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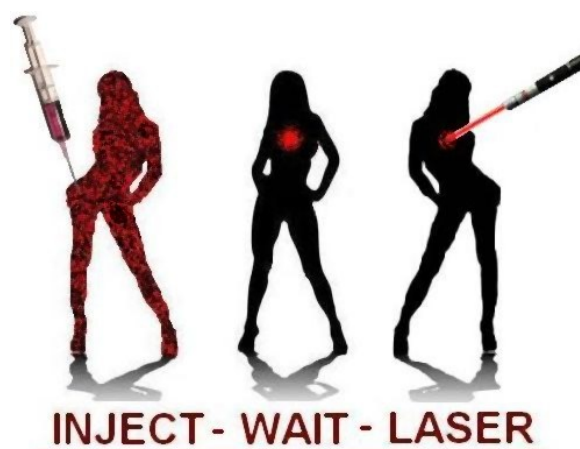
A study of Pyropheophorbide-a based photosensitisers for photodynamic therapy

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and the Diploma of Imperial College London

Abstract

Photodynamic therapy (PDT) is an approved but under-development modality for cancer therapy. Conventional, non-targeted PDT consists of administration of a photosensitiser to the patient, passive accumulation in the tumour, localised photosensitiser activation using visible light and subsequent cellular destruction via reactive oxygen species (ROS) and/or free radicals. A major limitation of PDT is its poor selectivity for tumours and a suboptimal pharmacokinetic / biodistribution profile. This leads to low potency and patient photosensitivity. This is being addressed with the development of antibody targeted PDT; the conjugation of multiple photosensitisers to a tumour-specific antibody. For effective antibody conjugation photosensitisers are preferable if highly water soluble. PPa (Pyropheophorbide-a) is an effective photosensitiser and more soluble versions have been prepared to aid in its conjugation. The work presented in this thesis explores the differences these novel, soluble photosensitisers exhibit in terms of toxicity, cell death mechanisms and uptake in an *in vitro* model of traditional PDT and also in terms of improvements in their bio-conjugation. Data presented shows chemical manipulation of PPa significantly affects the biochemical properties important for effective PDT. PEG-modified PPa (PS-1, with enhanced solubility over PPa) as an un-conjugated drug exhibits similar overall toxicity to PPa, along with similar hallmarks for caspase-independent apoptosis, however this equivalent toxicity is achieved despite a significantly reduced cellular uptake. Its toxicity therefore is higher per internalised molecule than its parent compound. This is hypothesised to be due to enhanced retention of photo-physical properties in a cellular environment. Cationic PPa (PS4, with even greater enhanced solubility over PPa) exhibits a loss in cytotoxicity in comparable levels of free photosensitiser studied; this is hypothesised to be due to decreased uptake in addition to an altered cellular localisation. Derivatives of PS1 activated for conjugation showed greatly enhanced reaction with antibody compared to PPa showing its promise for improved targeted PDT, PS4 proved too difficult to chemically activate in the time frame of this project.



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In the Microscope

Here too are dreaming landscapes,
lunar, derelict.

Here too are the masses,
tillers of the soil.

And cells, fighters
who lay down their lives
for a song.

Here too are cemeteries,
fame and snow.
And I hear murmuring,
the revolt of immense estates.

Miroslav Holub (1923 – 1998) Czech poet and Immunologist.

Translation by Ian Milner

Declaration of Originality

I hereby declare that the work described in this thesis is the result of my own independent investigation and where others have made a contribution it is clearly acknowledged. This work has not been previously submitted for any degree and is not being concurrently submitted in candidature for any other degree.

Hayley Pye (October 2012)

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Abbreviations

$^1\text{O}_2$	Singlet Oxygen
$^3\text{O}_2$	Molecular Oxygen
A490	absorbance at 490nm
Ab	Antibody
Abs	Absorbance
ADC	Antibody Drug Conjugate
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADP	Antibody directed phototherapy
AE	Active ester
AIPcS ₄	Aluminum phthalocyanine tetrasulfonate.
ALA	5-aminolevulinic acid
AMD	Age-related macular degeneration
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
bp	Base pairs (measure of DNA size)
BPD-MA	Benzoporphyrin derivative monoacid
BSA	Bovine serum albumin
CD20	B-lymphocyte antigen CD20
CDK	Cyclin-dependent kinases
CDR	Complementarity-determining region
CDC	Complement-dependent cytotoxicity
Ce6-PVP	Chlorin e6-polyvinylpyrrolidone
CEA	Carcinoembryonic antigen
CMC	Critical micelle concentration
DAMP	Damage-Associated Molecular Pattern
DCM	Dichloromethane]
DFF	DNA fragmentation factor
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DT	DMSO/ Triton-X114 lysis solution
EGFR	Epidermal growth factor receptor
EndoG	Endonuclease G
ER	Endoplasmic reticulum
EtBr	Ethidium Bromide
EtOH	Ethanol
FC	Flow Cytometry
FcRn	Neonatal Fc receptor
FCS	Foetal Calf Serum
Fc γ R	Fc-gamma receptors
FITC	Fluorescein isothiocyanate
FL1/FL2/FL3/FL4	Fluorescent Filter/Detection channel for the FACSCalibur flow cytometer.
g/L	Grams per Litre
G ₀	Quiescent state (Cell cycle phase)
G ₁	Gap1 Phase (Cell cycle phase)
G ₂	Gap2 Phase (Cell cycle phase)
GFP	Green fluorescent protein
H ₂ O	Water

H ₂ O ₂	Hydrogen peroxide
Hb(H ₂ O)	Deoxygenated hemoglobin
Hb(O ₂)	Oxyhemoglobin
HCl	Hydrochloric acid
HeNe	helium–neon
HER2	Human Epidermal Growth Factor Receptor 2
HPD	Hematoporphyrin derivative
HPLC	High-performance liquid chromatography
HPMA	N-(2-Hydroxypropyl)methacrylamide
HPPH	2-(1-hexyloxyethyl)-20devinyl pyropheophorbide-a
Hrs	Hours (unit of time)
HSA	Human serum albumin
IC ₁₀	Concentration required for 10% inhibition (10% cell kill)
IC ₅₀	Concentration required for 50% inhibition (50% cell kill)
IC ₉₀	Concentration required for 90% inhibition (90% cell kill)
IgG	Immunoglobulin G
ITC	Isothermal titration calorimetry
IUPAC	International Union of Pure and Applied Chemistry/
J	Joules (unit of energy)
JNK	c-Jun N-terminal kinase
kb	Kilobase (measure of DNA size)
kDa / Da	Kilodalton / Dalton (Dalton = atomic mass unit)
L (μl / ml / L)	microliters / millilitres / litres (a unit of volume)
Laser	Light amplification by stimulated emission of radiation
LD ₅₀	Lethal dose required for 50% cell kill
LDL	Low density lipoprotein
Log D	Log of the distribution (D) coefficient.
Log P	Log of the partition (P) coefficient.
LUMO	lowest unoccupied molecular orbital
M (pM / nM / μM / mM / M)	picomolar / nanomolar / micromolar / millimolar / molar (a unit of concentration)
M phase	Mitosis (Cell cycle phase)
M ₀	Stress-induced senescence (Mortality phase)
M ₁	Cellular aging or telomere-initiated senescence (Mortality phase)
M ₂	Mitotic catastrophe (Mortality phase)
MACE	Mono-(L)-aspartylchlorin-e6
MAPK	Mitogen-activated protein kinases
MeCN	Acetonitrile
MOMP	Mitochondrial outer membrane permeabilisation
mTHPC	M-tetrahydroxyphenylchlorin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Mv	Millivolts
Mw	Molecular weight
MWCO	Molecular weight cut-off
NaCl	Sodium Chloride
NADH	Reduced nicotinamide adenine dinucleotide
NaOH	Sodium Chloride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	N-hydroxysuccinimide
NIR	Near infrared (certain wavelengths of light)
nm	nanometers (unit of length) also used for wavelength of electromagnetic radiation.

°C	Degree celsius (unit of temperature)
OG	n-Octyl-β-D-Glucopyranoside
OH	Hydroxyl
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCI	Photochemical internalization
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PES	Polyethersulfone
PGA	Polyglutamic acid
pH	$-\log_{10} a(\text{H}^+)$ (the negative logarithm of the activity of hydrogen ions in solution)
pI	Isoelectric point
PI	propidium iodide
PICs	Photoimmunoconjugates
pKa.	$-\log_{10} K_a$ (the negative logarithm of the equilibrium constant for acid dissociation)
PPa	Pyropheophorbide-a
PPa-ether	Pyropheophorbide a-ether
PPME	Pyropheophorbide a methyl ester
PS	Photosensitiser
PTFE	Polytetrafluoroethylene
PVA	Polyvinyl acetate
PVDF	Polyvinylidene fluoride,
QSAR	Quantitative structure-activity relationship
R ₂ NH	Secondary amine
RFU	relative fluorescence values
RNH ₂	Primary amine
ROS	Reactive oxygen species
S phase	DNA Synthesis (Cell cycle phase)
S ₀	Ground singlet state, (atomic/electronic energy level)
S ₁	Excited singlet state, (atomic/electronic energy level)
scFv	Single-chain variable fragment (antibody fragment)
SDS	Sodium dodecyl sulfate
SDT	Sonodynamic therapy
SH	Sulfhydryl
SIP	Small Immunoprotein (antibody fragment)
SnEt ₂	Tin ethyl etiopurpurin
Stauro	Staurosporine
T ₁	Excited triplet state. (atomic/electronic energy level)
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor alpha
UV	Ultraviolet (certain wavelengths of light)
UV-VIS	Ultraviolet-Visible (certain wavelengths of light)
VDss	Steady state volume of distribution
VEGF	Vascular endothelial growth factor
v/J	Vibrational/rotational levels (atomic/electronic energy levels)

Chapter One

1.1 Introduction

1.2 Photodynamic therapy for cancer

1.2.1 Cancer and its clinical treatment.

The standard clinical treatment of cancer involves a combination of both cancer specific and non specific treatments. These include chemotherapy, radiotherapy, hormone therapy and immunotherapy (King and Robins, 2006) Not all cancer patients respond well to treatment and even those with non-metastatic cancers will show signs of relapse; during a study of over 2000 females being treated for various stages of non-metastatic, invasive breast cancer, 18% of these patients had local, regional or distant relapse, 79% of these occurring within the first 5 years after primary treatment (Elder et al., 2006).

The resistance of cancer cells to current treatment is thought to be both an endogenous and acquired characteristic and in some cases treatment induced. There is an inherent genetic and morphological instability of cancer cells, allowing adaptation and survival at a rate beyond that of a normal cell. Research has discovered multiple elements with the potential to contribute to this resistance of cancer, including defects in apoptosis pathways, superior detoxification mechanisms, acquired desensitisation, cell cycle status, hypoxia and poor vascularisation of the tissue mass (Agarwal and Kaye, 2003; Gottesman et al., 2002; Igney and Krammer, 2002; King and Robins, 2006; Trédan et al., 2007). In addition by the time of treatment variable progression of the tumour has led to a heterogeneous cell mass, this is reflected in a heterogeneous response of the cancer mass to treatment. The discovery of specific cells in solid tumours that exhibit self-renewal capacity has created the ‘cancer stem cell hypothesis’, this discovery has implications for both the origin of cancer and its resistance to therapy (Dean et al., 2005; Zhou and Zhang, 2008). All the above elements confer differing resistances to the various therapies and this has caused an increased interest in new and/or combinational approaches to treating cancer. This project will be exploring a promising new treatment for cancer; targeted photodynamic therapy.

1.2.2 Clinical photodynamic therapy.

Conventional, non-targeted photodynamic therapy (PDT) involves intravenous or topical administration of a photosensitiser (PS) to the patient, followed by its activation using visible light. The photosensitiser is transformed from its singlet state into its triplet state which can react both with cellular components directly, or with molecular oxygen to produce various radicals and reactive oxygen species which mediate cellular destruction (Palumbo, 2007; Robertson et al., 2009; Wilson and Patterson, 2008).

The first photodynamic therapy of cancer was carried out with a standard white light lamp around 100 years ago, since then, great advances have occurred and PDT is now an approved clinical modality for various types of cancer, (Agostinis et al., 2011; O'Connor et al., 2009; Triesscheijn et al., 2006). Table 1 lists selected approved photosensitisers for oncology and their indications. Now championed by some to be preferable to classical treatments options, it has been shown to be cost-effective, have superior cosmetic outcome along with equivalent or improved results, and provides the chance of a cure for some patients who would have limited treatment options, for those unsuitable for the classical treatments options or those who have previously failed with radiotherapy, surgery or systemic chemotherapy. (Hopper et al., 2004; Nyst et al., 2009).

In addition to anti-cancer applications PDT has the potential to be a platform technology over a wide spectrum of applications (Celli et al., 2010; Choudhary et al., 2009; Dai et al., 2009; Maisch, 2007; Tandon et al., 2008; van der Snoek et al., 2008; Wilson and Patterson, 2008; Wormald et al., 2007):

- Current clinical indications for PDT that have been approved or are in active clinical trials include; solid tumours, dysplasias, warts, rheumatoid arthritis, age-related macular degeneration, actinic keratosis, cosmetic application, psoriasis, endometrial ablation, localised infection, coronary atherosclerosis and prophylaxis of arterial restenosis.
- Current non-clinical indications include; diagnostic and prognostic imaging or as a sterilisation / antimicrobial agent for synthetic materials or donated human samples.

Clinical progression of PDT has in part been driven by the development of multiple branches of so-called 'second generation' photosensitisers; researchers have produced a vast toolbox of new synthetic and/or chemically modified photosensitisers with a range of specified photophysical and pharmacokinetic properties. This extension in photosensitiser repertoire expanded alongside a growth in the understanding of the mechanisms behind the structure-activity relationship of photosensitisers in all areas from photophysics to clinical application. Clinical progression is reviewed in (Agostinis et al., 2011; Huang, 2005; Mang, 2004; Mitton and Ackroyd, 2008).

1.2.3 Benefits and limitations of traditional PDT.

PDT lacks a specific cellular target and has multiple mechanisms of biological effect, this helps against the inherent variability of tumours and means treatments can be repeated without inducing significant resistance in the tumour, although a range of cell sensitivities to PDT has been shown (Casas et al., 2011). Additionally, it has the dual specificity of preferential tumour localisation and laser directionality, and it has influence on the immune system that could be very important as discussed below in 1.2.4. Other advantages of PDT include: It can be minimally invasive as the drug is given intravenously or topically and illumination is carried out at the surface or via endoscope or hollow needle. PS have low systemic toxicity when inactive, and treatment is low scarring as damage only occurs in target tissue- sparing tissue structures like collagen. Treatment time is low and has a rapid effect with the potential in some cases to be limited to a single treatment. The mechanism of action also means it is compatible with many other cancer treatments such as chemotherapy or immunotherapy allowing concurrent use.

PDT also has some limitations, some fundamental but others only limited by current technology and knowledge; they are outlined in Table 2. (Allison et al., 2008; Z. Huang et al., 2008; Walker and Andrew Maiden, 2009; Whitehouse and Rapley, 2012; Wilson and Patterson, 2008)

Table 1 : Selected approved PDT photosensitisers in oncology
Re-produced from (Agostinis et al., 2011).

Photosensitiser	Structure	Wavelength	Approved	Trials	Cancer Type
Porfimer sodium (Photofrin) (HPD)	Porphyrin	630	Worldwide		Lung, Esophagus, Bile duct, Bladder, Brain, Ovarian
ALA	Porphyrin precursor	635	Worldwide		Skin, Bladder, Brain, Esophagus
ALA esters	Porphyrin precursor	635	Europe		Skin, Bladder
Temoporfin (Foscan) (mTHPC)	Chlorin	652	Europe	United States	Head and Neck, Lung, Brain, Skin, Bile Duct
Verteporfin	Chlorin	690	Worldwide (AMD)	United Kingdom	Ophthalmic, Pancreatic, Skin
HPPH	Chlorin	665		United States	Head and Neck, Esophagus, Lung
SnEt2 (Purlytin)	Chlorin	660		United States	Skin, Breast
Talaporfin (LS11, MACE, NPe6)	Chlorin	660		United States	Liver, Colon, Brain
Ce6-PVP (Fotolon), Ce6 Derivatives (Radachlorin, Photodithazine)	Chlorin	660		Belarus, Russia	Nasopharyngeal, Sarcoma, Brain
Silicon Phthalocyanine (Pc4)	Phthalocyanine	675		United States	Cutaneous T-Cell Lymphoma
Padoporfin (TOOKAD)	Bacteriochlorin	762		United States	Prostate
Motexafin lutetium (Lutex)	Texaphyrin	732		United States	Breast

Abbreviations: ALA (5-aminolevulinic acid), AMD (age-related macular degeneration), Ce6-PVP (chlorin e6-polyvinylpyrrolidone), HPD (hematoporphyrin derivative), HPPH (2-(1-hexyloxyethyl)-20devinyl pyropheophorbide-a), MACE (mono-(L)-aspartylchlorin-e6), mTHPC (m-tetrahydroxyphenylchlorin), SnEt2 (tin ethyl etiopurpurin)

Table 2: Selected shortcomings in PDT.

Disadvantage	Reason
Poor selectivity / Skin photosensitivity	Although many PS exhibit significant tumour localisation, PDT drugs do not have high selectivity, in part due to their high hydrophobicity. Many PS-drugs remain in the body for weeks and accumulate in the skin; patients have to stay out of direct sunlight to prevent skin damage/inflammation. This is being addressed by the development of specifically targeted PS, like in antibody directed phototherapy (ADP).
Lack of approved drugs	Clinical development of new drugs is hindered by poor understanding and/or an unwillingness of pharmaceutical companies to explore the area. Complicated dosimetry and a requirement for equipped PDT centres has led to a lack of robust, randomised clinical trials.
Limited light penetration into tissues	Light propagation through tissues is heavily limited by refraction, reflection and scattering processes and the absorption patterns of tissue chromophores. This is being addressed by the continual improvement of laser and light delivery technology including interstitial PDT under image guidance, light diffusing fibres and use of near infrared (NIR) or ultrasound absorbing drugs.
Not being as immediately applicable to systemic diseases	PDT is not as immediately applicable to systemic/disseminated disease This is being addressed by the development of the specific targeting of PS, like in ADP, and the development and further understanding of PDT-induced immune responses in particular antitumor-specific immunity
A degree of oxygen dependence	Many of the effective mechanisms of PDT depend on the availability of molecular oxygen in the target tissue during light irradiation. This is being addressed as some PS can preferentially initiate oxygen independent pathways, and light-dose fractionation can allow time for tissue re-oxygenation
Difficult patient dosimetry	Due to the range of different PS available, its multiple applications and multiple factors involved in its administration, there are difficulties in prescribing the correct patient dosimetry for each situation. Over-illumination of some drugs can also lead to drug inactivation (photobleaching).

1.2.4 PDT and manipulation of the immune response

The ability of a patient's own immune system to recognise and manage cancer is backed up by many reported cases of spontaneous regression, often seen after an infection. This is especially important when you consider most of the commonly used cancer therapies; high dose chemotherapy, major surgery and high dose radiation are all immunosuppressive. The ability of PDT to manipulate both the innate and adaptive immune systems in an immune stimulatory way is of particular interest because if understood, it will challenge the idea that PDT cannot be used to treat disseminated disease. The potential for PDT-activated mechanisms to recognise remaining or disseminated cancer in the body after specific eradication of the main solid tumour mass is very appealing. Even more so when it is also suggested that PDT could provide future protection from relapse with the same cancer. The work is briefly discussed below and reviewed in (Ana P Castano et al., 2006; Firczuk et al., 2011; Korbelik, 2011; Mroz and Hamblin, 2011; Nowis et al., 2005; van Duijnhoven et al., 2003).

In vivo experiments in mice have shown not only that an intact immune system can improve the efficacy of PDT but when cured of their cancer via PDT mice can develop a resistance to any re-challenge with the same tumour, mice with the same intact immune system but cured of cancer by surgery did not survive the same re challenge (Canti et al., 1994). In addition, studies in mice have also shown that the immune response produced by PDT treatment of a tumour transplanted on one leg of a mouse (with the additional post PDT intra-tumour injection of autologous dendritic cells) can also cause regression in a distant and completely untreated tumour (Saji et al., 2006). Excitingly (Kabingu et al., 2009) presents clinical work carried out in human patients with basal cell carcinoma in which lesions were either treated with PDT or surgically removed, they showed that in humans too, local tumour PDT can induce a systemic immune response to the tumour in the patient. The balance between apoptosis and necrosis is thought to be important in the type and extent of immune activation after PDT, and can be manipulated by many factors including the clinical dosimetry method as well as the type and delivery method of the PS. Pyropheophorbide-a (PPa), the photosensitiser under study in this thesis, and its related molecules show this capability to enhance immune response. (Li et al., 2006).

1.2.5 PDT in combination with other therapies

In addition to the combination of PDT with the classical cancer treatments including chemotherapy (Khdaïr et al., 2009) and hormone therapy (Bhuvaneswari et al., 2007), there are a range of combination therapies based on immune modulation alongside PDT including co-administration of microbial adjuvants, cytokines or moieties to regulate T cell or other leukocyte balance. (Kwitniewski et al., 2008; Qiang et al., 2008; St Denis et al., 2011). Other combination therapies with PDT (Agostinis et al., 2011; Verma et al., 2007) include vascular manipulation (Ferrario et al., 2000) or changes in the tumour microenvironment (Gomer et al., 2006), co-administration of antioxidant inhibitors (Kimani et al., 2012) or conversely antioxidants (Jakus and Farkas, 2005).

1.2.6 ‘State of the art’ in PDT

One of the most successful variations from classical PDT is two photon PDT, this is listed below alongside other variations of note:

- Two photon PDT uses sub-picosecond pulsed lasers for simultaneous delivery of two photons in quick succession to a fluorophore, the package of energy delivered is additive, providing a way of accessing an excited state by using photons of half the energy / twice the wavelength of the corresponding one-photon transition. Two photon PDT can penetrate deeper into tissues as well as being incredibly spatially specific. Two photon PDT has been used to selectively close a single blood vessel (Collins et al., 2008; Khurana et al., 2007; Pawlicki et al., 2009).
- Sonodynamic therapy (SDT) involves the use of ultrasound to activate sonosensitisers, these sonosensitive molecules are often the same photosensitive molecules as used in PDT (Kuroki et al., 2007; Ma et al., 2009; Mitragotri, 2005; Yamaguchi et al., 2011).
- Gold containing photosensitisers or nanoparticles can also produce heat in response to light activation damaging tumour tissues via a different method to PDT (El-Sayed et al., 2006; Huang et al., 2007; X. Huang et al., 2008).

- The development of nanomaterials linked to photosensitisers that work as energy transfer molecules, including the development of systems that use ionising radiation for example X-rays or gamma rays which can penetrate much deeper in order to activate the photosensitiser. (Chen and Zhang, 2006; Juzenas et al., 2008; Liu et al., 2008; Samia et al., 2006).
- Specifically modified PS used as combined magnetic resonance imaging probes and PDT agents (Huang et al., 2011; Vaidya et al., 2008).
- Laser independent-PDT using chemiluminescence strategies to activate PS (Laptev et al., 2006).
- The development of a genetically encoded photosensitiser, named killer red, (Bulina et al., 2005; Vegh et al., 2011). This work was particularly influential on the field of bioconjugation as classical PS conjugation reactions that require harsh conditions and subsequent purification are completely negated if the PS can be encoded and expressed as a fusion protein. Work by (Serebrovskaya et al., 2011, 2009) produced such photo-immunoconjugates with killer red and showed specific photo activity and cell killing *in vitro* although currently this is not as potent as traditional PDT.
- The manipulation of photosensitisers to make them activatable at certain times or in certain environments, for example; after the exposure of a protease site to an enzyme, or a sequence of deoxyribonucleic acid (DNA) to its complementary partner, or an environmental induced conformational change, these effects can be made to initiate or relieve quenching of a PS. (Lovell et al., 2010; Zheng et al., 2007).
- Photosensitisers have also been used as ‘genetic switches’ using light activation (and the subsequent oxidative mediators) to switch off or inactivate selected proteins or pathways in living cells (Mitra et al., 2003; Yogo et al., 2008).
- PS also make excellent moieties to include in delivery vehicles in a technique called photochemical internalization (PCI), a well studied and established technique that uses photosensitisers which are known to preferentially localise in endosomal membranes. Once taken up and upon irradiation of the cell with light, the activated photosensitiser causes rupture of endosomal membranes and delivery of the payload which is normally a drug or a toxin. (Adigbli and Macrobert, 2012; Norum et al., 2009a, 2009b).

1.3 Important physiological factors in the efficiency of PDT

Currently there is a wealth of knowledge in the literature about the effectiveness of specific photosensitisers in various specific situations but there is a lack of any clear unifying model of photosensitiser clinical or *in vivo* efficiency; if such a thing is indeed possible. Progress in clinical effectiveness of PDT would surely benefit from the existence of such models; however it is likely there are two major reasons for the lack of any umbrella models:

- The first being the complex influences of systemic factors and pharmacokinetics on the delivery of the PS to cancer; factors including photosensitiser distribution, aggregation, absorption, specificity and localisation, metabolism, excretion, and the properties of the tumour itself (Caldwell et al., 1995; Castano et al., 2005a; Ferrari, 2010; Iyer et al., 2006; Ochsner, 1997; Smith et al., 2010; Trédan et al., 2007).
- The second being how clinical use of PDT can effect outcome; understandably the method of injection can effect outcome (Bachor et al., 1994), but interestingly also photosensitiser dose, total light dose, fluence rate (laser energy delivered over time), change of the drug–light interval, fractionation of the dose over different time points, and even temporal characteristics of the light (i.e. pulsed vs. continuous irradiation), can all show remarkable effects on overall efficiency (Henderson et al., 2004; Wilson et al., 1997).

The ability to modulate PDT efficiency using dosimetry in the clinic means that, with sufficient knowledge, treatment can be patient optimised, and if models can be developed for the consistent optimised delivery of PDT its therapeutic potential is massive. Some mathematical models are already being designed to help with various aspects of clinical dosimetry. (Grossweiner, 1997; Hu et al., 2005; N. Huang et al., 2008; Kogan, 2005, 2004, 2003).

1.3.1 How clinical use of PDT can effect outcome

Optimal dosimetry methods can be complicated and there are many factors that can change the effective light dose beyond manipulation of laser application, many of these are well discussed in (Wilson et al., 1997) and include:

- Intrinsic tissue sensitivity to oxidative damage.
- Variation in concentration and micro-distribution of photosensitiser in the tumour target and adjacent normal tissues.
- The range of absorption and scattering coefficients of different tissues as well as the spatial distribution of the light in the tissue volume, before and any changes during treatment.
- The variability in initial tissue oxygenation and any changes during treatment.
- Possible self shielding or quenching with highly absorbent PS.
- PS photo-bleaching and loss of efficiency of the PS during treatment.
- The vascular status before and during treatment.

However despite these variables it has been well documented that clinical manipulation of laser application can affect outcome significantly, in particular fluence rate modification and the dose light interval.

1.3.1.1 The drug-light interval

The drug-light interval can be a very important factor in therapy design because of the effect it can have on the location of the PS during irradiation and subsequently the type of tissues that are damaged. (Andrejevic-Blant et al., 1997; Borle et al., 2003; Chen et al., 2001; Dolmans et al., 2002; Ris et al., 1993). A highlighted paper from Chen et al (Chen et al., 2005) studied the PS VisudyneTM in an *in vivo* rat prostate tumour model and showed that 15 minutes after dosing the PS predominantly localised in tumour vasculature but by 3 hours it was predominantly in the tumour parenchyma. They carried out combination light dose experiments and showed the best anti tumour response when achieved when they irradiated at first 3 hours and then 15 minutes when compared to all other regime options. The inverse (15minutes then 3 hours) was shown to be less effective due to increased hypoxia in the tumour tissue by the second irradiation.

1.3.1.2 Fluence rate modification

In some systems, but not all, maintaining the light dose but lowering the fluence (laser energy delivered over time) can produce significant beneficial effects on overall PDT efficiency. The current explanation for this is that extended irradiation at lower laser doses causes significantly less oxygen depletion in the tissues, increasing the power of the laser can also increase the heat in the tissue, but this can occur with prolonged irradiation as well as high power irradiation. Photosensitiser type along with light dose will also affect the oxygen consumption through its photophysical characteristics, i.e. PS with higher extinction coefficients or higher quantum yields for singlet oxygen production will cause more rapid oxygen depletion. (Angell-Petersen et al., 2006; Babilas et al., 2003; Coutier et al., 2002; Ericson et al., 2004; Henderson et al., 2006, 2000; Xue et al., 2011).

Tissue oxygenation is of particular importance as solid tumours are often themselves hypoxic before any treatment (Freitas and Baronzio, 1991). Evidence for reduced PDT efficacy in hypoxic conditions are available (Henderson and Fingar, 1987; Istomin et al., 2006) and expression of HIF (hypoxia inducible factors) was correlated with a low PDT response rate in patients with early-stage oesophageal cancers (Koukourakis et al., 2001). However (Busch et al., 2004) looked at tumour hypoxia and PS uptake in human patients and showed no correlation. Because of this it is important to investigate the switch from Type II to Type I reactions in hypoxic areas of the tumour.

As well as prolonged fluence rate, fractionated fluence rate, a pulsed light delivery and also a lower total PS doses have all been used to also facilitate the recovery of tissue oxygenation. Wang and colleagues (Wang et al., 2007) showed changes such as these, in cases where they may not have any direct effect on therapeutic efficacy, can still be changing the mechanism of action and even the type of tissue damaged. The manipulation of other factors important to the mechanism of tumour cell death are also mirrored in papers that show inflammation or immune suppression can also be manipulated by light dose (Frost et al., 2011; Henderson et al., 2004).

1.3.2 Monitoring treatment before or during PDT

PDT has the specific advantage of utilising an inherently fluorescent drug and this has been used for fluorescence imaging and diagnosis as well as treatment, this is reviewed in Celli et al (Celli et al., 2010). In addition to PS fluorescence, other measurable endpoints from the PDT process have been widely investigated to try and provide a direct way to monitor treatment before or during therapy. (Dysart et al., 2005; Gill et al., 2009; Stefflova et al., 2006; Wilson et al., 1997; Yu et al., 2006; Z. Zhang et al., 2004; Zhu et al., 2005). Some of these endpoints are listed below, however their use has proving complicated, for example; the most popular endpoint, photo-bleaching, has been shown experimentally be both effective by (Dysart et al., 2005; Sheng et al., 2007) and ineffective by (Dysart and Patterson, 2006; Inuma et al., 1999).

- PS fluorescence / photo-bleaching,
- Singlet oxygen production,
- Photoproduct formation (i.e. a chemical modified by the PDT reaction/singlet oxygen),
- Uptake of PS into tissue.
- PS triplet-state concentration and lifetime.
- Tissue parameters such as temperature, optical properties or oxygenation, pH or redox potential.
- Changes in vascular permeability or tumour blood flow.
- The addition of fluorescence 'reporter' molecules for example for apoptotic activation

1.3.3 Tumour micro-environmental pH

Poor perfusion and high metabolic rates in tumour tissues leads to the acidification of the extracellular medium by about 0.5 pH units creating a typical pH of around 6.9, the intracellular pH remains normal and between pH 7-7.2. (Gerweck and Seetharaman, 1996; Zhang et al., 2010). This can significantly change cellular uptake of molecules with a carboxylic acid group or other ionisable group within the right range (Gerweck et al., 2006) (Raghu and Gillies, 2000) changes in pH can effect PS cellular membrane partition and/or self-aggregation (Bonneau et al., 2004a).

1.3.4 Serum proteins can influence drug uptake

Highly dividing cells like tumours have a high demand for cholesterol and there are two ways for them to supply this demand; either by higher uptake of low density lipoprotein (LDL) (major carrier of cholesterol in blood) or by increasing endogenous synthesis via HMG-CoA reductase (rate limiting enzyme). Many tumours have been shown to have increased levels of LDL receptor (Firestone, 1994; Gal et al., 1981; Vitols et al., 1992). Particularly hydrophobic photosensitisers, are often injected in a solubilising transport vehicle, e.g. liposomes, oil in water emulsions or lipid nanoparticles, and the nature of this delivery vehicle can also affect the relative transfer of the drug to other elements within the blood or target tissue. (Kongshaug et al., 1989; Sobolev et al., 2000a).

In terms of PDT, research has shown the extent and mechanism of tumour uptake can be changed by the presence of albumin, LDL or other serum factors in the media. Although most PS show they can interact with both various lipoproteins (including LDL) and albumin, there is a general rule that states; hydrophobic PS tend to associate with LDL and hydrophilic PS tend to associate with serum albumin, for PS that contains an asymmetric charge its serum distribution will favour LDL-binding above what is predicted based on its lipophilicity. Both LDL and albumin can interact with cellular membranes and can be taken up by receptor mediated endocytosis, albumin can also be taken up in liquid phase endocytosis. Changes in the tumour environment can cause release of the PS near the cell surface, this seems to be dependent on PS structure and its response to changing pH. LDL or albumin binding can alter cellular uptake and localisation as well as effect PS photo efficiency and/or PS aggregation. (Alarcón et al., 2010, 2009; Bonneau et al., 2004b; Brandis et al., 2005a; Guelluy et al., 2010; Mojzisova et al., 2007b, 2007c; Rinco et al., 2009; Siboni et al., 2002)

1.3.5 Cancer and its effect on drug bio-distribution.

Reviewed by (Ferrari, 2010) and others there are many transport differences between cancer and normal tissue, a field sometimes called ‘transport oncophysics’, and the drug development field has started to design macromolecules to manipulate this. Cancer tissues exhibit these changes most often alongside the progression of tumour angiogenesis, during which the presence of atypical vessel morphologies or endothelial gaps are thought to make pores with sizes ranging from tens of microns to hundreds of nanometers. Changes do not only effect direct extravasation but the dynamics of the blood flow. Together with unregulated proliferation of the tumour the insufficient co-development of lymphatic drainage can help to generate imbalances in hydrostatic, osmotic and interstitial fluid pressures in cancer. Changes toward tissue invasion and metastasis also drive changes in cell size, shape, and mechanical properties, as well as cell surface and vasculature biomarker changes in protein expression and modifications of the endothelial barrier all likely affecting any potential drug uptake. These factors can also be key players in drug resistance mechanisms. All this has to be manipulated for effective drug (including antibody) delivery. Depending on the properties of the drug in question these changes in tumour micro-environment and transfer processes can help to inhibit or promote non targeted drug uptake. In traditional PDT many of the PS accumulated non-specifically in the tumour in this manner. (Danhier et al., 2010; Iyer et al., 2006; Thurber et al., 2008; Trédan et al., 2007).

1.4 Photochemical factors in PDT

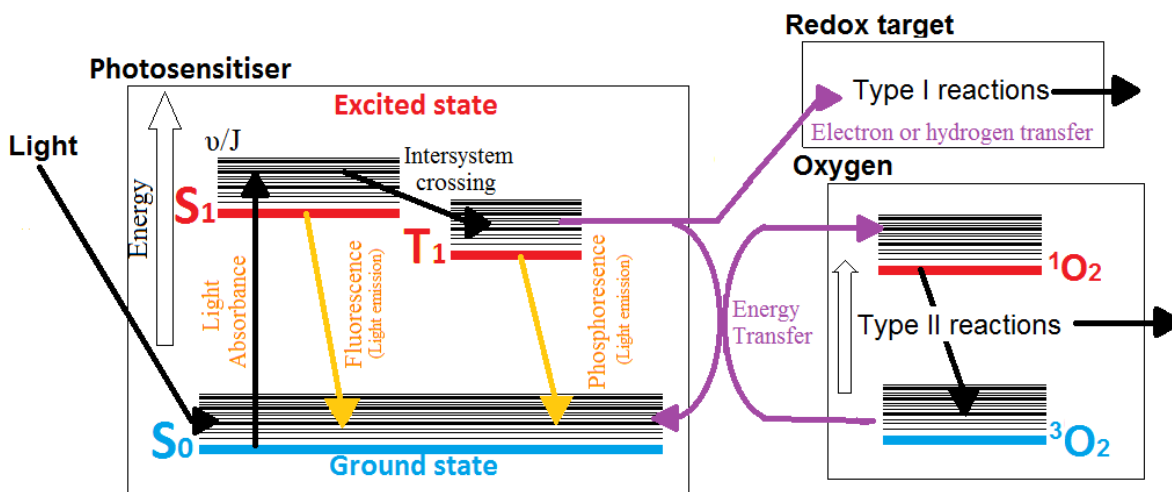
1.4.1 The photophysics behind PDT.

Photosensitisers are chromophore-containing compounds that can harness the energy in a photon of light for energy transfer reactions in biological systems. Photosensitiser photophysics is explained by the principles of molecular orbital theory. In terms of light absorbance, it is the wavelength, and hence the ‘quantum of energy’, that is absorbed by the molecule that is important as this will correlate with the energy difference between available ground and excited state energy levels within the molecule. It is because photosensitisers have extended regions of delocalised electrons that this correlates to areas of the spectrum uncluttered by more common bond energy transitions.

Absorption of a photon produces a short lived excited singlet state (excitation), the excited state reached depends on the energy contained in the light absorbed. (Transition between states corresponds to the movement of one electron from one orbital to another.) The transition back to ground state (relaxation) can then go via numerous paths, a modified Jablonski diagram, shown in Figure 1, can be used to detail some of the important excitation and relaxation paths of a photosensitiser. Absorbed photon energy of the singlet state can be lost through radiationless transition (a transition between two states of a system without photon emission or absorption) including vibrational and rotational motions of a molecule, or re-emitted as a photon (fluorescence). Certain molecules, including PS, can undergo intersystem crossing to a triplet excited state (when an electron changes its spin state). This triplet state has a longer lifetime than the singlet and the energy of the triplet state can also be lost through vibrational and rotational motions of a molecule or re emitted as a photon (phosphorescence). However it is the radiationless transfer of energy from this triplet state to other molecules that is crucial for effective PDT. (Bonnett, 2000; Macdonald and Dougherty, 2001; Plautzer et al., 2009) .

The path of radiationless transitions that occur in PDT can be classed as either Type I or Type II transfer reactions. In PDT both type I and II reactions can be occurring in the same system but may occur to varying extents. The energetic choice of relaxation path for a photosensitiser depends on the chemical characteristics of the photosensitiser itself, the local concentration of specific targets, as well as other environmental factors. Type II energy transfer refers to the transfer of energy, specifically the transfer of energy from the triplet excited state of the photosensitiser to the triplet ground state of molecular oxygen, causing the excitation of oxygen into its singlet state. Singlet oxygen can cause significant damage to key cellular components for example by direct oxidation of proteins and lipids. Type I energy transfers refer to the transfer of electrons, i.e. the reaction of the triplet state photosensitisers upon collision with other molecules. Energy is transferred via electron exchange creating free radicals within both photosensitiser and target molecule; these radical species can include the creation of superoxide as well as other more reactive chemical species. Radical production has a propagative nature in that reaction of radicals with non radical substrates creates an escalating number of highly reactive radicals throughout the cell (Bonnett, 2000; Plautzer et al., 2009).

Figure 1: Photosensitisation processes in PDT illustrated by a modified Jablonski diagram.
Adapted from (Agostinis et al., 2011)



Absorption of a photon of light converts a PS from S_0 = ground singlet state, to S_1 = excited singlet state, it can then undergo intersystem crossing to a T_1 = excited triplet state. From either the S_1 or T_1 energy can be lost as emission of a photon of light (orange arrows) or various paths of radiationless transition (not all shown). The paths of radiationless transition that are important in PDT are shown as purple arrows and are characterised as either Type II reactions; the transfer of energy to the triplet ground state of $^3O_2$ = Molecular Oxygen, causing excitation into its excited singlet state, $^1O_2$ = Singlet Oxygen. Or Type I reactions; the electron or hydrogen transfer of the triplet state photosensitisers upon collision with other molecules. v/J = vibrational/rotational levels, loss of energy by movement between these vibrational/rotational levels are what cause light to be emitted at a lower energy, this is called the Stokes shift, energy transfer arrows are not associated with any discrete v/J level. Singlet Oxygen can also lose energy via phosphorescence (not shown on this diagram).

1.4.2 Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) is a phrase used to describe a number of reactive molecules and free radicals derived from molecular oxygen. Many are free radicals as they can be defined as molecules containing a single unpaired electron in atomic or molecular orbits. ROS can readily interconvert between themselves and all have different redox potentials and reactivity. A species with particular importance to PDT is singlet oxygen ($^1O_2$), see 1.4.1. (Redmond and Kochevar, 2006; Winterbourn, 2008). During PDT oxidative damage occurs to a range of biomolecules due to the relatively non specific type of oxidation reactions carried out by the mixture of ROS that are found in different microenvironments within the cell. (Davies, 2003; Ott et al., 2007; Valko et al., 2006). Work presented by (Sakharov et al., 2003) used these oxidized markers left behind after PDT to look at which proteins are most effected and trace the path of cell death through the cell.

In eukaryotic cells the mitochondria are a major source of endogenous ROS, where they play important roles as subcellular signalling molecules (Esposito et al., 2004). An imbalance toward the pro-oxidative state and accumulation of ROS within the cell is often referred to as 'Oxidative stress'. This is kept in balance by the numerous cellular antioxidant defences in place to remove excess ROS (Kohen and Nyska, 2002; Turrens, 2003; Valko et al., 2006; Winterbourn, 2008). Through PDT one is attempting to overcome this balance with the introduction of excessive singlet oxygen and other ROS. In cancer the levels of basal oxidative stress are already found to be higher in tumours over normal tissue where it has been implicated in the pathology and progression of cancer (Behrend et al., 2003; Valko et al., 2006).

Due to the highly reactive nature of ROS its lifetime within the cell and subsequent diffusion distance is transient and decreases with more reactive ROS. Distances can range from <10nm ($^1\text{O}_2$) to ~1.5 mm (H_2O_2). Singlet oxygen as it is an excited state molecule will always return to the ground state so its reactivity within the cell is given an upper limit by its inherent excited state lifetime. Many attempts have been made to model its intracellular maximum lifetime and spherical diffusion range but it has proved difficult as it can encompass a range of environments within the cell and the different compartmentalisation of any scavengers. Estimates have been made ranging from <10 nm to 125 nm, (125nm is the upper limit assumed if in aqueous solution and without reaction with any other bio-molecules). For reference a typical whole cell diameter is 10 to 30 μm , a mitochondria around 500 nm, and a lipid membrane or average globular protein is around 10 nm across. Any secondary ROS beyond singlet oxygen will have a greater and more variable range of diffusion paths to propagate the signal. (Kuimova et al., 2009; Redmond and Kochevar, 2006; Winterbourn, 2008). The behaviour of singlet oxygen can also change dependent on where it is produced, and as changes occur in the cell during the PDT process (da Silva et al., 2012).

1.4.3 Light for PDT

Light propagation through tissues is heavily affected by refraction, reflection, absorption and scattering processes. Diffusion and loss of light energy in refraction and reflection can be minimised by perpendicular light application, scattering however is harder to minimise. Mathematical models have shown that over 4/5ths of the fluence can be lost in the first 0.5 cm, and cancerous tissue can present different optics than normal tissue (Jacques, 2010; Salomatina et al., 2006; Wilson and Patterson, 2008). Another contributing factor to loss of light intensity is light absorption by tissue chromophores. Levels of chromophores vary dependent on tissue type but the most relevant are water, oxyhemoglobin, deoxyhemoglobin and melanin. When plotted as seen in Figure 2 the absorption spectra of these chromophores show an optimum window for PDT which diminishes tissue absorbance, this window corresponds to outer limits of 650-1200 nm this range is further reduced since PS triplet-state molecules excited by light >850 nm are predicted to have a low triplet energy level so be unable to produce singlet oxygen efficiently (Plaetzer et al., 2009), however alternative type I processes could still occur.

Porphyrins, Chlorins and Bacteriochlorins have proven widely popular as photosensitisers because alongside a good singlet oxygen quantum yield, (defined as the average number of molecules of singlet oxygen produced per photon of light absorbed, 100% efficiency is defined as 1), they also have inherent spectral properties suitable for PDT applications; i.e. absorption bands in the red. These sorts of photosensitisers have a characteristic absorption pattern consisting of a strong Soret band around 400nm and a series of weaker satellite Q bands between 600 and 800nm, it is these Q bands that are used for PDT treatment (Macdonald and Dougherty, 2001). Below in Figure 3 is the absorption spectrum of Pyropheophorbide-a, the Chlorin based photosensitiser used in this thesis, it is presented here to show its typical spectral bands and the standard spectral nomenclature for Porphyrins and related molecules as discussed above. There is a significant body of work on interpreting spectral pattern shifts in relation to molecular orbital theory, including the effect of chemical addition of new groups onto the core ring structure, particularly those with electron withdrawing or donating properties that would affect the conjugated electron system and have a pronounced effect on the intensity and wavelength of the Q bands (Bonnett, 2000).

Figure 2: The ‘optical window’ for PDT.

Taken from (Plaetzer et al., 2009) Absorption spectra of common tissue chromophores. H_2O = water, $Hb(H_2O)$ = deoxyhemoglobin, $Hb(O_2)$ = oxyhemoglobin.

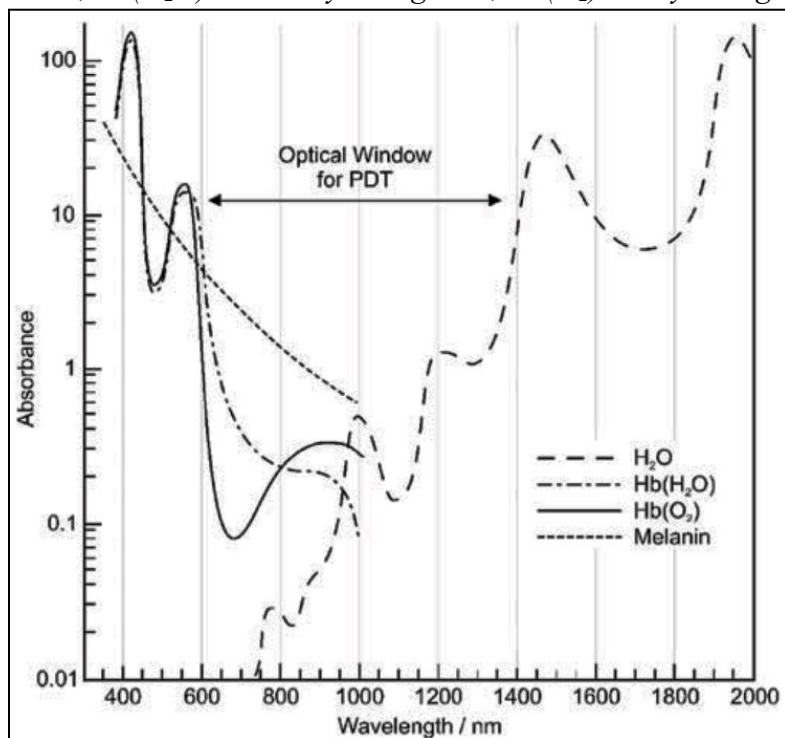
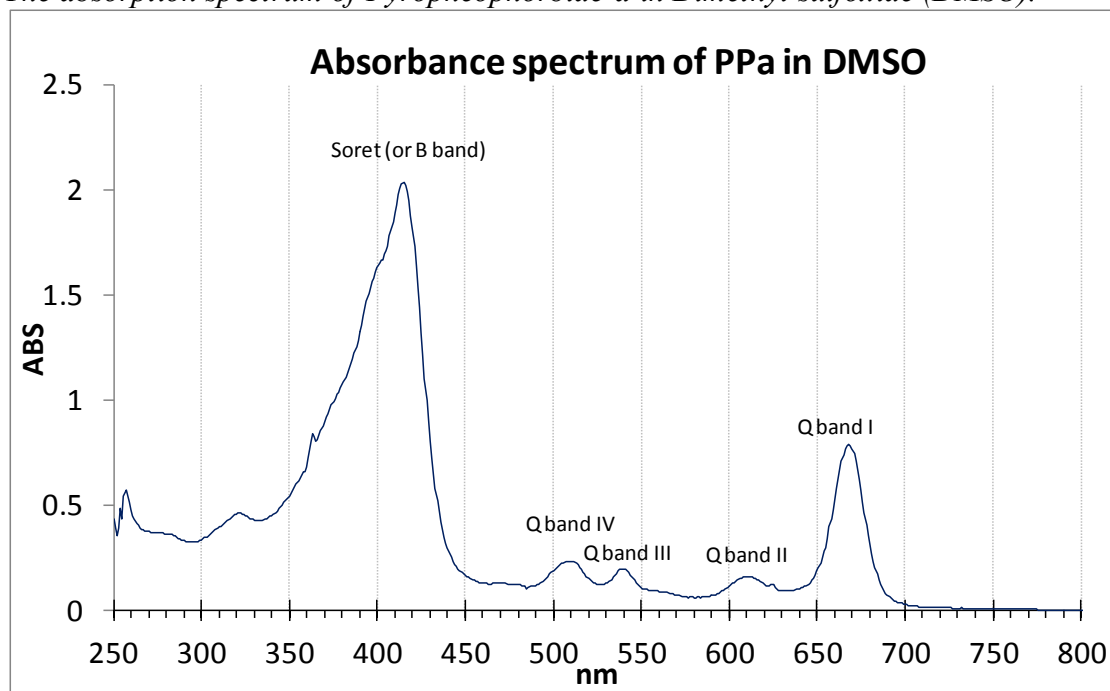


Figure 3: Absorption spectrum of PPa and its standard spectral nomenclature

The absorption spectrum of Pyropheophorbide-a in Dimethyl sulfoxide (DMSO).



1.4.4 Photosensitisers for PDT.

Many of the classical photosensitisers were originally derived from natural substances. For example pheophorbides, like the one used in this thesis, were originally derived from chlorophyll-a. The first photosensitiser to achieve regulatory approval in 1993 was hematoporphyrin derivative or HPD, derived from haemoglobin. A partial purification from HPD, PhotofrinTM is one of the most commonly used and studied photosensitisers to date, but its research and application was hindered by the fact it is in fact an impure mixture of porphyrin oligomers of varying lengths, linkages and stereo-chemistries. (Allison and Sibata, 2010; Detty et al., 2004).

New photosensitisers are developed via chemical synthesis techniques with preferred photophysical or pharmacokinetic characteristics designed into them. Preferable characteristics can vary upon application of PS but can include (Ochsner, 1997; Plaetzer et al., 2009):

- Photosensitisers that absorb in the red as tissue penetration of light is optimal with red / NIR light, aiding activation of photosensitisers in deeper tissue. See 1.4.3.
- Emission photophysics that favour more efficient radiationless transition to molecular oxygen or other specified cellular targets. Optimised triplet state excitation energy (110-130 kJ.mol⁻¹) being the theoretical triplet state energy required to promote oxygen transfer mechanisms, a lower energy however can still promote transfer to other molecules.
- Increased triplet state quantum yield (defined as the average number of times that photosensitiser triplet state formation occurs per photon of light absorbed, 100% efficiency is defined as 1).
- Triplet state lifetime (the average time a molecule spends in its first excited triplet state, optimum time is 500ns or longer).

Among such a wide repertoire of natural or synthetic photosensitisers and the numerous chemically-modified derivatives that have been produced from them, scientists and clinicians have a great deal of choice. This means theoretically, suitable photosensitisers could be chosen for the best possible applications based solely on their individual photophysical or

pharmacokinetic properties (Allison et al., 2004; Palumbo, 2007; Plaetzer et al., 2009). However ideal, having just one photosensitiser that will fit all applications looks less achievable than having a toolbox of different photosensitisers, tuneable with clinical endpoints (dose, light etc) to different patients and disease states. For example one photosensitiser; a monoacid benzoporphyrin derivative called verteporfin, branded clinically as VisudyneTM, has a half-life in the body too short for tumour accumulation. However the half-life is ideal for the treatment of neovascularisation in the eye during treatment of the wet-form of age related macular degeneration (Macdonald and Dougherty, 2001). The effect of any specific chemical modification on a PS is hard to predict and not always synergistic for all the important factors that influence *in vivo* efficiency, although many labs do carry out quantitative structure-activity relationship to try and predict this (QSAR) studies (Banfi et al., 2006; Gryshuk et al., 2007; Henderson et al., 1997; MacDonald et al., 1999).

1.4.5 Chemical modifications of PPa

Pyropheophorbide a (PPa) is the PS under investigation in this thesis. One QSAR study (MacDonald et al., 1999), looked at a molecule very similar to PPa, Pyropheophorbide a-ether (PPa-ether) the same molecule as PPa but with a ether group in place of the vinyl group. PPa-ether was then modified with side chains with increasing alkyl carbon chain length from propyl (n=3) to dodecyl (n=12). Increasing chain length increased hydrophobicity. The intracellular localisation of these PS showed the octyl, decyl and dodecyl derivatives localised to the lysosomes at extracellular concentrations below the LD₅₀ (PS concentration required to kill 50% of the cells). At extracellular concentrations equal or greater than LD₅₀, the compounds localised mainly to mitochondria. The propyl, pentyl, hexyl and heptyl ether derivatives localised mainly to the mitochondria at all concentrations studied. This change in localisation correlated with the change in cell kill efficiency, (mitochondrial localisation being more lethal than lysosomal). They concluded an inverse relationship between the aggregation state and the LD₅₀ concentration, compounds were shown to have a higher tendency to aggregate at low concentrations with increasing alkyl chain length, hence effecting their mechanism of uptake, sequestering aggregated PS in the lysosomes after endocytosis while monomeric PS were internalised by some other pathway.

Two molecules from this same group of PPa-ether molecules above were further investigated to examine whether the PS that aggregated could disaggregate once inside tumour cells (Kelbauskas and Dietel, 2002). They looked in particular at the hexyl and nonyl derivatives which were both shown to aggregate and accumulate in lysosomes. Over time, both hexyl and nonyl derivatives underwent disaggregation in the cell interior, the extent of disaggregation was faster and more complete for the more hydrophilic hexyl derivative which corresponded with its potency as a more effective photosensitiser.

As in this thesis Guelluy and colleagues (Guelluy et al., 2010) were trying to improve the solubility of a similar molecule to PPa, PPME (Pyropheophorbide a methyl ester), which differs from PPa in the replacement of the carboxylic acid group on the propionic acid side chain to a methyl ester. They chose a liposomal delivery method to aid PS solubilisation. Free PPME dissolved in phosphate buffered saline (PBS) exhibited spectra designated to be typical of aggregates, with a strongly reduced fluorescence and absorbance efficiency, whereas PPME dissolved in a liposomal formulation (Lipo-PPME) retained high fluorescence and absorbance efficiency comparable to monomeric PS (observations from the PS in Ethanol). The use of a liposome improved cellular uptake in HCT-116 cells by a factor of 5 but over time both reached the same maxima, interestingly all the internalised molecules (delivered either way) showed a fluorescence lifetime measurement that matches monomeric PPME. When they looked at subcellular localisation they found that in both cases PPME located to many intracellular organelles including endoplasmic reticulum, Golgi, lysosomes and mitochondria (although levels were limited in mitochondria to only when the higher concentrations of extracellular PS were used). They then showed the liposomes themselves did not enter the cells by internalisation or fusion mechanisms but they released PPME at the plasma membrane by inter-membrane contact. Other work by this group showed they could alter the balance from apoptotic cell death to necrotic cell death by changing concentration of PPME; propensity for necrotic cell death varied from 8% (1 mM) to 79% (5 mM) for PPME alone, and from 10% (0.25 mM) to 68% (1 mM) for Lipo-PPME. After irradiation of cells incubated with both forms of PS, they also characterised the types of ROS generated inside the cell via ESR spin trapping. Their experiments revealed that $^1\text{O}_2$ is the major ROS detected after activation of both forms of intracellular PPME. Others types of ROS (H_2O_2 , O_2^- , $\text{OH}^\cdot$) represented only 30% of the ROS detection.

1.4.6 Addition of a positive charge or Polyethylene glycol chains to a PS

In this thesis the novel PS under investigation have been modified either by the addition of a positive charge and/or small Polyethylene glycol (PEG) chains in order to try and improve their aqueous solubility.

Cationic porphyrin molecules often show improved cellular uptake; for example Ball and colleagues (Ball et al., 1999) investigated 3 substituted zinc phthalocyanines, one anionic, one cationic and one neutral. They showed increased aqueous solubility for both the anionic and cationic PS over the neutral PS. Quantified levels of cellular uptake after 2 hours was as follows; cationic > neutral > anionic, and the concentration of each PS needed to kill 50% tumour cells (LD₅₀) showed cationic to be by far the most potent at 0.028 µM, the anionic and neutral PS were similar at 2.1 µM and 2.6 µM respectively. In terms of localisation all showed an initial punctuate distribution indicative of lysosomes that under low light conditions (1000 lower than cell kill irradiation) re-localised to different places depending on the charge of the PS, the neutral PS stayed punctuate, the anionic PS showed a more diffuse mostly nuclear pattern, and the cationic showed a more diffuse but cytosolic pattern.

PEGylation is a popular choice to improve water solubility; for example Grahn and colleagues (Grahn et al., 1999) synthesised a PEGylated PS where they modified the clinically licensed PS FoscanTM. Four PEG chains were either attached by carbonate (liable linker) or triazine (stable linker) linkages via the 4 phenol hydroxyl groups of the FoscanTM. They noted however that preparations did contain some un-reacted (free) FoscanTM and a range of incompletely conjugated FoscanTM (1–3 PEG chains per FoscanTM). Their work showed FoscanTM PEG had a longer circulating half-life than free FoscanTM, but potency was not improved over the original molecule. The stable linker showed better bio-distribution but insufficient potency in tumours and the liable linker showed increased non specific toxicity at early dose light intervals and an equivalent profile to FoscanTM at later time points. Continuation of this work (Krueger et al., 2003; Reuther et al., 2001) further developed the linkages of FoscanTM to the PEG and did show some improvements in *in vivo* effectiveness.

A different group PEGylated a series of Chlorin e6 photosensitisers which were loaded onto a Poly L-Lysine polymer (Hamblin et al., 2001), they showed PEGylation changed the mechanism of PS deactivation from majority type II (singlet oxygen) to majority type I (radicals or electron transfer) and that PEGylation increased the selectivity and potency *in vitro*, showing it had an increased tendency to disaggregate, an altered cellular localisation and a more apoptotic mechanism of action. When injected intra-peritoneally into mouse tumour models the PEGylated PS-Poly Lysine conjugates also gave a higher photosensitiser tumour uptake and higher tumour to normal tissue ratios as well as increased depth of penetration into the peritoneal wall.

1.5 Antibody targeted PDT

1.5.1 Antibodies in modern medicine

Cancer cells exhibit altered and/or unregulated expression profiles of numerous genes, proteins, carbohydrates and lipids compared to the endogenous cells in their surrounding environment. These have become useful markers in aiding diagnosis, treatment and research. Antibodies against these molecules can be raised and recombinant antibody therapy for cancer is well established; four of the top ten cancer therapy drugs are antibodies including Trastuzumab (Herceptin[®]), and Bevacizumab (Avastin[®]). (Adams and Weiner, 2005; Deonarain, 2008; Scott et al., 2012). In terms of antibody engineering the field has expanded exceptionally since its beginnings (Carter, 2006; Nelson et al., 2010; Sidhu and Fellouse, 2006).

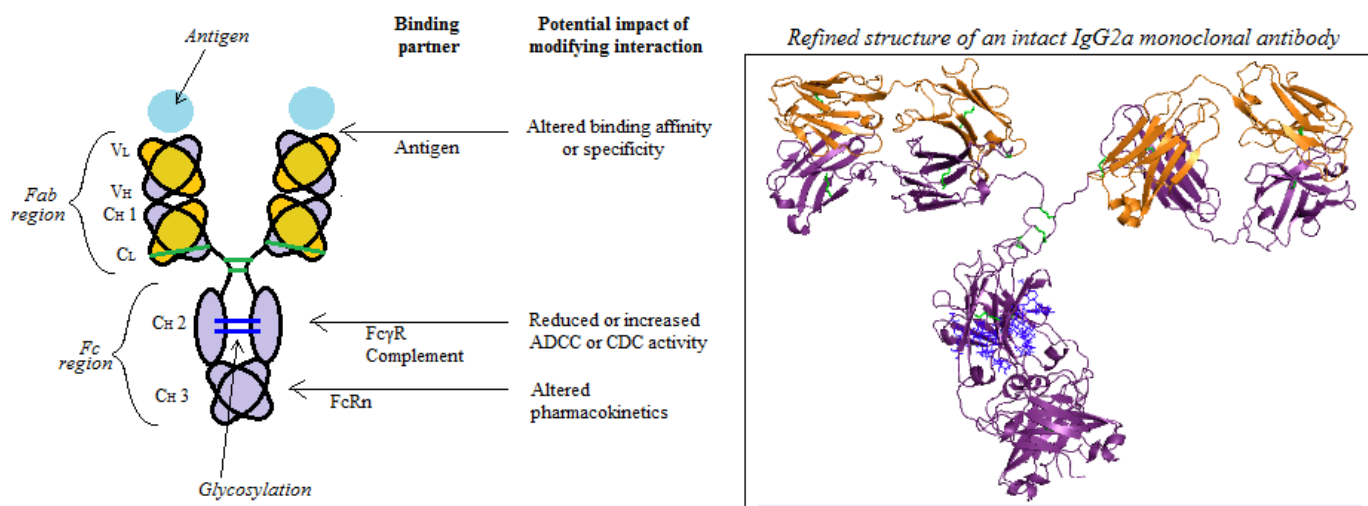
Humans express five isotype forms of antibodies which are designated IgG, IgA, IgM, IgD, and IgE, they differ in their physicochemical properties, i.e. charge, size, and solubility as well as their behaviour and interaction with antigens and other bio-molecules. The IgG (Immunoglobulin G) constitutes about 80% of the antibodies in the serum, and as such is the ‘classic’ antibody. Many of the current therapeutic antibody drugs are of this format or a fragment thereof (Elgert, 2009). The influence of selecting the most suitable Isotype is discussed well by Salfeld et al (Salfeld, 2007).

Antibodies are typically made up of a series of repeating immunoglobulin units that are roughly 110 amino acid residues in length and share significant structural homology, each domain has the characteristic tertiary structure consisting of two β -pleated sheets. Layers are covalently linked by a disulfide bridge near the centre of the domain. All proteins that exhibit this structural motif belong to the immunoglobulin gene super family (Elgert, 2009). A schematic representation of an IgG molecule is shown in Figure 4 next to the structure of an intact IgG solved by X-ray diffraction. The IgG molecule consists of two heavy and two light chains linked by disulfide bonds and non-covalent interactions. The light chain of IgG has two domains called VL and CL. The heavy chain of IgG has four domains: one VH and three in the CH region numbered 1 to 3. When the VL and VH chain framework residues assume their native arrangement, three loops from each domain come together and are held out into the solvent, these loops are called complementarity determining regions or CDRs. The size and shape of the loops are defined by their amino acid residues. It is these CDRs that form the antigen-binding site of the antibody. An antibody can also be designated in terms of its Fc portion or its Fab portion, an IgG is made up of one Fc and two Fab portions (Elgert, 2009).

Each immunoglobulin domain contributes different properties to the molecule, whether it is intra or inter-dimerisation, binding to cellular molecules or encoding regions for glycan extension. Each domain folds independently of each other into a compact globular structure so can be 'cut up' and rearranged like biological Lego bricks. These isolated fragments of the original antibody molecule or novel multimeric antibody formats all have their own properties, and can be adapted to fulfil a chosen role. Figure 5 shows a schematic representation of some of the antibody formats used. Antibody fragments such as these are fast replacing monoclonal antibodies in the preclinical pipeline providing a much greater potential for intelligent design and engineering toward both diagnostic and therapeutic applications. (Holliger and Hudson, 2005; Nelson and Reichert, 2009).

Figure 4 : Structure and nomenclature of an IgG antibody

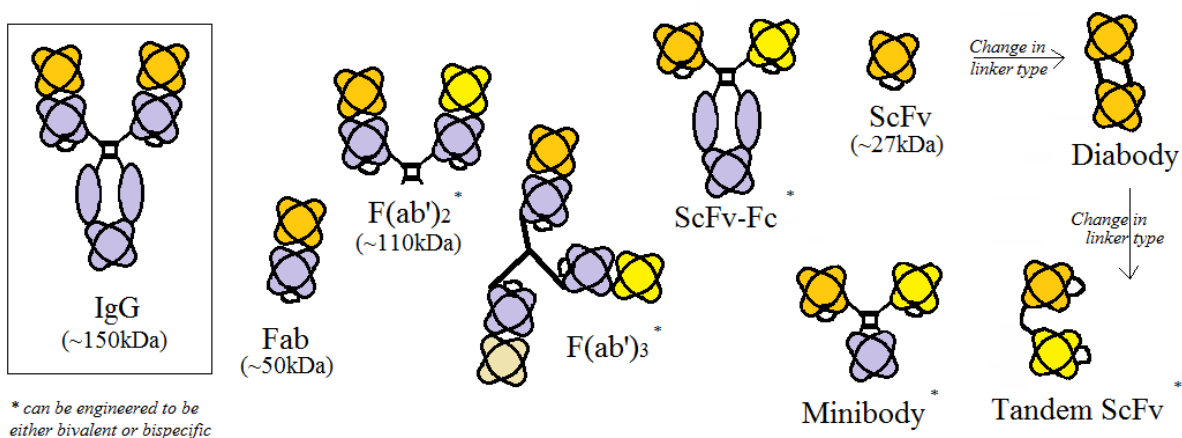
Reproduced from (Carter, 2006) and structure file taken from <http://www.rcsb.org/pdb>.



IgG molecules comprise a pair of identical heavy (purple) and light (orange) chains linked by interchain disulphide bonds (green bars). Selective binding of antigen (light blue) is mainly mediated by six loops three of which are present in each of the VH and VL domains. Effector functions require the presence of the Fc-region and its glycosylation (dark blue bars) and are crucially influenced by its structure. The structure of an actual immunoglobulin was taken from the RCSB protein databank at <http://www.rcsb.org/pdb> (PDB number 1IGT) and recoloured in Pymol www.pymol.org/. Abbreviations: FcRn = neonatal Fc receptor, FcγR = Fc-gamma receptors, ADCC = Antibody-dependent cell-mediated cytotoxicity, CDC = complement-dependent cytotoxicity

Figure 5 : Schematic representation of selected antibody formats

Adapted from (Holliger and Hudson, 2005) and (Carter, 2006)



Schematic representation of an intact 'classic' IgG molecule and a variety of antibody fragments and novel multimeric formats including Fab, F(ab')₂, F(ab')₃, ScFv-Fc, Minibody, ScFv, Diabody and Tandem ScFv. Sizes given in kDa are approximate. Abbreviations: ScFv = single-chain variable fragment, IgG = Immunoglobulin G, kDa = Kilodaltons.

1.5.2 Considerations for antibody selection

The target to which the antibody is raised will affect its efficiency, despite more than 30 different antibodies already approved worldwide for therapeutic use. More than 80% of these are against only five main targets (TNF- α , HER2, CD20, EGFR, and VEGF) (Shim, 2011). The choice of target will not only effect therapeutic considerations but also pharmacokinetic ones because it can affect retention and distribution throughout in the tissue.

Inclusion of the Fc domain has significant influence on many important biological properties including binding of the neonatal Fc receptor (FcRn) which extends the half-life (Roopenian and Akilesh, 2007), and Fc mediated activation of the immune system by antibody dependent cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) (Golay and Introna, 2012).

Bacterial expression systems for recombinant antibody expression will produce non glycosylated antibodies while a mammalian expression system will produce a range of glycoforms (Raju, 2003). Inclusion of glycans can provide useful conjugation handles or physiological properties however the lack of glycans will produce a more standardised product (Arnold et al., 2007).

Stability of the fragments is important (Quintero-Hernández et al., 2012; Röthlisberger et al., 2005) as well as domain order (Carmichael et al., 2003; Kipriyanov et al., 2003) and the inclusion of any linkers. In particular with scFv; molecules with a linker of 3–12 residues cannot fold into a functional scFv domain and instead associate with a second scFv molecule to form a divalent dimer, called a diabody. Reducing the linker length below 3 residues can further force scFv association into trivalent triabodies, or tetravalent tetrabodies, depending on composition and domain orientation (Todorovska et al., 2001) Interestingly even scFv with the traditional (Gly₄-Ser)₃ peptide linker, such as used in this work, often thought to produce scFv in a fully folded monomeric form, can also allow reversible formation of domain swapped dimers in solutions above 1mg/ml. (Lee et al., 2002).

1.5.2.1 The effect of antibody size

The effect of antibody size on its pharmacokinetics can both be modelled mathematically (Schmidt and Wittrup, 2009) and has repeatedly been shown experimentally (Batra et al., 2002; Kenanova and Wu, 2006; Yuan et al., 1995), including for anti HER2 antibodies like the one used in this thesis (Deyev and Lebedenko, 2008).

Smaller antibody fragments are able to penetrate the tumour quickest and achieved peak uptake early within the first few hours, the blood clearance of these molecules is also relatively quick, so in terms of the tumour to blood ratio the best ratios are normally found with these smaller fast clearing fragments. However the highest tumour uptake levels are normally always seen for the full IgG, the increase over all other fragments even those of similar size is due to Fc interactions and FcRn mediated salvage. Evidence that it is the increased rate of serum clearance that is limiting small fragment tumour uptake is in part backed up by (Adams et al., 1998) who showed that in a standard mouse tumour model, tumour retention of an scFv was of a sufficient but predictably much lower level when compared to the equivalent molecule in its IgG format. However when they looked at a mouse model in which renal clearance was blocked by surgically nephrectomisation the peak tumour retentions for scFv were similar to the tumour retention levels achievable by full IgG molecules in the normal model.

A general rule is that larger fragments such as the IgG fragments are retained at high levels in human tumours over longer time periods (days) but with a lower degree of specificity while smaller fragments such as scFv are taken up into the tumour more rapidly (hours) and with greatly improved specificity but at comparatively lower levels and will show quicker clearance times both from the serum and the tumour.

1.5.2.2 The effect of affinity and avidity

Properties inherent in vascular or tumour tissue as well as the endogenous properties of the antibody such as size, shape, lipophilicity and charge can all have an impact on localisation and bio-distribution of the antibody. Of particular importance are the affinity and avidity of the binding interactions especially in reference to any engineered increased valency (Cuesta et al., 2010) and the idea of a 'binding site barrier' (Rudnick and Adams, 2009). Another factor of importance in tumour penetration related to antigen binding is the influence of antigen expression, density and the rates of internalisation and any subsequent antibody catabolism. (Ackerman et al., 2008).

Heterogeneous antibody (Ab) distribution within a tumour can arise in part from heterogeneous antigen expression, vasculature, or necrosis but it can also be affected by the 'binding site barrier'. This is a model which predicts that diffusion of high-affinity antibodies into a tumour is limited because slow rates of dissociation decrease the local concentration of diffusible, free antibody. As the strength of the bond between the Ab and its target increases, the amount of Abs available to diffuse into tumour decreases, leading to a reduction in penetration into the tumour. This effect is made even more pronounced in a tumour microenvironment where the lack of draining lymphatics hinders further the diffusion of macromolecules (Rudnick and Adams, 2009; Rudnick et al., 2011; Zuckier et al., 2000). This 'binding site barrier' has been shown with the antibody used in this thesis; C6.5 in both the scFv and IgG formats, (Adams et al., 2001; Rudnick et al., 2011) as well as for the clinical competition HerceptinTM (Baker et al., 2008).

1.5.3 Photo-Immunoconjugates

Many academic and industrial researchers are now developing antibody targeted PDT for treatment and tumour detection by conjugating either whole antibodies or antibody fragments to photosensitisers. As antibody conjugation delivers photosensitisers to the tumour via tumour-associated cell receptors it is hypothesised to improve both specificity and efficiency, removing some of the current limitations of non-targeted PDT see 1.2.3, including the prolonged sensitisation and non specific uptake of PS into the skin, and the problem with complicated dosimetry in the clinic. The antibody targeting of PS to well characterised disease state epitopes will hopefully provide a more predictable, more specific and hopefully more effective clinical outcome.

The first work carried out with photo-immunoconjugates (PICs) was in 1983, inspired by the recent development of monoclonal antibody technology- this technology was to grow to represent the realisation of the ‘magic bullet’ hypothesis proposed by Paul Ehrlich back at the beginning of the 20th century. Mew and colleagues (Mew et al., 1983) synthesised Hematoporphyrin (HPD) / IgG conjugates via creation of an amide bond between an acid group of the photosensitisers and amine groups on the antibody. Their hypothesis was antibody conjugation would lower the effective dose of PDT required in cancer therapy, reducing the current problematic side effects caused by low tumour specificity. When compared to equivalent concentrations of free Photosensitiser (PS) or Antibody (Ab) in antigen positive over antigen negative tumour lines they were indeed able to demonstrate both superior specificity and efficiency in tumour targeting and reduction in tumour growth both *in vitro* tumour cell lines, (efficiently killing 95% of tumour cells compared to 0%) and *in vivo* mouse tumour models, (significantly increasing mean time for tumour development).

In between the first steps and today, significant scientific developments have helped shaped the field of PIC production, both in terms of antibody engineering and photosensitiser development. The vast toolbox of new synthetic and/or chemically modified photosensitisers with a range of specified photophysical and pharmacokinetical properties has improved alongside a growth in the understanding of the important aspects of mechanisms behind effective PDT. Interest in the application of these photo-immunoconjugates is growing and recently work has been published in some well recognised journals; e.g. *Nature Medicine*, (Mitsunaga et al., 2011) and *PNAS* (Serebrovskaya et al., 2009).

Antibody photosensitiser conjugates have now been proven to increase the rate of cellular PS uptake and prolong cellular PS retention, along with an ability to be more selective and more effective at cancer destruction than free PS both *in vitro* and *in vivo* producing complete cures in a number of different mouse models of cancer. More detail of the scientific work carried out on PICs is discussed further in a later chapter see 5.1.4.

Despite these successes the only PIC studied at a clinical level is an old feasibility study to determine whether immune-photodiagnosis could be applied in the clinic; a FITC conjugated human-mouse chimeric IgG directed against carcinoembryonic antigen (CEA) was studied in six patients with primary colorectal carcinoma, (Folli et al., 1992) in which they showed some promising results. Another small piece of PIC clinical work is a short communication by (Schmidt, 1993) in which they used Anti-CA125 and Anti-TAG 72 Phthalocyanine-zinc(II) conjugates alongside surgical techniques. The delay in clinically successful PICs is likely due to the fact that despite the substantial increase in the scientific understanding of PDT processes, funding for clinical trials is much harder to find for complex drugs and there are still PIC production problems, namely non specific toxicity and impurity of the product due to non covalently bound hydrophobic PS, alongside incorrect or un-reproducible dosimetry calculations, both things resulting in an inability to always produce a reliable well-characterised product. These problems are discussed further see 5.1.4.

1.5.3.1 'State of the art' with photoimmunoconjugates.

Most PICs are produced against solid tumour antigens but the use of PIC to manipulate other systems includes: the targeting and shutdown of tumour vasculature (Palumbo et al., 2011), PICs against non solid-tumours for example lymphomas, (Mew et al., 1985; Oseroff et al., 1986), induction of immune regulation and induced cancer immunity (Palumbo et al., 2011), the targeted removal of one cell population from a mixture i.e. *ex vivo* in bone marrow transplants (Berki and Németh, 1998), or *in vivo* manipulation of wound healing (Strong et al., 1997). PICs have also been developed for infectious disease (Berthiaume et al., 1994; Bhatti et al., 2000) including against drug resistant bacteria like MRSA, (Embleton et al., 2004), as well as being manipulated to target on a much smaller scale to destroy specific intracellular structures while sparing the rest of the cell (Conlon and Berrios, 2001; Conlon et al., 2002).

1.5.3.2 Targeted PDT without the antibody

Other targeting methods or methods that improve the level and/or specificity of delivery include (Bechet et al., 2008; Chatterjee et al., 2008; Konan et al., 2002; Sharman et al., 2004; Solban et al., 2006):

- The encapsulation in other carrier systems such as liposomes, oil-dispersions, dendrimers, or polymers to facilitate drug delivery.
- The pre-association or direct conjugation with serum protein carrier molecules or serum protein binding moieties.
- Conjugation to peptides, glycans or lipids that mimic the natural ligands or mitogens of over expressed tumour receptors.
- Conjugation to substrates for metabolic pathways altered in tumours, e.g. some steroids, amino acids, or sugars.
- Conjugation to chemicals with signals for specific cellular uptake and localisation, e.g. signals for scavenging macrophages, nuclear localisation sequences, or cell penetrating peptides.
- Conjugation to non endogenous moieties such as some bacterial toxins, viral peptides or plant lectin molecules that have been shown to selectively bind tumour associated targets.

1.6 Previous work with C6.5-k targeted PPa.

The work presented in this thesis is based on original work from this lab (Bhatti et al., 2008; Kuimova et al., 2007). This work was recently extended by Stamati et al (Stamati et al., 2010).

For the original body of work an scFv fragment of medium affinity for good tissue penetration and rapid clearance was chosen for conjugation to PS. Bhatti and colleagues (Bhatti et al., 2008) showed that the conjugation of PPa to available lysine residues on the surface of a number of different scFv antibody fragments led to effective PICs. Conjugation ratios of 2-8 PS per scFv molecule were obtained, and final PDT cytotoxicity was hypothesised to be influenced by the number and position of these lysines. The most successful PIC was with C6.5-k, a scFv that binds HER2. This PIC was tested *in vitro* against cell lines positive and negative for HER2, results demonstrated improved specificity and potency over free PPa, again this was proposed to be due to the optimal lysine spacing and therefore PS loading of this antibody. This enhanced potency was rare in the literature at a time where it was becoming common to see a loss in potency once PS were conjugated at this level to antibodies, see 5.1.4. They also looked at blood clearance in mice of the conjugates compared to scFv and PPa alone, scFv clears rapidly and PPa very slowly, the conjugate was found to clear at a rate intermediate to these two parent compounds – providing a therapeutically more attractive profile over free PPa. The serum half life calculations from this work are reproduced in Table 3 below. The PIC was also effective *in vivo* at eradicating tumour burden in mice.

Kuimova and colleagues (Kuimova et al., 2007) looked in more detail at two of these conjugates, both C6.5-k as either a PIC with PPa or with the clinically licensed PS VisudyneTM. They used fluorescent spectroscopic techniques to confirm that there was ~20% non-covalently bound PS associated with the PICs. Interestingly there was a difference in the fluorescent profiles of VisudyneTM conjugates and PPa conjugates, PPa becoming twice as fluorescent (emission intensity) once bound to C6.5-k where as VisudyneTM became 3 times less emissive. Confocal fluorescent microscopy experiments were also carried out on these conjugates in cell lines positive and negative for HER2, the results showed specificity for PIC over free PS and

significantly different rates of uptake and leaching for the free PS vs. the PIC – the PIC exhibiting faster initial uptake and prolonged retention in the cells.

The conclusions of this work was that although promising, there were still some problems with solubility of the PS PPa, it was thought to be affecting PIC pharmacokinetics, conjugation efficiency and driving the non-covalent contamination of free PS after conjugation. Work initiated by these two papers was therefore followed by Stamati and colleagues (Stamati et al., 2010), in the characterisation of two new water soluble photosensitisers, based on PPa and synthesised with either 3 Polyethylene glycol (PEG) chains, called PS1, or with a small single PEG and a permanent positive charge, called PS4. The structures of these compounds are shown below in Figure 6. Fluorescence quantum yields and singlet oxygen quantum yields were calculated for the new PS and these are reproduced in Table 4 below. The results show that in toluene PPa and PS1 share similar fluorescence quantum yields, PPa (0.3) and PS1 (0.26) whereas PS4 is lower (0.15). The pattern for singlet oxygen quantum yields in toluene show that PPa and PS1 are again similar; PPa (0.50) and PS1 (0.56) but PS4 this time shows an increase (0.75). An indication of water solubility is also demonstrated in this data; when fluorescent quantum yield is examined in water compared to toluene, PPa quantum yield drops dramatically, PS4 shows a slightly better quantum yield retention between toluene and water but it is PS1 that shows the greatest retention of quantum yield, inferring that both PPa and PS4 extensively aggregate in water, whereas this is reduced in PS1 formulations. Also presented are cell kill experiments comparing the new PS to their parent molecule, the demonstrated potency of the new photosensitisers was PS1 > PPa >>> PS4. IC₅₀ values for this work are reproduced in Table 4 below. They also looked at confocal fluorescent microscopy co-localisation of all three PS in SKOV-3 cells, and concluded PS4 likely had a more lysosomal localisation compared to the co-localisation of PPa and PS1, which included mitochondria, ER and Golgi.

Table 3 : Serum half life calculations for PPa, C6.5-k and their PIC.
Taken from (Bhatti et al., 2008)

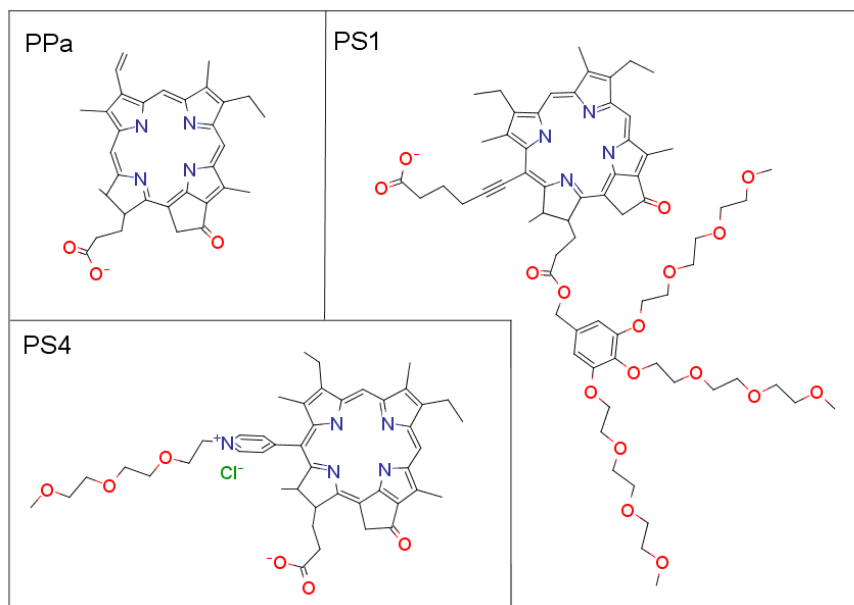
Molecule	half life α phase* (hrs)	half life β phase* (hrs)
IgG	1.1	76.2
PPa	1.6	20.9
C6.5-k ScFv	0.14	4.1
C6.5-k PPa PIC	0.17	12.3

*fit to a Bi-phasic exponential clearance curve

Table 4 : Quantum yields and IC₅₀ values for PPa, PS1 and PS4
Taken from (Stamati et al., 2010).

Molecule	Fluorescent quantum yield (toluene)	Singlet oxygen quantum yield (toluene)	Fluorescent quantum yield (water)	Potency; IC ₅₀ SKOV-3 cells (μ M)	Potency; IC ₅₀ KB cells (μ M)
PPa	0.3	0.5	4×10^{-3}	14.5 +/- 3.2	1.2 +/- 0.4
PS1	0.26	0.56	0.01	1.1 +/- 0.2	1.2 +/- 0.5
PS4	0.15	0.73	2×10^{-3}	259.6 +/- 31	24.7 +/- 11.7

Figure 6: Chemical structure of PPa, PS1 and PS4 at pH 7.4



Major Species at pH 7.4 of all three Photosensitisers under investigation in this thesis, produced using MarvinSketch Freeware Version: 5.7.0, (ChemAxon Ltd.)

1.7 Key aims and objectives

During this project the aim is to investigate the effect of increased PS solubility on efficiency to induce cytotoxicity in cancer cells *in vitro* and the molecular mechanisms behind any changes seen. This project will have a focus on elucidating the molecular mechanisms behind any altered potency, particularly in respect to the processes of intracellular trafficking and the mechanism of the cell death. Another aim is to investigate the potential of these new PS for antibody conjugation, and how this further affects its properties. The work aims to explore the possibilities of targeted PDT while improving understanding of the influence of PS solubility on PDT cell kill mechanisms. This knowledge will hopefully help make the development of PDT and targeted PDT agents for cancer therapy more informed and hopefully help modulate current cancer treatment modalities around the endogenous and treatment induced resistance seen in patients.

1.8 Work presented in each of the results chapters

Chapter 3: Chemical characterisation of PS and testing of PDT in an *in vitro* model of cancer

Characterisation and evidence for the increased solubility of novel PPa based photosensitisers, alongside further development of a traditional non-targeted PDT cancer cell model *in vitro*, and testing of the new PS compounds within this model.

- *New PS modifications are hypothesised to make the new PS more water soluble. Presented are studies to check this hypothesis that also give an insight into PS photophysical efficiency in physiological conditions.*
- *Initial testing of the cytotoxicity of new PS compounds is presented to show if the enhanced solubility affects the potency of the PS as a free molecule for PDT.*

Chapter 4 : The effect of increased PS solubility on cellular mechanisms important for PDT

Analysis of the effect on PS on cellular uptake and localisation and the mechanism of cell death induced upon irradiation.

- *Experiments are presented that investigate why changing the solubility can alter the potency of the free PS. For example looking in more detail at some of the factors beyond photophysical efficiency that can impact potency in vitro.*

Chapter 5: The conjugation of novel photosensitisers to the antibody fragment C6.5-k

Optimisation of the conjugation of photosensitisers to the antibody fragment C6.5-k and attempts made toward a consistent purification and analysis of conjugates.

- *New PS modifications are hypothesised to make the new PS more water soluble and so have improved conjugation efficiency and purity, presented is work carried out to investigate the extent of any such improvements.*

Chapter Two

2 Materials and Methods

2.1.1 Equipment

2.1.1.1 Laser System

HPD 7401 laser system, (High Power Devices Inc)

Model HPD5325-HHLF-TEC (High Power Devices Inc)

Semiconductor laser diode light, Wavelength 671.6nm FWHM 0.6nm.

PMax = 2.05 Watts, I_{max} = 5.00 Amps, V_{max} = 2.95 Volts

Laser temperature = 15-30°C

Fibre Optic Cable

SMA to SMA Patch Cable: 600µm/0.39 NA FT600EMT Fibre (M29L01 ThorLabs)

Length = 1meter

Collimating lens

λ=633nm SMA Collimation Package, NA 0.25, F=11.0mm (F220SMA-B ThorLabs)

1/e² Output beam diameter = 2.2mm

Full angle beam divergence = 0.021 °

Aligned at 633nm

2.1.1.2 Microplate Spectrophotometer (Absorbance)

SPECTRAmax 340PC Microplate Spectrophotometer (*Moleculardevices*)

340-850nm Monochromator tuneable in 1nm increments. 5nm FWHM

5Watt Xenon Flash Lamp,

Photodetectors = Silicon photodiode

2.1.1.3 Microplate Spectrophotometer (fluorescence)

Cary Eclipse Fluorescence Spectrophotometer (Varian)

Excitation monochromator (Czerny-Turner) 190-900nm +/- 1nm

Emission monochromator (Czerny-Turner) 190-900nm +/- 1nm

15W Xenon pulse lamp (pulse width 2-3µs), Power 60-75kW

Photodetector = photomultiplier (X2)

Fluoroskan Ascent FL; Type 374. (Labsystems)

Fluorometric filters :

Excitation = 320nm HBW 40nm / 390nm HBW 20nm / 485nm HBW 14nm.

Emission = 405nm HBW 50nm / 510nm HBW 10nm / 538nm HBW 25nm / 555nm HBW 25nm

Quartz-halogen lamp (30W)

Photodetector = photomultiplier

2.1.1.4 Cuvette Spectrophotometer (Absorbance)

Agilent 8453 UV Visible Spectrophotometer (Agilent Technologies)

Combined deuterium-discharge lamp (190-800nm) and tungsten lamp (370-1100nm)
Photodetector : photo diode array, 190nm-1100nm , nominal spectral slit width= 1nm

2.1.1.5 Luminescent image analyse

Las-3000 (Fujifilm)

CCD camera of 3,200,000 pixels with cooling function

Incident light source: Blue LED (460nm) / White

Transmitted light source 312nm UV / White

Filters: 605DF40 detection between 565 and 645 nm / Y515 for detection over 515 nm

2.1.1.6 Inverted confocal laser scanning microscope

LEICA TCS SP5 (LEICA)

Lasers : Argon (max30mW): 458, 476, 488, 496, 514 / Diode: 405 / HeNe1: 543 / DPSS 561
HeNe2: 633.

Objectives: 10x Air (HC PL APO 10x 0.40 CS) 20x Air (HC PL APO 20x 0.70 CS) 20x Imm Corr (HC PL APO 20x 0.70
IMM CORR CS) 40x Oil (HCX PL APO 40x 1.25-0.75 OIL CS) 63x Oil (HCX PL APO 63x 1.40-0.60 OIL CS) 63x
Water (HCX PL APO 63x 1.20 W CORR CS)

Detectors : 3 x normal PMT detectors / 1 x FLIM PMT detector / 1 x PMT detector (bright-field)
/ NDD1: FITC (BS RLD + TLD/RLD) / NDD2: TRITC (BS RLD + TLD/RLD)

2.1.1.7 Flow Cytometer

FACSCalibur flow cytometer (BD Biosciences)

Laser : 15 mW 488 nm, air-cooled argon-ion laser / optional red diode laser 635 nm

Forward Scatter Detector and Filter: solid-state silicon detector; 488 nm bandpass filter

Side Scatter Detector : Photomultiplier using Brewster-angle beam splitter

Fluorescence Detectors and Filters : photomultipliers with bandpass filters

FL-1 = 530/30, FL-2 = 585/42, FL-3 = 650/LP, FL-4 = 661/16

2.1.1.8 SDS-Page Gels / Western Blot Equipment

Various short and spacer glass plates and combs for Hand Cast Gels (BioRad)

Mini-PROTEAN Tetra Electrophoresis System (BioRad)

Trans-Blot SD Semi-Dry Transfer Cell (BioRad)

ECL Hybond nitrocellulose membrane (GE Healthcare RPN203D)

Blotting paper (BioRad 170-3965)

2.1.2 Buffers

(dH₂O = deionised water) (RT = room temperature)

10X TG buffer (2L) Store at RT

	Fw	Final;	To make;
Tris	121.14	0.25M	60.4g
Glycine	75.07	2.5M	376g
dH ₂ O			Up to 2L

1X SDS-PAGE Running buffer (2L) Store at RT

	Stock	Final;	To make;
TG buffer	10X	1X	200ml
SDS	20%	0.1%	10ml
dH ₂ O			Up to 2L

1X Transfer buffer (2L) Store at RT

	Stock	Final;	To make;
TG buffer	10X	1X	200ml
SDS	20%	0.04%	4ml
methanol	100%	20%	400ml
dH ₂ O			Up to 2L

10X PBS (10L) Store at RT

From this make 1 X PBS and 1 X PBS+0.1% Tween 20

	Fw	Final 10X;	To make up;
NaCl	58.44	1.37M	800g
KCl	74.55	0.027M	20g
KH ₂ PO ₄	138.08	0.018M	24g
Na ₂ HPO ₄	141.96	0.0101M	144g
dH ₂ O			Up to 10 Litres

TE buffer (pH 8) (100ml) Store at RT

	Stock	Final;	To make;
Tris	100mM	10mM	10ml
EDTA	0.5M	1mM	0.2ml
dH ₂ O			Up to 100ml
pH to 8.0 with HCL			

NB: when making up 0.5M EDTA warm slightly and continually pH > pH8 with NaOH during dissolution

FC buffer

	Final	Supplier and Cat no
FBS	2%	Gibco 10108-165
EDTA	1mM	Sigma E7889
PBS(-Mg-Ca)		PAA HIS-002
Make up under sterile conditions with sterile stocks		

12% Acrylamide SDS Gels

(mixed in the order listed below, APS then TEMED last)

Acrylamide and TEMED have to be stored in the toxic cabinet at RT. APS is only stable for a few months at 4°C, re-make if not used within this time. Tris and SDS buffers can be stored at RT for longer periods of time.

	Resolving Gel				Stacking Gel		
	1 Gel	2 Gel	3 Gel		1 Gel	2 Gel	3 Gel
dH ₂ O	1.7ml	3.4ml	5.1ml	dH ₂ O	1.5ml	3.0ml	4.5ml
1.5M Tris pH 8.8	1.25ml	2.5ml	3.75ml	1.0M Tris pH 6.8	0.25ml	0.5ml	0.75ml
30% Acrylamide	2.0ml	4.0ml	6.0ml	30% Acrylamide	0.3ml	0.6ml	0.9ml
10% SDS	50ul	100ul	150ul	10% SDS	20ul	40ul	60ul
10% APS	50ul	100ul	150ul	10% APS	20ul	40ul	60ul
TEMED	2ul	4ul	6ul	TEMED	2ul	4ul	6ul

Coomassie (and de stain) 500ml

Ingredient (stock)	FINAL	Coomassie	De-Stain
Methanol(100%)	40%	200ml	200ml
Acetic acid (100%)	10%	50ml	50ml
De-Ionised water	Made up to 500ml	250ml	250ml
Brilliant Blue R (Sigma B0149)	0.1%	0.5g	/

Laemmli Loading buffer 50ml (for reducing and denaturing conditions)

Ingredient (stock)	FINAL (1x)	FINAL (5x)	For 50ml
Tris pH6.8 (Fw 121.14)	0.0625 M	0.3125 M	12.5 ml of 0.3125 M pH'd to 6.8
SDS (solid)	2%	10%	5g
Glycerol (100%)	10%	50%	25 ml
Brilliant Blue R (Sigma B0149)	0.01%	0.05%	0.025g
β-mercaptoethanol	5%	25%	12.5 ml

2TY media

Make up 2.5L in dH₂O and send to autoclave, aliquot at 500ml plus 3 X 20mls (storable at RT unopened for a couple of months)

	Amount (g in 1L)	Supplier and Cat no
Tryptone	16g	Merck 1.11931.1000
Yeast	10g	Merck 1.11926.1000
NaCl	5g	BDH 102415K

2TY agar

Make 1L 2TY agar in dH₂O and send to autoclave, aliquot at 200ml (around 10 plates, ~10ml per plate) (storable at RT unopened for a couple of months)

	Amount (g in 1L)	Supplier and Cat no
Tryptone	16g	Merck 1.11931.1000
Yeast	10g	Merck 1.11926.1000
NaCl	5g	BDH 102415K
Agar	15g	Merck 1.01614.1000

NB doesn't completely go into solution at RT so heat it up on stirrer for a bit to get as much in as possible and try to pour evenly for aliquots. Re heat in Microwave until all in solution before pouring plates under flame. NB wait to cool until it's just bearable to touch before adding antibiotic.

2.1.3 Computation Based Methods

2.1.3.1 Curve fitting and equations

Where fitted used = SigmaPlot for Windows Version 11.0 (Systat Software, Inc.)

Name	Equation	Constraints	Figure
4 parameter logistic curves	$y = \min + \frac{(\max - \min)}{1 + (x/EC_{50})^{-Hillslope}}$		Figure 11 Figure 14
2 parameter power curve	$y = aX^b$	$b < 0$	Figure 15 Figure 31
Exponential Decay, Double, 4 Parameter	$y = ae^{(-bx)} + ce^{(-dx)}$		Figure 12
Sigmoidal, Sigmoid, 4 Parameter	$y = y_0 + \frac{a}{1 + e^{-((x-x_0)/b)}}$		Figure 12
Exponential rise to maximum	$y = a(1 - e^{-bx})$	$b > 0$	Figure 30

2.1.3.2 Computational pK_a prediction

The programme used to present data in 3.2.3.3 is:

MarvinSketch Freeware Version: 5.7.0, (ChemAxon Ltd.) www.chemaxon.com

It utilizes a QSPR method the details to which can be found at:

<http://www.chemaxon.com/marvin/help/calculations/pKa.html>

Models for computational pK_a

Different algorithms have been developed to calculate the pK_a. Standard methods for pK_a prediction can be purely empirical and based on observation or experimentation; or based on quantum methods which utilize 3D structure determinants and calculated quantum mechanical properties. The more commonly used empirical methods include 1) linear free-energy relationships (LFER), based on the Hammett and Taft equations which aim to correlate atomic properties to biological activity. 2) Quantitative structure-property relationships (QSPR), based on correlating calculated structural groups with predefined pK_a values. Or 3) Database similarity relationships, methods that search for similar whole structures in a database of molecules with known experimentally measured pK_a values. It is still not clear which of these is best and often different methods favour the chemical type upon which they were originally developed. (Liao and Nicklaus, 2009)

2.1.3.3 Computational Log P Prediction

The programmes used to present data in 3.2.3.4. are:

ACD/Log P

Calculated with ACD/Chemsketch Freeware, Version 11.02, (Advanced Chemistry Development Inc. ACD/Labs) www.acdlabs.com

Uses a fragmental based method details to be found at <http://www.acdlabs.com/resources/freeware/chemsketch/logp/>

ALOGPs, AC LogP, KOWNN, XLOGP2 and XLOGP3

calculated with the ALOGPS 2.1 freeware (Virtual Computational Chemistry Laboratory, VCCLAB) www.vcclab.org

Use various methods, details to be found at <http://www.vcclab.org/lab/alogps/>

VG, KLOP, and PHYS

Calculated with MarvinSketch Freeware Version: 5.7.0, (ChemAxon Ltd.) www.chemaxon.com

All uses atomic level fragmental based methods utilising 3 databases published by Viswanadhan et al (VG), Klopman et al (KLOP) and the PHYSPROP database (PHYS) details to be found at <http://www.chemaxon.com/marvin/help/calculations/logPlogD.html>

Models for computational LogP

Computational Log P predicting software is readily available as freeware online, there have been multiple algorithms designed over the years but all are being continually improved, many can provide some impressive correlations with experimental data (Xing and Glen, 2002a) The standard methods can be grouped as either substructure methods or property based methods. Substructure methods will break a molecule into fragments or single atoms and assign each a contribution value which are then summed, most fragmental methods also apply additional correction factors to compensate for intermolecular interactions. Property based methods use structural features and various considerations of the entire molecule to calculate its predicted behaviour in a solvent. It is normally suggested that one uses multiple programmes. (Faller and Ertl, 2007; Mannhold and van de Waterbeemd, 2001; Mannhold et al., 2009; Xing and Glen, 2002b).

2.1.3.4 Protein Homology modelling

Protein fold recognition server used = PHYRE Version 0.2 Structural Bioinformatics Group, (Imperial College, London) (<http://www.sbg.bio.ic.ac.uk/~phyre/>)

More information on PHYRE can be found within the case study published in *Nature Protocols* (Kelley and Sternberg, 2009).

The amino acid sequence was trimmed to remove tags and linker regions and the heavy and light variable regions put through independently. The top models for the two domains were downloaded, heavy chain top model was based on SCOP code d1n0xh1; (Immunoglobulin heavy chain variable domain, VH from Human (*Homo sapiens*), cluster 1) with 50% amino acid ID and an E value of 4.4×10^{-18} , Estimated precision was 100%. The top model for the light chain was based on SCOP code d1rzfl1; (Immunoglobulin light chain lambda variable domain, VL-lambda from Human (*Homo sapiens*), cluster 2) with 77% amino acid ID and an E value of 3.3×10^{-16} , Estimated precision was 100%. (NB high-confidence homologs have E values < 0.001). These two models were then aligned to the variable heavy and light chains in an endogenous IgG with a known structure taken from the RCSB Protein Data Bank or PDB (<http://www.rcsb.org/pdb/home/home.do>) structure ID =1IGT, which is of a intact IgG2a monoclonal antibody refined to Resolution of 2.80 Å and an R value of 0.209. (Harris et al., 1997).

The results from this modelling work for C6.5-k were manipulated in The PyMOL Molecular Graphics System, Version: Open-Source PyMOL 1.1eval. Schrödinger, LLC. (<http://pymol.org/>)

The 3D model was also put through the PROPKA 3.0 server (Jensen Research Group, Department of Chemistry, University of Copenhagen) (<http://propka.ki.ku.dk/>).

PROPKA calculates the predicted pK_a values of ionisable residues (including lysines) taking into consideration extra information from the 3D structure file such as spatially neighbouring residues and solvent exposure (Bas et al., 2008).

2.1.4 Cell Based Methods

2.1.4.1 Cell culture

SKOV3 cells (human ovarian adenocarcinoma)

ATCC number = HTB-77TM (<http://www.lgcstandards-atcc.org>)

Cells were cultured in DMEM media (PPA E15-883) or phenol red free DMEM media (PAA E15-877) supplemented with 10% FBS (Gibco 10108-165) , incubated at 37°C / 5%CO₂ and split twice a week along standard cell culture techniques for an adherent cell line.

U-87 cells (human brain glioma)

ATCC number = HTB-14TM (<http://www.lgcstandards-atcc.org>)

Cells were cultured in DMEM media (PPA E15-883) or phenol red free DMEM media (PAA E15-877) supplemented with 10% FBS (Gibco 10108-165) and 1% NEAA (SIGMA M7145), incubated at 37°C / 5%CO₂ and split twice a week along standard cell culture techniques for an adherent cell line.

Jurkat E6-1 cells (human acute T cell leukaemia)

ATCC number TIB-152TM (<http://www.lgcstandards-atcc.org>)

Cells were cultured in RPMI media (PPA E15-885) or phenol red free RPMI media (PAA E15-848) supplemented with 10% FBS (Gibco 10108-165) and 1% NEAA (SIGMA M7145) 1mM Sodium Pyruvate (SIGMA 58636) and 50uM β-mercaptoethanol, incubated at 37°C / 5%CO₂ and split twice a week along standard cell culture techniques for a suspension cell line.

Initially all cell lines were grown up and multiple stocks made and stored in 20 % DMSO in FCS under liquid nitrogen along standard cell culture techniques. Cell used in all experiments presented in this thesis were of passage numbers no more than 15 more than this original stock.

Genetic abnormalities in SKOV-3 cells

It is worth noting that SKOV-3 are a hypoploidy cell line, information taken from ATCC (<http://www.lgcstandards-atcc.org>) states the modal chromosome number for this cell line is 43, occurring in 63.3% of cells with a range from 42-45.

Listed are the coding gene mutations mentioned on the Wellcome Trust/Sanger Institutes COSMIC database: SKOV-3 COSMIC I.D. = 905959. (Forbes et al., 2006), Care has to be taken using such databases as they can contain both cancer causing mutations and passenger mutations. Additionally due to the complexity of signalling pathways post gene transcription, these mutations in single genes, although interesting, are currently unlikely be able to tell us everything about how our cell line will behave.

- Homozygous mutation in both p53 genes. (triggers cell cycle arrest and apoptosis in response to diverse stress stimuli)
- Homozygous deletion mutations in the gene coding for Cyclin-dependent kinase inhibitor 2A (negative regulator of proliferation of normal cells)
- Heterozygous substitution missense mutation in the gene coding for Phosphoinositide-3-kinase (PI3K), (Involved in the generation of PIP3 in the cellular membrane which activates signalling cascades involved in cell growth, survival, proliferation, motility and morphology)
- Homozygous whole gene deletion of the gene coding for mismatch repair protein Mlh1, (a component of the post-replicative DNA mismatch repair system).

2.1.4.2 Control compounds

Staurosporine (Merck 569397)

(Mw = 466.5) Stock solution made up at 1mM in DMSO and stored at -80°C

A small molecular weight compound (466.5 Da) that is a cell permeable promiscuous kinase inhibitor (Karaman et al., 2008). Due to the promiscuous nature of staurosporine- it can induce apoptosis in a wide range of cell lines, and has an ability to initiate apoptosis via different mechanisms, both caspase dependent and independent (Belmokhtar et al., 2001; X. D. Zhang et al., 2004). This means inherent mutations within cancer cell lines that convey protection from apoptosis will be less influential. Staurosporine at sub apoptotic doses can induce cell cycle arrest at various points; relatively low dose can cause G1 cell cycle arrest and subsequent accumulation at this phase and relatively high dose can induce G2/M arrest (Bernard et al., 2001).

Triton X 141, (Sigma X114)

(Av Mw =537) Stock solution made up at 10% in Water and stored at room temperature.

Triton X 114 is an non-denaturing non-ionic detergent and for cell lysis is used at levels sufficient to break open cellular membranes and form mixed cell lipid/detergent micelles, i.e. above the critical micelle concentration (Koley and Bard, 2010), the CMC for Triton X 114 is approx 0.2mM or 0.009% in water), below CMC triton has been shown to be able to induce an apoptotic like phenotype including DNA laddering (Strupp et al., 2000) .

Cisplatin (Sigma P4394)

(Mw = 300.05) Stock solution made up at 10mM in DMF and stored at -80°C

A platinum containing clinical chemotherapy compound (Mw 300), thought to induce apoptosis primarily via its ability to form cross links between DNA bases (Jamieson and Lippard, 1999; Siddik, 2003). The influence of additional protein adducts is also suggested, and like staurosporine, a number of different cell death mechanisms can be initiated (Berndtsson et al., 2007). Cisplatin at low doses is also known to be able to induce cell cycle arrest at various points in particular an S phase block that in some cases appears to be transient and leads to a delayed G2 block (Qin and Ng, 2002; Siddik, 2003)

2.1.4.3 Photodynamic therapy;

Pyropheophorbide-a (PPa) (Frontier Scientific Inc 24533-72-0)

PS4 and PS1 synthesised in house. (Dr G. Yahioğlu and Dr. I. Stamati)

PS stored in 100% dry DMSO at 4°C in a dark dry environment.

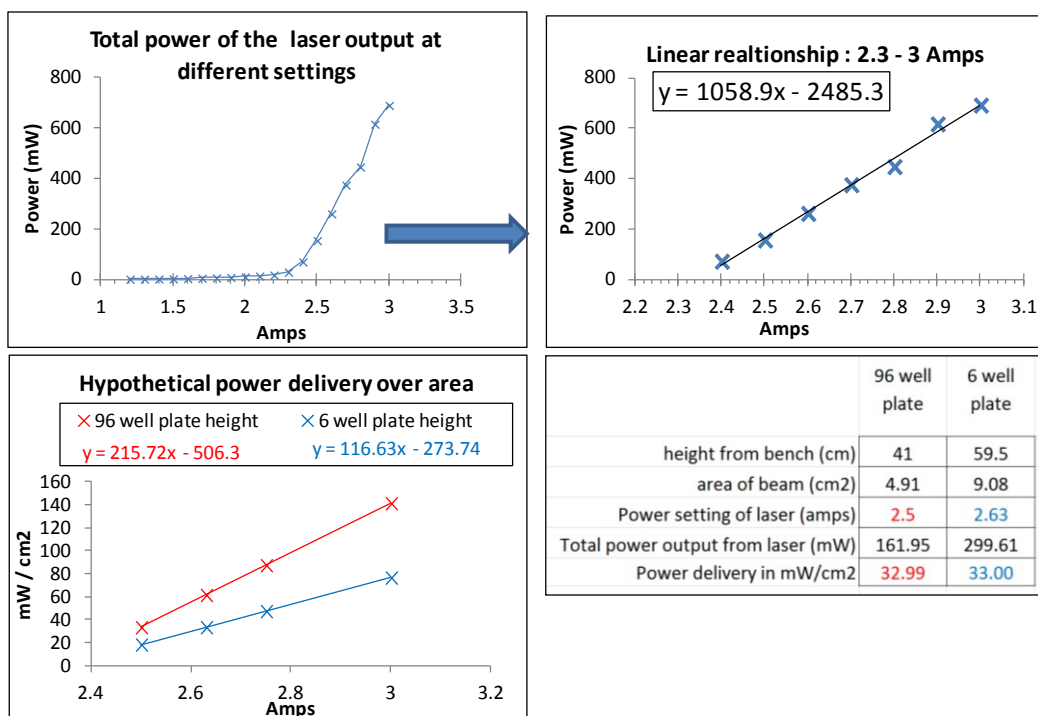
HPD 7401 laser system

Cells were plated in black 96 or 6 well plates, replicate plates were set up for +/- laser. The day after plating, media was replaced with new media containing PS. PS was diluted into supplemented phenol red free cell culture media (PS stock in 100% DMSO, PPa 18.7mM, PS1 24.52mM, PS4 25.11mM) and final DMSO made up to 2%, DMSO vehicle and compound controls were also plated. Plates were protected from ambient light with foil and where necessary assays were carried out in subdued lighting. Cells were incubated for a further 2 hours then washed twice with PBS, media re-added then cells were lasered.

Calibration of laser power:

The laser power output over the whole beam was measured and optimised to deliver 33 mW/cm² in both microplate formats, see below. The standard laser dose was 10 seconds of 670nm laser light at a final fluence of 0.33 J/cm².

(1 Watt = 1 Joule / Second)



2.1.4.4 MTS assay

CellTiter 96 Aqueous One Solution Cell Proliferation Assay (PROMEGA G3580).

Read on : SPECTRAmax 340PC Microplate Spectrophotometer

Cells were plated in a 96 well plate at 2500 cells/well in 200 µl supplemented DMEM phenol red free media (phenol red interferes with absorbance readings at 490nm.) After treatment 20 µl MTS assay reagent was added directly to each well and the plate incubated at 37°C / 5%CO₂, protected from light, for a further 2 hours. The absorbance at 490nm was then read.

What the MTS assay measures

The MTS assay is a well used standard end point for the *in vitro* cyto-toxicity assay. It is an adaptation of a relatively old technique used to measure cell viability via metabolic capacity (the MTT assay) (Mosmann, 1983). It is based on the fact that metabolism in viable cells produces reducing equivalents- these will indirectly reduce the MTS reagent into a soluble coloured formazan product. At death, cells rapidly lose this ability to reduce MTS, and so the production of the coloured formazan product is proportional to the number of viable cells in culture. Reduction occurs at various locations throughout the cell (Bernas and Dobrucki, 2002). Key reducing agents thought to be responsible for the change include the reduced pyridine nucleotides NADH and NADPH (Takahashi et al., 2002; Vistica et al., 1991).

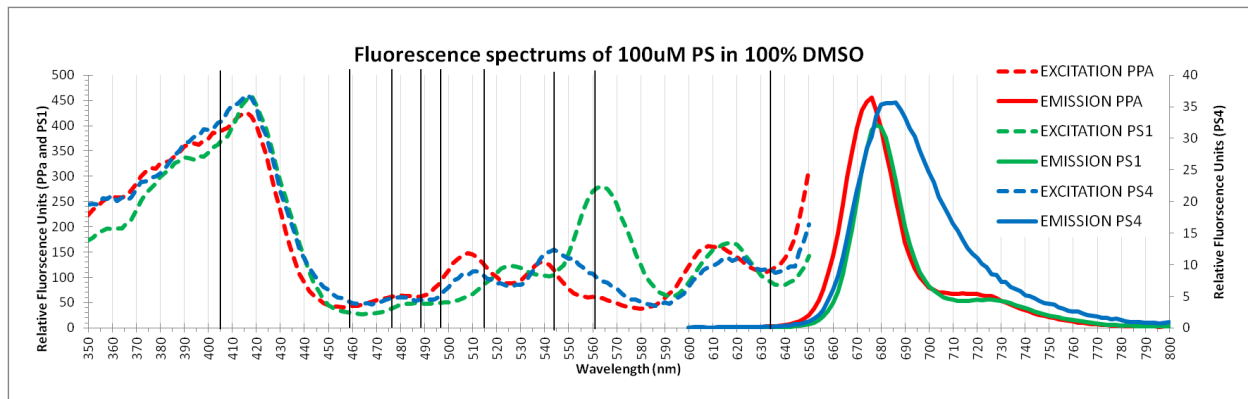
2.1.4.5 Confocal fluorescent microscopy

Image management: LAS AF Lite: Version 2.6.0 (Leica Microsystems CMS GmbH)

Cells were plated in a Lab-Tek 8 well chambered borosilicate cover glass (NUNC 155411) at 10,000 cells/well in 200 µl supplemented DMEM phenol red free media, replicate wells were set up for dye free cell controls for endogenous fluorescence (negative control). After treatment and depending on experimental set up, cells were either; imaged directly, washed once in PBS prior to imaging, or washed with PBS, fixed with 4% methanol free formaldehyde in PBS (Thermo Scientific 28906) for 10 minutes at room temperature, washed again and then imaged. Lasers and detector set up was optimised for each experiment so no fluorescence was seen from negative control and the control predicted to have the highest fluorescence was not saturated. See individual experiment details for the objectives, laser and detector settings used. Between comparable images, laser parameters and detector gain and offset were not adjusted.

PS fluorescent spectra overlaid with the available lasers

Fluorescence spectra of equivalent concentrations of photosensitisers read in 100% DMSO on Cary Eclipse Fluorescence Spectrophotometer. Due to its lower fluorescent efficiency PS4 is plotted on a different axis to PPa and PS1 to show the optimal spectral pattern.



LASERS = NearUV: 405, Argon: 458/476/488/496/514, DPSS: 561, HeNe: 543/633

Quantification of confocal fluorescent microscopy image

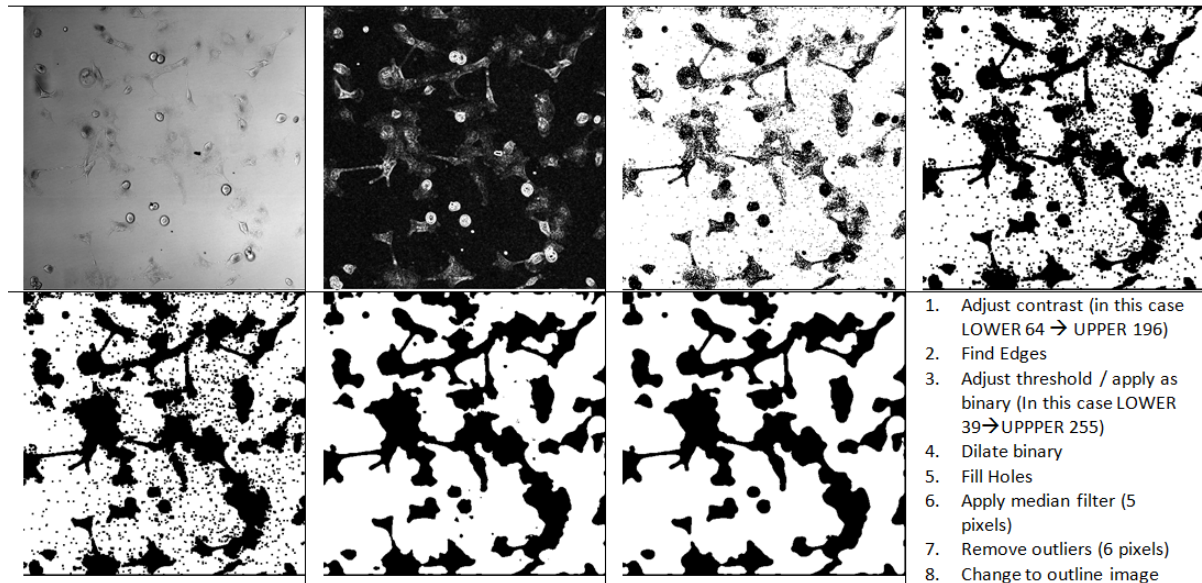
SOFTWARE = Image J, Version 1.45s, (Wayne Rasband, National Institutes of Health)
<http://rsbweb.nih.gov/ij/>

To quantifying the images for fluorescent intensity a mask was made from the brightfield image to cover the area of the image not covered by cells. One of the macros written for this in Image J software and examples of what is happening to the image is shown over the page. Average fluorescent intensity can then be measured from the fluorescence images but restricted to the area prescribed by the brightfield mask. The fluorescent image is opened alongside the brightfield binary image in image J. The fluorescent image needs to be converted to an 8-bit greyscale image and if actual cell area is required the scale can be set to μm with the known image width (found in original image information in LAS AF Lite software). Using Image Js analyze particles plugin the measurements need to be re-directed from the binary image to the fluorescent one and the mean grey value for each independent 'particle' on the binary image is calculated, this can then be averaged relative to particle size to obtain average fluorescence intensity for the cell only area.

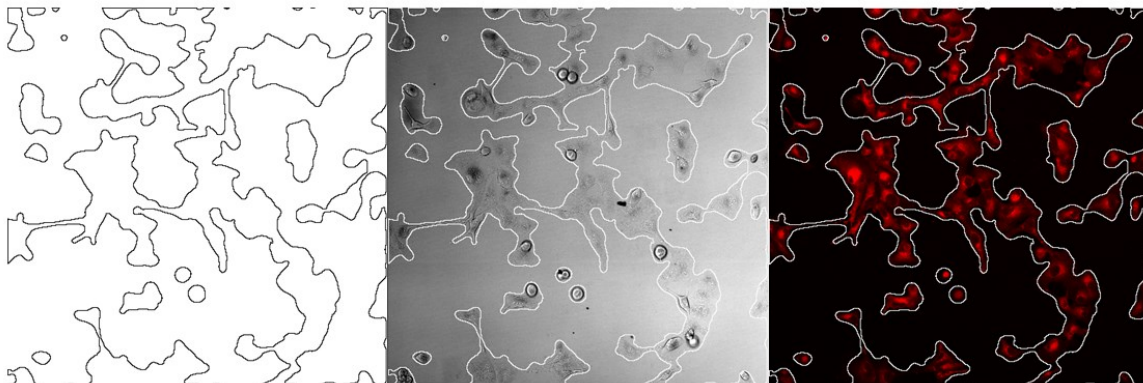
Macro for brightfield image conversion to a binary image:

```
run("8-bit");  
//run("Brightness/Contrast...");  
run("Enhance Contrast", "saturated=0.35");  
run("Apply LUT");  
run("Find Edges");  
setAutoThreshold("Default");  
//run("Threshold...");  
setThreshold(40, 255);  
run("Convert to Mask");  
run("Dilate");  
run("Median...", "radius=5");  
run("Remove Outliers...", "radius=6 threshold=50 which=Dark");
```

What is happening during macro

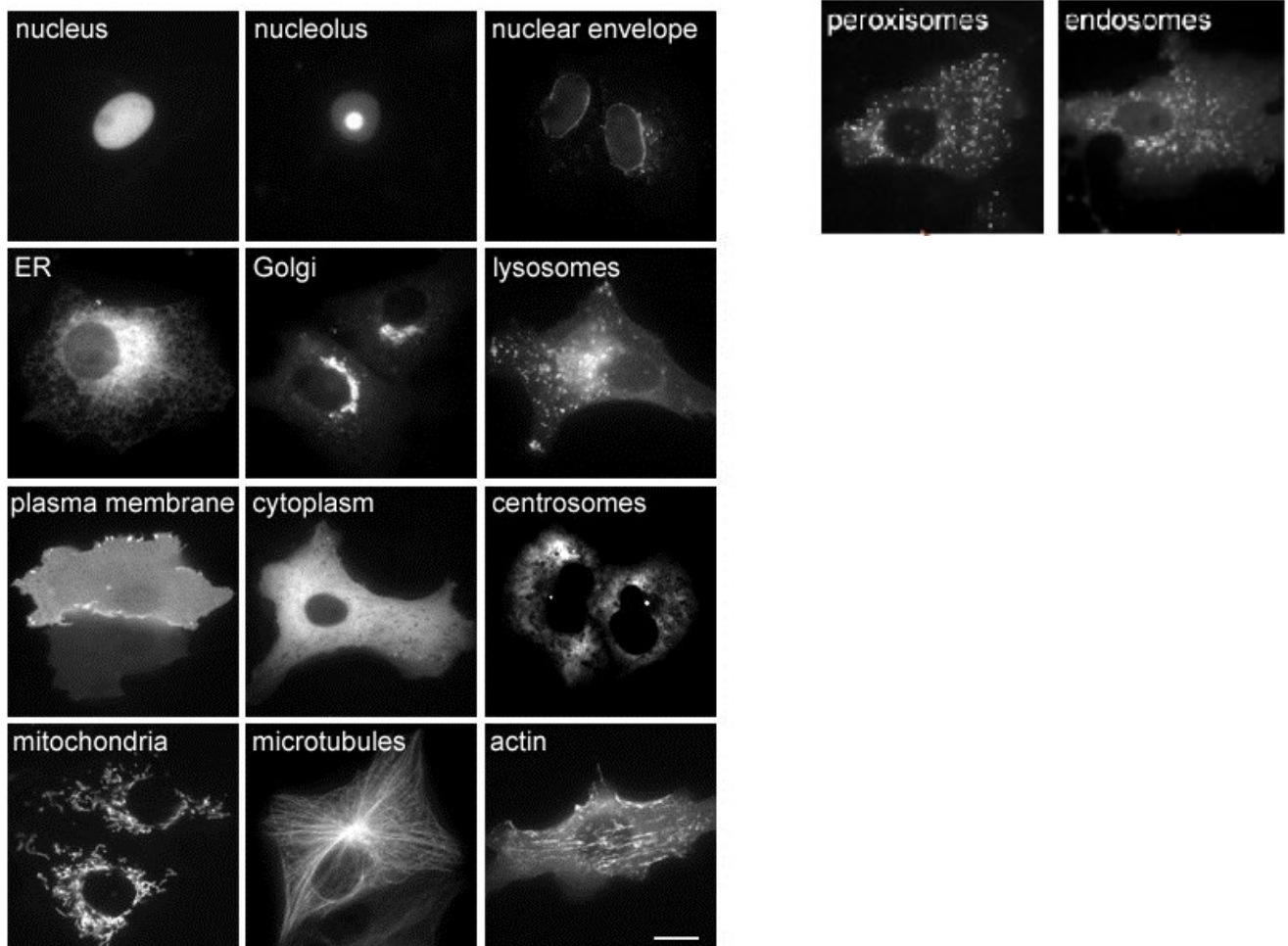


Below is a representation of the binary image above converted to an outline and overlaid with the original cell images.



Recognising patterns of intracellular localisation

Identification of intracellular localisation was based on fluorescent images of proteins with prescribed localisations, in particular images from the work by both the Pepperkok lab and the Wiemann lab in their GFP-cDNA Localisation Project. Example images from this are shown in below or can be found online (<http://gfp-cdna.embl.de/>). or in papers such as (Simpson et al., 2000). Examples of such images produced using GFP tagged proteins are shown below



2.1.4.6 PS Uptake experiment

Read on : Cary Eclipse Fluorescence Spectrophotometer

Uptake experiment in a 96 well microplate

Cells were plated in 96 well plate at 8000 cells/well, cell containing plates were also set up for the standard curves. The day after plating, media was replaced with new media containing PS. PS was diluted into supplemented phenol red free cell culture media (PS stock in 100% DMSO, PPa 18.7mM, PS1 24.52mM, PS4 25.11mM) and final DMSO made up to 2%, DMSO. For the standard curves known dilutions of PS in 100µl DT lysis solution (DT=0.25%triton x 114 in DMSO) were plated directly over the cells. Plates were protected from ambient light with foil and where necessary assays were carried out in subdued lighting. Cells were incubated at the desired condition for the chosen time and then washed twice with PBS before adding 100ul DT lysis solution (DT=0.25%triton x 114 in DMSO) to each well and the plates read alongside the standard curve plates. The fluorescence was read using Excitation at 410nm and Emission at 680nm.

Uptake experiment in cell culture flask

Adaption to a larger cell number; cells were grown in small flasks covering an area of 25cm², exposed to PS in cell culture media as before and then (as quickly as possible and under subdued lighting) washed twice, harvested with trypsin (via normal cell culture technique), and washed with PBS again, all via centrifugal wash steps, the cell pellet was then lysed into 350ul DT lysis buffer. This was then divided into 100ul aliquots over 3 wells in a 96 well microplate and the fluorescence read from the 96 well plate as before. For the standard curves known dilutions of PS in 100ul DT lysis solution (DT=0.25%triton x 114 in DMSO) were plated in empty 96 well microplates to be read at the same time as the sample plates

2.1.4.7 Protein assays

Micro BCA Protein assay kit – Thermo scientific (23235)

Coomassie (Bradford) Protein assay kit – Thermo scientific (23200)

Additional information BCA Assay

Bicinchoninic acid (BCA) is the detection reagent for Cu^{+1} , which is formed when Cu^{+2} is reduced by protein in an alkaline environment. A purple-coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations. The macromolecular structure of the protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) have all been reported to be responsible for colour formation with BCA <http://www.piercenet.com>

Additional information Bradford Assay

When coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465nm to 595nm with a concomitant colour change from brown to blue. NB: Differences in amino acid sequence, isoelectric point, structure and the presence of certain side chains or prosthetic groups can alter the protein's colour response <http://www.piercenet.com>.

2.1.4.8 Trypan Blue assay.

Trypan Blue (Sigma T8154).

Cells were plated in a 96 well plate at 8000 cells/well, and the day after cells are treated. After treatment media is removed, and cells washed in PBS, Trypan blue was added in PBS at a final concentration of 0.2% and left at room temperature for 5 minutes, cells were then washed again and then fixed with 4% methanol free formaldehyde in PBS (Thermo Scientific 28906) for 10minutes room temperature, solution was then removed and 50 μl PSB added. Cells could then be stored at 4°C until they could be imaged on an inverted light microscope at x63 objective under white light from a mercury bulb.

2.1.4.9 Caspase assay.

Apo-ONE Homogeneous Caspase-3/7 Assay(PROMEGA G7790).

Read on : Cary Eclipse OR Fluroskan Ascent FL Fluorescence Spectrophotometer.

Cells were plated in a black 96 well plate at 8000 cells/well. During treatment final volume was adjusted to 100 µl. After treatment 70 µl media was removed from each well and 25 µl caspase reagent added, the plate was incubated in foil at 37°C / 5%CO₂, protected from light for 6 hours. The fluorescence was read using Excitation at 500nm and Emission at 520nm.

The caspases detected in the caspase assay

To measure caspase 3 and 7 activation cells are permeabilised and a profluorescent substrate specific for caspase 3 and 7 is delivered, any active caspase 3 and 7 that is present can then cleave the assay substrate into its fluorescent form – this can then be measured. The main caspases that participate in apoptosis can be divided into the initiator caspase group, which includes caspases 2, 8, 9 and 10; usually activated by dimerisation, and the effector caspase group to which caspases 3, 6 and 7 belong; usually activated by cleavage. Effector caspases can be activated by a number of different upstream apoptotic signalling pathways (traditionally by cleavage by initiator caspases) and so the presence of activated effector caspases is a convenient way to look for activation of a number of apoptosis pathways in parallel. (Huai et al., 2010; Kroemer and Martin, 2005; Pop and Salvesen, 2009).

2.1.4.10 DNA fragmentation assay

For each condition cells were plated in an entire 6 well plate at 80,000 cells per well. After treatment cells were trypsinised and pooled, the cell pellet obtained from both well supernatant and trypsinised cell layer was snap frozen in a 100%EtOH/Dry ice mixture and stored at -80°C. Subsequently genomic DNA was extracted using a Phenol/Chloroform extraction technique detailed over the page. After extraction DNA (4 µg) was made up in DNA loading dye (Fermentas R0611) and loaded into a 1.5% agarose gel and ran slowly at 60V for 1 hour. The gel was then stained in either ethidium bromide (SIGMA E7637) or GelRedTM (Biotium) in TE buffer at 0.5 µg /ml EtBr or 1X GelRedTM respectively. Gels were visualised under UV light. If necessary used two 8 well combs stuck back to back to widen the well so it could take a capacity of ~50µl, (single comb was only ~15µl.)

Detailed method: Extraction of Genomic DNA from Cells

Note: Do all of the following transfers of the DNA-containing supernatant with a wide-bore pipette tip or a tip with the end cut off. This is because the DNA will be viscous, and it can be easily sheared.

Extraction buffer (pH8) =10mM Tris-Cl / 0.1 M EDTA / 0.5% SDS / MilliQ Water

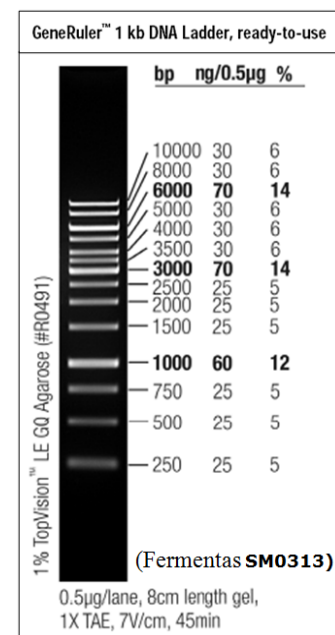
Just prior to experimentation supplement extraction buffer with final concentration 20µg/ml RNase (SigmaR5125) and 100µg/ml Proteinase K (SigmaP2308). Resuspend cells (from frozen pellet) in 1ml extraction buffer. Detach cell pellet by carefully flicking tube with finger or gentle pipetting. Incubate at 55°C for 3 hours or until pellet is dissolved. Cool to room temp. Add 1ml of phenol equilibrated to pH 8.0 (SigmaP4557) to each eppendorf. Mix two phases by slowly turning tubes over for 10 minutes. Centrifuge at 2000rpm for 2mins to separate the phases on a bench top centrifuge. Transfer the top aqueous phase (DNA-containing) to new tubes. Repeat the extraction using 1ml of phenol+chloroform(1:1). Again save the top phase. Repeat extraction using 1ml of chloroform. Again save the top phase. Divide the top phase into 2 (~500ul/eppi) into 2ml eppendorfs. Add 1ml 100% Cold EtOH (-20°C) and 100ul 5M NaCl to each. Put at -20°C for at least two hours (overnight also ok). Spin on high (~14,000rpm) at 4°C in microfuge to precipitate DNA for 15 minutes. Remove EtOH and resuspend pellet in 700ul 70% ethanol. Spin on high (~14,000rpm) at 4°C for 5 minutes. Remove as much EtOH as possible and invert on tissue and allow any remaining EtOH to evaporate (can put on 37°C heat block with lid open for 10/20min to help.) Add 100ul TE buffer (pH8) to all tubes and resuspend pellet, recombine split samples as one here.

QUANTIFICATION

Take a 10ul sample of DNA and mix with 190ul TE buffer (1:20 dilution) and measure absorbance on UV-VIS spec reader over 190-400nm and record values for A260. 1 x A260 unit of double stranded DNA = 50ug DNA/ml so use the following equation to calculate DNA concentration of sample.

$$A_{260}(1\text{cm}) \times 50 \times \text{dilution factor} = \text{DNA in ng/}\mu\text{l}$$

DNA marker (Fermentas SM0313) →



2.1.4.11 Cell cycle assay

FACSCalibur flow cytometer (BD Biosciences)

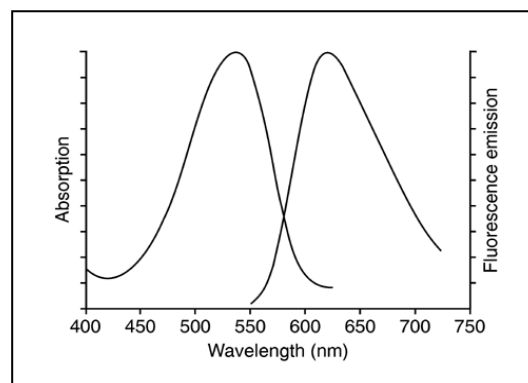
Software = FlowJo. Version: 7.6.5 (Tree Star, Inc.) <http://www.flowjo.com/>

For each condition cells were plated in an entire 6 well plate at 80,000 cells per well. Include additional conditions exposure to control compounds (positive controls) and an untreated control for endogenous fluorescence (negative control). Replicate plates were set up for each condition for a saponin free PI stained cell control to check for any cell death. After treatment cells were removed with Accutase solution (PAA L11-007) and pooled, the cell pellet obtained from both well supernatant and cell layer was resuspended and washed once in PBS then resuspended in 200ul PBS+5%FCS. For negative control leave unsupplemented, for cell death controls supplement with 50ug/ml Propidium Iodide (Sigma P4864), for cell cycle analysis supplement with 50ug/ml Propidium Iodide and 0.05% Saponin (Sigma S7900). Store on ice and run directly on a flow cytometer, readjusting detection channels between cell death analysis and cell cycle analysis. For cell cycle analysis set up to include FL-2 as the DDM (see below)

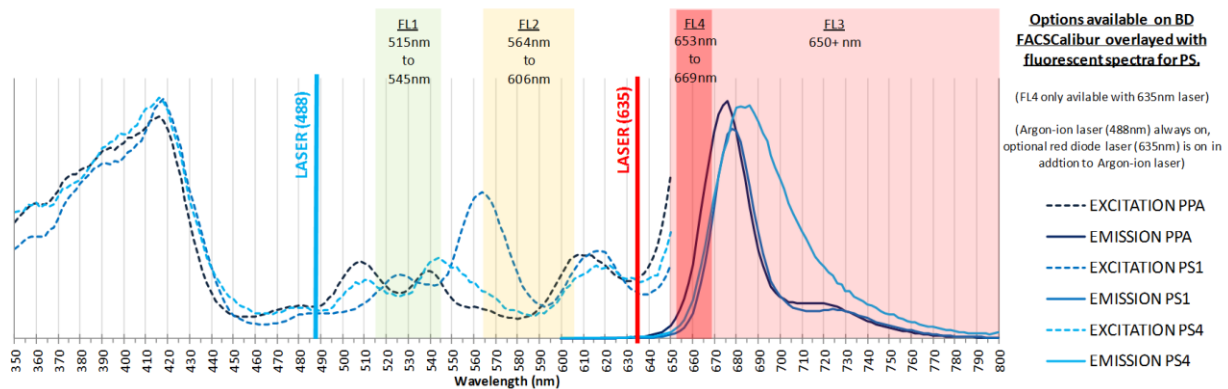
Background information -Cell cycle assay

To identify what part of the cell cycle a population of cells are in one of the most established methods involves cell permeabilisation and subsequent staining of genomic DNA with propidium iodide, multi-parametric analysis by flow cytometry can then determine the difference in fluorescence between individual cells with 1 set of chromosomes (cell cycle position G1), 2 full sets of chromosomes (cell cycle position G2) or mid synthesis of the second set, i.e. somewhere in between G1 and G2 (cell cycle position S) NB: any cells actively undergoing Mitosis (M) will appear with G2 and any cells in quiescence (G0) will occur with G1. Each cell is measured independently and so can be assigned to a cell cycle position depending on the amount of DNA they contain (the intensity of their fluorescence). Any apoptotic or necrotic cells with partially degraded or fragmented DNA will be shown within the DNA histogram as a sub-G1 peak (Ormerod, 2002) .

Fluorescence of propidium iodide bound to DNA →
<http://www.invitrogen.com/site/us/en/home/support/Product-Technical-Resources/Product-Spectra.1304dna.html>

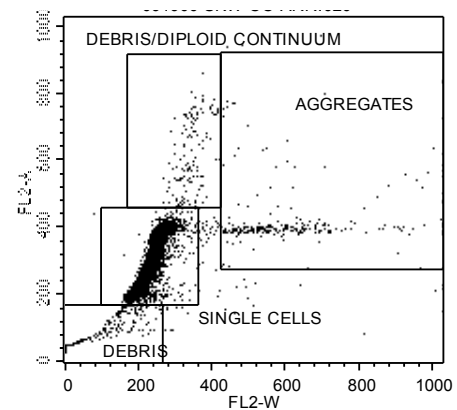


PS Fluorescence spectra of PS overlaid with BD FC Calibur setup options.



Data manipulation for cell cycle analysis

Fluorescence intensity is detected in channel FL-2 as the DDM parameter (doublet discrimination mode), DDM means it will record FL2- W (width of fluorescent pulse, i.e. time taken to travel through detector) FLA-H (height of pulse, i.e. maximum fluorescence intensity) and FL2-A (area, i.e. total fluorescence of pulse, $\text{area} \times \text{height} / 2$). FL2-H which is recorded on a log scale normally is sufficient to see shifts positive and negative between populations, but as cell cycle data requires a greater sensitivity it is recorded on a linear scale and so 2 cells which are in G1 travelling together will give the same signal as one cell in G2 travelling alone. For this reason it is important to separate artefacts such as these. To do this, as well as separating signal from larger aggregates or cell debris, data can be gated out by plotting FL2-A vs. FL2-W and gating out all outliers as shown on the right. (Taken from : <http://www.ucl.ac.uk/wibr/services/docs/cellcyc.pdf>)



After gating FlowJo was used to fit a Watson Pragmatic model to the FL2-A histogram in order to define the G1, S, and G2 phases of the cell cycle. This model fits G1 and G2 with Gaussian curves and no assumptions are made about the shape of the S-Phase distribution. When cell cycle curves were modelled in all cases G2 was constrained to be twice the fluorescence of G1 – the centres of these peaks at the constrained distance are marked as lines in the figure. More information on the software can be found at:

<http://www.flowjo.com/home/tutorials/TN/cellcycleweb2010.pdf>

2.1.5 Protein Based Methods

2.1.5.1 Cloning of C6.5-k

C6.5 is a fully human anti-HER2 single-chain Fv isolated from a filamentous phage antibody library, first described by (Schier et al., 1995), see 5.1.1. The original paper by (Schier et al., 1995) presented the full amino acid sequence of the heavy chain and light chain (without any tags or linkers, the linker they used was a 'single chain linker' (G₄S)₃ like ours). Later work produced a range of higher affinity versions by light chain shuffling, heavy chain shuffling, and molecular evolution of the VL CDR3 region (Schier et al., 1996a, 1996b).

Previous work within this lab had cloned the full length scFv antibody fragment C6.5 (obtained from Prof J. Marks (University of California, San Francisco) with an N terminal pelB secretion sequence, C terminal His and Myc tags into a PUC119 vector under control of the lac promoter. An annotated sequence confirmed by recent sequencing for the inserted antibody is shown in section 2.1.5.2. Various mutations to the amino acid sequence were carried out including: The lysine in CDR3 was changed to an alanine (K111A) and two leucine to valine mutations were introduced at the N terminal - it was then termed C6.5-k. This sequence compared to the sequence of the original C6.5 antibody is shown below. (Mutagenesis, expression and Biacore analysis for kinetic constants (see 5.1.1.) were carried out at within the lab of M.P.Deonarin at Imperial College)

Changes CDR regions			
C6.5-K	QVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPGDSDTKY	60	
C6.5_Schier	QVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPGDSDTKY	60	

C6.5-K	SPSFQGGVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHDVGYCSSSNCAWPEYFQHWG	120	
C6.5_Schier	SPSFQGGVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHDVGYCSSSNCAWPEYFQHWG	120	

C6.5-K	QGTLTVTSVSGGGSGGGSGGGSGSVLTQPPSVSAAPGQKVTISCSSSSNIGNNYVSW	180	
C6.5_Schier	QGTLTVTSV-----QSVLTQPPSVSAAPGQKVTISCSSSSNIGNNYVSW	165	

C6.5-K	YQQLPGTAPKLLIYGHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYCAAWDDSL	240	
C6.5_Schier	YQQLPGTAPKLLIYGHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYYCAAWDDSL	225	

C6.5-K	SGWVFGGGTKLTVLGAAAEQKLISEEDLNGAAHHHHHH	278	
C6.5_Schier	SGWVFGGGTKLTVLG-----	240	

2.1.5.2 Annotated amino acid and DNA Sequence of C6.5-k

KEY

Start /Stop

His Tag (HHHHHH) Myc Tag (EQKLISEEDL)

pelB leader sequence (MKYLLPTAAAGLLLLAAQPAMA)

Nco1(C/CATGG) Not1 (GC/GGCCGC)

Linker region

Heavy chain framework regions (1,2,3 and 4)

/ Light chain framework regions (1,2,3 and 4)

Heavy chain CDR (1,2, and 3)

/ Light chain CDR (1,2, and 3)

							ATG Met	AAA K	TAC Y	CTA L	TTG L	CCT P	ACG T	GCA A	GCC A
							PelB leader Sequence								
GCT A	GGA G	TTG L	TTA L	TTA L	CTC L	GCG A	GCC A	CAG Q	CCG P	GCC A	ATG M	GCC A	CAG Q	GTG V	
							Nco1								
CAG Q	CTG L	GTG V	CAG Q	TCT S	GGG G	GCA A	GAG E	GTG V	AAA K	AAG K	CCC P	GGG G	GAG E	TCT S	
CTG L	AAG K	ATC I	TCC S	TGT C	AAG K	GGT G	TCT S	GGA G	TAC Y	AGC S	TTT F	ACC T	AGC S	TAC Y	
TGG W	ATC I	GCC A	TGG W	GTG V	CGC R	CAG Q	ATG M	CCC P	GGG G	AAA K	GGC G	CTG L	GAG E	TAC Y	
ATG M	GGG G	CTC L	ATC I	TAT Y	CCT P	GGT G	GAC D	TCT S	GAC D	ACC T	AAA K	TAC Y	AGC S	CCG P	
TCC S	TTC F	CAA Q	GGC G	CAG Q	GTC V	ACC T	ATC I	TCA S	GTC V	GAC D	AAG K	TCC S	GTC V	AGC S	
ACT T	GCC A	TAC Y	TTG L	CAA Q	TGG W	AGC S	AGT S	CTG L	AAG K	CCC P	TCG S	GAC D	AGC S	GCC A	
GTG V	TAT Y	TTT F	TGT C	GCG A	AGA R	CAT H	GAC D	GTG V	GGA G	TAT Y	TGC C	AGT S	AGT S	TCC S	
AAC N	TGC C	GCA A	GCG A	TGG W	CCT P	GAA E	TAC Y	TTC F	CAG Q	CAT H	TGG W	GGC G	CAG Q	GGC G	
ACC T	CTG L	GTC V	ACC T	GTC V	TCC S	TCA S	GGT G	GGA G	GGC G	GGT G	TCA S	GGC G	GGA G	GGT G	
							Linker region								
GGC G	TCT S	GGC G	GGT G	GGC G	GGA G	TCG S	CAG Q	TCT S	GTG V	TTG L	ACG T	CAG Q	CCG P	CCC P	

(continued over page.....)

TCA	GTG	TCT	GCG	GCC	CCA	GGA	CAG	AAG	GTC	ACC	ATC	TCC	TGC	TCT
S	V	S	A	A	P	G	Q	K	V	T	I	S	C	S
GGA	AGC	AGC	TCC	AAC	ATT	GGG	AAT	AAT	TAT	GTA	TCC	TGG	TAC	CAG
G	S	S	S	N	I	G	N	N	Y	V	S	W	Y	Q
CAG	CTC	CCA	GGA	ACA	GCC	CCC	AAA	CTC	CTC	ATC	TAT	GGT	CAC	ACC
Q	L	P	G	T	A	P	K	L	L	I	Y	G	H	T
AAT	CGG	CCC	GCA	GGG	GTC	CCT	GAC	CGA	TTC	TCT	GGC	TCC	AAG	TCT
N	R	P	A	G	V	P	D	R	F	S	G	S	K	S
GGC	ACC	TCA	GCC	TCC	CTG	GCC	ATC	AGT	GGG	TTC	CGG	TCC	GAG	GAT
G	T	S	A	S	L	A	I	S	G	F	R	S	E	D
GAG	GCT	GAT	TAT	TAC	TGT	GCA	GCA	TGG	GAT	GAC	AGC	CTG	AGT	GGT
E	A	D	Y	Y	C	A	A	W	D	D	S	L	S	G
TGG	GTG	TTC	GGC	GGA	GGG	ACC	AAG	CTG	ACC	GTC	CTA	GGT	<u>GCG</u>	<u>GCC</u>
W	V	F	G	G	G	T	K	L	T	V	L	G	A	A
<u>GCA</u>	GAA	CAA	AAA	CTC	ATC	TCA	GAA	GAG	GAT	CTG	AAT	GGG	GCC	GCA
A	E	Q	K	L	I	S	E	E	D	L	N	G	A	A
CAT	CAC	CAT	CAT	CAC	CAT	TAA	TAA							
H	H	H	H	H	H	stop	stop							

<http://web.expasy.org/protparam/>

Number of amino acids: 278

Theoretical pI: 6.52

Extinction coefficients are in units of $\text{M}^{-1} \text{cm}^{-1}$, at 280 nm measured in water.

Abs 0.1% (=1 g/l) 2.235, assuming all pairs of cysteine residues form cystines

Abs 0.1% (=1 g/l) 2.222, assuming all cysteine residues are reduced

2.1.5.4 Expression of antibody fragment C6.5-k

Stocks of transformed HB2151 E.Coli bacterial cells containing the antibody vector were stored in 10-15% Glycerol in 2TY Media at -80°C. NB all work carried out using full bacterial sterile technique as standard. Single colonies from this were picked for carbenicillin selection (Melford C0109) and plated on 2TY agar plates (recipe in 2.1.2) supplemented with carbenicillin 0.1mg/ml. A single colony was subcultured into 10ml 2TY media (recipe in 2.1.2) then grown up at 37°C / 250rpm for ~16hours under high (2%) glucose and carbenicillin (0.1mg/ml) conditions overnight until it reached stationary growth phase. At this point bacteria were resuspended in 10ml fresh 2TY, and 5ml subcultured into 500ml fresh 2TY + 0.1% Glucose + 0.1mg/ml Carbenicillin + a drop of Antifoam 204 (Sigma A8311). Bacteria were cultured at 37°C 250 rpm until O.D 600nm was 0.6-0.8 then expression was induced by introduction of 1mM IPTG (Isopropyl- β -D-thio-galactoside) (Melford MB1008) and temperature reduced to 30°C and left to shake for no more than 16 hours

Inclusion of the *PeI*B leader sequence should direct the protein to the periplasmic space where a membrane anchored peptidase will cleave the sequence completely (after “PAMA”) (Hauser and Ryan 2007) (Lei et al 1987). Previous work in our lab (also supported by other work in the literature), show that the majority of the protein produced escapes the periplasm and accumulates in the extracellular culture media. Therefore supernatant was collected and concentrated 10X using a 200 cm² 10,000 MWCO PES crossflow cassette (VIVAFLOW 200, Sartorius Stedium VF20P0) with a SciQ 323 bench top pump system (Watson Marlow). Supernatant was then dialysed into PBS through MWCO 8 kDa cellulose dialysis tubing (Fisher scientific BID-030-040K). From this fully folded C6.5-k was purified via its Myc tag using columns pre-prepared with an Anti Myc antibody (9E10 IgG) isolated on Protein G activated sepharose beads (an anti Fc Protein), elution from column was carried out with 0.2M Glycine buffers of decreasing pH (pH 5, pH 3 and pH 2.5). *Column was previously prepared by Dr Antony Constantinou (Imperial College).*

Analysis of expression

Various short and spacer glass plates and combs for Hand Cast Gels (BioRad)

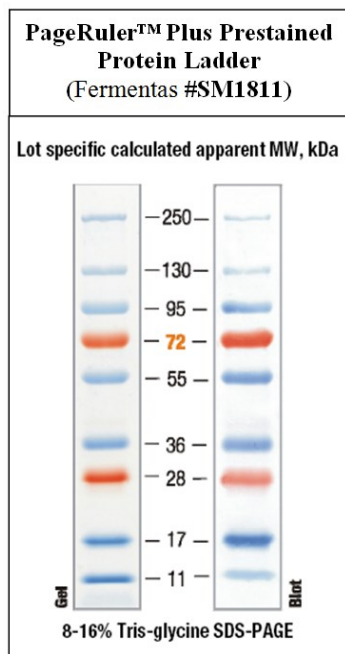
Mini-PROTEAN Tetra Electrophoresis System (BioRad)

Trans-Blot SD Semi-Dry Transfer Cell (BioRad)

ECL Hybond nitrocellulose membrane (GE Healthcare RPN203D)

Blotting paper (BioRad 170-3965)

Samples taken from all wash and elution fractions from the purification process were mixed with Laemmli Loading buffer (recipe in 2.1.2) and separated via SDS PAGE. Gels were cast of 0.75mm thickness, with resolving and stacking gel made up to the 12% Acrylamide SDS Gels recipe above (recipe in 2.1.2). Gels were run under 1X running buffer made up as (recipe in 2.1.2) at constant current (40mA per gel) or a constant voltage (180V) for ~40 minutes or until dye boundary is ~1cm from the bottom of the gel. Two sets of samples were run alongside each other and one gel subsequently stained with coomassie (recipe in 2.1.2) and the second gel was transferred to nitrocellulose via transfer buffer (recipe in 2.1.2) and a semi-dry transfer blotter. The membrane was subsequently blocked (5% Marvel in PBS) for at least an hour then blotted with a peroxidase-conjugated anti-polyHistidine IgG used at 1:5000 for 1 hr in 1% Marvel in PBS (AntiHis HRP Sigma A7058) Washed membrane could then be imaged using an ECL detection reagent (Amersham ECL plus RPN2132) and chemiluminescence detected and imaged on the Las-3000 system. See 2.1.1.5



Protein marker (Fermentas SM1811)

Final protein preparation

The purified fractions containing C6.5-k alone were collected, dialysed into PBS through MWCO 8 kDa cellulose dialysis tubing (Fisher scientific BID-030-040K), concentrated via a 5000 MWCO polyethersulfone membrane using centrifugal pressure (Vivaspin 20, Sartorius Stedium VS2012) and clarified through a 0.2 µm PVDF membrane (Generon # GEN-MSF500) before storage at -20°C.

QUANTIFICATION

Sample was diluted into PBS and absorbance was measured on a UV-VIS spec reader over 190-400nm and values recorded for A280. The molar extinction coefficient (ϵ) is 65235 M⁻¹ cm⁻¹, at 280 nm measured in water, see 2.1.5.3. The Beer-Lambert law states :

$A\lambda = \epsilon c L$ (Thermo Scientific, 2008) so when the cuvette used has a path length of 1cm the following equation can be used to calculate C6.5-k concentration of sample:

$$(A280 / 65235) \times \text{dilution factor} = \text{concentration of protein in Molar}$$

NOTE:. The extinction coefficient was chosen assuming all pairs of cysteine residues form cystines because predicted tertiary structure for immunoglobulin type structures such as the VH and VL domain have two cystine bridges, this accounts for the 4 cysteine residues in the framework regions, another 2 cysteine can be found in the CDR 3 region of the heavy chain, this is a solvent accessible flexible loop region important for antigen recognition so it is likely these other cysteine also form a bond to help give specific structure and therefore specific recognition. (Cystine is the name given to the dimerisation of two cysteine residues via oxidation, the bond is referred to as a disulfide bond, and can contribute to absorbance at 280nm.)

2.1.5.5 C6.5-k binding via Flow cytometry

FACSCalibur flow cytometer (BD Biosciences)

Software = FlowJo. Version: 7.6.5 (Tree Star, Inc.) <http://www.flowjo.com/>

Cells were grown as usual and on the day of the experiment Accutase (PAA L11-007) was used to remove cells from flask. Cells were counted and aliquoted at 300,000 per eppendorf / per condition. Include conditions for unstained and secondary/tertiary only controls. Cells were subjected to centrifugal force and washed in PBS then resuspended in 50µl FC buffer plus the primary antibody. For C6.5-k it was determined to be optimal at 60nM C6.5-k final concentration, for FC buffer (recipe in 2.1.2) and for details on the titration experiment to determine this see 5.2.1.1. Cell were stained on ice in the primary staining solution for 1 hour before washing twice in FC buffer before re-suspending in 50ul FC buffer plus the secondary antibody at 3 times molar excess over primary. (180nM anti-c-Myc monoclonal mouse IgG (9E10) (Sigma M4439)) or (180nM anti- Penta-His monoclonal mouse IgG (Qiagen 34660 made up to 100ug/ml)). Cell were stained on ice in the secondary staining solution for 1 hour before washing twice in FC buffer before re-suspending in 50ul FC buffer plus the tertiary antibody at 3 times molar excess over secondary. (540nM goat anti mouse IgG (whole molecule) FITC conjugate Sigma F2012). Cell were stained on ice in the tertiary staining solution for 30 minutes before washing twice in FC buffer and re-suspended in 300µl FC buffer. Signal from the FITC fluorophore was then detected through channel FL-1 on the BD FACSCalibur machine. See 2.1.4.11.

2.1.6 Chemical Based Methods

2.1.6.1 Spectral analysis

Absorbance spectra carried out using Agilent 8453 UV Visible Spectrophotometer

Fluorescent spectra carried out using Cary Eclipse Fluorescence Spectrophotometer

2.1.6.2 Experimental Log P

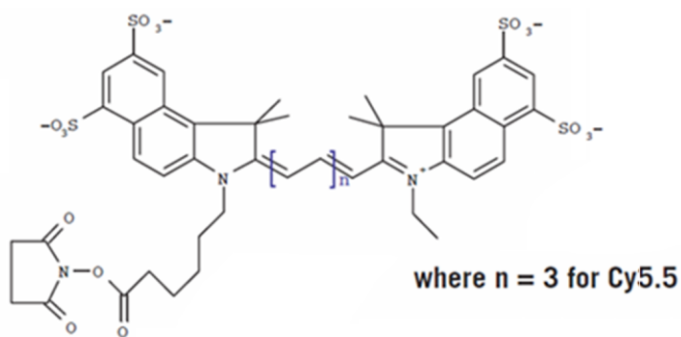
Photosensitisers were diluted 1:1000 into either 1-Octanol or PBS pH7.4 and then added to the opposite solvent. Alongside photosensitisers were also mixed under the same conditions and concentrations in either purely PBS pH7.4 or 1-Octanol. Experiments were carried out in glass vials and solvent mixtures were upturned over 24 hours before layers were separated and UV spectra read of each partition and peak values compared to extinction coefficients previously calculated for each PS in the relevant solvent. Data was calculated utilizing the equation;

$$\text{Apparent LogD} = \text{Log}_{10}([\text{Organic}]/[\text{Aqueous}])$$

Where square brackets correspond to absolute concentrations calculated from both the Octanol and PBS fractions using their extinction coefficients.

2.1.6.3 Cy5.5 AE structure and fluorescent spectra

Amersham Cy5.5 Mono NHS Ester (GE healthcare PA15605) Images and information taken from the product booklet

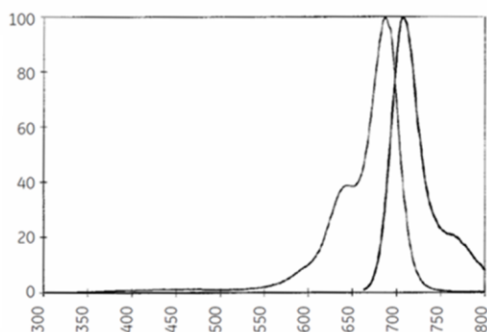


Monofunctional dye characteristics

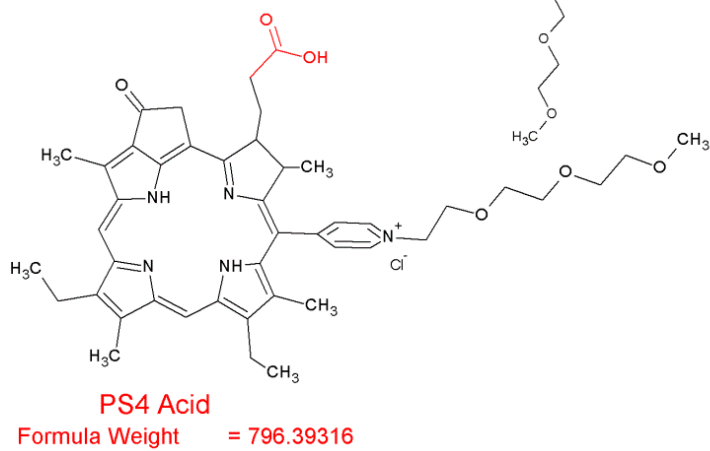
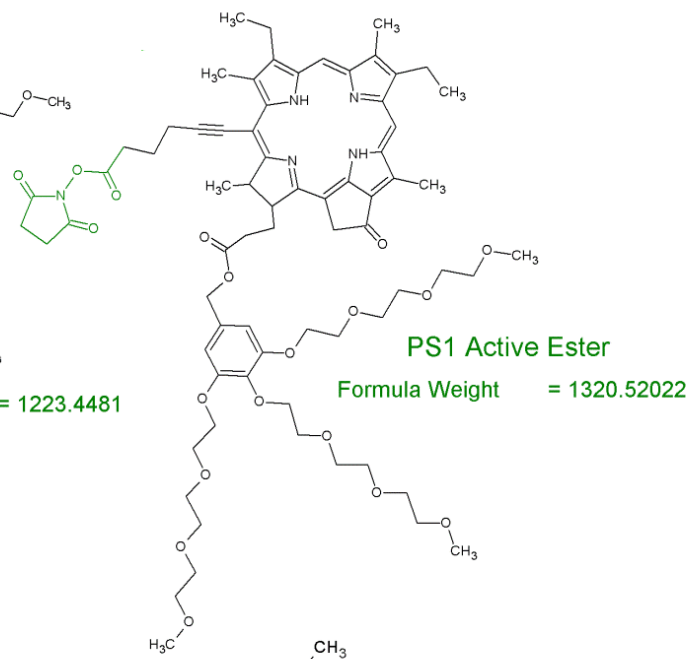
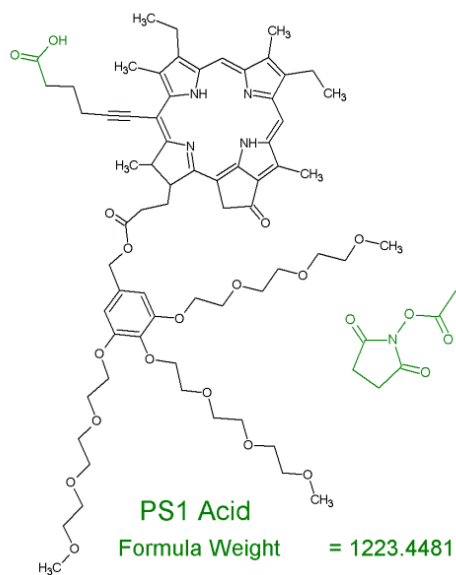
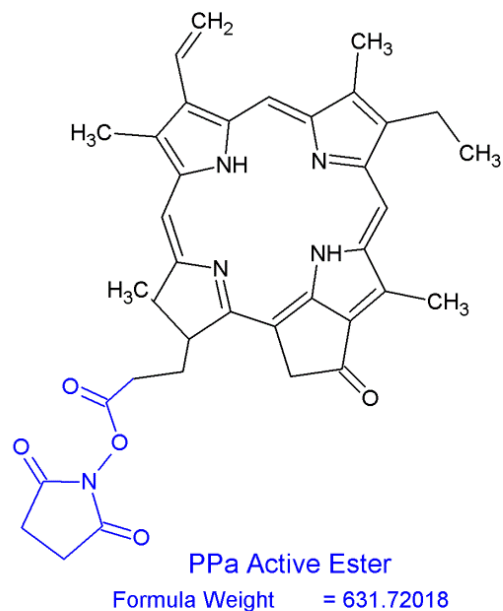
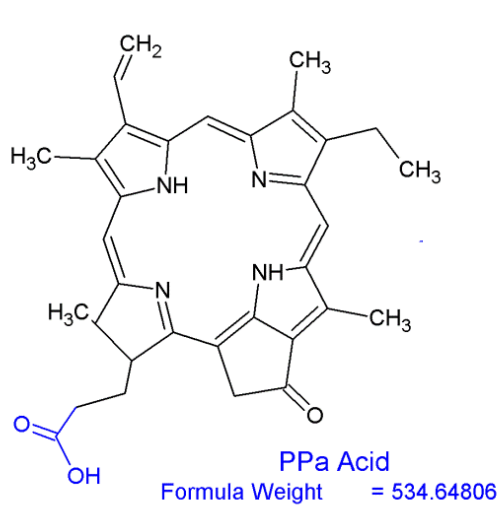
	Cy5.5
Formula weight	1128.42
Absorbance max	675 nm
Extinction max	250 000 M ⁻¹ cm ⁻¹
Emission max	694 nm
Quantum yield	>0.28*

* for labelled proteins, D/P=2

Cy5.5 dye absorption and fluorescence spectra



2.1.6.4 PS Acid and AE structure and Mw



2.1.6.5 Photo-Immunoconjugate production

Amersham Cy5.5 Mono NHS Ester (GE healthcare PA15605)

*NHS Ester forms of PPa, PS1 and PS4 synthesised in house. (Dr G. Yahioğlu and Dr. I. Stamati)
Reconstituted in small batches in 100% dry DMSO and stored at -20°C in a dark environment
and tested periodically via TLC for the extent of any hydrolysis (see 2.1.6.8).*

The reaction conditions previously used within this lab (Stamati et al., 2010) were; C6.5-k at 10 µM in PBS at pH8, PPa AE (active ester) at 16 times molar excess over C6.5-k with additional DMSO added to make the final DMSO 2% of total reaction volume, and MeCN at 6% of total reaction volume. MeCN then PS/DMSO was added to the C6.5-k drop wise in a 15°C water bath over one hour, all the while stirred and protected from light. Conjugation conditions changed throughout the work presented in this thesis but a general method is described below. See main text for more details. The main changes were an increase in the antibody concentration to help encourage interactions between reactants, and changing the buffer in order to raise the pH was so more of the lysine amine groups would be reactive for conjugation, although the rate of hydrolysis of the active ester is also increased which could compromise reactivity.

General method:

C6.5-k was buffer exchanged and concentrated to 160 µM in Carbonate buffer at pH 10.5 (buffer made with varying ratios of 0.1M Na₂CO₃ and 0.1M NaHCO₃) (Delory and King, 1945). PS AE (active ester) dissolved in 100% dry DMSO at a stock of 25.11 mM was added at 1/10th of the volume of the C6.5-k solution along with the same volume of 100% DMSO. (For example: 100 µl C6.5-k + 10 µl DMSO + 10 µl PS AE) The final molar ratio of PS:C6.5-k was 16:1 with 145 mM C6.5-k in the final conjugation mixture and the final DMSO concentration was 16.67%. Mixtures were added immediately and reaction was left shaking and protected from light at RT for 2-3 hours then left overnight at 4°C before analysis and purification.

2.1.6.6 Analysis of photo-immunoconjugate:

Spectral analysis

Spectral analysis of the absorption spectra was undertaken in various different buffers. Absorbance of the conjugate from its peak absorbance in the red (for PS this is Q band I ~680nm) should be solely due to the absorbance of the dye – this can be used to calculate final concentration of dye in the conjugate, the extinction coefficient of the dye at 280nm (as it also absorbs in this part of the spectra) can be used to calculate the contribution to the absorbance at 280nm from the PS and so therefore also from the protein and therefore also the protein concentration. Before spectra could be used to calculate conjugation efficiency, extinction coefficients for 280nm and the peak absorption were calculated for the free dyes in the relevant solution. See figure Figure 51 for Cy5.5 and Figure 57 for PPa and PS1.

Gel analysis

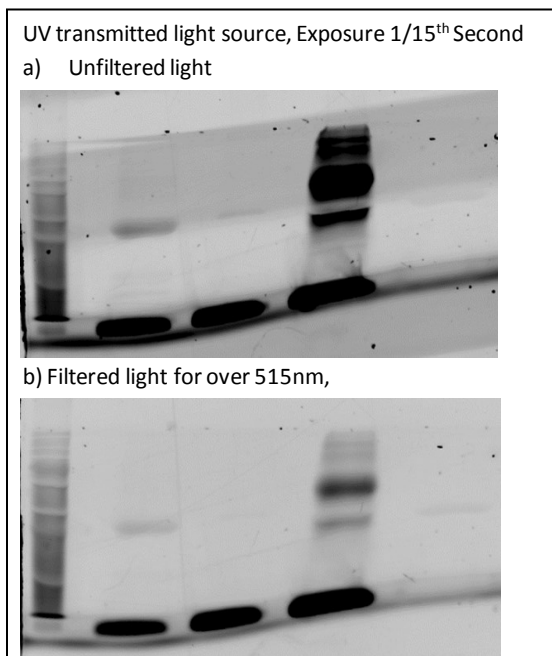
Conjugates were separated on SDS Page gel as described before in 2.1.5.2 the only exception being the protein loading buffer used was made without the blue dye (recipe in 2.1.2) as this travels at the dye front along with any free PS and interferes with the fluorescent signal from the PS. Directly after the run had ended gels were directly imaged under UV light in the Las-3000 see 2.1.1.5, using a filter to remove light below 515nm, the gel was then stained in coomassie.

Image J software has macros designed to calculate band intensity on SDS page gels
SOFTWARE = Image J, Version 1.45s, (Wayne Rasband, National Institutes of Health)
<http://rsbweb.nih.gov/ij/> .

The gel is manually divided into equally sized wells and the intensity of each band converted into a graphical format with the X axis of each well representing the distance travelled down the gel, and the Y axis for the fluorescent intensity of that distance down the well. The areas of these curves can then be calculated and correspond to the total intensity of the band, see 5.2.10.2.

The effect of using a filter to image gels under UV

Unfiltered light shows a seemingly more heavily conjugated sample but also a ‘banding’ pattern from detection of the UV bulbs themselves; shown right, and shows how manipulation of the equipment can change how conjugation effectiveness is perceived. All other conjugate gels presented in this thesis are imaged with filtered light to avoid this banding.



2.1.6.7 Purification of photoimmunoconjugate

Many different methods were tried for PIC purification, see main text for a full discussion. Listed here are the source information for selected pieces used.

- Dialysis tubing : MWCO 8 kDa cellulose (Fisher scientific BID-030-040K),
- Vivaspin 20 ; 5000 MWCO polyethersulfone membrane /centrifugal pressure - (Sartorius Stedium VS2012)
- Vivapure Ion Exchange centrifugal Spin Columns -Q mini H. (Sartorius Stedium VS1X01QH24)
- Ion exchange chromatography columns – HiTrap CaptoG 1ml (GE healthcare 11-0013-02)

2.1.6.8 TLC plates of free PPa and PS1

PS samples were cleaned in a H₂O/DCM mixture, so any DMSO would partition to the H₂O and PS to the DCM. This was important to prevent the PS running inside a DMSO solvent bubble up the plate. Separation obtained by using Silica gel 60 glass TLC plates with F254 indicator (Merck) in a 10% Methanol in DCM solvent system and once removed the solvent front was marked by a pencil line before it evaporated. Images of the plates were taken on a standard computer scanner and RF values were calculated from the plate image in Image J software according to the equation below:

$$\text{RF value} = \text{distance travelled by the compound} / \text{distance travelled by the solvent front}$$

SOFTWARE = Image J, Version 1.45s, (Wayne Rasband, National Institutes of Health)
<http://rsbweb.nih.gov/ij/>

NB PS4 requires a different solvent system; 80 % MeCN plus 10% H₂O and 10% K₂NO₃.

Chapter Three

3 Chemical characterisation of PS and testing of PDT in an *in vitro* model of cancer

3.1 Introduction

3.1.1 Considerations for an effective PDT model.

Chapter 0 introduced some clinical factors that influence PDT. This thesis however will mostly focus on the influences on a chemical and cellular level. Cellular processes that are well validated in the literature to be important for effective PDT include cellular uptake rates, the uptake mechanism, the final intracellular localisation and the type of cell death mechanisms induced. These processes are discussed in more detail in chapter 0.

3.1.1.1 The end point

Drugs in preclinical use are often compared in their efficacy by an *in vitro* cytotoxicity measurement, in many cases this is described as its IC₅₀ value. To calculate this parameter, *in vitro* cytotoxicity assays are set up using an appropriate cell line and the drug is tested for the concentration required to kill 50% of the cells. IC is actually the ‘inhibitory concentration’, here it is used in the context that it ‘inhibits proliferation or viability’, so for example an IC₉₀ is the dose required for 90% of cells to have died. A parameter that is possibly more relevant but essentially the same is LD₅₀ (lethal dose required for 50% cell kill) but it is perhaps not so ubiquitous in the literature so IC values will be used instead. The MTS assay is a standard end point for an *in vitro* cytotoxicity assay, it is an adaptation of a technique used to measure cell viability via metabolic capacity (the MTT assay) See (2.1.4.4).

3.1.1.2 The cell line

The cell line chosen to carry out this work is SKOV-3, a cell line originally derived from a sample of human ovarian adenocarcinoma tissue. The choice of cell line is worth prior investigation as all have inherent sensitivities and bias, especially in pathways involving cell death (X. Li et al., 2001; Marcelli et al., 2000). SKOV-3 cells have non-functional p53 due to a homozygous mutation in both genes causing a complete lack of detectable mRNA or protein

(Berglind et al., 2008; Yaginuma and Westphal, 1992). p53 is a tumour suppressor protein that is mutated in up to 50% of cancers and its most famous role is that it can trigger cell cycle arrest and apoptosis in response to diverse stress stimuli including DNA damage and hypoxia (Stegh, 2012). Other genetic aberrations are listed in 2.1.4.1. SKOV-3 have also been shown to have low levels of FAS receptor cell surface expression making them more FAS resistant (FAS induces apoptosis) than endogenous ovarian tissue (Baldwin et al., 1999). They have also been shown to over express Notch 1 intracellular domain, (an important role in cellular differentiation and proliferation) (Rose et al., 2010), and have relatively low level Bcl-2 expression, an anti-apoptotic protein from the Bcl-2 family of proteins (Beale et al., 2000).

3.1.1.3 The light dose

PDT is able to be dosed in two ways, the dose of drug and the dose of light. The standard light dose used in our laboratory is 33mW/cm^2 which over 10 seconds corresponds to a power delivery of 0.33 J/cm^2 . The choice to use almost the lowest power setting available on the laser, (which is of the type and power that would be used in a clinical setting), is deliberate. Cells in a dish will be exposed to a lot more of the laser light than one could deliver *in vivo* due to tissue absorbance factors, see 1.4.4. The light dose used *in vivo* and in the clinic commonly ranges between $50\text{-}100\text{ J/cm}^2$. In previously published work (Bhatti et al., 2008; Stamati et al., 2010), the photosensitiser was left on the cells for two hours at $37^\circ\text{C}/5\%\text{CO}_2$ before removal and subsequent immediate irradiation of the cells. This timepoint was originally designed so to be sufficient for most mechanisms of cellular uptake *in vitro*, but not so prolonged as to create a situation irrelevant to the *in vivo* situation where cells are unlikely to experience the prolonged exposure to such high levels of drug. As mentioned in 1.3.1.1, the drug-light interval can be a very important factor in therapy design and have significant impact on overall effectiveness of the therapy, however the main factors that drive the influence of the drug-light interval on toxicity, i.e drug presentation to a target site and systemic clearance processes, are not likely to have any effect in our *in vitro* assay.

3.1.1.4 Other considerations

An example of the differences seen between *in vitro* and *in vivo* data in PDT include work by Kessel (Kessel et al., 1997) who looked *in vitro* with two photosensitisers, both structurally similar with similar absorbance profiles, however one had an amine side chain and the other carboxyl. *In vitro* the PS with the amino group demonstrated a much more efficient and quick apoptotic cell kill however *in vivo* they both had a similar efficacy. Other work by Moore (Moore et al., 2007) looked at the PDT susceptibility of endothelial cells vs. tumour cells *in vitro* compared to *in vivo*. They saw *in vitro* no differences in susceptibility to PDT however *in vivo*, the endothelial cells routinely appeared to be more heavily targeted.

The use of 3D structures made from mixtures of *in vitro* cell lines (multi-cellular tumour spheroids) are designed to better represent the a tumour environment, however one problem with structures like this, particularly tumour spheroids is that they can only grow to a certain size since there is no vasculature inside the spheroids to provide tissue oxygen, this also means that PDT can easily deplete oxygen and so PDT experiments can show much lower toxicities on cells in the spheroid centre. Models such as these can show us factors important for PDT not seen in a monolayer cell culture, for example the balance between apoptotic and necrotic cell death in a population and PS intra-tumour distribution (Marchal et al., 2005; Obrigkeit et al., 2009b; Xiao et al., 2005).

Even *in vivo* tumour models can show bias, Chen et al (Chen et al., 2005) looked at the same tumour model implanted ectopically in subcutaneous space or orthotopically in the prostate and showed differences in PS uptake, even at time points where uptake was similar in the two models they showed different responses to PDT.

Despite all the care in setting up a comparable *in vitro* assay, all the cells within an actual tumour are going to be exposed to different levels of drug and light as well as factors that include possible levels of de-differentiation, areas of hypoxia or local pH change, and differential supply of nutrients and removal of waste. All these things are capable of changing how, and to what extent, a cell will respond to PDT. The bystander effect will also play a role *in vitro*. Various

experiments have shown that when cells die they often die in groups, independent of the location of the death source and beyond the probability of the group all dying independently by chance. This bystander effect indicates that communication between neighbouring cells plays a role in the induction of cell death, in both apoptosis and necrosis, (although evidently to a larger extent in necrosis). This has been shown to be particularly relevant for PDT (Dahle et al., 2000; Rubio et al., 2009).

3.1.2 Making Drugs more soluble

Solubility as a term is dependent on its context. The IUPAC Gold Book definition for solubility (IUPAC, 2011) defines its use linked intrinsically to the solution in which you are trying to dissolve said component in and the fact that the solute should be at saturation to infer any information about its solubility. In the context of this project however the term solubility is taken in a much broader sense to fit its application to clinical ‘pharmacokinetic solubility parameters’ and in these the solvent is the human body.

One of the most common solubility terms when studying pharmacokinetic and pharmacodynamic properties of drug molecules is a term better used to describe a molecules lipophilicity; the log of a partition coefficient- LogP. It sees prolific use in both bench experiments and computational analysis. Experimentally it is most commonly measured as the Log of the ratio of the distribution of solute in a aqueous-organic / liquid-liquid biphasic system, (commonly 1-octanol and water) under conditions in which the unionised species is both in a saturated and non aggregated condition (Sangster, 1989). Also available are computational approaches of LogP calculation (Faller and Ertl, 2007), which try to improve upon bench made predictions via the sheer number of structures that can be put through the system while avoiding difficult un-biased experimental set-ups. As important as Log P is in giving an indication of the molecules intrinsic lipophilicity, when this is related to its use in the clinic, information about a molecule in its ionised form (for example at the pH of blood plasma), has encouraged the additional use of a LogP derivative LogD (D for distribution coefficient) which takes into account its degree of ionisation at given pHs. Other ‘LogP like’ systems that utilise biological

solvents are also common; including replacing the octanol partition with lipid systems that model biological membranes (Avdeef, 2001).

Solubility forms a key aspect of the Biopharmaceutics Classification System and there have been many attempts by pharmaceutical companies to design wish lists of physiochemical parameters to help predict and therefore produce drugs with ideal pharmacokinetic profiles; *Pfizer* (Lipinski et al., 2001) *GlaxoSmithKline* (Veber et al., 2002) *AstraZeneca* (Waring, 2009). LogP is almost always included in some form and traditionally it is thought that the lower this can be the better (Leeson and Springthorpe, 2007). The average LogP of most approved drugs is ~2.5 (Proudfoot, 2005), or 1.92 for intravenous drugs (Obach et al., 2008), and can vary from -2 to +8 (Avdeef, 2001). Typical LogP values in the context of drug development are thought of as follows: very hydrophilic (LogP <0), hydrophilic (LogP 0-1), moderate (LogP 1-2), lipophilic (Log P 2-4), very lipophilic (>4), (Obach et al., 2008).

The data of Obach (Obach et al., 2008) also shows a positive correlation with increased lipophilicity and higher distribution in tissues (as opposed to blood plasma) this was measured by steady state volume of distribution (VDss). High VDss levels are not always an indication that the drug is in the specific target tissue just that it is no longer in the blood plasma. Many papers argue that high lipophilicity promotes high promiscuity (Leeson and Springthorpe, 2007) but the role of lipoprotein-mediated drug transport from the blood to the tissues is also important, especially as metastatic tumours have an increased demand for the cholesterol contained within these lipoproteins (Wasan et al., 2008). The intelligent 'formulation' of medicines can help insoluble drugs fit the solubility parameters set out by the pharmaceutical industry but it is no guarantee of success, what happens to drug and formulation once inside the body can be two independent events. It is more sensible to be able to design intrinsic solubility into drugs via chemical manipulation methods.

3.1.3 Making Photosensitisers more soluble

Solubility design with respect to PDT drugs needs to consider the inherent requirement of a photosensitiser for delocalised electrons -they often have large areas of conjugated planar ring systems which contribute heavily to making the molecule more hydrophobic in character. It is also generally believed that strong hydrophobic interactions will drive toxicity- caused by high levels of unspecific binding and poor metabolic clearance. PDT itself is recognised to exhibit this problem through the commonly seen side effect of high skin accumulation and prolonged photosensitivity, see 1.2.3. Evidence suggests therefore that making PS more soluble will have significant influence on PDT efficiency.

One reason to improve photosensitiser water solubility has been the desire to improve PDT by the conjugation of photosensitisers to proteins such as antibodies. This is the main driving force behind the work presented in this thesis. Two hypotheses are behind the theory that aqueous soluble photosensitisers will provide better photoimmunoconjugates (PICs): The first is physiological; the conjugation of too many heavily hydrophobic molecules to an antibody may disrupt its inherent pharmacokinetic and targeting properties. The second is chemical; the conjugation process to antibody has to be carried out in aqueous conditions as antibodies like most proteins can be irreversibly damaged if they unfold when exposed to organic solvents (Pace et al., 2004; Ugwu and Apte, 2004).

There is a lot of research that looks at the relationship between changing a PS structurally, calculating its lipophilicity via LogD and LogP and comparing this to uptake, whether into cells or artificial cellular membrane models. There is a general trend between increased lipophilicity and increased cellular or liposomal uptake. Engelmann and colleagues (Engelmann et al., 2007) looked at a series of 20 cationic *meso*-phenyl(pyridyl)porphyrin derivatives and showed a general correlation between an increase in the number of positive charges introduced to the rings and an decrease in LogP with values ranging between -4 and 2. Other factors such as the type of positive charge and where it was on the molecule were also shown to be influential. They also looked at binding to negatively charged synthetic liposomes as a model of cell interaction and showed a positive correlation with an increase in LogP, the only outlier being a PS with an

asymmetric distribution of charges which showed enhanced binding above the trend predicted by its LogP. Meng and colleagues (Meng et al., 1994) measured LogP for a series of *meso*-substituted porphyrins via sulfonation of the phenyl or methylation of the pyridyl groups, all derivatives had varying numbers of both positive and negative charges, and the LogP value correlated closely with data measuring increased uptake into HT-29 cells with increased lipophilicity. Kepczynski and colleagues (Kepczyński et al., 2002) also show a trend between increased lipophilicity and increased cellular uptake but suggested this relationship is much stronger within related groups of PS, for example modification of the same core as opposed to comparing different PS entirely. They also suggested a rule of thumb that a molecule should have a log P greater than 0 in order to bind cellular membranes minimally but larger than 3 for significant binding.

It has however been suggested that there is an upper limit where PS that are too lipophilic will form aggregates in solution and that this will compete with cellular uptake. Ben-Dror and colleagues (Ben-Dror et al., 2006) investigated particularly hydrophobic porphyrins over a range of computationally predicted LogP values between 8.89 and 11.81, they showed a negative correlation between LogP and Liposome binding indicating the more hydrophobic molecules bound to liposomes less, their overall hypothesis was that for molecules with high hydrophobicity (in their experience LogP >7) the usual positive correlation becomes inverted due to a tendency of the molecules to aggregate.

These rules may not correlate exactly with tumour uptake *in vivo* as they do not consider the effect lipophilicity will have on serum pharmacokinetics. Work by Henderson and colleagues (Henderson et al., 1997) is of particular relevance as it looked at a range of PPa derivatives, their C1 compound being very close to the PPa used herein. The rest of their panel differed from each other only in the number of carbon atoms in and the shape or flexibility of an alkyl ether side chain. To measure hydrophobicity they looked at retention times on a reverse phase HPLC column, computationally calculated LogD at pH 7.4, and for some compounds they attempted to measure an experimental logD. They then used this panel of PS to look directly at anti-tumour activity in mice: Upon treatment with PS, speed of tumour growth was fastest at the extreme ends of LogP (3 and 9) with a peak for slowest tumour growth around LogP 6 or 7, a similar

pattern was seen when they plotted LD₅₀, with the highest tumour cytotoxicity seen around LogP 5.6-7. Interestingly they also looked at plasma and tumour PS levels 24hrs after injection (the time point used for irradiation in the above mentioned experiments), both tumour and plasma showed an increase in plasma PS with increasing LogP until a plateau around LogP=8. This doesn't match the cytotoxicity observed as a distinct drop in toxicity is seen with the PS above LogP=6, that is not due to a lack of uptake into tumour tissue or sufficient blood retention times. They excluded molar extinction coefficients and quantum yields as a reason as they stated these were the same as the parent compound for all derivatives, they therefore concluded differential intracellular localisation is likely to blame, and hypothesised an optimal compound has midrange lipophilicity allowing it to readily enter and leave different cellular compartments on its way to the target compartment.

3.1.4 Measuring the solubility of PS via spectral shifts

In addition to experiments to determine logP, chromophore containing molecules like photosensitisers provide a more direct way to investigate their properties in a given environment - their absorption profiles. This relationship between absorbance profile and molecular orbital theory, see 1.4.1, gives us a way to visualise the effect various solvents have on PS atomic structure and can therefore give us important information about PS solubility (Lakowicz, 1999).

Shifts in spectral profile can be described by four common terms; Bathochromic or red-shift (shift to lower energies), Hypsochromic or blue-shift (shift to higher energies). Hyperchromic (increase in intensity of a spectral band) and hypochromic (decrease in intensity of a spectral band). Common factors that can cause solvent induced spectral shifts are the influence of polarity of a solution in the stabilisation of energy states (Solvatochromism), aggregation/ interaction of a molecule with itself or other moieties, an altered ionisation state of the molecule, metal ion co-ordination, or charge-transfer interactions (inter molecular or with solvent ions). Unfortunately spectral shifts are a complex phenomenon and often involve the interaction of multiple elements, also not all changes to molecular orbitals will result in visible spectral effects (Lakowicz, 1999).

3.1.5 Aggregation of Photosensitisers

Aggregation is a solution phenomenon involving the non covalent interaction of solute molecules, in this case PS, to give assemblies of molecules, they can be dimers, trimers, multimers or mixtures of all these. In some cases the aggregates become so extensive they will precipitate out of solution. Aggregation arises when the intra-molecular interactions between solute and solute become more energetically favourable than the interactions between solvent and solute. Aggregates can be hetero-aggregation (with other solutes in the media) or homo-aggregation (with other molecules of the same type within the solution). Aggregate formation is reversible with monomer and multimer formation reaching a point of equilibrium. The equilibrium point can be affected by various factors such as temperature, solute concentration, changing ratio of solvents in a mixed solvent system or manipulation of molecular conformation; for example via metal ion binding. Weak forces are responsible for the aggregative interactions for example hydrogen, electrostatic, van der Waals, or hydrophobic interactions.

For PS it is often the delocalised π electrons from the ring, with themselves or with electrons from σ orbitals that make a significant contribution to aggregative interactions. The molecular orientation of aggregation can in part be determined by the various substituents made around the ring (Bonnett, 2000). Research by Kasha, Jelly and Scheibe over the years have all been important in the development of a model and subsequent nomenclature for different aggregative formations seen with Porphyrins. A basic interpretation of the model put forward here is that if two monomers aggregate, exciton splitting occurs and two energy levels are formed of different energies, one higher and one lower than the original energy level, the probability of each of these energy levels being used is predicted by molecular orbital rules influenced by the spatial relationship of the two molecules to one another. Aggregates that form side by side aggregation exhibit a blue shift in their spectra and are termed J-aggregates. Aggregates that form face to face aggregation and exhibit a red shift in their spectra are termed H-aggregates (Bonnett, 2000). This model, alongside other methods, was used by Fabiano and colleagues to investigate the aggregation of cationic PPa molecules in aqueous buffer (Fabiano et al., 1997).

Both fluorescence quantum yield and singlet oxygen quantum yield of aggregated photosensitisers are often significantly lower than those of the monomeric form. This is predicted to be due to increased radiation-less energy transfer between the photosensitiser molecules within aggregates. This fast energy relaxation channel shortens the lifetime of the lowest excited level of the photosensitiser, thus reducing the probability of the molecule converting to the triplet level, the level from which most important photochemical processes for PDT start.

Serum mediators are capable of monomerising PS *in vivo* and once intracellular the aggregated PS have also been shown to revert to a monomeric form. Some researchers have even carried out work to design molecules that are more photo-physically efficient when aggregated (Baba et al., 2007; Chang et al., 2012; Kim et al., 2007).

3.2 Results

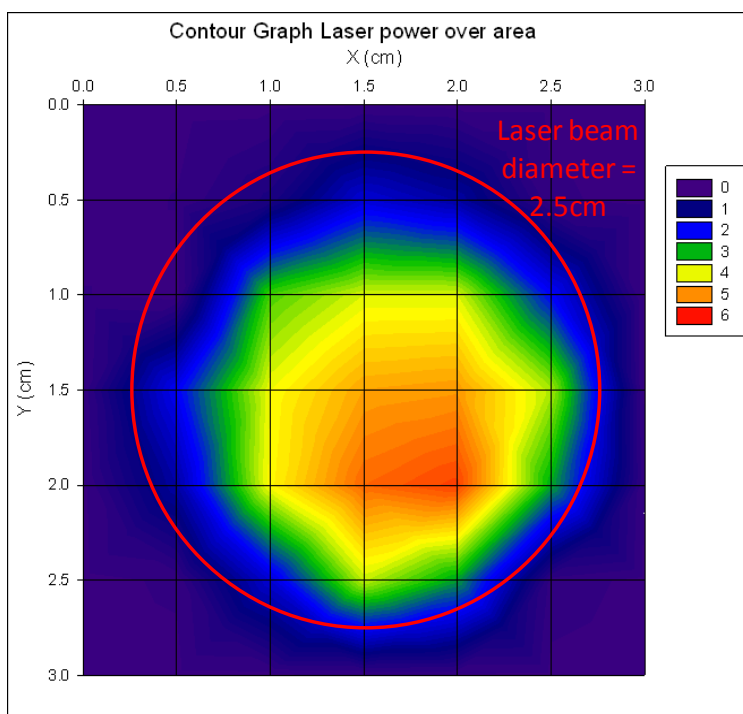
3.2.1 Further development of the *in vitro* PDT assay

To check if the new, more water-soluble photosensitisers PS1 and PS4 retain their efficiency they were tested on cancer cells grown *in vitro*. Some changes to the earlier published method (Stamati et al., 2010) were made:

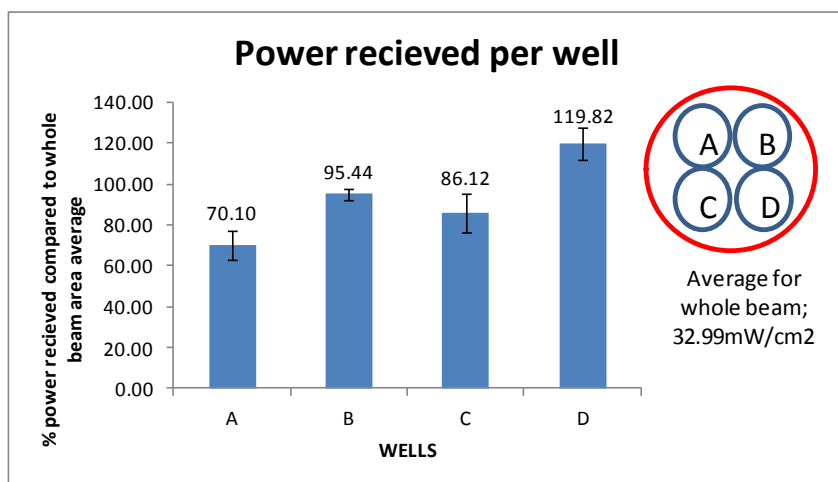
Black cell culture plates were used instead of clear plates, and the lids are removed during irradiation. Previously when using clear plates during the irradiation process the scattering of laser light was observed both throughout the entire plate and through the lid. Control experiments were carried out and results show the change to a black plate and lid-off irradiation didn't have any significant difference on final toxicity parameters (Data not shown).

Laser calibration experiments were carried out, see 2.1.4.3; with a new slightly larger beam diameter than used previously to ensure the irradiated wells were covered more evenly by the laser beam. The experimental method allows irradiation of 4 wells in a 96well plate simultaneously and to investigate if they were receiving a similar energy, measurements were taken with an apparatus device to try to discover any 'hotspots' within the laser. This data is shown in Figure 7 and shows there are slight differences in the amount of energy each well receives. The method was also adapted for a larger beam diameter ideal for 6well plate formats (required to obtain enough irradiated material to analyse DNA and protein lysates). Calibration with a power meter ensured the same energy was delivered to the cells in this format as in the 96well experiments. This calibration is shown in 2.1.4.3.

Figure 7: Hot spots within the laser light effects the amount of energy delivered.



Using an appature device power at different points within the projected beam of laser light were measured and plotted over area to show the off-centered hotspot from the laser.



During the PDT of 96well microplates 4 wells are irraiated simultaneously and when power was measured over the area that would project onto each individual well in a 96 well plate setup differences in the ammount of light each would recieve was seen.

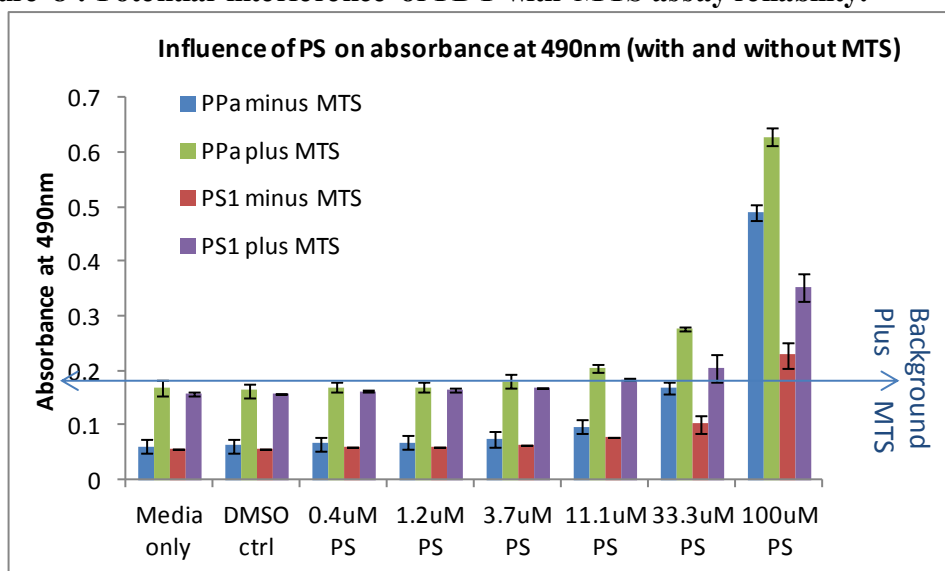
3.2.1.1 Interference of PDT with MTS assay reliability

MTS is not a direct measure of cell viability and can be reduced in the absence of cells (Ishiyama et al., 1997; Peng et al., 2005). It was important to have confidence in our endpoint so a few additional PDT-specific checks were carried out.

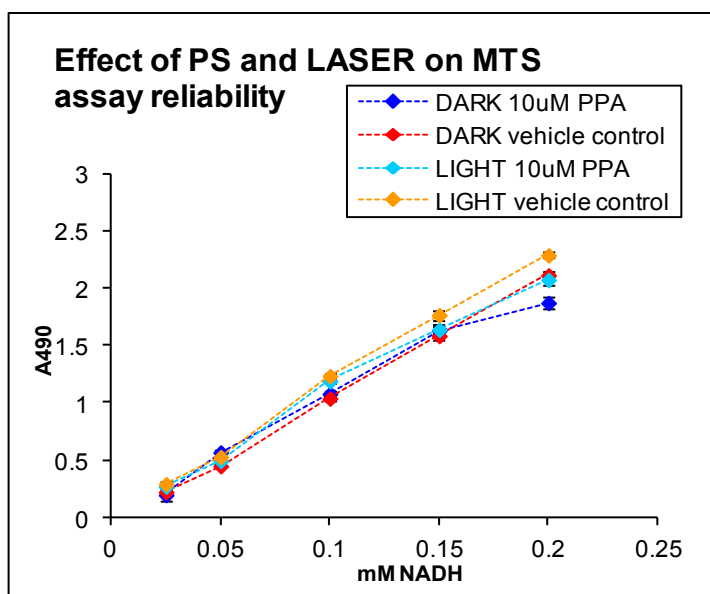
PS are promiscuous absorbers of light, so experiments were done to check that the photosensitisers didn't interfere with absorbance at 490nm (end point absorbance of MTS). These are shown in Figure 8 and show that PS does not contribute to absorbance at 490nm below concentrations of 10 μ M, this should be sufficient as wells containing this level of PS or above are washed prior to irradiation and subsequent MTS.

Photosensitisers are chemicals that can carry out redox activities so there was potential for the PS to oxidise cellular NADH either directly or *via* its reactive oxygen products, this could reduce the signal seen on MTS without any actual cell death. Figure 8 shows an experiment in which a standard curve of recombinant NADH in a suitable buffer produces a range of absorbance at 490nm when mixed with MTS, this was incubated with PS (at the maximum level that did not directly absorb at 490nm) with and without irradiation by laser to see if any signal is lost. No signal loss was seen, from which one can conclude that PPa-based PS cannot directly oxidise NADH under these conditions.

Figure 8 : Potential interference of PDT with MTS assay reliability.



Different dilutions of PS were mixed with and without MTS solution over a range of PS concentrations, when absorbance at 490nm (endpoint of the MTS assay) was read it shoed that PS does not contribute to absorbance at 490nm when below 10 μ M.



A standard curve of recombinant NADH in a suitable buffer can produce a range of absorbance at 490nm when mixed with MTS, when this was incubated with and without irradiation by laser to see if any signal is lost, no significant signal loss was seen.

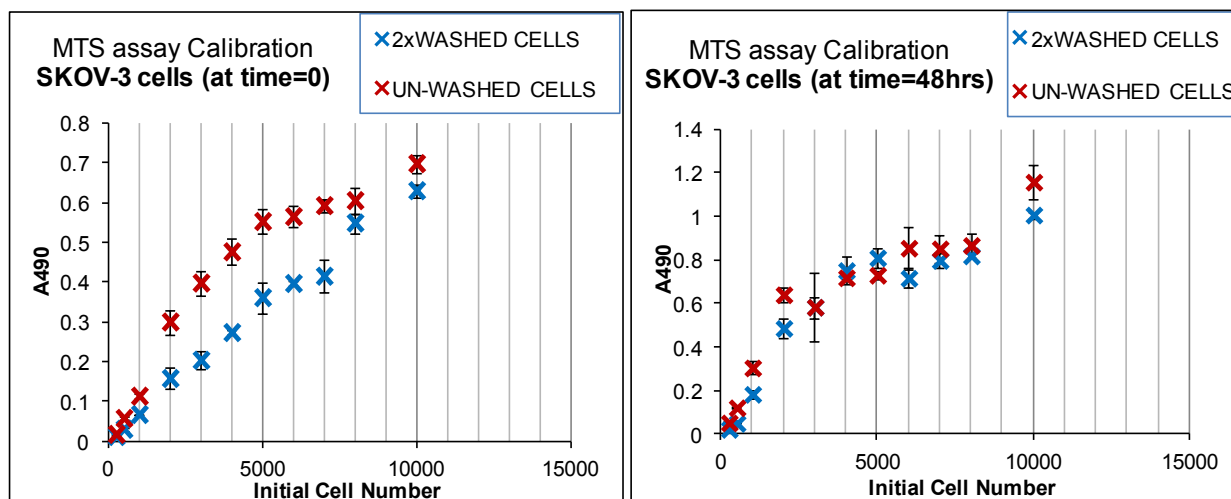
3.2.1.2 Calibration of MTS assay for chosen cell line

The only further calibration needed in respect to the MTS assay was that it would need to be of a suitable sensitivity to follow cell death over time; to obtain this an initial cell plating number was needed that would give a positive reading in the linear region of the MTS assay for both timepoints 0hr and 48hr with no saturation of signal or growth inhibition of cell lines. As shown in Figure 9 this was chosen to be 2500 SKOV3 cells per well. The data also considers un-washed cells vs washed cells because the PDT method requires a fair amount of washing/media exchange steps. An additional cell line was required for a negative control (for later work) this presented some problems as many cell lines tested at this stage had insufficient plate adherence for intensive wash steps and so couldn't be used reliably, a few were tested (data not shown) but eventually a less adherent cell line was chosen (U87) and calibrated but with plate pre treatment with attachment factor (TCS cellworks ZHS-8949) (data not shown). Whilst carrying out this work it was noticed that the time between addition of MTS and the reading of absorbance was important, also shown in Figure 9, particularly relevant as the plate reader available was not immediately accessible, therefore all future data shown will be presented as a % of untreated control to make data more comparable, although care was taken to keep as close as possible to a 2 hour incubation time.

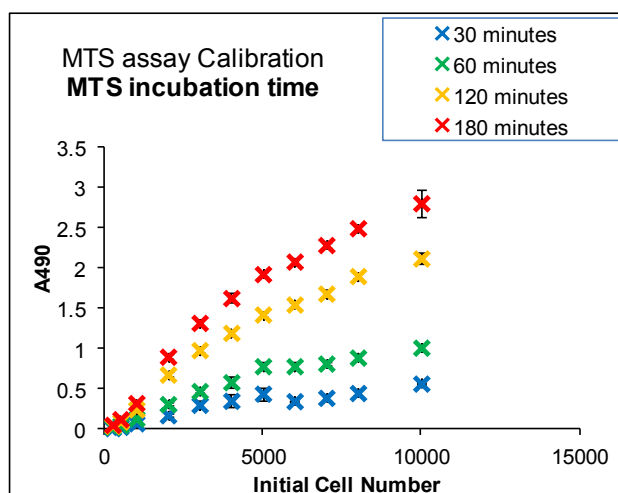
3.2.1.3 Effect of DMSO in the cell culture media.

A final concentration of 2% DMSO was maintained in all the cell culture media solutions containing PS, this was included to help PS solubilisation. Any possible cytotoxic effects were investigated. This is presented in Figure 10, where 2% DMSO was shown to have no significant effect on SKOV-3 cell culture. For interest, spectral analysis was carried out and is also shown in Figure 10. Interestingly the only effects of DMSO on the PS that were visible via spectral change was with PS4, thought to be the most water soluble PS.

Figure 9 : Calibration of MTS assay for SKOV-3 cells

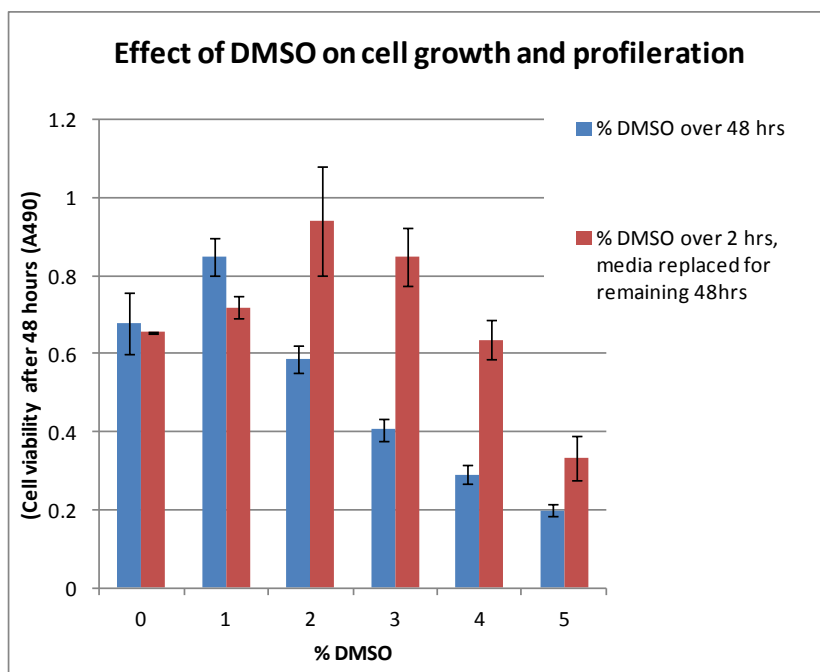


Optimisation of the number of SKOV-3 cells that would produce a non saturated signal with the MTS assay at two timepoints within the 'PDT assay', these were 0hrs, around 24hours after plating, the time at which cells would be irradiated, and 48hrs, which was 48hrs after irradiation.

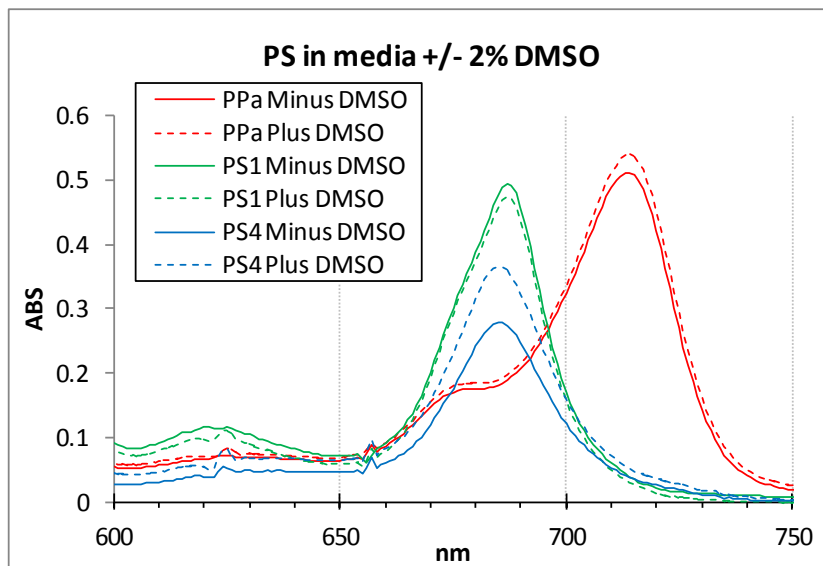


Increasing the time between addition of MTS reagent to the well and subsequent reading of absorbance at 490nm increases the signal recorded.

Figure 10 : Effect of DMSO in the cell culture media.



The toxicity of different amounts of DMSO contained within the cell culture media on SKOV-3 cells, when exposed either transiently for two hours before leaving cells to grow for a further 48hours or leaving the DMSO in for the entire 48hours.



Spectra of PPa, PS1 and PS4 in cell culture media with and without 2% DMSO.

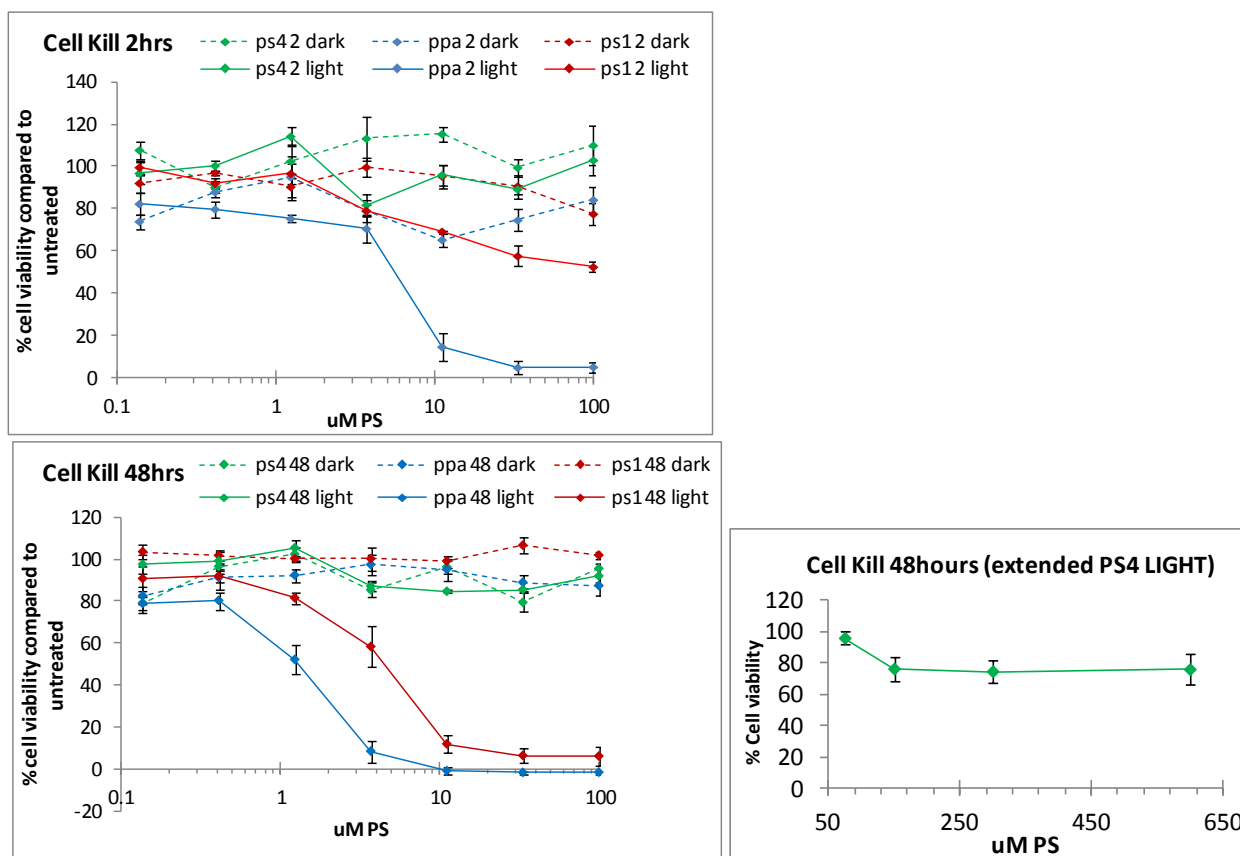
3.2.2 Potency of the new photosensitisers.

3.2.2.1 Cyto-toxicity of the new photosensitisers in the in-vitro PDT model

Cell kill experiments were carried out in the PDT *in vitro* model described above and in 2.1.4.3 with PPa and each of the new photosensitisers alone and in parallel. Figure 11 shows a representative experiment of results obtained when running all PS in parallel. Cell viability 2 and 48 hours after irradiation is shown. This data shows that PS4 exhibits no cytotoxicity at any of the examined doses under these experimental conditions, in a separate experiment the doses for PS4 were escalated to over 500 μM but still there was no observed cytotoxicity. At 48 hours after irradiation PPa and PS1 both exhibited dose dependant cytotoxicity, PPa showing slightly higher potency than PS1. Two hours after irradiation high dose PPa can reduce cell viability levels to around 0% whereas PS1 showed only limited potency at the same high doses. Cell kills for each PS were carried out numerous times at 48 hours, and dose response curves were fitted see 2.1.3.1. From all the collected values, averages were taken for the dose needed to kill 10%, 50% and 90% of cells, termed IC_{10} , IC_{50} and IC_{90} respectively. These average values are shown in Table 5.

No PS exhibited any ‘dark’ toxicity at any of the doses studied, i.e. toxicity independent of activation by laser but this data suggests that the new, more water soluble, photosensitisers exhibit a loss in potency compared to their parent compound PPa. Of interest is the differences seen between PPa and PS1 in the time they take to kill the cells, PPa seems capable of killing immediately with the dose response curve shifting slightly after a further 48 hours of time has passed, whereas with PS1 a much larger difference is seen between 2 hours, when very little toxicity is recorded, and 48 hours when it is almost as cytotoxic as PPa. This suggests that different mechanisms of cell death could be being initiated. Because the PS was washed off the cells after two hours and then cells immediately irradiated, only PS that was intercellular or cell associated at that point will have been contributing to potency, additionally the ‘dark’ data shows only activated PS is toxic.

Figure 11: Cell viability after PDT with PPa, PS1 and PS4



PPa, PS1 and PS4 tested in the *in vitro* PDT assay on SKOV-3 cells. Viability measured 2 hours and 48 hours after PDT.

Table 5 : IC₁₀, IC₅₀ and IC₉₀ of PPa and PS1

		IC10	IC50	IC90
SKOV (PPa)	AVERAGE	0.51	2.10	12.64
	StDev	0.42	0.95	7.00
	SE	0.19	0.36	2.86
	95% Confidence limits	0.38	0.72	5.71
	n	5	7	6
		IC10	IC50	IC90
SKOV (PS1)	AVERAGE	0.45	3.45	26.73
	StDev	0.40	2.54	15.92
	SE	0.18	1.14	7.12
	95% Confidence limits	0.36	2.27	14.24
	n	5	5	5

3.2.2.2 Indirect evidence for uptake and leaching kinetics from cell viability studies

In an *in vitro* model, although influential factors such as serum pharmacokinetics, tissue delivery and blood clearance are absent, it is their absence that allows cellular uptake and cellular leaching processes of PS to be studied. These cellular processes will of course still occur *in vivo* but the tissue level processes are likely to have an equally or more significant influence on outcome. In this section some initial work looking at cellular uptake and leaching of PS is presented which will be expanded in the next chapter. Figure 12 shows the standard cell kill experiment carried out at IC₉₀ doses of PS but adapted to show cellular uptake by varying the amount of time PS was left over the cells before it was washed off and cells then immediately irradiated. It also shows a similar experiment but designed to look at cellular leaching: The PS were left over the cells for 2 hours, washed off and then the time before irradiation was varied.

Results from the uptake experiment showed that by two hours both PPa and PS1 have reached a plateau at around 10% cell viability, but PPa reaches this point by around 1 hour, much quicker than PS1. Two different conclusions could be taken from this data, if one assumes a linear relationship between uptake of PS into the cell and its toxicity then it could be concluded that PPa is taken up quicker than PS1. However because this experiment does not look at uptake directly but rather final toxicity as an endpoint, this result could also be due to inherent toxicity differences between PS: For example, if both are taken up at the same rate but PPa is more toxic, at low doses it would seem more toxic than PS1. There are also additional factors such as a possible change in intracellular localisation of photosensitiser, if this occurred over time it could also affect toxicity over time. It will be important, as shown later, to try and answer these questions.

Results from the leaching experiment showed that after PS was removed from the surrounding medium enough PS leached back out of the cells over two hours that it caused a complete loss in toxicity. This data was very influential in further assay design as when many plates are being processed, the time between initiation of washing and irradiation can easily stray into 10-15 minutes which according to this result can significantly reduce potential toxicity. This data also confirms that it is only internal PS that can induce cell kill. This is because the cells

were not re-washed after the first wash step, meaning any leached PS would still be in the well during irradiation. However it is also possible that other processes could be involved; for example if once PS is taken up it relocates or converts to a non toxic form, this would also cause a decrease in toxicity over time after cells are washed, something that wouldn't be seen at 2 hours in the standard cell kill experiments as the PS is continually replaced by PS in solution. This data may also explain the difference in standard deviations seen associated with the IC values in Table 5, PS1 has larger variation values than PPa, possibly a result of slight changes in the wash-irradiation interval having more of an influence in PS1 over PPa. This argument also fits to explain the general increase in standard deviation seen with $IC_{10} < IC_{50} < IC_{90}$ as increased PS dose would show increased amounts lost in leaching.

3.2.2.3 Cell kill with increasing light dose

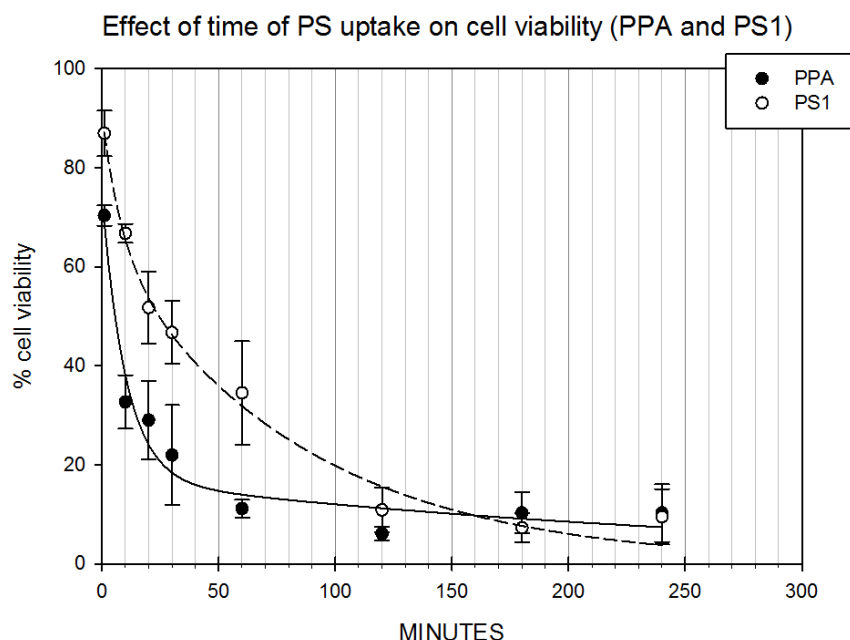
All of the standard cell kills above were performed at a relatively low laser dose, to see the full potential of these photosensitisers it was of interest to carry out cell kill experiments at the full range of light doses. There are two ways to increase the overall energy of light delivered to the cells, you can increase the power of the laser, or you can increase the amount of time spent lasering. Differences in this 'fluence rate', is of particular clinical relevance as discussed more in the introduction see 1.3.1.2.

Figure 13 shows an experiment carried out to see if our model was affected by a change in fluence. The top graph shows all the possible laser doses available on our laser, traced over the top are three blue 'paths' that cover the range of possible energies; one for changing time, another for changing power (amps) and the final for a combination of both. Standard cell kills were carried out with constant doses of PS whilst changing the energy delivered over each of these paths. The results show for both PPa and PS1 there is no significant difference in which of these paths are taken, it is only the final energy received by the cells that has an effect on toxicity.

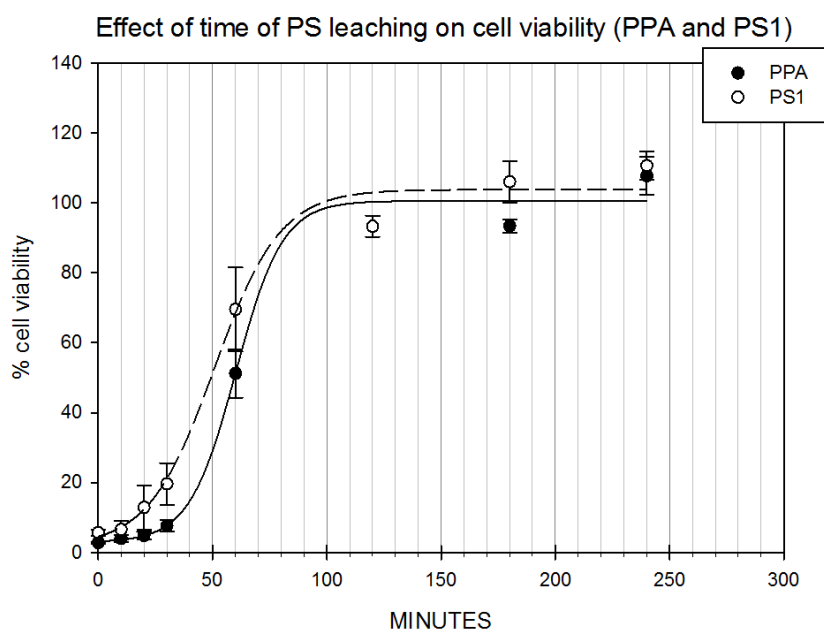
Changes in the PS drug dose response curve over a range of laser doses could then be studied. The results are shown in Figure 14. The data shows that PS4 has not lost its cyto-toxicity completely and at 23 J/cm^2 can exhibit potent toxicity with an IC_{50} of 337nM. At this dose PPa

and PS1 have IC_{50} values of 61 nM and 65 nM respectively. PPa and PS1 are now only a factor of around 5 times more potent than PS4. At the laser doses used above in the standard assay ($0.33 J/cm^2$), if PS4 were simply 5 times less cyto-toxic, cell death would have been observed. Figure 15 shows this influence of light more clearly by plotting the IC_{50} value of each PS over increasing light dose. This graph gives an indication of the ‘rate’ at which PS4 becomes toxic compared to PPa and PS1. Each curve was fitted with a 2 parameter power curve following the equation ($Y = aX^b$) see 2.1.3.1. The constants from this equation are a and b; constant a refers to a scaling factor, used to convert the units to the appropriate amount, but constant b corresponds to the slope or rate of change, (a higher value indicating a steeper slope or higher rate of change, the negative sign before the b shows it is a decay function not a growth function). PS4 has a higher value for b (2.7), than PPa (0.7) and PS1 (0.6), this could indicate the PS4 ‘population’ of PS within the cell is responding in a more sensitive way to an increase in light than PS1 or PPa, i.e. it is exhibiting more efficient photophysical behaviour. However what is still not known is how many molecules of PS are actually contained within this population, if this was known one could make a stronger conclusion about how effective each PS is relative to the amount of light delivered. This is discussed later in the thesis, see 4.2.3.5.

Figure 12 : Indirect evidence for uptake and leaching kinetics from cell viability studies.

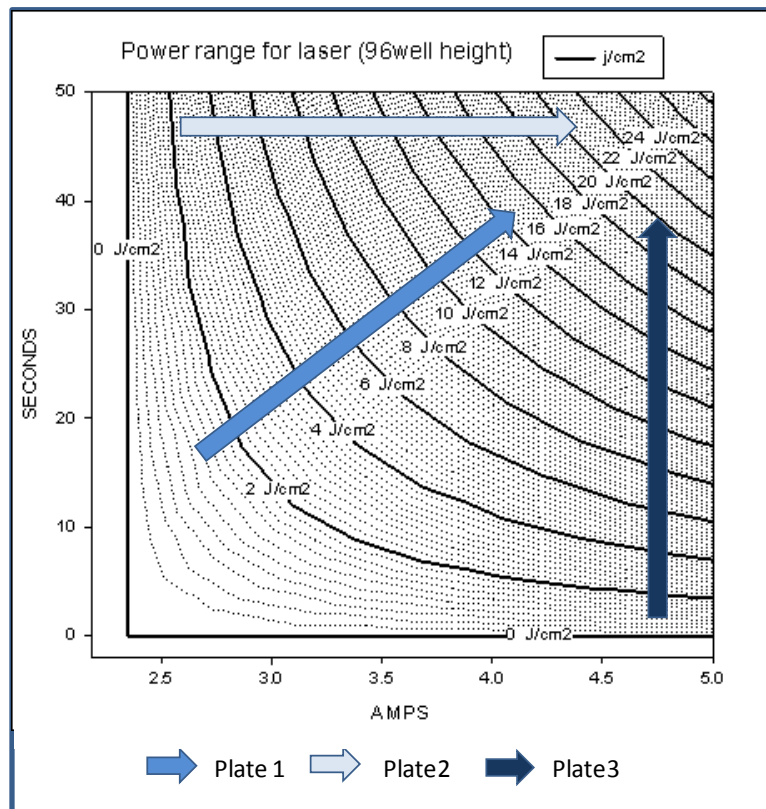


The standard cell kill experiment carried out at IC_{90} doses of PS but adapted to show cellular uptake by varying the amount of time PS was left over the cells before it was washed off and cells then immediately irradiated.

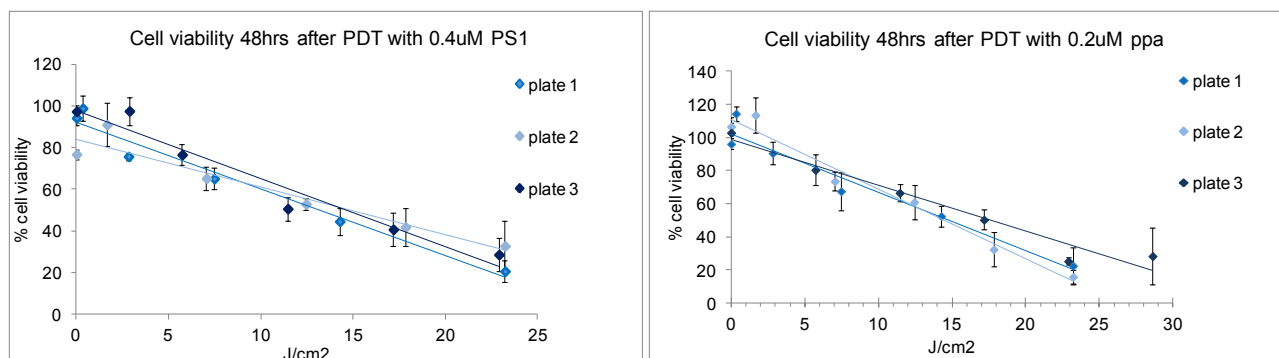


The standard cell kill experiment carried out at IC_{90} doses of PS but adapted to show cellular leaching by leaving the PS on the cells for 2 hours, washing it off and then the time before irradiation varied.

Figure 13 : Light dose delivery, and the importance of overall dose.

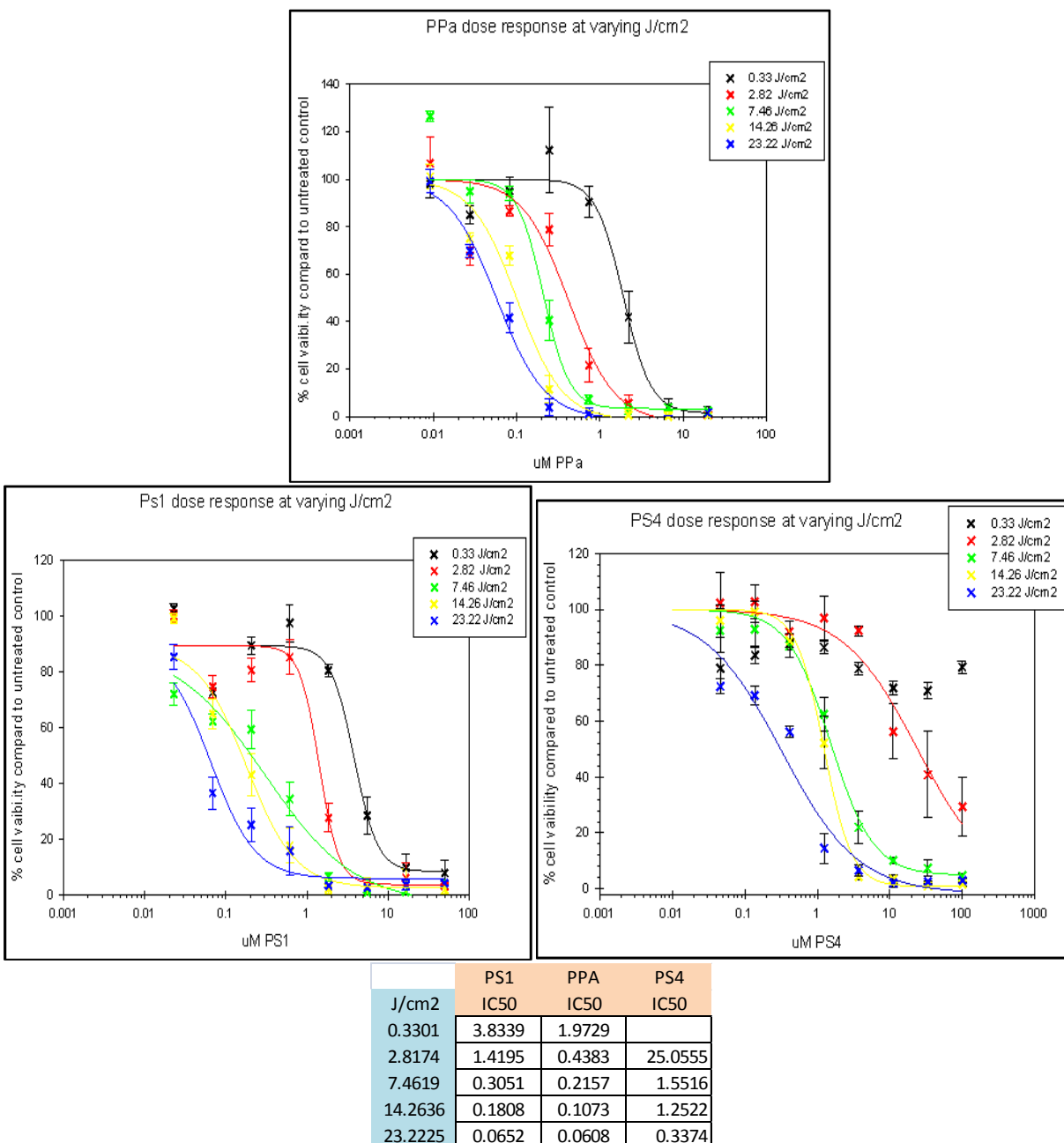


All the possible laser doses available on our laser, traced over the top are three blue 'paths' that correspond to three ways to increase the overall light dose; one for just changing the time lasered, another changing the power (amps) and the final changing a combination of both.



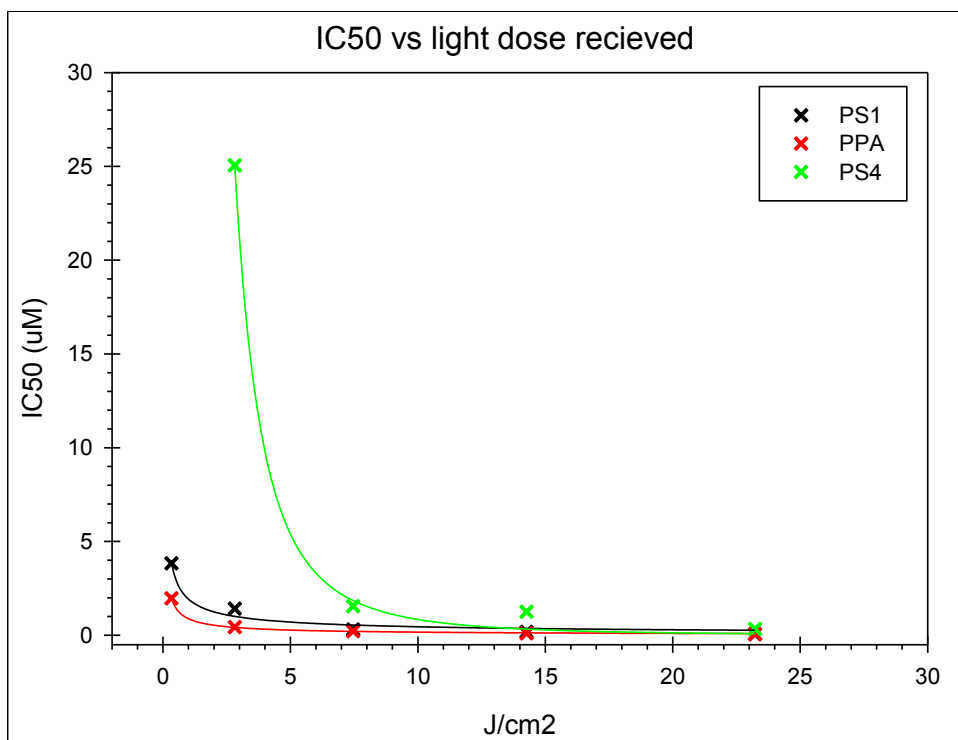
Standard cell kills were carried out with constant doses of PS whilst changing the total energy delivered over each of the paths shown above. The results show for both PPa and PS1 there is no significant difference in which of these paths are taken, it is only the final energy received by the cells that has an effect on toxicity.

Figure 14 : Cell kill with increasing light dose



The standard cell kill experiment carried with PPA, PS1 and PS4 out over a range of increasing laser doses. IC₅₀ values calculated from 4 parameter logistic curves fitted to each dataset.

Figure 15 : IC₅₀ value of each PS over increasing light dose



NonLinear Regression - Global Curve Fitting
Equation: Power, 2 Parameter
 $f=a*x^b$
Constraint = $b<0$
Global Goodness of Fit: Rsqr = 0.9976

Data Set 1: PS1
Rsqr = 0.9699
 $a=1.9313$
 $b=-0.6285$

Data Set 2: PPA
Rsqr = 0.9993
 $a= 0.8862$
 $b= -0.7230$

Data Set 3: PS4
Rsqr = 0.9977
 $a= 400.1415$
 $b= -2.6753$

The standard cell kill experiment carried with PPA, PS1 and PS4 out over a range of increasing laser doses. X axis = energy delivered, and Y= axis μ M PS needed to kill 50% of cells. The IC₅₀ value from each experiment plotted over increasing light dose. Curves were fitted with a 2 parameter power curves and the constants from each curve shown alongside the graph.

3.2.3 Investigating the solubility of the new PS.

3.2.3.1 Spectral changes in different solvent environments.

To get an initial indication of solubility, all 3 photosensitisers were diluted to the same concentration into various solvents. The solvents were chosen to be relevant to future biological work and to cover a range of solvent polarity, osmolarity and protein content. Solvents properties as specified by supplier are tabulated below the spectra in Table 6. The dielectric constant (or relative permittivity) gives an indication of solvent polarity, an increase in polarity exhibits as an increase in permittivity. Osmolarity was chosen instead of molarity as it is more useful in the comparison of 'salty' solutions because it represents the number of moles of solute particles taking into account dissociation of any salts.

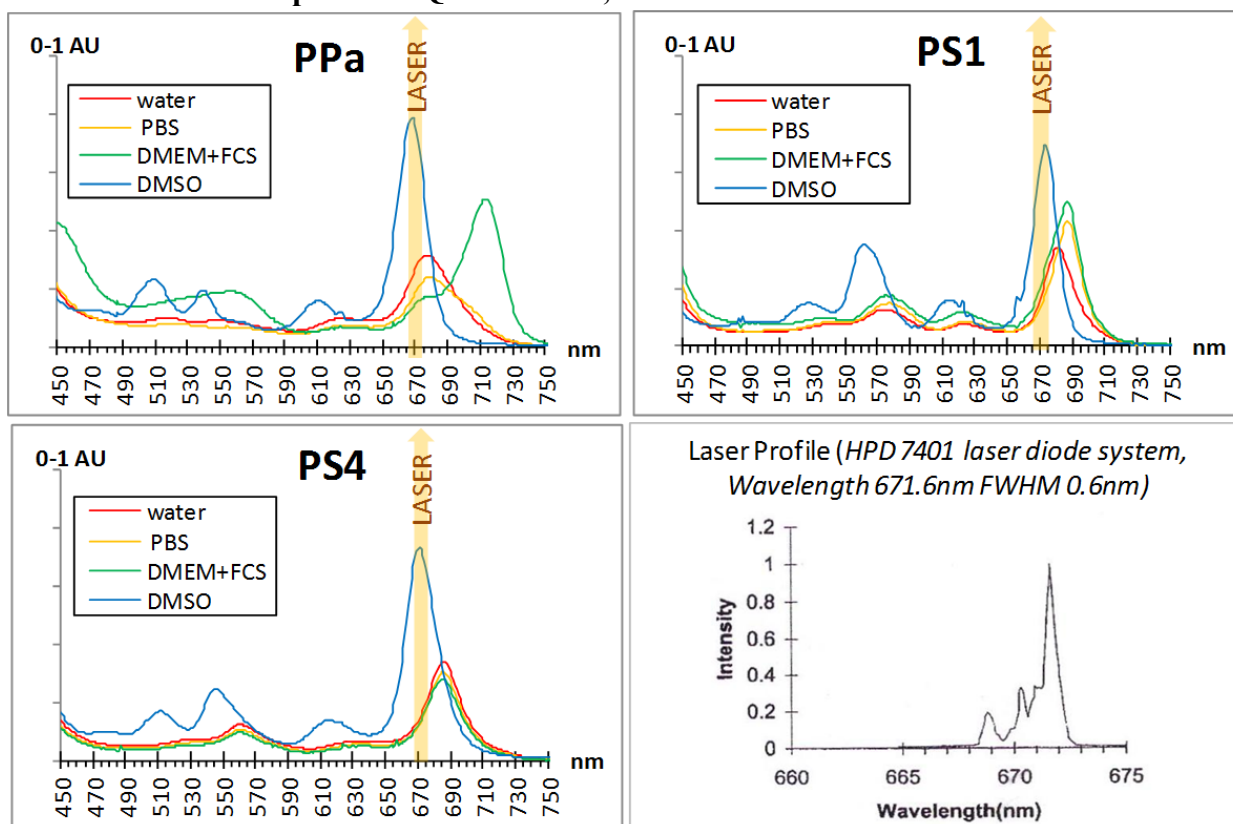
It is the Q bands (in particular Q band I) that were of most interest because they are nearest to the relevant irradiation wavelengths that will be used to carry out PDT, however all wavelengths were studied. The PS concentration chosen gives between the values of 0 and 1 AU (absorbance units) to avoid saturation of signal often caused by particle shadowing in more concentrated solutions. Additionally as solid PS were initially solubilised in 100% DMSO the final DMSO concentration in other solvents was maintained at $\leq 0.1\%$ at which was expected to have the same contribution, if any, to spectral shifts in each condition (Pan et al., 2001).

In the spectra shown in Figure 16 all three photosensitisers showed notable changes in Q band I upon dissolution in different solvents, all three photosensitisers showed red-shifts and reductions in intensity when dissolved in water compared to the less polar aprotic solvent DMSO. Upon dissolution in PBS compared to water shifts of PPa and PS4 showed little change, perhaps a slight decrease in intensity, PS1 exhibits a more notable further red shift and an increase in intensity. Upon dissolution in foetal calf serum (FCS) supplemented media compared to PBS, this time PS4 and PS1 show little change but PPa exhibits a significant red shift and an increase in intensity. In all cases when in more aqueous solutions Q bands II-IV seem to flatten and red-shift appreciably as to make them difficult to distinguish.

A common way to determine if shifts are due to aggregative processes or solvation processes is to look at the shifts change over different concentrations. Aggregation is concentration dependant where as solvatochromatic effects are not. In theory if there is aggregation you should acquire a linear correlation between absorbance and concentration at low concentrations and the point at which this changes is seen as evidence of aggregation, care must be taken not to confuse a saturating signal with the effect of particle shadowing mentioned above. For the data shown in Figure 16 there are no major peak changes or changes to the linear relationship of absorbance with concentration suggesting effects are due to solvatochromism. Fluorescence is actually a more sensitive measure of this effect but that will be discussed later see 4.2.2.1.

For analysis of this data the assumption was made that when a chromophore molecule is 'monomeric' in a solvent, i.e. when it is fully dissociated and has less intra and inter molecular interactions that reduce photo efficiency, it will show a more intense and more distinct absorbance pattern. When interactions with the solvent aren't favourable the molecule will undergo either normally unfavourable conformation changes or form interactions with other molecules in the solution more preferably than interactions with the solvent. These changes will often change the atomic orbitals available for the absorption of light and hence the absorption spectra. Most PS spectral solubility studies are done in reference to an assumed 'monomeric' conformation in an organic solvent like DMSO or Ethanol. As can be seen from the spectra in Figure 16 all PS in this study are most 'monomeric' in DMSO. As the shifts away from DMSO are less pronounced in PS4 over PS1, and both exhibit less shifts than PPa when they are dissolved into more physiological buffers, one can assume they also undergo less conformational changes / soluble aggregate formation, and therefore they are more soluble in these environments than their parent molecule.

Figure 16: Absorbance spectra of Q bands. PPa, PS1 and PS4 in various solvents



Absorbance spectra over 450nm to 750nm of PPa PS1 and PS4 dissolved in de-ionised water (red), PBS(yellow), DMEM media with phenol red and 10% FCS (green) and 100% DMSO (blue). All PS at a final concentration of 20 μ M. Spectrums of absorbance read over a blank of the relevant solvent and normalised to absorption at 900nm. Graphs overlaid with an indication of laser irradiation wavelength at 671.6nm shown more accurately alongside.

Table 6: Solvent properties taken from details specified by supplier

SOLVENT	Supplier (Cat No)	pH	Dielectric constant (20°C)	Osmolarity (mOsmol/kg)	Protein content (g/100mL)
Water	Purite System	6.9 -7.2	78.4	0.1	0
PBS	PAA HIS-002	7.0 – 7.5	(as aqueous)*	240 – 320	0
DMEM+10% FBS	DMEM =PPA E15-883 FCS=Gibco 10108-165	DMEM =6.8 -7.5 FCS= 6.90 - 7.80	(as aqueous)*	DMEM = 280 – 350 FCS = 280 - 340	DMEM = 0 FCS = 3.50 - 5.50
DMSO	Sigma D2650	none	46.7	none	none

(DMEM = Dulbecco's modified Eagle's medium)

(FCS = Foetal Bovine Serum)

(PBS = Phosphate buffered saline)

(DMSO= Dimethyl sulfoxide)

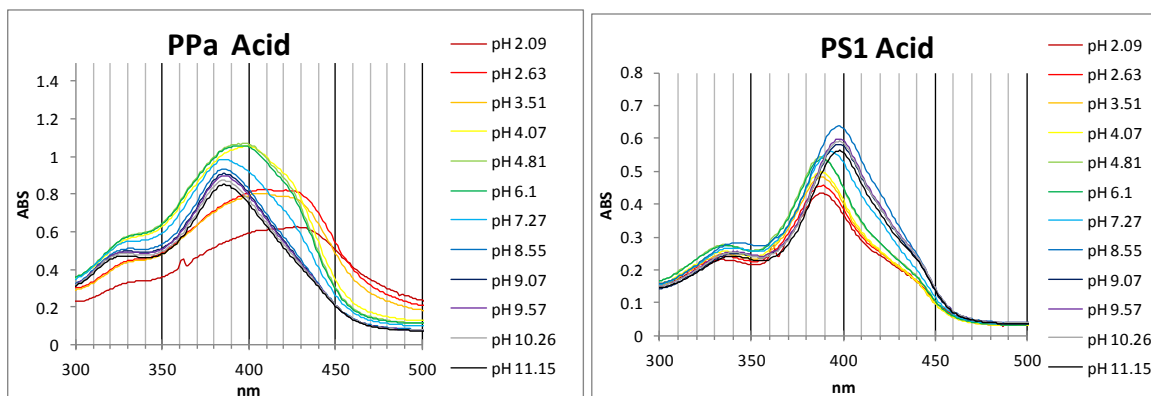
*unknown - proteins and salts will increase it but not vastly

3.2.3.2 PS spectral changes over pH change

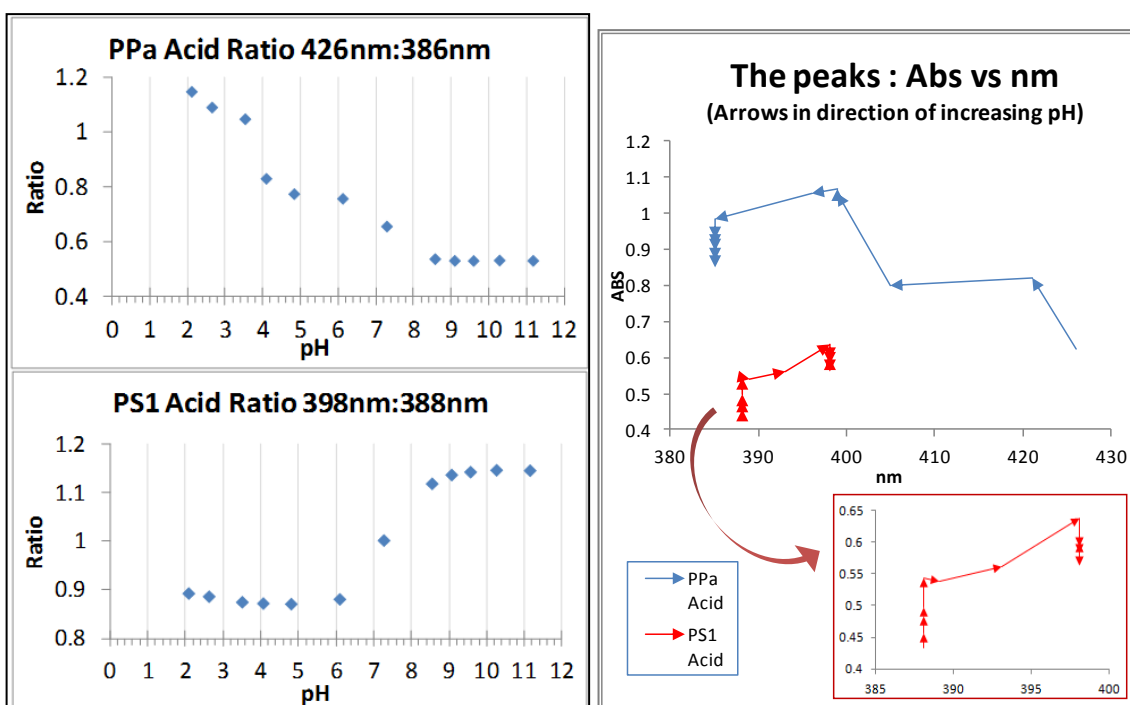
To further extend the spectral investigation a set of photosensitiser solutions were prepared at different pHs. In chromophoric compounds with ionisable groups, protonation or deprotonation of each group can affect the molecular orbitals within the compound as with solvation or aggregation. If you consider each ionisable group will have a pK_a , when pH is at this pK_a the species will be 50% ionised and 50% unionised, so this point should be at the mid-point of a spectrum changing between plateau areas where the majority species is either ionised or unionised and where you see no spectral changes (excluding speciation that doesn't show viable spectral change). Spectrums themselves represent averages of the population and can show the range of species present in the broadness of a band, (note; even single species bands exhibit some broadness due various internal rotational and vibrational levels providing a range of energy levels available).

PPa photosensitisers are multiprotic and two of the ionisation sites are within the delocalised electron system so likely to show visible spectral effects when altered. PS concentration was kept constant and pH was adjusted from a 0.2 M glycine solution with NaOH or HCl to prepare a range of solutions from pH 2 to pH 11. Figure 17 shows how the Soret band spectrum shifts with changing pH. The work could not be done with PS4 due to insufficient compound availability. Changes in species of PPa and PS1 over pH induces changes in intensity as well as shifts in wavelength. To make plateaus and transitions easier to visualise the ratio between peak absorbance at each pH extreme was plotted over pH and is also shown in Figure 17. However this method is biased to presenting shifts in wavelength over intensity; PPa seems to have 3 species with transitions over pH 3-4 and pH 6-8. But PS1 seems to have only two species with a transition over pH 6-8. However when you look at the spectrums themselves there does appear to be a matching shift over lower pH with PS1 to match PPa however it shifts over intensity not wavelength. Identical shift patterns were also seen in Q band I (not shown). The shifts seen between the PPa species are more dramatic than in PS1, this is shown more clearly by the graph in Figure 17 which shows a plot of change in peak (nm) vs. change in absorbance at that peak. It also highlights the direction of the shift; as pH increases, PPa shows a blue shift and PS1 a red shift.

Figure 17 : PS spectral changes over pH.



PS concentration was kept constant and pH was adjusted from a 0.2 M glycine solution with NaOH or HCl to prepare a range of solutions from pH 2 to pH 11 showing how the Soret band spectrum shifts with changing pH for PPa and PS1.



The same data presented as a ratio of peak absorbencies at each pH extreme (left) and change in peak wavelength vs. change in absorbance at that peak (right)

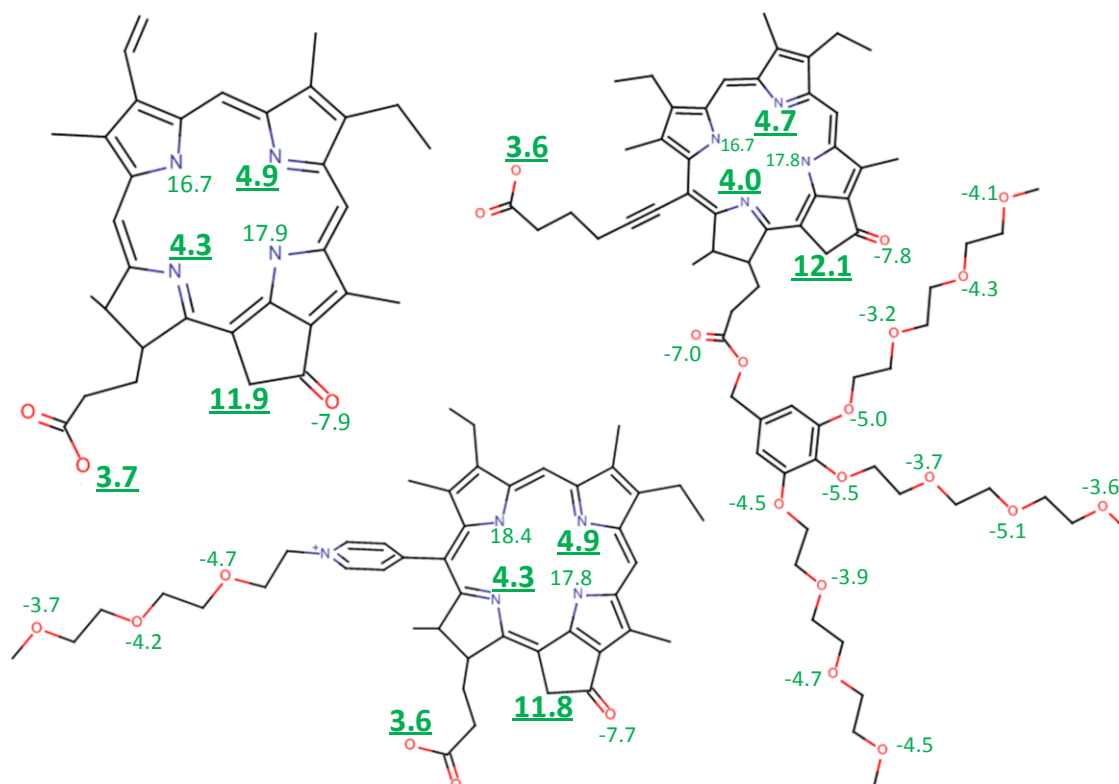
3.2.3.3 .Computational prediction of pK_a values within PS

To help understand the spectral data presented above some *in-silico* computer predictions of the pK_a of PS ionisable groups was done. The programme used here is Marvin, see 2.1.3.1. To carry out computational pK_a calculations a lot of computer programmes are available, (McLaughlin and Fisk, 2007). It is normally suggested that you use multiple programmes, preferably one from each algorithm family. Unfortunately many could not provide pK_a values for our compounds (ACD, SPARC, PHYSPROP, VCCLab), this is likely due to either the complexity of our compounds and number of possible sites, or lack of similar data in experimental databases. Additionally freeware options are more limited.

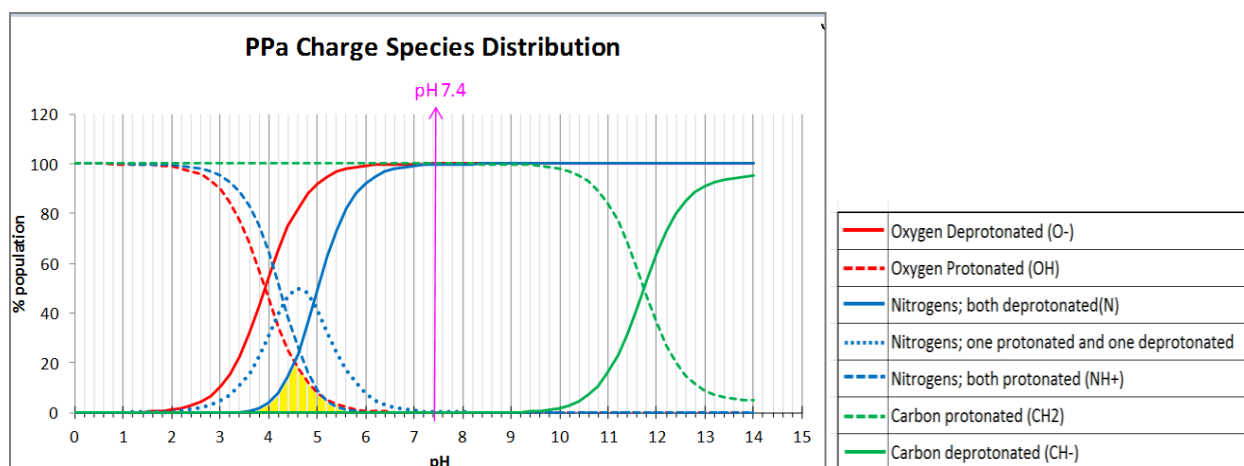
Figure 18 shows all three structures with their predicted pK_a Values, the ones that fall between pH 1 to 14 are in bold and underlined. In all three photosensitisers it is predicted to be the same groups that become ionised and the pK_a do not vary to any great extent between PS, although it is important to remember pK_a is a log scale so small changes will make big differences in the percentage of species that will be present. One should however also be wary of forming conclusions using computer prediction alone unless backed up with experimental data. The four ionisation atoms predicted are; two of the four nitrogens on the central pyrrole rings ($pK_a \sim 4.5$), the oxygen on the propionic acid side chain ($pK_a \sim 3.5$) and the allylic carbon in the cyclopentanone ring ($pK_a \sim 12$).

Because all photosensitisers are predicted to show a similar ionisation pattern only the charge species distribution graph for PPa is presented, this graph shows the percentage of each ionised form that is likely to be found at each pH for each of these three sets of atoms (nitrogens, carbon and oxygen). If these predictions are accurate it is likely the spectral shifts seen in the spectra above over pH 3-4 may be due to central N ionisation but the shifts over pH 6-8 must be due to a different pH induced effect. The yellow area in the charge species distribution graph shows where the unionised photosensitiser is predicted to appear. Importantly for LogP the unionised form is not predicted to be present as a majority species at any pH, so LogP would experimentally always be compromised. A logD may be a more relevant and obtainable indication of solubility.

Figure 18 : Computational prediction of pK_a values within PS.



In-silico computer predictions of the pK_a of ionisable groups on PPa, PS1 and PS4 was carried out with Marvin (see 2.1.3.1.). Structures are presented with their predicted pK_a values next to each ionisable group. The ones that fall between pH 1 to 14 are in bold and underlined.



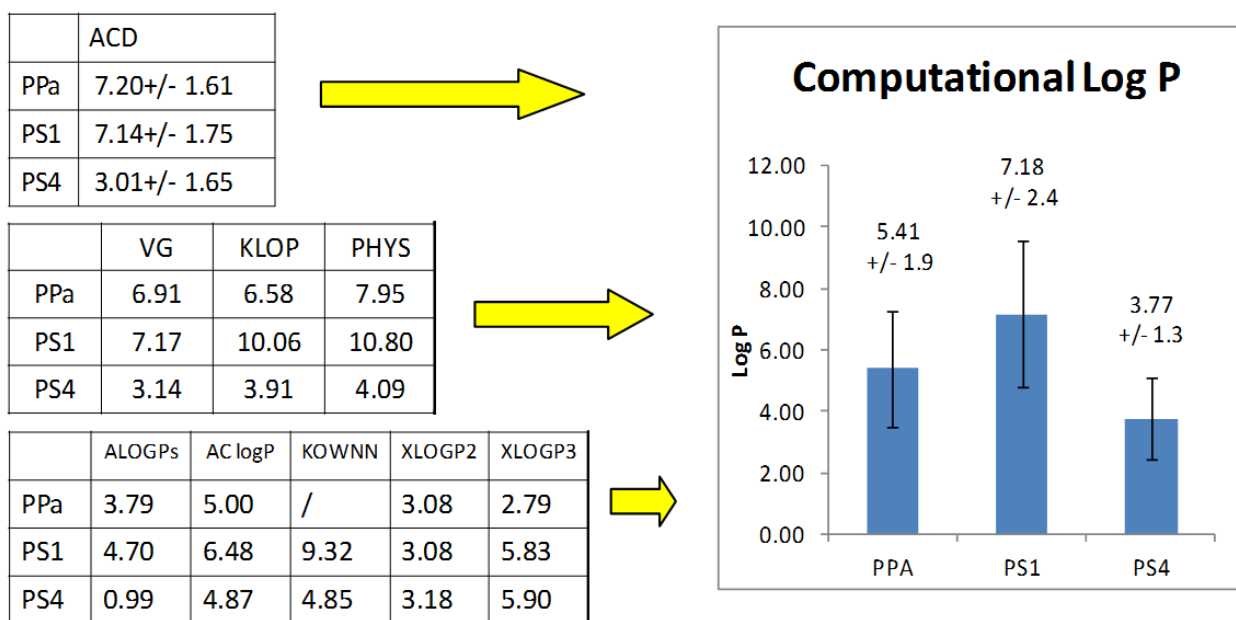
For PPa the charge species distribution graph for PPa is presented, this graph shows the percentage of each ionised form that is likely to be found at each pH for each of these three sets of ionisable atoms (nitrogens, carbon and oxygen).The yellow area in the charge species distribution graph shows where the unionized photosensitiser is predicted to appear.

3.2.3.4 Computational prediction of LogP values for PS

Computational LogP predictive software is also readily available as freeware online, multiple algorithms have been designed over the years and all are being continually improved. Many can provide impressive correlations with experimental data. Again it is normally suggested that you use multiple programmes, here three programmes were used to make predictions of LogP, two of which provide results for multiple different algorithms. The results are presented in Figure 19 individually in tables and as an average of all in graphical format. For more detail on the methods and software see 2.1.3.1. As can be seen in Figure 19 the range of calculated LogP values is large, (around 2 LogP units), this seems particularly unreasonable as in one report (Faller and Ertl, 2007) it was suggested as little as 0.5 would be the expected deviation. The apparent difficulty in computational prediction faced here is likely due to the number of ionisation group and the extensive delocalisation of electrons which will propagate interactions over longer distances than usually encountered by these computational models. The fact that these compounds are also unlikely to inhabit an completely unionised form, and that these compounds can also be prone to soluble aggregate formation when neutralised, makes LogD a more relevant parameter for this work.

Within the data the PS-1 predictions tended to appear more hydrophobic than their parent molecule, this does not agree with previous spectral data. PS1 was designed with short PEG chains; chains synonymous in the literature as a technique used to impart water solubility (Allen et al., 2002). One possible reason for the confusion of the computation software could be that PEG chains help to provide water solubility by helping to order the water molecules around the protein, creating a hydration shell, these complex global interactions may not be fully considered by the models employed by the various programs. Or perhaps because a majority of the prediction methods used to calculate LogP were of a fragmental method, the software is likely to work out all possible micro species (each micro species being a combination of possible ionisations) and PEG chains will provide lots of potential domains for ionisation, despite not being physiologically relevant ionisations to our work they could be altering the weighting of influence from the physiologically relevant ionisations in the overall calculation.

Figure 19 : Computational prediction of LogP values within PS



Three different programmes used to predict computational LogP of PPa, PS1 and PS4, two of which provide results for multiple different algorithms. Each programme is represented in its own table containing all the predicted LogP Values. Alongside an average of all these value is presented in graphical format. For more detail on the exact methods and software see 2.1.3.1.

3.2.3.5 Computational prediction of LogD values

LogD combines predicted LogP and pK_a information. For this reason Marvin see 2.1.3.1 was the only software that could be used to produce a predicted graphical LogD and this is presented in Figure 20. The peaks shown on the predicted LogD graph seems to match the experimental pH influenced spectral changes that were unexplained by predicted ionisation domains earlier 3.2.3.2. Between pH 2 and pH 8 the LogP is predicted to increase with a peak around pH 4.5, likely due to the prevalence of neutral form, resulting in a dramatic drop in solubility. This means that the data shown in Figure 17 represents not just the effect of ionisation domains but of a molecule becoming less soluble in its chosen solvent and forming different species of soluble aggregates.

3.2.3.6 Experimental prediction of LogD

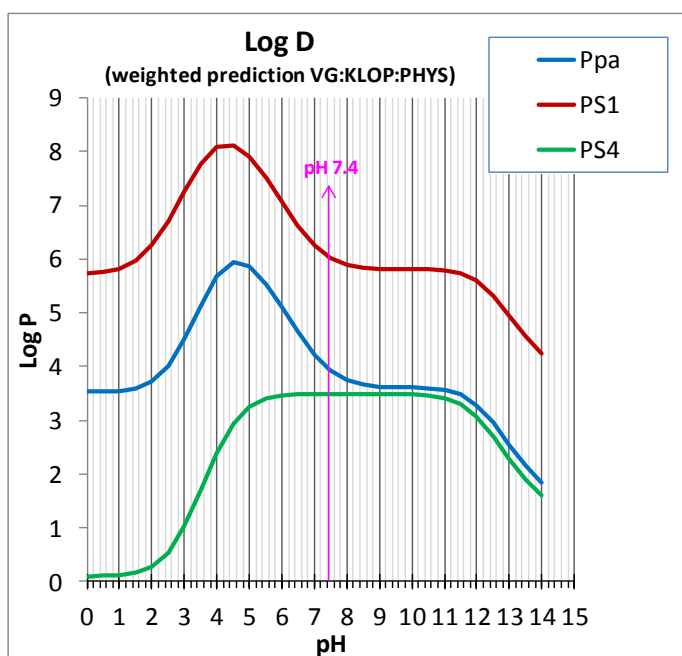
To attempt to provide more experimental evidence for any increased solubility of our new compounds an experiment like a LogP ‘shake flask’ experiment was carried out. As previously mentioned this sort of experiment requires a unionised form of the photosensitisers, which in isolation is unlikely, so an experiment was designed to predict LogD rather than LogP and the photosensitisers were partitioned in an aqueous-organic / liquid-liquid biphasic system consisting of 1-octanol and PBS at pH7.4 (Any ionisation of PS therefore will be physiologically relevant). Parallel experiments were carried out in which solute was diluted initially in either 1-octanol or PBS pH7.4 and then added to the opposite solvent, providing information on possible movement in both directions and hopefully reducing any bias to the solvent the PS was initially dissolved in. Photosensitisers were also diluted 1:1000 into the relevant solvent to try and avoid any influence of original preparatory solvent (DMSO) acting as a conduit where PS could exist in bubbles of DMSO as it travels between each solvent.

Table 7 shows the average ‘apparent LogD’ value of the two vials, each with the chosen solute travelling in a different direction. Due to low absorbance values for the PBS fraction absolute concentrations of PS could not be accurately calculated for this fraction so the calculation shown in Table 7 involved using just the data taken from the 1-octanol solvent

partitions in which it was assumed all photosensitiser is in a non aggregated state and absorbance values were within an accurate range. Control vials of the same concentration of PS in purely 1-octanol were calculated alongside and therefore any loss in the biphasic system could be calculated as a fraction of this and the corresponding hypothetical fraction that moved into the aqueous fraction also calculated.

The experimental 'apparent LogD' data shows PS4 and PS1 exhibit lower values of LogD compared to PPa (PPa>PS1>PS4). However the errors are large and for this data to provide any useful indication of solubility one would need to carry out subsequent repeats of the experiment and at higher PS concentrations, the dilution factor meant very small volumes of stock photosensitiser were handled and absorption values low.

Figure 20 : Computational prediction of LogD values



LogD combines predicted LogP and pK_a information. Previous predictions from Marvin for these two values were combined by the same software to provide a graphical LogD. Physiological pH (pH7.4) is marked by a pink arrow, at pH 7.4 LogD is 3.92 (PPa) 6.01 (PS1) and 3.48 (PS4).

Table 7: Experimental prediction of LogD.

	Apparent LogD	Standard deviation between vials
PPa	1.266	0.64
PS1	1.086	0.36
PS4	0.748	0.12

An experiment like a LogP 'shake flask' experiment was carried out in which PPa, PS1 and PS4 were partitioned in a aqueous-organic / liquid-liquid biphasic system consisting of 1-octanol and PBS at pH7.4. See 2.1.6.2 for method details.

3.3 Discussion

3.3.1 Potency of the new PS in the *In-vitro* PDT model

Changes were made to the methodology of the *in vitro* PDT cell kill assay used in previously published work (Stamati et al., 2010). When directly compared in the same assay, the altered conditions demonstrated no significant change to the cytotoxicity of PS, but when IC₅₀ values from the new assay were compared to those previously published the PS have different potencies: **Stamati et al** : IC₅₀ = PPa 14.5 µM, PS1 1.1 µM and PS4 259.6 µM, **This Thesis**; IC₅₀ = PPa 2.1 µM and PS1 3.5 µM with PS4 non toxic even up to 500 µM. This could be due to unknown changes made in the protocol between researchers, changes in batches of PS, storage conditions or even an adaption of the cell line over time. The experiments were repeated a number of times over the course of this thesis and the IC values were reproducible. The cells were divided into stock vials and kept within a limited passage for all experiments.

A particular problem with the development of a successful *in vitro* assay for PDT is the increased number of variables over a standard drug toxicity model. This also makes it hard to compare PS cytotoxicity across the literature and to clinical standards. As demonstrated in this chapter; changing the light dose can change the cytotoxicity of a photosensitiser (PPa) from 2-3 µM to 60-70 nM, and a PS (PS4) which could have been abandoned due to complete loss of cytotoxicity has been retained for further analysis because when light dose is increased it actually showed more potential for an improved relationship between toxicity and delivered light over its parent molecule, a hypothesis backed up by PS4s increased singlet oxygen quantum yield in toluene (shown in 1.6).

It will be of interest to investigate what changes the chemical manipulations of PPa (i.e. PS1 and PS4) has had on cellular uptake, intracellular localisation and the mechanism by which these new photosensitisers initiate cell death. All these things will be investigated in the next chapter but it was initially desirable to also try to obtain an experimental demonstration of any increase in solubility of these new PS.

3.3.2 Spectral investigation of PS solubility.

Stamati and colleagues (Stamati et al., 2010) designed two new photosensitisers to exhibit more water solubility than their parent compound PPa. In the work presented here an indication of the increase in solubility provided by these chemical modifications was investigated.

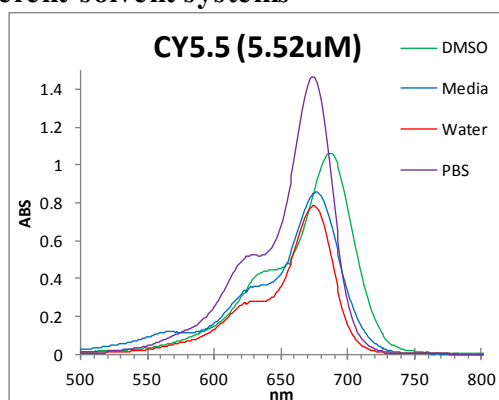
PS contain chromophores which can function as inherent solubility indicators, shifts in their absorbance spectrums when dissolved in different solvents can help indicate how well the molecules interact with the solvent. In the data presented in this chapter larger shifts away from monomeric PS were seen with PPa than with its derivatives after dissolution in more physiological solvents such as water, PBS and supplemented cell culture medium. For reference a chromophore known to have good water solubility; Cy5.5 dissolved in the same solvents as PS is shown below in Figure 21. From the spectral data one could conclude that PS1 and PS4 are both more soluble in aqueous solution than their parent compound, PS4 more so than PS1. Another important thing to note from the spectral data is that some of the shifts in absorbance take the spectrum a significant distance, in some cases outside the range of the emitted light from the laser being used for therapy, if similar shifts occur intracellularly it would have an influence on the cytotoxicity of these compounds.

The spectra for the new photosensitisers all show a notably lower intensity red-shifted spectra for Q band I when dissolved in water compared to the less polar aprotic solvent DMSO. An increase in the intensity of a band would be more preferable (as seen in Figure 21) because if the molecules are absorbing more photons and the deactivation pathways are unaffected it will increase the efficiency of photophysical processes. The impact of a red or blue shift is less predictable of photophysical efficiency unless the shift to the red is to such an extent that the energy level inhabited is now so low as to not be sufficient to be able to react with the preferred target molecules required for effective PDT induced toxicity.

Similar work was carried out by Aveline and colleagues (Aveline et al., 1995) with benzoporphyrin derivative monoacid ring A (BPDMA), another chlorin-type molecule, they showed concentration dependent self association into non covalent aggregates in aqueous media,

this was exhibited through the absorbance spectra with a broadening of all bands along with a reduction in intensity, the Q bands were red shifted and the Soret band was blue shifted. Addition of BSA to the aqueous media returns the spectra (blue shifts) to that of a monomeric PS (i.e. similar to its spectra in organic solvent). Kelbauskas and Dietel (Kelbauskas and Dietel, 2002) conducted a study of pyropheophorbide-a ether (PPa-ether) and chlorin e6 derivatives possessing alkyl chains of 1–9 carbons. Lipophilicity of these compounds increased with increasing alkyl chain length. The PS which were shown to aggregate in aqueous solution (PBS) vs. monomerisation in organic solvent (methanol) also showed in their absorbance spectra a broadened Soret band and broadened and red shifted Q bands.

Figure 21: Cy5.5 in different solvent systems



A chromophore known to have good water solubility; Cy5.5 dissolved in the same solvents as PS presented in Figure 16.

The spectral shift of PS in media-supplemented with FCS when compared to PBS is particularly intriguing; large changes were seen with PPa compared to much smaller changes with the new photosensitisers. The introduction of serum proteins (in FCS) could indicate the formation of non-specific interactions between serum protein and PS, visualised by shifts in the PS spectra, these would affect both any future antibody conjugation reactions and the PS's pharmacokinetic behaviour in the blood. Nevertheless the introduction of PS to an environment as complicated as the human body can sometimes be unpredictable. . It is worth considering that there are other differences between media and PBS that could account for this change other than serum proteins, but there is a significant body of work that supports the observation that these molecules (PS) often form associations with BSA or LDL and that this can affect its spectra, these are discussed in part below and in more depth in 1.3.4.

Fluorescence emission profiles have previously been used to investigate the same thing in other PS however the data tends to show a return to a monomeric type spectra with the introduction of serum proteins: Rinco and colleagues (Rinco et al., 2009) looked at a range of different porphyrin molecules and their fluorescent emission profile in different solutions including toluene, methanol, 0.04M phosphate buffer (pH 7.4) and phosphate buffer supplemented with human serum albumin (HSA). Most showed a blue shift in their fluorescence spectra with increasing solvent polarity (toluene < methanol < phosphate buffer), and a red shift once HSA was added to the phosphate buffer solution. Mojziso and colleagues (Mojziso et al., 2007c) looked at Chlorin e6, a PS which is based on same macrocycle as PPa, is also derived from methyl-PPa but has three additional carboxylic acid groups on one side of the ring. Their work demonstrated PS fluorescent emission profiles showed a red shift and increased intensity with association with human serum albumin or LDL.

Seemingly most papers that show a shift in PS spectra will state aggregation of the molecule as its cause, but there are many other factors that can influence spectral shifts, see 3.1.4. Although no precipitates were seen in any of the solutions used in this work, it may be useful to carry out more work to confirm if the formation of soluble aggregates is indeed occurring. As aggregation is concentration dependant, aggregates will present a spectral shift back to a monomeric spectral pattern at lower PS concentrations. This was not seen in the work carried out for this thesis; however it could be that PS were not diluted sufficiently, (although absorbance was approaching the limit of detectability). To check this idea more thoroughly fluorescence can be used and is a much more sensitive measure of this aggregative effect, fluorescence has the additive sensitivity of measuring not just miss-absorption of laser light but the quenching of fluorescence due to the close interaction of another molecule. Quenching occurs as either the emitted light is re-absorbed or the energy transferred in non-radiative transition to the other molecule without any emission of light. Fluorescence as a result of changing PS concentration is looked at in the next chapter, see 4.2.2. Isothermal titration calorimetry (ITC) could also be used to confirm aggregation as it is able to detect the change in heat energy as aggregates or micelles form in a solution (Heerklotz and Seelig, 2000). This could be especially interesting in the case of PS-1 as its chemical structure suggests it could form micelles via its 'soapy' PEG-like tail.

3.3.3 The partition and distribution coefficient of PS (LogP and LogD).

A well recognised measurement of solubility is the partition coefficient LogP, to calculate this an unionised form of our compound was needed but computational software predicted that this would be unlikely (see Figure 18). Another complication in carrying out a LogP partition experiment with a molecule like PPa is ion pair partitioning; where the charged species pairs with a reagent ion or even in certain cases itself. Instead LogD, an ionisation dependent form of LogP, was decided more suitable, in addition LogD has been shown to be a more physiologically relevant parameter for drug discovery and more predictive of pharmacological outcome than LogP (Bhal et al., 2007).

As presented in this chapter the experimentally derived LogD values, see Table 7, agree with the spectral data that the order of aqueous solubility from least soluble to most soluble is, PPa (LogD=1.266), PS1 (LogD=1.086) then PS4 (LogD=0.748), but as discussed there were problems with data reliability. Problems seen with the reliability are echoed in work by Engelmann and colleagues (Engelmann et al., 2007) whom carried out a similar initial experiment but improved their data by carrying out the experiment in a butanol–water system. The results from the LogD experiment presented in this thesis were a lot lower than any values predicted by computation software. Published LogP and LogD values for similar molecules in the literature cover a large range so both numbers presented here could be in line with what would be expected. Other researchers too have seen variations between LogP/LogD values for the same molecule when they are calculated using different models, however most also show the trend between different molecules will be reliable when using the same model, see 3.1.3.

It is often hard to match up computational data and experimental data because there are many elements not always easy to factor into computational calculations. For LogP/LogD values these can include ion pair interactions, interactions between the molecules themselves and consideration of the solvent molecules not just the structure of interest (McLaughlin and Fisk, 2007; Xing and Glen, 2002b). This leads to both difficulties in prediction as well as the design and interpretation of experimental data.

Other simple methods to look at aqueous solubility beyond partitioning or spectral shifts also exist, for example Pan and colleagues (Pan et al., 2001) added a compound to aqueous solution and sieved it through nylon or PTFE filters to measure how much remained in solution via spectrometric analysis at 245nm (most organic compounds will have strong UV absorbance here) or you can measure turbidity using a Nephelometer.

3.3.4 Spectral shifts with pH change

The importance of looking at spectral shifts over pH may initially be overlooked but the work carried out in order to help design a LogP experiment (i.e. trying to visualise ionisation domains) may also have given us hints about pharmacokinetic behaviour. Computational prediction of ionisation domains showed very little difference between PS1 and PPa, but the spectral changes upon changing pH were markedly different: Both PPa and PS1 show 3 species with transitions over pH 3-4 and pH 6-8, but the shifts occur in opposite directions and to different extents. PS1 transitions were smaller and as pH increased, light of a lower energy was absorbed, i.e. it was red shifted. In comparison PPa showed a blue shift as pH increased and the changes that occurred were of a more dramatic nature.

Other labs that have studied pH dependent changes in PS including Mojzisoova and colleagues (Mojzisoova et al., 2007c) who showed pH dependant spectral changes when they looked at Chlorin e6, a PS based on same macrocycle and also derived from Methyl-PPa. Their work examined its fluorescence emission spectra in PBS at pH 6.5, 7.4 and 8.0 and they showed a decrease of the fluorescence intensity and a small blue shift (3nm) when the pH was decreased. Bonneau and colleagues (Bonneau et al., 2004a) looked at membrane diffusion rates for an asymmetrical porphyrin with 2 carboxylic acid groups on the same side of the ring. Dimerisation of the PS was shown to be pH dependent and accompanied by changes in absorption spectra and a strong inhibition of the fluorescence. The monomer and dimer forms could be identified by bands at 395 and 370 nm respectively, this shift was in keeping with the exciton theory that a blue shift is interpreted as the formation of dimers with face to face stacking, the structure of the PS also suggested this formation as the polar groups would be arranged in opposite directions to

reduce repulsion. This arrangement meant the distribution of polar chains in the dimer was roughly symmetrical this reduced its subsequent incorporation into cellular membranes.

These pH dependant shifts, as with the shifts seen in different solvents, can also help predict behaviour in a cellular environment. PS1 shifted less than PPa over pH so it is more likely to retain optimum photophysics in the range of pHs found within cellular environments see 4.1.2. Pharmacokinetics may also be affected as the pH of the environments a drug can be exposed to will vary considerably, some examples are as follows: blood plasma 7.4, tumour extracellular media 6.9, stomach 2.0, kidneys 4.2, small intestine (food 5.0; fasted 6.8), duodenal mucus 5.5, (Xing and Glen, 2002b).

3.3.5 Chapter conclusions

Key proposition put forward from work;

PPa derivatives PS1 and PS4 were shown to be more water soluble than their parent compound and show less spectral shifts in a range of physiological environments which could suggest improved photo-physical characteristics for effective traditional PDT in a biological environment. However in an *in vitro* non-targeted PDT cancer cell model, initial data suggested these new more soluble PS became less potent.

Main question raised from this work;

Are there any other experiments looking in more detail at some of the factors beyond photophysical efficiency that can be carried out to find out why changing the solubility in this way has caused a loss in observed potency?

Chapter Four

4 CHAPTER 4: The effect of increased PS solubility on PDT cellular mechanisms

4.1 Introduction

The work presented in this thesis on cell death mechanisms is being undertaken in order to see if changes to PS structure can modulate the type of cell death induced. However there are additional benefits to looking at cell death mechanisms: If one can identify the cell death pathway that is implicated, or the importance of particular biochemical cascades or molecular targets that are being inhibited or activated, this can allow prediction of possible *in vivo* effects, including any possible immune activation, any possible resistances that could be pre-determined in certain sets of patients or help in the design of combination treatments.

Regulation of cell survival, cell division, and all the forms of cell death are highly interconnected. Manipulation of just one variable is sometimes enough to switch cellular fate between these paths. (King and Cidlowski, 1995; Maddika et al., 2007; Vermeulen et al., 2003). However the mechanism and rate of PS cellular uptake and any subsequent intracellular localisation will also have a significant effect on PDT efficiency and the type of cell death initiated.

4.1.1 The different cell uptake mechanisms

Mechanisms controlling the membrane transport and uptake of compounds into cells can be divided into passive mechanisms, (single molecule with concentration or electrochemical gradient), active mechanisms, (single molecule against the concentration or electrochemical gradient) or bulk flow mechanisms (multiple molecules via vesicle mediated transport). Passive mechanisms occur via direct diffusion or can be facilitated by protein channels or carriers. Active mechanisms are defined by the requirement of carriers or pumps that are coupled to a source of metabolic energy, for example adenosine triphosphate (ATP) hydrolysis or an ion gradient. Bulk flow can be categorised as active or passive; for example endocytosis as a process requires cellular energy but can at the same time take up specific molecules against their

concentration gradient or non specifically 'drink' molecules from the extracellular environment. NB these processes exist in two directions.

Theoretically any molecule can diffuse across a lipid bilayer down a concentration gradient but for larger, more polar or charged molecules the rate of this becomes inhibitive slow. The extent of diffusion of molecules across the membrane is controlled by the interaction between the composition and structural arrangement of the membrane and the physicochemical characteristics of the molecule including its hydration and/or solvation. Passive facilitated transport of molecules through channels or carriers also follows this same gradient, but the facilitation lowers the activation energy for the transport process.(Brandl et al., 2007) .

Passive and active transport can occur directly over the plasma membrane into the cell cytosol, but in bulk transfer vesicles containing patches of the cellular membrane, often containing a high concentration of protein or lipids acting as receptors for extracellular ligands are 'budded off' and invaginated forming an intracellular vesicle, this vesicle then travels on microtubules and links up to a pathway of vesicles and organelles that share membranes and cargo. This vesicle mediated transport can carry cargo in both directions between the cell surface and internal organelles including endoplasmic reticulum (ER), Golgi, Endosomes and Lysosomes. Endocytosis travels from the cell surface in, and exocytosis from intracellular organelles out. Cargo can be either part of the vesicle wall, (lipids themselves or lipid associated macromolecules), or soluble cargo carried in the aqueous centre of the vesicle. Organelles outside this system (inc mitochondria, peroxisomes, nucleus) have independent trans-membrane transport mechanisms between them and the cytosol for protein and other molecular movement, membranes for these compartments are not shared directly with other organelles as with the vesicle system mentioned above but phospholipid exchange proteins can move lipids around specifically between organelles (Alberts et al., 2002).

Endocytosis can be divided into phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and the multiple remaining mechanisms that are clathrin- and caveolae independent. See Figure 22. Although cargo selection and uptake mechanistically is different, all pathways appear to feed into the same early endosome system,

but with differences seen on final locations and potential interactions occurring during the trafficking process. Cargo taken from the cell surface by endocytosis will be, if not otherwise directed, confined within the endocytic pathway. The default process follows transport of cargo through early endosome to late endosomes and finally the lysosome. (Conner and Schmid, 2003; Doherty and McMahon, 2009; Luzio et al., 2007; Maxfield and McGraw, 2004; Sorkin and von Zastrow, 2009).

4.1.2 Cell uptake and drug design

Although it has long been established that transporters across the plasma membrane exist for various endogenous compounds such as glucose, amino acids, nucleosides, water soluble hormones and neurotransmitters, cellular uptake for non endogenous drugs was traditionally thought to be exclusively via passive diffusion through the lipid bilayer, and many pharmaceutical processes designed drugs around this idea. Many novel techniques have been designed to overcome the cell membrane barrier and drive cellular uptake (Rajendran et al., 2010a; Sobolev et al., 2000b).

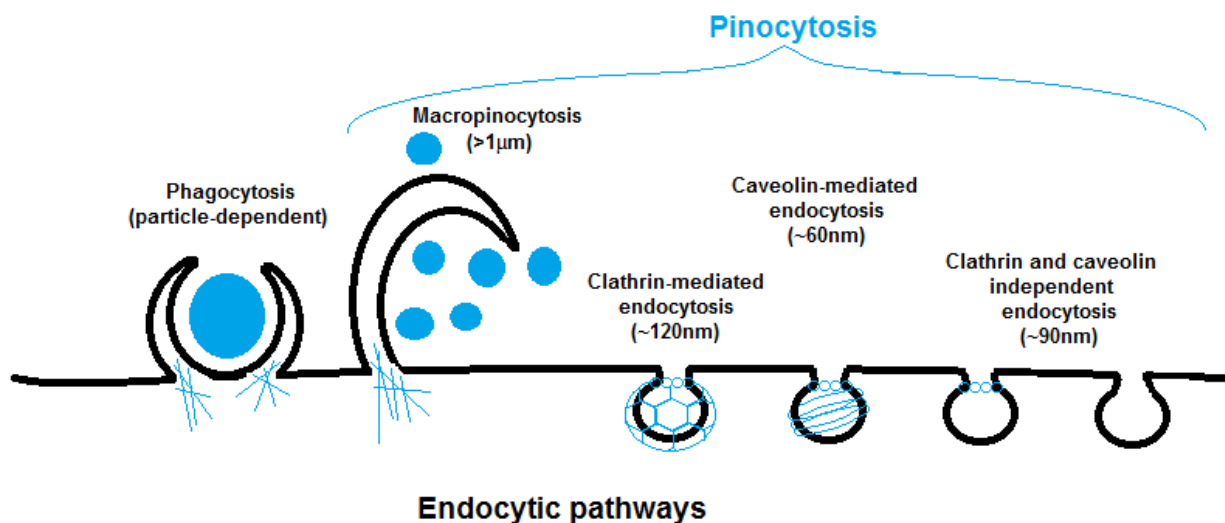
A series of opinion papers published relatively recently put forward the theory that successful membrane crossing could in fact be dominated almost solely by carrier-mediated cellular uptake via transporters, they argue what is thought to be uptake via passive diffusion is due to either unknown transporter interactions, transporters as yet unknown with uncharacteristic substrate specificity or due to the hitchhike of drugs on endogenous molecules. (Di et al., 2012; Dobson and Kell, 2008; Kell et al., 2011; Sugano et al., 2010).

After endocytotic uptake, drug macromolecules designated for target organelles, i.e. nucleus, mitochondria can undergo what is called endosomal entrapment and end up stuck in the lysosome. There have been numerous methods designed to overcome this and free the drug to the cytosol (El-Sayed et al., 2009; Rajendran et al., 2010b; Torchilin, 2006).

Particularly useful for manipulation in drug design is the trans-membrane pH gradients that exist between various organelles, covering a range from pH 8 to 5 for example: cytosolic and ER

(pH 7.1-7.2) golgi (pH 6.2-7) secretory granules (pH 6-5) cell surface (pH 7.2-7.4) endosomes (pH 5.5-6) lysosomes (pH 5.0) and mitochondria (pH 8). Changes in proton concentration between microenvironments therefore can cause changes in the physiochemical behaviour of drugs -especially those with groups with a pK_a between 5 and 8. For example; as pH decreases below the pK_a of molecule containing a weak acid group the anionic charge is neutralised driving an increase in membrane partitioning of that group. A molecule with a weak base would show the opposite behaviour and membrane partitioning with the neutral species would occur as pH increases above the pK_a . The different concentrations of other ions (e.g. potassium, sodium, calcium) between the inside and outside of the cell, as well as within different intracellular compartments, creates membrane potentials across cellular membranes (also called membrane voltages); the plasma membrane typically has a membrane potential of -60 to -80 Millivolts (mV) in amplitude, and for the mitochondria this membrane potential is thought to be between 180 and 200 mV. Any molecules with a net positive charge are therefore favoured in their attraction for these slightly negatively charged membranes and should have improved uptake efficiency (Asokan and Cho, 2002; Rajendran et al., 2010b).

Figure 22 : Mechanisms of Endocytosis



Reproduced from (Conner and Schmid, 2003) The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo, the type of lipids and receptors in the vesicle, and the mechanisms of vesicle formation. (NB: monomeric PS is $\sim 1\text{-}2\text{nm}$)

4.1.3 Categorised cell death

Cell death can be categorised into many forms. The field of cell death has however perhaps suffered from the rapid and un-controlled introduction of new terms for types of cell death, leading to confusion and incorrect generalisations. In the few years there have been various publications to try and re-address this, (Galluzzi et al., 2007; Golstein and Kroemer, 2007; Kroemer et al., 2009). Cell death categorisation is defined by observed morphology, this in turn is a reflection of the pathway taken to death. An official commentary from the ‘nomenclature committee on cell death’ in 2009 defined cell death into four distinct categories, Apoptosis, Autophagy, Cornification and Necrosis, and these are shown below in Table 8. Interestingly since their last commentary in 2005 this has reduced drastically from the eight categories presented then (Kroemer et al., 2005). This new categorisation of cell death vocabulary has made the process of carrying out experiments into cell death a little more challenging, no longer can one molecular end point such as caspase activation be sufficient to define cell death and many conventional assay interpretations now no longer stand (Galluzzi et al., 2009).

4.1.4 Practical considerations

Many of the key biological markers of cell death are transient and will occur at different time points so the timing of a cell death assay is an important consideration. The cell line in use is also important, as its immortality means it will likely have mutations or an altered phenotype that mean some cell death hallmarks or receptors for apoptotic stimulus will be missing or non functional. Also factors such as cell density, differences in cell cycle phases, passage number and the density to which the parental culture was grown can affect a cells ability to respond to a stimulus. Care also needs to be taken when comparing data from populations of cells vs. single cells (Sundquist et al., 2006) (Goldstein et al., 2000). Finally *In vivo* observations show the removal of the apoptosing cells by phagocytosis, with the absence of these immune cells *in vitro* apoptotic cells will continue through cell death after apoptosis into a state that more resembles necrosis, this transition is termed secondary necrosis. (Krysko et al., 2006; Silva et al., 2008).

Table 8: Modalities of cell death (nomenclature committee on cell death).

Taken from (Kroemer et al., 2009).

Cell death mode	Morphological features	Notes
Apoptosis	Rounding up of the cell	'Apoptosis' is the original term introduced to define a type of cell death with specific morphological features. Apoptosis is NOT a synonym of programmed cell death or caspase activation.
	Retraction of the pseudopodes	
	Reduction of cellular and nuclear volume (pyknosis)	
	Nuclear fragmentation (karyorrhexis)	
	Minor modification of cytoplasmic organelles	
	Plasma membrane blebbing	
	Engulfment by resident phagocytes, <i>in vivo</i>	
Autophagy	Lack of chromatin condensation	'Autophagic cell death' defines cell death occurring with autophagy, though it may misleadingly suggest a form of death occurring by autophagy as this process often promotes cell survival
	Massive vacuolisation of the cytoplasm	
	Accumulation of (double-membraned) autophagic vacuoles	
	Little or no uptake by phagocytic cells, <i>in vivo</i>	
Cornification	Elimination of cytosolic organelles	'Cornified envelope' formation or 'keratinisation' is specific of the skin to create a barrier function. Although apoptosis can be induced by injury in the basal epidermal layer (e.g., UV irradiation), cornification is exclusive of the upper layers (granular layer and stratum corneum).
	Modification of plasma membrane	
	Accumulation of lipids in F and L granules	
	Extrusion of lipids in the extracellular space	
	Desquamation (loss of corneocytes) by protease activation	
Necrosis	Cytoplasmic swelling (oncosis)	'Necrosis' identifies, in a negative fashion, cell death lacking the features of apoptosis or autophagy. Note that necrosis can occur in a regulated fashion, involving a precise sequence of signals.
	Rupture of plasma membrane	
	Swelling of cytoplasmic organelles	
	Moderate chromatin condensation	

4.1.5 Molecular markers for apoptosis

Popularly reported molecular markers for apoptosis include; initiator and effector caspase activation, mitochondrial membrane permeabilisation and release of cytochrome c, externalisation of phosphatidylserine on the plasma membrane, and internucleosomal DNA fragmentation. Not all of these biochemical events are specific to apoptosis, and even within apoptosis may not always occur.

Caspases consist of a family of proteases and activation of the ‘caspase cascade’ was a classical marker of apoptosis. This was until the discovery of types of caspase independent apoptosis and the involvement of some caspases in non lethal signalling pathways for inflammation, differentiation and migration. Caspase activation is now no longer a requirement for the categorisation of apoptosis but as effector caspase targets still include the various proteins important for the induction of cell death morphologies they are still a well respected marker (Earnshaw et al., 1999; Huai et al., 2010; Pop and Salvesen, 2009). Malfunction of apoptosis plays an important role in the pathogenesis of tumours therefore it is unsurprising that many cancers show mutations in one or more of the key caspases or regulatory proteins, these mutations are also often also a factor in the development of treatment resistance (Ghavami et al., 2009).

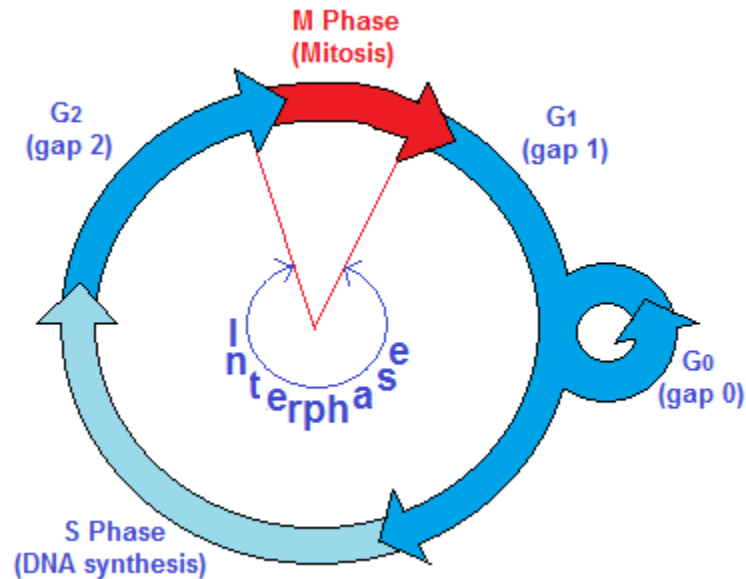
DNA fragmentation, unlike caspase activation is still a hallmark fundamental to the classification of apoptosis, and can be seen as a united endpoint for various different upstream apoptotic initiating pathways. During apoptosis, DNA fragmentation usually occurs in two stages: initial cleavage of chromatin loop domains (creates 50–300kb fragments) then subsequent cleavage of exposed internucleosomal DNA (creates fragments in approximate multiples of 180bp) (Kitazumi and Tsukahara, 2011). Several nucleases have been shown to be able to induce degradation of DNA during apoptosis, (Counis and Torriglia, 2006; L. Y. Li et al., 2001; Nagata et al., 2003) but the two main ones are endonuclease G (EndoG) and DNA fragmentation factor (DFF) (Candé et al., 2002; Widlak and Garrard, 2005). DNA damage is fundamental to the progression of cancer and many cancers have been shown to lack aspects of the DNA damage response pathways (Aziz et al., 2012; Jackson and Bartek, 2009).

4.1.6 The cell cycle and cellular senescence

Many cells in the human body are capable of cell division and proliferation but reside in a non-proliferative state, called G_0 , which despite its name is in fact quite an active phase in which cellular functions still occur as the cell awaits the stimulus to enter the cell cycle which commits a cell to a path resulting in cell growth and cell division. The cell cycle is divided into four phases (G_1 , S phase, G_2 , and M phase) and these are shown in Figure 23 (Alberts et al., 2002; Norbury and Nurse, 1992; Sherr, 2000). Progression through the cell cycle is monitored by checkpoints which will prevent or delay cell cycle progression if various criteria are not satisfied, they are also involved in the initiation of repair mechanisms. If cells aren't able to re enter the cycle, cells can be induced to enter cellular senescence or undergo apoptosis. There are currently thought to be numerous checkpoints within the cell cycle, found in all parts of the cell cycle (Khodjakov and Rieder, 2009; Nyberg et al., 2002).

Cellular senescence is in most cases an irreversible process during which cells acquire widespread changes in gene expression and can acquire certain resistances to apoptotic cell-death signals. Cells can undergo cellular senescence due to various cellular stresses including DNA damage and oxidative stress, this is termed stress-induced senescence or (M_0) (M =mortality phase) and is independent of both telomere-initiated senescence (cellular aging or M_1) and mitotic catastrophe (M_2). Cell cycle arrest can be hard to study *in vitro* as by necessity tumour cells have mutations in pathways thought to be responsible for normal cell ageing and cellular senescence, although many papers have shown many they retain the ability to undergo stress induced senescence and there is a long history of cell cycle regulation for cancer treatment (Campisi and d'Adda di Fagagna, 2007; Malumbres and Barbacid, 2009, 2001; Schwartz and Shah, 2005). The cellular stresses responsible for the induction of cellular senescence are very similar to those that induce apoptosis. Importantly for PDT, ROS are thought to be able to play an active role in induction of cellular senescence in both prolonged exposure or short bouts of oxidative stress, (Colavitti and Finkel, 2005).

Figure 23 : The cell cycle



The cell cycle can be separated into two major phases that alternate with each other: Interphase (shown in blue), during which the cell grows, prepares for mitosis and duplicates its DNA, and the mitotic (M) phase (shown in red), Interphase can be further divided into three phases: G_1 , S phase, and G_2 . G_1 phase is the gap phase which leads to a cells commitment to entry into the cell cycle (the restriction point) and subsequent S phase, S phase is the synthesis phase in which chromosomal DNA replication occurs, G_2 phase (gap 2) determines the time of entry into M phase, and M phase consists of the division of two cells into two daughter cells (Mitosis). It is in G_1 phase in which cells can respond to cellular signals that determine exit (or entry) from the cell cycle into the quiescent state (G_0)

4.1.7 Cell death in PDT.

There is a very fine balance within the cell, and PDT is capable of manipulating the balance between different cell pathways with only a small change in parameters. The original papers published back in 1996/1997 (Dellinger, 1996; Luo and Kessel, 1997) first showed that changing PS incubation time or dose can change the induced cell death from an apoptotic morphology to a necrotic morphology. Nowadays manipulation of this is regularly seen in PDT papers, even during clinical treatment. The preference of apoptosis vs. necrosis isn't always clear, and although some argue for a preferably less immunogenic/inflammatory controlled cell death - recently the interest in the recruitment of immunogenic mediators to improve long term PDT efficiency has challenged this view.

PDT can induce cell death via a number of different routes and all the three main modalities - apoptosis, necrosis and autophagy have been linked to PDT. No single PDT factor is responsible for control over cell death mechanism, some of the responsible factors include photosensitiser type, localisation and dose, PS physicochemical behaviour in specific microenvironments, light fluence/wavelength and timing, the oxygen concentration and the disease state itself. The process of intracellular signalling after PDT is complex but much work has been published on the subject. A review from (Almeida et al., 2004) tabulated major molecular events during specifically apoptotic death induced by PDT, this single table contained 181 references, covering 20 different 'major' cellular events, each one investigating a different photosensitiser or tumour cell line. This example emphasises the difficulty in compiling a model molecular pathway for PDT. When large scale studies looking at proteome changes post PDT are carried out (Baglo et al., 2011; Tsaytler et al., 2008) the scale of the number of pathways involved is apparent. However attempts have been made and numerous in-depth reviews have been written on the subject (Almeida et al., 2004; Buytaert et al., 2007; Castano et al., 2005b; Moor, 2000; Nowis et al., 2005; Oleinick et al., 2002; Ortel et al., 2009).

Pathways that are commonly manipulated by PDT alongside apoptosis, necrosis and autophagy, include the unfolded protein response, calcium homeostasis, lipid metabolism/ceramide signalling, transcriptional regulation including NF- κ B signalling, tyrosine kinase signalling including mitogen-activated protein kinases (MAPK), Cyclin-dependent kinases (CDK) and cell cycle control, heat shock protein expression, ROS and antioxidant pathways, hypoxia induced pathways and DNA-damage associated signalling. These are just some of the examples of known and well studied intracellular events that can occur after PDT. Extracellular signalling processes will also have an effect on cell survival, whether via the direct effect of PDT or via a secondary response of neighbouring cells and tissues. Reviews on extracellular effects such as these include the effect of PDT on the extracellular matrix, the immune system, surrounding vasculature, including effects on cell adhesion, cytokine expression, and angiogenesis. (Ana P Castano et al., 2006; Castano et al., 2005a, 2005b; Pazos and Nader, 2007).

4.1.8 PPa specific cell death mechanisms

Although changeable using experimental parameters, some common pathways of cell death are upregulated in more comparable cases. Work on examination of the specific upregulation of cell death pathways after PDT with PS derived from or similar to PPa, are reviewed in part by Piette et al (Piette et al., 2003) and briefly discussed below.

Li and colleagues (Li et al., 2007) present the induced cell death response from PPa PDT as a mixture of necrosis and apoptosis with cytochrome c release from the mitochondria into the cytosol alongside caspase-9 and caspase-3 activation.. Matroule and colleagues (Matroule et al., 2001, 1999) also show a mixture of necrosis and apoptosis, but focus on the involvement of reactive oxygen species and NF-kB activation, interestingly they also identify the lysosomal release of ceramide. Cytochrome c release is frequently observed by others (Choi et al., 2004) alongside involvement from p38 and c-Jun N-terminal kinase (JNK) pathways and subsequent activation of caspase-3 (Lee et al., 2004). Li and colleagues (Li et al., 2006) show a release of cytochrome c alongside the collapse of mitochondrial membrane potential, the up-regulation of endoplasmic reticulum stress proteins (calnexin, Grp 94 and Grp78), and the activation of caspases-3 and -9. Interestingly this paper also shows up-regulated gene expression of pro-inflammatory cytokines and beta-chemokines. Collapse of Mitochondrial membrane potential is common (Tan et al., 2009), and possibly related to evidence for the direct deactivation of the mitochondrial electron transport chain (Kim et al., 2004).

4.1.9 Intracellular localisation and PDT

As mentioned in section 1.4.2 the reactive oxidative mediators in PDT have a short lifetime due to their inherent instability and reactivity. This means that the intracellular localisation of the photosensitiser will have a big influence on the type of photo-damage that occurs. This subcellular localisation can be influenced by; the chemical nature of the PS (inc size, lipophilicity, amphiphilic character, ionic charge, structural symmetry), macromolecule binding characteristics (inc protein albumin, LDL), the local and serum concentration of the PS,

incubation time or time after injection, even the phenotype of the target cell and state of lipid bilayer (inc presence of cholesterol).

Different PS have been found to localise in a wide range of intracellular localisations and different cell death pathways can be induced from these various intracellular sites. Most organelles can be targeted and PS have been seen to locate in the plasma membrane, nucleus, mitochondria, ER, Golgi, Endosome/Lysosomal, and Cytosol. PS at all localisations are capable of killing but at varying efficiencies, this is due to the range of reactive mediators that can be induced by PS and their promiscuity and highly reactivity capability..

There are some good reviews on PS subcellular localisation and uptake (Boyle and Dolphin, 1996; Castano et al., 2004; Mojzisova et al., 2007a), these reviews aim to pull together some rules for a collection of molecules that are incredibly structurally variable from the mass of data available. Some of these rules include: The main factors for PS structural changes affecting intracellular localisation are hydrophobicity, ionic charge, and structural asymmetry. Mitochondria and endosomes/lysosomes are the most common PS localisation sites and apoptosis prevails over necrosis when death is induced from these sites. The localisation of PS in the Golgi apparatus and endoplasmic reticulum is less frequent and more likely related to a non-specific solubilisation of the photosensitiser in the membranes of intracellular organelles. Compounds that localise in the plasma membranes or nucleus are possible but relatively uncommon. Plasma membrane localisation has been linked with inducing a quick, necrotic style death. Lysosomal localised PS can lead to cell killing upon illumination, however the relative efficacy is significantly lower than that seen with PS localised in mitochondria and other organelles. It is often seen that highly aggregated PS are internalised by endocytosis and remain sequestered in the lysosomes, while PS that remain as (or are induced to be) monomers can be internalised by other pathways. PS that accumulate in mitochondria are usually hydrophobic with an asymmetric cationic charge (this rule is less exclusive). PS which are hydrophobic and have two or less negative charges can likely freely diffuse across the plasma membrane, and then relocate to other intracellular membranes. Those PS which are less hydrophobic and have more than 2 negative charges tend to be too polar to diffuse across the plasma membrane, and are taken up by endocytosis. PS which are hydrophilic or have many positive or negative charges

also tend to be too polar to diffuse across the plasma membrane, and are therefore also taken up by endocytosis. (Boyle and Dolphin, 1996; Castano et al., 2004; Mojzisova et al., 2007a).

Intracellular localisation can change over time (Fabris et al., 2001) and it is also worth noting that the process of irradiation itself can cause not only photo-modification and photo-degradation of the PS but has been shown to cause photo-relocalisation of some photosensitisers, often thought to be either due to photodynamic permeabilisation of the various membranes and release of PS or the lysosomal autophagy of damaged organelles (Moan et al., 1994; Scully et al., 1998; Wood et al., 1997).

Localisation is also important in a multi-cellular model, Xiao and colleagues (Xiao et al., 2005) compared commonly used photosensitisers, and looked at the intra-tumour distribution in tumour spheroids. All PS showed concentration-dependent, diffusion driven penetration and accumulation and all showed greater accumulation at the spheroid rim than the spheroid centre. Contrary to single cell studies in which higher lipophilicity often means increased cell uptake (see 3.1.3), here they showed better distribution with the more hydrophilic PS, hypothesised to be because a lipophilic PS would have higher affinity for the network of hydrophilic extracellular matrix found on a spheroid surface and not seen in monolayer cell culture, this could slow its penetration into the spheroid centre. However PS size too was also an important factor in their results.

4.2 Results

4.2.1 Intracellular localisation by confocal fluorescence microscopy

To visualise the localisation of the intracellular photosensitiser, cells were exposed to 20 μ M photosensitiser over two hours in conditions matching those used in the *in vitro* cell kill assays. Confocal fluorescence microscopy images were taken from the brightest part of the cell (generally found to be the centre). A laser excitation wavelength helium–neon (HeNe) laser at 633 nm was used instead of the 405 nm diode laser. This was because with the 405 nm laser, even at the lowest useable settings, irradiation of the image would initiate cell morphology changes over the whole plate. For example one saw cell shrinkage and/or what seemed to be the development of intracellular vesicles (data not shown). It is likely cells were undergoing conformational cell death changes.

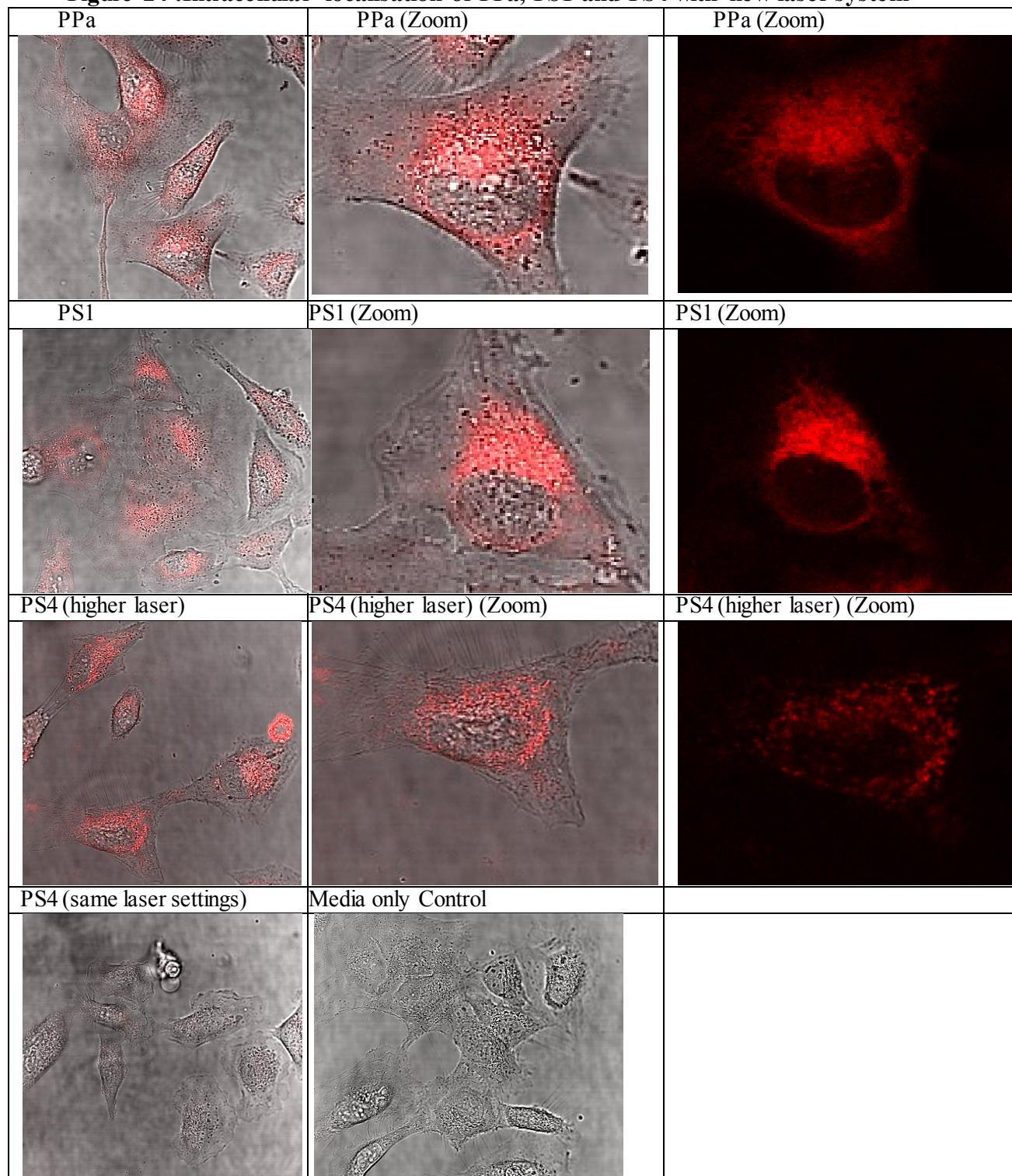
Cells were imaged directly and not fixed, although fixing was shown to reduce irradiation induced morphology change (data not shown) the introduction of formaldehyde can compromise images in other ways: For example it can introduce artefacts from precipitated salts or paraformaldehyde, formaldehyde can have endogenous auto fluorescence, it can also cause morphological changes, loss of immuno-reconition, and the re-localisation of unfixable molecules. Its presence also has the potential to change the fluorescent efficiency of any neighbouring fluorophores in its vicinity by changing their immediate environment (Ganguly et al., 2011; Grizzle, 2009; Meade et al., 2010; Schmiedeberg et al., 2009; Tate and Cooper, 2008).

In the set of confocal fluorescent microscopy images shown in Figure 24 all images were taken using the same camera sensitivity, however PS4 staining required higher laser intensity settings than used for PPa and PS1 due to lower fluorescent yield (Stamati et al., 2010). Presented are images of cells containing the three PS at their optimal laser settings, in these images PPa and PS1 are imaged using the same laser settings but PS4 at a higher laser intensity. For interest included at the bottom of the figure is an image of PS4 containing cells taken at the same settings as used for PPa and PS1 and an image of cells in just media alone demonstrating no cellular auto-fluorescence at these camera settings.

In all the images obtained PS1 and PPa both show the same localisation pattern; dotted throughout the cell but concentrated in an area around the nucleus, this peri-nuclear localisation seems most similar to that of an Endoplasmic Reticulum (ER) localised protein. ER is an area of dense membranes within the cell and its most recognised function being protein folding, sorting and modification. PS4 displays a different localisation pattern, more punctuate and less centred close to the nucleus, perhaps of a more endosomal or lysosomal pattern. Conclusions on localisation were based on fluorescent images of proteins with prescribed localisations; for more information and example images see 2.1.4.5.

Throughout this work it was also noticed that upon leaving the PS containing media in with the cells during imaging, no difference would be seen between these images and images taken where PS containing media was removed and cells washed prior to imaging (data not shown). Due to this and the decrease in imaging induced morphological changes with the HeNe laser, it was possible to image the same cells 4-5 times before any morphological changes were seen. If the same image was taken every few minutes after PS addition in the media one could see the cells over time start to fluoresce over their background. PPa demonstrated this to a greater extent than PS1, (data not shown). This raises questions over whether the pattern of fluorescence observed is only an artefact of the change in fluorescent efficiency in different microenvironments within the cell, for example the PS could be in other compartments to those seen but reduced fluorescent efficiency will make it invisible, as it is the surrounding cell culture media.

Figure 24 :Intracellular localisation of PPa, PS1 and PS4 with new laser system



SKOV-3 cells exposed to supplemented cell culture media containing 20 μ M photosensitiser solution, after 2 hours cells imaged directly. Confocal fluorescent microscopy imaging carried out under irradiation of 633nm laser at 20% (PPa and PS1) or 49% (PS4), through a X63 water objective with detection channels for brightfield and fluorescence between 660-770nm.

4.2.2 PS fluorescent spectral profiles

4.2.2.1 Extracellular fluorescent spectral profiles

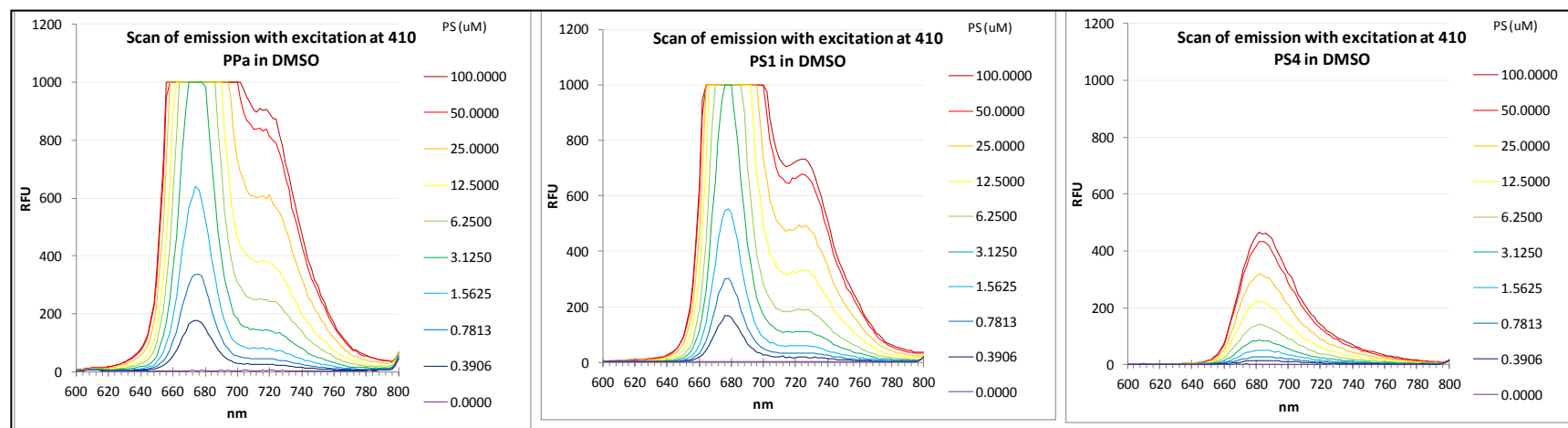
PS fluorescent spectra outside the cell was studied, particularly PS emission spectral changes over concentration in DMSO (in an assumed non aggregative and optimal photophysical state) and in cell culture media containing FCS and 2% DMSO. As mentioned in 3.2.3 this will also give a better indication of the existence of any aggregation induced spectral shifts and at what concentration they occur. The data is shown in Figure 25 and shows that for none of the PS in DMSO are any shifts or losses in intensity to the spectra seen as concentration changes, peaks occur in DMSO at PPa (675nm), PS1(678nm) and PS4 (684nm). When in supplemented cell culture media however all PS show some change in spectra between 3µM and 6µM: At concentrations below 3µM all PS show similar peaks to that of PS in DMSO, for PS4 the peak at all concentrations is the same as in DMSO and it is only a gradual drop in fluorescence intensity after 3µM that is seen. PPa exhibits increasing peaks at 675 nm until between 3µM and 6µM where it begins to decrease in intensity at 680 nm and increase at 718 nm, at around 25µM the peak at 718 nm is the dominant peak. PS1 also exhibits increasing peaks at 678 nm until between 3µM and 6µM where it begins to shift over to 694 nm but unlike PPa and PS4 it continues to increase in fluorescence intensity as concentration increases. The extent of the fluorescent shifts to lower energy wavelengths (red-shifts) tell us that the lowest unoccupied molecular orbital (LUMO), the one responsible for the amount of energy released in both fluorescence and non radiative processes important for PDT induced toxicity, is also at a lower energy level. The extent of shifts in spectra are as follows; PS4 (684nm→684nm), PPa (675nm→718nm), PS1 (678nm→694nm). It is thought photosensitisers that absorb above 850nm cannot efficiently excite oxygen (Plaetzer et al., 2009), and so the energy contained within even the aggregated red shifted states of each PS should still be sufficient to excite molecular oxygen if relevant deactivation processes are still maintained, however in some cases type II dissipations of energy could become less favourable.

4.2.2.2 Intracellular fluorescent spectral profiles

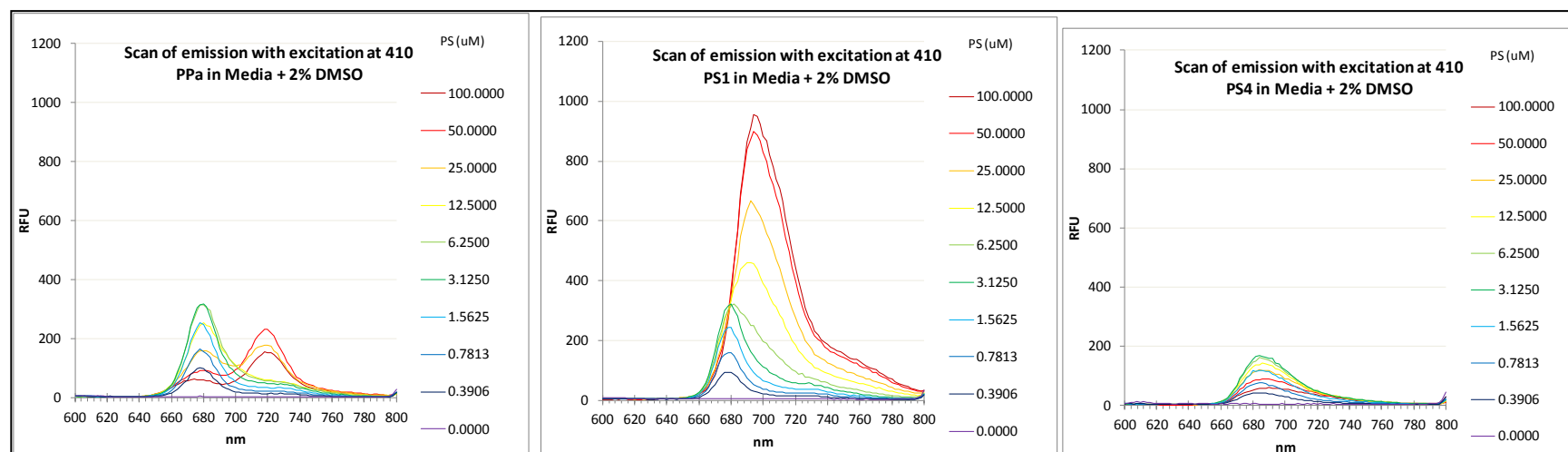
Within the confocal fluorescence microscopy system a lambda scan can read the emissive profile of the fluorophore coming from within the cells under irradiation. Lambda scan results are shown in Figure 26. The data has been represented in two different ways; one graph shows the raw relative fluorescence readings of all three PS under the same laser intensity and camera detection settings. The values have been normalised to take into account for the cell covered area of the image via a method described in 2.1.4.5. Another graph shows comparative optimal spectra normalised to 1 for peak emission, for this PS4 was imaged under increased laser power conditions as to get a more comparative signal range. PPa and PS1 show similar emissive spectra with peaks at around 680 nm whereas PS4 shows a broader red shifted spectra when compared to PPa and PS1, with a peak around 685 nm. When compared to the 'optimal' emission spectra of the three PS in DMSO (see 4.2.2.1) and considering the accuracy of the Lambda scan (band width = 10 nm, overlap = 5 nm), the spectra match well, suggesting the PS detected in their cellular environment are suitably dis-aggregated and in an optimal photophysical state.

Although the imaged cells exhibit much lower fluorescence with PS4 than with PPa and PS1, seen quantifiably on the lambda scan, this cannot be directly correlated with the level of internalised PS for a number of reasons including a differences in inherent PS fluorescence efficiency as well as the influence the cellular environment can have on fluorescent efficiency, also dense packing in organelles could cause self quenching of PS.

Figure 25 :Extracellular fluorescent profiles of PPa, PS1 and PS4



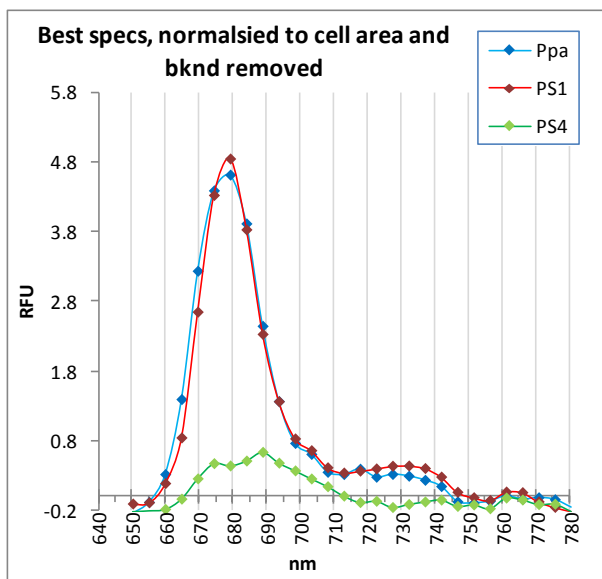
PS emission spectra measured with excitation at 410nm over changes in concentration of PS in 100% DMSO



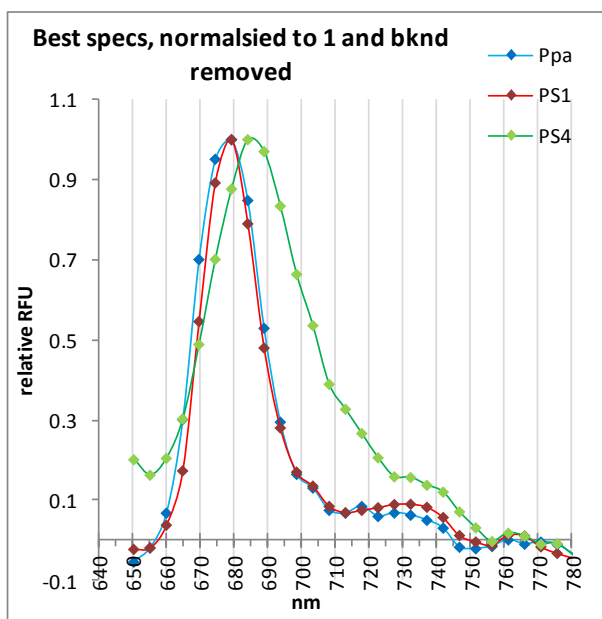
PS emission spectra measured with excitation at 410nm over changes in concentration of PS in cell culture media containing FCS and 2% DMSO.

Figure 26 :Lambda scans of intracellular PS spectral profiles

SKOV-3 cells exposed to supplemented cell culture media containing 20 μ M photosensitiser solution, after 2 hours cells were imaged directly. Confocal fluorescent microscopy imaging carried out under irradiation of a 633nm laser at 69% or 100%, through a X63 water objective and with the lambda scan reading the emissive profile of the fluorophore within the cells between ~640-780nm in 30 sections of 10nm bandwidth.



Graph showing the raw relative fluorescence readings of all three PS under the same laser intensity and camera detection settings. The values have been normalised to take into account for the cell covered area of the image via a method described in 2.1.4.5.



Graph showing comparative optimal spectra normalised to 1 for peak emission, for this PS4 was imaged under increased laser power conditions as to get a more comparative signal range.

4.2.3 A quantifiable measurement of PS uptake and leaching

4.2.3.1 Assay design

An assay was designed to measure absolute PS uptake. SKOV-3 cells were cultured in a microplate and exposed to PS containing cell culture media before being washed with PBS and a solution added to lyse the cells and release the PS into the solution, fluorescence could then be read and compared to a standard curve of PS in the same solution. As seen earlier in Figure 25 PS in cell culture media does not provide a linear relationship between concentration and fluorescent intensity, therefore the lysis solution used was DMSO supplemented with 0.25% Triton X 114, see 2.1.4.6. As well as providing a linear relationship, PS in DMSO fluoresces more brightly than in aqueous buffers so allowing greater sensitivity within the assay. This is of particular importance for PS4 as the sensitivity of any fluorescence based assay will be reduced for PS4 compared to the other PS due to its inherent lower fluorescent efficiency.

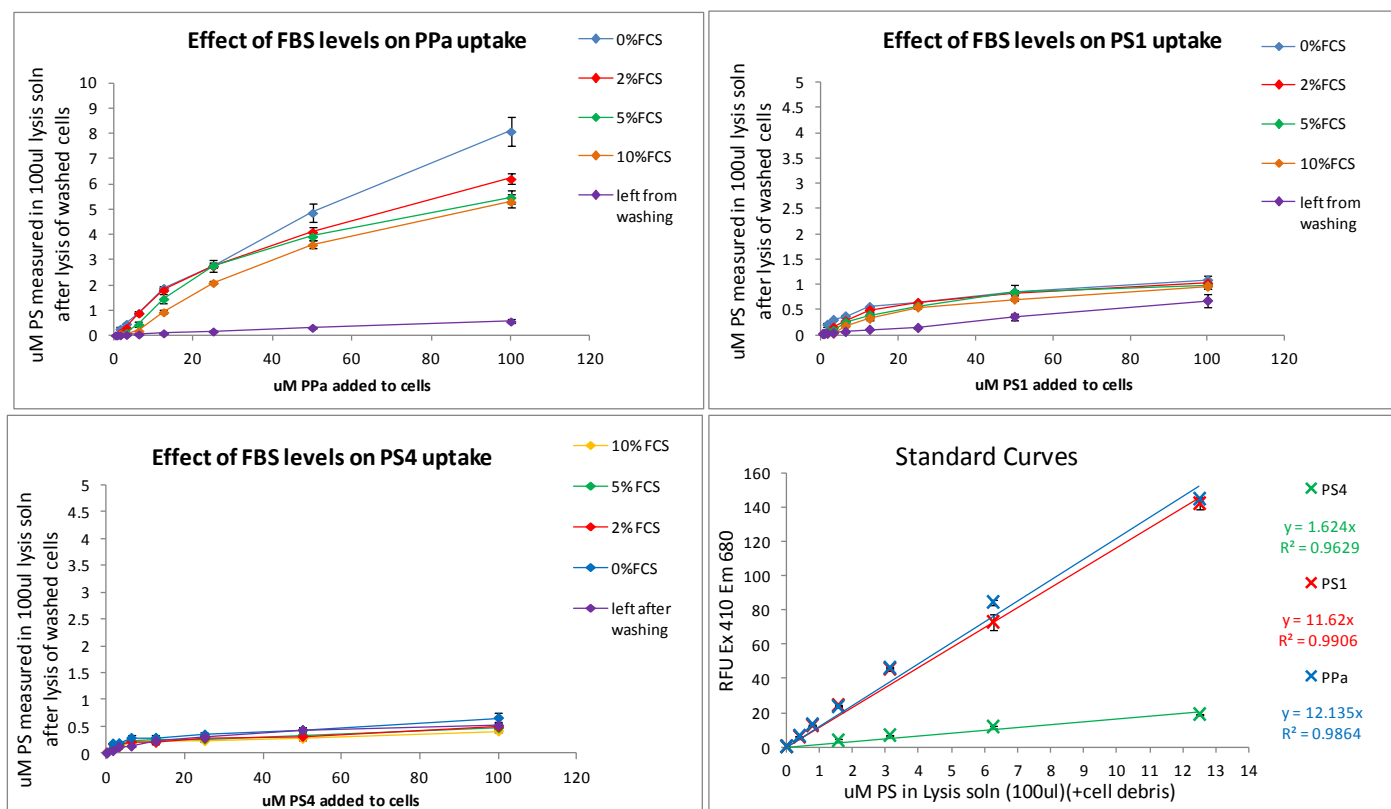
The lysis solution was tested with the MTS assay to confirm complete cell destruction occurred before 5 minutes (data not shown). Photosensitiser dilutions in the DT lysis solution (DT = 0.25% Triton X 114 in DMSO) were shown to be linear over concentrations of PS required for this assay, these dilution series were exposed to regular laser irradiation and then re-measured but no loss in signal that would indicate photo bleaching was observed (data not shown). Standard curves in DT were also left sealed at 4°C for a few days and re-read and no significant loss in intensity was seen (data not shown). Standard curves of PS in DT were also plated with and without 8000 cells per well to see if the presence of cell debris had any effect, a small decrease in signal was seen with PS1 at higher PS concentrations but not PPa or PS4 (data not shown), because of this all experimental standard curves were set up on the day of the experiment over cell debris and read at the same time as the data plates. The main source of error was the correction for how much PS was left in the wells after washing; two PBS washes was shown sufficient to reduce the background to manageable levels (<10 RFU) (data not shown). In addition; for all assays a washed standard curve was included alongside the normal standard curve to monitor PS levels left after washing. It is important to carry out this control and work highlighting this problem has recently been published by (Engelhardt et al., 2011).

4.2.3.2 Uptake of PPa, PS1 and PS4 into cells and the effect of serum depletion.

Experiments were carried out to look at the uptake of PS over two hours, mirroring the time used in the *in vitro* assay. Additionally as this experiment is relatively high throughput, dissolution of the PS in cell culture media containing varying amounts of foetal calf serum (FCS) was also studied. This is of interest because as discussed in 1.3.4 PS are known to bind serum proteins and this can have an effect on cellular uptake. Previous work carried out within this lab (Kuimova et al., 2007) showed the addition of bovine serum albumin (BSA) to a PPa solution did exhibit a BSA concentration dependant increase in PPa fluorescence intensity which was suggested to be an indication of the association of the PPa with the hydrophobic pockets on a BSA molecule. If PS favourably interact with serum proteins or other elements in the FCS this interaction could both compete against or help encourage cellular uptake.

The results of this experiment are shown in Figure 27, raw relative fluorescence units (RFU) have been converted to 'concentration of PS in 100µl lysed cell solution' using the standard curves also shown in the figure. On each graph the relevant 'left from washing' curve is also plotted. Results show the amount of internalised PPa increases with the amount of PS added, the relationship is not linear and the rate of this increase slows as concentration of PS added increases. PS1 and PS4 both show cellular uptake levels barely above the values measured for PS 'left from washing'. PS1 shows slightly higher increase over 'left from washing' compared to PS4 but it is at the limit of detectability. With respect to decreasing the FCS concentration in the media, this didn't increase uptake of PS4 or PS1 above baseline, suggesting it is not sequestration of PS by serum proteins preventing detectable uptake. PPa does show some increased uptake at low FCS but only at 0% FCS and only at higher PS levels. This experiment was carried out at least twice on two independent occasions with the same result.

Figure 27 : Uptake of PPa, PS1 and PS4 into cells and the effect of serum depletion.



SKOV-3 were incubated for 2 hours in supplemented cell culture media containing PS with varying amounts of FCS. Cells were washed twice in PBS then lysed into 100 μ l DT solution. Fluorescence was read at Ex410Em680 and values converted to concentration in μ M with standard curves seen alongside. 'left from washing' controls are shown on each graph.

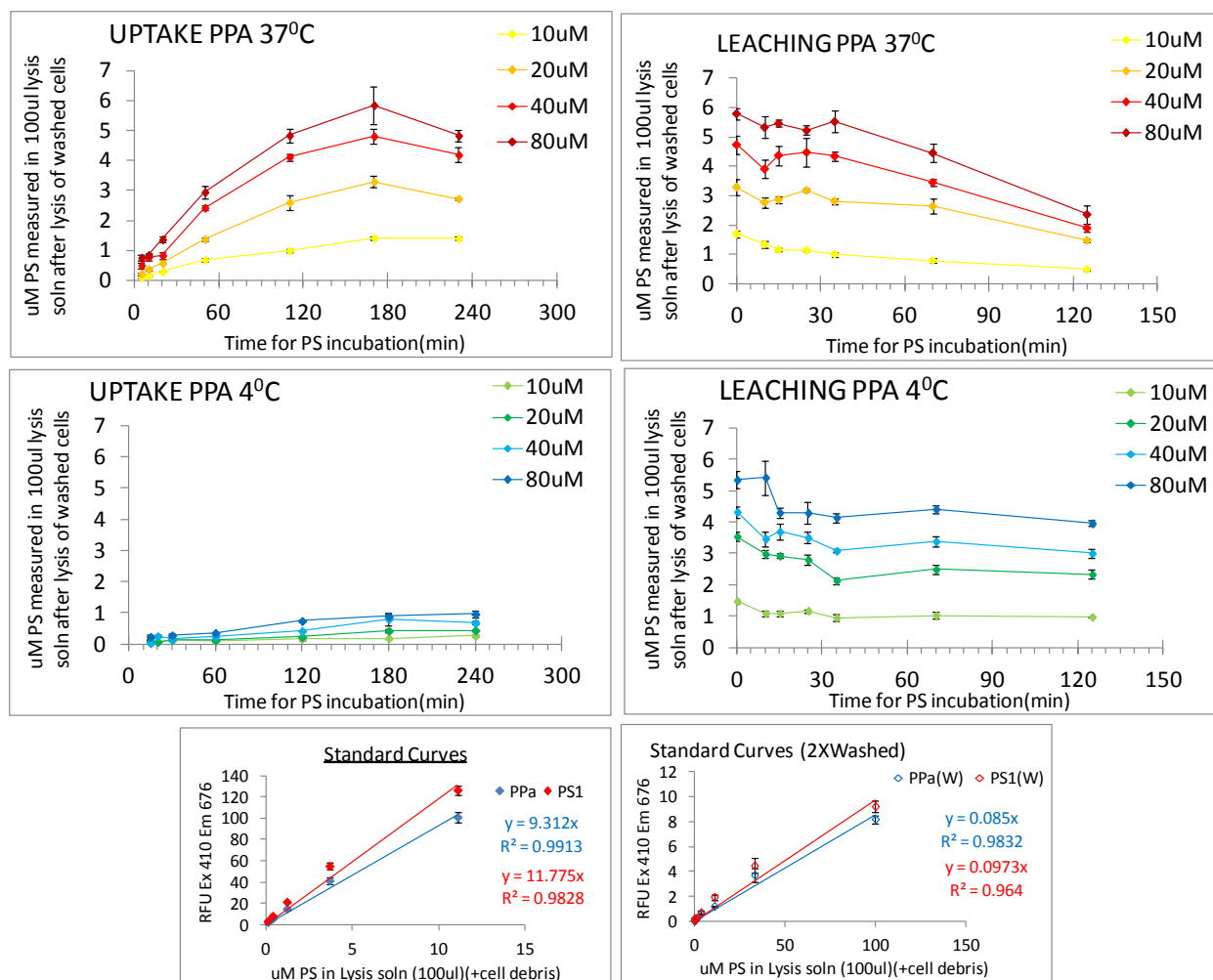
4.2.3.3 Uptake and leaching of PPa and PS1 over time and the effect of temperature.

This assay was also used to look at the uptake of PPa and PS1 over time at 4°C compared to 37°C. The results of these experiments are shown in Figure 28. PS4 was not tested due to lack of material. This experiment was carried out at least twice on two independent occasions with the same result. Again the assay was too insensitive to pick up any internalised PS1 at any time point (data not shown), meaning that PS1 must be taken up at a much lower level than PPa or is rapidly degraded once internalised, although degradation would be surprising considering the results obtained by confocal microscopy 4.2.1 and 4.2.2.

With PPa, the signal is again strong enough to be detected and uptake over time is seen to saturate between 2 and 3 hours, this is later than was seen in via confocal microscopy in which the quantified fluorescent signal saturated within 1 hour for both PPa and PS1 (data not shown). The saturation seen in this assay occurs with all concentrations of PPa around the same time. When the temperature is reduced, the uptake of PPa is drastically reduced, to just above an undetectable level. In the same model leaching of PPa over time was also investigated, this is also shown in Figure 28. PPa leaching occurred at a slower rate than uptake and after two hours levels had only reached around half of what they were originally. Again this is not what was seen previously via confocal where leaching reached undetectable levels within 2 hours (data not shown). Reducing the temperature to 4°C slowed leaching rates slightly but not by much.

An explanation for this data not correlating with the confocal fluorescent microscopy data is explained because in confocal microscopy fluorescent detection is of intracellular PS within different cell microenvironments, this can effect PS photophysical efficiency for example PS could be in an aggregated or densely packed form, however once released in the 96 well plate assay PS can monomerise and/or recover comparable photophysics in the lysis solution. As the confocal system is also measuring single cells (compared to thousands in the 96 well plate assays) it may also be less sensitive. Despite the fact sensitivity levels were kept below pixel saturation on the confocal, intracellular PS quenching and fluorescent saturation could still have occurred, a fact controlled in the 96 well plate assays using standard curves.

Figure 28: Uptake and leaching of PPa and the effect of temperature



UPTAKE: SKOV-3 were incubated for varying amounts of time at either 37°C or 4°C in supplemented cell culture media containing PPa. Cells were washed twice in PBS then lysed into 100µl DMSO.

LEACHING: SKOV-3 were incubated for 2 hours in supplemented cell culture media containing PPa at 37°C then media was removed and replaced with media only, plates were then left for varying amounts of time at either 37°C or 4°C. Cells were washed twice in PBS then lysed into 100µl DMSO.

Fluorescence was read at Ex410Em676 and values converted to concentration in µM with standard curves seen alongside. 'left from washing' values taken from equations fitted to a washed standard curve were subtracted from the data presented.

4.2.3.4 Increasing the cell number to increase sensitivity

To extend this work to be able to detect PS1 and PS4, experiments on a much larger scale were attempted; see 2.1.4.6 for the adapted method. For this experiment an additional control was required; after reading the plates 50µl was taken from each well and the BCA assay was used to calculate how many cells were in originally in each well, the fluorescence data could then be normalised to any fluctuations in the BCA values to make it more accurate. Additionally after harvesting and before lysis a small sample was taken from some of the samples to count via haemocytometer, this allowed a rough calculation of the estimated number of cells plated – the average count from this was around 25,000-30,000 expected cells per well, which matched the values obtained from the BCA assay ($AV=28072 \pm 11084 (2 \times SD)$).

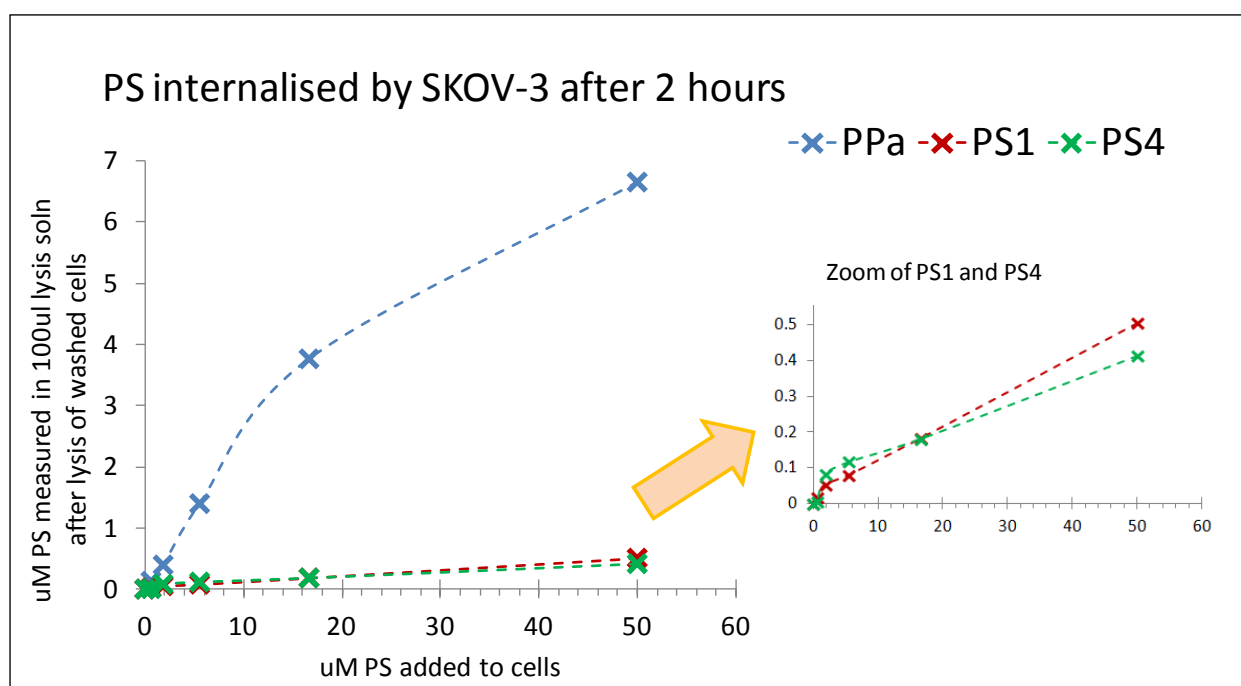
Both the BCA and Bradford assays were investigated for their sensitivity in DT lysis buffer, see 2.1.4.7. The background for the Bradford assay went up unsuitably high with DT solution and so was deemed unsuitable, whereas the BCA assay showed alteration in signal only at higher levels of protein while the linear relationship still seemed intact, albeit less sensitive. (Data not shown). Direct PS interference with the end point of the BCA assay (absorbance at 562nm) was also checked and results showed that PS do exhibit concentration dependent absorption at 562nm but it was of a sufficiently low level that it was below the variability of the assays itself. (Data not shown).

As cell number is increased in this assay the amount of cell debris that is required to interfere with PS fluorescent signal in DT buffer was investigated, and over 100,000 cells were required to cause interference in fluorescent signal of 5µM PS in 100µl DT lysis buffer (the highest PS concentration obtained from previous uptake experiments) (data not shown).

Data obtained from this higher sensitivity uptake assay is shown in Figure 29, the data is presented after normalisation with the BCA assay and after conversion using standard curves to a value for final concentration of PS in 100µl lysis solution. The experiment showed a similar relationship to the original lower sensitivity uptake assay in that uptake increased with concentration, at a rate which slowed over higher PS concentrations. The signal is about double

what was detected from the original uptake assay that used only 8000 cells, as calculated cell number from this assay was about 3 to 4 times that amount, signal is surprisingly low. This is most likely due to additional PS lost in leaching as it takes longer to prepare the cells in the higher sensitivity experiment. It is unlikely caused by PS saturation as results are still within the linear range. Despite this, values from PPa PS1 and PS4 from within this experiment should still be comparable with each other. PPa again shows greatly enhanced signals compared to PS1 and PS4 which show some signal but still very low, PS1 and PS4 show similar uptake levels.

Figure 29 : Higher sensitivity cellular uptake assay for PPa, PS1 and PS4.



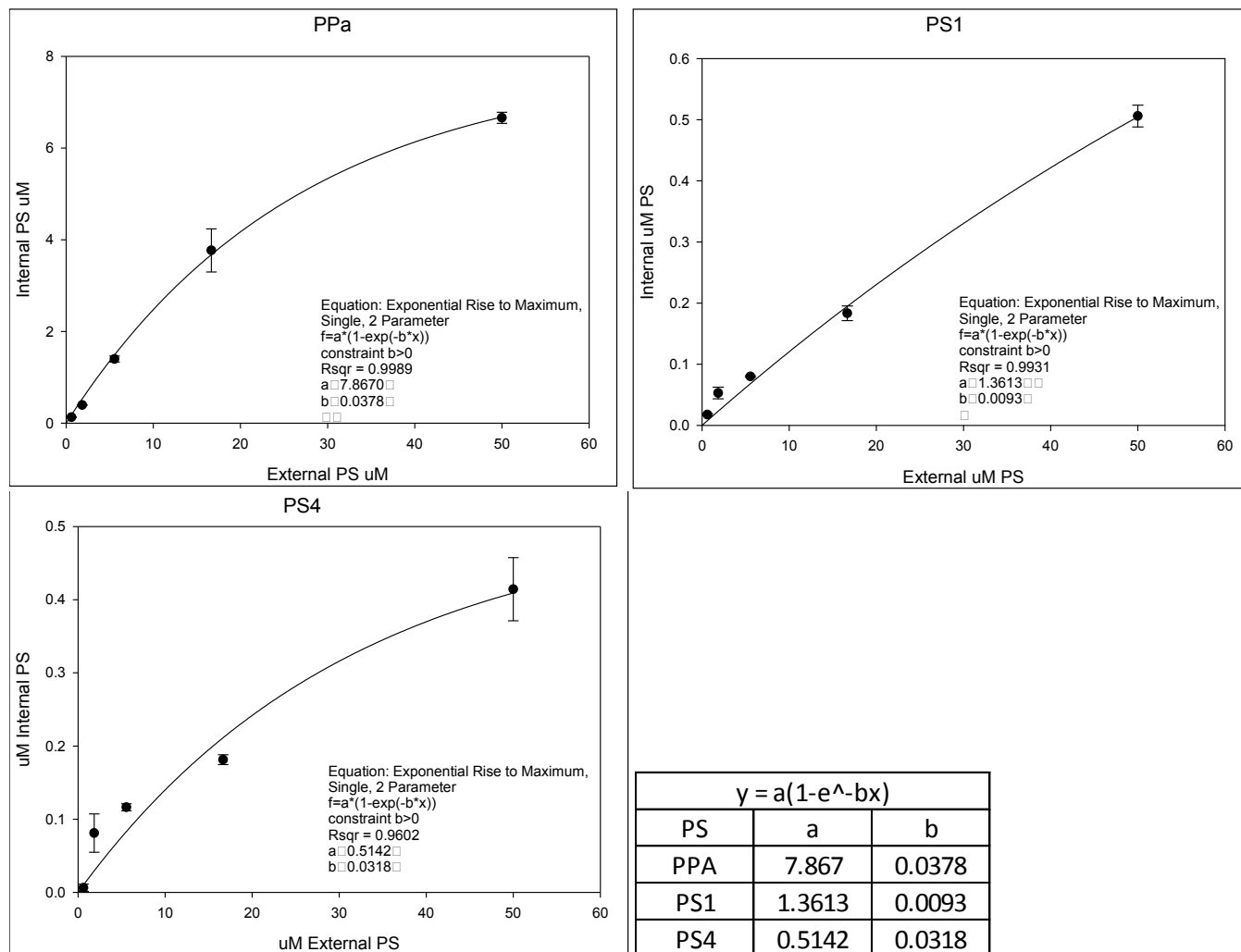
SKOV-3 were incubated for 2 hours in supplemented cell culture media containing PS. Cells were washed twice in PBS then lysed into DT solution. Fluorescence was read at Ex410Em680 and values normalised to cell number and converted to concentration in μM with standard curves. see 2.1.4.6 for full method.

4.2.3.5 Using absolute uptake values to re plot cyto-toxicity graphs

In Figure 30 the data from Figure 29 on absolute PS uptake into cells in was plotted against the concentration of PS that was added externally to the experiment. These graphs could be fitted with a 2 parameter exponential rise to maximum curve see 2.1.3.1. From this equation parameters a and b can be derived for each PS and used to calculate predicted internalised PS from external PS added. The parameters derived from Figure 30 were used to re plot the cell kill data from Figure 15. This is shown in Figure 31 and shows the IC_{50} value of each PS, which has now been converted to the concentration of PS that will be found internalised, plotted over increasing light dose. As in Figure 15 each curve is fitted with a 2 parameter power curve and as before constant b can give an indication of the 'rate' at which PS becomes toxic as light dose is increased. PS4 has a higher value for b (2.3) than PPa (0.7) and PS1 (0.6), these values are very similar to the values obtained with external IC_{50} in Figure 15 but now the data uses the internalised PS concentration it validates the conclusion that PS4 is responding in a more sensitive way to an increase in light than PS1 or PPa. The values for this sensitivity match with the increase in singlet oxygen quantum yield with PS4 compared to similar values for PPa and PS1, see 1.6.

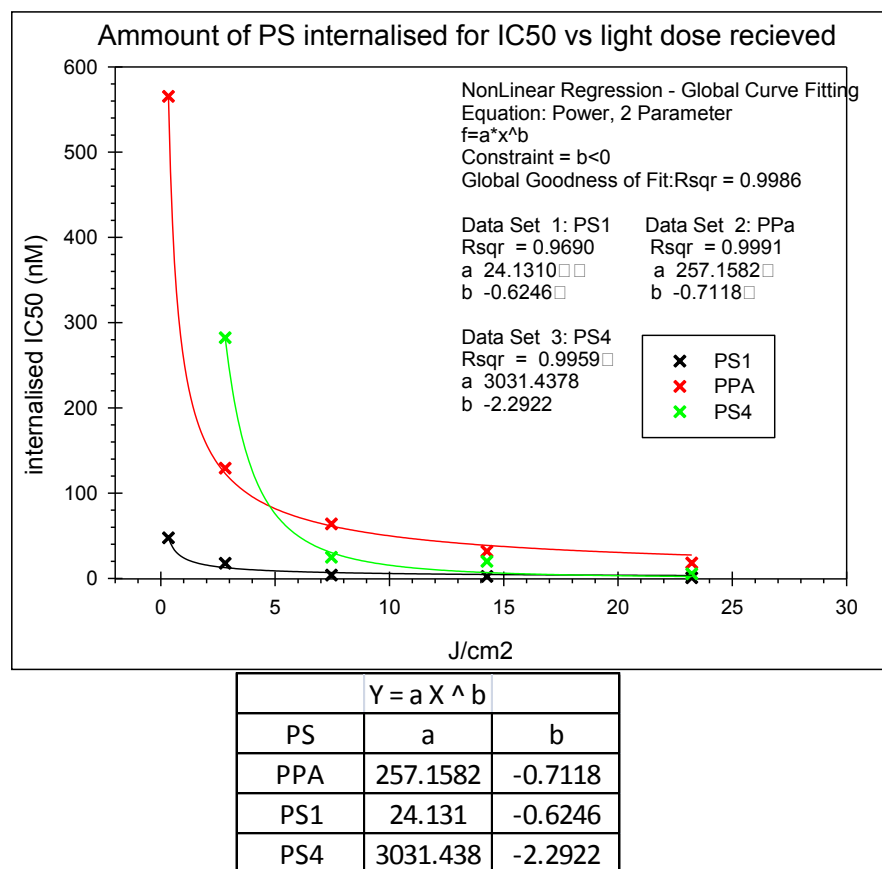
Despite similar curve gradients when you compare the two graphs in Figure 15 and Figure 31, the changes in the placement of the curves on the X axis do change dramatically. In Figure 15 it was shown that PPa and PS1 sat close to one another and close to the Y axis with PS4 sitting much further to the higher end of the X-axis. Now internalisation has been taken into account, PPa has shifted to join PS4 on the right hand side, and it is only PS1 retaining its toxicity at very low laser doses. In fact at higher light doses PS4 exhibits more cyto-toxicity than PPa in terms of internalised PS levels.

Figure 30: Predicted internalised PS from external PS added to cells.



Data from Figure 29 on absolute PS uptake into cells was plotted against the concentration of PS that was added externally to the experiment. These graphs could be fitted with a 2 parameter exponential rise to maximum curve see 2.1.3.1. From this equation parameters a and b can be derived for each PS (shown in the table) and used to calculate predicted internalised PS from external PS added.

Figure 31 : Internalised IC₅₀ of each PS over increasing light dose.



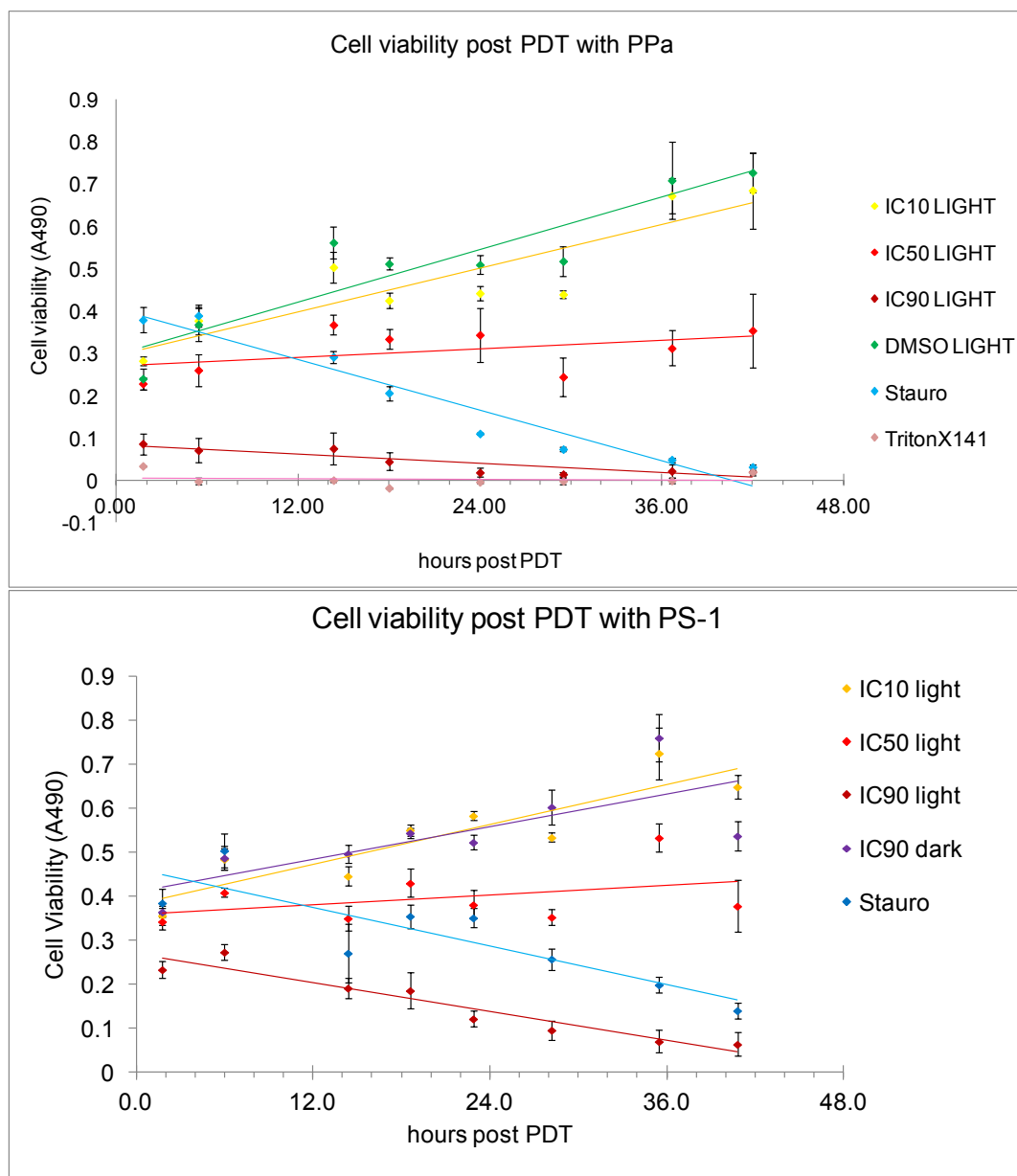
Parameters derived from Figure 30 used to re plot the cell kill data from Figure 15.

4.2.4 Mechanism of cell death, cell viability over time.

The data presented in 3.2.2.1 indicated there may be some differences in the type of cell death initiated by PPa and PS1: In the standard *in vitro* MTS assay model presented in chapter 0 PPa caused significant loss of cell viability within two hours whereas PS1 required longer to exhibit a similar final toxicity. To investigate this further cell viability was measured at various time points after irradiation when concentrations of PS required for 10% 50% and 90% cell death (at 48hrs) were used. In Figure 32 the data is presented in raw 490nm absorbance (A490). For both PPa and PS1 the IC₅₀ dose shows no change in A490 over time suggesting cell growth arrest, if the data had been presented as % viability as previously this would have been interpreted as a slow decrease over time. The IC₉₀ dose for PPa confirms that a high proportion of the cell population death occurs within 2 hours, whereas for the IC₉₀ dose PS1 the amount of cells dead within 2 hours is much lower and the majority of the population will die at various points over the 48 hours. This suggests a more necrotic style of death for PPa and a more apoptotic style of death for PS1 with the final same potency. This experiment was carried out at least twice on two independent occasions with the same result.

In these experiments new controls have been introduced (see 2.1.4.2), these include 0.25% TritonX114 which provides a reading for immediate 100% cell death and 1µM staurosporine (Stauro) which was chosen for its ability to induce an apoptotic style death. Prior to its inclusion in this assay a dose response cell kill experiment was carried out in SKOV-3 cells, this is presented in Figure 37. It should be mentioned at this point that for two reasons cell death mechanisms induced by PS4 was not investigated in the following part of the chapter: Firstly to try and control variables in this work it was desirable to limit the work to looking mainly within the confines of the already introduced *in vitro* cell model, for example 2 hours PS incubation, laser irradiation at 0.33 J/cm² and so on. Within these constraints PS4 is not cytotoxic enough. Changing PDT assay variables are known to be able to have significant impact on the toxicity and even mechanism of death and to modulate them all would, at this point in time, make the experiment unreasonably large. Secondly the mechanism of synthesis for PS4 involved a particularly labour intensive purification step so there was limited material.

Figure 32 : Cell viability over 48hours post PPa and PS1 PDT.

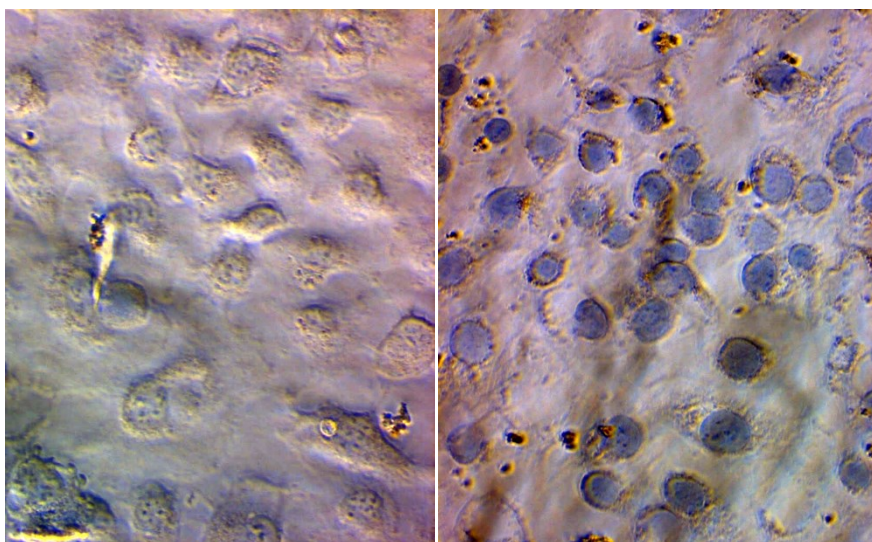


The standard cell kill experiment carried out with concentrations of PPa or PS1 required for 10% 50% and 90% cell death by 48hours. At various points over the 48hours post irradiation cells were assayed for cell viability via the MTS assay; viable cells contribute to absorbance at 490nm.

4.2.5 Trypan blue staining of high dose PPa-PDT

Trypan blue (Mw 960.81) is a blue acid dye containing two negatively charged chromophore groups. The dye is taken up into cells upon membrane damage and binds cellular proteins. It is used routinely as a marker of cell death. To confirm the immediate cell death seen with PPa PDT was in fact cell death and not an artefact of the PDT process, cells were exposed to 10 μ M PPa for 2 hours, lasered and subsequently stained with Trypan blue, this is shown in Figure 33 next to a photograph of cells exposed to 10 μ M PS but not lasered. Positive staining of the cells treated with PPa PDT confirmed the MTS assays result. The experiment was carried out at least twice on two independent occasions with the same result.

Figure 33 :Trypan blue staining of high dose PPa-PDT.



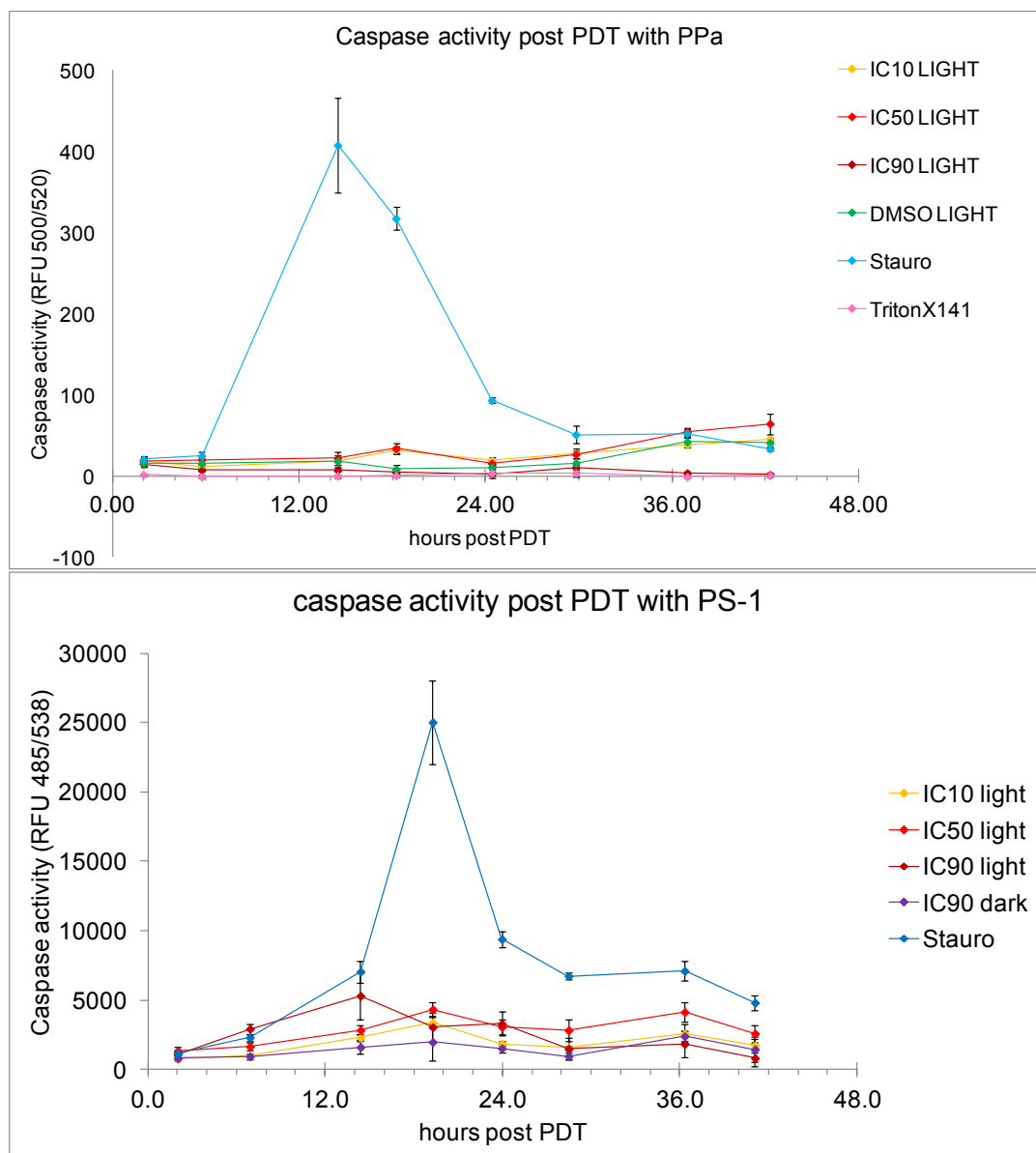
SKOV-3 cells exposed to supplemented cell culture media containing 10 μ M PPa solution, underwent PDT as normal, 2 hours after irradiation cells were washed, stained with Trypan Blue, fixed with formaldehyde and a photo taken (RIGHT), cells that underwent the same treatment but not lasered are also shown (LEFT).

4.2.6 Caspase 3/7 activation post PDT.

As mentioned in 4.1.5 a key hallmark of apoptosis is caspase activation. An assay to measure the activation of effector caspases 3 and 7 via cleavage of a pro-fluorescent substrate, (see 2.1.4.9) was calibrated in a similar way to the MTS assay (see 3.2.1.2), to obtain a suitable cell number and incubation time to get a detectable linear signal range (data not shown). Additionally as PS are fluorophores themselves a control was carried out in which cells were incubated with PS for 2 hours, washed to remove PS but not lasered and then induced into apoptosis with staurosporine – this should reflect the amount of PS that would be present inside the cells and could possibly interfere with the assay. Results show PS at this level does not significantly interfere with the assay (data not shown).

Caspase activation is transient so has to be studied over time at frequent intervals; staurosporine at 1 μ M was used as a positive control. The effect on caspase activation of PPa PDT or PS1 PDT at concentrations required for 10% 50% and 90% cell death at 48 hours was measured at various time points throughout 48 hours after irradiation. The results are presented in Figure 34 and show no detectable caspase activation with PPa or PS1 at any dose, MTS assays were carried out alongside to confirm cells died to the extent expected (data not shown), and different plate readers were used for the two figures – this accounts for the differences seen in RFU and sensitivity. The experiment was carried out at least twice on two independent occasions with the same result.

Figure 34 : Caspase 3/7 activation post PDT



The standard cell kill experiment carried out with concentrations of PPa or PS1 required for 10% 50% and 90% cell death by 48hours. At various points over the 48hours post irradiation cells were assayed for activation of caspase 3 / 7. Staurosporine is used alongside as a positive control for activation of caspases 3 / 7.

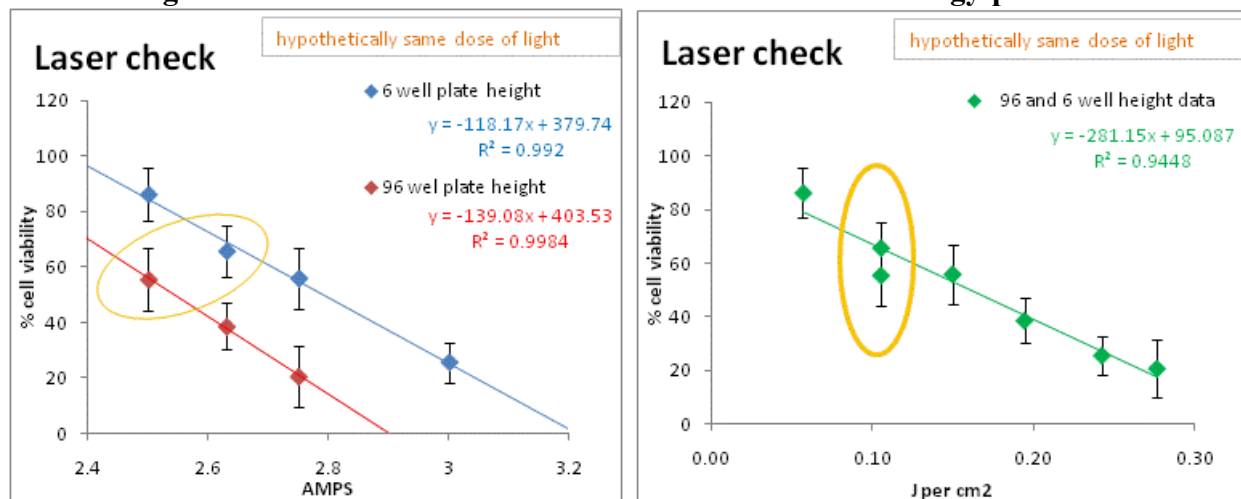
4.2.7 DNA fragmentation post PDT

Another important hallmark of apoptosis is DNA fragmentation, see 4.1.5. An established method for visualisation of DNA fragmentation is by running isolated cellular DNA through an agarose gel so separating it by size and subsequent staining to visualise a ‘DNA Ladder’ under UV light.

In order to extract enough DNA for visualisation the standard cell kill model had to be adapted to a 6 well format. The predicted increase in laser electric current required to maintain J/cm^2 intensity when the beam is used over a larger area is shown in 2.1.4.3. In order to verify the predictions, 96 well plates were set up in the standard cell kill assay and irradiated with either the small beam or the larger beam at different laser power settings, the amount of cell death resulting from each laser setting is shown in Figure 35. Both beams show a positive correlation between increased laser power and increased cell death, the smaller beam (96 well plate height) showing increased cell kill over the larger beam at the same laser settings because the same laser energy is concentrated over a smaller area. Ringed in yellow are the two settings predicted to be equivalent J/cm^2 for each of the beams. When data is plotted over J/cm^2 as opposed to laser electric current (amps) all points fall on the same line and the two settings predicted to be the same overlap – this indicates that the predictions made are correct and the two beams are delivering the same amount of energy per cm^2 .

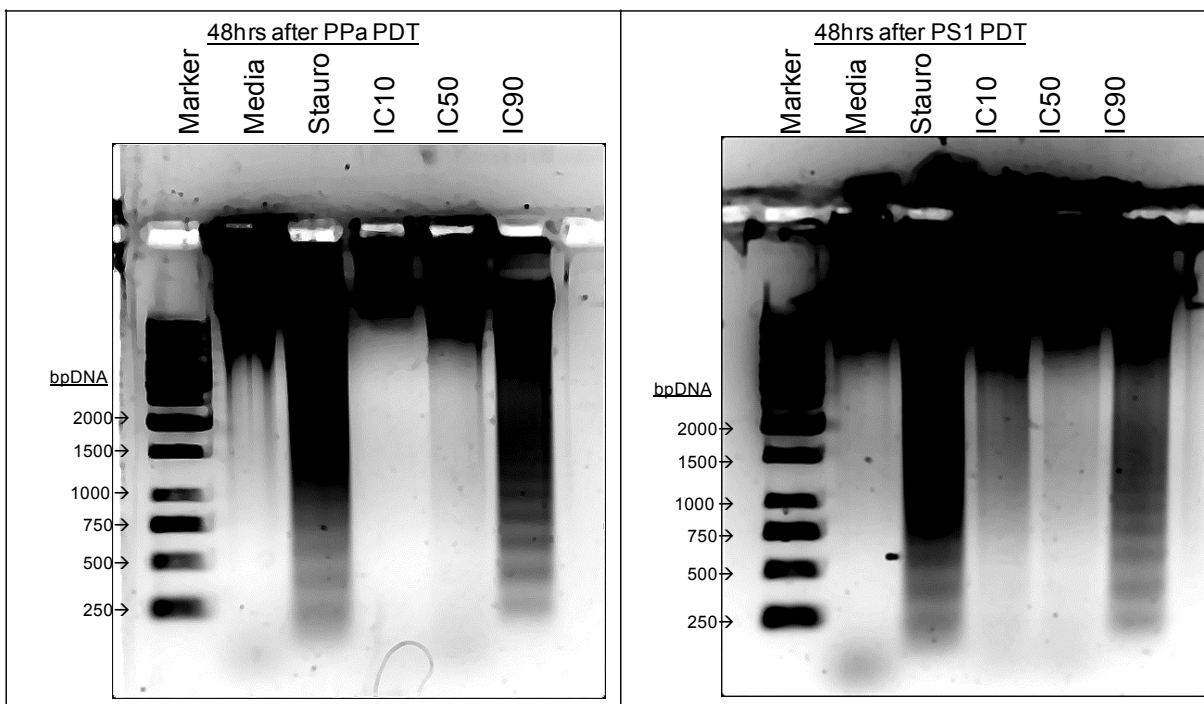
To visualise DNA fragmentation after treatment with either PDT or staurosporine (the positive control) DNA was isolated from cells as detailed in 2.1.4.10. The data from this set of work is presented in Figure 36. DNA laddering is seen with staurosporine controls as well as IC_{90} levels of PS for both PPa and PS1 treatments. The experiment was carried out at least twice on two independent occasions with the same result.

Figure 35 : Large and small laser beams deliver the same amount of energy per cm^2 .



96 well plates were set up in the standard cell kill assay and irradiated at different laser power settings with either the small or large laser beam; for 6 and 96 well plates respectively. The amount of cell death resulting from each laser setting is presented. When plotted over predicted energy delivered over the laser area (J/cm^2) all points fall on the same line indicating that the two beams are indeed delivering the same amount of energy per cm^2 .

Figure 36 : DNA fragmentation post PDT.



The standard cell kill experiment carried out with concentrations of PPa or PS1 required for 10% 50% and 90% cell death by 48hours. After 48hours DNA was extracted as detailed in 2.1.4.10 and ran through an agarose gel to visualise any DNA Ladders under UV light.

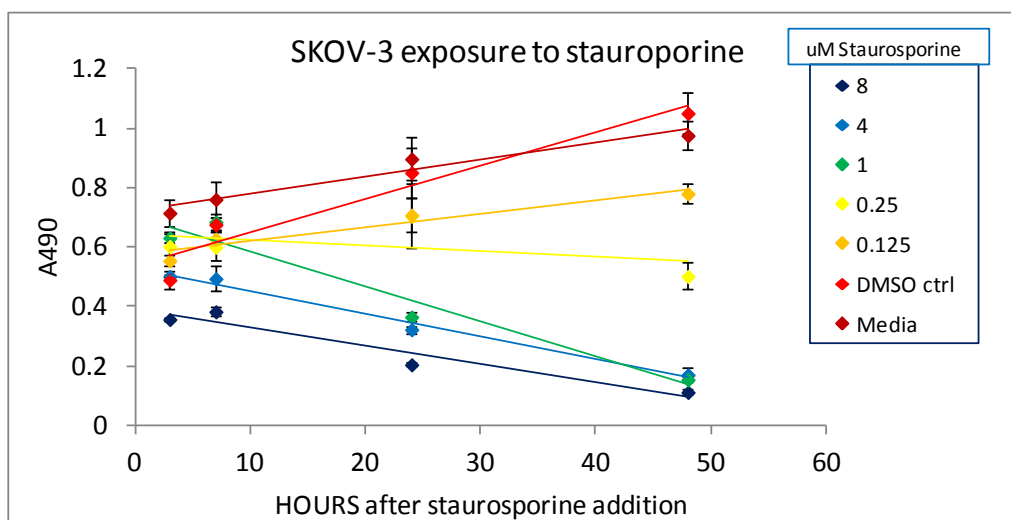
4.2.8 Cell cycle arrest after mid dose PDT

An aspect of interest from the data presented in 4.2.4 was the ability of IC₅₀ levels of PS to induce cell growth arrest in both PPa and PS1. In order to confirm this cells undergoing this arrest were examined to see if they exhibited accumulation in any stage of the cell cycle – this would suggest that it was stress induced checkpoint controlled cell cycle arrest and not an artefact.

As a control for chemically-induced cell cycle arrest, staurosporine was used at sub apoptotic doses as it can cause either G1 or G2/M cell cycle arrest, see 2.1.4.2. The dose chosen was 0.25µM, which as seen in Figure 37 shows evidence of cell growth arrest in our cell line SKOV-3. An alternative compound was Cisplatin which at sub apoptotic doses can induce an S phase block that can be transient and lead to a further G2 block, see 2.1.4.2. A dose response cell kill experiment for cisplatin was carried out to calculate the correct dose for SKOV-3 cells, which was chosen to be 40µM. This data is shown in Figure 38.

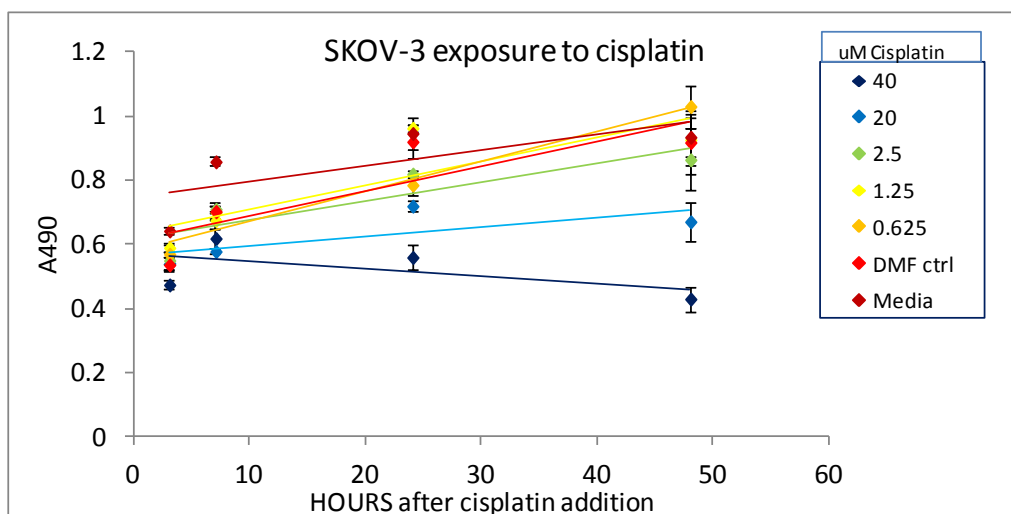
To identify which part of the cell cycle a population of cells are in, one of the most established methods involves cell permeabilisation and subsequent staining of genomic DNA with propidium iodide, analysis by flow cytometry can then determine the cell cycle position of individual cells, see 2.1.4.11 for more information. Before running this experiment it was important to check that fluorescence from any residual intracellular PS wouldn't interfere with analysis, especially as the propidium iodide (PI) fluorescent spectra overlaps with that of the PS. The work was carried out with Jurkat E6-1 cells (see 2.1.4.1) a suspension cell line useful for setting up flow cytometry (FC) assays due to ease of handling. The data presented in Figure 39 shows channels FL3 and FL4 are unusable as the background from internalised PS is unacceptably high, unfortunately these are the also the best channels for PI detection, however PI is also detectable in FL2 and in this channel PS doesn't have as much 'bleed through'. To check if the bleed through from the PS would be enough to effect the distinction of different cell cycle positions in the cell cycle assay Jurkat were pre-incubated with either PPa or cell culture media and stained with PI for cell cycle analysis, results showed no difference between pre incubation with PS or not as PI staining is very bright (data not shown).

Figure 37 : Dose response cell kill with staurosporine



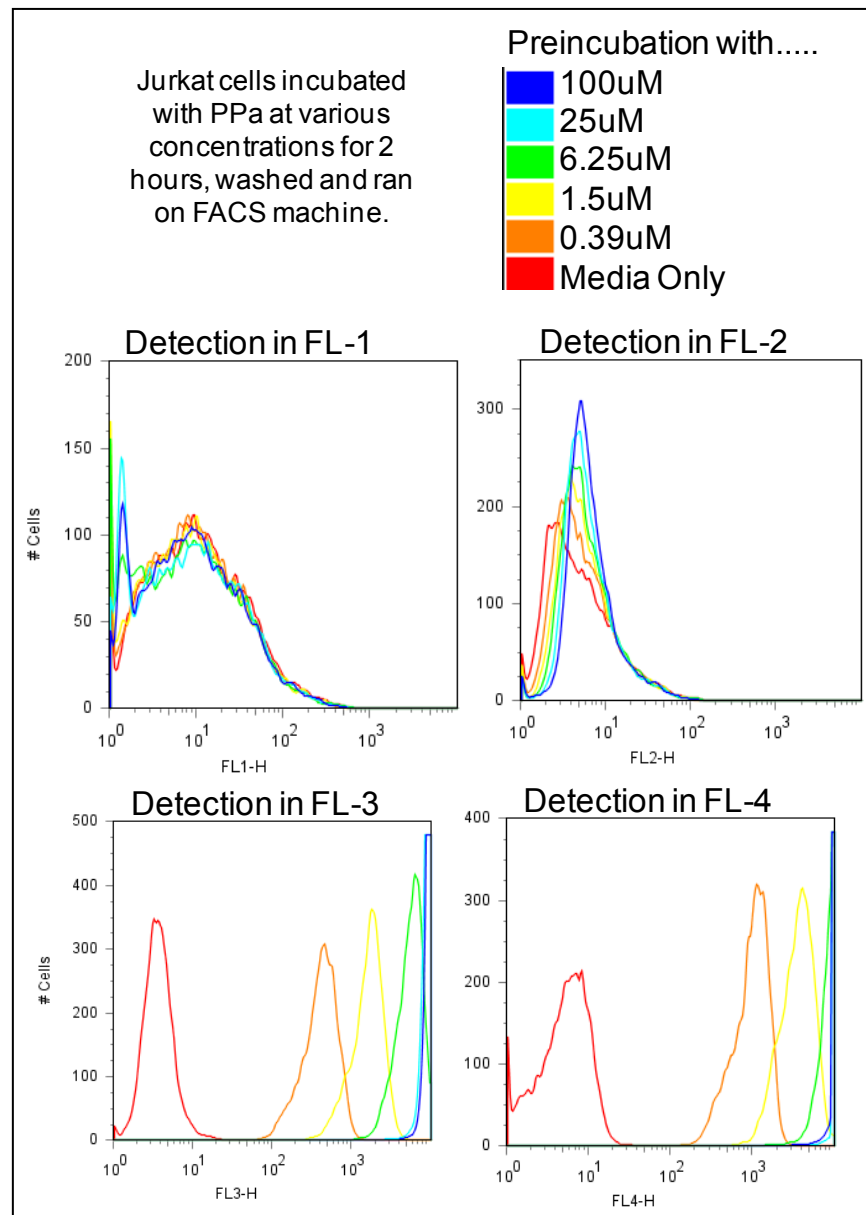
SKOV-3 cells were exposed to different amounts of Staurosporine in supplemented cell culture media over 48hours and at various points assayed for cell viability via the MTS assay; viable cells contribute to absorbance at 490nm.

Figure 38 :Dose response cell kill with Cisplatin.



SKOV-3 cells were exposed to different amounts of Cisplatin in supplemented cell culture media over 48hours and at various points assayed for cell viability via the MTS assay; viable cells contribute to absorbance at 490nm.

Figure 39 : Interference of internalised PPa with flow cytometry analysis.

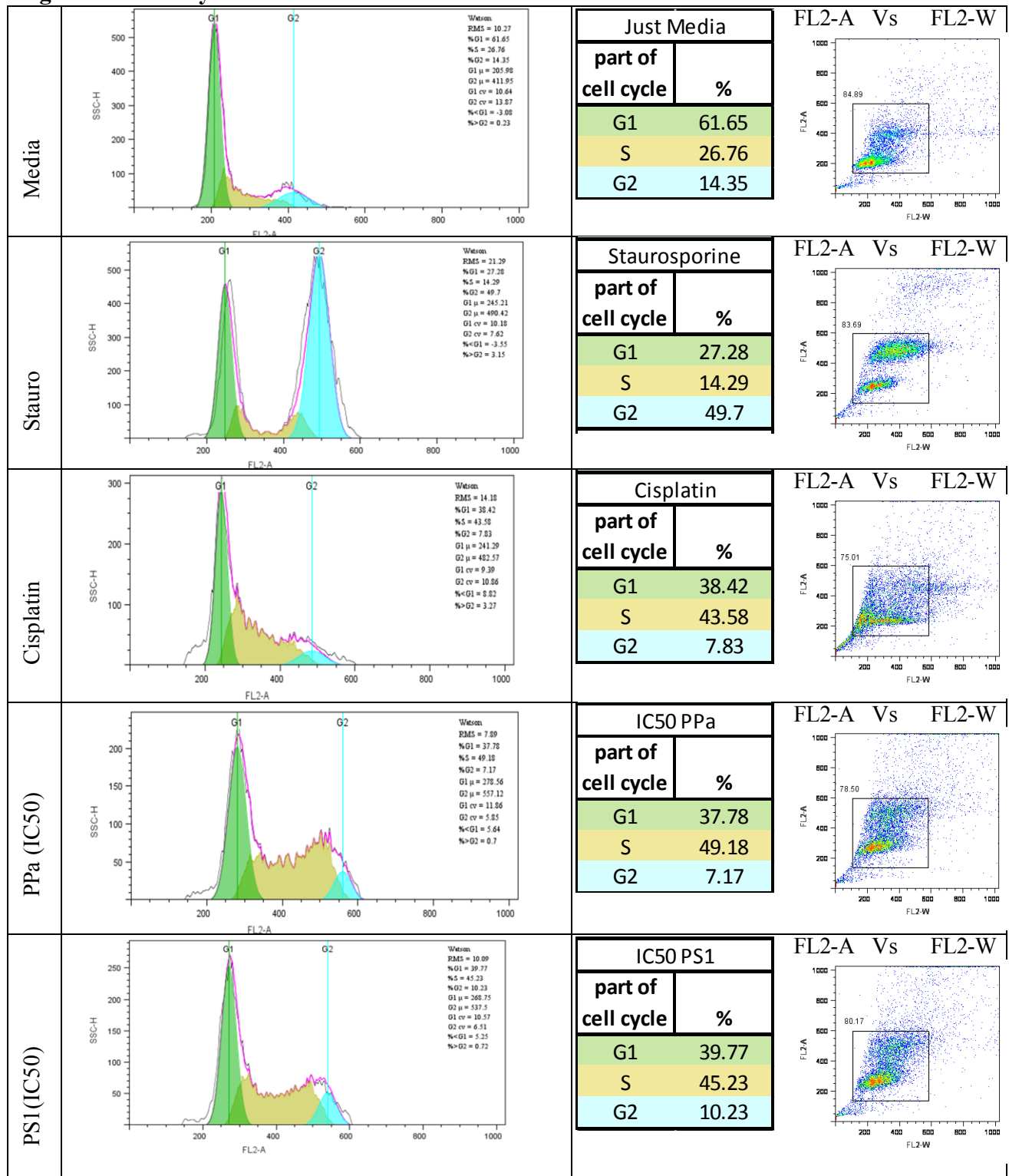


Jurkat cells were incubated with different amounts of PPa for 2 hours then washed and ran directly on the FC machine to see what channels the internalised PS was detectable in and to what extent.

Experiments were carried out to determine the cell cycle position of cells 48hrs after treatment with IC₅₀ levels of PPa or PS1, as well as low dose staurosporine or cisplatin. The results are shown in Figure 40. Data manipulation specifically for cell cycle analysis was carried out as described in 2.1.4.11. Results from this experiment show untreated cells distributed between G1 (62%), S (27%) and G2 (14%). Compared to this; staurosporine causes accumulation of cells in G2 as predicted from the literature (Bernard et al., 2001), and cisplatin caused an accumulation in S phase, also as has been predicted in the literature (Qin and Ng, 2002). IC₅₀ levels of PPa and PS1 showed a distribution between the cycles similar to that induced by cisplatin; cells seemed to have accumulated in the S phase. The pattern showed some slight difference in that there seemed to be an increase of cells with an amount of DNA that was close to but not exactly at the G2 level, however this could be seen as a transition to G2 arrest as can occur upon cisplatin treatment and as described in (Siddik, 2003). On some traces there was also the presence of a small sub G1 peak, likely apoptotic cells.

It is worth noting that SKOV-3 are a hypoploidy cell line, (see 2.1.4.1) their modal chromosome number is 43 occurring in 63.3% of cells with a range from 42-45. Normal chromosome number for human diploid cells is 46. Chromosome imbalance (aneuploidy) in cancer is not an unusual occurrence, in fact chromosome aberrations have been found in 90% of all solid human tumours (Fröhling and Döhner, 2008; Manchado and Malumbres, 2011) but this variation in chromosome number could have some impact in the interpretation of signals from a cell cycle study, the doubling of intensity from G1 to G2 should however still remain. For comparison Jurkat E6-1 cells are only pseudodiploid with the modal chromosome number being 46, occurring in 74% of cells.

Figure 40 : Cell cycle arrest after mid-dose PDT.



Cell cycle position of cells 48hrs after PDT treatment with IC₅₀ levels of PPa or PS1, as well as low dose staurosporine or cisplatin. Data manipulation specifically for cell cycle analysis was carried out as described in 2.1.4.11.

4.3 Discussion

4.3.1 Modulation of cellular uptake or localisation by novel photosensitisers

4.3.1.1 Intracellular PS localisation

Work presented in this chapter showed the visualisation of endogenous PS fluorescence under a confocal microscope to determine PS localisation within cells. The data suggested the localisation of both PPa and PS1 into membrane rich areas of the cell such as the endoplasmic reticulum. PS4 localisation was also centred close to the nucleus but displayed a different, more punctuate, localisation pattern, it was suggested to be more of an endosomal/lysosomal localisation. Other work within the lab (Stamati et al., 2010) looked into this further by co-localising commercial 'tracker' dyes for the mitochondria and lysosomes with these PS. Their data showed PPa and PS1 co-localised with but not exclusively to mitochondria whereas PS4 did not, and PS4 and PS1 co-localised with but not exclusively to lysosomes but PPa did not. It seems likely that the PS are associating with multiple areas within the cell. This matches other published work for PPa based PS which have been shown to localise membrane rich areas including Endoplasmic Reticulum/Golgi, Mitochondria, and Endosomes/Lysosomes. (Li et al., 2006; Matroule et al., 2001; Sun and Leung, 2002; Tan et al., 2009).

From ideas presented in the literature it is thought that a PS that localises in membrane rich environments, particularly the mitochondria and other key cellular organelles, will be more cytotoxic than those in a less lipid environment for example PS contained within endosomes or in the cytosol (see below). This could explain why PPa and PS1 are more cytotoxic than PS4, despite PS1 and PS4 being taken up into the cell to the same level.

PS have been shown to be capable of inducing death from almost all subcellular localisations. Discussed briefly below are those relevant to this work:

- The mitochondria is often stated as the most important site for induction of apoptosis as either a primary or secondary target. Although also capable of inducing necrosis, for apoptosis the mitochondria forms the convergence and amplification point for a number of signalling pathways. ROS are formed as a natural part of mitochondrial action, and endogenous levels are managed for cellular function. Excessive levels however have been

linked to membrane permeabilisation and release of various apoptotic factors. (George, 2002; Morgan and Oseroff, 2001).

- The Endoplasmic Reticulum (ER) is also linked to apoptosis via its ability to modulate calcium levels within the cytosol as well as containing various members of the Bcl-2 family. (Buytaert et al., 2006; Ogata et al., 2003; Oleinick et al., 2002).
- Lysosomal leakage of enzymes has been shown can initiate apoptosis via mitochondrial damage. Lysosomal damage by oxidative stress, in particular the Fenton reaction, will be enhanced by a low internal pH (~4–5) and high level of Fe(II) found within the organelle (Gèze et al., 1993; Kurz et al., 2008; Rodriguez et al., 2009).

Although relatively closed to protein the ER membrane has been shown to be more permeable to small molecules than other cellular organelles. A paper by Le Gall and colleagues (Le Gall et al., 2004) showed the energy independent permeation of ER by multiple small molecules of a similar size to PPa that were unlikely to have endogenous transporters, they hypothesised this was via either by protein conducting channels that are kept open by the association with non-translating ribosomes or a more leaky membrane due to its high protein content and the low level of cholesterol, known to fill “gaps” in lipid bilayers. The mitochondrial outer membrane is also thought to be permeable to all small molecules under 5 kDa in size due to the presence of membrane spanning proteins called porins, the inner membrane however is relatively impermeable for a cellular membrane as the membrane polarity is important for mitochondrial function (Malhi and Murthy, 2012).

One of the difficulties encountered with confocal fluorescent microscopy when studying localisation was its un-wanted ability to induce cell death. This in some cases can be used to an advantage in the direct use of the confocal laser to irradiate and monitor cell death or look at ROS production post PDT (Alexandratou et al., 2005; Rollan et al., 1996).

There were some observations during confocal fluorescent microscopy that raised questions over whether the pattern of fluorescence seen was artificially simulated in some way by the change in PS fluorescent efficiency in different microenvironments within the cell. This argument has further implications for potency. On one hand it could be said that increased

fluorescent efficiency in a particular microenvironment could indicate a more efficient absorptive processes or a reduction in energy dissipation processes such as aggregative induced quenching either with itself or other molecules within the cell, therefore indicating that a more fluorescent PS will be a more cytotoxic PS. Yet one could also make a similar counter-argument that a loss in fluorescence quantum yield could indicate an increase in the non radiative transfers important for PDT for example an increase in singlet oxygen quantum yield of PS in that microenvironment.

It also may be interesting to look at intracellular localisation at more time points than the arbitrarily chosen two hours (chosen to match *in vitro* data). There is a chance the data presented here is not showing a change in final localisation of PS but a change in uptake mechanism and given time PS4 could re-locate itself to represent the same as PPa or PS1 or visa-versa. This has in part already been answered in the work by other colleagues within the lab (Stamati et al., 2010) as their localisation experiments were carried out twenty hours after addition of PS and look similar to the ones presented here. Questions still remain however about once inside the cell, can the PS move around freely or is it fated to a destination prescribed by the uptake mechanism, in particular is it trapped within the protein trafficking pathway? It is generally thought that once inside the cytosol, i.e. via direct plasma membrane transport or endocytic escape, the PS are free to associate with their preferred compartment. Using this theory then PS1 and PPa have at some point then travelled through the cytosol as they exhibit some mitochondrial localisation and this cannot be reached by endosomal transport alone. These ideas will also have implications once an antibody is used to deliver the PS.

4.3.1.2 Intracellular and Extracellular fluorescent spectral profiles of PS

As part of this work PS fluorescent profiles were investigated in DMSO vs. supplemented cell culture media. In 3.2.3.1 the absorbance profiles of PS in these same solutions were presented and although shifted, the relationship between absorbance and concentration was of a linear nature. Fluorescent curves in supplemented cell culture media however exhibited dramatic changes over concentration, especially PPa and PS1. Differences between absorbance and fluorescence spectra can occur for a number of reasons and many solvation effects are

exaggerated in fluorescent emission profiles compared to absorption profiles. Excited state molecules can encourage different interactions in the solvent than the ground state, this is because the time required for solvent relaxation is longer than the time needed to absorb a photon and excite a molecule but shorter than the excited state lifetime, solvent interactions can therefore move to accompany the excited state and will change respective energy levels before a photon is emitted (Lakowicz, 1999).

Because of this fluorescence is also a much more sensitive measure of the aggregative effect than absorbance. Concentration dependent shifts that are only visible in the fluorescence profile and not the absorbance profile suggest a change in the excited state, and when shifts are caused by an increase in the concentration of the molecule itself it is likely indicating PS self-aggregation. The extent of the fluorescent shifts, like seen with absorbance, are less dramatic with PS1 and PS4 than with PPa and although the extent of the shifts varied between the different PS, they all occurred at a similar concentration (between 3 μ M and 6 μ M) with all three PS. So although modification of the PS may have made the new PS more soluble in aqueous solution, shown by the retention of electronic configuration more effectively in aqueous solvent, multimerisation still occurs. This extent of aggregation is unknown and still may vary between PS, no extensive precipitate forming aggregates were visualised in any solutions.

Looking at extracellular PS in different solvents gives a good idea of how a molecule behaves in different environments, but it is worth remembering that these solvent environments are not completely comparable to intracellular environments. The principle of ‘macromolecular crowding’ (Ellis, 2001a, 2001b; Verkman, 2002) should always be kept in mind.

Confocal fluorescent microscopy also produced fluorescent emission spectra from intracellular PS. The spectra for PPa and PS1 matched that for disaggregated and relaxed PS (i.e. in DMSO or at low concentrations in supplemented cell culture media), this was particularly obvious for PPa and PS1 as they exhibit shifts upon aggregation. PS4 however only showed a loss in fluorescent intensity at higher concentrations and not a shift, this means the data is less conclusive on whether PS4 exists in a disaggregated form or not within the cell. It is possible that as these lambda scans were averages of whole images containing a number of cells, more

information on environment specific shifts could be deduced from this data if lambda scans were taken from different areas within the cell, but for this, laser intensity would have to be increased and spectral changes could occur in the dying cell during irradiation.

To support the suggestion PS becomes monomeric once internalised by cells, there are many examples in the literature, including a paper by Sun and Leung (Sun and Leung, 2002) that present evidence of this idea with PPME using simple spectra techniques. Spectra of our PS within a lipid environment have not been studied here but it would be possible to mix synthetic lipid vesicles in with PS solutions in order to study this. Experiments like this are often carried out with PS to model uptake and it is usually seen that lipid vesicles or serum proteins will induce a more monomeric spectra.

PS1 and PS4 modifications were made outside the main ring and when structures were analysed with computer software to predict lowest energy conformers (data not shown) they were all shown to have groups projecting above or below the ring; for PPa and PS4 this was the propionic acid side chain and for PS1 it is the PEG-like chains. This would have steric influence on the way the molecules arrange during aggregation and could explain the spectra which suggests side by side aggregation (J aggregates / red shifted) and not face to face stacking (H aggregates / blue shifted) NB In different solvent environments molecules can interconvert readily between a number of conformers.

4.3.1.3 PS uptake into cells

Although the data presented in this chapter suggests cellular uptake is unaffected by serum protein binding, it does not remove the possibility of serum protein binding occurring. PPa has already show evidence for binding in work presented from this lab previously (Kuimova et al., 2007) and presented in this thesis are significant spectral shifts induced with the introduction of FCS to an aqueous buffer, see 3.2.3.1. In a similar assay to the one used in this thesis Siboni and colleagues (Siboni et al., 2002) saw differences in uptake of their PS with 10% serum, however for Brandis and colleagues (Brandis et al., 2005b) their *in vitro* assay levels of FCS had to be 50% to show any significant loss in cytotoxicity even though they had already shown shown

significant albumin binding with their 'water soluble' PS. It could therefore be of interest to study further this relationship of the new PS with different isolated serum or cellular components. It may also be interesting to see if changes seen in the fluorescent spectra are translated into effects on singlet oxygen quantum yield.

The data produced by the PS uptake experiments can give us an insight into what type of uptake mechanism is in use: Saturation of a receptor-driven process can be seen typically as an increase in intracellular concentrations with increasing incubation concentrations before reaching a plateau as available sites on the cell surface are saturated. (NB some mechanisms of endocytosis can also show this as there are limited numbers of sites for endocytosis on the cell surface.) If at high dosing concentrations in which these processes exhibit saturation some continued increased uptake is observed but at a lower rate, this indicates there may also be some passive diffusion of the complex across the cell membrane. If uptake is occurring only by a passive diffusion mechanism, uptake would be expected to increase linearly with concentration for all dosing concentrations. Additionally cell uptake by passive diffusion is most likely to show rates that mirror trends in logP (Sugano et al., 2010).

Looking at the PPa data presented it is most likely visualisation of a system like the one described above with contribution from two mechanisms; a saturable process evident at low concentrations driving the majority of the uptake, but once this is saturated another mechanism continues to show its relationship between extracellular PS concentration and uptake. Uptake of PPa shows saturation of uptake much more prominently with time, rather than concentration. Saturation being dependent on time can infer a depletion of a requirement for uptake; this is unlikely to be ATP as the cells should be growing healthily in the provided cell culture conditions, it could be an indication the external PS source is running low – but as it is in high excess this is also unlikely. It is most likely an indication that flow in, flow out and internal degradation of the PS are in balance at the point of saturation and therefore no longer providing a change in internal PS concentration.

The data presented on PPa shows the leaching rate and the uptake rate to be different (leaching < uptake) and reducing the temperature to 4°C almost completely prevents uptake and barely effects leaching – this suggests that the mechanisms for these two paths are different. Due to the reduced uptake at 4°C compared to 37°C the most likely mechanism of uptake that could be responsible for PPa uptake is an active mechanism (i.e. one that requires ATP). Attenuation of uptake by reduction in temperature to 4°C is a common and well established method to determine the presence of an active mechanism of uptake. However there is also evidence in the literature that a reduction in temperature can induce stiffening of cellular membranes so will also to some extent reduce cellular uptake that is controlled by simple membrane diffusion processes (Poirier et al., 2008; Sugano et al., 2010). As there are reductions at 4°C in both leaching and uptake but the extent is very different, this indicates that the slight reduction seen with leaching is due to membrane stiffening -suggesting the PS are removed out by a non-active mechanism, and with uptake the almost complete reduction is due to the presence of an active mechanism for uptake like endocytosis. As suggested above a slower non-active mechanism of uptake is also likely occurring alongside endocytosis as uptake remains above-baseline even at 4°C.

A similar PS to PPa, PPME was shown to cross the membrane passively by Guelluy and colleagues (Guelluy et al., 2010). PPME alters from PPa only by the exchange of a carboxyl group for a methyl ester on the propionic acid side chain, it is probably the presence of this carboxyl group (at pH 7.4 predicted to be in the ionised form) that is reducing the passive diffusion capability of PPME and instead be forcing PPa to rely more exclusively on endocytosis.

The chemical modification of PPa into its more soluble forms PS1 and PS4, causes a significant reduction in cellular uptake, this would fit into a model describing the loss of recognition by a specific receptor, but as PPa is not an endogenous molecule and at the moment has no known specific receptor it is more likely that it is endocytosis that drives its uptake, either by passively being taken up as part of the solvent surrounding cells or as piggyback on another molecule.

The literature primarily suggests that with less aggregative PS you achieve increasing uptake due to the higher proportion of monomeric PS in the solution which can then undergo passive diffusion across the membrane (Guelluy et al., 2010; Kelbauskas and Dietel, 2002). However with our less aggregative PS (PS1 and PS4) less uptake is seen. The predicted pK_a data 3.2.3.3 also shows a neutral species cannot exist in any of these molecules as an independent species and neutral species are usually the driving force for both PS aggregation and membrane integration. Alongside the evidence above for endocytosis playing a dominant role in PPa uptake this suggests that the reduction in uptake of PS1 and PS4 is not likely being driven by a change from monomerised passive diffusion to aggregative endosomal uptake.

The levels of PPa taken up into the cell at 4°C is a reduction to a similar level of PS1 or PS4 at both 37°C and 4°C. A hypothesis could then be provided that PS1 and PS4 have significantly reduced ability to get as involved with an active form of endocytosis compared to PPa, and passive diffusion mechanisms for example pinocytosis are responsible for the baseline uptake for all three PS. For endocytosis, having a more hydrophobic character, i.e. like PPa, can increase levels of endocytosis in two main ways. Firstly, it can lead to increased PS interaction with serum proteins which are taken up more favourably, although for the PS studied here this is unlikely to be the reason as when FCS is removed completely the uptake relationship doesn't change. Secondly; via increased direct PS interaction of hydrophobic PS with the cellular membrane and so a subsequently higher local concentration at the cell surface. PS1 has a few Polyethylene glycol (PEG) chains and PS4 has a small one. PEG is widely used for drug *in vivo* applications because it has been shown to prolong blood circulation time and decrease nonspecific interaction with the reticuloendothelial system, but, as shown by El Sayed and colleagues (El-Sayed et al., 2009), PEG chains can also decrease cellular uptake by prevention of direct cell membrane interaction by steric hindrance. PS4s additional positive charge, although predicted to increase membrane interaction may with the addition of the carboxyl be making the molecule carry too many charges to encourage any membrane integration.

To make a more definite conclusion on uptake mechanism there are a lot of tools available to assess the contribution of various endocytotic pathways to PS uptake that could be used to look at this further, for example this paper by New and colleagues (New et al., 2010) used a diverse

panel of chemical inhibitors or activators of the various endocytotic processes and showed despite the differential localisation seen within a panel of different dyes, a common uptake mechanism of macropinocytosis was in place for all. Also Isothermal titration calorimetry (ITC) with lipid vesicles in particular, can be used to measure direct interactions/ integration into the membranes allowing easier measurement of kinetic calculations (Heerklotz and Seelig, 2000; Tsamaloukas et al., 2007), this can be very useful in understanding uptake mechanisms and would be hopefully more sensitive than the fluorescence based method used in this thesis. Another way to dissect data obtained from the uptake experiments could be by the application of a mathematical model. This can help separately address passive vs. active reaction kinetics providing such parameters for active transport processes such as Michaelis-Menten (V_{max} and K_m), and for passive diffusion processes (P_{dif}). One such model that could possibly be applied to my data is described here (Poirier et al., 2008).

4.3.1.4 Possible endosomal escape mechanisms

If the hypothesis above that PS are entering the cell via endocytosis and/or pinocytosis can be accepted the question still remains about final localisation of PS: PS1 and PPa seem able to escape and travel to the cytosol where they then partition into all sorts of cellular membranes whereas PS4 cannot.

The computational prediction of pK_a groups in our PS are shown in Figure 18, and this along with data looking at fluorescence over pH (3.2.3.2) suggests the following: The pK_a values predict that at pH 7.4 the major species shows an ionised carboxylic acid and deionised pyrrolic nitrogens. The pH of the cytosol (pH7.1-7.2) vs. endosomes (pH5.5-6) and lysosomes (pH5.0) means endosomal escape could be mediated by the nitrogens in the tetrapyrrole ring becoming protonated on a higher proportion of the PS population in lysosomal pH. Protonation would take free H^+ out of solution and cause an imbalance, possibly causing more protons to be pumped into the lysosome and subsequent osmotic imbalance making the lysosome more leaky. However there is no significant difference in predicted pyrrolic nitrogen pK_a between PPa, PS1 and PS4, so the difference in PS4s inability to escape could be that PS4 cannot pass through the membrane due to its additional constant positive charge. For all PS once protonated there will be positive

charges on the ring so it could be that one proportion of the population causes the leakiness (protonated PS) and the rest that are able to leak out (un-protonated), PS4 however has a constant positive charge so none of its population are able to cross the membrane.

The importance of the ionisation of PS in cellular transit is emphasised in work carried out by Bonneau and colleagues (Bonneau et al., 2004a) who looked at membrane diffusion rates for an asymmetrical porphyrin with 2 carboxylic acid groups on the same side of the ring. Small unilamellar vesicles were used to simulate membranes and they looked at PS transition into them at different pHs. Interestingly they found that whatever the pH of the initial solution membrane incorporated porphyrins in the membranes were not protonated on their ring nitrogens. They also observed a decreased rate of entry with decreasing pH, this decrease of the entry rate was thought to be due to dimerisation of neutral porphyrins. They also observed the movement of these molecules from a moderately acid to a neutral aqueous compartment was found to be strongly favoured kinetically, mirroring the tumour environment *in vivo*.

4.3.1.5 Conclusions for cellular PPa, PS1 and PS4 uptake and localisation

Quantified images of cell fluorescence from the confocal fluorescent microscopy showed cell fluorescence with PS has a relationship as follows $PPa > PS1 \ggg PS4$ but when this was compared to actual uptake data it was seen that cellular uptake actually follows the relationship $PPa \gg PS1 = PS4$. Potency in an *in vitro* assay follows the relationship $PPa > PS1 \ggg PS4$, matching the confocal fluorescent microscopy data almost exactly, intracellular PS fluorescence in this case then could be used as a more accurate indicator for potency than cellular uptake.

When considered together and in the context of toxicity it seems clear that despite massively reduced cellular uptake PS1 actually exhibits improved death per dose effects than its parent molecule PPa, this is hypothesised to be due to enhanced intracellular photophysics reflected in its enhanced fluorescence seen in confocal fluorescent microscopy and in extra cellular studies with PS in aqueous solution. What is still not known is if this enhanced photophysical retention is actually directly responsible for increased toxicity per dose, it could be that the increased

fluorescence per dose is in fact an artefact and not causative and is it instead through the induction of an alternative cell death mechanism that PS1 increases in potency.

4.3.2 Modulation of cell death by novel photosensitisers

4.3.2.1 Changes seen in the cell death induced by PPa and PS1

Earlier in the chapter it was discovered that much lower levels of PS1 were responsible for the same toxicity at forty-eight hours as higher levels of PPa. Localisation experiments showed that PPa and PS1 seem to be localised in similar parts of the cell, so the question addressed in this part of the chapter asked could an altered mechanism of cell death be responsible for improved PS1 toxicity once inside the cell.

Doses of PPa and PS1 that caused 90% cell death over 48 hours were used to look at two popular hallmarks of apoptosis, caspase 3/7 activation and DNA fragmentation. Neither PPa or PS1 seemed to induce any caspase 3/7 activation in SKOV-3 cells but there was evidence for DNA fragmentation for both PS. Could it be possible then, that both PPa and PS1 have similar mechanisms of action even though cell death occurs much quicker for PPa than PS1? The cell death that was observed within two hours with PPa was confirmed by the loss of outer membrane integrity at this point, i.e. cells were trypan blue positive. Despite this, the DNA fragmentation data suggests death is still of an apoptotic mechanism as opposed to necrosis, necrosis being the more likely mechanism to cause such sudden loss in membrane integrity and reducing ability (MTS). DNA damage when seen with necrosis results in non-specifically degraded DNA visualised as a smear rather than a ladder of DNA. Two hours, although incredibly quick, could still be sufficient time for apoptosis to initiate, however it is more likely that a population of cells is being measured in which both necrosis and apoptosis are occurring and there has been some bias in each experiment to one type of cell death. For example in the DNA fragmentation experiment, cells and cell debris are recovered after 48 hours and the DNA subsequently isolated, if some cells underwent necrosis 2 days previously there may not be much DNA left to collect and so when DNA is loaded on the gel it is only representative of a few of the cells that were in the PDT treated population. (Although during collection care was taken to collect both

floating and dead cells and debris but if cells were completely degraded DNA would have been lost).

The idea of a heterogeneous population of cell death mechanisms is backed up by data presented in Figure 7 in which the laser profile was shown not to be evenly distributed in that it delivered a range of energy doses through the irradiated area. Additionally with both PPa and PS1 the cell kill profiles via MTS show some evidence of a 'gradual' decrease in cell viability over time, although a smaller proportion of the cell population in PPa that shows this some cells still take longer to die. This gradual slope is not a direct measure of the cells dying slowly, i.e. losing reducing ability slowly but more likely a representation of cells dying at different times over the period of analysis as this experiment measures an average of a population, not single cells.

Some work was initiated to address this bias in the development of a flow cytometry assay utilising a dual Annexin V / Propidium iodide staining technique (van Engeland et al., 1996), that can distinguish on a single cell level the difference between apoptotic, necrotic and living cells, assigning all individual cells within a population to one of these categories simultaneously within the same experiment. This experiment was successfully set up for control compounds but once PS were introduced to the cells the compensation between channels became too convoluted and it had to be manipulated to such an extent that the data lost any significance or relation to the original data (data not shown).

4.3.2.2 Possible cell death mechanisms initiated

There is a significant body of work investigating mechanisms of cell death that are caspase independent and still cause DNA fragmentation, some are reviewed here (Kitazumi and Tsukahara, 2011; Kroemer and Martin, 2005). A favoured contender for our model is perhaps EndoG/AIF mediated DNA fragmentation. (L. Y. Li et al., 2001; Susin et al., 2000; Widlak and Garrard, 2005). This would fit with the slightly smearyness of the higher Mw DNA fragmentation seen because it is known that there is higher internucleosomal DNA background upon Endo G digestion compared to CAD.

The first figure presented in a review paper by Kitazumi and Tsukahara (Kitazumi and Tsukahara, 2011) describes well the main regulation pathways for DNA fragmentation. From it you can see mitochondrial permeability linking both EndoG/AIF mediated DNA fragmentation alongside activation of the caspase cascades, however in our model these pathways are not both activated- despite the predicted mitochondrial damage caused by both PS1 and PPa. This result may be SKOV-3 specific, SKOV-3 cells have non functional p53 mutations, see 2.1.4.1, and p53 is responsible for relocating BAX and formation of the mitochondrial pore that releases cytochrome c, if this isn't properly formed it would prevent apoptosome formation and subsequent effector caspase activation. Cellular pathways however are very complicated and this brief description is merely of a selection of the pathways potentially involved.

Mitochondrial permeability, an independent process to membrane permeabilisation (MOMP), is often associated with a number of apoptotic pathways, and is linked to EndoG release. A paper on PDT by (Kessel and Luo, 1998) describes various PS that initiate different rates of cell death but all initiate mitochondrial permeability at some point. They hypothesise that this mitochondrial permeability can occur either as direct and rapid radical damage during irradiation or a subsequent delayed permeabilisation after the initiation of apoptosis. Something similar could be happening with PPa and PS1, both initiating mitochondrial permeability and release of EndoG but at different times.

To confirm the influence of this pathway in both PPa and PS1 induced cell death one could use the assay set up for DNA fragmentation but instead of isolating DNA from the cell pellet, the pellet could be resuspended in fractionation buffers and nuclear, mitochondrial and cytosolic fractions separated. Standard western blot techniques can then be used to look for relocalisation of EndoG from the cytosol to the nucleus. Looking for evidence of mitochondrial outer membrane permeabilisation (MOMP), or the distinct mitochondrial permeability transition could also prove interesting. It would also be interesting to repeat this work in another cell line exposed to its own IC₉₀ doses of PPa and PS1.

4.3.2.3 Theories on difference in cell death between PPa and PS1

The intracellular localisation of PS is very influential on the type of cell death mechanism induced upon irradiation but PPa and PS1 were shown to have a similar localisation. There was no sufficient evidence from this work that suggests a change in apoptotic mechanism for PPa vs. PS1, only an apparent shift in balance between apoptosis and necrosis and/or how soon cell death is initiated. It has been shown that PS1 has more effective intracellular fluorescent photophysics than PPa, and that there are more molecules of PPa than PS1 contained within the cell; based on this a few alternative theories are presented below:

- Although the localisations look similar by confocal microscopy, it could be a matter of orientation in the lipid bilayer that could change either the type of reactive species produced or the lifetime of the singlet oxygen produced.
- As there are more PPa molecules they could be spread wider throughout the target organelle. PS1 will take longer to initiate cell death as it has to work via other reactive mediators to spread throughout the organelle via oxidative stress pathways.
- As could be predicted by its hydrophobic nature PPa is forming aggregates inside membranes and these localised regions of PS are able to make larger holes in the membranes, inducing a more sudden necrotic death.
- There is a pH difference between the cytosol/ER (pH7.1-7.2) and Mitochondria (pH8). Back in chapter 3, Figure 17 it was shown that between pH 7 and pH 8 PPa shows a decrease in absorbance and PS1 an increase. Since mitochondria are a particularly sensitive spot for inducing an apoptotic cell death this could be providing PS1 with a greater ability to induce apoptotic cell death compared to PPa.
- That although the enhanced fluorescent photophysics suggest PS1 may be more effective at producing PDT mediators within the cell, because there are less molecules of PS1 the rate at which they are produced may be slower (a lower number of molecules can be excited at one time), favouring an apoptotic mechanism of death.

It is an inherent benefit of PDT that it produces reactive oxidative mediators that can kill in a multitude of non-specific ways. This idea, alongside the fact that SKOV-3 cells are ‘uniquely

different' along with all other cancer cell lines, and therefore inherently biased to certain cell death mechanisms and its markers, means that this work is perhaps not worth chasing without a more specific hypothesis in mind. Alongside this are the additional two ideas that a) it is possible that as soon as one changes variables in the *in vitro* kill assay everything could change and b) cells in a real tumour are subject to far more variations in light and drug dose than one would currently have the ability to accurately model.

4.3.2.4 Cell cycle arrest with mid-dose PDT

An additional investigation was undertaken to confirm the observation that IC₅₀ levels of PPa and PS1 induced cell cycle arrest. This experiment confirmed that cells were arrested in the S phase similar to treatment with low dose cisplatin. Cell cycle arrest induced by low level PDT isn't unusual (Sacková et al., 2006; Vantieghem et al., 2002) and has been seen with PPa (Tian et al., 2006), low level ROS are also known to induce cellular senescence (Esposito et al., 2004).

The question remains whether cell cycle arrest is a desired side effect of PDT? Staurosporine has the potential for cell cycle arrest at low dose, and has anticancer properties with a number of analogues undergoing clinical trials (Gescher, 2000). Cisplatin, a proven successful chemotherapeutic drug, also has this effect at low dose. Potential may rest on whether one is seeing a transient cell cycle quiescence that can lead to apoptosis or a more permanent cellular senescence, which has been shown to provide cells with resistance to apoptosis and a reduction in non-specific drug uptake (Wang, 1995). It may also be useful to try PDT and Cisplatin and/or staurosporine together as a combination therapy. A paper by Duska and colleagues (Duska et al., 1999) has previously used full dose PDT to successfully re-sensitise cisplatin resistant cancer cells to cisplatin.

4.3.3 Chapter conclusions

Key proposition put forward from work ;

Investigation into the mechanism of cellular uptake and localisation of the modified PS showed that despite being as potent as PPa, PS1 intracellular concentrations were much lower suggesting PS1 actually exhibits improved death per dose over PPa, this was hypothesised to be due to its enhanced intracellular photophysics. Despite showing significantly different uptake kinetics, PPa and PS1 at lethal doses seem to be able to initiate similar mechanisms of apoptotic action, likely due to their similar intracellular localisations but the balance is shifted between the time and type of cell death that is initiated. PS4s reduced potency over PPa seems to be driven by both the substantially reduced cellular uptake, and an altered cellular localisation, although the potential for PS4 upon antibody conjugation still remains high.

Main question raised from this work;

If the cellular uptake of PS1 and PS4 were re-established on a similar level to PPa, for example by conjugation to an antibody fragment and subsequent utilisation of different uptake pathways (antibody internalisation), would this make them significantly more toxic than PPa? Other questions will arise over whether the PS or the antibody would provide the dominant determinants in uptake and localisation, and would antibody conjugation be able to change the cell death mechanism?

Chapter Five

5 CHAPTER 5: The conjugation of novel PS to the antibody fragment C6.5-k

5.1 Introduction

5.1.1 HER 2 as a target

The protein HER2 (Human Epidermal Growth Factor Receptor 2), also known as ErbB-2, Neu, CD340 or p185 is encoded by the ERBB2 gene. It is a member of the epidermal growth factor receptor (EGFR/ErbB) family and although thought to have no natural ligand, (an orphan receptor), it has a constitutively exposed dimerisation arm and through its role as a binding partner for itself and other members of the family can initiate downstream signalling important in many cellular pathways including proliferation, differentiation, angiogenesis and cell survival. Its ability to homo-dimerise is limited but it is the preferred hetero-dimerisation partner for all the other family members. (Gutierrez and Schiff, 2011; Hynes and Lane, 2005; Roepstorff et al., 2008) HER2 is expressed in clusters on the cell surface (Kaufmann et al., 2011) and exhibits more resistance to down regulation than other members of the family. Due to the binding between family members the influence of the expression level of different members of the same family is also important. (Emlet et al., 2006; Roepstorff et al., 2008; Sorkin and Goh, 2009).

First shown by Slamon and colleagues (Slamon et al., 1989) the amplification of the HER2 gene and / or overexpression at the messenger RNA or protein level occurs in about 20% of patients with breast cancer and correlates with poor patient prognosis. HER2 is also implicated in other cancers including bladder, pancreatic, lung, ovarian, endometrial, colorectal, renal, head and neck, gastric, oesophageal, and prostate cancers (Ménard et al., 2001). It has provided and continues to provide a basis for many targeted therapeutics. (Arteaga et al., 2012; Yarden and Pines, 2012).

Trastuzumab, also known as HerceptinTM (Roche), is the most successful anti HER2 therapy and is a humanised monoclonal immunoglobulin G (IgG) antibody against the extracellular region of HER2. Alone and in combination therapies it has shown significant success as a therapeutic agent in treating breast cancer. The lack of down regulation of HER2 after binding of antibody means cell populations will not develop a loss in sensitivity as often seen with other

targets when receptors are down regulated after prolonged antibody binding. Despite this, some resistance (treatment acquired and endogenous) has been observed, the reason for this resistance, seen in patients with HerceptinTM, is still unknown, but it is usually ascribed to modulation of intracellular downstream signalling pathways or the initiated ADCC response (Bailey et al., 2011; Wong and Lee, 2012).

C6.5-k is the chosen fragment for antibody conjugation in this thesis and is based on C6.5, see 2.1.5.1, a fully human anti-HER2 scFv isolated from a filamentous phage antibody library, first described by Schier et al in 1995 (Schier et al., 1995). C6.5 targets an epitope on the extracellular region of HER2, distinct from that bound by HerceptinTM (Reddy et al., 2011) Initial studies (Schier et al., 1995). showed C6.5 exhibited good tumour localisation over normal tissue and bound to the immobilised extracellular domain of HER 2 with a K_D of 16 nM and to native antigen expressed on SKOV-3 with a K_D of 20 nM. For reference HerceptinTM is stated as having a K_D of 5 nM in a cell based assay (HerceptinTM packet insert). Later work from this lab produced a range of higher affinity versions of C6.5 which were used to answer some fundamental questions on antibody targeting, see 1.5.2.2. The mutated version of C6.5 used within the lab of M.P.Deonaraian at Imperial College, and for this thesis; (C6.5-k), shows binding affinity to the immobilised extracellular domain of HER2 with a K_D of 3.1nM ($K_{on} = 1.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $K_{off} = 4.3 \times 10^{-3} \text{ s}^{-1}$) as measured by surface plasmon resonance (Biacore), see 2.1.5.1 for a comparison for the sequence to the original C6.5.

5.1.2 Conjugation techniques

There has been significant development in the technology for antibody-drug conjugate (ADC) production over the years, and this has applied directly to the field of photoimmunoconjugates (PIC). As the photo efficiency of PS has improved with second generation PS, the field generally moved in the direction of the low level specific and direct conjugation of one or two PS to each antibody molecule, however as the solubility of PS has been improved some, including ourselves, are returning to more highly loaded less specific direct conjugations techniques. Other choices include conjugation via a spacer or pre conjugation of PS to a polymer that is then subsequently attached to the Ab. See 5.1.4. for more details.

There are many techniques for potential bioconjugate reactions, enough to fill a 1200 page book by Hermanson (Hermanson, 2008). The amino acids that make up protein provide a range of key reactive groups that can be specifically manipulated to provide a tailorable approach to protein-drug conjugation. Although the amino acids are often the most common providers of reactive groups, lipids, glycans and nucleic acids are also possible bioconjugation partners. The easily disrupted 3D tertiary protein structure however adds a level of complexity and sensitivity to chemical bond formation, a field traditionally carried out in a myriad of solvent and environmental conditions. The ideal reaction will go to near completion without any side products or damage to the protein resulting in the production of a pure and well described product. It is also important to consider how the linker will react in an *in vivo* environment, linkers can be designed to have independent functionality alongside the two molecules being conjugated. The most commonly manipulated linker functionality is the predicted breakage of this bond in a chosen physiological environment. In the case of PIC however the reasons for introducing an antibody are primarily to reduce the side effects of free PS so a stable, essentially irreversible, bond is usually preferred.

For PIC production the bioconjugation methods that have been used in the field so far include direct conjugations via carbodiimide coupling, activated esters, reductive amination, conjugations via isothiocyanate, maleimide or acryloyl moieties, and indirect conjugation of PS onto polymeric linkers such as dextran, polylysine, polyvinyl acetate (PVA), polyglutamic acid (PGA), fullerene or HPMA before subsequent conjugation of this PS/linker to the antibody. The use of all these different techniques and their use over the development of the field has been well discussed recently (Bullous et al., 2011; Giuntini et al., 2011). The use of specifically targeted endogenous thiols within the antibody fragment has attracted particular attention recently as a method for exquisite specificity of both conjugation (Junutula et al., 2008), and controlled release of the drug (Shen et al., 2012). Investigation into different conjugation chemistries has already shown to, and will further help prevent, common problems encountered with PS conjugation.

5.1.3 Chosen conjugation technique for our system

The reaction most commonly carried out in the context of PIC formation is the reaction between an NHS (*N*-hydroxysuccinimide) ester reactive group and the ϵ -amine found on the amino acid lysine. This same reaction was used in work preceding this project, see 1.6. The reaction provides an easy one step method to make highly loaded covalent conjugates with very little manipulation of the antibody itself. The antibody used in this thesis was itself partially chosen because of its high number of well spaced and solvent exposed lysine residues, see 5.2.2. A reaction mechanism for this reaction is shown in Figure 41.

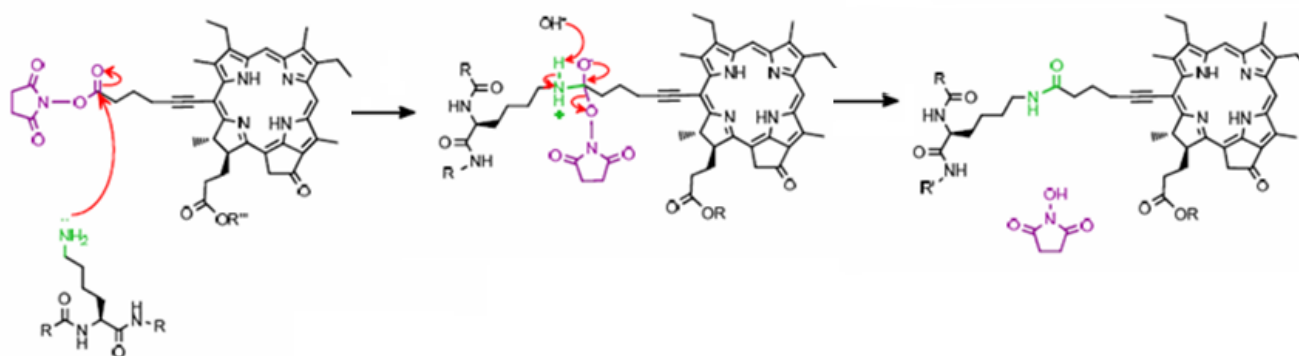
Before conjugation the PS have to be chemically modified with the NHS ester group, this is formed away from the central ring by modification of the carboxylate group on the propionic acid side chain of each PS (Stamati et al., 2010). NHS ester derivatives of the PS to be investigated were isolated as purified dry solids to be later reconstituted into DMSO and added to the conjugation reaction as opposed to generation of the NHS *in situ* during the reaction. An NHS ester will react with nucleophiles with the release of the NHS leaving group to form an acylated product. Its reaction with primary (RNH_2) and secondary (R_2NH) amines creates stable amide and imide linkages respectively. In respect to protein; the reactive groups for an NHS ester are the primary α -amine at the N terminus and the primary ϵ -amine in the side chain of lysine residues. The amide bonds formed from this reaction are very stable, it is amide bonds that link the individual amino acids together in endogenous protein. (Hermanson, 2008)

It is worth noting that the NHS ester can also react with other nucleophiles on the protein surface including the nucleophiles found within sulfhydryl (SH) or hydroxyl (OH) groups, however this will not yield stable conjugates as the bonds formed are thioesters or ester linkages respectively both of which are hydrolysed and broken down in aqueous environments or exchanged in the presence of reactive amine nucleophiles. It is also possible for the ring nitrogens on histidine and tryptophan to react with an NHS ester but again the linkages are predicted to hydrolyse very rapidly in an aqueous solution. Of all the nucleophiles found in proteins a list of the strongest and most reactive nucleophiles to the weakest is as follows; the sulfhydryl group of cysteine, particularly in the ionised, thiolate form, the lysines ϵ amine and N

terminal α amine groups in their uncharged, un-protonated forms, the secondary amines in the histidine imidazolyl groups or tryptophan indol rings, then the guanidino amines of arginine residues and finally the oxygen containing ionisable groups including the α -carboxylate at the C-terminal, the β -carboxyl of aspartic acid, the γ -carboxyl of glutamic acid, and the phenolate of tyrosine residues (Hermanson, 2008). The ionisation state of these groups is very important and will change NHS reactivity dramatically. In respect to lysine, it is the un-protonated / un-ionised form that is reactive and as pH increases toward and over the pK_a of this group this reactive un-protonated form becomes more prevalent. The pK_a of the ϵ -amine of lysine is stated as being 9.3-9.5 and so increasing the pH of the conjugation reaction toward or above this should help drive it forward, although the micro-environmental effects occurring within the proteins can effect lysine pK_a , meaning even at neutral pH this reaction can, and has been shown to, progress effectively. In terms of competing reactions from other more or similarly reactive nucleophiles for the NHS ester, histidines imidazolyl nitrogen has a predicted pK_a of 6.7-7.1 so will be more reactive at pH above this, cysteines sulfhydryl has a predicted pK_a of 8.8-9.1 so will also be more reactive at pH above this, and the Tryptophan indole ring isn't ionisable at physiological pH so is always in its reactive uncharged form. (Hermanson, 2008)

Multiple factors other than the pK_a must be considered when predicting the reactivity and selectivity of the reaction, in the case of the NHS ester this has been studied and discussed elsewhere (Anjaneyulu and Staros, 1987; Cuatrecasas and Parikh, 1972; Mädler et al., 2009). However it could be of interest to carry out competition experiments with the isolated reactive groups as done by Alonso and colleagues with a similar reactive group the maleimide group (Alonso et al., 2010). This reactive group can also be used in PIC preparation, and can exhibit potential conjugation to both protein thiol and amine groups, the experiments carried out by this lab ensured they could redirect antibody conjugation to the preferred sites, in this case the thiols. Another issue also worth consideration is the spontaneous hydrolysis of the NHS ester and its subsequent inactivation. In aqueous physiological conditions the half life of an NHS ester group is predicted to be in the order of hours and will decrease as the pH increases, at pH 8.6 the half life could be as short as 10 minutes (Hermanson, 2008). Therefore the pH of the conjugation mixture has to balance NHS ester hydrolysis and lysine de-protonation.

Figure 41 : Chosen conjugation technique for our system.



Anticipated mechanism for the formation of an amide bond between an NHS ester on a PPa molecule and a primary amine on a lysine amino acid side chain. The lysine is de-protonated and acts as the nucleophiles for the NHS ester forming a tetrahedral intermediate state. The de-protonation of the nitrogen in this intermediate state catalyses the formation of an amide bond and release of the leaving group NHS. Adapted and re-produced with the permission of Dr. Ioanna Stamati (Imperial College London).

5.1.4 Photo-immunoconjugates; progression of the field.

As discussed in the introduction (1.5.3), despite the initial development of photo-immunoconjugates (PICs) thirty years ago, not one has undergone clinical evaluation. Some of the problems seen with PIC preparation are detailed below through a description of how various labs have progressed PIC production and the problems they each encountered.

The high loading of hydrophobic PS can cause an antibody to aggregate and become insoluble in aqueous solution. This was shown in initial work carried out under Van Dongen (Vrouenraets et al., 2002, 2001, 2000, 1999) in which they produced direct amide linkage conjugations between various internalising and non internalising IgG antibodies and various PS of different solubilities; including the hydrophobic chlorin mTHPC (the active drug in the commercial product FoscanTM) and the hydrophilic tetrasulfonated aluminium phthalocyanine AlPcS₄. For all the PS used they found conjugation efficiencies above 2-3 molecules of PS per Ab was limited by aggregation of the IgG and a subsequent loss during purification. When tested *in vitro* the results showed that generally the more hydrophobic PS showed lower or equal uptake and toxicity when conjugated compared to its comparable free PS and the more hydrophilic PS

showed equal or higher uptake and toxicity conjugated than their comparable free PS. *In vivo* results showed selective tumour localisation of the PICs but for nearly all the conjugate combinations a higher coupling ratio would clear faster and/or was taken up into the tumour less, liver accumulation of PS was also seen to increase, especially with hydrophobic PS.

The changes seen above in *in vivo* antibody bio-distribution upon conjugation of PS was also seen by other labs: PIC production carried out under Pèlegri (Carcenac et al., 2001, 1999; Folli et al., 1994, 1992; Pèlegri et al., 1991) achieved much greater conjugation efficiencies between the same hydrophilic PS AIPcS4 Van Dongen used above, and IgG antibodies against either CEA (non internalising) or HER2 (internalising), again the linker was via an amide bond. With a FITC conjugate to the same anti CEA IgG (non internalising) they tested ratios of between 0 and 19 FITC per IgG and showed when conjugation was below 10 FITC per IgG it affected neither tumour localisation or tissue bio-distribution but when above 10 both half life and tumour localisation fell dramatically. When the same antibody was conjugated to PS, they produced conjugates with ratios of 5, 12 and 16 PS per IgG and showed that compared to the free IgG all conjugates retained their tumour retention and specificity *in vivo*. However when studied by *in vitro* assay, they showed no enhanced cytotoxicity over a similar conjugate with an irrelevant antigen. *In vitro* they also compared uptake and toxicity of both the non-internalising (CEA) and internalizing (HER 2) antigen with similar PS conjugates of between 6-9 PS per IgG. They engineered a cell line to express equimolar amounts of each antigen on the surface and showed much higher toxicities could be obtained with the internalising conjugate against the non-internalising conjugate. Both groups mentioned above have subsequently moved onto different ADCs with payloads other than PS.

In the ideal PIC PS will acquire targeting and macromolecular blood clearance and the antibody will acquire cytotoxicity. However there can be other changes in the PS caused by the proximity of antibody and changes in the antibody by the proximity of PS. These changes are in particular seen with highly hydrophobic PS and/or smaller Ab fragments, these changes are what drive the balance between highly loaded highly toxic Ab fragments and the loading of only a few well spaced PS to ensure more effective retention of antibody and PS functionality. Overloading of PS onto an antibody will cause a loss of PIC toxicity, most often caused by a loss of Ab

binding to antigen, however it can also be caused by a loss of PS photo efficiency due to quenching by the Ab or the other conjugated PS molecules.

The very first conjugates produced under the group of Levy (Mew et al., 1985, 1983; Steele et al., 1988) between HPD (PhotofrinTM) and various IgG molecules produced conjugates of high loading ratios, although this was difficult to confirm accurately because HPD is a mixture of oligomers, but if the oligomer is assumed to be on average 5 units long the ratios would be around 5 – 27 PS per IgG. Interestingly even in this very first paper they predicted they may have high levels of non-covalently bound PS within the conjugate, the group later stated that these conjugates made with HPD suffered from poor reproducibility and significant loss of antibody binding before changing to preloading of a PVA carrier molecules with the PS benzoporphyrin derivative monoacid (BPD-MA / Verteporfin / VisudyneTM). Using this technique they could load up to 25 PS molecules per carrier and could load 1 carrier per IgG (Jiang et al., 1992, 1991, 1990). Unfortunately they also showed these conjugates included significant levels of non-covalently bound PS and conjugation of more than one carrier was limited due to extensive cross linking of the Ab under the conjugation conditions used. *In vitro* testing of the compounds showed killing of cells not expressing the target antigens, a sign of non-covalently bound PS, and when a conjugated irrelevant IgG was used in the same cell line, although toxicity was lower it was too significant to disregard.

Even in these early papers the problem of hydrophobic PS forming non-covalent interactions with the antibody or itself meant a pure PIC was hard to obtain. This is a problem for the future application of any successful therapies if success is in part more due to this non-covalent PS dissociating *in vivo* and less due to the PIC itself.

The Hasan group initially studied the conjugation of the PS chlorin e6 to a shedable non-internalising IgG against CA 125, a glycoprotein over expressed on 85% of non-mucinous epithelial ovarian carcinomas. The conjugation was carried out via pre loading of a PGA linker. Loading ratios were high with 1-2 linkers per IgG and so 24-40 PS per IgG in total. (Goff et al., 1996, 1994, 1991). However with these conjugates there was evidence of cross linking and the formation of a mixture of products, along with evidence for significant levels of non-covalently

bound PS. Additionally some loss of binding was seen when compared to IgG alone, despite this they did show some specificity and improved toxicity over free PS *in vitro* and when tested *in vivo* they showed lower toxicity and higher specificity for tumour accumulation than for that of the free PS. Higher dosage levels however induced fatal side effects but use of a fractionated light delivery did something to improve survival rates without having to increase PIC dose.

Their work then moved on to try and increase tumour uptake by the use of a smaller fragment version of the same antibody (OC125), a F(ab')₂ fragment. The chlorin e6 was now conjugated to a new poly-L-lysine linker prior to its conjugation to the antibody. Conjugation ratios achieved were around 15-20 PS per Ab. An additional factor was introduced in that the linker could be modified to bear a positive or negative charge, shown to affect intracellular localisation (cationic was vesicular and anionic was diffuse) and cellular uptake (up to 17 times more for cationic vs. anionic and un-conjugated antibody). (Duska et al., 1997; Hamblin et al., 1996; Molpus et al., 2000). *In vitro* the cationic conjugates showed good specificity and higher uptake than all other PICs and controls, cytotoxicity was also respectively higher even when compared to the cationic conjugate of the full IgG molecule. Binding to cells expressing antigen was also maintained relative to unmodified Ab. *In vivo* although they did show some improvement in survival times, and despite the fact difficulties such as reticulo-endothelial uptake and extravasation were removed because the PIC was injected intraperitoneally rather than intravenously, these PICs still had a problem with non specific tissue toxicity. Despite this cationic PIC were seen to deliver more PS to the tumour than any other species at both time points, overall tumour to normal tissue ratios however were similar to PS conjugated to a non specific IgG, alongside a marked separation of Ab and PS post injection this suggests high levels of non-covalently bound PS.

They then changed the antigen they were targeting to epCAM, this time an internalising antigen. They conjugated chlorin e6 against this full IgG molecule but limited it to an extent of 4-5 PS per lysine linker molecules (anionic or cationic) and only 1 (cationic) or 2 (anionic) linkers per IgG. (Del Governatore et al., 2000a, 2000b; Hamblin et al., 2000). Again the cationic conjugate showed greater uptake *in vitro* but there was evidence for a loss in native antigen binding and evidence for it not being antigen-dependent uptake. Internal localisation showed

similar but not identical patterns between un-conjugated IgG, anionic PIC and cationic PIC, and data this time suggested the Ab and PS stayed bound together. *In vitro* toxicity was shown to be greatest in cationic PIC but still significant toxicity was still seen with irrelevant antigen PICs. *In vivo* intravenous injection showed greatest tumour uptake was seen with the anionic PS, the cationic PS accumulating highly in the lungs, both accumulated highly in the liver. Treatment experiments showed only a small increase in survival over free alongside significant evidence for collateral liver damage.

More recent work from this group has focused back on the direct coupling of the PS to the Ab and a move back to full size IgG (Savellano and Hasan, 2005, 2003; Soukos et al., 2001) (Soukos et al., 2001). More recently they conjugated chlorin e6 and the non toxic dye Cy5.5 to Cetuximab (ErbixTM); a chimeric anti-EGFR IgG licensed for the treatment of metastatic colorectal and head and neck cancer. The initial work with chlorin e6 and Cy5.5 was designed to be used as non invasive pre-diagnostic treatment that could be used for detection (Cy5.5) and subsequent removal (chlorin e6) of pre cancerous cells for oral squamous cell carcinoma. They used the same conjugation reaction as used in this thesis and created conjugates with 4.8 chlorin e6 per IgG and 2.1 Cy5.5 per IgG, stating that for chlorin e6 if the ratio was carried beyond 5 the molecules aggregated and could not be used. When tested in a hamster pouch cheek model fluorescent levels of Cy5.5 dye correlated with disease stage when an irrelevant conjugate (BSA Cy5.5) did not, they then went in with the PDT PIC and irradiated, when re probed with the Cy5.5 PIC they saw lower levels in the irradiated area compared to both dark and normal control areas. This work shows despite relatively low level PS conjugation, levels were sufficient for both detection and induction of cell damage, although they did have to wait significant amounts of time (days) for the highest tumour to normal tissue retention levels.

Later work (Savellano and Hasan, 2005, 2003) used a different PS, verteporfin, with the same Ab and the emphasis of this work was to create water soluble PICs that are free of any aggregates and non covalently bound material. They made a few adaptations to the conjugation method but as before it was NHS ester/lysine ϵ -amine mediated as in this thesis, they first PEGylated the Ab with 2 PEG chains (via the same method) to improve PIC solubility, if not PEGylated then when they tried to transfer the conjugated PICs to 100% aqueous solution they

would precipitate out of solution. The Ab was stated as stable in up to 50% DMSO but it aggregated and denatured at 60%. At 40% DMSO verteporfin was shown to be primarily monomeric via spectral methods, because of this they carried out the conjugation and purification in a 50% DMSO in aqueous solvent system to prevent aggregation and NCB. The PIC conjugation ratios achieved by this method were 7-10 verteporfin with 2 PEG per Ab, if they achieved more than 11 they lost Ab binding and yields dropped during purification, less than 7 saw a loss in toxicity. They achieved <5% non covalently bound PS and an *in vitro* toxicity assay showed specificity for the target antigen and cell death that could be almost completely competed with free IgG showing its reliance on antigen dependant uptake. However the light dose needed was much higher than that needed for free un-conjugated PS (PIC needed around 20J/cm²) and to obtain equivalent uptake levels to free PS the PIC had to be incubated for 40hours (vs. 4hrs for the free PS). They showed this was due to loss of photophysical efficiency with the PIC when compared to predicted levels of free verteporfin.

The success of PIC therapy when non-covalently bound PS is deliberately minimised and when PS loading is reduced to a level that ensures almost complete Ab binding retention, is shown best by the work of Boyle and Neri, this work shows that a PIC need not be excessively conjugated or internalising to produce some impressive results.

The Boyle labs first PIC paper; (Sutton et al., 2002), put primary focus on the development of synthetic PS specifically for PIC production, they synthesised a panel of porphyrin based PS, all with an isothiocyanate reactive group for conjugation. Like an NHS ester, isothiocyanate also reacts with protein nucleophiles such as ϵ -amine on lysine but creates a stable isothiourea linkage instead of an amide bond. They tried to identify structural features that would minimise non-covalent binding and studied its effect on not just SDS PAGE gel but also with preferential binding experiments, like those carried out with BSA within our own lab by Kuimova (Kuimova et al., 2007). Of particular relevance is that they saw free PS transfer from the antibody was especially rapid and efficient onto whole HeLa cells, reinforcing the emphasis of why the non-covalently bound PS should be as low as possible in the final PIC, if present at all. One of the results in this paper also supports the hypothesis of the work in this thesis in that the PS that were more water soluble showed a higher % removal of unbound PS by size exclusion

chromatography making the final PIC purer. After this study they chose a few different PS, many with 1 or 3 positively charged Methyl-pyridinium groups, to create and test a number of different PICs (Hudson et al., 2005; Malatesti et al., 2006; Smith et al., 2011; Staneloudi et al., 2007). Two PS frequently under investigation were a PS with 3 dihydroxyl phenyl groups and a PS with 3 positively charged Methyl-pyridinium groups. With IgG, Ab conjugation ratios were similar for both PS, around 1.5-2.5 PS per IgG, although during conjugation if the amount of PS used in excess with the dihydroxyl PS (the more hydrophobic PS) was increased, it would cause a decrease in the final conjugation ratios - thought to be due to a loss in solubility of the more highly conjugated PIC and its loss upon purification. Increased ratios of the pyridinium PS to Ab (the more hydrophilic PS) increased conjugation efficiencies as expected. No loss of antigen binding on immobilised sepharose was seen for these PICs however a small loss for all PICs was seen with native cell expressed antigen on flow cytometry. *In vivo* all dihydroxyl IgG PIC showed equivalent tumour retention to the free IgG, whereas the pyridinium PS showed that PIC with increased conjugation ratios caused a correlated decrease in tumour uptake, though to be due to increased blood clearance. The same PS were also conjugated to scFv antibody fragments and the conjugation ratio increased for the hydroxyl PS; 4-20 PS were found per scFv but all PIC completely lost their binding affinity to antigen. For the pyridinium PS ratios were 0.67-1.2, lower in a molar sense than for IgG but in terms of mass, higher. These conjugates all retained effective binding efficiency. Nevertheless, despite some success in *in vitro* cell kills they saw significant non specific toxicity with both IgG and scFv conjugates with various IgG both internalising and non internalising.

The Neri group were also independently working on PICs, (Birchler et al., 1999; Fabbrini et al., 2006; Trachsel et al., 2007) They were using a successful and well studied, high affinity antibody in its SIP or scFv fragment formats. The antibody is called L-19 and it is a vascular targeted non internalising Ab which targets EDB fibronectin, a splice variant expressed during angiogenesis. They were conjugating the non toxic but fluorescent Cy3 or the toxic but weakly fluorescent Sn-chlorin e6. Conjugation ratios were around 1.5 PS per scFv or 2 PS per SIP monomer (4 per SIP dimer), conjugations above these ratios was claimed to cause a loss in binding efficiency. For these PIC conjugation was carried out at a ratio so that antigen binding was maintained above 80%, all conjugates were purified via size exclusion column and

specifically shown to be free of any non-covalently bound material. Experiments carried out in models of ocular vascular growth (scFv PIC) and cancer (SIP PIC) both showed specific targeting, in the case of ocular vascular growth they showed effective vessel occlusion and in the case of the tumour models they showed significant tumour growth delay and even cures in a number of the mice. However in both models when mice were allowed to live past the treatments end, the blood vessels or the cancer would grow back.

The two labs under Neri and Boyle came together for their most recent work on PICs, (Alonso et al., 2010; Palumbo et al., 2011) in which they created PICs using the L-19 SIP antibody from the Neri lab and the PS with 3 Methyl-pyridinium groups from the Boyle lab. These conjugates were made in two different ways but both showed promising results when tested *in vitro* or *in vivo*:

Alonso and colleagues (Alonso et al., 2010) used a site specific conjugation of the PS, in this case derived with a maleimide group, to two C terminal cystines residues, one genetically engineered onto the end of each SIP monomer. Tertiary folding of the antibody means these two groups are close together in space in the final molecule so an awareness of any possible PS quenching was important. As a tool to investigate this they changed the length of linker between the porphyrin and maleimide. One PS was linked directly, another had a short hydrocarbon spacer (CH₂)₄, and another had a longer hydrocarbon spacer (OCH₂CH₂)₇, this longest linker was also designed like a PEG to increase hydrophilicity a bit further. Conjugation was shown to go to completion by mass spectrometry analysis i.e. one PS per SIP monomer, they also showed conjugation did not affect SIP dimerisation or its ability binding to antigen. The conjugates were clean and showed no non-covalently bound PS on SDS PAGE. Photo-toxicity *in vitro* showed the PIC with the longest linker was significantly more toxic (factor of 10) then the mid length linker and this in turn was more toxic then the direct PS. Specificity was maintained and there was lack of any killing in a cell line not expressing the antigen. Interestingly, and an argument for the influence of quenching affecting spectral conjugation ratios, despite all PS showing on mass spectrometry that a conjugation ratio of 2 was obtained, spectral quantification results give ratios 0.76, 0.9, and 1.75 increasing with linker length, re-enforcing the idea of quenching between the PS with smaller linkers.

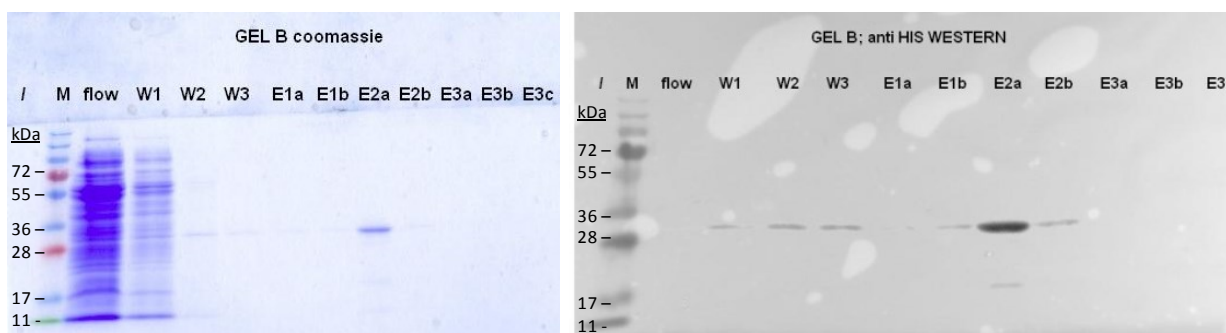
Palumbo and colleagues (Palumbo et al., 2011) also used L-19 SIP and a 3 Methyl-pyridinium group PS, but instead used the same conjugation technique as presented in this thesis, via an NHS ester (no spacer) to lysine ϵ -amines. Their conjugates were shown by mass spectrometry to present as a poisson distribution over a number of molecular masses with an average of 3 PS per monomeric SIP (6 per dimer). The PIC could still form dimers, had no free PS, and binding to immobilised antigen on sepharose was over 90% retained. *In vivo* experiments showed the PIC retained its highly selective tumour vascular targeting compared to the free Ab. And in therapy experiments (in which they added 10 fold less PS within the PIC compared to the standard treatment dose for free PS), PICs showed a very strong anti-cancer response: Complete tumour eradication was shown alongside complete and prolonged cures in 4/4 mice treated. Comparatively an irrelevant SIP-PIC, un-irradiated PIC or un-conjugated L19 showed no impairment in tumour growth. Upon closer inspection tumour histology showed disruption of tumour blood vessels, extensive haemorrhage, oedema and widespread cell death. Of additional interest is that when they carried this out in mice deficient in NK cells; only transient tumour growth arrest was seen, implying an immune element was important to the complete cures.

5.2 Results

5.2.1 Expression of antibody fragment C6.5-k

The full length scFv antibody fragment C6.5-k with N terminal secretion sequence and C terminal His and Myc tags was expressed in HB2151 E.Coli bacterial cells and purified from bacterial supernatant via its Myc Tag. See 2.1.5.1. Wash and elution fractions from this purification process separated via SDS PAGE are shown in Figure 42. A single band of the predicted size (29185 Da), with a small amount of what is thought to be degradation products, is shown in both coomassie stained gels and in gels transferred to nitrocellulose and blotted with a peroxidase-conjugated anti-poly Histidine IgG.

Figure 42 : Expression of antibody fragment C6.5-k



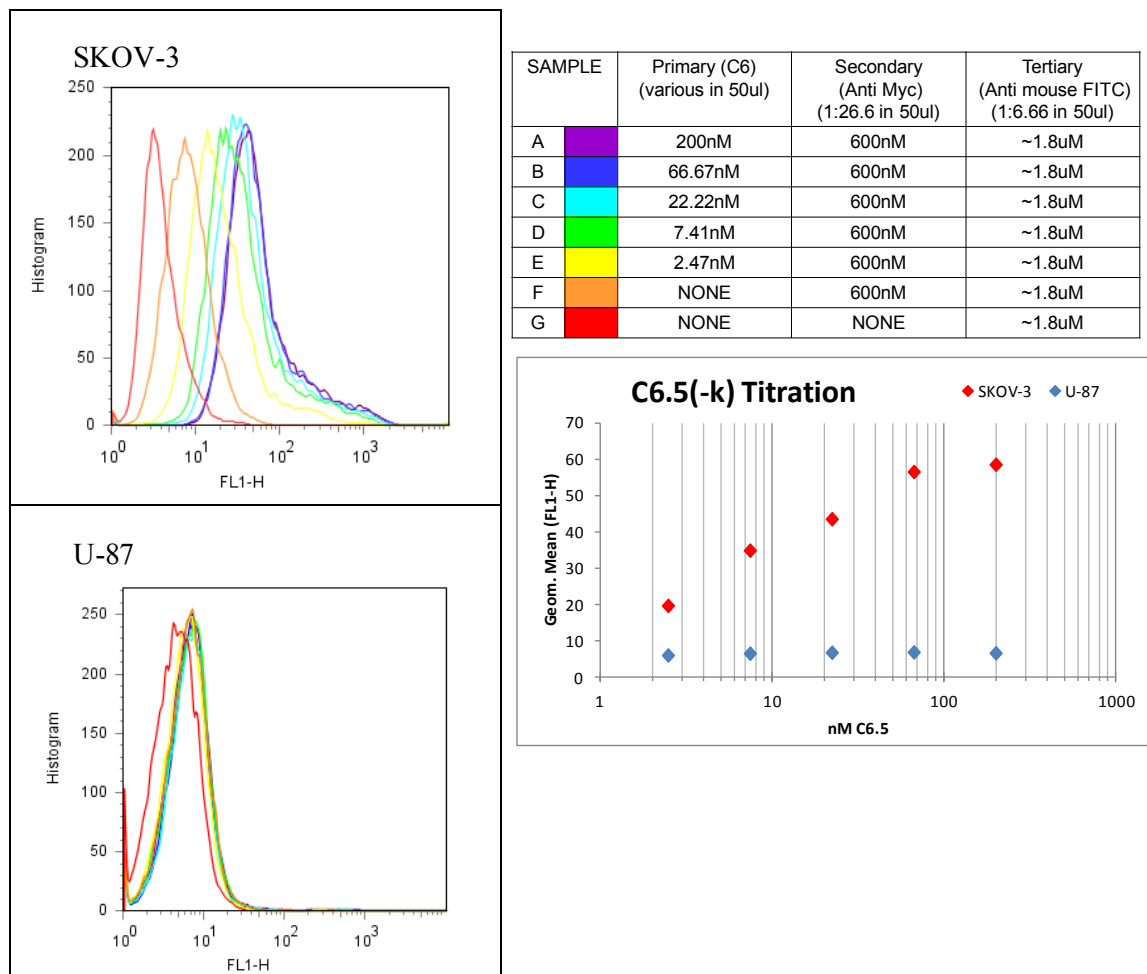
C6.5-k with C-terminal His and Myc tag was expressed in HB2151 E.Coli bacterial cells and purified from bacterial supernatant via its Myc Tag. Wash and elution fractions from this purification process are shown separated via SDS PAGE. One gel (LEFT) is stained with coomassie and another (RIGHT) is transferred to nitrocellulose and blotted against the His Tag. Flow = flow-through from purification column, W = washes with PBS, E1-3 = glycine washes with decreasing pH for elution of antibody.

5.2.1.1 Binding of antibody fragment to native antigen

To test the ability of the C6.5-k antibody fragment to specifically recognise native cell surface expressed antigen, in this case HER2, a binding titration experiment was carried out. This experiment was also required to identify the concentration of C6.5-k needed to show a significant but not saturated fluorescent shift upon binding, important when carrying out later flow cytometry (FC) experiments to investigate PIC binding. Two cell lines SKOV-3 and U-87, were chosen to act as a positive and negative control for HER2 expression respectively. Suspension preparations of these cells were incubated with C6.5-k over a range of concentrations chosen considering published expression levels, cell number and staining volume. It is commonly stated in the literature that breast cancers can have up to 2 million receptors expressed at the tumour cell surface, the reference trail for this figure normally leads back to a paper by Kallioniemi et al (Kallioniemi et al., 1992) however that figure is not contained within this paper, which doesn't even look at protein expression level but gene copy number. Despite this and for the purpose of setting up this experiment it gave a figure to start from, from this a average maximum number of receptors expressed from all the cells in the sample could be calculated and therefore roughly the number of scFv molecules to have in the primary solution to create a suitable range that should cover receptor saturation.

Figure 43 shows the data from this flow cytometry experiment and the calculated genomic mean of each result is plotted in a graph over initial C6.5-k concentration. The data shows our C6.5-k binds specifically to SKOV-3 cells and not U-87. In this system saturation of signal occurs around 60nM (SKOV-3) with low levels of non specific binding (U-87). This dose will be used for all further work with secondary and tertiary at 3 time molar excess over each other respectively.

Figure 43 : C6.5-k binding titration assay.



Two cell lines SKOV-3 and U-87 were chosen to act as a positive and negative control for HER 2 expression respectively. Suspension preparations of these cells were incubated with a range of C6.5-k concentrations and then relevant secondary and tertiary antibodies to allow detection of binding via flow cytometry, see 2.1.5.5 for method. (C6.5-k shortened to C6 in figure).

5.2.2 Homology modelling of C6.5-k.

Antibodies are made up of linked immunoglobulin-like domains see 1.5.1. High levels of homology between the amino acid sequence of the various antibody domain families and isotypes allows the reliable modelling of the tertiary structure of our antibody (Ab) fragment against the known structure of other antibodies. It is clear that certain areas of the antibody will have less successful modelling, in particular the CDR loops. For one; these are the areas that will exhibit most structural differences from other Ab and for two; even in a similar Ab these are solvent accessible flexible loop domains so would be harder to get reliable crystal structures for. However the modelling carried out for this thesis did aim to look at changes in atom orientation but more generally at the spacing of lysines around the whole structure, for this it should be reliable. For the prediction of the three-dimensional (3D) structure of C6.5-k from its amino acid sequence a protein fold recognition server called PHYRE was used, see 2.1.3.4 for an overview of the process and PDB files used to align the final model. Alignment to an experimentally solved endogenous IgG antibody structure ensured the orientation of the final model was optimal; however scFv molecules are expressed independent of the rest of the IgG that might provide some structural influence on domain orientation as well as the additional structural influence of an amino acid linker. Maintained antigen binding can be a good indication that the variable light and heavy chain are interacting correctly as the binding site for antigen recognition is formed by a mixing of CDR loops from both domains.

5.2.2.1 Prediction of spacial orientation and reactivity of lysines on C6.5-k

The images shown in Figure 44 show the modelled C6.5-k antibody, presented as a space fill model from 4 angles as well as a ribbon and stick model. The model has been coloured to show; Red = heavy chain framework regions, Pink = light chain framework regions, orange = CDR for both heavy and light chains, yellow = lysines from both heavy and light chains. From this model the lysines residues seem well-spaced around the molecule, this should help avoid PS self quenching. It should be noted however that one of the Lysine residues is within the center of the CDR region (Lysine 59) and this could cause problems with steric hindrance of antigen recognition if it gets conjugated by a PS.

To further investigate this model, Figure 45 shows the results for all the Lysine residues when the 3D model was put through the PROPKA server, see 2.1.3.4. Alongside is a simple annotated amino acid sequence with all lysines labelled in red and numbered in the order they appear in the amino acid sequence (N to C). PROPKA calculates the predicted pK_a values of ionisable residues (including lysines) taking into consideration extra information from the 3D structure file such as spatially neighbouring residues and solvent exposure (Bas et al., 2008). The data presented here confirms most of the lysines are 100% solvent accessible, a good sign for effective conjugation reactions. Three of the lysines however are predicted to be partially buried in the protein core, including the one in the CDR (underlined) hopefully this will be energetically less likely to get conjugated, particularly with our more water soluble PS. In terms of pK_a prediction the ϵ -amine of lysine side chain is usually stated as having a pK_a of 9.3-9.5 (Hermanson, 2008). When $pH = pK_a$ 50% of the groups will be ionised and 50% will be unionised, the nature of the Log unit means that 1 pH unit above or below the pK_a 91% of the groups will be un-protonated or protonated respectively, (in lysines case unionised or ionised respectively). The prediction by PROPKA that average antibody lysine pK_a is a pH unit higher (average value of 10.3) could have implications on conjugation efficiency.

5.2.2.2 Model Images of a fully conjugated fragment

Figure 46 models each PS in stick format next to the modelled C6.5-k, this gives an idea of the relative size and contribution to surface cover/character each PS will make. C6.5-k is shown in blue with lysine residues highlighted in pink. PPa is in Green, PS4 in orange and PS1 in red. In Figure 47 PPa molecules as shown as if conjugated to the antibody. C6.5-k is shown in space fill (orange with yellow = lysine, white = reactive ϵ -amine), where as PPa molecules are in stick format, PPa molecules 'conjugated' to exposed lysines are shown in red, and those that would be conjugated to the hypothetically buried lysines are in pink. Although orientation of PS is likely to change upon interaction with protein surface this diagram does give us an idea to how possible distribution of hydrophobic character on one side of the fully conjugated molecule could occur and how the face containing the CDR could be spared as all PPa here are shown in pink not red.

Figure 44 : Homology modelling of C6.5-k.

The prediction of the 3D structure of a C6.5-k from its amino acid sequence using a protein fold recognition server was used called PHYRE, see 2.1.3.4 Red = heavy chain framework regions, Pink = light chain framework regions, orange = CDR for both heavy and light chains, yellow = lysines from both heavy and light chains.

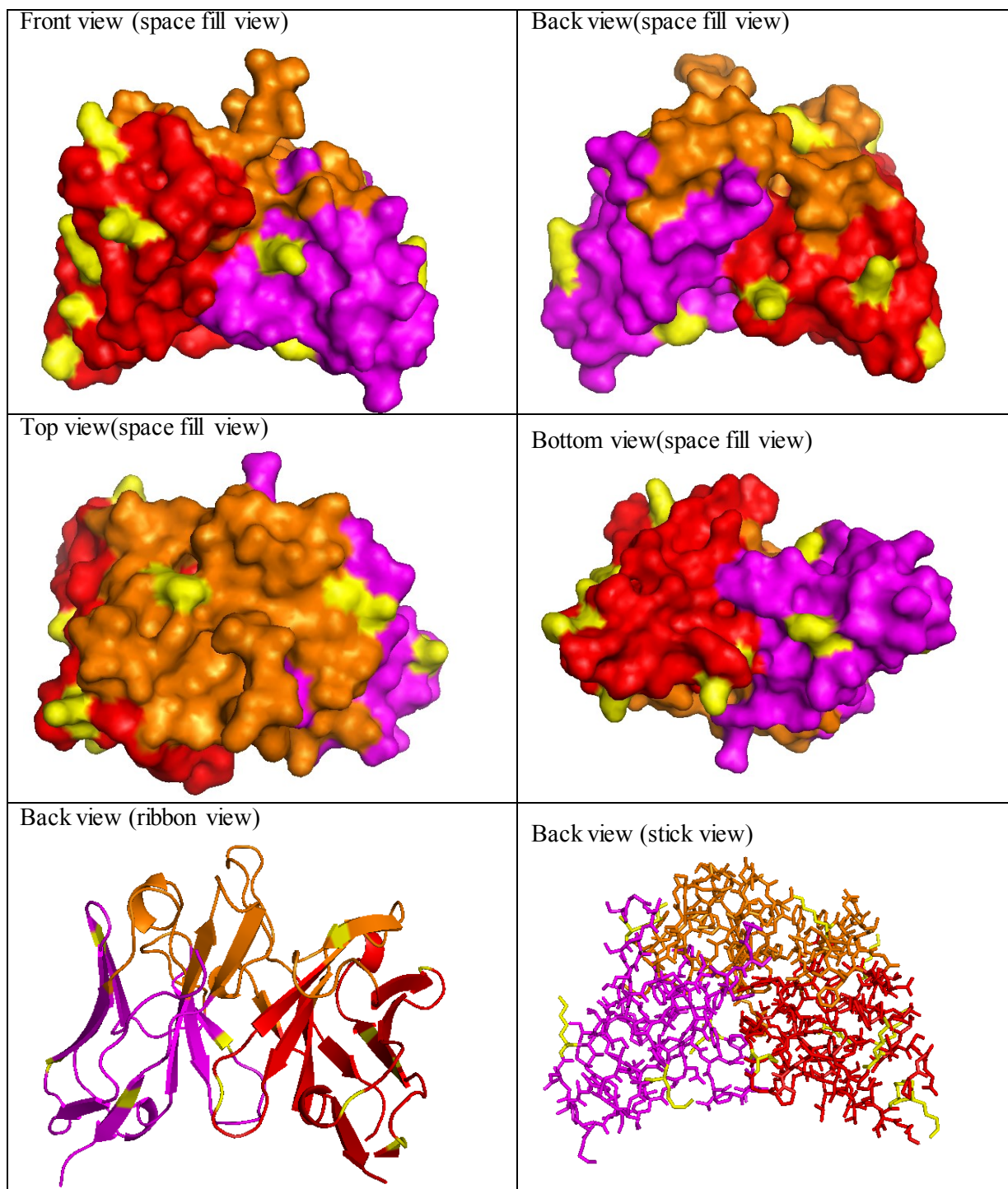


Figure 45 : Prediction of pK_a for lysines in 3D model of C6.5-k.

3D model of C6.5-k was put through the PROPKA server, see 2.1.3.4 (The underlined lysine falls in the CDR loop region). PROPKA calculates the predicted pK_a values of ionisable residues (including lysines) taking into consideration extra information from the 3D structure file such as spatially neighbouring residues and solvent exposure.

RESIDUE	pK_a	BURIED
LYS 12 A	10.45	0%
LYS 13 A	10.49	0%
LYS 19 A	10.21	0%
LYS 23 A	10.37	0%
LYS 43 A	10.43	0%
<u>LYS 59 A</u>	<u>9.81</u>	<u>35%</u>
LYS 74 A	10.46	0%
LYS 87 A	10.47	0%
LYS 161 A	10.36	0%
LYS 190 A	10.18	25%
LYS 211 A	9.03	33%
LYS 250 A	11.48	6%

A annotated amino acid sequence of C6.5-k with all lysines labelled in red and numbered in the order they appear in the amino acid sequence (N to C).

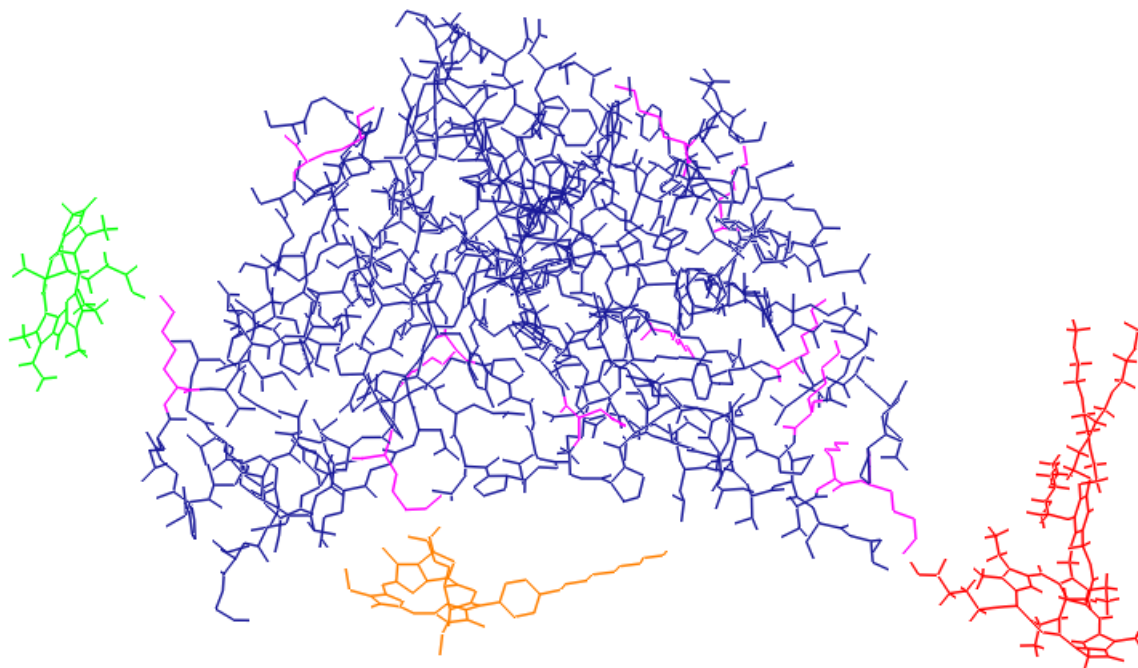
Heavy chain variable region - linker - light chain variable region - Myc tag - His Tag
(Red = lysines, Underlined = CDRs)

QVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPGDSDTKYSPSFQGGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHDVGYCSSN
CAAWPEYFQHWGQGLTVTVSSGGGSGGGSGGGSGSVLTQPPSVSAAPGQKVTISCSGSSNIGNNYSWYQQLPGTAPKLLIYGHTNRPAGVPDRFSGSKSG
TSASLAISGRSEADYYCAAWDDSLSGWVFGGGTKLTVLGAAAEQKLISEEDLNGAAHHHHHH

Numbered aa (1-278)

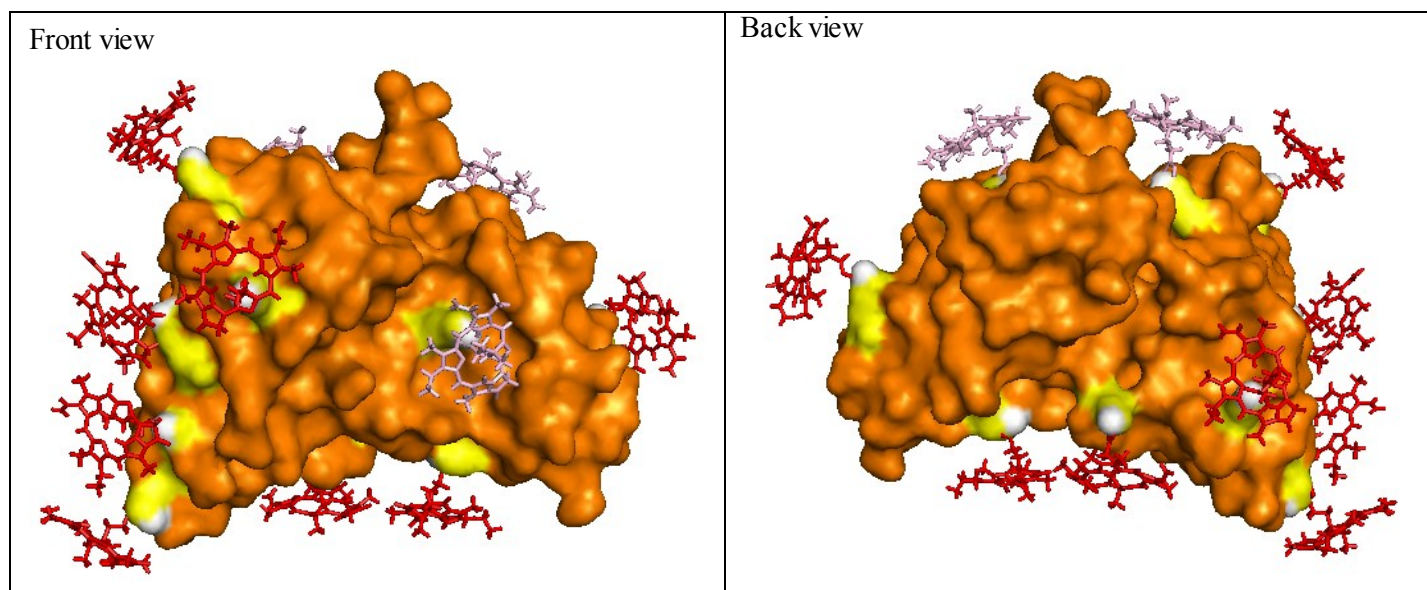
1-50)	QVQLV	QSGAE	12/13 VKKPG	19 ESLKI	23 SCKGS	GYSFT	<u>SYWIA</u>	WVRQM	43 PGKGL	EYMGL
51-100)	<u>IYPGD</u>	59 SDTKY	SPSFQ	GQVTI	74 SVDKS	VSTAY	LQWSS	87 LKPSD	SAVYF	CAR <u>HD</u>
101-150)	<u>VGYCS</u>	SSNCA	AWPEY	FQHWG	QGTIV	TVSSG	GGGSG	GGGSG	GGGSQ	SVLTQ
151-200)	PPSVS	AAPGQ	161 KVTIS	CSGSS	SNIGN	NYVSW	YQQLP	190 GTAPK	LLIYG	HTNRP
201-250)	<u>AGVPD</u>	211 RFGS	KSGTS	ASLAI	SGFRS	EDEAD	YYCAA	WDDSL	SGWVF	250 GGGTK
251-278)	LTVLG	AAAEQ	KLISE	EDLNG	AAHHH	HHH				

Figure 46 : Model images of PPa, PS1 and PS4 in relation to C6.5-k



Each PS next to the modelled C6.5-k, all in stick format. C6.5-k is shown in blue with lysine residues highlighted in pink. PPa is in Green, PS4 in orange and PS1 in red.

Figure 47 : Model images of a fully conjugated fragment



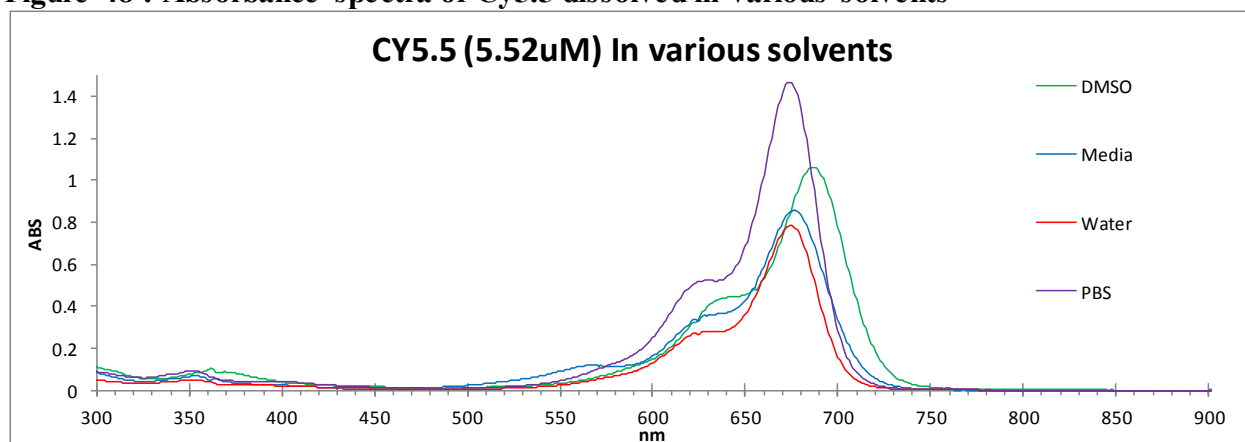
PPa molecules lined up next to each ϵ -amine of Lysines on the model of C6.5-k (yellow = lysine, white = reactive ϵ -amine), C6.5-k is shown in space fill (orange) where as PPa molecules are in stick format, PPa molecules corresponding to exposed lysines are shown in red, and corresponding to the predicted buried lysines are in pink

5.2.3 Introduction of a new dye to establish a model conjugation system.

Previous work within this laboratory had found the level of C6.5-k conjugation with activated PPa to be inadequate, this was hypothesised to be due to the insolubility of activated PPa in the conjugation reaction. In order to see if higher levels of conjugation of the C6.5-k was possible and if it could be improved by more water soluble dyes, a commercial dye was used; a highly water soluble cyanine dye Cy5.5 derivatised with a mono-activated NHS ester group (2.1.6.3). As with the PS previously (3.2.3.1) Cy5.5 was dissolved in different solvents and the extent of any shifts in absorption spectra examined, this work is shown in Figure 48 and reflects the high water solubility of this dye.

The reaction conditions previously used within this lab (Stamati et al., 2010) were changed slightly for the Cy 5.5 conjugations (see 2.1.6.5) in that concentration of C6.5-k was increased to 145 μ M, the buffer C6.5-k was dissolved in was also changed from PBS pH8 to a carbonate buffer made from 0.1M Na₂CO₃ and 0.1M NaHCO₃ to a pH of 9.4. (Delory and King, 1945) The Dye to Ab ratio was maintained at 16:1 but the addition of DMSO was increased to 10% of final volume and no additional MeCN was added to the mixture. Mixtures were added immediately and reaction was left shaking and protected from light at room temperature for 2-3 hours then left overnight at 4°C before purification.

Figure 48 : Absorbance spectra of Cy5.5 dissolved in various solvents



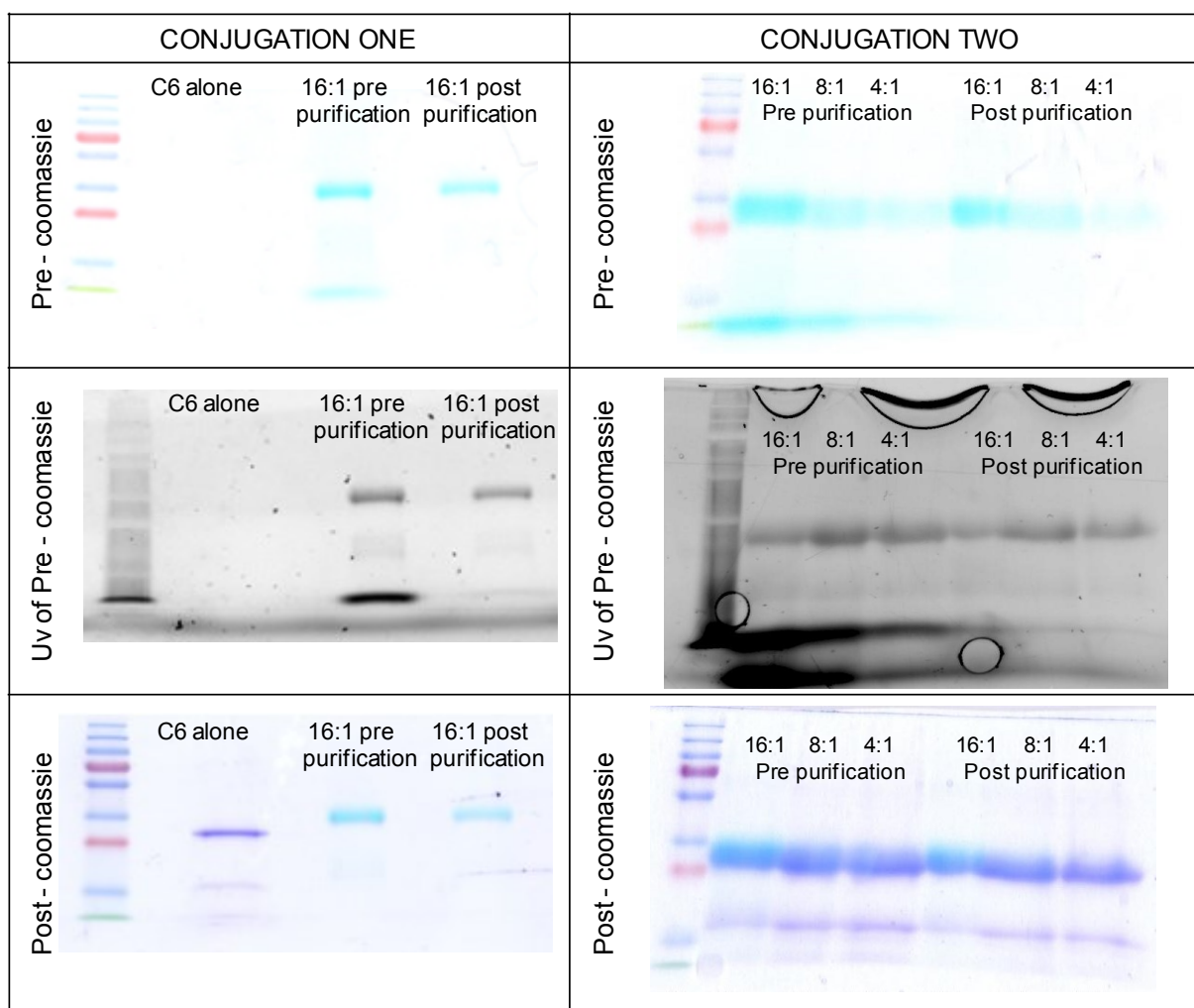
Absorbance spectra over 300nm to 900nm of Cy5.5 dissolved in de-ionised water (red), PBS(purple), DMEM media with phenol red and 10% FCS (blue) and 100% DMSO (green). Cy5.5 is at a final concentration of 5.52 μ M. Spectrums of absorbance read over a blank of the relevant solvent and normalised to absorption at 900nm.

5.2.3.1 C6.5-k conjugates with Cy5.5

Two sets of conjugation reactions were carried out between C6.5-k and Cy5.5. The first ‘conjugation one’ was a small scale experiment carried out as above and the second ‘conjugation two’ was of a larger scale in which 3 reactions were carried out in parallel with Cy5.5 at 16:1, 8:1 and 4:1 molar excess over C6.5-k. All conjugations were carried out as above and purification was achieved via repetitive washing with PBS through a 5000 MWCO polyethersulfone membrane using centrifugal pressure, progression was followed by examination of absorbance of dye at 674nm in the filtrate and was continued until level of Cy5.5 in the wash was undetectable, the final product was then concentrated back to the original volume.

Figure 49 shows the photoimmunoconjugates (PIC) before and after purification separated via SDS page gel. In the pre stained gels the light blue from the Cy5.5 can already be seen in two places, running around the predicted height for the antibody and at the dye front, the band at the dye front corresponding to unconjugated Cy5.5 is removed in the purified sample. When imaged under UV light to visualise fluorescence from the Cy5.5 and compared to the intensity of the blue in the unstained gel images it seems free dye fluoresces more than bound dye, and bound dye fluorescence can be quenched at higher conjugation ratios. When stained for protein (dark blue) the gels now show the unconjugated C6.5-k which runs at a similar place in the gel to the PIC. Interestingly the coomassie dye does not bind the PIC as effectively as free C6.5-k, perhaps due to steric hindrance of the binding site for coomassie. The PIC in ‘conjugation one’ seems to have conjugated better than the equivalent 16:1 PIC in ‘conjugation two’ and can be seen to run higher in the gel. The conditions were similar for both conjugations and this loss in efficiency could be due to hydrolysis of the Cy5.5 NHS ester, even though it was stored for only a short time at -20°C and in anhydrous DMSO. NB: during staining with coomassie free Cy5.5 leaches out of the gel, the coomassie staining solution contains acetic acid and methanol which will fix the protein by causing it to precipitate out of solution and get stuck within the gel. The same must not happen with the free dye.

Figure 49 : C6.5-k conjugates with Cy5.5.



Two sets of conjugation reactions carried out with C6.5-k and Cy5.5. The first 'conjugation one' was a small scale experiment carried out at 16:1 Cy5.5 to C6.5-k molar ratio. The second 'conjugation two' was of a larger scale and 3 conjugations were carried out in parallel with Cy5.5 at 16:1, 8:1 and 4:1 over C6.5-k. SDS PAGE gels are presented showing separation of PIC samples before and after purification alongside the predicted equivalent level of un-conjugated protein if no protein was lost during the conjugation procedure. The gels are shown un-stained, unstained and imaged under UV light and stained with coomassie. (C6.5-k shortened to C6 in figure)

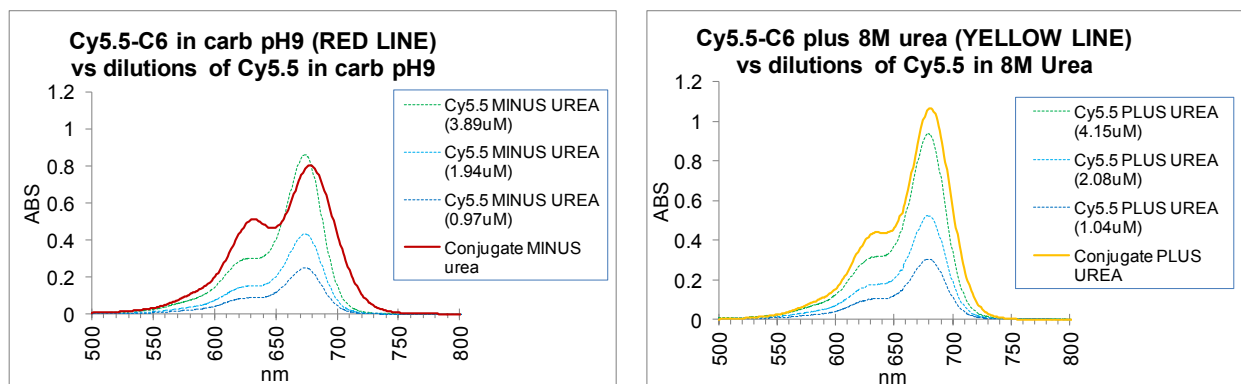
5.2.3.2 Quantification of Cy5.5 conjugates with C6.5-k

In order to quantify the conjugation efficiency of the PICs the UV spectra of the conjugate was used, see 2.1.6.6. This method to calculate conjugation ratios is seen frequently in the literature, however within our system the standard method was compromised and had to be modified: In the spectra of the PIC the dye peak had a different ratio between the maxima and its shoulder peak when compared to the free dye. This shift was hypothesised to be due to interactions between the protein and the Cy5.5 dyes now conjugated to its surface. To circumvent this PIC was denatured with urea and spectra were re read. Figure 50 shows that PIC dissolution in urea induces a return of the PIC dye spectra to that of a free dye.

Using extinction coefficients of Cy5.5 at 280nm and 674nm in urea, shown in Figure 51. Final concentration ratios of purified PIC could now be calculated by a spectral method; Conjugation one produced an average molar ratio of 10:1 (dye: protein), whereas the conjugations in Conjugation two produced final conjugation ratios of 4:1, 2:1 and 1:1 for initial conjugation ratios of 16:1, 8:1 and 4:1 respectively.

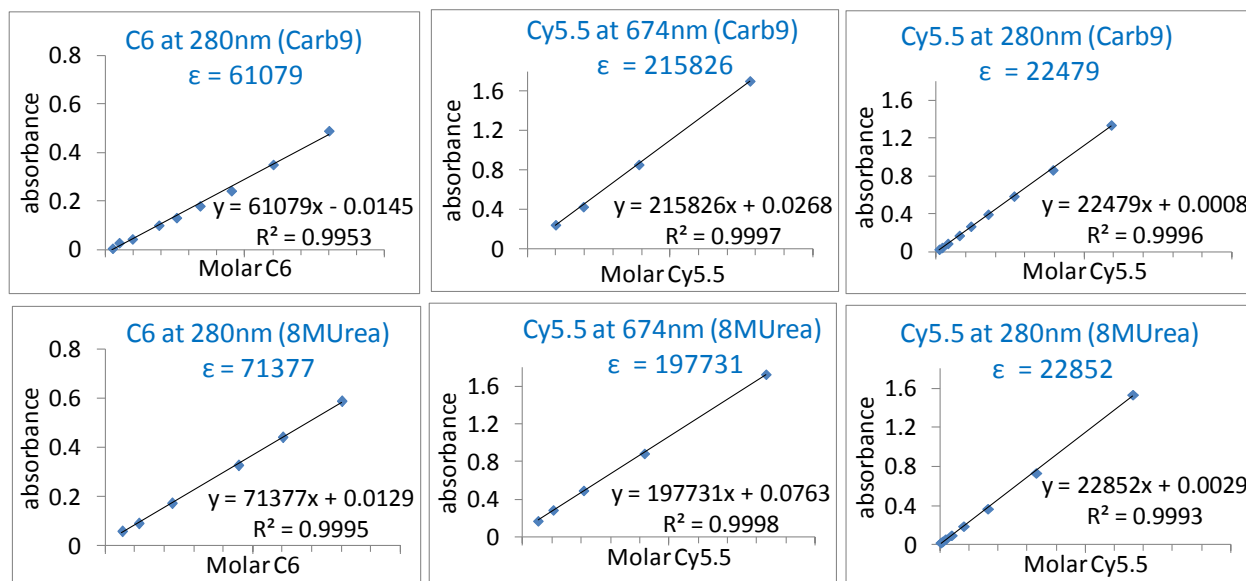
As discussed above conjugation can quench the photophysical properties of the Cy5.5. It can also affect the absorbance of residues within a protein responsible for its absorbance at 280nm; tryptophan (contribution of one residue to ϵ at 280nm = 5500), tyrosine (1490) and cysteine (125) (Thermo Scientific, 2008) so an alternative method of protein quantification was investigated. Figure 52 shows an experiment carried out to look at interference of Cy5.5 with two other methods of protein concentration determination, the Bradford assay and the BCA assay see 2.1.4.7. The BCA assay was unusable as the absorption spectra of Cy5.5 directly interferes with the spectra from the BCA. The Bradford assay spectra was also affected but would be fine if the reading was taken at a shorter wavelength. However, as was seen with coomassie staining of the SDS page gels, Bradford staining may be also be inhibited by extensive conjugation. This is because the Bradford assay and coomassie staining utilise different forms of the same protein. Different forms of coomassie exist; an cationic (red), anionic (blue) and neutral (green) form, the neutral (Bradford) and the anionic (Gel stain) species can both bind proteins but do so in different ways (Georgiou et al., 2008).

Figure 50: Spectral quantification of Cy5.5 conjugates with C6.5-k



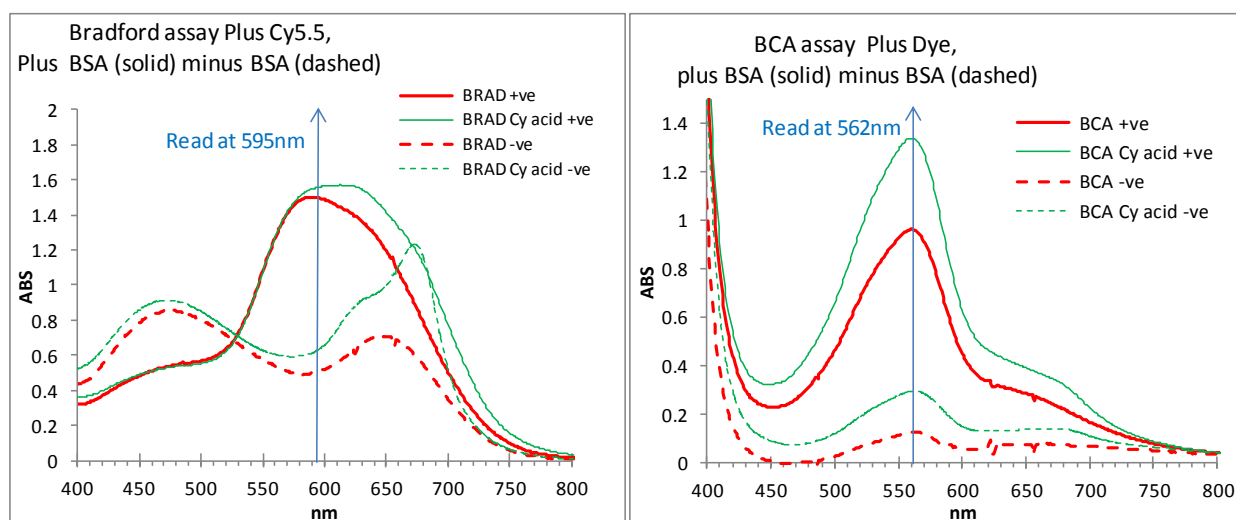
The spectra of the conjugates in their either 8M Urea or Carbonate buffer pH 9.4 overlaid with dilutions of free Cy5.5 in the same buffer. NB concentration unknown in conjugate so not directly comparable with any dilution of free Cy5.5 (C6.5-k shortened to C6 in figure)

Figure 51 : Extinction coefficients of Cy5.5 and C6.5-k in carbonate buffer or 8M urea.



Extinction coefficients for Cy5.5 were calculated by measuring different molar solutions of C6.5-k or Cy5.5 in either carbonate buffer at pH 9.4 or 8M Urea solution. (C6.5-k shortened to C6 in figure).

Figure 52 : The interference of Cy5.5 with the BCA or Bradford assay

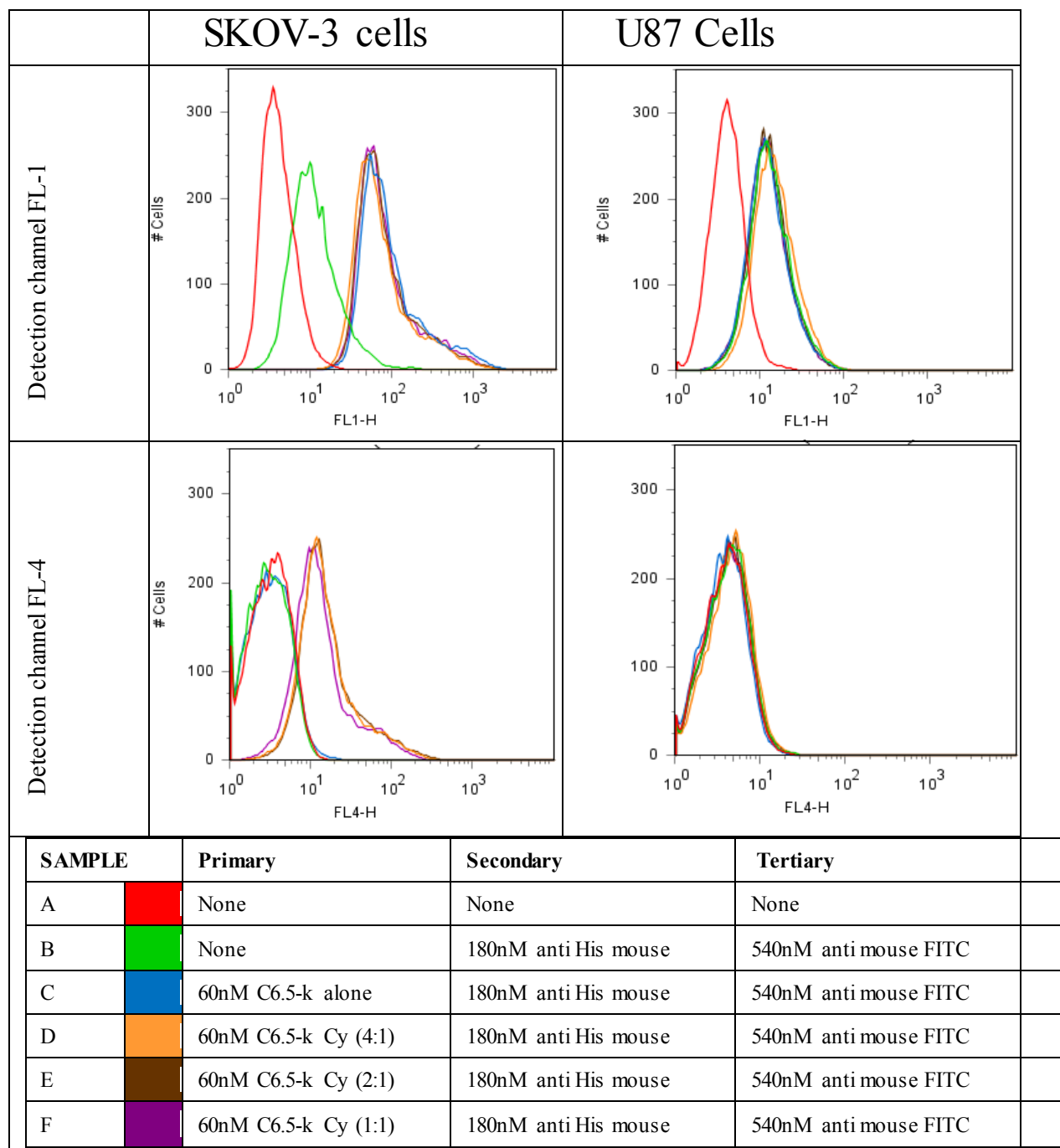


Cy5.5 diluted into either BSA in H₂O or H₂O alone. Cy5.5 was at 10 times molar excess over the protein. BCA or Bradford assay were used on these solutions as normal and absorbance spectra were read alongside solutions with no Cy5.5.

5.2.4 Binding of purified conjugates to native antigen

From 'conjugation two' presented above, three conjugates were produced with different final Cy5.5 to C6.5-k conjugation ratios: 1:1, 2:1 and 4:1. In order to see if changes in conjugation level affect the ability of the PIC to bind to antigen two cell lines SKOV-3 and U-87 were used as a positive and negative control for HER2 expression respectively and binding of PIC to these cells analysed by flow cytometry. PIC from 'conjugation one' could not be tested due to lack of material. Figure 53 presents this experiment. Fluorescent shifts that would indicate binding were monitored in two channels; the FL-1 channel (for the C6.5-k) and FL-4 channel (for Cy5.5). To the HER2 negative U-87 cells all PIC showed no binding in either channel. To the HER2 positive SKOV-3 cells all three conjugates show the same shift in the FL-1 channel as the C6.5-k only control indicating there is no loss in binding affinity for the PICs. In the FL-4 channel positive shifts are also seen but this time only for the Cy5.5 conjugated C6.5-k and not the C6.5-k alone. All shifts are similar despite the differences in the number of Cy5.5 conjugated per C6.5-k, this suggests fluorescence emission from the fluorophore is quenched above a certain conjugation level and all PIC conjugates have the same fluorescent efficiency, this was also seen on the SDS page gels.

Figure 53 : Binding of purified Cy5.5 / C6.5-k conjugates to native antigen.



Three Cy5.5 / C6.5-k PICs with final conjugation ratios as follows : 1:1 , 2:1 and 4:1 (dye: protein) were tested for native antigen binding. SKOV-3 and U-87 were used as a positive and negative controls for HER2 expression respectively. Cells were incubated with 60nM of each of the PICs and then relevant secondary and tertiary antibodies to allow detection of binding via flow cytometry, fluorescence was measured in two channels. FL- 1 to detect the C6.5-k and FL-4 to detect the Cy5.5.

5.2.5 Cellular uptake and localization of conjugates.

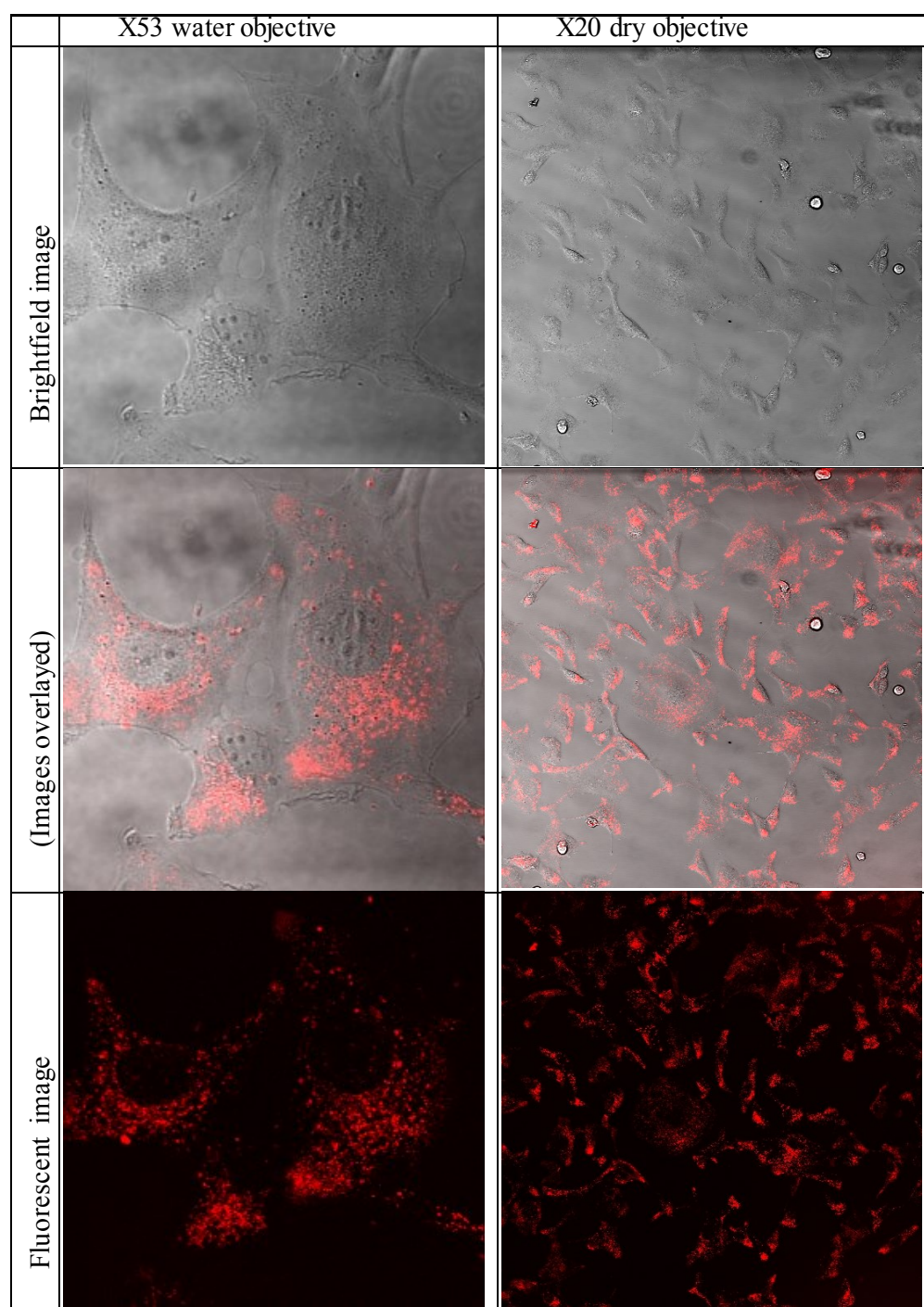
Confocal experiments were carried out with Cy5.5/C6.5-k PIC and HER2 positive SKOV-3 cells to investigate PIC intracellular localisation. The PIC with a final Cy5.5 to C6.5-k conjugation ratio of 10:1 (dye: protein) from the reaction ‘conjugation one’ above was used in this experiment and incubated with SKOV-3 cells at 2µM PIC for 2-3 hours, after this time cell were imaged directly without fixation see 2.1.4.5. Presented in Figure 54 are the images of intracellular localisation visualised by Cy5.5 fluorescence, the PIC localisation is punctuate and spread throughout the cytosol.

Further experiments to study cellular uptake of the Cy5.5/C6.5-k PIC at different time points as well as the leaching/degradation of the PIC over time were carried out with the PIC with a final Cy5.5 to C6.5-k conjugation ratio of 4:1 (dye: protein) from the reaction ‘conjugation two’ above. The results are presented in Figure 55 and the data shows a slow uptake of conjugate over 2-3 hours, after washing the PIC remains inside the cell with no evidence of any extensive leaching or degradation over the 3 hours studied. A matching dose of free Cy5.5 dye alone over 4 hours didn’t show any uptake. This matches data presented with the same antibody but conjugated to verteporfin by Kuimova and colleagues (Kuimova et al., 2007).

5.2.6 Summary of work with model conjugation

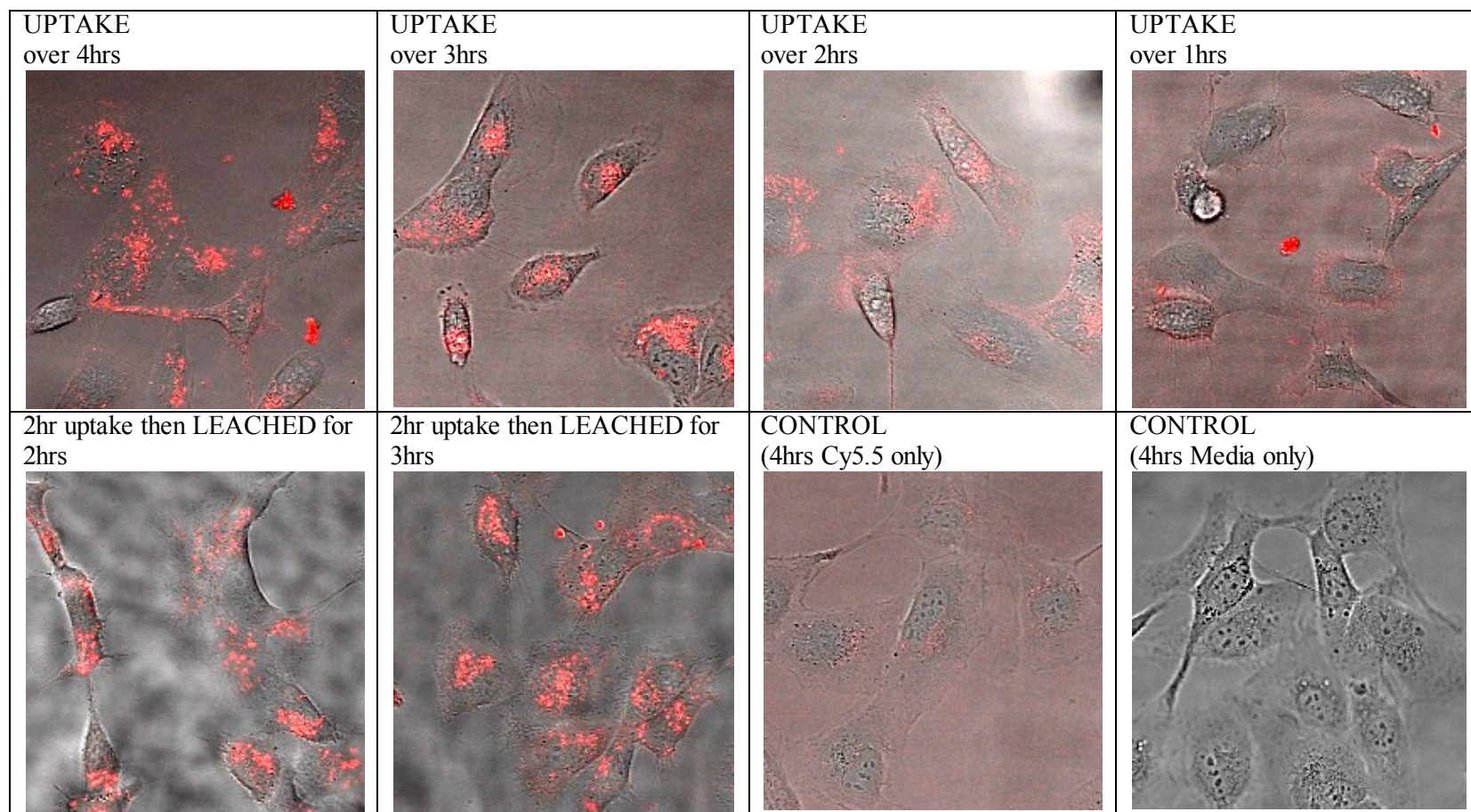
Introduction of a commercial aqueous dye to establish a model conjugation system was carried out in order to see if extensive conjugation of the C6.5-k was possible, data has shown that it is possible to conjugate 10 Cy5.5 molecules to C6.5-k, the upper limit predicted by homology modelling of the availability of reactive lysine residues on the proteins surface. PICs could be successfully purified and successfully used in assay designed to look at native antigen binding and cellular uptake and localisation.

Figure 54 : Cellular localisation of Cy5.5 / C6.5-k conjugates.



SKOV-3 cells exposed to supplemented cell culture media containing 2 μ M C6.5-k (within a Cy5.5 / C6.5-k PIC), after 2-3 hours cells washed once in PBS and media re-added and imaged. Confocal fluorescent microscopy imaging carried out under irradiation of 633nm laser at 28%, through a X20 dry objective or X53 water objective with detection channels for brightfield and fluorescence between 640-780nm.

Figure 55 : Cellular uptake and leaching of Cy5.5 / C6.5-k conjugates



UPTAKE = SKOV-3 cells exposed to supplemented cell culture media containing 2 μ M C6.5-k (within a Cy5.5 / C6.5-k PIC) for varying times before cells were imaged. LEACHING = SKOV-3 were incubated for 2 hours in supplemented cell culture media containing 2 μ M C6.5-k (within a Cy5.5 / C6.5-k PIC) then exchanged to media alone for varying times before being imaged. Confocal fluorescent microscopy imaging carried out under irradiation of 633nm laser at 20%, through a X40 dry objective with detection channels for brightfield and fluorescence between 660-770nm.

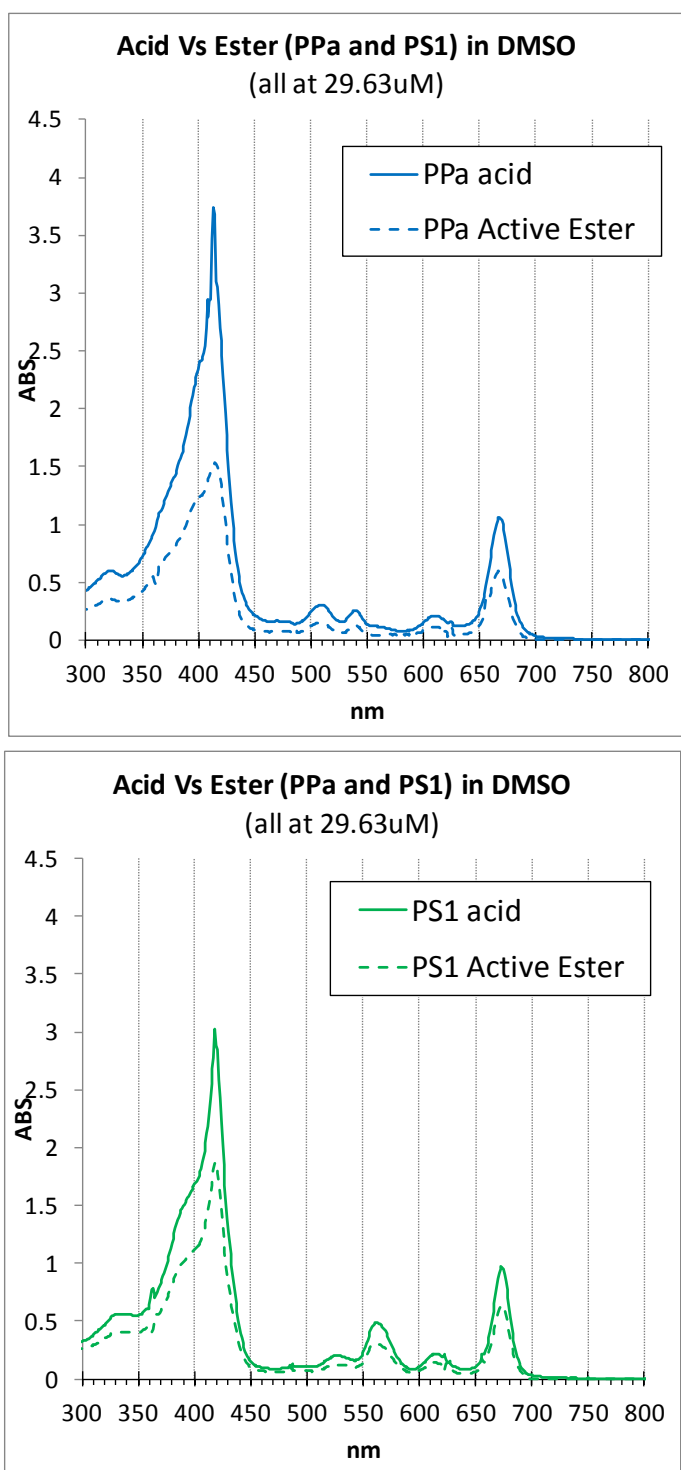
5.2.7 Activation of PPa, PS1 and PS4 for conjugation.

In order to conjugate the free PPa, PS1 and PS4 they first had to be converted into their active form; synthetic chemists within the laboratory (Dr G. Yahiloglu and Dr. I. Stamati) converted the carboxylic acid group on the propionic acid side chain of the PS to an activated NHS ester. See 2.1.6.4 to see the change in structure of the activated PS along with the relevant molecular weight changes. Unfortunately PS4 proved a harder conversion and it was not optimised in the time frame of this work.

Figure 56 shows the absorbance spectra of PS acid vs. PS active ester (AE) in DMSO for PPa and PS1. No shifts were seen in any of the peaks upon PS activation but a slight loss in intensity is seen in both PPa and PS1 between acid and AE.

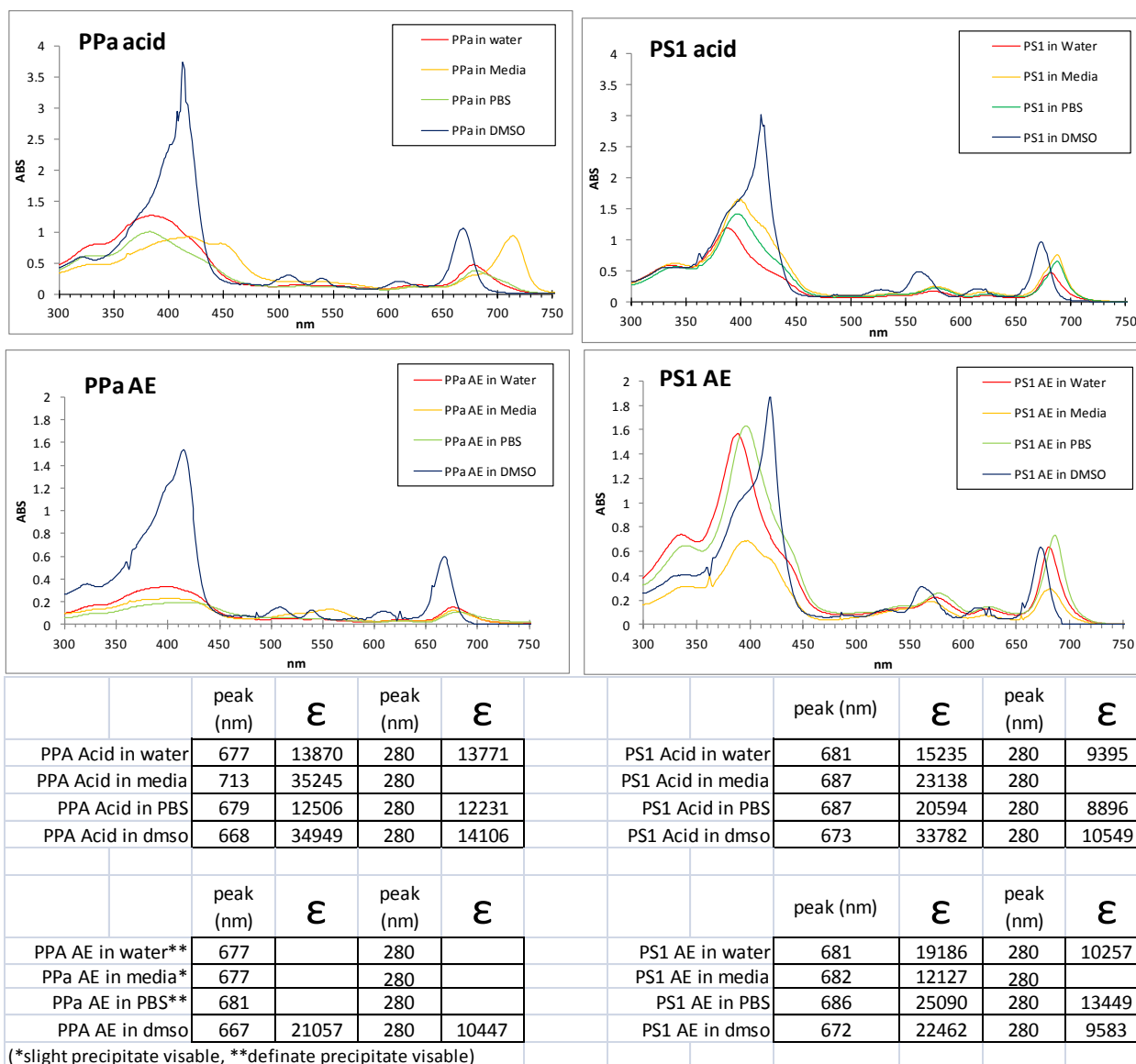
Figure 57 looks at the difference between the acid and AE of PPa and PS1 in more detail, presented is comparative spectra of all four PS at the same concentration in 4 different solutions, PBS, Water, supplemented cell culture media and DMSO. As it may have been required for quantification of conjugates later, the extinction coefficients at the peak of Q band 1 and 280nm were calculated and recorded and are shown alongside. NB. for PS in supplemented media extinction coefficient at 280 nm cannot be calculated as the FCS in the solution also contains proteins that absorb at 280nm. The difference between PPa acid and PPa AE in aqueous buffers is striking, for all aqueous solutions some or extensive precipitate was seen and the spectra flattened considerably. This meant no extinction coefficients could be calculated. Comparatively PS1 AE easily dissolved into solution with no precipitate for any of the solvents. In fact it showed a better retention of spectral shifts than the PS1 acid form in water and PBS, but slightly worse in media. Both PS AE retained the characteristic red shift upon dissolution in aqueous solution over DMSO.

Figure 56 :Absorbance spectra of PS acid vs. PS AE in DMSO for PPa and PS1



Absorbance spectrums over 300nm to 800nm of PS dissolved in de-ionised 100% DMSO at a final concentration of 29.63 μ M. Spectrums of absorbance read over a blank of the relevant solvent and normalised to absorption at 900nm.

Figure 57 : Absorbance spectra of PPa and PS1 acid and AE forms in various solvents.



Absorbance spectrums over 300nm to 750nm of PS dissolved in de-ionised water (red), PBS(green), DMEM media with phenol red and 10% FCS (yellow) and 100% DMSO (blue). PS is at a final concentration of 29.63μM. Spectrums of absorbance read over a blank of the relevant solvent and normalised to absorption at 900nm. Extinction coefficients for Q band 1 and 280nm calculated over a range of concentrations of PS in the same solvents are shown below.

5.2.8 Conjugation of C6.5-k with PS1 AE and PPa AE.

The first conjugation between C6.5-k and either PPa AE or PS1 AE was carried out at a 16:1 PS to C6.5-k molar ratio under the same conditions as for the cyanine dye in 5.2.3. After the reaction samples were collected and subjected to centrifugal force. The PPa conjugation reaction produced a large pellet where as the PS1 conjugation reaction had no pellet at all. Equivalent amounts of the supernatants were separated on an SDS PAGE gel and are shown in Figure 58. The PS1-PIC shows a greater intensity of fluorescence and so it seems evident that the PS1 AE has reacted more extensively with the antibody than PPa AE. Both PS1 and PPa fluoresces to a similar efficiency in the gel environment, see 5.2.10.2. However both reactions show the majority of PS is in an unconjugated form and runs at the dye front.

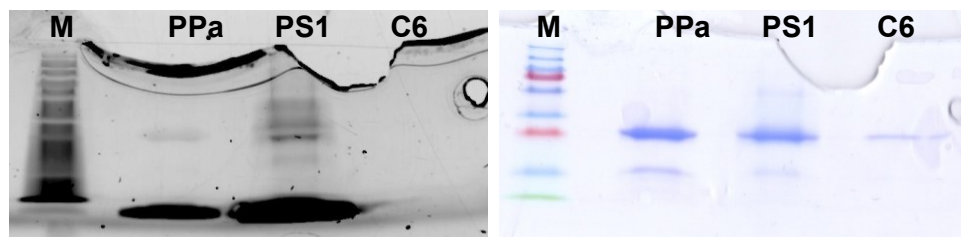
To try and improve conjugation efficiency some small scale conjugations were carried out in which the conditions of conjugation were adjusted. The DMSO was increased to 20% and the pH of the carbonate buffer was increased to 10.6. These conjugate trials were ran directly on a gel and compared to a band of the equivalent amount of un-conjugated C6.5-k. The SDS PAGE gels of these PIC are shown in Figure 59. For PPa AE the increase of pH to 10.6 caused a big improvement in conjugation efficiency, this was less evident with PS1 AE. Increased DMSO had no obvious effect for either PS. A reaction like this will typically produce a range of PIC conjugated to different extents, these will be normally distributed around a mean (Palumbo et al., 2011). The PS1-PIC that migrated highest in the gel, so hypothetically most highly conjugated, were not stained at all by coomassie. This mirrors what was seen with the highly conjugated Cy5.5 PICs see 5.2.3. The different levels of conjugated PS1-PIC are separated more effectively by the gel than PPa-PIC, this is most likely due to the gel retardation effect of PEG chains (Savellano and Hasan, 2003). All future conjugations were carried out in carbonate buffer pH 10.6 and 16.67% final DMSO, all other conditions were maintained as previously.

Prior to this study, experiments were carried out to check the effect of increased pH and organic solvent on C6.5-k stability and PS absorbance spectra.

Figure 60 shows a native antigen binding experiment for C6.5-k that has been exposed to different proposed conjugation solvents for the same time and temperatures as a typical conjugation reaction. Results showed C6.5-k binding to SKOV-3 is maintained in all pH conditions (9.4, 10.2 and 10.7) when compared to C6.5-k that was in PBS pH 7.4. However exposure to different levels of DMSO showed that binding was only maintained with DMSO levels of 10% or 20% but not 50% or 80%.

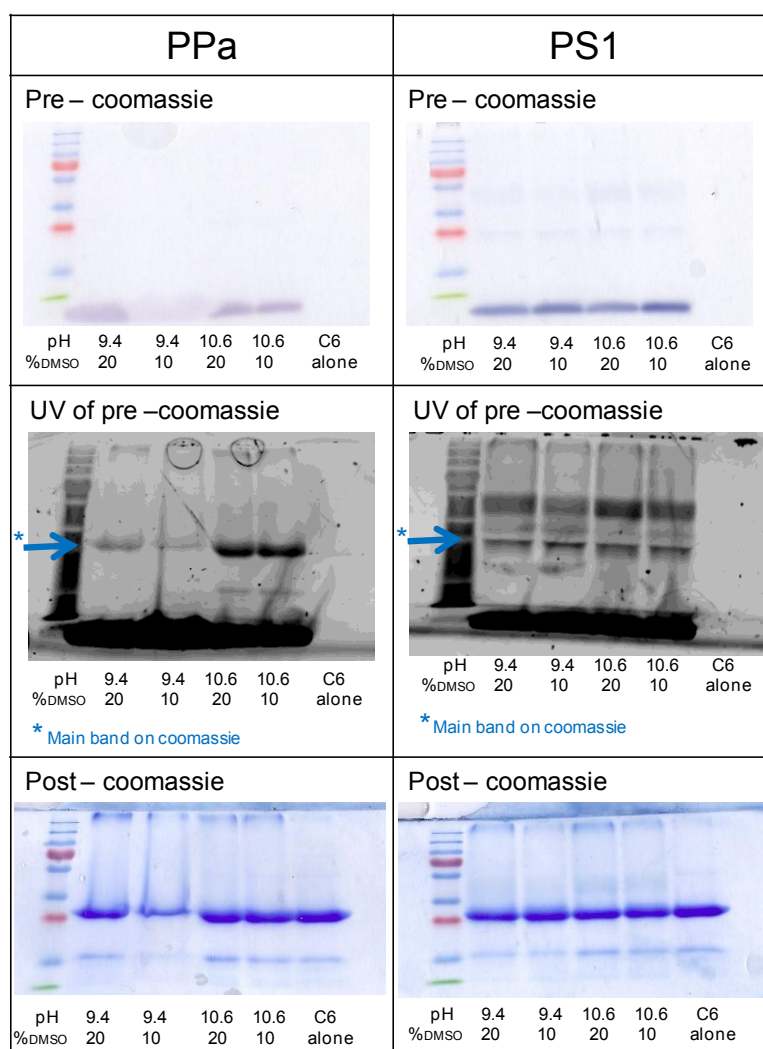
Figure 61 shows the absorbance spectra of PPa AE or PS1 AE in the same proposed conjugation solvents. For PPa AE changing the pH of the carbonate buffer does not improve spectra at all but with increasing DMSO small spectral changes are seen at lower wavelengths with 10% and 20% DMSO, 50% shows a slight improvement in Q band spectra but it requires 80% to see a significant recovery of absorbance. In terms of PS1 AE, the changes in pH exhibit only slight spectral shifts, and with increasing DMSO the pattern is similar to PPa AE in that increasing DMSO increases absorbance efficiency albeit any improvements are of a smaller extent.

Figure 58 : First conjugation of PPa and PS1 with C6.5-k



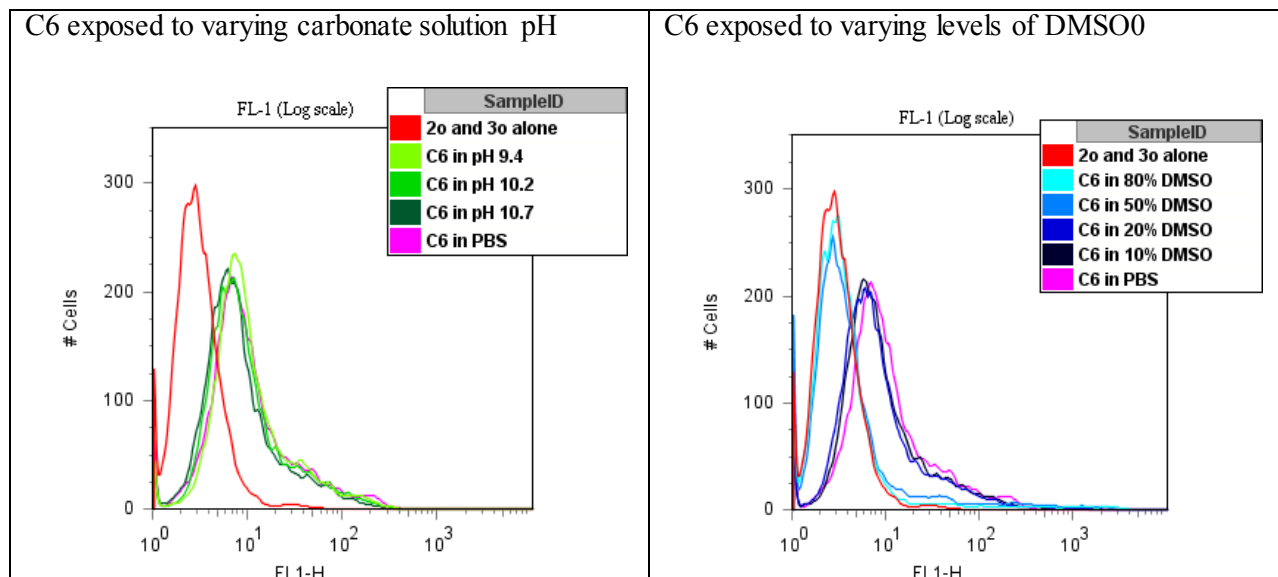
Conjugation reactions carried out with C6.5-k and PPa AE or PS1 AE, experiment was carried out at 16:1 PS to C6.5-k molar ratio with conditions used as in 5.2.3. SDS PAGE gels are presented showing the supernatant taken from a PIC conjugation mixture in which insoluble material was removed via centrifugal force. Alongside un-conjugated C6.5-k has been ran (not equivalent amount). The gels are shown un-stained and imaged under UV light and stained with coomassie. . (C6.5-k shortened to C6 in figure).

Figure 59 : Trials of modified conjugation buffers on conjugation efficiency.



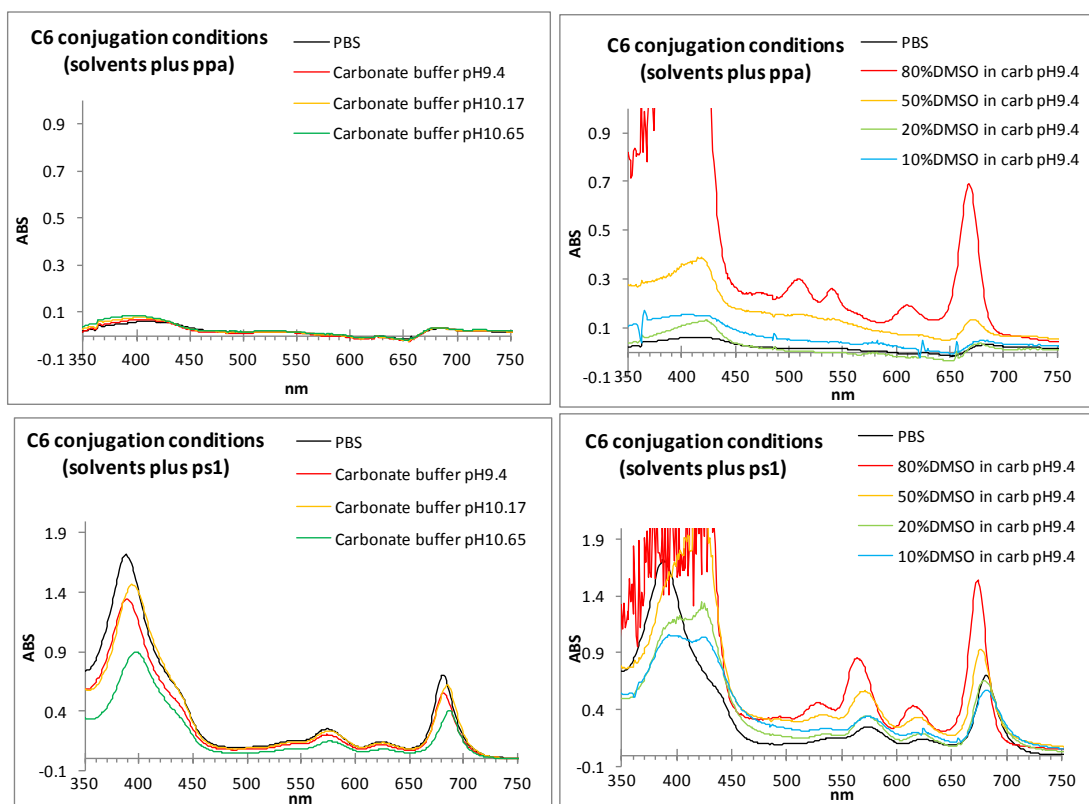
Conjugation reactions carried out with C6.5-k and PPa AE or PS1 AE, experiment was carried out in varying conditions explained in (5.2.8). SDS PAGE gels are presented showing separation of a direct sample of the PIC conjugation mixture alongside the equivalent level of un-conjugated protein. The gels are shown un-stained, un-stained and imaged under UV light and stained with coomassie. (C6.5-k shortened to C6 in figure).

Figure 60 : C6 stability in modified conjugation conditions



A native antigen binding experiment (see 2.1.5.5) for C6.5-k that had been exposed to the various possible conjugation solvents. . (C6.5-k shortened to C6 in figure).

Figure 61 : PS solubility in modified conjugation conditions

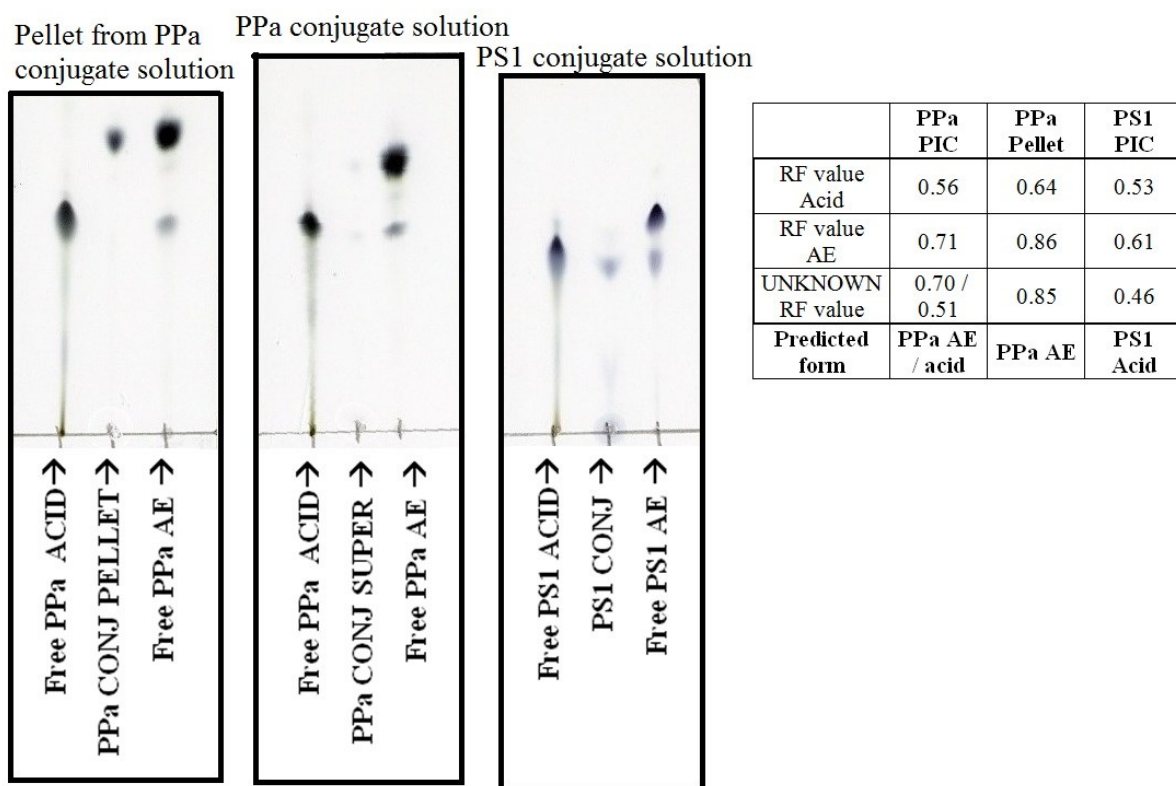


The absorbance spectra of PPA AE or PS1 AE in the various possible conjugation solvents. . (C6.5-k shortened to C6 in figure).

5.2.9 TLC plates to determine PS state after conjugation

It was of interest to work out if the free PS left after conjugation was of an acid or AE form. A sample of PS1 PIC and PPa PIC, (the PPa conjugate was separated into pellet and supernatant), was ran on a TLC plate alongside free PPa or PS1 in its acid or AE form. Only free PS is expected to run. Separation between acid and ester was obtained by using Silica 60 TLC plates in a 10% Methanol in DCM solvent system see 2.1.6.8. Images of the plates and calculated RF values are presented in Figure 62. Results provide evidence that in the PS1 conjugate mixture, the free PS is in the acid form, in the PPa conjugate supernatant the free PS is present in both the acid and the ester form and in PPa conjugate pellet all the free PS is present in the ester form. The presence of the ester form suggests not all the PPa AE was in solution and therefore not available for conjugation.

Figure 62 : TLC plates to determine PS state after conjugation



PS1 PIC, PPa PIC supernatant and PPa PIC pellet were ran alongside free PPa or PS1 in its acid or AE form. Separation between acid and ester was obtained by using Silica gel 60 glass TLC plates in a 10% Methanol in DCM solvent system see 2.1.6.8. Identification of RF values for each moiety listed in the table alongside. Attempted purification of PICs

There was a significant amount of unconjugated PS that ran at the dye front, with the Cy5.5 conjugate this could be removed in aqueous solution via a semi permeable membrane. But when samples of the PPa or PS1 PIC were washed with PBS against a 5000 MWCO polyethersulfone membrane none of the free or conjugated PS was able to pass through the membrane, this was seen by both spectral and gel analysis of the samples from both sides of the membrane (data not shown). Small losses seen could be due to non specific binding to plastic surfaces, loss upon liquid transfer or insoluble PS AE being trapped in the membrane. PICs were also clarified through a 0.2µm PVDF syringe driven membrane which would remove any large aggregated PIC or PS, and again only small losses are seen (data not shown). The inability to purify the free PS could be due to a number of factors, including the possible complete non covalent association of all of the free PS with the antibody or that the free PS is unable to interact with the semi permeable membrane and/or traverse it, even with its concentration gradient or under centrifugal force.

5.2.9.1 The introduction of n-Octyl-β-D-Glucopyranoside to promote purification.

Experiments were carried out in order to see if free unconjugated PS were able to cross a semi permeable membrane. To help solubilise the free PS the inclusion of a dialysable non ionic alkyl-glucoside detergent called n-Octyl-β-D-Glucopyranoside (OG) (Sigma 08001) was used. CMC for OG is around 0.73% solution in water (24-26mM) and micellar molecular weights of 8000-29000 have been reported.

Figure 63 shows PPa and PS1 diluted with varying concentrations of OG. All four PS were tested and the AE forms showed similar behaviour to their acid forms (acid forms not shown). Spectral shifts are evident as OG concentration increases. When compared to spectra of PS as seen in Figure 57, PS at low OG concentrations matched the spectra of PS in PBS and at high OG the spectra of PS in DMSO. For future work it was decided to use 2% OG as for both PS this was sufficient to return the spectra to a more 'DMSO like' spectra.

To see if the presence of 2% OG can aid semi-permeable membrane transition. Free PPa AE or PS1 AE were made up as mock solutions, i.e. up to a similar concentration with co-solvent as would be found in a conjugation reaction, and then diluted into buffer with or without 2% OG

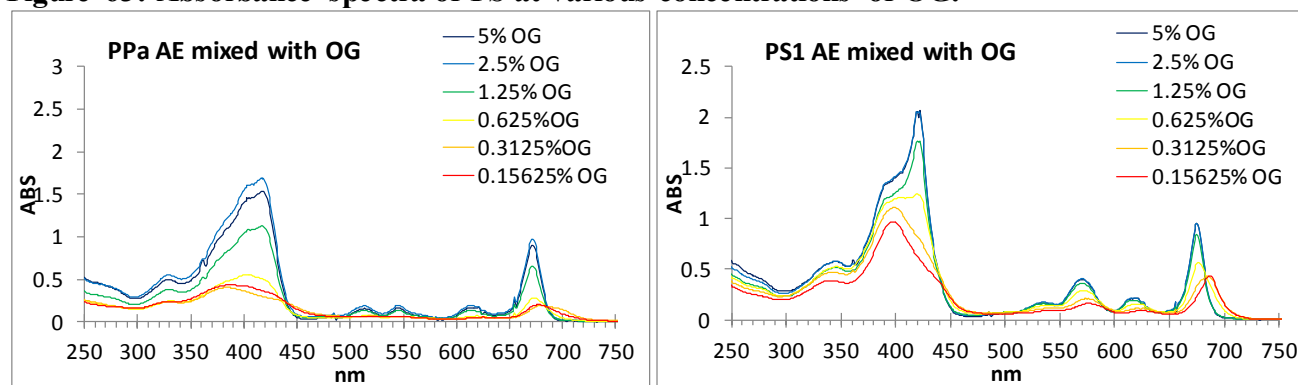
and passed through the 5000 MWCO polyethersulfone membrane. Results are shown in Figure 64; both with and without OG, no PS was able to flow through the membrane. NB the PPa AE mock solution was not submitted to centrifugal force before being used in the experiment and over half of the PPa AE got stuck in the membrane. Although large OG micelles predicted to contain free PS could not have passed through the membrane themselves it was hoped that as micelle formation is in equilibrium and its presence would help encourage PS interaction with the membrane or co-transport with OG monomers.

To extend this work a small scale dialysis experiment in which free PPa AE or free PS1 AE were diluted in and dialysed into different solvents through a 8000 MWCO Cellulose Dialysis Tubing. These solvents included; PBS, Tris pH8, Tris pH 9, Carb pH 10 and Glycine pH 2, in combinations with different supplements: 0.6% OG (below CMC) 1% OG (above CMC) and 20% DMSO. None of the samples in any of the supplemented solvent systems equilibrated across the membrane. Many of the samples, especially those with PPa AE showed evidence of significant precipitation. Example data for the supplemented PBS solvents is shown in Figure 65. Other solvents systems showed very similar data (data not shown).

5.2.9.2 A solvent exchange system for purification of PIC.

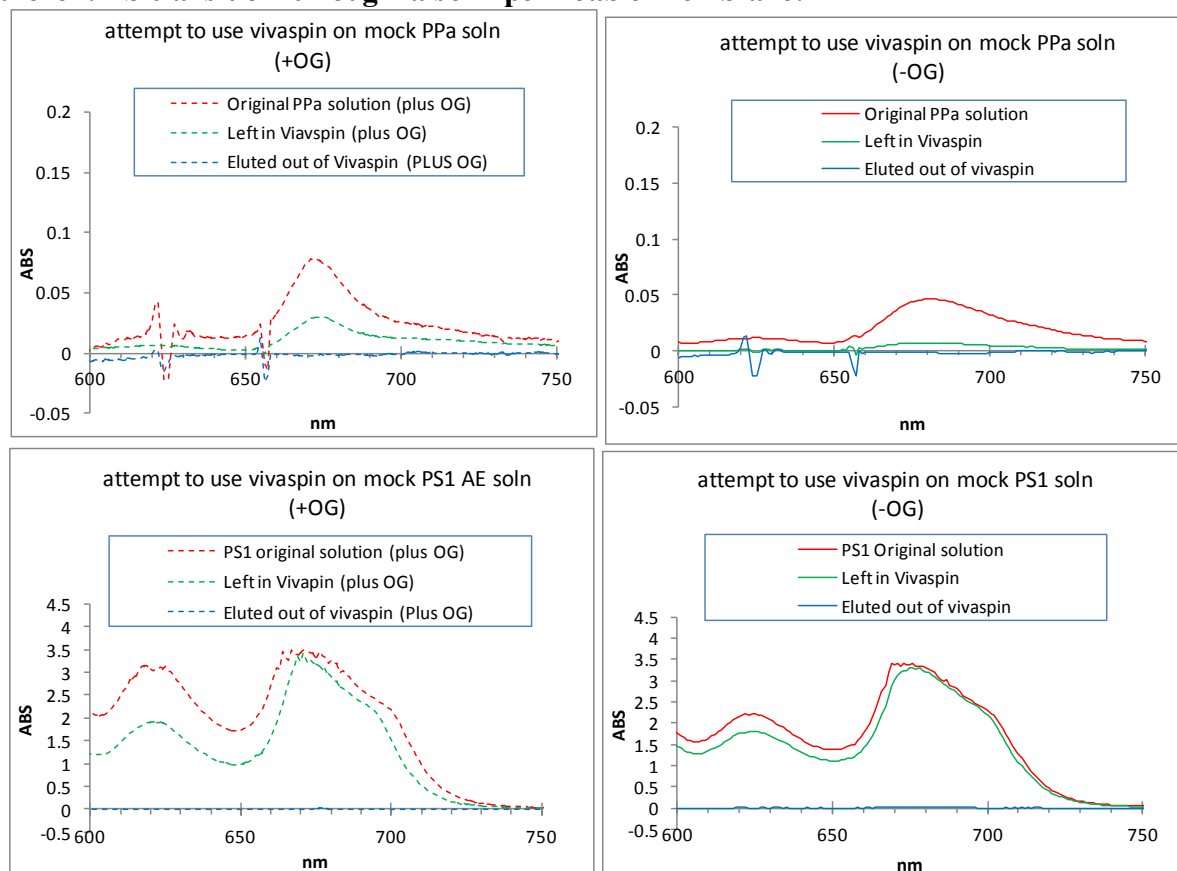
Another idea to purify the un-conjugated PS from the mixture was by solvent extraction. A trial using free PS and free BSA was carried out with a chloroform / PBS two-solvent system in which BSA or PS were dissolved in PBS, a layer of chloroform added over the top and both gently mixed overnight before spectral analysis of the two layers for evidence of transition between them. A similar experiment but with compound containing PBS mixed with activated charcoal, mixed overnight and then separated by centrifugation was also tested. For the chloroform / PBS solvent system the BSA remained entirely in the PBS (top fraction) where as the PS transitioned entirely into the chloroform. For the activated charcoal experiment the charcoal extracted both BSA and PS from the solution. When the chloroform extraction technique was attempted with PIC samples, the PS showed no transition into the chloroform to any extent, when this was extended over a further day, PS partially transitioned but so did the PIC. (data not shown)

Figure 63: Absorbance spectra of PS at various concentrations of OG.



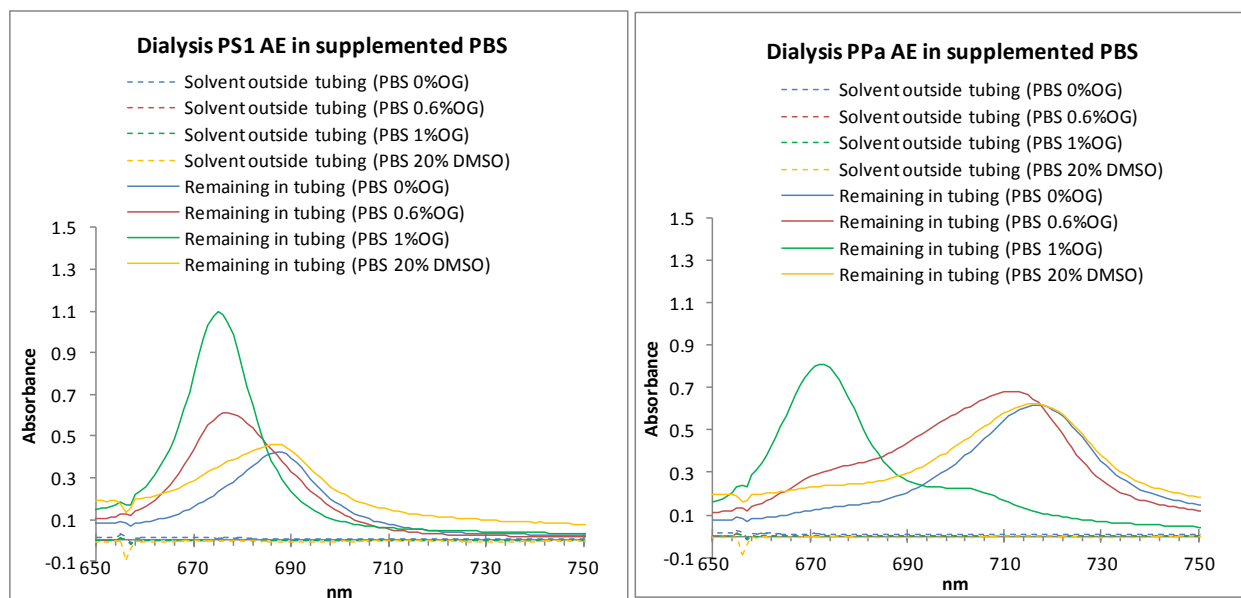
Absorbance spectrums over 250nm to 750nm of PS dissolved in aqueous solution supplemented with varying concentrations of *n*-Octyl- β -D-Glucopyranoside. PS is at the same final concentration. Spectrums of absorbance read over a blank of the relevant solvent and normalised to absorbance at 900nm.

Figure 64: PS transition through a semi permeable membrane.



Free PPa AE or PS1 AE were made up as mock solutions, i.e. up to a similar concentration with co-solvent as would be found in a conjugation reaction, and then diluted 1:3 into buffer with or without 2% OG. Absorbance spectra was taken before and after the mixtures were passed through a 5000 MWCO polyethersulfone membrane, remaining solutions were then made up to the original volume and re analysed against the flow through.

Figure 65 : Dialysis of free PS in a number of different supplemented solvent systems.



Small scale dialysis experiment with a number of different 500ul PS AE solutions (either PPa AE or PS1 AE) were dialysed into 30ml of the same solution minus the PS through a 8000 MWCO Cellulose Dialysis Tubing. All solvents PBS, Tris pH8, Tris pH 9, Carb pH 10 and Glycine pH 2, were tested in combinations with all solvent supplements 0.6% OG (below CMC) 1% OG (above CMC) and 20% DMSO alongside un-supplemented solvent. Dialysis was carried out over 4 days in the dark at RT. Samples from inside and outside the dialysis tubing were recovered, all insoluble material was removed and discarded before UV spectrums were ran of the samples. Only data from the PBS system is shown, similar data was obtained for all other solvent systems.

5.2.9.3 Ion exchange columns to aid purification of PICs.

The next step was to use a column based system to specifically retain the antibody while washing with supplemented solvents to remove free PS, before buffer exchange and release of the now purified antibody. Centrifugal Ion Exchange Spin Columns coated with a quaternary ammonium ($R-CH_2-N^+-(CH_3)_3Cl$) strong basic anion exchanger, are designed to reversibly bind to negatively charged molecules, i.e. molecules at a pH above their pI value, and elute at increased salt concentrations or by lowering the pH below the pI. The pI of C6.5-k was predicted at 6.52, although this could have changed upon PS conjugation.

The ion exchange columns were shown to bind and release un-conjugated C6.5-k as predicted. However when PPa and PS1 PICs were tested samples became stuck on the column. Columns were washed with increasing amounts of OG in 0.02M Tris pH 8.5. Initial washes of 0.6% were increased to 0.9% then 1% over 13 washes, during these washes free PS was observed in the wash fractions by both spectral analysis and SDS PAGE. Elutions steps were with 1M NaCl + 0.65% OG in 0.02M Tris. Small amount of un-conjugated C6.5-k were eluted but none showed any fluorescence under UV light on SDS PAGE. Despite various harsh elution techniques; i.e. decreasing the pH from 7 to 5 over the elution period, using 5M NaCl, or 0.2M Glycine pH 2 or 2% OG (in 0.02M Tris pH 8.5) still no release of the PIC was observed. Upon dissection of the spin column it was clear the conjugate was stuck in the upper few layers of the column system, this is shown in Figure 66. UV spectra and SDS PAGE separation of all the fractions from this attempted purification are shown in Figure 67.

The same idea was applied with further conjugates but with Ion exchange columns based on a chromatography system made of high flow agarose with a dextran surface extender containing anion $N^+(CH_3)_3$ the idea being that for these if the conjugate didn't bind they would remain in the bulk flow and not be trapped. However, the same trapping in the top layers of the column was observed, see Figure 66, extreme denaturing washes in a range of solvents were not able to remove it.

Figure 66 : Images of PICs stuck within ion exchange chromatography columns.

Centrifugal Ion Exchange Spin Columns coated with a quaternary ammonium ($R-CH_2-N^+-(CH_3)_3Cl^-$) strong basic anion exchanger: layers separated from the column show PIC stuck in the top few layers.



Chromatography system made of High flow agarose with a dextran surface extender containing anion $N^+(CH_3)_3$: PIC stuck visibly in the top of the column.



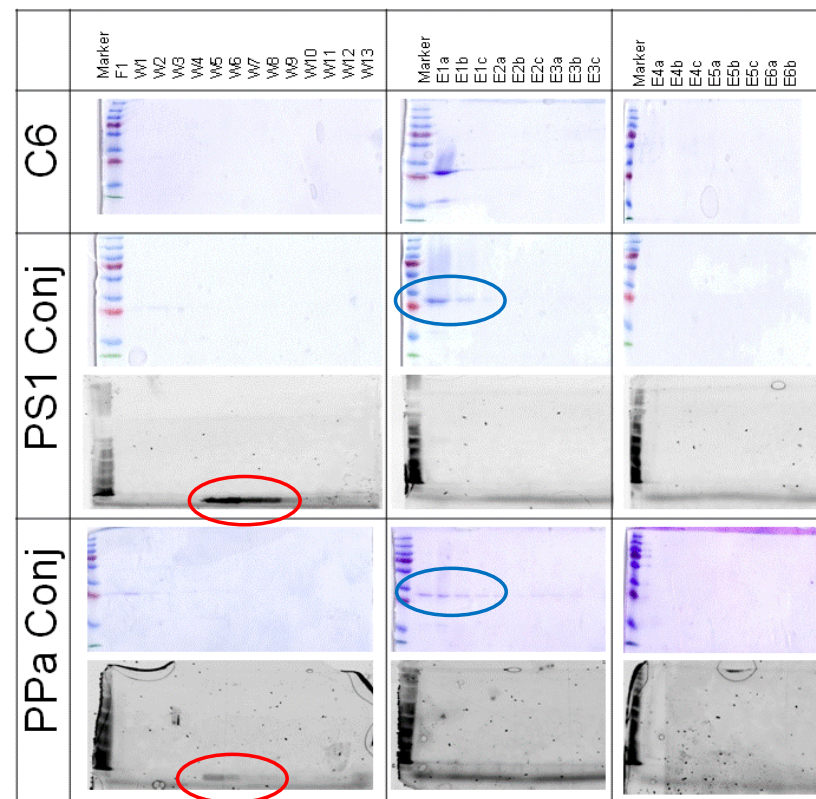
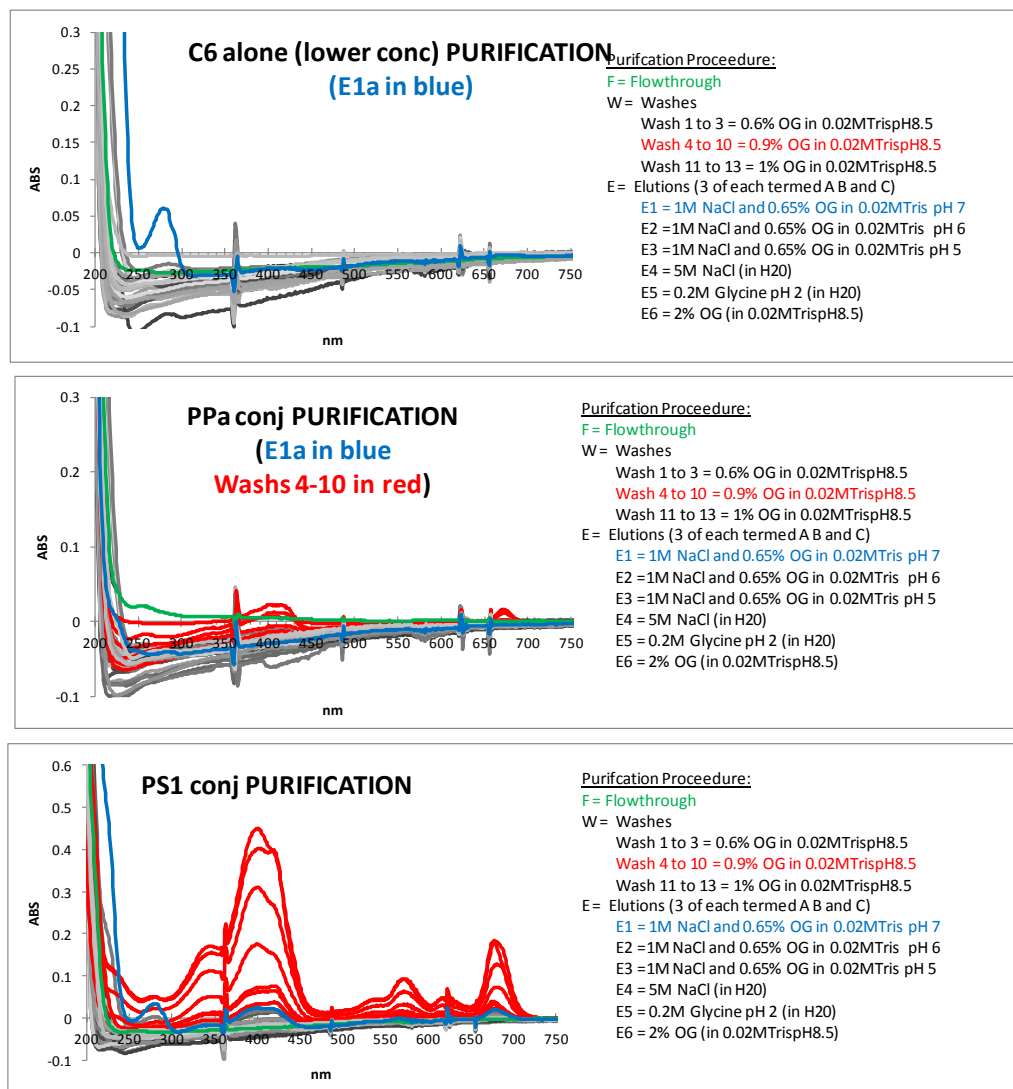


Figure 67 : Ion exchange chromatography for PICs

Insoluble material was removed from PPa PIC and PS1 PIC and the remaining solution dialysed into 0.02M Tris pH 8.5 before addition to the Ion Exchange Spin column. NB: the amount of un-conjugated C6.5-k ran as a control is much lower than the amount contained within the conjugates. Samples were added to the column and washed with increasing amounts of OG in 0.02M Tris pH 8.5. Initial washes of 0.6% were increased to 0.9% then 1% over 13 washes; initial elutions were with 1M NaCl and 0.65% OG in 0.02M Tris solutions decreasing the pH from 7 to 5 over the elution period. UV spectra and SDS PAGE separation of samples of the fractions was monitored. (C6.5-k shortened to C6 in figure).

5.2.10 Quantification of the extent of PS conjugation

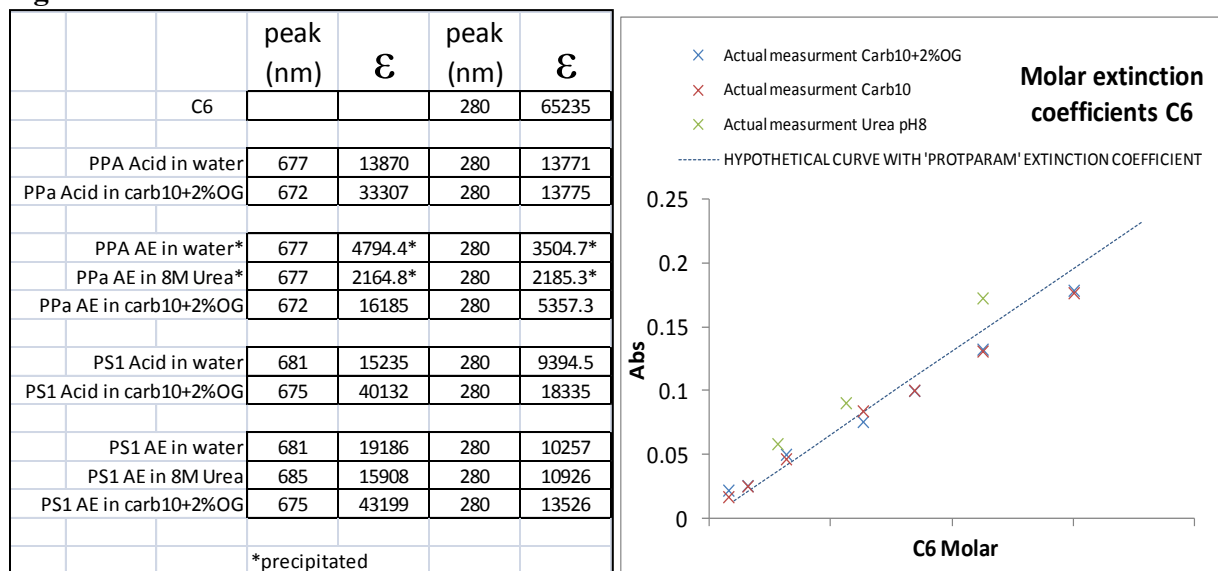
Despite the problems with purification shown above, work was carried out toward development of a method to ensure reliable analysis of the PIC. The extinction coefficients can change when PS is activated, this was shown in Figure 57, and the PS that has conjugated will not either of these two forms, instead it will have formed an amide bond in position of the NHS ester or carboxylic acid group on the propionic acid side chain. This position is far from the central ring and so changes to it are unlikely to alter the absorption spectra directly but via its effect on PS aggregation and/or solubility.

5.2.10.1 Spectral methods to calculate conjugation ratios

Extinction coefficients of the four PS in water were determined and are shown in Figure 57. Extinction coefficients were also calculated for the PS in 8M Urea and in 2% OG in Carbonate buffer pH10, these solvents were used to dissolve PIC in an attempt to circumvent possible protein-PS interactions interfering with PS spectra like for Cy5.5 See 5.2.3.2. The extinction coefficients for PS in these buffers are shown in Figure 68 alongside a graph that plots the predicted relationship of C6.5-k and absorbance at 280nm using the extinction coefficient predicted by ProtParam ($\epsilon=65235$) (See 2.1.5.3), and actual readings taken diluting C6.5-k into carbonate pH10, carbonate pH10 + 2% OG and 8M Urea. For C6.5-k all diluted curves matched that of the predicted value, and so it was used for all subsequent calculations.

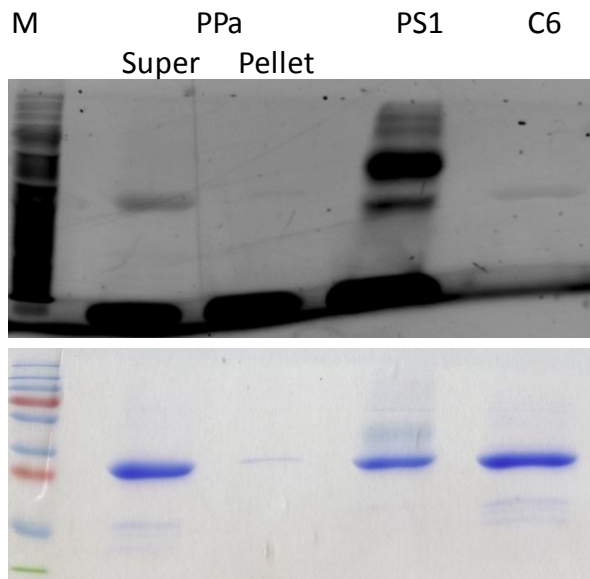
An additional complication is the inability to remove free PS from the PIC mixture, the spectra produced will therefore be a mixture of PS acid, PS ester (in the case of PPa) and conjugated PS forms. For all the following work on spectral and gel quantification Figure 69 shows the SDS Page analysis of the PIC that were used. Conjugation reactions carried out with C6.5-k and PPa AE or PS1 AE at a 16:1 PS to C6.5-k molar ratio along the conditions detailed in 2.1.6.5. PIC were subjected to centrifugal force to separate any insoluble PIC/PS then dialysed extensively into PBS.

Figure 68: Extinction coefficients of PS and C6.5-k in 8M urea and in 2% OG.



Extinction coefficients of PS in 8M Urea and in 2% OG in Carbonate buffer pH10, alongside a graph that plots the predicted relationship of C6.5-k and absorbance at 280nm using the extinction coefficient predicted by ProtParam ($\epsilon=65235$) See 2.1.5.3, and actual readings taken diluting C6.5-k into carbonate pH10, carbonate pH10 + 2% OG and 8M Urea. . (C6.5-k shortened to C6 in figure).

Figure 69 : Conjugation of PPa and PS1 with C6.5-k for quantification experiments



Conjugation reactions carried out with C6.5-k and PPa AE or PS1 AE, experiment was carried out at 16:1 PS to C6.5-k molar ratio with conditions used in 2.1.6.5. insoluble material was removed and is shown alongside as 'pellet', dialysed into PBS and subsequently separated via SDS page gel. Final PIC samples are shown alongside an equivalent amount of un-conjugated C6.5-k. The Gel was imaged under UV light before staining with coomassie. . (C6.5-k shortened to C6 in figure).

The absorbance spectra of the final PPa PIC and PS1 PIC samples were analysed after dilution in either water, 8M Urea or 2% OG in carbonate buffer. Spectra are shown in Figure 70 and Figure 71 alongside tables showing the actual vs. predicted Q band 1 peak for free PS in the same solution. NB; around twice as much PIC was used for the OG vs. water dilutions (1 in 20) in Figure 71 compared to the Urea vs. water dilutions (1 in 40) in Figure 70, and OG solutions have some absorbance at 280nm so it was important to produce accurate blanks including the 5µl or 10µl of PBS that would be added alongside PIC.

When Comparing PIC dissolved in water or 8M Urea; PIC spectra doesn't change and PS peaks still match the peak expected for free PS. This could mean that there is no change of PS spectra upon conjugation, that any changes that do occur urea can't reverse, or that the free PS is at too high a concentration compared to bound that any effects are masked. When Comparing PIC dissolved in water or 2%OG; the PIC spectra changes significantly when dissolved in 2% OG and peaks match what would be expected for free PS in those solutions. However, for both systems when absorbance values were taken and respective concentrations of PS and C6.5-k calculated via extinction coefficients the following was observed.

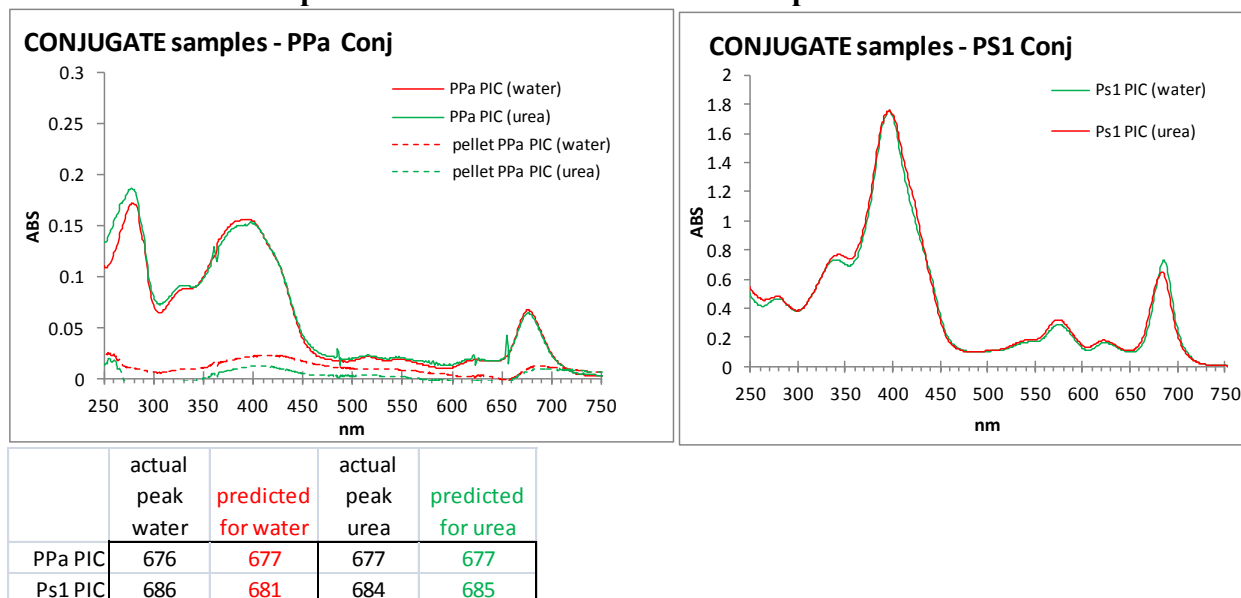
- For PPa Conjugates; even though PS is in both an acid and AE form (see 5.2.9), acid PS coefficients had to be used for water and urea as AE coefficients couldn't be calculated due to extensive precipitation. This caused a large underestimation of PS in all urea and water calculations. For example the results in the 1 in 20 dilution into water predicted the total PS (pellet + supernatant) was 10µM as it is known none or very little PS has been removed from the sample since conjugation (completely in-effective purification) the total PS split between pellet and conjugate should be around 72µM. This was improved with dissolution in 2%OG and total PS was calculated at a more believable 60µM (predicted around 72µM). However; for the OG dilutions PPa AE coefficients could also now be used as dissolution produced no precipitate, but use of this extinction coefficient calculated impossible values for total PPa at 119 µM. The mixture of AE and Acid would need to be resolved or quantified for future accurate quantification.

- For PS1 conjugates; acid coefficients could be used with confidence (see 5.2.9), but when the calculations were carried out predictions in all three solutions, (water, 8M Urea and 2%OG), all predicted the contribution to the absorbance at 280nm from C6.5-k to be unusually low. For example for the 1:40 dilutions into water or urea the absorbance at 280nm for protein was calculated at less than 0.06 units, almost undetectable. This seemed unlikely because if no C6.5-k was lost the sample should contain around 2.5µM C6.5-k, which would produce absorbance at 280nm of 0.16 units. Perhaps the conjugated PS has quenched not its own absorbance but that of C6.5-k. Because of this PS:C6.5-k ratios predicted by these calculations were 43:1 for water and 74:1 for urea - impossible values as only 16:1 was added. Even when C6.5-k concentration was increased to a 1 in 20 dilutions they again predicted C6.5-k to be unusually low with impossibly high ratios of PS 30:1 (Water) and 71:1 (2% OG).

Despite seeing very little C6.5-k is lost on coomassie and knowing PS can't escape during the dialysis process having manipulated the samples above significantly post conjugation the levels of PS or C6.5-k that one would predict to be contained within the samples couldn't be guaranteed. For this reason a sample was taken directly from the conjugation mixture and analysed in the same way; this sample had undergone no manipulation since the conjugation was set up so the exact concentration of PS and C6.5-k added was known. If the spectral method showed agreement with this 'known' sample then the spectral method for quantification of PIC could be deemed accurate. Known amounts of C6.5-k and PS in these solutions were 3.3µM C6.5-k and 53.3µM PS. When absorbance values were taken and respective concentrations of PS and C6.5-k calculated via extinction coefficients the sampled diluted in 2% OG coefficients again underestimated the amount of C6.5-k, at 2µM or 1.3µM for PPa and PS1 respectively, predicting PS:C6.5-k ratios of 22:1 and 34:1, again impossible as the mixture only contains 16:1.

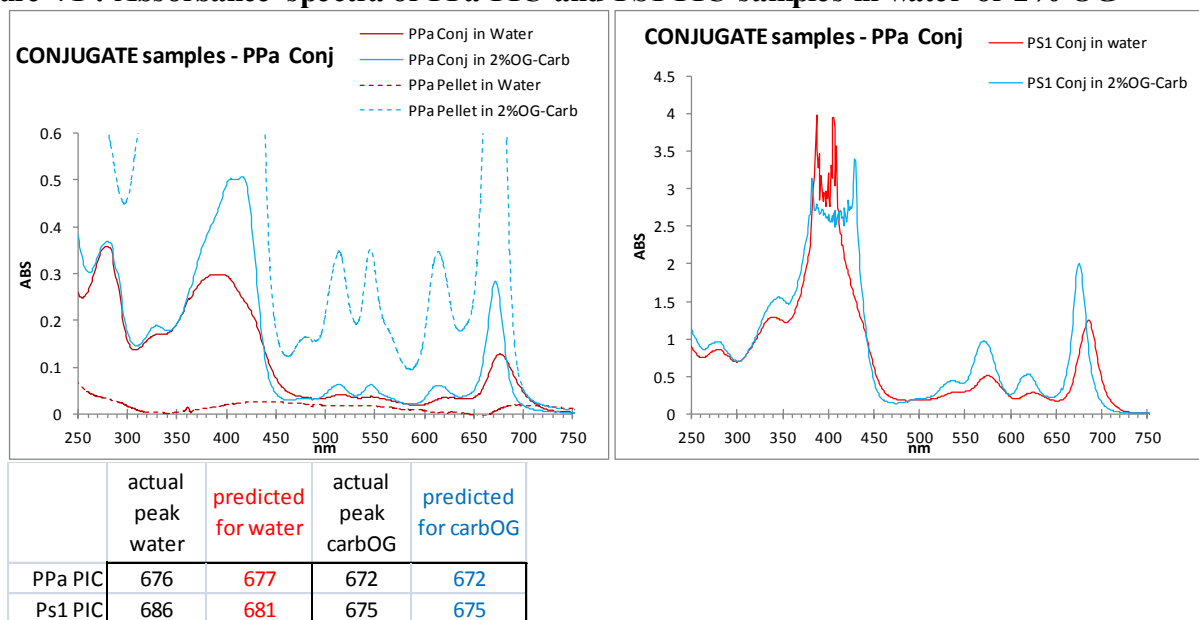
None of the spectral analyses presented above are reliable and there is still a lot of contaminating un-conjugated PS effecting readings and possibly masking any shifts of PS upon conjugation. Purification of the conjugates should be addressed before this is investigated any further.

Figure 70 : Absorbance spectra of PPa PIC and PS1 PIC samples in water or 8M urea.



The absorbance spectra of the final PPa PIC and PS1 PIC samples were analysed after dilution in either water or 8M Urea, alongside a table showing the predicted Q band I peak for free PS in the same solution and the actual peak nm at Q band I of the PIC.

Figure 71 : Absorbance spectra of PPa PIC and PS1 PIC samples in water or 2% OG



The absorbance spectra of 10ul the final PPa PIC and PS1 PIC samples were analysed after dilution in either 190ul water or 2% OG in Carbonate buffer pH10.6, alongside a table showing the predicted Q band I peak for free PS in the same solution and the actual peak nm at Q band I of the PIC.

5.2.10.2 Quantification of PS by UV imaging of SDS PAGE gels.

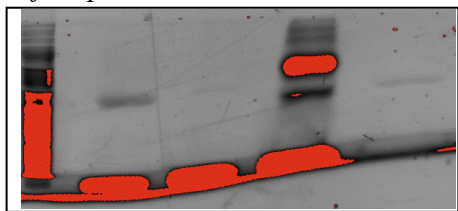
Although PIC and free PS cannot yet be separated in their native form, separation is seen in a denatured form on the SDS Page gels. The images of these taken under UV light show us the relative distribution of PS between the protein-conjugated and free forms of PS. To further investigate if this was quantifiable at the same time as conjugate gels were ran gels with a dilution series of free PS AE was also ran, they were then imaged at the same time. The PIC gel as presented in Figure 69 is shown again in Figure 72 but shown in red is any camera saturation in terms of its pixels. Exposure times was adjusted until none of the bands were saturated on either the gel containing PIC or the gels containing free PS. These images are shown in the larger image in Figure 72 and were taken under the same camera settings.

Gel band intensity was quantified using Image J software, see 2.1.6.6. Figure 73 shows the PS 'standard curve' gels and a graph of PS concentration over band intensity. In this graph PPa and PS1 show similar fluorescent efficiency in the gel environment but even though the image pixels are not saturated only band intensities below around 8000 show any linearity. Figure 74 presents the gel containing the PICs, and from the calculated band intensity values the amount of PS conjugated compared to the amount free at the dye front could be predicted. Values were normalised for the increased intensity from dye front alone and any endogenous C6.5-k fluorescence. The PS1 PIC shows twice as much PS1 is free than is conjugated. For the PPa supernatant there is 25 times more free PPa than is conjugated. For the PPa PIC when you calculate a ratio of the distribution of PS between conjugated, free and pelleted, free PPa in pellet is (36), free PPa in supernatant is (25) and PPa in conjugate is (1).

If it is assumed no PS has been lost and PS at the dye front is not saturated (as suggested may be possible by the free PS gels), the amount of conjugated PS out of the total PS added to conjugation mixture is 1 part out of 3 for PS1 and 1 part out of 62 for PPa. If it is assumed no C6.5-k is lost from these conjugates this would provide PS:C6 molar ratios of 0.3:1 for PPa and 5:1 for PS1, but too many assumption are made to take these values reliably; for example conjugated PS is assumed to have similar fluorescent properties to free PS in a gel environment, and it is unknown if any free PS could have diffused out of the gel over time.

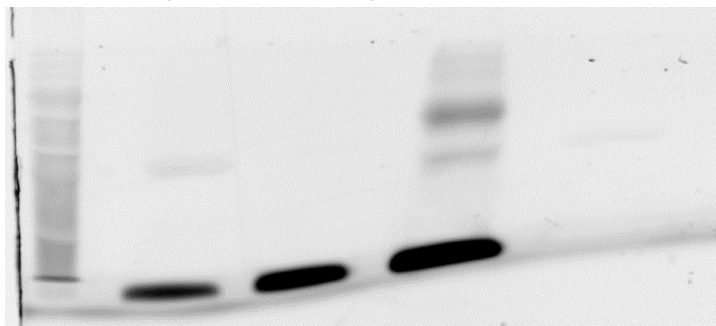
Figure 72 : PIC gel re-imaged for no saturation alongside free PS AE standard curves.

The PIC gel as presented in Figure 69 is shown again but in red is any camera saturation in terms of its pixels:



Gels were ran with a dilution series of free PS AE and were imaged at the same time as the gel containing the PICs. Exposure times were adjusted until none of the bands were saturated on either the gel containing PIC or the gels containing free PS and all images recorded under the same camera settings:

A (CONJ)



B (PPa AE)



3000uM
1000uM
333.33uM
111.11uM
37.04uM
12.35uM

C (PS1 AE)

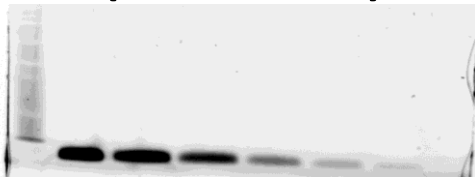
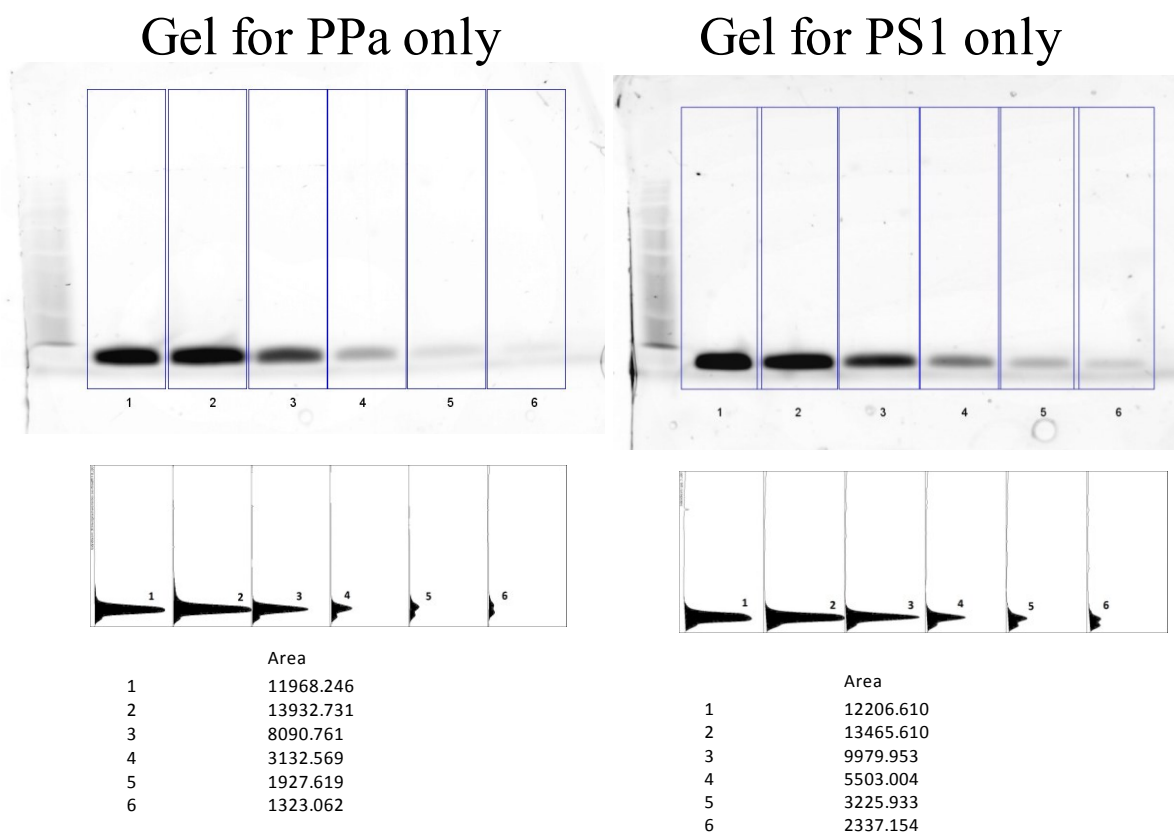


Figure 73 : Quantification of band intensity of free PS AE standard curve gels



A graphical version of each gel is shown below actual image of the gel with the X axis of each well representing the distance travelled down the gel, and the Y axis for the fluorescent intensity of that distance down the well. The areas of these curves shown below correspond to the total intensity of each band. These figures are plotted against PS concentration in the graph below.

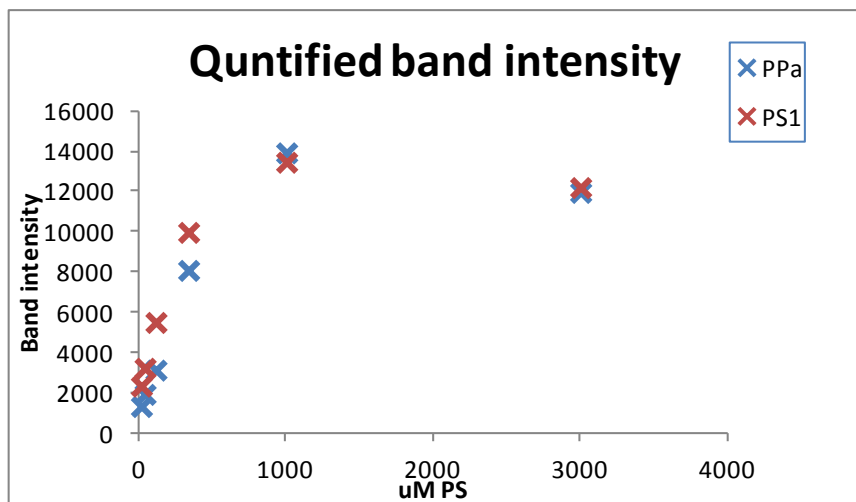
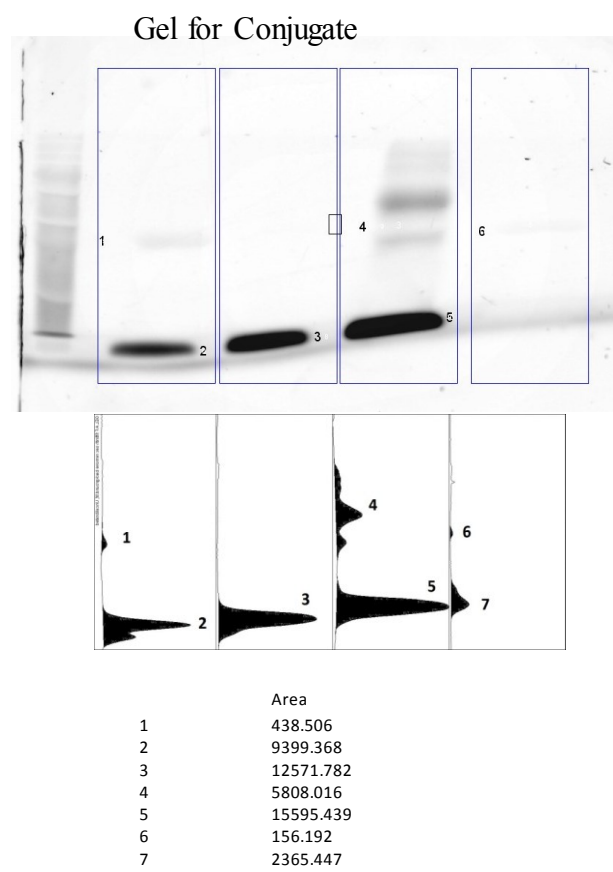
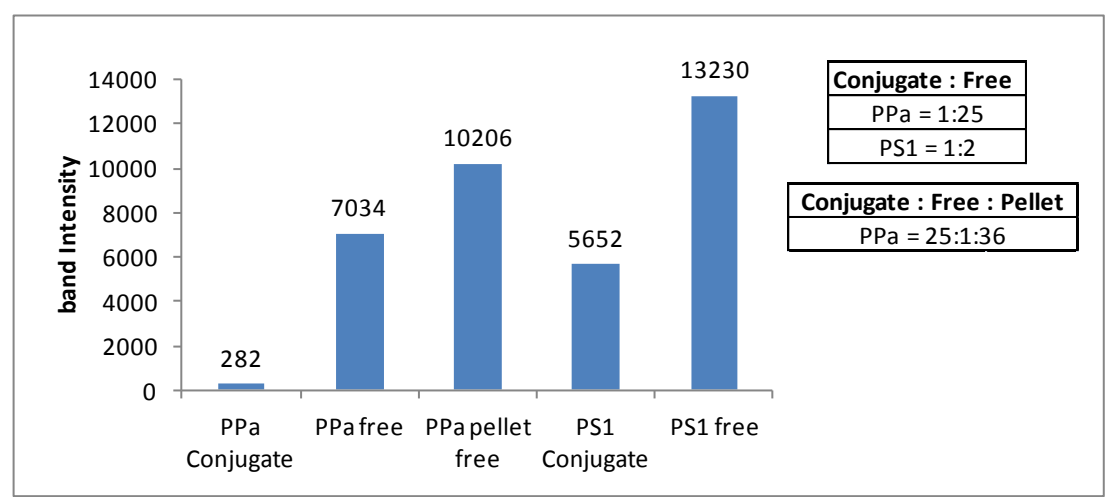


Figure 74 : Quantification of band intensity of PICs gel.
A graphical version of the PIC gel :



From the band intensity values the amount of PS conjugated compared to the amount free at the dye front has been calculated and is shown in the graph below, values at the dye front have been normalized for the increased intensity from dye front alone (seen above as number 7 in the C6.5-k only well) and the conjugate band normalised against the endogenous C6.5-k fluorescence (seen as number 6 in the C6.5-k only well)



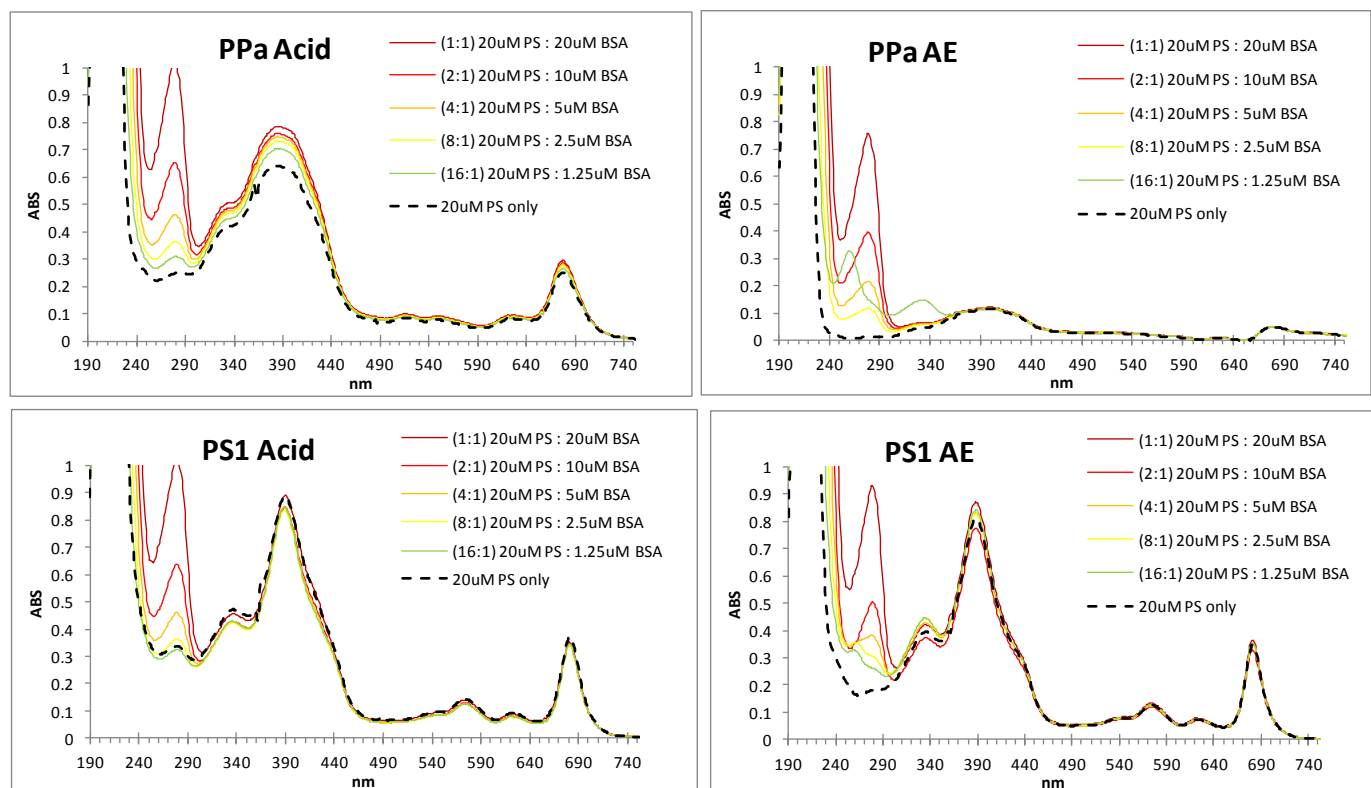
5.2.10.3 Evidence for the quenching of endogenous protein absorbance by PS.

A continual issue encountered throughout this chapter has been the possibility that PS can quench the endogenous absorbance of protein at 280nm, to investigate this possibility BSA was incubated at different concentrations in water with a constant concentration of PS over molar ratios relevant to conjugation efficiencies. The spectra are shown in Figure 75 alongside the spectra produced by the 20 μ M PS alone. The Q band 1 of all PS is unaffected by an increase in concentration of BSA. However results show inclusion of PS in its AE form do affect the absorbance at 280nm possibly due to conjugation reactions between the active ester and the protein. The change in A₂₈₀ (minus the contribution of the relevant 20 μ M PS) is shown plotted over protein concentration and compared to a protein only control (shown in red), this graph shows significant deviations from the normal relationship with all PS, even the acid forms.

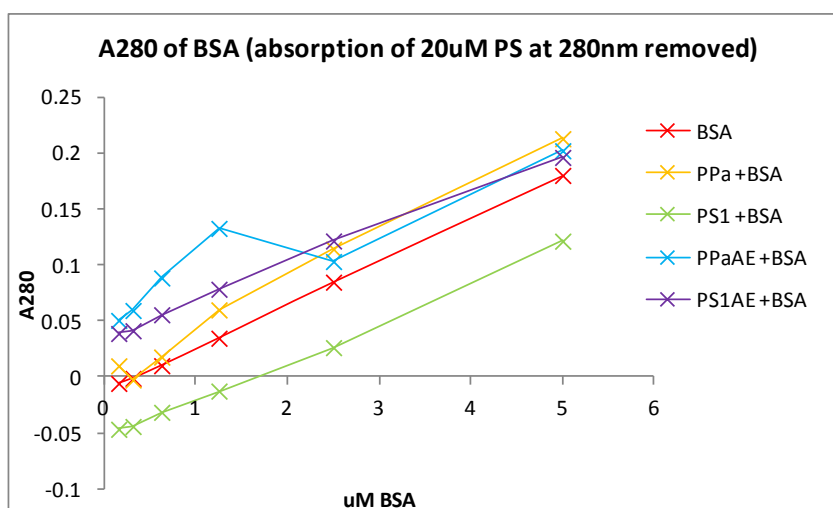
5.2.10.4 Interference of PS with the Bradford and BCA assays.

As with Cy5.5 an alternative way to measure protein could be via BCA or Bradford assay, (see 5.2.3.2)Figure 52. The interference of PS with these assays is presented in Figure 76. It is shown, as with Cy5.5, the BCA assay is unusable due to absorption spectral overlap whereas the Bradford assay is less affected but again ineffective coomassie binding (seen in gel formats) has been shown for highly conjugated PIC.

Figure 75 : Evidence for the quenching of endogenous protein absorbance by PS.

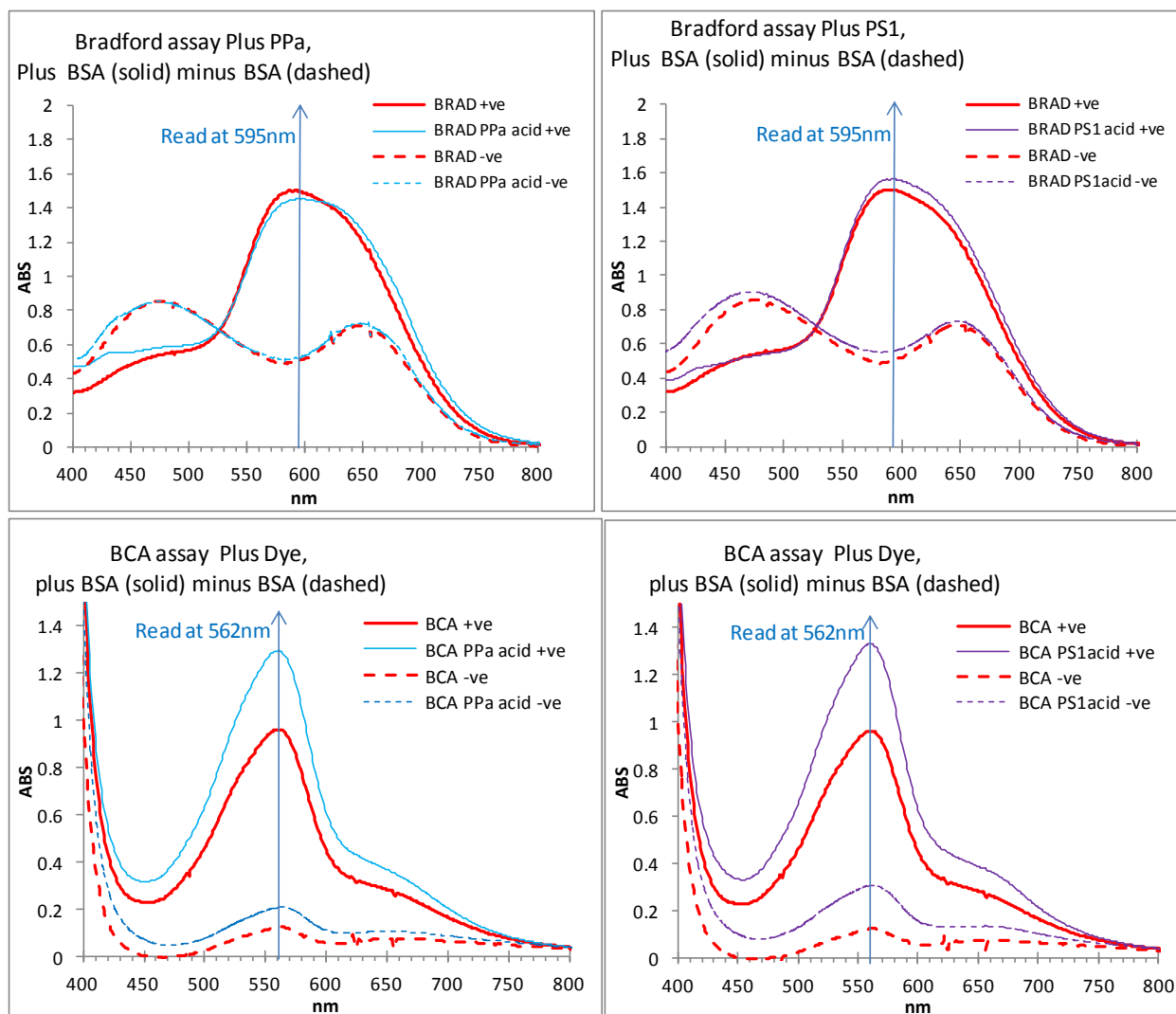


BSA was incubated at different concentrations in water with a constant concentration of PS over molar ratios relevant to conjugation efficiencies. The absorbance spectra of each mixture was read alongside the spectra produced by the 20 μ M PS alone -shown by a black dotted line.



Data above plotted as the absorbance at 280nm (minus the contribution of the relevant 20 μ M PS) over BSA concentration. In red is the same dilutions of BSA with no PS present.

Figure 76 : Interference of PS with the Bradford and BCA assays.



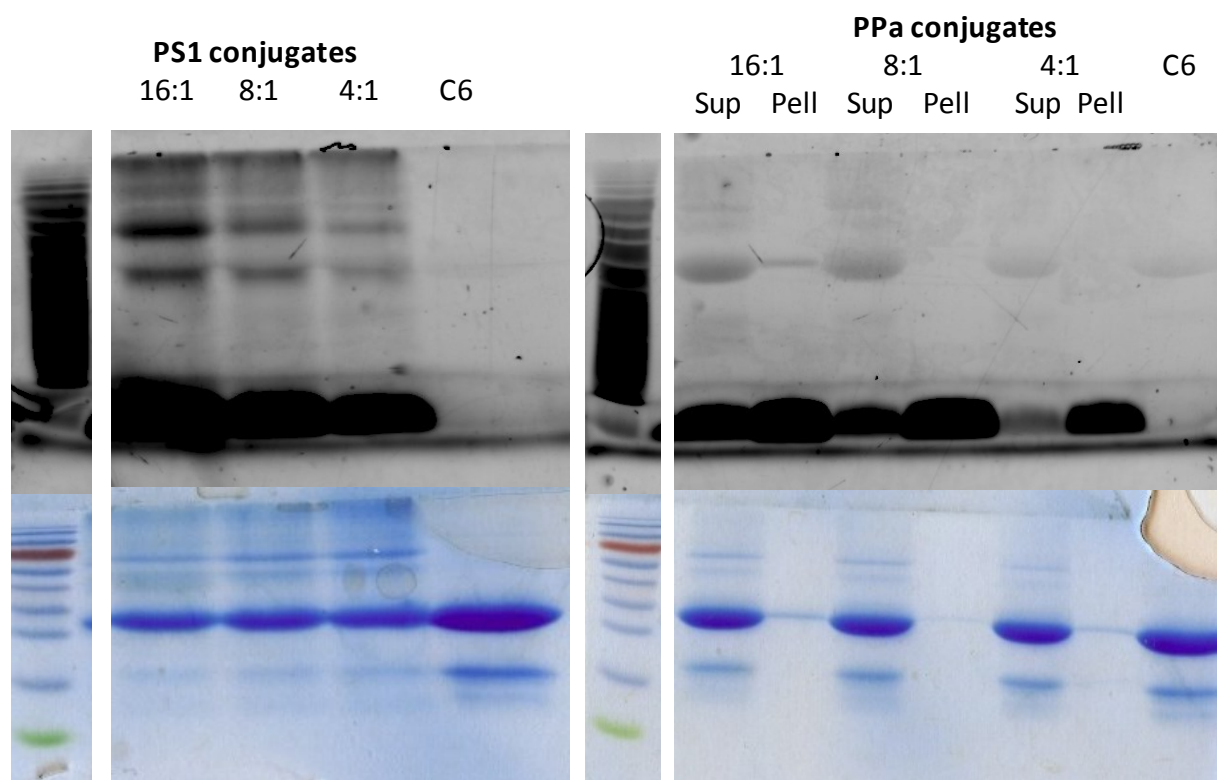
PPa acid or PS1 acid diluted into either BSA in H₂O or H₂O alone. PS was at 10 times molar excess over the protein. BCA or Bradford assay were used on these solutions as normal and absorbance spectra were read alongside solutions with no PS.

5.2.11 Evaluating conjugate binding on native antigen.

Despite the conjugate not being pure, it can be used to look at native HER 2 binding as any free PS not attached to the bound antibody should be washed away before analysis. As some free PS could be taken up into the cell non-specifically, an in-direct method against an antibody tag and not endogenous PS fluorescence was used to study binding. For these experiments new conjugations were set up at PS:C6.5-k ratios of 16:1, 8:1 and 4:1, after reaction the mixtures were dialysed into PBS then subjected to centrifugal force to remove any insoluble material in a pellet. The SDS Page gels of the PIC are shown in Figure 77.

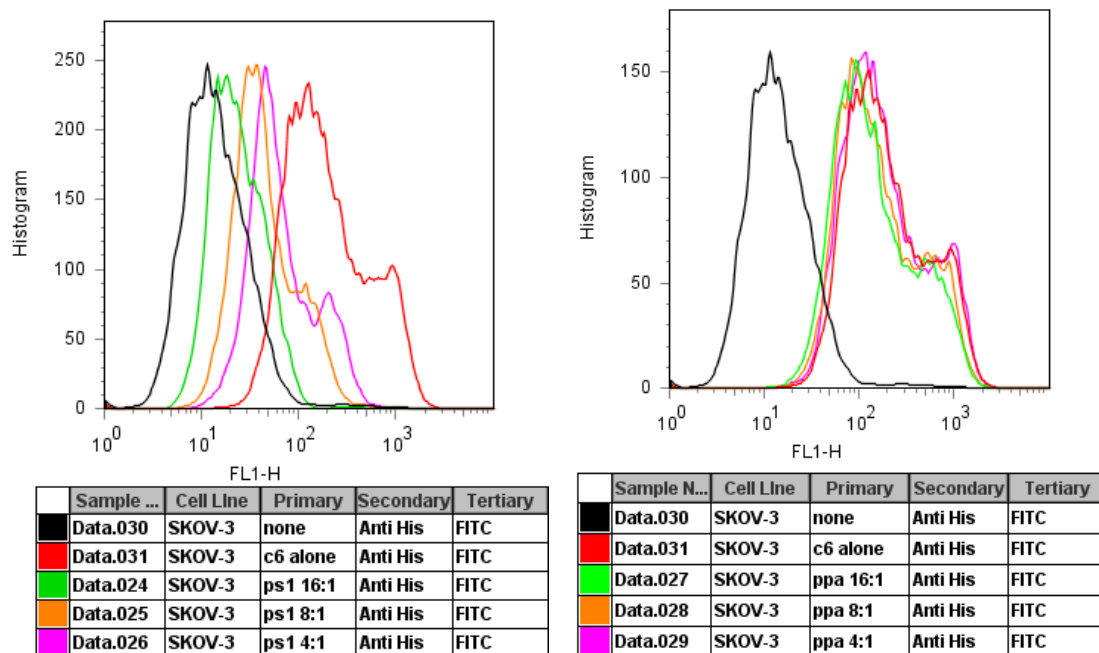
Figure 78 shows the results from the native antigen binding experiment set up as in 2.1.5.5. All the same samples were also incubated with U-87 cells that do not express any antigen and were all negative for any shifts (data not shown). All conjugates were incubated at levels predicted to be 60nM if no protein was lost in the conjugation and dialysis procedure. Each conjugate was separately investigated with either a secondary antibody against the His or the Myc tag on C6.5-k. Samples assayed against the C6.5-k His tag shows all three PPa PIC bind as well as un-conjugated C6.5-k but PS1 PIC show a decreased binding efficiency with increased conjugation efficiency, with the 16:1 PSI-PIC showing a considerable loss. The same samples assayed against the C6.5-k Myc tag show a similar pattern for PPa-PIC but a different pattern for the PS1-PIC; here it is only the 16:1 PS1-PIC conjugate that shows a loss in binding and the extent of the loss is greatly reduced. There could be a number of reasons for a loss in PIC binding; PS conjugation may be interfering with tag recognition and/or antigen recognition, this could be either via a steric interaction or PS quenching when brought into close contact with the FITC on the tertiary antibody. The change in binding patterns between the different tags does suggest a loss of tag rather than antigen recognition. The Myc tag contains a Lysine residue and the Histidines that make up the His tag are both possible PS conjugation sites. Native tag availability could be tested by retention on anti Myc or anti HIS columns similar to those used for purification. A purified PIC would allow use of the PS endogenous fluorescence to visualise binding more directly.

Figure 77 : PPa/C6.5-k and PS1/C6.5-k PICs used for native anitgen binding experiment.

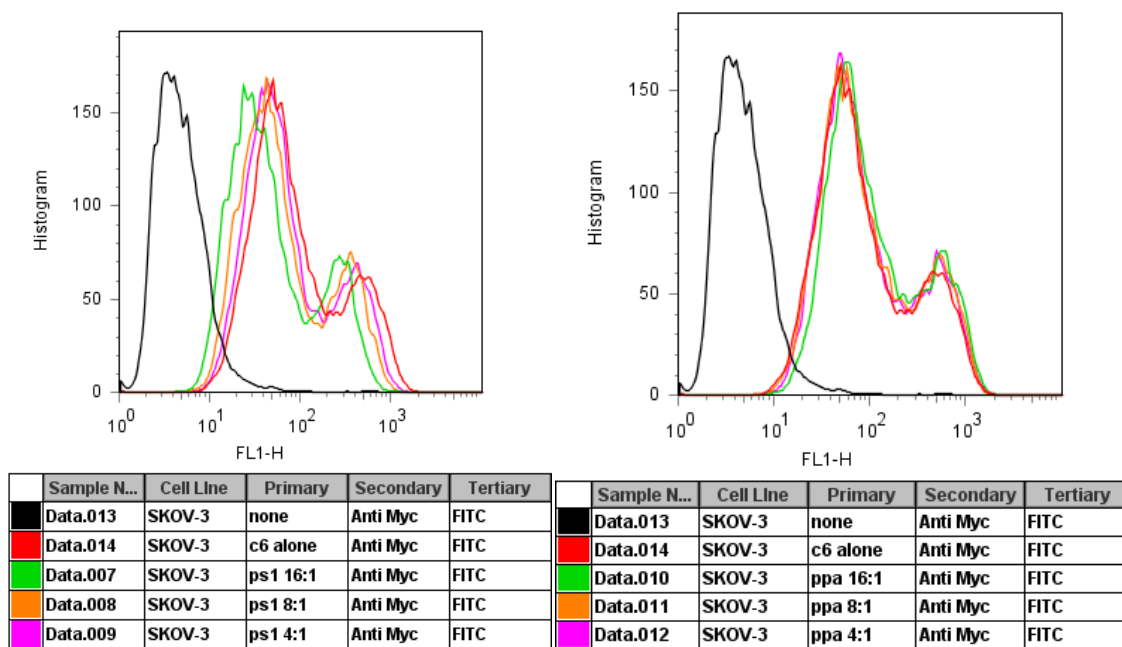


Two sets of conjugation reactions carried under conditions mentioned in 2.1.6.5 with C6.5-k and either PS1 AE or PPa AE. For each set 3 conjugations were carried out in parallel with PS at 16:1, 8:1 and 4:1 over C6.5-k. SDS PAGE gels are presented showing separation of PIC samples alongside the predicted equivalent level of un-conjugated protein. The gels are shown un-stained and imaged under UV light and subsequently stained with coomassie. . (C6.5-k shortened to C6 in figure).

Figure 78 : Native antigen binding experiment of PPa / C6.5-k and PS1 / C6.5-k PICs.



All PIC from Figure 77 were incubated at levels predicted to be 60nM if no protein was lost in the conjugation and dialysis procedure, and followed by secondary mouse anti His and tertiary FITC anti mouse as described in 2.1.5.5. . (C6.5-k shortened to C6 in figure).



All PIC from Figure 77 were incubated at levels predicted to be 60nM if no protein was lost in the conjugation and dialysis procedure, and followed by secondary mouse anti Myc and tertiary FITC anti mouse as described in 2.1.5.5. . (C6.5-k shortened to C6 in figure).

5.3 Discussion

This chapter presented work on the detailed analysis and optimisation of the conjugation of photosensitisers to the antibody fragment C6.5-k. The chosen conjugation strategy makes use of the numerous endogenous reactive ϵ -amines exposed on the antibodies surface, for this reason *in silico* modelling was carried out to get an idea of the 3D arrangement of these reactive groups. From this model, the lysines residues were seen to be well spaced around the molecule, this should help the final photo-immunoconjugates (PICs) avoid PS quenching upon high loading. It was noted at this point however that one of the lysine residues was to be found within the centre of the CDR region – a region important for antigen recognition. This 3D model was also analysed within the PROPKA server which predicts the pK_a and solvent accessibility of amino acids of interest, and also therefore their reactivity. PROPKA predicted that 9 of the 12 lysines were almost entirely solvent accessible and 3 were ~30% buried in the proteins core, including the lysine in the CDR, hopefully discouraging its conjugation. The prediction that the pK_a of the lysines was a pH unit higher than free lysine also had implications on the optimisation of the conjugation procedure and the pH used. (NB: The model did not include linker or tags, therefore it didn't consider the lysine found inside the Myc tag which brings the total lysines in the sequence to 13).

To help develop and understand the conjugation reaction the extent to which an optimal highly water soluble dye could be conjugated to C6.5-k was investigated. Conjugates were produced and successfully purified through a semi-permeable membrane. Conjugation ratios were obtained of up to 10 dye molecules per C6.5-k when quantified in a modified solvent system (8M Urea) that was able to reduce the dye spectra shifts induced by conjugation. C6.5-k/Cy5.5 conjugations of final efficiencies 4:1, 2:1 and 1:1 (ratio of PS to Ab), were shown to all retain their ability to bind native antigen expressed on cells, and intracellular conjugate showed a widely distributed punctuate pattern that corresponds to the endosomal pattern seen with other anti-HER2 antibodies or similar PICs (Carcenac et al., 2001; Savellano and Hasan, 2005). In addition, after media containing the PIC was removed from the cells there was a prolonged high level retention of the PIC many hours after removal of solution. A hopeful sign toward successful PIC tumour retention *in vivo*.

In cultured cells expressing low or moderate levels of HER2, the internalisation half-life ($t_{1/2}$) (time it takes to internalise half of the molecules) is reported to be in the range of 6–10 hrs, whereas in cells highly over-expressing HER2 the $t_{1/2}$ could be 24 hrs or longer (Sorkin and Goh, 2009). As cell surface expression levels of HER2 have been shown not to be affected by binding, uptake levels of the antibody after antibody binding to HER2 are likely to be similar to that of the endogenous HER2 internalisation. This has already been seen with Trastuzumab and C6.5-k(IgG) where upon binding of HER2 uptake rate was similar to that of naked HER2 (Austin et al., 2004; Rudnick et al., 2011). This matches the uptake rates seen with our C6.5-k/Cy5.5 PIC as well as other anti-HER2 or similar PIC data in the literature (Kuimova et al., 2007; Savellano and Hasan, 2003).

Conversion of the new water-soluble PS to the active form could only be carried out with PS1 as the synthesis of PS4 active ester (AE) was more complicated and could not be completed in the time frame of this work. When the active ester forms of PPa and PS1 were compared to their acid forms differences were seen; PPa AE was a lot less soluble than its acid form and showed visible precipitate in all aqueous solution. PS1 AE dissolved well and was comparable to the acid form, if anything it showed shifted but more preferable spectra in physiological solution than its acid form. The difference between PPa AE and PS1 AE is now even greater in terms of aqueous solubility. Under all conjugation conditions investigated, C6.5-k was more highly conjugated when reacted with PS1 than with PPa, validating the initial idea behind the development of more water soluble PS for conjugation. This was shown via SDS PAGE gel analysis, both free PPa and PS1 were shown to fluoresce to a similar extent in the gel environment but the band corresponding to PIC fluoresced much more brightly for PS1-PIC than for PPa-PIC, in addition it migrated more slowly in the gel but this could be over-enhanced by the PEG chains ability to attract water molecules. However without further analysis, for example mass spec of the purified product, it is still possible that both PIC are conjugated to the same extent but there is significantly more quenching of PPa than for PS1 once conjugated, whether between itself or by interaction with the proteins surface, the larger PEG chains on PS1 possibly keeping the PS away from the proteins surface. It has already been seen that for C6.5-k/Cy5.5

PIC fluorescence intensity as seen on a gel was more effected by quenching than the PICs absorbance profile.

There is a lot of work presented in this chapter on the attempted removal of free PS from PIC solution, no reliable method was found during the short period of study presented here. It was shown that in the case of semi permeable membrane purification it is not just non-covalent interactions between PS and protein but the inherent inability of the PS on its own to cross a semi permeable membrane that causes a purification problem. When purification was changed to a ion-exchange based system a lot of the free PS was successfully removed but the PIC became trapped in the matrix of the column. In future size or affinity based chromatography along with a supplemented solvent system with a different matrix could be the solution.

Lack of purification of the PICs hampered any further work that could be done on development of a quantification method as results from spectral analysis or in-gel fluorescence quantification were shown to be unreliable. Purification of the conjugate would allow more reliable analysis and even the possibility for mass spectrometry experiments which would resolutely show the conjugation efficiency without any bias of spectral quenching. Many of the experiments performed hinted that bound PS could be quenching the endogenous protein absorption, compromising any spectral quantification of the protein concentration. Two other options for protein quantification were studied; the BCA assay and the Bradford assay. The BCA unfortunately was affected by PS spectral interference. The spectral interference of PS with the Bradford assay was lower but it is a low sensitivity assay and SDS Page gels have already shown there may be a problem with high level PS conjugation inhibiting binding sites for coomassie. For quantification of PIC most published articles, see 5.1.4, use a similar spectral method to the one described herein but other published options include use radio-labelling of the antibody, (Carcenac et al., 1999; Vrouenraets et al., 2002) or the use of the Lowry method of protein detection (cupric ion reaction with peptide bond) (Goff et al., 1991).

Many published PIC papers show spectral changes associated with the PS upon PIC formation; Savellano and Hassan showed a red shift of PIC over free PS after direct conjugation to the antibody (Savellano and Hasan, 2003), Goff and colleagues showed blue spectral shifts of

5nm from the PIC compared to free PS (Goff et al., 1991) and Del Governatore and colleagues showed that once conjugated PS Soret band is reduced and Q band is red shifted by ~12nm (Del Governatore et al., 2000b). Interestingly these last two papers used the same PS and the same antibody but conjugation occurred via a different linker. The spectral changes can not only affect quantification but PIC potency, many papers show a direct loss in PS photophysics when conjugated, see 5.1.4, including Savellano and Hasan (Savellano and Hasan, 2003) who looked at fluorescence decay signal as an indication of a PS' ability to intersystem cross, they showed a reduction in maximal intensity as well as a longer lived decay signal in PIC vs. free PS, which became more exaggerated as PS loading increased.

Despite being impure, some PPa PICs and PS1 PICs were produced of varying but unknown conjugation ratios and were tested for native antigen binding. The PPa conjugates all exhibited retention of binding compared to the un-conjugated Ab, but PS1 conjugates showed a loss of antigen binding as conjugation ratio increased. There were some questions remaining over this experiment and whether it was actually loss of tag recognition. The purification and accurate determination of C6.5-k content in the sample is required before any reliable conclusions can be drawn. An alternative binding experiment independent on tag recognition or fluorescent detection, for example by surface plasmon resonance (Biacore) would be preferable but that also requires accurate protein concentration determination.

5.3.1 Chapter conclusions

Key proposition put forward from work;

Activated PS1 (PS1 in its NHS ester form) shows further increased aqueous solubility compared to the activated form of PPa, and as hoped, PS1 greatly improve conjugation efficiency to the antibody fragment C6.5-k. There remain problems with purification of the unconjugated PS from the mixture and hence attempts at quantification of the conjugation efficiency were hampered. Using a water soluble activated model dye it was to be possible to conjugate around 10 molecules of dye onto each C6.5-k molecule with retained antigen binding, but this needs to be confirmed by non-spectroscopic methods. Any further increases in solubility of the PS may provide these high conjugation ratios seen with Cy5.5 alongside more effective purification, for this reason the successful activation of PS4 for use in this work would be of interest.

Main question raised from this work;

If the cellular uptake of PS1 and PS4 were re-established on a similar level to PPa, for example by conjugation to an antibody fragment and subsequent manipulation of different uptake pathways (antibody internalisation), would this make them significantly more toxic than PPa? Other questions will arise over whether the PS or the antibody would provide the dominant determinants in uptake and localisation. Once internalised would conjugation be retained or will PS and antibody return to their independent pathways? Successful purification of the PICs and application into many of the assays developed in previous chapters will help answer these questions.

6 General Discussion.

6.1 Original aims and objectives

Targeted PDT is an emerging treatment area that promises to overcome the limitations of current, non-targeted PDT. Most photosensitisers (PS) for PDT are poorly soluble and could be improved if made more compatible with an aqueous protein environment. The work presented in this thesis showed that PPa-based photosensitisers could be chemically modified to increase their solubility and this in turn could facilitate bio-conjugation with a view to their use as antibody targeted drugs. Increasing PS solubility can also alter PS potency and intracellular localisation; this must therefore be taken into account when assessing their utility as conjugates. The objectives of this work were to establish systems to characterise the free PS (potency, localisation, etc.) and measure these properties in the context of targeting to learn what the important attributes are. These objectives were not fully met during the work presented because pure photo-immunoconjugates were never obtained. Both PPa and PS1 derived PICs contained a high amount of non-covalently bound material which meant any comparison with the free PS was compromised. However, much was learnt about how manipulating the chemical properties of the free PS affects its solubility in physiological solutions and therefore its potency as a free PDT agent.

6.2 Photosensitiser solubility and potency

PPa is an excellent PS and serves as a good building block for improved derivatives. It is often stated as being a very effective photosensitiser, and although not an absolute indication of efficiency, the singlet oxygen quantum yield for PPa was measured to be 0.5 (Stamati et al., 2010). Compared to other photosensitisers this is at the higher end of the range, the range being stated as ~0.3 to 0.5 (Plaetzer et al., 2009) or 0.009 – 0.83 (Bonnett, 2000). By no means does this exclusively predict PS potency, efficient Type I reactions can also occur with PS, for example Ding and colleagues (Ding et al., 2011) presented some work in which the micelle carrying the PS could manipulate the tendency for either Type I (radicals or electron transfer) or Type II (singlet oxygen) reactions to occur. They showed similar potencies in three cell lines

under air but increased potency for the type I preferable PS in cells under argon, they suggest such manipulation of the PS would make it more effective in a hypoxic tumour environment.

The considerable influence of multiple systemic and pharmacokinetic factors, see 1.3, will always play a role in PS potency. For this reason direct comparison of potency of a PS is difficult, numerical physical measurements of a PS are the most often used but there is a lack of any single comparable model for photosensitiser clinical efficacy, especially as manipulation of clinical application factors such as laser dose, see 1.3, can also enhance or diminish potency dramatically. Berlanda and colleagues (Berlanda et al., 2010) tried to compare a series of clinically relevant PS including Hypericin, Foscan, AIPcS4 and ALA in the same *in vitro* PDT model in the same cell line (A431 cells). Multiple factors were investigated including cellular uptake, caspase activation and calculation of an IC_{50} both for dark cytotoxicity and cytotoxicity with laser activation. The results for the IC_{50} values range from $0.03\mu M$ to $393\mu M$. The IC_{50} values presented in this thesis (see 3.2.2.3) for PPa range from $1.97\mu M$ (at $0.33J/cm^2$) to $0.06\mu M$ (at $23.22J/cm^2$) with no observed dark toxicity at any light dose at PS up to $100\mu M$. Compared to the values presented in Berlanda et al (Berlanda et al., 2010) dark toxicity is greatly improved for PPa over these PS and at higher laser doses PPa is nearly as potent as the best PS they studied especially considering the laser dose used by Berlanda et al was $1.5 J/cm^2$ and PS were incubated with the cells for 20 hours in serum free media. Despite this comparing the data presented in this thesis to that presented in (Berlanda et al., 2010) is flawed. As discussed for SKOV-3 in 3.1.1.2 all cell lines are inherently biased. It may also be worth remembering that in this *in vitro* situation, one has removed some of the more complicated but highly influential effecting mechanisms, and it can be misleading to set certain variables at set levels. The question remains what parameters really would be important for when PDT is chosen for use in the clinic? What would a clinician need to know? One may have to resort back to more comparable ‘optimum physiochemical factors’ as an indication of the potential of a PS that can be brought out with clinical optimisation. These ‘optimum physiochemical factors’ could then be presented alongside some thorough pharmacokinetic dosimetry data for PS blood clearance and tissue distribution in humans.

The work presented in this thesis showed the novel PS1 and PS4 to be more water soluble than their parent compound (PPa), the molecules showed less spectral shifts in a range of physiological environments which suggests improved photo-physical characteristics for effective traditional PDT. However in an *in vitro* non-targeted PDT cancer cell model, initial data suggested these new more soluble PS became less potent. In 3.1.3 and 5.1.4 ways in which other researchers have approached the creation of more water soluble PS was discussed, both the addition of a positive charge (PS4) and PEG chains (PS1) are well supported methods to improve solubility, however the literature results on overall potency from more water soluble PS like these are variable. Unfortunately our new PS lost some potency, significant amounts in the case of PS4. However this may change *in vivo* and work studying blood clearance and tumour uptake in an *in vivo* mouse transplanted tumour model has been carried out by other members of this lab (unpublished data) and did show despite lower tumour uptake PS4 had improved specificity over PPa and PS1 and increased blood clearance times, PPa exhibited the highest tumour uptake but it was equally specific to PS1 which cleared from the system twice as fast. Free PS have not yet been tested against each other for their potency in this model.

Despite showing increased solubility in aqueous solutions the new PS presented some fluorescent spectra that could suggest aggregation. Aggregation is normally associated with a loss in photophysical efficiency, and work investigating a polymer carrying a number of closely linked PPa molecules did show a decrease of the fluorescence, intersystem crossing, and singlet oxygen quantum yields (Helmreich et al., 2005), however other work carried out by Dairou and colleagues (Dairou et al., 2002) showed in fact in aqueous solution the dimers formed by PPa, although exhibiting shifted and loses in intensity of their fluorescent spectra, maintained their singlet oxygen quantum yield and therefore are still effective photosensitisers. This work also has implications for the spacial arrangement of PS over a protein surface upon antibody conjugation.

6.3 Photosensitiser uptake and cell kill mechanisms

Investigation into the mechanism of cellular uptake and localisation of the modified PS showed that despite being as potent as PPa, PS1 intracellular concentrations were much lower

suggesting PS1 actually exhibits improved death per dose over PPa, this was hypothesised to be due to its enhanced intracellular photophysics. Despite showing significantly different uptake kinetics, PPa and PS1 at lethal doses seem to be able to initiate similar mechanisms of action, likely due to their similar intracellular localisations. The reduced potency of PS4 over PPa seems to be driven by both the substantially reduced cellular uptake, and an altered cellular localisation, although the potential for PS4 upon antibody conjugation still remains high.

There is a lot of evidence to support increased water solubility reducing cellular uptake, see 3.1.3, but one is reminded of the importance of other factors for potency, by Henderson and colleagues (Henderson et al., 1997), who showed that *in vivo* PS with decreased solubility would show increased absolute tumour uptake, however this potency would only increase alongside decreased solubility up to a point despite further increased tumour uptake. After a point the potency was limited by other more influential factors like internal localisation and photophysical efficiency. In relationship to the work presented herein the addition of a PEG or a cationic charge could manipulate internalised PS efficiency in a number of ways, for example cationic PS have been shown to more likely localise in the mitochondria (Dummin et al., 1997; Woodburn et al., 1991), although in our case this seems to not be the case. PEG chains can speed up intracellular diffusion (Suh et al., 2007) or as Hamblin and colleagues (Hamblin et al., 2001) showed, when they PEGylated a series of Chlorin e6 photosensitisers, the mechanism of PS can be changed from majority type II (singlet oxygen) to majority type I (radicals or electron transfer), when they tested these PS *in vivo* PEGylated PS were more potent than the non PEGylated controls.

Cell death mechanisms activated upon illumination presented mixed results for the novel PS. From the limited work carried out, it suggested the multiple cell death mechanisms that were activated for PPa and PS1 were similar but the balance between these could be changing. In terms of clinical PDT the type of cell death required for effective therapy is not always clear. It is also very likely that throughout the tumour and its microenvironment cell death mechanisms will be different due to changes in local PS dose, light dose, and tumour environmental factors. Despite this however various rules are often stated for example Mikes and colleagues (Mikes et al., 2007) say that “*Oxygen-conserving low fluence rate protocols favouring apoptosis and should be recommended when PDT is applied for the curative treatment of in situ neoplasia*

or/and as a palliative de-obstructing treatment modality. In both cases, a rapid shrinking of the tumour mass is expected and apoptosis without accompanying inflammatory reaction and swelling should be favoured above complete tumour eradication. On the other hand, when the goal of PDT is to cure an infiltrative tumour that is possibly already micro-spreading beyond the margins of the illumination spot, necrosis with its accompanying inflammatory reaction may be recommended. However, the protocols employing high fluence rates should be finely tuned, since oxygen-depleting regimens may significantly limit treatment outcome.”

There exist factors that can be brought up to dispute this idea but as a generalisation it may be worth bearing in mind. It is now understood that apoptosis can manipulate the immune system just as necrotic cells can be cleared away quietly via phagocytosis, phagocytosis itself is a complicated process that can also manipulate the immune system (Galluzzi et al., 2012; Krysko and Vandenabeele, 2010; van der Most et al., 2008; Zitvogel et al., 2010). So although the preference of apoptosis vs. necrosis isn't always clear - recently the interest in the recruitment of immunogenic mediators to improve long term PDT efficiency, see 1.2.4, has revitalised the area. One unifying factor seen frequently with PDT is the idea of soluble attraction and danger signals (Damage-Associated Molecular Patterns, DAMPs) being released and/or secreted by both apoptotic cells and primary and secondary necrotic cells. There is also a body of work to back up theories that PDT process can induce photo-oxidative changes that provide new antigens for recognition and therefore make the cells more immunogenic (Galluzzi et al., 2012; Korbélik, 2011). The requirement of an antigen for recognition in this process was shown to be important in mouse models transplanted with tumours with or without engineered expression of the foreign jellyfish GFP protein, a difference in cure rate was seen in that the tumours that GFP were much more effectively cured (A P Castano et al., 2006).

6.4 Soluble PPa for bio-conjugation

Activated PS1 (PS1 in its NHS ester form) showed further increased aqueous solubility compared to the activated form of PPa, and as designed, PS1 greatly improve conjugation efficiency to the antibody fragment (C6.5-k). There remain problems however with purification of the un-conjugated PS from the mixture and hence attempts at quantification of the conjugation

efficiency were hampered. Using a water soluble activated model dye it was shown to be possible to conjugate around 10 molecules of dye onto each C6.5-k molecule with retained antigen binding, but this should be confirmed by non-spectroscopic methods. It would be interesting to see, once pure, the ratios achieved with the PS1 conjugates which may now be providing these same high conjugation ratios due to PS1s enhanced solubility, however a yet further increase in solubility may be required for effective purification, for this reason the successful activation of PS4 for use in this work would be of interest.

The problem of PIC purity is evident throughout the progression of PIC development. Different approaches to address this include attempts to pre conjugate the PS to a polymer which can then be treated to more harsh solvent conditions to facilitate purification before conjugation, or the adaptation of the conjugation method and the antibody itself to take the antibody to the limits of its solubility in organic solvents, see 5.1.4. In terms of purification both PEGylation and the introduction of positive charges have been used as tools to improve this. The PEG chains on PS1 are hypothesised to increase solubility as well as sterically hinder binding to any hydrophobic patches on the protein, in (Savellano and Hasan, 2003) PEG were indeed shown to help effective PIC production. Reports also cite cationic PS as providing less contamination with non-covalently bound PS (Sutton et al., 2002) and in recent PIC work presenting very successful and pure PIC; it was cationic PS that were used (Palumbo et al., 2011).

Adaption of the purification techniques to manipulate the free PS and PIC into an environment where their differences, for example in size, could be used for purification purposes is still an alive and viable option. Although of course, as was the initial theory of this work, the re-design of PS themselves to make them even more water soluble is also a possible solution. When structures were analysed with computer software to predict lowest energy conformers (data not shown) , all showed one group projecting either above or below the ring. Perhaps redesigning PS1 to include a top and bottom PEG extending above and below the plane of the molecule as suggested by Dr G. Yahioğlu would be worth consideration.

6.5 Summary of work

Introduction of chemical factors to increase the aqueous solubility of a photosensitiser can change both the cellular uptake and localisation and hence alter the modality and efficiency of cell death in cancer cells treated with PDT. Despite significant reductions in cellular uptake of more soluble photosensitisers a more effective retention of photophysical efficiency of the more soluble photosensitiser (PS1) could more than compensate for reduced uptake. However when final intracellular localisation was changed alongside the significant reduction in cellular uptake (PS4) possible retention of photophysics could no longer provide any potency of the administered PS. The introduction of increased water solubility to the photosensitiser was also able to improve conjugation to an antibody fragment. Once purified if antibody driven uptake of the PIC will help override the loss in cellular uptake seen with the free PS the potential for selective and highly effective PDT is a real possibility.

6.6 Future work

During this project the aims were to investigate the effect of increased solubility on photosensitiser efficiency with an emphasis on antibody conjugation for targeted-PDT. Previous work had already shown targeted PDT exhibits enhanced specificity and cell kill over conventional, non-targeted PDT. This project therefore had a focus on elucidating the molecular mechanisms behind this improvement, particularly in respect to the processes of intracellular trafficking and the mechanism of the cell death. Increased solubility of the free PS was shown to have significant effects on these properties and so once pure conjugates can be made, it will be of interest to test the PIC in the same experiments as the free to find out what further differences or similarities are seen upon antibody conjugation. It is likely if the cellular uptake of PS1 and PS4 were re-established on a similar level to PPa this would make PS1 and PS4 significantly more toxic than PPa.

The hypothesis that antibody conjugation can be used to control and override natural uptake and localisation patterns of any photosensitiser could be of particular interest for the greater acceptance of PDT in the clinic. Once an understanding of the respective contribution of PS and

antibody to the cellular localisation and cell death pathways initiated by a photo-immunoconjugate has been improved, knowledge could be utilised for a more predictable and controllable PDT experience, i.e. a more intelligent modulation of PDT efficiency via the effective combination of antibody, targeted cell surface receptor, conjugation technique and synthetically optimised photosensitisers.

The drug discovery field is currently thriving with interest in antibody drug conjugates in general. It was over a half a century ago that the first antibody drug conjugates were used and radio-labelled antibodies showed their exquisite potential for targeting specific tissues within the human body. However the delivery of successful antibody drug conjugates as clinically approved drugs has taken a long time and alongside advances in linker technology and increased drug potencies, only now in the last few years a successful class of biopharmaceuticals with an exciting and growing pipeline of candidates has been seen; there have been numerous reviews on this subject over the last few years three of which with specific emphasis on the history of the ADC field and its recent explosion are detailed here (Adair et al., 2012; Goldenberg and Sharkey, 2012; Hughes, 2010).

Personally an area of particular potential is the use of photosensitisers in contexts out of the restriction of the localised laser light, as discussed in 1.2.6, the use of ultrasound in Sonodynamic therapy (SDT). (Kuroki et al., 2007; Ma et al., 2009; Mitragotri, 2005; Yamaguchi et al., 2011). The development of quantum dots or other nanomaterials that act as energy transfer molecule so systems can use ionising radiation for example X-rays or gamma rays which can penetrate much deeper into tissue in order to activate the photosensitiser. (Juzenas et al., 2008; Liu et al., 2008; Samia et al., 2006). Or PS conjugates that can convert the energy from radiotherapy (Chen and Zhang, 2006), or magnetic resonance imaging scans (Huang et al., 2011; Vaidya et al., 2008). After all porphyrin molecules are incredible machines for the conversion and delivery of packets of energy with possibly unlimited potential, as well as curing our cancer they could be found in our computers as molecular electronic components (Jurow et al., 2010) or provide our energy in solar panels (Campbell et al., 2007).

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