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Spatial measurement of oxygen levels during photodynamic therapy using time-resolved optical spectroscopy

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Abstract

Tissue oxygenation is one of the key dosimetric factors involved in the application of photodynamic therapy (PDT). However, quantitative studies of oxygenation levels at and surrounding the treatment site have been lacking both before, during and after treatment. With the recent development of sensitive, non-invasive, optical spectroscopic techniques based on oxygen-dependent phosphorescence quenching of probe compounds, oxygenation levels can now be measured quantitatively at selected sites with spatial resolution on the millimeter scale. We present results using the phosphorescent compound, palladium *meso*-tetra(carboxyphenyl)porphine, for measurement of in vivo microvascular oxygen tensions in rat liver during PDT. Time-resolved phosphorescence detection was carried out using fibre-optic sensoring, and oxygen tensions were determined from the phosphorescence lifetimes using Stern–Volmer analysis. During PDT treatment using 5-aminolaevulinic (ALA) acid-induced protoporphyrin IX (PPIX) with a 50 mg/kg ALA dose, oxygen levels near the irradiation fibre placed on the surface of the liver showed a significant decrease by a factor of ten from 20 to 2 torr after an energy dose of 60 J using 100 mW at 635 nm. Areas farther from the treatment site which were exposed to lower light doses exhibited lower reductions in oxygen levels. This spectroscopic technique is a highly sensitive means of investigating tissue oxygenation during and after treatment, and should help not only to advance the understanding of hypoxia and microvascular damage in the PDT mechanism but also contribute to improving the dosimetry of PDT.

Keywords: Photodynamic therapy; Tissue oxygenation; Phosphorescence

1. Introduction

Photodynamic therapy (PDT) is a non-thermal technique for inducing necrosis of tissue with light following administration of a light-activated photosensitising drug which can be selectively retained in malignant tumours (or diseased tissue in the case of non-malignancies) relative to normal adjacent tissue. There are three fundamental requirements for obtaining a photodynamic effect in tissue: a photosensitiser, molecular oxygen and excitation light of the appropriate wavelength, with phototoxicity most likely mediated through the reactions of the cytotoxic oxygen species, singlet oxygen, with cellular substrates [1]. It is well established that vascular damage induced by PDT resulting in hypoxia and deprivation of nutrients is a key factor in the response of tissue to PDT [2], although the time-course and acuteness of the effects vary between different sensitisers. Recently it has become clear that the rate of light delivery can have an important effect on the treatment with higher fluence rates resulting in less tumour damage [3]. There are many possible explanations for this effect but the most probable is that due to the high fluence rate, local oxygen levels become depleted so limiting the extent of the photocytotoxic effect. Fractionation of irradiation for potentiating PDT is a further option that has only been considered by a few research groups but which could well have a major effect on the ultimate results, as has been shown in many studies with ionising radiation. A recent study [4] on fractionation during PDT of normal rat colon using 5-aminolaevulinic acid has demonstrated that a major increase in the size of lesions (factor of three in diameter) can be produced when irradiation is divided into two doses separated by an interval of 5 min. Enhanced effects were also found using modulated irradiation with 50 s on/off periods. Other workers have also found similar results using Photofrin with a modulation period of 30 s, and it has been proposed that the increase in PDT efficacy may be due to transient

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recovery of the vasculature and accompanying reoxygenation of the tissue during the dark intervals [5].

A few studies of PDT-induced hypoxia have been carried out using oxygen electrodes but this technique has problems with electrode calibration, sensitivity, zero current drift and the diffusion barrier, although newer devices offer improved performance; electrodes also consume oxygen, which introduces artefacts at low oxygen levels and it is difficult to avoid surface compression of the tissue which might perturb blood flow. Efforts to minimise variations in sensitivity have included step-wise advancement/withdrawal of the electrode but only a very small portion of the tissue can be probed. Nevertheless, the electrode technique remains the standard approach for tissue oxygen measurement. A study was carried out in rabbits sensitised with Photofrin using a transcutaneous electrode [6] placed on the ear. Intermittent irradiation was applied to the reverse side of the ear and the oxygenation levels were observed to decline during irradiation and then recover during the intervals; however only relative oxygenation values were measured and, owing to the size of the electrode, no spatial information could be obtained.

Recently, Wilson and co-workers have developed a non-invasive optical spectroscopic technique which is capable of quantitative and spatially resolved measurements of tissue oxygenation [7]. The technique relies on oxygen-dependent quenching of phosphorescence of an injected porphyrin probe compound and it has been shown to be capable of measuring oxygen levels and consumption accurately from air saturation to 0.1 torr. The great advantage of this optical technique is that it can also be used to image oxygen distributions using time-gated camera systems. The oxygen probe most commonly used is a palladium porphyrin: palladium *meso*-tetra-(4-carboxyphenyl)porphine (PdTCPP) which has a good phosphorescence quantum yield (7%), long triplet lifetime (ca. 0.7 ms under anoxic conditions), and can be administered to animals (typically at 10–20 mg/kg) in aqueous solutions containing serum albumin [7,8]. Since the technique relies on a lifetime measurement rather than just signal intensity, the derived oxygen partial pressures (pO_2) are independent both of the circulating concentration of the probe and the intensity of the excitation light, and immune to changes in tissue optical properties caused by alterations in haemoglobin levels. Calibration of the probe is absolute in that the lifetime is fully characterised by the microvascular oxygen concentration, as oxygen is the only quencher present at significant concentrations. Since the pO_2 in perfused tissue is dependent on the level of pO_2 in the microvasculature, this technique has found wide application for studies of hypoxia-induced injury to the microvasculature and reperfusion studies [9] but has not as yet been applied to PDT.

In this work we have adapted this technique to PDT for investigating the role of tissue oxygenation, both before, during and after treatment. Photosensitisation was carried out using a natural porphyrin precursor, 5-aminolaevulinic acid (ALA), which is metabolised within cells to produce the photosensitiser, protoporphyrin IX (PPIX), via the haem biosynthetic pathway. We have previously carried out studies in normal tissues and tumour models [10–12], and selective mucosal sensitisation of several organs has been observed [10,11] combined with short-lived (24 h) cutaneous photosensitivity in contrast to HpD.

Since our main aim at this stage was to develop the instrumentation and validate the technique, we have confined the study to normal rat liver rather than a tumour model. Another advantage of using liver is that is it relatively large and homogeneous which makes it ideal for investigating the spatial dependence of the response of tissue oxygenation to PDT. For the spatially resolved measurements across the tissue surface surrounding the treatment site, a single fibre-optic probe was employed both to excite and detect the phosphorescence.

2. Materials and methods

2.1. Compounds

Experiments were initially carried out on solutions of the PdTCPP in cuvettes and subsequently in animals. For the phosphorescence emission studies, PdTCPP (obtained from Porphyrin Products) was dissolved in a phosphate buffered (pH 7.4) aqueous solution containing bovine serum albumin (BSA, Sigma). Binding of the probe to serum albumin is necessary to prevent aggregation and thus self-quenching effects. PPIX (dimethyl ester) was obtained from Porphyrin Products. A spectrofluorimeter (Perkin-Elmer LS50B) was used to record delayed phosphorescence spectra and absorption spectra were measured using a Perkin-Elmer Lambda 15 spectrophotometer.

For animal studies, more concentrated solutions were required, and PdTCPP was obtained from Digitimer Corporation as a lyophilised powder (Oxypor™) containing by weight 10% PdTCPP, 80% BSA and 10% buffering salts.

2.2. Apparatus

Tissue oxygenation was measured before, during and after PDT treatment using a single fibre-optic probe to both excite and detect the phosphorescence. A bare-end, high NA, 600 μm core diameter fibre was used and positioned using a micromanipulator just above the tissue surface to excite and detect phosphorescence over an area of less than 1 mm^2. The excitation source was a a low power pulsed nitrogen pumped dye laser (Laser Photonics) with a 1 ns pulse-width and 10 μJ pulse energy at a repetition rate of ~ 10 Hz. An excitation wavelength of 527 nm was used thus confining phosphorescence detection to the surface layer. This fibre-optic technique could thus be used for spatially-resolved surface measurements at pre-determined distances from a laser irradiation fibre placed in contact with the tissue. A schematic diagram of the apparatus is shown in Fig. 1. The excitation light pulse was passed through a dichroic mirror (650DRSP, Omega

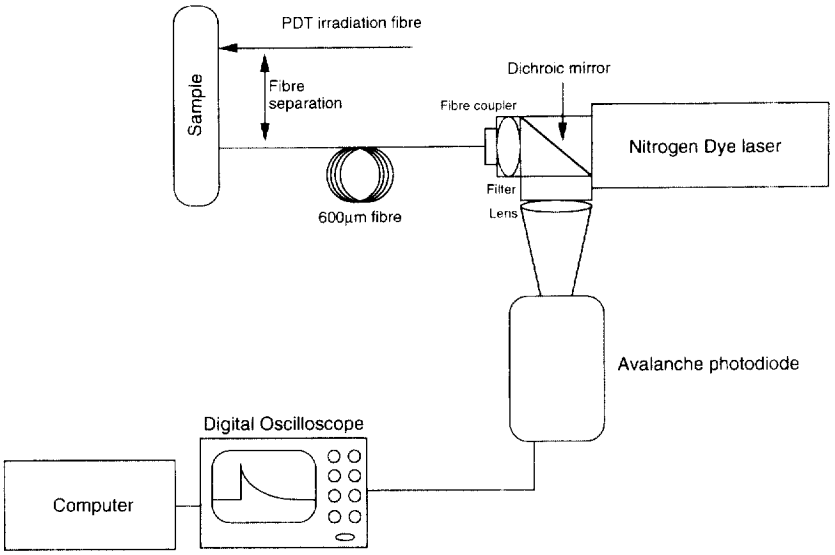

Fig. 1. Schematic layout of time-resolved phosphorescence detection apparatus. The excitation pulse was focused into the probe fibre which also collected the phosphorescence emission. Phosphorescence was reflected from a dichroic mirror onto an avalanche photodiode via long pass filters. The irradiation PDT fibre and phosphorescence probe fibre were set normal to the liver surface. The probe fibre could be positioned at an adjustable separation from the irradiation fibre.

Optical Inc.) and focused into the fibre. The emission was collected using the same fibre, reflected from the dichroic mirror, passed through two long-pass filters (Schott RG630 and RG9), and focused onto a 3 mm avalanche photodiode (APD) with an integrated pre-amplifier (Hamamatsu Photonics, model C5460-01) with a rise-time of ~3 μs which was adequate for these studies. The APD exhibits good sensitivity over the detection wavelengths between 700–800 nm with a larger dynamic range than photomultiplier detectors, making it less prone to saturation. The APD output was acquired by a digitising oscilloscope (Tektronics 460, 1024 channels, bandwidth set to 20 MHz) and integrated over a pre-set number of decays (typically 50–100) to give 12-bit resolution. The averaged decay trace was then passed to a PC for data analysis. The oscilloscope was triggered by a fast photodiode (Thorlabs, model 201-579) monitoring the laser pulse and baseline levels were set using the pre-trigger channels. The lifetimes were then calculated with the PC using non-linear least-squares analysis (Jandel Scientific). Since we did not wish to interrupt the PDT treatment which might have allowed reoxygenation, phosphorescence decays were acquired during PDT laser irradiation which resulted in minor interference from photosensitiser fluorescence induced by the PDT laser although this contribution could be averaged out satisfactorily. A more serious problem was a short-lived fluorescence spike from photosensitiser emission induced by the probe laser and this was a persistent problem despite using filters transmitting at longer wavelengths beyond the peak sensitiser emission. To avoid interference from this transient fluorescence contribution, decays were fitted after a 20 μs delay from the laser pulse.

Since oxygen is the only quencher present at significant concentrations in plasma, the oxygen partial pressure pO_2 can be related to the phosphorescence lifetime (τ) by the standard Stern–Volmer equation:

$$\tau_0/\tau = 1 + k_q \tau_0 (pO_2)$$

$$pO_2 = (1/k_q)(1/\tau - 1/\tau_0)$$

where the rate of phosphorescence quenching $k_q = 381$ torr^{-1} s^{-1} and the phosphorescence lifetime in the absence of oxygen $\tau_0 = 637$ μs [13]. These values apply to PdTCPP at 38°C and pH 7.4 bound to serum albumin (2% by weight). In plasma from mammalian species the albumin concentration by weight exceeds 2% which ensures that the PdTCPP is fully bound upon injection [13]. Measurements of pO_2 using this spectrocopic technique have been compared with standard electrode techniques [13] and blood-gas analysis [14] and good linear correlation was found from 0.1 to 140 torr (1 torr = 133 Pa) in the case of the electrode measurements. Fortunately the values of k_q and τ_0 are insensitive (< 5%) to variations in pH from 6.4 to 7.8. However there is also a temperature dependence with k_q reduced to 334 torr^{-1} s^{-1} at 33°C [13] so it is important to maintain the animal body temperature during treatment. Another advantage of this technique is that as the oxygen concentration is reduced the lifetime increases which enables greater experimental precision.

2.3. Photodynamic therapy and clamping experiments

For the animal studies we used Wistar rats (female) weighing 120–180 g. 50 mg/kg of ALA.HCl (Sigma) dissolved in PBS was administered intravenously via the tail vein 75 min before PDT irradiation. This time was selected on the basis of in vivo fibre-optic fluorescence spectroscopic detection of PPIX (data not shown) which reached a maximum around this time-point. The liver was exposed via laparotomy

under general inhaled anaesthesia (Halothane) and the body temperature was maintained using a heat-pad. The exposed liver of the animal was covered with an oxygen impermeable thin transparent plastic film. Each rat was injected with 25 mg/kg of PdTCPP via the tail vein (above the ALA injection point) and was left for 20 min for the compound to circulate in the animal before PDT irradiation. Control animals injected with PdTCPP only were also included for study.

The laser irradiation fibre (400 μm diameter, bare-end) was positioned orthogonally to the liver surface as shown in Fig. 1. The irradiation power was set to 100 mW which was supplied by a Cu-vapour pumped dye laser (Oxford Lasers) tuned to the appropriate wavelength for PPIX, 635 nm. The probe fibre was also positioned orthogonally to the liver surface at pre-determined distances from the irradiation fibre using a micromanipulator enabling a histogram distribution of oxygenation to be collected before, during and after the treatment. Note that the probe fibre did not touch the surface in order to prevent any local pressure perturbations. A lifetime measurement was taken immediately before the 635 nm light irradiation and readings were then taken at the noted time intervals during the PDT treatment. The effect of clamping the blood supply to the liver was also investigated in unsensitised animals by applying a pair of forceps covered in gauze to one of the liver lobes.

3. Results

3.1. In vitro spectroscopy

Fig. 2 shows the absorption and phosphorescence spectra of solutions of PdTCPP. The main Q-band absorption maximum is at 420 nm (not shown) and the B-band maximum is at 525 nm. There is negligible absorption beyond 600 nm. The phosphorescence spectrum was recorded using the delayed detection facility of the LS50B spectrometer which employs pulsed excitation (ca. 80 μs pulse width). With the detection gate width set to 1 ms at a delay of 0.2 ms, the short-lived fluorescence PdTCPP emission near 620 nm and PPIX emission near 635 nm were completely eliminated. Phosphorescence emission (corrected spectrum) occurs from about 650 to beyond 800 nm with the maximum near 700 nm. Fig. 3 shows a time-resolved PdTCPP phosphorescence decay recorded from a cuvette containing an aqueous solution of the probe. The laser output was focused into a bare-end 600 μm core fibre which was immersed in the air-tight cuvette containing a deoxygenated (by nitrogen bubbling) PBS pH 7.4 solution of the probe (at 10 μM) and bovine serum albumin (100 μM). The solution also included protoporphyrin IX (10 μM), but the lifetime was unaffected by the PPIX fluorescence which is emitted on a much shorter nanosecond time-scale (although convoluted by the rise-time of the detector) and is only present in the first few channels of the trace. The measured lifetime of 690 μs is in good agreement with the literature value at 298 K [13].

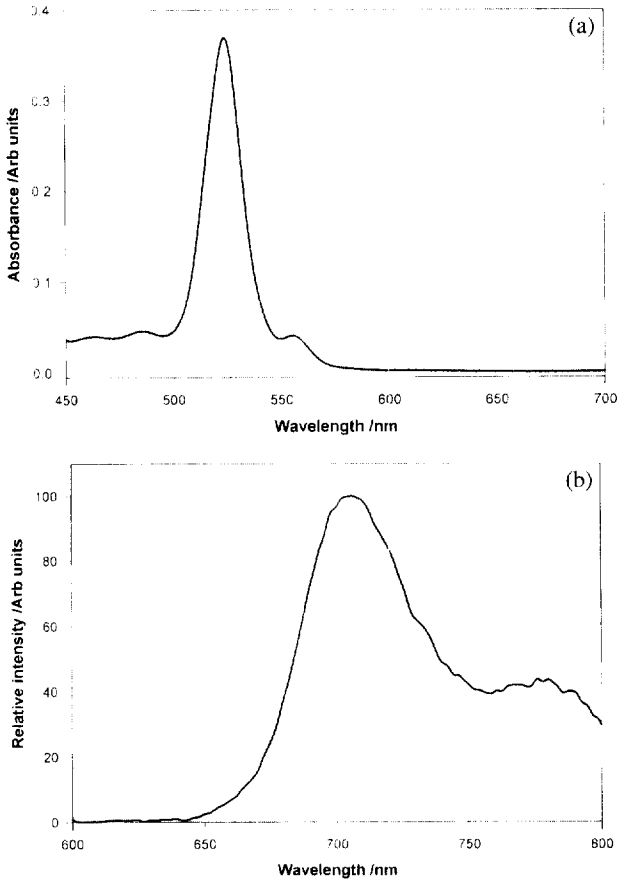

Fig. 2. (a) Absorption spectrum of 25 μM PdTCPP in DMSO; (b) phosphorescence emission spectrum of 10 μM PdTCPP bound to BSA at 100 μM in deoxygenated PBS solution using excitation at 525 nm and 10 nm slit widths. The solution also contained 10 μM PPIX but short-lived PPIX fluorescence is not present since the spectrum was recorded 0.2 ms after the excitation pulse. The intensity has been corrected for the photomultiplier response.

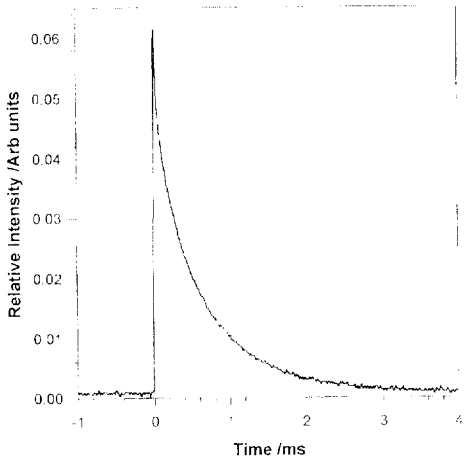

Fig. 3. Phosphorescence decay of 10 μM PdTCPP bound to BSA at 100 μM in de-oxygenated PBS solution using excitation at 527 nm. The solution also contained 10 μM of PPIX bound to the BSA.

3.2. Clamping of liver

The effect of clamping the blood supply to the liver using forceps applied to one lobe was also investigated in unsen-

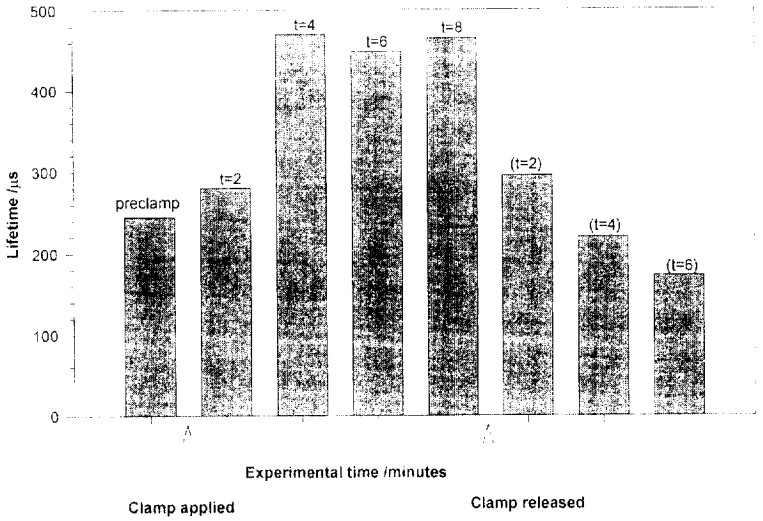

Fig. 4. The effect on phosphorescence lifetime of clamping the blood supply to the liver following the application and release of a pair of forceps covered in gauze to one of the liver lobes. The figures in parentheses give the period in minutes following the application and release of clamping respectively.

sitised animals in order to check that the lifetime changes that we observed were reflecting changes in oxygen levels and that laser irradiation with PdTCPP alone did not perturb the lifetime values. Upon clamping the lifetime rose to near 500 μs and then declined to baseline levels when the clamp was removed (Fig. 4).

3.3. Effect of PDT

Laser irradiation was carried out with the detection fibre at different radial separations from the treatment fibre in both sensitised and control unsensitised animals. Lifetimes were measured sequentially during irradiation and Fig. 5 shows the lifetime dependence in sensitised animals during PDT with a total light dose of 60 J (at 100 mW) for two fibre

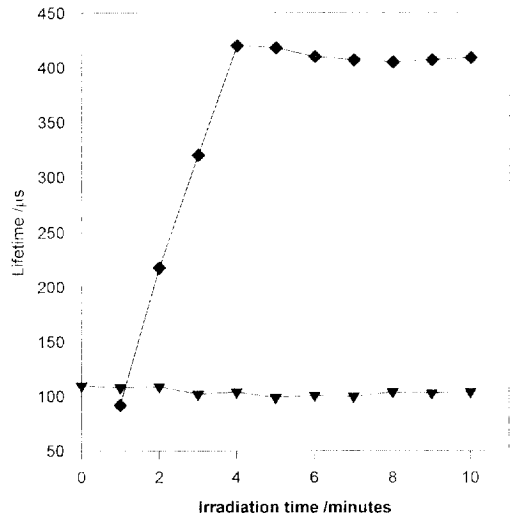

Fig. 5. Lifetime dependence vs. irradiation time from liver sensitised with 50 mg/kg ALA recorded during PDT with 100 mW 635 nm light giving a total dose of 60 J for two fibre separations, 2 and 9 mm, i.e. between the fixed PDT irradiation fibre and the phosphorescence probe fibre; 2 mm separation (diamonds) and 9 mm separation (inverted triangles).

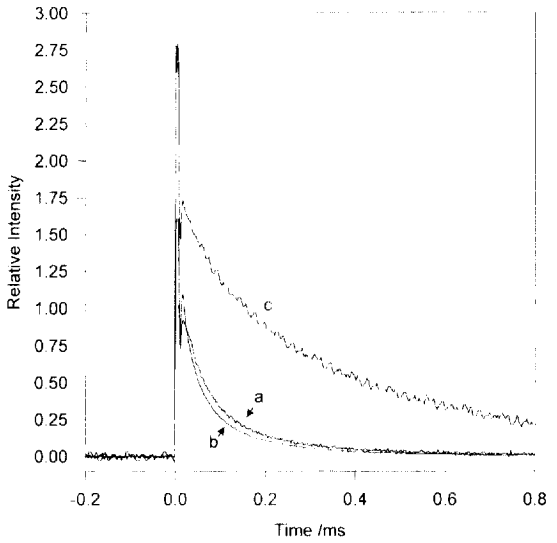

Fig. 6. (a) Time-resolved control trace from unsensitised liver; (b) trace observed after an energy dose of 60 J (10 min irradiation time) with a fibre separation of 9 mm. The lifetime observed, 100 μs, corresponds to a pO_2 of 22 torr; (c) trace after the same energy dose in the same animal, but with the two fibres adjacent at a separation of 2 mm; the lifetime recorded was much longer at 420 μs corresponding to a pO_2 of only 2.1 torr.

separations, 2 and 9 mm, i.e. between the PDT irradiation fibre and the phosphorescence probe fibre. Fig. 6 shows the recorded traces in unsensitised (a), and sensitised liver (50 mg/kg ALA) during PDT with a total energy dose of 60 J (100 mW) for two fibre separations 9 and 2 mm, ((b) and (c)). At a separation of 9 mm between the irradiation and probe fibre no significant change in the lifetime was observed during treatment. The average lifetime observed, 100 μs, corresponds to a pO_2 of 22 torr. However, when the two fibres were adjacent at a separation of 2 mm a dramatic increase in the life-time was observed after only 2–3 min irradiation, corresponding to a sharp decrease in pO_2. The lifetime reached a value of 420 μs corresponding to a pO_2 of only 2.1 torr; little change was evident thereafter between 5–10 min

irradiation. Fig. 6 demonstrates the slower rate of decay and increased lifetime observed with the probe fibre adjacent to the PDT laser fibre (plot (c)) over the unsensitised case (a) and a 9 mm fibre separation (c) which showed comparable decay rates. These results were reproduced in four other animals. A spike was present at the start of the traces which resulted from short-lived porphyrin fluorescence emission; in the case of the control (a) a weaker spike is still observed owing to the natural porphyrins present in the liver. The palladium porphyrin also fluoresces weakly but with peak emission at shorter wavelengths near 620 nm, which is out of the detection range [7]. However, the fluorescence spike is short-lived and is negligible after 20 μs in animals which did not receive ALA, so lifetime fits were therefore performed after a delay of 20 μs. The greater noise for the longer lifetime trace is due to the higher PPIX fluorescence intensity induced by the PDT irradiation fibre which was adjacent to the detection fibre. We used an averaged baseline level derived from the pre-trigger data points, which also included the same fluorescence contribution, to subtract out the fluorescence from the phosphorescence which was of comparable intensity; however, this procedure does not affect the noise present in the trace. With the PDT laser switched off the same lifetime was observed but without the extra noise. The effect of photobleaching and thus variation in the fluorescence level can be discounted since subtracting the pre-trigger baseline compensates for this effect. In any case, recording each decay trace only takes a few seconds so photobleaching would be minimal.

At intermediate fibre separations of 4–5 mm significant increases in the lifetimes were only seen after about 5 min irradiation (30 J). In control studies, lifetimes were also measured from unsensitised, irradiated animals with the fibres placed adjacently but no increase in lifetime was observed from baseline levels (ca. 100 μs). Therefore the increase in lifetime and reduction in pO_2 can be attributed to an ALA-PPIX PDT-induced effect. The initial intensity of the Pd-TCPP emission recorded at the beginning of the decay traces appeared to increase during PDT but this effect occurred too slowly to affect the lifetime measurements which only took about 5 s to record in each case. The intensity increase most probably reflects changes in the tissue optical properties as a result of PDT.

Fig. 7 shows data recorded from an animal that had received a light dose of 40 J. The lifetimes were recorded as a function of the separation between the fixed PDT irradiation fibre and the probe detection fibre. Phosphorescence lifetimes and corresponding pO_2 values are plotted on the same graph for comparison. The longer lifetimes and lower oxygen tensions are observed nearer the irradiation fibre (on each side) whereas beyond 7 mm separation there is little increase over the baseline levels.

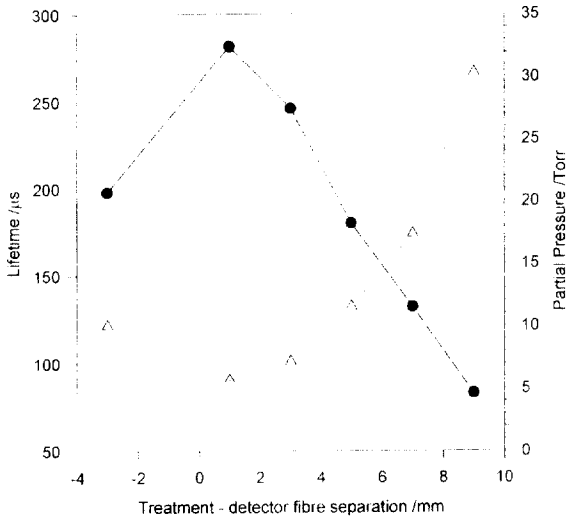

Fig. 7. Phosphorescence lifetimes (filled circles) and corresponding pO_2 values (open triangles) observed in an animal after an energy dose of 40 J as a function of the separation of the fixed PDT treatment fibre and the probe detection fibre. The longer lifetimes and lower oxygen tensions are observed with the probe nearer the irradiation fibre.

4. Discussion

One of the main challenges facing PDT is the development of real-time monitoring techniques for improving the control of dosimetry and the treatment reliability. Attention to date has been largely devoted to measurements of photosensitiser fluorescence and photobleaching and the light dose delivered at the treatment site. Whilst these measurements are obviously necessary, the tissue level of molecular oxygen is also an important dosimetric parameter but much less work has been carried out in this area. The main aim of this investigation was to establish a method of monitoring the oxygen concentration within and near the boundary of the region of necrosis caused by a photodynamic treatment. A sensitive real-time non-invasive spectroscopic technique is described for the quantitative monitoring of microvascular oxygen tensions during photodynamic therapy using the oxygen dependent phosphorescence lifetime of palladium *meso*-tetra(4-carboxyphenyl)porphine. The PdTCPP probe compound exhibits negligible absorption above 600 nm as shown in Fig. 2 and control studies elicited no change in phosphorescence lifetimes during PDT irradiation at 635 nm. The power of the N_2/dye laser used to excite the probe at 527 nm is very low (ca. 0.1 mW) and was only used for a fraction of the total time of the PDT experiment. Phosphorescence was excited and detected using a 600 μm fibre-optic probe which could be positioned at different points on the tissue surface with respect to the irradiation fibre. Thus the effects on oxygen tension could be monitored selectively with 1 mm resolution at points within and surrounding the treatment zone for assessment of the spatial response to PDT. The phosphorescence technique could equally well be applied to other photosensitisers which absorb further to the red than PPIX. Although the technique in its present form can only be applied to surface measurements it is nevertheless very suitable for experimental measurements on thin hollow organs such as the colon which we have found to be a useful model for studying and quantifying PDT effects [4].

Since the lifetimes are relatively long, up to nearly 1 ms under hypoxic conditions, acquisition and data analysis is straight-forward (unlike fluorescence studies) and rapid: an averaged lifetime measurement over 50 decays using the pulsed excitation source operating at 10 Hz would take less than 10 s. A major advantage of using this time-resolved method is that it is essentially a ratiometric technique relying on lifetime decay measurements instead of steady-state intensities as is the usual case in optical measurements. During PDT tissue can undergo significant changes in optical properties which inevitably affects steady-state emission intensity measurements but not emission lifetimes provided the lifetimes are measured promptly. The phosphorescence lifetime measurements should also in principle be unaffected by photosensitiser fluorescence which is emitted on a much shorter time-scale and requires different excitation/detection combinations. However a short-lived but intense fluorescence spike from photosensitiser emission induced by the probe laser was a persistent problem occasionally resulting in saturation of the input amplifier of the oscilloscope. To alleviate this problem we restricted detection of phosphorescence to the 700–800 nm range where PPIX fluorescence is relatively weak but PdTCPP phosphorescence is still moderately intense, as shown in Fig. 2, and can be detected efficiently using an avalanche photodiode. This problem has also been encountered in time-resolved studies of sensitised singlet oxygen emission and can be eliminated using an electronic switch to temporarily ground the oscilloscope input during the laser pulse [15]. Another solution would be to gate the operation of the detector itself [16].

The results of this pilot study on normal rat liver show that PDT using ALA-PPIX can induce a rapid and significant reduction of tissue oxygenation in the zone surrounding the irradiation fibre as shown in Figs. 5–7. Although a relatively low ALA dose was used in this work (50 mg/kg) PPIX synthesis is particularly efficient in the liver [17]. For high light doses near the irradiation fibre, the oxygen partial pressure was reduced to 2 torr which is comparable to values obtained by clamping the blood supply as shown in Fig. 4. As would be expected, the rate of oxygen consumption is lower at farther distances from the irradiation fibre since these areas receive a smaller light dose, although a reduction was still evident at 7 mm from the irradiation fibre after a dose of 40 J, as shown in Fig. 7. The power dependence will be investigated in a future study, but we would expect when using lower powers to deliver the equivalent light dose, that the longer irradiation times would result in a lower rate of oxygen consumption and consequently a higher pO_2 value. There have been several reports on the occurrence of vascular effects in normal tissue and tumours induced by ALA-PDT [18,19] which has recently been reviewed [17]. Leveckis et al. [19] studied the response of rat cremaster muscle microcirculation to ALA-PDT using quantitative microscopic analysis and observed a rapid reduction (by about 80%) in the mean arteriole diameter during irradiation; after the end of irradiation the diameter slowly recovered to pre-treatment values. The observed reduction in vascular perfusion must result in a decrease of the pO_2 which has been confirmed in the present work on normal liver. However it remains unclear whether the degree of vascular constriction is the major factor resulting in tissue destruction since direct cellular kill should also be present with ALA-induced PPIX sensitisation [17]. In the liver it is possible that the mechanism of vascular damage includes a combination of phototoxic effects to endothelial cells directly and to hepatocytes which could become swollen during PDT resulting in constriction of the capillaries. One potential problem with the PdTCPP phosphorescence technique is macromolecular leakage into the interstitium induced by PDT. It should be possible to assess the extent of this effect using PdTCPP fluorescence microscopy of cryosections since the fluorescence efficiency is insensitive to oxygen variations. The presence of free-base porphyrin impurities should be checked for since depending on the irradiation wavelength these may cause photosensitisation.

These results indicate that spatially resolved tissue oxygenation measurements during treatment may be useful for quantification of PDT effects although much work remains to be done in comparing extents of PDT damage under different irradiation regimes and their relation to pO_2. If replicated using tumour models, then methods for obtaining pO_2 tensions at defined points within or on tissue should be included in the repertoire of real-time monitoring systems for PDT. The spatial resolution attainable would depend on the detection technique: using a single fibre to excite and detect phosphorescence a lateral resolution of 1 mm should be possible, with probing limited to a depth of about 1 mm with excitation at 527 nm. One problem that might be encountered with a tumour model is that in severely hypoxic regions the concentration of the probe will be limited by the poor circulation. However for treatment monitoring the critical region to probe would be the boundary between tumour and normal tissue which would be better vascularized. Ideally the method presented here, which measures the microvascular oxygen tension, should be calibrated versus an electrode technique. However present devices are too large for comparable measurements in small vessels. Reflectance oximetry is another possibility for assessing tissue oxygenation during PDT using the ratio of oxygenated to deoxygenated haemoglobin. Although this technique is much less sensitive and only provides relative rather than absolute values, these studies would nevertheless be complementary to the phosphorescence studies. Although as yet PdTCPP is not licensed for routine clinical use it is possible that fibres containing an oxygen-dependent luminescent probe immobilised in an oxygen-permeable tip may find applications for pO_2 measurements during clinical PDT [20]. This technique has been recently demonstrated by Zilberstein et al. [21] using a fibre-optic sensor containing pyrene in an experimental study on murine melanoma tumours sensitised with a bacteriochlorophyll derivative. They observed a rapid decrease in tumour oxygenation during PDT and spontaneous reoxygenation during

dark periods confirming the observations of Tromberg et al. [6].

Previous studies using electrode techniques [6,22] have found significant reductions in tissue oxygenation induced by PDT using Photofrin followed by recovery of oxygen levels on cessation of irradiation. Regeneration of oxygen levels possibly accompanied by reperfusion injury may be responsible for the enhancement of PDT damage observed using modulated or fractionated irradiation [4,5,23]. The reperfusion of oxygen into the treatment zone could be easily monitored using the phosphorescence technique since the derived pO_2 values do not depend on the PdTCPP concentration itself. It would then be possible to quantify the recovery in oxygenation during dark intervals and assess the effects of different fractionation light dose regimes and irradiation powers in relation to the heterogeneity and kinetics of tissue oxygenation at the treatment site. Since each fibre-probe is relatively inexpensive, an array of probes at strategic points could be used although imaging would be a more elegant means of acquiring spatial data. Using pulsed excitation in combination with time-gated imaging of PdTCPP phosphorescence it is possible to obtain high-resolution two-dimensional maps of oxygen pressure in the surface 1 mm of tissue and this technique has recently been used in studies of tissue reperfusion [9] and evaluation of tissue oxygenation [24,25].

5. Abbreviations

PDT	photodynamic therapy
ALA	5-aminolaevulinic acid
PPIX	protoporphyrin IX
PdTCPP	palladium tetra(carboxyphenyl)porphine
BSA	bovine serum albumin

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