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Review of the role played by the photosensitizer's photobleaching during photodynamic therapy

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Photodynamic therapy (PDT) is based on the combined administration of photosensitizing drugs and light to treat a variety of malignant, pre-malignant and also non-malignant diseases. The reactive species resulting from the excitation of the photosensitizers are at the origin of their photodegradation, which has an influence on the treatment outcome. PDT has a long history, including the improvements of photosensitizers and light delivery systems, which led to approved clinical applications. A knowledge of the photobleaching processes occurring during PDT will help implicit dosimetry, as it describes multiple photophysical, photochemical and photobiological factors involved in PDT. Due to this the clinical efficacy of PDT and optimisation of treatment regimes will be allowed.
This article draws a light on key concepts like importance of photosensitizer photobleaching, the central role of light administration parameters (dosimetry, fluence, fluence rate, drug-light interval and light fractionation), and the critical role of oxygen to optimize the PDT treatment regimes for improved clinical efficacy. The main conclusions from this review work is that the efficiency of PDT can be, in certain cases, significantly enhanced by light fractionation; that most photobleaching processes involve singlet oxygen and obey second-order kinetics; and that the rate of photosensitizer photobleaching depends on initial drug concentration. This review also concludes that there is no straightforward approach to optimizing PDT given the complexity of the mechanisms involved, as well as the variety of diseases, organs, photosensitizers and PDT regimes that have been studied and reported.

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1. Introduction

1.1 Basic principles of PDT

Photodynamic therapy (PDT) is a well-established modality for the treatment of cancer, as well as some non-cancerous diseases, based on the interaction of a photosensitizer (PS) and light, in the presence of molecular oxygen [1]. The PDT treatment protocol involves the administration of a photosensitising drug (termed Photosensitizer (PS)) to the patient, orally, intravenously, or for specific superficial conditions, topically. In certain conditions, the PS may preferentially accumulate in the hypermetabolic or fast proliferating tissues. When it reaches a certain concentration and localization, light at wavelengths corresponding to an absorption peak of the PS is delivered to the tissues.

Light is most commonly obtained from coherent sources i.e. lasers, but different varieties of incoherent sources are also used. The light absorption by the PS starts a process of molecular energy transfer that results in the formation of highly reactive species. The principal species are: superoxide anion and and other reactive species such as the hydroxyl radical and hydrogen peroxide (Type I reaction) [2], singlet oxygen (Type II reaction) [3]. Oxidative damages to various critical cellular components close to the site of the highly reactive species formation, or necrosis generated by vascular damages, result in the destruction of the tissue, in certain cases by apoptosis, leaving the healthy non-illuminated surrounding tissue undamaged [4].

Type I reaction (see Figure 1) involves the excitation of the PS to one of its triplet states and its subsequent direct interaction with a biological substrate followed by hydrogen atom (or electron) transfer, producing transient radicals. These radicals may interact with other molecules, such as locally-present molecular oxygen. A direct reaction of the excited PS with molecular oxygen generates superoxide radical anions (O_2^{-}), which can also react with, and damage, the substrate. Finally, electron transfer from the triplet state PS to the oxygen may occur, again creating superoxide radical anions (O_2^{-}) and an oxidised version of the photosensitizer, which again can cause damage to the substrate.

In the Type II reaction, there is energy transfer from the excited triplet state of the PS to molecular oxygen naturally present in the cells to produce singlet oxygen. The cytotoxic singlet oxygen ($^1O_2$) thus generated may react with the substrate (e.g., surface of individual cells or organelles within the cell), destroying the targeted cells as shown by the equations.

$$S + h\nu \rightarrow S^*_1$$
$$S^*_1 \rightarrow T^*_1$$

$T^*_1 + O_2 \rightarrow S + {}^1O_2$

${}^1O_2$ + cellular target→ Cell death (apoptosis or necrosis)

Both type I and II reactions may take place in parallel, their respective contributions being in particular dependent upon the concentrations of oxygen and PS, specific physical-chemical characteristics of the PS (triplet quantum yield; rate of triplet decay; quantum yield of reactive species generation; etc), and the reaction rate of the triplet photosensitizer with its target. However, it is important to indicate that the type II reaction is usually dominant in the PDT process [2].

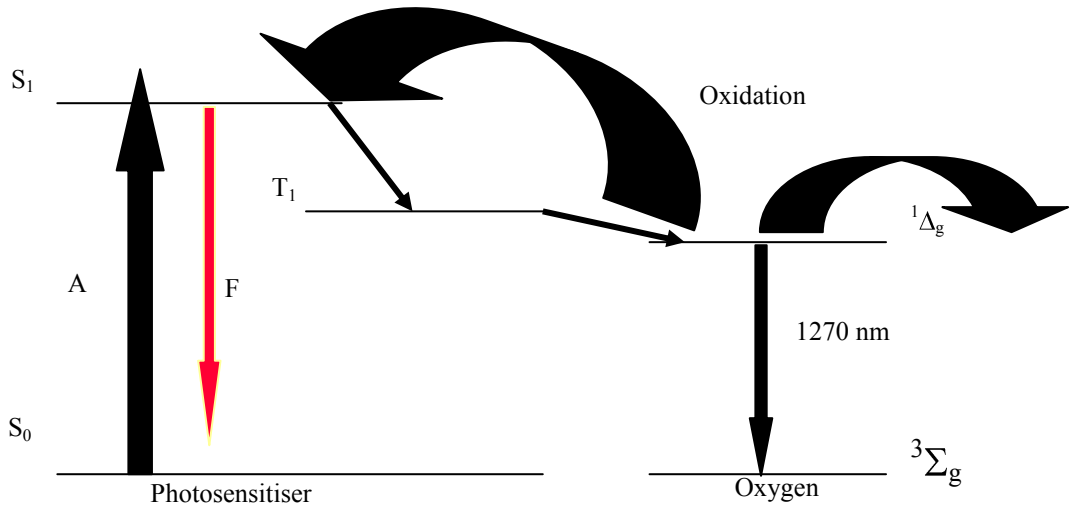

Fig. 1. Modified Jablonski diagram representing Type I and Type II processes. (Self sensitised photo-oxidation may be monitored via the progressive reduction in photosensitizer fluorescence intensity).

Generally speaking, the therapeutic outcome of PDT depends upon a large number of parameters including the PS used, its microscopic and macroscopic distribution and dose at the time of treatment, the light wavelength, the irradiance and the optical properties of the tissue at the treatment wavelength. It should be noted that the situation is more complex if PDT is combined with other therapeutics, including the use of angiogenic and vasoconstricting agents, drugs inducing a modilation of the immune system, or (photo) biostimulation.

The PDT therapeutic effect also critically depends on the presence of molecular oxygen to generate the highly reactive species responsible in most cases for the tissular damages. Its presence can be analysed by several ways that are beyond the scope of this paper. One can, however, briefly mention:

- Scavengers: Scavengers inhibit reactions dependent on singlet oxygen. For example, azide acting as a physical scavenger reacts with ${}^1O_2$ to give a reactive azide radical, $N^{3-} + {}_1O^2 \rightarrow N^{3*} + O_2$. Other scavengers including carotene, ascorbate, thiols and histidine act as chemical scavengers.
- D_2O as far as in vitro studies are concerned: the lifetime of singlet oxygen in D_2O is ten-fold higher than H_2O. Hence, if a reaction in aqueous solution is dependent on singlet oxygen, carrying it out in D_2O instead will greatly enhance the reaction.
- Luminescence: As singlet oxygen undergoes radiative decay to the ground state, some of its energy is emitted in the form of light. The light from ${}^1O_2$ appears in the near-infrared wavelength at around 1270 nm.

Nonetheless, the distribution of molecular oxygen *in vivo* has proved to be a critical factor in PDT. It is well known that this parameter is closely linked to the cellular metabolic status and the effectiveness of PDT is directly related to the concentration of available molecular oxygen at the time of treatment. This is the reason why the oxygen consumption rate within the tumour during PDT affects the potency of this treatment (Chen et al and Piffaretti et al) [5-6].

One crucial parameter that impacts the PDT effects is the photobleaching of the PS itself. Because the highly reactive species generated by the PS+light interaction induce damages locally (Peng et al) [7], the possibility exists that the photodynamic action will damage the PS itself, thus hindering further photodynamic activity.

Whilst the local concentration of oxygen is critically important both before and during treatment (Fuchs and Thiele) [8], the local concentration of PS is also of crucial importance, as mentioned above. The values of these concentrations are difficult to determine *in vivo*. As presented in details below, various techniques have been developed to monitor oxygen concentration, light distribution and PS concentration during PDT. A number of studies performed by different groups proposed monitoring the depletion in PS fluorescence intensity or photobleaching during light exposure and correlating this with the local concentration of singlet oxygen, the tissue damages or the clinical treatment outcome. In this paper, we propose to review this body of work. Our review on the role played by photobleaching is preceded by a brief coverage of the various PSs, light delivery systems and roles played by oxygen.

1.2 Photosensitisers/drugs used for PDT

Several types of PSs exist in various stages of development for use in the clinical environment, but relatively few are extensively used in clinical applications [9-10].

1.2.1 First generation photosensitisers

Although photosensitizes have been used for medical applications since centuries, if not millennia, it is well accepted that the term of first generation PSs involves the haematoporphyrin derivative (HpD) family, a mixture including haematoporphyrin, protoporphyrin, as well as dimers and oligomeric fractions. In depth reviews suggested that the oligomeric material was able to penetrate into certain solid tumours and accounted for the tumour localising activity of HpD *in vivo*. That led to the establishment of the most commonly used and approved photosensitiser, Photofrin®. It also sparked the race to develop new PSs with optimized properties, to overcome some drawbacks of Photofrin®. These include:

(1) Low or zero toxicity in the dark

(2) A high triplet quantum yield, a triplet energy > 94 kJ/mol and a long lifetime allowing efficient singlet oxygen production.

(3) The ability to accumulate preferentially in tumour tissue rather than in normal tissue.

(4) Fast clearance from the body after treatment to reduce skin photosensitivity.

(5) Constant composition, reasonable stability of the single substance, and a straightforward synthesis method.

(6) Absorption in the "deep red" (wavelength > 650 nm) part of the visible spectrum to treat "massive" lesions.

1.2.2 Second generation photosensitisers

A variety of new PSs, known as "second generation", have been developed following the partial success of Photofrin®-mediated PDT. These are modified porphyrins, chlorines and bacteriochlorins. Their improved photophysical properties result in their longer activation wavelengths (further in the "red") allowing these PSs to be activated by light penetrating deeper into tissue, thus substantially enhancing their PDT efficiency.

Chlorins and bacteriochlorin have a tetrapyrrole molecular structure, similar to Photofrin®, but the hydrogenation of one or more of the double bonds results in an intense absorption band at wavelengths greater than 650 nm and 740 nm respectively.

Meso-tetra hydroxyphenyl chlorin (mTHPC), marketed under the name Foscan®, is one of the most promising chlorins developed. In October 2001, Foscan® was approved in several countries of the European Union and Norway as a local therapy for the palliative treatment of patients with advanced head and neck cancer who have failed prior therapies and are unsuitable for radiotherapy, surgery or systemic chemotherapy.

Foscan® exhibits a large extinction coefficient in the red around 652 nm, and appears to be approximately 200 times more potent than Photofrin® in terms of photodynamic dose [15]. Therefore, it requires low drug (typically 0.1 mg kg^{-1}) and light (10 J cm^{-2}) doses. Preclinical studies indicate that this drug is more tumour-selective than photofrin in certain cases [11-12]. The benefits of this drug are also evident clinically, but mTHPC induces a long period of skin photosensitivity (days or weeks) [13]. Studies reported by Grosjean et al, and Savary et al, [14-16] have shown that the drug is also effective in the treatment of bronchial and oesophageal early tumours.

The most commonly used PDT photosensitizer in ophthalmology is benzoporphyrin derivative monoacid ring A (BPD-MA), or verteporfin (Visudyne®), a second-generation photosensitizer, which shows absorption at 690 nm. The "ring A" consists of the conjugation to cyclohexadiene ring on the "A pyrrole" position of the chlorin structure. BPD-MA is a racemic mix of two regioisomers that possess different location of the carboxylic acid and methyl ester on the C and D rings of the chlorin macrocycle. Both of these monoacid regioisomers are transformed into the diacid in the liver. It is found that, the plasma half-life of BPD-MA is 5-6 hours in humans. An important characteristic of BPD-MA is that it is hydrophobic and therefore its liposomal formulation is soluble. The PDT selectivity is an important aspect for its accumulation within microvasculature, and most precisely its capability to cause damage to endothelial cells. Verteporfin possesses a very high affinity for plasma lipoproteins and hence is taken up by cells having low-density lipoprotein receptors e.g., neovascular endothelium. In most cases, tissue destruction is due to vascular damage and thrombosis [17]. Light of appropriate fluence and drug dosing are optimally used for the treatment of the retina if we want to achieve the closure of the choriocapillaries and choroidal neovascular tissue without damage to the overlying retinal tissue or vasculature. Hence the selective and localized treatment of PDT has an important prospective for visual function while macular photocoagulation for AMD resulted frequently in instant and intense damage to the retina. It should be noted that the development of novel technologies, such as Lucentis®, to treat certain forms of AMD significantly reduced the clinical use of Visudyne® during the past years.

Phthalocyanines are synthetic benzo-porphins originally developed as dyes and pigments (extensively used in ballpoint pen inks), but also investigated for their possible use in PDT (Ruck et al; Moan et al) [9, 10, 18, 19]. The structure of the phthalocyanine is similar to the porphyrin, which causes the longer-wavelength absorbance in the 650-700 nm regions potentially allowing increased treatment depth.

Because of their large extinction coefficients in the "red", some phthalocyanines are extremely potent PDT sensitizers (Stephen et al; Barr et al; Bonnett) [20-22]. Phthalocyanines can be conjugated with different types of metals. The most effective of these include atoms such as zinc and aluminium, which lengthen their triplet lifetime

thus enhancing their potency, probably in part due to type I process [23].

Different types of other synthetic compounds have been developed to enhance tumour selectivity and photophysical properties, and especially to promote tissue penetration by using longer wavelength absorption ranges. Purpurins are composed of the porphyrin ring with an absorbance band at 660 nm. Recently an expanded porphyrin structure, termed texaphyrins, has been described. It shows a strong absorption at 732 nm and can be used successfully to treat a variety of experimental animal tumours (Young et al; Woodburn et al) [24-25]. Palladium-bacteriopheophorbide, also called Tookad, is a photostable compound, and has an absorbance band at 763 nm, with a high extinction coefficient (Chen et al) [5]. Pd-bacteriopheophorbide, also known as Tookad is used to treat small cell caricinoma of prostrate (SCCP) a relatively rare form of aggressive protrate cancer. In another application white Landrace male piglets were given intravenous Tookad followed by laser light illumination to treat mainstream bronchus.

1.2.3 Third generation photosensitisers

The PSs, which are coupled to delivery vehicles like liposomes or antibodies are called third generation PSs. Various types of drug delivery vehicles for example polymeric micelles, liposomes, lipoprotein-based drug carriers, nano-particle drug carriers, dendrimers are available. Important characteristics are required for an ideal drug delivery vehicle: it must be non-toxic, biocompatible, non-immunogenic and biodegradable [26-27].

Further PSs exist in the family of the PS precursors. Whilst it is not the aim of this paper to review them all, it should be mentioned that a large body of work has been produced on Aminolaevulinic Acid (ALA) and its derivatives as precursors of Protoporphyrin IX (PPIX), a potent PS [28-30] which is discussed in the following chapter (1.2.4) on the endogenously induced PSs.

1.2.4 ALA-induced protoporphyrin IX

The cellular synthesis of endogenous photosensitisers can be used as an alternative to the administration of exogenous photosensitising compounds. 5-Aminolaevulinic acid (ALA – figure 2) is a naturally occurring amino-acid. ALA is an intermediate in the haem biosynthesis pathway as reported by Kennedy and Pottier, 1992 [28]. The pre-cursor to haem in this pathway is protoporphyrin IX (PpIX – figure 2) that has long been known to have photosensitising capabilities, but heterogeneous production in tissues, thus limiting its clinical use. The haem cycle is regulated by enzymatic control under normal circumstances, with PpIX to haem as one rate-limiting step. Therefore, if ALA is applied in excess, the negative-feedback mechanism is bypassed, causing the build-up of phototoxic levels of PpIX (Kennedy et al, 1990) [29].

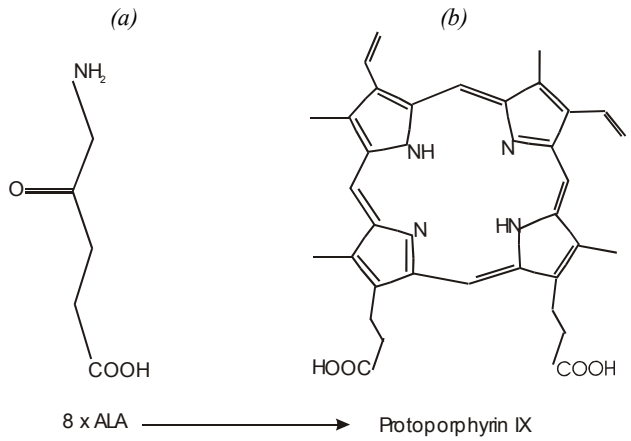

Fig. 2. Chemical structures of (a) ALA and (b) PpIX.

Advantages of ALA-induced PpIX include a rapid systemic clearance from the body, reduced skin photosensitivity, very low dark toxicity and repeatability without risk of damaging normal tissue. The topical administration of ALA, or some of its derivatives, reveals strong usefulness of the latter for the local treatment of superficial skin lesions [31].

The comparison of the advantages of ALA-induced PpIX over Photofrin® shows that there are still some drawbacks associated with this precursor. PpIX is produced heterogeneously in most lesions, it has a long wavelength absorption peak centred at 635 nm, so offers a similar penetration depth as Photofrin®. 5-Aminolaevulinic acid is also hydrophilic, which limits drug penetration through the skin layers, but there is a possibility of lipophilic ALA-esters being able to penetrate the cells more easily (Gaullier et al, 1999) [30]. This strategy is also used for the detection of superficial bladder cancers by fluorescence imaging [32]. ALA is especially useful for eradication of actinic keratosis. ALA and its methyl-ester is approved for this treatment in the EU and in the US under the trade names Metvix® and Levulan®, respectively.

1.3 Light delivery and dosimetry in PDT

Since the photobleaching of photosensitizes strongly depends on the fluence and fluence rate in the tissues, mastering the light delivery and dosimetry is of crucial importance in this field. Modelling the propagation of light for a given illumination geometry is also important and require, in most situations, the knowledge of the tissue optical properties.

Treatment and research within the PDT field has been facilitated by the development of reliable and ‘easy-to-use’ medical light sources and delivery systems. In particular, semiconductor light sources (lasers; LEDs) play an increasingly important role because of their compactness, low cost, robustness and convenience

of use. In addition, the introduction of semiconductor light sources allowed for the development of portable devices that produce stable output at a particular wavelength. Diode lasers can also be engineered to produce wavelengths that match the requirements of most

PSs. The main benefit of the diode lasers over LEDs, gas discharge and thermal light source is their suitability for coupling light into a fibre, allowing efficient delivery in hollow organs or interstitially. Indeed, the light can be accurately and efficiently delivered through an optical fibre, allowing not only the treatment of surface conditions, but also internal lesions if introduced into the body *via* an endoscope, or directly implanted into the tumour mass [33-34]. Attachment of a micro lens to the fibre allows a homogenous illumination over a defined area. Optical fiber-based sticks or cylinders are also used to illuminate hollow organs or for interstitial PDTs. In all cases, the purpose of these frontal or cylindrical light distributors is to administer controlled irradiance and light doses in the treated tissues as homogenously as possible. Finally, for superficial treatments, light devices using diode arrays were developed and are now also commercially available.

Tissular optical properties have also been widely studied [35]. The determination of the tissue optical parameters is important since this enables to model the propagation of light and to predict the light dose delivered at different locations in the tissues.

In PDT, the most commonly used wavelengths are 690 nm for the treatment of age-related macular degeneration (AMD-PDT) with Visudyne®, 652 nm for mTHPC and 635 nm for ALA-induced PPIX. These laser wavelengths correspond to the peak of light absorption in the "red" region for each compound and ensure maximum penetration of tissue (the typical tissue penetration of light in soft tissues is around 3 mm at 630 nm) [36-39]. It should be noted that the non-uniform in-depth distribution of the fluence rate results in a heterogeneous excitation, and consequently photobleaching, of the PS.

Dosimetry allows for a homogeneous therapeutic dose distribution over the region requiring PDT and quantitatively evaluates the dosing of pathologic and normal tissues. All the parameters shown in Figure 3 are interrelated to one another and point to why the tissue response is complex.

Traditionally, photodynamic dose measurement has been carried out in terms of four explicit parameters:

- Administered PS dose (in units of $mg.kg^{-1}$ of body weight),
- Incident light dose or fluence (in units of $J.cm^{-2}$),
- Drug to light time interval (in hours) and
- Irradiance or fluence rate (in units of $mW.\ cm^{-2}$).

However, this approach of dosimetry does not account for inter and intra-patient variability in PS uptake, tissue oxygenation or tissue optical properties, for the inter-dependence of PS-light-tissue interactions, or for various other factors influencing the therapeutic result of PDT (self-shielding of the PS, which limits the light penetration due to added absorbance by the photosensitiser itself; photochemical depletion of oxygen during laser exposure, especially at high fluence rates, resulting in a reduced photodynamic effect; vasoconstriction or shut-down of the blood flow; etc). Presently, one aim of the research in this field is to improve dosimetry models, and to use PS photobleaching as an index to monitor the effective dose [40].

The dosimetry is called "implicit" when it relies on the use of PS photobleaching to provide a measure of the light dose. If we measure photobleaching as a loss in PS fluorescence, and assume that this corresponds to a loss of photodynamic activity, then relative fluorescence measurements can be utilised since the photobleaching is linked to the photochemical activation of the drug. For relatively low PS concentrations fluorescence spectroscopy is highly sensitive, and the dosimetry can be analysed continuously.

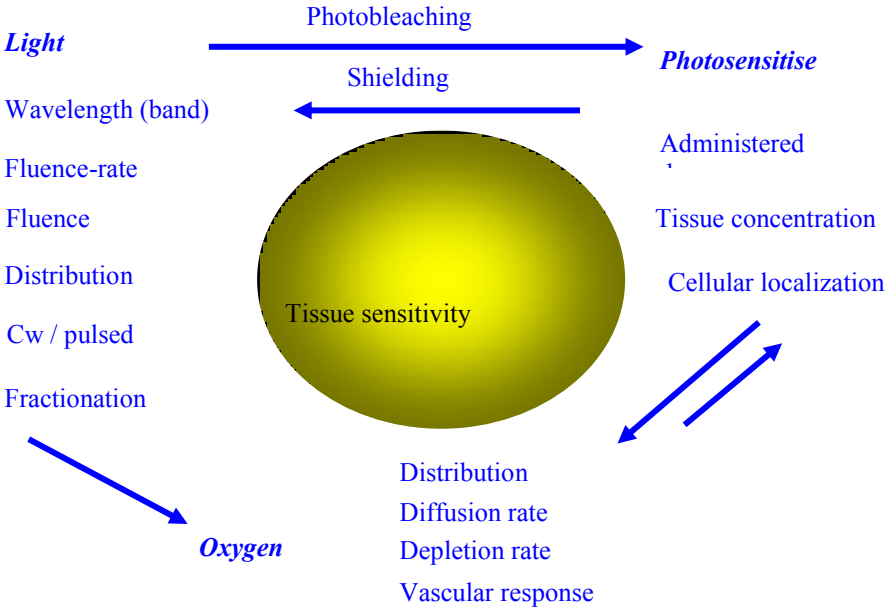

Fig. 3. Interrelating factors in Photodynamic therapy Dosimetry.

2. Photosensitizer photobleaching

As mentioned above, the photodynamic interactions require the simultaneous presence of PS, light and oxygen. However, many PSs are themselves prone to photodestruction when exposed to light, a process called 'photobleaching'.

Photobleaching is a term used to indicate that the concentration of a chromophore decreases during light exposure. In PDT, it is defined as the photo-degradation of the fluorescence intensity or loss of fluorescence. Photoproducts can be generated alongside the photobleaching process during the destruction of the photosensitiser molecule, and therefore should also be included in the definition of photobleaching.

During PDT the destruction of the photosensitizer molecule results in both beneficial and detrimental effects. Indeed, a threshold photodynamic dose must be delivered to ensure tumour necrosis. Therefore, a lesion can be undertreated if the PS is photobleached before reaching this threshold photodynamic dose. However, no tissue damage may occur in the surrounding normal tissues if significant photobleaching takes place before this threshold is reached, even if this tissue is over-illuminated. Therefore the possibility exists to enhance the therapeutic contrast and the treatment safety from differential photosensitizer uptake between the lesion and normal tissue.

A detailed investigation has to be performed on each photosensitiser before photobleaching can be used in an implicit dosimetry model. The degradation of different PSs occurs by different mechanisms and this needs to be correlated to photodynamic damage. Some PSs do not show enough fluorescence intensity to make it possible to monitor dose in this way. In these cases, the simplest dose metric would be to measure singlet oxygen luminescence directly. However, the technology to be developed in this approach is relatively new and, despite initial studies in vitro [41] showing that it is promising, its use may not be translated to clinical applications in the near future (Niedre et al) [42].

Knowledge of the photobleaching processes occurring during PDT helps implicit dosimetry, as it describes multiple photophysical, photochemical and photobiological factors involved in PDT. Due to this, the clinical efficacy of PDT and optimisation of treatment regimes will be allowed.

There have been major efforts to describe the mechanism of the photobleaching both *in vivo* and *in vitro* (Forrer et al; Blant et al; Ma et al; Robinson et al; Finlay *et al*; Georgakoudi and Foster; Hadjur et al; Coutier et al, Atif et al). In all investigations [43-63], a singlet oxygen-mediated bleaching of the fluorescence intensity has been found, which could not be adequately described *via* first-order fluence-dependent decay kinetics.

2.1 Photobleaching studies in vitro

Georgakoudi and Foster [48] have reported a study addressing the rate of PpIX photobleaching in a tumour spheroid. The analysis revealed that the rate of photobleaching was in excellent agreement with results obtained from in vivo and in vitro studies of the singlet oxygen-mediated photobleaching of Photofrin®. When $^1O_2$ is responsible for photobleaching, photosensitiser reduction progresses gradually from the regions closest to the oxygen source (outer radius of tumour spheroid), towards the centre of the spheroid. Therefore, singlet oxygen mediated photobleaching is confined to areas of oxygen abundance. It is suggested that there is the potential to enhance the photodynamic effect if photobleaching does not occur at a rate that depletes the PS concentration before the threshold dose of singlet oxygen is deposited.

Evidence of involvement of $^1O_2$ was provided by Hadjur et al [49] during the photodegradation of mTHPC in 10 % fetal calf serum in solution. Their experimental results confirmed that mTHPC was able to generate singlet oxygen, which then caused self-photobleaching.

Coutier et al [50] reported the impact of fluence rate on cell survival and photobleaching in Colo 26 multicell spheroid photosensitised by mTHPC. The spheroids were irradiated with 650 nm light at 5, 30, 90 $mW.cm^{-2}$. The experimental results demonstrated that the rate of photobleaching increased as the irradiance level was reduced and that there was a corresponding decrease in the fraction of cells surviving treatment. Their findings also show that fluence and oxygenation play a major role in the photobleaching of mTHPC sensitised tumour spheroids and in PDT–induced cell toxicity. Moreover, these experimental results were analysed by comparing them with a mathematical model of photobleaching, dependent upon self–sensitized oxygen reactions, with the PS ground state. Modelling was performed by incorporating photophysical parameters determined from microelectrode measurements of oxygen depletion at the surface of mTHPC-sensitized spheroids and was improved by introducing the inhomogeneous distribution of mTHPC in spheroids and oxygen depletion in the bulk medium. The consistency of this model with experimental results suggests that the fluence rate dependence of the cell survival and of mTHPC photobleaching are due to photochemical oxygen consumption and readily observable singlet oxygen mediated mechanism of mTHPC photobleaching. In this system, the threshold dose of reacting singlet oxygen was measured to be 7.9 ± 2.2 mM.

Atif et al, [51-63] applied a micro-spectroscopic technique in order to record the laser-induced fluorescence emission of mTHPC from micron-scale locations within individual formalin-fixed keratinocytes. They demonstrate that mTHPC is highly photolabile in a cellular environment, and that the process of photobleaching can be monitored *via* the depletion in fluorescence emission during continuous irradiation with 410 nm laser light. The progressive reduction of the characteristic 652 nm mTHPC fluorescence peak can be described with bi-exponential decay kinetics, consistent with a singlet oxygen mediated process. The rate of photobleaching, when plotted as a function of light dose, shows inverse fluence-rate

dependence. Specifically, the rate of photobleaching induced by the higher laser powers appears to be limited by oxygen availability, as demonstrated by an increase in the (1/e) bleaching dose. Fractionated irradiation provides evidence of intracellular re-oxygenation. These results are in qualitative agreement with previous *in vitro* and *in vivo* studies, which indicate that the photodynamic dose delivered during light irradiation is critically dependent upon local fluence-rate and oxygen partial pressure.

In a recent study, Tekrony et al [64] used photobleaching kinetics of two PSs (Verteporfin and Lemuteporfin) in vitro following two-photon excitation, to clarify the effect of structural differences between the two PSs. Interestingly, they hypothesize that the differences they observe in the photobleaching kinetics of the two PSs could be explained by their respective localization in the lipid bilayer (and corresponding availability of molecular oxygen in the same region), and not by the intrinsic photophysics of the two molecules. This finding opens the door to approaches correlating the photobleaching constants with the micro-localization of a given PS in a given environment.

In all investigations to date, oxygen concentration and fluence rate dependent intracellular photobleaching [57-59, 65-71] was found. This is known as inverse dose-rate behaviour, i.e. a reduction of irradiance (fluence rate) resulted in more photobleaching at comparable light doses (time integrated irradiance). These studies do however, illustrate that the widely used fluence-dependent single exponential decay description of sensitizer degradation is not always an adequate model of photobleaching during PDT.

Although in vitro studies provide valuable information regarding the physico-chemical mechanisms involved during photobleaching, in vivo experiments must be conducted to address numerous effects related with the oxygen supply by passive diffusion or vie the blood stream, the metabolic activity, the immune response, etc.

2.2 Photobleaching studies in vivo

Forrer et al [43] studied the mTHPC bleaching kinetics in vivo in patients with early squamous cell carcinomas in the oesophagus, irradiating at 514 nm (fluence rate 100 $mW.cm^{-2}$). They found a 60% decay of the fluorescence of mTHPC at 652 nm, when a light dose of 100 $J.cm^{-2}$ was delivered. Their investigations also demonstrated that the decay of mTHPC fluorescence was consistent with the assumption that $^1O_2$ was the agent responsible for bleaching.

Blant et al [44] described in vivo fluence rate effect in photodynamic therapy of early cancers using mTHPC. Their in vivo studies demonstrated more efficient treatment of Syrian hamster cheek pouch tumours at low fluence rate.

Ma et al [45] observed that the rates of photobleaching of mTHPC and mTHPBC [meso-Tetrahydroxyphenyl-bacteriochlorin] were identical when mice bearing human colon adenocarcinoma implants were exposed to 652 and 740 nm wavelength light having a fluence of ~ 10 $mJ\ cm^{-2}$. Since mTHPBC contains a small amount of chlorin (<5%), a small peak due to mTHPC is detected in the fluorescence spectra of mTHPBC in mice. It was proposed that by irradiating the tumour first at 740 nm and then at 652 nm the damage to normal tissue could be avoided, since mTHPBC could be easily cleared, leaving mTHPC unaffected. On the other hand, if the amount of mTHPC left was high enough, exposing to 652 nm wavelength light could then affect tumour necrosis.

Robinson et al [46] demonstrated an experiment in which UVB-induced tumour tissue was subjected to ALA-induced PPIX-mediated PDT. The detected fluorescence intensity taken from the tumour tissue was observed to be of a much higher intensity than that of the normal mouse skin despite the tumour being optically thicker. It was found from the analysis of results that the bleaching rates (with respect to fluence) of PpIX and the amount of photoproducts increase with the decreasing irradiance. Moreover, the rate of photobleaching in the normal skin varies at a greater rate as compared to tumour tissue. It was also observed that at low values of irradiance on normal mouse skin, the increased rate of photobleaching corresponded to enhanced homogeneous photodynamic damage across the treatment site. On possible mechanisms is that the reduced photochemical oxygen consumption places less demand upon the vascular system, allowing a critical supply of oxygen to be maintained throughout the illuminated area.

Finlay et al [47] demonstrated the impact of mTHPC photobleaching in normal rat skin. On laser exposure at 650 nm, loss of mTHPC fluorescence was detected using in vivo fluorescence spectroscopy during photodynamic therapy. The bleaching was analyzed for irradiances of 5, 20, 50 $mW.cm^{-2}$. Two different phases of mTHPC photobleaching were examined. The first phase did not show obvious irradiance dependency (plotting loss of fluorescence against fluence). In the second phase the photobleaching rates show irradiance dependency consistent with an oxygen dependent reaction process. In order to investigate the unusual shape of the in vivo bleaching curves, they observed the PDT induced changes in O_2 concentration in mTHPC sensitized spheroids exposed with 5, 20, 50 $mW.\ cm^{-2}$ at 650 nm. The data of oxygen concentration pointed to no unusual features within this range of fluences where the discontinuities in fluorescence were monitored during in vivo spectroscopy. Moreover, this analysis reveals that higher irradiance causes more significant oxygen depletion, which is consistent with a singlet oxygen-mediated bleaching mechanism.

Ascenio et al, [72] described the histological parameters and the PpIX photobleaching associated with i.p. HAL-induced PDT [ALA-hexylester] in a rat model. Their results showed that HAL is a new promising PpIX precursor for PDT of advanced ovarian cancer. PpIX fluorescence measurement and its photobleaching is a feasible and reliable parameter to predict the outcome in HAL-PDT in vivo. The calculation of the PpIX residual fluorescence threshold, which reflects the photobleaching of the photosensitizer and may indicate a second light

fraction to be achieved, must be considered as a predictive tool that could enhance the tissue response to PDT.

Wang et al, [73] used a comprehensive, previously published, mathematical model to simulate the effects of therapy-induced blood flow reduction on the measured PpIX photobleaching. This mathematical model of in vivo PDT incorporates a singlet oxygen-mediated photobleaching mechanism, dynamic unloading of oxygen from hemoglobin, and integrates blood flow velocity changes. It permits simulation of the in vivo photobleaching of PpIX in this patient population over a broad range of irradiances and fluences. Their results propose that the physiological equivalent of discrete blood flow reductions is necessary to simulate successfully the features of the bleaching data over the entire treatment fluence regime. Furthermore, the magnitude of the blood flow changes in the normal tissue margin and lesion is consistent, for a wide range of irradiances, with a nitric-oxide-mediated mechanism of vasoconstriction.

Piffaretti et al. [74] have reported that the magnitude of PpIX's photobleaching during Metvix®-mediated PDT treatment of Actinic Keratosis (AK) is a valuable parameter to provide information on the clinical outcome including, to a certain extent, in the long term (33.6 months following PDT). When the clinical outcome is evaluated, high levels of fluorescence intensity or PpIX photobleaching correspond to a more potent therapeutic effect. The PpIX's photobleaching is linearly correlated to the normalized fluorescence before PDT. This study shows that, in well-oxygenated conditions, the PpIX and the PpIX's photoproducts can be efficiently and completely bleached during the illumination, with the exception of a small, unbleachable fraction of the accumulated PpIX (or alternatively, with the presence of unbleachable photoproducts). The exact origin of this effect is unknown. These preliminary clinical results support that a satisfactory clinical outcome can only be reached if the PpIX fluorescence intensity or the PpIX fluorescence photobleaching are above a certain threshold. These encouraging results are supported by other publications that report correlations between the photobleaching and clinical outcome. It should be noted that most of these studies reported that the fluorescence photobleaching is better correlated with the clinical outcome than the fluorescence intensity.

2.3 Fluence rate effects

Foster and his co-workers (Foster *et al*., 1991 [75]; Nichols and Foster, 1994 [76]; Georgakoudi *et al*., 1997 [77]; Georgakoudi and Foster, 1998 [48]) reported a number of studies on in-vitro tumour spheroid system to study the effects of oxygen consumption and photobleaching of Photofrin®.

Several studies have also been carried out by different researchers (Coutier et al [50]; Kunz et al [78]; Dysart et al [79]; Finlay et al [47]) to study the impact of the fluence rate on the oxygen consumption and PS photobleaching. Coutier et al [50] discussed the impact of fluence rate on cell survival and photobleaching in mTHPC photosensitized Colo 26 multicell tumour spheroids (a non-metastasizing mouse colorectal tumour cell line). The mTHPC photosensitized spheroids were irradiated with 650 nm light at 5, 30, 90 mW. cm^{-2}. Fluorescence decay of mTHPC was measured in cells. This fluorescence rapidly reduced after laser exposure and was expressed as a percentage of controls (drug, no light). The experimental results showed more important bleaching at lower fluence rate.

It is important to mention here that for a low fluence (5 $J.cm^{-2}$) delivered at 5 mW cm^{-2}, approximately 50% of the mTHPC fluorescence intensity remained. This differs from the results obtained with 90 mW. cm^{-2}, where 85% of the fluorescence intensity remained. Using fluence rates of 90, 30, and 5 $mW.cm^{-2}$the loss of fluorescence was 50%, 80% and 90% respectively. The therapeutic efficacy and photosensitizer degradation increased dramatically and progressively when the fluence rate was reduced over the range from 90 to 5 $mW.cm^{-2}$. The cytotoxicity of mTHPC based PDT also showed strong fluence rate dependence. However, the reduced fluence rate significantly altered the therapeutic effect in certain conditions.

In subsequent works, Kunz et al [78] studied the intracellular photobleaching of mTHPC in the murine macrophage cell line J744A.1. They used quantitative fluorescence imaging microscopy, microspectrofluorometry and microspectrophotometry in these investigations. Using 652 nm laser exposure, it was observed that mTHPC demonstrates oxygen- and fluence rate-dependent intracellular photobleaching. The rate of photobleaching, when plotted against the light dose, shows inverse dose-rate dependence, i.e. a low fluence rate leads to more photobleaching at comparable fluences. In additions, the effect of de-oxygenation was observed to be more critical with decreased bleaching at low fluence rates and increased bleaching at high fluence rates.

Jarvi et al [41] used in vitro conditions to compare photobleaching of mTHPC and the luminescence of singlet oxygen for individualized PDT dosimetry purposes, thus addressing inter- and intra-treatment variability. Interestingly, they observed that, at low oxygen concentrations, singlet oxygen concentration estimates, based on photobleaching only, cannot always be considered reliable, although it seems to be the only relevant option for clinical use. These authors also identified a mTHPC-specific luminescence signal that indicates the oxygen levels underneath which photobleaching measurement is no longer reliable. They suggest that this could be used as a signal to stop illumination for the tissue to re-oxygenate.

It is concluded from this discussion that, in order to influence the therapeutic response, treatment strategies must be devised with fluence rates that allow deposition of a threshold dose of singlet oxygen throughout the system.

2.4 Photobleaching effects upon oxygen consumption and singlet oxygen production

The experimental results of Georgakoudi *et al*., (1997) supported a theory of self-sensitised singlet oxygen

mediated bleaching of Photofrin® in the spheroid model mentioned above [77]. Later on, Georgakoudi and Foster (1998) [48] described the mechanisms of action for Nile blue selenium and ALA-induced PpIX. It was observed that the photobleaching was mediated by singlet oxygen for PDTs based on the use of ALA-induced PpIX and Photofrin®, while it was not the case when Nile blue selenium was used as PS. This work has a particular significance in dosimetry for measuring the accurate photosensitiser photobleaching mechanisms.

Dysart et al [79] discussed mTHPC photobleaching in DP16 cells using 514 nm laser exposure. mTHPC concentration, fluence rate and oxygenation effects were independently controlled and studied during in vitro experiments. PDT experiments were carried out over a range of treatment conditions, thus demonstrating that PS photobleaching obeyed second order bleaching kinetics.

A dosimetry model that relates bleaching of photosensitizer fluorescence to biological damage during PDT was tested for varying sensitizer concentrations, fluence rates and medium oxygenation. The prediction about the cell viability from mTHPC fluorescence photobleaching could be made using a single curve for a range of treatment parameters used except at higher concentration. Fluorescence of mTHPC was continuously examined via a CCD coupled spectrometer during treatment and at selected fluences. Cell viability was determined using a trypan blue exclusion assay. These investigations also suggest that fluorescence bleaching may be utilized in order to predict PDT damage in vitro [80].

As reported above in 2.2, Finlay et al [47] reported, in another investigation, the impact of mTHPC photobleaching on normal rat skin.

This investigation led to the following two conclusions.

1. Photobleaching relates to the degradation of active photosensitizers caused by photochemical reactions with $^1O_2$ or other reactive species.

2. The loss of fluorescence is more efficient with respect to fluence at lower irradiance and this behavior supports the view that higher irradiance causes more significant oxygen depletion, which is consistent with a singlet oxygen-mediated bleaching mechanism.

In the next chapter observations of the influence of the fluorescence photobleaching and light fractionation during photodynamic therapy are described. A number of studies [81-92] have also revealed that PDT efficiency can be enhanced by short term and long-term light fractionation. This scheme allows re-oxygenation of irradiated tissue, thereby enhancing singlet oxygen production in the second and subsequent fractions.

2.5 Fractionation of light

Different approaches have been considered to enhance photodynamic efficacy and to reduce treatment fluence rate. The favoured approach is based on maintaining the ambient oxygen concentration in such a way as to insure that threshold conditions are met across the target tissue volume. However, identical results can be achieved by punctuating continuous irradiation with dark intervals, which allows the re-oxygenation of anoxic regions. This is termed fractionation of light, and is, at the present time, being investigated for various applications of PDT (Hua et al; Van der Veen et al; de Bruijn et al; Robinson et al; Cunrow et al; T. A. Middelburg et al; H. S. de Bruijn et al) [81-87] as an emerging technique due to its enhanced photodynamic response. It should be noted that some authors report that it can be non-significant in certain cases [86].

In a theoretical analysis, Fuchs et al [8] proposed that during PDT oxygen partial pressure in cell walls away from the capillary was sufficiently low to minimise $^1O_2$-mediated effects. At high irradiance and oxygen depletion, this effect is readily observable and could be partially overcome by light fractionation. Since fractionation increases the singlet oxygen concentration in cells relatively “far” from the capillary wall, the analysis of the results shows that fractionating the irradiation dose should result in improved therapeutic response for PDT. This effect has been observed by Foster et al [93].

Fuchs et al [8] point out that in ALA-PDT, protoporphyrin IX photobleaches rapidly. The effectiveness of PDT is then determined by the PpIX concentration in the tissue rather than optical dose as discussed by Kennedy et al [28]. In the case of PDT using ALA, fractionating the light dose may allow the tissue to regenerate adequate levels of protoporphyrin IX in order to continue PDT. This is in agreement with the works of Messmann et al [71] who reported that fractionating the light dose significantly enhanced the cytotoxic effect of PDT with ALA in normal rat skin (colon).

The photobleaching dynamics of keratinocytes was also investigated by Atif et al [57] using fractionated exposure at constant laser power. The fractionation regime involved 4 exposures, each of 10 seconds duration, separated by a dark period of 200 seconds. A sequence of 10 spectra were recorded during each exposure, each acquired over 0.5 second. This reduced acquisition time was adopted in order to increase the number of data points recorded during each exposure. The data from 10 separate cells were averaged and normalised with respect to intensity, as described above. In this study the fractionated light exposure regime results in a deviation from continuous bleaching kinetics following immediately the dark periods. Specifically, there is an increased bleaching rate during the early part of the second, third and fourth light fractions which suggests some recovery of oxygen partial pressure during the dark periods.

These results are in general agreement with those from previous in *vitro* and *in vivo* reports of fluorescence bleaching analysis, using a variety of photosensitisers in different chemical and biological environments. Hence it was concluded that during PDT, light fractionation may also help maintain high tissue porphyrin concentrations resulting in an enhancement of PDT efficacy.

Fractionation of light is a technique compatible with clinical practice that can influence the effectiveness of PDT and this possibility is demonstrated by a number of

studies. It can be divided into two different approaches of light fractionation depending on the timing of the dark period between irradiation. The first approach is based on short (a few minutes) dark intervals. This method allows tissue re-oxygenation, thereby increasing singlet oxygen generation in the subsequent irradiations.

The second approach is based on longer intervals of one hour or more to allow not only re-oxygenation but also a re-localization of the PS and/or an important re-synthesis of PpIX within the target tissue. In addition, this may involve either a fresh application of precursor, or it may rely upon renewed synthesis of PpIX from remaining supplies of precursor in the tissue following initial irradiation.

2.5.1 Short-term fractionation

Several investigations have been performed to examine the effects of short-term light fractionation on PDT. Robinson et al [94] studied short-term fractionation in UVB-induced tumours of the hairless mouse skin. The PpIX photobleaching rate was observed to significantly increase upon renewed irradiation after a defined dark period due to an increased oxygen concentration during the dark period. Fractionation of light was also observed to increase the damage in normal mouse skin, as a result of enhanced oxygen concentration and homogeneity across the exposed area.

Analysis of this study suggested that the use of low irradiances rather than fractionation at high irradiances would be more beneficial, although an optimal fractionation scheme had not been established. The timing of the dark period has been considered critical to improve the effects of fractionation. Moreover, the photobleaching rate of PpIX has been found to increase with decreasing irradiance in both normal and UVB treated skin. This effect could then be enhanced by introducing periods of light fractionation which results in the increased rate of bleaching and photodynamic damage.

Curnow *et al* [95] observed oxygen levels at the treatment site using a micro-electrode in another study using a short-term fractionation technique. They examined the oxygenation to be particularly crucial when investigating the mechanism of light dose fractionation. Continuous illumination for 25 J was evaluated with fractionation after 5 J, followed by a 150 second dark period, followed by the remaining 20 J. It was found that oxygen levels in the continuous illumination scheme close to the irradiation fibre dropped quickly after laser exposure, whilst further away the oxygen levels decreased more slowly. Hence the kinetics of oxygen reduction was modified in this fractionation scheme such that the partial pressure dropped to zero only after the second irradiation had started. These observations were discussed in terms of PS relocalisation and reoxygenation at the treatment site during the dark period, and possible reperfusion injury. It is suggested that by turning off the light, the consumption of oxygen is temporarily interrupted, and as long as the microcirculation remains viable, the tissue should re-perfuse. When the light is "on" after the dark period, the photochemical oxygen consumption should proceed. It was also proposed that the timing and length of dark period play a significant role in maintaining viable microcirculation, to re-perfuse the treated area in as short a dark period as possible. A small increase in PpIX fluorescence was also observed during the dark interval, pointing out that re-synthesis can occur within this short time. Consequently, the damage produced by the treatment may be enhanced with additional photosensitiser, but it is very unlikely that such a small increase in PpIX levels could account for the differences between the regimes.

2.5.2 Long-term fractionation in PPIX-mediated PDT

In order to analyse the effects of long-term fractionation on PPIX-mediated PDT, different studies have been carried out. Van der Veen et al [82] reported that two illuminations separated by a dark period of 6 hours acted to enhance skin damage by 2.5 to 4.3 times over the same continuous exposure. The authors suggested that this effect is due to cells (damaged or not) having the capacity to form PpIX after the first illumination.

De Bruijn et al [83] studied a 75-minute dark interval introduced between the first and second light fractions. The damage of skin produced by this interval was not increased compared to a single illumination, but discoloration was more readily found due to haemorrhage of blood vessels around and at the border of the tumour site. Hence the tumour response was improved drastically using a two-tiered illumination scheme.

In another study Robinson *et al* [84] discussed the kinetics of PpIX fluorescence during and after laser exposure, the relationship between laser exposure parameters (fluence and fluence rate), the photobleaching of PpIX during irradiation, and the photodynamic effect of two-tiered irradiation separated by a dark period of 2 hours. The results of post-ALA topical application were compared with constant illumination light schemes at 4 and 6 hours. Two-tiered illumination was found to result in a re-synthesis of PpIX during the dark period, and a considerable increase in photodynamic damage. Reducing the fluence of the initial irradiation, allowing the re-synthesis of relatively more PpIX during the dark period, which is then used during the second illumination, enhances this effect. However, the second illumination needs to be delivered at a large fluence so as to achieve greater photodynamic damage than a lower fluence at the second illumination stage. Hence the first irradiation renders cells in the illuminated volume sensitive to a second illumination, perhaps by inducing repair mechanisms that are subsequently damaged by a large fluence during the second illumination. It is also possible that after a small first dose the mechanisms of cell death will be different, or that the initial light dose might sensitize the cells, making them more susceptible to subsequent damages [48].

In a recent study, De Vijlder et al [96] report that for patients treated for superficial Basal Cell Carcinoma (sBCC), the rates of complete response 5 years after ALA-

PDT treatment are significantly greater following a 2-fold irradiation scheme (2 hours interval between irradiations) than a single irradiation. A similar study with a shorter follow-up reports similarly encouraging results for Actinic Keratosis (AK) [96]. This study follows previous works by the same group on pre-clinical models [97], and early clinical work [96]. Whilst this study shows clearly how patients can benefit from light fractionation, it should be noted that this approach might not be easy to translate to organs other than the skin, and that the clinical feasibility must also be taken into consideration. Interestingly, the authors report that their use of photobleaching of PpIX as a metric for PDT monitoring demonstrates that oxygen recovery during the dark intervals is not significantly contributing to the enhanced response after fractionated illumination. Therefore, this enhanced response may be due to a sensitization of tissues resulting from the initial illumination, and to the micro-localization of PpIX in the tissues. The mechanism behind the enhanced response is unlikely to be solely explained by the amount of PpIX present before each irradiation and the extent to which it is photobleached during exposure [96].

Since studies tend to explore various models and various illumination schemes, it is not an easy task to point to mechanisms underlying the enhanced effect of fractionated illumination. In a tentative explanation, three mechanisms are thought to be involved: re-oxygenation of the treatment site; re-perfusion of injured tissues; PS relocalization and PpIX re-synthesis. All were studied by Curnow et al [85,95, 98]. Other factors such as the light-induced changes of metabolic activities and the induction of inflammations are also likely to play a role in the enhanced effect of fractionated illumination. Generally speaking, it is not straightforward to optimize the PDT response through light fractionation. It is possible that multiple dark periods may be of help, as would be an illumination scheme starting with less than half the total fluence, before a longer illumination after the dark period [85], thus preventing extensive damage to the micro-vasculature before the latter part of the treatment.

3. Conclusions

Based on our analysis of the reviewed body of works, we can conclude:

1. There have been a number of studies on the photobleaching of PDT photosensitizers [99-101], mostly spectral and kinetic studies, which measured loss of absorbance or fluorescence. Both photo-modifications (where loss of absorbance or fluorescence occurs at some wavelengths but the chromophore is "minimally" modified) and true photobleaching (where "major" chemical changes take place and result in fragments which no longer have appreciable absorption or fluorescence in the spectral region of interest) have been encountered.

2. The efficiency of PDT can be enhanced by fractionation of light. There is evidence of an increased bleaching rate following a dark period.

3. Most of the processes involved in photobleaching are oxidative and they involve singlet oxygen [102].

4. Following light exposure, the photosensitizer can re-localize from one environment (the compartment where the sensitizer is located) to another, possibly with a change of its optical spectroscopy as well as its potency.

5. Although many PDT experiments have been reported covering a broad range of treatment conditions, fluorescence photobleaching obeyed a second-order kinetics, as discussed by Forrer et al. [43]. The second-order photobleaching model is based on the concept that the photosensitizer is photobleached through reactions with singlet oxygen.

6. The rate of photobleaching, when plotted as a function of light dose, shows inverse fluence-rate dependence in most cases.

7. Implicit dosimetry has been observed to be photosensitiser-specific. ALA-induced PpIX has been found to photobleach in an identical manner than Photofrin®, that is, *via* a singlet oxygen mediated process. It has been observed that there are complex interactions, *in vivo* and *in vitro*, which hinder implicit dosimetry models.

8. Cells with higher drug concentration exhibit faster fluorescence bleaching than those with lower concentration.

9. When it comes to optimizing results with PDT treatment, there is no straightforward answer given the variety of diseases, organs, photosensitizers and PDT regimes that have been studied and reported.

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