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Attempts to measure sensitiser photophysics in opaque tissue

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Abstract

Diffuse reflectance laser flash photolysis has been used in an attempt to measure transient triplet spectra of the sensitisers, disulphonated aluminium phthalocyanine and porphyrin IX (derived from 5-amino laevulinic acid), in opaque tissue and models. The latter consisted of tissue phantoms; the former included rat liver and red blood cells. In all cases, triplet–triplet absorption spectra with relatively poor signal-to-noise were obtained providing some encouragement in the application of this technique to in vivo studies on photosensitisers.

Keywords: Diffuse reflectance laser flash photolysis; Transient triplet spectra; Disulphonated aluminium phthalocyanine; Porphyrin IX; Rat liver; Red blood cells

1. Introduction

The increasing success in the treatment of cancer by photodynamic therapy [1] PDT has not been accompanied by a major understanding of the underlying photophysics of the sensitisers in vivo. Conventional wisdom favours the type II process involving energy transfer from triplet sensitiser to ground state oxygen, producing cytotoxic singlet oxygen [1]. However, in bacterial cells, there is evidence that a type I electron-transfer process may also be involved [2–4]. The mechanism would be expected to depend upon the choice of sensitisers and its microenvironment.

Clearly, measurement of triplet-state absorption photophysics, and the detection of species derived from this state, such as singlet oxygen and radical ions of sensitisers in vivo will be required if photochemical mechanisms are ever to be elucidated fully. The experiments below represent attempts to make such measurements, using principally the sensitiser disulphonated aluminium phthalocyanine [4–6] in a variety of opaque media.

A previous direct measurement of triplet state absorption in macroscopic tissue has been reported by Roberts et al. [7] who exploited the transparency of bovine lenses.

The lens protein environment reduced the rate constant of quenching of the (*p*-sulphonatophenyl)-porphyrin triplet state by oxygen and caused a red shift in the triplet absorption spectrum.

The only report of $^1O_2$ luminescence detection in sensitised tumour tissue is that of Parker [8] who reported the detection of $^1O_2$ luminescence from Photofrin II sensitised subcutaneous mouse tumours. The tumour $^1O_2$ signal was ≈0.6 μV, compared with 25 μV autofluorescence and 200 μV residual sensitiser fluorescence. Patterson et al. [9] were unable to repeat this work using a very similar detection system. Parker's tumour emission was centred at 1260 nm, a larger shift than any other observed for $^1O_2$ [10] and it is possible it may have been due to the second-order image of back-scattered 630 nm laser-excitation. Patterson et al. [9] avoided this artefact by using 624–625 and 670 nm excitation and two interference filters instead of a monochromator. All the reports of $^1O_2$ in cell suspensions have detected it after it has escaped into the surrounding medium. As Gorman and Rogers have pointed out, detection of $^1O_2$ luminescence in tissue will be very difficult, owing to the inherently low quantum efficiency of $^1O_2$ emission, and the high probability of $^1O_2$ being quenched by substrates within the tissue [10].

2. Experimental

Diffuse-reflectance laser flash photolysis DRLFP mea-

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surements on the triplet states of sensitisers, and species derived from them were performed as described elsewhere in full [2–4].

Diffuse reflectance spectroscopy is of particular use with strongly scattering media, where incident light is partially absorbed and multiply scattered. Kubelka–Munk theory holds for many opaque diffuse reflectors, there being two limiting cases (i) for an optically thick sample of a homogeneous distribution of ground-state absorbers, and (ii) for a low concentration of absorbers such that the exciting laser pulse causes complete conversion of the sample to the excited state. The two cases lead to different kinetics; in the present case, it has to be stated that conditions may be intermediate between the two limiting cases.

The sensitiser used here was clinical disulphonated aluminium phthalocyanine $AlPcS_2$, as the sodium salt, Batch 18/1/91 synthesised by Bishop et al. [5]. Purity was verified by high-performance liquid chromatography (HPLC). The major fraction in this preparation is the adjacent α,α-isomer shown below (Fig. 1). In some experiments, the sensitiser 5-aminolaevulinic acid, ALA, which produces endogenous protoporphyrin IX, was used.

2.1. Tissue phantoms

Tissue phantoms were prepared to mimic the near-IR optical absorption and scattering properties of human breast tissue. These consisted of a scattering compound (amorphous silica spheres) and an absorber incorporated into the epoxy resin substrate [11]. The dye absorption peaked at 850 nm and was minimal in the visible region of the spectrum, so did not mimic tissue properties across the entire spectrum. Varying concentration of $AlPcS_2$ (0.02–1.66 mM) were also added. The phantoms were supplied as 2.5×2.5×0.5-cm blocks and these were placed directly in the DRLFP sample mount with the lower, matt, face exposed. (The top face of the preparation was shiny. No difference was observed in kinetics obtained from this surface; however, the signal intensity was lower-due to greater specular reflection.)

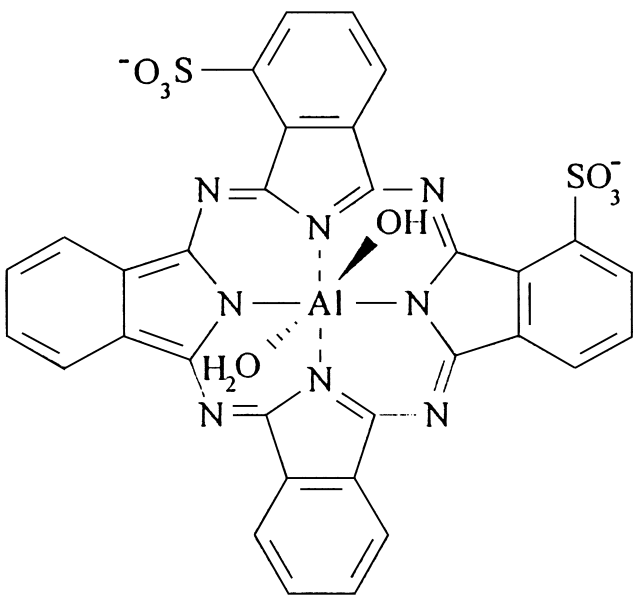

Fig. 1. Structure of clinical aluminium disulphonated phthalocyanine major component.

Oxygen levels in the phantoms have been measured using the oxygen-dependent quenching of palladium *meso-tetra* (4-carboxyphenyl)porphine phosphorescence [12,13] and found to be very low or zero [14], equivalent to less than 5×10^{-5} M concentration.

2.2. Rat liver

Male Wistar rats were given 200 mg kg^{-1} δ-amino-laevulinic acid (ALA) or 5 mg kg^{-1} $AlPcS_2$ intravenously and sacrificed 2 and 6 h later, respectively. The dissected liver was stored on ice until used (ALA) or sectioned into 5-mm cubes and frozen in liquid nitrogen ($AlPcS_2$). Lateral sections were taken through single lobes of the ALA-treated livers and the sections were mounted in solid sample holders with the internal surface exposed for DRLFP. $AlPcS_2$ sections were thawed, sliced in half and two sections were then mounted in a solid sample holder for DRLFP.

No attempt was made to control oxygen levels during DRLFP. They were assumed to be low.

2.3. Red-blood cells

Red blood cells (RBC) extracted from the whole blood of healthy human subjects were obtained as a 50-ml suspension in phosphate-buffered saline in (PBS) and foetal calf serum (FCS). Pelleting this suspension at 600× *g* for 10 min yielded approximately 8 ml of RBC. Whole RBC were stored at 37°C until required and used within 24 h. To mimic blood banking applications of photodynamic action, the cells were mixed with $AlPcS_2$ solution and measured immediately. Best DRLFP results were obtained by suspending 0.5 ml RBC pellet in 2 ml 53 μg ml^{-1} $AlPcS_2$.

2.4. Red-blood cell ghosts

RBC ghosts were prepared and sensitised using modifications of the methods of Steck and Kant [15] and of Pooler [16]. The intention was to produce preparations similar to those described by Rywkin et al. and Kanofsky [17,18], for comparison purposes. Two ml of cells was washed twice with 4 ml PBS at 25°C and then incubated at 37°C with 10 ml of 21, 42 or 63 μg ml^{-1} $AlPcS_2$ for 60 min with periodic gentle shaking. Cells were recovered by centrifugation at 600×*g* for 10 min, and washed three times with 10 ml PBS pH 8.0, removing any yellowish

residues on the pellet surface each time. The pellet was divided into four parts and each was thoroughly and rapidly washed three times at 25°C with 15 ml of 5P8 (5 mM $N_{a2}HPO_4$, pH 8.0), centrifuging at 10 000×*g* between washes and removing the supernatant and the harder deep-red button of proteases at the base of the pellet each time. The resulting ghosts were recombined and washed at 25°C with 15 ml 5P8 and stored as a pellet at 4°C for no more than 72 h. The resultant pellet was pale red, rather than the 'creamy white' described by Steck and Kant [15] (who centrifuged at 22 000×*g* rather than 10 000×*g*), and it was concluded that a significant amount of haemoglobin remained trapped within the ghosts.

3. Results and discussion

3.1. Tissue phantoms

A tissue phantom sensitised with 0.83 μM $AlPcS_2$ was used to generate the transient absorption spectrum shown in Fig. 2. The decay shown in Fig. 2 illustrates the low signal/noise ratios obtained in the experiment. The signal/noise ratios are comparable to those obtained from sensitised RBC ghosts as described below, although the limitation in that case was low diffuse reflectance, whereas in this case it appeared to be due to low transient absorption. Bleaching of the signal was generally insignificant over the course of these measurements. The transient spectrum closely matches that of the $AlPcS_2$ triplet in solution, with a maximum at 490 nm. No signal was observed from phantoms which did not contain $AlPcS_2$, nor when the $AlPcS_2$ concentration was reduced to 0.12 μM. At 1.7 μM the transient intensity was independent of laser power over the range 3.1–9.4 mJ $pulse^{-1}$.

No evidence of radical species was found in the *time*-dependence of the spectrum. Global analysis using a single exponential decay with offset yielded a lifetime of 410 μs. The offset displayed random behaviour and could not therefore be given any physical significance. When the offset was excluded from the fit, a lifetime of 680 μs was obtained which is consistent with triplet lifetimes in oxygen-free organic solvents. Oxygen concentrations in the phantoms were found to be more than two orders of magnitude lower than in air-saturated solution [14]. The apparent shortening of the lifetime when an offset was used could be attributed to artefacts resulting from the

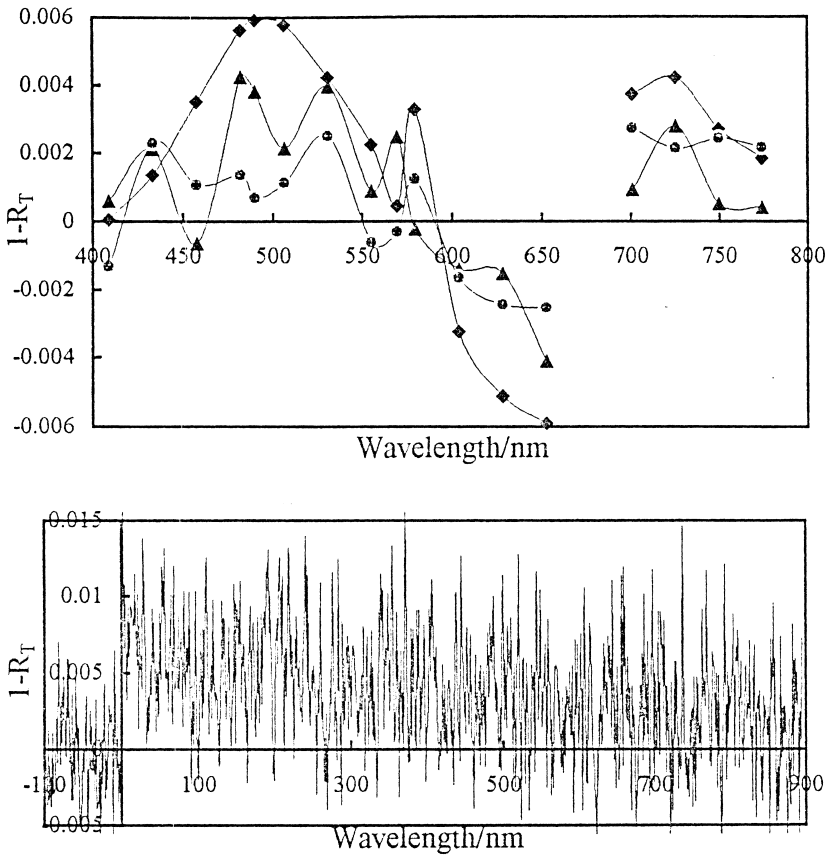

Fig. 2. Transient absorption spectrum of 0.83 μM $AlPcS_2$ in a human tissue phantom at 20 μs (diamonds), 400 μs (triangles) and 850 μs (circles) after the laser pulse. Points are the average of 30 channels. The signals are very weak. No importance should be attached to apparent differences in spectrum. The lower panel shows the transient decay at 490 nm (average of eight shots at 673 nm, 18.1 mJ $pulse^{-1}$).

decay noise or the presence of a distribution of triplet lifetimes due to different local environments in the phantoms. Noise levels were too high to distinguish between these explanations; however, the former is preferred on the basis that phantom preparation would be expected to result in a uniform medium composition with no compartmentalisation effects.

Since typical $AlPcS_2$ concentrations achieved in various tissue types during PDT are higher than those in the phantoms (e.g., 3–5 μg g^{-1} in rat tumour, skin and muscle [19] or approximately 4–6 μM), these results suggest that in vivo detection may in principle be realised.

3.2. Rat liver

With a view to making the extension to in vivo studies, a number of pilot measurements of sensitiser triplet states were made on excised rat livers. Liver was chosen due to the relatively high concentrations of sensitiser which accumulate in organ. Measurements were made more than 3 h post mortem and so, though it was not measured, the oxygen concentration was expected to be low [20]. Due to tissue absorption, baseline reflectance was less than 10% of that achieved using cell suspensions. No attempt was made to detect $^1O_2$ from the rat livers.

In rat livers sensitised with 200 mg kg^{-1} ALA, triplet absorption at 450 nm and singlet depletion at 420 nm characteristic of the ALA metabolite protoporphyrin IX (PpIX) was expected [21,22]. Excitation of livers at 630 nm initially produced no transient absorbance or depletion. Continued irradiation produced the transient spectrum similar that shown in Fig. 3a. However, the same effect was observed in liver from a control rat which had *not* received ALA. The intensity of the transient was linearly

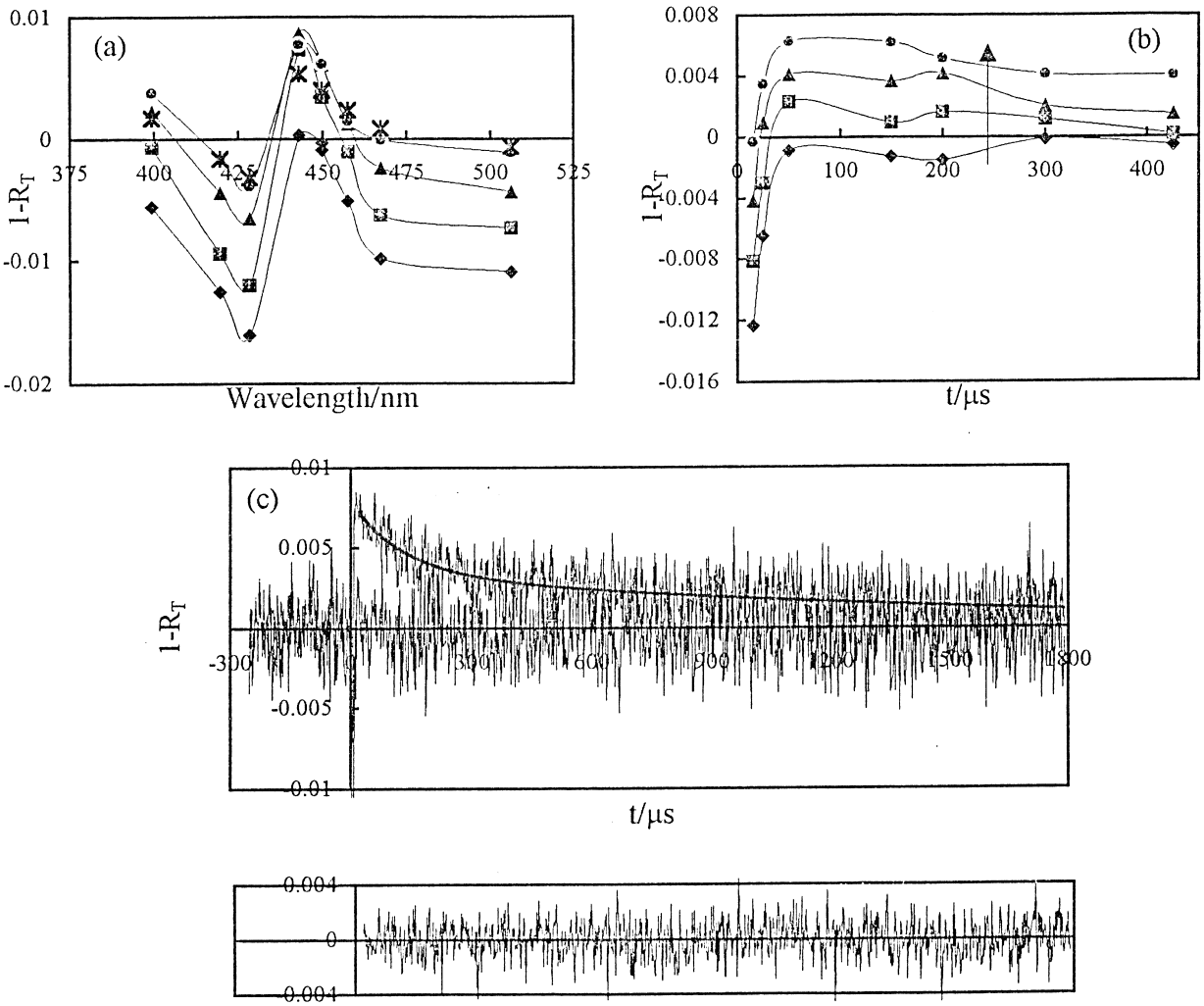

Fig. 3. (a) Transient spectrum obtained following excitation of unsensitised rat liver. Delays: 15 μs (diamonds), 25 μs (squares), 50 μs (triangles), 150 μs (circles) and 425 μs (stars). The spectrum from ALA-sensitised livers was identical. (b) Evolution of the 450-nm transient absorption as a function of irradiation time: 0 min (diamonds), 1 min (squares), 3 min (triangles), 6 min (circles). (c) Transient decay observed at 450 nm in irradiated liver (sensitised with ALA). The lower signal was observed in unirradiated liver. The biexponential fit parameters were: τ_1=129 μs, τ_2=1 580 μs, A_1=0.0045, A_2=0.0034, χ^2=1.03. Excitation at 630 nm, 10.9 mJ $pulse^{-1}$.

dependent upon the length of liver irradiation by the Xe lamp. No macroscopic damage to the liver was observed during the course of the experiments.

The nature of the species producing the transient absorption remains undetermined. Since the transients were independent of ALA administration they cannot be attributed to administered PpIX nor any of its photoproducts. The initial transient depletion evident in Fig. 3b is not due to scattered laser light since the contribution of the laser in the absence of the probe beam has been subtracted in all cases and the spike due to scattered laser light was never wider than 10 μs. This suggests than more than one species gives rise to the observed transient spectrum, although PMT derived artefacts due to the combination of laser scatter and DC Xe lamp have not been completely eliminated. Excitation of ALA sensitised liver at 670 nm (to the red of most porphyrin Q-bands) failed to produce the transient, although the specimen was subject to less Xe lamp irradiation than those excited at 630 nm. The peak of the transient absorption is typical of porphyrin species. The long-term stability of the photoproduct was not determined.

These results suggest the transient might be due to the photoproduct of a naturally occurring liver porphyrin. PpIX is a candidate for the parent porphyrin, although naturally occurring levels in liver will be much lower than those when ALA has been administered. The porphyrin in the ALA metabolic pathway, including coproporphyrin, uroporphyrin, PpIX, and the mono- and di-cations and two photo-oxidation products of PpIX show a singlet depletion centred at 400 nm, which is considerably broader than that in Fig. 3 (which is centred at 420 nm) [21,23,24]. PpIX is the only one of these with a triplet lifetime in the absence of oxygen in excess of 500 μs and the lifetimes of its known photoproducts are much shorter [23]. Contributions from haem can be eliminated since triplet states have not been reported for it or any other iron-containing porphyrins; however, the photophysics of its degradation products are not known [25].

A weak, short-lived transient depletion was observed at 650–660 nm in liver from rats given 4 mg kg^{-1} $AlPcS_2$ (Fig. 4). This was detected without prior irradiation by the Xe lamp and was assigned to $A1PcS_2$ (Fig. 4) singlet depletion. The lifetime was relatively short (80–150 μs) which may have been due to residual oxygen levels or simple the signal noise coupled with limited decay time available for kinetic analysis. The irradiation time dependence of the decay was not recorded. No signal was observed at 490 or 715 nm.

The experiments confirm that transient detection in tissue is possible, although sensitivity improvements must be made before useful information will be provided and assignment of transient species is likely to be complicated in some cases by unknown photochemical reactions. Liver was chosen for this pilot study due to the relatively high levels of sensitiser accumulating there. Liver is also a relatively strongly absorbing tissue and so penetration depths for both pump and probe beams were relatively low and diffuse reflectance was low. Better results might be obtained in muscle, brain or other tissues where penetration depths are 2–3 times greater [20]. Probing to the red of the haemoglobin absorption band at 600 nm will also be

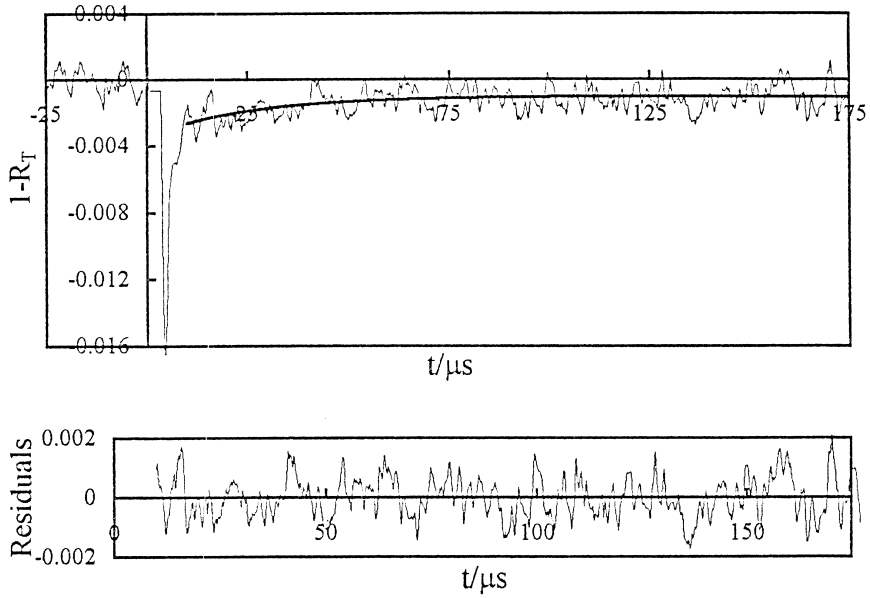

Fig. 4. Transient depletion observed at 656±6 nm following excitation of thawed rat liver sensitised with 5 mg kg^{-1} $AlPcS_2$. Excitation at 673 nm, 11.6 mJ $pulse^{-1}$. The decay is the average of three decays. The best fitting model was a single exponential with offset, giving $A=-0.0025$, $R=-0.0010$, $\tau=24$ μs, $\chi^2=1.11$. The signal due to the laser in the absence of probe light was subtracted from each decay before averaging.

advantageous, suggesting the use of phthalocyanine sensitisers rather than ALA, and the monitoring of singlet depletion rather than triplet absorption.

3.3. Red blood cells

An attempt was made to monitor $AlPcS_2$ triplet states in red blood cells (RBCs) and RBC ghosts. This system is of particular importance because there is good indirect evidence that both type I and type II mechanisms contribute to RBC photodamage, dependent upon type of sensitiser and environment [26,27]. A range of important blood-borne pathogens have been shown to be susceptible to photoinactivation including hepatitis viruses (type B and non-A–non-B), human immunodeficiency virus (HIV), human T cell lymphotropic virus (a leukaemia virus), herpes simplex virus, cytomegalovirus, Epstein–Barr virus, *Trypanosoma cruzi* (Chagas disease) and *Plasmodium falciparum* (malaria) [26–32]. Phthalocyanines have proved to be particularly effective sensitisers in this application.

Photosensitisation also induces haemolysis of RBCs and reduces their circulatory lifetime. Quenching studies suggest that the mechanism of viral inactivation and RBC damage are different. Rywkin et al. [17] achieved 5-log reductions in vesicular stomatitis virus (VSV) infectivity in RBC concentrates using 13 μM $AlPcS_4$. The addition of radical quenchers (10 nM mannitol, glycerol or reduced glutathione or 200 units ml^{-1} of superoxide dismutase) had no appreciable effect on VSV inactivation, while $^1O_2$ quenchers (2 mM NaN_3 or 4 mM tryptophan) reduced the rate of inactivation by 50% and VSV was inactivated 3 times faster in D_2O suspension than in H_2O suspension. Similar observations have been made by other groups [30,33,34]. RBC damage was reduced by the addition of 1 mM mannitol or reduced glutathione and also by NaN_3.

These results strongly suggest that RBCs are damaged by both type I and type II processes, whilst viruses are inactivated primarily by type II processes (though there is some evidence that a superoxide (type I) pathway may contribute in the MB inactivation of herpes simplex virus [30,35]. Experiments were conducted here in an attempt to identify spectroscopically type I inactivation of $AlPcS_2$ triplet states by RBCs. If confirmed this would be the only mammalian cell line studied which induced a type I reaction.

The first experiments mimicked the conditions used to inactivate viruses in blood products by suspending whole RBCs in a solution of $AlPcS_2$ and performing DRLFP immediately. Best results were obtained by suspending 0.5 ml RBC in 2 ml of 52 μg ml^{-1} $AlPcS_2$, in PBS and using a chemical scavenger to remove all oxygen from the system. When excited at 673 nm using a 3-mJ $pulse^{-1}$, a transient reduction in diffuse reflectance was detected in the region 700–750 nm with a single exponential lifetime of 420±60 μs. A very weak singlet depletion was observed in the region 650–600 nm. At shorter wavelengths, the strong absorption of haemoglobin reduced the amount of diffuse reflectance to levels such that expected transient intensities were well below the sensitivity of the instrument. In particular transients could not be detected at 570 or 490 nm, the $AlPcS_2^{-\cdot}$ and $AlPcS_2$ triplet absorption maxima. No transients were detected when the sample was deoxygenated by argon purging, and so it was concluded that oxygen bound to haemoglobin remained an effective triplet quencher.

In an attempt to eliminate the masking contribution of haemoglobin, RBCs were incubated with 21, 42 or 63 μg ml^{-1} $AlPcS_2$ and the membranes isolated. A transient spectrum was obtained from the ghosts; however, residual haemoglobin in the preparation resulted in a low signal intensity which did not permit a conclusion to be drawn regarding the presence or absence of sensitiser radical ions. The transient lifetime was extended to 590 μs. A similar extension of lifetime was observed when $AlPcS_2$ was taken up by the microbe *S. mutans* (data not shown here) and may be assigned to the sensitiser being taken up into the membrane which is of lower polarity. The signal intensity was proportional to the sensitiser concentration in the ghosts and no signal was observed from unsensitised ghosts.

Understanding the mechanism of RBC photodamage is necessary in order to minimise damage during photoinactivation of viruses and other infectious agents in blood products. The results presented here suggest that spectroscopic investigations of the RBC/virus system may be a useful probe of the photodynamic mechanism. The photophysics of $AlPcS_2$ clearly can be measured in RBC ghosts, although an improved preparation free of haemoglobin is needed. Kanofsky has shown that $^1O_2$ can be detected from sensitised RBC ghosts and that ghosts quench a large part of $^1O_2$ generated in the membrane [18]. Beyond the confirmation of the mechanism suggested by the quenching studies, this system may provide the opportunity to quantify spectroscopically the relative contributions of type I and type II processes. Corresponding studies of $^1O_2$ and $AlPcS_2$ photophysics in viral suspensions may prove fruitful to the understanding of the different sensitivity of viruses and RBCs.

4. Conclusions

The measurements of $AlPcS_2$ transient spectra in tissue phantoms and singlet depletion in sensitised liver demonstrate the possibility for determining photophysical properties in intact tissues. This has obvious application in the study of PDT mechanisms in tumours, but may also be useful for studying other photoprocesses such as the photoisomerisation of bilirubin in jaundice therapy. At this stage, sensitivity is low and there are difficulties confirming assignments and identifying photochemistry; however,

further studies can be directed to addressing the latter issues once the sensitivity problem is solved. One step towards this is the use of tissues with low absorption coefficients and high reflectance as an alternative to the liver used in this study.

At this point some potential limitations on the application of the DRLFP techniques on living specimens should be noted. The high energy density of both the laser and more particularly the analysing light are likely to generate extensive local heating and PDT induced necrosis during a measurement. Light delivery and reflectance collection are potentially difficult using the instrument described here, although modification to use optical fibre or liquid light guide light delivery and reflectance collection may be envisaged. The photophysics of sensitisers in skin and subcutaneous tumours are in principle measurable using the present instrument in, for example, nude mice or rabbit ears. Studies on whole or sectioned excised organs are technically and ethically feasible, since the organ or tissue may be mounted directly in the sample holder of the current instrument. It is relatively easy to obtain ethical approval to administer a sensitiser, particular one such as ALA, prior to surgery to remove an organ, thereby making a broad range of tissues (including human tissue) accessible.

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