Time-resolved fluorescence spectroscopy and intracellular imaging of disulphonated aluminium phthalocyanine

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Abstract
Spectroscopic studies were carried out on the photosensitizer disulphonated aluminium phthalocyanine (AlS₂Pc) which has prospective applications in photodynamic therapy. The fluorescence lifetimes of AlS₂Pc were measured in a range of model systems and cultured leukaemic cells using laser excitation and time-correlated, single-photon-counting detection. In an investigation of non-covalent protein binding, we studied AlS₂Pc in the presence of human serum albumin (HSA) in 0.1 M phosphate-buffered saline at pH 7.4. On addition of excess concentrations of HSA, small red shifts in the fluorescence and absorbance spectra were observed, together with an increase in fluorescence polarization anisotropy, consistent with binding of the phthalocyanine. Fluorescence decays could be resolved into two lifetimes for bound AlS₂Pc with a dominant component of 5.5 ns and a minor component of 1 ns. Fluorescence imaging and time-resolved microfluorometry were carried out on intracellular AlS₂Pc using leukaemic K562 cells. Microscopic imaging with a charge-coupled device (CCD) camera revealed that AlS₂Pc fluorescence predominated in a discrete perinuclear region which was then probed selectively by a focused laser spot for fluorescence lifetime measurements. Bi-exponential decays with lifetime components of 6.1 and 2.2 ns were observed. On irradiation at 633 nm, the fluorescence intensity increased initially and subsequently declined due to photodegradation.

Key words: Phthalocyanine; Fluorescence lifetime; Fluorescence imaging; Photodynamic therapy

1. Introduction
Photodynamic therapy (PDT) is a promising treatment for certain cancers and is based on the systematic administration of a photosensitizer followed by laser irradiation of the sensitized tumour. Preparations based on haematoporphyrin derivative (HpD) are being used at present as clinical photosensitizers, but it is now widely recognized that these compounds suffer from several disadvantages. Consequently, there has been a vigorous search for new photosensitizers, and among the most actively investigated are phthalocyanine dyes [1], in particular the water-soluble aluminium sulphonated phthalocyanine (AlSPc). The principal advantage over porphyrin sensitzers is the much stronger absorption of AlSPc \( \varepsilon \approx 10^5 \text{ M}^{-1} \text{ cm}^{-1} \) at longer wavelengths near 700 nm. Furthermore, AlSPc is a good fluorophore with a peak emission near 680 nm which has enabled in vitro and in vivo detection [2, 3]. Promising results have been obtained with experimental animal tumours [4, 5] but, until recently, the majority of these studies employed AlSPc in the form of a mixture comprising mono-, di-, tri- and tetrarsulphonated components. This compound can now be prepared with a defined degree of sulphonation and a considerable amount of work [6, 7] has been devoted to comparative studies of these components, AlSPc \( (n = 1, 2, 3, \text{ and } 4) \). The degree of sulphonation affects the lipid solubility and aggregation properties, both of which are known to

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influence cellular uptake and phototoxicity, with the lesser sulphonated, more lipophilic components exhibiting higher in vitro photoactivities [6]. The disulphonated aluminium phthalocyanine component (designated hereafter as S2) exhibits the optimum combination of in vitro pharmacokinetics and phototoxicity and appears to be the most suitable for clinical PDT [6-8], whereas the monosulphonated component (the most active component in vitro) undergoes relatively high uptake in vivo by the reticuloendothelial system, particularly the liver, resulting in poor tumour sensitization.

The fluorescence from intrinsic fluorophores of biomolecules (e.g. the aromatic amino acid residues in proteins) or from fluorophores added to biological samples has been widely used to probe the structure, environment and dynamics of a variety of systems of biological importance. In particular, the fluorescence spectrum, intensity, polarization and lifetime of fluorophores may all be influenced by environmental factors. Thus such studies can provide information on the binding of photosensitizing fluorophores to biological substrates and localization within membranes, which are believed to be among the primary targets of PDT owing to lipid peroxidation. The application of fluorescence microscopy and, more recently, scanning confocal fluorescence microscopy has enabled the direct observation of fluorescence from isolated cells and intracellular components. With a few exceptions, microfluorometric studies have been concerned with monitoring only the fluorescence intensity and spectra of fluorophores in cellular systems. However, the steady state fluorescence intensity is a function of both the concentration and fluorescence quantum yield, which is dependent on the fluorescence lifetime and may vary from site to site owing to quenching and aggregation processes. Therefore, in order to quantify microscopic distributions of a given fluorophore, it is necessary to measure the fluorescence lifetimes at the microscopic level. A sensitive technique for fluorescence microscopic lifetime measurements has now been developed using time-correlated single-photon counting with laser excitation [9] and several groups have employed this method in studies of intracellular porphyrins. Streak camera studies have also been reported although this technique is generally less sensitive. Research in this field has been reviewed recently [10].

This work is concerned with fluorescence spectroscopic studies of disulphonated aluminium phthalocyanine, the aim being to determine how the fluorescence properties of the sensitizer are influenced by microenvironmental factors. Such studies are of practical relevance since fluorescence detection of sensitizers in vitro and in vivo is a highly sensitive means of quantifying cellular uptake and localization as demonstrated recently for S2 [7, 11, 12]. Two complementary experimental approaches have been adopted here as part of a comprehensive study of the photoproperties of sulphonated phthalocyanines: (i) measurements in model systems; and (ii) direct microscopic studies on individual cells containing the sensitizer. In an investigation of binding to serum proteins, we have made steady state and time-resolved studies of S2 fluorescence in the presence of an excess of human serum albumin (HSA), and similarly with micelles, which act as rudimentary models of membrane lipid environments. For the work on cells, we have combined fluorescence imaging of intracellular S2 with spatially resolved measurements of fluorescence decays.

2. Experimental details

2.1. Materials

Aluminium sulphonated phthalocyanine of mixed sulphonation was prepared by oleum sulphonation of chloroaluminium phthalocyanine. The mixture was then separated into the AIS₄Pc components using reverse-phase medium-pressure liquid chromatography, with purity assessed using reverse-phase high-performance liquid chromatography (HPLC) [13]. Similar procedures have been used elsewhere and the preparation of the S2 component as a well-defined peak in HPLC chromatograms (the most hydrophobic) appears to be reproducible between different laboratories [14]. This S2 component is commonly supposed to contain sulphonates with an adjacent configuration [13, 14]. AIS₄Pc and AIS₃Pc were prepared as described previously [13]. HSA, essentially fatty acid free, was obtained from Sigma; Triton X100, Nonidet P40 detergent and cetyl ammonium bromide (CTAB) were obtained from BDH; air-equilibrated aqueous solutions were prepared using distilled water at pH 7.4 in 0.1 M phosphate-buffered saline (PBS) (Sigma).

For the microscopy experiments, cells from the K562 line suspended in RPMI-1640 with 2% foetal calf serum (FCS) at a concentration of 2 × 10⁶ ml⁻¹ were incubated with AIS₄Pc at 10 μM for 60 min at 37 °C in the dark. The cells were then washed in RPMI-1640 with 2% FCS, centrifuged, resuspended in PBS and then placed onto a glass slide for microscopic examination at ambient tem-
perature (25 °C). Further details may be found elsewhere [15].

2.2. Methods

Absorbance spectra were measured using a Perkin–Elmer Lambda 15 spectrometer and fluorescence spectra with a Perkin–Elmer LS-5B spectrophuorometer controlled by a PC which could generate emission spectra corrected for variations in wavelength response and polarization anisotropy. From the parallel \( I_\| \) and perpendicular \( I_\perp \) anisotropy components, the steady state anisotropy of each sample was evaluated using the standard equation, 
\[
r = \frac{(I_\| - GI_\perp)}{(I_\| + 2GI_\perp)},
\]
where \( G \) is the polarization correction factor derived from the ratio \( I_\| /I_\perp \) using horizontally polarized excitation.

2.2.1. Fluorescence decay measurements

For fluorescence decay lifetime studies in model systems, phthalocyanine concentrations of 0.5 µM in 0.1 M PBS (pH 7.4) were employed using laser excitation at 610 nm. Lifetimes were measured using time-correlated single-photon counting, and emission was detected via a long-pass Schott RG645 filter with a Hamamatsu red-sensitive R928 photomultiplier tube, with a convoluted instrument response time of approximately 350 ps. The laser apparatus consisted of a frequency-doubled, mode-locked Nd:YAG laser (Coherent, Antares 76-S) which synchronously pumped a cavity-dumped dye laser (Coherent 701-3CD, rhodamine 6G dye) supplying output pulses of less than 10 ps at a repetition rate of 3.8 MHz. Time-resolved fluorescence decays \( I(t) \) and polarization anisotropy decays \( r(t) \) were analysed as a sum of exponentials using eqns. (1) and (2), where for the \( i \)th component, \( \tau_{\text{Cor}} \) is the rotational correlation time and \( r_o \) is the fraction of the total anisotropy
\[
I(t) = \sum_i A_i \exp(-t/\tau_i)
\]
\[
r(t) = \sum_i r_o \exp(-t/\tau_{\text{Cor}})
\]

Data were recorded to a minimum of 20 000 counts in the channel of maximum intensity for the model system studies and analysed by a non-linear, least-squares iterative reconvolution program using the reduced \( \chi^2 \) coefficients, plots of weighted residuals and autocorrelation function for assessment of the data. Global analysis was also employed in certain cases [16].

For the fluorescence decay microscopic studies on individual K562 cells, the dye laser beam (wavelength, 610 nm) was reflected off a dichroic mirror (DF650, Omega Optical Inc.) mounted on an Olympus IMT-2 microscope and directed through a 40× objective (NA 0.95) to produce a laser spot of 1 µm radius on a pre-selected cell. Fluorescence was collected by the same objective and transmitted through the dichroic mirror to an R928 photomultiplier for time-correlated single-photon-counting analysis via barrier filters (Schott RG665) and an iris to eliminate scattered light; both s and p polarizations were equally transmitted by the dichroic mirror over the detection wavelength band (660–700 nm). In order to minimize AlSPc photodegradation, the power of the excitation beam was attenuated to less than 1 µW, which nevertheless yielded fluorescence counting rates up to \( 10^4 \) s\(^{-1} \) due to the high detection efficiency of the microscope objective.

2.2.2. Fluorescence imaging

An inverted microscope (Olympus IMT-2), with epifluorescence and phase contrast attachments, was used with excitation light provided by an 8 mW helium–neon laser emitting at 632.8 nm. Fluorescence was imaged using a cooled, slow-scan, charge-coupled device (CCD) camera (Wright Instruments, model 1, resolution 600×400 pixels, 14 bit). A liquid light guide and a condenser were used to direct the laser output (via a 10 nm bandpass filter centred at 633 nm to remove extraneous light) into the dichroic mirror housing for standard epifluorescence excitation [6, 7]. The phthalocyanine fluorescence was detected in the range 660–700 nm covering the main fluorescence band of these sensitizers. The advantages of using a cooled, slow-scan, CCD camera over video imaging systems include much higher sensitivity, direct digital image integration and a high dynamic range of over \( 10^4 \). The high sensitivity allows low-power excitation and short integration times minimizing sensitizer bleaching that may distort the fluorescence image. An IBM PC with a high-resolution colour monitor controlled the camera operation and was used for digital image processing, display and storage. Autofluorescence from control cells amounts to only 1–2 counts on an image scale of \( 10^3 \) employed in this work. Fluorescence was digitally quantified by either line or box superimposition on areas of interest.

3. Results

3.1. Model systems

3.1.1. Human serum albumin (HSA)

Binding of porphyrins to this protein has been widely studied owing to its importance in the
pharmacokinetics of the circulating sensitizer and delivery to tissues. The interaction of S2 with HSA was investigated using a combination of steady state and time-resolved techniques, namely measurements of alterations in absorbance and fluorescence spectra, and changes in fluorescence decay and fluorescence emission anisotropy. Since the main objective of this work was to investigate the fluorescence properties of S2, the S2 concentrations were in general restricted to 0.5 μM in order to minimize re-absorption over the 1 cm path length of the cuvettes (optical density (OD)<0.1 at 675 nm). Therefore, for these binding studies, it was found necessary to use excess concentrations of HSA which were added to phthalocyanine solutions in successive aliquots, freshly prepared in 0.1 M PBS, to give HSA concentrations ranging from 0.5 to 100 μM. For the absorbance experiments, a cuvette with HSA at the same concentration was placed in the reference beam to compensate for light scattering. Experiments at higher HSA concentrations up to physiological values were not attempted owing to the marked light scattering which could have affected polarization anisotropy, amongst other effects. Figure 1 shows that the S2 absorption spectra in the presence of HSA are consistent with monomeric phthalocyanine, with the Q-band absorbance slightly broadened and red shifted from 672 to 676 nm at the highest HSA to S2 concentration ratio; an isosbestic point is apparent at 676 nm, although closer examination reveals that the traces are not exactly coincident. Fluorescence emission spectra (Fig. 2) also exhibit a small red shift on addition of excess HSA, reaching a maximum at 685 nm compared with 680 nm in 0.1 M PBS. Steady state fluorescence emission anisotropy was observed with increasing HSA concentration and converged to an anisotropy value of \( r = 0.06 \). For the higher HSA concentrations, a small reduction (typically 10%) in the integrated fluorescence intensity (corrected for changes in emission wavelength response and absorbance at 610 nm) was found in these measurements; however, some variation was evident between experimental runs probably because of the residual aggregated S2 before addition of HSA. From the bathochromic shifts in the S2, absorbance and fluorescence emission spectra, we estimate that greater than 90% association was present in 50–100 μM of HSA. Although we did not attempt a definitive study with a range of different S2 and HSA concentrations, the binding constant for S2 may be estimated as \( K \approx 10^5 \text{ M}^{-1} \) which is an order of magnitude lower than for a haematoporphyrin [17]. A limited number of experiments were carried out with the tri- and tetrasulphonated components (S3 and S4) which both exhibited lower binding affinities to HSA judging from the spectral shifts. It is interesting to note that the S4 component, which is present in both monomeric and dimeric...
forms of 0.1 M PBS [13], did not monomerize on addition of excess HSA, in contrast with solutions containing aggregated S2. Systematic studies will be reported elsewhere [18].

From time-resolved measurements, S2 in PBS alone exhibited a mono-exponential fluorescence decay with a lifetime of 5.0 ± 0.1 ns, but at high HSA to S2 concentration ratios (50 and 100) bi-exponential decays were observed with lifetimes of $\tau_1 = 5.5 \pm 0.2$ ns and $\tau_2 = 1.0 \pm 0.2$ ns, with normalized pre-exponential $A$ factors converging to $A_1 = 0.92 \pm 0.1$ and $A_2 = 0.08 \pm 0.01$ ($\chi^2$ coefficients of 1.05–0.95). At intermediate HSA to S2 ratios (1, 2, 5, 10 and 25), we observed multi-exponential decays which could be fitted satisfactorily using the $\tau_1$ and $\tau_2$ values together with the free dye lifetime of 5 ns. It should be noted that these decays were measured without a monochromator under conditions which were independent of polarization anisotropy ($G$ factor of unity); a contribution from scattered light can also be eliminated. Of course, even at the higher HSA concentrations the dye is never completely bound, but it is difficult to resolve the relatively small contribution from the free dye especially when the lifetime does not differ significantly. At high HSA to S2 ratios the time dependence of the polarization anisotropy [19] was studied, and we derived a rotational correlation time of $\tau_{cor} = 45 \pm 5$ ns; the value for free dye in PBS was $\tau_{cor} = 0.3$ ns corresponding to out-of-plane rotation (in-plane would exceed the resolution of our system).

3.1.2. Micelles

The incorporation of S2 was examined in two micellar solutions: CTAB $(10^{-2}$ M) and Triton X100 (1%) in 0.1 M PBS. A 20 $\mu$l aliquot of S2 stock solution was added to the micellar solutions to give an S2 concentration of 0.5 $\mu$M. In both cases a small spectral red shift was observed: the peak absorbance in Triton X100 is at 675 nm compared with 672 nm in aqueous solution. The addition of Triton X100 to a more concentrated S2 aqueous solution (10 $\mu$M), in which the S2 was partially aggregated, resulted in monomerization. The fluorescence lifetimes were found to be: $\tau = 5.9 \pm 0.1$ ns and $\tau_{cor} = 8.0 \pm 0.7$ ns for Triton X100; $\tau = 6.0 \pm 0.1$ ns for CTAB. For comparison, in glycerol $\tau = 5.4$ ns and $\tau_{cor} = 5.7 \pm 0.5$ ns.

3.2. Microspectrofluorometry of K562 cells

3.2.1. Steady state spectroscopy and imaging studies

Spectroscopic studies were carried out on cultured leukemic cells, the K562 human erythromyelogenous leukaemic progenitor line. This particular cell line was selected for investigation because it has been shown to be highly susceptible to AIS$_2$Pc photosensitized destruction [15, 20] which is hoped may provide a means of selective elimination of leukemic cells from infected bone marrow (photopurging). In this work, cells were incubated under the same conditions as used in the photosensitization studies, i.e. 10 $\mu$M of S2 for 1 h. Using the CCD imaging system to examine the intracellular S2 fluorescence distribution, it is clearly evident from Fig. 3(a) that the dye is taken up inside the cell (diameter, approximately 20 $\mu$m) at extranuclear sites. The conspicuous feature is a highly fluorescent region adjacent to the nucleus contributing over 50% of the integrated intensity, which proved ideal for spatially selective probing by a focused laser spot in subsequent time-resolved studies. Owing to the high sensitivity of the CCD, which afforded a short exposure time of 1 s, no perturbation in the S2 distribution due to photodegradation was apparent. A few cells were examined using a 100$\times$ objective which suggested a granular structure in the fluorescence distribution. Further studies using cytospins demonstrated good correlation between the fluorescence distribution and histochemical staining for acid phosphatase, implying that the S2 was associated with lysosomes. In comparative studies with S4, a much weaker fluorescence intensity was observed, whereas S3 exhibited an intensity distribution which appeared to be intermediate between the S2 and S4 components.

On prolonged irradiation at 633 nm (40$\times$ objective), it was apparent that the S2 fluorescence distribution became perturbed, and a series of images were taken during irradiation of the same cell as shown in Fig. 3(b) (recorded with a 1 s exposure) after 30 s irradiation) and 3(c) (after a further 60 s). The power incident on the cells at 633 nm was estimated to be 2 mW over 0.2 mm$^2$, giving an irradiance of about 1 W cm$^{-2}$. Comparing Figs. 3(a) and 3(b), it is clearly seen that the perinuclear feature intensified and expanded: quantitative analysis showed an increase in overall intensity of this feature by about 50% between the two images. This intensity increase was reproducible in all cells examined after 20–40 s irradiation and, in exceptional cases, a factor of two was found (but a 50% increase was the typical value). In Fig. 3(c) the fluorescence has spread throughout the cell and appears to have penetrated into the nucleus. Figure 3(d) shows a three-dimensional intensity plot of Fig. 3(b). These observations, which were found in the many cells
Fig. 3. Using the CCD fluorescence imaging system a series of false colour images (white, highest intensity) with an exposure time of 1 s were recorded during irradiation at 633 nm (1 W cm$^{-2}$) of the same K562 cell (diameter, approximately 20 μm) incubated for 1 h with 10 μM S2: (a) 1 s irradiation; (b) 30 s irradiation; (c) 90 s irradiation. Comparing (a) and (b), it is clearly seen that the perinuclear feature intensifies and expands; (d) shows a three-dimensional plot of (b), in which the low-intensity intranuclear region is distinctly delineated. In (c) the fluorescence distribution has become more diffuse and weaker as the S2 undergoes photodegradation. Scale in micrographs, 50×35 μm.
examinined from different batches and which were apparently irreversible, would be consistent with the photosensitized destruction of organelles associated with S2 such as mitochondria and lysosomes. The integrated cellular fluorescence intensity in Fig. 3(c), and for yet longer irradiation times, declined progressively signifying that photodegradation of S2 was taking place in parallel with its redistribution.

The fluorescence emission spectrum of cell suspensions excited at 610 nm closely resembled that observed with HSA with a peak intensity at 683 nm. The addition of Nonidet P40 detergent (1%), which lysed with cells releasing the dye in monomeric form, resulted in up to a twofold increase in fluorescence emission, and this effect was observed over a range of S2 incubation concentrations from 2 to 20 μM using 2 ml suspensions containing approximately 10^6 cells. With a calibrated plot of S2 fluorescence intensity vs. concentration in the detergent solution, we were able to estimate the mean cellular S2 uptake to be approximately 10^7 molecules per cell.

3.2.2. Time-resolved studies

For time-resolved microfluorometry, the focused laser beam was aligned initially by adjusting the position of the cell under phase contrast relative to the laser spot. The CCD camera, which was attached to another port on the inverted microscope orthogonal to the photomultiplier tube, was then used to assess the fluorescence distribution and thus guide the positioning of the laser spot over the selected intracellular region taking care to minimize irradiation of the cell. In addition to its inherent spatial resolution, this microscopic technique is more reliable than bulk measurements on a cell suspension, which are susceptible to interference from sensitizer leakage from the resuspended cells and polarization anisotropy effects.

The irradiation power of the laser spot was approximately 10 W cm^{-2} at 610 nm and experiments were confined to cells incubated with 10 μM of S2 for 1 h in a series of studies conducted over several days. In most of this work, S2 fluorescence decays were recorded with the laser spot (2 μm in diameter) incident on the highly fluorescent region (typically 4 μm in diameter) as shown in Fig. 3(a). Counting rates up to 5000 s^{-1} were detected and decays were accumulated to a maximum of 10,000 counts in the peak channel in the first instance. It was found that, under these conditions, the S2 fluorescence at this site exhibited bi-exponential decays with lifetime components near 2 and 6 ns. Modelling of the data from 15 cells including global analysis confirmed values of 2.2 ± 0.4 and 6.1 ± 0.2 ns, with similar amplitudes for each component; good fits were obtained with the normalized A factor of the shorter component in the range 0.3-0.5 (χ^2 coefficients of 1.2-1.4), although in some cases [4] tri-exponential fits gave a slightly better agreement with the inclusion of a minor 1 ns component. The data quality was limited by the relatively short accumulation times; these were required because, on prolonged irradiation, the bi-exponential dependence evolved essentially to a mono-exponential decay with just the longer component of 6.1 ns remaining, i.e. counting to 20,000 in the peak channel reduced the A factor of the shorter component to less than 0.1. Thus the time-resolved data may be characterized by two regimes of fluorescence decay behaviour with case 1 (initial excitation) giving a bi-exponential dependence and case 2 (excitation after irradiation) giving a mono-exponential dependence. The relative fluorescence yields for case 1 compared with case 2 may be estimated from the respective A factors (taking equal values for case 1) and lifetimes by the equation: Y = ΣA/τ_i; for case 1, Y_1 = (0.5×2) + (0.5×6) = 4 and Y_2 = (1×6) = 6, giving a 50% increase from case 1 to 2. However, in general, the counting rate from the laser spot showed little change, presumably due to diffusion and photodegradation, although in a few cases it did increase by 10% when the laser intensity was sufficiently attenuated. This is an important point, since it has often been assumed in previous studies that a near-constant counting rate implies no perturbation to the fluorophore lifetime dynamics. Cells examined by the CCD system after laser spot probing exhibited fluorescence distributions similar to those shown in Figs. 3(b) and 3(c) demonstrating that, during the course of the lifetime measurements, S2 underwent intracellular redistribution. Lifetime measurements were attempted at other subcellular sites but with varying success due to signal limitations: probing at the cell periphery outside the highly fluorescent region yielded a lifetime of about 6 ns.

4. Discussion

In previous papers, we have investigated the excited singlet and triplet state properties of the AIS_{nPc} components using steady state and time-resolved techniques [13, 21-23]. Comparative studies were carried out in methanol and phosphate-buffered saline which demonstrated that pertur-
bations to the phthalocyanine photoproperties, induced by the addition of varying numbers of sulphonate groups, are negligible [13]. However, differences in the fluorescence quantum yields and lifetimes of S2 in these solvents were found: in aqueous solution $\tau = 5.1 \pm 0.1$ ns, $\Phi = 0.4 \pm 0.04$; in methanol $\tau = 6.1 \pm 0.1$ ns, $\Phi = 0.56 \pm 0.05$. It was concluded that this effect was due to an increase in the quantum yield of internal conversion to 0.4 in aqueous solution, consequently limiting the triplet state yield to 0.2. In this work, the interaction of S2 with HSA has been studied at pH 7.4 in 0.1 M PBS. Both the steady state and time-resolved spectroscopic studies provide convincing evidence of binding, although the affinity of S2 for HSA is weaker than found for haematoporphyrin. Bathochromatic spectral modifications have also been observed for several porphyrins on binding to HSA [24] and have been attributed to the lower overall dielectric constant of the protein microenvironment. HSA is predominantly an $\alpha$-helical globin protein comprising three major domains, each of which contains two subdomains. This morphology offers a variety of binding sites, with two particular subdomains separated by approximately 8 nm identified as being suitable conformations for small molecules [25]. From absorbance and fluorescence spectroscopic studies [24], the binding of haematoporphyrin to HSA is characterized by one high-affinity site, which is adjacent to the sole tryptophan residue (204-Trp) on the basis of fluorescence energy transfer from this residue to haematoporphyrin, together with several other unspecified lower affinity sites. We attempted the same fluorescence energy transfer experiment using S2 but the results were unconvincing. Nevertheless, the presence of one dominant decay component clearly favours the presence of a specific binding site for S2. Our results may be compared with another study [26] of HSA binding with AlS2Pc containing a mixture of components giving an average sulphonation of about three. Although these results are not strictly comparable with those presented here, because the mixture components have different binding affinities, similar spectral changes were reported in the presence of HSA; time-resolved studies were confined to the triplet state and they concluded that the bound phthalocyanines were accessible to quenching species residing in the solvent phase. Binding of S2 to HSA to form a non-covalent complex is an important factor influencing the biodistribution of the sensitiser, since it is thought that HSA delivery favours interstitial localization, and S2 is certainly known to partition to these regions, especially submucosal sites [7, 12]. Binding of S2 with lipoproteins also occurs at similar concentrations [21] but, given the large excess of HSA in serum, binding to albumin should predominate in vivo.

In the time-resolved fluorescence studies at the highest HSA concentrations, S2 fluorescence decays could be fitted reproducibly within the time resolution of the system using two lifetimes: one major component of 5.5 ns and one minor component of 1 ns. These results imply two binding sites for S2. Time-resolved fluorescence studies of haematoporphyrin complexed with HSA at pH 7.4 have also shown the presence of two lifetime components with decay times near 9 and 17 ns [24]. It has been established that protein structural fluctuations occur in the nanosecond time scale; thus single lifetimes resolved from simple exponential fitting may represent mean values for the various conformations in the dye/protein microenvironment [19]. As shown in Table 1 and the accompanying references, the 5.5 ns value of the major S2 lifetime component is intermediate between the lifetimes measured in H2O and other solvents including micelles for which higher values around 6 ns are consistently found. As mentioned above, it has been shown recently that the fluorescence quantum yield and lifetime in H2O are reduced owing to an enhancement in the rate of internal conversion. We therefore propose that this effect is inhibited partially when the phthalocyanine is protein bound, yielding the intermediate lifetime value observed here in the presence of HSA, with the assumption that the intrinsic singlet state lifetime is not perturbed significantly on binding. A similar shielding effect has been observed in the triplet state kinetics of AlS2Pc studied using a tunable nanosecond flash photolysis system [21]. In argon-saturated PBS, S2 exhibits a triplet–triplet absorption band centred at 480 nm which decays as a single exponential with a lifetime of 600 ± 40 $\mu$s. However, in the presence of excess HSA, the lifetime of the triplet state undergoes a substantial increase: in 100 $\mu$M HSA,

<table>
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<tr>
<th>Solvent</th>
<th>$\tau$ (ns)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>MeOH</td>
<td>6.1 ± 0.1</td>
<td>[13, 22]</td>
</tr>
<tr>
<td>0.1 M PBS (pH 7.4)</td>
<td>5.0 ± 0.1</td>
<td>[13]</td>
</tr>
<tr>
<td>Triton X100/PBS</td>
<td>5.9 ± 0.1</td>
<td>This work</td>
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<tr>
<td>50 $\mu$M HSA/PBS</td>
<td>5.5 ± 0.2 (0.92), 1.0 ± 0.2 (0.08)</td>
<td>This work</td>
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$\tau = 875 \pm 50$ $\mu$s. This stabilization of the triplet state was ascribed to a reduction in the rate of intersystem crossing to the ground state of the protein-bound dye compared with the free dye in H$_2$O. In addition, the rate constant for oxygen quenching of the triplet state is decreased by a factor of ten when AISPc is protein bound (vs. unbound) as a result of the lower rate of oxygen diffusion through the protein matrix. It should be noted that the S2 fluorescence lifetime is insensitive to quenching by oxygen and this could not explain our results [19].

However, the minor 1 ns lifetime component ($A = 0.1$) appears to be anomalous and has no convincing explanation; it is also intriguing to note that the $A$ factors for both components exhibited a similar correlation with HSA concentration, indicating comparable affinities assuming the presence of two binding sites. The short lifetime could arise from binding at a site which induces efficient quenching by distortion of the phthalocyanine macrocycle, resulting in an enhanced rate of internal conversion. An alternative explanation would involve interaction with amino acid residues, but a series of experiments in aqueous solution with several amino acids (tryptophan, tyrosine, cysteine) did not provide any evidence for singlet state quenching, although admittedly we are comparing different microenvironments. It is conceivable that S2 may contain two isomers with distinctly different binding properties, but reproducible results were obtained between different S2 preparations with different isomeric compositions. In an attempt to resolve the components from differences in the polarization anisotropy, we studied the time-resolved polarization anisotropy of the bound dye. However, optimal fitting yielded a rotational correlation time of 45 ns which is the expected value for an immobilized fluorophore bound to a globular protein of 65 kDa [19]. Of course, fitting with two lifetimes does not necessarily preclude another type of decay function, and the weak shorter component found here may merely allude to more subtle decay dynamics of a single population. For example, deviations from simple exponential behaviour can result from excited state dipole relaxation processes in the protein matrix, and diffusion-controlled quenching which introduces an extra $t^{1/2}$ dependence [18]. The latter explanation is a possibility if the surrounding H$_2$O solvent molecules may be considered to be a quenching species, since solvation (and complexation of H$_2$O with the central Al) enhances the rate of internal conversion. MCP detection, with its superior time resolution, may offer further refinement to these provisional conclusions. The slight degree of steady state fluorescence quenching (typically 10%), observed on addition of HSA, was near the limit of the experimental resolution and difficult to reproduce satisfactorily. In principle, from the small counterbalancing changes in lifetime behaviour, observed on binding, the overall fluorescence yield of bound S2 should be indistinguishable from the free dye. However, in practice, the increased scattering of the spectrometer excitation beam at higher HSA concentrations could have resulted in a small apparent attenuation of the detected fluorescence. Adsorption of HSA onto the sides of the silica cuvette and subsequent binding of S2 is another possible factor: with orthogonal excitation/detection geometry, the fluorescence of S2 on the cuvette would be undetectable. Adsorption on silica is known to occur for bovine serum albumin and was investigated recently using the technique of evanescent-wave-induced fluorescence spectroscopy [27].

As expected for an amphiphilic dye, S2 is incorporated into Triton X100 and CTAB micelles; the partition coefficient ($K$) of S2 between Triton X114 and water has been studied previously [14] as a function of pH: at pH 7 and 37°C, $K = 200$, with the value increasing at lower pH as the sulphonate groups with $pK_a = 3$ become progressively acidified. The longer fluorescence lifetime observed in micellar solutions (approximately 6 ns) compared with aqueous solutions is consistent with exclusion from the aqueous phase, which was also demonstrated by a correlation time (approximately 8 ns) indicative of hindered rotation and similar to that observed in glycerol. For comparison, time-resolved studies of haematoporphyrin in Triton X100 revealed a single fluorescence lifetime of 16 ns typical of the monomeric species [24].

These studies on model systems were followed by an in vitro investigation of intra-cellular S2 using the leukaemic K562 cell line. Imaging with a CCD camera revealed that S2 fluorescence predominated in a discrete perinuclear region which was then selectively probed in isolated cells by a focused laser spot for fluorescence decay measurements. Two-component fits were observed giving lifetimes of 2.2 and 6.1 ns with comparable pre-exponential factors. However, on extended irradiation, the fluorescence decays were characterized by a single lifetime of 6.1 ns, with the shorter 2.2 ns component being excluded. This evolution from bi-exponential to mono-exponential decay behaviour suggests that a major change in the S2 microenvironment occurs during irradiation, which must be due in part to photosensitized
oxidation of the subcellular sites (e.g. organelles) associated with S2, and probably combined with diffusion of S2 away from these sites. The fluorescence lifetimes of AlSPc (mean sulphonation of two) have been measured in murine ascitic tumour cells [28] incubated in vivo but studied ex vivo in a suspension. The bulk fluorescence decays were multi-exponential with lifetimes close to the microscopic values found here. The presence of the shorter lifetime components in that study and ours shows that AlSPc may undergo a significant degree of fluorescence quenching in vitro at certain intracellular sites with lysosomes being prime candidates. Assuming that lysosome localization is significant, the observed fluorescence redistribution can be explained by photo-oxidation of lysosomal membranes leading to the release of S2 together with hydrolytic enzymes into the cytosol which would ultimately result in cell autolysis. The mechanism of AlSPc sensitization of cell membranes has recently been studied [29] using erythrocyte ghosts, which were shown to sustain damage from extensive lipid peroxidation in addition to cholesterol degradation specific to a type 2 process mediated by singlet oxygen. The subcellular localization of S2 and S4 has been studied previously [30] in carcinoma cells (NHIK 3025) which exhibited uptake mainly by lysosomes; furthermore, it was proposed that S2 partitioned mainly to lysosomal membranes, whereas the hydrophilic tetrasulphonated component resided in the central aqueous compartment. In another recent study [31] on S2 localization in a human melanoma cell line using confocal scanning, uptake in mitochondria as well as lysosomes was implicated. We also note that localization in the Golgi apparatus was suggested in a recent study of AlSPc (mixed sulphonation) photosensitization of K562 cells [32]. In view of these potential complications, the partitioning of S2 between subcellular sites remains unresolved and further studies using confocal scanning of S2 in K562 cells would be desirable.

Regarding the origin of the fluorescence quenching mechanism, we can only speculate which substrates might be involved. Assuming that localization in lysosomes is important, the influence of pH should be considered since the intralysosomal compartment is relatively acidic (about pH 5). Such an effect, resulting in a marked lifetime shortening of lysomotropic sulphonated tetraphenyl porphyrin in RR1022 epithelial cells [33], has been observed using time-resolved microfluorometry and was ascribed to the presence of a protonated monocationic species at the lysosomal pH. However, from previous studies [13] we can eliminate any perturbation in the S2 lifetime at this pH; a shorter lifetime is observed under more acidic conditions (below pH 3), but this is accompanied by pronounced spectral changes which were not observed here. A more plausible quenching mechanism would involve energy transfer or electron transfer between S2 and substrates at or near the S2 sites. For example, we already know that highly efficient quenching of the AlSPc singlet state occurs with the electron acceptors methyl viologen and anthraquinone in aqueous solution [34]. On photosensitization, the binding sites would presumably be destroyed, thus eliminating the quenching, and resulting in the longer fluorescence decay observed on extended irradiation. The difficulty with this explanation is that, for a high proportion (nearly half) of the heterogeneous S2 sites, a suitable substrate must reside in close proximity to S2; moreover, without a precise knowledge of S2 localization it is obviously impossible to identify a specific class of substrates in cells which could account for either of these quenching processes.

A further possibility is the interaction between adjacent phthalocyanine molecules, resulting in concentration quenching of the excited state by ground state S2. In the preceding analysis, we have assumed that fluorescence from sulphonated phthalocyanine aggregates is undetectable, as other workers have concluded [30, 31], and would therefore exhibit an unresolvable lifetime on the picosecond time scale. Certainly the S2 fluorescence and excitation spectra observed from suspensions of K562 cells appeared to be consistent with monomeric S2, although it should be noted that the emission spectrum of dimeric unsulphonated zinc phthalocyanine in liposome membranes closely resembles that of the ZnPc monomer [35]. From an analysis of the fluorescence decays as a function of ZnPc concentration, it was concluded that the dimeric species in liposomes exhibits a lifetime approximately half that of the monomer. Some degree of aggregation of intracellular S2 would be expected: the estimated cellular uptake was approximately $10^7$ molecules per cell which would give a local concentration approaching $10^{-3}$ M in the region probed. At such a concentration in aqueous solution, S2 would be completely aggregated, but intracellular binding to substrates and/or association with membrane structures would be expected to promote partial monomerization [30]. It is therefore conceivable that self-quenching between S2 molecules either associated or in close proximity (e.g. in membranes) may be possible at such high concentrations; alternatively, a distinct
type of intracellular fluorescent aggregate could be invoked. It would be desirable to replicate such behaviour in model systems, such as liposomal membranes, with high dye to lipid ratios in order to induce S2 aggregation within the membrane. Studies of the photophysics of S2 in reversed micelles [36] may also aid the interpretation of the non-exponential fluorescence decay dynamics of S2 in heterogeneous environments.

The attractive feature of a concentration quenching mechanism is that it could explain the transition to the longer mono-exponential lifetime behaviour when S2 undergoes dilution following photosensitized redistribution. In order to verify conclusively the contribution of such a mechanism, experiments at lower concentrations would have been desirable; however, the limited fluorescence intensities available with the present detection system precluded a more complete investigation. The increase in S2 fluorescence intensity after the addition of detergent to unirradiated cellular suspensions is also consistent with the presence of fluorescence quenching in whole cells. The lack of any distinct concentration dependence using S2 concentrations in the range 2–20 µM (although only a factor of four difference in cellular uptake was observed) would appear to argue against a self-quenching mechanism, but it is possible that localization in the relevant sites becomes saturated even at the lowest concentration, thus limiting correlation with concentration. The small Stokes shift of the fluorescence emission from the S2 Q-band may favour an energy transfer mechanism, and fluorescence depolarization studies (not attempted here) could, in principle, provide evidence of such a mechanism.

It is interesting to note that, from transient absorption studies [21, 22], we have established that ground state quenching limits the S2 triplet state lifetime with a rate constant of approximately $2 \times 10^8$ M$^{-1}$ s$^{-1}$ in water.

Following extended irradiation, the evolution of the lifetime to just one longer component near 6 ns may be interpreted in terms of the photosensitized elimination of the nascent fluorescence quenching process; equivalently, we can infer that (before photodegradation becomes significant) the overall S2 fluorescence efficiency is increased during irradiation. The transition between these two lifetime regimes appears to be consistent with fluorescence imaging studies on whole cells in which photosensitized alterations in fluorescence intensity and distribution were observed using extended irradiation at 633 nm, although these changes occurred over a longer time scale than with the focused laser spot at 610 nm which had a much higher irradiance (factor of ten); moreover, the S2 absorbance at 610 nm is twice that at 633 nm. It is also pertinent to note that irradiation at 633 nm for over 90 s (see Fig. 3(c)) at 1 W cm$^{-2}$, giving an energy dose of 90 J cm$^{-2}$, is equivalent to approximately 10 J cm$^{-2}$ at 675 nm, which is comparable with that employed in previous in vitro studies [15] of AISPc photosensitization of K562 cells: irradiation with approximately 30 J cm$^{-2}$ (using 675 nm) was lethal to more than 95% of the cells under the same incubation conditions as used here. Although a precise comparison between the different sets of microscopic data is somewhat conjectural, the increases in microscopic steady state fluorescence, as shown in Figs. 3(a) and 3(b), are similar to the approximate 50% increase in the fluorescence yield from case 1 to 2 in the lifetime studies. We therefore propose that the transition between the two lifetime regimes occurs concomitantly with the intensification and redistribution of intracellular S2 as a result of the photosensitized rupture of organelles. This view is reinforced by the observation that, after the acquisition of case 2 data, the intracellular distribution closely paralleled that of Fig. 3(b). These results may have phototherapeutic implications since, if significant fluorescence quenching to the ground state is present, the triplet and singlet oxygen yields will be correspondingly reduced and a lower photosensitization efficiency will be obtained. Moreover, differential fluorescence quenching at various sites would affect the correlation of sensitizer localization with fluorescence imaging of cells and frozen sections. Further studies should investigate the degree of quenching in other cell lines and tissue explants.

Similar photosensitization effects have been reported with AISPc in other cell lines using quantitative microscopic imaging. The behaviour of a mixture of S3 and S4 was studied [37] in cells at comparable concentrations as in this study, but after a much longer incubation time of 24 h for two cell lines: RR1022 rat epithelial cells and 3T3 murine fibroblasts. Following irradiation at 675 nm with 3.8 W cm$^{-2}$, a fluorescence increase (factor of two) was observed for RR1022 cells, but not for the fibroblast line (this disparity may be related to different subcellular localization), and photobleaching was subsequently observed in each line with prolonged irradiation. In another study, comparative experiments were performed on a human melanoma cell line with S2 and S4 [31]; a large initial increase in cellular fluorescence was observed for both S2 and S4 components, but this effect was attributed to disaggregation to form...
fluorescent monomers following photosensitization of organelles containing non-fluorescent aggregates. This explanation is reasonable since an 18 h incubation time was used, which should result in much higher intracellular concentrations and therefore favour aggregation. Our results indicate a slightly different conclusion, since our studies investigated a much lower concentration regime, but in the absence of time-resolved data from the other cell lines we believe that the two interpretations are compatible. The common feature of all these in vitro studies is the ultimate process of photodegradation or photobleaching. Photodegradation of AISPC in aqueous solution is minimal with a quantum yield of only approximately 10^{-6} [38], since phthalocyanines are relatively resistant to singlet oxygen attack. In the present case, we irradiated the cells with about 1 W cm^{-2} giving an incident fluence of approximately 10^{20} h\nu \text{ cm}^{-2} delivered over an estimated photodegradation half-life of approximately 10^2 s. In combination with the absorption cross-section of S2 (10^{-16} \text{ molecule}^{-1} \text{ cm}^2 at 633 nm), we can estimate that the photodegradation quantum yield in K562 cells is in excess of 10^{-5}, most probably occurring via type 1 processes [24]. Photodegradation of AISPC has also been shown to occur in vivo at therapeutic light doses, and this process is now recognized to be a key factor in improving the selectivity of PDT, since it allows normal tissue adjacent to tumour to be spared if the AISPC dose is below a certain threshold level. However, in contrast with the in vitro results reported here and elsewhere, an initial increase in fluorescence intensity has not been observed upon irradiation in vivo. Several factors may be involved in this disparity: firstly, much lower sensitizer concentrations are generally present in vivo and the presence of non-fluorescent aggregates is consequently lower, and secondly, a substantial amount of the sensitizer may be bound to extracellular components. In the case of AISPC we know from CCD fluorescence imaging of frozen tissue sections that a major fraction is retained in interstitial regions containing macromolecular substrates such as collagen [12].

5. Conclusions

This work has demonstrated the value of the combination of time-resolved microfluorometry and quantitative fluorescence imaging to unravel complex processes in cellular systems. This technique has many other potential applications in studies of heterogeneous samples (not only biological) on the microscopic scale with the next stage being to assemble a time-resolved fluorescence imaging system with subnanosecond time resolution and submicrometre spatial resolution using confocal optics. Fluorescence decays and time-gated spectra would be acquired using single-photon-counting techniques with detectors (e.g. microchannel plates and photon-counting avalanche photodiodes) offering enhanced time resolution and sensitivity.

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