - triplet-state decay profiles in ethanol at 440 nm (A), 400 nm (B e C) in the presence and absence of oxygen.

Flash photolysis was also used to spectroscopically identify the triplet state of the photosensitizers and determine their lifetimes. Figure 2.5 shows the triplet absorption spectra of the porphyrin  $Cl_2PEt$ , the chlorin  $F_2CMet$  and of the bacteriochlorin  $Cl_2BHep$  obtained after excitation at 355 nm in ethanol.

The triplet-triplet absorption spectra for all the analyzed halogenated photosensitizers closely resemble that of Figure 2.5 and that of other related tetraphenyl macrocycles reported in literature.<sup>11,16</sup> Both porphyrins and chlorins show absorption triplet bands around 330 and 450 nm. While bacteriochlorins show three distinct absorption bands at 400, 600 and 790 nm.

The decays at the triplet absorption wavelengths maxima (Figure 2.5, right-side) give triplet lifetimes longer than 28  $\mu$ s in deaerated solutions, for all the tetraphenylmacrocycles studied. Long lived triplet states provide high sensitivity to the photosensitizers concerning the presence of quenching species in the environment. The presence of oxygen reduces significantly the triplet lifetime of porphyrins and more efficiently of bacteriochlorins and chlorins (Table 2.2). Bacteriochlorins and chlorins have their lifetime reduced to about 250 ns and 280 ns, respectively, while porphyrins triplet lifetimes are more sensitive to halogenation. Porphyrins with fluorinated atoms have triplet lifetimes in the 300-500 ns range, about 100 ns higher than chlorins and bacteriochlorins. Dichlorinated porphyrins triplet lifetimes are in the 600-900 ns range.

The quenching rate constant of the triplet excited state by molecular oxygen was estimated from equation 2.2, considering the oxygen concentration in air-saturated ethanol at 20 °C as  $[O_2] = 2.1 \times 10^{-3}$  M.<sup>26</sup>

$$\mathbf{k}_{q} = \left(\frac{1}{\tau_{\mathrm{T}}} - \frac{1}{\tau_{\mathrm{T}}^{0}}\right) \cdot \frac{1}{[0_{2}]}$$
(2.2)

Where,  $\tau_T$  is the triplet lifetime in air saturated ethanol solution and  $\tau_T^{0}$  is the triplet lifetime in the absence of oxygen. Calculated  $k_q$  are presented in Table 2.2. The quenching rate constants measured for bacteriochlorins are generally faster than those of the porphyrins and chlorins analogues.

Spin statistics associated with interaction between one triplet excited state (sensitizer) and another triplet state (as oxygen ground-state) require  $k_q$  to be  $\leq 1/9k_{diff}$  for a process where quenching proceeds via singlet channel. However, if both the singlet and the triplet channels contribute to the deactivation of the triplet excited state by molecular oxygen, then it is expected that  $k_q \leq 4/9k_{diff}$ .

		$\tau_{T}\left(N_{2}\right)$	$\tau_{\rm T} \left( air \right)$	$k_q \times 10^9$	$\Phi_{\mathrm{T}}$	φ <sub>1</sub> <sup>0</sup>	E <sub>T</sub>
		/µs	/ns	$/M^{-1}s^{-1}$			/kcal mol <sup>-1</sup>
	TPP <sup>a</sup>		349	1.6	$0.73 \pm 0.1$		33.0
	mTHPP <sup>c</sup>			1.9	0.69		
	FPMet		309	1.5			
	$\mathbf{F}_{2}\mathbf{P}^{b}$	16.6	493	1.1			
yrins	F <sub>2</sub> PMet <sup>f</sup>		389	1.2	$0.71\pm0.04$		
orph	$Cl_2P^b$	16.5	641	0.87			
Ч	Cl <sub>2</sub> POH	40	855	0.55			
	Cl <sub>2</sub> PEt	38	710	0.66		$0.60\pm0.04$	$33.0 \pm 2$
	Cl <sub>2</sub> PHep	41	758	0.62		$0.56\pm0.06$	$36.1 \pm 5$
	Cl <sub>2</sub> PMet		674		$1.02\pm0.08$		
	mTHPC <sup>c</sup>			1.8	0.89		
s	FCMet <sup>f</sup>	43	285	1.7			
llorin	$F_2C^b$	5	305	1.8			
G	F <sub>2</sub> CMet <sup>f</sup>	50	283	1.7	$0.58 \pm 0.05$		
	$Cl_2C^b$	8.2	393	1.4			
	mTHPB <sup>c</sup>			2.5	0.83		
	FBMet <sup>f</sup>	55	200	2.4			
	F <sub>2</sub> B <sup>e</sup>	33	216	2.6			
	F <sub>2</sub> BMet <sup>f</sup>		226	2.2	$0.65\pm0.05$	$0.45 \pm 0.01$	$26\pm 4$
orins	$\mathbf{F}_2\mathbf{BOH}^{\mathrm{f}}$	61	268	1.8			
iochl	<b>ClBOH</b> <sup>d</sup>	39	246	1.9			
Bacteri	ClBEt <sup>d</sup>	36	228	2.1			
	Cl <sub>2</sub> B <sup>e</sup>	32	254	2.1			
	Cl <sub>2</sub> BOH <sup>f</sup>	33	226	2.1			
	Cl <sub>2</sub> BEt <sup>f</sup>	38	265	1.8		$0.69\pm0.02$	$25.7 \pm 3$
	<b>Cl<sub>2</sub>BHep</b> <sup>f</sup>	28	295	1.6		$0.66\pm0.03$	$27.4\pm4$

**Table 2.2** Triplet states lifetimes, quantum yield and energy measured by flash photolysis and photoacoustic calorimetry in ethanol solutions for halogenated tetraphenyl porphyrins, chlorins and bacteriochlorins.

a) from ref. <sup>12</sup>, b) from ref. <sup>16</sup>, c) from ref. <sup>15</sup>, d) from ref. <sup>27</sup>, e) from ref. <sup>11</sup> f) published at <sup>3,4,17-19</sup>  $\Phi_T$  errors obtained from the standard error associated to the linear fits for  $\Delta$ OD determination or from the error associated to the  $\mathcal{E}_{max}$ , whichever was higher.  $\phi_1^{0}$  and  $\mathcal{E}_T$  errors calculated from the average of two independent experiments. A value of  $k_{diff}$  was estimated from the difference in the singlet lifetimes of FPMet in oxygen- or air-saturated ethanol solutions and in degassed ethanol solutions (Figure 2.6) and the concentration of oxygen in oxygen- and air-saturated ethanol. The average value of the two determinations gives  $k_{diff} = 9.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ . The values of  $k_q$  for porphyrins are  $\leq 1/9k_{diff}$ confirming a singlet deactivation channel by energy transfer to molecular oxygen. Chlorins show a  $k_q$  slightly higher than  $1/9k_{diff}$  and in bacteriochlorins  $1/9k_{diff} \leq k_q \leq 4/9k_{diff}$  which can be related to a process of charge-transfer. The possibility of charge transfer in the deactivation of bacteriochlorins and possibly also of chlorins, would mean the possibility of electron transfer to molecular oxygen, e.g. a deactivation channel that can give rise to a Type I reaction.



**Figure 2.6** Single photon counting of FPMet after excitation at 373 nm in degassed, air-saturated or O<sub>2</sub>-saturated ethanol solutions. Weighted residuals (W.R.), autocorrelation functions (A.C.) and  $\chi^2$  values are also presented. The instrument response function is presented by the dashed line.

Porter and Wilkinson,<sup>28</sup> observed that when the energy of the donor triplet was considerably higher than that of the acceptor, the process would be controlled by diffusion. As the triplet energies became comparable, the energy transfer efficiency decreases and no quenching by molecules with triplet levels higher than that of the donor were observed.

The ability of oxygen to quench the triplet excited state of porphyrin derivatives is obvious from the substantial reduction on the triplet lifetime in the presence of  $O_2$ . In order to have an efficient quenching of the triplet excited states of the photosensitizer by molecular oxygen, the

reaction has to be energetically favorable. Additionally, the efficiency of energy transfer from a triplet sensitizer to molecular oxygen is also dependent of spin statistics.

The triplet state energies of various photosensitizers were determined by photoacoustic calorimetry (PAC) and the values obtained are presented on Table 2.2.



**Figure 2.7** Jablonski representative diagram of the processes involved in the generation and removal of the triplet excited state of a photosensitizer. Nonradiative processes involved in the photoacoustic wave generation, are represented by dashed arrows. Radiative processes are represented by full arrows.

When a system of absorbing molecules is exposed to an appropriate exciting light, a population of electronically excited states is produced. Deactivation of these excited states can occur by a radiative or a nonradiative process. The last one is a source of heat and the only one that contributes to the photoacoustic effect. Structural volume changes may also contribute to the PAC signal but they are not relevant in our systems.

The processes that contribute to the generation of photoacoustic waves are represented in Figure 2.7 by dashed arrows. The first process ( $\phi_1$ ) measured by PAC is the decay of the <sup>1</sup>S state and the formation of the triplet state of the sensitizer (<sup>3</sup>S) and the second process ( $\phi_2$ ) is the decay of the triplet state. These processes are translated into two-sequential decays, each one described by a lifetime ( $\tau_i$ ) and a fraction of energy released in that lifetime ( $\phi_i$ ). The first exponential corresponds to the formation of the triplet state in the lifetime ( $\tau_1$ ) and by the fraction of energy released in this lifetime ( $\phi_1$ ). The lifetime  $\tau_1$  is very short and is out of the temporal profile of the transducer (2.25 MHz). In the deconvolution process we set  $\tau_1$  to 1 ns.

The second process is the decay of the triplet state. In the absence of quenchers, this is a long process and will not be detected in the time window of the PAC experiment with megahertz transducers. The energy balance between the absorbed energy (experimentally measured using a photoacoustic reference) and the heat decay associated with  $\phi_1$ , gives the energy stored in the triplet state ( $\Phi_T E_T$ ). Deconvolution of the PAC signal obtained with the reference and with the sample gives  $\phi_1$  and  $\phi_2$  when a two-sequential exponential model is employed.



**Figure 2.8** Photoacoustic waves generated at 355 nm in ethanol: for  $Cl_2PHep$  (solid), for the reference MnTPP (dash) and residuals (dots). Photoacoustic waves normalized and subtracted from the solvent wave. The calculated wave is superimposed with the sample wave and is indistinguishable.

An indistinguishable overlap between experimental and calculated wave is observed for all the photosensitizers analyzed (Figure 2.8), and the residuals confirm the adequacy of the fitting model. An algorithm described by Melton et al.<sup>29</sup> was used to deconvolute the values of  $\phi_1$ . To avoid the possible contribution of biphotonic processes, the fraction of energy released  $\phi_1$ , was measured as a function of power intensity (Figure 2.9), and extrapolated to zero laser energy.

The energy balance for the kinetic scheme, presented in Figure 2.7, in the absence of oxygen can be translated into the following equation.

$$\mathbf{E}_{\mathrm{T}} \Phi_{\mathrm{T}} = (1 - \phi_{1}) \mathbf{E}_{\mathrm{hv}} - \Phi_{\mathrm{F}} \mathbf{E}_{\mathrm{S}}$$

$$(2.3)$$

Where,  $E_T$  is the triplet energy,  $\Phi_T$  is the triplet quantum yield,  $\phi_1$  is the fraction of laser energy released in the formation of the triplet state,  $E_{hv}$  is the laser energy at 355 nm,  $\Phi_F$  the fluorescence quantum yield and  $E_S$  is the energy of the singlet state. We have analyzed dichlorinated porphyrins and bacteriochlorins by photoacoustics. The triplet state energy is given by equation 2.3. The values obtained are registered on Table 2.2 and show that bacteriochlorins triplet states are about 10 kcal mol<sup>-1</sup> lower in energy than porphyrins. The production of  ${}^{1}O_{2}$  occurs via energy transfer from the sensitizer triplet state to molecular oxygen and requires a minimum of 22.5 kcal mol<sup>-1</sup>, the energy of the first electronic excited state of molecular oxygen ( ${}^{1}\Delta_{g}$ ). Both porphyrins and bacteriochlorins triplet energies exceed this limit and can thus generate singlet oxygen irreversibly by energy transfer.



**Figure 2.9** Fraction of energy released as a function of the laser relative intensity for  $Cl_2PHep$ . Full points correspond to experimental values, and open point corresponds to the extrapolated value for a zero value of energy.

## 2.2.3 Singlet oxygen generation in solution

The most common path for the generation of singlet oxygen is photosensitization or, more precisely, quenching of the excited state of the photosensitizer by molecular oxygen. Singlet oxygen photosensitization can occur by energy-transfer quenching of both excited singlet and triplet states. The singlet excited states lifetime are short lived for chlorinated and difluorinated related dyes (~0.5 ns and 3-9 ns, respectively),<sup>13</sup> so quenching by molecular oxygen is most certainly negligible. FPMet has however, a singlet excited state relatively more long lived (11 ns) and some quenching by molecular oxygen can be possible. Phosphorescence emission was found at 1270 nm for all the photosensitizers. This is characteristic of the transition from the first excited singlet state ( ${}^{1}\Delta_{g}$ ) of oxygen to the ground triplet state ( ${}^{3}\Sigma_{g}$ ). Typical lifetimes for the deactivation of singlet oxygen in ethanol, ~14 µs,<sup>30</sup> were found (Figure 2.10). Interestingly, for bacteriochlorins and fluorinated porphyrins the rate constants obtained from fitting the rise on the singlet oxygen generation traces gives lifetimes larger than the ones predicted by the triplet decay in aerated solutions (Table 2.3).

The amplitudes obtained from the monoexponential fit of the decays at 1270 nm extrapolated for t=0 at different laser energies are plotted on Figure 2.11. Singlet oxygen quantum yields were determined, from the ratio of the slopes of the reference ( $m_{ref}$ ) and of the sensitizer ( $m_s$ ), obtained at low laser energies where the linearity is kept, according to,

$$\Phi_{\Delta}^{\rm s} = \frac{m_{\rm s}}{m_{\rm ref}} \Phi_{\Delta}^{\rm ref}$$
(2.4)

The curvature observed from the plot  $I_{\Delta}^{0}$  versus the relative laser energy may reflect the singlet ground state depletion as the laser energy is increased, and as it was also evident from the triplet state absorption data (Figure 2.4).

Porphyrins systematically show higher values of singlet oxygen quantum yield than chlorins and bacteriochlorins. Moreover, the chlorinated dyes yield higher amounts of singlet oxygen in ethanol than the fluorinated ones.

Comparing porphyrins, chlorins and bacteriochlorins, we found that, as the triplet state energy increases, the rate constant of oxygen quenching decreases and singlet oxygen quantum yield increases. The generation of a charge transfer (CT) state  $({}^{3}S^{\delta+}...O_{2}{}^{\delta-})$  from quenching of the triplet excited state of a sensitizer by oxygen can accelerate the rate of triplet quenching by oxygen and reduce the singlet oxygen quantum yield.<sup>31-33</sup> Bacteriochlorins and chlorins have the lowest singlet oxygen quantum yields and also the higher quenching rate constants, suggesting the existence of a charge transfer channel for these photosensitizers. For molecules with

tendency to undergo oxidation, CT interactions with  $O_2$  can strongly influence the rate and efficiency of singlet oxygen formation. McGarvey et al.<sup>34</sup> found that an increase in the  $E_{ox}$  results in a systematic increase on the efficiency of singlet oxygen formation and in the triplet quenching rate constant ( $k_q$ ).



**Figure 2.10** Singlet oxygen 1270 nm phosphorescence traces recorded in ethanol from  $F_2POH$  (left panel) and  $Cl_2BEt$  (right panel). The inset shows with more detail the rise on singlet oxygen generation. Singlet oxygen traces were obtained after excitation at 420 nm ( $F_2POH$ ) and 750 nm ( $Cl_2BEt$ ) with a femtosecond laser with 1 kHz of repetition rate.



Figure 2.11 Singlet oxygen phosphorescence intensity extrapolated to t = 0 in ethanol, as a function of the laser pulse energy.

In order to make a better and more reliable judgment of a possible charge transfer mechanism we compared the oxidation potentials for a number of photosensitizers studied in this work, which are presented in Table 2.4.

		$\overline{\tau_{risetime}}^{f}$	$\tau_{decay}$	$\Phi_{\Delta}{}^{g}$
		/μs	/µs	
	TPP <sup>a</sup>			0.71
	mTHPP <sup>c</sup>			0.46
	<b>FPMet</b> <sup>e</sup>	$0.62 \pm 0.06$ <sup>3</sup>		$0.64\pm0.07$
	$\mathbf{F}_{2}\mathbf{P}^{b}$			$0.84\pm0.04$
ins	F <sub>2</sub> POH	$0.87 \pm 0.02^{3}$		$0.71\pm0.09$
hyri	F <sub>2</sub> PMet <sup>e</sup>	$0.60 \pm 0.01^3$		$0.71\pm0.07$
Porp	ClPEt			$0.68\pm0.08$
	$Cl_2P^b$			$0.98\pm0.04$
	Cl <sub>2</sub> POH <sup>e</sup>	$0.81 \pm 0.01 \ ^1$	14.6	$0.98\pm0.03$
	Cl <sub>2</sub> PEt	$0.74 \pm 0.01 \ ^1\!/1.2 \pm 0.02^3$	14.7	$0.85\pm0.10$
	Cl <sub>2</sub> PHep <sup>e</sup>	$0.84 \pm 0.01 \ ^1$	14.5	$0.85 \pm 0.04$
	mTHPC <sup>c</sup>			0.43
IS	FCMet <sup>e</sup>			$0.58 \pm 0.04$
lorir	$F_2C^b$			$0.88 \pm 0.04$
Ch	F <sub>2</sub> CMet <sup>e</sup>	$0.41 \pm 0.01^{3}$		$0.54 \pm 0.04$
	$Cl_2C^b$			$0.93 \pm 0.05$
	mTHPB <sup>c</sup>			0.43
	<b>FBMet</b> <sup>e</sup>	$1.00 \pm 0.02^{3}$	14.6	$0.63\pm0.05$
	$\mathbf{F}_2 \mathbf{B}^{\mathbf{d}}$			0.48
rins	F <sub>2</sub> BOH <sup>e</sup>	$0.95 \pm 0.01^3$	14.6	$0.44\pm0.08$
chlo	F <sub>2</sub> BMet <sup>e</sup>	$0.98 \pm 0.01^3$	14.6	$0.43\pm0.06$
erioo	ClBEt	$0.78 \pm 0.01^3$	14.7	
Bact	Cl <sub>2</sub> B <sup>d</sup>			0.60
	Cl <sub>2</sub> BOH <sup>e</sup>	$0.22 \pm 0.02/0.49 \pm 0.01^3$	14.8	$0.85\pm0.11$
	Cl <sub>2</sub> BEt <sup>e</sup>	$0.69 \pm 0.01^3$	14.9	$0.66 \pm 0.10$
	Cl <sub>2</sub> BHep <sup>e</sup>	$0.33\pm0.02^2$	14.3	$0.63 \pm 0.12$

 Table 2.3 Singlet oxygen rise time, decay, and quantum yield generated by halogenated tetraphenyl porphyrins and bacteriochlorins in ethanol, and of related compounds.

a) From ref.<sup>2</sup>, b) from ref.<sup>16</sup>, c) from ref.<sup>15</sup>, d) from ref.<sup>11</sup>, e) published at<sup>3,17-19</sup> f) errors obtained from the standard error associated to the rise fit. Rise traces presented in Appendix I. Rise times obtained after excitation solutions of the photosensitizers in ethanol with: 1- nanosecond laser ( $\lambda_{exc}$ =355 nm), 2-picosecond laser ( $\lambda_{exc}$ =355 nm), 3-femtosecond laser ( $\lambda_{exc}$ =420 nm for porphyrins and  $\lambda_{exc}$ =748 nm for bacteriochlorins). g) Standard errors obtained from the average of at least 2 experiments or from the linear fits to obtain m<sub>s</sub>.

Compared to porphyrins and chlorins oxidation potentials found for bacteriochlorins are lower than those of chlorins, and these are lower than the oxidation potentials of porphyrins. Two or three irreversible oxidations were found. The third one, at less positive potentials, is related to the oxidation of the -NHSO<sub>3</sub>R group. Photosensitizers that have an electron withdrawing group in the phenyl ring show an increase in the first oxidation potential, from +1.23 to +1.38 for Cl<sub>2</sub>P and Cl<sub>2</sub>Pet respectively. In bacteriochlorins, this is observed from +0.65, to +0.80 and to +0.82 for F<sub>2</sub>B, F<sub>2</sub>BMet and Cl<sub>2</sub>BEt, respectively. Bacteriochlorins, F<sub>2</sub>BMet and Cl<sub>2</sub>BEt, have only two oxidations because the oxidation of the sulphonic group ( $E_{ox}^{0}$ ) is probably overlapped with the first oxidation of the macrocycle ( $E_{oxl}^{0}$ ).

Table	2.4	Oxidation	potentials,	vs	SCE,	of	some	studied	photosensitizers	and	of	other	selected
macroo	cycle	s. Cyclic vo	ltammograi	ms a	and red	ucti	on pote	entials ar	e presented in the	Appe	endi	x I.	

		$E_{\rm ox}^{0}$ /V	$E_{\text{ox1}}^{0}$ /V	$E_{\text{ox2}}^{0}$ /V	E <sub>CT</sub> /kcalmol <sup>-1</sup>	ΔG <sub>CT</sub> /kcalmol <sup>-1</sup>
	<b>TPP</b> <sup>a</sup>		0.95	1.32		
	$\mathbf{F}_{2}\mathbf{P}^{b}$		1.23	1.51		
phyrins	СІРОН	0.70	1.28	1.88		
Porj	$Cl_2P^b$		1.23	1.52		
	Cl <sub>2</sub> PEt	0.85	1.38	1.60	33	8.6
orins	<b>TPC</b> <sup>a</sup>		0.88			
Chlo	Cl <sub>2</sub> CEt	0.85	1.36	1.59		
	<b>TPB</b> <sup>a</sup>		0.40			
Bacteriochlorins	$\mathbf{F}_{2}\mathbf{B}^{\mathrm{b}}$		0.65	1.24		
	F <sub>2</sub> BOH	0.55	0.70	1.11	25.9	
	F <sub>2</sub> BMet		0.80	1.18	28.2	0.05
	Cl <sub>2</sub> BEt		0.82	1.24	28.7	0.01

a) oxidation potentials in dichloromethane vs. SCE, from ref.,<sup>8</sup> b) oxidation potentials in benzonitrile, from ref.<sup>35</sup>

The energy of the full electron transfer from the photosensitizer to molecular oxygen is given by:

$$\Delta G_{CT} = E_{ox}^* - E_{red}^{O_2} \tag{2.5}$$

Where,  $E_{ox}^{*}$  is the triplet state oxidation potential and  $E_{red}^{O_2}$  the half-wave reduction potential of oxygen ( $E_{red}^{O_2} = -0.425$  V vs. SCE in hydrogen-bonding solvents).<sup>36</sup>  $E_{ox}^{*}$  is given by: $E_{ox}^{*} = E_{ox1}^{D} - E_{T}$ . Table 2.4 shows the calculated values for  $\Delta G_{CT}$ . Bacteriochlorins full electron transfer to molecular oxygen is nearly isothermic. A negative  $\Delta G_{CT}$  is a thermodynamically favorable electron transfer from the photosensitizer to oxygen, with the generation of radical species, such as superoxide ion. The energy for a radical ion pair formation for porphyrins is higher and so the endothermicity of these reactions makes it less probable to happen. These observations are consistent with the quenching of the triplet excited states by oxygen for porphyrins which follows essentially the energy transfer channel. On the other hand, the quenching of the bacteriochlorins triplet state is consistent with the involvement of charge transfer interactions in the quenching process.

Assuming the existence of a charge transfer state, we can use PAC, to estimate the quantum yield of species resulted from electron transfer ( $\Phi_{CT}$ ) between the triplet excited state of the photosensitizer and molecular oxygen following the energy balance on figure 2.7.<sup>12</sup>

$$(\phi_1 + \phi_2)E_{hv} = E_{hv} - \Phi_F E_S - \Phi_\Delta E_\Delta - \Phi_{CT} E_{CT}$$
(2.6)

Where, CT refers to full electron transfer with formation of free ions. The energy of radical ion pair that gives rise by charge separation to free ion can be estimated from the free energy of the charge separated state and is around ~28 kcal mol<sup>-1</sup> for the bacteriochlorins analyzed (Table 2.4). The triplet quantum yield of Cl<sub>2</sub>BEt was not determined, but a value close to unity would be expected considering the enhancement on the intersystem crossing provided by the heavy atom effect. All the other parameters have been described previously in this chapter. The sum of the quantum yields,  $\Phi_{\text{ET}}$  and  $\Phi_{\Delta}$  for F<sub>2</sub>BMet and Cl<sub>2</sub>BEt, is consistent with the triplet quantum yield measured for the first and expected for the last one.

 Table 2.5 Free ions quantum yield in ethanol.

	$\phi_1^{0}$	$\phi_2^{0a}$	$\Phi_{CT}^{b}$
F <sub>2</sub> BMet	$0.45\pm0.01$	$0.09\pm0.04$	$0.16\pm0.03$
Cl <sub>2</sub> BEt	$0.69\pm0.02$	$0.03\pm0.02$	$0.26{\pm}\ 0.04$

a) Error obtained by the average of two experiments, b) Estimated error of 15 %.

As the photosensitizer oxidation potential decreases and the CT process becomes more competitive against non-CT process,  $k_q$  increases and  $\Phi_{\Delta}$  decreases. In the absence of a charge transfer process it would be expected, as it was previously discussed, that the quenching rate for the triplet excited quenching to be  $1/9k_{diff}$ . In such conditions, the generation of singlet oxygen should be equal to unity ( $\Phi_{\Delta} = 1$ ), in a purely energy transfer process. When a CT process is also involved, the quenching rate should be higher,  $4/9k_{diff}$ , and singlet oxygen quantum yield should be reduced to 0.25, since a new CT channel is possible leading back to ground state oxygen or to radical ions.



Figure 2.12 Quenching of triplet excited states of a photosensitizer by molecular oxygen.

The mechanism in Figure 2.12, expanded from Wilkinson's mechanism presented in the Introduction, compiles most of the possibilities described in literature for quenching of the triplet states of an organic molecule by molecular oxygen.

Porphyrins and chlorins generate essentially singlet oxygen by quenching of their triplet states. Although, the quenching rate constants of their triplet states by molecular oxygen can differ significantly, where porphyrins  $k_q \le 1/9k_{diff}$  and chlorins $1/9k_{diff} > k_q < 4/9k_{diff}$ , their singlet oxygen efficiency (defined as:  $S_{\Delta} = \Phi_{\Delta}/\Phi_{T}$ ) is close to 1 and there is no evidence for a charge transfer complex. Thus, the direct channel for generation of singlet oxygen by energy transfer, with a rate constant of  $k_{\Delta}$ , dominates the triplet decay.

Bacteriochlorins, on the other hand, show  $1/9k_{diff} < k_q < 4/9k_{diff}$ , higher than chlorins, and only part of the triplet excited states that are quenched by molecular oxygen lead to singlet oxygen ( $S_{\Delta}$ ~0.7 for F<sub>2</sub>BMet), this relation between k<sub>q</sub> and  $S_{\Delta}$  has been considered as an evidence for charge transfer in the quenching mechanism.<sup>37</sup>

Energy transfer in the encounter complex formed in the singlet pathway has been interpreted as an internal conversion of  ${}^{1}({}^{3}S^{*...}O_{2}{}^{3}\Sigma_{g}^{-})$  into  ${}^{1}(S^{...}O_{2}{}^{1}\Delta_{g})$ , following an early suggestion by Kearns and co-workers.<sup>38</sup> This requires that the encounter complex decay rate constant should follow the energy-gap law and become slower as the triplet energy of the sensitizer increases,  $\Delta E = E_{\Delta} - E_{T}$ . Figure 2.13 compares the energy transfer rates from the triplet states of our dichlorinated porphyrins with those photosensitizers reported by Bodeisheim and Schmidt.<sup>39</sup> The rate constants for the porphyrin triplets have reached the maximum possible value of the nCT channel,  $1/9k_{diff}$ .



**Figure 2.13** Energy gap law for triplets of TPP, ketones, quinones and other aromatic molecules measured in  $CCl_4$  (**n**) and for the halogenated porphyrins in ethanol ( $\circ$ ). Energy of 35 kcal mol<sup>-1</sup> was considered for the porphyrins which triplet energy was not measured experimentally. Dotted line corresponds to  $1/9k_{diff}$  for ethanol.

Charge-transfer induced quenching of triplet states is clearly observed in the quenching of substituted naphthalene triplet states by molecular oxygen in acetonitrile, Figure 2.14.<sup>37</sup> The quenching of triplet bacteriochlorins also follows the inverse correlation between the rates and

efficiencies of singlet oxygen generation observed for the naphthalene sensitizers when quenching proceeds through the CT channel.



**Figure 2.14** Dependence of the rate constants for quenching of the triplet excited state by oxygen,  $k_q$  ( $\circ$  - bacteriochlorins Cl<sub>2</sub>BEt, F<sub>2</sub>BMet, and  $\bullet$  - naphthalene derivatives), and the quantum yield of singlet oxygen,  $\Phi_{\Delta}$  ( $\Box$  - Cl<sub>2</sub>BEt, F<sub>2</sub>BMet and  $\blacksquare$  - naphthalene derivatives), on the free energy change  $\Delta G_{CT}$  for electron transfer. Dotted line corresponds to 4/9k<sub>diff</sub> in ethanol.

## 2.2.4 Generation of radical species in solution

Spin trapping is often used for the detection and characterization of transient radicals. <sup>40,41</sup> A spin trap (SP) serves as an efficient scavenger of reactive free radicals to produce a more stable adduct and facilitate the measurement of electron paramagnetic resonance (EPR) spectra.

We have made use of two spin traps in this work, DMPO (5,5-dimethyl-1-pyrroline-N-oxide) and BMPO (5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide), to detect radicals generated by bacteriochlorins.



Figure 2.15 Structures of DMPO (A) and BMPO (B).

Irradiation of  $Cl_2BHep$  in DMSO and in the presence of DMPO for 10 s with a diode laser resulted in the spectrum presented in Figure 2.16. Similar spectra were recorded for  $Cl_2BEt$ ,  $Cl_2BOH$  and  $Cl_2BMet_2$  under the same experimental conditions (Appendix I). From computer simulation of the line shape and the hyperfine (hf) splitting of the signal, these EPR spectra were assigned to the adduct DMPO-OOH (Table 2.6). The EPR spectrum of F<sub>2</sub>BMet after irradiation in these conditions resulted in a mixture of DMPO-OOH and a carbon-centered localized radical adduct (Figure 2.16 B). When EPR spectra are obtained after illumination of the sensitizers in solution of Triton X-100 and in the presence of DMPO the formation of a spin adduct between this spin trap and hydroxyl radical (DMPO-OH) is observed, Figure 2.17. The same adduct was detected for  $Cl_2BOH$  when irradiated in PBS and in the presence of BMPO (Appendix I).<sup>18</sup> The hyperfine constant values for the hydroxyl radical adducts are presented on Table 2.6. When BMPO spin trap was used, in combination with  $Cl_2BOH$  and in PBS, irradiation induced the generation of superoxide ions which were trapped and detected as adduct BMPO-OOH (Table 2.6).

We were unable to detect superoxide radical from the direct  $O_2^{\bullet}$  trapping with DMPO under diode laser irradiation in TritonX-100, the adduct DMPO-OH was observed instead (Figure 2.17). There are at least two possible explanations for this observation:  $O_2^{\bullet}$  may act as a precursor of 'OH or the DMPO/BMPO-OOH adduct may be formed, but cannot be observed due to its lifetime being too short. <sup>42</sup>



**Figure 2.16** A) EPR spectrum of DMPO-OOH observed during illumination of  $Cl_2BHep$  (A) and  $F_2BMet$  (B) in DMSO solution with DMPO. Top (A, B) - after 10 sec of irradiation at 748 nm, Middle (A) and bottom (B) - simulation of EPR spectra, Bottom (A) - in the presence of superoxide dismutase (SOD) and after 10 sec of irradiation.<sup>34</sup>

To confirm that the superoxide ion is at the origin of the observed signal, superoxide dismutase, a known scavenger of the superoxide ion, and which products are oxygen and hydrogen peroxide, was added to the first system in which DMSO was used as solvent, prior to irradiation. The generation of the radical adduct DMPO-OOH was inhibited, meaning that superoxide dismutase is an efficient scavenger of  $O_2^{\bullet}$  and confirms that superoxide ion is, responsible for the observation of the DMPO-OOH EPR spectrum for each of the photosensitizers.

The reaction of  $OH^{\bullet}$  with the solvent DMSO can generate the radical adduct DMPO-CH<sub>3</sub>. This carbon-centered methyl radical, forms a more stable adduct than the  $OH^{\bullet}$  itself.<sup>43</sup> (Appendix I), and could be responsible for the mixed EPR spectrum observed for F<sub>2</sub>BMet.

Catalase splits hydrogen peroxide into water and molecular oxygen. Irradiation of sensitizer solution in TritonX-100 or PBS where catalase is also present inhibited the generation of DMPO-OH adduct. This observation reflects the importance of hydrogen peroxide in the mechanism to generate hydroxyl radical.

In other experimental conditions such as in the presence of catalase, absence of light or when the solution was saturated with nitrogen, no EPR signal was detected.



**Figure 2.17** A) EPR spectrum recorded (top) and simulated (bottom) of  $Cl_2BOH$  (50  $\mu$ M) in PBS and in the presence of BMPO (50 mM). B) EPR spectrum observed from a solution of  $F_2BMet$  (50  $\mu$ M) in TRTX-100 and in the presence of DMPO (50 mM). Bottom – Simulation.

	Spin adduct	Hyperfine coupling constant				
		$\mathbf{a}_{\mathbf{N}}$	$\mathbf{a}_{\mathrm{H}\beta}$	<b>a</b> <sub>HY</sub>		
<b>Cl<sub>2</sub>BOH</b> <sup>a</sup>	BMPO-OH	14.2	15.8			
	DMPO-OOH	13.3	10.6	1.34		
Cl <sub>2</sub> BEt	DMPO-OH	14.9	14.7			
	DMPO-OOH	13.5	8.37	1.29		
Cl <sub>2</sub> BHep	DMPO-OH	14.9	14.7			
	DMPO-OOH	13.5	8.37	1.35		
Cl <sub>2</sub> BMet <sub>2</sub>	DMPO-OH	14.9	14.6			
	DMPO-OOH	13.6	8.40	1.27		
F <sub>2</sub> BMet	DMPO-OH	14.9	14.6			
	DMPO-OOH/DMPO-CH <sub>3</sub>	14.9	21.3	1.1		

 Table 2.6 EPR hyperfine coupling constants for hydroxyl radical and superoxide radical adducts.

a) Published at $^{18}$ .

The photogeneration of superoxide ion by bacteriochlorins has been already reported <sup>44,45</sup> and its subsequent reactions to produce hydrogen peroxide in hydroxylic solvents can be mechanistically described as follows:

$${}^{3}S + O_{2} \rightarrow S(H)^{+\bullet} + O_{2}^{\bullet}$$

$$(2.7)$$

$$S(H)^{+\bullet} + O_2^{\bullet-} \rightarrow S(H)^{\bullet} + HO_2^{\bullet}$$
 (2.8)

$$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2 \tag{2.9}$$

$$\mathrm{HO}_{2}^{\bullet} + \mathrm{HO}_{2}^{\bullet} \to \mathrm{O}_{2} + \mathrm{H}_{2}\mathrm{O}_{2} \tag{2.10}$$

$$HO_2^{\bullet} + O_2^{\bullet} + H^+ \to O_2 + H_2O_2$$
 (2.11)

The rate of superoxide disproportionation at pH 7.4 is reported as  $2.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  (equation 2.9) <sup>46</sup> and the disproportionation rate of the perhydroxyl radical is  $8.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  (equation 2.10) <sup>47</sup>. The oxidation of superoxide by the perhydroxyl radical reflects the fact that HO<sub>2</sub>• is the more potent oxidant. The sensitizer radical cation is indicated as the proton source in 2.8 but the proton may also come from the solvent. When the proton source is the sensitizer, reactions 2.7 and 2.8 may be concerted in a single proton coupled electron transfer step.

The subsequent formation of the hydroxyl radical may proceed directly by equations 2.12 and 2.13, but their rate constants, 16 and 3.7  $M^{-1}$  s<sup>-1</sup> in water, are much slower than the relatively rapid dismutation of the HO<sub>2</sub><sup>•</sup>/O<sub>2</sub><sup>•-</sup> radicals<sup>48</sup>.

$$O_2^{\bullet} + H_2 O_2 \rightarrow O_2 + OH^{\bullet} + OH^{\bullet}$$
(2.12)

$$HO_2^{\bullet} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + H_2O$$
(2.13)

The Haber-Weiss reaction (equation 2.12) has been observed in gas phase<sup>49</sup> but is still remains controversial in water. An alternative path for bacteriochlorins that may also lead to hydroxyl radical *via* photocatalysis can be described by the following reaction:

$${}^{3}\text{Cl}_{2}\text{BOH} + \text{H}_{2}\text{O}_{2} \longrightarrow \text{Cl}_{2}\text{BOH}^{+\bullet} + \text{H}_{2}\text{O}_{2}^{\bullet-} \longrightarrow \text{Cl}_{2}\text{BO}_{3}^{\bullet} + \text{OH}^{\bullet} + \text{H}_{2}\text{O}$$
(2.14)

In view of the facile electron transfer from  ${}^{3}Cl_{2}BOH$  discussed above we propose the generation of hydroxyl radical through photocatalysis, although hydrogen peroxide is a worse electron acceptor than molecular oxygen. It has been suggested that the transient specie  $H_{2}O_{2}^{\bullet-}$  is accessed as a Franck-Condon transition in a dissociative electron attachment to  $H_{2}O_{2}$  that produces OH<sup>•</sup> and HO<sup>-.50</sup> Given the very weakly exothermic electron transfer between the triplet excited state of Cl<sub>2</sub>BOH and O<sub>2</sub>, we expect electron transfer to H<sub>2</sub>O<sub>2</sub> to be endothermic and slow. Nevertheless, the relatively long  $\tau_{T}$  of Cl<sub>2</sub>BOH in water gives us the opportunity to investigate photoinduced electron transfer to H<sub>2</sub>O<sub>2</sub>. Before addressing the excited state reaction with H<sub>2</sub>O<sub>2</sub>, it is important to note that aqueous solutions of Cl<sub>2</sub>BOH are remarkably stable in the dark even in the presence of H<sub>2</sub>O<sub>2</sub>. We measured the triplet state lifetimes of Cl<sub>2</sub>BOH in N<sub>2</sub>saturated PBS solutions with various amounts of H<sub>2</sub>O<sub>2</sub>. Figure 2.18 shows the reciprocals of the triplet lifetimes as a function of [H<sub>2</sub>O<sub>2</sub>], from which we estimate the rate constant for electron transfer to H<sub>2</sub>O<sub>2</sub>, k<sub>H2O2</sub>=3x10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup>. This is much higher than the rate constants of reactions 2.12 and 2.13 and makes of photocatalysis the preferred mechanism for hydroxyl radical generation by bacteriochlorins. This rate should correspond to an outer-sphere electron transfer reaction that is 5 kcal mol<sup>-1</sup> endothermic,<sup>51</sup> as expected from the low electron affinity of H<sub>2</sub>O<sub>2</sub>. It must be emphasized that in aerated solutions electron transfer to H<sub>2</sub>O<sub>2</sub> competes with quenching by O<sub>2</sub>, which occurs with rate constant of <sup>4</sup>/<sub>9</sub>k<sub>diff</sub>. The production of hydroxyl radical only becomes effective with the depletion of O<sub>2</sub> from the solution and concomitant accumulation of H<sub>2</sub>O<sub>2</sub>.



**Figure 2.18** Decays of triplet  $Cl_2BOH$  absorption at 790 nm in  $N_2$ -saturated PBS solutions with the following concentrations of  $H_2O_2$ : 0, 1 and 4 mM. The inset shows the  $H_2O_2$  concentration dependence of the reciprocal of the triplet lifetimes.

The generation of hydroxyl radical is highly favorable in cells according to the Haber-Weiss/Fenton reaction which consists of an iron reduction step by  $O_2^{-1}$  (equation 2.15) and an OH<sup>-</sup> generation step through the Fenton reaction <sup>52</sup> (equation 2.16).

The reduction of the iron metal can be accomplished by superoxide, but also by biological reductants such as ascorbate and glutathione.<sup>53-55</sup> Also, iron is the most likely but not the only biological metal able to catalyze formation of hydroxyl radicals.

$$\operatorname{Fe}^{3+} + \operatorname{O}_2^{\bullet^-} \to \operatorname{Fe}^{2+} + \operatorname{O}_2$$
 (2.15)

$$\operatorname{Fe}^{2+} + \operatorname{H}_2\operatorname{O}_2 \rightarrow \operatorname{Fe}^{3+} + \operatorname{OH}^- + {}^{\bullet}\operatorname{OH}$$
 (2.16)

Hydroxyl radical is a highly reactive oxidant with a lifetime of about 2 ns in aqueous solution and a diffusion radius of 20 Å.<sup>56</sup> Thus, after generation it will induce damage only to targets in its close proximity. In a system where superoxide radical and hydrogen peroxide are generated, the reaction of Haber-Weiss/Fenton is more favorable to happen and can result in a clear advantage for enhancing photodynamic therapy.<sup>57</sup>

# **2.3 Conclusions**

We found distinct differences between the photochemistry of the studied halogenated tetraphenyl porphyrins, chlorins and bacteriochlorins in ethanol solutions. Reduction of the macrocycle results in a remarkable increase in the absorption along the series. Halogenated porphyrins and bacteriochlorins show low fluorescence quantum yields but highly efficient intersystem crossing to generate very long lived excited triplet states.

Bacteriochlorins are particularly interesting for photodynamic therapy because of their strong absorption in the phototherapeutic window, long lived triplet states and high quantum yield of ROS.

Quenching of the triplet excited states of porphyrins by molecular oxygen was found to follow essentially an energy transfer path that leads to the quantitative generation of singlet oxygen. Bacteriochlorin excited triplet states, on the other hand, are quenched with a quenching rate constant that is  $>1/9k_{diff}$  and  $\Phi_{\Delta}$  tends to be lower than of related porphyrin.<sup>18</sup> This reciprocal combination is regarded as an attribute of charge transfer processes. Singlet oxygen is the main reactive oxygen species generated by these dyes, but a significant quantum yield of ~0.2 was determined for the generation of other reactive oxygen species by electron transfer. Superoxide ion and hydroxyl radicals were found as the result of full electron transfer in hydroxylic solvents. Hydroxyl radicals are highly toxic to cells and might enhance the efficacy of PDT.<sup>27,58</sup>

A photocatalytic pathway for the generation of hydroxyl radical was found for bacteriochlorins.<sup>18</sup> In biological conditions where oxygen depletion after PDT occurs very quickly in the tissue, accumulation of  $H_2O_2$  would favor the generation of hydroxyl radicals. We speculate that generation of this ROS may be further enhanced by using ferrous iron as an adjuvant to PDT.

Chlorins, FCMet and F<sub>2</sub>CMet, have strong absorption in the infrared and show fluorescence intensities about 2.7 times higher than that of Foscan.<sup>15</sup> Moreover, they combine high fluorescence quantum yields and,  $\Phi_F + \Phi_T$  approaches unity. Considering that they have  $\Phi_{\Delta} > 0.5$  and  $\Phi_F \approx 0.4$ , these dyes may exert both functions of a photosensitizer in PDT and fluorophore in fluorescence imaging, and be explored as templates for theranostics.<sup>17</sup>

Porphyrins have high yield of singlet oxygen, which makes them interesting to study as singlet oxygen generators in cells.

# 2.4 Materials and methods

### 2.4.1 Materials

DMPO, BMPO, phenalenone, methanol, Triton X-100, dimethyl sulfoxide, hydrogen peroxide, and ethanol p.a. were obtained from Sigma-Aldrich. MnTPP, TPP and Cl<sub>2</sub>B were kindly offered by the synthesis group of Prof. Mariette Pereira.

### 2.4.2 Methods

#### Determination of absorption coefficients

Absorption spectra were recorded at room temperature with a Shimadzu UV-2100 spectrophotometer. Absorption coefficients were determined for photosensitizer samples with absorbance values below 1. We found no evidence of sensitizer aggregation at these concentrations. Solutions were prepared in ethanol by either dilution of a stock solution or by weight each point for the desired concentrations.

#### Fluorescence quantum yields

Fluorescence spectra were recorded with a SPEX Fluorolog 3.22 spectrophotometer. The fluorescence yields were determined by matching the absorption of both sample and reference at 0.2 at the excitation wavelength, and then the solutions were diluted by a factor of 10 before collecting the fluorescence. The fluorescence quantum yields were obtained from the ratio of the fluorescence bands of the sample and reference, multiplied by the fluorescence quantum yield of the reference, after correction for the difference in the refractive indexes between the sample and the reference solutions. The reference employed for porphyrins fluorescence quantum yield determination was (TPP,  $\Phi_F = 0.1 \pm 0.01$ ) and for bacteriochlorins 5,20,15,20-tetrakis(2,6-dichlorophenyl) bacteriochlorins (Cl<sub>2</sub>B,  $\Phi_F = 0.012 \pm 0.02)^{11}$ , was used. Both references, TPP and Cl<sub>2</sub>B were prepared in toluene to avoid any possible aggregation in ethanol, and a correction for the refractive index of the solvent was applied.

## Determination of triple state quantum yield by Flash Photolysis

Transient triplet-triplet absorption was obtained with an Applied Photophysics LKS. 60 flash photolysis spectrometer with a R928 photomutiplier from Hamamatsu for detection and HP Infinium (500 MHz, 1GSas-1) or Tektronix DPO 7254 (2.5 GHz, 40 GSas-1) oscilloscopes.

Ethanol solutions were prepared with absorption of ~0.2 and excitation of the samples was achieved at 355 nm with a Nd:YAG laser (Spectra-Physics Quanta Ray GCR 130, 5-6 ns FWHM, or EKSPLA PL 2143 A, 30 ps pulse width).

Triplet quantum yield ( $\Phi_T$ ) were determined according to the following equation,

$$\Phi_{\rm T}^{\rm s} = (\epsilon_{\rm T}^{\rm ref}/\epsilon_{\rm T}^{\rm s})(\Delta 0{\rm D}^{\rm s}/\Delta 0{\rm D}^{\rm ref})((1-10^{-{\rm A}({\rm ref})})/(1-10^{-{\rm A}({\rm s})}))\Phi_{\rm T}^{\rm ref}$$
(2.17)

Where, the superscripts s and ref denote sample and reference, respectively.  $\Delta OD_T$  is the preexponential parameter obtained from the monoexponential fitting of the transient triplet decay in ethanol  $\varepsilon_T$  is the triplet molar absorption coefficient and is determined from:

$$\varepsilon_{\rm T} = \varepsilon (\Delta 0 D_{\rm T} / \Delta 0 D_{\rm s}) \tag{2.18}$$

Being  $\varepsilon$  is the ground-state molar absorption coefficient.  $\Delta OD_{T,S}$  are the pre-exponential factor obtained from the monoexponential fit of either the triplet absorption or the singlet depletion. The values of  $\varepsilon_T$  were measure at 445, 450 and 440 nm for F<sub>2</sub>PMet, F<sub>2</sub>CMet and Cl<sub>2</sub>PMet, respectively. The singlet depletions were measure at 405 nm for F<sub>2</sub>PMet and at 410 nm for F<sub>2</sub>CMet and Cl<sub>2</sub>PMet. The linear range of the laser energy dependence of these  $\Delta OD$  values was used to obtain a more precise measurement of their ratio. Benzophenone ( $\varepsilon_T = 7300M^{-1}$  cm<sup>-1</sup> at 545 nm and  $\Phi_T = 1$ ) was used as reference.<sup>59</sup>

### Triplet energy determination by Photoacoustic Calorimetry

Photoacoustic calorimetry (PAC) was performed in a front-face cell design.<sup>60</sup> The sample, reference and solvent solutions were flowed separately with a 1 mL min<sup>-1</sup> rate by a pump (SSI chromatographic pump) through a cell of thickness 0.2 mm. They were irradiated at 355 nm with a Spectra-Physics Quanta Ray GCR 130 at a frequency of 10 Hz. A small fraction of the laser beam was reflected to a photodiode, used to trigger the transient recorder (Tektronix DSA 601, 1 GSas-1). The photoacoustic waves detected with a 2.25 MHz Panametrics transducer (model 5676) and captured by the transient recorder, were transferred to a PC for data analysis. In a typical PAC experiment, 200 waves of the sample, reference and of pure solvent were recorded and averaged in the same experimental conditions. Four sets of averaged sample, reference and solvent waves were used for the data analysis at a given laser intensity, and four laser intensities were employed in each experiment. The laser intensities are obtained by interposing neutral density filters with transmissions between 25 and 100 %. The measurements were made using manganese 5,10,15,20-tetraphenylporphyrin (MnTPP) as photoacoustic reference.<sup>12</sup>

### Single photon counting

Fluorescence lifetime was measured at 293 K with a home-built time correlated single photon counting (TCSPC) apparatus described in detail elsewhere.<sup>61</sup> The excitation source consisted of a Horiba-Jobin-Yvon pulsed nanoled,  $\lambda_{exc}$ =373 nm. Deconvolution of the fluorescence decay curves was performed using the modulation function method as implemented by Stricker in the SAND program.<sup>62</sup>

#### Singlet oxygen quantum yield

An adaptation of the spectrometer used in flash-photolysis for time-resolved singlet-oxygen phosphorescence allowed the detection of singlet oxygen phosphorescence. The modification of the spectrometer involved the interposition of a Melles Griot cold mirror (03MCS005), which reflects more than 99 % of the incident light in the 400-700 nm range, and Schott RG665 filter. A 600 line diffraction grating was mounted in place of a standard one to improve spectral resolution and sensitivity in the NIR. The emission was detected using a Hamamatsu R5509-42 photomultiplier, cooled to 193 K in a liquid nitrogen chamber (products for Research, model PC 176TSCE005). This equipment allows for spectral identification of the singlet oxygen phosphorescence and measurement of the singlet oxygen lifetime in the nanosecond and microsecond ranges.

By extrapolating to time-zero the decays of the singlet molecular oxygen emissions measured in ethanol for the reference (phenalenone,  $\Phi_{\Delta}=0.95 \pm 0.02)^{63}$  and for the sensitizers, at a given laser intensity, we obtain a relation between emission intensities, that is identical to the relation between the singlet molecular oxygen quantum yields. The singlet oxygen quantum yields were obtained from fitting the linear dependence between the intensities and the energies of the laser pulse.

### Singlet oxygen formation – Rise time determination

Ethanol solutions were prepared with absorbance close to 0.2. Singlet oxygen phosphorescence at 1275 nm was detected by a cooled near-IR sensitive photomultiplier tube, PMT (Hamamatsu model R5509-42) by interposing a 1270 nm band-pass filter. Excitation for bacteriochlorins (except Cl<sub>2</sub>BHep) was achieved by using a Ti:Sapphire laser system (Spectra Physics, Tsunami and Spitfire), that delivers femtosecond pulses at a repetition rate of 1 kHz that are tunable over the range ~765-850 nm. The generation of 750 nm, a exciting wavelength was achieved by pumping an optical parametric amplifier (Spectra Physics, OPA-800CF), resulting in linearly polarized, femtosecond pulses tunable over the range ~300-3000 nm. A more detail description of the setup is given in the section 4.4.2 of Chapter 4. For Cl<sub>2</sub>BHep excitation was achieved at 355 nm by using a picosecond laser (35 ps) EKSPLA model PL2143A and using the same detection system.

## Electrochemistry

Cyclic voltammetry experiments were performed in the Chemistry Department of the University of Coimbra by Madalina M. Barsan.

Experiments were done at 25°C on a computer-controlled  $\mu$ -Autolab type I potentiostatgalvanostat with GPES software (Metrohm-Autolab, Utrecht, Netherlands). The experiments were performed in de-aerated electrolytes (nitrogen bubbled through the solvent and then into the cell solution for at least 10 min before experiments and then as blanket during experiments). The solvents were either dichloroethane or acetonitrile containing 0.1 M tetra-*n*butylammonium perchlorate as supporting electrolyte. A three-electrode system was utilized and consisted of a glassy carbon working electrode (1 mm diameter), a platinum wire counter electrode, and a saturated calomel electrode (SCE) as reference. The reference electrode was separated from the solution in the cell by a fritted-glass bridge filled with the solvent/supporting electrolyte mixture.

## Detection of radicals by Electron Paramagnetic Resonance

Reactive oxygen species generated by irradiation of the sensitizers in solution, form adducts with various spin traps more stable than the radicals itself making it possible to detect by EPR.

EPR spectra were recorded under in situ irradiation with a Hamamatsu diode laser, type LA0873, S/N M070301, delivering 100 mW at 748 nm. This diode laser was controlled by a ThorLabs 500 mA ACC/APC Laser Diode Controller. The laser energies were regularly checked with an Ophir model AN/2E laser power meter.

The spin trap used in all the experiments was 5,5-dimethylpyrroline-N-oxide (DMPO). For detection of hydroxyl radical solutions of the sensitizer in PBS/TRTX-100 (3/100) were prepared. Just before irradiation DMPO (50 mM) was incorporated in solution.

For detection of superoxide radical, DMSO was used as solvent. The sensitizer (50  $\mu$ M) was previously dissolved in DMSO, and DMPO (50 mM) was added just before measuring.

The PBS employed in these measurements was previously treated with chelating resin, Chelex 100, in order to remove any contaminating metal ions that may catalyze the decomposition of peroxides.

# *Triplet excited state quenching by* $H_2O_2$

A solution of  $Cl_2BOH$  was prepared in PBS with different concentrations of  $H_2O_2$  (0 mM to 5 mM) and was deoxygenated for ~30 min. Flash-photolysis (previously described) was used to record the triplet-triplet absorption decays at 790 nm after excitation at 355 nm.

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# Photobleaching and photomodification of dyes

- 3.1 Introduction
- 3.2 Results
  - 3.2.1 Photobleaching of photosensitizers with potential application in photodynamic therapy
  - 3.2.2 Subcellular localization of porphyrins and bacteriochlorins: Photobleaching, relocalization and photomodification
- **3.3 Conclusions**
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# **3.1 Introduction**

As presented before, in the context of the properties of a "perfect" photosensitizer, it is expected that high stability towards light, or pH changes, would contribute to an increase in efficiency of a sensitizer.<sup>1</sup>

However, photobleaching it is also a tool to limit the damage to healthy tissue, as it is markedly dependent on dose of photosensitizer, and to reduce the sensitivity of patients to light after PDT.<sup>2,3</sup> It is difficult to achieve the right balance between photobleaching and photoaction, but with appropriate sensitizer dosages and bleaching rates, injury to normal tissues surrounding the tumor can be significantly decreased without compromising treatment efficacy.

Porphyrins typically undergo slow photobleaching over time. Bonnett et al<sup>4</sup> compared the photobleaching in solution for mTHPP, mTHPC and mTHPBC. mTHPC and mainly mTHPBC showed pronounced photodegradation under laser irradiation. The photoproducts were mainly dipyrrin derivatives and colorless fragments. Irradiation of mTHPBC resulted also in a small but detectable yield of mTHPC. The most stable of these macrocycles, mTHPP, generated benzoquinonylporphyrins as photoproducts.<sup>5</sup>

Bacteriochlorins have shown some advantages and higher photodynamic actives *in vivo* over the first generation of sensitizers and many other clinically approved sensitizers.<sup>6-8</sup>The interest in bacteriochlorophyll *a* derivatives as sensitizers for medical applications, especially PDT, has long been explored in view of their promising photophysical and photochemical properties.<sup>9,10</sup> However, the use of these or other bacteriochlorins as photosensitizers was discredited because they were readily oxidized and difficult to prepare <sup>11</sup>. Recently, new methods of synthesis have been described to yield very stable and tunable bacteriochlorins with high phototoxicity towards undesired tissue, reopening the interest in this class of compounds.<sup>8,12-16</sup>

A number of different factors have been found to influence the reactions leading to the photodegradation of sensitizers. These may be related to the structure of the sensitizer (chemical modification of substituents or central metals, oxidation potential) or to their environment (solvent, presence of quenchers, pH, aggregation, oxygen level). The photodegradation quantum yield ( $\Phi_{pb}$ ) provides a quantitative measure of the resistance of sensitizers towards light induced structural changes.

The photobleaching quantum yield can be expressed as:

$$\Phi_{pb} = \frac{rate \ of \ disappearance \ of \ photosensitizer \ molecule \ (v_m)}{rate \ of \ absorption \ of \ photons \ (v_p)}$$
(3.1)

The disappearance rate of the photosensitizers under irradiation gives,

$$v_m = \frac{\Delta n}{\Delta t} \tag{3.2}$$

Where the number of photosensitizer molecules is given by  $n=CVN_A$ , and *C* is the concentration in mol/dm<sup>3</sup>, *V* the volume in dm<sup>3</sup> and  $N_A$  the Avogadro number. The disappearance of the number of photosensitizer molecules can be obtained by the decrease in absorption per unit time (i.e., per second)

$$\Delta C = \frac{\Delta A}{\varepsilon l} \tag{3.3}$$

Thus,

$$\Delta n = \frac{V N_A \Delta A}{\varepsilon l} \tag{3.4}$$

The initial rate of absorption of the photons is given by the difference between the number of incident  $(I_0)$  and transmitted  $(I_t)$  photons, by unit time

$$v_p = I_0 - I_t = I_0 (1 - 10^{-A_i}) \tag{3.5}$$

Where  $A_i$  is the initial absorbance of the medium at the wavelength  $\lambda$  of the (monochromatic) incident light. The number of incident photons per unit time is determined by the power (*W*) of the light taking into consideration the energy of each incident photon

$$I_0 = \frac{W}{hc/\lambda} \tag{3.6}$$

Thus,

$$v_p = \frac{\lambda W (1 - 10^{-A_i})}{hc}$$
(3.7)

From these two initial rates, weobtain

$$\Phi_{pb} = \frac{V N_A h c}{\varepsilon l \lambda W (1 - 10^{-A_i})} \frac{\Delta A}{\Delta t}$$
(3.8)

The photobleaching quantum yield relies on the fact that it accounts for the fluence rate of the light source, the volume of the irradiated solution and the photodecomposition rate constant. Indeed, the decomposition rate can also be written as:

$$V_m = \frac{dn}{dt} = N_A V \frac{dC}{dt} = N_A V k_{pb} C = \Phi_{pb} v_p$$
(3.9)

$$\Phi_{pb} = \frac{hcN_A V A_0 k_{pb}}{\varepsilon l \lambda W (1 - 10^{-A_i})}$$
(3.10)

In this chapter the photobleaching for a number of halogenated porphyrins and bacteriochlorins, is followed by absorption changes in solution. Most of the existing knowledge on photobleaching mechanisms and kinetics derive from solution based studies. It is generally assumed that the photobleaching of dyes in solution can be directly translated into the behavior one would expect in cells. However, this assumption does not take into consideration that cells are very complex chemical systems where incorporated dyes can interact and bound to a number of targets, such as DNA, proteins, or other cellular components.

We have incorporated some of the sensitizers tested in solution into mammalian cells and followed the fluorescence changes under light irradiation. Upon light irradiation, depending of the photosensitizer, reactive oxygen species will be generated, such as singlet oxygen, superoxide and/or hydroxide radical. The intracellular diffusion of these ROS will depend on their lifetimes, but in the case of porphyrins most of the ROS are singlet oxygen molecules with a lifetime of a few microseconds,<sup>17-19</sup> and diffusion between different environments in the cell is possible causing not only damage at the generation site but also in other organelles. On the other hand, bacteriochlorins may generate reactive oxygen species with different reactivities. Singlet oxygen and hydrogen peroxide have long lifetimes and therefore higher diffusion range, whereas superoxide or hydroxyl radical are short lived and will cause the most damage at the generation site. Thus, the localization of the photosensitizer in the cell can have a major influence on the mechanism and efficiency of photodynamic therapy.

The complexity of the photobleaching mechanisms and PDT efficacy is further increased by the fact that photosensitizers will be localized in different spatial domains in a cell where they have different lipophilicities, and we can expected to observe changes in their photobleaching kinetics due to their different environment.

Since all the photosensitizers studied in this work have some fluorescence, this feature was used to analyze the light induced changes on dye localization, and photobleaching in cells. Confocal microscopy, as a more sensitive technique which uses lower fluencies, was used for subcellular localization of  $F_2PMet$  and  $F_2BMet$  by overlapping the fluorescence of the dyes with the fluorescence of specific organelle trackers.

The intracellular light-induced fluorescence changes for hydrophobic and hydrophilic porphyrins and bacteriochlorins was followed by imaging and spectroscopically from directing the detected emission light of the photosensitizers from the microscope into a fiber optic spectrometer.

# **3.2 Results**

# **3.2.1** Photobleaching of photosensitizers with potential application in photodynamic therapy

The resistance of our dyes to light was tested, their photobleaching quantum yields determined and the values obtained are presented on Table 3.1. Light induced modification of photosensitizers produced significant changes in the spectral characteristics. Very different photobleaching rates and very distinct pathways were observed.



**Figure 3.1** Light induced changes in the absorption spectra of  $F_2POH$  in PBS (A) and DMSO (B) when irradiated at 508 nm (pulsed laser, 77 mW), of  $F_2CMet$  (C) and of  $F_2BMet$  (D) in MeOH/H<sub>2</sub>O (3/2) after irradiation at 653 nm (pulsed laser, 6 mW) and 748 nm (diode laser, 71 mW), respectively. The arrows show the absorption changes during irradiation time.

Figure 3.1 shows the absorption spectra of porphyrin  $F_2POH$ , chlorin  $F_2CMet$  and of bacteriochlorin  $F_2BMet$  before and after irradiation. After irradiation of  $F_2POH$  in PBS or DMSO different photoproducts are formed. Absorbing products at ~650 nm and 750 nm are observed when irradiation is performed in DMSO. In PBS no increase in absorption is observed above 450 nm. Under specific conditions of pH, presence of surfactants, temperature and even

light porphyrins can aggregate to rearrange their monomeric molecules in parallel units stacked face-to-face or edge-to edge to generate J-aggregates, which is normally identified by the appearance of red-shifted absorption bands. <sup>20-25</sup> Rotomskis et al. have similarly observed the formation of red absorbing products after irradiation of haematoporphyrin, haematoporphyrin derivative, meso-tetraphenylporphyrin tetrasulphonated, Photofrin II, Photosan-3 and dimethoxyhaematoporphyrin in aqueous solution. After chromatography those photoproducts were identified as porphyrin, chlorin and bacteriochlorin type of molecules.<sup>23</sup> The authors concluded that the formation of red absorbing products is favored by the presence of sandwich type structures and suggested a mechanism of light-induced reduction of porphyrins. However, to our knowledge light-induced aggregation and/or reduction of porphyrins in DMSO has not been observed before.

Irradiation of  $F_2BMet$  at 748 nm causes a decrease in all absorption bands characteristic of this dye. A slight increase is recorded at 404 and 652 nm, typical absorption bands of chlorins. The closely related chlorin  $F_2CMet$  could be a possible photoproduct. If this is true, we can conclude that not all of the photobleached bacteriochlorin was converted into chlorin (from the molar absorption of each dye and considering the absorption changes observed, the percentage of bacteriochlorin that is converted into chlorin can be determined and is smaller than the percentage of bacteriochlorin bleached). The observation of isosbestic points in the absorption spectrum along with the irradiation suggests that a single reaction is responsible for the degradation of  $F_2BMet$ . Similar behavior was observed to all other bacteriochlorins studied (Appendix II).

Light induced irradiation at 653 nm of  $F_2CMet$  (Figure 3.1 C) in MeOH/PBS solution resulted in decay of all the absorption bands.  $F_2CMet$  suffers photomodification with the appearance of a new absorbing product at ~640 nm. Most probably the new absorbing photoproduct is also a chlorin.<sup>5</sup>

The photobleaching kinetics was followed for a number of bacteriochlorins and monitored by the light induced changes in their near-infrared absorption band (~748 nm), Figure 3.2. First order kinetics was observed for all bacteriochlorins studied (data shown in Appendix II). For porphyrin,  $F_2POH$  and chlorins,  $F_2CMet$  or FCMet no clear photobleaching kinetics was identified. The formation of absorbing photoproducts with absorption bands close/under the bands of the photosensitizers might be pointed as a possible explanation.

Photodecomposition quantum yields,  $\Phi_{pb}$ , were determined and are presented in Table 3.1. For bacteriochlorins,  $\Phi_{pb}$  values were calculated using equation 10. Since, no first-order rate constants were obtained for porphyrin, F<sub>2</sub>POH and chlorins, FCMet and F<sub>2</sub>CMet,  $\Phi_{pb}$  was determined by using equation 8 instead and considering the absorption change that occur for the first bleaching points (with less contribution of photoproducts generated at the same wavelength).



**Figure 3.2** Photostability kinetics of halogenated bacteriochlorins in aerated methanol (Cl<sub>2</sub>BHep, FBMet<sub>2</sub>, FBMet) solutions and PBS (Cl<sub>2</sub>BOH) solution. Irradiation was achieved with a 748 nm diode laser with 40 mW.

Our results follow the same trend as observed by Bonnett et al. <sup>4</sup> for a series of porphyrin, chlorin and bacteriochlorins. Porphyrin, F<sub>2</sub>POH, shows higher stability followed by the chlorins, F<sub>2</sub>CMet and FCMet, and finally by the bacteriochlorins. Nevertheless, the photostability of our halogenated bacteriochlorins are one to three orders of magnitude higher than those other bacteriochlorins, such as TOOKAD<sup>26</sup> or mTHPBC<sup>4,27</sup>. Photobleaching quantum yield of Cl<sub>2</sub>BHep is quite remarkable ( $\Phi_{pb}$ = 0.4×10<sup>-6</sup>), exceeding the stability of any other known bacteriochlorin, better than THPP ( $\Phi_{pb}$ =3.8×10<sup>-6</sup>), and comparable to that of phtalocyanines.<sup>28,29</sup>

From Figure 3.2, we can easily see that the photostability of the photosensitizers can be tuned and  $\Phi_{pd}$  is highly dependent of the solvent used. The following trend for photobleaching quantum yield was found: H<sub>2</sub>O>MeOH/H<sub>2</sub>O>MeOH. Interesting to note that photobleaching in DMSO occurs faster than in any of the other solvents. As a polar aprotic solvent, DMSO has shown to appreciably increase the rates of many reactions.<sup>30</sup> We found that, not only that the photobleaching rate on DMSO is faster but also that the photobleaching products generated in this solvent are different from PBS/MeOH.<sup>5</sup> From the irradiation of F<sub>2</sub>POH in DMSO two new bands at ~660 and 750 nm are observed, suggesting the generation of photoproducts that resulted from the photomodification of the porphyrin. On the other hand, in PBS the breakage of the porphyrin structure is the most likely option.<sup>28</sup>

Similar dependence was also observed by Bonnett et al. in MeOH and MeOH/ $H_2O^4$  and by Spikes when studying the photobleaching of haematoporphyrin, Photofrin, tetraphenylporphyrin tetrasulfonic acid and uroporphyrin in different organic solvents and buffered aqueous solutions.<sup>31</sup>

	Φ <sub>pb</sub> ×10 <sup>-6</sup>				
	Solvent	PBS	MeOH/ PBS (3/2)	MeOH	DMSO
Porphyrins	F <sub>2</sub> POH	$4.2\pm0.7$			$65.7 \pm 7$
	Cl <sub>2</sub> PEt		$6.5 \pm 1.1$		
Chlorins	F <sub>2</sub> CMet		$1.9\pm0.1^{a}$		
	FCMet		$3.3 \pm 0.2^{a}$		
	Cl <sub>2</sub> BHep			$0.4 \pm 0.2$	
	Cl <sub>2</sub> BEt		$6\pm 2$		
SI	Cl <sub>2</sub> BOH	$152 \pm 39$			
Bacteriochlorin	CIBOH	$284\pm28$	$296\pm59$	$35 \pm 1.8$	
	ClBEt		$82 \pm 7.4$		
	F <sub>2</sub> BOH	$203\pm52$			
	F <sub>2</sub> BMet		$10\pm0.2$ $^{b}$	$0.69 \; (EtOH) \pm 0.07$	$22 \pm 1$
	FBMet		$81\pm 6.5$	$17\pm0.7$	
	FBMet <sub>2</sub>			$12 \pm 0.3$	

 Table 3.1 Photobleaching quantum yields of halogenated porphyrins, chlorins and bacteriochlorins in different solvents.

a) in MeOH/H<sub>2</sub>O (3/2), b) from ref <sup>32</sup>. Errors estimated from the average of at least 2 experiments or from the monoexponential fitting from the kinetics, whichever was higher.

		$\Phi_{ m pb}\!\! imes\!10^{-6}$	Solvent
Commercial photosensitizers	Foscan ® <sup>4,27</sup>	33	PBS/MeOH
	Photofrin® <sup>28,34</sup>	55	PBS
	Tookad® <sup>26</sup>	1800	MeOH
Others	mTHPP <sup>28</sup>	3.8	PBS/MeOH
	mTHPBC <sup>4,27</sup>	1500	PBS/MeOH

**Table 3.2** Photobleaching quantum yields of commercial photosensitizers and of mTHPP and mTHPB recalculated from <sup>33</sup>.



**Figure 3.3** Absorption spectra of  $Cl_2BOH$  dissolved in a buffered aqueous solution at different pH values. Data were recorded as a function of the elapsed time of irradiation at 750/40 nm (Xenon lamp, 8.2 mW/cm<sup>2</sup>). A)  $Cl_2BOH$  at pH 7 over an irradiation time of 165 min, B)  $Cl_2BOH$  at pH 5 over an irradiation time of 177 min and C)  $Cl_2BOH$  at pH 10 over an irradiation time of 145 min. Arrows indicate the direction of irradiation-induced spectral changes.

Different photoproducts are also observed when the irradiation of bacteriochlorins is followed at different pH values (Figure 3.3).

	pH	$\Phi_{ m pb}\!\! imes\!10^{-6a}$	Solvent
	5	$1298\pm78$	
F <sub>2</sub> BOH	7.4	$189\pm10$	
	10	$157\pm9$	PBS
	5	$707 \pm 42$	
Cl <sub>2</sub> BOH	7.4	$78 \pm 4$	
	10	$79\pm4$	

Table 3.3 Photobleaching quantum yield dependence with pH for F<sub>2</sub>POH and Cl<sub>2</sub>BOH.

a) Standard errors determined from the monoexponential fitting of the kinetics decays.

In an acidic solution (pH 5) the sensitizers are less stable than at pH 7. A significant increase in absorption at ~410 nm and ~650 nm is observed at any pH value studied. At pH 10 the bleaching rate is slower for  $F_2POH$  and for  $Cl_2BOH$  we found no change in the photobleaching rate when compared to pH 7. The light induces spectral changes at pH 10 that are similar to the ones observed at pH 7. The kinetics variability under different pH conditions fits well with our proposed model for hydroxyl radical generation, on Chapter 2. The rate of superoxide disproportionation (equation 3.11) is higher at pH 5 promoting the reaction in the direct way to generate more hydrogen peroxide and consecutively more hydroxyl radical (equation 3.12). At pH 10 we find a slight decrease in the photobleaching quantum yield for  $F_2BOH$  and no changes in the kinetics for  $Cl_2BOH$ . The excess of OH<sup>-</sup> can neutralize H<sup>+</sup> and reduce the bleaching rate because less  $H_2O_2$  will be produced. With  $Cl_2BOH$  the excess of OH<sup>-</sup> might still not be enough to observe a significant effect on the bleaching rate.

$$2O_2^{-} + 2H^+ \rightarrow O_2 + H_2O_2 \tag{3.11}$$

$$O_2 + H_2O_2 \rightarrow O_2 + OH + OH$$
(3.12)

Takeushi et al. reported that by lowering the energy of the HOMOs strongly disfavors oxidative destruction of the macrocycle, and should contribute to increase the stability with respect to oxidation.<sup>35</sup> Incorporating halogen atoms, such as F or Cl atoms in the *ortho*-positions of the phenyl rings should raise the oxidation potential <sup>36</sup> of the photosensitizer and provide extra stability towards degradation. We do observe an increase in photostability on our dyes compared to non-halogenated photosensitizers such as Photofrin,<sup>28,34</sup> Foscan<sup>4,27</sup> and Tookad.<sup>26</sup> The nature of the substituents can also influence  $\Phi_{pb}$ . We also observe a steric protection of the

photosensitizers to bleaching when bulky substituents are introduced in the phenyl ring, as observed from FBMet<sub>2</sub> to Cl<sub>2</sub>BHep<sub>2</sub>

#### 3.2.1.1 Mechanistic considerations

The photo-oxygenation processes which lead to photobleaching are expected to be complex. No major mechanistic studies were performed to understand the photobleaching of these dyes, although there is some evidence to support a Type II (singlet oxygen) photobleaching mechanism for  $F_2POH$  and for chlorins ( $F_2CMet$  and FCMet) based on their high singlet oxygen quantum yields. Bacteriochlorins, on the other hand, generate additional reactive oxygen species which most certainly contribute to enhance their bleaching rates. The contribution of a Type I reaction was further investigated by Dabrowski et al.<sup>6</sup> by following the photobleaching of CIBOH in PBS/MeOH and in the presence of sodium ascorbate, an antioxidant. A dramatic increase in photostability for this dye was observed in the presence of this antioxidant, suggesting the participation of the hydroxyl radical in the photobleaching mechanism.<sup>6</sup> When the photobleaching of our bacteriochlorins is held in methanol, known to be a radical scavenger, a decreased in the photobleaching quantum yield is registered compared to conditions that water is present. The faster photobleaching of the bacteriochlorins in PBS can be attributed to the generation of highly reactive oxygen species as hydroxyl radical, that we have shown to be detected under similar conditions and in the present of a spin probe by EPR (Chapter 2).

# **3.2.2** Subcellular localization of porphyrins and bacteriochlorins in cells: Photobleaching, relocalization and photomodification.

Lipophilicity, often correlated to the bioactivity of drugs, is a factor to predict the ability of molecules to cross cellular membranes. It can be quantified by the partition coefficient between an organic solvent and water, log  $P_{ow}$ , which is defined as the concentration ratio between these two phases when the solute and the solvent are in equilibrium.<sup>37</sup>

The partition coefficient of the photosensitizers (Table 3.4) show that sulphonated tetraphenyl macrocyles have a higher affinity to aqueous environment ( $P_{ow}$ < 1) while sulphonamide photosensitizers with high partition coefficient values have more propensity to diffuse over membranes and localize in a more lipophilic environment.

**Table 3.4** Partition coefficient values of the photosensitizers studied in this work, values obtained from references <sup>38</sup> and <sup>12</sup>.

		log P <sub>OW</sub>
	CIPEt	2.23
~	СІРОН	-2.71
nyrins	Cl <sub>2</sub> PEt	1.84
Porpł	Cl <sub>2</sub> POH	-1.80
	FPMet	2.33
	FPOH	-2.49
	Cl <sub>2</sub> BOH	-1.69
S	Cl <sub>2</sub> BEt	1.83
llorin	CIBOH	-1.70
nioch	ClBEt	2.50
3acte	F <sub>2</sub> BOH	-1.44
I	F <sub>2</sub> BMet	1.9
	FBMet	2.7

# Hydrophobic photosensitizers

# F<sub>2</sub>PMet and F<sub>2</sub>BMet



**Figure 3.4** Intracellular distribution of  $F_2PMet$  (5  $\mu$ M) in A-549 cells. Left panel - Fluorescence of  $F_2PMet$  and overlap image with ER-tracker, Mito-tracker and Lyso-tracker. Arrows indicate the analyzed area for topographic construction. Right panel - Topographic profiles.

Confocal microscopy was used to determine the subcellular localization of  $F_2PMet$  by overlapping the fluorescence image of the photosensitizer with specific organelle trackers for ER (endoplasmatic reticulum), mitochondria and lysosomes.

The fluorescence micrographs obtained for  $F_2PMet$  (Figure 3.4) reveal a diffuse pattern of dye localization throughout the cytoplasm with a more intense fluorescence in the perinuclear area of the cell. According to the topographic analysis their subcellular localization is more coincident with the endoplasmatic reticulum and mitochondria while overlapping with the lysotracker is almost nonexistent. Taking into account the high affinity of  $F_2PMet$  to interact with cell membranes and to be localized in a lipid environment, binding to Golgi apparatus should not be excluded.



**Figure 3.5** Time-lapse fluorescence images of  $F_2PMet$  (5  $\mu$ M) incubated in HeLa cells under light irradiation at 420/40 nm (100 W broadband *Xe* lamp) and emission recorded at 650/40 nm. Bottom panel-

Bright field image of HeLa cells incubated with  $F_2$ PMet before irradiation. The inset corresponds to the time the cells were exposed to light.

From the continuous incidence of light in the cells we can observe an intensification of the fluorescence and the appearance of a punctate fluorescence in the cytoplasm (Figure 3.5). An increase in the fluorescence intensity has been previously observed for mTHPC<sup>39</sup>, hypericin<sup>40</sup> and several phtalocyanines<sup>41</sup>, upon irradiation. The results were interpreted as a possible photoinduced disaggregation/relocalization of the sensitizers but in none of these sensitizers the light induced changes on the intracellular fluorescence evolved to a punctate profile. From comparing the contrast image of the same group of cells before irradiation (Figure 3.5, bottom picture) and the fluorescence image after irradiation we can observe that the punctate fluorescence is localized in defined round structures (Figure 3.5) present in the cytoplasm of the cell. Similar morphology, with distinct granules in the cytoplasm of the cell, was observed for derivatives of bacteriochlorin *p* on HeLa cells and for derivatives of chlorin *p*6 in A549 cells.<sup>42-44</sup> The authors have associated these vesicles to lipid droplets, by co-staining the cells with Nile Red. We have not used this tracker in our subcellular co-localization, since no evidence of the localization of the sensitizers in these granular structures was evident before irradiation. Other possibilities such as dye aggregation in the cytoplasm should not be excluded.

The lipid droplets are able to accumulate large amounts of dye photoproduct. At high concentrations the already low fluorescence of  $F_2$ PMet will be quenched, and even if some fluorescence is still generated in these structures could be under the detection limit of our system, which could explain the lack of fluorescence initially.

The fluorescence spectra recorded from cells reveals two characteristic porphyrin bands at 672 and 702 nm. Interesting, the ratio between the fluorescence intensity of the Q(0,0)/Q(0,1) bands is opposite to what is observed in solution. Continuous irradiation results in a ~20 nm shift of the first Q(0,0) band, which correlates in time with the appearance of the fluorescence from the granulated spots in the cytoplasm (Figure 3.5). This behavior was reproducible, although the intensity of this band depends on the group of cells being analyzed. In most of the cases the intensity of the band increases with irradiation, nevertheless a decrease and/or stabilization of the fluorescence (Figure 3.6) were also registered for some group of cells. Because the fluorescence is collected from an area which covers a group of cells in the focal plane, the different events that occur simultaneously as the cells are under light irradiation, such as bleaching, relocalization and/or dye leakage, will contribute to the spectra construction.

The increase in the first emission band could be explained by the generation of a photoproduct more fluorescent than  $F_2PMet$ . A chlorin closely related to  $F_2PMet$  as  $F_2CMet$  has a fluorescence quantum yield that is about 7 times higher that of  $F_2PMet$ . The photoreduction of porphyrins is not documented in such conditions, however, recently Friaa et al.<sup>45</sup> have shown that under irradiation m-THPC (Foscan) is reduced to its anion radical in the presence of Trolox, a standard oxidant.

Relocalization of the porphyrin or photoproduct into these granular structures should be also considered. Nevertheless is difficult to justify how the sensitizer would move in the cytosol (a hydrophilic area) to accumulate preferentially in these structures.



**Figure 3.6** Left -Time-lapse fluorescence spectra of  $F_2PMet$  (5  $\mu$ M) in HeLa cells. Excitation at 420/40 nm and emission recorded above 600 nm. Right- fluorescence kinetics followed at 701 nm and 672 nm (data averaged for at least 5 different groups of cells). Arrows indicate the direction of irradiation-induced spectral changes.

The subcellular localization of bacteriochlorin,  $F_2BMet$ , in A-549 cells was determined using the same protocol and trackers as used for  $F_2PMet$ . The fluorescence images show a distribution profile of  $F_2BMet$  in the endoplasmic reticulum and in the mitochondria, similar to  $F_2PMet$ (Figure 3.7).

Under prolonged irradiation, the fluorescence of  $F_2BMet$  fades and a punctate pattern is revealed (Figure 3.8). Once again the fluorescence observed comes from structures similar to the ones observed on the bright field image on Figure 3.5. Hamblin and co-workers<sup>46</sup> observed a similar clustered profile at specific sites in the cells when studying the subcellular localization of a group of novel bacteriochlorins. The authors have also ruled out the possibility of dye aggregation because otherwise inter-bacteriochlorin interactions would result in fluorescence quenching.



**Figure 3.7**  $F_2BMet$  (5  $\mu$ M) subcellular localization in A-549 cells. Left panel - Confocal overlay pictures of A-549 cells double-stained with  $F_2BMet$  and ER, Lyso or Mito tracker. Arrow indicates the analyzed area for topographic construction. Right panel - Topographic profiles.



**Figure 3.8** Fluorescence microscope images of  $F_2BMet (10 \ \mu M)$  incubated in HeLa cells, under elapsed irradiation tine at 500/40 nm (100 W broadband Xe lamp), and with emission at 750/40 nm. The inset corresponds to the time the cells were exposed to light.



**Figure 3.9** Left -Time-lapse fluorescence emission spectra of  $F_2BMet$  (19  $\mu M$ ) in HeLa cells. Fluorescence spectra recorded after excitation at 425/40 nm and emission over 600 nm. Right-fluorescence kinetics followed at 749 nm and 658 nm (data averaged for 5 different groups of cells). Arrows indicate the direction of irradiation-induced spectral changes.

Following the fluorescence spectrum recorded under light irradiation, two emission bands are observed at the minimum light exposure of 1s, Figure 3.9, with maxima at 658 and 749 nm. Light induces bleaching of the bacteriochlorins manifested by a fast decrease of its band at 749 nm, which is concomitant with an increase of fluorescence at 658 nm. The most probable

bleaching photoproduct is a chlorin. The emission band of the bacteriochlorin is shifted about  $\sim$ 3nm to the emission maximum observed in solution.

Compared to  $F_2PMet$ ,  $F_2BMet$  has similar subcellular localization and similar behavior under light irradiation. However, the photostability of the dyes are clearly different: the bacteriochlorin bleaches faster than the porphyrin, but a quantitative assessment would require the knowledge of cellular concentration of these photosensitizers and correction for the intensity of the incident light absorbed.

### Cl<sub>2</sub>PEt and Cl<sub>2</sub>BEt

The detection of fluorescence from cells incubated with chlorinated porphyrin or bacteriochlorins was less readily detectable. Chlorinated porphyrin or bacteriochlorins have very low fluorescence quantum yield, about a third of that from difluorinated sensitizers (Chapter 2), and no fluorescence spectrum was recorded.

The fluorescence images show initially a punctate fluorescence pattern for  $Cl_2PEt$  in HeLa cells which under continuous irradiation gets diffuse over the cytoplasm (Figure 3.10, top panels).

 $Cl_2BEt$  (Figure 3.10, lower panel) revels a more diffusive fluorescence all over the cell with higher intensity in the periphery of the nucleus. The subcellular localization was shown to be preferably in the ER of the cells.<sup>6</sup>





**Figure 3.10** Time-lapse fluorescence microscope images of  $Cl_2PEt$  (top panel) and  $Cl_2BEt$  (lower panel) incubated in HeLa cells with a concentration of 10  $\mu$ M, under irradiation at 425/40 nm and 500/40 nm (100 W broadband Xe lamp), and with emission at 650/40 nm and 750/40 nm, respectively. The inset corresponds to the time the cells were exposed to light.

#### Hydrophilic photosensitizers

#### F<sub>2</sub>POH and F<sub>2</sub>BOH

The light induced changes on the fluorescence images and spectral profile in HeLa cells are presented on Figures 3.11 and 3.12 for  $F_2POH$  and on Figures 13 and 14 for  $F_2BOH$ . Initially a granular fluorescence pattern is observed for both the sensitizers in the cytoplasm of the cells. Under light irradiation a diffusion pattern of fluorescence all over the cell with some extracellular leaking, and preferential accumulation of the dye in the nucleus is observed for  $F_2POH$ . A similar behavior and fluorescence distribution pattern is observed for other hydrophilic dyes as TPPS or TMPyP and their subcellular localization has been attributed to lysosomes.<sup>47,48</sup> The preferential accumulation in the nucleus was suggested by Patito et al<sup>47</sup> to occur because of an association of the photosensitizer to proteins which translocate to the nucleus under oxidative stress conditions. Not knowing exactly the subcellular location of  $F_2POH$ , it is nevertheless an educated guess to assume that it is the lysosomes and the loss of the punctate fluorescence may be related to lysosomal/endosomal photodamage.

The fluorescence changes under irradiation on HeLa cells incubated with  $F_2BOH$  (Figure 3.13) are different from what was observed for  $F_2POH$ . The fluorescence fades very quickly not being possible to observe any dye relocalization. This is consistent with the higher photobleaching quantum yield of  $F_2BOH$  in solution.



**Figure 3.11** Fluorescence images of HeLa cells incubated with  $F_2POH$  (10  $\mu$ M). Time-lapse irradiation with excitation at 420/40 nm and emission at 650/40 nm (100 W broadband Xe lamp). The inset corresponds to the time the cells were exposed to light.



**Figure 3.12** Light-induced fluorescence changes of  $F_2POH$  (10  $\mu M$ ) incubated in HeLa cells, with irradiation at 420/40 nm and emission above 600 nm with a 100 W Xe lamp. Left panel – fluorescence spectra, right panel - fluorescence intensity analyzed as a function of time at 654 and 705 nm, for an average of 5 groups of different cells.

Following the light-induced changes in the fluorescence spectra of  $F_2POH$  (Figure 3.12), we can observe an increase in the fluorescence intensity of both emission bands Q(0,0) band and Q(0,1) band.

Although as the first one increases continuously over time the last band fades after  $\sim 25$  s of irradiation. This behavior can be associated to the relocalization of the dye and change in the environment, or to the generation of a more fluorescence photoproduct with emission at  $\sim 650$ 

nm (as it was possible to occur to  $F_2PMet$ ). The second hypothesis would also explain the change in the relative intensities of the two emission bands.



**Figure 3.13** Light induced fluorescence changes on HeLa cells incubated with  $F_2BOH$  (10  $\mu$ M). Fluorescence images recorded after excitation with a Xe lamp (100 W broadband) at 500/40 nm and emission at 750/40 nm for Cl<sub>2</sub>BOH. The inset corresponds to the time the cells were exposed to light.



**Figure 3.14** Light induced changes on fluorescence in HeLa cells incubated with  $F_2BOH$  (10  $\mu$ M) with excitation at 500/40 nm and emission recorded above 600 nm. Left panel - fluorescence spectra, right panel - fluorescence analysis over time at the emission maximum of 749 nm and at the photoproduct maximum at 658 nm (data averaged for 5 different groups of cells).

From the fluorescence spectra on HeLa cells incubated with  $F_2BOH$  a typical band of this bacteriochlorin at 749 nm is observed (Figure 3.14). Following the light induced changes on the fluorescence spectra, the fluorescence maximum at ~749 nm of  $F_2BOH$  fades as observed from the microscope images. The fluorescence intensity of the band centred at 658 nm, typical of a chlorin and that most probably corresponds to  $F_2COH$ , increases during the first 18 s of irradiation thus and further irradiation causes its photobleaching. It is known that  $F_2COH$  is present as a contamination in the samples and comes from the synthesis and that as we pointed before are expected to have higher fluorescence quantum yield than the corresponding porphyrin.

## Cl<sub>2</sub>POH/Cl<sub>2</sub>BOH

The substitution of the two fluorine atoms in the phenyl ring of the porphyrins or bacteriochlorins by two chlorine atoms in  $Cl_2POH$  and  $Cl_2BOH$  does not introduce many changes on the initial fluorescence profile observed in the cells. The same applies to the behavior under irradiation, where  $Cl_2POH$  fluorescence diffuses all over the cell under irradiation, while  $Cl_2BOH$  fluorescence intensity quickly fades over time.



**Figure 3.15** Light induced fluorescence changes on HeLa cells incubated with: top panel- Cl<sub>2</sub>POH (10  $\mu$ M) and bottom panel - Cl<sub>2</sub>BOH (10  $\mu$ M). Fluorescence images recorded after excitation with a Xe lamp (100 W broadband) at 425/40 nm and emission at 650/40 nm for Cl<sub>2</sub>POH and excitation at 500/40 nm and emission at 750/40 nm for Cl<sub>2</sub>BOH. The inset corresponds to the time the cells were exposed to light.

The irradiation time required to collect the fluorescence pictures also leads to oxidative stress in the cells. Just based on the contrast images, necrosis was recognized as the mechanism of cell death under our experimental conditions. This evaluation was based on immediate and massive production of surface evaginations (bubbles) clearly visible by brightfield images after irradiation of the photosensitizer and by fluorescence when sulphonated dyes were irradiated. <sup>49</sup> Nuclear and plasma membrane deformation (defined nucleus) developed rapidly and most of the blebs detach concomitantly from the cell membrane into the medium (Figure 3.16).



**Figure 3.16** Bright field images of HeLa cells incubated with  $Cl_2BEt$  (10  $\mu$ M) before (left panel) and after 290 s of irradiation (right panel) with a Xe lamp (100 W broadband) at 500/40 nm.

## **3.3 Conclusions**

We have examined the photobleaching behavior, in solution and in cultured cells, of a group of sensitizers.

Solution studies reveal that photobleaching is highly dependent on the solvent used and pH of the solution. Chlorinated dyes have shown a relatively higher photoresistance to degradation over the fluorinated. Increasing the size of the side chain in the phenyl substituent of the macrocycle has shown to contribute for some steric resistance to degradation by light. Halogenated sulphonamide bacteriochlorins revealed a photostability without precedents and can be as stable as some chlorins, while retaining some amphilicity.

By establishing the intracellular localization of a photosensitizer we can predict the initial PDT targets. It is interesting to note that we found similar intracellular distribution pattern for sensitizers with similar octanol/water partition coefficients. An exception was  $Cl_2PEt$  with a punctate pattern observed initially, but was not observed for other hydrophobic sensitizers.

The sulfonamide sensitizers have a preferential accumulation in the endoplasmic reticulum of the cells, but some localization in the mitochondria is also possible.<sup>6</sup> Some clinical photosensitizers that are also localized in the ER, such as Foscan or Photofrin®, have shown to trigger an apoptotic response after the generation of ROS.<sup>50,51</sup> Most of these photosensitizers that cause photochemical damage to the ER have shown excellent in vitro PDT efficacy.<sup>52</sup> Photosensitizers localized in the lysosomes have shown to induce cell death via the release of lysosomal enzymes in the cytosol, or via the relocalization of the sensitizer after irradiation to other targets.<sup>53</sup> It has been reported for phtalocyanines and Nile blue derivatives that damage of the lysosomal membrane damage does not seem to cause cytotoxicity to the cells.<sup>54</sup> In some cases, apoptosis induced by photosensitizers localized in lysosomes was reported, but this was found to be a very slow process.<sup>55</sup> If the sulphonic photosensitizers studied are indeed localized in the lysosomes, this could contribute to explain their lower efficacy in vitro and in vivo, when compared to the sulphonamide ones.<sup>7,56</sup> Based on our results, the main differences found between these sensitizer and that might dictate their PDT efficacy are: photostability (higher for sulphonamide dyes than in the sulphonated ones), different subcellular localization (different primary site of action) and light-induced fluorescence changes (relocalization increases the number of action sites).

Most of the light induced changes observed in the fluorescence of the dyes in the cells occur in the first seconds of exposure to light. This may be a consequence of the generation of reactive oxygen species which cause degradation of the sensitizer itself and/or the degradation of

molecules in the intracellular environment and that immediately surround the sensitizer. With this in mind, it is important to ascertain the subcellular localization of the photosensitizers to recognize the primary site of action, though no predictions on the PDT efficiency of these sensitizers should be based on this premise since under light irradiation the sensitizer may relocalize to new targets or is photodegraded into new reactive photoproducts.

# **3.4 Materials and methods**

#### **3.4.1 Materials**

F<sub>2</sub>POH, F<sub>2</sub>CMet, FCMet, Cl<sub>2</sub>BHep, Cl<sub>2</sub>BEt, Cl<sub>2</sub>BOH, ClBEt, ClBOH, F<sub>2</sub>BMet, FBMet<sub>2</sub>, FBMet, F<sub>2</sub>BOH, were kindly offered by Luzitin S. A.

Methanol (MeOH) and dimethyl sulfoxide (DMSO) were used as received from Sigma-Aldrich.

#### 3.4.2 Methods

#### Photodecomposition quantum yields

A 3mL sample in a quart cuvette of the photosensitizer dissolved in a given solvent was placed in front of the irradiation source. The loss of volume by evaporation during irradiation was compensated by weight, adding the necessary methanol volume to the sample before each time interval. Absorption spectra were recorded at different times from the sample that was constantly stirred during irradiation. The photodecomposition quantum yields were obtained according to equation 8 or 10. Absorption spectra were recorded with spectrophotometers Shimadzu models 2100 and 2450.

Phosphate buffer saline was prepared as 137 mM NaCl, 2.7mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH=7.

Hamamatsu (max power 120 mW) and Sacher (max power 40 mW) diode lasers at 748 nm were used to irradiate bacteriochlorin solutions. Irradiation of porphyrin ( $F_2POH$ ) and chlorins ( $F_2CMet$  and FCMet) was achieved by a pulsed laser OPO EKSPLA model PG/122/SH (ca. 6ns) pumped by a Nd:Yag laser EKSPLA model NL301G at 508 nm and 653 nm, respectively. A power meter (power meter detector 818P) was used to quantify the incident photon flux. Photobleaching quantum yields were determined for bacteriochlorins by equation 10 and by equation 8 for porphyrins and chlorins.

The photobleaching quantum yield of  $Cl_2BOH$  solutions at pH 7, 5 and 10 were irradiated at 750 nm with a Xe lamp. A 40 nm bandpass filter centered at 750nm was interposed between the lamp and the sample. The solution was continuously stirred under irradiation. Photobleaching quantum yield was determined by equation 10.

#### Intracellular distribution

The intracellular distribution of bacteriochlorins  $F_2BMet$  and porphyrin  $F_2PMet$  was assessed in A-549 cell line. A-549 cells were plated at a density of  $15 \times 10^3$  cells per well in eight-well slides (IBIDI, Germany) and were kept at 37 °C in a 95 % atmospheric air and 5% CO<sub>2</sub> humidified atmosphere, for 24 h. After being washed with fresh medium, the cells were incubated in the dark with 5  $\mu$ M sensitizers, diluted in cell medium, for  $\approx$ 18 h, at 37°C in a CO<sub>2</sub> incubator (5 % (v/v) CO<sub>2</sub> in air). After being washed with HBSS/Hepes buffer, the cells were incubated with specific intracellular organelle probes: 100 nM of Mito-tracker green, 1 mM ER-Tracker green, 75 nM Lyso-Tracker green (Molecular Probes, Invitrogen Life Technologies), diluted in HBSS/HEPES buffer. After ~ 30 min incubation, at 37 °C, in the dark, the cells were washed with HBSS/HEPES buffer and the slide was transferred to the microscope stage. Cells were visualized under a confocal microscope LSM 510 Meta; Carl Zeiss, Jena, Germany) with a 63× oil immersion objective (Plan-Apochromat, 1.4 NA Carl Zeiss). Images from the porphyrin and bacteriochlorin were obtained by exciting at 514 nm using an argon laser (45 mW) and fluorescence was detected after passage through a long-pass filter 575 nm and 700 nm, respectively. To obtain the fluorescence profile of the fluorescence probes a helium-neon laser (5 mW) was used as light source at 633 nm for visualization of cell morphology.

#### Light induced fluorescence changes in HeLa cells

HeLa cells, a cervical cancer subline, was used for single cells experiments. Cells were grown in Eagle's Minimum essential medium (EMEM, Sigma, Deutschland) supplemented with 2 mM L-glutamine (Sigma), 1 % nonessential aminoacids (Sigma), 1 % penicillin-streptomycin (Sigma) and 10 % fetal calf serum (Sigma). The cells were maintained at 37 °C in a humidified 5 % CO<sub>2</sub> atmosphere and platted when 80 % of confluence was achieved. After washing them with phosphate buffered saline (PBS) and trypsinizing (0.25 % trypsin, Sigma), they detached from the bottom of the cultivation flask. The cell suspension was collected and centrifuged (2 min, 1000 rpm) washed with cultivation medium, re-suspended in the cultivating medium and plated onto poly-D-lysine coated cover slips in 12 well plates. The cell density can vary from experiment to experiment. After seeding, the cells were left to settle and restart growth for at least 24 h before the sensitizer was incubated.

Hydrophilic sensitizers:  $F_2POH$  (10  $\mu$ M),  $F_2BOH$  (10  $\mu$ M),  $Cl_2POH$  (10  $\mu$ M),  $Cl_2BOH$  (10  $\mu$ M) and hydrophobic sensitizers:  $F_2PMet$  (10  $\mu$ M),  $F_2BMet$  (10  $\mu$ M),  $Cl_2PEt$  (10  $\mu$ M),  $Cl_2BEt$  (10  $\mu$ M) were added to the cell medium, and left to incubate for 24 h.

After incubation cells were washed with PBS and the cover slip was transferred to a microscope slide, and visualized under an Olympus IX70 inverted microscope. The irradiation of the entire cell and surroundings was achieved by a steady-state Xe Lamp using interference filters to

select the appropriate excitation wavelength. Light emitted by the sample was detected through interference filters using a CCD camera (Evolution QEi controlled by ImagePro software, Media Cybernetics) placed at the image plane of the microscope. For bright-field images a tungsten lamp as an accessory of the microscope was used.

For the detection of fluorescence spectrum from cells, the emitted light was collected by the microscope objective and then coupled onto an optical fiber connected to a spectrometer and CCD detector (USB 2000-FLG Ocean Optics, Florida, USA).

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# Detection of reactive oxygen species in mammalian cells

- 4.1 Introduction
- 4.2 Results
  - 4.2.1 Microscopy based singlet oxygen phosphorescence detection in single HeLa cells.
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# **4.1 Introduction**

Over the years, a number of different and sophisticated optical techniques have been developed to detect singlet oxygen from bulk ensembles. Only more recently singlet oxygen direct detection from single mammalian cells was demonstrated by Ogilby et al.<sup>1-3</sup>

Ogilby exploited the  $D_2O/H_2O$  solvent isotope effect to collect appreciable singlet oxygen phosphorescence signals from single cells.<sup>4-6</sup> Singlet oxygen lifetimes in the range 15-30 µs were obtained in  $D_2O$ -incubated cells, and the lifetime was found to be dependent on the sensitizer subcellular localization.<sup>6</sup> The authors have estimated the singlet oxygen lifetime to be ~3 µs in H<sub>2</sub>O-incubated cells. This value of lifetime would significantly increase the radial diffusion of singlet oxygen in cells from what it was previously presumed (~ 10-300 ns).<sup>7,8</sup>

Singlet oxygen is the reactive oxygen species most efficiently produced by photosensitizers used in photodynamic therapy and also by most of the photosensitizers considered in this work. Because of its great importance in mediating cell death, the singlet oxygen mechanism of action and kinetics has been extensively studied.

We have independently incorporated hydrophobic and hydrophilic photosensitizers into Hela cells and recorded the lifetime of singlet oxygen generated intracellularly in different spatial domains. We analyzed the results with the kinetic model exemplified in the scheme below. The mechanism of singlet oxygen generation and removal in a solution has already been discussed in chapter 2. When we try to address this same issue in the cell environment, we need to consider that singlet oxygen removal can now occur by physical quenching but can also involve chemical reactions with the surroundings in its site of action.

 $T_{1}+O_{2}(X^{3}\Sigma_{g}) \xrightarrow{k_{form}} O_{2}(a^{1}\Delta_{g}) \xrightarrow{k_{\Delta}} Singlet \text{ oxygen removal}$ 

The photosensitized production of singlet oxygen from the triplet excited state of the photosensitizer should follow the kinetics described by equation 4.1. In a phosphorescence experiment, the 1275 nm signal will be described as a difference of two exponential functions,

$$\begin{bmatrix} {}^{1}O_{2} \end{bmatrix}_{t} = \frac{k_{form} {}^{3}O_{2}[T_{1}]_{0}}{k_{\Delta} - k_{T}} \{ exp(-k_{T}t) - exp(-k_{\Delta}t) \}$$
(4.1)

We consider the sum of all processes by which singlet oxygen can be removed (i.e., chemical and physical channels) through the first order constant,  $k_{\Delta}$ . The reciprocal of this rate constant,  $1/k_{\Delta}$ , defines the lifetime of singlet oxygen,  $\tau_{\Delta}$ .

If the rate constant for singlet oxygen removal,  $k_{\Delta}$ , is much smaller than the rate constant for singlet oxygen generation, then the lifetime of singlet oxygen is obtained from the falling portion in the time-resolved phosphorescence signal. However, when working with cells, the rate constant for singlet oxygen deactivation may become much larger and the rate constant for the decay of the singlet oxygen precursor much smaller. Depending on the working conditions the lifetime of singlet oxygen may appear in the rising portion of the signal.

To avoid possible misunderstandings, in the conditions where it becomes difficult to accurately determine if the singlet oxygen lifetime is defined by the rising or by the falling part of the phosphorescence signal, we have made use of the solvent isotope effect by preferentially record singlet oxygen traces from  $D_2O$  based systems. The lifetime of singlet oxygen in  $D_2O$  (~67 µs) is substantially longer than that in  $H_2O$  (3.5 µs).<sup>9</sup>

# 4.2 Results

# **4.2.1** Microscopy based singlet oxygen phosphorescence detection in single HeLa cells.

Time-resolved singlet oxygen phosphorescence traces obtained from single cells incubated independently with hydrophilic and hydrophobic porphyrins (TMPyP,  $F_2POH$  and  $F_2PMet$ ) and a hydrophilic bacteriochlorin  $F_2BOH$ , in a D<sub>2</sub>O-based medium, are presented in Figure 4.1.



**Figure 4.1** Time-resolved singlet oxygen phosphorescence traces recorded in single cell experiments using HeLa cells in a D<sub>2</sub>O-based medium. A) Traces recorded for TMPyP after 3 min ( $\blacksquare$ ) and after 9 min ( $\bullet$ ) of irradiation at 420 nm, B) Data recorded for F<sub>2</sub>POH after 3 min ( $\blacksquare$ ) and 9 min ( $\bullet$ ) of irradiation time at 420 nm, C) Data recorded for F<sub>2</sub>PMet after 3 min of irradiation at 420 nm ( $\blacksquare$ ), and D) Data recorded for F<sub>2</sub>BOH after 3 min of irradiation at 745 nm ( $\blacksquare$ ). Data presented by ( $\nabla$ ) were recorded for the respective sensitizer in the presence of BSA and data presented by ( $\Box$ ) were recorded in the presence of NaN<sub>3</sub>.

It is clear that the kinetics of the singlet oxygen phosphorescence traces recorded for the hydrophilic dyes (TMPyP and F<sub>2</sub>POH) evolve as a function of the elapsed irradiation time. Both the time constants for the rise  $(1/k_T)$  and fall  $(1/k_{\Delta})$  get increasingly longer as light is delivered to the system. The values of  $\tau_{\Delta}$  obtained can vary from 15-20 µs to 40 µs after several minutes

of irradiation for porphyrins TMPyP and F<sub>2</sub>POH. For the hydrophobic F<sub>2</sub>PMet and the hydrophilic F<sub>2</sub>BOH, the evolution in the singlet oxygen lifetime under irradiation was much less pronounced. We were not able to resolve the rising portion on the phosphorescence trace and the decay gives  $\tau_{\Delta}$  of ~30 µs and 15 µs, respectively. F<sub>2</sub>PMet, is subcellularly localized mainly in membrane-based structures, and it is well known that the triplet state lifetime in hydrocarbon solvents is smaller compared to water, because of the higher concentration of oxygen, i.e., k<sub>T</sub> (aqueous) <k<sub>T</sub> (hydrocarbon).

A similar phenomenon of irradiation-induced changes in  $\tau_{\Delta}$  in H<sub>2</sub>O incubated cell suspensions was observed by Scholothauer<sup>10</sup> and Hackbarth<sup>11</sup> for pheophorbide-*a* and by Kuimova et al <sup>12,13</sup> from single cells. Kuimova et al considered that these observations were consistent with light induced viscosity changes during cell death.

#### Spatial localization and scattered light

For the detection of singlet oxygen phosphorescence in a single cell experiment, the excitation laser light is typically focused by the microscope objective into a beam waist of ~1  $\mu$ m, at the sample, which is smaller than the cell diameter (30-40  $\mu$ m).<sup>14</sup>

In the laser focal volume, singlet oxygen can mediate significant changes in the reactive substrates (e.g., protein oxidation) which could certainly have a significant effect on the kinetic profile of the singlet oxygen phosphorescence signal recorded.<sup>15</sup> However, the excitation light is appreciably scattered by the cell,<sup>16</sup> and although a portion of the 1275 nm phosphorescence signal detected will come from a localized intracellular domain in or near the laser focal volume, an appreciable amount of the signal still comes from singlet oxygen produced in other parts of the cell and possibly also from other cells, where scattered light is absorbed by the sensitizer (Figure 4.2). Because the scattered light is less intense, the singlet oxygen phosphorescence emitted from these spatial domains will likely contribute to the overall signal observed with a kinetic profile that is different from that in the laser focal volume. In short, pronounced oxygenation of reactive substrates within the localized spatial domain of the focused laser will not be the only thing that influences singlet oxygen phosphorescence data in the single cell experiments.



**Figure 4.2** Laser induced irradiation of HeLa cells incubated with TMPyP in  $D_2O$ -based medium. Fluorescence detected under laser irradiation at 420 nm: A) after a few seconds and B) after 18 min. The spot in the figure represents the laser focal point.

#### Irradiation power and elapsed irradiation time

To collect a time-resolved  $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g)$  phosphorescence trace from a single cell experiment, we have used a fluence of 7nJ per pulse and collect the 1275 nm emission for a period of time of 3 min using a multichannel scaler and a laser repetition rate of 1 kHz. Under these conditions, we generally observe morphological changes in the cell typical of a necrotic cell death (Figure 4.3, left-hand panel). No specific cell viability assays were done, but from the bright field images before and after irradiation we can identify morphological changes as vacuole formation, loss of membrane adhesion to the plate or condensation of chromatin in the nucleus.<sup>17-20</sup>In the control experiment of HeLa cells irradiated under the same conditions but without being incubated with a photosensitizer we have not observed any signs of cell damage.

#### Influence of incubation medium.

In order to avoid complications related to the presence of certain proteins and/or indicators in the normal cultivating medium, it can be more advantageous to use a specially-composed maintenance medium.<sup>21</sup> It was shown that HeLa cells tolerate  $D_2O$  quite well for the first ~5 h of application in the absence of light. <sup>4,16,18</sup> Nevertheless, if the cell handling and incubation protocol involves periods of time longer than ~5-6 h, this can have adverse effects in the cell population when the maintenance medium is used.<sup>4</sup> We have always tried to ensure that we use live and viable cells at the start of a given experiment though this can be a non-trivial issue given the difficulties in the subcellular incorporation of some photosensitizers and the perturbations caused by replacing H<sub>2</sub>O with D<sub>2</sub>O when we optimize the weak singlet oxygen phosphorescence signal.

We have adopted two different incubation protocols to record singlet oxygen phosphorescence. The singlet oxygen traces presented in Figure 4.1 were recorded from HeLa cells incubated with the sensitizer in the specially-composed maintenance medium, and show signs of perturbations after incubation (Figure 4.3 right-hand column). In the left-hand column, HeLa cells were incubated with TMPyP in a cultivating medium and were exposed to the maintenance medium during the  $H_2O/D_2O$  exchange (~3 min) and experiment. Under these last conditions, and after the irradiation time used to record the singlet oxygen traces, vacuole formation from cells it is readily observed (right-hand column on Figure 4.3). The appearance of defined vacuoles under irradiation was already discussed in this work as a one of the visible responses to light induced oxidative stress caused on a viable cell in the process of cell death. The lack of a similar response to the cells which have been incubated with maintenance medium shows that most likely the cell "machinery" that gives rise to the formation of vacuoles is no longer working. The selection of cells to be used to detected singlet oxygen is limited to a bright field image, no viability assays are executed because they would interfere with the experiment itself, and so this can lead to perform the experiment in a dead or dying cell.

A consequence from working with cells that are dead or dying at the start of the experiment is that we detect more easily the singlet oxygen phosphorescence signals. When cells are incubated in maintenance medium, we have observed that there is an increase in the uptake of the sensitizer and that in the case of TMPyP and  $F_2POH$  there is a preferential migration to the nuclei of the cells.<sup>22</sup>





**Figure 4.3** Bright field images obtained from cells incubated in  $H_2O$  (left-hand column) and  $D_2O$  (righthand column) medium with TMPyP. After moderate exposure of cells with TMPyP to focused laser light (for 3 min.), vacuole formation indicative of cell necrosis was apparent for the cells that were initially alive (left panel), whereas essentially no morphological changes were observed upon irradiation of dead/dying cells (right panel). Kinetic traces recorded from cells incubated with TMPyP as described in the left side panel after 3 min (dotted line) and 9 min of irradiation time (solid line).

#### Quenching of singlet oxygen: BSA and H<sub>2</sub>O

It is important to ascertain that the recorded singlet oxygen signal is only a reflection of an intracellular singlet oxygen population. We have made use of bovine serum albumin (BSA), a protein that quenches singlet oxygen with a rate constant of  $k_q = \sim 5.0 \times 10^8 \text{ s}^{-1} \text{ M}^{-1} \text{ }^{23}$  and does not cross the outer membrane of HeLa cells,<sup>2,5</sup> in order to avoid the detection of singlet oxygen that may have diffused or that may have been generated extracellularly. Extracellular singlet oxygen can be generated from photosensitizer molecules that cross the extracellular membrane under irradiation and are excited by scattered light.

We incubate both live and, independently, dead/dying cells for over 1h in a medium containing fluorescein-labeled BSA. Upon washing the cells with fresh medium, no fluorescein fluorescence was detected from HeLa cells. This not only confirms that BSA does not enter HeLa cells but also that is not adsorbed onto the cell membrane.

In the presence of BSA, singlet oxygen lifetimes are now reduced to ~13-20  $\mu$ s which are consistent with the values obtained by Snyder et al.<sup>5</sup> and with the value of singlet oxygen lifetime obtained from a live cell (trace on Figure 4.3). Additionally, in the presence of BSA, the rising portion of the singlet oxygen phosphorescence signal, observed for the hydrophilic sensitizers, generally disappears or gets appreciably faster. The singlet oxygen traces obtained in the presence of BSA are presented on Figure 4.1 ( $\nabla$ traces) and the values of  $\tau_{\Delta}$  are summarized on Table 4.1. These results show that a large fraction of the singlet oxygen. Nevertheless the reduction on the singlet oxygen lifetime in the presence of BSA shows that a non-negligible fraction of the singlet oxygen detected is detected from outside the cell. This can result from the propensity of hydrophilic dyes, to diffuse out of the cell under irradiation (enhanced by possibly a more leaky membrane of the perturbed cells) into a D<sub>2</sub>O-based medium which has low concentration of singlet oxygen quenchers. This would give rise to a longer lived population of singlet oxygen.

Increasing the percentage of H<sub>2</sub>O (~3.5  $\mu$ s)<sup>24</sup> in a D<sub>2</sub>O-based (~67  $\mu$ s )<sup>25</sup> system will result in singlet oxygen quenching. It was previously reported that singlet oxygen lifetimes obtained from TMPyP sensitized single cell experiments indeed get shorter as the amount of H<sub>2</sub>O added to the system is increased.<sup>4</sup> The rate constant for singlet oxygen quenching obtained from these experiments,  $3.0 \pm 0.7 \times 10^3$  s<sup>-1</sup> M<sup>-14</sup> was consistent with that expected for H<sub>2</sub>O (i.e., 3.5  $\mu$ s × 55M)<sup>-1</sup> ~ 5 × 10<sup>3</sup> s<sup>-1</sup> M<sup>-1</sup>) quenching. We have performed H<sub>2</sub>O quenching experiments using TMPyP and, independently, the hydrophobic porphyrin F<sub>2</sub>PMet, as sensitizers under conditions where singlet oxygen traces were recorded from HeLa cells in the presence of BSA. For the data recorded using the hydrophilic sensitizer TMPyP (Figure 4.4), the plot of k<sub>Δ</sub> against the

concentration of H<sub>2</sub>O is reasonably linear and yields a quenching rate constant of  $2.5 \pm 0.2 \times 10^3$  s<sup>-1</sup> M<sup>-1</sup>, consistent with the data recorded in the absence of BSA and also for the general quenching by H<sub>2</sub>O. Importantly, singlet oxygen in D<sub>2</sub>O is more cytotoxic than in H<sub>2</sub>O, and irradiation of a system with more D<sub>2</sub>O gives rise to a population of cells "more dead", and its components should appear less effective as quenchers of singlet oxygen, potentially originating in a curvature in the plot on Figure 4.4. However, the data on Figure 4.4 gives a linear plot, which indicates that for cells that were already dead/dying at the start of the experiment, most of the pertinent intracellular damage was already done and/or the damage is irrelevant to the intracellular domains from which a significant fraction of our singlet oxygen signal is originated. This latter conclusion is supported by our BSA studies that show that  $\tau_{\Delta}$  recorded in the presence of BSA does not depend appreciably on the time of sensitizer irradiation.



**Figure 4.4** Plot of  $k_{\Delta}$  against the concentration of H<sub>2</sub>O of TMPyP incubated in HeLa cells in a D<sub>2</sub>O-based medium containing 0.75 mM BSA. Each point represents the average of data from at least 5 cells, and the slope yields  $k_q$ = 2.5 × 10<sup>3</sup> s<sup>-1</sup> M<sup>-1</sup>.

It was difficult to determine the quenching rate constant by  $H_2O$  of singlet oxygen generated intracellularly in HeLa cells incubated with  $F_2PMet$ , and the results were non-systematic. Nevertheless, the presence of  $H_2O$  on HeLa cells incubated with this sensitizer resulted in a consistent decrease of the singlet oxygen lifetime, which indicates that singlet oxygen generated in lipophilic intracellular domains is still quenched by  $H_2O$ . Also consistent with this latter point, is the observed decreased on singlet oxygen lifetime generated by this hydrophobic sensitizer, when NaN<sub>3</sub> is present in the cells. NaN<sub>3</sub> is a hydrophilic quencher which subcellular localization should not meet  $F_2PMet$ . These results are consistent with the model that singlet oxygen readily crosses the interface between hydrophobic and hydrophilic domains.

		$ au_{\Delta}$ /μs	
		Single cells	Cell
			suspensions
Hydrophilic sensitizers			
TMPyP	Irradiation dependence	$\sim 15 \pm 6 \rightarrow \sim 40 \pm 4$	~40 ± 4
	with BSA	$\sim 15 \pm 3 - 20 \pm 5$	~4.5 ± 1
	with BSA		$6\pm2^1$
F <sub>2</sub> POH		$\sim 25 \pm 6 \rightarrow \sim 40 \pm 4$	
	with BSA	$\rightarrow$ ~20 ± 6	
F <sub>2</sub> BOH		~15 ±	
	with BSA	~13.6 ±	
Hydrophobic sensitizers			
F <sub>2</sub> PMet	Irradiation dependence	$\rightarrow \sim 30 \pm 6$	
	with BSA	→~17 ± 3	
PPa	Irradiation dependent		$\rightarrow \sim 27 \pm 5$
	with BSA		<~5 ± 2
Chlorin <sup>2</sup>		$\rightarrow \sim 17 \pm 2$	

1) skin fibroblasts from  $^{26}$ , 2) from  $^{6}$  Standard deviation obtained for at least 5 cells (single cells) or two independent experiments (cell ensemble).

#### Effect of localized photoxidative chemistry.

Proteins play a significant role in the removal of intracellular singlet oxygen due to their high abundance in a cell and large rate for interaction with singlet oxygen.<sup>27</sup> Proteins generally remove singlet oxygen via chemical reactions and not physical deactivation,<sup>27</sup> and singlet oxygen could, over time, deplete the local concentration of effective singlet oxygen quenchers, which would, give rise to increasingly longer singlet oxygen lifetimes. Under focused laser irradiation one could think that in our microscope based single cell experiments we would deplete a pool of good intracellular quenchers of singlet oxygen. Protein structural changes which may accompany singlet oxygen mediated cell death can have an effect on singlet oxygen lifetime.<sup>15,28</sup> However, our data in the presence of BSA seems to indicate that the effects of localized photo-oxidative chemistry do not appear to have a pronounced influence in the observed values of  $\tau_{\Delta}$ . Even upon prolonged irradiation, we still record values of  $\tau_{\Delta}$  that are equivalent to those recorded from an "unperturbed cell" (phosphorescence traces on Figure 4.4  $\tau_{\Delta}$ ~ 15-20 µs)

#### Sensitizer light-induced relocalization and bleaching.

Given the laser fluences required for the microscope based single cell experiments of 7 nJ per pulse, photobleaching of the photosensitizer during the irradiation time can have a marked effect on the intensity of the singlet oxygen signal recorded. <sup>13,29</sup> The photoinitiated reactions can result not only in the degradation of the sensitizer but can also induce changes in the surrounding environment leading to the relocalization of the photosensitizer in the cell and extravasation to the extracellular medium. TMPyP and F<sub>2</sub>POH are clear examples of lightinduced intracellular relocalization and leakage out in the extracellular medium (Figure 4.21 on section 4.2.4, and figure 3.11 on section 3.2.2, respectively)<sup>17,22,30-32</sup> confirming the conclusion made on the basis of the BSA quenching experiments. Thus the kinetics and signal intensity for singlet oxygen generation and removal may change over the course of the experiment influenced by the photobleaching products and by relocalization.<sup>33</sup> No evolution in the rate constant of singlet oxygen formation and removal is observed for the bacteriochlorin F<sub>2</sub>BOH with increasing the irradiation time. A possible explanation for this is the low photostability of  $F_2BOH$  in cells, as it was demonstrated in chapter 3, which compromises the relocalization of the dye in the cell and leakage to the extracellular medium.  $F_2PMet$ , on the other hand, is a very stable dye, and in this case the most likely explanation is related to the local environment of where singlet oxygen is generated.

#### 4.2.2 Cuvette-based singlet oxygen phosphorescence detection in cell suspensions

To complement the microscope-based singlet oxygen experiments, we carried out independent experiments using cell suspensions. For these studies, we used nonadherent HL-60 cells, the experiments were run in D<sub>2</sub>O-based medium and sensitizers were incubated in culture medium and in the specially-composed maintenance medium. The choice of a D<sub>2</sub>O based medium instead of a H<sub>2</sub>O-based medium is related to the difficulty of decoupling the rate constant for singlet oxygen removal,  $k_{\Delta}$ , from the rate constant of oxygen generation,  $k_T$ , because  $k_T \sim k_{\Delta}$  in H<sub>2</sub>O-based media. Additionally the time constants involved (i.e.,  $1/k_T$  and  $1/k_{\Delta}$ ) are comparatively short, and it is difficult to accurately quantify  $k_{\Delta}$ , in H<sub>2</sub>O-based media even with the appropriate control experiments. We have performed experiments with hydrophilic (TMPyP and PPa) and hydrophobic (F<sub>2</sub>PMet) sensitizers, and Figure 4.5 shows representative singlet oxygen phosphorescence traces recorded for TMPyP in cell suspensions.



**Figure 4.5** Time-resolved  $O_2$  ( $a^1\Delta_g$ )  $\rightarrow O_2$  ( $X^3\Sigma_g$ ) phosphorescence traces obtained from suspensions of HL-60 cells in a D<sub>2</sub>O-based medium and incubated with TMPyP. The sensitizer was irradiated at 420 nm using an average power of 1 mW (laser operated at 1 kHz). Data were acquired over a period of 1 min. A fit of eq. 4.1 to the data is shown as a solid line in each trace. A) Trace recorded after 1 min of irradiation, yielding  $\tau_{\Delta} = 40 \ \mu$ s. B) Trace recorded under the same conditions, only with 0.75 mM BSA present in the medium,  $\tau_{\Delta} \sim 4.5 \ \mu$ s.

In the absence of BSA or NaN<sub>3</sub>, we recorded singlet oxygen lifetimes in the range of 39 - 42  $\mu$ s and time constants for the singlet oxygen formation of ~2.0 - 4.5  $\mu$ s (Figure 4.5). The rise time is consistent with the time constant obtained by independent measurements of TMPyP phosphorescence in cells at 900 nm, a wavelength where there is no emission from singlet oxygen. An irradiation-dependence increase in  $\tau_{\Delta}$  was observed, although this was a subtle increase of only ~3  $\mu$ s. Cells incubated in the maintenance medium instead of the cultivating

medium revealed similar lifetimes. In the presence of 3.2 mM NaN<sub>3</sub>, we observed a substantial decrease in the intensity of the singlet oxygen and the expected reduction on  $\tau_{\Delta}$  to ~0.6 µs, which correspond to a quenching rate constant of singlet oxygen by NaN<sub>3</sub> of 5 × 10<sup>8</sup> s<sup>-1</sup> M<sup>-1</sup> in water <sup>13</sup>. No change in the time constant for oxygen formation occurred.

Experiments performed in the presence of 0.75 mM of BSA in the cell medium, likewise resulted in an appreciably decrease of singlet oxygen signal intensity and lifetime to  $\tau_{\Delta}$ ~ 4.5 µs (Figure 4.5 B).This value is consistent with what is expected for the quenching of singlet oxygen by BSA in a homogeneous solution (i.e., 0.75 mM BSA and  $k_q = 3 \times 10^8 \text{ s}^{-1} \text{M}^{-1}$ ).<sup>2,23</sup>

Once again, we have confirmed by an independent experiment with HL-60 cells incubated with BSA-fluorescein that BSA does not enter the cells in the course of our experiments. These observations show that an appreciable amount of the singlet oxygen phosphorescence signal detected in sensitized cell suspensions comes from an extracellular population of singlet oxygen.

Such pronounced effect is consistent with data recorded from suspension of skin fibroblasts incubated with TMPyP by Jimenez-Banzo<sup>26</sup> and from leukemia cells incubated with a hydrophilic aluminium phtalocyanine<sup>34</sup>.

Some possible explanations related to cell preparation and maintenance during the experiment can be proposed: i) after incorporation of the sensitizer the procedures used to wash the cells with sensitizer-free medium are ineffective and do not completely remove residual extracellular sensitizer, ii) once the cells are washed and resuspended in a sensitizer-free medium, the intracellular sensitizer diffuses out to the extracellular domain along the newly established concentration gradient and stirring of the cells can exacerbate the permeability of the membrane.<sup>35</sup> These effects are enhanced by light-induced perturbation of the cells, because the cell membrane tends to become more permeable to diffusion.

It is important to recognize that the effect of sensitizer diffusion into the extracellular medium will be more pronounced in suspension cell measurements because light propagates through a 1 cm thick sample that is dominated by the medium rather than by the cells. In microscope-based single cell experiments, the incident light is focused into the cell and the extracellular medium is irradiated only as a consequence of scattered light (i.e., the fraction of extracellular excitation is much smaller in the microscope-based study).

In order to avoid some of the problems associated with recording singlet oxygen signals in cell suspensions using a hydrophilic dye, we have recorded data from the lipophilic sensitizer  $F_2PMet$  (Figure 4.6). Although, we found it difficult to obtain consistent results with this photosensitizer in cells suspension, a 1275 nm emission signal from  $D_2O$ -based suspension

cells, upon irradiation at 420 nm and in the presence of BSA resulted in a decay with a  $\tau < \sim 4.5$  µs. When NaN<sub>3</sub>, a hydrophilic quencher, was added to the medium, no effect was observed on the lifetime recorded, possibly because the lifetime observed was already very short.



**Figure 4.6** Time-resolved singlet oxygen trace from HL-60 cells incubated with F<sub>2</sub>PMet in a D<sub>2</sub>O-based medium. The data recorded after 4 min of elapsed irradiation time in the presence of BSA (0.75 mM), fit with a single exponential yield  $\tau_{\Delta} = 4.5 \ \mu s$ .

We have used PPa as another lipophilic sensitizer which readily incorporates into a variety of cells and it is known to localize in the plasma membrane and other membranes in the cytoplasm.<sup>17,36-38</sup>

Despite the facile bleaching of this dye, we have been able to record singlet oxygen phosphorescence signals from HL-60 cells incubated with PPa (Figure 4.7).

Upon an elapsed irradiation time of 1 min of HL-60 cells incubated with PPa, singlet oxygen phosphorescence signal was detected at 1275 nm and fit to the sum of two exponential decays with  $\tau_1 = 2.9 \pm 0.1 \ \mu s \ \tau_2 = 18 \pm 1 \ \mu s$ . After irradiation for additionally 4 min, the rate of appearance and disappearance of singlet oxygen clearly decrease ( $\tau_{rise} = 0.8 \pm 0.1 \ \mu s$ ;  $\tau_1 = 4.1 \pm 0.1 \ \mu s$ ;  $\tau_2 = 27 \pm 3 \ \mu s$ ). In the presence of 50 mM NaN<sub>3</sub> the signal is reduced to  $\tau = 3.2 \pm 0.1 \ \mu s$ . The addition of BSA to the surrounding medium removes the long-lived component (after 1 min irradiation the trace is best fit with a single exponential decaying function with  $\tau_{decay} = 3.7 \pm 0.1 \ \mu s$ , after 4 min of irradiation the trace is best fit as the difference of two exponential functions with  $\tau_{rise} = 1.0 \pm 0.1 \ \mu s$  and  $\tau_{decay} = 5.4 \pm 0.1 \ \mu s$ ). With added NaN<sub>3</sub>, and after 4 min of irradiation a single exponential decaying fit gives  $\tau = 3.0 \pm 0.1 \ \mu s$ .

The interpretation of these data is not trivial, we are in a domain where  $k_T \sim k_{\Delta}$  but also appears that both  $k_T$  and  $k_{\Delta}$  change during the course of the experiment. Nevertheless, an appreciable amount of the 1275 nm emission signal can be attributed to singlet oxygen phosphorescence on

the basis of the  $NaN_3$  quenching. Moreover, from the BSA experiment, it appears that the bulk of the signal that we assigned to singlet oxygen phosphorescence is originated inside the cell.



**Figure 4.7** Time resolved 1275 nm emission traces recorded upon 420 nm irradiation of PPa in suspensions of HL-60 cells in a D<sub>2</sub>O-based medium. A) Data recorded in the absence of added BSA. B) Data recorded in the presence of 0.75 mM BSA. ( $\circ$ ) Traces recorded after an elapsed irradiation period of 1 min ( $\blacksquare$ ) Traces recorded after an elapsed irradiation period of 4 min and ( $\blacktriangledown$ ) trace recorded in the presence of NaN<sub>3</sub> (50 mM).

Even though we are working with  $D_2O$ -incubated cells, the lifetime of singlet oxygen recorded from suspension cells appears to be shorter than that recorded in our single cell experiments. Since the radiation fluence required to record a singlet oxygen signal from cell suspensions is less than that used in our single cells experiments, the shorter values of  $\tau_{\Delta}$  recorded from suspension cells containing PPa could reflect a higher concentration of effective intracellular singlet oxygen quenchers (e.g. proteins) that have yet to be fully oxygenated/oxidized upon elapsed irradiation, compared to our single cell experiments. However, this interpretation contradicts the conclusions obtained through a variety of independent single cell experiments which point a value of  $\tau_{\Delta}$  ~15-20 µs to the inherent intracellular lifetime in a D<sub>2</sub>O incubated cell. A more likely explanation is that a high concentration of PPa co-localized in membrane domains would yield singlet oxygen with short  $\tau_{\Delta}$ . This is support by an independent experiment of PPa (with 1% added DMSO to facilitate PPa dissolution) prepared on unilamellar vesicles (i.e., liposomes). Both the absorption and fluorescence spectra of this system of PPa in liposomes were characteristic of PPa aggregation (i.e., spectrally broadened absorption bands, weak fluorescence intensity), and most importantly no singlet oxygen signal was detected. This efficient quenching could be due to high local concentrations of PPa and/or to the inability of PPa aggregates to make appreciable amounts of singlet oxygen. However, upon the addition of Triton X-100 to the PPa-liposomes system an appreciably singlet oxygen signal was observed correlated to liposomes fragmentation into smaller units in which presumably PPa was less packed. Considering this interpretation, experiments performed with incubation times and

different concentrations in the medium of the HL-60 cells did not yield the expected changes on the kinetics of singlet oxygen, which may indicate that PPa concentration in the HL-60 cells membranes may not appreciably change.

The irradiation dependence change in  $k_T$  could reflect changes in the extent to which PPa binds to proteins as the proteins are oxidized by singlet oxygen and/or the viscosity of the environment around the sensitizer.

#### 4.2.3 Detection of oxygen radicals in cells

Bacteriochlorins generate singlet oxygen as the main reactive oxygen specie in ethanol solution ( $\Phi_{\Delta} \sim 0.4$ -0.8). Nevertheless, a significant fraction of the triplet states generated by these molecules can also generate charge transfer complexes with molecular oxygen that ultimately lead to the generation of superoxide and hydroxyl radical in DMSO and TRTX-100 solutions, respectively.



**Figure 4.8** Reaction of 3'-(*p*-aminophenyl) fluorescein (APF) with ROS (more specific to OH') resulting in a fluorescent form.

By direct detection of  ${}^{1}O_{2}$  phosphorescence at 1275 nm in HeLa cells, we have ascertained that bacteriochlorin, F<sub>2</sub>BOH, generates singlet oxygen also intracellularly. We had no direct way to evaluate if also other reactive oxygen species would be generated at the cellular level. Indirectly, we have made use of 3'-(p-aminophenyl) fluorescein, APF, a nonfluorescent probe that reacts rather selectively with the hydroxyl radical to release fluorescein which is fluorescent near 520 nm.<sup>39</sup>

#### Before irradiation



## After 15 min of irradiation



#### After 45 min of irradiation with 505 nm LED



**Figure 4.9**Fluorescence micrographs of A549 cells co-incubated with  $F_2BMet$  and APF. Left-panel shows bright field images of the cells before irradiation. Middle-panel shows red fluorescence from the photosensitizer when excited at 514 nm and with emission above 700 nm. Right-panel shows the fluorescence of fluorescein (excitation at 490 nm and emission at 510 nm) obtained after illumination of the cells with 505 nm LED (0.6 mW/cm<sup>2</sup>).

Figure 4.9 shows the fluorescence of F<sub>2</sub>BMEt in cells and the fluorescence of fluorescein observed after the irradiation of the cell culture at 505nm. Prior to that illumination, only a residual fluorescence from fluorescein emission was observed. Over the two sets of irradiation time (15 and 45 min) an increase on fluorescence intensity was recorded. It is clear that excitation of F<sub>2</sub>BMet in cells leads to the generation of a ROS that are capable of reacting with APF and generate the fluorescent fluorescein. Considering the preferential reaction of APF with hydroxyl radical rather than other reactive oxygen species such as singlet oxygen and superoxide,<sup>40</sup> one can conclude that, in addition to singlet oxygen, F<sub>2</sub>BMet promotes also the generation of hydroxyl radical in cells. These radicals are extremely reactive and can be expected to explore only a small reaction volume before being consumed. F<sub>2</sub>BMet is localized in the ER and mitochondria and, we would be expected these to be the primary targets of the hydroxyl radicals. F<sub>2</sub>BMet may initially generate hydroxyl radical by the mechanism proposed for solution, but the presence of Fe<sup>2+</sup> in cells will enhance the production of this specie by the Fenton reaction. The increased phototoxicity of bacteriochlorins over many sensitizers that exclusively follow a Type II photoensitization process has been associated to their ability to generate reactive oxygen species more cytotoxic than singlet oxygen, such as the hydroxyl radical.41

#### 4.2.4 Microscope based singlet oxygen detection – photobleaching considerations

To record a singlet oxygen trace from individual cells we make use of a focused laser to initiate a photosensitized reaction that produces singlet oxygen in the subcellular domain that is being irradiated. <sup>42-45</sup> At the laser fluences used to detect and monitor singlet oxygen phosphorescence, bleaching of the photosensitizer generally occurs over the elapsed irradiation time. The generation of ROS and the bleaching of the dye is not the only consequence of its electronic excitation. As we have observed in Chapter 2 for our porphyrins and bacteriochlorins, photomodification and relocalization are commonly observed after continuous light induced irradiation of these dyes at appropriate wavelengths.

Bleaching and relocalization can also occur in microscopy with fluorescent probes, used to assess cell structure and activity, under the conditions in which the cell is being examined, and this is a major limitation for their use.<sup>46-51</sup> This is a characteristic of fluorescein and other fluorescent probes which partly reflects the fact that, under many biologically-pertinent conditions, an excited state of the probe itself can sensitize the production of singlet oxygen in appreciable yield. <sup>52,53</sup>

Singlet oxygen traces recorded from dyes continuously changing over the irradiation time are collected without temporal or spatial control of the cytotoxic species created in situ. The amount of singlet oxygen produced varies over time, which is reflected in the intensity of the singlet oxygen phosphorescence signal recorded.<sup>45,54</sup> Moreover, the photobleached products can also influence the decay kinetics of singlet oxygen.<sup>25,55,56</sup>

In light of the bleaching-dependent problems inherent to working with cells, we set out to identify from a group of sensitizers, which ones are sufficiently stable to enable collecting reproducible data under the conditions of our single cell experiments. Additionally we also investigate whether solution experiments can be used to predict the behavior of a given sensitizer inside a cell.

Krieg and Whitten<sup>57</sup> reported in 1984 that the oxygen-dependent photobleaching of protoporphyrin IX in solution is significantly enhanced in the presence of selected amino acids and, independently, in the presence of erythrocyte ghosts which contain lipids as well as membrane proteins. A mechanism was proposed in which singlet oxygen creates, for example, a protein-based oxygenated intermediate (*e.g.*, a long-lived peroxide) which, in turn, can then oxidize the porphyrin. These observations have been substantiated in a number of studies where the photobleaching of selected chlorins was enhanced upon the addition of bovine serum albumin to the solution.<sup>58,59</sup> This indicates that the rate and extent of sensitizer bleaching in a

cell will depend on the immediate environment and, hence, intracellular location of that sensitizer.

We investigated the following non-porphyrin singlet oxygen sensitizers, that in view of their low cytotoxicity, and reasonable stability under irradiation in solution: benzo[cd]pyrene-5-one(BP),<sup>60</sup>N,N-dimethylfulleropyrrolidinium iodide (DMP-C<sub>60</sub>),<sup>61</sup> and 1,4-Bis[4-(N,N-diphenylamino)phenylethynyl]-2,5-dibromobenzene (BBB).<sup>62</sup>

<u>BP</u>



**Figure 4.10** Absorption spectra of BP as a function of the elapsed irradiation time in: A) benzene (1 kHz fs laser irradiation at 400 nm with 57 mW/cm<sup>2</sup>) and B) DMSO (irradiation at 420 nm with 43 mW/cm<sup>2</sup>). Arrows show the direction of irradiation-induced changes in the spectra.

The polycyclic aromatic molecule BP has an absorption profile that extends to wavelengths longer than 500 nm (Figure 4.10) and with a maximum centered at ~483 nm. The carbonyl group facilitates  $S_1 \rightarrow T_1$  intersystem crossing,<sup>60</sup> resulting in a weak fluorescence ( $\Phi_F \sim 0.004$  in toluene) at ~560 nm, and a singlet oxygen quantum yield close to unity.<sup>60</sup>



**Figure 4.11** Absorption spectra of BP in aqueous phosphate-buffered solutions, PBS, as a function of the elapsed irradiation time with a 1 kHz fs laser at 420 nm. A) Data were recorded over an elapsed irradiation period of 219 min with 17 mW/cm<sup>2</sup>. B) The sample also contained 0.75 mM Bovine Serum Albumin, BSA, and data were recorded over an elapsed irradiation period of 220 min with 29 mW/cm<sup>2</sup>. In both experiments, a small amount of DMSO (0.25% by volume) was added to facilitate BP solubilization.

BP is reasonably stable upon prolonged irradiation at 400 nm in oxygenated solutions of benzene (Figure 4.10 A). On the other hand, appreciable photoinitiated changes are rapidly observed when BP is dissolved in DMSO (Figure 4.10 B), which is reflected in the

comparatively large photodegradation quantum yield of  $7.02 \times 10^{-4}$ . This phenomenon was also observed for F<sub>2</sub>POH (Chapter 3), proving once again that DMSO appreciably increases the rates of many reactions.<sup>63</sup>

The isosbestic points in the spectra shown in Figure 4.10 suggest that, over the time period examined, only one photoproduct of BP is formed in each case. However, the absorption spectra shows that the photoproduct formed in DMSO may be different from that in benzene.

BP is hydrophobic, and it was previously dissolved in DMSO (~ 0.25% by volume) and then mixed with Phosphate buffer, PBS, aqueous solutions. The absorption spectra of BP in these conditions show a broadening of the absorption bands, when compared to the absorption spectra in organic solvents, explained by dye aggregation. Under irradiation (Figure 4.11 A) no major changes occurred over time. When Bovine Serum Albumin, BSA, is added to the solution in PBS, BP becomes more sensitive to irradiation (figure 4.11). A substantial increase of the absorption is observed in the U.V. which could be related to the photooxidation of BSA<sup>64</sup> by singlet oxygen generated by BP. It is expected that BP will not be particularly photostable under intracellular conditions where other oxidative mechanism can also play a role,. The data obtained presented in figure 4.12 are consistent with this expectation. The corresponding emission spectra recorded from HeLa cells incubated with BP indicate that the emitting molecules are not the same as those seen in solution experiments (Figure 4.13).



**Figure 4.12** Images of BP-containing HeLa cells based on emission detected at wavelengths longer than 500 nm upon excitation at 480 nm ( $4.1 \text{ mW/cm}^2$  from a *cw*Xe lamp). Images were recorded as a function of the elapsed irradiation time at 480 nm.



**Figure 4.13** BP-containing HeLa cells emission spectra detected at wavelengths longer than 500 nm and upon excitation at 480 nm (8 mW/cm<sup>2</sup> from a cwXe lamp). Images were recorded as a function of the elapsed irradiation time at 480 nm.

## BBB

BBB was the focus of earlier studies by Ogilby and co-workers, <sup>65</sup> that have shown that molecules containing alkyne-conjugated phenyl moieties are more stable than the corresponding molecule with alkene-based units upon exposure to singlet oxygen. Also, the rate of singlet-oxygen-mediated photobleaching markedly decreases by introducing electron-withdrawing substituents on such an alkene-based unit.<sup>66</sup>

However, upon incorporating the dye in cells it becomes extremely labile toward irradiation (Figure 4.14). The hydrophobicity of the dye would make us predict that it is likely to be localized in a lipophilic environment and clearly the local environment of the molecule will most likely play a role on the photostability of the molecule. The generation of singlet oxygen will rapidly lead to cell damage by oxidizing cellular components, such as lipids and proteins, that consecutively will initiate chain reactions.



**Figure 4.14** Images of BBB-containing HeLa cells based on emission detected at wavelengths longer than 500 nm upon excitation at 425/40 nm (6 mW/cm<sup>2</sup> from a *cw*Xe lamp). Images were recorded as a function of the elapsed irradiation time at 480 nm.

#### $DMP-C_{60}$

The construction of fullerene derivatives has been of great interest to biological fields because they may overcome the hydrophobicity of  $C_{60}$  without loss of the interesting photochemical and photophysical properties of this molecule.<sup>67-70</sup>

However, introducing perturbations in the conjugated system of  $C_{60}$  is known to change its photophysical properties.  $C_{60}$  itself has a singlet oxygen quantum yield of ~1.0 and is highly resistant to light induced degradation <sup>68,71,72</sup>

The derivative we selected to study, DMP-C<sub>60</sub> has been reported to have a singlet oxygen quantum yield of 0.27  $\pm$  0.02 in CH<sub>3</sub>OD (with 1% added DMSO).<sup>73</sup> In a D<sub>2</sub>O-based PBS solution (with 4% added DMSO), these same investigators were not able to detect a O<sub>2</sub>(a<sup>1</sup>Δ<sub>g</sub>)  $\rightarrow$  O<sub>2</sub>(X<sup>3</sup>Σ<sub>g</sub><sup>-</sup>) phosphorescence signal upon irradiation of DMP-C<sub>60</sub> implying that, under these latter conditions, the singlet oxygen quantum yield is appreciably less than 0.27.<sup>73</sup> Although the latter may partly reflect the aggregation of DMP-C<sub>60</sub>,<sup>73</sup> these observations also contribute to the suggestion that, under physiological conditions, irradiation of C<sub>60</sub> and its derivatives may be cytotoxic as a consequence of the production of oxygen-related radicals, and not singlet oxygen.<sup>61,73,74</sup> We can nevertheless still use this compound to address aspects of the photoinitiated oxygen-dependent degradation of intracellular sensitizers. The derivative we opted to study, DMP-C<sub>60</sub>, can be incorporated into a cell and can initiate cell death upon irradiation.<sup>61</sup>



**Figure 4.15** (A) Absorption spectra of DMP-C<sub>60</sub> in: A)  $H_2O/DMSO$  (9-to-1, by volume) as a function of the elapsed time with 355 nm irradiation (10 Hz ns laser at 45 mW/cm<sup>2</sup>). (B) Data were recorded under similar conditions from a solution that also contained 0.75 mM BSA. Arrows show the direction of irradiation-induced changes in the spectra.

Irradiation of DMP-C<sub>60</sub> at 355 nm in a bulk solution of H<sub>2</sub>O/DMSO (9-to-1, by volume) shows a modest degradation-dependent change in the absorption spectrum (Figure 4.15 A). This modest change is consistent with the comparatively small quantum yield of DMP-C<sub>60</sub> photodegradation in this solvent,  $\Phi_{pd} = 4.6 \times 10^{-7}$ . Upon irradiation of a corresponding solution containing 0.75 mM BSA, more pronounced changes in the absorption spectrum are observed (Figure 4.15 B). Most interestingly, however, these latter changes only influence the spectra at wavelengths shorter than ~ 400 nm, likely reflecting photoinduced changes in BSA. Thus, one might be able to exert some control in a cell-based DMP-C<sub>60</sub> experiment by irradiating at wavelengths longer than 400 nm.

Upon incubation of HeLa cells with DMP-C<sub>60</sub>, this dye was readily incorporated into the cells (Figure 4.16). Images obtained of these cells based on the fluorescence of DMP-C<sub>60</sub> show a punctate diffusional pattern of dye localization and a non-negligible amount of the dye appears to be randomly dispersed in the cytoplasm (Figure 4.16). Upon irradiation of these cells at 450 nm, only slight changes in the broad-band intensity of emitted light were observed, with much of the change occurring at early irradiation times. Most notably, there appears to be minimal irradiation-induced relocalization of the dye suggesting that if the dye is localized subcellularly inside an organelle no damage occurs when reactive oxygen species are produced by DMP-C<sub>60</sub>. This latter observation is in contrast to what is observed upon irradiation of TMPyP sensitizer that likewise initially is localized in lysosomes which appear to suffer a facile rupture of the lysosome upon irradiation (*vide infra*). It appears that DMP-C<sub>60</sub> might indeed be a sufficiently stable sensitizer for selected oxygen-dependent intracellular experiments, which generates reactive oxygen species capable of effectively induce cell death but that may do not involve singlet oxygen.



**Figure 4.16** Fluorescence images of HeLa cells that had been incubated with 25  $\mu$ M DMP-C<sub>60</sub> recorded as a function of the elapsed irradiation time at 450 nm (8.2 mW/cm<sup>2</sup> from *cw*Xe lamp). Emission was detected at wavelengths longer than 600 nm.

#### *TMPyP*

Porphyrin-based molecules have long been used as singlet oxygen sensitizers, particularly for PDT-related applications.<sup>75</sup> As it was discussed on chapter 3 the properties of these molecules are readily tuned by changing substituents and/or the extent to which the macrocycle is reduced (*i.e.*, chlorins and bacteriochlorins). Most importantly, these compounds are generally not cytotoxic in the absence of light.

The hydrophilic porphyrin TMPyP has been the basis for much of the single cell singlet oxygen phosphorescence work over the years.<sup>43,76,77</sup> It is readily incorporated into cells, produces singlet oxygen efficiently with a quantum yield of  $0.77 \pm 0.04$ ,<sup>78</sup> and is sufficiently fluorescent to allow for imaging experiments to ascertain its localization.<sup>45</sup> Indeed, these properties of TMPyP have made it a popular sensitizer in experiments to record singlet oxygen phosphorescence from cells.<sup>43,45,79</sup>



**Figure 4.17** Absorption spectra of TMPyP dissolved in a buffered aqueous solution containing 0.75 mM BSA. Data were recorded as a function of the elapsed time of irradiation at 420 nm (1 kHz fs laser, 50  $mW/cm^2$ ). Arrows indicate the direction of irradiation-induced spectral changes.

In a solution model where TMPyP is dissolved in a buffered solution containing BSA, photoinduced bleaching is readily apparent (Figure 4.17). As with other sensitizers, the quantum yield of TMPyP photodegradation in the presence of BSA  $(1.2 \times 10^{-5})$  is appreciably larger than that in a PBS solution lacking BSA  $(3.3 \times 10^{-6})$ .



**Figure 4.18** Fluorescence images of HeLa cells that had been incubated with 20  $\mu$ M TMPyP recorded as a function of the irradiation time at 425/40 nm (2.6 mW/cm<sup>2</sup> from *cw* Xe lamp). Emission was detected at 650/40 nm.



**Figure 4.19**TMPyP fluorescence emission from Hela cells. Images were recorded as a function of the elapsed irradiation time at 425 nm. Emission was detected at wavelengths longer than 500 nm (2.6  $\text{mW/cm}^2$  from a *cw*Xe lamp).

Upon incubation of a cell with TMPyP, this molecule first localizes in lysosomes (Figure 4.18, left frame).<sup>45</sup> Upon irradiation of the TMPyP, the singlet oxygen produced presumably facilitates lysosome rupture. Depending on the experimental conditions, TMPyP thus released may then localize in the nucleus, binding to the DNA, and/or it may cross the plasma membrane into the extracellular medium (Figure 4.18, right frame).<sup>45,80</sup> Moreover, intracellular TMPyP bleaches upon prolonged irradiation.<sup>80</sup> All of these processes are manifested in TMPyP-sensitized singlet oxygen phosphorescence signals.<sup>43,45,80</sup>

Based solely on the photobleaching kinetics of each of the studied photosensitizers and comparing them with TMPyP, from which singlet oxygen traces are easily recorded, we can speculate that any dye more resistant to light degradation than TMPyP should be sufficiently stable to be analyzed under the experimental conditions necessary to observe a singlet oxygen

signal (Figure 4.20). Based on that data, one could additionally predict that it would be possible to detect an intracellularly generated singlet oxygen signal from most of the sensitizers studied in this work, including BP and DMP-C60.

However, all the other porphyrins show photoinduced intracellular relocalization, just like TMPyP, followed by photobleaching upon irradiation, <sup>45,80-82</sup> and light induced fluorescence changes upon irradiation are dramatic. Considering that we would be able to detect singlet oxygen from these sensitizers, they are far from meeting the characteristics of an ideal intracellular sensitizer for singlet oxygen detection with spatial and temporal control.



**Figure 4.20** Fluorescence image of HeLa cells incubated with TMPyP recorded as a function of elapsed irradiation time over 3 min at 420 nm with a femtosecond laser (7 nJ per pulse). Emission detected at 650/40 nm.

DMP- $C_{60}$ , is the tested dye with the most stable fluorescence behavior upon irradiation. However, its low efficiency on generating singlet oxygen species might not be sufficient for meaningful results.

We might be facing a paradox: we look for a photosensitizer that is simultaneously stable and does not suffer relocalization and, at the same time, generates reactive oxygen species that reacts directly with the sensitizer or with the surrounding environment causing its destruction.

An alternative approach to obtain the stability of the chromophore could be its encapsulation. Evidence has been presented to indicate that encasing/encapsulating a sensitizer or fluorophore can increase its stability towards oxygen-dependent degradation.<sup>83-85</sup> Although this observation may reflect a number of phenomena, it is principally a consequence of shielding the chromophore from reactive species that can diffuse over a finite distance (*e.g.*, singlet oxygen, hydroperoxides, etc.)

	$\Phi_{pb}{}^a$	Solvent
BP	$7.02 \pm 1 \ge 10^{-4}$	DMSO
DMP-C60	$4.56 \pm 2 \ge 10^{-7}$	DMSO/H <sub>2</sub> O (1/9)
ТМРуР	$1.22 \pm 0.1 \text{ x } 10^{-5}$	PBS (with BSA)
	$3.25 \pm 0.3 \times 10^{-6}$	PBS

**Table 4.4** Photobleaching quantum yields determined in solution.

a) Standard errors obtained from the kinetics fitting.

# **4.3 Conclusions**

It is particularly challenging to directly record  $O_2 (a^1 \Delta_g) \rightarrow O_2 (X^3 \Sigma_g)$  phosphorescence from single live cells in a time-resolved laser-based experiment. Most of this challenge comes from the fact that singlet oxygen is cytotoxic. Thus, the system on which the experiment is made is perturbed continuously as the experiment is performed. Chemical reactions of singlet oxygen with proteins, for example, can readily change the local intracellular environment which consecutively can influence the production and removal of singlet oxygen kinetics. Regardless of these expected environmental changes, we were able to detect singlet oxygen from HeLa cells incubated with porphyrins and bacteriochlorins and have demonstrated that, once we account for the effects of certain irradiation dependent phenomena (i.e., sensitizer leaking out of the cell), the inherent intracellular lifetime of singlet oxygen does not appear to change dramatically as the cells progresses towards death.

In experiments performed with hydrophobic and hydrophilic dyes, where singlet oxygen is generated in different subcellular locations, the data obtained are consistent with long-lived species that crosses phase-separated domains. Nevertheless, key aspects of singlet oxygen behavior still depend on the local environment in which it is produced.

From our BSA based experiments we have confirmed that hydrophilic dyes can diffuse out of the cell, giving rise to the detection of a population of singlet oxygen generated in the extracellular medium.

The evaluation of the cell suspension data, where the overall rate of singlet oxygen removal is roughly equivalent to singlet oxygen formation, can be challenging and should be discussed with care since it can lead us to misleading conclusions.

Experiments performed on single cells and cell suspensions follow from very different handling procedures and employ different cells line, which per se can influence the data obtained in a time-resolve phosphorescence experiment.

In the second part of this chapter we have focused our study on the photobleaching problems associated to the sensitizers used to generate a singlet oxygen signal under our experimental conditions. In addition to generate singlet oxygen, we used the fluorescence probe APF to demonstrate that bacteriochlorins also generate other radical species, namely hydroxyl radical.

We can conclude that predictions about intracellular stability based solely on the chemical structure of the sensitizer can be erroneous. Rather, one is best advised to first examine the behavior of a given sensitizer with an appropriate solution experiment (*e.g.*, a solution with an

added protein) before proceeding to an *in vitro* test. Indeed, we state that attempts to design a stable sensitizer based solely on principles of functional group reactivity will continue to be a challenge. The data obtained in this work contributes in a meaningful way to the ultimate goal of designing and synthesizing singlet oxygen sensitizers whose intracellular behavior can be controlled under a variety of oxidizing conditions.

# 4.4 Materials and methods

#### 4.4.1 Materials

5,10,15,20-Tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphine (TMPyP, Sigma-Aldrich), and pyropheophobide-a (PPa, Frontier Scientific), phosphate-buffered saline solution (PBS, Sigma-Aldrich) and bovine serum albumin (BSA, MW ~65 kDa, Sigma-Aldrich), BSA-fluorescein conjugate (Molecular Probes/Invitrogen), and D<sub>2</sub>O (99% D, EurisoTop), dimethyl sulfoxide (DMSO, Sigma-Aldrich) and benzene (Sigma-Aldrich), sodium azide (Sigma-Aldrich), were used as received. F<sub>2</sub>POH, F<sub>2</sub>BOH, F<sub>2</sub>BMet were kindly offered by Luzitin S.A. Benzo[*cd*]pyrene-5-one, BP,<sup>86</sup> 1,4-bis[4-(*N*,*N*-diphenylamino)phenylethynyl]-2,5-dibromobenzene, BBB,<sup>87</sup> and *N*,*N*-dimethylfulleropyrrolidiniuim iodide, DMP-C<sub>60</sub>,<sup>88</sup> were prepared as outlined in the papers respectively cited.

#### 4.4.2 Methods

The output of a continuous wave (cw) diode-pumped Nd:YVO4 laser (Millenia V, 5W; Spectra Physics/Newport) is used to pump a Ti:Sapphire laser (Tsunami 3941, Spectra Physics/ Newport, Irvine, CA). The Tsunami, operating at a repetition rate of 80 MHz, delivers tunable pulses over a spectral range of ~725 to 910 nm and a Gaussian temporal profile with full width at half maximum (FWHM) from ~75 to 95 fs depending on the wavelength. The pulse energy is amplified by approximately a factor of 105 in a 1000 Hz pumped regenerative amplifier (Spitfire pumped by an Evolution Nd:YLF laser). The Spitfire operates with a repetition rate of 1 kHz, this ultimately results in tunable pulses from ~760 to 850 nm (~100-150 femtoseconds with a spectral bandwith of ~ 15 nm fwhm). Laser powers can be adjusted by rotating the polarization of the beam with a half-wave plate (model WPH05M-780, Thorlabs) and then passing the resultant beam through a fixed polarized optic (Thorlabs model GT-10B Glan-Taylor Polarizer). For experiments with porphyrins, the laser output was frequency-doubled by a β-barium borate (BBO) crystal (giving 380-425 nm output) and associated filters were placed after the polarizer to remove light that it is not frequency-doubled.

Microscope-based Singlet oxygen detection



**Figure 4.21** Schematic drawing of the femtosecond optical system used to directly detect singlet oxygen with the associated beam paths. The cuvette holder used for solution and cell ensemble experiments can be removed such that the output from the microscope can be sent directly to the PMT.

For the bacteriochlorin, an optical parametric amplifier (OPA-800CF) is used in order to achieve an excitation at 745 nm. The output of the Spitfire is directed to pump the OPA, delivering tunable femtosecond pulses from ~ 300-3000 nm, thereby significantly improving the spectral range. The average light power was measured with a power meter (Field Max-II controller, Head model No. P519Q, Coherent, Santa Clara, CA).

The output of the laser is directed into an inverted microscope (Olympus IX 71) and focused (diameter ~1  $\mu$ m at the beam waist) using a water immersion, long working distance (~ 2 mm) 60× objective (Olympus LUMPLFL60×W/IR/0.90).<sup>2,14</sup>

A CCD camera (Evolution QEi controlled by Image-Pro software, Media Cybernetics) is attached to the microscope and placed at the image plane of the microscope. The position of the laser spot is ascertained by imaging with the CCD camera a scattered/reflected light from a glass plate. A motorized stage (Prior, model: CS152DP) and controller (Prior, ProScan II) are used to move the cells relative to the laser spot. The microscope lamp (Olympus, TH4-200) and
a band pass filter at 700 nm (FWHM 40 nm) are used for bright field illumination to avoid induced damage of the cells.

The 1275 nm  ${}^{1}O_{2}$  phosphorescence emitted from the sample upon irradiation is isolated with an interference filter at 1270 nm (FWHM 50 nm, bkInterferenzoptikElektronikGMbH, Germany) and detected by a liquid nitrogen cooled near-infrared photomultiplier tube (PMT, Hamamatsu model R55509-42, Hamamatsu City, Japan) used in a time-resolved photo counting mode. The response time of the PMT is 3 ns, it has an active area of  $3 \times 8$  mm and a spectral response that covers the range from 400 nm – 1500 nm. The output of the PMT is amplified (model 445, preamplifier, Stanford Research Systems, Sunnyvale, CA) and sent to a multiscaler photon counter (MSA 300, Becker Hickl, Germany).

#### Singlet oxygen detection on single cells

HeLa cells, a cervical cancer subline, was used for single cells experiments. Cells were grown in Eagle's Minimum essential medium (EMEM, Sigma, Deutschland) supplemented with 2 mM L-glutamine (Sigma), 1 % non-essential aminoacids (Sigma), 1 % penicillin-streptomycin (Sigma) and 10 % fetal calf serum (Sigma). The cells were maintained at 37 °C in a humidified 5 %  $CO_2$  atmosphere and platted when 80 % of confluence was achieved. After washing them with phosphate buffered saline (PBS) and trypsinizing (0.25 % trypsin, Sigma), they detached from the bottom of the cultivation flask. The cell suspension was collected and centrifuged (2 min, 1000 rpm) washed with cultivation medium, re-suspended in the cultivating medium and platted onto poly-D-lysine coated cover slips in 12 well plates. The cell density can vary from experiment to experiment. After seeding, the cells were left to settle and restart growth for at least 24 h before the sensitizer was incubated.

The specially-composed maintenance medium was prepared by using 140 mMNaCl, 3.5 mMKCl, 2mM MgCl<sub>2</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM glucose and 10 mM HEPES.<sup>4</sup>

The sensitizers TMPyP ( $\mu$ M), F<sub>2</sub>POH ( $\mu$ M), F<sub>2</sub>BOH ( $\mu$ M) were added to the medium, and left to incubate for 20 - 24h. Hydrophobic dye F<sub>2</sub>PMet (12.5  $\mu$ M) was diluted in the medium by adding a maximum of 1 % ethanol.

The exchange of intracellular  $H_2O$  with  $D_2O$  was achieved by exposing the cells to a hypertonic solution (double concentration of KCl and NaCl in the maintenance medium). The cover slip containing the cells was removed from the culture medium and washed with PBS. The cells were incubated for 3-5 min with the hypertonic  $D_2O$ -based solution. After incubation the cells were washed three times with maintenance medium after washing with PBS. The cells were held in a  $CO_2$  incubator at 37 °C for ~ 24 h in an isotonic maintenance medium containing the

sensitizer to incubate. The cells were washed three times with  $ABM/D_2O$  after incubation, and transferred to the microscope.

For some experiments, cells were only exposed to  $D_2O$ -based maintenance medium for a period that did not exceed ~15 min and the laser experiments were performed immediately thereafter.

#### Singlet oxygen detection on cells suspensions

Nonadherent HL-60 cells (Human promyelocytic leukemia cells) were used. Cells were maintained in 75 cm<sup>2</sup> cultivation flasks in RPMI 1640 medium supplied with 10% fetal calf serum, 1 % L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at a concentration of ~5 × 105 cells/ml. In a typical experiment, the cells were collected from the flask, centrifuged at 1000 rpm for 2 min, resuspended, and incubated with 50  $\mu$ M solution of TMPyP dissolved in the cultivating medium for 24 h at 37 °C. When the hydrophobic sensitizer F<sub>2</sub>PMet was used, a maximum 1% ethanol was added from the stock solution to a final concentration of 12.5  $\mu$ M in the medium. PPa was dissolved in the medium by adding a maximum of 1 % DMSO to a final concentration of 5  $\mu$ M, and left to incubate for 3 h.

Some experiments were performed by incubating cells with a solution of the sensitizer dissolved in maintenance medium. After incubation, the cells suspension was centrifuged (1000 rpm for 2 min) and the medium was removed. Samples were resuspended in D<sub>2</sub>O-based hypertonic maintenance medium for 3 min to remove intracellular H<sub>2</sub>O via osmosis <sup>4</sup> and then centrifuged and suspended again in D<sub>2</sub>O-based maintenance medium with or without BSA, depending on the experiment to be performed. Cells were present at a concentration of ~  $10^6$  cells/mL. During experiment cells were continuously stirred to maintain their suspension.

#### PPa preparation on liposomes

Experiments on liposomes were performed by Thomas Breitenbach. Unilamellar vesicles were prepared from 1,2-dimyristoylglycero-3-phosphocholine (Sigma-Aldrich) by an established procedure.<sup>89</sup>

#### Intracellular detection of hydroxyl radicals

The 3'-(p-aminophenyl)fluorescein probe was employed for intracellular detection of hydroxyl radicals. A-549 cells were plated at a density of  $15 \times 10^3$  cells *per* well in 8 wells slides (IBIDI, Germany) and were kept at 37 °C in a 95% atmospheric air and 5% CO<sub>2</sub> humidified atmosphere, for 24h. After washing the cells with fresh medium, cells were incubated in the dark with 5 µM F<sub>2</sub>BMet, diluted in cell medium, for ≈16 h, at 37 °C in a 95% atmospheric air and 5% CO<sub>2</sub> humidified atmosphere, and 10 µM of 3'-(p-aminophenyl)fluorescein was added and incubated

for additional 1-2 hours. After washing the cells with HBSS/HEPES buffer, the slide was transferred to the microscope stage and control images of the cells with excitation at 488 nm and 530 nm emission were recorded under a confocal microscope (LSM510 Meta, Carl Zeiss, Jena, Germany) with a 63x oil immersion objective (Plan-Apochromat, 1.4 N.A., Carl Zeiss, Jena, Germany). The cells slide was irradiated at 505 nm with a LED and fluorescein fluorescence images were recorded immediately after illumination with the confocal microscope as described above.

The bacteriochorin images were recorded by exciting at 514 nm, using an Argon laser (45 mW); emission light was collected after passage through a long-pass filter of 575 nm. A Helium–Neon laser (5mW) was used as light source at 633 nm for visualization of cell morphology.

## **4.5 References**

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# 5

# **Overall conclusion**

5.1 Overall conclusion

5.2 References

#### **5.1 Overall conclusion**

The work described in this dissertation explores the photochemical and photophysical properties of halogenated tetraphenyl macrocycles that were designed to meet the requirements of PDT photosensitizers. This project was developed and progressed along with the work of synthesis and biology teams in order to understand and optimize the photosensitizers to improve the chances of succeeding in Photodynamic Therapy.

As photosensitizers for PDT, bacteriochlorins have favorably distinguished themselves from porphyrins and chlorins by the intense absorption in the red, where light penetration in the tissues is optimal, and by the ability to interact with oxygen through energy and charge transfer channels leading to the generation of oxygen species with different reacitivities. Singlet oxygen and hydroxyl radical were generated and detected in solution and intracellularly. Intracellular singlet oxygen was found to have a comparatively long lifetime and readily crosses phaseseparated domains. We found that, porphyrins mainly generate singlet oxygen and their PDT targets will be selectively chosen in the whole of the cell, rather than be correlated to their initial intracellular localization. On the other hand, the very reactive hydroxyl radical generated by bacteriochlorins will induce damage at the generation site. As so, bacteriochlorins can initiate cell death processes that are associated to their initial subcellular localization.

An important achievement of the design of this new group of macrocycles, especially bacteriochlorins, is their high photostability. As the charge-transfer interaction between the photosensitizer and oxygen becomes stronger  $\Delta G$ , for full electron transfer from the sensitizer and oxygen, becomes negative (Figure 5.1 A).  $\Delta G$  decreases as the oxidation potential of the photosensitizer increases. However, this is associated with an increase in the photobleaching quantum yield (Figure 5.1 B). According to Jori,<sup>1</sup> the upper limit for the photobleaching quantum yield of a useful photosensitizer is 10<sup>-5</sup>, and this sets a lower limit of -0.5 V for the oxidation potential of the excited state of the photosensitizer. The high charge-transfer interaction between molecular oxygen and the triplet excited state of the sensitizer and the photobleaching quantum yield below 10<sup>-5</sup> are best combined in F<sub>2</sub>BMet. This combination of parameters can have a decisive role in determining the most effective photosensitizer.

For bacteriochlorins, generation of reactive oxygen species occurs by deactivation of the triplet state by singlet and triplet channels. In conditions where the quenching rate constant approaches the diffusion controlled limit, the actual rate constant for the deactivation of the triplet excited state of the photosensitizer by molecular oxygen should be corrected for diffusion.

$$k_{\rm D} = k_{\rm -diff} k_{\rm q} / \left( k_{\rm diff} - k_{\rm q} \right) \tag{5.1}$$

In order to use the appropriate diffusion limit,  $k_D$  was calculated considering an estimated value of  $k_{diff} = (1/9 + 4/9)/2k_{diff}$ , which represents an average of the relevant spin statistic factors.

The biology team from Coimbra determined the phototoxicity of bacteriochlorins in vitro. The lethal light dose required to kill 90 % of cells ( $LLD_{90}$ ) incubated with a given concentration of a photosensitizer increases in the following order: ClBOH<ClBEt<Cl<sub>2</sub>BEt<FBMet<F<sub>2</sub>BMet.

We found that the photodynamic efficiency of these bacteriochlorin photosensitizers is correlated with  $k_D/\Phi_{pb}$  (Figure 5.2). In conditions where other important criteria for PDT efficacy are met, the ratio between the rate constant of the interaction between the photosensitizer and molecular oxygen ( $k_D$ ), and photosensitizer photostability ( $\Phi_{pb}$ ) can determine the order of PDT efficacy of bacteriochlorin photosensitizers.

The higher efficacy of sulfonamide dyes over the sulfonated ones in killing cancer cells should be also evaluated considering their subcellular localization. Sulfonic dyes have a lysosomal like accumulation while sulfonamides, distribute mainly into the mitochondria and endoplasmic reticulum (ER). ER and/or mitochondria targeting PDT has been shown to be highly effective and in addition, ER co-localization has been considered to play an essential role on inducing an innate inflammatory response in tumor cells.<sup>2,3</sup>



**Figure 5.1** A) Dependence of the quenching rate constant of the triplet excited sate by molecular oxygen  $(k_q)$  with the oxidation potential for some porphyrins and chlorins. B) Dependence of the photobleaching quantum yield with the triplet state oxidation potentials of some halogenated bacteriochlorins (Cl<sub>2</sub>Bet, F<sub>2</sub>BMet, FBMet) and mTHPP, mTHPC and mTHPB. Graphs were constructed using the values from table S2 in the Appendix III.



**Figure 5.2** Dependence between light doses required to kill 90% of S91-I3 cells  $(LLD_{90})^{4-6}$  and the ratio of the triplet-oxygen interaction rate constant  $(k_D)$  and photodegradation quantum yield  $(\Phi_{pb})$  for halogenated bacteriochlorins, using  $k_{diff} = 2.64 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ . Values presented in table S2 in the Appendix III.

An important part of this work was to directly quantify the intracellular singlet oxygen lifetime. Time-resolved singlet oxygen detection is the most desirable and accurate approach to do it, although to perform these experiments at the single cell level can be very challenging. We work on a dynamic system which is being perturbed during the experiment, and this will be reflected in the phosphorescence data. Despite the environmental changes, we have demonstrated that singlet oxygen lifetime does not appear to change dramatically as the cells progresses to death. Also, singlet oxygen produced from intracellular localized hydrophilic or hydrophobic sensitizers was consistent with a model where it readily crosses between phase separated domains.

With the fluences used with the present methodology to directly detect singlet oxygen at the single cell level, bleaching of the sensitizers was currently observed. Ideally, singlet oxygen sensitizers should have high singlet oxygen quantum yield and photostability. Our results show that in order to achieve a predictable behavior in cells, appropriate photobleaching studies in solution should be performed.

This work aimed to optimize the photodynamic effect that results from the combination of oxygen, a photosensitizer and light of appropriate wavelength. We are now better informed on how photochemistry plays a role in cellular damage and how we can take advantage of this knowledge to improve new drugs for PDT. The properties of the ideal photosensitizer are very well described in the literature. However, in the process of selecting the best drug candidate it is useful to consider the results of this work: the type of interaction between oxygen and the photosensitizer excited state, under a specific environment, will dictate the success of the photosensitizer.

## **5.2 References**

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# Appendix I

## Supporting material for data in Chapter 2

- Rise time decays of singlet oxygen emission at 1275 nm
- Cyclic voltammograms of F<sub>2</sub>BMet and Cl<sub>2</sub>Bet and reduction potentials
- EPR spectra of Cl<sub>2</sub>Bet, Cl<sub>2</sub>BHep, F<sub>2</sub>BMet, Cl<sub>2</sub>BMet<sub>2</sub> and Cl<sub>2</sub>BOH.



**Figure S1** Rise-time of singlet oxygen emission at 1275 nm for bacteriochlorins (top), porphyrins and chlorin (bottom) in ethanol. Excitation was achieved with a femtosecond laser at 750 nm for bacteriochlorins and 420 nm for porphyrins.



**Figure S2** Upper panel: Cyclic voltammogram recorded in 0.1 M TBAP dissolved in ACN containing 0.5 mM  $F_2BMet$ ; scan rate 25 mV s<sup>-1</sup>. Lowe panel: Cyclic voltammogram recorded in 0.1 M TBAP dissolved in ACN containing 0.5 mM  $Cl_2PEt$ ; scan rate 25 mV s<sup>-1</sup>.

	$E_{\rm red2}^{0}$	$E_{\rm red1}^{0}$
	V	V
Cl <sub>2</sub> PEt	-1.40	-0.94
TPC		-1.12
Cl <sub>2</sub> CEt	-1.44	-0.84
ТРВ		-1.10
$F_2B$	-1.46	-0.95
F <sub>2</sub> BOH		-0.93
F <sub>2</sub> BMet	-1.18	-0.74
Cl <sub>2</sub> BEt	-1.15	-0.79

**Table S1** Reduction potentials vs SCE, of selected macrocycles and their photodecomposition quantum yields in PBS:MeOH (2:3, V:V).



**Figure S3** EPR spectrum observed from a solution of  $Cl_2BEt$  (50  $\mu$ M) in TRTX-100 (top panel) or in DMSO (lower panel) and in the presence of DMPO (50 mM), recorded after 10 s of irradiation with a diode laser at 748 nm. Signal simulation on the right side.



**Figure S4** EPR spectrum observed from a solution of  $Cl_2BHep$  (50  $\mu$ M) in TRTX-100 and in the presence of DMPO (50 mM), recorded after 10 s of irradiation with a diode laser at 748 nm. Signal simulation at the right side.



**Figure S5** EPR spectrum observed from a solution of  $F_2BMet$  (50  $\mu$ M) in DMSO and in the presence of DMPO (50 mM), recorded after 10 s of irradiation with a diode laser at 748 nm. Signal simulation at the right side.



**Figure S6** EPR spectrum observed from a solution of  $Cl_2BMet_2$  (50 µM) in TRTX-100 (top panel) or in DMSO (lower panel) and in the presence of DMPO (50 mM), recorded after 10 s of irradiation with a diode laser at 748 nm. Signal simulation on the right side.



**Figure S7** EPR spectrum observed from a solution of  $Cl_2BOH$  (50  $\mu$ M) in DMSO (top panel) and in the presence of DMPO (50 mM), recorded after 1 min of irradiation with a diode laser at 748 nm. Signal simulation on the right side.

# Appendix II

## Supporting data to Chapter 3

 Light induced changes in the absorption spectra and photobleaching kinetics of F<sub>2</sub>POH, Cl<sub>2</sub>BHep, Cl<sub>2</sub>BEt, F<sub>2</sub>BMet, FBMet, FBMet<sub>2</sub> and ClBOH.



**Figure S8** Left panel - Light induced changes in the absorption spectra of  $Cl_2PEt$  in MeOH/PBS solution (3/2) when irradiated at 512 nm with a diode laser with 52 mW. Right panel – Photobleaching kinetics followed by absorbance at 512 nm. No clear kinetics was found.



**Figure S9** Left panel - Light induced changes in the absorption spectra of FCMet in MeOH/PBS solution (3/2) when irradiated at 653 nm with a diode laser with 6.3 mW. Right panel – Photobleaching kinetics followed by absorbance at 653 nm.



**Figure S10** Left panel - Light induced changes in the absorption spectra of  $F_2POH$  in PBS solution when irradiated at 748 nm with a diode laser with 95 mW. Right panel – Photobleaching kinetics followed by absorbance at 746.5 nm, and fitted to a mono exponential decay with  $k = 3.81 \times 10^{-3}$ .



Figure 3.11 Light induced changes in the absorption spectra of  $Cl_2BHep$  in methanol solution when irradiated at 748 nm with ~40mW. Left panel- before irradiation, right panel – after 315 min of irradiation.



**Figure 3.12** Photobleaching kinetics of  $Cl_2BHep$  in methanol, followed by absorbance at 746 nm and fitted to a monoexponential to yield  $k = 1.7 \times 10^{-6}$ .



**Figure 3.13** Light induced changes in the absorption spectra of  $Cl_2BEt$  in MeOH/PBS (3/2, v/v) solution before (left panel) and after 180 min of irradiation (right panel) at 748 nm diode laser with ~40 mW.



**Figure S14** Photobleaching kinetics of Cl<sub>2</sub>BEt in MeOH/PBS (3/2, v/v), followed by absorbance at 746 nm and fitted to a monoexponential to yield  $k = 6.9 \times 10^{-5}$ .



**Figure S15** Left panel - Light induced changes in the absorption spectra of  $F_2BMet$  in MeOH/PBS (3/2, v/v) solution at 748 nm with a diode laser with ~40 mW.Left panel - Photobleaching kinetics followed by absorbance at 744 nm and fitted to a monoexponential to yield  $k = 7.6 \times 10^{-5}$ .



**Figure S16** Light induced changes in the absorption spectra of FBMet in methanol solution before (left panel) and after 182 min of irradiation (right panel) at 748 nm diode laser with ~40 mW.



**Figure S17.** Photobleaching kinetics of FBMet in methanol, followed by absorbance at 742 nm and fitted to a monoexponential to yield  $k = 5.86 \times 10^{-5}$ .



**Figure S18** Light induced changes in the absorption spectra of FBMet in MeOH/PBS (3/2, v/v) solution before (left panel) and after 65 min of irradiation (right panel) at 748 nm diode laser with ~40 mW.



**Figure S19** Photobleaching kinetics of FBMet in MeOH/PBS (3/2, v/v), followed by absorbance at 743 nm and fitted to a monoexponential to yield  $k = 3.3 \times 10^{-4}$ .



**Figure S20** Light induced changes in the absorption spectra of FBMet<sub>2</sub> in methanol solution before (left panel) and after 225 min of irradiation (right panel) at 748 nm diode laser with  $\sim$ 40 mW.



**Figure S21** Photobleaching kinetics of FBMet<sub>2</sub> in methanol, followed by absorbance at 742 nm and fitted to a monoexponential to yield  $k = 4.7 \times 10^{-5}$ .



**Figure S22** Photobleaching kinetics of ClBOH in MeOH/PBS (3/2, v/v), followed by absorbance at 743 nm and fitted to a monoexponential to yield  $k = 3.9 \times 10^{-3}$ .



**Figure S23** Photobleaching kinetics of ClBOH in PBS, followed by absorbance at 746.5 nm and fitted to a monoexponential to yield  $k = 4.3 \times 10^{-3}$ .



**Figure S24** Photobleaching kinetics of CIBOH in methanol, followed by absorbance at 741 nm and fitted to a monoexponential to yield  $k = 4.2 \times 10^{-4}$ .



Figure S25 Light induced changes in the absorption spectra of  $F_2BOH$  in PBS at pH7.4.



Figure S26 Light induced changes in absorption spectra of F<sub>2</sub>BOH in PBS at pH5.



Figure S27 Light induced changes in absorption spectra of F<sub>2</sub>BOH in PBS at pH10.

# Appendix III

Supporting data to Chapter 5

Table S	52
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	E* <sub>ox</sub>	$\mathbf{k}_{\mathbf{q}}$	$\Phi_{ m pb}$	LLD <sub>90</sub>
	/V	/s <sup>-1</sup>		/Jcm <sup>-2e</sup>
TPP <sup>a</sup>	-0.48105	1.6×10 <sup>9</sup>		
$\mathbf{F}_{2}\mathbf{P}^{b}$	-0.20105	1.1×10 <sup>9</sup>		
$Cl_2P^b$	-0.20105	$8.7 \times 10^{8}$		
Cl <sub>2</sub> PEt	-0.14105	6.7×10 <sup>8</sup>		
FBMet	-0.62254	$2.4 \times 10^{9}$	8.1×10 <sup>-5</sup>	0.17
F <sub>2</sub> B <sup>c</sup>	-0.62254	2.6×10 <sup>9</sup>		
F <sub>2</sub> BMet	-0.47254	2.2×10 <sup>9</sup>	1.0×10 <sup>-5</sup>	0.14
CIBOH		1.9×10 <sup>9</sup>		0.26
ClBEt		2.1×10 <sup>9</sup>		0.22
Cl <sub>2</sub> BEt	-0.39749	1.8×10 <sup>9</sup>	6.0×10 <sup>-6</sup>	0.19
mTHPP <sup>d</sup>	-0.48105	1.9×10 <sup>9</sup>	3.8×10 <sup>-6</sup>	
mTHPC <sup>d</sup>	-0.55105	1.8×10 <sup>9</sup>	3.3×10 <sup>-5</sup>	
mTHPB <sup>d</sup>	-0.81422	2.5×10 <sup>9</sup>	1.5×10 <sup>-3</sup>	

a) From ref. <sup>1</sup>, b) from ref. <sup>2</sup>, c) from ref. <sup>3</sup>, d) from ref. <sup>4</sup> and redox potentials considered the same as those of TPP, TPC and TPB according to ref. <sup>5</sup>, e) from ref. <sup>6-8</sup>

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