Time-resolved fluorescence studies of porphycene and tetrasulfonated phthalocyanine dyes in varying solvents

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ABSTRACT

Various chemicals used for photodynamic therapy, a promising treatment for cancer, exhibit high fluorescence yields, which can be used for tumour detection. For a chosen few such compounds fluorescence lifetimes, absorption, and steady state fluorescence spectra were measured at different concentrations (ranging from 10^{-5} M to 10^{-8} M) in a variety of solvents. The lifetimes were repeated at three different concentrations (ranging from 10^{-5} M to 10^{-6} M) in suspensions of 3T3 fibroblast cells.

Keywords: fluorescence lifetime, tumour detection, porphycene, phthalocyanine

1. INTRODUCTION

Photodynamic therapy has been found to be a promising treatment for cancer.[1,2,3] Some of the chemicals used for therapy also exhibit high fluorescence yields, which can be used for tumour detection.[4,5,6] Steady-state fluorescence can be used in detection of malignancies, but the heterogeneity of tissue and the tendency of photosensitisers to photobleach can make measurements extremely difficult to quantify. Background autofluorescence also complicates the ability to quantify intensity images.[7] Ratio methods have been developed that can be used to exploit autofluorescence to improve contrast. In spite of extensive efforts, useful ratiometric photosensitisers require shorter wavelength excitation (which does not penetrate tissues as deep) for optimum contrast.[8] Also, the fluorescence spectra of the photosensitiser and tissue autofluorescence often overlap, reducing selectivity.[9] In conclusion, even with the aid of ratiometric methods, the ability of steady-state fluorescence techniques to detect early invisible cancerous lesions has not been indisputably demonstrated.

Alternatively, one can enhance contrast by measuring the characteristic fluorescence lifetime of a photosensitiser.[10] Time-resolved techniques are based on three parameters (excitation and emission wavelengths and fluorescence lifetime) in order to discriminate endogenous and exogenous sensitisers, whereas steady state methods only use two of these criteria.[11] A major factor which limits the usefulness of steady-state fluorescence imaging of tumours is the optical contrast between the tumour and surrounding, healthy tissue. Improved contrast can be obtained with time-resolved techniques because of the differing lifetimes between endogenous and exogenous photosensitisers. Autofluorescence mainly extinguishes within 3-5 nanoseconds of excitation whereas most photosensitisers fluoresce for longer. For example, hematoporphyrin derivative, a commonly researched photosensitiser, has a lifetime of about 15 nanoseconds. Taking this into account, a nanosecond-pulsed excitation source can be used and then the fluorescence signal can be detected after a 20-30 nanosecond delay. By using this method, only the longer-lived emission signal of the photosensitiser is detected.[12]

Until recently, most biomedical researchers did not have access to the technology that is required to image on these time scales. However, advances in ultrafast lasers and high-speed imaging detectors, such as CCD cameras, have allowed lifetime imaging to be applied to living organisms. The described method has been shown to be an effective way of imaging neoplastic areas.[12] In order to perfect this imaging technique, photophysical properties (absorption spectra, fluorescence spectra, and lifetimes) of chemicals and how they might change after entering a cellular environment need to be investigated. This work presents results that will contribute to the improvement of nanosecond fluorescence lifetime imaging methods.

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The first generation photosensitisers (hematoporphyrin derivatives such as Photofrin) that have been investigated thus far have two negative aspects. First, these chemicals are retained in the tissues for up to ten weeks after injection into the body, which can lead to considerable patient discomfort. Second, Photofrin has a very low absorption at about 630 nm which is the wavelength most often used to excite photosensitisers as the tissue penetration of light increases with increasing wavelength.[13]

A number of second-generation photosensitisers have been developed to overcome these deficiencies. These chemicals are rapidly excreted from the body and have a higher absorbance at the desired longer wavelengths.

2. METHODS

2.1 Materials

A literature survey was conducted to review the photophysical properties of the second-generation photosensitisers were considered and as a result, a number were rejected for the following reasons: a) a low extinction coefficient above 630 nm, and/or b) a poor fluorescence yield (See Table 1). Some Porphycenes and Phthalocyanines based photosensitisers do suit the above criteria having relatively high extinction coefficients above 630 nm and relatively high quantum yields (See Table 2). Also, some derivatives of these materials give fluorescence yields that are relatively high compared to the other photosensitisers considered. A wider range of phthalocyanines and porphycenes were considered, but were rejected because of their low fluorescence yields or of their non-availability.

Photosensitiser	**Reason Not Appropriate**
5-Aminolaevulinic acid (protoporphyrin IX)	Extinction coefficient too low =<5000 $M^{-1}cm^{-1}$ (Sharman et al., 1999)
BPD-MA (Verteporfin)	Fluorescence yield too low =0.002-0.051 (Aveline et al., 1995)
Hypericin	Absorption peak too low =590 nm (Sharman et al., 1999)
Methylene Blue	Fluorescence yield too low =0.16-0.04 (Van der Putten, 1987)
N-aspartyl chlorin e6	Fluorescence yield too low =0.05-0.192 (Bonnett and Berenbaum, 1989)
Naphthalocyanines	Fluorescence yield too low =0.2 (Owens et al., 1998)
Rhodamines	Absorption peak too low =511 nm (Sharman et al., 1999)
Temoporfin (m-THPC)	Fluorescence yield too low =0.19-0.22 (Morlet, 1997)
Texaphyrins	Fluorescence yield too low =0.01 (Sessler et al., 1997)
Tin etiopurpurin	Fluorescence yield too low =0.014-0.03 (Pogue et al., 1998)

Table 1: Inappropriate Photosensitisers

Photosensitiser	Absorption Peaks (Extinction coeff.)	Fluorescence Yield
2,7,12,17-tetra-n-propylporphycence (compound 1)	634 nm (42,300 M^{-1} cm^{-1})[a]	0.35 (degassed)[a] 0.38 (aerated)[a]
2,7,12,17-tetrakis(2-methoxyethyl)-9-acetoxyporphycene (compound 18)	638 nm (37,000 M^{-1} cm^{-1})[a] 647 nm (35,700 M^{-1} cm^{-1})[a]	0.30 (degassed)[a] 0.27 (aerated)[a]
Phthalocyanine Tetrasulphonate (PcTs)	698 nm (126,000 M^{-1} cm^{-1})[b]	0.6[e]
Chloroaluminum Phthalocyanine Tetrasulfonate (AlPcTs)	674 nm (178,000 M^{-1} cm^{-1})[c]	0.559[e]
Zinc Phthalocyanine Tetrasulfonate (ZnPcTs)	677 nm (N/A)[d]	0.277[e]

Table 2: Appropriate Photosensitisers

[a]*Braslavsky et al., 1997*
[b]*Leznoff and Lever, 1989*
[c]*Lang et al., 1998*
[d]*Howe and Zhang, 1997*
[e]*Owens et al., 1998*

Chloroaluminum phthalocyanine tetrasulfonate (AlPcTS), zinc phthalocyanine tetrasulfonate (ZnPcTS), and phthalocyanine tetrasulfonate (PcTS) were purchased from Frontier Scientific Europe, Ltd (Carnforth, England). The porphycenes 2,7,12,17-tetra-n-propylporphycence (porphycene 1) and 2,7,12,17-tetrakis(2-methoxyethyl)-9-acetoxyporphycene (porphycene 18) were graciously donated by Professors Silvia Braslavsky (Max-Planck-Institut fur Strahlenchemie, Germany) and Emanuel Vogel (Universitat Zu Koln, Germany).

Solutions of differing concentrations were made from stock solutions of $1x10^{-4}$ M. Phosphate buffer solutions (pH=7.2) were made from 36.0 mL 0.2 M Na_2HPO_4, 14.0 mL 0.2 M NaH_2PO_4, and 50.0 mL deionised H_2O. For absorbance spectra, 50:50 EtOH/buffer solutions (pH=7.6) were made from 13.25 mL 0.2 M Na_2HPO_4, 36.75 mL 0.2 M NaH_2PO_4, 150.0 mL deionised H_2O, and 200.0 mL ethanol obtained from Fluka (Analar grade). For all other experiments, the 50:50 EtOH/buffer solutions were made using ethanol obtained from Sigma (Spectrophotometric grade). Solutions for porphycene 1 for all experiments except cell suspensions were acquired by adding amounts of stock solution, composed of the porphycene and acetone, to appropriate amounts of acetone. For cell suspensions, amounts of the stock solution were added to appropriate amounts of phosphate buffer (pH=7.2) instead of acetone in order to increase cell viability. Solutions for porphycene 18 were obtained by adding amounts of stock solution, made up of the photosensitiser and DMSO purchased from Sigma (HPLC grade), to appropriate amounts of phosphate buffer, pH=7.2. All chemicals were used without any purification.

Absorption spectra, fluorescence spectra and fluorescence lifetimes were taken for all solutions using the following concentrations: 1.0×10^{-7} M, 1.0×10^{-6} M, 5.0×10^{-6} M, 1.0×10^{-5} M, 5.0×10^{-5} M, 1.0×10^{-4} M.

2.2 Cell Suspensions

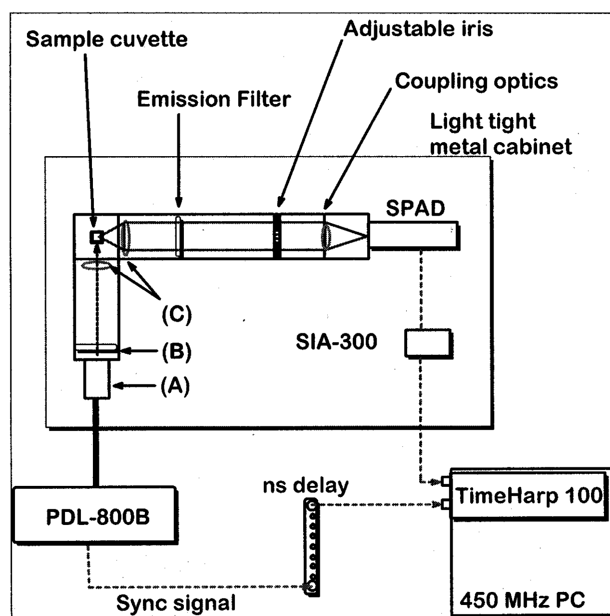
3T3 Fibroblasts were grown in 10 mL of Dulbeccos Modified Eagle's Media (1 glutimate and sodium pyruvate) supplemented with 10% fetal calf serum and 1% penicillin-streptomyocin (Sigma). Cells were incubated for 24-48 hours in 5% carbon dioxide and 95% humidity at 37° C. The old media was removed and the cells were rinsed with Hanks' Balanced Salt Solution (HBSS). The HBSS was removed and 1-2 mL of a 10% trypsin and HBSS (Sigma) were added. The cells were incubated for about 5 minutes until detachment from the flask occurred. Addition of 1-2 mL of the media was used to rinse cells from the sides of the flask and the contents were then transferred into sterile, capped test tubes. The tubes were centrifuged at 2500 rotations per minute for 5 minutes after which the top media was removed. Foil was then wrapped around each test tube to avoid light from reaching the photosensitisers. Addition of 4 mL of the chosen concentration of photosensitiser and solvent was used to resuspend the cells. Cells were resuspended in photosensitiser solutions of the following concentrations: $1x10^{-5}$ M, $5x10^{-5}$ M, $1x10^{-4}$ M.

2.3 Absorption and Fluorescence Spectra

All solutions were measured at room temperature in 1 cm or 1 mm pathlength quartz cuvettes (Spectro-Service, Ltd., Brackley, England). Absorption spectra were taken using a Shimadzu UV-1601 spectrophotometer. Fluroescence spectra were obtained using a Perkin-Elmer LS 50B luminescence spectrometer using excitation wavelengths near the maximum absorbance for each photosensitiser and solvent.

2.4 Fluorescence Lifetimes

Fluorescence lifetimes were recorded using the Time Correlated Single Photon Counting (TCSPC) technique with a system that was assembled in house (See Figure 1).[14] The excitation source was a pulsed laser diode with a centre wavelength of 635 nm powered by a PDL-800B LED/laser diode driver (PicoQuant, Berlin, Germany). The emission of the laser diode was filtered using an interference filter of 632 nm to remove spurious, longer wavelength emissions. The laser diode was operated at a variety of frequencies ranging from 5 MHz to 20 MHz depending on the lifetime of the photosensitiser. For most lifetime measurements of photosensitisers and cell suspensions, the laser diode was operated at a frequency of 5 MHz. The fluorescence emission wavelength was selected with narrow band pass interference filters of 650 nm, 700 nm, and 750 nm and detected using a Perkin-Elmer SPCM-AQR-14 Single Photon Counting Module (SPAD). The output transistor-transistor logic (TTL) pulses from the SPAD were inverted and attenuated to nuclear instrument module (NIM) format using a SIA 300 inverter module before being fed into a TimeHarp 100 TCSPC module (both from PicoQuant). Light intensity at the detector was varied using an adjustable iris in front of the detector and by the use of ND filters. Excitation light was prevented from reaching the detector by placing an additional interference filter between the emission filter and iris.



(A)Removable laser diode head, (B) Short pass filter, (C) focusing Optics

Figure 1: Fluorescence Lifetime apparatus[13]

The Instrument Response Function (IRF) was obtained from a non-fluorescing suspension of alumina in water held in a 1 cm path length quartz cell and was assumed to be wavelength independent. Decay data were acquired until between $5x10^3$ and $3x10^4$ counts had been registered in the channel of maximum intensity. For all cell suspensions, decay data were acquired until a maximum count in one channel reached $1x10^4$ counts. The determining factor as to the count maximum was the count rate, which was always <1% of the pulse rate. Count rates between $8x10^2$ and $6x10^4$ counts per second were obtained for the photosensitisers dissolved in neat solvent and between $4x10^2$ and $4x10^4$ counts per second while in cell suspensions. For very weakly fluorescing samples, (<$1.5x10^2$ counts per second) decay data was not recorded. Lifetimes were obtained by deconvolution of the decay curves using the FluorFit Software program (PicoQuant GmbH, Germany). All lifetimes were fit to a χ^2 value of less than 1.2 and with a residuals trace that was fully symmetrical about the zero axis.[14]

All lifetime measurements for cell suspensions were recorded within 3 hours after suspending the cells in the dye solutions. A fluorescence spectrum was recorded for cells suspended in media alone to reassure that the cells or media did not fluoresce without dye. Measurements for a concentration of $1x10^{-4}$ M porphycene 1 in acetone were not possible due to immediate observable cell death.

3. RESULTS AND DISCUSSION

3.1 ZnPcTS

ZnPcTS in phosphate buffer displays a maximum absorbance at 635 nm with a minor peak at 334 nm. In 50:50 EtOH/buffer solution, the absorbance maximum shifts to 675 nm, and a minor peak is observed within the range of 340 nm to 347 nm. The spectra for ZnPcTS in phosphate buffer agree with previous studies when water is used as a solvent.[17] Spectra for ZnPcTS in 50:50 EtOH/buffer solution are similar to earlier studies using DMSO as a solvent.[17] A change in absorption spectra when going from a non-polar solvent to a more polar solvent is observed (See Figure 2). While in the phosphate buffer, the absorption peak in the red is broadened, decreased, and blue shifted which is evidence for the formation of H-aggregates.[18] Aggregation is likely to be promoted by the more polar solvent.

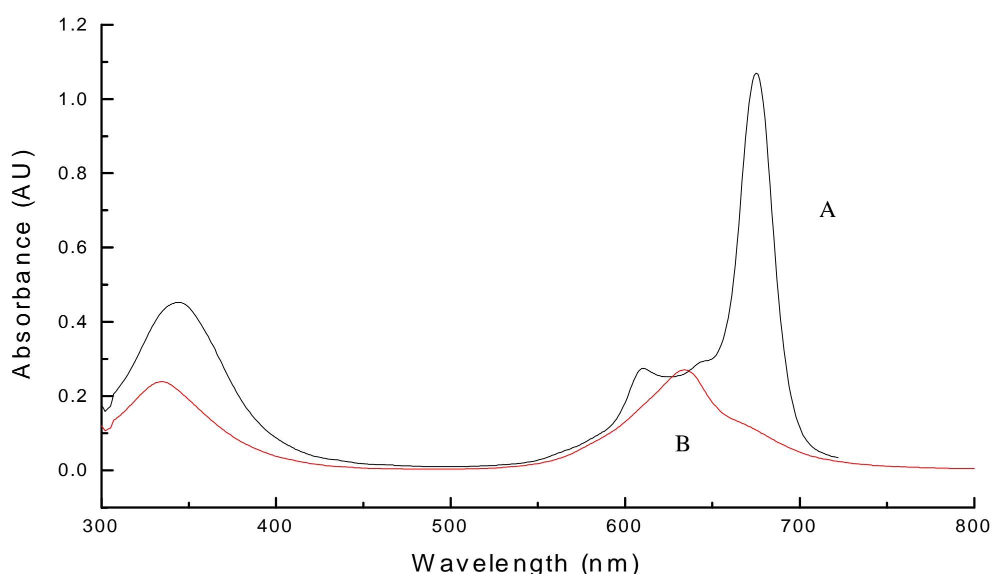


Figure 2: Absorption spectra of ZnPcTS in various solvents at a concentration of $1x10^{-5}$ M; A) Absorption peak in 50:50 EtOH/buffer solution (pH=7.6), B) Absorption peak in phosphate buffer (pH=7.2)

A change in the fluorescence spectra of the photosensitisers is also observed when increasing the polarity of the solvent. For both solvents the fluorescence peaks are observed at 685 nm, but are of much lower intensities in the more polar solvent. Also, when in the 50:50 EtOH/buffer solution a peak intensity is reached at a concentration of $5x10^{-6}$ M with the peak decreasing at higher concentrations. This is believed to be due to re-absorption rather than aggregation because there is no evidence of dimer formation from the absorption spectra.

The fluorescence lifetime measurements of ZnPcTS in neat buffer and in cell suspensions display two species at all concentrations and emissions (See Table 3). For most measurements there is no difference between the decay rates whether the ZnPcTS is in buffer alone or in cell suspensions, but there is a difference observed at a concentration of $1x10^{-5}$ M. The longer-lived species exhibits a shortened lifetime and a doubling of amplitude when placed in cell suspension, which could possibly be explained by collisional quenching. This effect is not observed for the shorter-lived species or for any other decays at the other concentrations. The presence of bi-exponential decays corresponds with previous evidence for the presence of dimers and/or higher aggregates.

	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)
$\lambda_{Emission}$ (nm)	700	700	750	750
$1x10^{-5}$ M	4.7 (0.265), 2.6 (1.085), τ_{Avg}=3.3	4.1 (0.585), 2.4 (0.999), τ_{Avg}=3.2	4.7 (0.327), 2.6 (1.108), τ_{Avg}=3.3	4.0 (0.715), 2.3 (0.940), τ_{Avg}=3.3
$5x10^{-5}$ M	4.5 (0.510), 2.4 (0.802), τ_{Avg}=3.5	4.3 (0.556), 2.4 (1.122), τ_{Avg}=3.3	4.4 (0.590), 2.3 (0.778), τ_{Avg}=3.8	4.6 (0.480), 2.5 (1.135), τ_{Avg}=3.5
$1x10^{-4}$ M	4.6 (0.517), 2.4 (0.946), τ_{Avg}=3.5	4.4 (0.497), 2.4 (1.054), τ_{Avg}=3.4	4.5 (0.618), 2.3 (0.914), τ_{Avg}=3.6	4.6 (0.531), 2.5 (0.980), τ_{Avg}=3.5

Table 3: Fluorescence lifetimes (amplitudes given in parentheses) of ZnPcTS in neat phosphate buffer (pH=7.2) and in cell suspensions at varying concentration. No measurements were made at an emission wavelength of 650 nm.

Fluorescence lifetime measurements of ZnPcTS in 50:50 EtOH/buffer mixture or in cell suspensions indicate the presence of only one species for all concentrations and emissions (See Table 4). There is no difference between the decay rates whether the ZnPcTS is in 50:50 EtOH/buffer alone or in cell suspensions. The presence of mono-exponential decays corresponds with previous evidence for the absence of dimers and/or higher aggregates.

	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)
$\lambda_{Emission}$ (nm)	650	650	700	700	750	750
$1x10^{-5}$ M	3.4	3.5	3.4	3.5	3.5	3.6
$5x10^{-5}$ M	3.7	4.0	3.7	3.9	3.8	4.0
$1x10^{-4}$ M	3.9	4.2	4.0	4.0	3.9	4.0

Table 4: Fluorescence lifetimes of ZnPcTS in 50:50 EtOH/buffer solution (pH=7.6) and in cell suspensions at varying concentrations. All decays were mono-exponential.

3.2 PcTS

The absorption spectrum of PcTS in phosphate buffer shows two peaks of about equal intensity at 323 nm and 598 nm which does not agree with previous studies where the longer wavelength absorption is reported at 630 nm.[17] A higher amount of aggregation in the present study could be the cause of the discrepancy. A change in absorption spectra when going from a non-polar solvent to a more polar solvent is observed (See Figure 3). Two narrow peaks can be observed at 660 nm and 695 nm while in 50:50 EtOH/buffer solution. Previous studies that use DMSO as a solvent indicate two more pronounced peaks at these wavelengths.[17] The inconsistency with the earlier studies is probably due to the dye still being aggregated in the 50:50 EtOH/buffer solution. While in the phosphate buffer, the absorption peak in the red is broadened, decreased, and blue shifted which is evidence for the formation of H-aggregates.[18] It is believed that the more polar solvent is promoting aggregation.

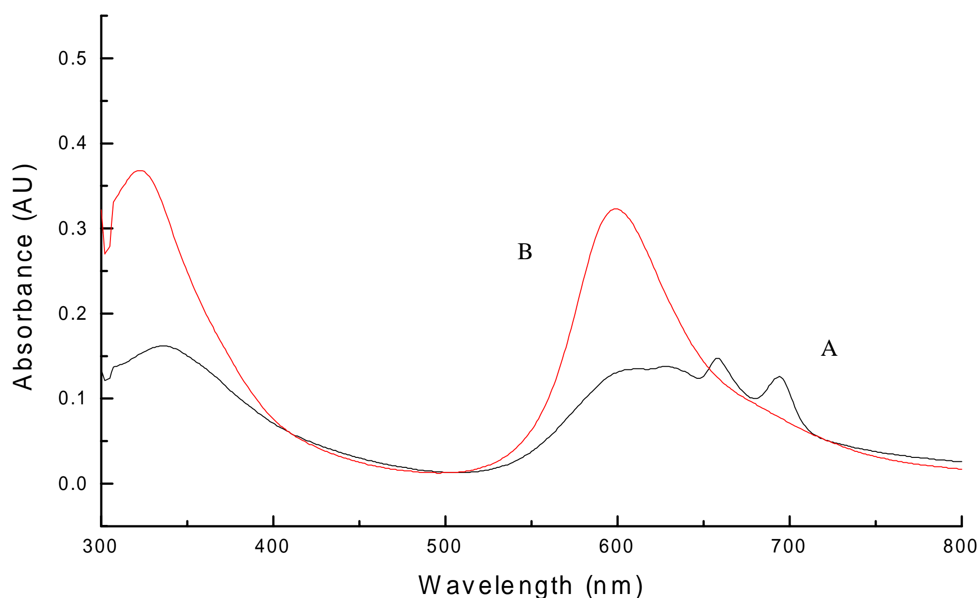


Figure 3: Absorption spectra of PcTS in various solvents at a concentration of $1x10^{-5}$ M; A) Absorption peak using 50:50 EtOH/buffer solution (pH=7.6), B) Absorption peak in phosphate buffer (pH=7.2)

More evidence of the more polar solvent having an aggregating effect can be seen in the fluorescence spectra. As much as a 1,000 fold increase in the fluorescence peak (695 nm) intensities is observed when the 50:50 EtOH/buffer solution is used instead of the phosphate buffer.

For PcTS and phosphate buffer a bi-exponential decay is apparent in both pure solvent and in cell suspensions, which is evidence for the presence of dimers and/or higher aggregates (See Table 5). Fluorescence lifetimes for PcTS and phosphate buffer are somewhat longer in cell suspensions than in solvent alone, which could be indicating a larger amount of aggregation.

	τ_{buffer} **(ns)**	$\tau_{cell\ suspension}$ **(ns)**	τ_{buffer} **(ns)**	$\tau_{cell\ suspension}$ **(ns)**
$\lambda_{Emission}$ **(nm)**	700	700	750	750
$1x10^{-5}$ M	4.8 (0.621), 1.3 (0.666), τ_{Avg}=4.0	5.5 (0.979), 2.0 (0.481), τ_{Avg}=5.0	4.8 (0.617), 1.3 (0.235), τ_{Avg}=4.5	5.5 (1.004), 2.0 (0.496), τ_{Avg}=5.0
$5x10^{-5}$ M	4.7 (0.793), 1.3 (0.371), τ_{Avg}=4.3	5.7 (0.979), 2.6 (0.436), τ_{Avg}=5.2	4.9 (0.918), 1.6 (0.257), τ_{Avg}=4.6	5.5 (1.120), 1.8 (0.398), τ_{Avg}=5.1
$1x10^{-4}$ M	4.8 (0.850), 1.7 (0.254), τ_{Avg}=4.5	5.4 (0.978), 2.1 (0.373), τ_{Avg}=4.9	4.9 (0.739), 2.4 (0.117), τ_{Avg}=4.7	5.4 (0.998), 2.1 (0.344), τ_{Avg}=5.0

Table 5: Fluorescence lifetimes (amplitudes given in parentheses) of PcTS in neat phosphate buffer (pH=7.2) and in cell suspensions at varying concentrations.

Fluorescence lifetime measurements for PcTS in 50:50 EtOH/buffer solution alone and cell suspensions are about the same at 700 nm and 750 nm (See Table 6). The lifetimes remain the same with varying concentration and emission filter. When the 650 nm filter is used, two lifetimes are observed for the dye in cell suspensions, but not for the solvent alone, which could be due to the dye aggregating more within the cell. The aggregate that is formed could have an emission wavelength of 650 nm or less, which is why the lifetime may not be detected using the 700 nm or 750 nm filter for the other cell suspensions.

	τ buffer (ns)	τ cell suspension (ns)	τ buffer (ns)	τ cell suspension (ns)	τ buffer (ns)	τ cell suspension (ns)
λ Emission (nm)	650	650	700	700	750	750
1x10-5 M	4.8	4.9	4.6	4.6	4.6	4.6
5x10-5 M	4.9	5.8 (0.715), 4.0 (0.724), τ_{Avg}=5.1	4.7	4.6	4.7	4.6
1x10-4 M	5.0	5.8 (0.596), 3.8 (0.483), τ_{Avg}=5.1	4.6	4.6	4.7	4.7

Table 6: Fluorescence lifetimes (amplitudes given in parentheses) of PcTS in 50:50 EtOH/buffer solution (pH=7.6) and in cell suspensions at varying concentrations

3.3 AlPcTS

AlPcTS with phosphate buffer shows a maximum absorbance around 675 nm with two minor peaks around 349 nm and 608 nm, which is in agreement with previous studies.[15,16] The absorption spectra of AlPcTS in phosphate buffer do not show any signs of aggregation because the peaks remain at the same wavelengths over a range of concentrations. The fluorescence spectra show a peak intensity at 680 nm being obtained at a concentration of $1x10^{-6}$ M and decreasing at more concentrated solutions. This is believed to be due to re-absorption and not aggregation due to there being no evidence of dimer formation from the absorption spectra. Fluorescence lifetime measurements for AlPcTS in phosphate buffer alone and in cell suspensions are about equal except for a slight variation in the more concentrated solutions (See Table 7). The lifetimes for the dyes do not vary with the differing emission filters, but seem to increase with increasing concentration. This increase is probably not due to aggregation since there is only one species present at all concentrations for both the dye in solvent alone and in cell suspensions and is the subject of further investigation.

	τ buffer (ns)	τ cell suspension (ns)	τ buffer (ns)	τ cell suspension (ns)	τ buffer (ns)	τ cell suspension (ns)
λ Emission (nm)	650 nm	650 nm	700 nm	700 nm	750 nm	750 nm
1x10-5 M	6.0	6.3	6.0	6.1	6.0	6.2
5x10-5 M	7.1	7.4	7.1	6.8	7.1	6.8
1x10-4 M	7.9	8.3	7.9	7.5	7.9	7.5

Table 7: Fluorescence lifetimes of AlPcTS in neat phosphate buffer, pH=7.2 and in cell suspensions at varying concentrations

3.4 Porphycene 1

Porphycenes are characterised by an envelope of three absorption bands in the red region,[18] which is found here. Porphycene 1 with acetone displays a major peak at 368 nm with three minor peaks (in order of decreasing intensity) at about 632 nm, 560 nm, and 599 nm. (Acetone had to be used as a solvent because porphyence 1 is highly hydrophobic). Spectra collected for Porphycene 1 and acetone are in agreement with earlier studies when CH_2Cl_2 is used as a solvent.[19] There does not appear to be any signs of aggregation, which is evident because of the absorption peaks remaining at the same wavelengths over a range of concentrations.

The fluorescence spectra of the porphycenes are difficult to interpret because the small stokes shift (~ 20 nm). With an excitation wavelength of 633 nm and an emission peak of 641 nm expected,[20] the fluorescence peak is probably masked by the excitation light.

While in cell suspensions, two decays appear for porphycene 1, which is probably due to the formation of dimers/and or higher aggregates within the cells (See Table 8). The shorter-lived species increases with increasing concentration and decreasing polarity, which most likely causes a larger amount of aggregation.

	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)	τ_{buffer} (ns)	$\tau_{cell\ suspension}$ (ns)
$\lambda_{Emission}$ (nm)	650 nm	650 nm	700 nm	700 nm	750 nm	750 nm
1x10-5 M 1:10 acetone/buffer	9.6	9.6 (0.076), 2.3 (0.197), τ_{Avg}=6.8	9.5	9.5 (0.057), 2.2 (0.339), τ_{Avg}=5.3	9.5	8.4 (0.022), 2.1 (0.539), τ_{Avg}=3.0
5x10-5 M 50:50 acetone/buffer	10.7	9.0 (0.520), 4.8 (0.615), τ_{Avg}=7.8	10.7	9.0 (0.311), 5.3 (0.210), τ_{Avg}=8.0	10.6	8.5 (0.233), 2.9 (0.344), τ_{Avg}=7.1
1x10-4 M 100% acetone	12.1	N/A	12.2	N/A	12.0	N/A

Table 8: Fluorescence lifetimes (amplitudes given in parentheses) of porphycene 1 and acetone and in cell suspensions at varying concentrations

3.5 Porphycene 18

Porphycene 18 also exhibits three absorption bands in the red (561 nm, 631 nm, and 602 nm), but only at higher concentrations when there is a smaller ratio of phosphate buffer in the solution. The higher fraction of buffer in the lower concentration solutions could be causing aggregation that changes the absorption spectra considerably. Formation of the three absorption peaks in the red region can be seen at a concentration of $5x10^{-5}$ M. Spectra of less concentrated solutions (containing a higher percentage of buffer) display peaks that are red shifted compared to those believed to be less aggregated.

A major peak appears for porphycene 18 at 650 nm at a concentration of $5x10^{-5}$ M (See Figure 4), which agrees with previous studies.[20] This peak shifts to the red at higher concentrations, which is evidence that at lower concentrations the fluorescence peak may be blue shifted and masked by the excitation light.

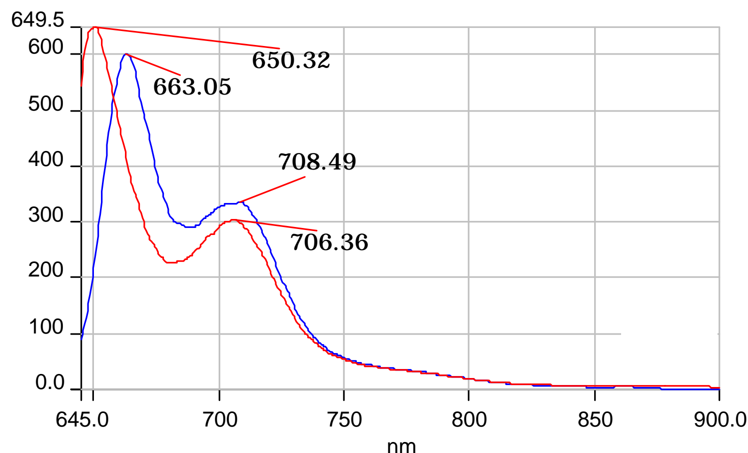


Figure 4: Fluorescence spectra of Porphycene 18 and DMSO and phosphate buffer, pH=7.2 (excitation=645 nm); A) 650.32 nm and 706.36 nm fluorescence peaks at a concentration of $5x10^{-5}$ M, B) 663.05 nm and 708.49 nm fluorescence peaks at a concentration of $1x10^{-4}$ M

For porphycene 18 in cell suspensions a second lifetime is visible at the two lower concentrations, which is probably due to the aggregating effect of being placed within cells and of the more polar solvent used (See Table 9). The shorter-lived species has very minute amplitudes at a concentration of $5x10^{-5}$ M, which is probably to the decrease in polarity of the solvent decreasing the amount of aggregation.

	τ solvent alone (ns)	τ cell suspension (ns)	τ solvent alone (ns)	τ cell suspension (ns)	τ solvent alone (ns)	τ cell suspension (ns)
$\lambda_{Emission}$ (nm)	650 nm	650 nm	700 nm	700 nm	750 nm	750 nm
1x10-5 M 1:10 DMSO/buffer	7.3	6.9 (0.482), 2.3 (0.485), τ_{Avg}=5.7	7.2	6.6 (1.053), 4.0 (0.525), τ_{Avg}=5.6	7.2	6.6 (0.425), 3.4 (0.496), τ_{Avg}=5.4
5x10-5 M 50:50 DMSO/buffer	8.3	8.1 (0.927), 1.7 (0.071), τ_{Avg}=8.0	8.3	8.1 (0.909), 2.8 (0.068), τ_{Avg}=8.0	8.3	8.0 (0.875), 0.95 (0.296), τ_{Avg}=7.7
1x10-4 M 100% DMSO	11.1	10.1	11.3	10.2	10.6	10.2

Table 9: Fluorescence lifetimes (amplitudes given in parentheses) of porphycene 18, DMSO, and phosphate buffer (pH=7.2) and in cell suspensions at varying concentrations

4. CONCLUSIONS

This study provides evidence that aggregate formation is likely to occur once the dye is located within the cell, which can have considerable effects on the photophysical properties of photosensitisers. The changes in the fluorescence decays once placed in a cellular environment must be taken into account if fluorescence lifetime imaging techniques are to be applied to live organisms.

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