Targeted Photodynamic Therapy of cancer using photoimmunoconjugates based on pyropheophorbide $\alpha$ derivatives

By

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For my family, especially my grandfather...
“I never did anything worth doing by accident, nor did any of my inventions come by accident; they came by work”. Thomas Alva Edison
Abstract

Photodynamic therapy (PDT) utilises light, oxygen and organic macrocycles, called photosensitisers, to produce reactive oxygen species that can kill malignant cells. Conventional PDT is associated with side effects that have stifled its advance and widespread use. These include low tumour selectivity, slow blood clearance and poor formulation. We proposed that an antibody fragment could be used to carry the photosensitiser to the target cells, significantly overcoming these limitations.

Pyropheophorbide-a (PPa) was synthetically modified to enhance its water solubility obtaining two compounds, PS1 and PS4 each more water soluble than PPa. The use of Sonogashira coupling and short polyethylene glycol chains gave PS1, whereas the use of Suzuki coupling and a single positive charge gave PS4. The singlet oxygen quantum yields of these were improved compared to PPa with that of PS4 being 1.5 times higher than PPa. The in vitro characterisation of PPa, PS1 and PS4 using cytotoxicity assays did not correlate with their photophysical characterisation. PS4 was significantly less potent than PPa and PS1 on SKOV3 and KB human cancer cell lines. Confocal microscopy aided further characterisation using stains for intracellular organelles. PS1 was found to localise primarily in the ER and Golgi apparatus, similarly to PPa, while PS4 was found to localise mainly in the lysosomes.

PS1 was conjugated to C6.5(-k), an anti-HER2 single chain variable fragment (scFv) using lysine coupling, to obtain a photoimmunoconjugate that was characterised in vitro and subsequently in vivo. In vitro characterisation showed increased potency and specificity but non-specific cell death attributed to the non-covalently bound photosensitiser was observed. However, in vivo therapy studies showed that the C6.5(-k)-PS1 photoimmunoconjugate could be used to cure SKOV3 subcutaneous tumours in nude mice, validating the use of targeted PDT as a successful targeted therapy with the potential to lower the effective drug dose and minimise side effects.
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 that 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.

Ioanna Stamati

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<th>Definition</th>
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<tr>
<td>ADC</td>
<td>Antibody-drug conjugate(s)</td>
</tr>
<tr>
<td>AE</td>
<td>Active ester</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium per sulphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BCC</td>
<td>Basal cell carcinoma</td>
</tr>
<tr>
<td>BOP</td>
<td>(Benzotriazol-1-yl oxy)tris(dimethylamino) phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>BPD</td>
<td>Benzoporphyrin derivative</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement dependent cytotoxicity</td>
</tr>
<tr>
<td>CDRs</td>
<td>Complementarity determining regions</td>
</tr>
<tr>
<td>Ce6</td>
<td>Chlorin e6</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>Complete Media</td>
<td>DMEM (10 % FBS, 1 % P/S)</td>
</tr>
<tr>
<td>Cp6</td>
<td>Chlorin p6</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Dbα</td>
<td>dibenzylidenacetone</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N' Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCHU</td>
<td>Dicyclohexylurea</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N'-Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMPM</td>
<td>dimethylpimelimidate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Federal drug administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HP</td>
<td>Haematoporphyrin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>i.v</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ID/g</td>
<td>Injected dose per gram of blood/tissue</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>iPrOH</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-1-thio-β-D-galactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Loading buffer</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LR</td>
<td>Loading Ratio</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MBR</td>
<td>Mitochondrial benzodiazepine receptor</td>
</tr>
<tr>
<td>MePPa</td>
<td>Methyl Pyropheophorbide a</td>
</tr>
<tr>
<td>min/s</td>
<td>Minute/s</td>
</tr>
</tbody>
</table>
MS Mass spectrum
mTHPC meta-tetrahydroxyphenylchlorin
MW Molecular weight
MWCO Molecular weight cut off
NCB Non-covalent binding, non-covalently bound
NHS N-hydroxysuccinimide
NMR Nuclear Magnetic Resonance
OG Octyl glucopyranoside
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PEG Polyethylene glycol
PIC(s) Photoimmunoconjugate(s)
POTE Polyoxyethylene 10 tridecyl ether
PPa Pyropheophorbide a
PPS Pyridiniok1kpropanesulfonate
PS(s) Photosensitiser(s)
QSAR Quantitative structure activity relationship
RIC(s) Radioimmunoconjugate(s)
RIT Radioimmunotherapy
RT Room temperature
scFv Single chain variable fragment
SDS Sodium dodecyl sulphate
SIP Small immunoprotein
SM Starting material
SN Supernatant
TC Tissue culture
TEA Triethylamine
 TEMED N, N, N', N'-tetramethylethlenediamine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TPDT</td>
<td>Targeted photodynamic therapy</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultra violet/ visible</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Variable heavy chain</td>
</tr>
<tr>
<td>V&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Variable light chain</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction
1.1 What is Photodynamic Therapy (PDT)

It is a bitter truth that most of us have lost a loved one to cancer. In Europe, an alarming one in three people will get cancer in their lifetime (Europa, 2009, Statistics, 2009). Yet, despite ongoing worldwide efforts in search for treatments and cures, countless people keep losing their fight with the disease, spend long periods suffering from side effects caused by existing treatments and see their quality of life deteriorating dramatically. The diversity and complexity of the various cancer types and characteristics make it difficult to find a single line of defence. Is there something that will kill all cancer cells more effectively? We consider that photodynamic therapy (PDT) may be a strong contender by selectively targeting malignant cells without damaging the surrounding healthy cells (Brown et al., 1999, Dolmans et al., 2003).

1.1.1 History

Photodynamic therapy is the tripartite process that occurs between a light-activatable compound, a ‘photosensitiser’ (PS), molecular oxygen – which is present in a biological cell – and light to produce biologically lethal species.

PDT is believed to have originated three to four thousand years ago in ancient India, with the treatment of vitiligo using plants containing psoralen that were ingested and then activated by exposing the sufferer to the sun. At around the same time, the Egyptians and Chinese were using the same treatment for vitiligo (Edelson, 1988, Epstein, 1990). The therapeutic properties of light go as far back as Herodotus in ancient Greece who used heliotherapy (sun treatment) as illustrated in the diagrammatic milestones of PDT in figure 1.1 (Epstein, 1990). The use of light without a chemical drug is referred to as direct phototherapy whereas indirect phototherapy takes place in the presence of both light and a sensitising agent (Bonnett, 2000).

In 1903, Danish Niels Ryberg Finsen won the Nobel prize in medicine for his work on treating diseases such as lupus vulgaris and small-pox using light (Nobel, 1903, Dolmans et al., 2003). Finsen is therefore considered to be the father of phototherapy, albeit direct phototherapy. Today, the remains of direct phototherapy are seen in the treatment of inflammatory skin diseases such as psoriasis and for killing microorganisms using UV light (Bonnett, 2000, Dolmans et al., 2003).
Even though the photodynamic effect was first observed and used thousands of years ago, systematic investigation only began in the early 20th Century. In 1903, von Tappeiner used eosin and visible light to treat basal cell carcinoma (Von Tappeiner et al., 1903, Bonnett, 2000, Dolmans et al., 2003). This was the first report of the use of PDT to treat cancer but the therapy was relatively unexplored in subsequent years until 1970s when Dougherty used fluorescein to treat in vivo mammary tumour implants, with the first clinical trials conducted in London in 1976 (Bonnett, 2000).

While PDT has advanced significantly since 1976 with the approval of several PSs for clinical use and is slowly becoming more accepted as a therapeutic modality and offered to patients, its adoption rate by physicians has been relatively low. Those who use PDT are still trying to ‘spread the word’ as well as optimise the therapy in terms of efficacy, practicalities and applicability. When considering the toxicities and side effects of chemotherapy it is perhaps surprising that PDT has not yet gained a wider acceptance (Mang, 2004).

It is interesting to point out that since 2001 there have been three times as many publications on PDT compared to the period 1990-1999 (http://apps.isiknowledge.com/).

**Figure 1.1 Historical milestones in the development of PDT.** Taken from (Celli et al., 2010).

### 1.1.2 Fundamentals of PDT

In PDT, the photosensitiser is administered (intravenously or topically) and following a time gap, termed ‘drug-to-light interval’, the site of interest is irradiated with light of appropriate wavelength (Brown et al., 2004, Celli et al., 2010). Three interdependent components need to come together to produce cytotoxic species leading to a therapeutic outcome. In the absence of light, an ideal PS should not exhibit any cytotoxic properties and this is defined as the dark toxicity, a property of each PS.

The production of the cytotoxic species is restricted at the site of activation where the three components are co-localised. Cells can undergo necrosis where they swell and lyse to
release their intracellular content causing inflammation or can undergo apoptosis where the cell shrinks and condenses and the nuclear content breaks up into fragments. Apoptotic cells get taken up by phagocytes clearing them from the body. Cells can also undergo autophagy where double membrane enclosed vacuoles are formed within the damaged cell to destroy some organelles or depending on the damage the entire cell (Hamblin, 2008)

Photosensitisers are commonly referred to as drugs but in fact they are not drugs in the conventional sense as they are not cytotoxic on their own (requiring light to be activated). Conventional PDT is considered to be a targeted therapy due to its bimodality which is one of the strengths of PDT. Light, can be delivered specifically to the site of interest in the form of non-thermal laser causing limited temporary damage to surrounding healthy cells which do not contain the photosensitiser. The primary cytotoxic species generated during PDT is singlet oxygen, a high energy form of molecular oxygen with a very short lifetime. This property of singlet oxygen restricts it to the site of generation, selectively targeting cells that both contain the PS and are illuminated with light (Brown et al., 2004). Engineering the PS to localise in malignant cells can lead to their death without affecting surrounding healthy cells (Brown et al., 2004). The bimodality of PDT is therefore important and only compares slightly with neutron capture therapy.

In an optimal setup, light of any wavelength would have been suitable to activate the PS. However, in the human body choice is restricted to red light due to the interference that will occur at most other wavelength regions from naturally occurring chromophores. These chromophores are melanin (present in the skin) and haemoglobin (present in the blood). Water can also absorb energy and these are shown in figure 1.2.

**Figure 1.2 Absorption spectra of naturally occurring chromophores.** This diagram shows the strong absorption of oxy- and deoxy-haemoglobin and melanin which are the main chromophores in the human body alongside water that absorbs further into the infrared. There is a gap-a therapeutic window- around 620 nm up to 1300 nm where neither show strong absorption. These wavelengths are suitable for PDT. Wavelength is plotted against absorption on a logarithmic scale. Taken from (Castano et al., 2004).
There is a therapeutic window between 600-800 nm where irradiation can take place without affecting these chromophores (Celli et al., 2010). Ideally, using a light source of wavelength beyond 630 nm would allow a greater percentage of the energy to reach the drug than at any other wavelength. Even though it is ideal in this respect, red light will still not penetrate the skin and tissue sufficiently to reach deep lying tumours. Scattering of the energy before it gets absorbed by the photosensitiser (Star, 1990) leading to unavoidable energy loses, is also a major issue.

Maximum tissue penetration of ~1 cm is achieved with lasers of wavelengths 700-800 nm. Red light (600-700 nm) will only travel ~5 mm in the body prohibiting the use of red light activated PSs to treat deep lying tumours. Using 700-800 nm activatable PSs is less feasible than 600-700 nm as will become evident when looking at the characterisation of most PSs and their absorption maxima used for their activation (see section 1.1.3 onwards) (Pandey et al., 1998). This is why using the most appropriate light delivery methods is important for effective PS activation and subsequent therapeutic effect (see section 1.1.9). As seen in Figure 1.3 red light will penetrate tissue more efficiently reaching the photosensitiser and thus activating it compared to blue light (Brown et al., 1999).

Figure 1.3 Light penetration through tissue. Deepest penetration is achieved with the longest wavelengths (800 nm) where up to 1 cm depth is observed. It is also important to point out the major increase in penetration from 600 nm to 700 nm. Taken from (Sternberg et al., 1998).

1.1.3 PDT in the clinic

Advantages over other therapies

When comparing PDT to other treatment modalities, there are distinct benefits that allow the patient to have a better quality of life. The following apply (Brown et al., 2004):

- it is not as invasive as radiotherapy and surgery
- it can be specific and partly targeted towards diseased cells
Looking at the invasive, aggressive nature and poor outcome that surgery, chemotherapy and radiotherapy have on cancer patients such as those with head and neck cancers it becomes apparent that alternative therapies are required (Nyst et al., 2009). Treatments that allow head and neck cancer patients to maintain a good quality of life are limited. PDT can treat and cure early stage disease providing the patient with excellent quality of life in the process (Brown et al., 2004). The laser used is non-thermal and should not cause burns or significant discomfort or scarring (Schweitzer et al., 2010). The treatment usually consists of local or IV injection of the formulated drug and subsequent irradiation of the area with either a laser or broad light lamp. The treatment can be painful but reports mostly refer to mild pain or a tingling sensation (Miller et al., 2007). Irradiation during treatment can take as little as 5-20 mins (Brown et al., 2004).

As PDT is a two step process, there are more than one conventional drug dose optimisations that need to be carried out to obtain maximum efficacy. The PS dose, drug-to-light time, light dose, fractionation, light delivery, light wavelength need to be optimised making the therapy complicated (Brown et al., 1999). Let us first consider the PSs that are currently used to treat patients as shown in figure 1.4.

**Photosensitisers currently used in the clinic**

PDT has mostly been approved and used in palliative and advanced stage cancer treatments (Celli et al., 2010). In 2000, PDT was approved as the first line of treatment for age-related macular degeneration (AMD) and today counts 2 million treatments (Celli et al., 2010). The approved PSs for use in clinical PDT are shown in figure 1.4.

**Hematoporphyrin derivative (HpD, Photofrin®, porfimer sodium)**, a mixture of porphyrins, was the first PDT drug to be used in the clinic to treat advanced and early stage lung cancers, gastric cancer, oesophageal adenocarcinoma, cervical and bladder cancer (Schuitmaker et al., 1996, Brown et al., 2004). It remains to date the gold standard for PDT. It is a lyophilized concentrated mixture of hematoporphyrin derivatives (Dougherty et al.,...
In the case of Photofrin, the FDA approved form of haematoporphyrin (1995), the patient is injected and following a two day drug-to-light interval, the patient is treated with a 630 nm laser (Pandey et al., 1998, Fda, 2010). Photofrin was approved by the FDA for treating esophageal and endobronchial tumours, early stage endobronchial nonsmall cell carcinoma and Barrett's oesophagus (Schweitzer et al., 2010). It is also used but not approved for, treating brain tumours, prostate cancer, cholangiocarcinoma, metastatic breast cancer, bladder cancer and head and neck cancers. Schweitzer also reports its use to treat juvenile laryngeal papillomatosis, laryngeal carcinoma, AIDS-related Kaposi’s sarcoma and aggressive skin tumours (O'connor et al., 2009, Schweitzer et al., 2010).

Perhaps surprisingly, phase III clinical studies - where a new drug would be compared against the best standard care - have yet to be carried out for PDT (Nyst et al., 2009), a fact that emphasizes the difficulties faced. Results of a trial conducted in 1985 where patients were treated with HpD for recurrent or metastatic head and neck cancers were ambiguous as the 24 patients were treated with mixed responses and side effects (Nyst et al., 2009). On the contrary, another study looking at the treatment of nasopharyngeal carcinomas gave positive results (Zhao et al., 1990, Nyst et al., 2009).

The low extinction coefficient of Photofrin® at its activation wavelength which only allows penetration of light up to 5 mm depth makes it an unlikely candidate for treating deep tumours (Macdonald et al., 2001). Nonetheless, the benefits of treating patients with Photofrin®-PDT include no known drug resistance and no cumulative dose ceiling (Macdonald et al., 2001).

Foscan® (Temoporfin, 5,10,15,20-tetrakis-(m-hydroxyphenyl)chlorin (mTHPC)), a second generation synthetic chlorin is one of the most potent PSs available. It was approved in Europe ten years ago for the treatment of head and neck cancers (Sharman et al., 1999). In a study where it was used to treat advanced head and neck carcinomas with 128 patients taking part proved that PDT can offer improvement in quality of life and no severe toxicities were detected (D'cruz et al., 2004). Another Foscan® study showed that it can be an excellent choice for interstitial tumours via the use of fibre optics to deliver the light to the site. Head and neck cancers are generally difficult to treat therefore good outcomes from the trials would be hard to obtain (Nyst et al., 2009). Another limiting factor in these trials is the fact that the patients treated had exhausted the benefits of other treatments and PDT was a last resort with the disease having become regional or systemic (Nyst et al., 2009). Following treatment with Foscan®, patients exhibited moderate to significant pain (Nyst et al., 2009,
Clinical trials are now more widespread than twenty years ago. Nyst reviews the head and neck cancer trials in 2009 where Foscan® PDT data are emphasized and points out the benefits of PDT over surgery and radiotherapy that can both severely affect the patient’s way of living (Nyst et al., 2009). Temporary pain, necrosis and photosensitivity that can persist for 2-4 weeks are the most commonly reported side effects while the requirement of a 4-day drug-to-light interval is another deterrent (Nyst et al., 2009, Allison et al., 2010). In another study, the cost-effectiveness of Foscan® PDT was compared to palliative chemotherapy and surgery for patients with advanced head and neck cancers (Hopper et al., 2004). The study showed increased life expectancy and increased chances of remission compared to no treatment and the overall cost effectiveness of Foscan®-PDT compared to the other two modalities that are offered to some of these patients.

Verteporfin (Visudyne®) (benzoporphyrin derivative BPD) is successfully being used for treating acute macular degeneration (Mellish et al., 2001). BPD, synthetically made from protoporphyrin was the first chlorin be approved for clinical use (Qlt, 2010). It benefits from rapid drug-to-light interval at only 15 mins, quick blood clearance and no skin photosensitivity (Brown et al., 2004). It has a longer absorption maximum and at 690 nm, better tissue penetration is observed compared to Photofrin® (Allison et al., 2010). Visudyne® has filled a vacant spot treating a condition that leads to blindness. Following successful and well designed clinical trials, Visudyne® is finding new applications for the treatment of ophthalmic astrocytoma (Eskelin et al., 2008), choroidal melanoma (Barbazetto et al., 2003) and other cutaneous malignancies (Kalka et al., 2000, Allison et al., 2010). Twenty clinical trials have recently been completed testing the application of Verteporfin in several of the mentioned diseases (Trials.Gov, 2010a). There is a staggering range of possible applications of a single drug which could apply to most PSs!

ALA or Levulan® - It was the discovery that a precursor (5-aminolevulinic acid) to an endogenous PS can be applied to skin cells and initiates its bioconversion to protoporphyrin IX that brought ALA or Levulan® to the clinic in the USA as an FDA approved pro-drug. ALA is a building block towards the biosynthesis of haem, which is not photoactive (Fien et al., 2007) and chlorophyll (Brown et al., 1999). When ALA is applied topically (usually as a cream) and is taken up by the cells, it enters the metabolic pathway that leads to haem whose penultimate step is the biosynthesis of protoporphyrin IX which acts as the PS (Blume et al., 2007). It shows high specificity up to 10 times compared to healthy cells in the treatment of actinic keratoses. Drug-to-light interval according to the FDA is 14-18 hrs after
which the patient is irradiated with a 630 nm red laser (O’connor et al., 2009). More uses of ALA are being found in Bowens disease and superficial basal cell carcinoma (BCC) where it has been used to treat patients successfully. The obvious advantages of ALA are its efficacy, low photosensitivity and excellent cosmetic effect with the only disadvantage being the limited light penetration depth required for the activation of protoporphyrin IX (O’connor et al., 2009). Derivatives of ALA have been produced to facilitate its more efficient penetration into the skin. This was achieved by increasing the hydrophobicity of ALA with the introduction of a methyl ester improving its penetration through the phospholipid bilayer of the cell's plasma membrane (Sieber et al., 1984). The resulting compound, Metvix was approved by the FDA in 2004 and is also in phase III clinical trials for the treatments of BCC (Fda, 2010) and actinic keratoses, showing improved deeper skin penetration, higher selectivity towards the tumours due to its increased hydrophobicity and reduced pain (Fien et al., 2007). Both Metvix® and ALA have become widely used in skin cancer therapy in Europe and the USA (Fien et al., 2007, O’connor et al., 2009). Some side effects are reported with the use of ALA which include pain, erythema, oedema, postinflammatory changes and a period of skin photosensitivity (Fien et al., 2007).

![Figure 1.4 Structures of photosensitisers currently being used in the clinic. Visudyne® and Foscan® are chlorins and Photofrin® which is a mixture of Hp derivatives is a porphyrin.](image)
1.1.4 Other applications of PDT

Even though PDT is mainly focused towards cancer treatment, it has potential applications in a wide array of other therapies and treatments.

Photodiagnosis is a sister-like application where PSs are used for imaging. Using PDT as a double agent for both imaging and treatment is an emerging field of considerable interest that has recently been on the rise (Celli et al., 2010, Lovell et al., 2010). Most photosensitisers would be suitable for such use, assuming their fluorescence quantum yield (see section 1.1.6 for definition) is high enough. A recent review outlines the fundamentals and prospects of using the fluorescence characteristics of photosensitisers to image disease (Celli et al., 2010).

A field where PDT is also emerging, at a slower perhaps pace, is in the deactivation of antibiotic resistant bacteria and other microorganism-related diseases and infections (Jori et al., 2004). PDT can potentially act as both a therapy and a sterilisation method (Jori et al., 2004, Costa et al., 2008) deactivating Gram positive bacteria which are susceptible to PDT using already available PSs (Jori et al., 2004). However, Gram negative bacteria (which are negatively charged and do not uptake neutral and anionic PSs) can be inactivated using a cationic compound to formulate an existing neutral or anionic PS (Jori et al., 2004). PDT can also be used to disinfect blood and water from viruses and other microorganisms (Jori et al., 2004, Wainwright, 2004). The 2004 issue 5 of Photochemical and Photobiological sciences has a good selection of papers in this field. An excellent review was also given by Dai (Dai et al., 2009). The deactivation of viruses in a therapeutic setting is less well advanced and currently does not offer any significant advantages over traditional therapies (Wainwright, 2004).

Lastly, PDT can be used in cardiovascular disease for treatment of stenosis, in dermatology for the treatment of cancerous and non-malignant inflammatory disorders such as acne, psoriasis and scleroderma and even hair removal (Hamblin, 2008).

1.1.5 Limitations of conventional PDT

Due to the localised effect of PDT it is unlikely that it can be used to treat disseminated disease (Brown et al., 2004, Nyst et al., 2009) although key recent findings discussed in section 1.7 show that PDT can trigger an immune response leading to antitumour immunity.
The use of the first PDT drug, HpD in the clinic highlighted a few issues that include side effects in the form of skin photosensitivity (Moriwaki et al., 2001) and low tumour to healthy tissue specificity (Gilson et al., 1988). In addition, the need to maintain the patient protected from light for several hours or days following administration depending on the PS were suggested (Brown et al., 2004). Especially with Foscan where a staggering 96-hour drug-to-light interval, persistent skin photosensitivity (4-12 weeks(Nyst et al., 2009)) and low initial selectivity are observed (Brown et al., 2004). Irradiation time at 30 mins is an additional negative point (Nyst et al., 2009). For example, HpD requires very high light doses due to its low absorption of red light (Nyst et al., 2009).

The recurring suggestions for facilitating the progress of PDT evolve around the need for targeted-thus specific photosensitisers which require longer wavelength for activation and exhibit limited skin photosensitivity (Schweitzer et al., 2010). It is reported that Photofrin contains 60 components (Allison et al., 2006) greatly disadvantaging itself as a viable photosensitiser.

Robust and randomised clinical trials that could lead to the approval of PDT drugs have been few with low numbers of participating patients. Limited understanding of the mechanisms involved in PDT-induced cell death adds to the scepticism of physicians to routinely use the treatment (Brown et al., 2004). Trials early in this decade focused mainly at using these pre-approved drugs to treat types of disease different to those they were approved for. Diseases that PDT could treat include Barrett’s oesophagus, bladder cancer, early non-small-cell lung cancer, intraepithelial neoplasias, pituitary tumours and glioblastoma (Brown et al., 2004).

The side effects and limitations associated with the drugs mentioned above, are the key obstacles preventing the more widespread use of PDT. Raising awareness and helping oncologists and physicians to better understand the role of PDT is another necessity if the benefits of the therapy are to be further realised. Furthermore, as Brown et al. conclude, drugs and light sources have to be optimised while the benefits of PDT compared to other therapies need to be established. There is a need for drugs that:

- Do not exhibit skin photosensitivity i.e. have rapid blood clearance
- Have higher tumour/malignant cell selectivity vs. healthy cells
- Have short drug-to-light periods
- Have absorption maxima in the red region of the spectrum (650-800 nm)
- Have good singlet oxygen quantum yield inferring potency
before PDT can become a successful mainstream therapy (Brown et al., 1999, Brown et al., 2004).

1.1.6 Photophysics

Understanding the fundamental mechanism through which the photoactivation of a molecule (the photosensitiser) leads to cell death, is imperative and will lead to the development of more effective drugs. PDT begins with the activation of the PS using appropriate light which is absorbed, the PS is excited and upon collision with surrounding molecules can produce cytotoxic species.

More specifically, as shown in figure 1.5, the Jablonski diagram shows the energy states of the photosensitiser and the potential movements to higher energy levels. When a photosensitiser absorbs a photon of appropriate wavelength, it can take an electronically excited state, the excited singlet state. The singlet excited state ($S_1$) has a very short lifetime and can easily relax to a lower energy, still excited state - the triplet excited state ($T_1$), via intersystem crossing ($S \rightarrow T$). $T_1$ is responsible for most of the photobiological reactions that take place as it can react with ground state molecular oxygen ($^{3}$O$_2$) via radiation-less transition, to form singlet oxygen ($^{1}$O$_2$) or with other organic substrates to form reactive oxygen species (ROS) (Macdonald et al., 2001). The reaction with molecular oxygen is termed a type II photoreaction and the reaction with other substrates is called a type I photoreaction as shown in figure 1.6. Type I reaction has several possible products such as a photosensitiser radical that can further react with oxygen producing superoxide anion ($O_2^-$) and hydroxyl radical (OH).
Figure 1.5 Modified Jablonski diagram showing the energy levels involved in one photon PDT. $S^0$ is the PS ground state, $S^1$ is the PS in an unstable excited state, $T^1$ is the PS’s triplet excited state which can react with either molecular ground state oxygen ($^3\Sigma_g^-$) to produce singlet oxygen ($^1\Delta_g$) (type II reaction) or react with other substrates (type I reaction). $h\nu$ denotes photon/energy; abs: absorption; fl: fluorescence; ic: internal conversion; ph: phosphorescence; isc: intersystem crossing.

Type II is the most crucial reaction in PDT and it is generally thought and accepted as the prevailing one. The energetic states of the photosensitiser are characteristic of each molecule. The relaxation of the photosensitiser from $S_1$ to the ground state $S_0$ is accompanied with emission of a fluorescent photon. This transition is characterised by the singlet state lifetime and usually ranges from $10^{-9}$– $10^{-6}$ seconds (Josefsen et al., 2008a). The decay from the triplet state to the ground state is accompanied by emission of a photon via phosphorescence. During the collision of the photosensitiser in its triplet state with oxygen in its triplet state (ground state), a triplet triplet annihilation process takes place. The photosensitiser rests at its ground singlet state ($S_0$) and singlet oxygen is produced which is highly cytotoxic. The photosensitiser can then undergo more cycles of excitation (Lovell et al., 2010). A single photosensitiser molecule can be used many times as it goes through the activation back to the ground state cycle (Hamblin, 2008).

However, continuous cycles of activation/relaxation and the generation of singlet oxygen and ROS can lead to photobleaching of a PS which is defined as “the loss of absorption or emission intensity caused by light” (Bonnett et al., 2001). Photobleaching can lead to the appearance of new peaks in the absorption spectra or can chemically destroy the PS resulting in its fragmentation and complete loss of characteristic absorption spectra which can be observed as discoloration of solutions (Bonnett et al., 2001, Macdonald et al., 2001). Photobleaching is another photophysical characteristic specific to each compound and can be initiated by type I or type II photoreactions (Macdonald et al., 2001). In cases of
severe photobleaching, the stability of the PS in a biological oxygenated environment may not be guaranteed long enough to use the PS.

The Stark-Einstein Law of photochemical equivalence states that one photon excites one molecule and that can subsequently produce one product. Therefore the singlet oxygen quantum yield (Φₘ), which describes the efficiency with which a molecule (PS) absorbs a photon and converts molecular oxygen to singlet oxygen, cannot be greater than 1 (Bonnett, 2000).

It is believed that most potent PSs bring about cell death via type II PDT mechanism. Good singlet oxygen quantum yield is therefore desired (Gorman et al., 2006).

In the area of measuring and detecting singlet oxygen, Peter Ogilby in Denmark has conducted several important experiments. The controversial work by Ogilby has shed doubts as to the lifetime and diffusion range of singlet oxygen which they reported as being 3 µs and 134 nm respectively in water (Skovsen et al., 2005). Diffusion is generally believed to be 20 nm with a lifetime of 40 ns in biological systems (Sternberg et al., 1998, Josefsen et al., 2008a) or 10 nm diffusion diameter with 2 µs lifetime in aqueous environments (Macdonald et al., 2001). Elsewhere it was reported that singlet oxygen remains within 0.1 µm of the point of formation which is closer to the figure obtained by Skovsen (Akhlynina et al., 1997).

Assuming a cancer cell is ±10 µm across, then the diffusion range is between 75 and 500 times less than the size of the cell making the generation of singlet oxygen highly localised to 1-2 cells (Van Dongen et al., 2004). Even though these photophysical values do not seem to correlate with the actual biological efficacy of a photosensitiser, they are a good approximation (Josefsen et al., 2008a).
Photosensitiser excited singlet and triplet lifetimes are crucial characteristics as they define how long the PS can occupy an excited state, enough for a collision with either molecular oxygen or other biological components to occur (Wainwright, 2008).

Singlet oxygen quantum yield with high accumulation in tumour cells at sufficient concentrations can help predefine the potency of a PS in humans (Brown et al., 1999). Understanding the mechanisms via which PDT is cytotoxic on a cellular level and is therapeutic on a systemic level, is important for the design of future photosensitisers.
1.1.7 Mechanisms of tumour and cell annihilation

Tumour destruction post-PDT treatment is considered to take place in one of two ways—directly, following initiation of apoptosis or necrosis and indirectly by damaging the tumour associated vasculature depriving tumour cells of oxygen and nutrients leading to tumour infarction (Dolmans et al., 2003, Castano et al., 2005, Nowis et al., 2005, Robertson et al., 2009). There is increasingly more supporting evidence that subsequent initiation of an immune response is paramount to tumour regression.

On a cellular level, singlet oxygen and ROS initiate the above cascades. Cell death mechanism (apoptosis or necrosis) depends on the subcellular localisation of the PS, cell type and the light dose applied (Pervaiz et al., 2006, Robertson et al., 2009). Lower doses of PDT are believed to lead to apoptosis whilst higher doses cause severe damage and can lead to increased necrotic compared to apoptotic cells (Ketabchi et al., 1998).

Only the key points of this vast area are covered here as reviewing it extensively would be beyond the scope of this thesis.

Direct (apoptosis, necrosis and immune response)

The ROS generated by either type I or type II photoreactions are shown in figure 1.7. Even though hydroxyl radical is considered to be perhaps more reactive than singlet oxygen, its extremely short lifetime limits its reactivity to its immediate surroundings. Therefore, singlet oxygen is considered to be the most toxic species that can be produced in PDT and can lead to direct apoptosis or necrosis of cells. Whether one or the other will occur mainly depends on the PS and its subcellular localisation (see chapter 5) (Oleinick et al., 2002).

![Diagram of ROS reactivity, lifetime and diffusion distance](image_url)

Figure 1.7 Diagrammatic comparison of ROS – comparing the reactivity, lifetime and diffusion distance of the main cytotoxic species in PDT. Hydroxyl radical is very unstable and extremely short lived. Reproduced from (Hamblin, 2008).
Apoptosis is a programmed cell death process initiated when a cell given the appropriate signals begins to shrink, chromatin and DNA fragment and eventually the cell is taken up by phagocytes (Tardy et al., 2006). Necrosis is caused by a sudden cell damage which leads to the rapture or damage of plasma membrane, cell lysis and tissue inflammation (Oleinick et al., 2002). Contrary, apoptosis is not associated with inflammation due to the packaging of cellular components (Tardy et al., 2006).

The mechanisms of PDT induced cell death are now the subject of extensive research (Miller et al., 2007). An interesting and thorough study on treating cutaneous malignancies as part of a phase I clinical trial was reported by Miller where they observed the induction of apoptosis and the increased levels of caspase-3 following PDT (Miller et al., 2007). However, correlation between induction of apoptosis and therapeutic efficacy was not established for PDT (Miller et al., 2007). On the cellular level, the gold standard Photofrin® works by damaging the mitochondria and inducing apoptosis (Schweitzer et al., 2010). It is generally believed that PDT leads to apoptosis.

Following the initial annihilation of tumour cells, cell debris is released in the tumour microenvironment and subsequently cleared from the body. During this process tumour antigens (debris) can be picked up by antigen presenting cells thus initiating the adaptive immunity and generating a systemic response.

**Indirect (vascular damage, hypoxia and immune response)**

Indirect tumour regression can be initiated by vascular damage and shutdown leading to hypoxia and starvation and eventually cell death (Conway et al., 2008, Nyst et al., 2009, Garg et al., 2010). Coagulation, vessel shrinkage and leakage are all signs of vascular damage (Castano et al., 2005).

Overall, tumour hypoxia is welcomed in PDT as it can lead to ischemia related cell death. In an ischemic environment, it is possible that vasodilating mediators are released, reopening the constricted/coagulated vessels. ROS including superoxide anion can then be released by the cells. This process is called oxidative stress and leads to complement activation (Nowis et al., 2005). Complement activation accompanied by neutrophils and other inflammatory cells is followed by further immune responses such as release of heat shock proteins, transcription factors and expression of cytokines (Nowis et al., 2005, Castano et al., 2006). The complement system activation is followed by an acute phase response where neutrophils and cytokines are released eliciting antitumour actions (Castano et al., 2005, Nowis et al., 2005, Nyst et al., 2009).
Despite the immune response activation, hypoxia is at the same time a limiting factor for PDT efficiency as the lack of oxygen does not allow photoreactions to take place. Presence of high oxygen levels in the tumour combined with high levels of PS concentration and light dose delivery have been shown to increase the consumption of tissular oxygen. This high oxygen consumption can lead to hypoxic areas within the tumours protecting some of these cells from PDT damage (Foster et al., 1991, Castano et al., 2005). To avoid this effect from occurring, fractionation of the light dose can help regulate the oxygen levels in the tumour allowing a more uniform oxygen concentration within the tumour (Castano et al., 2005).

For example, PDT with Photofrin\textsuperscript{®} induces type II photoreaction leading to tumour cell death in rats via vascular damage and shutdown leading to ischemic necrosis (Leach et al., 1992). (Reed et al., 1988, O'connor et al., 2009). Following treatment with chlorin p6 (see section 1.2.2) the response included congestion, oedema and coagulation necrosis (Leach et al., 1992).

The first study to investigate the activation of the immune system post PDT treatment in humans was carried out on patients with basal cell carcinoma (BCC). The study involved 25 patients with nodular and superficial lesions, 21 of which were treated with porfimer sodium and ALA; 4 patients were treated surgically. Blood was collected prior and 7-14 days after treatment and the collected lymphocytes were analysed for MHC class I surface antigens (Kabingu et al., 2009). Of the 21 patients treated with PDT, 17 showed increased immune response with those treated for superficial lesions being higher than those with nodular. Also noting that patients treated on a smaller area showed higher immunity as well as those treated with lower laser doses. It is suggested that high laser doses lead to vascular shutdown obstructing immune cells from reaching the tumour. Higher immunoreactivity appeared to correlate with better overall response to PDT with no distinction being made between the two drugs. Only one patient treated surgically showed some immunoreactivity (Kabingu et al., 2009). These findings form an important basis for further clinical studies.
Generating singlet oxygen and ROS following PDT, can cause excessive damage to lipids, proteins (amino acids) and DNA leading to individual cell death followed by initiating a cascade of reactions on a cellular level (Lovell et al., 2010). These are only briefly covered here.

Direct reactions of singlet oxygen with proteins are perhaps the most important as they are also the most likely to occur with the amino acids histidine, tryptophan, methionine, cysteine and tyrosine being labile to forming endo- or hydro-peroxides (Davies, 2003, Klotz et al., 2003, Cadet et al., 2006, Hamblin, 2008). Contrary to the other ROS, singlet oxygen is not a commonly occurring species in cells and cannot interconvert to other ROS but can lead to their formation. Crucially, there aren’t any known cell defence mechanisms against singlet oxygen and only chemical singlet oxygen scavengers could protect a cell from it. These would have to be present in high concentrations in order to be effective (Hamblin, 2008).

Since ROS are naturally occurring in cells, being produced as part of their signalling pathways and as byproducts of normal aerobic metabolism, cells have developed defence mechanisms against them which include vitamins, flavanoids and enzymes such as superoxide dismutases, catalases and glutathione peroxide (Garg et al., 2010). If present in the cell in excessive and uncontrollable amounts then the cells can undergo DNA, protein and lipid damage. The inability of a cell to deal with the sudden surge of release of such reactive species (from an exogenous source such as PDT) leads to oxidative stress (Garg et al., 2010).

Whereas singlet oxygen production within a cell is most likely to lead to cell death, production of ROS can have both proliferative and non-proliferative outcomes. Following the generation of ROS, mitogen-activated protein kinases and heat shock proteins are expressed whilst several pathways related to apoptosis and proliferation are activated (Martindale et al., 2002). ROS can help activate the tumour suppressor protein, p53 by damaging the DNA as shown by many chemotherapeutic agents (Martindale et al., 2002). Cells with reduced p53 activity have been shown to survive hydrogen peroxide therefore becoming resistant to ROS toxicity.
Figure 1.8 Main routes to tumour destruction by PDT. Upon excitation of the PS and production of singlet oxygen, pathways that lead to apoptosis, necrosis, vascular shutdown leading to tumour infarction and inflammation are activated. Taken from (Castano et al., 2006).

Remarks

In *in vivo* PDT, it is important to illuminate the tumour as a whole by perhaps using several fibre optics at the same time to ensure various angles of the tumour are illuminated. The efficiency of illumination is crucial as non-uniform light application can lead to hypoxic areas within the tumour which become resistant to the treatment (Hamblin, 2008).

Inhomogeneous accumulation of the PS in the tumour is also a limiting factor in the efficiency with which a tumour is treated (Dolmans et al., 2003, Castano et al., 2005). With targeted PDT (TPDT) the accumulation of the PS should be governed by the targeting moiety, focusing the binding of the PS to tumour cells over normal healthy cells.

Other parameters affecting the outcome include cancer type being treated, the subcellular localisation of the PS and the irradiation method (Akhlynina et al., 1995). A further discussion on the importance of subcellular localisation of a photosensitiser can be found in chapter 5.

Considering the importance of damaging vasculature in tumour response, it is likely that PSs targeting the blood vessels will be developing, which is indeed true (see section 1.3).

The overall stimulation of inflammatory response observed in PDT is remarkable and offers a great advantage compared to chemotherapy and radiotherapy that are mainly immunosuppressive (Nowis et al., 2005). Additionally, cells at any point of their cell cycle can be damaged by PDT which does not apply in chemotherapy (Morgan et al., 1989).
1.1.8 Cellular uptake of photosensitisers

Upon administration into the body via intravenous injection (i.v), a PS binds albumin and other serum proteins and is trafficked around the body, from the bloodstream to endothelial cells to vessels then resides in tumour cells. It is cleared by lymphatics and excreted by glomerular filtration or the liver (Castano et al., 2005, Nowis et al., 2005, Robertson et al., 2009).

Photosensitisers can be divided into three categories depending on how they behave upon entering the bloodstream: those that bind albumins (human serum albumin (HSA)), the outer layer of lipoproteins (high density lipoprotein (HDL)) and those that reside in the very hydrophobic inner core of lipoproteins (e.g. low density lipoprotein (LDL)) (Castano et al., 2005). By binding to HSA PSs can be trafficked through the endothelium of the tumour vasculature (Hamblin, 2008). Many drugs have an affinity for HSA, binding either of its sites (I and II) with those binding site II being more potent (Sudlow et al., 1975, Tsuchida et al., 1997).

Hydrophobic PSs also bind strongly and non-covalently to serum proteins such as LDL. In doing so, they are trafficked into the tumour cell via receptor mediated endocytosis, the pathway via which LDL internalises. Cancer cells overexpress LDL receptors in order to obtain the necessary nutrients for their increased metabolism (Kessel, 1986, Hamblin, 2008). LDL is therefore suitable to act as a carrier of PSs and facilitate their internalisation.

Additionally, photosensitisers can become “trapped” around the tumour vasculature due to the poor lymphatic drainage of tumours inhibiting their clearance. They can also be phagocyted by macrophages and transported to the tumour, as macrophages tend to infiltrate solid tumours (Castano et al., 2005). For example, Ce6 was conjugated to BSA and successfully delivered to the tumour by macrophages following intra-tumoural injection in nude mice (Anatelli et al., 2006).

Lastly, endogenous porphyrins in human blood such as proporphyrin IX and heme have high affinity for receptors called peripheral-type benzodiazepine receptors (Verma et al., 1988). Potent porphyrins were shown to have high affinity for mitochondrial benzodiazepine receptors (MBR). Tumours high in MBR have also been shown to be prone to PDT induced death (Verma et al., 1998). Affinity for MBR can be an early indication of a mitochondrial localising PS capable of efficiently killing tumour cells (Tsuchida et al., 1997, Verma et al., 1998).
1.1.9 Light Sources

Suitable light sources used to be one of the factors limiting the success of PDT as delivery of a known light dose at a specific site was difficult. The first lasers were large and inefficient (Nyst et al., 2009) with the first laser used in PDT being a 630 nm high-powered argon/dye laser that was immobilised on a wall (Mang, 2004). This has changed in the last 10-20 years with several options being available (Brown et al., 2004, Mang, 2004). Light in the form of a cold laser can be administered locally, even endoscopically using fibre optics whereas non-laser sources are used for superficial treatments. Lasers coupled to fibre optics are efficient at delivering the necessary dose to the patient.

Just ten years ago, the diode laser was approved for use with Photofrin PDT. Diode lasers are compact, at the size of a computer tower and highly portable. These have a fixed wavelength (630 (Diomed Inc.) and 652 (Biolitec) nm) and can give out up to ~2.5 W of energy. They are cheaper to buy and run and are more stable than pre-existing types of lasers (Mang, 2004). In order to reach deep tumours, laser light is delivered using endoscopic cylindrical or spherical diffusers, depending on the tumour size being irradiated (Schweitzer et al., 2010). For intraperitoneal tumours, the fibre optic is inserted endoscopically. New developments include the use of a transparent balloon that guides the fibre optic for the treatment of the oesophagus allowing for uniform and efficient irradiation of the site (Star, 1990, Macdonald et al., 2001, Mang, 2004).

Light emitting diodes (LEDs) are very small, light and easy to run and can provide low energy at several wavelengths (630, 670, 690 nm). They are not tunable and they only work on flat surfaces. For superficial tumours, lamps with appropriated filters or arc lamps have also been used (Star, 1990). However, these are not as efficient as a laser in activating photosensitisers.
1.2 Photosensitisers

Photosensitisers mediate the photochemical reaction for PDT as described in section 1.1.6 and ideally should have the characteristics as listed in table 1.1.

Table 1.1 Characteristics of an ideal photosensitiser (Gorman et al., 2006, O’connor et al., 2009):

| Purity and stability at room temperature |
| Minimal/ no dark toxicity |
| Biodecomposable, should not burden the body with toxic smaller fragments |
| Absorption in the red |
| Localisation in tumours |
| Rapidly cleared and metabolisable |
| High singlet oxygen quantum yield |
| Easy synthesis |
| Subcellular localisation such that it promotes apoptosis vs. necrosis |
| Low general toxicity |
| Minimal skin photosensitivity |

A wide range of PSs exist that posses many of these features. Finding or synthesising one that possess all is the challenge.

Photosensitisers are usually divided into two categories which are porphyrins and non-porphyrins.

1.2.1 Porphyrins, chlorins and bacteriochlorins

Porphyrins, chlorins and bacteriochlorins are the most dominant PDT agents currently under investigation (Allison et al., 2010). They prevail in the line of synthesis, targeting and clinical investigations. Porphyrinic PSs are further divided to first, second and third generation (O’connor et al., 2009). These three closely-related macrocycles are suitable for PDT as they have many of the features listed in table 1.1 (Macdonald et al., 2001).

Porphyrinic macrocycles are aromatic cyclic tetrapyrroles connected by four methine bridges and include porphyrins, chlorins, bacteriochlorins and isobacteriochlorins. These PSs and related compounds have planar structures and are often characterised by hydrophobicity rendering them insoluble or slightly soluble in aqueous solutions (Bonnett, 2000, Macdonald et al., 2001, Dolmans et al., 2003). Their solubility in organic solvents varies depending on the substituents on the periphery of the macrocycle.
Being heavily conjugated, these macrocycles have very characteristic absorption spectra, indicative of each member of the family as shown in figure 1.9. It is important to note that a single bond reduction causes a significant change between the spectral properties of the three groups. They all have a main strong absorption peak around 400, called the Soret band and four smaller peaks called the Q bands. Porphyrins generally have the weakest last Q-band with the bacteriochlorins having the strongest. This last, deep red Q-band is usually the choice of wavelength for use in therapy (Macdonald et al., 2001), as it allows maximum depth of penetration. The Soret is the most intense band and is typically a narrow peak around 400 nm. The Soret band reflects the transition from the ground state to the second singlet excited state (S₀ to S₂) (Josefsen et al., 2008a). The Q bands are a result of the transition between S₀ and S₁ (Josefsen et al., 2008a). The fluorescence of a PS reflects only the Stokes shift of the Q band (the transition of S₁ back to S₀) as the transition from S₂ is too fast and occurs via internal conversion and cannot be observed (Josefsen et al., 2008a).
First generation PSs were covered in section 1.1.3 therefore the development of newer ones is described here.

Figure 1.9 UV/Vis absorption spectra and structures of the main porphyrinic PSs; a simple porphyrin (top), chlorin (centre) and bacteriochlorin (bottom). The reduction of a double bond as the transition from a porphyrin to a chlorin and to the bacteriochlorin occurs is also shown. The blue arrow (●) shows the Soret band, a characteristic strong absorption peak around 400 nm, and a set of 4 bands in the region of 500-800 nm called the Q bands (●). Taken and modified from (Sternberg et al., 1998).
1.2.2 Second generation photosensitisers

**Tin etiopurpurin** (Purlytin) is a purpurin, with an absorption maximum of 650 nm and is shown in figure 1.11 (Josefsen et al., 2008a, O'connor et al., 2009). Purlytin was used in clinical trials exhibiting good results comparing well to HpD, however it also exhibited photosensitivity which combined with its low water solubility requiring lipid-based formulations for its administration are likely to hinder its progress. It is being tested for the treatment of cutaneous metastatic breast cancer and Kaposi’s sarcoma as well as psoriasis and restenosis (Josefsen et al., 2008a).

**Lutetium texaphyrin, Lutrin, Lu-Tex** is a tripyrrolic pentaazakexpanded porphyrin that tends to localise in tumour vasculature (figure 1.11) (Mody et al., 2001). It has a strong absorption in the near infrared at 732 nm, it's water soluble (O'connor et al., 2009) and has a good singlet oxygen quantum yield. It has been shown to localise in lysosomes leading to apoptosis in a murine EMT-6 sarcoma model (Woodburn et al., 1997) and overall, the *in vivo* cure rates are higher when compared to Photofrin (O'connor et al., 2009). It is in clinical trials for the treatment of recurrent breast cancer, acute macular degeneration and treatment of atherosclerosis showing minimal photosensitivity with the only downside being the pain that the patients exhibited but which was manageable with topical anaesthetics (Josefsen et al., 2008a, O'connor et al., 2009). Finally, it appears to be a good second generation photosensitiser with the long absorption maximum and the preferential tumour uptake making it a good candidate for PDT (Macdonald et al., 2001).

**Tookad®, (WST11)** a palladium bacteriochlorin is one of the most promising PSs currently in clinical trials for prostate cancer currently recruiting patients for more extensive studies (O'connor et al., 2009, Trials.Gov, 2010c). Clinical trials for localised prostate cancer are ongoing in the UK and Canada (Steba, Trachtenberg et al., 2007). A derivative of bacteriochlorophyll-a, Tookad has a strong absorption at 760nm exhibiting quick blood clearance *in vivo* with a short 20 minute drug-to-light interval followed by 30 mins illumination (Allison et al., 2010). Its absorption maximum makes it a very attractive PS for use in patients as it allows deeper tissue penetration. Figure 1.10 compares the absorption spectra of Tookad® to Photofrin. There is a significant difference in the absorption maxima between the porphyrin and the bacteriochlorinins. Several clinical trials have been - or are in the process of being - conducted. So far the results have been positive with patients showing complete tumour regression (O'connor et al., 2009). Tookad® is probably the most promising photosensitiser currently in clinical trials. It is worth noting that WST11 is the second derivative of Tookad to enter clinical trials which is an improvement to the previously less water soluble WST9.
Figure 1.10 Comparing the absorption spectra of Photofrin® and TOOKAD, showing the distinct differences in their absorption maxima. Taken from (Wilson et al., 2008).

NPe6 (mono-L-aspartyl chlorin e6, talaporfin sodium, MACE, LS11, Laserphyrin, Litx™, Photolon and Apoptosin™) is a hydrophilic chlorophyll a derivative with an absorption maximum of 664 nm (O’connor et al., 2009, Allison et al., 2010). It tends to localise in the lysosomes and triggers the immune system, the production of VEGF and proto-oncogenes c-jun and c-fos through the activation of p38 MAPK (Nakagawa et al., 2007). Its drug-to-light interval is 2-4 hr (O’connor et al., 2009) and skin photosensitivity is minimal compared to Photofrin®. Npe6 shows rapid clearance enabling treatment to begin soon after injection (Macdonald et al., 2001) while several doses can be administered. In clinical trials it led to complete cure of subcutaneous tumours while further clinical trials (phase I and II) on subcutaneous tumours and lung cancer are being carried out with very promising results showing minimal side effects (O’connor et al., 2009).
Figure 1.11 Structures of third generation photosensitisers currently in clinical trials or advanced pre-clinical studies.

Photochlor (HPPH) is another chlorin, a chlorophyll a derivative with an absorption maximum at 665 nm (112). It is currently in phase I clinical trials in the US for treatment of basal cell skin carcinoma (Trials.Gov, 2010b). In a different phase I trial it has shown very low photosensitivity when used to treat cancers of the oesophagus and Barrett’s syndrome. In a further phase I trial for endobronchial lung cancer, HPPH exhibited high potency and mild photosensitivity. This trial was suspended but several others are about to commence.
including one for oesophageal and head and neck cancer (Allison et al., 2010, Trials.Gov, 2010b).

**Porphyecenes**

Other types of second generation photosensitisers include porphyecenes which are porphyrin isomers with an 18 \( \pi \) electron cloud (see figure 1.11). Porphyecenes were discovered more than twenty years ago by Vogel. They can be synthesised using McMurry coupling obtaining products that can be both hydrophilic and hydrophobic depending on the side chain. Yields are generally low but the variety of compounds that can be synthesised makes them appealing. Modifying the side chain can affect the photophysics, clearance and uptake (Bonnett, 2000, O’connor et al., 2009). Such hydrophilic derivatives have been shown to have quick blood clearance but relatively low tumour uptake (Richert et al., 1994). Potency has been shown to be up to 220 times higher than Photofrin. The porphycene shown in figure 1.11 exhibited 200 fold increased potency on SSK2 cells when encapsulated in a liposome (Richert et al., 1994). Additional modifications such as the introduction of hydroxyl or methoxy groups lead to improved pharmacokinetics which when compared to existing photosensitisers included the very minimal photosensitivity, rapid clearance, efficient photophysics and potency.

**Phthalocyanines**

Another family of macrocycles are phthalocyanines which are also flat aromatic compounds. Due to the attached benzene rings directly on the periphery of the macrocycle they are more conjugated than a porphyrin and have a longer absorption maximum which is more suitable for PDT as it allows deeper light penetration (Boyle et al., 1996, Miller et al., 2007). For example, a silicon derivative phthalocyanine, Pc 4, was administered topically for the treatment of skin malignancies and lesions as part of a phase I clinical trial. Initial studies showed promising results with no dark toxicity or photosensitivity mentioned and no associated pain (Miller et al., 2007). Photosens is a mixture of sulphonated phthalocyanines with an absorption maximum at 670 nm developed in Russia. It has been used to treat patients with early esophageal cancer with complete response of small tumours. The effectiveness of the treatment decreased with increasing tumour size. Overall, it was shown to prolong life expectancy by 4.59 years (Filonenko et al., 2008). Due to their very hydrophobic nature, phthalocyanines are more challenging to modify into water soluble usable PDT agents (Wainwright, 2008).
Phenothiazinium dyes

Methylene blue is the representative of this category of singly positively charged dyes with strong absorption in the red (600-660 nm) and good singlet oxygen quantum yields. (Gorman et al., 2006). They are easy to synthesise with methylene blue being relatively hydrophilic and exhibiting high dark toxicity levels in vitro making it less attractive to use in anti-cancer PDT (Gorman et al., 2006). They are mainly used as disinfectants such as blood sterilisation and for the deactivation of microorganisms (Wong et al.).

1.2.3 Third generation photosensitisers

First generation photosensitisers included the gold standard Photofrin discussed previously. In the second generation PSs, synthetic modifications were applied to existing PSs in order to improve their characteristics based on the knowledge and understanding of an ideal PS. Third generation PSs include the currently under development PSs that are synthesised having “the ideal PS” criteria (section 1.2) in mind. These can be targeted using chemical, (nano)particle and biological means. Biological means, include antibody targeting by directly or indirectly linking the PS to the antibody. Antibody targeted PSs will be covered in section 1.3.

Current trends in PDT drug design include taking existent PSs and formulating or encapsulating them in liposomes and nanoparticles to aid their solubility and administration. Other areas focus on covalently attaching existing PSs to small molecules such as sugars and receptor binding ligands (Nyman et al., 2004). Perhaps more advanced is the use of proteins to deliver PSs to target cells. These can be peptides, whole antibodies and antibody fragments. The latter two are the most frequently reported, due to the success of antibodies and antibody conjugates in cancer therapy.

An even smaller area of research is focused on engineering an ideal TPDT drug by firstly synthetically optimising a chosen PS then a targeting species and finally producing a conjugate that solves the specificity, dark toxicity and persistent photosensitivity issues exhibited by first and second generation PSs.

Designing third generation photosensitisers

Considering that even the most successful PSs mentioned in section 1.1.3 (BPD and mTHPC) have significant limitations, it is by approximation and correlation of the existing literature and feedback from clinicians that new PSs are designed and synthesised. The
specific structural characteristics required in an ideal PS are still under investigation. These could be neutral or charged, cationic or anionic, amphiphilic or symmetrical (Boyle et al., 1996). If it will be targeted, would maximum load or site specific controlled conjugations be more appropriate? If antibodies are the targeting moiety, use whole antibodies or fragments?

When designing new photosensitisers with improved biological efficacy and trying to meet the criteria of a good PS, quantitative structure activity relationship studies (QSAR) have been conducted (Henderson et al., 1997, Potter et al., 1999). These are based on the additive contribution of steric, hydrophobic and charge interactions of a molecule with various biomolecules and solvents (Macdonald et al., 2001). Even though a rational and mathematically based design of a PS is possible, novel improved PSs are usually discovered by trial and error modification of an existing one (Macdonald et al., 2001). Some more specific QSAR findings are discussed in chapter 5.

Some of the emerging studies on 3rd generation PSs include examples that contain a chelated metal in their cavity. Inserting a metal into the porphyrinic cavity changes the photophysics of the macrocycle. Some PSs showed improved photophysics whereas others such as haematoporphyrin (Hp) were photoinactive. Transition metals are preferred such as zinc (II), aluminium (III) and tin (IV) as the heavy atom effect can increase the rate of intersystem crossing improving the photophysics (Josefsen et al., 2008a).
**Unconjugated 3rd generation photosensitisers**

When considering charge, PSs can be cationic, anionic or neutral and in terms of water solubility they can be described as hydrophobic and hydrophilic. Amphiphilic compounds contain both a hydrophilic and hydrophobic region.

Cationic PSs tend to have very quick tissue clearance *in vivo* which is unfavourable as the effective drug dose can be low, not allowing enough time for preferential accumulation to occur followed by treatment (Villanueva et al., 1993). Negatively charged PSs are generally less potent as PDT agents as shown by several groups with efficacy declining with increasing charge number (Ali et al., 1988, Woodburn et al., 1992, Macdonald et al., 2001). As for the cationic, they also clear very rapidly from tissue (Woodburn et al., 1992). An amphiphilic character might provide more appropriate clearance profiles combining characteristics of both hydrophobic and hydrophilic PSs. Hydrophobic PSs remain in circulation for an extended period of time which is not ideal as it can lead to photosensitivity and potentially systemic side effects (Macdonald et al., 2001).

Amphiphilic PSs are still considered to be more potent than symmetrical ones in terms of photodynamic activity. This could be explained by their localisation in between hydrophobic and hydrophilic membranous environments and in the hydrophobic pockets of proteins (Macdonald et al., 2001). Aggregation, which is closely related to amphiphilicity affects the photophysical properties of a PS and subsequently its potency, reducing its singlet oxygen quantum yield (Boyle et al., 1996, Macdonald et al., 2001).

Trying to synthesise photosensitisers that preferentially localise in specific compartments of a cell such as the mitochondria or lysosomes without a designated targeting species is one of the approaches used in improving PDT agents (Macdonald et al., 2001). Dyes such as nile blue, rhodamine and bodipys can localise in a “targeted” way in organelles. This is further discussed in chapter 5.

Porphyrinic PSs tend to exhibit low water solubility and form aggregates in aqueous solutions due to their flat hydrophobic structures thus limiting their efficient use in a biological setup. Low water solubility can hinder the administration of the PS to the patient. It is often not possible to obtain sufficiently high enough therapeutic concentrations of the PS in an aqueous based solution. However, it is possible to manipulate the working conditions to enhance the solubility of a hydrophobic photosensitiser without affecting its structure. Polyethylene glycol, lipid emulsions, polysorbates can be used to enhance solubility. Water miscible organic solvents of high polarity such as DMSO and short chain alcohols (ethanol) are also used (Macdonald et al., 2001).
Similarly, aggregation can alter the photophysics of the PDT agent, affecting the singlet oxygen quantum yield by quenching the singlet excited state of the PS. Therefore, the PS relaxes to its ground state instead of colliding with molecular oxygen to generate singlet oxygen (Macdonald et al., 2001). Aggregation is a solution phenomenon and is observed when aromatic flat molecules are in solution. Large planar aromatic structures like porphyrins can interact non-covalently to form aggregates by association of two molecules ((homo)dimers) or several to form oligomers. The electrostatic interactions that develop between the aromatic rings (π-π), can lead to the formation of aggregates that have various geometries. Aggregation is further discussed in chapter 3. Aggregation is also observed in the presence of proteins where PSs tend to non-covalently bind (NCB) to their hydrophobic pockets with strong non-covalent interactions. For example, formulation of BPD with low density lipoprotein (LDL) showed that the binding of the PS to the LDL is strong and does not dissociate in vitro where it was shown to internalise via receptor mediated endocytosis via the LDL receptor (Allison et al., 1994). Generally, neutral, hydrophobic porphyrin photosensitisers strongly interact non-covalently with serum proteins especially LDL (Macdonald et al., 2001).

To overcome the photophysical quenching and insolubility of many PSs, encapsulation in dimysristoyl-L-α-phosphatidylcholine (DMPC) lipids is an attractive option as it offers tumour selectivity due to the high lipoprotein content of tumour cells and can deliver the PS without altering its structure or synthetic manipulation. MePPa, the methyl ester derivative of PPa, a third generation PS, was encapsulated in DMPC and delivered to colon carcinoma cells in vitro (Guelluy et al., 2010). The liposomes delivered the PS to the cell membrane where it was released by intermembrane contact and internalised by diffusion showing a 5-fold increased intracellular localisation compared to the MePPa. As the authors comment, this could lower the effective administered dose as less PS needs to be injected to acquire equal intracellular concentration exhibiting similar cytotoxicities.
Figure 1.12 Principles of targeted photodynamic therapy (TPDT). The patient is injected with the PIC which targets, binds and accumulates specifically on/in the antigen expressing cells. Light in the form of a non-thermal laser or LED is then used to activate the ‘drug’ by direct illumination of the site which produces singlet oxygen and other ROS leading to the death of malignant cells sparing healthy cells.

Conjugated 3rd generation photosensitisers

Targeted photodynamic therapy (TPDT) tries to address most of the limitations and problems faced by conventional PDT as previously described in section 1.1.5, which include skin photosensitivity, low tumour specificity and long drug-to-light periods. Two years ago, Josefsen and Boyle critically reviewed the future of conventional PDT, “Is there any future without highly selective photosensitisers that specifically target diseased cells?” (Josefsen et al., 2008b). The concept of TPDT (illustrated in figure 1.12) is based on the use of a targeting moiety. Targeting can be based on a drug delivery system, such as liposomes and nanoparticles, or molecular targeting and recognition, such as antibodies, peptides and sugars. Drug delivery systems can help modulate the release and pharmacokinetics of a photosensitiser but with limited specificity for diseased cells when compared to a molecular targeted approach. In many cases, drug delivery systems rely on the EPR (enhanced permeability retention) effect, a characteristic of tumour physiology, which facilitates the uptake and retention of macromolecules (Lyer et al., 2006). Drug delivery systems can be modified to include a molecular targeting species such as antibodies that will enhance the specificity of the PDT agent.

The molecular targeting approach for PDT where antibodies, peptides and sugars as well as other small molecules are used to deliver photosensitisers to tumour cells is further discussed here and in section 1.3. The PS is covalently attached to the targeting species,
binds and internalises in cells that express a characteristic antigen thus specifically killing diseased cells (Josefsen et al., 2008b). The most promising approach is the use of directly linked PSs on antibodies. Just under ten years ago when the first antibody-PS photoimmunoconjugates (PICs) were reported, either the photophysical properties of the PS or the bioactivity of the antibody were compromised upon bioconjugation. Despite the observed limitations and problems reported, antibody targeting remains a popular and promising approach (Macdonald et al., 2001, Van Dongen et al., 2004). Some other approaches to TPDT include the use of sugars, peptides and polymers which will be looked at very briefly here.

Third generation photosensitisers are increasingly being conjugated to targeting moieties, including small molecules such as sugars and large molecules such as BSA. Research by the Pandey group is focused on PPa derivatives such as HPPH (Photoclor) which was delivered to lysosomes using sugars, including β-galactose. The sugars were covalently attached to the propionic acid chain of the chlorin. The inherent localisation of HPPH is primarily in mitochondria, yet with these studies it was shown that it was possible to alter the subcellular localisation of the PS (Zheng et al., 2009). Conjugates of carbohydrates with high affinity for galectin-3 (an overexpressed lectin on tumour cells) were shown to bind preferentially to galectin-3 than galectin-1. They synthesised conjugates with multivalent galactose, glucose and combinations of both in order to observe their targeting specificity with differing structure hydrophobicity (Zheng et al., 2009). The conjugates showed higher efficacy in vitro compared to the PS alone with no appreciable potency differences between the conjugates.

PPa analogues were synthesised by the same research group to target the peripheral benzodiazepine receptor (PBR) which is overexpressed on the outer mitochondrial membrane of some cancer cells. The derivatives studied incorporated features such as NBD chloride and indium analogues for targeting the PBR. Increased singlet oxygen quantum yield and selective PBR binding led to an increase in in vitro and in vivo potency in particular for the indium(III) complexes they prepared (Chen et al., 2005).

Peptides are suitable candidates for targeting over-expressed receptors on the surfaces of cancer cells. Peptides can be synthesised to mimic the natural ligands of these receptors therefore binding to the cells (Solban et al., 2006).

For example, purpurin-18 was activated by its carboxylic acid side chain and conjugated to a nuclear localising sequence (NLS) linear peptide obtaining conjugates of known consistency.
and purity (Walker et al., 2004). Importantly, the resulting conjugate retained its photodynamic activity. Similarly, a 5 amino acid cyclic cRGDfK peptide targeting tumour vasculature, by binding αvβ3 integrins was conjugated to protoporphyrin IX (PPIX). Conjugates were purified on HPLC obtaining a 1:1 loading ratio (Conway et al., 2008). The peptide, still attached to the solid phase resin was stable in the organic solvents that the reaction took place in (DCM and DMSO) resulting in a pure conjugate analysable by mass spectrometry (Conway et al., 2008). It is important to note that the photodynamic activity of PPIX was maintained after conjugation although the binding capacity of the peptide was somewhat reduced. However, the conjugate exhibited different intracellular localisation compared to the free PS but similar in vitro cytotoxicity. In vivo studies showed higher uptake for the conjugate, however it did not correlate to significant potency difference compared to free PPIX. The authors comment on the multi-component aspect of an in vivo photodynamic therapy raising questions as to what effect subcellular localisation, tissue oxygenation levels and PS type have on efficacy (Conway et al., 2008).

Prostate specific membrane antigen (PSMA) is another attractive target for prostate cancer therapy. Using a PSMA inhibitor, Liu et al. conjugated pyropheophorbide a (PPa) using lysine coupling to form a singly labelled inhibitor. The small molecule conjugate exhibited specificity towards the antigen expressing cells successfully inducing apoptosis in vitro. However, the in vitro efficacy of the conjugate was reduced compared to the free PS attributing this difference to different subcellular localisation (Liu et al., 2009).

Comparing peptides and small molecules to antibodies (see section 1.3), the former are easier to use as they are more stable in organic solvents leading to pure conjugates. However they can lack the specificity and affinity of antibodies. Being a lot smaller than antibodies, it is also difficult to obtain high loading ratios of PS to targeting moiety.

Lastly, BSA was coupled to chlorin e6 (Ce6) (shown previously in figure 1.11) and subsequently to insulin to obtain a conjugate capable of binding insulin receptors and internalising by receptor mediated endocytosis. It was found to localise endo and perinuclearily on human hepatoma cells as verified by fluorescence microscopy. The potency of the conjugate was 100 times higher than free Ce6 requiring less energy for activation compared to the free PS to have the same cytotoxic effect. The conjugate contained 5 % free/non-covalently bound Ce6 and the loading ratio was 1:13:16 (BSA:insulin:Ce6) as analysed by thin layer chromatography (TLC) (Akhlynina et al., 1995). Other conjugates to Ce6 were prepared using a nuclear targeting signal-insulin or nuclear targeting signal-peptide, using BSA as the carrier molecule to test their conjugates in vitro. Potency was improved 2000 times compared to free Ce6 with a loading ratio of up to 7 molecules of Ce6.
per conjugate (Akhlynina et al., 1997). This attempt suggested that the nucleus is an ideal intracellular target. However, insulin might not be very appropriate when targeting cancer cells as insulin receptors are not highly overexpressed in liver tumours (Solban et al., 2006).

These examples of conjugates describe successful attempts that improved the specificity of a free PS, some improving its potency and at the same time highlighting some of the limitations that TPDT is faced with such as compromising the photophysical properties of the PS and non-covalent binding.

1.3 Photoimmunoconjugates (PICs) for TPDT

Antibodies are used in successful targeted therapies and cytotoxic conjugates with drugs, toxins and radionuclides have been studied for many years. For 3rd generation PDT, targeting with antibodies is a natural and viable option due to this wealth of information.

The conjugation of a PS to an antibody to form a PIC, is very similar and has been based mostly on the pre-existing antibody-drug conjugates (ADC) literature, techniques and methods (Senter, 2009). Until June this year there was a single ADC in the clinic approved (Deonarain, 2008) for the treatment of elderly people with acute myeloid leukemia, Gemtuzumab Ozogamicin (GO) known as Mylotarg®, which is a humanised murine CD33 antibody with a calicheamicin-g1 derivative covalently attached using a labile bifunctional linker (Pagano et al., 2007). Calicheamicin which is the cytotoxic component of the conjugate is an anti-tumour antibiotic. The monoclonal antibody (mAb) is an antineoplastic agent and does not exhibit any cytotoxicity or trigger any immune system reactions. The cytotoxic effect of the conjugate is therefore attributed to the antibiotic. The benefits of Mylotarg® treatment as reported in extended clinical trials and from the results during the approved use of it were overshadowed by the extensive and severe side effects experienced by the patients. Life expectancy was marginally prolonged with Mylotarg but side effects which included myelosuppression, hepatic veno-occlusive disease, neutropenia, thrombocytopenia, infections, hyperbilirubinemia (Pagano et al., 2007) lead Pfizer to voluntarily withdraw it from the US market (Hughes, 2010). Considering the discouraging results of Mylotarg, it appears that for a potential ADC to be successful, the activity of the cytotoxic component needs to be controlled and restricted to the targeted cells. Could TPDT using a PIC prove suitable to do so by delivering a seemingly non-toxic PS to the cells which is only activated upon light illumination?
Delivering light activatable molecules to tumour cells using targeting vehicles offers a dual selectivity which includes the specificity offered by the mAb and the requirement of light to activate the ‘drug’ in order to kill the cells (Oseroff et al., 1986). The tumour area is carefully illuminated restricting the irradiation of other tissues as much as possible. Antigen expressing healthy cells that could also be targeted by the PIC would be (in a properly designed therapy) outside the illumination zone and subsequently a non-dark toxic PIC would spare them of any damage (Oseroff et al., 1986).

Similarly to ADCs, in order to synthesise a PIC, a reactive amino acid that is accessible to solvent (on the antibody) and an activating group on the “drug” under the right conditions are required (Senter, 2009). The conjugation of a photosensitiser to biomolecules can be achieved in many ways (N-hydroxysuccinimide, maleimide, isothiocyanate activated groups) but is predominantly carried out using an N-hydroxysuccinimidyl activated ester (usually on the photosensitiser) and an amine on the biomolecule to form an amide bond (Lovell et al., 2010). Several issues can arise that are also seen with ADCs such as overloading an antibody with a “drug” which can cause it to aggregate and precipitate out of solution (Senter, 2009). Other issues, characteristic of PIC synthesis include quenching of the photophysical properties of the PS and the presence of non-covalently bound PS impurities in the PIC sample. These are further discussed in chapter 4.

**Early work**

The progress of PDT had been slow due to the somewhat disappointing results in the clinic which included low tumour specificity and associated side effects that had been observed with HpD. At the same time, magic bullets in the form of monoclonal antibodies (mAbs) were being widely used in various therapeutic concepts (Deonarain, 2008).

Photoimmunotherapy was first described in 1983 in the Journal of Immunology where leading the group, Julia G. Levy published the first paper on photoimmunoconjugate synthesis (Mew et al., 1983). The first PIC synthesis involved the activation of hematoporphyrin using a carbodiimide and its reaction with an anti DBA/2J myosarcoma M-1 mAb followed by quenching using a primary amine. This was followed by a 4 day dialysis procedure to obtain the final PIC (Mew et al., 1983). It was freeze dried and purified on G-25 size exclusion columns but the purification step had limited success. The concept of free and covalently bound PS was introduced and the problem with non-covalent binding (NCB) and purification were acknowledged. Non-covalently bound Hematoporphyrin was determined to be less than 10 % of the sample. Both antibody and PS were found to retain their reactivity...
and selectively kill the antigen positive cell line whilst not exhibiting any effect on the antigen negative cell line. Appropriate controls (mAb, PS, PBS) were also run. Radiolabelled PIC showed specificic targeting of the tumour in vivo and the treated animals showed excellent response, up to 6 months post treatment. It was concluded that mAbs on their own are unlikely to be cytotoxic therefore they can be used as delivery vehicles (Mew et al., 1983). Two years later, further conjugates employing different antibodies were reported showing potency and emphasising the possibility to eliminate or minimise the side effects seen in clinical trials with conventional PDT agents. At the same time, the technologial advances allowed them to use a laser to activate the PS compared to a fluorescent light previously used (Mew et al., 1983, Mew et al., 1985).

In subsequent work they used the then newly developed benzoporphyrin derivative (BPD). Conjugating BPD to 5E8 mAb using hexane diamine-modified polyvinyl alcohol (PVA) facilitated loading 25-50 molecules of PS per antibody. The carrier molecule was used (PVA) to increase batch to batch reproducibility for the conjugate and to enhance its water solubility. When tested in vitro, it demonstrated enhanced specificity and 15-fold increased cytotoxicity compared to free BPD. However, non-specific cytotoxicity was observed when using a non-specific antibody conjugate. BPD was also conjugated to LDL showing 6-fold increased cytotoxicity compared to free BPD but less specificity compared to the mAb conjugates (Jiang et al., 1990, Jiang et al., 1991, Jiang et al., 1992). These studies highlight the use of a carrier molecule to obtain conjugates that can be reproducibly prepared and the non-specific cytotoxicity observed even with the use of a relatively well characterised PIC.

It is important that bioconjugation reactions provide a reproducible synthesis of a PIC that has the characteristic binding specificity of the mAb and the photophysical properties of the PS. Theoretically, attaining this specificity will allow the PIC to bind its antigen in vitro and in vivo and the photosensitiser to kill the cells upon activation. The resulting PIC is characterised by the loading ratio (the amount of PS molecules covalently attached on the mAb), the theoretically unchanged targeting specificity, affinity, biodistribution of the mAb and the photophysical properties of the PS (Van Dongen et al., 2004). The methods used for PIC characterisation are discussed in chapter 4.

One of the first PICs reported was Ce6 coupled to dextran (a branched glycan) and subsequently coupled to the anti-Leu-1 mAb to obtain conjugates loaded with 24-36 PS per mAb in a multistep synthesis. It was shown that the singlet oxygen quantum yield of the PS remained unaffected by the conjugation and the binding of the antibody was also retained (Oseroff et al., 1986). It was also suggested that antibody internalisation was not necessary
for an efficient PDT effect and that the membrane was a suitable cell component for targeting (Oseroff et al., 1986).

Although a methodical study looking into the benefit of using an internalising compared to a non-internalising antibody has not been conducted, it is considered likely that internalising PICs will be more potent than non-internalising ones. The effect is expected to differ depending on antigen, cell type and antibody (Carter, 2006). Antibody targeted photosensitisers will internalise via receptor-mediated endocytosis and are likely to traffic to the lysosomes and endosomes (Savellano et al., 2005a). The effect of subcellular localisation on the therapeutic outcome in vitro is discussed in chapter 5.

Photodiagnosis is another area where PICs can be used for imaging tumours. In an interesting approach, Soukos et al. prepared conjugates of an anti-EGFR mAb with a fluorescent dye for imaging and then using the same mAb with Ce6 for use in therapy. They successfully imaged the tumour showing specificity towards the tumour and successfully continued imaging post PDT with the Ce6 PIC.

The poor water solubility of existing PSs is one of the restricting factors to the success of PICs (Van Dongen et al., 2004). Complications that can occur upon conjugating a PS to an antibody include decrease of PDT efficacy which could be explained by increased aggregation (Van Dongen et al., 2004). Additionally, specificity of the PIC compared to free PS is not always observed because of the non-covalently bound (NCB) PSs. An illustration of a PIC containing non-covalently bound PS together with an 'ideal' PIC is shown in figure 1.13. An ideal PIC should contain only covalently attached PSs and the ratio of antibody to PS must be fully characterised and reproducible. The importance of non-covalent binding in the efficacy of PDT is described in chapters 4 and 5.

In vivo studies in the area of PICs are very limited (see chapter 6). The slow progress of PICs is probably due to the multi-component nature of TPDT as previously mentioned. In order to design a potentially ideal PIC, firstly, the PS must be synthesised. This can be either by total synthesis (e.g. porphyrins) or modified from an existing PS to introduce characteristics that enable more efficient conjugation, higher loading ratios and pure, well characterised conjugates. Secondly, an antibody (whole or fragment) needs to be identified as a suitable carrier for the PS which must be stable to the conjugation reaction conditions, have sufficient sites for conjugation and must not contain sites of conjugation at its antigen recognition site to ensure PIC bioactivity. In summary, many factors require optimisation.
before a PIC can even be tested *in vivo* and twenty-five years since the report of the first PIC, the key prerequisites are still being explored and defined (Savellano et al., 2005b).

**Figure 1.13 Illustration of a photoimmunoconjugate** made of an scFv antibody fragment. Dark shaded areas indicate hydrophobic pockets where photosensitisers are likely to reside during NCB interactions. On the left, an impure PIC containing a mixture of covalently attached and free/NCB and aggregated photosensitisers is depicted and on the right an ideal PIC that contains only monomeric, covalently attached photosensitisers.

Vrouenraets, led by van Dongen in Denmark, has contributed four papers on TPDT. These firstly described the use of mAbs conjugated to mTHPC via a relatively complicated route to treat head and neck squamous cell carcinoma in tumour bearing nude mice. This study showed that a loading ratio of 4 PS per mAb was tolerated by both components and showed tumour selectivity compared to free PS (Vrouenraets et al., 1999). This was also the first report comparing internalising to non-internalising antibodies for use in TPDT with the internalising showing increased potency *in vitro*. They pointed out that poor solubility of the PS can lead to low loading ratios and non-covalent binding of the PS to the antibody which they addressed by introducing carboxylic acids onto the PS to increase its water solubility but at the same time complicating the synthesis of the PS further which was a mixture of mono-activated and di-activated esters. Making conjugates of higher loading ratios caused the resulting PICs to precipitate during purification (Vrouenraets et al., 1999). The three main issues that need to be addressed when synthesising PICs were highlighted as low water solubility of the PS leading to low loading ratios and non-covalent binding, high loading ratios leading to precipitation of the PIC and the difficulty in obtaining a pure PS for bioconjugation (Vrouenraets et al., 1999). Their subsequent work described the use of a mono-activated tricationic porphyrin conjugated to the same antibodies as previously, showing increased solubility when conjugated with up to 3 PS per mAb. Efficacy *in vitro* was again higher for the internalising antibody (Vrouenraets et al., 2000). Subsequent work used a water soluble phthalocyanine with low efficacy as a free PS which when conjugated to the same mAbs showed significant increase in potency *in vitro*. Decreasing solubility of the PIC above 4 PS per mAb loading ratio was observed again. However, the efficacy of these PICs
was higher than the previously reported mTHPC ones (Vrouenraets et al., 2001) and showed selective tumour targeting in vivo. Finally, they conjugated mTHPC and the water soluble phthalocyanine to 3 different mAbs and compared their efficacy in vitro using 5 different squamous cell carcinoma cell lines (Vrouenraets et al., 2002). The water soluble phthalocyanine conjugates were found to be more potent in all cell lines used compared to the mTHPC conjugates. Importantly, the observed potencies were in the nanomolar range. Lastly, with this work they contradicted their previous work and concluded that it is not the internalisation of the antibody that is crucial but its binding capacity and probably affinity (Vrouenraets et al., 2002).

When a drug (such as a radionuclide) circulates in the body longer than necessary, side effects have been observed. Antibody fragments offer improved pharmacokinetics that can help reduce these side effects by clearing faster from the circulation compared to an IgG (see section 1.4). Antibodies modulate the pharmacokinetics of the ADC/ PIC and subsequently the pharmacokinetics of the PS/ drug (Adams et al., 2000, Adams et al., 2004). At the same time, the targeting properties of an IgG are maintained, especially in the case of a FAb₂ which only lacks the Fc domain (see section 1.4). The application of antibody fragments in TPDT has been gaining support with an increasing number of research groups using them to target and deliver PSs.

Studies of photoimmunoconjugates using antibody fragments (see section 1.4) were first reported by the Hasan group in 1996 where a Fab₂-PS PIC was described (Hamblin et al., 1996). Following a relatively complicated method, the PS, Ce6 N-hydroxysuccinimide ester was first reacted with polylysine, purified by dialysis and subsequently anionised or quaternised before reacting with a heterobifunctional cross-linker, pyridyldithiopropionic acid NHS ester. The resulting polylysine-Ce6-linker conjugate was reacted with the partially reduced antibody to obtain the final PIC (Hamblin et al., 1996). It was a conjugate of Ce6 coupled to a murine FAb₂ using site specific cysteine residue conjugation (Duska et al., 1997). They observed that the positively charged PIC was better internalised by ovarian cancer cells and that potency was proportional to the uptake (Hamblin et al., 1996). The conjugation had no effect on the immunoreactivity of the Fab₂ and the free PS control showed no cytotoxicity. This conjugate was then used in vivo in an ovarian cancer tumour xenograft model and the results correlated with the in vitro studies verifying that the cationic PIC was more specifically uptaken (Duska et al., 1997). Haematoporphyrin was also conjugated to the same antibody fragment using the cationic route giving purer conjugates than Ce6. However, the haematoporphyrin PIC was less soluble, aggregated and suffered
from the poor absorption properties of the PS as well as observed dark toxicity (Hamblin et al., 1998). Further Ce6 conjugations were carried out using BSA to produce better characterised conjugates which showed improved cytotoxicities with higher loading ratios (Hamblin et al., 2000).

The same group also coupled Ce6 to an anti-colon cancer mAb using their earlier method (Del Governatore et al., 1999). The resulting cationic PIC internalised four times more efficiently which showed a slightly improved affinity (ELISA) than the anionic PIC which showed a slightly reduced affinity (ELISA) and both showed specificity when compared to the non-specific mAb PIC. Free PS showed some cell kill activity with the cationic PIC being about 3 times more potent (Del Governatore et al., 1999).

Polyethylene glycol (PEG) chains were used to facilitate the purification of PICs from insoluble aggregates. The same research group conjugated BPD (Verteporfin) to a chimeric anti-EGFR mAb modified using PEG chains. The PEG chains were first covalently attached on less than 3 of the antibody’s lysines and then the BPD-NHS ester was coupled to the remaining free lysines of the antibody (Savellano et al., 2003). The resulting PIC showed increased solubility, cytotoxicity on the antigen positive cell line and minimal efficacy on the antigen negative cell line (Savellano et al., 2003). The free PS showed no specificity between the two cell lines but was more potent than the PICs (Savellano et al., 2005a). With this work they showed it was possible to introduce solubility in a normally insoluble PIC retaining its efficacy.

Conjugations of antibodies with other third generation photosensitisers included anti-HER2 mAbs with pyropheophorbide a (PPa). PPa is a third generation photosensitiser derived from synthetic modification of chlorophyll a with a long absorption at 667 nm making it a good candidate for PDT. PPa is a relatively hydrophobic chlorin with sites for potential synthetic manipulation including an acid side chain for the introduction of an activating group for bioconjugation (as shown in figure 1.14) such as an NHS ester used by Savellano.

PPa conjugates were tested on HER2 positive cell lines of ovarian and breast cancer origin but were shown to be less cytotoxic than the corresponding equimolar amount of free PS as previously reported for BPD (Savellano et al., 2005a, Savellano et al., 2005b). The authors suggested two possible reasons for the potency difference. These were the photophysical quenching of the PS on the conjugate and the difference in intracellular localisation of the PIC compared to the free PS (Savellano et al., 2005b).
The research of a group led by Boyle focuses on the use of porphyrins and their development into improved bioconjugatable PSs. They successfully synthesised, purified and conjugated a tricationic water-soluble porphyrin to BSA to form thiourea bonds. They also synthesised chlorins and bacteriochlorins following an extensive synthesis (Sutton et al., 2002).

These conjugates contained non-covalently bound PS which was successfully removed by purification using size exclusion chromatography and SDS-PAGE electroelution. Importantly they highlighted the limitations of using conjugates containing non-covalently bound PS in vitro as these can detach from the protein surface and non-covalently bind to cell proteins inducing non-specific effects (Clarke et al., 1999, Sutton et al., 2002). Further work compared conjugates of a tricationic and a neutral porphyrins with an anti-HER2 and an anti-EpCam antibodies. The conjugates, loaded with 1-3 PSs were characterised in vitro showing specificity towards the antigen positive cell line. All antibodies retained their bioactivity upon conjugation as shown by FACS analysis. Comparing the internalising with the non-internalising PIC, the former appeared to be more potent in vitro. In vivo studies on human colon carcinoma showed tumour specificity as high as 33.5 tumour to healthy colon cells (Hudson et al., 2005). These were very promising results proving that tumour specificity, pure PICs starting from a pure PS are possible to achieve although a therapy in vivo would be required in order to verify the potency of the PICs.

In subsequent work, dicationic porphyrins were synthesised and conjugated to whole mAbs using an isothiocyanate group for bioconjugation. The resulting PICs showed antigen
specificity \textit{in vitro} and a lower effective drug dose compared to the free PSs (Malatesti et al., 2006).

The same tricationic and neutral porphyrins mentioned previously have also been conjugated to single chain Fvs (scFvs) (Sutton et al., 2002, Staneloudi et al., 2007). An anti-colon scFv was conjugated to the porphyrins using isothiocyanate conjugation and purified using size exclusion chromatography (Staneloudi et al., 2007). The tricationic conjugate with a loading ratio of 5:1 (PS:scFv) maintained its antigen binding as shown by FACS analysis and it was specifically cytotoxic towards antigen positive cells. Higher loading ratios of up to 40:1 completely destroyed the antibody binding (Staneloudi et al., 2007). Attempts to conjugate the neutral hydrophobic porphyrin failed to give a PIC (Staneloudi et al., 2007). Overall, the work by Boyle pointed out that it is possible to obtain PSs that exhibit low non-covalent binding, have a single reactive group for conjugation and of high purity. However, the efficacy of these conjugates has not been demonstrated \textit{in vivo}. The use of porphyrins with low absorption in the red may restrict their significant progress towards the clinic.

An alternative to coupling to lysine residues, which by its nature is random (see chapter 4), is site specific conjugation providing better characterised conjugates. In site-specific conjugation the conjugation site and loading ratios can be controlled-to an extent. This has mostly been used in ADC (see section 1.4).

Recently, water soluble cationic porphyrins were conjugated to L19 vasculature - targeting small immune protein (SIP) using a maleimide activating group to couple onto the cysteine residues of the mAb to form a carbon-sulfur bond (Alonso et al., 2010). The process resulted in PICs loaded with up to 1.75 molecules of PS to SIP. These were well characterised, probably the best characterised PICs currently reported and were tested \textit{in vitro} to show that both components were unaffected by the conjugation and toxicity was specific to antigen expressing cells (Alonso et al., 2010).

Prior to this work, the scFv format of L19, that binds the EDB domain of fibronectin was conjugated to bis(triethanolamine) Sn(IV) chlorin e6 (SnCe6). The PIC efficiently caused occlusion to newly formed blood vessels of a rabbit eye model by promoting apoptosis. (Birchler et al., 1999, Fabbrini et al., 2006). Crucially, they observed that healthy vasculature was unharmed demonstrating the actual potential of vasculature TPDT (Birchler et al., 1999). The human SIP L19 was also conjugated to SnCe6. When targeting tumour vasculature, thrombosis is one of the signs of blood vessel damage. The tumour becomes hypoxic and starves (Fabbrini et al., 2006). Subsequent tumour infarction can be observed followed by tumour mass regression. On this occasion, the treatment could not eradicate all the cancerous cells with few remaining at the border between the healthy and diseased cells.
(Fabbrini et al., 2006). The use of porphyrins with low absorption in the red is likely to hinder the therapeutic application of these PICs in the clinic. Importantly, they demonstrated that a non-internalising antibody can be very photodynamically potent as part of a PIC. The higher affinity SIP was found to be a lot more potent than the scFv (Fabbrini et al., 2006). The exceptional response observed is likely to be due to the L19 antibody. It is a very high affinity antibody, one of the highest known and likely to modulate the overall PDT efficacy of the PIC. The use of a vasculature binding antibody does not negate use of it solely for tumour targeting, but for any other disease where neovasculature needs to be targeted-such as ocular disorders, arthritis and macular degeneration.

Summarising, several issues are associated with PIC synthesis. These include (1) the aggregation of the PSs, (2) the lack of sufficient water solubility of the PS and/or the resulting PIC, (3) the presence of non-covalently bound PS in the final PIC and (4) the low reproducibility of the bioconjugation reaction (Sutton et al., 2002, Savellano et al., 2003, Savellano et al., 2005a, Savellano et al., 2005b, Alonso et al., 2010). The low reaction reproducibility can partly be addressed by site-specific conjugation which leads to reduced loading ratios. The rest are a direct result of the inherent properties of PSs that are flat aromatic molecules that tend to aggregate in aqueous solvents. Some research is focused on developing PS tailored for bioconjugation such that they are water soluble, the resulting PICs can be purified and contain a “handle” where an activating group can be added for coupling to amino acid side chains (Alonso et al., 2010). This is not straightforward for all types of porphyrinic PS especially non-symmetrical ones.

**Internalising vs non-internalising antibodies**

Despite the initial work by Vrouenraets and some work by Hudson (Hudson et al., 2005) that showed internalising antibodies to be superior targeting moieties for TPDT (Vrouenraets et al., 1999) there is not sufficient evidence to support this argument (Jiang et al., 1992, Sobolev et al., 1992, Vrouenraets et al., 2002, Hamblin, 2008). In the important work by Carcenac et al. further referred to in chapter 5, SKOV3 cells were transfected to express the carcinoembryonic antigen in order to have a single cell line that expresses both an internalising and non-internalising antigen. Whereas some work showed strong correlation towards internalising antibodies, Neri are showing positive results using a very high affinity antibody fragment that is non-internalising. It is difficult to make assumptions at this point regarding the role of internalisation of an antibody carrying a PS but it is likely to depend on the affinity and target of the antibody as well as the potency of the PS (Schliemann et al., 2009, Alonso et al., 2010).
Antibody targeted liposomes

Nanoparticles conjugated to antibodies are also being used to encapsulate and subsequently deliver PSs to cells. This area of TPDT will be very briefly discussed here.

Liposomes and other nanoparticles conjugated to antibodies are also being used as carrier and targeting species for targeted PDT. Liposomes are “multilamellar or unilamellar phospholipidic submicroscopic vesicles” often used to carry hydrophobic drugs by encapsulating them in their core (Konan et al., 2002). Composed of phospholipids and cholesterol they offer good biocompatibility and have been shown to offer some tumour selectivity. Aggregation of the tightly packed PSs can occur when incorporating PSs in liposomes which can lead to photophysical quenching. In order to overcome the limited selectivity of liposomes for tumours these have been conjugated to antibodies.

Using liposomes coupled to a mAb to encapsulate AlSPc, a water soluble phthalocyanine, it was noted that non-covalent binding (NCB) of the PS to the liposome was an issue that was only partially solved upon purification of the charged liposomes (Morgan et al., 1989). The small difference in potency between the antigen positive cell line and controls was attributed to the non-covalent binding. A key benefit of using liposomes to deliver a PS is their ability to carry high doses of PS to the cells (53 per liposome) (Morgan et al., 1989). However, some leakage of the PS from the liposomes upon storage has been observed.

Perhaps liposomal or other nanoparticles use to encapsulate the PS followed by bioconjugation will prove to be too complicated. As this area of TPDT appears to face similar problems as direct PS-mAbs conjugates, it might remain relatively unexplored.

PICs in humans

Probably the first PIC to enter a patient was an anti-CEA chimeric mAb conjugated to fluorescein which was tested on patients with colorectal cancer for imaging purposes (Van Dongen et al., 2004). Even though the results were positive, it was realised that fluorescein is not a suitable PS and was dismissed (Folli et al., 1992). A group in Germany then used a phthalocyanine-mAb conjugates to treat women with recurring breast, ovarian and cervical cancer (Schmidt, 1992, Schmidt et al., 1992, Schmidt, 1993). These studies showed some specificity and evidence of response. However, they were conducted on a small number of patients drawing few reliable conclusions thus their validity may be argued. Currently there are no clinical studies using PICs (as until 2006 (Fabbrini et al., 2006)).
1.4 Antibodies - antibody engineering

1.4.1 Antibody formats

Antibodies are large complex proteins produced in B lymphocytes and are part of a powerful defence mechanism of the human body against disease (Reichert, 2001). Antibodies can recognise and bind their antigens eliciting an immune response such as phagocytosis, cytolysis and triggering of the complement system (Reichert, 2001). There are five main classes of antibodies, IgG, IgD, IgE, IgA and IgM. IgG is the most commonly used in a therapeutic context (Weiner et al., 2010).

Immunoglobulin G (IgG) is a 150 kDa glycoprotein made up of four chains held together by disulphide bonds as shown in figure 1.15 (Scott, 2008, Nelson et al., 2009). It contains two identical light chains (each 25 kDa) which are attached via disulphide bonds to the heavy chain. The light chain consists of the variable light (\(V_L\)) which is the N-terminal and the constant light (\(C_L\)) which is the C-terminal, domains. There are two heavy chains (each 50 kDa), which make up the two constant domains of the Fc (fragment crystallisable) domain and a further constant domain in the Fab fragment. The heavy chain part of the Fab fragment (fragment antigen binding) is made up of the variable heavy chain (\(V_H\)) which is the N-terminal and a constant domain which binds the Fc portion via the hinge region. The two variable domains, \(V_H\) and \(V_L\) are responsible for antigen recognition and binding via their unique sequence. An IgG is bivalent as it can bind the same epitope using each Fab arm (Scott, 2008, Nelson et al., 2009). The Fc region primarily governs the pharmacokinetics of an antibody. It can elicit an immune response by activating immune cells by binding to their Fc receptors. Critically, the Fc elicits an immune response and binds to other cell types.
Figure 1.15 Schematic representation of an IgG molecule. A whole IgG antibody consists of two chains, the heavy chain (●) and the light chain (●). The various sub-domains are shown including the variable light and variable heavy chains as well as the 3 sets of constant domains. Glycosylation sites on CH2 are also shown as well as disulphide bonds (●).

Monoclonal antibodies were initially isolated from murine hybridomas by fusion of B-lymphocytes and myeloma cells (Reichert et al., 2005). The development of hybridoma technology in 1975 by Kohler and Milstein set the foundations for a booming antibody field both commercially and scientifically (Kohler et al., 1975, Carter, 2006). B cells from immunised (with antigen) mice are fused with immortalised myeloma cells to produce a stable cell line that produces the antibody of interest (Kohler et al., 1975, Carter, 2006).

Use of murine antibodies in humans highlighted their limitations; they were immunogenic with human anti-mouse mAbs (HAMA) being detected in the body minimising their efficacy and increasing side effects (Reichert et al., 2005). Figure 1.16 highlights the chronological development of mAbs. Chimeric antibodies followed, which eventually led to humanised then to fully human. The high immunogenicity observed with the murine mAbs was improved with the chimeric mAbs by replacing the murine constant domains with human IgG constant
domains. The DNA containing mouse and human genes was transfected into mouse myeloma cells to produce the chimeric antibodies containing both human and murine genes (Morrison et al., 1984). To produce humanised antibodies, the CDRs of a mouse antibody were grafted into the CDRs of a human protein with the resulting humanised antibody exhibiting binding properties of the murine but containing mostly (>95 %) human DNA sequences (Jones et al., 1986).

Transgenic mice (Lonberg, 2005) and phage display (Hoogenboom, 2005) technologies were the tools for the development of fully human mAbs in the late 1990s (Reichert et al., 2005, Carter, 2006). From these human formats sprung the various recombinant fragments such as scFvs and antigen-binding fragments. Some are shown in figure 1.17. (Reichert et al., 2005). Antibodies in their various formats have shown that they can successfully bind their antigens-ligands or receptors- and mediate an immune response or regulate receptors providing therapeutic outcomes (Deonarain, 2008).

Figure 1.16 Historical milestones in antibody engineering development and their clinical applications. Key therapeutic antibodies are listed. Green areas indicate murine regions and blue regions are human. Within 30 years, mAbs have gone from non-existent in the clinic to fully human with an array of recombinant forms. Taken from (Lonberg, 2005).
Antibodies in the clinic

The production of mAbs in the early 1980s was hailed as a therapeutic breakthrough (Reichert, 2001). The so-called “magic bullets” capable of targeting and eliminating foreign or diseased biological components were first introduced as murine whole antibodies (Reichert, 2001).

Developing mAbs for therapy begun in the 1980s with big pharmaceutical companies being sceptical and hesitant to move into that line of research (Reichert et al., 2005). However it has now paid off with more than 450 mAbs having entered clinical trials (Nelson et al., 2009) with recombinant antibodies being more likely to end up in the clinic than small molecules. As of 2008 22 mAbs were approved for therapeutic use, 10 of which were for cancer treatments (Deonarain, 2008, Reichert, 2008). Of these, only three are fragments with the rest being whole IgGs (Nelson et al., 2009). In the 1980s there was a surge of murine antibodies entering clinical trials which were eventually substituted by human mAbs (Reichert et al., 2005). Murine monoclonals had less chances of being approved compared to human and fully human antibodies, which are now the main focus (Reichert et al., 2005).
Figure 1.17 Schematic representation of various human antibody formats produced by antibody engineering and bioconjugation. Green spheres indicate glycosylation and red curves indicate peptide linkers connecting the variable heavy with the variable light chains. Disulphide bonds are shown on the IgG and small molecules are shown on the scFv conjugate. Reproduced and modified from (Deonarain, 2008).

Whole Antibodies, fragments and cancer

Most approved mAbs are for cancer or immunological treatments (Reichert et al., 2005). In cancer, approved or advanced mAbs are against a handful of validated and well understood targets-HER2, CD20, EGFR, VEGF, but there are many other targets being exploited and the next decade should see dozens of mAbs against new and potentially more effective targets (Griggs et al., 2009). For example, there is a continuous search for new biomarkers for breast and prostate cancer (Normanno et al., 2009, Radpour et al., 2009, Larkin et al., 2010).

Whole antibodies are large molecules associated with slow perfusion into tumours and slow blood clearance with half-lives in the order of days. This leads to low tumour to normal tissue ratios as whole mAbs do not accumulate at high enough doses to induce a therapeutic effect (1:1 by 24 hrs and 1:3 by 3 days) (Jain, 1990). Fragments do not suffer from this and exhibit
faster clearance times and better tumour ratios (Schier et al., 1996). Whole IgGs have long
half-lives in the order of more than 10 days. The Fc receptor can bind the neonatal receptor
(FcRn) which protects the antibody from being destroyed and helps trafficking across cells
(Holliger et al., 2005).

Fragments clear faster than whole IgGs as they have shorter half lives in the human body
(Larson et al., 1983). The benefit of fast clearance depends on the treatment type and the
drug potency (Nelson et al., 2009). Their faster clearance is attributed to the lack of Fc-Rn
binding and to their smaller size allowing them to go through the glomerular filtration
whereas whole antibodies go through the liver (Nelson et al., 2009). In order to increase a
fragment’s half-life and blood circulation, various engineering techniques have been
developed such as PEGylation and polysialylation (Constantinou et al., 2010).

Side effects associated with whole mAbs include toxicity caused by the effector function of
the Fc domains by activating antibody dependent cell mediated cytotoxicity/complement
dependent cytotoxicity (ADCC/CDC). The Fc portion can activate immune cells (natural killer
cells, phagocytes and neutrophils) which bind the antibody and elicit an immune response
leading to the release of growth factors and cytokines, inhibition of tumour angiogenesis,
antigen presentation and overall tumour immunogenicity and cytolysis (Deonarain, 2008).
Potent therapeutic antibodies including rituximab, cetuximab and trastuzumab are all
able to activate ADCC and/or CDC (Deonarain, 2008). As this response is associated
with the Fc receptor, fragments cannot elicit ADCC or CDC (Nelson et al., 2009).

The statistics regarding fragments and their clinical success are as follows: 54 entered
clinical trials, 3 were approved in the USA, 1 in China, 19 still in clinical trials and 31 (57 %)
were discontinued (Nelson et al., 2009). It takes 6-7 years for antibodies to go through the
clinical trials until approval (Nelson et al., 2009). Of these, 24 (44 %) were conjugates
against cancer targets. Immunoconjugates in clinical trials are mostly scFvs (Nelson et al.,
2009).

**Single chain variable fragments (scFvs)**

Fragments are genetically modified derivatives of IgGs. Single chain Fvs were first reported
in 1988 and have become particularly favourable with various formats being engineered
facilitated by being easily selectable by phage display (Hoogenboom, 2005). Figure 1.17
shows some of the main fragments commonly used. Single chain Fvs are made up of the
variable light and variable heavy chains of the respective IgG. A peptide linker connects the C-terminus of the V<sub>L</sub> with the N-terminus of the V<sub>H</sub> chain (Bird et al., 1988, Huston et al., 1988). They are 5-fold smaller than an IgG and exhibit higher and faster tumour penetration at the same time maintaining the affinity and specificity of the parent molecule (Holliger et al., 2005). An excellent review by Holliger describes the various fragments (Holliger et al., 2005).

Engineering antibodies to produce agents that are more stable, easier to produce in higher yields and have high affinity is becoming the norm. These are some of the tools provided by recombinant techniques allowing the design and synthesis of an array of biomolecules. These include smaller fragments, monovalent, divalent, trivalent, mono-specific, bis-specific, fusions, conjugates and so on (Holliger et al., 2005).

**Immunoconjugates**

The idea of a drug behaving like a “magic bullet” capable of targeting specific diseases in the body was conceived by Ehrlich in the 1900s (Strebhardt et al., 2008). The affinity of dyes for biological components was part of Ehrlich’s studies (Strebhardt et al., 2008). He combined observations on synthetic dyes, cells and medicine. He believed that it was necessary to “learn how to aim chemically” thus setting the foundations of chemotherapy (Strebhardt et al., 2008).

It is clear that the ideas of Ehrlich some 110 years ago are being realized as therapy-oriented projects are becoming increasingly interdisciplinary and the understanding of biology, chemistry, medicine and physics are being bridged towards the same goals.

Antibody therapy in the 60-70s or even the 50s when polyclonal and mouse Abs were used, focused on conjugates but unconjugated Abs eventually superseded because they were simpler to develop and at the time more successful. But now conjugates are returning to fill the gap left by mAbs.

Antibodies conjugated to chemotherapeutic or radiation agents can deliver a cytotoxic drug specifically to the site of interest improving the potency of the therapy (Reichert et al., 2007). Even though in theory they sound ideal, synthesis of immunoconjugates is more complicated as intricate chemistry is required making them more difficult to manufacture and thus only accounted for 44 % of the anticancer mAbs in clinical trials in 2008 (Reichert et al., 2007, Deonarain, 2008) some of which are shown in figure 1.18.
Radioimmunotherapy (RIT) was first reported in the early 1950s with anti-globulin antibodies labelled with iodine-131 (Pressman et al., 1950). Radioimmunoconjugates (RICs) are mostly used for haematological malignancies and are less suitable for solid tumour treatments (Sharkey et al., 2005). There are two approved RICs, ⁹⁰Y-based Bexxar® and ¹³¹I-based Zevalin® (Davies, Sharkey et al., 2005). The former delivers “beta particles with 0.8 mm path and a half-life of 64 hrs and the latter delivers beta particles and gamma radiation with a 5.3 mm path length and a half-life of 8 days”. Isotopes in the pipeline include Bi-213 emitting alpha particles which have a path length of 80 µm (Deonarine, 2008). Shorter half lives and shorter path lengths can potentially reduce side effects.

<table>
<thead>
<tr>
<th>Drug (developer)</th>
<th>Antibody-drug conjugate</th>
<th>Indication (phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glembatumumab vedotin (Celldex Therapeutics)</td>
<td>A fully human mAb specific for GPNMB conjugated to monomethyl auristatin E ¹</td>
<td>Metastatic breast cancer and melanoma (II)</td>
</tr>
<tr>
<td>Trastuzumab emtansine (Roche/Genentech/Chugai)</td>
<td>A humanized mAb specific for HER2 conjugated to the maytansine derivative DM1 ³</td>
<td>HER2-positive metastatic breast cancer (I/III)</td>
</tr>
<tr>
<td>Lorvotuzumab mertansine (ImmuGen)</td>
<td>A humanized mAb specific for CD56 conjugated to the maytansine derivative DM1</td>
<td>Small cell lung cancer, Merkel cell carcinoma, ovarian cancer and multiple myeloma (II)</td>
</tr>
<tr>
<td>SAR-3419 (Sanofi-Aventis)</td>
<td>A humanized mAb specific for CD19 conjugated to the maytansine derivative DM4 ⁵</td>
<td>Non-Hodgkin’s lymphoma (II)</td>
</tr>
<tr>
<td>Brentuximab vedotin (Seattle Genetics/ Millenium Pharmaceuticals)</td>
<td>A chimeric mAb specific for CD30 conjugated to monomethyl auristatin E</td>
<td>Anaplastic large cell lymphoma (II), relapsed or refractory Hodgkin’s lymphoma (II) and Hodgkin’s lymphoma following autologous stem cell transplant (III)</td>
</tr>
<tr>
<td>Inotuzumab ozogamicin (Pfizer)</td>
<td>A humanized mAb specific for CD22 conjugated to calicheamicin</td>
<td>Diffuse large B-cell lymphoma, indolent non-Hodgkin’s lymphoma (II)</td>
</tr>
</tbody>
</table>

GPNNB, glycoprotein non-metastatic melanoma protein B; HER2, human epidermal growth factor receptor 2 (also known as ERBB2); mAb, monoclonal antibody. ¹This list only includes antibody-drug conjugates that link an antibody to a cytotoxic agent; it does not include antibodies conjugated to radioisotopes or immunotoxins. ²Licensed from Seattle Genetics. ³Licensed from ImmunoGen.

Figure 1.18 Antibody-drug conjugates in clinical trials. Phase II or III. An increasing number of ADCs are expected to enter clinical trials in the future as their potential is being investigated. Reproduced from (Hughes, 2010).

RICs are administered to patients at doses that cause “severe myelosuppression” leading to patients requiring supportive therapy to overcome the side-effects (Sharkey et al., 2005). The administered dose and the possibility of repeated treatment depend on the response of the patient and the severity of the experienced side-effects (Sharkey et al., 2005). RIT has been successful in treating haematological cancers but less so at treating solid tumours mainly due to the poor pharmacokinetics of whole mAb conjugates (Sharkey et al., 2005, Jain et al., 2007). Newer ones are currently more successful in clinical trials with yet more hurdles to overcome (Jain et al., 2007).

The use of whole antibodies with long blood half-life and poor tumour penetration has stifled its advance (Jain et al., 2007). The use of antibody fragments, with shorter half-lives than whole mAbs, for RIT is not ideal either as the nature of the therapy requires the radioisotope
to remain at the tumour site long enough to be cytotoxic. The faster clearance associated with fragments minimising side effects outweighs the benefits by decreasing potency (Deonarain, 2008). In an attempt to solve this issue, a pre-targeting approach has been used where the antigen binding antibody is allowed to bind the tumour and only after this step is the radioisotope introduced by linking it to a small molecule that binds the antibody. Therefore the clearance of the radioisotope is not governed by the antibody (Deonarain, 2008).

Some issues that the area of antibody-drug conjugates (ADCs) is faced with overlap with those of TPDT such as those shown in figure 1.19. In the field of ADC, it is important that there is a significant discrepancy between the expression levels of the antigen in healthy and diseased cells in order to avoid targeting healthy cells (Deonarain, 2008). The concept for TPDT, RIT and ADCs is to deliver the cytotoxic drug to the cell which becomes active once within the cell therefore restricting its cytotoxicity to the diseased cells (Damle, 2008). The advantage of TPDT is its bimodality (Mellish et al., 2001). TPDT has the potential to be very effective and to exhibit many benefits over other treatments.

One consistent issue with immunoconjugates (whether for RIT, TPDT or other antibody-chemotherapy drug conjugate) is the heterogeneity of the resulting sample which will inevitably consist of a range of multiply loaded antibody molecules (Damle, 2008, Goldmacher, 2010). Attempts to improve the variability within the resulting distribution can minimise it but as reactions are never 100% complete it would be difficult to assume a scenario where an exactly homogeneous sample is obtained. THIOMABs are mAbs engineered to contain exactly known sites available for thiol conjugation at sites on the antibody where no biological function will be obstructed and have been shown potent with improved in vivo tolerance (Damle, 2008, Junutula et al., 2008). Minimising the uncertainty with regards to the conjugation efficiency, site-specific conjugations have been utilised. In PDT the L19 SIP-porphyrin PICs mentioned previously are an example (Alonso et al., 2010). Site specific conjugates with known loading ratios are preferred by the FDA.
Figure 1.19 Key parameters in antibody-drug conjugate synthesis. These are important for both the synthesis and the efficiency of the conjugate to act as a drug in vivo. Modified from (Alley et al., 2010).

TPDT can possibly offer advantages over RIT and targeted chemotherapy especially as there are no restrictions on how often a patient can be treated. With aggressive chemotherapy and radiotherapy treatment is restricted to one session (Nyst et al., 2009). Patients treated with PDT for head and neck cancer showed benefits compared to surgery and radiotherapy with respect to reduced morbidity and good cosmetic outcomes (Nyst et al., 2009). PDT has an added advantage compared to chemotherapy and radiotherapy in that it does not appear to be carcinogenic (Allison et al., 2010). In tumours where antigens are not solely expressed by diseased cells—which rarely is the case—targeted PDT can still be applied if the healthy tissue expressing the antigen is difficult to reach or if it is isolated from daylight and far from the irradiation site (Carter, 2006).

The “age of ADCs” is upon us with ADCs being developed extensively and big pharmaceutical companies at the forefront. In July 2010, Genentech submitted for approval a conjugate of trastuzumab (Herceptin) with DM1 (T-DM1) a potent chemotherapeutic which is currently the most advanced ADC in the clinic (Hughes, 2010). This ADC is trying to address the limitations of Herceptin, a highly potent mAb (see section 1.4.4) in treating HER2 positive breast cancer. In their phase II clinical trials, they found that 30 % of the treated women showed a strong response with the clinicians pointing out the benefits of the ADC primarily being the specificity in delivering chemotherapy to the diseased cells (Hughes, 2010).

With increasing understanding of antibodies, antigens, the importance of drug load, technologies on linking drugs to antibodies these are likely to further improve and be more successful compared to 40 years ago. The potency of the conjugate drug is also highly important as only a small percentage (0.01 % ID/g of tumour) actually reaches the tumour but at the same time it needs to be “dormant or inactive” whilst circulating the blood to minimise side effects (Hughes, 2010).
ImmunoGen has carried out extensive and meticulous research in the field of ADCs looking at the linkers and their stability in serum emphasising the need for the drug to be attached on the mAb at least until it reaches its target (Doronina et al., 2008, Hughes, 2010). Lysine coupling is preferred due to the accessibility of lysines usually on the surface of mAbs which can be modified without affecting the structure of the biomolecule (Hughes, 2010). One area of concern is that even with ADCs cells can still develop resistance to the delivered drug (Hughes, 2010).

1.4.2 Cancer therapy in the context of PDT

Cancer is the disease of the 21st century; many disciplines such as genetics, cell biology, immunology and therapeutics are involved in the research for possible treatments and cures. It is beyond the scope of this thesis to review cancer and will therefore suffice at looking at some key characteristics of tumours related to the tumour model used here (HER2). It can be seen above that whole mAbs targeting photosensitisers towards cancer related antigens have dominated the area of targeted PDT.

Cancer cells are normal cells that have attained an immortalised status through mutations and although they cannot cause death on their own, their interactions with the body at the tumour-host level can inhibit organ function and slowly inhibit vital functions leading to death (Deonarain, 2008). Establishing their own blood supply is one of the crucial and rate-determining steps in the disease progression of solid tumours. Angiogenesis plays an important role in cancer growth and metastasis as it supplies the cells with nutrients allowing them to grow, invade crucial organs and metastasise (Deonarain, 2008).

Tumour physiology is characterised by high interstitial pressure and heterogeneous and disorganised blood supply (Jain, 1990). Tumours are highly vascularised with the exception of necrotic areas. The perfusion of tumours is much less than healthy cells (Van Dongen et al., 2004). When treating tumours with mAbs, the antibody needs to cross the blood vessels to reach the cancer cells. This is why mAbs have been more successful for haematological cancers. Internalisation of whole antibodies in healthy lung and skin endothelium is not easily feasible (Van Dongen et al., 2004). Contrary to healthy cells, cancerous cell endothelium is much more permeable (Van Dongen et al., 2004). Interstitial pressure is yet another tumour characteristic that can affect the penetration of an antibody. Increasing tumour size correlates with increased pressure which lowers the uptake of an antibody due to the competing fluid forces.
Targeting the vascular endothelial growth factor (VEGF) which binds to VEGF receptors providing angiogenic signalling and new blood vessel growth is one approach to cancer therapy which overcomes some of the above issues (Deonarain, 2008). Neutralising VEGF has been proven to be effective in treating metastatic colorectal cancer as well as non-small cell lung and breast cancers (Panares et al., 2007). Bevacizumab works by inhibiting vasculature growth leading to cell starvation, normalises the tumour microenvironment and interstitial pressure making the tumour susceptible to drugs and reduces the number of progenitor cells available (Jain et al., 2006). There are many anti-angiogenic therapies under development, including anti-growth factors or directly targeting the tumour vasculature with a cytotoxin. For example, using a vasculature targeting SIP to bind the extra domain B of fibronectin on B-cell non-Hodgkin lymphoma with a fusion antibody-IL2 gave improved efficacy compared to IL2 alone and when combined with rituximab complete remissions were observed in mice xenografts. The fusion L19-IL2 is currently in clinical trials for patients with solid tumours (Schliemann et al., 2009).

Targeting cell surface antigens is the most common approach to anti cancer immunotherapy with some of the most common being epithelial adhesion molecule (EpCam), epidermal growth factor receptor (EGFR), CD20, mucin 1(MUC1), CD22, CD23, Lewis Y, EGFR, PSMA, human epidermal growth factor receptor 2 (HER2), carcinoembryonic antigen and TAG-72 (Harari, 2004, Reichert et al., 2007, Deonarain, 2008).

The main approach to treating tumours with unconjugated antibodies is to disrupt the signalling pathways downstream/ related to the survival of the antigen. The role and importance of mediating ADCC and/or CDC responses is another approach that has been less well explored (Weiner et al., 2010). For example, targeting MUC1 overexpressing tumours, a humanised antibody that binds a specific mucin 1 related peptide sequence is in clinical trials. Other approaches include promoting ADCC by modifying Fc domains such as in the case of CD20 targeting with ocrelizumab. CD20 is also targeted with ofatumumab which mediates CDC causing tumour cell lysis in the treatment of chronic lymphocytic leukaemia (Weiner et al., 2010).
1.4.3 The ErbB family and the Human epidermal growth factor receptor 2 (HER2)

The ErbB family of transmembrane receptors is made up of four type I tyrosine kinase receptors. These are EGFR, (HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) (Baselga et al., 2009). These receptors bind ligands extracellularly that initiate a cascade of signals by homo and heterodimerization intracellularly associated with vital healthy cell functions such as proliferation, survival, migration, differentiation and angiogenesis as shown in figure 1.20 (Deonarain, 2008, Baselga et al., 2009).

The ErbB2 family and cancer

The over-expression of EGFR has been linked with 50 % of all cancers and 80 % of metastatic colon cancers with poor therapeutic outcomes. HER2 over-expression is related to 25 % of breast cancers, some ovarian cancers, gastric carcinoma and salivary gland tumours (Baselga et al., 2009). It is also found over-expressed in non-small cell lung cancers and in androgen resistant prostate cancers (Baselga et al., 2009). Formation of HER2 homodimers (in the absence of a ligand) or heterodimers (in the presence of a ligand for one of the other erbB family members) activates signals for phosphorylation which turns on a cascade of reactions that can lead to angiogenesis, proliferation, cell cycle control, apoptosis suppression and survival as shown in figure 1.20 (Baselga et al., 2009).

HER2, closely related to the other members of the ErbB family, is a glycoprotein which again is associated with drug resistance and poor therapeutic prognosis for 30 % of invasive breast cancers (Slamon et al., 1987). There are 25-fold more HER2 receptors on the cancer cell surface than healthy ones which can be more than 2 million receptors/cell (Press et al., 2002). The receptors have different functions as HER4 has no known tumourigenic ability and it is possible that its role is anti-proliferative (Baselga et al., 2009). HER2 on the other hand has an always active tyrosine kinase binding site but no known ligand (Deonarain, 2008, Baselga et al., 2009). HER2 and HER3 form the strongest heterodimer pair which activates phosphorylation and hence cell proliferation (Deonarain, 2008, Baselga et al., 2009). There is strong evidence that shows this heterodimer to be the primary cause to HER2 dependent tumourigenesis (Baselga et al., 2009). Figure 1.20 describes these processes.
1.4.4 Anti-HER2 treatments and their limitations

Anti-HER2 immunotherapy

Herceptin® (trastuzumab) is a humanised mAb developed by Genentech Inc. and is one of the most successful commercially available antibodies. It is used for the treatment of HER2 positive breast cancers. Even though it is a highly potent mAb, patients relapse within a year from treatment and develop resistance to the therapy. There are several possible pathways that when activated can inhibit the effect of trastuzumab (Baselga et al., 2009). These are not fully understood but their elucidation is important in developing treatments to combat the resistance developed in patients treated with Herceptin® (Nahta et al., 2006).

Herceptin binds the extracellular domain of HER2 (ErbB2) and causes the downregulation of HER2 which leads to its degradation by inhibiting its signalling as shown in figure 1.21 (Deonarain, 2008) (Nahta et al., 2006). There are also indications that Herceptin has an anti-angiogenic function by normalising tumour vasculature (Deonarain, 2008). Trastuzumab antagonises HER2 signalling whereas newer antibodies against HER2 block its dimerisation (Franklin et al., 2004, Deonarain, 2008). As the members of the ErbB2 family are proven to
be oncogenic-with the exception of HER4- many therapeutic efforts focus on inhibiting dimer formation and tyrosine kinase inhibitors (Baselga et al., 2009).

Antibody based therapies require patients to express or overexpress an antigen in order to be potentially effective (Deonarain, 2008). A significant amount of patients (15 %) treated with Herceptin (following verification of antigen expression) become resistant and do not respond to the therapy (Nahta et al., 2006).

**Herceptin**

On a patient level, Herceptin® has been used to treat patients with HER2 positive tumours in parallel with chemotherapy. In combination with surgery can further enhance the survival rates up to 50 %. There are currently more anti-HER2 mAbs in the therapeutic pipeline such as Pertuzumab which is humanized and binds the dimerisation domain of ErbB2, a distinct epitope to trastuzumab. It inhibits the dimerisation of HER2 with HER3 blocking the pathway to the cell’s survival and proliferation (Baselga et al., 2009).

Combination treatments where both Herceptin® and pertuzumab were administered have been shown to be efficacious. However, significant side effects could develop as Herceptin® is known to affect the heart causing cardiac dysfunction due to the role of HER2 in the heart (Hudis, 2007). Combining two anti-HER2 drugs might not prove to be prudent.

Other anti-erbB2 drugs include bi and tri-specific Abs and a conjugate of Herceptin with an anti-microtubule agent-a chemotherapeutic- which is released into the HER2 positive cell. Other routes include tyrosine kinase inhibitors (TKI) -one such example is lapatinib (GlaxoSmithKline) and has shown promising results when used with capecitabine. Herceptin has limitations including patients relapsing and requiring a combination of lapatinib and trastuzumab (Baselga et al., 2009). Combination therapies, using either two antibodies or two modalities, conjugates, TKIs are some of the developing approaches in the area (Baselga et al., 2009). There is a need for a potent anti-HER2 treatment that is less prone to resistance. Drug development efforts are aimed at addressing this and include the previously mentioned trastuzumab-DM1 conjugate developed by Genentech (see section 1.4.1).
Figure 1.21 Mechanisms of selected anti-HER2 therapies showing antibody binding and subsequent inactivation of tyrosine kinase activity. Trastuzumab is shown in (a) where it binds the extracellular domain and inhibits signalling as well as initiating ADCC through its Fc receptor. Other approaches b-f are shown.

1.4.5 C6.5(-k) - a human anti-HER2 scFv

C6.5 is a fully human anti-HER2 scFv obtained by phage display by Schier et al. in 1995 (Schier et al., 1995). They started from a naive human library containing variable heavy and variable light chain genes which were displayed on phage as pIII fusions. Selection of the scFv was performed using immobilised antigen, c-erbB-2 extra-cellular domain (Schier et al., 1995). The gene was cloned into pUC119SfiI/Not1mycHis with the tags present at the C-terminal of the protein (Nielsen et al., 2002). The scFv was expressed in E. coli HB2151 and was purified by periplasmic isolation and subsequent IMAC purification to obtain pure scFv that could bind the native antigen on live cells (Schier et al., 1995). The $K_d$ was determined via BIAcore and Scatchard analysis and was found to be 16 nM (Schier et al., 1995). It was also found that the $k_{off}$ rate was fast ($6.3 \times 10^{-3}$ s$^{-1}$) which means it does not associate with the antigen for very long (Schier et al., 1995).
C6.5K-A, as referred to by Adams, was one of the C6.5 mutants obtained during affinity maturation of the scFv (Schier et al., 1996). They constructed a library containing mutations in nine amino acids in the CDR3 region of the V_L chain. Following four selection rounds they obtained a total of 11 clones that successfully bound the antigen. The mutant with the highest affinity was further mutated by converting selected amino acids to alanines in the V_H CDR3 (Schier et al., 1996). A smaller V_H library was constructed and the final scFvs were obtained following four selection rounds. The best one had 145 fold higher affinity than C6.5. C6.5(-k), as will be referred to here, has lysine100 in the CDR3 region of the heavy chain mutated to an alanine (Schier et al., 1996, Adams et al., 2000). Its $K_d$ was determined to be 9.8 nM (Schier et al., 1996). C6.5(-k) is an ideal candidate for lysine-directed bioconjugation reactions. The presence of a labile to conjugation amino acid in the CDR3 region of an antibody is not desirable as this can inhibit antigen binding (Adams et al., 2000). This was therefore removed leading to fully active RICs when conjugated via lysines and hence was considered a good choice for NHS-coupled PICs (more in chapter 4).

1.5 Summary

Even though PDT has yet to reach its full potential (Brown, 2008) the booming research interest is the first step towards improved targeted drugs exhibiting no side effects and excellent therapeutic outcomes. Reviews more than ten years ago referred to the suitable photosensitiser for use in PDT with higher specificity and longer wavelength absorption (Pandey et al., 1998). PDT is now converging towards TPDT with associated limitations and newly introduced criteria. Using conjugates that can specifically target the molecule of interest can result in lower doses of the drug being administered (effective drug dose) reducing the side effects associated with high dose therapies (Wartlick et al., 2004).

“There is still no “magic bullet”” as Wainwright points out in an attempt to put PDT into perspective in the clinical setting. Chemotherapy, radiotherapy and surgery, the main options available for cancer patients leave at best, traces of the disease in terms of scarring and side effects. Side effects can be as severe as immunosuppression, morbidity and lowering of the immune defence of the patient (Wainwright, 2008). PDT is milder than both radiation and chemotherapy.
Using mostly first and second generation PSs, conjugates were prepared using mainly antibodies to target PSs to antigen expressing cells. Focus is also perhaps shifting away from porphyrins whose absorption profiles are not ideal and focusing more on chlorins and bacteriochlorins (Hamblin et al., 1998).

In the attempts to make PICs, solubility, aggregation and non-covalent binding were found to be limiting the loading ratios and purity of the resulting conjugates. It is possible to design and synthesise a PS that will facilitate the preparation of a pure PIC as shown by Boyle (Malatesti et al., 2006). An ideal one has yet to be described. That would entail: strong absorption in the red (>630 nm), good generator of singlet oxygen, targeted or capable of being conjugated to a carrier molecule, exhibit rapid clearance to minimise photosensitivity, must be a single pure component and easy to make synthetically especially on a gram scale, stable and soluble in aqueous solutions (Josefsen et al., 2008a). From this extended list of prerequisites one can see that the (re)search for an ideal TPDT drug will involve a multidisciplinary project with the input of the chemist in design, synthesis and initial characterisation being crucial followed by extensive biological testing and then the enthusiasm of the clinician to use it (Wainwright, 2008).
1.6 Aims and Objectives

PDT has made significant advances in recent years. Concurring research data from both the clinic and the laboratory point towards the need to develop highly specific, targeted and water soluble agents that can be easily administered and the patient treated quickly without experiencing any significant side effects. Key characteristics of the resulting TPDT drug should be purity, absorption in the red and specificity with no dark toxicity.

The overall aim of this research project was to produce well characterised, water soluble PICs of high purity and efficacy using pyropheophorbide $a$ (PP$a$) as the core macrocycle and an anti-HER2 scFv and subsequently test one of these in a HER2 over-expressing cancer cell line model.

Therefore, the objectives of the work were:

1. Synthesis of water soluble PP$a$ derivatives by incorporating short polyethylene glycol chains and/or positive charges and characterising these for aggregation and photophysical properties
2. Conjugate these to an anti-HER2 scFv to make high concentration conjugates and subsequently purify and characterise the loading ratio of the resulting PICs
3. Characterise both the PICs and free PSs in vitro using cell viability assays to estimate potency, confocal microscopy and FACS analysis
4. Lastly, characterise both the free PSs and a chosen PIC in vivo using a HER2 expressing cancer tumour xenograft on nude mice. Determine the potency of the PIC and either prove or disprove the benefits of TPDT.
Chapter 2 Materials and Methods
2.1 Chemistry

2.1.1 List of Materials

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-pyridine boronic acid</td>
<td>Maybridge®</td>
<td>Temperature sensitive-keep cold</td>
</tr>
<tr>
<td>6-hexynoic acid</td>
<td>Sigma (544000)</td>
<td>Used as purchased</td>
</tr>
<tr>
<td>Oxygen free nitrogen and argon gas</td>
<td>BOC</td>
<td>Pass through a drierite gas drying unit</td>
</tr>
<tr>
<td>Pd/ C (10 %)</td>
<td>Sigma</td>
<td>-</td>
</tr>
<tr>
<td>Potassium phosphate tribasic</td>
<td>Sigma</td>
<td>Only use the powder-keep desiccated</td>
</tr>
<tr>
<td>Preparative TLC plates 2000 µm</td>
<td>Sigma (Z513024)</td>
<td></td>
</tr>
<tr>
<td>Pyridinium perbromide hydrobromide</td>
<td>Sigma</td>
<td>Ensure it is dry- if necessary dry before use</td>
</tr>
<tr>
<td>Pyropheophorbide-a</td>
<td>Frontier Scientific, Inc</td>
<td>Kept at 4 °C in the dark</td>
</tr>
<tr>
<td>Pyropheophorbide-a methyl ester</td>
<td>Frontier Scientific, Inc</td>
<td>Kept at 4 °C in the dark</td>
</tr>
<tr>
<td>Spectroscopic grade toluene</td>
<td>Sigma</td>
<td>use for all photophysical measurements</td>
</tr>
<tr>
<td>Tetrakis (triphenylphosphine) palladium (0)</td>
<td>Strem Chemicals, Inc.</td>
<td>Yellow crystalline solid. Store desiccated under nitrogen/argon at 4 °C</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>Sigma-Aldrich (T6508)</td>
<td>Ensure it is fuming</td>
</tr>
<tr>
<td>Tris(dibenzylideneacetone)dipalladium (0)</td>
<td>Strem Chemicals, Inc.</td>
<td>Store desiccated under nitrogen/argon at 4 °C</td>
</tr>
</tbody>
</table>

Other chemicals were purchased from Sigma-Aldrich®, Fluka, Acros Organics, VWR®, Merck. Common solvents were purchased from VWR®. Dry solvents were purchased from Sigma. TEA and DCM were distilled from calcium hydride. TEA was kept under vacuum at room temperature, in an amber container. THF (CHROMASOLV® Plus) and diethyl ether (spectrophotometric grade, inhibitor free) were distilled from sodium/ benzophenone.

Silica gel 60, aluminium oxide 90 active neutral or active basic, aluminium oxide 60 F254 neutral sheets and TLC Silica Gel 60 F254 glass plates were purchased from Merck.

Aluminium oxide grade III was prepared by equilibrating with 6 % water overnight.

TLC plates were visualised under a UV-lamp (365 and 254 nm) or developed in an iodine tank.
2.1.2 Equipment

UV/Vis spectra were recorded on an Agilent 8453 UV Visible Spectrophotometer.

Fluorescence spectra were recorded on a Varian Cary Eclipse Fluorescence spectrophotometer. All spectra were recorded using a 1 cm path quartz cuvette.

NMR spectra were recorded and analysed on Bruker Topspin 1.3 software.

Analytical LC MS were recorded on an LCT Premier instrument with ESI as the ionisation method. A Waters Atlantis C18 reverse-phase column, 30 mm x 2.1 mm, 3 microns particle size with a linear solvent gradient, going from 95% Water (0.1% formic acid) : 5% MeCN to 5% Water : 95% MeCN over 10-15 mins.

Preparative LC MS were run on an XBridge™ C18 5 µm, OBD™, 19 x 100 mm column with gradient elution using MeOH/ water (0.1 % FA) (0 mins 95 % water going to 15 mins 5 % water). The HPLC was connected to a Waters 2767 sample manager and a Waters 3100 Mass detector.

Electron Ionisation (EI) and Chemical Ionisation (CI) spectra were recorded on a Micromass AutoSpec Premier instrument. Electrospray Ionisation (ESI) spectra were recorded on a Micromass LCT Premier instrument coupled to a time-of-flight (ToF) analyser. MALDI spectra were recorded on a Micromass MALDI instrument. Masses for MS results are given within the accepted range (measured to <5 ppm).
2.1.3 Building Block synthesis

Toluene-4-sulfonic acid 2-[2-(2-methoxy-ethoxy)-ethoxy]-ethyl ester (1) (Snow et al., 2003)

To a stirred ice cooled solution of sodium hydroxide (6.25 g, 0.16 mol) in water (25 ml) a solution of triethylene glycol monomethyl ether (20 g, 0.122 mol) in THF (30 ml) was added and left to stir for 10 min. at 0-5 °C. To the resulting mixture p-toluenesulfonyl chloride (24.6 g, 0.129 mol) dissolved in THF (56 ml) was added dropwise over 1 hour whilst maintaining the temperature <5 °C. Upon completion of addition, it was left to stir for a further 1 hour and then allowed to warm to RT. The reaction mixture was quenched by the addition of water and extracted with diethyl ether (3 x 15 ml). The combined organic layers were acidified by the addition of 1M HCl (30 ml), washed with water (2 x), dried (MgSO₄), filtered and the solvent removed to give the product as a yellow oil (31.31 g, 81 %). The crude product was used without further purification. ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 7.82 (d, 2H, aromatic), 7.36 (d, 2H, aromatic), 4.18 (t, 2H, J 4.8 Hz, CH₂TS), 3.7 (t, 2H, CH₂O, J 4.9 Hz), 3.6 (m, 6H, CH₂O), 3.5 (m, 2H, CH₂O, J 4.9 Hz), 3.39 (s, 3H, Me ar), 2.47 (s, 3H, Me). MS El (m/z)= 318 [M]+ (Calc for C₁₄H₂₂O₆S 318.39)

methyl 3,4,5-tri(triethylene glycol monomethyl ether)benzoate (2) (Oar et al., 2005)

Potassium carbonate (17.268 g, 0.125 mol), 18-crown-6 (1.315 g, 0.000498 mol) and 3,4,5-trihydroxybenzoate (4.596 g, 0.0249 mol) were added to a solution of (1) (25.00 g, 0.0785 mol) in acetone (220 ml). The reaction mixture was heated under reflux under nitrogen for 40 hrs, filtered and the solvent removed to give a yellow oil. This was dissolved in chloroform (370 ml) and washed with saturated sodium carbonate (5 x 320 ml), saturated sodium hydrogen carbonate (3 x 180 ml) and brine (1 x 200 ml). The combined organic layers were dried (MgSO₄), filtered and the solvent removed to give a yellow oil (19.94 g). This was purified on silica gel eluting with methanol/chloroform (5 %) giving the desired product as a clear oil (14.66 g, 95 %). ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 7.31 (s, 2H, aromatic), 4.22 (m, 6H, ar-OCH₂), 3.9 (s, 3H, MeO), 3.89 (t, 3H, CH₂CH₂Oar), 3.81 (t, 3H CH₂CH₂Oar), 3.74
(m, 6H, CH₂), 3.66 (m, 12H, CH₂) 3.56 (m, 6H, CH₂), 3.39 (s, 9H, Me). MS ES (m/z)= 622[M⁺], 623 [M+H⁺] (Calc for C₂₉H₅₀O₁₄ 622.70).

3,4,5-tri(triethylene glycol monomethyl ether)benzyl alcohol (3) (Oar et al., 2005)

To a cooled (0-5 °C) slurry of LiAlH₄ (0.2536 g) in dry THF (30 ml), a solution of (2) (4.99 g, 8.03 x 10⁻³ mol) in dry THF (50 ml) was added dropwise over 1 hr. Once addition was complete the reaction mixture was allowed to warm to RT and stirred under nitrogen overnight. TLC (SM Rₜ 0.56, product Rₜ 0.38) showed the presence of two spots at this point the reaction was again cooled to 0-5 °C and a further batch of LiAlH₄ (0.25 g) was added and stirred at RT until the reaction went to completion. (IR C=O at 1450 is absent in the product). The mixture was quenched (CARE!) using celite/ Na₂SO₄.10H₂O (1:1) and filtered. The solvent was removed to give the product as a yellow oil (4.42 g, 93 %). ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 6.64 (s, 2H, aromatic), 4.59 (s, 2H, CH₂OH), 4.17 (m, 6H, ar-OCH₂), 3.85 (t, 3H, CH₂CH₂Oar), 3.73 (t, 3H CH₂CH₂Oar), 3.66 (m, 12H, CH₂), 3.38 (s, 9H, Me). MS ES (m/z)= 617 [M+Na⁺] (Calc for C₂₉H₅₀O₁₃ 594.69).

Iodo triethylene oxide (4) (Holmes et al., 2007)

To a cooled (0-5 °C) slurry of LiAlH₄ (0.2536 g) in dry THF (30 ml), a solution of (2) (4.99 g, 8.03 x 10⁻³ mol) in dry THF (50 ml) was added dropwise over 1 hr. Once addition was complete the reaction mixture was allowed to warm to RT and stirred under nitrogen overnight. TLC (SM Rₜ 0.56, product Rₜ 0.38) showed the presence of two spots at this point the reaction was again cooled to 0-5 °C and a further batch of LiAlH₄ (0.25 g) was added and stirred at RT until the reaction went to completion. (IR C=O at 1450 is absent in the product). The mixture was quenched (CARE!) using celite/ Na₂SO₄.10H₂O (1:1) and filtered. The solvent was removed to give the product as a yellow oil (4.42 g, 93 %). ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 6.64 (s, 2H, aromatic), 4.59 (s, 2H, CH₂OH), 4.17 (m, 6H, ar-OCH₂), 3.85 (t, 3H, CH₂CH₂O), 3.73 (t, 3H CH₂CH₂Oar), 3.66 (m, 12H, CH₂), 3.38 (s, 9H, Me). MS ES (m/z)= 617 [M+Na⁺] (Calc for C₂₉H₅₀O₁₃ 594.69).
2.1.4 Chlorophyll Extraction

Obtaining MePPa from Spirulina Pacifica® Section 2.2.4 was carried out by following known procedures (Smith et al., 1985, Garrido et al., 2003, Hargus, 2005)

Chlorophyll-a (5)

Dried Spirulina Pacifica algae (Cyanotech, Hawaii) (300 g) was slurried with acetone (1 L) and added in liquid nitrogen (1.5 L). It was left to stand for 2 hrs before more acetone (1 L) and liquid nitrogen were added and left to stand in the dark o/n. The slurry was transferred to a 3 L RBF equipped with a mechanical stirrer and heated under reflux with vigorous stirring for 2 hrs light protected under nitrogen. The resulting mixture was filtered hot and the filter cake re-extracted with acetone for 2 hrs. This procedure was repeated 4 times. The solvent was removed to give a dark viscous oil (20.95 g). This was divided into three and purified on silica gel eluting with DCM (to elute the carotenoids) and then with EtOAc/DCM (2/8) to give pure pheophytin (1.7 g).

Methyl pheophorbide-a (6)

Methanol (300 ml) was degassed with nitrogen and conc. H₂SO₄ (15 ml) was added followed by chlorophyll-a (5) (1.7 g). The mixture was allowed to stir at RT, light protected, under nitrogen, o/n after which it was diluted with DCM (200 ml), washed with water (200 ml) and back extracted with DCM. The combined organic layers were washed with saturated NaHCO₃ (100 ml), dried over Na₂SO₄, filtered and concentrated to obtain a blue solid, methyl pheophorbide-a (1.54 g) Rf: 0.64.

Methyl Pyropheophorbide-a (7)

Collidine (150 ml) was added to crude methyl pheophorbide-a and the mixture heated under reflux maintaining the internal temperature at 165 °C for 6 hrs. The collidine was distilled off to give a viscous oil. This was purified on silica gel eluting initially with DCM and then
EtOAc/DCM (10 %) to give pure MePPa (0.275 g) as a purple solid. $^1$H NMR (CDCl$_3$, 400 MHz, 25 °C) δ= 9.51 (s, 10-meso-H), 9.40 (s, 5-meso H), 8.58 (s, 20-meso H), 8.02 (dd, 1H, 3a), 6.29 and 6.19 (2 x dd, 2H, 3b), 5.22 (q, 2 H, 13b), 4.51 (m, 1 H, 18H), 4.31 (m, 1H, 17H), 3.70 (s, 3H, 17d), 3.69 (m, 2 H, 8a), 3.64 (s, 3H, 12a), 3.43 (s, 3H, 7a), 3.26 (s, 3H, 2a), 2.72 (m, 2H, 17a), 2.6 (m, 1H, 17b), 2.32 (m, 1H, 17b'), 1.84 (t, 3H, 8b), 1.72 (t, 3H, 18a), 0.48 (br s, 1 H, NH), -1.7 (br s, 1H, NH). UV/Vis (CH$_2$Cl$_2$): $\lambda_{\text{max}}$ 667 nm. MS (FAB): (m/z)= 549 [M$^+$], (Calc. for C$_{34}$H$_{36}$N$_4$O$_5$ 548.67) LCMS (C$_{18}$) 11.38 min (549.28)

2.1.5 Pyropheophorbide-a Main Derivatives

Pyropheophorbide-a succinimidy l ester (8) (Bhatti et al., 2008)

N-hydroxysuccinimide (0.0132 g, 0.115 mmol) and N,N’-dicyclohexylcarbodiimide (0.025 g, 0.12 mmol) were added to a solution of pyropheophorbide-a (0.050 g, 0.0935 mmol) in dry dichloromethane (9 ml) and dry THF (1 ml) and stirred at RT, under nitrogen protected from light for 18 hrs. The solvent was removed to obtain a blue residue. This was dissolved in chloroform and precipitated with hexane and filtered. The resulting solid was dissolved in a minimum amount of chloroform and purified on silica gel eluting with hexane/EtOAc (20 %) to give the desired product (35.5 mg, 60 %). R$_f$ (EtOAc/Hexane, 80 %) = 0.6; MS LCMS 10.77 mins (m/z)= 632.3 [M+H]$^+$ (Calc. for C$_{37}$H$_{37}$N$_5$O$_6$ 631.3)

3-devinyl-methyl pyropheophorbide-a (9)(Li et al., 2003)

A solution of zinc acetate dihydrate (304 mg, 1.39 mmol) in methanol (12.5 ml) was added to a solution of methyl pyropheophorbide a (Frontier scientfic) (289 mg, 0.526 mmol) in dichloromethane (21 ml) and stirred at RT, light protected under Nitrogen for 3 hrs. The reaction was followed by UV/Vis spectroscopy monitoring the disappearance of a band at $\lambda_{\text{max}}$ 669 nm and the appearance of a band at $\lambda_{\text{max}}$ 656 nm. It was washed with water (4 x 30
ml), the combined aqueous layers were back extracted with DCM (1 x) and dried (MgSO₄), filtered and concentrated to obtain a blue crystalline solid. It was dissolved in dry THF (27 ml) and triethylamine (75 µl) and Pd/C 10 % (29.3 mg) were added. The resulting mixture was hydrogenated using a balloon filled with hydrogen at RT, light protected for 18 hrs. The mixture was filtered through a pad of celite and the solvent was removed. To the obtained solid TFA was added and stirred at RT, under nitrogen for 1.5 hrs. The reaction was quenched with iced water (150 ml) and extracted with DCM. The organic layer was washed with water (2 x 55 ml), 5 % NaHCO₃ (55 ml) and the combined organic layers dried (Na₂SO₄), filtered and concentrated. The crude product was purified on neutral alumina (Brockmann grade III) eluting with EtOAc/DCM (10 %) to give the product as a dark purple solid (275.5 mg, 92 %). Rf (10 % EtOAc/DCM)= 0.66. ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 9.51 (s, 10k meso), 9.24 (s, 5k meso), 8.49 (s, 20-meso), 5.22 (q, 2 H, 13b CH₂CO), 4.49 (m, 1 H, 18-H), 4.30 (m, 1H, 17-H), 3.85 (q, 2H, 3a), 3.69 (m, 2 H, 3b), 3.64 (s, 3H, 17d), 3.62 (s, 3H, 12a), 3.31 (s, 3H, 7a), 3.28 (s, 3H, 2a), 2.72 (m, 2H, 17a), 2.6 (m, 1H, 17b), 2.32 (m, 1H, 17b'), 1.82 (d, 3H, 18a), 1.74 (t, 3H, 8b), (m, 2H, 8a), 0.63 (br s, 1 H, NH), -1.57 (br s, 1H, NH). UV/Vis (CH₂Cl₂): λₘₐₓ 656 nm. MS ES: m/z= 551.3 [M+H], (Calc. for C₃₄H₃₈N₄O₅ 550.69)

3-devinyl-20-bromo methyl pyropheophorbide-a (10) (Kenner et al., 1973)

Pyridinium perbromide (198 mg, 0.618 mmol) and pyridine (500 µl) were added to a solution of (9) (271 mg, 0.492 mmol) in dry DCM (75 ml). The resulting mixture was stirred for 20 mins at RT, light protected under argon and the solvent removed to give the crude product as a brown solid. The reaction was followed by UV/Vis spectroscopy monitoring the disappearance of a band at λₘₐₓ 656 nm and the appearance of a band at λₘₐₓ 668 nm. This was purified on neutral alumina (Brockmann grade III) eluting with DCM. The final product was obtained as a purple solid (242 mg, 77 %). Rf (DCM): 0.3. ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 9.56 (s, 1H, 10-meso), 9.45 (s, 5-meso), 5.0 (q, 2 H, 13b), 4.89 (m, 1 H, 18H), 4.25 (m, 1H, 17H), 3.85 (m, 2H, 3a), 3.69 (m, 2 H, 3b), 3.68 (s, 3H, 17d), 3.60 (s, 3H, 12a), 3.56
Concentrated hydrochloric acid (100 ml) was slowly added to (10) (202 mg, 0.37 mmol). The resulting green solution was stirred at RT, light protected with a nitrogen bubbler for 1.5 hrs. The reaction was diluted with iced water (600 ml) and extracted with chloroform until the washings were clear. The combined organic layers were washed with 5 % NaHCO₃, water, dried over Na₂SO₄, filtered and the solvent removed to give a dark blue solid. This was used purified on silica gel eluting with CHCl₃ followed by gradient elution with MeOH/CHCl₃ up to 15 % MeOH to give a dark blue solid. Rf (10 % MeOH/CHCl₃): 0.4 ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 9.43 (s, 1H, 10-kmeso), 9.17 (s, 1H, 5-kmeso), 8.45 (s, 1H, 20-kmeso), 5.18 (q, 2H, 13b), 4.38 (m, 2H, 17H and 18 H), 3.80 (q, 2H, 3a), 3.66 (m, 3H, 3b), 3.63 (s, 3H, 12a), 3.28 (s, 1H, 7a), 3.24 (s, 1H, 2a), 2.62 (m, 2H, 17a), 2.34 (m, 2H, 17b), 1.81 (d, 3H, 18a), 1.69 (m, 3H, 8b), 1.27 (br s, 1H, NH), -1.5 (br s, 1H, NH). UV/Vis (CH₂Cl₂): λmax (log ε) 410 (4.8), 503 (4.1), 656 (4.8).
2.1.6 *Pyropheophorbide-a Derivatives (PS1 route)*

3,4,5-tri(triethylene glycol monomethyl ether)benzyl ester meso pyropheophorbide-a (12) (Huber et al., 2007)

To a solution of 3-devinyl pyropheophorbide-a (11) (250 mg, 0.46 mmol) in dry DCM (150 ml) and dry THF (15 ml), under nitrogen DIC (3 mmol, 469 µl), DMAP (1.8 mmol, 222 mg), DPTS (1.7 mmol, 512 mg) and N-ethyl-diisopropylamine (280 µl) and finally 3 (0.698 mmol, 416 mg) were added and stirred at RT overnight, light protected under nitrogen. The reaction was followed by TLC (silica gel, 5 % MeOH/CHCl₃) by observing the consumption of the starting material. The reaction mixture was washed with HCl (0.5 M) and twice with water combining the organics. The water layer was backextracted with DCM twice before combining the organics and drying over Na₂SO₄ and concentrating. The crude was redissolved in DCM, layered with hexane and left to stand at 4 °C overnight. The precipitated white solid was collected by filtration through cotton wool, and the filtrate concentrated and purified on silica gel eluting with 2 % MeOH/CHCl₃ followed by gradient addition of MeOH up to 5 %. The concentrated sample was redissolved in DCM and layered with hexane leaving to stand overnight at 4 °C at which point it was filtered through cotton wool again this time obtaining the pure product as a viscous brown oil (457 mg, 88 %). Rₜ (5 % MeOH/CHCl₃) = 0.37. ¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 9.47 (1H, s, 10kmeso), 9.21 (1H, s, 5-meso), 8.47 (1H, s, 20-meso), 6.52 (2H, s, 17e), 5.17 (2H, dd, 13b), 4.95 (dd, 2H, 17d), 4.48 (1H, m, 18H), 4.39 (2H, m, 17H), 4.09 (6H, m, 17f), 3.86 (2H, m, 8a), 3.77 (6H, m, 17g), 3.70-3.49 (30H, m, 3a, 3b, 17h), 3.67 (3H, s, 12a), 3.34 (9H, s, 17l-OMe), 3.30 (3H, s, 7a), 3.26 (3H, s, 2a), 2.71 (m, 1H, 17a), 2.61 (1H, m, 17a), 2.33 (2H, m, 17b), 1.79 (3H, d, 18a), 1.73 (3H, m, 8b), 0.97 (1H, s, NH), -1.59 (1H, s, NH). MS LCMS (C₁₈) retention time 11.23 min 1113.60 [M]+ (Calc for C₆₁H₈₄N₄O₁₅ 1113.33). UV/Vis (CH₂Cl₂): λₘₐₓ (log ε) 410 (5.11), 503(3.99), 600 (3.9), 656 (4.74).
3,4,5-tri(triethylene glycol monomethyl ether)benzyl ester 3-devinyl-20-bromo-pyropheophorbide-a (13)

3,4,5-tri(triethylene glycol monomethyl ether)benzyl ester meso pyropheophorbide-a (12) (395 mg, 0.355 mmol) was dissolved in dry DCM (160 ml) under nitrogen. Pyridinium perbromide (148 mg, 0.461 mmol) and dry pyridine (376 µl, 4.61 mmol) were added and the mixture stirred under nitrogen, light protected at RT for 20 mins. The reaction was followed by UV/Vis spectroscopy monitoring the disappearance of a band at $\lambda_{\text{max}}$ 656 nm and the appearance of a band at $\lambda_{\text{max}}$ 668 nm. The mixture was concentrated and purified on silica gel eluting with 2 % MeOH/ CHCl$_3$. The product was isolated as a dark brown viscous oil (405 mg, 96 %). R$_f$ (10 % MeOH/ CHCl$_3$):0.38 UV/Vis (CH$_2$Cl$_2$): $\lambda_{\text{max}}$ 668 nm. $^1$H NMR (CDCl$_3$, 400 MHz, 25 °C) $\delta=$ 9.54 (s, 1H, 10-kmeso), 9.43 (s, 1H, 5-meso), 6.51 (2H, s, 17e), 5.25 (2H, d, 13b), 4.9 (3H, m, 17d, 18H), 4.26 (1H, m, 17H ), 4.10 (6H, m, 17f), 3.92 (2H, m, 8a), 3.80-3.50 (36H, m, 3a, 3b, 17g-k), 3.68 (3H, s, 12a), 3.56 (3H, s, 2a), 3.37 (3H, s, 17l OMe), 3.36 (6H, s, 17l-OMe), 3.30 (3H, s, 2a), 2.64 (3H, m, 7a), 2.24 (2H, m, 17a), 1.72 (2H, m, 17b), 1.60 (3H, d, 18a), 1.17 (3H, d, 8b), -1.74 (1H, s, NH). MS ES (m/z)= 1191.51[M]+, 1215.49 [M+Na]+ (Calc for C$_{61}$H$_{83}$N$_4$O$_{15}$ 1192.23)
3-devinyl-20-hexynoic-acid-17-O-(3,4,5-tri-ethyleneoxide)benzyl ester pyropheophorbide-a (14) (PS1 acid) (Kelley et al., 2006b)

Dry DMF and dry triethylamine were separately degassed with dry nitrogen for 30 mins. Degassed DMF (4.5 ml) was added to (13) (109 mg, 0.092 mmol) under nitrogen followed by tri(otolyl)phosphine (0.0325 g, 0.107 mmol). After degassing for a further 5 mins, degassed TEA (0.5 ml) and tris(dibenzylideneacetone)Pd2 (0) (12.77 mg, 0.014 mmol) were added whilst maintaining a constant flow of dry nitrogen. Hexynoic acid (93.35 µl, 0.92 mmol) was then added and the reaction stirred under nitrogen, light protected at RT overnight. The reaction mixture was diluted with diethyl ether and washed once with dilute citric acid and once with water before drying over Na2SO4 and concentrating. The crude product was purified on silica gel eluting with CHCl3 followed by gradient addition of MeOH up to 15% to obtain the pure product as a brown viscous oil (78.5 mg, 74%). Rf (7% MeOH/ CHCl3) = 0.35. UV/Vis (CH2Cl2): λmax (log ε) 419 (4.6), 672 (4.2); 1H NMR (CDCl3, 400 MHz, 25°C) δ= 9.39 (1H, s, 10-meso), 9.22 (1H, s, 5-meso), 6.56 (2H, s, 17e), 5.17 (2H, dd, 13b), 4.98 (2H, dd, 17d), 4.74 (1H, m, 17H), 4.14 (7H, distorted m, 18H, 17f), 3.8-3.4 (39H, distorted m, 3a, 3b, 20c,d, 17g-k), 3.37 (9H, dist. s, 171-OMe), 3.17 (3H, s, 12a), 2.97 (2H, m, 20e), 2.77 (2H, m, 17a), 2.56 (4H, m, 17b), 2.27 (3H, m, 18a), 1.71 (3H, m, 8b), 0.1 (1H, s, NH), -1.0 (1H, s, NH). (Note that the region 1-4.2 ppm is quite broad, some protons could not be assigned as could not distinguish them) 13C NMR (CDCl3, 400 MHz, 25°C) (195), (176), (175.8), (173) (159) (154) (152) (151) (149) (144) (143.3), (143), (138) (136) (134) (131.8), (131) (130) (128) (108) (106) (104) (97) (96) (92) (83) (72) (71) (71) (70.6) (70.5) (70.4) (70) (69). (69) (68) (66) (58.92) (58.9) (51) (50) (48) (45) (33) (32) (31) (29.9) (24) (20.9) (19.9), (19.3), (19.2) (17.7) (17) (16.8) (14) (11.9) (11.1) (8.4) (0.9). MS LCMS (C18) retention time 11.38 min (1245.61 [M+Na]+, 1223.63 [M+H]+) (Calc. for C67H90N4O17 1223.45).
3-devinyl-20-hexynoic acid-17°-(3,4,5-tri-triethyleneoxide) benzyl ester
pyropheophorbide-a succinimidy ester (15) (PS1 AE)

3-devinyl-20-hexynoic acid-17°-(3,4,5-tri-triethyleneoxide) benzyl ester pyropheophorbide-a
(20 mg, 0.016 mmol) was dissolved in dry DCM (9 ml) and dry THF (1 ml) under nitrogen. N-
hydroxysuccinimide (2.26 mg, 0.019 mmol) and diisopropylcarbodiimide (5.06 µl, 0.033
mmol) were added and the reaction was left to stir under nitrogen light protected at RT
overnight. The solvent was removed and the crude oil was washed with warm hexane
repeatedly and was then purified on silica gel eluting with CHCl₃ followed by the gradual
addition of MeOH up to 5 %. The resulting oil was redissolved in dry DCM and was filtered
through cotton wool to obtain the product as a dark viscous oil (16.6 mg, 77 %). UV/Vis
(CH₂Cl₂): λₘₐₓ 671 nm; ¹¹H NMR (CDCl₃, 400 MHz, 25 °C) δ= 9.37 (1H, s, 10-meso), 9.20
(1H, s, 5-meso), 6.47 (2H, s, 17e), 5.12 (2H, dd, 13b), 4.89 (2H, dd, 17d), 4.71 (1H, m, 17H),
4.12 (1H, m, 18H), 4.05 (6H, m, 17f), 3.81 (3H, m, 3b), 3.75-3.46 (32H, m, 3a, 17g-k), 3.6
(3H, s, 12a), 3.34 (3H, s, 17I-Ome), 3.31 (6H, s, 17l-OMe), 3.21 (3H, s, 7a), 3.05 (3H, m),
2.85 (4H, broad s, NHS), 2.55 (2H, m), 2.37 (2H, m), 2.19 (2H, m), 1.67 (11H, m), 0.08 (1H,
s, NH), -1.01 (1H, s, NH). ¹³C NMR (CDCl₃, 400 MHz, 25 °C) (195.9) (175.9) (173) (169.1)
(168.2) (159.5) (156.8) (154.3) (152.6) (151.1) (148.8) (144.8) (143.5) (143.2) (138.4)
(138.3) (135.9) (134.7) (131.8) (131) (130.7) (128.3) (108) (106.6) (104.6) (97) (96.2) (91.8)
(83.8) (77.6) (72.2) (71.9) (71.8) (70.7) (70.6) (70.58) (70.5) (69.6) (68.8) (66.4) (58.9) (51)
(50.1) (48.2) (42.2) (30.9) (30.3) (29.9) (29.7) (25.6) (24) (23.5) (21) (19.5) (19.4) (19.3)
(17.3) (16.9) (14.8) (14) (11.9) (11.1) (0.99); MS LCMS (C₁₈) retention time 11.34 min Found
1343.6 [M+Na]+, 1320.6 [M+H]+ (Calc. for C₇₁H₈₃N₅O₁₉ 1320.52).
2.1.7 Pyropheophorbide-a Derivatives (PS4 route)

3-devinyl-20-pyridyl-methyl pyropheophorbide-a (16) (Kelley et al., 2007b)

(10) (0.2 g, 0.32 mmol) and 4-pyridyl boronic acid (0.39 g, 3.18 mmol) were degassed with dry nitrogen for 15 mins before adding dry THF (80 ml) and degassing for a further 30 mins with the nitrogen bubbling through. Pd(PPh₃)₄ (~80 mg) was added and continued degassing the mixture for 15 mins. K₃PO₄ (1.35 g, 6.35 mmol) was added and the reaction was heated under reflux for 15 hrs under nitrogen, light protected. Pd(PPh₃)₄ (~40 mg) was added and continued heating under reflux for 9 hours before diluting the mixture with chloroform and washing with water, sat NaHCO₃, brine and water and drying over Na₂SO₄. The crude product was purified on silica gel eluting with CHCl₃/THF (9/1) to obtain a dark oil which was recrystallised from CHCl₃/Hexane at 4 °C overnight to obtain long purple crystals (63 % yield). Rf (CHCl₃/THF): 0.3. UV/Vis (CH₂Cl₂): λₘₐₓ (log ε) 414 (4.7), 512 (3.6), 543 (3.73), 611 (3.5), 668 (4.3). ¹H NMR (CDCl₃, 400 MHz, 25 °C) 9.55 (1H, s, 10kmeso), 9.43 (1H, s, 5kmeso) 9.07 (1H, d, J₄.92 Hz, 20b), 8.9 (1H, d, J₄.92 Hz, 20b'), 8.15 (1H, d, J₄.72 Hz, 20a), 7.67 (1H, d, J₄.7 Hz, 20a'), 5.21 (2H, s, 13bk CH₂CO), 4.23 (1H, q, 18k H), 4.12 (1H, dd, 17 CH), 3.84 (2H, q, 3a-CH₂), 3.75 (2H, m, 8a CH₂CH₃), 3.7 (3H, s, 17d), 3.58 (3H, s, 12a) 3.31 (3H, s, 7a), 2.55 (2H, m, 17a), 2.37 (3H, s, 2a), 2.21 (2H, m, 17b), 1.75 (3H, t, 8b CH₂CH₂), 1.67 (3H, t, 3b CH₃CH₂), -1.45 (1H, s, NH), MS ES (m/z) (Calc. for C₃₉H₄₁N₅O₆ 627.32) Found 628.32 M⁺+H. LCMS (C₁₈) retention time 11.12 min (628.3306).
3-devinyl-20-pyridyl pyropheophorbide-a (17)

3-devinyl-20-pyridyl-methyl pyropheophorbide-a (16) (108 mg, 0.172 mmol) was added to an oven dried RBF and purged briefly with nitrogen. Concentrated HCl (22 ml) was added to dissolve under nitrogen and stirred at room temperature light protected for 8 hrs. The reaction was quenched by into iced water and extracted with chloroform followed by washes with sat. NaHCO$_3$ and water. It was dried over Na$_2$SO$_4$, filtered and concentrated before purifying on silica gel with gradient elution of 5-10 % MeOH/CHCl$_3$ to obtain a dark purple solid (71 % yield). $R_f$ (5 % MeOH/ CHCl$_3$): 0.5. The $^1$H and $^{13}$C NMR were broad and could not be analysed but the hydrolysis of the methyl ester was observed as the disappearance of the singlet peak at 3.58 ($^1$H NMR) and change in $R_f$ value. MS ES (m/z) (Calc for C$_{38}$H$_{39}$N$_5$O$_3$, 613.75) Found 614.31 [M + H]$^+$; $^1$H NMR (CDCl$_3$, 400 MHz, 25 °C) as observed for most of the acid PP$_a$ derivatives this was broad leading to indistinguishable peaks making it difficult to interpret and assign. LCMS (C$_{18}$) retention time 9.51min (614.3132) UV/Vis (CH$_2$Cl$_2$): $\lambda_{max}$ 668 nm

3-devinyl-20-(4-triethyleneoxide pyridinium) pyropheophorbide-a iodide (18)

3-devinyl-20-pyridyl pyropheophorbide-a (17) (46.6 mg, 0.076 mmol) was dissolved in dry DMF (5 ml). Iodo triethylene oxide (1.15 g, 4.2 mmol) was added and the reaction was stirred at 80 °C under nitrogen light protected for four days. The reaction was monitored by TLC (silica gel, MeCN:H$_2$O: sat.K$_2$NO$_3$ 60:10:10) by following the consumption of the starting material. The solvent was removed and the residual oil purified on basic alumina
(Brockmann grade III) eluting with MeOH/DCM (5 %) gradually to 100 % MeOH and then 10 % H$_2$O/MeOH to obtain a brown oil. UV/Vis $\lambda_{\text{max}}$ 675 nm. MS ES (m/z) Found 760.41 [M – I]$^+$ (Calc for C$_{45}$H$_{54}$IN$_5$O$_6$ 887.84). Some product was obtained via this method. However, it was not time efficient and the purity of the final product was not significantly improved.

3-devinyl-20-(4-triethyleneoxide pyridinium) pyropheophorbide-a iodide (19)

3-devinyl-20-pyridyl pyropheophorbide-a (17) (60 mg, 0.098 mmol) was dissolved in dry DMF (5 ml). Iodo triethylene oxide (1.48 g, 5.4 mmol) was added and the reaction was stirred at 80 °C under nitrogen light protected for four days. The reaction was monitored by TLC (silica gel, MeCN:H$_2$O: sat.K$_2$NO$_3$ 60:10:10) by following the consumption of the starting material. The solvent was removed and the residual oil redissolved in dry DCM and after cooling down filtered through cotton wool. This was repeated several times. The crude was redissolved in dry DCM and carefully layered with dry ether and left to stand at 4 °C. The precipitated brown solid was collected by centrifugation and dried over P$_2$O$_5$ in a vacuum desiccator. MS ES (m/z) Found 760.41 [M – I]$^+$ (Calc for C$_{45}$H$_{54}$IN$_5$O$_6$ 887.84). LCMS (C$_{18}$) retention time 6.84 min (760.4090) UV/Vis $\lambda_{\text{max}}$ 677 nm.
3-devinyl-20-(4-triethyleneoxide pyridinium)-methyl pyropheophorbide-a iodide (20)

3-devinyl-20-pyridyl-methyl pyropheophorbide-a (16) (0.100 g, 0.159 mmol) was weighed into a dry RBF under nitrogen and dissolved with dry DMF (10 ml). Iodo triethylene oxide (1.853 g, 6.76 mmol) was added and stirred at 80 °C light protected, under nitrogen for 24 hrs. The reaction was monitored by TLC (silica gel, MeCN:H2O: sat.K2NO3 60:10:10) by following the consumption of the starting material. The solvent was removed and the residual oil was washed with dry ether. This was repeated several times. The crude was redissolved in dry DCM and filtered through cotton wool. Finally, the crude was redissolved in dry DCM, carefully layered with dry ether and left to stand at 4 °C. The precipitated brown solid was collected by centrifugation and dried over P2O5 in a vacuum desiccator. Rf (MeCN:H2O: sat.K2NO3 60:10:10): 0.6; UV/Vis λmax (log ε) 414 (4.7), 512 (3.6), 559 (3.9), 620 (3.6), 675 (4.3). 1H NMR (CDCl3, 400 MHz, 25 °C) 9.63 (1H, d, J6.12 Hz, 20b), 9.55 (1H, s, 10kmeso), 9.49 (1H, s, 5kmeso), 9.38 (1H, d, J6.24 Hz, 20a), 8.98 (1H, dd, J6.16, 1.76 Hz 20a'), 8.18 (1H, dd, J5.84, 1.28 Hz, 20a'), 5.34 (2H, dd, 2ck CH2), 5.18 (2H, dd, 20dkCH2), 4.47 (1H, q, 18H), 4.39 (2H, m, CH2), 4.16 (1H, dd, 17H), 3.89 (2H, t, CH2), 3.83 (2H, q, 8a), 3.74 (4H, m, 3a), 3.70 (4H,m.), 3.67 (3H, s, 17d), 3.57 (2H, m, ), 3.56 (3H, s, 12a), 3.29 (3H, s, 7a), 3.23 (3H, s,7a), 2.76 (3H, s, 20i), 2.63 (2H, m, 17a), 2.46 (3H, s, 2a), 2.37 (2H, m, 17b), 1.70 (6H, dt, 8b 3b), 1.35 (1H, bs, NH), -1.30 (1H,s, NH). 13 C NMR (CDCl3, 400 MHz, 25 °C) 196.9, 173.6, 168.1, 160.8, 159.9, 153.6, 151.8, 148.9, 145.6, 145.2, 145.1, 143.9, 139.5, 139.0, 136.8, 134.1, 133.7, 131.7, 131.0, 130.5, 128.8, 106.7, 105.4, 104.6, 99.8, 71.9, 70.8, 70.4, 70.4, 69.3, 61.9, 58.9, 52.4, 51.8, 48.5, 47.9, 34.9, 31.6, 29.7, 29.6, 21.4, 19.6, 19.4, 17.4, 17.0, 16.0, 12.1, 11.2. LCMS (C18) retention time 7.38 mins Found 774.42 [M-I]+ (Calc for C46H56IN5O6 901.87)
3-devinyl-20-(4-triethyleneoxide pyridinium) pyropheophorbide-a iodide (21)

Concentrated HCl (8 ml) was added to 3-devinyl-20-(4-triethyleneoxide pyridinium)-methyl pyropheophorbide-a iodide (20) (0.0736 g, 0.082 mmol) under nitrogen and allowed to stir at room temperature light protected overnight. The reaction was monitored by TLC (silica gel, MeCN:H2O: sat.K2NO3 60:10:10) by following the consumption of the starting material. A 5 M solution of NH4PF6 (~5 ml) was added and stirred briefly. Iced water was added followed by CHCl3 and extracted the aqueous layer several times until it was clear. The organics were further washed with water until the aqueous layer was neutral. The solvent was removed to obtain a crude product. This was purified on a preparative TLC plate (20 x 20 cm², 2000 µ) coated with silica gel 60 using MeCN:H2O: sat.K2NO3 60:10:10 as eluent. The product was dissolved in CHCl3 and washed with water and concentrated. This was redissolved in dry DCM, carefully layered with dry ether and left to stand at 4 °C. The precipitated brown solid was collected by centrifugation and dried over P2O5 in a vacuum desiccator. Rf (MeCN/H2O/KNO3 8/1/1): 0.33; LCMS (C18) retention time 6.87 mins Found 760.4 [M-I]+ (Calc for C45H54IN5O6 887.84)

3-devinyl-20-(4-triethyleneoxide pyridinium) pyropheophorbide-a chloride (22)

3-devinyl-20-(4-triethyleneoxide pyridinium) pyropheophorbide-a iodide/ PF6 (21) (0.010 g, 0.011 mmol) was dissolved in dry MeOH (3 ml) under nitrogen. Dowex 1x8-400 (~10 mg) was added and stirred at RT light protected for 3hrs before filtering through cotton wool and concentrating. This was redissolved in dry DCM, carefully layered with dry ether and left to
stand at 4 °C. The precipitated brown solid was collected by centrifugation and dried over P₂O₅ in a vacuum desiccator to give the final product (8.3 mg, 95 %). UV/Vis (DCM) λmax (log ε) 414 (4.7), 512 (3.8), 551 (3.9), 620 (3.9), 674 (4.4); ¹H NMR (CDCl₃, 400 MHz, 25 °C) This was quite broad and difficult to assign. 10.27 (1H, s, 20b), 9.52 (1H, s, 10 meso), 9.37 (1H, s, 5 meso), 9.18 (2H, s, 20a,20a'), 8.02 (1H, s, 20b'), 5.48 (1H, m, 13b),5.21 (1H, m, 13b'), 4.71 (1H, m, 18H), 4.30 (4H, m, CH₂), 4.19 (1H, m, 17H), 3.78 (2H, m, CH₂), 3.74 (2H, q, 3a), 3.66 (2H, m, 8a ), 3.61 (3H, s, 12a), 3.49 (2H, m, 17a), 3.28 (3H, s, 7a), 3.16 (3H, s, 2a) 2.61 (6H, s, 20i ), 2.32 (3H, m, 17b ), 1.73 (3H, t, 8b), 1.53 (3H, t, 3b), -1.51 (1H, bs, NH). ¹³C NMR (CDCl₃, 400 MHz, 25 °C) 134.1, 132.6, 132.2, 131.5, 128.8, 128.5, 126, 106.4, 104.9, 99.2, 71.8, 70.6, 70.3, 69.5, 61.2, 58.6, 54.1, 47.8, 46.0, 42, 40.9, 32.5, 29.7, 28, 22.7, 20.7, 19.5, 19.3, 17.3, 16.8, 15.3, 14.1, 12.1, 11.2. LCMS (C₁₈) retention time 6.80 mins Found 760.41 [M- Cl]⁺ (Calc for C₄₅H₅₄ClN₅O₇ 796.39).

3-devinyl-20-(4-methylpyridinium) pyropheophorbide-a iodide (23)

(17) (20.3 mg, 0.033 mmol) was dissolved in dry DMF (2.5 ml) under nitrogen. Methyl iodide (6.5 mmol) was added and stirred under nitrogen at room temperature for 3.5 hrs until the starting material was consumed as shown by TLC (5 % MeOH/ CHCl₃). Most of the solvent was removed, dry ether was added and left at 4 °C overnight before filtering to obtain a dark brown solid (74 %). ¹H NMR (CDCl₃, 400 MHz, 25 °C) (this was quite broad and partially assigned) 9.78 (1H, d, pyridyl H), 9.52 (1H, s, 10-meso), 9.44 (1H, s, 5-meso), 9.10(1H, d, pyridyl H), 9.02 (1H, d, pyridyl), 5.31 (2H, s, 13b), 4.33 (1H, q, 17- H), 3.9 (1H, dd, 18 CH), 3.73 (4H, m, 8a, 3a), 3.64 (3H, s, 12a), 3.50 (3H, s, 7a), 2.74 (3H, s, pyr Me), 1.73 (3H, t, 8b), 1.64 (3H, t, 3b), -1.38 (1H, s, NH). MS ES (m/z) (Calc for C₃₉H₄₂N₅O₅, 755.65) Found 628.33 [M – I]⁺; UV/Vis (DCM) λmax 675 nm.
3-devinyl-20-(4-methylpyridinium) pyropheophorbide-a succinimidyl ester iodide (24)

3-devinyl-20-(4-methylpyridinium) pyropheophorbide-a iodide (23) (13 mg, 0.017 mmol) was dissolved in dry DCM (3 ml) and dry THF (1 ml). DCC (0.043 mg, 0.021 mmol) and n-hydroxysuccinimide (3 mg, 0.021 mmol) were added and left to stir at room temperature, light protected under nitrogen for 17 hours. Followed by TLC (Rf (10% MeOH/CHCl₃): 0.1). The solvent was removed and the solid was washed repeatedly with hexane followed by dry ether to obtain a brown solid (with traces of white powder). ¹HNMR (CDCl₃) 9.53 (1H, s, 10-meso), 9.46 (s, 5-meso), 9.47 (1H, d, pyridyl H), 9.16 (1H, d, pyridyl H), 8.95 (1H, d, pyridyl H), 8.20 (1H, d, pyridyl H), 4.99 (2H, s, 13b), 4.42 (1H, q, 17H), 4.12 (1H, dd, 18H), 3.80 (2H, q, 8a), 3.74 (2H, m, 3a) 3.67 (3H, s, 12a), 3.29 (3H, s, 2a), 3.20 (2H, q, 17a), 2.9 (4 H, m, 17d), 2.74 (3H, s, pyr Me) 2.75 (1H, m, 17b), 2.44 (1H, m, 17a) 2.49 (3H, s, 7a), 2.44 (1H, m, 17b), 2.19 (1H, m, 17b), 1.74 (3H, t, 8b), 1.64 (3H, t, 3b), -1.36 (1H, s, NH). MS ES (m/z) (Calc for C₄₃H₄₅N₆O₅I) Found 725.76 [M-I]⁺ plus DCHU.

3-devinyl-20-(4-methylpyridinium) methyl pyropheophorbide-a (25)

5-pyridyl meso MePPa (25mg, 0.039 mmol) was dissolved in dry DMF (2.5 ml), MeI (7.97 mmol) was added and stirred at RT under nitrogen for 20 hours and monitored by TLC (disappearance of the starting material, 5% MeOH/ CHCl₃). Most of the solvent removed, dry
ether was added and the solution cooled to give a brown solid (30 mg, 99 %). $^1$H NMR (CDCl$_3$) 9.56 (1H, s, 10kmeso), 9.48 (s, 5kmeso), 9.52 (1H, d, pyridyl H), 9.35 (1H, d, pyridyl H), 8.95 (1H, d, pyridyl H), 8.20 (1H, d, pyridyl H), 4.99 (2H, s, 13b- CH2CO), 4.42 (1H, q, 17- H), 4.12 (1H, dd, 18 CH), 3.80 (2H, q, 8aCH2), 3.74 (2H, m, 3a CH2CH3) 3.67 (3H, s, 17d), 3.57 (3H, s, 12a) 3.29 (3H, s, 7a), 3.20 (2H, q, 17a), 2.74 (3H, s, pyr Me) 2.63 (1H, m, 17b), 2.44 (1H, m, 18H) 2.49 (3H, s, 2a), 2.44 (1H, m, 17b), 2.27 (1H, m, 17b), 1.73 (3H, t, 8b CH3CH2), 1.68 (3H, t, 3b CH3CH2), -1.31 (1H, s, NH); MS ES (m/z) (Calc. for C$_{40}$H$_{44}$N$_5$O$_3$I 768.9) Found 642.34 [M – I]$^+$ UV/Vis (DCM) $\lambda_{max}$ (log $\varepsilon$) 411 (5.0), 512 (3.8), 554 (4.1), 620 (3.8), 675 (4.6).

3-devinyl-20-[(4-benzyloxy carbonyl)phenyl]-pyropheophorbide-a

(10) (20 mg, 0.032 mmol) and 4-(benzyloxy carbonyl)benzene boronic acid (81 mg, 0.32 mmol) were degassed with dry nitrogen for 15 mins before adding dry THF (15 ml) and degassing for a further 30 mins with the nitrogen bubbling through. Pd(PPh$_3$)$_4$ (~10 mg) was added and continued degassing the mixture for 15 mins. K$_3$PO$_4$ (0.137 g, 0.635 mmol) was added and the reaction was heated under reflux under nitrogen, light protected o/n. Pd(PPh$_3$)$_4$ (~10 mg) was added and continued heating under reflux for 8 hours before diluting the mixture with chloroform and washing with water, sat NaHCO$_3$, brine and water and drying over Na$_2$SO$_4$. The crude product was purified on silica gel eluting with CHCl$_3$ to obtain a dark oil. MS ES (m/z) (Calc. for C$_{48}$H$_{48}$N$_4$O$_5$ 768.36) Found 761.37 [M+H]$^+$.

3-devinyl-20-[(4-methoxy carbonyl)phenyl]-pyropheophorbide-a

(10) (30 mg, 0.048 mmol) and 4-(methoxy carbonyl)phenyl boronic acid (88 mg, 0.49 mmol) were dissolved in dry THF (12 ml) and degassed for 30 mins with the nitrogen bubbling through. Pd(PPh$_3$)$_4$ (~14 mg) and K$_3$PO$_4$ (0.209 g) were added and heated under reflux under nitrogen, light protected o/n. The mixture was diluted with chloroform and washed with water, sat NaHCO$_3$, brine and water and dried over Na$_2$SO$_4$. The crude product was purified on silica gel eluting with CHCl$_3$/ EtOAc (9/1) (R$_f$ on silica 0.65) to obtain a dark oil (25 %). ($\lambda_{max}$ 667 nm) MS ES (m/z) (Calc. for C$_{48}$H$_{48}$N$_4$O$_5$ 684.82) Found 685 [M+H]$^+$.

3-devinyl-20-phenyl- pyropheophorbide-a (Kelley et al., 2006a)

(10) (30 mg, 0.048 mmol) and phenyl boronic acid (62 mg, 0.51 mmol) were dissolved in dry THF (10 ml) and degassed for 30 mins with the nitrogen bubbling through. Pd(PPh$_3$)$_4$ (~12
mg) and K$_3$PO$_4$ (0.197 g, 0.93 mmol) were added and heated under reflux under nitrogen, light protected o/n. The mixture was diluted with chloroform and washed with water, sat NaHCO$_3$, brine and water and dried over Na$_2$SO$_4$. The crude product was purified on silica gel eluting with CHCl$_3$/Acetone (75/25) ($R_f$ on silica 0.4) to obtain a dark oil (30 %). ($\lambda_{\text{max}}$ 667 nm) MS ES (m/z) (Calc. for C$_{48}$H$_{48}$N$_4$O$_5$ 626.33) Found 627.33 [M+H]$^+$.

3,4,5-tri(triethylene glycol monomethyl ether)benzyl chloride (Oar et al., 2005)

3,4,5-tri(triethylene glycol monomethyl ether)benzyl alcohol (3) (2.02 g, 3.36 mmol) was dissolved in dry DCM (100 ml) under nitrogen and thionyl chloride (3.33 g, 28 mmol) in dry DCM (40 ml) was added dropwise over an hour at RT and allowed to stir for a further 1 hr 30 min. The solvent was removed (KOH trap) to obtain a dark oil. It was used without further purification ($R_f$ 0.31, silica MeOH/CHCl$_3$), MS ES (m/z) (Calc. for C$_{28}$H$_{49}$O$_{12}$Cl 612.5) Found 635.3 [M+Na]$^+$ . $^1$H NMR (CDCl$_3$) 6.64 (2H, s, benzyl), 4.17 (6H, m, CH$_2$O), 3.86 (6H, m, CH$_2$O), 3.8 (2H, m, CH$_2$O), 3.74 (6H, m, CH$_2$O), 3.66 (12H, m, CH$_2$O), 3.56 (6H, m, CH$_2$O) 3.39 (9H, s, Me).
2.1.8 Photophysical Measurements

UV/Vis Spectra

For measuring extinction coefficients and obtaining spectra for determining aggregation in various solvents, the compound under study was dissolved in the appropriate solvent/ buffer and its absorption was measured using a 1 cm path quartz cuvette.

For extinction coefficients at a certain wavelength, an average of absorptions was taken for each wavelength for various known concentrations then plotted and a linear curve was fitted. The gradient of this curve was then used as the extinction coefficient.

For the aggregation/disaggregation curves, photosensitiser solutions were made up from a DMSO stock (usually 10 mg/ml) into the relevant DMSO/ PBS/ iPrOH solvent resuspending and ensuring that the solution was clear before scanning.

Singlet oxygen quantum yield

Air-saturated photosensitiser solutions in spectroscopic grade toluene at a concentration corresponding to absorption ~0.15 (at excitation wavelength) were excited at 420-430 nm using a frequency-tripled Nd:YAG (Continuum Surelite I-10) pumped dye laser (Lambda Physik, Coumarin 120 laser dye) at 0.01-1.0 mJ per pulse (10 ns pulse). Singlet oxygen was detected by its phosphorescence at 1270 nm using a North Coast Scientific EO-817P germanium photodiode detector. Measurements were obtained for a range of power settings. The 1270 nm phosphorescence intensity was plotted vs power to obtain linear curves which were fitted to a linear function hence obtaining the gradient at A420nm.

Φ∆ was determined using the following equation:

\[ \Phi_{\Delta} = \frac{\varepsilon_s A_{420 \text{ st}}}{\varepsilon_{st}} \times \Phi_{\Delta \text{ st}} \]

where \( \Phi_{\Delta} \) is the singlet oxygen quantum yield; \( \varepsilon \) is the gradient of the curve and A is the absorption in absorption units; s indicates sample and st indicates standard. PPa was used as the standard with a known \( \Phi_{\Delta} \) in toluene of 0.5.

Fluorescence quantum yield and fluorescence studies

The optical density of the solutions in spectroscopic grade toluene (anhydrous) or water was adjusted to ca. 0.1 – 0.5 at the excitation wavelength (420 nm). Emission was detected at 550-800 nm on a Cary Eclipse fluorescence spectrophotometer using a 1 cm x 1 cm quartz cuvette. Octyl-glucopyranoside was added to the water samples as a solid to a final
concentration of 50 mM and allowed to dissolve before scanning the samples. The UV/Vis absorption spectra of the samples were re-scanned following the completion of the fluorescence measurements. Fluorescence quantum yields in toluene and water were determined comparatively using the fluorescence quantum yield for PPa.

Emission quantum yields, $\Phi_{em}$, were calculated from the equation below, using a solution of PPa (toluene) as emission standard,

$$
\Phi_{em} = \Phi_{st} \frac{I}{I_{st}} \frac{A_{st}}{A}
$$

where $\Phi_{st}$ = quantum yield of emission standard (PPa 0.3 in toluene), $I$ = integrated emission intensity of the sample, $I_{st}$ = integrated (Origin 8.1) emission intensity of the emission standard, $A$ = absorbance of the sample at the excitation wavelength, $A_{st}$ = absorbance of the emission standard at the excitation wavelength.

**Octanol- water partition coefficient**

The octanol-water partition coefficient (P) which is referred to as the logarithmic ratio of octanol: water is used to give a quantitative description of the lipophilicity of a compound in relation to its structure. Theoretical values were obtained using the Pallas 3.7.1.1 software from Compudrug International Inc.
## 2.2 Biochemistry

### 2.2.1 List of Materials

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier (catalogue number)</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue Culture</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM high glucose (4.5g/L) with L-Glutamine without phenol red</td>
<td>PAA (E15-877)</td>
<td></td>
</tr>
<tr>
<td>DMEM high Glucose (4.5g/L) with stable Glutamine</td>
<td>PAA (E15-883)</td>
<td>Unless otherwise stated, cells were cultured using these buffers supplemented as indicated in the methods.</td>
</tr>
<tr>
<td>Dulbecco’s PBS (1x) without Ca &amp; Mg</td>
<td>PAA (H15-002)</td>
<td></td>
</tr>
<tr>
<td>Hank’s BSS (1x) without Ca &amp; Mg without phenol red</td>
<td>PAA (H15-009)</td>
<td></td>
</tr>
<tr>
<td>CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS Assay)</td>
<td>Promega</td>
<td>See methods for details</td>
</tr>
<tr>
<td>DMSO Hybri-Max®</td>
<td>Sigma-Aldrich (D2650)</td>
<td></td>
</tr>
<tr>
<td>Endothelial attachment factor</td>
<td>TCS Cell Works (ZHS-8949)</td>
<td>Coat plate for 30 mins at 37 °C</td>
</tr>
<tr>
<td>Heat inactivated FBS, P/S, L-Glu, Trypsin EDTA free</td>
<td>Invitrogen™ (10108165)</td>
<td>Use at 10 % in Media</td>
</tr>
<tr>
<td>HEPES Buffer (1x)</td>
<td>Sigma-Aldrich (H0887)</td>
<td></td>
</tr>
<tr>
<td>KB</td>
<td>European Cell Culture Bank (ECCB)</td>
<td>Cells were grown in DMEM (see methods) up to a passage #15.</td>
</tr>
<tr>
<td>Penicillin/ Streptomycin</td>
<td>Invitrogen™</td>
<td></td>
</tr>
<tr>
<td>SKOV3 human ovarian carcinoma cell line</td>
<td>Purchased from Cancer Research UK</td>
<td>Cells were grown in DMEM (see methods) up to a passage #15.</td>
</tr>
<tr>
<td>Triton® X-100</td>
<td>Promega</td>
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</tr>
<tr>
<td>Trypan Blue Solution (0.4%)</td>
<td>Sigma-Aldrich (T8154)</td>
<td>-</td>
</tr>
<tr>
<td>Trypsin, EDTA free</td>
<td>Invitrogen™ (25200072)</td>
<td>-</td>
</tr>
<tr>
<td>Water (TC grade)</td>
<td>PAA</td>
<td>-</td>
</tr>
<tr>
<td><strong>Microscopy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HER2 FITC</td>
<td>BD Biosciences</td>
<td>-</td>
</tr>
<tr>
<td>Antikmouse IgG Alexa 488</td>
<td>Invitrogen™</td>
<td>See methods for details</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Bodipy® FL C₅-ceramide complexed to BSA</td>
<td>Invitrogen™ (B22650)</td>
<td>See methods for details</td>
</tr>
<tr>
<td>Chambered #1.0 Borosilicate Coverglass System</td>
<td>Lab-Tek® (155411)</td>
<td></td>
</tr>
<tr>
<td>DAPI nucleic acid stain</td>
<td>Invitrogen™</td>
<td>See methods for details</td>
</tr>
<tr>
<td>ER-Tracker® Green (BODIPY® FL glibenclamide)</td>
<td>Invitrogen™ (E34251)</td>
<td>See methods for details</td>
</tr>
<tr>
<td>LysoTracker® Green DND-26</td>
<td>Invitrogen™ (L-7526)</td>
<td>See methods for details</td>
</tr>
<tr>
<td>MitoTracker® Green FM</td>
<td>Invitrogen™ (M-7514)</td>
<td>See methods for details</td>
</tr>
<tr>
<td>Photosensitisers</td>
<td>Prepared in-house</td>
<td>a stock solution in 100% anhydrous DMSO was stored at 4°C in the dark</td>
</tr>
<tr>
<td>Transferrin Alexa 488</td>
<td>Invitrogen™ (T13342)</td>
<td>See methods for details</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IODOGEN tubes</td>
<td>Pierce</td>
<td>-</td>
</tr>
<tr>
<td>Ketaset</td>
<td>Fort Dodge Animal Health 100 mg/ml solution</td>
<td>-</td>
</tr>
<tr>
<td>Matrigel</td>
<td>BD/VWR (356231)</td>
<td>-</td>
</tr>
<tr>
<td>Rompun</td>
<td>Bayer Healthcare</td>
<td>23.32 mg/ml Xylazine HCl; 1 mg/ml methyl-4-OH benzoate</td>
</tr>
<tr>
<td>Na¹²⁵I</td>
<td>MP biomedicals</td>
<td>-</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-(1-pyridinio)-1-propanesulfonate</td>
<td>Fluka (82804)</td>
<td></td>
</tr>
<tr>
<td>9E10 antibody</td>
<td>Prepared in-house by Dr Antony Constantinou, Imperial College London.</td>
<td>Used for making antigen columns; for Western blots it was used 1 in 3000</td>
</tr>
<tr>
<td>Acrylamide 30 %</td>
<td>National Diagnostics (EC-890)</td>
<td>-</td>
</tr>
<tr>
<td>Anhydrous DMSO and Acetonitrile</td>
<td>VWR or Sigma</td>
<td>Store under nitrogen and take out as needed</td>
</tr>
<tr>
<td>Anti–mouse IgG (Fab specific) peroxidise conjugate</td>
<td>Sigma</td>
<td>Used for Western blots at 1 in 10000</td>
</tr>
<tr>
<td>Anti-HIS HRP</td>
<td>Sigma (A7058-1VL)</td>
<td>Used 1 in 4000 for blots</td>
</tr>
<tr>
<td>BCA Assay</td>
<td>Pierce/ Fisher Scientific</td>
<td>-</td>
</tr>
<tr>
<td>Bio-Beads® SM-2 Adsorbent</td>
<td>BioRad</td>
<td>152-8920</td>
</tr>
<tr>
<td>BioDesign Dialysis Tubing™</td>
<td>Fisher</td>
<td>Unless stated otherwise a MWCO of 8000 Da was used.</td>
</tr>
<tr>
<td><strong>Bacterial Cultures/protein Expression</strong></td>
<td><strong>Chemical name</strong></td>
<td><strong>Vendor</strong></td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>-------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>Melford (CO109)</td>
<td>Use at 100 µg/ml; store -20 °C</td>
</tr>
<tr>
<td>Dimethylpimelimidate</td>
<td>Sigma</td>
<td>-</td>
</tr>
<tr>
<td>Isopropyl-1-thio-β-D-galactopyranoside (IPTG)</td>
<td>Melford (MB1008)</td>
<td>Use at 1 mM; store -20 °C</td>
</tr>
</tbody>
</table>

Standard chemicals were purchased from Sigma-Aldrich®, Fluka, Acros Organics, VWR®, Merck. Common solvents were purchased from VWR®. Dry solvents were purchased from Sigma-Aldrich.
2.2.2 Equipment

Spectroscopy

UV/Vis spectra were recorded on an Agilent 8453 UV Visible Spectrophotometer.

Gels/ Blots

Western Blots and nitrocellulose blots fluorescent detection were visualised on a FujiFilm Las-3000. SDS-Page Gels were run on a BioRad Mini-PROTEAN 3 system and transferred onto nitrocellulose using BioRad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell. AIDA Image Analyzer v.3.52 (Raytest) software was used for densitometry measurements.

Laser

PDT treatment was carried out using a High Powered Devices-HPD 7401 laser with $\lambda_{\text{max}}=690$ nm, 2W output coupled to a fibre optic cable. The laser was calibrated (see section 2.3.11) and used according to the experimental design.

Confocal microscopy

Imaging was conducted at the Facility for Light Microscopy (FILM) Imperial College London using an inverted confocal laser scanning microscope (LEICA TCS SP5) coupled to a CW argon-ion laser (488 and 496 nm line), and a diode laser (405 nm line). The fluorescence emission of the photosensitisers in the cells was spectrally dispersed using a prism and detected using a photomultiplier tube. A water immersion 63 x objective (NA=1.23) was used for imaging.

Other

Bacterial supernatant was concentrated using a 323S/RL Watson-marlow peristaltic pump.

Water was distilled from a Purite Select system. It was autoclaved where necessary.
### 2.2.3 Formulae

#### (Gel) Loading Buffer (5x)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/ Mass</th>
<th>Final Concentration</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>2.5 ml</td>
<td>6.8 M</td>
<td></td>
</tr>
<tr>
<td>1 M TrisHCl pH 6.8</td>
<td>0.3 ml of 1 M</td>
<td>60 mM</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>1 ml of 10 % solution in dH₂O</td>
<td>69 mM</td>
<td>Omit for Native/ Non-reducing gels. Substitute with water.</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>250 µl</td>
<td>0.7 M</td>
<td></td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>traces</td>
<td>7.5 µM</td>
<td>Omit from conjugation samples</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Made up to 5 ml. Store at -20 °C in aliquots.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Phosphate Buffered Saline (PBS) (1L, 10 x) Filtered for most applications through a 0.2 µm PES membrane.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/ Mass</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>80 g</td>
<td>1.37 M</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>2 g</td>
<td>0.027 M</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>2.4 g</td>
<td>0.018 M</td>
</tr>
<tr>
<td>Sodium hydrogen phosphate</td>
<td>14.4 g</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Made up to 1 L</td>
<td></td>
</tr>
</tbody>
</table>

#### 2TY Agar (autoclaved) 2TY Media (autoclaved)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/ Mass</th>
<th>Reagent</th>
<th>Volume/ Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>16 g</td>
<td>Tryptone</td>
<td>16 g</td>
</tr>
<tr>
<td>Yeast</td>
<td>10 g</td>
<td>Yeast</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Made up to 1 L</td>
<td>Distilled water</td>
<td>Made up to 1 L</td>
</tr>
</tbody>
</table>

#### Resolving Gel for PAGE (12 %) (2 mini gels)

<table>
<thead>
<tr>
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<th>Volume/ Mass</th>
<th>Final Concentration</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M TrisHCl pH 8.8</td>
<td>2.5 ml</td>
<td>0.375 M</td>
<td>Filtered through 0.2 µm</td>
</tr>
<tr>
<td>30 % Acrylamide</td>
<td>4 ml</td>
<td>12 %</td>
<td>Filtered through 0.2 µm Omit from native gels. Substitute with water</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 µl</td>
<td>0.1 %</td>
<td>Filtered through 0.2 µm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.4 ml</td>
<td></td>
<td>Filtered through 0.2 µm</td>
</tr>
<tr>
<td>10 % APS</td>
<td>100 µl</td>
<td>0.1 %</td>
<td>Filtered through 0.2 µm</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Stacking Gel for PAGE (for 2 mini gels)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/ Mass</th>
<th>Final Concentration</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M TrisHCl pH 6.8</td>
<td>500 µl</td>
<td>0.075 M</td>
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</tr>
<tr>
<td>30 % Acrylamide</td>
<td>600 µl</td>
<td>1.8 %</td>
<td></td>
</tr>
<tr>
<td>10 % SDS</td>
<td>40 µl</td>
<td>0.04 %</td>
<td>Omit from native gels. Substitute with water</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3 ml</td>
<td></td>
<td>Filtered through 0.2 µm</td>
</tr>
<tr>
<td>10 % APS</td>
<td>40 µl</td>
<td>0.04 %</td>
<td>Filtered through 0.2 µm</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Running Buffer for PAGE (1 x)

<table>
<thead>
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<th>Reagent</th>
<th>Volume/ Mass</th>
<th>Final Concentration</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma base</td>
<td>3.02 g</td>
<td>24.9 mM</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>18.8 g</td>
<td>250 mM</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>1 g</td>
<td>3.47 mM</td>
<td>Omitted for Native gels</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Made up to 1 L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Transfer Buffer for PAGE (1 x)

<table>
<thead>
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<th>Reagent</th>
<th>Volume/ Mass</th>
<th>Final Concentration</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma base</td>
<td>3.02 g</td>
<td>24.9 mM</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>18.8 g</td>
<td>250 mM</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>0.4 g</td>
<td>1.39 mM</td>
<td>This is NOT omitted for Native gels</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>Made up to 1 L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Gel Destain/ Fixing Solution (1 x)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/ Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>450 ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>450 ml</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
2.2.4 Protein Expression

Transformation

C6.5 scFv in pUC119 was obtained from Prof J. Marks (University of California, San Francisco). A100K mutation was introduced in the antibody binding site, to make the clone C6.5(-k) (Bhatti et al., 2008). This was expressed and purified as follows.

E. coli, XL1 blue cells (chemically competent) were transformed with the pUC119 vector containing the gene for C6.5(-k). Two aliquots of competent cells (50 µl) and the DNA were thawed on ice for approximately 15 minutes. DNA (1 µl, 10 ng) was added to the cells (50 µl) and incubated on ice for 30 minutes. This was then heat-shocked at 42 °C for 1 minute and placed immediately on ice for 2 minutes. 2 TY Media (300 µl) was added and grown in an orbital shaker (250 rpm, 1 hour, 37 °C. This was plated on 2TY agar plates (+100 µg/ml carbenicillin, 1 % glucose) with a positive and negative-control and then incubated at 37 °C overnight (at least 16 hours).

Expression

A single colony was picked and was used to inoculate 5 ml of 2TY (100 µg/ml carbenicillin, 0.1 % glucose). The culture was grown overnight (37 °C, 280 rpm) in an orbital shaker.

The overnight culture was centrifuged (5 mins, 4000 rpm, RT), resuspended in fresh 2TY media (+100 µg/ml carbenicillin, 0.1 % glucose) (5 ml) and transferred to a 2 L conical flask containing 500 ml of 2TY media (+100 µg/ml carbenicillin, 0.1 % glucose). This was grown shaking (37 °C, 250 rpm) and was induced at OD₆₀₀ ~ 0.7 using IPTG at a final concentration of 1 mM. The induced culture was grown overnight at 30 °C, 250 rpm. The culture was centrifuged (8500 rpm, 30 mins, 4 °C), the supernatant was collected and was concentrated ten-fold on Vivaflow 200. This was dialysed extensively against PBS at 4 °C.

2.2.5 Protein Purification and Analysis

Purification Methods

- Using the HIS₆ tag- Talon® Resin

Following dialysis the culture supernatant was incubated with prewashed (PBS 2 x 3 bed volumes) Talon® beads (1 ml packed beads for 1 L bacterial culture) at RT for 1.5 hrs or 4 °C o/n rolling gently. The mixture was then packed onto a 10 ml polypropylene column and the
flow through was collected followed by washes (2 x 3 bed volumes PBS pH 7.34). The scFv was eluted by incubating for 5 mins with 500 mM imidazole (in PBS) collecting fractions of 0.5-1 ml. This was tested by the Bradford assay reagent to determine the amount of protein present. Following SDS-PAGE analysis, the corresponding fractions were combined and dialysed extensively in PBS. For long term storage, 0.005 % sodium azide and 5 % glycerol were added and kept at -20 °C or -80 °C.

Using the c-myc tag

Antibody that was previously purified on Talon\textsuperscript{®} was further purified if required. Depending on the estimated capacity of the 9E10 column (1 mol of 9E10 theoretically binds 2 mol of C6.5(-k)), scFv in PBS was incubated with the beads at RT for 1.5 hrs or 4 °C o/n rolling gently. The mixture was then packed onto a 10 ml polypropylene column and the flow through was collected followed by washes (2 x 3 bed volumes PBS pH 7.34). The column was then eluted using 0.2 M glycine buffers in the following order: pH 5.0 (3 x 1 ml), pH 3.0 (3 x 1 ml), pH 2.5 (0.5 ml volumes, checking the elutions with the Bradford reagent). For all the elutions, the column was incubated with the glycine buffer for ~4 mins before eluting and the fractions were neutralised in 1 M TrisHCl pH 8.0 (1/20\textsuperscript{th} of the volume for pH 5, 1/10\textsuperscript{th} of the volume for pH 3.0 and 1/5\textsuperscript{th} of the volume for pH 2.5). Following SDS PAGE analysis, the corresponding fractions were combined and dialysed extensively in PBS. For long term storage, 0.005 % sodium azide and 5 % glycerol were added and stored at -20 °C or -80 °C.

Protein concentration and quantification

As appropriate, the fractions of either the Talon\textsuperscript{®} -or 9E10-purified scFv. This was done using Vivaspin\textsuperscript{™} concentrators according to the manufacturers instructions. Concentration could also be combined with buffer exchange by concentrating the antibody and then diluting with the desired buffer then concentrating and repeating ensuring that overall the buffer exchange was ~10000x.

The concentration of the purified protein was calculated using either the BCA (Pierce) assay according to the manufacturer’s instructions or using its absorption intensity at 280 nm as measured on the NanoDrop (Thermo Scientific) or UV/Vis spectrophotometer using a 1 ml quartz cuvette. The extinction coefficient for C6.5(-k) was calculated using its sequence (shown in figure 4.4) and the Expasy ProtParam software. Some of the main characteristics of C6.5(-k) are shown in Table 4.2. Yields were 5 mg L\textsuperscript{-1} and consistent with published data.
**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

A resolving gel (see section 2.3.3) was poured into the glass plates (0.75 mm thickness), layered with ethanol and allowed to set for 45 minutes. The stacking gel was poured, a 10 or 15 well comb was inserted and allowed to set for a further 15-30 mins. The gels were loaded (usually 10 µl in a 15 well gel and 15-20 µl in a 10 well gel) and run at 30 mA per gel on the BioRad MiniProtean II system. Protein ladders (Fermentas/ Biorad) were used as standards.

When running gels of conjugates, the gel dye front was stopped when 2 cm from the bottom to allow imaging of the free dye which migrates slightly faster than the 10 kDa protein marker.

Protein samples were prepared for electrophoresis by diluting with gel loading buffer (5x) and subsequently heating at 100 °C for 4 mins. For non-reducing native gels, the samples were not heated and used non-reducing LB.

- **Coomassie staining**

  The gel was immersed in fixing solution for 30 mins before washing in water (3 x 5 mins) followed by staining with Coomassie stain for 1 hour. The gel was rinsed in distilled water and imaged.

- **Western blotting**

  Following electrophoresis, the gel, nitrocellulose membrane and blotting paper were soaked in transfer buffer for 15 mins. The gel was placed on top of the nitrocellulose membrane and sandwiched between the blotting paper. It was transferred onto the nitrocellulose using the BioRad semi dry cell for 45 mins at 15 V (constant).

  The membrane was blocked (5 % marvel in PBS (MPBS)) overnight at 4 °C followed by the antibody/ antibodies (3 % MPBS) for 1 hour each at RT with shaking followed each by washes with PBS/ 0.1 % Tween-20 (3 x 10 mins) and PBS (3 x 10 mins). Finally, it was developed using the ECL kit according to manufacturer’s instructions and visualised on the FujiFilm Las-3000 (by chemiluminescence).
• Visualising inherent fluorescence

The membrane following the transfer was kept in PBS and visualised directly on the FujiFilm Las-3000 (SyBr Green settings). The membrane could then be treated as above for Western blotting.

NB: Coomassie stained SDS-PAGE gels were scanned and labelled using Corel PhotoPaint X3. Western blots and fluorescence blots were visualised using the Fujifilm LAS3000 Imager (chemiluminescence and SYBRGreen (480-670 nm) settings respectively) and processed using Aida Image Analyzer v.3.52 and Corel PhotoPaint X3. The blots were analysed using AIDA densitometry and visually enhanced using CorelDraw. 2 µg of protein was loaded onto the gels when the concentration of the sample was known.

Preparation of a 9E10 Immuno affinity Column

The 9E10 monoclonal whole antibody was prepared by Dr Antony Constantinou (Imperial College) from a hybridoma cell line and subsequently purifying the combined medium on protein G. Antibody, 9E10 (10 mg; pH 7.3, 2 mg per 1 ml of packed beads) was added to pre-washed protein G-Sepharose (5 ml packed beads) (PBS pH 7.3, 2 x 2 mins, 2000 rpm, 4 °C) and incubated at RT for 1 hr - 1.5 hrs, tumbling. The beads were collected by centrifugation (2 mins, 2000 rpm, 4 °C) and were washed (PBS, 2 mins, 2000 rpm, 4 °C). The mixture was further washed using 0.2 M borate buffer at pH 9.0 (2 x 2 mins, 2000 rpm, 4 °C). DMPM (dimethylpimelimidate dihydrochloride) (0.269 g, in borate buffer) was added to the column and incubated for 45 mins at RT rolling gently. The beads were collected by centrifugation (2 mins, 2000 rpm, 4 °C) and were washed twice with 0.2 M ethanolamine buffer pH 8.0 followed by a 2 hour wash at RT before centrifugation (2 mins, 2000 rpm, 4 °C) and a final wash (PBS, 2 x 2 mins, 2000 rpm, 4 °C). The column was stored in PBS/NaN3 (0.01%) at 4 °C.
Figure 2.1 SDS-PAGE- making the 9E10 affinity column by linking mouse anti-myc 9E10 antibody to protein G coupled to Sepharose beads. Coomassie blue-stained 12 % SDS-PAGE reducing gel. Samples are M (pre-stained protein ladder), 1 (9E10 antibody before conjugation), 2 (unbound 9E10), 3 (resin sample before cross-linking), 4 (resin sample following cross-linking step), 5 (ethanolamine wash step), 6 (final resin sample). Blue arrow shows the full length whole IgG which appears as two bands when reduced due to the cleavage of disulfide bonds whereas the red arrow shows the light chain of the antibody. This is the only visible band upon successful conjugation to the beads as the heavy chain is cross-linked to the beads.

The 9E10 antibody was used as part of the antigen column as well as for Western blots for detecting the c-myc tag present on the N-terminal of C6.5(k). The IgG1/κ, 9E10 monoclonal antibody was purified using a protein A or protein G- Sepharose column (Evan et al., 1985, Hilpert et al., 2001). Specific for human p62c-myc protein, the mouse mAb was isolated from hybridomas and has high affinity for its antigen, the c-myc (Evan et al., 1985). 9E10 recognises EQKLISEEDL (Evan et al., 1985).

As shown in figure 2.1, 9E10 efficiently bound the protein G as there was no detectable level of unbound material in the unbound sample. After the cross linking step where the heavy chain of the IgG was covalently bound to the protein G epitope it recognises, the sample run as a single band of low molecular weight (~25 kDa) corresponding to the antibody light chain only. The heavy chain (~55 kDa) could not enter the gel as it was attached to the Sepharose beads in a covalent bond unbreakable under the reducing conditions of SDS-PAGE electrophoresis.

The cross-linker used to covalently attach the 9E10 mAb to protein G is a homobifunctional imidoester which forms amidine bonds upon reaction with primary amines. In this manner, a lysine side chain of the 9E10 antibody binds the linker on one side and a lysine side chain on protein G binds the linker on the other side. As the linker is only 7 carbons long the two side chains need to be close to each other for the cross linking to work which occurs during protein-protein affinity interactions (Schneider et al., 1982, Hermanson, 1996).
2.2.6 Preparation of Photoimmunoconjugates

C6.5(-k)-PPa Conjugates

Purified protein was dialysed and concentrated into PBS as required. The photosensitiser succinimidyl ester stock (in 100 % DMSO) was warmed and resuspended prior to use to ensure it is in solution. PPa AE (supplementing if necessary to make up final DMSO content to 2 % of reaction volume) was combined with anhydrous grade acetonitrile (6 % to overall reaction volume). The scFv (up to 500 µg/ml, 0.017 mM) in PBS pH 7.34 was transferred to a 5 ml RBF and the photosensitiser solution was added in small portions over half an hour (roughly 5 µl every 5 minutes) under vigorous stirring and protecting from light. After addition, the reaction mixture was stirred at RT for 1 hr protected from light. The conjugate was dialysed extensively against PBS pH 7.34. The samples were recovered and centrifuged (5 mins, 13000 rpm). The supernatant was obtained and used for further experiments and the pellet was resuspended in equal volume of PBS as the SN for subsequent analysis. The conjugation reaction was followed by UV/Vis spectroscopy and SDS-PAGE.

C6.5(-k)-PS1 Conjugates

Purified protein was dialysed and concentrated into PBS as required. The photosensitiser succinimidyl ester stock (in 100 % DMSO) was allowed to warm to RT prior to use to ensure it is in solution. Typically, PS1 AE (supplementing if necessary to make up final DMSO content to 2 % of reaction volume) was combined with anhydrous grade acetonitrile (6 % to overall reaction volume). The scFv (up to 4 mg/ml, 0.137 mM) in PBS pH 7.34 was transferred to a 5 ml RBF and the photosensitiser solution was added in small portions over 45 minutes (roughly 5 µl every 5 minutes) under vigorous stirring and protecting from light. After addition, the reaction mixture was stirred at RT for 2 hrs protected from light. The conjugate was dialysed extensively against PBS pH 7.34. The sample was recovered and centrifuged (5 mins, 13000 rpm). The supernatant was obtained and used for further experiments and the pellet was resuspended in equal volume of PBS as the SN for subsequent analysis.

The conjugation reaction was followed by UV/Vis spectroscopy and SDS-PAGE.

NB: Purification attempts will be discussed in the results section.
Typically, to make 2 ml of 1 mg/ml C6.5(-k)-PS1 conjugate:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock conc.</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6.5(-k) scFv</td>
<td>4.21 mg/ml</td>
<td>475.1</td>
</tr>
<tr>
<td>PBS pH 7.34</td>
<td>-</td>
<td>1324.9</td>
</tr>
<tr>
<td>MeCN (dry)</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>DMSO (dry)</td>
<td>-</td>
<td>43.6</td>
</tr>
<tr>
<td>PS1 AE</td>
<td>30.3 mM</td>
<td>36.4</td>
</tr>
</tbody>
</table>

### 2.2.7 Cell Culture maintenance

This is a general procedure followed for **cell maintenance** and **passaging** as well as for **plating cells** for cell kill assays and confocal microscopy.

Human tumour cell lines were grown- unless otherwise noted- in DMEM (with 10 % FBS, 1 % P/S), at 37 °C, 5 % CO₂ in a humidified atmosphere (150 cm³ flasks). When confluency was 70-80 %, cells were washed with TC grade PBS (2 x 25 ml) and incubated with trypsin (10x) (2 ml per 150 cm³ flask) for 5-7 min. DMEM (10 % FBS, 1 % Penicillin/streptomycin) (10 ml) was added and the cells were resuspended by pipetting. The cells were transferred to a falcon tube and centrifuged (2 mins, 2000 rpm). The supernatant was discarded and the pellet was resuspended in DMEM (5 ml) (with 10 % FBS, 1 % Penicillin/streptomycin). The cells were counted using a haemocytometer, diluted accordingly (number of cells plated was found experimentally-SKOV3 3000 cells/ well and KB 2000 cells/ well) and plated 200 µl per well. NB: KB cells were grown over two nights before being used for an assay or imaging.

**Complete media:** DMEM supplemented with 10 % FBS, 1 % Penicillin/streptomycin.

**Cell freezing**

Cells were split as above and counted. They were divided in aliquots of ~1.5 million cells (DMEM) and diluted two-fold with 20 % DMSO/ FBS. They were allowed to cool down using a cryopreserver before storing in liquid nitrogen.
Photosensitiser solution preparation for use in cell kill assays, confocal microscopy and UV studies

Photosensitiser solutions were prepared from a stock solution in 100 % anhydrous DMSO. The stock was diluted accordingly into phenol red-free DMEM (10 % FBS, 1 % Penicillin/streptomycin) maintaining an overall 2 % of DMSO.

It was found that the more concentrated PPa solutions required extensive heating in order to completely solubilise in media. This was done either by a heat gun or water bath followed by resuspending using a pipette. For PS1 and PS4 this was not an issue.

2.2.8 Imaging by Confocal Microscopy

Imaging took place at the facility for imaging by light microscopy (FILM) Imperial College London on a LEICA SP5 MP inverted confocal microscope, at 37 °C and under a CO₂ supplemented atmosphere. The water objective (63 x) was used in all the experiments. Images were processed using the LAS AF Lite Leica software, Image J, CorelDraw and Powerpoint.

Note: in any experiment where a photosensitiser is used on cells, the experiment is done under subdued lighting exposing the cells to as little light as possible and incubating wrapped in foil. Following addition of PS to cells, cells were no longer observed on the microscope. For brief outline of microscope set up see table 1.2. A summary of the stains and conditions used is shown in table 1.1.

Cells were washed (3 x 100 µl media) and plated 25000 cells per chamber in 200 µl of phenol red free complete media and allowed to grow overnight (it was found that less confluent (60-70%) cells attached better for imaging experiments). Cells were plated on a Lab-Tek® 8 Chambered #1.0 Borosilicate Coverglass System. With some cell lines, it is often useful to use an attachment factor (200 µl, 30 mins, 37 °C) to coat the wells first.

Cellular staining using photosensitisers (PPa, PS1 and PS4)

Photosensitiser solutions in phenol red free complete media (0.5 % DMSO) were prepared fresh and pre-warmed to 37 °C for 15 mins. The cells were washed with media (1 x 200 µl)
before adding the solutions (200 µl per chamber). Unless otherwise stated, the cells were grown over 20 hrs in a humidified atmosphere (37 °C, 5 % CO₂). The cells were washed (2 x 200 µl) with pre-warmed phenol red free DMEM prior to imaging. (absorption and fluorescence emission spectra of the 3 PSs are shown in figure 2.2 in toluene).

![Normalized UV/Vis Absorption spectra and Fluorescence Emission spectra in Toluene](image)

Figure 2.2 UV/Vis absorption and fluorescence emission spectra of the three main PSs normalised at the λ<sub>max</sub>. Samples were excited at their absorption maxima (Absorption maximum/emission maximum) PPa (414, 669/ 676 nm), PS1 (419, 675/ 678 nm) and PS4 (417, 674/ 687 nm).
Staining cellular organelles

- Lysosomal staining

Lysotracker® Green DND-26 was diluted according to manufacturers indications (1 mM stock) and further diluted to twice the working concentration in complete media. For use for single colour staining, this was diluted with complete media to a final concentration of 1 µM. When it was used for two colour staining with photosensitisers, a 2 µM solution was twice diluted in photosensitiser solution to give a 1 µM final concentration.

The cells were incubated for 15 mins at 4 °C and a further 30 mins at 37 °C before replacing with fresh medium and imaging.

- Mitochondrial staining

MitoTracker® Green or Orange CMTMRos was diluted according to manufacturers indications (1 mM stock) and further diluted to twice the working concentration in complete media. For use for single colour staining, this was diluted with complete media to a final concentration of 1 µM. When it was used for two-colour staining with photosensitisers, a 2 µM solution was twice diluted in photosensitiser solution to give a 1 µM final concentration.

The cells were incubated for 15 mins at 4 °C and a further 1 hr at 37 °C before replacing with fresh medium and imaging.

- Endoplasmic Reticulum staining

ER-Tracker™ Green (BODIPY® FL glibenclamide) was diluted according to manufacturer’s indications (1 mM stock) and further diluted to twice the working concentration in HBSS buffer (+ 2 % HEPES). For use for single-colour staining, this was diluted with HBSS (+ 2 % HEPES) to a final concentration of 3 µM. When it was used for two-colour staining with photosensitisers, a 6 µM solution was twice diluted in photosensitiser solution to give a 3 µM final concentration.

The cells were incubated for 15 mins at 4 °C and a further 1 hr at 37 °C before replacing with fresh buffer and imaging.

- Golgi apparatus staining

Bodipy® FL C₆-ceramide complexed to BSA was diluted according to manufacturer’s indications (0.5 mM stock) and further diluted to twice the working concentration in HBSS
buffer (+ 2 % HEPES). For use for single-colour staining, this was diluted with HBSS (+ 2 % HEPES) to a final concentration of 5 µM. When it was used for two-colour staining with photosensitisers, a 10 µM solution was twice diluted in photosensitiser solution to give a 5 µM final concentration. The cells were incubated for 15 mins at 4 °C and a further 30 mins at 37 °C before washing with cold HBSS/HEPES (3 x 100 µl) and replacing with either HBSS/HEPES or photosensitiser solution and incubating at 37 °C for a further 30 mins before replacing with fresh buffer and imaging.

- DAPI Nuclear Staining

DAPI nucleic acid stain was diluted according to the manufacturer's indications (HEPES/HBSS) and further diluted to twice the working concentration in HBSS buffer (+ 2 % HEPES). For use for single-colour staining, this was diluted with HBSS (+ 2 % HEPES) to a final concentration of 5 µg/ml. When it was used for two-colour staining with photosensitisers, a 10 µg/ml solution was twice diluted in photosensitiser solution to give a 5 µg/ml final concentration. The cells were incubated for 15 mins at 4 °C and a further 60 mins at 37 °C before replacing with fresh buffer and imaging.

- Staining with Transferrin Alexa 488 conjugate

Transferrin Alexa Fluor® 488 conjugate was dissolved according to the manufacturer's indications and kept at 4 °C light protected. The solution was diluted in complete media to twice the working concentration (100 µg/ml) for two-colour staining and subsequently twice diluted to the final 50 µg/ml concentration. For single colour staining the sample was diluted to a final 50 µg/ml. Samples were incubated at 37 °C for 80 mins before washing twice with pre-warmed PBS and fixing with ice cold formaldehyde (4 %, PBS) for 15 mins at 4 °C, washing with cold PBS and storing in PBS/ NaN₃ light protected at 4 °C.

- Staining with Photoimmunoconjugates

Freshly prepared C6.5(-k)- PS1 photoimmunoconjugates (PBS) was diluted to twice the working concentration (200 µg/ml) in DMEM (10 % FBS, 1 % P/S) for dual colour staining and subsequently twice diluted to the final 100 µg/ml concentration. For single-colour staining the sample was diluted to a final 100 µg/ml. Samples were incubated at 37 °C for 80 mins before washing twice with pre-warmed PBS and fixing with ice cold formaldehyde (4 %, PBS) for 15 mins at 4 °C, washing with cold PBS and storing in PBS/ NaN₃ light protected at 4 °C.
Table 2.1 Summary of the stains and conditions used in confocal microscopy experiments

<table>
<thead>
<tr>
<th>Stain</th>
<th>Conc. (µM)</th>
<th>Media/ Buffer</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPa</td>
<td>25</td>
<td>complete</td>
<td>20 hrs 37 °C</td>
</tr>
<tr>
<td>PS1</td>
<td>25</td>
<td>complete</td>
<td>20 hrs 37 °C</td>
</tr>
<tr>
<td>PS4</td>
<td>25</td>
<td>complete</td>
<td>20 hrs 37 °C</td>
</tr>
<tr>
<td>MitoTracker® Green</td>
<td>1</td>
<td>complete</td>
<td>15 mins 4 °C, 1 hr 37 °C</td>
</tr>
<tr>
<td>Lysotracker® Green DND-26</td>
<td>1</td>
<td>complete</td>
<td>15 mins 4 °C, 30mins 37 °C</td>
</tr>
<tr>
<td>ER-Tracker™ Green (BODIPY® FL glibenclamide)</td>
<td>3</td>
<td>HBSS (+ 2 % HEPES)</td>
<td>15 mins 4 °C, 1 hr 37 °C</td>
</tr>
<tr>
<td>Bodipy® FL C5-ceramide complexed to BSA</td>
<td>5</td>
<td>HBSS (+ 2 % HEPES)</td>
<td>15 mins 4 °C, 30mins 37 °C, wash, 30 mins 37 °C</td>
</tr>
<tr>
<td>DAPI</td>
<td>18</td>
<td>HBSS (+ 2 % HEPES)</td>
<td>15 mins 4 °C, 1 hr 37 °C</td>
</tr>
<tr>
<td>PICs</td>
<td>3.45 (scFv)</td>
<td>complete</td>
<td>80 mins 37 °C then fix</td>
</tr>
<tr>
<td>Transferrin Alexa Fluor® 488</td>
<td>0.63</td>
<td>complete</td>
<td>80 mins 37 °C then fix</td>
</tr>
</tbody>
</table>
Table 2.2 Summary of the stains and microscope set up used in confocal microscopy experiments

<table>
<thead>
<tr>
<th>Stain</th>
<th>Laser(s) Wavelength (nm)/ and intensity (%)</th>
<th>PMT Detection Range (nm)</th>
<th>Mode of internalisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS and PICs</td>
<td>405 / 10-15</td>
<td>640-800</td>
<td>Receptor mediated endocytosis/ passive diffusion</td>
</tr>
<tr>
<td>MitoTracker® Green</td>
<td>488/ 10-15</td>
<td>500-590</td>
<td>Passive diffusion¹</td>
</tr>
<tr>
<td>MitoTracker® Orange CMTMRos</td>
<td>543/ 40</td>
<td>565-647</td>
<td>Dye is bound to a weak base-cell membrane permeant. Becomes protonated and binds lysosome membranes²</td>
</tr>
<tr>
<td>Lysotracker® Green DND-26</td>
<td>496/ 25</td>
<td>506-600</td>
<td></td>
</tr>
<tr>
<td>ER-Tracker™ Green (BODIPY® FL glibenclamide)</td>
<td>496/ 25</td>
<td>506-600</td>
<td>Binds sulphonylurea receptors if ATP-sensitive K⁺ channels on the ER³</td>
</tr>
<tr>
<td>Bodipy® FL C5-ceramide complexed to BSA</td>
<td>496/ 15</td>
<td>508-600</td>
<td>Metabolised to sphingomyelin and to glycosylceramide in the Golgi apparatus⁴</td>
</tr>
<tr>
<td>DAPI nucleic acid stain</td>
<td>405/ 14</td>
<td>415-570</td>
<td>Non-intercalative binding to DNA. High affinity for the minor groove⁵</td>
</tr>
<tr>
<td>Transferrin Alexa Fluor® 488 conjugate</td>
<td>488/ 15</td>
<td>500-600</td>
<td>Receptor-mediated endocytosis. Delivers iron to the cell and recycles back to the cell surface⁶</td>
</tr>
</tbody>
</table>

Sequential scanning was used for images containing more than one colour. The water objective was used for all samples. Scanning was xyz and the pinhole was 1 airy unit (111.5 µm).

¹ (Probes, 2008), ² (Probes, 2007), ³ (Hambrock et al., 2002), ⁴ (Pagano et al., 1991), ⁵ (Kubista et al., 1987), ⁶ (Rothenberger et al., 1987)
2.2.9 In vitro cytotoxicity assays

The cells were washed with phenol red-free complete media (1x200 µl), the lights were dimmed and the appropriate solutions (100 µl per well in quadruplicates) were added and incubated in the dark (covered in foil) for 2 hrs in a humidified atmosphere (37 °C, 5 % CO₂). The cells were then washed with PBS (3 x 100 µl), DMEM phenol red-free complete media (100 µl) was added and irradiated at 0.5 W for 10 secs (0.6 J) using a High Powered Devices laser (laser dosage was estimated using a calibration obtained for the specific laser and diameter using a power meter- see section 2.3.11). The cells were supplemented with DMEM phenol red-free media (200 µl) (10 % FBS, 1 % P/S).

For the non-lasered cells, DMEM (300 µl) was added following the washes. Controls used included a set of cells treated with 1 % Triton X-100 and a set of untreated cells.

The plates were incubated in the dark (covered in foil) in a humidified atmosphere (5 % CO₂, 37 °C) over two nights.

Cell viability assay

This was done in low lighting throughout.

Cell viability was measured using the Promega Aqueous Cell-titre-96™ kit according to manufacturer’s instructions. Briefly, 200 µl of the media was removed per well and 20 µl of reagent was added and gently resuspended. The plates were read on an Elisa plate reader at 490 nm after a 2 hr incubation in the dark (5 % CO₂, 37 °C).

Data analysis

The data (absorption units) were converted to % cell survival by using the untreated controls as the 100 % cell survival and the Triton X-100 controls as the 100 % cell death. The average absorption value for the latter was subtracted from all the rest of the data in order to get a suitable baseline. Then, the averages were converted to survival and standard error values were obtained for each n value (as a % cell survival). The data were plotted and fitted to a dose-response sigmoidal logistic 3-parameter curve using the equation y = y₀ + a/ (1 + (x/x₀)b) where x₀= IC₅₀ and x₀>0 and a=100 using SigmaPlot 11.0. Experiments were repeated at least 3 times for each compound tested and a set or an average of the data was plotted and fitted to obtain a dose response curve.
**Calibration experiments**

Prior to starting the *in vitro* PDT experiments, the cell number to be plated (per cell line) and the laser dose had to be optimised. Two sets of experiments were run.

First, cells were plated as above (see section 2.2.7) with a known but varied cell number per quadruplicate set. The cells were then grown overnight, washed with PBS (3 x 100 µl), supplemented with phenol red-free complete media (200 µl) and incubated in a humidified atmosphere (5 % CO$_2$, 37 °C) over two nights. The MTS viability assay was then carried out as previously. The cell number for each cell line that gave an absorption unit near 1 (MTS assay) was used thereon.

A further two experiments were then run where firstly the cells were plated at the optimum number. These were either treated with a maximum PP$_A$ concentration (400 µM) only or with DMEM only. In both experiments, a DMEM non-lased control was used. The cells were irradiated (in quadruplicates) with a range of laser doses. The laser dose that corresponded to maximum cell death in the PP$_A$ (400 µM) experiment but gave no cell death for the media only experiment was chosen and subsequently used throughout.

**2.2.10 In vivo experiments**

All *in vivo* work was carried out under Home Office project licence to MPD Reference 70/6982. Procedures were carried out by Mr Daniel Wells, CBS, Imperial College London. Dr Mahendra P. Deonarain, Imperial College London carried out all the radiolabelling and biodistribution work as well as supervised and irradiated the mice during the therapy experiments.

**Cell preparation**

SKOV3 human ovarian carcinoma cells were grown as indicated in section 2.2.7 washed and detached with trypsin, washed twice with phenol red free DMEM centrifuging (1200 rpm, 6 mins, RT) to collect the cells. The pellet was resuspended in ice cold matrigel (2 ml) making up to 4 ml with phenol red free DMEM (not supplemented) and kept on ice. Approximately, 15-20 million cells were used to implant 20 mice.
**Tumour implantation**

BALB/c nude female mice (6-8 weeks) were briefly anaesthetised using isofluorine. They were inoculated by injecting subcutaneously on the flank with 0.2 ml of the cell suspension. The tumours were monitored and measured on a daily basis. Mice were kept under continuous sterile conditions (individually-vented cages) and had constant food and water access.

**Biodistribution studies**

For the biodistribution studies and uptake pharmacokinetic experiments of C6.5(-k), PPa, PS1, PS4 and PICs C6.5(-k)-PPa and C6.5(-k)-PS1 radiolabelling reactions were carried out using IODOGEN (Pierce) according to the manufacturer’s indications.

Firstly, sodium iodide-125 (Na^{125}I) (solution in PBS) was activated using the supplied iodination tubes. The sample to be labelled was added to the tubes; C6.5(-k) and the PICs in PBS and the free PS in methanol. The reaction was allowed to stand for 7 mins and the C6.5(-k) and PIC samples were purified on a PD10 column eluting with PBS collecting the first major fraction (coloured). The free PS samples were purified using a Sep-Pak® cartridge eluting with methanol. The PS samples were concentrated to dryness and reconstituted in PBS for injections.

Injections: this was done avoiding direct light exposure, room lights were subdued.

When the tumour size reached 4-6 mm diameter, 50-100 mm³, the mice, in groups of 3-5 (per time point) were injected in the tail vein with the corresponding radiolabelled solution (5-10 µg, 0.2 ml per mouse) and transferred back to the cages protecting from direct light exposure and were left for 0-24 hrs. Ketaset/ Rompun (2:1, 20 µl) was injected intramuscularly to anaesthetise the mice in groups and culled by cardiac puncture. Blood and tissue samples were collected by dissection at 0.25, 1, 2, 4, 8 and 24 hrs. The samples were weighed and the gamma count for each sample was measured in a LKB Gamma counter. These were compared to the initial gamma count (100 % injected dose) and expressed in percentage injected dose per gram of tissue (%ID/ g). The data were plotted and fitted to a double exponential decay 4 parameter curve using the equation

\[ f = a \times e^{-bx} + c \times e^{-dx}, \]

where \( a \) phase \( t_{(1/2)} = \ln 2/b \) and \( \beta \) phase \( t_{(1/2)} = \ln 2/d \) using SigmaPlot 11.0.
Therapy

This was done avoiding direct light exposure, room lights were subdued.

When the tumour size reached 4-6 mm diameter, 50-100 mm$^3$, the mice, in groups of 3-5 were injected in the tail vein with the corresponding solution (0.2 ml per mouse) and transferred back to the cages protecting from direct light exposure and were left for 3-6 hrs. Ketaset/ Rompun (2:1, 20 µl) was injected intramuscularly to anaesthetise the mice in groups of two and subsequently placed on a heated mat and irradiated the tumour site a High Powered Devices (HPD) laser (2.3 A, 15 x 1 mins, 110 J/cm$^2$) coupled to a fibre optic cable. The irradiation was done in doses of 1 min to avoid potential skin damage. Higher power led to skin damage.

Observation/ follow up

The mice were observed taking tumour measurements thrice a week for 2-8 weeks.

Mean tumour volume (3-5 mice) was plotted using the equation $volume = \frac{w \times d \times l}{2}$ for the tumour dimensions. Tumour measurements were repeated 2-3 times/week and the % tumour size was calculated using the equation $\% = \frac{size \text{ (mm}^3)\text{ }}{original \text{ size (mm}^3)\text{ }} \times 100$, averaged and the standard deviation obtained. Mice were culled when tumours got bigger than 12 mm in diameter (>700 mm$^3$), if mice were infected or if they were distressed. Statistical analysis included standard deviations and analysis of variance (ANOVA) between samples.

2.2.11 Laser calibration

The red laser by High Powered Devices was calibrated using a power meter and adjusting the output diameter to suit the area of either the in vitro assays or the tumour size for the in vivo experiments. Energy was calculated as follows: $E(J) = P(W) \times t(s)$
*note that “intensity” refers to our laser only. It does not correspond to amplitude.
Chapter 3 Photosensitiser synthesis and characterisation
3.1 Introduction

This chapter describes the rationale behind and execution of the various synthetic attempts to make improved PPa derivatives for bioconjugation. The steps, obstacles, limitations and successful synthetic examples are discussed, together with both physical and photophysical characterisation.

3.1.1 Choosing Pyropheophorbide a (PPa) as our photosensitiser

Pyropheophorbide a is a semi-synthetic photosensitiser obtained by three steps from Spirulina algae a rich source of chlorophyll a. It has a long wavelength absorption at 667 nm and a singlet oxygen quantum yield of 0.5 coupled with minimal dark toxicity (Pandey et al., 1996). These properties make it a suitable candidate for PDT and its derivative HPPH (Photoclor®) is currently in phase II clinical trials for the treatments of non-small cell lung cancer, oesophageal cancer and cancer of the oral cavity (Health, 2010). The total synthesis of chlorins for applications in PDT and imaging has recently been published by Lindsey’ s group but their elegant multistep approach was in our opinion not feasible for commercial synthesis. Other examples in the total synthesis of chlorin macrocycles have been published and all suffer from being complicated, low yielding and long.

Other approaches to obtaining chlorins include the direct reduction/oxidation of a porphyrin (Galezowski et al., 2007). These include the cis-dihydroxylated derivatives obtained by reaction with osmium tetroxide and the diimide reduction of porphyrins. However, both approaches lead to a mixture of chlorins, bacteriochlorins and regioisomers (Sternberg et al., 1998, Bonnett, 2000, Sutton et al., 2000, Sutton et al., 2002).

This is further complicated when trying to synthesise unsymmetrical chlorins with a suitable handle for bioconjugation (Ptaszek et al., 2007).

A porphyrin can be reduced to the corresponding chlorin using metal-acid reduction or by reaction with diimide in pyridine. These reactions are hard to control and do not always stop at the chlorin stage and bacteriochlorins are often obtained as by-products. Using nature derived chromophores as starting materials is a more direct but not necessarily an easier route. These can be chlorophylls a/b, bacteriochlorophylls c/d (Kureishi et al., 1998, Pavlov et al., 2004, Chen et al., 2005, Zheng et al., 2009).
3.1.2 *Introducing water solubility and minimising aggregation*

It is generally accepted that in order to inhibit the aggregation of flat aromatic molecules such as porphyrins, chlorins, bacteriochlorins and phthalocyanines one needs to introduce solubilising groups that cover all sites of a molecule. The solubilising groups are likely to be more efficient if positively charged or bulky or a combination of both (Wagner et al., 1994). One such example are the 2,5 di-substituted benzene rings, used by Lindsey’s group, known as swallow tails (Borbas et al., 2008b, Muresan et al., 2008) where they introduce a water solubilising unit based on an alkyl di-phosphonate above and below the macrocycle plane (Borbas et al., 2006, Borbas et al., 2008a, Borbas et al., 2008c). They found that the di-phosphonate swallow tail moieties provided the highest water solubility compared to carboxymethoxy phenyl groups or N,N’-dialkylimidazoium-2-yl groups (Muresan et al., 2008). The swallow tail moiety encumbers the structure on both faces, imparts solubility and steric hinderance on both sides of the molecule inhibiting aggregation (Borbas et al., 2006). In another approach they introduced aminoalkyl groups to synthetic bacteriochlorins which upon quaternisation afforded di, tetra and hexa methyl-cationic water soluble bacteriochlorins, however these compounds were without a bioconjugation handle (Ruzié et al., 2008).

Other similar approaches include the work of Inoue where they recently published the synthesis of a range of chlorophyll a/b derivatives that carry one, two, four and six positive charges as shown in figure 3.1 (Taima et al., 2009). In this key publication they synthesised cationic PPa derivatives where they successfully esterified the propionic chain with a primary alcohol containing a tertiary nitrogen. Their studies pointed to the difficulty in suppressing π-π stacking and the importance of the positioning of substituents with (c) in figure 3.1 showing no aggregation in water (Taima et al., 2009).
Fabiano et al. made a monocationic derivative of PPa where they introduced an ammonium group at the end of the propionic acid chain. However, despite its improved water solubility they found that the compound aggregated severely in water forming face to face dimers (Fabiano et al., 1997). It appears that in order to inhibit the aggregation of PPa charges or solubilising groups need to be introduced on several positions around the molecule to increase steric hinderance and hinder π-π stacking.

Examples of other cationic substituents based on tertiary and quaternary nitrogens are described by (Roncucci et al., 2004) where various charges are introduced at the meso positions of porphyrins. The building blocks they used are particularly interesting highlighting branched ones similar to those used by Inoue.

Overall, work of others has shown that the most suitable groups for imparting solubility are either neutral or cationic such as oligoethylene glycol chains (OEG) (Grahn et al., 1999, Hornung et al., 2000), OEG dendrimers (Zingg et al., 2002), sugars (Schell et al., 1999), alkyl amine chains (Zhuo et al., 1999), branched polycarboxy groups (Subbarayan et al., 2001), as well as the phosphonate and imidazolium groups used by Lindsey (Bhaumik et al., 2006, Borbas et al., 2006).
Deciding which features to incorporate into developing successful PPa derivatives for targeted PDT relies on a host of factors. As it was previously emphasised (discussed in chapter 1), the ideal photosensitisers for use as part of a conjugate would have to:

1. Be sufficiently water soluble to allow bioconjugation at high concentrations
2. Exhibit no or low aggregation in aqueous solvents
3. As a result of (2) would not interact non-covalently with biomolecules or if they did they would be separable
4. Have a single reactive carboxylic acid group for activation and bioconjugation onto lysines
5. Would maintain or have improved photophysical properties compared to PPa

In summary, the key structural features that we wanted to incorporate onto our PPa derivatives that would allow both a high concentration bioconjugation to antibodies and result in a bioconjugate that displayed decreased levels of non-covalent binding were a suitable handle and groups to impart solubility in aqueous solution.
3.2 Results

3.2.1 Nomenclature of structures

There are two naming systems for porphyrins and related macrocycles; the IUPAC and Fischer systems (Bonnett, 2000, Nyman et al., 2004). The former is the most widely used but in order to avoid long, extensive names according to IUPAC, shorter names will be used from this point onwards as indicated in chapter 2 along with the numbers indicated on the schemes (below the structures (x)) in this chapter for clarity. The main MePPα structure and the numbering system used are indicated in figure 3.2.

![Figure 3.2 Numbered positions on MePPα structure. The positions on the chlorin are referred to using these numbers based on IUPAC numbering system.](image)

3.2.2 Extraction of chlorophyll a from Spirulina algae

Chlorophyll a was extracted from the dried algae Spirulina Pacifica by first cryogenically fracturing the cells followed by large scale acetone extraction at reflux. This was followed by the purification of the crude chlorophyll a (1) extract (figure 3.3 shows a representative TLC showing the major extracts from spirulina) which was then converted to pheophorbide a (2) using degassed sulphuric acid and methanol to remove the phytol chain and perform a transesterification. The last step, the thermal decarboxylation (as shown in figure 3.4) in collidine was found to be the least reproducible and gave the lowest in yields. Attempts to improve the yield by performing the collidine step before the transesterification were unsuccessful and yields were consistently lower than those in the literature. In our hands, 300 g of algae gave 275 mg of pure MePPα (3) (literature: 500 g of dried algae gave 1.8 g of pure MePPα (Smith et al., 1985, Hargus, 2005)). The problem encountered in obtaining
sufficient amounts of starting material was addressed by obtaining material from a commercial supplier (Frontier scientific).

**Figure 3.3 Chlorophyll extraction from Spirulina- TLC plate** showing the composition of the first extracts from Spirulina. This is prior to the isolation of the chlorophyll which is part of the lower running spot.

**Figure 3.4 Extraction of chlorophyll-a from Spirulina Pacifica and its conversion to MePPa**
3.2.3 Synthesis of the main pyropheophorbide-a derivatives

The preparation of PPa succinimidyl AE (4) (figure 3.5) has been reported by several groups (Zhang et al., 2003, Bhatti et al., 2008). However we found that the purification of this product is crucial and required several washes and filtration steps prior to flash chromatography to remove the urea by-product in order to obtain a pure material for bioconjugation. Also, we found that with the use of the liquid N,N'-diisopropylcarbodiimide (DIC) instead of the traditionally used N,N'-dicyclohexylcarbodiimide (DCC) we could obtain purer products due to the increased solubility of the N,N'-diisopropylurea by-product and this therefore became the preferred reagent for all esterification reactions.

![Chemical structure of PPα succinimidyl AE (4) and PPa NHS active ester preparation.](image)

Figure 3.5 Synthetic scheme of PPα NHS active ester preparation.

Starting from MePPα (3), two key intermediates, meso-MePPα (7) and bromo-meso-MePPα (8) had to be made in order to allow both functionalisation and the introduction of solubilising groups as shown in figure 3.6. The starting point for the synthesis of Meso-MePPα was the reduction of the vinylic side chain of MePPα. This was necessary to prevent overbromination when attempting to form the bromo-meso-MePPα (8). The reduction was achieved via a three step continuous process and meso-MePPα (7) was obtained in high purity and very high yields (>95 %) following the literature procedure as shown in figure 3.6 (Li et al., 2003). which involved initial metallation of MePPα using zinc acetate in methanol/chloroform. This allowed the hydrogenation process to be carried out under atmospheric pressure by simply using a balloon filled with hydrogen and 10% Pd/C as the catalyst instead of high pressure hydrogenation (Smith et al., 1985). The next step was the demetallation using fuming TFA. The intermediates (figure 3.6) were not isolated but the reaction was monitored by the shifts...
in the UV/Vis absorption spectrum. The zinc insertion into PPa was observed with a shift from 668 nm to 656 nm. After the reduction the Q band shifts further, to 645 nm, and following the demetallation back to 656 nm for the free base meso-MePPa (7). The formation of the demetallated meso-MePPa was observed by the overall slight blue shift in the UV/Vis and the shift of the 3a and 3b protons in the NMR (figure 3.7).

Following the reduction of the double bond, the next step (figure 3.6), depending on the final intermediate was either bromination of the 20-meso position (step e) or hydrolysis of the methyl ester (step d). The meso bromination reaction proceeds well following the published method using pyridinium perbromide with the main proviso being the apparent sensitivity of the reaction to both moisture and air (Kenner et al., 1973). It was also important to maintain the ratio between the pyridine and the pyridinium perbromide hydrobromide salt constant. The product was identified by the disappearance of the 20-meso proton in the $^1$H NMR (figure 3.7) and a characteristic shift in the UV/Vis spectrum of the Q band (from 656 nm to 669 nm). A slight $R_f$ change on TLC was observed accompanied by the loss of fluorescence. The bromo-meso-MePPa (8) was found to be relatively unstable compared to other derivatives in solution upon storage even in the dark at 4 °C.

The subsequent hydrolysis (step f) of the propionic methyl ester (17d) side chain following optimisation of the reaction conditions gave yields (>70 %) of very pure material. The hydrolysis of the meso-MePPa (7) derivative gave slightly purer and more stable product than the bromo-MePPa (8) derivative. It was important to start with dry starting materials, under an inert atmosphere. The addition of enough fuming hydrochloric acid was also crucial, together with maintaining the reaction under constant positive pressure with the inert gas. When quenching the reaction mixture it was important to ensure that this was done efficiently (colour change to a dark blue/grey colour) otherwise it was too difficult to extract the desired acid. The crude acids were purified even though generally, acid phophorbidies are used crude after hydrolysis. In our hands following very slow purifications on silica, very pure (> 90 % by LCMS for the meso-PPa (9)) products were obtained. It was imperative to allow the column to run under gravity to allow the first front running bands to elute with chloroform before slowly increasing the methanol percentage to up to 10-15 % to elute the product. Significant $R_f$ differences were observed between starting materials and products and the disappearance of the methyl group is seen on the $^1$H NMR as well (figure 3.8). Shifts were not seen in the UV/Vis spectrum as the main chromophore is not affected by this modification. Although the base hydrolysis using LiOH was explored, it was found to be less efficient and the acid catalysed hydrolysis of the ester was used in all subsequent
conversions (Pandey et al., 1996). As a note it was found that the use of old batches of HCl (non-fuming) resulted in incomplete hydrolysis.

Figure 3.6 Synthetic steps to key intermediate PPa derivatives: meso-MePPa (7) (●), meso-PPa (9) (●), bromo meso-MePPa (8) (●) and bromo-meso-PPa (10) (●).
Figure 3.7 $^1$H NMR spectra of the main PPa derivatives. MePPa (3) (●), meso-MePPa (7)(●) and bromo-meso-MePPa (8)(●). Centre, the entire spectrum is shown. Top and bottom, areas pointed out were magnified.
the hydrolysis step

Assignment of the methyl groups MePP a (●) (8)

Figure 3.8 ¹H NMR spectra of PPa derivatives observing the disappearance of the methyl group following the hydrolysis step. Top shows entire spectrum, bottom shows the magnified region 2.2-4.1 ppm focusing on the assignment of the methyl groups. MePP a (●) (3), meso-MePP a (●) (7), meso-PP a (●) (9), pyridyl-meso-MePP a (●) (15) and pyridyl-meso-PP a (●) (16). The pyridyl derivatives are discussed in section 3.2.5.
NMR assignment

Proton NMR analysis and assignment of pheophorbides is complex and Smith has previously worked meticulously on it (Smith et al., 1980). However, it is a fact that not all published proton NMR assignments of PP\textsubscript{a} are concise, accurate or even the same. Proton NMR analysis and electrospray were used for the analysis of the PP\textsubscript{a} derivatives. Electrospray was preferred as MALDI and FAB gave more complicated/heavily fragmented spectra.

3.2.4 The synthesis of PS1

Introducing solubility

The incorporation of the short triethylene glycol chains to the PP\textsubscript{a} core was straightforward and carried out by slightly modifying the published method as seen in figure 3.9, (steps b\textsubscript{1} or a\textsubscript{2}) (Huber et al., 2005, Huber et al., 2007). The use of DIC instead of DCC for the esterification of the propionic acid side chain gave very pure material in yields over 80 % (in the case of the meso-PEG-PP\textsubscript{a} (11)). The synthesis of the PEGylated benzyl alcohol (34) is described in section 3.3.7. Characteristic differences between the meso-PP\textsubscript{a} (9) and its corresponding PEGylated derivative (11) included R\textsubscript{f} changes as the products’ hydrophilicity changed and the absence of the acid allowed it to move faster on silica. The product was a viscous sticky oil which was readily soluble in PBS and water.

Introducing the bromine

Once the meso-PEG-PP\textsubscript{a} (11) derivative was obtained and characterised a conjugation handle had to be introduced and the best route to the active ester (AE) (14) had to be found. As mentioned previously in section 3.2.3 for bromo-meso-MePP\textsubscript{a} (8), it was also found that the bromo-meso-PEG-PP\textsubscript{a} (12) is unstable and can readily debrominate to the corresponding meso-PEG-PP\textsubscript{a} (11) which was very stable. Working with the brominated derivative became problematic as with each reaction purification a degree of debromination was observed resulting in overall lower yields and products containing the meso-derivative as a contaminant. The meso-derivative (as for meso-MePP\textsubscript{a} vs bromo-meso-MePP\textsubscript{a}) run very closely on a column and just behind the corresponding bromo-derivative. Its presence was verified by UV/Vis spectroscopy with the shifts being the same as for meso-MePP\textsubscript{a} to bromo-meso-MePP\textsubscript{a} as shown in figure 3.10.
Figure 3.9 Synthetic scheme of the two possible routes to PS1 that were initially examined. Route 2 was eventually favoured as it lead to purer, more stable products.
Figure 3.10 UV/Vis absorption spectra of compounds leading to PS1 acid (13). Absorption is normalised at the last Q band for clarity. Solvent is DCM. An overall shift from 667 nm (MePPa) to 671 nm (PS1) was observed.

Use of the Sonogashira coupling for introducing the bioconjugation handle

The synthetic availability of the brominated meso-PPa (10) derivative gave us a way to introduce a handle for bioconjugation through metal catalysed carbon carbon bond formation using the Sonogashira reaction. This has previously been described for PPa by Wasielewski using an aryl alkyne however obtained yields were low partly we think as a result of the steric crowding around the brominated meso centre. We therefore postulated that the use of linear (alkyl) alkynes would lead to more efficient carbon-carbon bond formation and chose hexynoic acid as our potential handle. By using hexynoic acid (step d, figure 3.9) the Sonogashira reaction proceeded more smoothly especially when a number of key procedures were followed (Kelley et al., 2006b). The separate degassing of the re-distilled TEA and the dry DMF were imperative to the success of this reaction. If the solvents were degassed together the volatility of the TEA compared to that of DMF would not allow the ratio of 1:10 to be maintained. Their separate degassing and introduction to the reaction flask ensured that this was retained. A large excess of dba₃Pd₂ catalyst was also required.
compared to the published method and a tenfold excess of the acetylene was necessary as homocoupling can occur as a side reaction in the presence of oxygen. The co-catalyst, tri(otoly)phosphine was used at a near tenfold excess to the catalyst which was added to the bromo-meso-derivative first followed by the solvent, the base, the catalyst and lastly hexynoic acid. It was then important to maintain the reaction under argon with merely positive pressure applied with argon to inhibit the evaporation of TEA. It was found best to estimate and not weigh out the amount of catalyst added to the reaction to avoid its prolonged exposure to air and moisture. The coupling was monitored by UV/Vis spectroscopy and TLC analysis and the formation of the product observed by a shift from 668 nm to 673 nm for the Q-band as some aromatic character lost with the reduction of the vinyl group is restored by the insertion of the triple bond (figure 3.10). The work-up and purification included a mild acid wash using citric acid. This was a small modification to the published method and ensured that the acid derivative was extracted efficiently to obtain PS1 acid in very good yields (>70 %) as a very pure dark purple oil (>95 % pure as observed by LCMS).

The final step in the synthesis of PS1 (step e) was preparing the active ester (14) using the same method as described for PP\textsubscript{A}E (4) (section 3.2.3). The reaction in THF/DCM using DIC was followed by TLC to observe the consumption of the starting material and was complete overnight. Several washes with hexane and purification on silica gave 75 % yield of pure material ready for bioconjugation as can be seen in the HPLC trace (figure 3.11). PS1 acid and PS1 AE were found to be stable when kept at 4 °C light protected and sealed over an extensive period of time. Their corresponding solutions in dry DMSO were also very stable when kept as indicated above.
Figure 3.11 A representative LCMS of the PS1 AE (14). The main peak observed at 11.34 mins corresponded to MW 1342.6 which corresponds to the M⁺ plus sodium. Nothing else was observed after 15 mins.

3.2.5 The synthesis of PS4

The starting point for the synthesis of PS4 was the known (Kelley et al., 2007b) pyridyl derivative (15) as shown in figure 3.12. This involves reacting bromo-meso-MePPa with 4-pyridyl boronic acid (Suzuki coupling) using Pd(PPh₃)₄ as the catalyst. In our hands, it was initially impossible to reproduce the published procedure. The desired product was finally synthesised after a more detailed and thorough procedure was disclosed to us by Prof. Wasielewski’s group (personal communication) for which we were extremely grateful.

The problem appeared to be the enhanced sensitivity of the reaction to both moisture and oxygen. These were overcome by adding the large amount of catalyst necessary through approximation and without weighing to avoid prolonged exposure to air and moisture. It was also critical that the pyridyl boronic acid was stored correctly and used before prolonged storage as batches that were not properly stored gave a set of completely unsuccessful reactions. For the reaction to go to completion, a second batch of catalyst was added after 12 hours. This and the use of, dry, freshly distilled THF and pump dried starting material sealed under an inert gas were very important parameters. Following these modifications the
reaction became reproducible and was scaled up compared to the published method giving comparable yields after recrystallisation (50-60 %). The coupling reaction which is almost complete within 18 hrs at 70 °C was monitored by UV/Vis spectroscopy and TLC. The formation of the product was observed by a very slight shift of the Q-band, from 668 nm to 667 nm and the appearance of a fluorescent spot on the TLC with a lower Rf. In the 1H NMR spectra the aromatic protons are distinctly visible. The reaction mixture was worked up, purified on a silica column and recrystallised from chloroform/hexane at 4 °C to give long purple crystals.

Figure 3.12 Carbon-carbon bond formation using the Suzuki coupling to form pyridyl-meso-MePPa, a key intermediate to the synthesis of PS4.

Following the successful Suzuki coupling, two routes were explored to our target molecule (see in figure 3.13). The initial approach followed was the hydrolysis of the methyl ester (step a1). This was carried out in conc. HCl as previously discussed and gave the pyridyl PPa derivative (16) as a pure product following chromatography on silica. This novel PPa derivative was identified using MS, LCMS and UV/Vis spectroscopy. NMR analysis however gave broad signals which were difficult to assign (figure 3.8).
The subsequent step involved the quaternisation of the pyridyl nitrogen of pyridyl-meso-MePPa (15) (steps \( b_1 \) and \( a_2 \) figure 3.13) using the iodo triethyleneoxide (35) (figure 3.22). This proved to be complicated. The quaternisation was initially carried out with an excess of the alkylating agent but was found not to go to completion (step \( b_1 \)). The reaction was performed at \( \approx 70 \) °C monitoring the disappearance of the starting material by TLC over several days using silica (10% MeCN, 10 % H₂O, 80 % saturated KNO₃). Attempts to push the reaction to completion by allowing the reaction to proceed over several days lead to significant amounts of the double alkylated product which was observed on LCMS. An optimum period was found to be approximately two days at 70 °C. The starting material (pyridyl meso-PPa (16)) was visible on TLC and on the LCMS.
One of the main challenges we faced was in how to isolate this quaternised acid derivative (17) obtained in step b₁. Following dissolution in dry dichloromethane it was precipitated with dry ether. Ensuring that both solvents were dry and allowing the mixture to set at 4 °C under argon for a few hours to overnight and then collecting the product by centrifugation in a glass vial became the routine. The removal of the starting material from the mixture was achieved following the optimisation of the wash/precipitation method which successfully lead to a very pure product with iodide as the counter ion. However, it was not possible to remove the doubly alkylated by-product.

In parallel, a second approach was followed as shown in route 2 (scheme 3.13), which involved quaternisation of the pyridyl nitrogen (a₂). The alkylation of pyridyl-MePPa (15) was carried out using the same procedure as described in step b₁ to give PS₄ Me I⁻ (18) in very high purity following the same wash/precipitation procedure as for step b₁. However, the subsequent hydrolysis (step b₂) proved to be problematic and gave rise to several problems. When using the optimised hydrolysis route (as previously described), the acid mixture would be quenched in iced water and extracted to obtain the neutralised material in an organic solvent. This proved to be difficult for the already quaternised derivative as it was partially water soluble leading to the formation of severe emulsions when attempting to separate and extract. This was eventually resolved by using ammonium hexafluorophosphate and forcing the cationic derivative to precipitate out of the acidic solution as the hexafluorophosphate salt (19) (step b₂) (Anderson et al., 1998, Batinick-Haberle et al., 2004, Batinić-Haberle et al., 2006). Compounds having PF₆⁻ as the counter ion are highly lipophilic and are thus soluble in organic solvents. Using this procedure these quaternised derivatives could be isolated, washed and neutralised following the hydrolysis step. However, one drawback was the co-precipitation of the hexafluorophosphate salt of the starting material (18).

The presence of either starting material or double alkylated by-product in the products obtained by either route in figure 3.13 led us to consider either using HPLC or preparative TLC to purify these mixtures. Initial attempts at purification involved preparative TLC. By using a system consisting of saturated potassium nitrate, water, acetonitrile (1:1:8) (Batinic-Haberle et al., 1999) as the eluent gave the acid derivative (17 or 19) in moderate yields. The separation was carried out on a commercially available 20x20 cm preparative TLC plate which was run giving distinct separated bands (see figure 3.14). The plate was then thoroughly air dried and following several attempts, the product obtained by slurrying in chloroform (sonicating to enhance the dissolution of the compound in the solvent) and washing with water. The organic layer was then separated, filtered to remove traces of silica and the product precipitated from dry DCM/dry ether as described above.
Purification was also attempted using HPLC however poor yields of the desired material (17) which still contained small amounts of starting material were obtained on C18 reverse phase silica (gradient elution MeCN/H$_2$O, 0.1 % TFA) as shown in figure 3.15.

As a final step for both routes, the iodide counter ion was exchanged to a chloride using an ion exchange resin by stirring in dry methanol for 6 hours under nitrogen followed by filtration and washes with dry DCM/ dry ether. The LCMS shown in figure 3.16 corresponds to this final product (20) obtained by preparative TLC and subsequent ion exchange. The route in figure 3.17 is suggested as the optimum for making PS4. Figures 3.18 and 3.19 show UV/Vis and $^1$H NMR spectra of key intermediates in the synthesis of PS4.
Figure 3.15 LCMS analysis of PS4 acid Cl following HPLC purification and anion exchange. The main product is seen at retention time 6.80 mins corresponding to MW 760.4 ([M – Cl]+) and at 7.32 mins the double alkylated by-product at MW 906.5 ([M-Cl]+). The rest of the peaks could not be identified.

Figure 3.16 LCMS trace of the final PS4 as obtained from preparative TLC. The main product is seen at retention time 6.85 mins corresponding to MW 760.4 ([M – I]+) and at 7.24 mins the double alkylated by-product at MW 906.5 ([M-I]+).
Figure 3.17 Proposed final route to PS4 following optimisation of the steps shown in Figure 3.13. Dashed arrow indicates reaction that was not attempted.

UV/Vis absorption spectra of compounds leading to PS4

Figure 3.18 UV/Vis absorption spectra towards the synthesis of PS4. Bathochromic changes were observed during the synthesis of PS4 starting from MePPa. The overall shift is from 667 nm (MePPa) to 674 nm (PS4).
3.2.6 Alternative routes

Alternative uses of the Suzuki coupling route

Once the Suzuki coupling was optimised the synthesis of other derivatives like PS2 was attempted as shown in figure 3.20. The active ester (24) of the pyridyl-meso-PPa was prepared from the corresponding pyridyl PPa (16) by activation with DCC (this was prior to the use of DIC) and NHS and identified by MS. This AE derivative was then quaternised using excess methyl iodide to introduce a charge on the nitrogen as for PS4 but using methyl iodide as the alkylating group. This however gave an unidentifiable main product. Quaternisation of the pyridyl-meso-PPa (16) with methyl iodide was then attempted and the product was isolated (22) (PS2 acid I') as identified by MS and $^1$HNMR using a dry DCM/dry ether wash precipitation step. The active ester of this was prepared using DCC as the dehydrating agent. The product contained substantial amount of DCHU which could not be removed with the wash steps previously mentioned.
Figure 3.20 Routes investigated in the early steps towards the synthesis of PS2 and PS4. Conversion of (15) to (16) was carried out as before; (16) was converted to (24) as for (4); attempts to make (23) from (24) gave products that could not be identified; (16) to (22) was carried out as for the PS4 route (17); (23) was obtained from (22) but with substantial amount of DCHU impurities present; (25) was one of the first cationic derivatives made. Yields are indicated as % in brackets.
The initial design for the synthesis of PS1 involved the introduction of a conjugation handle by Suzuki coupling of a carboxy phenyl group on the 20-meso position followed by conversion to the AE (31) (figure 3.21). Since a majority of the planned PPa derivatives were based on the success of the Suzuki coupling and the formation of a single carbon-carbon bond it was crucial that this reaction worked. Many attempts were made to couple the 4-carboxy phenyl boronic acid and subsequently 4-(2-carboxyethyl)benzene boronic acid but all of them failed without any product being isolated. In contrast, the reactions between the bromo-meso-MePPa (8) and the boronic acids (phenyl boronic acid, 4-methoxycarbonyl phenyl boronic acid, 4-(benzyloxy carbonyl) benzene boronic acid) (figure 3.21) were carried out on a small scale successfully. The products gave fluorescent spots on TLC and slightly shifted UV/Vis spectra as expected and verified by mass spectroscopy.
Figure 3.21 Suzuki couplings at the 20-meso position of PPa using three different boronic acids potentially leading to the free carboxylic acid and subsequently the active ester. Route a. shows introduction of a free carboxylic acid group. This reaction was not successful. Route b. shows the successful reaction introducing an ester (27) followed by a potential hydrolysis to give the free acid (29). Route c. shows the most promising route in which a protected carboxylic acid is successfully coupled to the macrocycle (28) and subsequently deprotected with Pd/C. Dashed arrows indicate reactions that were not attempted. R is solubilising group.
3.2.7 Synthesis of key building blocks

The synthesis of the building blocks used in the preparation of PS1 and PS4 was through literature routes. The key toluene-4-sulfonic acid 2-[2-(methoxy-ethoxy)-ethoxy]-ethyl ester (32) (figure 3.22) was produced in excellent yields (95 %) and characterised by MS and NMR as reported in chapter 2 (Snow et al., 2003).

The synthesis of the benzyl alcohol PEG moiety (34) was carried out in two steps as shown in figure 3.22 (Oar et al., 2005). The first step was the base catalysed alkylation of 3,4,5-trihydroxybenzoate using the tosylated ester derivative (32) which gave the trialkylated ester (33) in excellent yields (95 %). The following step was the subsequent reduction of this benzoate ester (33) to the corresponding benzyl alcohol (34). This reduction was carried out using LiAlH₄ in dry THF and was monitored by IR and TLC to observe changes in Rf and the loss of the carbonyl peak (C=O peak at 1750 cm⁻¹). The alcohol was obtained in a yield >95 % as a yellow oil and was used without further purification.

The short iodo alkylating group (35) was synthesised using the commercially available NH₄I salt in a nucleophilic substitution with the (32) in DMF (Holmes et al., 2007). The product was obtained as a yellow oil following a 5 hr reaction at 80 °C which was monitored by TLC. A simple plug purification using neutral alumina gave the desired pure product in excellent yields (>80 %).

Figure 3.22 Synthetic outline to the solubilising building blocks for PS1 (34) and PS4 (35).
Looking at PS1, the linking group between the solubilising moiety and the core photosensitiser is a hydrolytically labile ester group. An alternative was therefore considered which would allow the coupling product between the two reactants to give a more stable bond, such as an amide (figure 3.23). Following a published method (Middel et al., 2002b), the chloride derivative (36) was successfully obtained as a dark oil and characterised by MS and $^1$H NMR. This reaction was followed by an attempt to substitute the chloride with an amine using again a published method (Middel et al., 2002a), however the desired product (37) could not be isolated.

**Figure 3.23 Attempted synthetic route to an alternative solubilising moiety** (37) for coupling to PS1 to allow the formation of an amide bond upon bioconjugation which is hydrolytically more stable.
3.2.8 Photophysical characterisation-singlet oxygen, fluorescence, LogP

Note: in this section “PS1” refers to (13) and “PS4” refers to (20)

Singlet oxygen quantum yield

Singlet oxygen quantum yields were obtained in toluene for PPa, PS1 (13), PS4 (20), meso-PEG-PPa, bromo-meso-PEG-PPa and pyridyl-MePPa. These are shown in table 3.1. The concentration of solutions was adjusted so that the absorbance at the excitation wavelength (430 nm) matched at 0.15 AU. The phosphorescence intensity values were recorded by monitoring the intensity after 4 µs of the decay curves of the singlet oxygen phosphorescence at 1270 nm to obtain a calibration curve for each compound (see chapter 2). The curve gradient with the absorption values of each solution at 420 nm and 800 nm (baseline) were used to calculate the singlet oxygen quantum yield using the formula indicated in chapter 2. PPa was used as the standard and its $\Phi_\Delta$ is 0.5 (Pandey et al., 1996).

Fluorescence quantum yield

For the fluorescence experiments, solutions were prepared in either 100 % toluene or 2 % DMSO/H$_2$O and were diluted accordingly to match the absorbance at 430 nm to 0.1, 0.2 and 0.3 AU. The 2 % DMSO/H$_2$O or PBS system is used throughout this thesis as it marries well the hydrophobicity of PPa with the stability and tolerability of the biological components of the project. The integrated area of the fluorescence curves for the different samples was used to obtain a calibration curve for each compound. The fluorescence quantum yield was calculated using the formula indicated in chapter 2. PPa was used as the standard ($\Phi_f$ is 0.3, toluene).

Octanol-water partition coefficients LogP

As part of the characterisation of the main derivatives it was considered necessary to try and briefly understand their behaviour with respect to biological membranes and in an aqueous environment. Obtaining octanol/ water partition coefficients using the equilibrate/ shake flask method did not provide any useful data and on all occasions the results were unclear. Hence, a computational approach was considered. These were obtained as a mere approximation using the Pallas 7.3 software and are shown in table 3.1 alongside the extinction coefficients and quantum yields.
<table>
<thead>
<tr>
<th>Compound</th>
<th>PPa</th>
<th>PS1 (13)</th>
<th>PS4 (20)</th>
<th>meso-PEG-PPa (11)</th>
<th>bromo-meso-PEG-PPa (12)</th>
<th>pyridyl-MePPa (15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption λ_{max} (nm)</td>
<td>667</td>
<td>671</td>
<td>674</td>
<td>656</td>
<td>668</td>
<td>667</td>
</tr>
<tr>
<td>Emission λ_{max} (nm)</td>
<td>722</td>
<td>731</td>
<td>755 (shoulder)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φ&lt;sub&gt;f&lt;/sub&gt; (Toluene)</td>
<td>0.3</td>
<td>0.26</td>
<td>0.15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φ&lt;sub&gt;f&lt;/sub&gt; (2% DMSO/Water)</td>
<td>0.004</td>
<td>0.01</td>
<td>0.002</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>φ&lt;sub&gt;Δ&lt;/sub&gt; (Toluene)</td>
<td>0.5</td>
<td>0.56</td>
<td>0.73</td>
<td>0.4</td>
<td>0.72</td>
<td>0.14</td>
</tr>
<tr>
<td>t&lt;sub&gt;Δ&lt;/sub&gt; / ms</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ε (DCM)</td>
<td>43976</td>
<td>22265</td>
<td>22685</td>
<td>48726</td>
<td>36646</td>
<td>20514</td>
</tr>
<tr>
<td>ε (2% DMSO/PBS)</td>
<td>11933 (678 nm)</td>
<td>19986 (681 nm)</td>
<td>13213 (280 nm)</td>
<td>10386</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>log P</td>
<td>3.2 ± 0.6</td>
<td>3.6 ± 1.7</td>
<td>0.4 ± 0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1 Key photophysical characterisation of the main PPa derivatives. Both λ_{max} refer to the maximum wavelength in DCM or toluene; log P was estimated using Pallas 7.3 software and it is an approximation; extinction coefficients (ε) are in M\(^{-1}\) cm\(^{-1}\).

**Aggregation studies using UV/Vis absorption and fluorescence spectroscopy**

Studies of the aggregation behaviour of the PSs (PPa as a control, PS1 and PS4) were conducted using UV/Vis absorption spectroscopy observing shifts and spectral shape changes. The samples were prepared in various quantities of DMSO and PBS (phosphate buffered saline), starting from 100 % DMSO observing the changes down to 2 % DMSO/PBS whilst maintaining the concentration of the samples constant. The mixture, 2 % DMSO/PBS is utilised throughout this thesis (see also chapters 4 and 5). The spectra for each photosensitiser are shown in figures 3.24-3.26. Starting with 100 % DMSO, the spectra are sharp and have well defined Soret and Q bands. As the PBS content is increased, a red shift is observed for all three PSs, reaches an isosbestic point at 40 % DMSO/ PBS for PPa and PS1 and is completely shifted at 2 % DMSO/ PBS for all 3. The isosbestic point for PS4 appears to be less pronounced than that of PPa and PS1. The bathochromic shifts observed as well as the broadening of the spectra are indicative of aggregation (Osuka et al., 1996) which becomes most pronounced at 2 % DMSO/PBS.
Fluorescence and UV/Vis absorption spectra of the PSs (PPa as a control, PS1 and PS4) were also obtained (as described in chapter 2) in toluene, a solvent commonly used for singlet oxygen and fluorescence quantum yield measurements (figure 3.28). The PSs are marginally soluble in toluene and therefore required sonication and heating to obtain clear solutions.

Fluorescence and UV/Vis absorption spectra were also obtained for the PSs in water. PPa has very low solubility in water and required sonication and heating to dissolve the particulate aggregates formed. To the water samples was then added, octyl β-D-glucopyranoside (OG) (final concentration 50 mM) and rescanned in order to observe the disaggregation of the PSs without changing the sample’s concentration. This detergent was chosen with the aim to observe the disaggregation of the PSs in their aqueous solutions. Octyl glucopyranoside is an amphiphilic non-ionic dialyzable detergent with a critical micelle concentration of 23-25 mM used for the solubilisation of proteins. The spectra obtained in the three different solutions (toluene, water and water/detergent) were combined and are shown in figure 3.27. The absorption spectra indicate that the PSs are aggregated in the 2 %DMSO/ H2O solution and disaggregate upon addition of detergent exhibiting a blue shift and sharper spectra. The same is observed for the fluorescence spectra where a distinct shoulder is visible for PPa and PS1 and not so severely for PS4. In all three PSs the most aggregated sample is the 2 % DMSO/H2O.

The three PSs in the aqueous solutions are compared in figures 3.29 and 3.30 and after considering their fluorescence quantum yields in water and toluene, we suggest that PS1 is the least aggregated one with PPa and PS4 showing similar levels of aggregation.

Focusing on PS1, two more aqueous conditions were used and analysed by UV/Vis spectroscopy. Isopropanol was used in the same manner as DMSO observing the aggregation of PS1 as the amount of the organic solvent is decreased to 2 % iPrOH/ H2O (see figure 3.31). Similarly to the DMSO/PBS system, a red shift is observed with broadening of the spectra. At the concentrations scanned, an isosbestic point was not observed. The various aggregation states of PS1 in buffer and detergent are shown in figure 3.32 suggesting that 20 mM OG in 2 % DMSO/PBS partially disaggregates PS1.
Figure 3.24 UV/Vis absorption spectra of PPa in various amounts of PBS/ DMSO whilst maintaining the concentration of the solutions constant. The experiment was run at 25 °C, avoiding prolonged light exposure. The aggregation is observed with a bathochromic shift and an isosbestic point at 40 % DMSO/PBS and a maximum shift from 667 nm to 706 nm.

Figure 3.25 UV/Vis absorption spectra of PS1 in various amounts of PBS/ DMSO whilst maintaining the concentration of the solutions constant. The experiment was run at 25 °C, avoiding prolonged light exposure. The aggregation is observed with a bathochromic shift and an isosbestic point at 40 % DMSO/PBS and a maximum shift from 672 nm to 686 nm.
Aggregation of PS4 in PBS

100 % DMSO
80 % DMSO/ PBS
60 % DMSO/ PBS
40 % DMSO/ PBS
20 % DMSO/ PBS
10 % DMSO/ PBS
6 % DMSO/ PBS
4 % DMSO/ PBS
2 % DMSO/ PBS

Figure 3.26 UV/Vis absorption spectra of PS4 in various amounts of PBS/ DMSO whilst maintaining the concentration of the solutions constant. The experiment was run at 25 °C, avoiding prolonged light exposure. The aggregation is observed with a bathochromic shift with the maximum shift was from 669 nm to 686 nm.
Figure 3.27 UV/Vis absorption spectra of the three photosensitisers corresponding to the fluorescence spectra in figure 3.38. Samples in toluene and 2% DMSO/H2O were prepared and matched for absorption at 430 nm. Detergent (OG) was subsequently added to a final concentration of 50 mM (without diluting the sample) and the sample was rescanned. Inserts show the λmax region of each PS magnified.
Figure 3.28 Fluorescence emission spectra of the three main photosensitisers in toluene, 2 % DMSO/H$_2$O and 2 % DMSO/H$_2$O and 50 mM OG (detergent). Samples in toluene and 2 % DMSO/H$_2$O were prepared and matched for absorption at 430 nm. Detergent (OG) was subsequently added to a final concentration of 50 mM (without diluting the sample) and the sample was rescanned. Inserts show normalised emission. For each photosensitiser, the samples for the three solvents were matched for absorption intensity at the excitation wavelength (430 nm).
Figure 3.29 UV/Vis absorption spectra of the three photosensitisers in top, 2 % DMSO/ PBS and bottom, 2 % DMSO/H₂O with 50 mM OG. Spectra were normalised at the Soret. Inserts show magnification of the last Q band.
Figure 3.30 Comparing the normalised emission spectra of the three PSs in toluene (main graph) and in 2 % DMSO/H$_2$O (insert). $\lambda_{\text{max}}$ are as follows (toluene, water): PPa (675 nm, 667 nm), PS1 (677 nm, 762 nm) and PS4 (686 nm, 767 nm).

Figure 3.31 UV/Vis absorption spectra of PS1 in iPrOH showing its disaggregation with increasing amounts of organic solvent; concentration of PS1 maintained constant at 20 µM. A red shift was observed from 672 nm to 687 nm.
Figure 3.32 UV/Vis spectra showing the disaggregation of PS1 using detergents. PS1 were diluted to 0.020 mM. The detergent octyl β-D-glucopyranoside (with a CMC of 20-25 mM) was used at 10 and 20 mM in PBS. A blue shift was observed upon addition of detergent; PBS 674 nm, 20 mM OG 676 nm.
3.3 Discussion

3.3.1 Chlorophyll a extraction from Spirulina algae

Spirulina Pacifica algae was chosen as it is a chlorophyll-a rich source containing no chlorophyll-b making isolation easy and can be easily obtained in a freeze dried powder form. Chlorophyll-a can be converted to methyl pyropheophorbide-a following a 3 step literature process and was briefly considered as a cost-effective solution to the need for large amounts of PPa as the starting material for the various synthetic schemes.

However, attempts to extract and synthesise MePPa from Spirulina Pacifica were time consuming and inefficient mirroring observations by other groups describing the process as a “cumbersome extraction”, and “laborious natural product isolation” (Pandey et al., 1991). Groups report being able to isolate of 1.2 g of methyl pheophorbide from 500 g of algae (Pandey) but we were not able to reproduce this.

In this work, collidine was used for the thermal decarboxylation step (Pandey et al., 1996) rather than pyridine (Kenner et al., 1973, Tauber et al., 1997) as the use of pyridine gave poorer yields and more byproducts. Pandey et al. were the first group to use chlorin e6 trimethyl ester as the starting material for the synthesis of MePPa (Pallenberg et al., 2004). They prepared methyl pheophorbide-a by a Dieckmann condensation on the exocyclic ring of the macrocycle. This successful one pot synthesis of PPa from ce6 was completed by performing the condensation in collidine to close the ring and hydrolyse the methyl ester and by subsequently raising the temperature to reflux to promote the decarboxylation step and give MePPa (3). The use of collidine for the decarboxylation step is governed by its high boiling point at 172 °C.

3.3.2 Main Derivatives

Based on the initial scope of this project, the main derivatisations of the macrocycle were going to take place at the 17d or 20-meso positions. It was therefore crucial to obtain successfully, reproducibly and in good yields the key intermediates.

The vinyl bond of PPa, following the insertion of zinc in the macrocycle cavity, behaves as an isolated double bond and can more easily undergo reduction, oxidation, addition and
substitution (Helaja et al., 2000, Pavlov et al., 2004). Reagents used to reduce the double bond included PtO$_2$ (Seely, 1966), Pd/C and Raney Nickel. Pd/C is the most widely used as it is a clean reaction, reproducible and under atmospheric pressure.

Meso-MePP$_a$ (7) and bromo-meso-MePP$_a$ (8) were routinely obtained in high yield and purity following their purification on alumina grade III by flash chromatography. The hydrolysis of the methyl ester derivative of both compounds can be carried out either through base or acid catalysed hydrolysis. In our hands, the acid catalysed hydrolysis using a large excess of conc. HCl consistently gave excellent yields of the corresponding acids.

During the course of this work it was realised that although most derivatives were stable in their solid state when light protected and maintained at 4 °C. This was not the case with those derivatives containing a bromine atom at the 20-position. These tended to debrominate to the corresponding meso-derivatives. This process was even more rapid when samples were in chlorinated solvents. The fluorescence of the bromo derivatives is quenched due to the heavy atom effect and it’s only restored when meso-PP$_a$ is reformed or if the bromine atom is replaced by a carbon bond (Hermanson, 1996). This observation helped in monitoring reaction progress especially when following the introduction of the pyridyl moiety using the Suzuki coupling where the observed UV/Vis spectrum shift is very small.

These intermediates were all purified using traditional gravity flash chromatography. However, a successful purification could take up to 12 hours giving extremely pure products. Attempts at using HPLC to purify these products were not explored.

**Ester bond formation-use of DPTS and carbodiimides**

The preferred route for forming the ester linkage between the propionic acid side chain and the benzyl alcohol involved DIC catalysed dehydration in dry THF. The main problem with this approach was the formation of the urea by-product which was especially troublesome when DCC was used as the dehydrating agent. The use of DIC markedly improved the isolation and purification of the corresponding ester derivatives due to the solubility of the urea. Inoue reported forming the same ester linkage using pivoyl chloride in basic conditions with or without DMAP. The key advantage of this approach is that it avoids the need to remove urea byproducts formed from the DCC/DIC route (Taima et al., 2009). Other reagents used for acid/ alcohol coupling especially in the case of more sterically demanding
alcohols is the BOP reagent (Taima et al., 2009). The formation of urea can be suppressed by the addition into the reaction mixture of DPTS as claimed by (Moore et al., 1990). It is likely that all the components used in the esterification reaction are not necessary and that sufficient amount of DPTS with the carbodiimide would suffice but we did not investigate this. The choice of carbodiimide esterification was based on the literature as well as the fact that it is a one step reaction that does not require the acid to be pre-activated in a separate step.

Wasielewski and Svec were the first to publish an ester bond formation at the propionic side chain (Wasielewski et al., 1980). They concluded that DCC/DMAP activated coupling was the most efficient.

### 3.3.3 $^1H$ NMR Assignment

![Figure 3.33 Reminder of the numbering of the MePPa structure](image)

The meso protons in porphyrins are always the most aromatic and appear between 8-10 ppm downshifted from TMS. In a chlorin, the presence of the reduced pyrole ring causes a characteristic splitting into three peaks corresponding to the meso protons. However, the assignment of these protons has varied from group to group. Kevin Smith assigns the 10-meso proton at 9.52 ppm, the 5-meso proton at 9.40 ppm and the 20-meso proton at 8.56 ppm for MePPa (Smith et al., 1985). However, other groups have labelled these in the order of 5, 10, 20 (Nikkonen et al., 2009), and 20, 5, 10 (Helmreich, 2005) meso protons. The second point is the 4 methyl groups (2a, 7a, 12a and 17d). These have been assigned in the following order 17d, 12a, 2a, 7a (Smith et al., 1985, Helmreich, 2005), 12a, 7a, 2a (Kenner et al., 1973) or not clarified at all (Pallenberg et al., 2004). It was therefore unclear as to which assignment was correct so we based our assignment on Smith and on our own observations taking into account the chemical shifts with each modification of the ring.
The meso protons were assigned as 10, 5 and 20 (figure 3.7). Proton 20 was considered to be the most upfield one as it shifted slightly upon double bond reduction as expected and following bromination at the 20 position the signal disappeared. Also, being next to the reduced pyrole group, it’s the least aromatic. Proton 5 was considered to be the second most downfield of the 3 meso protons as it shifted upfield slightly upon reduction and subsequently downfield following the bromination of the 20-position as expected. Lastly, the 10 meso-proton is furthest away from all the modifications hence it is the least affected and shows the least shift. Therefore, the meso proton assignment based on our data correlated with the assignment by Smith.

Comparing the methyl groups in the various derivatives as shown in figure 3.7, one group shifts by 0.15 ppm upfield (compared to MePPa) following the double bond reduction. The closest groups to the double bond that could be affected were 2a and 7a. Going from the meso to the bromo derivatives, the same proton showed a downfield shift of >0.2 ppm which was likely caused by the insertion of an aromatic group nearby. Hence this was assigned as 2a. This is further supported by the shift of the same peak to 2.4 ppm (figure 3.8) (pyridyl MePPa (15)) which could only correspond to the nearest group which would be 2a.

The disappearance of the singlet peak at 3.7 ppm when converting the Meso-MePPa to Meso-PPa is indicative of the presence of 17d at the furthest downfield position. Concluding, the proton order based on our observations was assigned as follows: for MePPa is 10-meso, 5-meso, 20-meso and 17d, 12a, 7a and 2a. Note that 7a and 2a can swap positions depending on the nature of the substituents at positions 20 and 3.

**Broad $^1$H NMRs**

It was very interesting to observe the broadening of the proton NMR spectra for most of the acid derivatives, especially that of the pyridyl (16). This was also observed by Helmreich who attributed it to interactions, most likely π-π between adjacent rings. It is very likely that here too, the broadening and the lack of distinct peaks is due to aggregation. It is worth noting that Kelley made the pyridyl MePPa to deliberately form tetramers which they successfully formed (Helmreich, 2005, Kelley et al., 2007a). The presence of the free carboxylic acid (propionic side chain) is probably introducing additional intermolecular interactions either via the ketone (C13 and the COOH hydrogen) or in fact the nitrogen of the pyridyl group and the hydrogen. This is supported by the fact that upon quaternisation of the pyridyl group (and the loss of the nitrogen’s availability to form hydrogen bonds) the $^1$H NMR spectra becomes
It is also worth noting that various solvents were used to run these $^1$H NMRs without any improvement. It was not possible to assign them but the identification of these derivatives was based on their accurate MS, $R_f$ changes and changes in the UV/Vis spectra.

### 3.3.4 The Suzuki and Sonogashira coupling - synthesis of PS1 and PS4

Both the Suzuki and the Sonogashira reactions have become key tools for the synthesis of carbon-carbon bonds. Their versatility and wide applicability has been summarised in a number of reviews (Stanforth, 1998, Chemler et al., 2001, Littke et al., 2002) and their applicability has grown with the availability of ever more sophisticated catalysts and boronic acids.

The formation of products in both the Suzuki and Sonogashira reactions was observed with a change in $R_f$, a red bathochromic shift in the UV/Vis spectra and the visible fluorescence of the products compared to the non-fluorescent bromo-derivatives.

Boyle developed and applied the use of the Suzuki coupling without the use of aqueous bases in the synthesis of meso-phenyl porphyrins when a phenyl group was introduced on the 20-position (Shi et al., 2002). This variation has been extensively used by Wasielewski’s group to couple substituted phenyl boronic acids to brominated PP$_a$ derivatives and has opened up the possibility of synthesising many new derivatives. Subsequently Kelley cross-coupled the 4-amino phenyl boronic acid at the same 20-position (Kelley et al., 2006a).

Boyle et al. observed two side reactions during the palladium catalysed coupling of an aryl-boronic acid to the meso-position of their porphyrin. These included the deborylation of the boronic acid and more importantly the debromination of the porphyrin. The latter was also consistently observed in our work as seen both on TLC and the UV/Vis spectroscopy. As mentioned before, initially it was difficult to isolate and identify the desired product in any of our Suzuki coupling attempts. Upon re-examining the published method and introducing seemingly minor changes in the synthesis following personal correspondence, the reaction worked well, improving on published yields. The synthesis of the pyridyl derivative of PP$_a$ opened up the possibility of introducing a positive charge as it contained a nitrogen centre which could be alkylated. This would be expected to improve both solubility in aqueous solution and to an extent minimise aggregation (Alonso et al., 2010).

Even though cationic compounds are generally aimed at targeting bacterial rather than mammalian cells as they are better internalised (Hamblin et al., 2004, Maisch et al., 2004), it
was not considered an issue when designing the cationic derivatives here. The eventual conjugation of the chosen photosensitisers to an antibody fragment should minimise the importance of passive diffusion as receptor mediated trafficking will be available due to the antibody’s targeting ability.

In an effort to synthesise a cationic derivative of PPa which also contained a suitable acid group for activation the ester side-chain had to be hydrolysed to the corresponding acid followed by ‘activation’ through active ester synthesis. Initial studies concentrated on synthesising a suitable ‘model’ compound PS2 (figure 3.20) in which the numerous combinations of hydrolysis, quaternisation and active ester synthesis could be studied.

During these studies, it was also realised that the order in which the positive charge was introduced was key and had to be prior to the active ester synthesis, contrary to the work of Tome (Tome et al., 2004). Attempts to quaternise the pyridyl nitrogen (15) in the presence of the active ester gave a mixture of products together with starting material and a prevailing and unidentified compound. In all subsequent preparations of the cationic derivatives the synthesis of the active ester was the final step in the synthesis. Although PS2 was an interesting compound, the observation that it exhibited lower aqueous solubility in both water and PBS as compared to PS4 (when left standing at 4 °C) meant that it was not studied any further and its synthesis was not optimised.

Establishing the best route for making PS4 was time consuming and required relatively large amounts of compounds to be used in order to identify problematic reactions, optimise steps and characterise products. Finding the best route to the quaternised derivatives involved trying several routes that could potentially lead to the same products. Whether to hydrolyse the methyl ester prior to charge introduction and when to make the active ester were some of the initial dilemmas. Small scale attempts (10-20 mg) leading towards both PS2 and PS4 improved our understanding of the reactions and helped identify problems.

Overall, the proposed synthesis of PS4 is a six step route starting from MePPa to the AE that was not obtained. However, we do not foresee any major issues with this last step as the use of DIC should make purification easier than with PS2 where DCC was used. The most challenging steps were the hydrolysis/quaternisation attempts which afforded pure products after extensive optimisation of the purification process (Casas et al., 1993). The long work up involved makes PS4 and potential similar derivatives less attractive than PS1.
Opimising the HPLC purification of these derivatives may offer a quick solution however unlikely as analytical rather than preparative HPLC is more often used with PPa derivatives as it is not always possible to elute these compounds from a C8 or C18 column (Zheng et al., 2009). The preparative TLC method may prove to be a better option if the elution of the product from the silica is reproducibly resolved.

Sonogashira coupling and the synthesis of PS1

The use of the Sonogashira reaction to introduce acetylene bonds onto the ring of a porphyrin macrocycle has been extensively used by Lindsey (Taniguchi et al., 2004, Taniguchi et al., 2008) in the functionalisation of synthetic chlorins and by Wasielewski to make complex PPa derivatives (Kelley et al., 2006a, Kelley et al., 2007a). In both cases the major improvement was the use of the copper-free Sonogashira coupling at room temperature, first reported by Lindsey. Copper readily inserts into free-base porphyrins but is difficult to remove, usually requiring a mixture of sulphuric acid and TFA. The presence of small amounts of copper porphyrins is a non trivial problem when studying photosensitisers as copper porphyrins are generally non-fluorescent. The use of copper in conjunction with palladium would have severely limited the scope of the Sonogashira reaction. The use of the copper free Pd-coupling method facilitated the direct introduction of a hydrolytically stable C-C bond (through an alkyne), and a carboxylic acid for making the AE together with a welcomed red-shift in the UV/Vis spectrum in one step. This coupling method became very reproducible following optimisation of the overall route towards PS1. Again, for PS1, significant amounts of compounds were required to allow chemical characterisation and their subsequent use for in vitro and in vivo studies so reproducibility was imperative.

One of the most important observations during the optimisation of the PS1 route was that depending on whether the bromo-meso-PEG-PPa (12) was obtained from route 1 or 2 (figure 3.9) the yields and the purity of the final product varied from 25 % to 75 %. Overall, it was more straightforward to produce the meso-PEG-PPa (11) derivative in very high purity contrary to the bromo-meso-PEG-PPa (12) derivative. It was therefore decided to leave the bromination step last, just before the Sonogashira coupling especially as the PS1 acid (13) was also found to be very stable. The bromo-PEG-PPa (12) was obtained in good yields following normal bromination conditions using pyridinium perbromide and subsequent slow purification which allowed the separation of the meso starting material from the product. Hence route 2 was chosen and optimised.
Once the bromo meso-PEG-PPa (12) was obtained as well as the successful Sonogashira coupling we also attempted to introduce an aryl acid as a potential handle through a Suzuki coupling. However all attempts to couple a carboxylic acid substituted aryl boronic acid failed resulting in unidentified material. Numerous attempts using 4 carboxyphenyl boronic acid resulted in unidentifiable mixtures (26), despite reports in the literature of carboxy-boronic acids being successfully used in Suzuki couplings (Anderson et al., 2005, Kumar et al., 2006). Some limited success was observed when carrying out the Suzuki coupling with the methyl ester (27) derivative of the 4-carboxyphenyl boronic acid and the benzyl protected derivative (28) but these were not pursued any further.

The Suzuki coupling was carried out with a limited number of boronic acids where the carboxylic acid group was protected as either the methyl or benzyl esters to try and understand the underlying problem with the direct conjugation of a carboxylic acid aryl boronic acid. The successful synthesis of the benzyl protected carboxyphenyl PPa derivative gave us an indirect alternative to the unsuccessful direct carboxyphenyl PPa derivative. The benzyl protected compound can be deprotected using Pd/C to obtain the free acid. However as this was only obtained on a small scale and at the same time the Sonogashira reaction was successful it was not pursued further due to time limitations. It does however remain an alternative solution if the coupling of a carboxy group is not favourable. Most references in the literature refer to acid containing boronic acids being coupled under aqueous conditions (Anderson et al., 2005).

The attempts to introduce a bioconjugatable handle through the Suzuki coupling were dropped in favour of the Sonogashira coupling which when the alkyne was a sterically undemanding alkyne worked efficiently and led to the synthesis of PPa derivative namely PS1 with a hexynoic acid handle which then could be activated and conjugated.

### 3.3.5 Other approaches

#### Addition to the vinyl group

Another possible point for derivatisation on PPa is the vinyl group. PS5 (scheme 1 appendix 1) was initially conceived and designed in order to introduce in the first instance the same solubilising moiety as for PS1 but at the vinyl position, at the top of the photosensitiser with one carbon atom space away from the aromatic ring. The introduction of a bulky hydrophilic group close to the macrocyclic ring should inhibit π-π interactions between these macrocycles. The synthesis (scheme 1, see appendix 1) involves the addition of HBr to the double bond using HBr/HOAc (Zheng et al., 2009) followed by treatment of the intermediate
perbromide derivative (not isolated) with benzyl alcohol (34) giving the alkoxy derivative. The use of a large excess of the alcohol caused severe problems and even though it appeared to work as observed by TLC with a small change in $R_f$, the work up was challenging. The wash steps used to remove the potassium carbonate and neutralise the sample lead to the formation of an emulsion. Eventually a crude product which could not be further purified was isolated as a dark viscous oil but not characterised. This approach, although promising was not pursued due to time constraints. A less direct but more practical route towards PS5 could involve oxidising the vinyl group to a formyl followed by reduction to form the alcohol. The alcohol could then be coupled via a carbodiimide coupling to a suitably substituted acid to form an ester bond. This approach would involve using stoichiometric amounts of reactants making work up and isolation easier.

### Introducing multiple positive charges

The fact that we observed a significant increase in water solubility going from PS2 to PS4 encouraged us to try and introduce multiple charges. The synthesis of PS6 is outlined in scheme 3, appendix 1.

Firstly, the benzyl alcohol moiety (41) as shown in scheme 2 appendix 1 had to be made. It was not possible to identify the tosylated tertiary amine chain (39) even after several attempts. Some of these included using methane sulfonyl chloride instead of toluenesulfonyl chloride and changing the solvent. The role of the reactants was then reversed and instead of activating the primary alcohol (42) we decided to activate the hydroxybenzyl group (43). Following optimisation, both the mesylated (38) and tosylated (not shown) derivatives of trihydroxybenzoate (43) were isolated and characterised as white crystalline solids. However, numerous attempts were made to alkylate the trimesylate (38) with the desired alcohol (42) with no success. Attempts and variations included changing the solvent (acetone, DMSO, acetonitrile) using potassium hydroxide or potassium iodide to catalyse the reaction.

A test reaction was conducted in which we were able to couple toluene-4-sulfonic acid 2-[2-(2-methoxy-ethoxy)-ethoxy]-ethyl ester (32) to the alcohol (42) giving the ether (45). Therefore the problem was not the reactivity of the alcohol. The successful synthesis of the benzyl alcohol moiety (34) (as shown in appendix 1, scheme 2) by alkylating triethylene oxide alcohol (44) with the mesylated benzoate ester (38) proved that the trimesylated ester was reactive. Due to time limitations, and following a series of unsuccessful reactions, attempts to obtain (41) were not pursued any further.
We then decided to test the idea of conjugating an alcohol containing a terminal tertiary amine group on to the PPa ring at the 17d position (appendix 1 scheme 3, concept leading to PS6-B). However, coupling 2-dimethylaminoethanol using the same method as for PS1 did not produce anything identifiable. Using EDC.HCl, changing the alcohol to 2-[2-(Dimethylamino)ethoxy]ethanol, the solvent from dry DCM to dry THF, increasing the alcohol equivalents and combinations of these eventually gave the product which was identified by mass spectrometry.

This was significant and positive as it demonstrated that meso-PPa (9) could be successfully coupled with 2-[2-(Dimethylamino)ethoxy]ethanol (42) allowing the introduction of tertiary amines on the core of the chlorin macrocycle using the versatile and convenient alcohol/acid coupling.

The design of PS6 incorporated PEG groups (albeit short) and positive charges, combining characteristics found in substances that do not show protein adsorption (Mcpherson et al., 1998, Ostuni et al., 2001). The perseverance in the synthesis of PS6 was based on our assumption that it would exhibit improved solubility and aggregation. The design of PS5 was also based on the density of the PEG chains which would increase its hydration radius and perhaps make the interactions between protein and the hydrated PS energetically unfavourable. It is known that increasing the chain length that is introduced at the 3a position too much will decrease biological efficacy as shown by Pandey (Pandey et al., 1996).

3.3.6 Photophysical and physical properties characterisation/aggregation

The photophysical characterisation of the photosensitisers is essential when trying to understand how modifications on the periphery and the core macrocycle affect its cell killing efficacy which is, after all, the ultimate goal of this project.

The key parameters for an efficient photosensitiser to use in PDT or photodiagnosis, are high singlet oxygen quantum yield for efficient generation of singlet oxygen which should correspond to higher cytotoxicity as well as high quantum yield of fluorescence for use in photodiagnosis to image, for example tumours in vivo (Celli et al., 2010).

Aggregation is a solution phenomenon that involves the non-covalent interaction of two or more molecules forming dimers or oligomers (figure 3.34) (Bonnett, 2000). The aggregation of PSs in aqueous solutions can compromise the photophysical characteristics of a PS by decreasing the efficiency of singlet oxygen generation leading to a decrease in PDT efficacy.
(Kuimova et al., 2009). Hydrophobic PPa derivatives tend to be soluble and in monomeric form in organic solvents and insoluble and aggregated in aqueous solutions (Delanaye et al., 2006).

We identified that the aggregation and stability in aqueous solvents are introduced with PS4 being 70% fold higher than PPa. This would theoretically mean that PS4 should be a more potent derivative than PS1 in vitro. However, as several other parameters including subcellular localisation, aggregation and stability in aqueous solvents are introduced, this is not a conclusion that could be made so early on. To illustrate this, the in vitro potency of these 3 PS is described in chapter 5.

Singlet oxygen and fluorescence quantum yields

Singlet oxygen quantum yields of key compounds were either maintained near those of PPa value or in the case of PS4 significantly increased (see table 3.1). Comparing the \( \Phi_\alpha \) of the three main PSs, the order is as follows: PS4 (1.3)>PS1 (1.1)>PPa with PS4 being 70% higher than PPa. This would theoretically mean that PS4 should be a more potent derivative than PS1 in vitro. However, as several other parameters including subcellular localisation, aggregation and stability in aqueous solvents are introduced, this is not a conclusion that could be made so early on. To illustrate this, the in vitro potency of these 3 PS is described in chapter 5.

We identified that the photophysical measurements of the three photosensitisers have an approximately constant sum of \( (\phi_\alpha + \phi_t) \) which is 0.8. Therefore, an increase in the singlet oxygen quantum yield would infer a decrease in the fluorescence quantum yield. This was observed in practice as the PS4 derivatives did indeed show very low intensity fluorescence on either TLC plates or in solution. Even though fluorescence is important for PDT (for use in diagnostics) a high quantum yield is not essential and 0.2 is deemed sufficient.

The aggregation of the photosensitisers in water is obvious when considering the fluorescence quantum yield (table 3.1) and figure 3.28. The \( \Phi_t \) of the PSs in water compared to toluene is 75-fold less (PPa), 26-fold less (PS1) and 75-fold less (PS4). Comparing the three PSs in water, their \( \Phi_t \) is in the following order PS1(2.5fold)>PPa (2fold)>PS4. Therefore, PS1 shows reduced aggregation when compared to PPa of approximately 5-fold when comparing the aggregation (toluene/water) and the difference in their \( \Phi_t \). In contrast, PS4 shows aggregation levels similar to PPa.
With respect to the other derivatives, as shown in table 3.1, the most important observation is that there is a small (20 %) reduction in the $\Phi_\Delta$ of meso-PEG-PPa (11) and a significant (44 %) increase for the brominated derivative (12) probably due to the heavy atom effect quenching of the fluorescence quantum yield. Lastly, the singlet oxygen quantum yield of pyridyl-meso-MePPa (15) showed a significant decrease (72 %) which was restored upon quaternisation of the pyridyl group to form PS4 (20) (1.5x higher than PPa).

**Octanol water partition coefficients**

Log P values give numeric characterisation to the potential interaction of a compound in a cell membrane environment. Octanol and water mimic the biphasic membrane environment in terms of lipophilicities and is often used to characterise drugs (Collander, 1951, Zhang et al., 2003). It was not possible to obtain reproducible values for PPa and PS1 experimentally. Obtaining log P values experimentally is generally difficult and hard to reproduce (Hansch et al., 1964, Engelmann et al., 2007). The use of computational software, Pallas 7.3 provided approximations that were used as an indication of the behaviour of these derivatives (Zheng et al., 2009). The software’s limitations only allow for these values to be used with caution especially for values below 1 (as for PS4) (Engelmann et al., 2007). It remains to be seen whether there is any validity and correlation with an actual biological environment.

Ostuni et al. hypothesised that lipophilic compounds with log P values in the range of 0-5 heavily adsorb proteins whereas compounds with log P values <0 tend to be non-adsorbent (Ostuni et al., 2001). However their experimental evidence were not clear enough to validate the hypothesis. The values obtained computationally would indicate that PS1 is slightly improved compared to PPa. PS4 showed great promise as the low value suggested it to be the most hydrophilic of the two. However, the absorption and fluorescence studies show intense aggregation for both compounds suggesting that a quantitative description of the lipophilicity of the compounds does not necessarily correlate in practice.

**Aggregation studies**

Aggregation is a reversible process that occurs in solutions and is affected by temperature, concentration, solvent and solute solubility as well as steric hindrance (Bonnett, 2000). It is usually detected by UV/Vis spectroscopy where solutions of the sample in question do not
obey the Beer-Lambert law, a bathochromic (red shift) or hypsochromic (blue) shift, broadening of the Soret and Q bands can all be results of aggregation (Bonnett, 2000).

J aggregate formation is characterised by a red shift in the UV/Vis spectrum compared to that of the monomer. In order to form tubular J aggregates, a central metal atom, a hydroxyl group (e.g. 3 position), a hydrogen bond acceptor (13 position ketone) and/or extended aromatic system are necessary (Huber et al., 2005). Although studies by Huber and others have shown that the 13-ketone group is a possible point for aggregation, the resulting blue shift that would be caused by the loss of this group was undesirable in our case (Pandey et al., 1996, Huber et al., 2005, Huber et al., 2007, Huber et al., 2008). Hence, our rationale was to interrupt the π π stacking which prevents formation of J aggregates by physically introducing an obstacle. Highly aggregated structures can exhibit bathochromic shifts of 90 nm (Huber et al., 2005) accompanied by the splitting of the Soret and the broadening of the Q bands. Even though free OH groups are not present in either of our designed AE products (PS1 AE and PS4 AE) it was not possible to eliminate the free acid from the precursors that were used in the aqueous based aggregation studies. It is possible that aggregation is enhanced by their availability. A suggestion would be to synthesise a protected acid for these studies for each derivative. Highly ordered tubular J aggregates require a central metal ion, a free hydrogen bond donor and a hydrogen bond acceptor as well as a flat aromatic system (Huber et al., 2005). In the case of PS1 acid, the only component missing is the central metal ion. The exocyclic ketone acts as a hydrogen bond acceptor and the acid as the hydrogen bond donor.

The extinction coefficient of PP\textsubscript{a} in DCM that we obtained is identical to that of PP\textsubscript{a} AE and MePP\textsubscript{a} as found in the literature measured in DMSO and organic solvents (Savellano et al., 2005b). Extinction coefficients for the acids can be assumed to be the same as for NHS ester. This was found to be $4.75 \times 10^4$ (mol/L)$^{-1}$ cm$^{-1}$ in DMSO at the absorption maximum, 667 nm (Savellano et al., 2005b).

Disaggregating the photosensitiser in aqueous solutions could help understand how to handle it in an in vitro setting (see Chapter 4). Therefore, various solvents and conditions were used to first observe the disaggregation of the free photosensitisers and then subsequently attempt to disaggregate them, particularly so with PS1. These efforts included the use of polar organic solvent isopropanol and various detergents.

The red shifts observed for the Qy-band for all 3 PS's upon their dissolution in aqueous solutions, is indicative of aggregation often observed with these types of molecules (Lang et al., 2004, Kuimova et al., 2007, Tamiaki et al., 2008). Aggregation is usually defined by the characteristics of both the absorption and fluorescence emission spectra. Red shifts for the
former, combined with distorted shape and small Stokes shifts for the latter indicate J aggregate formation (Huber et al., 2005). Aggregation can be reversed by heating a sample or by adding a protic solvent to weaken the hydrogen bonds (Huber et al., 2005) such as isopropanol as shown in figure 3.34 (Kuimova et al., 2009). Detergents are also another good way to disaggregate samples in aqueous solutions. However detergents tend to form micelles and therefore there is a maximum critical micelle concentration (CMC) that one can work with. In a biological setting, detergents are not favourable as they are usually difficult to remove from the sample using dialysis. The use of detergent, in figures 3.27, 3.28, 3.29, 3.32 shows the disaggregation observed by the increase in absorption intensity accompanied by a blue shift and sharper spectra or a narrower and more intense fluorescence emission spectrum (Kuimova et al., 2009).

**Quaternisation and solubility-aggregation**

The work by Inoue is the closest to what we would ideally like in a PPa derivative. They synthesised (as mentioned in section 3.1) a non-aggregating water soluble derivative of PPa however, their derivative does not contain a handle for bioconjugation. During their studies, they also observed that the monocationic methyl quaternised PPa derivative precipitated out of an aqueous HEPES solution after prolonged standing at RT (Taima et al., 2009). We observed this for PS2 but not for PS4 when left standing at 4 °C in 2 % DMSO/PBS. More importantly, they observed aggregation for all of their di- to tetracationic derivatives. The only derivative found not to aggregate was the hexacationic which did not show any UV shifts, showed consistently high extinction coefficients in organic and aqueous solvents and similar circular dichroism (CD) spectra (Taima et al., 2009). In 2008 Lindsey had made a range of methyl cationic bacteriochlorins bearing up to four positive charges (Ruzié et al., 2008). They showed that most of their derivatives, that contained charges at diagonally opposite sites on the molecules, exhibited sharp UV spectra and small shifts hence indicating a low tendency for aggregation. Similar derivatives to PS4 were synthesised by trialkylating chlorin e6 pyridines that were monomeric in Tris-buffer (Taima et al., 2005).

**3.3.7 Future work**

Understanding the role of substituents at given positions on the macrocycle and their subsequent effect on the potency of the compound is vital to allow the design and synthesis of future PDT drugs (Muthiah et al., 2009).
Although the active ester of PS4 has not been made, proceeding from the acid to the active ester should be straightforward following the methods previously used for PPa and PS1.

An appealing and challenging idea would be to introduce a further halogen on the chlorin ring. For example, following the bromination on the 20-position, one could assume that allowing the bromination reaction to proceed further with higher excess of reagents would allow the introduction of another bromine atom on one of the remaining two meso positions. It is possible that under the right conditions, a further bromine could be inserted on 5, 10 or even 17 position. Two publications by Wang et al. have described bromine substitution reactions using N-bromosuccinimide as well as molecular bromine at low temperatures (Wang et al., 2004, Wang et al., 2008). It would require a balance of conditions, position reactivity and steric hinderance to allow this reaction to occur. Should it be successful, it could be further assumed that a Suzuki or Sonogashira coupling could introduce solubilising groups on two different areas on the molecule increasing steric bulkiness and solubility at the same time and block face to face interaction and the formation of aggregates. Using metallated (for example Zn) porphyrins to make any modifications on the meso positions has often been the norm. Osuka et al. reported a meso-iodination of Zn complexed porphyrins using silver hexafluorophosphate in the presence of base at room temperature for 15 mins (Nakano et al., 1998). Introducing an iodide atom on the chlorin periphery would be a useful tool however unlikely given the steric bulkiness of the alkyl chains next to the 20-position. It would then be interesting to incorporate a group containing ethylene oxy and tertiary amines combined branching off a central tertiary amine such as described by Koh et al. (Koh, 1996). This compound could be used to quaternise an iodide atom present on the chlorin macrocycle.

Possible sites for modification also include the C=O bond at the exocyclic ring. However, such modifications lead to an absorption maximum blue shift of about 15 nm, which is significant, and probably too much of a sacrifice (Pandey et al., 1996). Pandey et al. found that upon ring opening, the corresponding chlorin e6 analogues exhibit lower efficacy in vivo compared to the pyropheophorbide analogues. Another important note to consider is that secondary alcohol derived compounds are less effective than those prepared via the formyl route, primary alcohol precursor (Pandey et al., 1996).

The vinyl position can be converted to a formyl group (CHO) by using sodium periodate and osmium tetroxide. This can be then further oxidised to obtain the carboxylic acid using sodium chlorite and sulfamic acid. The double bond can also undergo oxidation to selectively form the diol (Gerlach et al., 1998). A range of reactions of the double bond (Gerlach et al.,
1998), the exocyclic ring and other modifications of chlorophylls are described in the review by Pavlov and Ponomarev (Pavlov et al., 2004).

The critical micelle concentration combined with direct light scattering studies for the main photosensitisers would aid the understanding of their aggregation in aqueous solutions. Direct light scattering might also provide answers regarding the aggregation states of the compounds and perhaps help us understand how it can be either avoided or reversed. It would also be prudent to further attempt to obtain Log $P$ values experimentally. Atomic force microscopy and circular dichroism can be used to study the aggregation of the dyes (Huber et al., 2005).

3.3.8 Concluding remarks

The aims of this part of the project included the synthesis of new PP$a$ derivatives which could be used for conjugation to antibody fragments. The characteristics that would be incorporated into these compounds included short PEG chains and positive charges to introduce water solubility. The use of PEG chains was considered a good option as they are neutral and will therefore not interfere with either the PS or the protein in bioconjugation reactions (see chapter 4). In order to accomplish these targets, the feasibility of the synthesis had to be explored, defined and refined. As discussed in section 3.3, several synthetic attempts failed (PS6 and introducing an acid group via the Suzuki coupling) leading to eventually PS1 and PS4.

Overall, two main photosensitisers based on PP$a$ were synthesised and their spectrophotometric and photophysical properties characterised. It was vital that the photophysical properties of the new derivatives would either be maintained or improved and this was successful. It was also crucial that the absorption maxima would not show blue shifts as the deeper red absorbing compounds are preferred in an in vivo setting for deeper light penetration for their activation.

The synthetic efforts presented here have provided an insight to increasing the solubility of PP$a$ in water and minimising its aggregation. It is generally not easy to afford aqueous solubility of large hydrophobic molecules and it is even more difficult to minimise their aggregation. The fact that PS1 and PS4 are readily soluble in only 2% DMSO/ PBS and do not precipitate is a major improvement. Others have spent years trying to achieve even the
latter (Sutton et al., 2002). PS1 and PS4 exhibit increased water solubility but at the same time persistent aggregation as shown by both absorption and fluorescence spectra in aqueous environments, PS4 to a similar extend to PPa with PS1 being slightly improved.

The synthesis of the aforementioned two PPa derivatives is rather laborious especially in the case of PS4. Scaling up to obtain gram amounts of each compound for either further study or commercial/clinical use will be costly. We know that it is possible to make PS1 in the hundreds of milligrams even on a laboratory scale. It would be more difficult to do this with PS4. It would also be particularly useful and time efficient if either or both derivatives could be purified on an HPLC on a larger scale to avoid those very time consuming purification steps. This is an obvious area to explore further.

PS1 has been synthesised and the route towards the AE was optimised and requires 6 overall synthetic steps. The synthesis is less laborious and less time consuming than PS4. Overall, PS1 is now an easier compound to produce than PS4 which has proven more challenging than expected. The use of both Suzuki and Sonogashira reactions for forming C-C bonds can become key and crucial reactions for use in the design and synthesis of future PPa derivatives. There are numerous C-C bond forming reactions and a vast variety of catalysts and building blocks commercially available therefore it is fascinating to consider the possibilities in synthesising new compounds. In retrospect, some of the failed reactions would probably be successful had they been attempted following our understanding and experience that was gained during this work.

PS1 is slightly more soluble than PS4 but the log $P$ values indicated that PS4 should interact less with hydrophobic biological membranes. Whether this has any validity, will be addressed in chapter 5. This can be determined practically, see chapter 5. For this project, complete inhibition of protein interaction and the photosensitiser is neither desirable nor probable. However, understanding the principles and the chemical groups involved in minimising protein adsorption onto surfaces would set the foundations in understanding what design features are essential and later how to purify the PICs.

The work described in this chapter was an attempt to understand the process and hurdles of producing soluble and low/non-aggregating PPa derivatives. The former was successful and
the two compounds are water soluble however the latter was not as straightforward to achieve as it was recognised throughout this work. The publication in 2009 by Inoue (see figure 3.1) who made a range of cationic PPa derivatives for use in DNA binding verify, that even though imparting solubility is relatively straightforward, the aggregation process is far more complicated to resolve.

The derivatives presented here contain elements of novelty (as this thesis is being written) such as the introduction of a positive charge on the 20-position of PPa and the use of the Sonogashira reaction to introduce a carboxylic acid for bioconjugation. Further to that, some of the unsuccessful attempts towards the synthesis of derivatives PS6 and PS5 and the knowledge gained trying to make them should form the basis of future targets. Even though these derivatives are based largely on published procedures by Wasielewski and Wurthner (Kelley et al., 2006b, Huber et al., 2007, Kelley et al., 2007b) their modification to allow bioconjugation and/or introduce solubilising groups, this work demonstrated a unique use of them and provided useful information regarding the structural requirements for disaggregating chlorophyll derivatives. Maintaining a single chemically independent moiety on the macrocycle for derivatisation into an activated ester such as the NHS would not be easily achieved with the nonetheless remarkable but symmetric hydrophilic molecules reported in the literature (Ruzié et al., 2008).

Basic photophysical characterisation of the derivatives was undertaken to verify or not that the synthetic modifications to the parent molecule did not destroy it. It was beyond the scope of this project to go into further in depth characterisation. If anything, the two main derivatives showed improved photophysical properties with both derivatives showing deeper into the red absorption maxima.

Aggregation studies showed that it is a necessary requirement for inhibiting face to face aggregation of the PPa core is to introduce solubilising, neutral or cationic groups at various positions around the molecule to inhibit electrostatic interactions. Cationic moieties that are introduced on meso-positions of porphyrins and chlorins are aimed at disaggregating the aromatic structures. This is based on the hypothesis/ evidence that these groups, especially in the case of pyridine will be perpendicular to the plane of the main structure hence inhibiting the intermolecular face to face interactions. The crystal structure obtained by
Neidle (Ford et al., 1987) confirms this as the four pyridyl groups at the meso-positions are indeed not coplanar to the rest of the molecule. More derivatives based on PS4 could provide improved derivatives with minimised aggregation.

As Pandey (Pandey et al., 1996) and others have shown, favourable photophysical characterisation cannot infer potency in vivo. Cellular localisation is an important parameter to consider when establishing structure/ activity relationships for compounds.

PPa is a natural product derivative with limited positions for introducing solubilising groups. Most research is focused in modifying the vinyl group, propionic side chain and more recently the 20-meso position and mostly in scope to understand and mimic the aggregation observed in nature in the chlorosome. Disaggregating PPa is after all an attempt to go against nature and the formation of beautifully stacked aggregates.
Chapter 4 Photoimmunoconjugate Synthesis
4.1 Introduction

4.1.1 Photoimmunoconjugates (PICs) and non-covalent binding (NCB)

Photoimmunotherapy has to deliver a sufficient amount of photosensitiser to the target cells or tissue. This may be a difficult-to-overcome obstacle, as it would require multiple loading of the targeting species. Conjugation of hydrophobic dyes to biomolecules has been hindered, firstly, because of their low solubility in aqueous solutions as described in chapter 1; secondly, the PSs’ tendency to bind non-covalently to proteins. As a result high concentration, multiply loaded PICs are difficult to obtain (Savellano et al., 2003, Bhatti et al., 2008).

A successful PIC must retain the binding capacity of the native antibody and the photophysical properties of the photosensitiser (Staneloudi et al., 2007). Various methodologies have been employed which include whole antibodies and fragments as described in chapter 1. In order to maintain the bioactivity, the reaction conditions need to be sufficiently mild and the resulting product pure enough to maintain its structural integrity and therefore function. Straightforward approaches where there is no need to modify the antibody to prepare it for conjugation, such as those used by Hasan and Boyle, should be preferred (Hudson et al., 2005, Savellano et al., 2005b).

Photosensitisers that are non-covalently bound to proteins in a PIC solution sample are an unwelcomed impurity. Such examples (described in chapter 1) indicated that most conjugation attempts where an antibody and a photosensitiser are involved will lead to some PS being non-covalently bound to the protein. The associated difficulties with purifying and characterising PICs have hindered their wider use and study (Savellano et al., 2003). What appears to differ among the various studies is the potential to purify these mixtures in order to obtain pure, characterisable PICs.

The non-covalent interactions between the proteins’ hydrophobic pockets and the hydrophobic PS are stable and cannot be easily broken nor can the PICs and free PS be resolved by size exclusion chromatography (Sutton et al., 2002). The non-covalently bound PS can dissociate and interact with serum proteins or cell membranes leading to non-specific effects (Sutton et al., 2002). When conjugating a neutral water soluble porphyrin to an scFv, non-covalently bound PS has been shown to affect the binding site of the antibody making the PICs ineffective (Staneloudi et al., 2007).

Savellano said that “impure PICs did not demonstrate that the PDT of the PICs was due to the activity of the conjugates and not due to the activity of non-covalently bound PS
impurities” (Savellano et al., 2003). Using existing protocols PICs that had large amounts of insoluble aggregates were being produced (Savellano et al., 2003). In order to make high quality PICs, PSs that do not interact non-covalently to proteins and have high water solubility are required. Whereas non-covalent binding does not necessarily mean that either the protein or the PS will be adversely affected it is the scope of developing these PICs for therapeutic purposes and commercial ‘drugs’ that creates the need to have pure well-characterised conjugates (Alonso et al., 2010).

Non-covalent interactions govern molecular assemblies and molecular recognition such as that of an antibody and its antigen. Even though they are considered to be weak bonds they are powerful tools responsible for many biological interactions. Hydrophobic and electronic interactions are both forms of non-covalent binding (Lang et al., 2004). Hydrophobic interactions occur in the presence of hydrogen bonds. Electronic interactions are more complex and involve electrostatic interactions (attractive or repulsive) and π interactions (Lang et al., 2004). Π-interactions include those with other aromatic systems (π-π), with a cation or with a C-H bond (Lang et al., 2004). These were described in chapter 3, figure 3.34.

When mixing capped (i.e. with no active site for conjugation) porphyrins with BSA and conducting “mock” conjugation reactions gave loading ratios of 2-1 and 0.6 molecules per BSA due to non-covalent binding. Similar results were obtained when mixing fluorescein with BSA. The same porphyrin-BSA mixtures (capped) caused HeLa cells to fluoresce. This was attributed to the non-covalently bound PS disassociating from the BSA and binding to the cell’s membranes (Sutton et al., 2002). This experiment nicely highlights the non-specific effects that can arise in vitro and perhaps in vivo when large amounts of non-covalently bound PS is present in a PIC.

**C6.5(-k)-PPa PIC and other studies**

The scFv, C6.5(-k) (section 1.4) has previously been conjugated to PPa NHS ester to obtain conjugates with an average loading ratio of 8.6 molecules of PPa per scFv. Two inhibiting factors restrict the use of this PIC in further trials. These are the aggregation of the photosensitiser leading to a large amount of free or non-covalent material associating with the protein and the low solubility of PPa in aqueous solutions which restricts the concentration of the PIC to up to 250 µg ml⁻¹ (Bhatti et al., 2008). Low PIC concentration is a limiting factor for clinical efficacy as injecting patients with high volumes of a drug is not a feasible option. A higher concentration PIC can contain more PS making the effective
injected dose higher assuming that the loading ratio at the higher concentration can be maintained. Non-covalently bound PPa remains as an impurity in the PIC sample, which is difficult to remove. PPa conjugates have yet to be purified as these non-covalent interactions between antibodies and the hydrophobic PPa are very strong (Savellano et al., 2005b).

Using a carrier molecule to attach several PSs which is subsequently linked to a biomolecule such as an antibody could be considered as a good idea. It would allow the characterisation and purification of the carrier/PS prior to its conjugation to the biomolecule. Such carrier molecules include dendrimers and fullerenes (Helmreich, 2005). Using a fullerene, Helmreich attached 10 PPa molecules onto a mAb using NHS chemistry purifying the product by size exclusion to obtain 60% coupled PIC 40% unreacted antibody (Helmreich, 2005). Forming various fullerene conjugates carrying up to 12 molecules of PPa they realised that the spatial proximity of the PS led to their aggregation and subsequent Forster energy transfer between them on the periphery of the fullerene. Consequentially, the photophysics of the macrocycle suffered substantially (Ermilov et al., 2004) with a five-fold decrease when compared to PPa (Helmreich, 2005, Helmreich et al., 2005). However, they did correlate and confirmed that in vitro potency tends to increase with increasing loading number of PSs (Rancan et al., 2005). The work conducted by this group verified two key points; that spatial separation is necessary to maintain the photophysical properties of a PS and specifically PPa, and secondly that loading more PSs is likely to infer increased potency—at least in vitro.

Pheophorbide a was conjugated to DAB dendrimers and effects similar to those reported by Helmreich were reported. The proximity of the molecules led to their facial stacking and subsequent decrease in both fluorescence and singlet oxygen quantum yields (Hackbarth et al., 2005). Similar observations were made when trying to conjugate porphyrins to an antibody. To resolve this, Alonso et al. synthetically modified their porphyrins to introduce linkers extending the distance between the macrocycle and the protein in order to keep the PSs spatially apart (Alonso et al., 2010).

It is believed that the spatial separation of the lysines on C6.5(-k) allows the covalently linked PSs to remain monomeric in solution. The 3D model shown in figure 4.1 indicates that the lysines on the scFv are well spaced apart. The covalently attached PSs remain monomeric as they cannot form oligomeric aggregates (Bhatti et al., 2008). Further work using various PSs can help clarify the validity of this argument. The promising results obtained with PPa in vitro and in vivo and the significant difference between free PPa potency and PPa PIC potency in vivo suggests that a process other than the specificity and uptake governs the efficiency of the PS. It could be hypothesised that the covalently attached PS is
disaggregated in vivo more so than the free PS favouring the photophysics and therefore increasing the efficacy.

![Diagram](image.png)

**Figure 4.1 Three-dimensional representation of the structure of C6.5(-k)-PPa PIC** pointing out the spatial separation of the lysines. The sequence of the scFv was used to obtain the model on Swiss PDB. The lysines are shown in red and the PS depicted in green.

The solubility of the resulting PIC has sometimes been compromised upon loading a macromolecule with several hydrophobic photosensitisers (Savellano et al., 2003). High loading ratios can often affect the binding of an antibody to its target antigen as observed by some groups when achieving loading ratios above 5:1 (Staneloudi et al., 2007). It was also found that loading 8-10 molecules of a fluorophore on a protein can cause fluorescence quenching and an increase in NCB (Bright, 1988).

Trying to resolve the non-covalent binding by purification methods is not always straightforward and depends on the protein and the photosensitiser being used. A chimeric anti-EGFR BPD conjugate was prepared by first PEGylating the antibody followed by an NHS ester conjugation. PICs were obtained by adding 28 equivalents of the ester to a 1.25 mg ml$^{-1}$ antibody solution. This was successfully purified using a size exclusion spin column (G-50) in a 50 % DMSO/ H$_2$O solution obtaining loading ratios of 2-11 (Savellano et al., 2003). A reaction system containing such large amounts of DMSO would not be well tolerated by many biomolecules especially scFvs. This solution system was chosen because a more aqueous reaction mixture gave lower yields and samples that could not be purified (Savellano et al., 2003, Bhatti et al., 2008). It was realised that it was important to purify conjugates in order to differentiate the specificity driven cytotoxicity in vitro. In order to purify
the PIC, the aggregation of the PS had to be minimised or reversed. At 40 % DMSO/H₂O BPD has an isosbestic point where it disaggregates making 50 % DMSO/H₂O a suitable solution for purification (Savellano et al., 2003). The use of detergent to disaggregate PS1 in the aggregation studies in chapter 3 was based on this approach. Understanding the disaggregation of a PS can potentially help modulate reaction conditions.

Overall, purification methods used for purifying photosensitiser-protein conjugates include electroelution (Sutton et al., 2002), PD10 columns (Sutton et al., 2002, Alonso et al., 2010), G25 columns (Hudson et al., 2005) and Sepharose 4B (Kirpotin et al., 1997) to list a few. Using a Sepharose 4B column and HEPES/HBS buffered solution it was possible to separate micelles from liposomes (Nielsen et al., 2002) indicating that there are several options available when attempting to purify PSs from proteins that are likely to form micelles or large aggregates.

4.1.2 Photoimmunconjugate synthesis

There are two approaches to synthesising PICs. These are direct covalent conjugation to the antibody’s amino acids and the use of a carrier molecule or linker (Hamblin, 2008). There are numerous activating groups, buffers, pH and temperature conditions that can be used in combination. Choosing the most appropriate will depend primarily on the stability of the biomolecule and the solubility of the PS. Some key examples are shown in table 4.1

Conjugates of BSA were prepared at pH 9.2 using either the NCS or NHS activated ester of the PS reacting for 16 hrs at room temperature (Sutton et al., 2002). However, not many antibodies will be stable at those conditions. Boyle synthesised porphyrins containing an isothiocyanate moiety as a single site for bioconjugation (Staneloudi et al., 2007). This reacts with primary amines to form thiourea bonds (see table 4.1). Kirpotin conjugated an anti-HER2 Fab’ to PS containing liposomes using a maleimide linker in HEPES at pH 7.4, at room temperature and under an inert atmosphere overnight binding to cysteines (Kirpotin et al., 1997).
Table 4.1 Summary of some of the key coupling methods used in the area of PDT and ADC. * this product forms when the maleimido group reacts with a primary amine when there is no competition from a thiol residue. C-S is reported to be the main product.

<table>
<thead>
<tr>
<th>Targeting Moiety</th>
<th>Linking group</th>
<th>PS (activated group)</th>
<th>Type of bond formed</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide (NH₂)</td>
<td>HATU</td>
<td>PPIX (COOH)</td>
<td>amide</td>
<td>(Walker et al., 2004)</td>
</tr>
<tr>
<td>Fab₂ (SH)</td>
<td>pyridydithiopropionic acid-NHS ester</td>
<td>Ce₆ (COOH)</td>
<td>disulfide and amide</td>
<td>(Hamblin et al., 1996)</td>
</tr>
<tr>
<td>mAb (NH₂)</td>
<td>NHS ester</td>
<td>BPD, PPa, AlPcs₄A₁ (COOH)</td>
<td>amide</td>
<td>(Savellano et al., 2003) (Savellano et al., 2005b) (Carcenac et al., 2001)</td>
</tr>
<tr>
<td>scFv (NH₂)</td>
<td>NCS</td>
<td>Porphyrin (NH₂)</td>
<td>thiourea</td>
<td>(Clarke et al., 1999, Staneloudi et al., 2007)</td>
</tr>
<tr>
<td>SIP (SH)</td>
<td>maleimide</td>
<td>porphyrin cationic (NH₂)</td>
<td>amide</td>
<td>(Alonso et al., 2010)</td>
</tr>
<tr>
<td>mAb (NH₂)</td>
<td>TFP</td>
<td>mTHPC (COOH)</td>
<td>amide</td>
<td>(Vrouenraets et al., 1999)</td>
</tr>
<tr>
<td>scFv (NH₂)</td>
<td>Sulfo NHS</td>
<td>PPa (COOH)</td>
<td>amide</td>
<td>(Bhatti et al., 2008)</td>
</tr>
<tr>
<td>mAb</td>
<td>Maleimide and NHS ester</td>
<td>nanoparticle</td>
<td>Thioether and amide</td>
<td>(Wartlick et al., 2004)</td>
</tr>
</tbody>
</table>
In situ preparation of the AE via EDC/ NHS at pH 9, for 18 hrs at 4 °C, with 40 equivalents of PS, followed by size exclusion chromatography on a G25 Sephadex column gave conjugates of a mAb-tetrasulfophthalocyanine with high loading ratios of 6-9 PS (Carcenac et al., 2001). Importantly, the migration of the PIC on SDS-PAGE gels did not differ to that of the parent antibody. The loading ratio was obtained using a spectrophotometric approach based on the extinction coefficient of the antibody and of the PS at 280 nm and the absorption maximum of the PS in the red.

Some of the most extensive bioconjugation attempts by Vrouenraets included the conjugation of mAbs with the tetrafluorophenyl esters (TFP) of porphyrins and phthalocyanines. These retained their immunoreactivity (Vrouenraets et al., 1999, Vrouenraets et al., 2000, Vrouenraets et al., 2001, Vrouenraets et al., 2002). Thiols can also react with iodoacetamides at basic pH. A 24 amino acid long peptide was reacted with a tricationic porphyrin in this manner and subsequently purified via HPLC on a C8 column to obtain a pure conjugate (Chaloin et al., 2001). Using HPLC to purify conjugates is not always possible when sensitive biomolecules are involved and most likely used for the analysis of PICs and not their purification.

Boyle conjugated water soluble porphyrins to an scFv at 0.5 mg ml⁻¹ at pH 9.2 using a molar excess of 2.5-60 equivalents for 1 hr at room temperature using the NCS route (Hudson et al., 2005, Staneloudi et al., 2007). Using site specific conjugation, maleimide activated PSs were used to specifically load 2 PSs per SIP (Alonso et al., 2010). The PIC retained the antibody's immunoreactivity (Neri, 2010).

An anti-HER2 scFv was engineered to include a fusion peptide in order to introduce threonine residues for multiple site-specific conjugations. The peptide could subsequently be glycosylated and conjugated to Alexa 488 retaining its binding properties (Ramakrishnan et al., 2009). A staggering 1000-fold excess of dye to scFv was used in the conjugation reaction overnight at room temperature (Ramakrishnan et al., 2009). The bioactivity of the mAb was retained. The PIC was obtained by precipitation using ammonium sulphate. Approaches such as these are unlikely to work for very hydrophobic PSs as they would lead to a large amount of non-covalent binding.

Engineering scFvs to allow efficient bioconjugation can involve inserting a C-terminal cysteine to specifically use in thiol coupling or a reducing agent to release the disulfide bonds (Nielsen et al., 2002, Alonso et al., 2010). Nielsen et al. used thiol coupling to conjugate to an anti-HER2 scFv by firstly reducing the disulfide bonds and subsequently
reacting a 4-fold excess of the scFv with a maleimide activated lipid to form the conjugate (Nielsen et al., 2002). A very interesting approach is the formation of an amide bond using an N-terminal cysteine (Dawson et al., 1994, Busch et al., 2008). The cysteine reacts with the thioester moiety via a transthioesterification reaction to form an amide bond (Busch et al., 2008). However use of this in the PDT setting is unlikely as it restricts binding to one molecule with the additional requirement to pre-engineer the terminal cysteine.

4.1.3 Aims and Objectives

The aims of this part of the project were to produce photoimmunoconjugates (PICs) using a model anti-HER2 scFv and pyropheophorbide a (PPa) derivatives with PPa as a control. These should exhibit increased aqueous solubility and loading ratios and be well characterised.

The conjugation method would be modified to optimise for high loading ratio and low non-covalent binding and the maximum concentration of the PICs would be investigated.

The objectives were to

- Express and purify active C6.5(-k) in large enough quantities to use for conjugations (gels, westerns, FACS)
- Set a reference point by making the PPa PIC
- Make the PICs and characterise them by UV/Vis spectroscopy and SDS Page gels
- Use Mass Spectrometric characterisation to determine accurate loading ratios and distribution.
4.2 Results

4.2.1 Antibody production

The expression and purification of C6.5(-k) was previously established by Dr Mitla Garcia-Maya and this work was based on the developed protocols and methods as described in chapter 2 (Bhatti et al., 2008). Native C6.5 as obtained by Schier et al. was subsequently mutated to C6.5(-k) (Schier et al., 1995, Adams et al., 1998, Adams et al., 2000). The C6.5 gene was kindly donated to us by Dr J Marks in pUC119mychis. The lysine-100 was mutated to an alanine by Dr Mitla Garcia-Maya and C6.5(-k) was provided for this work in the pUC119 plasmid with C-terminal his$_6$ and c-myc tags and the lac promoter for expression and selection.

The culture supernatant was purified by ion metal affinity chromatography (IMAC) using cobalt based Talon® resin. Figure 4.4 shows the amino acid sequence of C6.5(-k) highlighting the tags and more importantly the lysines. The scFv was further purified by antigen affinity chromatography on a 9E10 column which binds the c-myc tag of the scFv eluting with 0.2 M glycine pH 2.5.

The samples were analysed by reducing SDS-PAGE and these purification steps are shown in figures 4.2 and 4.3. The scFv should run as one entity around the 30 kDa molecular weight marker. The concentration of the scFv prior to conjugations was calculated using its absorption at 280 nm and the extinction coefficient as stated in table 4.2 alongside key characteristics of C6.5(-k).

Looking at both figures 4.2 and 4.3, the main band corresponding to the scFv is around 29 kDa as expected (see table 4.2 for more accurate MW). A band of slightly lower MW than expected can be seen on the Coomassie stained gels (fig. 4.2A and 4.3A). This is not detected on the anti-HIS Western blot (fig. 4.2B and 4.3B) but appears to be present on the anti-myc Western blot (fig. 4.3C). It is possible that this band corresponds to the scFv with the his$_6$ tag cleaved but with the c-myc tag still intact. There is a second prominent band at ~15 kDa in both figures corresponding to the tagged scFv light chain. The heavy chain would have the same MW as the light chain but would not be visible on a Western blot as it is not tagged.
Figure 4.2 SDS-PAGE-Purification of C6.5(-k) scFv by IMAC chromatography using Talon®. Gel A shows a Coomassie blue stained 12 % SDS-PAGE reducing gel and B shows an anti-his<sub>6</sub> western blot on a nitrocellulose membrane. Samples in both: M (pre-stained protein ladder), 1 (flow through), 2 (PBS wash 1), 3 (PBS wash 2), 4-9 (elutions using 100 mM imidazole). Blue arrows show full length scFv; green arrow shows the variable light chain of the scFv which remained tagged following the decomposition of the scFv. Red arrow shows untagged full length scFv.

Figure 4.3 SDS-PAGE-Purification of C6.5(-k) scFv using a 9E10 affinity column. Gel A shows a Coomassie blue stained 12 % SDS-PAGE reducing Gel, B shows an anti-his<sub>6</sub> and C. an anti-c-myc Western blot on nitrocellulose membranes. Samples in A and B: M (pre-stained protein ladder), 1 (C6.5(-k) +ve control), 2 (flow through), 3-7 (PBS washes), 8-10 (elutions with 0.2 M glycine pH 5.0), 11-12 (elutions with 0.2 M glycine pH 3.5) and 13-14 (elutions with 0.2 M glycine pH 2.5). Gel C samples: M (pre-stained protein ladder), 1 (flow through), 2 (PBS wash), 3 (elution with 0.2 M glycine pH 5.0), 4-5 (elutions with 0.2 M glycine pH 3.5) and 6-14 (elutions with 0.2 M glycine pH 2.5). Blue arrows show full length scFv; green arrows show the variable light chain of the scFv which remained tagged following decomposition of the scFv and red arrow shows the full length scFv which has possibly lost its his6 tag as this band is not detected on the anti-his<sub>6</sub> western blot (B.).
Figure 4.4 Shows the amino acid sequence of C6.5(-k). The lysines (13 total) are highlighted. Arrow points out the lysine present in the c-myc tag.

<table>
<thead>
<tr>
<th>C6.5(-k) characterisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (Da)</td>
</tr>
<tr>
<td>Extinction coefficient (M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>pI</td>
</tr>
<tr>
<td>Lysines</td>
</tr>
<tr>
<td>Cysteines</td>
</tr>
<tr>
<td>Histidines</td>
</tr>
<tr>
<td>Tyrosines</td>
</tr>
<tr>
<td>Serines</td>
</tr>
</tbody>
</table>

Table 4.2 Lists some of the physical characteristics of the C6.5(-k) scFv as obtained by ProtParam tool on the ExPASy Proteomics Server including the number of selected amino acids present in the antibody’s sequence.
4.2.2 Preparing C6.5(-k)-PPa PICs

Note: (x %) in some of the captions of PIC gels/blots corresponds to the percentage of conjugate compared to free/non-covalently bound PS for that particular sample. Also, some of the conjugates used in the various purification attempts were from lower yield reactions (low loading ratios) in order not to waste unwanted material. From section 4.1.3 onwards, PIC refers to C6.5(-k)-PS1 PIC unless specified otherwise.

C6.5(-k) was conjugated to PPa AE as described in chapter 2 with a slight modification of the published procedures (Kuimova et al., 2007, Bhatti et al., 2008). When conjugating PPa to the scFv, it was important to allow the PS to warm up to room temperature before diluting with the DMSO. This was then slowly added to the stirring scFv in PBS/MeCN. The conjugation reaction was carried out maintaining the sample protected from light throughout (using foil and subdued room lighting) and kept at 4 °C as much as possible to avoid protein degradation. Vigorous stirring and resuspending of the mixture as well as slow dropwise addition of the PPa AE solution to the protein were found to be crucial ensuring the solubility of PPa. It was also crucial to resuspend the mixture frequently as it was visibly precipitating out of solution and forming particulate aggregates. Running the reaction at a higher temperature to increase its solubility would not agree with the stability of the scFv.

Three main samples were obtained in every conjugation reaction which included the crude product obtained following the reaction completion prior to any processing, the pellet obtained after dialysis and centrifugation and the final PIC which was the supernatant separated from the pellet. Following dialysis in PBS, the green-coloured sample was gently resuspended before removing from the dialysis tubing. It was briefly centrifuged to obtain the final PIC as the supernatant and a dark coloured pellet. The pellet obtained did not readily dissolve in buffer and large aggregates were visible. The PIC solution required resuspension following storage at 4 °C.

The samples were analysed using UV/Vis spectroscopy and SDS-PAGE as shown in figures 4.5 and 4.6. On the nitrocellulose blot, the PIC samples run as two bands; one at the same molecular weight as C6.5(-k) around 30 kDa corresponding to the PIC and another at less than 10 kDa corresponding to the free or non-covalently bound PPa. Free PPa (mock sample) run at less than 10 kDa. In lane 1, the purity of the SN sample (after centrifugation) appeared improved compared to lane 2 (before centrifugation) with a higher % of covalently attached PS. Figure 4.6 shows the absorption spectrum of the final SN. It has the characteristic shape of the absorption spectrum of PPa (as shown in chapter 3) with a strong absorption at 280 nm corresponding to the scFv.
Figure 4.5 SDS-PAGE-Making C6.5(-k)-PPa PIC at 300 µg ml⁻¹ of scFv, 16 equivalents of PPa, pH 7.34. Fluorescence nitrocellulose blot showing the main samples following a conjugation reaction. M (pre-stained protein ladder), 1 (final conjugate, supernatant following dialysis and centrifugation) (60 %), 2 (conjugate prior to dialysis and centrifugation) (44 %), 3 (pellet obtained following centrifugation), 4 (mock sample of PPa only). Red arrow shows the PIC band which runs as the free scFv and blue arrow shows the fluorescence signal due to the free photosensitiser.

Figure 4.6 UV/Vis spectrum of C6.5 (-k)-PPa PIC, 300 µg ml⁻¹ of scFv, 16 equivalents of PPa. The sample was scanned in PBS following dialysis and centrifugation. The absorbances at 280 nm (blue arrow) and 678 nm (red arrow) were used in conjunction with the blot to determine the loading ratio of the antibody with PPa. Using both blot (figure 4.5) and the spectrum the loading ratio was calculated to be 3.4 mols PPa per scFv.
Characterisation of PICs- Loading ratios

Finally, following dialysis and centrifugation, the loading ratios were calculated as follows: from the fluorescence blot, the fluorescence intensity values for the PIC and free/ non-covalently bound PP\textsubscript{a} bands were obtained for the supernatant sample (following dialysis and centrifugation) (SN) using AIDA densitometry. Their sum corresponded to 100% fluorescence intensity of the sample and a % for the PIC band was obtained. Using the intensity of the absorption spectrum of the PIC (as shown in figure 4.6) at 678 nm, the concentration of PP\textsubscript{a} in the sample was obtained using its extinction coefficient in 2 \% DMSO/ PBS (see chapter 3) and applying the Beer-Lambert Law. Using this concentration for PP\textsubscript{a}, the absorption contribution of PP\textsubscript{a} at 280 nm was calculated and subtracted from the absorption intensity at 280 nm of the PIC (using the extinction coefficient of PP\textsubscript{a} at 280 nm). The protein concentration was then calculated for the PIC given the corrected absorption intensity using the scFv’s extinction coefficient at 280 nm. A ratio was obtained by dividing the concentration of PP\textsubscript{a} by the concentration of C6.5(-k) and subsequently corrected for the non-covalent material by multiplying with the fraction of the PIC band (here 0.6) as calculated using densitometry and was determined to be 3.4:1 molecules of PP\textsubscript{a} per scFv as shown in figure 4.5. Dialysis followed by centrifugation increased the % of covalently attached PP\textsubscript{a} from 44 % to 60 % as some of the free precipitated out partially purifying the PIC.
4.2.3 Preparing C6.5(-k)-PS1 PICs

C6.5(-k) was conjugated to PS1 AE as described in chapter 2 by slightly modifying the method for the PPa conjugation (Kuimova et al., 2007, Bhatti et al., 2008). Most importantly, under the standard PS1 coupling conditions, the antibody was used at 3.3 x higher concentration than for PPa and both dry co-solvents were pre-combined with the PS1 AE. The conjugation reaction was carried out maintaining the sample protected from light throughout and kept at 4 °C as much as possible to avoid protein degradation. Vigorous stirring and resuspending the mixture and slow dropwise addition of the PS1 AE solution to the protein were retained as the standard conditions. A clear dark green solution (SN) and a dark green pellet (pellet) that was mostly soluble in aqueous solution were obtained. The PIC solution remained clear upon storage at 4 °C. The enhanced water solubility of PS1 allowing it to remain in solution throughout the reaction as well as the higher concentration were an immediate improvement on the parent scaffold.

The samples were obtained and analysed as for the C6.5(-k)-PPa PIC as shown in figures 4.7, 4.8, 4.9. Pointing out that due to the higher concentration of the PICs, the samples used for the absorption spectra had to be tenfold diluted in order to allow their characterisation within the limits of detection. The extinction coefficients for PS1 at 681 and 280 nm were described in chapter 3.

Furthermore, a control experiment, as described by Bhatti et al. (Bhatti et al., 2008) for PPa was conducted for PS1 alongside a standard conjugation reaction. PS1 in its acid form (unreactive towards lysines at neutral pH) underwent the same treatment as PS1 AE in a conjugation reaction. This is shown in lanes 1-3 in figure 4.7. The UV/Vis spectra for the two reactions would not be expected to differ and this was confirmed as shown in figure 4.8 where the two samples after dialysis are nearly identical. From the gels, the protein band of the mock samples shows tight bands at the region of 29 kDa as for C6.5(-k) (lane 7). Contrary, the lanes corresponding to the PIC preparation contain bands that are polydispersed indicating the presence of several species of various MW. The UV/Vis spectrum of the SN (figure 4.9) shows a much sharper spectrum with distinct Soret and Q bands compared to PPa indicative of its higher solubility. The loading ratio for the mock coupling was in the region of 0.1 mols of PS1 per scFv whereas the loading ratio for the C6.5(-k)-PS1 was determined to be 5.4 mols of PS1 (40 % coupled) per scFv. There was an increase in non-covalently bound PS1 after dialysis and centrifugation from 48 % coupled (before) to 40 % coupled (after). C6.5(-k) and PS1 mock samples were run as controls most of the time.
Figure 4.7 SDS-PAGE-Synthesis of C6.5(-k)-PS1 PIC, 1 mg ml$^{-1}$ of scFv, 16 equivalents of PS1. The figure shows left, a Coomassie blue-stained 12 % SDS-PAGE gel and right, a fluorescence nitrocellulose blot showing the main samples obtained during conjugation. A control experiment was run in parallel where PS1 in its acid form underwent the same procedure as for the C6.5(-k) – PS1 AE. 2 µg of protein was loaded in each well. M (pre-stained protein ladder), 1 (C6.5(-k)-PS1 ACID sample before dialysis), 2 (C6.5(-k)-PS1 ACID sample after dialysis and centrifugation) (2 %), 3 (C6.5(-k)-PS1 ACID pellet after dialysis and centrifugation), 4 (C6.5(-k)-PS1 PIC before dialysis)(48%), 5 (C6.5(-k)-PS1 final PIC after dialysis and centrifugation) (40 %), 6 (C6.5(-k)-PS1 pellet after dialysis and centrifugation), 7 (C6.5(-k)), 8 (PS1). Red arrow shows the PIC band which runs as the free scFv and blue arrow shows the fluorescence signal due to the free photosensitiser.

![SDS-PAGE Image]

Figure 4.8 UV/Vis absorption spectra of mock conjugation samples showing the absorption profiles of samples as shown in the gels in figure 4.7. These are the final, C6.5(-k)-PS1 PIC and the control conjugation using PS1 acid before and after dialysis. C6.5(-k)-PS1 PIC before dialysis is not shown as it was the same as for C6- PS1 acid (mock) before dialysis (*).
Figure 4.9 UV/Vis absorption spectrum of C6.5(-k)-PS1 PIC as used for in vitro and in vivo experiments. The sample was diluted tenfold in order for the absorbance values to be within the linear boundaries. The blue arrow shows the absorption at 280 nm mainly corresponding to the protein absorption and the red arrow shows the absorption at 681 nm which corresponds to the photosensitiser. Using these values in conjunction with the fluorescence blot the loading ratio of the scFv with PS1 can be determined.

Native PAGE gels were also run as shown in figure 4.10 in an attempt to investigate whether the PIC is a single entity when folded in its native state. Gels were prepared and run without SDS for 20 mins at 30 mA. Two sets of samples were loaded; one where samples were not reduced (omitting the β-mercaptoethanol from the loading buffer and did not boil) and one where they were treated as normal. They were run from the anode to the cathode and the transfer onto nitrocellulose was carried out as usual (with SDS). From the gels on figure 4.10 the free PS1 (lane 3) appears polydispersed. Native SN (lane 2) appears more polydispersed than the free PS1. PS1 moved quite fast indicating that the lower band of the SN sample corresponded to the free/non-covalently bound PS1. The PIC, detected on both the fluorescence blot and Coomassie stained gel was moving slightly faster than C6.5(-k) towards the cathode (+) indicating a slightly more negative species. Nonetheless there is a difference between free PS1 and SN and, unconjugated C6.5(-k) and SN. Note that the Native gels were run only once. Both sets of gel data suggest that the conjugation process was working and that the protein and PS in the PICs were migrating with different properties.
Figure 4.10 Native PAGE gels of C6.5(-k)-PS1 PIC, 1 mg ml$^{-1}$ of scFv, 16 equivalents of PS1. The figure shows left, a fluorescence nitrocellulose blot and right a Coomassie blue-stained 12 % PAGE non-reducing gel. Both PAGE gels were run without SDS. SDS was used in the transfer buffer. Non-reduced samples were diluted with loading buffer without SDS and without β-mercaptoethanol and were loaded without boiling. M (pre-stained protein ladder), 1 (C6.5(-k) not reduced), 2 (C6.5(-k)-PS1 SN after dialysis and centrifugation not reduced), 3 (PS1 mock sample not reduced), 4 (C6.5(-k) normal loading buffer and boiled), 5 (C6.5(-k)-PS1 SN after dialysis and centrifugation normal loading buffer and boiled). Run from the anode(-) to the cathode (+).

4.2.4 C6.5(-k)-PS1 conjugation at 4 mg ml$^{-1}$

It was important to determine the limit of the concentration of the reaction in terms of scFv and PS1 solubility and reaction efficiency. At 4 mg ml$^{-1}$, the fourfold increase of scFv concentration led to the fourfold increase of PS1 AE concentration. Subsequently, there was a visible difference in the behaviour of PS1 under these conditions.

C6.5(-k) was conjugated to PS1 at 4 mg ml$^{-1}$ and the samples were analysed on SDS-PAGE as shown in Figure 4.11. Obtaining a higher concentration stock of the PS1 AE in DMSO was not an issue as it was readily soluble. The reaction was run at room temperature in an Eppendorf tube to allow vortexing of the mixture and shaking. The PS1 AE was added to the scFv slowly over 1 hr 30 mins, vortexing and shaking in between additions to ensure that everything was in solution. It was allowed to react for a further 2 hours at which point it was noticed that the photosensitiser was precipitating. The sample was centrifuged as usual to obtain a larger than normally pellet and a clear green supernatant. The supernatant was then dialysed as usual. The pellet could not be redissolved in PBS and was neither very soluble in chloroform. It is noteworthy that a TLC on silica gel (not shown) of the pellet in chloroform against PS1 AE and PS1 acid showed that the pellet was made up of primarily the AE. The samples were analysed by SDS-PAGE (figure 4.11) and UV/Vis (not shown). The bands corresponding to the PIC SN (lane 5) are less polydispersed than a lower concentration coupling such as that shown in figure 4.7 indicating that this higher concentration reaction is less efficient. The loading ratio was calculated to be 1.4 mols of PS1 per scFv with only 35 % coupled vs free (lane 5). The pellet band (lane 4) was less
intense than a standard conjugation due to its limited solubility, therefore less was loaded onto the gel. The scFv was used as a control (lanes 1, 6).

![Figure 4.11 SDS-PAGE-Synthesis of C6.5(-k)-PS1 PIC, 4 mg ml⁻¹ of scFv, 16 equivalents. The figure shows left, a Coomassie blue-stained 12 % SDS-PAGE gel and right, a fluorescence nitrocellulose blot showing the main samples obtained during conjugation. 2 µg of protein was loaded. M (pre-stained protein ladder), 1 (C6.5(-k)), 2 (C6.5(-k)-PS1 PIC before dialysis), 3 (C6.5(-k)-PS1 pellet after centrifugation), 4 (C6.5(-k)-PS1 pellet after dialysis and second centrifugation), 5 (C6.5(-k)-PS1 final PIC after dialysis and centrifugation), (35 %), 6 (C6.5(-k)). Red arrow shows the PIC band which runs as the free scFv and blue arrow shows the fluorescence signal due to the free photosensitisers.]

4.2.5 Purification attempts

Although higher concentration PICs could be obtained with PS1, the amount of non-covalently bound material was higher than the corresponding PPa PIC. As PS1 is readily water soluble it does not precipitate enough out of solution following centrifugation to partially improve the purity of the PIC. The precipitation of PPa following centrifugation leads to PICs with comparatively less non-covalent binding. A selection of purification attempts is described in this section.

Using dialysis as a method to purify the PICs

Attempts to remove the unbound/ free photosensitiser (PPa or PS1) from the PIC mixture by dialysis were unsuccessful as was observed consistently with all the samples that underwent the established conjugation procedure. The dialysis of mock solutions of free photosensitisers dissolved in the two co-solvents and PBS and their subsequent analysis by UV/Vis spectroscopy as shown in figure 4.12 confirms this. From the spectra, the absorption intensity for both sets of PSs does not significantly change following dialysis indicating that
the concentration remains approximately the same. Further to that, there is a slight red shift for PS1 after dialysis indicating some aggregation.

Figure 4.12 UV/Vis spectra of a dialysis of free PPα and PS1. PPα (0.126 mM) and PS1 (0.107 mM) were dissolved in 2% DMSO, 6% MeCN/PBS and dialysed in PBS pH 7.34. Curves show the samples before and after dialysis for each compound.

Attempts to improve PIC purity using dialysis under various conditions which included detergents and dialysis membranes of various molecular weight cut offs (8, 16 and 20 kDa) are shown in figure 4.13. Octyl β-D-glucopyranoside (OG) is a non-ionic dialyzable detergent with a critical micelle concentration of 23-25 mM used for the solubilisation of proteins used here in an attempt to disaggregate the PS in the solution (see section 3.2.8). Monomerising the PS could force it to disassociate from the protein allowing it to diffuse through the dialysis membrane. However even though the detergent disaggregates the PS as shown previously (chapter 3) it did not aid its dialysis here. From figure 4.13, it can be observed that there was no marked difference in the amount of PS1 present in the mock samples (lanes 1-3) following dialysis.

Heating is known to facilitate disaggregation (Kuimova et al., 2009) following the same rationale as for the use of detergent. In lane 4, a PIC sample (before dialysis) was heated and subsequently dialysed (lane 5). There was no improvement in non-covalent binding as shown by the blot and no degradation of the protein as shown on the Coomassie stained gel. Noting from figure 4.13 that the his₆ tag of the PIC cannot be detected on the Western
blot. In the Coomassie, the PIC bands appear to be slightly higher than the control scFv indicating a higher MW.

Figure 4.13 SDS-PAGE-Purification attempts of PS1 and C6.5(-k)-PS1 PIC using dialysis. The figure shows top, a fluorescence nitrocellulose blot, centre, an anti-his western blot on a nitrocellulose membrane and bottom, a Coomassie blue-stained 12 % SDS-PAGE reducing gel. M (pre-stained protein ladder), 1 (PS1 mock solution before dialysis), 2 (PS1 mock solution after dialysis in 20 mM OG/ PBS using 14 kDa cut off Slide-A-Lyzer); 3 (PS1 mock solution after dialysis in 20 mM OG/ PBS using 8 kDa cut off dialysis membrane); 4 (C6.5(-k)-PS1 PIC before purification); 5 (C6.5(-k)-PS1 PIC following heating at 50 °C for 10 mins followed by dialysis in PBS using a 14 kDa cut off dialysis membrane); 6 (C6.5(-k)).
Size exclusion chromatography

Size exclusion purification of the conjugate supernatant was attempted by loading onto a pre-equilibrated with PBS PD10 column and eluting with PBS pH 7.34 collecting fractions and analysing by UV/Vis absorption spectroscopy (not shown). The fractions were concentrated to dryness, dissolved in water and analysed by SDS-PAGE as shown in figure 4.14. Fraction 8 contains free PS as indicated by the presence of only a low MW fluorescent band and absence of a higher MW band in the Coomassie whereas fractions 9-14 contain a mixture of free/NCB PS and PIC (A and B). Unconjugated protein with free/non-covalently bound PS appears to be present in fractions 15-16 (gel D). The main fractions contain 50 % coupled/free PIC/PS. The heavily aggregated PS probably elutes first as the non-covalent amount is more for fraction 10 than 12. Therefore, using these conditions it was not possible to separate the PIC from free/non-covalently bound PS. An improvement of only 5 % was seen, with the starting PIC (not shown) being 40 % coupled.

![Figure 4.14 SDS-PAGE-Purification attempt of C6.5-(k)-PS1 PIC, 1 mg ml⁻¹ of scFv using a PD10 column.](image)

The figure shows 12 % SDS-PAGE reducing gels: A and C, fluorescence nitrocellulose blots and B and D, Coomassie blue-stained gels (visualised using white light on FujiFilm Las3000). The fractions were lyophilised and redissolved in water to ensure they were concentrated enough to visualise. M (pre-stained protein ladder), 1 (flow through), 2-18 (column fractions eluted with PBS) (Fr10: 30%, Fr11 and Fr12: 50 % and Fr13: 42 %).
Subsequently, a further attempt to purify a PIC on a PD10 column using detergents (OG and POTE as described in chapter 3 in the aggregation studies) did not improve the purity (not shown).

A PIC SN was also purified on a PD MiniTrap™ G25 column (GE healthcare) eluting with PBS pH 7.34 collecting fractions which were first analysed by UV/Vis and then by SDS-PAGE as shown in figure 4.15.

The first fraction contains mostly free PS (lane 1) although some conjugate is present as verified by the Coomassie stained gel. Having started with a PIC of 35 % and loading ratio of 1.85 molecules of PS1 per scFv (not shown), there was some improvement as seen in the second fraction at 53 %. The loading ratio for fraction 1 was 0.2 molecules of PS1 per scFv and for fraction 2, 3.3 molecules of PS1 per scFv and negligible for fraction 3. It is possible that the first fraction consists of mainly heavily aggregated free PS1 whereas the second fraction might contain non-covalently bound and oligomeric PS1 as would be suggested by the size separation. Therefore, fraction 2, showed a 33 % improvement.

![Figure 4.15 SDS-PAGE-Size exclusion purification with G-25 of C6.5(-k)-PS1 PIC](image)

BioBeads (BioRad), another type of size exclusion chromatography were also used, again in the presence of OG detergent/ 2 % DMSO. The column was pre-equilibrated with eluent and the sample eluted collecting fractions as shown in figure 4.16. As can be observed, there was no significant improvement in the non-covalent binding of the starting material.
compared to the elutions. The final fractions contained more free PS than conjugate or protein as seen by all three gels. It is interesting to point out that the his₆ tag is detected on the Western blot probably due to the removal of some of the free PS. Biobeads are probably more suitable for purifications where higher amounts of organic solvents are used as indicated by the manufacturer.

Figure 4.16 Purification attempts of PS1 and C6.5(-k)-PS1 PIC using Bio-Beads (Biorad). The figure shows top, a fluorescence nitrocellulose blot, centre, an anti-his₆ western blot on a nitrocellulose membrane and bottom, a Coomassie blue-stained 12 % SDS-PAGE reducing gel. M (pre-stained protein ladder), 1 (C6.5(-k)-PS1 PIC before purification), 2-8 (elutions using 20 mM OG/ 2 % DMSO/ PBS), 9 (C6.5(-k)).
Finally, a PD10 column was also run in 50 % isopropanol/PBS. Isopropanol was chosen as it was previously shown to disaggregate PS1 (section 3.2.8). The fractions were collected and analysed by UV/Vis spectroscopy and SDS-PAGE. However, the problem faced with this approach was the decomposition of the resin upon extended contact with the eluent. The results are shown in figure 4.17 alongside another PD10 purification using PBS as eluent. Elution with PBS only gave a main fraction with more non-covalent binding than the starting material. This fraction was detected by Western blot indicating that some non-covalent material was removed (lane 2). The rest of the fractions contained higher amounts of non-covalent binding (not shown). Elution with iPrOH successfully removed several fractions of free PS1 (lanes 4-7) but the antibody and/or the PIC could not be detected neither by SDS-PAGE nor by UV/Vis spectroscopy. It is possible that it precipitated on the column. Also, eluted degradation fragments would have been observed on the Coomassie.

![Figure 4.17 SDS-PAGE-Purification attempts using a PD10 column in 50 % iPrOH and PBS only of C6.5(-k)-PS1 PIC. The figure shows (A.), a fluorescence nitrocellulose blot, (B.), an anti-his6 Western blot on a nitrocellulose membrane and (C.), a Coomassie blue-stained 12 % SDS-PAGE reducing gel. M (pre-stained protein ladder), 1 (C6.5(-k)-PS1 PIC before purification) (44 %); 2 (main fraction collected from a PD10 column eluted with PBS only) (18 %); 3 (C6.5(-k)-PS1 PIC before purification) (35 %), 4-7 (PD10 column fractions 6-10 eluted with 50 % iPrOH/ PBS); 8 (C6.5(-k)).]
Changing the reaction conditions

Trying to improve the conjugation yield, the pH and the stirring method during the reaction were two parameters investigated as shown in figure 4.18. Reactions were set up as follows: (1) slow addition and stirring at pH 7.34 (standard conditions), (2) slow addition, stirring at pH 8.0, (3) one portion addition and agitation/shaking at pH 7.34 and (4) one portion addition and agitation/shaking at pH 8.0. All other parameters of the conjugation procedure were maintained as for "standard conditions".

The fluorescence blot and Coomassie stained gels are shown alongside the corresponding Western blot. Samples appeared polydispersed and residing slightly higher than control scFv (lane 7) indicating perhaps a difference in size. Again, the PICs could not be detected by Western blot and only the control sample gave a positive signal.

The final PIC loading ratios were calculated to be 6.5 (agitation at pH 7.34), 9.7 (slow addition stirring at pH 7.34), 4.6 (agitation at pH 8.0), 8.1 (slow addition stirring at pH 8.0) molecules of PS1 per C6.5(kk). The % coupled was 40 %, 43 %, 33 % and 40 % respectively with a change of ±7 % before and after dialysis. Overall, there is a significant advantage in using slow addition and stirring of the reaction mixture as the conjugation conditions. There is a less distinct difference when using pH 7.34 over pH 8.0 with the former being somewhat improved.

In another set of reactions, changes to the conditions included lowering the photosensitiser equivalents to 1:8 instead of 16, the reaction time from 1 hr to 6 hrs and a standard as a control as shown in figure 4.19. Minimising the amount of excess reagent added to the solution could theoretically lead to less non-covalent binding. Extending the reaction time can allow the reaction to proceed to nearer completion. The loading ratios were calculated as 0.6 (1:8 equivalents), 1.85 (6 hours), 1.85 (standard conditions) molecules of PS1 per scFv. These ratios were reflected in the non-covalent binding with % coupled being 16 %, 34 % and 35 % respectively. Overall, decreasing the number of equivalents of PS in the reaction is not an efficient approach as the overall reaction yield decreases. The extended reaction time from 2-6 hrs did not appear to have any effect to the overall yield of the reaction.

Organic solvents (immiscible with water) can be used to extract hydrophobic compounds from an aqueous solution. The sample obtained using standard conditions was stirred with ethyl acetate for 30 mins and centrifuged to obtain a clear purple organic layer. The aqueous layer was dialysed as usual and the samples were analysed as shown in Figure 4.19 (lanes 7, 8). The organic layer contained mostly free PS and the aqueous SN contained mostly
unconjugated scFv as there was no detectable fluorescence for the PIC band. A protein band was visible in the Coomassie for the same sample (lane 7). The smear seen in lane 8 is likely to be due to traces of EtOAc in the sample. Finally, the stained gel from the transfer is shown (middle gel) emphasising the large amount of protein that remains on the gel after transferring to nitrocellulose.

![Image of gel electrophoresis](image)

**Figure 4.18 SDS-PAGE-Synthesis of C6.5(-k)-PS1 PIC, 1 mg ml⁻¹ of scFv, 16 equivalents, altering pH and mixing method.** The figure shows **top**, a fluorescence nitrocellulose blot, centre, an anti-his western blot on a nitrocellulose membrane and bottom, a Coomassie blue-stained 12 % SDS-PAGE reducing gel. **M** (pre-stained protein ladder), 1-3 show samples where PS1 was added in 1 portion and the mixture agitated pH 7.34 (1: C6.5(-k)-PS1 PIC before dialysis (35%), 2: C6.5(-k)-PS1 pellet after dialysis and centrifugation, 3: C6.5(-k)-PS1 final PIC after dialysis and centrifugation)(40%). 4-6 show samples where standard conditions were used, stirring at pH 7.34 (4: C6.5(-k)-PS1 PIC before dialysis (43%), 5: C6.5(-k)-PS1 pellet after dialysis and centrifugation, 6: C6.5(-k)-PS1 final PIC after dialysis and centrifugation)(42 %) 7 (C6.5(-k)); 8-10 show samples where PS1 was added in 1 portion and the mixture agitated at pH 8.0 (8: C6.5(-k)-PS1 PIC before dialysis (40%), 9: C6.5(-k)-PS1 pellet after dialysis and centrifugation, 10: C6.5(-k)-PS1 final PIC after dialysis and centrifugation.
(33%)); 11-13 show samples where stirring at pH 8.0 was used (11: C6.5(-k)-PS1 PIC before dialysis(57%), 12: C6.5(-k)-PS1 pellet after dialysis and centrifugation, 13: C6.5(-k)-PS1 final PIC after dialysis and centrifugation (40%).

Figure 4.19 SDS-PAGE-Optimisation of reaction conditions and purification attempts of C6.5(-k)-PS1 PIC, 1 mg ml⁻¹, pH 8.0. The figure shows top, a fluorescence nitrocellulose blot, centre, the respective Coomassie blue-stained 12 % SDS-PAGE reducing gel following its transfer onto nitrocellulose, bottom, Coomassie blue-stained 12 % SDS-PAGE reducing gel. M (pre-stained protein ladder), 1 (PIC final SN, 8 equivalents(16%)), 2 (pellet after dialysis, 8 equivalents); 3 (PIC final SN, 16 equivalents, 6 hrs(34%)), 4 (pellet after dialysis, 6 hrs), 5 (PIC final SN, 16 equivalents, 2 hrs(35%)), 6 (pellet after dialysis, 16 equivalents, 2 hrs), 7 (aqueous SN following stirring with EtOAc and dialysis), 8 (organic SN following stirring with EtOAc), 9 (C6.5(-k)), 10 (PS1 mock solution).
Affinity purification

BSA (as well as HSA) has a high affinity for dyes and this has been described for PSs and PPα (see section 1.1.8) (Kuimova et al., 2007). Addition of BSA to a C6.5(-k)-PPα PIC solution led to the disaggregation of PPα from the same scFv (Kuimova et al., 2007). Using this affinity to purify the PIC by forcing the free/non-covalently bound PS1 to interact with BSA was considered as an approach to dissociating PS1 from C6.5(-k). In an effort to mimic an antigen affinity column, a BSA column was prepared by immobilising BSA onto a resin. BSA was reacted with CH activated Sepharose 4B (GE healthcare) containing an NHS ester, in 0.1 M NaHCO₃/0.5 M NaCl pH 8.0 for 2 hrs at room temperature according to the manufacturer’s guidelines. It was then quenched using an ethanolamine buffer and washed.

The crude PIC supernatant was added to the column resin and gently mixed for a few minutes. PBS pH 7.34 was used to elute the PIC from the column. The fractions obtained are shown in Figure 4.20 but do not appear to differ from any other purification attempts. The sample in lane 1 corresponds to the SN before purification which contains significant amount of free PS1. Elution from the column removed some free PS1 as shown on the blot. However, the PIC was diluted as confirmed by the Coomassie stained gel with protein present in all the major fractions.

![Figure 4.20 SDS-PAGE-Purification attempt of C6.5(-k)-PS1 PIC, 1 mg ml⁻¹ of scFv using a BSA column. The figure shows top left, a fluorescence nitrocellulose blot, top right, fluorescence image of an unstained 12 % SDS-PAGE reducing gel, bottom, a Coomassie blue-stained 12 % SDS-PAGE reducing gel. M (pre-stained protein ladder), 1 (C6.5(-k)); 2 (C6.5(-k)-PS1 PIC after dialysis and centrifugation), 3-5 (elutions 2-4), 6 (C6.5(-k)), 7 (PS1 only).]
Finally, in figure 4.21 a Talon® purification is shown. Affinity purification is a powerful method previously used to purify C6.5(-k) from bacterial cultures. It was therefore rational to consider it as a method to purify the PIC as it would allow the scFv/PIC to be reversibly bound on the resin and subsequently eluted. Theoretically, when bound to the resin it would be possible to wash the free/non-covalently bound PS off. 

The conjugate was incubated with the resin overnight at 4 °C and subsequently washed with a 20 mM OG/H₂O solution and eluted with 500 mM imidazole using a spin column. The fractions were collected and analysed by UV/Vis spectroscopy and SDS-PAGE. The results are shown in figure 4.21. From the image, the PIC before purification wasn’t detected on the anti-his₆ Western as observed previously. The conjugate was eluted from the column in the wash steps, prior to the addition of imidazole, indicating that it did not bind the resin during the incubation period. This can be observed on both the blot and Coomassie stained gel (lanes 2-4). The wash steps efficiently removed significant amount of free PS1 allowing the remaining PIC/scFv to be detected on the western blot as seen in lanes 4 and 5. A minimal amount of scFv bound to the resin as seen in lane 5 was bound and eluted from the column (elution 1 with imidazole) as detected by Western blot and Coomassie stain. It was not possible to improve non-covalent binding using affinity purification.

![Figure 4.21 SDS-PAGE-Purification attempts of C6.5(-k)-PS1 PIC using Talon® affinity chromatography.](image-url)

The figure shows (A.), a fluorescence nitrocellulose blot, (B.), an anti-his₆ Western blot on a nitrocellulose membrane and (C.) a Coomassie blue-stained 12 % SDS-PAGE reducing gel. M (pre-stained protein ladder).
(C6.5(-k)-PS1 PIC before purification), 2 (flow through); 3-4 (washes using 20 mM OG/ PBS); 5-7 (elutions 1-3 using 500 mM imidazole).

4.2.6 Mass spectrometry

In an attempt to apply the MALDI-TOF method by Alonso (Alonso et al., 2010) for characterising the PICs loading ratio it was realised that it would not be straightforward as the samples were not flying properly off the matrix and the detected peaks merely corresponded to the scFv and degradation products. Several sample preparation procedures were used including dialysing the samples in acetonitrile/ TFA prior to spotting as well as various chromatographic purification attempts that are normally used to desalt and purify samples before mass spectrometric analysis. The MALDI-TOF spectra of C6.5(-k) and PS1 are shown in figure 4.22 which were run as controls each corresponding to the estimated MW. The PIC sample gave mainly unconjugated C6.5(-k) and fragmentation products that could not be identified.

The spectra were run by Biopolymer Mass Spectrometry service using the method by Alonso (Alonso et al., 2010).

Figure 4.22 MALDI-TOF spectra of C6.5(-k) (left) and PS1 acid (right).
4.3 Discussion

In this chapter, the scFv C6.5(-k) was expressed, purified and used to prepare PICs with both PPα and PS1. The newly reported C6.5(-k)-PS1 PICs were prepared at an initial 4-fold higher concentration and subsequently at a more challenging 16-fold higher concentration than that previously described by Bhatti for PPα (Bhatti et al., 2008). Although higher PIC concentrations were achieved it was not possible to remove non-conjugated PS1 from the reaction mixture leading to a higher percentage of non-covalently bound contaminant.

4.3.1 C6.5(-k) expression and purification

C6.5 has been expressed and purified in our group for many years. However, the more recent C6.5(-k) proved to be less well expressed and less stable than the native antibody. As shown on Coomassie and Western blots (figures 4.2, 4.3), the his₆ and c-myc tags are easily cleaved resulting in two bands on the gels which are also present after Talon® purification. The light chain of the scFv is also easily cleavable with lower molecular weight bands present on both Coomassie gels and Western blots. This is more pronounced when looking at the anti-c-myc Western blot where three sets of bands are visible in figure 4.3. There are no reports from Adams confirming the degradation of the scFv but in the duration of this project, the cleavage of the c-myc and his₆ tags has been persistent and problematic. Pointing out the importance of minimising the decomposition of the scFv as these degradation products can all undergo bioconjugation reactions, reducing the reaction yield and purity. Subsequent work has identified a possible cleavage point which is in the process of being mutated (Deonarain, personal communication).

Following the second purification step using the anti-c-myc column, the purity of the scFv was greatly improved and thus considered sufficient for use in preparing PICs. Bypassing the first purification step and using only the anti-myc column was considered as an option but proved to be less efficient in terms of final product purity (data not shown).

These purification steps were based firstly on the affinity of the talon resin for the his₆ tag and then on the affinity of the 9E10 IgG antibody for the c-myc tag. The interactions involved in both cases are non-covalent interactions between the two complexes. The protein of interest is dissociated from the ligand by disrupting the affinity interaction (Pierce, 2009a). Antibody-antigen interactions can be interrupted by increasing or lowering the pH which is
the case with the 9E10 purification using glycine buffers at pH 5.0, 3.0 and 2.5. This is performed without interfering with the structure of either the antigen or the antibody. Talon® is a cobalt-based Sepharose resin that has high affinity for polyhistidines (Hermanson, 1996, Pierce, 2009a). Imidazole is a histidine analogue and competes for binding to the cobalt eluting the tagged protein.

Conjugating the scFv at 1 mg ml$^{-1}$ and attempting to purify these PICs demanded copious amounts of scFv. The periplasmic extraction of the scFv from the bacterial pellet as described by Adams was eventually also used to obtain scFv (not described in this thesis) (Schier et al., 1995). This gave purer product than the supernatant but the combined yields were only about 7 mg L$^{-1}$. It was beyond the scope and time limits of this PhD work to improve the expression of C6.5(-k) but it is nonetheless an issue that should be addressed (Bhatti et al., 2008). There are ways to improve the yield which include changing the vector and bacterial strain. Yields for native C6.5 were about 10 mg/L (Schier et al., 1995).

Confirming that the purified scFv is biologically active and can bind its antigen efficiently would be a natural next step. This could be verified by an ELISA using purified HER2 antigen or FACS analysis using a HER2 positive cell line and a HER2 negative cell line as a negative control. FACS analyses of the scFv using SKOV3 and SkBr3 as HER2 positive cell lines and DU145 and KB as HER2 negative cell lines were periodically conducted by other members of the group to confirm the antibody’s integrity (data not shown).
4.3.2 Conjugation reaction

As discussed in section 4.1.2 several methodologies exist for conjugating PS or drugs to antibodies. Considering a method that is simple, mild, well established and would facilitate loading several PSSs on the scFv, coupling to lysines was an obvious choice and the NHS ester was the activating group to use to do so. During the conjugation reaction the major process that occurs is the one between the NHS activated ester on the photosensitiser with the protein’s primary amines to form an amide bond via a tetrahedral intermediate. The formation of an amide bond on the lysine side chain is shown in Figure 4.23 and shows the nucleophilic attack on the activated carboxylic acid of the PS (Hermanson, 1996).

![Proposed mechanism for the formation of an amide bond](image)

Figure 4.23 Proposed mechanism for the formation of an amide bond between a primary amine on a lysine side chain and the NHS activated ester of the PS. This is the predominant reaction that occurs in solution at pH >7 during bioconjugation. The lysine is neutrally charged and acts as the nucleophile to form a tetrahedral intermediate (step a.). The deprotonation of the nitrogen (step b.) catalyses the bond cleavage- the oxygen is protonated to produce n-hydroxysuccinimide as the leaving group (Cline et al., 1987).

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Table 4.3 N-hydroxysuccinimide ester half-life in aqueous buffers of varying pH and temperature (Hermanson, 1996).
4.3.3 C6.5(-k)-PPa system

Reaction conditions

The conjugation of PPa to C6.5(-k) was reported in section 4.1.2 as developed by Bhatti (Bhatti et al., 2008). Their optimised conditions included sixteen equivalents of PPa NHS ester reacted with the scFv at pH 7.34 in PBS in the presence of co-solvents (6 % MeCN and 2 % DMSO). The use of co-solvents is crucial as they are used at a low enough concentration to maintain the scFv in its native conformation and high enough to ensure maximum possible solubility of PPa (Kuimova et al., 2007, Bhatti et al., 2008). The use of higher amounts of DMSO or other organic solvents would be ideal especially if it could be used around the isosbestic point of the PS (see chapter 3). However C6.5(-k) is not stable under such conditions. Elsewhere, a peptide-PPa conjugate was made using the NHS ester in 25 % DMSO for 6 hrs at room temperature to obtain a 1:1 ratio of the PSMA inhibitor peptide to PPa (Liu et al., 2008, Liu et al., 2009). Helmreich used a PPa derivative dissolved in 35 % DMF to conjugate it to an IgG (Helmreich, 2005) which was purified by Bio-Gel® P-60 eluting with PBS/DMF (8/2). Even though these groups acknowledged the presence of non-covalently bound material, the use of appropriate reaction conditions allowed them to resolve it.

This project was initiated with the preparation of the C6.5(-k)-PPa PIC at 300 µg ml⁻¹ whereas the published procedure referred to 100 µg ml⁻¹. Therefore in that respect it was slightly improved. The loading ratio obtained for C6.5(-k)-PPa was calculated to be 3.4 and it was not possible to replicate the 10:1 loading ratio that was previously reported (Kuimova et al., 2007, Bhatti et al., 2008). Increasing the coupling concentration 3-fold inherently increases the PPa aggregation which can lead to analogously less PPa being available for conjugation. Also, the extinction coefficient previously used for PPa, was 855 M⁻¹cm⁻¹ whereas it was determined experimentally for this work to be 11375 M⁻¹cm⁻¹ in 2 % DMSO/PBS. It would therefore be difficult to compare two sets of experiments obtained using different parameters. Furthermore, Bhatti et al. did not correct for the absorption contribution of PPa at 280 nm. We determined the extinction coefficient in 2 % DMSO/ PBS and subsequently based calculations for the loading ratios on what could be considered an accurate value. The loading ratios were previously calculated using the extinction coefficient of PPa at the Q band in PBS and corrected for non-covalent binding using densitometry. Subsequently it was compared to an alternative method using fluorescence spectroscopy. This approach was based on the affinity of PPa for BSA which was used to disaggregate it. PPa was shown to have a higher affinity for BSA compared to C6.5(-k) (Kuimova et al.,
An increase in fluorescence intensity of an aqueous PPa solution upon addition of BSA verifies its disaggregation. Using a standard curve for PPa/BSA interaction, the PIC solution was titrated with known amounts of BSA and the % of non-covalently bound PS was calculated for the conjugate. The ratio obtained was found to be very similar to the number obtained using spectrometry with densitometry (Kuimova et al., 2007) therefore validating the spectroscopy/densitometry method used throughout this work.

Comparing experiments where even slight modifications to the system are made is unreliable as the conjugation of a protein to a hydrophobic molecule is a dynamic process. Throughout the literature there are reports of conjugating PPa to biomolecules but these are not comparable as the biomolecules differ, reaction conditions differ and the characterisation methods differ. For example, Savellano used the extinction coefficient of PPa in DMSO which is 47500 M$^{-1}$cm$^{-1}$, four times higher than that in the aqueous system to calculate the loading ratios of their mAb PICs admitting that this is not an accurate representation of the PS in the PIC due to its aggregation (Savellano et al., 2005b). Savellano characterised PPa-mAb PICs by visualising SDS-PAGE gels with a CCD camera (Savellano et al., 2003). They then also calculated the PIC loading ratio of BPD-mAb in the same way (Hamblin et al., 2000), to get 2-11 molecules of PPa per mAb with less than 5 % free. They made these PICs controlling the excess PS to study the efficiency of the conjugation. They concluded that the reaction is more efficient for the lower reaction ratios than those above 10 equivalents where the yield decreases from 75 % to 45 % (Savellano et al., 2003). From figure 4.19, decreasing the excess of PS1 from 16 to 8 equivalents reduced the yield by more than half. It is obvious that it is not possible to generalise conclusions.

The free, non-covalently bound material is visible in the blot as shown in figure 4.5. Noting that dialysis and centrifugation led to a decrease in non-covalent material for the PPa PIC from 56 to 40 % as it precipitated out of solution allowing removal from the sample. The UV/Vis absorption spectrum further supports that the PS is aggregated in the PIC solution. The Soret band is broad and the Q bands are less well defined and blue shifted compared to a 100 % DMSO sample. Observing the fluorescence blot, the NCB/ free PS is seen which appears to be less intense in the final PIC than the crude material. The PIC fluoresces at the ~30 kDa band whereas the free PS runs at less than 10 kDa as expected.

As the aim of this project was to produce higher concentration PICs for clinical application. PPa could not be used further due to its low solubility and was only used to establish a reference point before proceeding with PS1. It was beyond the scope of this PhD work to attempt to purify or further optimise the C6.5(-k)-PPa conjugation. Previous work by Bhatti did not result in the purification of the PIC (Bhatti et al., 2008).
4.3.4 C6.5(-k)-PS1 PICs

Covalent bond formation

Even though the method for conjugating PS1 to C6.5(-k) did not differ from the one used for PPϕ, successful conjugations of PS1 were not straightforward and required several refinements. In the initial conjugations, the acetonitrile and DMSO co-solvents (supplement) were added to the aqueous solution prior to photosensitiser addition. This was later abandoned as the co-solvents were combined with the photosensitiser and the combined solution added to the aqueous buffer.

Obtaining evidence that (stable) covalent bonds were formed during the conjugation reaction was not straightforward. The most direct and reliable method would be to use mass spectrometry but that proved to be more complicated than expected. Therefore, SDS-PAGE was used as a method to visualise the denatured PIC.

Being able to observe a significant size increase on an SDS-PAGE reducing gel would be important. For example, conjugating an scFv to PEG\textsubscript{2000}-DSPE, a notable 3kDa increase in size was seen for a 1:1 loading ratio (Nielsen et al., 2002). The loading ratio for a mAb-mTHPC conjugate was determined using the iodinated antibody’s gamma count and the extinction coefficient of the PS at 415 nm (Vrouenraets et al., 1999). Combined with SDS page loading ratios of 2-2.5 were calculated. This mAb being an IgG1 should be about 150 kDa in size and upon addition of 2-2.5 molecules of PS should increase about 2kDa. The band appeared polydisperse on their non-reduced SDS-PAGE gel but the predominant band was at 150 kDa (Vrouenraets et al., 1999, Vrouenraets et al., 2001). This observation agrees well with what we have seen here for C6.5(-k)-PS1 PIC. It is also possible that a PEGylated protein will travel differently through an SDS-PAGE gel than a non-PEGylated protein of the same size thus leading to inaccurate comparison of proteins containing PEGs on an SDS gel (Chunyang et al., 2007). Also, PEG chains of minimum size 350 Da associate with SDS to form aggregates (Bernazzani et al., 2004). On PS1, the combined size of the three small PEG chains is 489 Da which is within the limit for SDS association. It was observed in the experiments shown in chapter 3 that detergents can disaggregate PS1. SDS is likely to help disaggregate PS1 explaining the lower MW band observed on denatured gels. The effect of SDS on the sample is could be further explored by running PEG as a control and UV/Vis absorption spectra in 20 % SDS. It is likely that it disaggregates PS1 and upon denaturation and protein unfolding the free/NCA PS runs according to its size, around 10 kDa. Our PIC samples were resolved on the gels as described by others. The conjugate
is seen on the fluorescence blot as a main fluorescence band at ~30 kDa and the free or non-covalently bound material is seen running lower at or above the bromophenol blue band (Hudson et al., 2005, Savellano et al., 2005b, Bhatti et al., 2008). Savellano run non-reducing, SDS containing gels that showed a minor size difference of the PIC (loaded with 7 molecules) against the unconjugated native mAb. On a non-reducing gel the mAb would still be folded in its native conformation thus unlikely any non-covalently bound PS would be observed and it is difficult to establish whether there is any size difference between the conjugate and free mAb. For the reducing SDS gels which they do not show they say that there is no difference in migration between the PEGylated (starter) and the PIC (Savellano et al., 2003). Generally when analysed by SDS-PAGE conjugates containing porphyrins/chlorins do not necessarily appear to migrate differently to their parent biomolecule regardless of the loading ratio and theoretical size difference between the conjugate and antibody (Cavanaugh, 2002).

The absence of a significant MW difference of the PIC band compared to the scFv on SDS-PAGE gels meant that a more indirect approach had to be used. A mock conjugation reaction was run where the PS1 acid instead of the AE was put through the same procedure and the samples were observed as shown in figure 4.7. Corresponding to the supernatant sample, a very faint, of negligible fluorescence intensity high MW band (30 kDa) was visible for the PIC and a very intense (over-saturated) band for the free PS1. On the Coomassie stained gel, the corresponding sample evidently verifies the presence of C6.5(-k) in the sample which run in the same way as control C6.5(-k) in lane 7.

The products of a standard conjugation reaction are shown in lanes 4-6. The intensity of the fluorescence band is much greater than the mock sample and on the Coomassie stained gel appears polydispersed and at a higher MW than the scFv. The intensity of the PS1 fluorescence band is less for the PIC than for the control sample and the mock PS1 sample (lane 8). The inherent fluorescence of C6.5(-k) is not strong enough to be observed on the blot as shown in lane 7. Therefore, comparing the “mock” samples with the standard PIC samples, there is an apparent fluorescence intensity difference. The stronger fluorescence intensity of the top band of the standard SN sample compared to the mock, corresponds to the presence of a greater amount of PS in the sample. It is therefore indirectly shown that under standard protocol conditions, the SN sample obtained contains PS1 covalently attached to C6.5(-k).

Others carried out similar experiments in their effort to address the same question. Alonso also conducted a similar control experiment (section 6.1.1) obtaining similar results where
the PIC band was slightly fluorescent (Alonso et al., 2010). Similarly, Hamblin combined BSA and chlorin e6 in its free form and run on a gel to observe the fluorescence of the free PS only, on the gel, concluding that the higher MW fluorescent signal they observed with their standard conjugate was attributed to covalently attached PS (Hamblin et al., 2000).

Another characterisation method that is not frequently used, is Native PAGE. It compares proteins primarily for charge rather than size difference by omitting the SDS from the process. By occupying the lysines of a protein, the overall protein charge changes if a sufficient number of lysines can be occupied by a neutral compound. This will change the protein’s pI and the way it migrates on a gel (Chunyang et al., 2007). If all the available lysines coupled to PS1, the protein would have 13 less positive charges and a ~13000 Da molecular weight increase. It is possible that the two can counteract each other and the protein can appear to run as the native scFv on a normal SDS reducing gel. However, on a native PAGE, the PIC would run faster than the native scFv as it would be more negatively charged than the uncoupled scFv. In an attempt to run a native PAGE gel, the PIC ran slightly faster than the scFv as seen on the Coomassie as expected (figure 4.10). The fluorescence blot was less clear showing a polydispersed band indicating a mixture of PICs throughout and the free PS1 running alongside the mock PS1 sample.

Changes in the overall scFv charge and pI could be responsible for the lack of notable MW difference on reducing gels. If on average 7 lysines take part in the conjugation reaction then that inherently implies that 7 positive charges are neutralised. Therefore, the overall protein will be overall more negative. This could mean that upon being coated with negatively charged SDS it will tend to run faster towards the anode than the native scFv and thus appear smaller than the PIC actually is.

**Loading ratios and efficiency**

It is likely that PS1 reacts with C6.5(-k) at a range of lysine sites and not always in the same manner (Chunyang et al., 2007), with the loading ratio being a distribution of differently loaded PICs. The hydrophobic character of the PIC might be affected when combining the two parameters. Results from the antibody-drug conjugate area verify this theory as they show a binomial probability distribution that describes lysine coupling. Using an IgG and maytansinoids Goldmacher described this distribution using mass spectrometry.
Lysine coupling appears to be a random process resulting in several species of conjugates with various degrees of labeling (Goldmacher, 2010).

In most conjugation reactions, a significant excess of the PS is used. For example, using a large excess of PS, Boyle et al. prepared 0.5 mg ml\(^{-1}\) conjugates by adding 2.5 to 60 equivalents of PS (Staneloudi et al., 2007). This approach is only reasonable when the purification of the resulting PIC is feasible. This approach would not work for our system as it would lead to a higher amount of non-covalently bound PS. Theoretically, as more PSs are coupled onto the antibody or targeting species the more photodynamically active and potent it will be. However, it has been shown that particularly in the case of antibodies, loading too many hydrophobic PS onto such biomolecules can cause them to precipitate. At 1 mg ml\(^{-1}\), mAb-mTHPC PICs, with a loading ratio higher than 4 were found to precipitate (Vrouenraets et al., 1999). Furthermore, bioconjugation reactions can affect the CDRs of an antibody by causing conformational changes or obstructing them leading to loss of antigen recognition (Hermanson, 1996). Overloading the antibody can increase this effect and it has been shown that in some instances a ratio of 1:5 is optimum for an scFv (Staneloudi et al., 2007).

We have not observed PIC precipitation at a loading ratio of 4 or above. An average of 6-7 PS1 per scFv has been most consistently obtained. The PIC was soluble and remained in solution. A balance between sufficient loading of the antibody for potency along with the retention of antigen binding, pharmacokinetics and stability \textit{in vivo} is required. The bioactivity of our PIC can only be determined once tested \textit{in vitro} (chapter 5) and \textit{in vivo} (chapter 6).

In order to improve the homogeneity of the loading ratios of the PIC, site-specific bioconjugation has been used. This approach partially controls the position where the drug binds the protein by using thiols. McDonagh engineered a protein to obtain defined loading ratios (Mcdonagh et al., 2006). Alonso et al. have also recently demonstrated highly homogeneous conjugates using site-specific conjugation onto a SIP's thiols (Alonso et al., 2010). They obtained conjugates with 2 porphyrins per SIP. However, it is possible that the low loading ratio will be a compromise on efficacy over purity and characterisation. It is feasible to bind all available groups of interest on an antibody as demonstrated by the conjugation of an IgG1 to auristatin E via cysteine coupling. It is also possible to control the conjugation by engineering the reactive groups present on the antibody such as the cysteines. McDonagh had to remove some of the cysteines present on their IgG in order to obtain stable conjugates via maleimide coupling (Mcdonagh et al., 2006). Further characterisation of our conjugate will demonstrate whether PICs can be consistently obtained with similar bioactivity and loading ratios using lysine coupling.
Comparing C6.5(-k)-PS1 PIC to C6.5(-k)-PPa PIC

The observations made during the analysis of the C6.5(-k)-PS1 PIC were similar to those made for C6.5(-k)-PPa PIC. The C6.5(-k)-PS1 PICs gave polydispersed bands which were not observed with the PPa PICs. Despite PS1 being larger than PPa (double), the MW of the PIC appears to run at 30 kDa consistent with C6.5(-k). As already mentioned, it was not possible to observe a significant MW difference (compare to C6.5(-k)) with neither PICs using SDS-PAGE. The UV/Vis spectrum of the C6.5(-k)-PS1 PIC was much sharper than that of the C6.5(-k)-PPa PIC. This could indicate that PS1 was less aggregated than PPa in PBS as shown by the UV/Vis studies in chapter 3. Also, the solubility of PS1 in PBS is considerably higher than that of PPa allowing conjugations to be carried out at 10 fold and even up to 40 fold higher to that published by Bhatti (Bhatti et al., 2008). The addition of PS1 into the aqueous reaction mixture gave a clear green solution which did not precipitate out of solution when conjugating at 1 mg ml\(^{-1}\). A pellet was obtained following centrifugation which was largely soluble in PBS and much smaller in size compared to PPa. The increased solubility of PS1 compared to PPa is responsible for the higher amounts of non-covalently bound PS1 than PPa. Following dialysis and centrifugation, the PPa NCB amount decreases by 14 % whereas for PS1 there was no change as stated for image 4.7 (an 8 % increase was noted for that experiment. However there is a ±7 % variation between experiments with PS1).

Focusing on the PS1 PICs, when increasing the concentration to 4 mg ml\(^{-1}\) precipitation was obvious and a large pellet was obtained. The C6.5(-k)-PS1 conjugation reaction has a solubility limit that lies between 1 and 4 mg ml\(^{-1}\). This was not investigated further but it pointed out that a further increase in the solubility of PS1 will be required if higher concentration PICs are to be obtained. The loading ratio obtained was very low at a maximum of 1.4 molecules of PS1 per scFv. It is possible that PS1 is forming oligomeric aggregates at a faster rate at the higher concentration or that micelles are forming instead. In either case, the hexynoic side chain is likely to be unavailable for conjugation thus leading to the lower yields. Overall, the PS1 conjugate is not only higher in concentration but it is also soluble and does not form insoluble aggregates at 1 mg ml\(^{-1}\) compared to the PPa PIC.

Reaction conditions and amino acid reactivity

The several parameters involved in a bioconjugation reaction can affect its efficiency. For example, it has been reported that the integrity of a mAb was compromised when incubating the PS with the antibody at room temperature, in the presence of light and air whereas it was...
unaffected under a nitrogen atmosphere (Akhlynina et al., 1997, Vrouenraets et al., 1999). During an attempt to establish the applicability of this on our system, a conjugation reaction was run in degassed PBS under a nitrogen atmosphere followed by dialysis in degassed PBS. There was no notable difference to the normally aerated reaction system (data not shown). Stringent use of dark conditions throughout the experiments is may be sufficient to protect the PIC from generating ROS and decomposing.

When the number of PS1 equivalents was reduced to 8 from the standard 16, the loading ratio was significantly reduced from the average 7 (40 % conjugated) (standard conditions) to 0.6 (16 %) as shown in figure 4.19. The two favourable processes that occur in the reaction mixture, the covalent bond formation between the AE and the amines of the antibody and the non-covalent interactions, compete (Savellano et al., 2005b) and upon lowering the reaction ratios the former appears to decrease further.

Time is also a contributing factor to a reaction’s efficiency. However, increasing the reaction time did not appear to improve the conjugation and an average of 3 hours was eventually established. Even though the loading ratio varied from 9 to 1.85, the amount of NCB/free PS was quite consistent at 40 %. From the middle gel, figure 4.19 it is important to observe the amount of protein that remains on the gel following semi-dry transfer onto nitrocellulose. The two components of the PIC sample (PIC and free PS) appear to transfer onto the blot with very different efficiencies. This is further verified by the more efficient transfer of the smaller molecular weight markers compared to the larger ones. The accuracy of our visualisation and quantification method is therefore questionable following this observation. The protein or PIC (≥30 kDa) appears to transfer less efficiently than smaller molecules such as free PS1. If PS1 transfers more efficiently than the PIC there is an inherent bias towards the free PS amount present in the sample. Identifying the extend of this bias would allow us to better characterise the PIC which could contain less free/NCB PS than what has been calculated so far by densitometry.

During transfer from a gel to a membrane, the proteins bind via hydrophobic interactions using SDS and methanol present in the buffer systems to do so. Nitrocellulose and PVDF are the most commonly used types of membranes. Nitrocellulose has the lowest fluorescence background and was thus used throughout this thesis. Using methanol in the transfer buffer aids the dissociation of the proteins from the gel and their interaction with the membrane (Dunbar, 1994). Increasing the amount of methanol in the transfer buffer can decrease the rate of elution from the gel of larger proteins over smaller ones. The second important component of the transfer buffer is SDS. SDS is a negatively charged detergent and can increase the elution of the proteins from the gel but can inhibit the binding of the
proteins to the membrane (Dunbar, 1994). Addressing the incomplete transfer onto the membrane is not straightforward. Longer transfer times were attempted but resulted in the PS passing through the nitrocellulose and staining the filter paper. Using a lower concentration gel, a buffer containing SDS and no methanol in the cathode buffer or reducing the methanol content altogether is another step worth considering (Dunbar, 1994).

In order to resolve the observed issue, the photosensitiser would have to be excited on the wet SDS-PAGE gel therefore omitting the transfer step. This approach would in theory be ideal as the wet gel represents the sample more accurately than the blot. We used the FujiFilm Las 3000 (as for the blots) in an attempt to visualise wet unstained gels (Figure 4.20), but it was difficult to obtain a reproducible and strong enough signal. In further attempts, the gels were fixed with methanol following electrophoresis, protected from light and dried immediately and then tried to visualise the dried unstained gel. The dried gel does not contain water, that can significantly affect the fluorescence quantum yield, but this did not work with the given apparatus (data not shown). A potential option would be to use a red or UV laser to achieve this as others have shown (Savellano et al., 2005b). Such devices include the LI-COR (LI-COR Biosciences) or a homemade instrument.

Further reaction conditions that can be modified are the method of combining the components together and pH. Some groups use shaking/agitation as a reaction method instead of stirring. The use of gentle shaking gave decreased yields when compared to stirring (figure 4.18). Increasing the pH from 7.34 to 8 also exhibited a decrease in reaction efficiency. The best loading ratio was obtained with pH 7.34 and vigorous stirring as shown in figure 4.18.

The amine and the active ester have optimum reactivity under different conditions. The half-lives of the ester at different pH are shown in table 4.3. The reaction kinetics of the ester at pH 6.3 and pH 8.6 vary by a factor of 10 with the latter being the fastest. Carrying out a reaction at pH 8.6 does not infer improved reaction yields. The time required for the NHS ester to significantly hydrolyse and its reactivity to decrease to 30 % was found to be 10 mins at pH 8.6. Its reactivity was reduced to 75 % at 50 mins at pH 6.3 at 4 °C. Therefore, a balance of pH, temperature and time is required to achieve near optimum conditions. At pH 6.4 it is beneficial to have a larger excess of the amine to the active ester.

Assuming that the main reaction is that of amines and the NHS ester, the conditions can be modified to optimise the reaction. The optimum conditions for an amino group to react vary
with its pK_a. For example, for alanine the optimum pH is 8.0 whereas for the ε-amine of lysine’s side chain it is 9.3-9.5. Lysine is positively charged at pH lower than 9.3 and is less likely to act as a nucleophile (Hermanson, 1996). Increasing the pH the pK_a of the targeted amino acid is not necessary and can be counteractive as proteins tend to be unstable at high pH (Hermanson, 1996). Additionally, even if the pH is optimised for the amine reactivity (where it can act as a nucleophile), increasing the pH of the reaction will increase the rate of hydrolysis of the NHS ester and subsequently decrease the efficiency of the reaction (Cuatrecasas et al., 1972). A balance between the two counteracting factors is necessary. Varying the pH of the reaction solution was previously shown to have a relatively minor impact on the efficiency and turnover of the coupling. Cuatrecasas has shown that when competing free amino acids, the alanine α-amine exhibits up to 20 times faster reaction kinetics than the lysine ε-amine (Cuatrecasas et al., 1972). Our attempts to improve the yields of the conjugation by increasing the pH to 8.0 did not provide consistently better results for the higher pH as was expected. It was therefore decided to use pH 7.34 as standard conditions to avoid the additional step of changing the protein’s pH. Slow addition of the PS whilst stirring were maintained as standard conditions.

Considering the prevailing reports in the literature and the extensive use of the NHS ester in bioconjugation reactions for the formation of stable amide bonds, it is probably safe to assume that the main bonds formed during the conjugation reaction are those between PS1 and primary amines. The second most likely reaction is that of histidines with the PS (Anjaneyulu et al., 1987, Mädler et al., 2009). Histidines have a pK_a at 6.7-7.1 (Hermanson, 1996), therefore at pH 7.34 they would be neutral and could theoretically act as nucleophiles. The NHS can also react with sulphydryl groups found in cysteines, hydroxyl groups found in tyrosine and serine side chains (Hermanson, 1996). However these bonds are not particularly stable and can undergo hydrolysis especially bonds formed with histidines (Cuatrecasas et al., 1972, Anjaneyulu et al., 1987, Mädler et al., 2009). Reactions with cysteines and tyrosines occur much more slowly than the reaction with amines and therefore less likely to occur (Anjaneyulu et al., 1987). One could hypothesise that the histidines consume a portion of the NHS activated PS from the reaction system not allowing it to react with the amines. The bonds formed with the histidines subsequently hydrolyse and the PS, no longer reactive remains in solution as free PS. It is unlikely that it could explain all the non-covalently bound material but it could account for some of it. Tyrosines are found near the surface of proteins and they can form ester bonds with acylating reagents (Hermanson, 1996) such as an NHS ester. It would be possible to form ester linkages with the PS and tyrosine (pK_a 9.7-10.1, above this). Also, if tryptophan can react with the NHS
activated photosensitiser then the absorption of the scFv could be affected as tryptophan is the main contributor to the absorbance of proteins at 280 nm (Hermanson, 1996). Should this be the case then the UV/Vis absorption measurements become unreliable, potentially affecting the way loading ratios have been calculated.

Competition reactions using free amino acid, alanine (α amine) and other amino acids with potentially reactive side chains showed cysteine and histidine as the only potential competitors to alanine when present at large excesses with the cysteine being the stronger. It is important to emphasise that even though histidine competes for the NHS ester, the bonds formed are not stable and tend to decompose (Cuatrecasas et al., 1972). Kinetically, the only competing amino acid for the NHS ester except for the primary amines is the histidine which forms unstable bonds that hydrolyse rapidly (Anjaneyulu et al., 1987).

A similar study with porphyrins was performed by Alonso looking at the reactivity of their maleimide activated porphyrin towards sulfides and amines. They concluded that under their reaction conditions and in the presence of both reactive groups, the thiol would react exclusively (Alonso et al., 2010). A useful future study should include a similar experiment to observe the reactivity of PS1 NHS ester towards the various reactive amino acids mentioned above at various conditions. Understanding the kinetics and the stability of the bonds formed might help improve the yields of the conjugations by altering the conditions to facilitate amide bond formation.

4.3.5 Purification attempts

The use of organic solvents to remove non-covalent or unreacted PS is often used as a purification step. A purpurinkpeptide conjugate was prepared using organic solvents to obtain a high purity conjugate following washes with organic solvents (Walker et al., 2004, Conway et al., 2008). The use of organic solvents is often a successful approach for purifying peptides and other stable to organic solvents biomolecules. We used EtOAc to wash away the unbound PS1. The protein remained in the SN (as shown in the Coomassie stained gel, figure 4.19) whereas a significant amount of free PS was extracted into the organic layer. The PIC was not detected. Again, low efficiency of transfer to nitrocellulose was observed as most of the 30 kDa band remained on the gel (fig. 4.19 middle gel). Therefore, even if there was a significant amount of PIC present in the SN following the washes, it would not be detected.

This approach could be repeated on a larger scale and the resulting SN concentrated prior to its analysis. However, a similar attempt using acetone to precipitate the PIC followed by
washes resulted in the complete degradation of the scFv (data not shown). The stability of C6.5(-k) in organic solvents would not allow their extensive use as part of a purification process.

**Using dialysis as a method to purify the PICs**

As shown in figures 4.12 and 4.13, dialysis was one of the first approaches for purifying the C6.5(-k)-PS1 PIC. The effect of dialysis in PBS on free PP\textsubscript{a} and PS1 is shown by the UV/Vis spectra. PS1 appeared red shifted following dialysis indicating further aggregation whereas PP\textsubscript{a} exhibited a small decrease in the absorption intensity which could be a sign of partial dialysis taking place. Savellano observed a broadening of the UV/Vis absorption spectra at the $\lambda_{\text{max}}$ due to dye aggregation in the PICs compared to the free PP\textsubscript{a} and detected up to 20 % non-covalent binding (Savellano et al., 2005b). Heating a sample can disaggregate it and similarly to the presence of dialyzable detergents (chapter 3). However, there was no significant improvement.

Dialysis can (theoretically) be used to remove the free photosensitiser. We used dialysis membranes with MWCO more than 100 times the MW of the PS. Even though dialysis is a process where smaller than the MWCO molecules diffuse through the membrane into the larger volume solution until an equilibrium is reached, it does not appear to significantly improve the % of free PS present in the PIC. Dialysis was not expected to completely remove the non-covalently bound PS but to remove low MW oligomeric PS (Pierce, 2004). Detergent was used (at a concentration below its CMC) to dissociate the aggregates hence allowing them to behave as monomers, which would be smaller, and thus able to cross the membrane. Dialysis was on several occasions allowed to proceed at room temperature for several hours with the buffer volume at least 1000 times the volume of the sample. Slide-A-Lyzers whose pores are different to the usual cellulose of tubing were also used (Pierce, 2004). Having tried these variations without success dialysis is an unlikely solution to the NCB/ free PS issue.

**Size exclusion chromatography**

Size exclusion chromatography was the most promising method for purification. Based on the wide use of PD10 columns in the purification of bioconjugates for PDT, several attempts were based on their use. Examples where PICs were purified to obtain a single fluorescent band on SDS-PAGE (Alonso et al., 2010) were promising. Usually, standard buffers were sufficient to obtain pure products as was the case with the mAb-THPC conjugate which was
purified on a PD10 column eluting with 0.9 % NaCl. A phthalocyanine PIC prepared and purified similarly, was also analysed by HPLC using a Superdex 200 HR column in phosphate buffer (Vrouenraets et al., 2001, Vrouenraets et al., 2002). Hasan found it was necessary to use 0.1 % SDS to elute a hematoporphyrin-IgG conjugate from a Sephadex 200 column, with the elution profile of the PIC and the native IgG being very similar even with a loading ratio of 5-6 HP per IgG (Hamblin et al., 1998).

Recently, using the tricationic PS prepared by Alonso (Alonso et al., 2010), we showed that C6.5(-k) conjugated to the tricationic PS via NHS chemistry was easily and successfully purified on a PD10 column eluting with PBS. Obtaining these data (not shown) was encouraging as it verified that the techniques and approach used towards purifying the PS1 PIC were appropriate. It also suggested that with an analogous, perhaps multicationic synthetic modification of PPa a pure conjugate can be obtained.

In size exclusion chromatography, the largest molecules elute first followed by the smallest ones (Chunyang et al., 2007). The conjugate loaded with most PSs would be expected to elute first. The elution profile of most PD10 purifications was free PS followed by impure PIC followed by more free PS with free scFv. These were shown by blot, Coomassie stained gel and Western blot (figures 4.14, 4.15, 4.17). It is important to note that a positive signal on the Western blot (anti his<sub>6</sub>) for the PIC was only obtained following size exclusion chromatography indicating that partial purification increased the availability of the his<sub>6</sub> tag to the anti-his<sub>6</sub> mAb.

There was a maximum 18 % improvement on the non-covalent binding when using the G-25 columns (figure 4.16). Nonetheless, this was not considered sufficient, with more than 30 % NCB PS remaining in the sample. Bio-Beads® is a neutral size exclusion resin that was used with detergent as shown in figure 4.16. This approach was not different to the others previously used. Conversely to the Sephadex based resins, Bio-Beads® are chemically resistant and can be safely used with organic solvents. This approach can be further explored once the scFv stability in organic solvents is determined.

Fluorescent dye removal columns (Thermo Scientific) were also used to remove the free PS1. These spin columns are pre-packed with an unnamed resin and claim to remove “almost any fluorescent dye” (Scientific, 2008). Using them to purify several samples gave impure PICs similarly to PD10 columns (data not shown). Purifying the same sample several times using these columns and combining this method with a PD10 column was not successful either.
Using RP-HPLC to purify PICs would probably destroy its biological activity as both organic solvents and the resin irreversibly denature the scFv (Mcdonagh et al., 2006, Chunyang et al., 2007). Nonetheless, attempts to mimic those conditions using a PepClean™ C-18 spin column (Pierce) gave the impure conjugate as the flow through. Free PS1 eluted in the subsequent washes (data not shown).

**Affinity Purification**

BSA is known to have a high affinity for dyes (Kuimova et al., 2007). Pandey showed that benzobacteriochlorins compete for binding to HSA’s ligand at Site II (Li et al., 2003). Conjugating BSA (5 mg ml\(^{-1}\)) to Ce6 using NHS ester followed by an acetone wash/precipitation system and then by Sephadex G50 purification eluting with PBS gave PICs that still contained a small amount of free PS (Hamblin et al., 2000).

The strength of the interactions was also observed when Verteporfin was completely disaggregated in the presence of albumin equally to when dissolved in an organic solvent (Mellish et al., 2001). It was noticed that solutions of PS1 in 10 % FBS/DMEM also appeared to be highly disaggregated and compared to its solutions in organic solvents (not shown) supporting both the BSA column concept and the Verteporfin observations.

BSA was covalently attached to Sepharose in an attempt to make an affinity column that could potentially bind the free/NCB PS. Such a purification attempt was shown in figure 4.20. Both the c-myc and his\(_6\) tags become unavailable following conjugation. This was attributed to the binding of a PS to the lysine in the c-myc tag (EQKLISEEDL (Hilpert et al., 2001)) and/or non-covalently bound PS (or less likely covalent) obstructing the his\(_6\) tag. Since the his\(_6\) tag can be detected following size exclusion purification, it can be assumed that non-covalently bound material accounted for the lack of signal of the crude PIC. During Talon\(^\circledR\) purification (figure 4.21) the impure PIC eluted from the column with the washes or the flow through whereas the elutions contained small amounts of free scFv and free PS (as shown in the Coomassie stained gel and Western blot).

Following multiple attempts to purify or improve the conjugation in order to minimise non-covalent binding, the most significant increase in covalently coupled PIC was an 18 % improvement using the G-25 column. Reviewing the purification attempts and pinpointing those that could be improved such as the use of a G25 column would be a next step.
Lastly the PICs used throughout this thesis were used within 2 days of their preparation. This is not a sustainable solution as this is counterproductive in terms of time efficiency and costs. However, methods of suitable prolonged storage were not examined. A storage method is required that would allow enough material to be available for constant use. It could involve freezing the solution at -20 °C (Staneloudi et al., 2007), freeze drying and freezing or keeping in solution at 4 °C. There are few other reports on the storage of PICs. A whole mAb- BPD PIC was stored at 4 °C and was stable for months in the presence of PEG (Savellano et al., 2003).

4.3.6 Mass spectrometry

The main products detected on the PIC mass spectra were the free scFv, its degradation products and free PS. As I am aware, the only published mass spectrometric analysis of a PIC is by Alonso et al. They obtained this on purified conjugates containing no NCB and low loading ratios (up to 1:2) which could allow the samples to fly more efficiently (Alonso et al., 2010). They made no correlation between the mass spectrometry data and the UV/Vis absorption/ SDS-PAGE conventional method, which would give an indication as to the validity of the latter. Attempts to apply the same protocol using the aforementioned pure C6.5(-k)-tricationic porphyrin pure conjugate (not shown) afforded inconclusive results similar to those obtained for the C6.5(-k)-PS1 PIC.

Characterising immunoconjugates containing dyes by MS is not straightforward. In an attempt to quantify the loading ratio of Alexa488® on an anti HER2 scFv, MALDI-TOF analysis was carried out but without success. Using the same conditions, the biotinylated scFv was successfully characterised by MALDI-TOF instead (Qasba et al., 2008, Ramakrishnan et al., 2009). MALDI-TOF characterisation of smaller biomolecules with dyes is probably easier to achieve such as a peptide-AlexaFluor conjugate where ratios of 1:1 were determined (Chaloin et al., 2001, Busch et al., 2008). A similar approach to the biotinylation could be used to characterise the loading ratio of C6.5(-k) with a small molecule other than a PS.

4.3.7 Limitations of the work

Kuimova et al showed that the ratios obtained using the spectroscopy combined with the blot densitometry approach, closely matched those obtained using BSA competition titration
(Kuimova et al., 2007). It was often found that when developing blots, the fluorescence signals exceeded the detection limits of the machine leading to unreliable results that could not be used (even though loading 2 µg of protein on gel was optimised). Previously, we described how densitometry of the blot is likely to favour the % NCB/free photosensitiser. Similarly, when spectroscopically measuring the absorption intensity of these samples, in order to avoid exceeding the detection limits of this method, the samples had to be diluted. However, a diluted sample does not give an accurate representation of the concentrated sample upon correction. Subsequent calculations could give lower loading ratios. Therefore, the most important limitation of the work is arguably the unreliability of the densitometry/spectroscopy approach when estimating the loading ratio.

4.3.8 Concluding remarks

C6.5(-k) is an internalising anti-HER2 scFv. It was hypothesized to be a good antibody for PDT due to the spatial separation of its lysines (as shown in figure 4.1) (Kuimova et al., 2007). In order for a PS to maintain its photophysical properties and ROS generation efficiency it must not be in spatial proximity to each other (Hamblin, 2008). Internalising antibodies are also considered better PDT candidates (Hamblin, 2008). Even though its $k_{off}$ is slightly faster than the parent scFv ($4.2 \times 10^{-3}$ s$^{-1}$ compared to $6.3 \times 10^{-3}$ s$^{-1}$) (Schier et al., 1996), it will be determined in the in vitro and in vivo studies whether this is an issue that should be addressed. Theoretically, the $k_{off}$ is not a critical parameter of an internalising scFv.

The aggregation of PP$a$ and PS1 in aqueous solutions was verified in chapter 3. In this chapter the effect it has on bioconjugation to C6.5(-k) was described. The C6.5(-k)-PP$a$ PIC was synthesised and characterised as a reference point. The C6.5(-k)-PS1 PIC was subsequently synthesised at 1 mg ml$^{-1}$, a 3.3 fold higher concentration than the C6.5(-k)-PP$a$ PIC. A maximum concentration of 4 mg ml$^{-1}$ was found to be possible to obtain but the efficiency of the reaction decreased significantly. Due to the lower water solubility of PP$a$ it was found that the centrifugation step was more effective at removing some of the free, non-covalently bound photosensitiser than with PS1. However the loading ratio was higher for PS1 than PP$a$ improving both the concentration and loading of the scFv.

It was not possible to purify the C6.5(-k)-PS1 PIC as attempted by size exclusion, affinity and hydrophobic interactions chromatography as well as dialysis, washes and the use of detergents to facilitate this. Slight improvement was seen with size exclusion but was not
considered sufficient. The main conclusion deduced from this part of the work is in accordance to that made by Savellano. It is not feasible to completely remove non-covalently bound PPa from PIC as these interactions are strong (Hamblin et al., 2000, Savellano et al., 2005b). The same, if not worse is true for PS1.

The characterisation of the C6.5(-k)-PS1 PIC using the most widely used methods (SDS-PAGE and UV/Vis spectroscopy) was achieved. Early efforts to characterise it by MALDI-TOF mass spectrometry were unsuccessful. The characterisation of the scFv and the PIC in terms of immunoreactivity was tested by FACS by other members in the group and was found to bind HER2 positive cells. The C6.5(-k)-PS4 PIC could not be synthesised as the synthesis of the PS4 AE was not completed within the timelines of this project. Retrospectively, a “mock” conjugation between PS4 acid Cl$^-$ and C6.5(-k) should have been attempted to observe how a monocationic PPa behaves in the presence of C6.5(-k). Future work will describe whether a cationic PPa derivative can exhibit reduced non-covalent binding when conjugated to C6.5(-k).

The in vitro and in vivo characterisation of the PIC will determine its efficiency, potency and improved pharmacokinetics compared to free PS1 and PPa. One of the most pertinent questions also raised by Savellano, is whether the PICs have the cell killing ability or the free non-covalently bound PS does (Savellano et al., 2003).

4.3.9 Future work

One of the first steps to facilitate improved characterisation of the PICs, would be to remove or move the his$_6$ tag. Replacing the c-myc tag with a tag that does not contain a lysine such as a HA tag would be ideal. The lysine present in the sequence of the c-myc tag is crucial for its binding to 9E10. Attempts to omit it or replace it with another amino acid resulted in much lower affinities (Hilpert et al., 2001). Therefore, the only option would be to either add another tag or replace the c-myc tag. The HA tag (YPYDVPDYA (Clontech Laboratories, 2008)) can be used in conjunction with the his$_6$ tag and does not contain a lysine or a histidine. Perhaps placing the his$_6$ tag prior to the HA tag would decrease the likelihood that the his$_6$ tag will form bonds with the PS (Cuatrecasas et al., 1972). This could allow the purification or at least detection of the PIC in various conditions. Further future work will
include engineering another scFv, targeting a different epitope relevant to PDT to introduce lysines spaced apart as on C6.5(-k).

Determination of the CMC followed by dynamic light scattering analysis could help modify the conjugation reaction conditions to improve the non-covalent binding. If possible, the conjugation reaction could be carried out below the CMC of PS1 in order for PS1 to remain monomeric in the solution. Theoretically, that should aid its purification and removal from the PIC sample. Once the PIC has been purified it could be freeze dried and reconstituted accordingly to obtain a more concentrated sample. Its bioactivity would have to be confirmed. Repeating the hydrophobic interaction chromatography, Bio-Beads® and looking into HPLC analysis for characterisation could be useful. Finding alternative means of visualising the wet unstained SDS-PAGE gels would also be helpful, such as a 670 nm laser (Savellano et al., 2005b).
Chapter 5 *In vitro* Characterisation
5.1 Introduction

5.1.1 In vitro cytotoxicity assays in PDT

Understanding the mechanisms via which PDT brings about cell death is important for the future of the field and although the area is currently under a lot of investigation, it is still in its infancy (Oleinick et al., 2002). Cell death is often loosely described so before trying to understand if and how it occurs during PDT treatment of cancer cells it would be prudent to review it. It is accepted that apoptosis, necrosis and autophagy are mechanisms of cell death; however when is a cell actually dead? Is there a clear “point of no return” when a cell can no longer recover? The Nomenclature Committee on Cell Death has recently suggested that a cell can be regarded as dead when its plasma membrane has been broken down, the cell has fragmented into apoptotic bodies including the nucleus and/or it has been phagocytosed (Kroemer et al., 2008).

Looking at cells post-PDT, apoptosis or necrosis markers can be detected and the route of cell death accounted for. FACS can be used in conjunction with propidium iodide and Annexin V assays to detect necrosis or apoptosis respectively (Piette et al., 2003). Laddering of DNA extracted from treated cells can also provide information regarding apoptosis (Oleinick et al., 2002, Sun et al., 2002). Studying the pathways and mechanisms of cell death in PDT, or indeed in any therapy is a complicated and difficult area and it will not be discussed extensively as it is beyond the scope of this thesis.

5.1.2 Measuring cell death in terms of cell viability - the MTS assay

Even though it might be more logical to measure cell death following PDT treatment, the assays used for the quantification and identification of cell death routes and mechanisms are complicated and time consuming. Subsequently, photosensitiser potency has been quantified using assays where the number of remaining viable cells is measured. These are common and largely accepted in the field of PDT and drug development. An example of such an assay is the colourimetric MTS assay which is linearly proportional to the live cell number present in a sample and was used to determine the potency of both photosensitisers and PICs in this work. The MTS assay is widely used as it is straightforward, quick and specific comparing well with conventional in vitro cell kill studies (Malich et al., 1997). Living and proliferating cells reduce the MTS tetrazolium reagent using NADH or NADPH to a
water soluble formazan compound that is coloured and whose absorption can be read at 490 nm. This absorption intensity is proportional to the number of live cells in the sample. There are limitations and potential artefacts due to background absorption and interference from the chemicals used in the initial part of the assay but these are quite limited.

PDT cell kill assays always include two sets of data/curves; one is for determining the PDT potency of the drug by laser/light activation of the drug. The second is a negative control curve corresponding to the dark experiment where the cells are pre-incubated with the PS but not subjected to laser treatment to activate the drug. As previously mentioned the bimodal nature of PDT means that the laser is required to excite the molecule in order to bring about cell death. Therefore, dark toxicity is an unwelcomed side effect often observed with various types of photosensitisers (Hamblin et al., 1998).

Throughout the literature the conditions and experimental set up of these types of assays vary significantly making it difficult to establish accurate comparisons between groups. Variability is seen in the incubation period, laser dose (intensity and time), time between post-PDT and cell viability quantification, cell lines and of course the growing repertoire of compounds being tested. Light sources also differ at both the in vitro and clinical setting with some groups using white light with appropriate filters or specific red lasers. Incubation periods of mammalian cells with standalone photosensitisers for various in vitro studies, range from 40 hrs (Sun et al., 2002, Savellano et al., 2005b), 24 hrs (Gariboldi et al., 2009, Zheng et al., 2009, Peng et al., 2010), 20 hrs (Sun et al., 2002), 12 hrs (Lim et al., 2009), 3 hrs, 2 hrs, 1 hr (Conway et al., 2008). In the case of photosensitiser-biomolecule conjugates the conditions also vary but as antibodies and other targeting molecules used also vary significantly, potency comparisons are difficult to make. When a targeting element is involved, the incubation period is governed by how long it requires to either internalise or to bind its target.

5.1.3 Subcellular localisation targets and imaging

In drug development, improving existing validated pharmaceuticals is important and in order to do so, potency comparisons need to be made. In PDT, subcellular localisation is critical as it is the first point of contact of ROS, including singlet oxygen, upon their generation within the cell and can determine the mode of cell death (Castano et al., 2004, Mojzisova et al.,
Singlet oxygen is short lived with a lifetime of 3 µs within a cell and only travels ~268 nm in 6 µs. Hence causing maximum damage at the first point of contact is of outmost significance (Egorov et al., 1989, Castano et al., 2004, Skovsen et al., 2005).

To understand the reasons for exhibited potency levels of different compounds, the intracellular levels and localisation of the given drug can provide useful information.

Using scanning laser confocal microscopes and organelle stains, a lot of information can be deduced about the localisation of a PS, its uptake and how that relates to the potency of the drug and the route to cell death (Castano et al., 2004).

**Mitochondria and Lysosomes**

Even though a large proportion of the existing successful photosensitisers were shown to localise in the mitochondria, it is not clear whether mitochondrial targeting is the key to an efficient PDT agent (Morgan et al., 2001). For example, pheophorbide a causes cell death to Jurkat cells via apoptosis which is triggered by the release of cytochrome c from the mitochondria following PDT damage of their membrane (Lee et al., 2004).

Although studies supporting the importance of mitochondrial localisation are fairly robust, studies in favour of lysosomal targeting are somewhat less clear.

Lysosomes are acidic organelles containing more than 50 hydrolases in order to allow for the degradation of macromolecules. These enzymes were initially considered as necrosis triggers but in the late 90s it was discovered that lysosomes release mainly Cathepsin D into the cytosol leading to necrosis (Johansson et al.). Lysosomal membrane permeabilisation which can lead to apoptosis initiation, can occur during oxidative stress and endoplasmic reticulum stress (Johansson et al.). There are many types and sizes of lysosomes and their roles and behaviour are still largely unclear. How does a cell respond to ROS being released within the lysosome? Lysosomal defence includes vitamins and antioxidant enzymes and can involve the inhibition of the release of cathepsin from the lysosome thus inhibiting apoptosis (Johansson et al.). Overall, subcellular localisation in lysosomes has not been considered a good PDT target. In most cases, lysosome-induced cell death was generally found to be less efficient than mitochondrial. Nonetheless there was evidence that a delayed apoptotic response was observed (Macdonald et al., 1999, Nagata et al., 2003). In some cases, following irradiation and subsequent lysosomal membrane permeabilisation, photosensitisers have been shown to re-distribute within the cells (Ambroz et al., 1994) and
relocalise in the cytoplasm or even the nucleus (Berg et al., 1991, Peng et al., 1991, Castano et al., 2004).

In contrast, one study showed that, a tetrapyrrrole, which accumulated primarily in the lysosomes exhibited higher potency via apoptosis compared to other derivatives with mitochondrial targeting and similar singlet oxygen quantum yields (Gariboldi et al., 2009). Similarly, Zheng also observed that compounds localising in the lysosomes appeared to be more potent than those localising mainly in the mitochondria (Zheng et al., 2009). Proteases present in the lysosomes should be able to induce apoptosis either by activating caspases or by damaging the mitochondria thus releasing cytochrome c followed by caspase activation (Kessel et al., 2000). Using N-aspartyl chlorin e6 and lysyl chlorin p6, two lysosomal localising photosensitisers, Kessel et al. monitored the effect their activation had on murine leukemia cells. It was obvious that lysosomal integrity was lost almost instantly whereas the mitochondria required at least 30 mins before the membrane potential started to decrease. DNA laddering was very clear within two hours post-treatment (Kessel et al., 2000). Therefore, there is clear evidence that lysosomal damage can lead to increased levels of caspase-3 in the cell and cause cell death (Kessel et al., 2000).

ER and Golgi

The significance of mitochondria has been studied extensively and that of the lysosomes is under investigation, but the significance of the ER and the Golgi apparatus is less clear. Being able to successfully trigger apoptosis from the ER and/or the Golgi apparatus (Matroule et al., 2001) is a positive prospect as many PPα derivatives tend to significantly localise in hydrophobic compartments which are protein-rich such as the ER and the Golgi. Foscan, one of the most potent PSs was found to localise primarily in the ER and Golgi with very little localisation in the mitochondria and lysosomes (Teiten et al., 2003). The potency and intracellular localisation of a PPα derivative, DH-II-24, was studied by a group looking at colorectal cancer therapy (Lim et al., 2009). The compound showed minimal dark toxicity and co-localisation studies showed that it targets mitochondria, lysosomes and the ER. This pattern is similar to others published for PPα (Sun et al., 2002, Chen et al., 2005). FACS analyses conducted by Matroule using MePPα and colon cancer cells monitoring mode of death showed that within 3 hrs of PDT the mode of cell death was primarily necrosis. Furthermore, MePPα localisation was found to be primarily in the ER/Golgi and lysosomes leading to the triggering of apoptosis (Matroule et al., 2001).
Chlorin e6 was shown to internalise primarily in the plasma membrane mainly via absorptive endocytosis and somewhat by passive diffusion (Mojzisova et al., 2007). Chlorin p6 was shown to localise mainly in the ER and the golgi apparatus with minimal localisation in the lysosomes and none in the mitochondria. It was also demonstrated that the ER localisation caused Ca\(^{2+}\) release inhibition (Begum et al., 2009). Monocationic cycloimide derivatives of cp6 were shown to localise in lysosomes compared to non-polar and anionic derivatives (Nazarova et al., 2007). Modifications in the structure of the monocationic derivatives only affected their uptake and not the intracellular target (Nazarova et al., 2007). These were also shown to internalise via caveolae-dependent endocytosis and were stable in the lysosomes even after a 7 hr incubation (Nazarova et al., 2007). These lysosomal targeting monocationic derivatives were also shown to be up to 10 times more potent than the parent cp6 but less potent than neutral and anionic cycloimide derivatives. Following irradiation and subsequent loss of lysosomal membrane integrity the PS leaked into the cytoplasm releasing proteases. There appears to be a barrier after which a cell can no longer cope with the release of lysosomal enzymes into its cytoplasm and undergoes necrosis as shown by PE-annexin and PI staining (Nazarova et al., 2007).

5.1.4 Structure activity relationship

Quantitative structure-activity relationship studies are fuelled by the need to understand which features are important when designing the synthesis of a new PS. These can be summarised as charge (neutral, anionic or cationic), hydrophobic character as determined by log \(P\) and the asymmetry of the molecule (Castano et al., 2004). It appears from the numerous emerging studies that designing a compound in order to facilitate a certain behaviour and actually achieving it is very different. When altering the hydrophobicity of a compound its internalisation route can change leading to diffusion or endocytosis (Castano et al., 2004).

Large aromatic structures such as porphyrins and phthalocyanines will be internalised by cells depending on their charge, hydrophobicity and charge distribution (Li et al., 2008). Octa-cationic phthalocyanines localised primarily in lysosomes exhibiting \textit{in vitro} potencies in the range of 2.2 µM with singlet oxygen quantum yields in the range 0.09-0.15 (DMSO) (Li et al., 2008). This perhaps indicates that high singlet oxygen quantum yield is not a necessary pre-requisite for a potent PS.

Recently Peng et al. conducted a study trying to relate structure, lipophilicity, localisation and potency. They observed that the hydrophilic derivatives of their cationic porphyrins showed
the lowest uptake by HeLa cells. These exhibited the lowest dark toxicity, whereas the other derivatives showed a significant amount of unwanted dark toxicity upon a 24 hr incubation with the cells (Peng et al., 2010). It is important to mention that their tetracationic derivative (also with the lowest log $P$) exhibited the worst potency on HeLa cells.

Dietel et al. looked at the potential disaggregation of a photosensitiser following its internalisation using time resolved spectroscopy and microscopy (Kelbauskas et al., 2002). They claimed that PS aggregates can internalise in cells via non-receptor mediated endocytosis and can partially disaggregate once in the intracellular environment (Kelbauskas et al., 2002). This was also shown by Joshi et al. (Begum et al., 2009). It also appears that aggregated PS tend to localise in the lysosomes and endosomes (Kelbauskas et al., 2002). A phthalocyanine was shown to relocate within a cell following a 24 hr incubation with its cytotoxicity improving when it was mainly located in the ER inducing apoptosis (Rück et al., 2000). It is therefore evident that potency varies with subcellular localisation, photosensitiser and incubation period.

### 5.1.5 Photoimmunoconjugates, C6.5(-k) and HER2

Depending on the antibody, PICs can be internalising or non-internalising. Whether internalising PICs are more potent is still under review (chapter 1). For example, in their initial studies, van Dongen showed that an internalising antibody PIC was more potent than a non-internalising PIC (Vrouenraets et al., 1999). Furthermore, the same group characterised phthalocyanines with increased hydrophilicity and showed that they exhibited reduced uptake and subsequently very poor cytotoxicity. Contradicting this, the same PSs, once conjugated the resulting PIC showed 9000 fold increased potency (Vrouenraets et al., 2001). The targeting ability of the antibody converted the non-potent tetrasulfonated phthalocyanines into highly potent photosensitisers by either binding and or internalisation to the cell (Vrouenraets et al., 2001, Vrouenraets et al., 2002).

Antibodies can be internalising or surface bound depending on their antigen. C6.5 internalises by receptor mediated endocytosis. Incubation of C6.5 scFv with SkBr3 for 2 hrs at 37°C in PBS showed its endosomal localisation (Becerril et al., 1999). Native C6.5 scFv binds to the extracellular domain of ErbB2 with an affinity of 16 nM (Schier et al., 1995) with $k_{on}$ 4x10$^5$ M$^{-1}$s$^{-1}$ and a $k_{off}$ 6.3x10$^{-4}$ s$^{-1}$. The mutated C6.5(-k) has an affinity of 9.8 nM with a $k_{on}$ 4.3x10$^5$ M$^{-1}$s$^{-1}$ and a $k_{off}$ 4.2x10$^{-4}$ s$^{-1}$ (Schier et al., 1996). The affinities and kinetic constants for the two scFvs are not significantly different. C6.5 diabody binds HER2 in a bivalent manner with an affinity of 0.4 nM (Adams et al., 1998). C6.5 was shown to bind
native c-erbB2 on SkBr3 cells (Schier et al., 1995). It is noteworthy that native C6.5 was found to bind native c-ErbB2 on cells with a high koff leading to quick dissociation in vitro leaving only less than 30% of scFv bound on the cells after 15 mins (Schier et al., 1995).

### 5.1.6 Aims and objectives

The aims of the work described in this chapter were to characterise the three main PSs described in chapter 3 in vitro. Further to that, the conjugates described in chapter 4 were also to be characterised in vitro. The intracellular localisation of both the PSs and their PICs would be determined in order to obtain basic information on structure-intracellular localisation-activity relationship.

Using SKOV3 HER2 positive cell line and KB HER2 negative cell line, the potencies of the three main PSs (shown in figure 5.1) would be determined using cytotoxicity assays. Using the same experimental setup, the efficacy of the PICs would be determined. Finally, using confocal microscopy and suitable intracellular organelle stains available commercially, the localisation of both the free PSs and PICs would be determined. Using the obtained information, an assessment of how potency and intracellular localisation relate will be performed would be made.

![Figure 5.1 Structures of the main PSs](image)

**Figure 5.1 Structures of the main PSs.** PPa (left), PS1 (centre) and PS4 (right) are shown. Their synthesis was described in chapter 3.
5.2 Results

5.2.1 In vitro cytotoxicity assays

Control experiments

Some of the first experiments conducted aimed at optimising the conditions for the cytotoxicity assays. Important control experiments included calibrating the MTS assay to each cell line as shown in figure 5.2 in order to adjust the cell number plated for each cell line accordingly. The aim was to have a number of cells that would give approximately the same absorption reading for the MTS assay for 100% cell survival readings. Following this, the laser dose was calibrated by finding an energy dose that was well tolerated by the control cells and which combined with a known high concentration of PPα (400 µM, (Bhatti et al., 2008)) would bring about a complete cell death as shown in figure 5.3. Following irradiation the cells were allowed to grow for 48 hrs before the cell viability assay (MTS) was conducted. This is considered as a suitable time gap to allow for both necrosis and apoptosis to take place (Goldstein et al., 2000, Berghe et al., 2010).

Figure 5.2 Cell number calibration curves of SKOV3 and KB for cell kill assays. The assay was conducted by plating various numbers of cells in quadruplicates (n=4) that underwent a “mock” cell kill assay where only PBS was used instead of a PS and their viability was measured. The cell number corresponding to ~1.5 AU was chosen for all cell kill assays thereon.
Finding the optimum Laser Dose for PS activation and subsequent complete cell death

Figure 5.3 Laser dose calibration using SKOV3 cells. A cytotoxicity assay with SKOV3 cells using a constant concentration of PPa (400 µM) whilst varying the laser dose was run in order to find the lowest laser dose that resulted in complete cell death. The chosen dose (2.5 AU (laser intensity units, arbitrary, see section 2.2.11)) should allow for complete activation of any photosensitiser which has similar photophysical properties to PPa. PPa does not exhibit any dark toxicity therefore the cell death observed at this concentration is due to the activation of the PS.

Free Photosensitiser Cell kills

Figure 5.4 shows the cytotoxicity assay of PPa on both cell lines, whereas figures 5.5 and 5.6 show the assays corresponding to PS1 and PS4 respectively. There was some dark toxicity observed in the case of KB with PS4. The rest of the assays showed insignificant dark toxicity up to 100 µM of photosensitiser. PS1 was found to be more potent than PPa and PPa more potent than PS4 on both cell lines. In SKOV3 cells, PS1 is 13 fold more potent than PPa and PPa is 18 fold more potent than PS4. In KB cells, PS1 and PPa showed similar potencies whereas they are both 20 fold more potent than PS4. Table 5.1 summarises all the IC \textsubscript{50} values for the various assays conducted in this section.

PICs cell kills

The dose-response curves for the PICs are shown in figures 5.7, 5.8 for both cell lines and corresponding to the conjugates of C6.5(-k) scFv and PPa, and PS1 respectively. The C6.5(-k)-PS1 conjugate, is 20 fold more potent on SKOV3 than the C6.5(-k)-PPa conjugate. On the antigen negative cell line, KB, the PS1 conjugate was over 40 fold more potent than the PPa conjugate. Even though the observed cytotoxicity on the antigen negative cell line is
unwelcomed, these figures show that the PS1 conjugate is at least 20 fold more potent than the parent PPa conjugate.

**Figure 5.4 Dose dependent response curves of PPa on cancer cell lines SKOV3 and KB.** Each experiment included a dilution series as a dark control to monitor dark toxicity, a 2% DMSO/ PBS sample as the 100% cell survival control and a Triton X-100 1% solution as the 100% cell death control. The smooth curves are a result of a fit to the data (● and ▲ symbols) in SigmaPlot using a 3-parameter sigmoidal logistic equation. The data points are an average of minimum 4 (n=4). Errors are standard errors.
Figure 5.5 Dose dependent response curves of PS1 on cancer cell lines KB and SKOV3. Each experiment included a dilution series as a dark control to monitor dark toxicity, a 2 % DMSO/ PBS sample as the 100 % cell survival control and a Triton X-100 1% solution as the 100 % cell death control. The smooth curves are a result of a fit to the data (● and ▲ symbols) in SigmaPlot using a 3-parameter sigmoidal logistic equation. The data points are an average of minimum 4 (n=4). Errors are standard errors.
Figure 5.6 Dose dependent response curves of PS4 acid Cl⁻ on cancer cell lines KB and SKOV3. Each experiment included a dilution series as a dark control to monitor dark toxicity, a 2 % DMSO/ PBS sample as the 100 % cell survival control and a Triton X-100 1% solution as the 100 % cell death control. The smooth curves are a result of a fit to the data (● and ▲ symbols) in SigmaPlot using a 3-parameter sigmoidal logistic equation. The data points are an average of minimum 4 (n=4). Errors are standard errors.
Figure 5.7 Dose dependent response curves of C6.5(-k)-PPa PIC on cancer cell lines KB and SKOV3. Each experiment included a dilution series as a dark control to monitor dark toxicity, a PBS sample as the 100 % cell survival control and a Triton X-100 1% solution as the 100 % cell death control. The smooth curves are a result of a fit to the data (● and ▲ symbols) in SigmaPlot using a 3-parameter sigmoidal logistic equation. The data points are an average of minimum 4 (n=4). Errors are standard errors.
Figure 5.8 Dose dependent response curves of C6.5(-k)-PS1 PIC on cancer cell lines KB and SKOV3. Each experiment included a dilution series as a dark control to monitor dark toxicity, a PBS sample as the 100 % cell survival control and a Triton X-100 1% solution as the 100 % cell death control. The smooth curves are a result of a fit to the data (● and ▲ symbols) in SigmaPlot using a 3-parameter sigmoidal logistic equation. The data points are an average of minimum 4 (n=4). Errors are standard errors.
5.2.2 Imaging by confocal microscopy

**Free Photosensitisers**

Images were taken by exciting the samples with the Argon ion laser (488 nm and 496 nm) as well as the diode laser (405 nm) using a water objective 63 x (NA=1.23). Further details were provided in chapter 2 including information on the organelle markers (2.2.8). The photosensitiser attributed fluorescence emission is depicted with the red pseudo colour and the co-stain, green dyes are depicted with the green pseudo colour in all samples. The overlap, i.e. co-localisation of two dyes in a sample is shown in yellow/orange. It is important to note that the shadows that are visible in some of the images (these are more apparent in the brightfield images and in the red channel) were due to a long running technical problem with the microscope. In some cases, the uneven colour intensity lead to two colour images containing areas of less intense red fluorescence and subsequently overlaid images were affected. The centre of the image was generally the best to use and when zooming in the problem appeared to be nearly non-existent therefore it did not overall impede with the accuracy of the experiments. Attempts to rectify the shadows by digital modification of the images were too time consuming, the resulting images were not significantly improved and it was considered best to use them as they were.

**Co-localisation studies**

During early imaging attempts, it became apparent that the inherent phototoxicity of our photosensitisers was going to be an obstacle in obtaining images of healthy looking cells. For example, PPa gave brightfield images that contained a significant amount of cell debris or aggregates possibly due to the low solubility of the photosensitiser in media (as seen in Figures 5.9A and 5.11). Following numerous optimisation steps, the protocols described in chapter 2 were obtained and finally applied. Very stringent conditions with regards to minimal light exposure were applied and the cells maintained as much as possible warm and under a CO₂ atmosphere.

**PICs and Transferrin**

Fluorescence images of the three main photosensitisers along with the corresponding brightfield image are shown in figure 5.9 where the photosensitisers are excited using the UV laser 405 nm and the emission observed at 600 nm. From the spectrum of the photosensitisers (figure 2.2, section 2.2.8) the 405 line is the most suitable as it is near the largest absorption maximum. The images obtained with the red laser were not bright enough. Once the conditions were set for the free photosensitisers, dyes that target and stain specific intracellular organelles such as MitoTracker® Green Fm, Lysotracker® Green
DND-26, ER-Tracker™ Green (BODIPY® FL glibenclamide), Bodipy® FL C5-ceramide complexed to BSA and DAPI nucleic acid stain were used to stain the mitochondria, lysosomes, the ER, the Golgi apparatus and nucleus respectively. See chapter 2, table 2.2 for details on their characteristics. Despite several examples of staining SKOV3 cells with various organelle specific dyes being published (Sun et al., 2006, Kosaka et al., 2009), we were not able to reproduce these and had to optimise the incubation times, temperature and concentrations until they reproducibly worked in our hands. The mitochondrial, Golgi apparatus and nuclear stains were the easiest to reproduce consistently whereas the ER and lysosome stains were more sensitive to changes in temperature and incubation periods. Staining with the transferrin conjugate was also very straightforward contrary to the commercially available anti-HER2 conjugate.

Sample images where these organelles were specifically stained using green coloured dyes are shown in figure 5.10. In some of the initial studies, the orange/ red analogues of the MitoTracker® and Lysotracker were used. Although on most occasions these did not overlap at all with the photosensitiser emission, it was decided to use the green dyes to ensure crosstalk was minimal throughout.

![Figure 5.9 Confocal fluorescence microscopy images of the three main photosensitisers on live SKOV3 ovarian cancer cells. Brightfield, white light transmission images (A, D, G) are shown on the left. Middle and right panels are fluorescence images with the third panel showing an example of magnified cells. Panels A-C show PPα, D-F show PS1 and G-I show PS4 (PS4 acid Cl⁻). Samples were excited at 405 nm and detected from 650 nm.](image-url)
Subsequently, co-localisation studies with SKOV3, the three photosensitisers and the four dyes were carried out as described in chapter 2. The various co-localisation images are shown in figures 5.11, 5.12, 5.13, 5.14 and 5.15 for the three main free photosensitisers. In figure 5.11, PPα is shown to co-localise mainly with the Bodipy dye and somewhat with the ER tracker and MitoTracker. Overlap was not observed for DAPI and LysoTracker.

For each experiment, the necessary controls were always run in parallel, i.e. photosensitiser only and co-stain only. The possibility of crosstalk between the two channels on each occasion was examined. A control sample containing cells only was often but not always run in conjunction with the other controls. It was not deemed necessary to always use this control as the fluorescence emission observed on those occasions in the channels that were monitored was insignificant especially since the fluorescence observed in the positive samples was always very strong and the gain was always kept below 1000 units.

Finally, for studying the targeted internalisation of PS1 via the C6.5(-k)-PS1 PIC (figures 5.16 and 5.17) both SKOV3 and KB cells were incubated with the conjugate at 100 µg/ml of PIC (corresponding to 100 µM PS1, 3.3 µM of scFv) or 100 µM of PS1 control for 1 hr 30 mins and then fixed (including PS samples). Other samples included the Transferrin Alexa Fluor® 488 conjugate which was also subsequently fixed. Attempts to study the internalisation of the scFv alone using an anti-myc mAb followed by an anti-mouse secondary were unsuccessful as were attempts to use an anti-HER2 FITC-labelled commercial mAb.
**Figure 5.10** Confocal fluorescence microscopy images of control stains on live SKOV3 cells. Panels A, D, G, J, M show white light transmission images. Middle and right panels are fluorescence images with the third panel showing an example of magnified cells. Panels A-C show MitoTracker® Green FM (mitochondrial staining), D-F show ER-Tracker™ Green (endoplasmic reticulum staining), G-I show Bodipy® FL C5-ceramide (golgi apparatus staining), J-L show Lysotracker® Green DND-26 and M-N show DAPI (nucleic acid staining).
Figure 5.11 Confocal fluorescence microscopy images studying the co-localisation of PPα on live SKOV3 cells. Panels A, E, I, P, W show white light transmission images. All other panels show fluorescence images. All samples are stained with PPα (in red, left fluorescence image); middle panels show the co-stain and the right panels show the overlaid image. Panels A-D are co-stained with DAPI nuclear staining, panels E-H are co-stained with ER-Tracker™ Green (endoplasmic reticulum staining); panels I-O are co-stained with Bodipy® FL C5-ceramide (golgi apparatus staining) (M-O magnified), panels P-V are co-stained with Lysotracker® Green DND-26 (lysosomal staining) (T-V magnified) and panels W-Z are co-stained with MitoTracker® Green FM (mitochondrial staining) and A’-C’ are magnified and stained with MitoTracker® Orange CMTMRos.

Figure 5.12 Confocal fluorescence microscopy images studying the co-localisation of PS1 on live SKOV3 cells. Panels A, E, L show white light transmission images. All other panels show fluorescence images. All samples are stained with PS1 (red, left panel); middle panels show the co-stains and the right panels show the overlaid images. Panels A-D are co-stained with DAPI nuclear staining, panels E-K are co-stained with ER-Tracker™ Green (endoplasmic reticulum staining) (I-K magnified), panels L-R are co-stained with Bodipy® FL C5-ceramide (golgi apparatus staining) (P-R magnified).
Figure 5.13 Confocal fluorescence microscopy images studying the co-localisation of PS1 on live SKOV3 cells. Panels A, H, L, S show white light transmission images. All other panels show fluorescence images. All samples are stained with PS1 (red, left panel); middle panels show co-stains and the right panels show the overlaid images. Panels A-K are co-stained with Lysotracker® Green DND-26 (Lysosomal staining). A-D show zoomed out image whereas E-G zoom into a particular cell (part of A-D) and then H-K zoom out again to the
Figure 5.14 Confocal fluorescence microscopy images studying the co-localisation of PS4 acid Cl⁻ on live SKOV3 cells. Panels A, E, L show white light transmission images. All other panels show fluorescence images. All samples are stained with PS4 (red, left panel); middle panels show co-stains (green) and right panels show overlaid images. Panels A-D are co-stained with DAPI nuclear staining, panels E-K are co-stained with ER-Tracker™ Green (endoplasmic reticulum staining) (I-K magnified), panels L-R are co-stained with Bodipy® FL C5-ceramide (golgi apparatus staining) (P-R magnified).
Figure 5.15 Confocal fluorescence microscopy images studying the co-localisation of PS4 acid Cl⁻ on live SKOV3 cells. Panels A and E show white light transmission images. All other panels show fluorescence images. All samples are stained with PS4 (red, left panel); middle panels show co-stains (green) and right panels show overlaid images. Panels A-D are co-stained with Lysotracker® Green DND-26 (Lysosomal staining); panels E-K are co-stained with MitoTracker® Green FM (mitochondrial staining) (I-K magnified).
Figure 5.16 Confocal fluorescence microscopy of fixed SKOV3 cells with C6.5-(k)-PS1 PIC. Panels A, C, F, M show white light transmission images. All other panels are fluorescence images. Fluorescence in B, G, J, N, Q is due to PS1 (red); fluorescence in D, E, H, K, O, R is due to green co-stains. I, L, P, S are overlaid images. A-B are C6.5-(k)-PS1 PIC; C-E show staining with Transferrin Alexa Fluor® 488 conjugate; F-L show the co-localisation of C6.5-(k)-PS1 PIC with Transferrin Alexa Fluor® 488 conjugate (J-L are magnified images); M-S show the co-localisation of PS1 free photosensitiser with the Transferrin Alexa Fluor® 488 conjugate.
Figure 5.17 Confocal fluorescence microscopy of fixed KB cells with C6.5(-k)-PS1 PIC. Panels A, C, E show white light transmission images. All other panels are fluorescence images. B, F, I fluorescence is due to PS1 (red); fluorescence in D, G, J is due to Alexa Fluor® 488 fluorescence (green); H, K are overlaid images. A-B are C6.5(-k)-PS1 PIC; C-D show staining with Transferrin Alexa Fluor® 488 conjugate; E-K show the co-localisation of C6.5(-k)-PS1 PIC with Transferrin Alexa Fluor® 488 conjugate (I-K are magnified images).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM) of SKOV3 ± Standard Error</th>
<th>IC₅₀ (µM) of KB ± Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laser</td>
<td>Dark</td>
</tr>
<tr>
<td></td>
<td>Rel. PPₐ</td>
<td>Rel. PS</td>
</tr>
<tr>
<td>PPa</td>
<td>14.5 ± 3.2</td>
<td>1</td>
</tr>
<tr>
<td>PS1</td>
<td>1.1 ± 0.2</td>
<td>13.2 ↓</td>
</tr>
<tr>
<td>PS4</td>
<td>259.6 ± 492.3</td>
<td>18 ↑</td>
</tr>
<tr>
<td>C6-PPa PIC</td>
<td>6.9 ± 0.8</td>
<td>2.1 ↓</td>
</tr>
<tr>
<td>C6-PS1 PIC</td>
<td>0.33 ± 0.1</td>
<td>44 ↓</td>
</tr>
</tbody>
</table>

Table 5.1 IC₅₀ values of the various PS/PICs as calculated using SigmaPlot. Standard Errors are given only for the phototoxicity values as the IC₅₀ values for the dark experiments contain very large errors due to the lack of data around the corresponding values. Rel. PPₐ columns indicate comparison of IC₅₀s in relation to PPa, Rel. PS columns indicate comparison of IC₅₀s with relation to the PS itself- for example C6(-k)-PPa PIC in relation to PPa and C6(-k)-PS1 in relation to PS1. ↑ indicates fold-increase, ↓ indicates fold-decrease.
5.3 Discussion

5.3.1 Controls used in in vitro cytotoxicity assays

A simple, effective and reliable method was necessary to quantitatively characterise the potency of the new photosensitisers and the respective photoimmunoconjugates. As it was beyond the scope of this work to establish the mode of cell death, the MTS assay was chosen as it is straightforward and quantitative. The MTS is an accepted assay of drugs throughout the literature. Cells were treated with the tetrazolium compound reagent which is reduced by the live cells to the coloured formazan.

Several factors can affect the efficiency, reproducibility and reliability of a PDT cell kill assay. These include the concentration and incubation time with the photosensitising agent, the laser dose and the cell type used as well as technical variability from person to person such as handling, protection from light etc. It is also important to note that the sensitivity of the assay can differ with cell line (Malich et al., 1997).

Control experiments were conducted in order to set up the assay in an efficient way. The number of cells that were going to be used for each cell line had to correspond to similar absorbance units during the MTS assay. The colorimetric change obeys the Beer-Lambert law and is therefore linearly proportionate to the cell number in the sample as verified by the control experiment shown in figure 5.2. The cell number corresponding to an MTS absorbance value of 1.5 was chosen for every cell line (see chapter 2). This value is large enough to allow significant readings to be obtained across the range of cell death samples and small enough not to exceed the limits of detection of the assay. Other control experiments included establishing a laser dose that would lead to complete cell death given a high enough concentration of PPa and verifying the laser is not cytotoxic. Finally, the use of DMSO at 2 % in the samples is well tolerated by the cells and was always used as part of the control samples.

These were important experiments to further establish the validity of the cell kill assays following the earlier work by Bhatti et al. (Bhatti et al., 2008).
5.3.2 Cytotoxicity assays with the free PSs

In order to establish a reference point, PPa was used as the standard PS. We have successfully improved on the IC\textsubscript{50} values (summarised in table 5.1) of PPa (41 µM for KB and 93 µM for SKOV3) but not of the C6.5(-k)-PPa (1.5 µM for SKOV3, no cell kill for KB) as previously published (Bhatti et al., 2008). The improvement could be attributed to the refinement of the experimental setup which included optimised cell number, media, DMSO content and laser dose. These factors combined could have lead to the differences in potencies.

Overall, KB cells were found to be more sensitive to PDT than SKOV3 and other cell lines used by other members of the group (not shown or discussed here) (Jiang et al., 1992). Head and neck cancers are considered more prone to free radical damage (Choi et al., 2009). An oversensitive control cell line is perhaps not a good control.

PS1 and PS4 photosensitisers exhibited very different potencies against SKOV3 and KB. Even though KB were found to be more sensitive compared to SKOV3 when treated with PPa, this was not true for PS1. Comparing the two cell lines, they appeared to be equally sensitive to PS1. On the contrary, despite PS4 being more than 18 times less potent than PPa on SKOV3 and 20 times less potent on KB, it was 12 times more potent on KB than SKOV3, similar to PPa which was 10 times more potent on KB than SKOV3. The dark toxicity of these three photosensitisers on the two cell lines tested was negligible and any shown was above the IC\textsubscript{50}.

5.3.3 Cytotoxicity assays with the PICs

Both C6.5(-k)-PPa and C6.5(-k)-PS1 anti HER2 conjugates were shown to be potent against HER2 positive SKOV3 cells. Using the C6.5(-k)-PS1 PIC, a 3.3 fold increase in potency was observed when compared to free PS1. C6.5(-k)-PPa PIC was only two-fold more potent than free PPa. This was not considered a significant improvement.

The PPa conjugate was more than two-fold less potent on KB than SKOV3 whereas the PS1 conjugate is slightly less potent on KB than SKOV3 (see table 5.1). Since KB cells are the antigen negative cell line, the difference in potencies should have been much bigger. Overall, the PS1 conjugate appears to be more potent than the PPa conjugate but the PPa conjugate appears to be slightly more selective than the PS1 conjugate. This is probably due to the higher aqueous solubility of PS1 compared to PPa, which could enhance leakage of the non-covalently bound material from the PICs. It could bind other proteins present in the
media and be internalised as a free PS. The more hydrophobic nature of the parent PPa would most likely favour its association-even non-covalent binding- with the scFv hence the increased targeting.

KB, a HER2 negative cell line was subjected to PDT with both PPa and PS1 conjugates. Unlike SKOV3, the difference in potency for the PPa PIC was 13 times worse than the free PPa whereas for PS1 it was 3 times worse. Ideally the antiHER2 PICs should not show any efficacy towards a HER2 negative cell line. There is however enough evidence to suggest some preferential toxicity due to targeting on SKOV3. The cell death observed on KB by both sets of PICs is attributed to the non-covalently bound PS that leaks out of the hydrophobic pockets of the PIC and can act as a free PS internalising and killing the cells. The amount of PS that internalises in the case of KB is limited to solely the non-covalently bound material whereas for the SKOV3 cells it is a combination of both. Considering the increased sensitivity of KB to the free PSs, one would expect the potency of the PIC to be greater for KBs compared to SKOV3 if KBs internalised the PIC in the same ways as SKOV3. In the case of PPa, SKOV3 were shown to be 12 times more resistant than KBs, which decreases to 0.4 times in the case of the conjugate. This can be attributed to the internalisation of less PS as non-covalent binding in the case of the PIC for KBs. Using free PS cell kills and antibody only samples as controls, one can reach the above conclusions with some certainty. C6.5 scFv does not exhibit dose dependent cytotoxicity (Poul et al., 2000).

It has previously been shown by Savellano that a PPa anti HER2 conjugate had no efficacy on an HER2 negative cell line (Savellano et al., 2005b). This was successful when they used conjugates containing <10 % NCB PPa and more importantly, their maximum PPa concentration in the conjugates was 300 nM. This is more than tenfold lower than the maximum concentration used in our studies. In another set up, comparing conjugates compared to free PS potency, there was a maximum 10 fold increase in potency for the conjugate (Malatesti et al., 2006). Cases have been reported where conjugates were less potent than the corresponding concentration of free photosensitisier even when the uptake levels were the same (Savellano et al., 2003, Savellano et al., 2005b).

When covalently attaching photosensitisers to biomolecules, such as an scFv the inhibition of the binding of the antibody to the antigen as well as reduced solubility of the conjugate can arise (Staneloudi et al., 2007, Bhatti et al., 2008). The latter has been discussed in
chapter 4. The C6.5(-k) antibody was engineered to replace the lysine to an alanine at the antigen binding site to address the former point. It was previously shown that the resulting PIC is active in vitro as well as in vivo, therefore minimising the possibility of reduced affinity or binding due to covalent modification of one of the CDRs amino acids (Schier et al., 1996, Adams et al., 2000, Bhatti et al., 2008).

Even though the scFv has been engineered to remove the lysine from the binding site (Schier et al., 1996, Adams et al., 2000, Bhatti et al., 2008) hence inhibit any covalent bonds from forming, it is still possible that non-covalently bound photosensitisers are present in the binding site (Savellano et al., 2005b). This would form an obstacle and diminish the ability of the scFv to bind to the antigen hence the difference between HER2 positive and negative cell line cytotoxicity would become even smaller. It might be extreme to suggest but there is a possibility that upon purification of the conjugate the absence of non-covalently bound material could improve the cytotoxicity of the PIC. The disaggregation of the covalently attached molecules and restoration of their photophysical properties could facilitate this (Alonso et al., 2010).

It was observed early on that non-covalently bound material present in the conjugates was going to affect the results of the in vitro cell kill assays. Hence, the extensive attempts described in chapter 4 of purifying the conjugate. Consequently, the benefits of targeted therapy were somewhat overshadowed by the non-specific cytotoxicity exhibited by these non-covalently bound molecules which remain active and potent regardless of whether they are covalently attached to the antibody fragment. On the contrary, the importance of targeting is exemplified by the fact that the antigen negative cell line appears to be comparatively less prone to cell death than the antigen positive cell line when using the PIC.

It is significant to point out that during the cell kill assays, the PICs solutions were prepared in PBS whereas the free PS were prepared in serum containing media. The use of PBS was chosen in order to restrict the non-covalently bound PS from disaggregating and leaking from the scFv once in the presence of large proteins (such those present in sera). Free PSs were prepared in media to minimise their aggregation. A strengthening argument towards the disaggregated state of the non-covalently bound PS in a cellular environment is that not only in the presence of BSA or FCS does the absorption peak shift but in the cells as well (Sun et al., 2002). The disaggregation of PPa in a protein rich environment formed the basis
of the calculations used to work out the loading ratio of C6.5(-k)-PPa PIC by Kuimova et al (Kuimova et al., 2007).

In summary, both PICs showed improved efficacy on SKOV3 compared to the free photosensitiser. On KB cells both PICs showed decreased efficacy compared to the free photosensitiser. Whether to attribute the cell kill effects to free/ NCB photosensitiser or covalently attached ones is one of the main questions that remain indirectly unanswered from this work. It has long been considered a controversial issue that holds targeted PDT back (Savellano et al., 2005b).

Transfecting a HER2 negative cell line with a HER2 expression vector to obtain cells that express the antigen would provide an ideally matched positive/ negative control. The negative control would consist of the cell line transfected with a control vector. Using this approach, the cytotoxicity results can be more accurately compared and cell death correlated to the targeting specificity. This would also eliminate the KB/SKOV3 susceptibility ambiguity. Similar methods have been used by others (Carcenac et al., 2001, Nielsen et al., 2002, Hudson et al., 2005). Another option would be to screen for an antigen negative cell line with similar susceptibility to free PPa. In vitro cytotoxicity does not necessarily translate to in vivo potency as one set up is static (plastic dish) and the other is flowing with numerous parameters such as the immune system affecting the outcome of a therapy (Kabingu et al., 2009).

Overall, the PIC experiments need to be further verified and optimised. A starting point would be to allow longer binding times for the PICs up to 24-48 hrs at 37 °C to allow receptor mediated endocytosis to occur more efficiently. Boyle et al incubated cells in serum free media for 15 mins at 37 °C obtaining maximum binding (Staneloudi et al., 2007). An anti-HER2 conjugate bound on SKOV3 within 30 mins, internalised within 90 mins and formed vesicles within 240 mins as observed by immunofluorescence microscopy (Carcenac et al., 2001). Therefore, optimisation using uptake experiments combined with FACS or confocal microscopy are necessary.

The non-specific cytotoxicity observed by the PIC on KB enforces the question; is the killing observed when using a PIC due to the conjugate and the conjugated PS or due to the non-
covalently bound material (Savellano et al., 2003)? Savellano also observed non-specific cell killing when using an anti-EGFR antibody conjugated to BPD with large amounts of non-covalently bound impurities. On the contrary, when using purified conjugates containing <5 % NCB photosensitiser, the negative control cell line did not exhibit any significant uptake of the PIC. When killing cells using these purified PICs, they observed a potency increases with increased incubation period of up to 40 hrs (Savellano et al., 2003).

5.3.4 Dark toxicity

Dark toxicity of a PS is acceptable when it is at a concentration 10-fold above its phototoxic equivalent (Gariboldi et al., 2009). Therefore, it can be assumed that the dark toxicity observed here is within the acceptable limits. Generally, dark toxicity in vitro does not necessarily correlate to dark toxicity in vivo as the pharmacokinetics and hence clearance is different. In vivo, dark toxicity would not be an issue as the working concentrations of the PS as part of the PIC would be much lower than the dark cytotoxic concentration. This argument is likely to be strengthened when using a targeting agent to deliver the photosensitiser. The dark toxicity observed particularly in the case of KB can be attributed to the more sensitive nature of the KB cells.

5.3.5 Confocal microscopy using free photosensitisers

Realising that PS4 did not behave as would be expected given its photophysical properties and that the difference in potency between PS4 and PS1/PPa is significant (see table 5.1), it was deemed necessary to look at the intracellular localisation of these molecules.

During the early microscopy experiments it was found that the published methods could not be applied in our set up/cell lines. The conditions detailed in the methods section are a result of extensive attempts to obtain sufficient staining of the cellular components. Initial confocal microscopy experiments looked at the uptake timelines and necessary concentrations of PPa and PS1 required to obtain sufficient staining of SKOV3. However, a clear distinction between the various timelines on live cells was not possible and a 20hr incubation period was chosen for all the experiments. This time gave sufficient staining, no observed dark toxicity and allowed for small incubation time increase/decrease to occur without having a significant effect on the uptake and localisation. The amount of DMSO (0.5 %) used in the solutions was found to be critical in the case of PPa as large aggregates and cell debris
were seen when it was omitted. The chosen concentration of photosensitisers provided reproducible staining. It is worth pointing out that at 25 µM, PPa and PS1 were very phototoxic (see figures 5.4, 5.8) whereas PS4 less so. Noting that there was a 2 hr uptake and incubation time prior to the cell kill assays. At the 20 hr point which was used for the imaging, there were equal amounts of PS4 as PPa and PS1 within the cells, however, conclusions cannot be made about the uptake at 2hrs. Therefore, at the 2 hr timepoint, it would be difficult to correlate on relative uptake and subsequent intracellular localisation and potency.

It is unlikely but possible that the internalisation patterns and subsequent localisation of the 3 photosensitisers changes with time or even that they have very different uptake times. This has not been studied at all as it is difficult to set strict incubation periods whilst imaging live cells as experiments are difficult to time-manage with accuracy. A prudent solution would involve fixing the cells and subsequently imaging. However, the green co-stains used for the co-localisation studies do not work well with fixed cells so it would initially require a more subjective observation and comparison of samples at various time points. Should a distinction be seen, one would proceed to co-localisation studies by using one sample per experiment. It is fair to say that this will require extensive time and effort as a single experiment takes 3 days to set up and run. Another possibility is to use the red equivalents of the intracellular markers which are compatible with fixing conditions but this could give rise to crosstalk issues.

One of the main obstacles encountered when the microscopy studies were initiated was the difficulty in reproducing the published procedures where PPa or other photosensitisers were used for confocal microscopy studies. We tried to apply similar experimental conditions from published work (on PPa studies) on SKOV3 but realised that it was not applicable in our setup. The same was found for the trackers staining the mitochondria, ER, Golgi apparatus and lysosomes. It took considerable time and effort to establish a working protocol that could be successfully and most importantly reproducibly applied in our setup with our photosensitisers.

Looking at the images of the free photosensitisers stained cells (figure 5.9), one can immediately distinguish the different patterns they form upon internalisation in SKOV3 cells. PPa and PS1 stain most of the membrane compartments; PPa colours the cells in a uniform
manner spread throughout the cell and PS1 appears to be sharper but very similar to PPa whereas PS4 is punctate and distinctly different to the former two.

5.3.6 Co-localisation studies with free PSs

To our knowledge, co-localisation studies of PPa in SKOV3 cells have not been previously described. A few examples of elsewhere described PPa and PPa derivatives co-localisation studies include PPa loaded fullerenes described by Helmreich on Jurkat cells (Helmreich, 2005), PPa on LNCaP cells (Liu et al., 2009), MePPa on HCT-116 cells (Matroule et al., 2001), PPa derivatives on RIF cells (Chen et al., 2005), PPa derivatives on RIF and FaDu cells (Macdonald et al., 1999) to list a few.

PPa co-localisation

The co-localisation studies aimed at explaining the difference in potency between the three molecules. We observed that PPa, co-localises (yellow colour in overlay) significantly with the Golgi apparatus as well as with the ER. There appeared to be no overlap in the case of the lysosomes and a slight overlap with the mitochondria. These results are in agreement with other similar studies based on PPa where these trackers have been used to monitor the internal trafficking of the chlorins (Matroule et al., 2001, Sun et al., 2002).

PS1 co-localisation studies

Yellow colour in co-localisation images indicates overlap between the tracker (green) and the photosensitiser (red). Judging by the yellow colour in the images, the second molecule of interest, PS1 appeared to significantly co-localise with the ER (figure 5.12, H, K). There was overlap observed with the Golgi apparatus and PS1. Up to this point, the patterns observed for PS1 matched those of PPa. We could not establish unambiguously whether there is significant overlap with the lysosomes figure 5.13 (B-K).

It is interesting to note that prolonged irradiation of cells incubated with PS1 and different fluorescent markers through the high magnification objective led to significant bleaching of the marker’s fluorescence.

This becomes clear if we inspect the cells shown in the sequence of images, figure 5.13, B-G which all show the same cell area. The cells were scanned in the order they appear in the figure; B-D were scanned first followed by ~3x zooming in (E-G) and then subsequently zooming out without changing the microscope’s settings (I-K). From these images it could be
seen that cells clearly visible in B and particularly C were no longer visible in J, i.e. the marker localised in those cells has been bleached. We have explained this observation based on the fact that PS1 is an efficient photosensitiser which we have shown to produce ROS upon excitation (chapter 3). Thus, upon zooming into a cell, the laser light intensity delivered to that particular cell increased (vs. the zoomed out scanning). This caused the activation of PS1 leading to the production of ROS which destroyed the green dye leading to its photobleaching whilst not affecting PS1 itself (hence the red fluorescence remained). We confirmed that the effect is due to the PS1 by zooming into a control cell containing lysotracker only, where no bleaching was observed. In order for the marker to be destroyed the green dye has to be localised within the ROS diffusion distance from the PS1 (few µm) and thus we take these observations as another proof of co-localisation.

A similar event took place and can be seen in figure 5.13 L-V where the cells were co-stained with PS1 and MitoTracker®. The lower magnification initial images showed some significant overlap with yellow patterns appearing within the cells. Subsequent higher magnification images P-R showed some overlap however not as significant as in the case of the Golgi apparatus and the ER. With the lower magnification T-V, it was observed that the fluorescence of the cell due to the green dye had significantly been reduced. This could be explained in terms of distance, i.e. that the two dyes are further apart than in the case of PS1 and the Lysotracker®. Alternatively, the MitoTracker® might be less susceptible to destruction by ROS.

It should be possible to further verify these two events using FRET to observe the interaction of the two dyes. An example where FRET was observed by FACS and verified the positive co-localisation data as obtained by confocal microscopy was published by Oleinick et al. (Morris et al., 2003).

**PS4 co-localisation**

The co-localisation studies of PS4 are shown in figures 5.14 and 5.15. In figure 5.14 E-K, PS4 is used to stain live SKOV3 cells co-stained with the ER specific dye observing the clear difference from what was observed for PS1 and PPα. There appears to be no or very little co-localisation of PS4 and the ER and that is even more pronounced in the case of the Golgi (L-R). These observations are the opposite to what was seen with PPα and PS1. In figure 5.15 (A-D) PS4 was tested against the lysosomal specific dye and the overlay shows significant overlap of the two. This would explain the punctate, lysosomal like pattern...
observed in the PS4 stained cells. Finally, the MitoTracker® and PS4 stained samples show very little co-localisation of the two.

The unexpected finding that PS4 and PS1 both localise in the lysosomes - probably due to increased hydrophilicity - does not help explain the differences in potency. However, one cannot ignore the markedly different co-localisation of PS1 with the ER to that of PPα and PS4. PPα showed some co-localisation with the ER marker but PS4 does not. It has been demonstrated that the ER is an important organelle for PDT induced apoptosis to occur and our data might support that. This can only be verified once the PIC with PS4 is made and tested. The intracellular localisation of PS4 will be governed by C6.5(-k) therefore the three conjugates should have the same localisation especially if they are successfully purified. From the above images, it is also clear that none of the photosensitisers localise in the nucleus.

5.3.7 Confocal microscopy with PICs

Obtaining images of C6.5(-k) scFv co-stained with either the commercial anti-HER2 or with the PIC could provide visual confirmation that KB are HER2 negative (shown by FACS by others) and that SKOV3 are HER2 positive. One would expect almost complete overlap between the scFv and the commercial anti-HER2 on SKOV3.

The PIC samples where there is a large amount of non-covalently bound photosensitiser, one would expect to see significant overlap of the PIC and the tag-detected scFv with some non-overlapping fluorescence due to the free PS1. Whether it would have been possible to detect the tag of the PIC will be seen once the conditions are optimised and the scFv has been further engineered to modify the tags as discussed in chapter 4.

The PIC showed intense staining of SKOV3 antigen expressing cells. It also showed significant overlap with transferrin (figure 5.16) indicating that the internalisation pathway of these two fluorophores is similar. Free PS1 showed minimal overlap with transferrin (figure 5.16) further supporting that the methods of internalisation of the PIC and the free PS1 differ. The overlap of the PIC and transferrin support receptor mediated endocytosis of the former, indicating that the fluorescence of the cells incubated with PIC is largely attributed to the conjugated PS1.

Furthermore, for a HER2 negative cell line, one would expect to see no co-localisation of either samples but simply detect the red fluorescence due to the non-covalently bound
photosensitiser. The attempts to image the scFv were unsuccessful despite the literature precedence. Therefore it was not possible to visualise the scFv component of the PIC to verify its absence from KB cells (and presence within SKOV3). In the case of the KB cells, there was significant staining of the cytoplasm with the PIC (figure 5.17) which would not have been expected as they are antigen negative. It was also noted that there was significant overlap between the PIC sample co-stained with the transferrin conjugate on KB cells. This was expected for SKOV3 cells as the PIC should internalise using HER2 receptor-mediated endocytosis and follow the same pathway as transferrin but it was not expected for KB. The staining observed for KB with the PIC sample is attributed to the non-covalently bound PS1 which is not believed to internalise via receptor mediated endocytosis hence it would not overlap with transferrin. This was verified with SKOV3 (as mentioned above), where free PS1 showed minimal overlap with transferrin. Retrospectively, KB cells stained with a sample containing free PS1 and transferrin would have been more informative. It is possible that free PS1 internalises via different pathways in the two cell lines explaining the free PS1 overlap with transferrin on KB. The PIC solutions used for the confocal studies were prepared in media containing FBS which contains BSA. KB cells are highly contaminated with HeLa cells (European Cell Culture Bank) which overexpress receptors that bind and internalise HSA (Sutton et al., 2002). It is likely that BSA will internalise similarly aiding the internalisation of free PS (chapter 1). If the non-covalently bound material disaggregates from the perimeter and pockets of the scFv and associates with the BSA present in the media it is possible that the non-covalently bound material will internalise and fluoresce in a similar manner as for SKOV3 (Sutton et al., 2002). Transferrin provides rapidly proliferating cells with essential iron hence tumour cells overexpress the transferrin receptor whereas it is found at low levels in healthy human cells (Cavanaugh, 2002). The transferrin iron carrier binds to the cell surface transferrin receptor and internalises delivering the iron to the cell. Internalisation of transferrin in both cell lines by receptor mediated endocytosis is expected and co-localisation of transferrin and scFv should be observed as shown by Kirpotin et al(Kirpotin et al., 1997).

These imaging data further emphasise the need to purify the PIC in order to better characterise and understand its mechanism of trafficking. The imaging experiments using the C6.5(-k)-PS1 PIC have not been completed. However, it has been verified that KB cells internalise PS1 when incubated with the PIC. This has been attributed to the non-covalently bound PS that is assumed to internalise upon binding to serum proteins. Previously, the cytotoxicity of the PIC on the KB was attributed to the non-covalently bound PS and these experiments further support this argument. It requires further investigation including verification that the scFv does not bind KB before further assumptions can be made. Lastly,
the PIC was shown to internalise and significantly stain SKOV3 cells as expected. Further investigation to clarify the nature (covalent or NCB) PS1 is required. To our knowledge, there are currently no published studies of fluorescence microscopy using PICs with co-stains.

5.3.8 Structure Activity relationship

Overall, it has been demonstrated that the three PPa derivatives exhibit different efficacies on mammalian cells that could not be explained based on their photophysical properties alone. They also exhibit different subcellular localisation patterns. The efficiency and extend to which the three photosensitisers are internalised in cells might not vary significantly but it is likely that the uptake mechanism will vary substantially in the case of the cationic PS4. In chapter 3, we obtained computationally the log $P$ values for the three molecules. These were for PPa ($3.20 \pm 0.58$), for PS1 ($3.63 \pm 1.66$) and for PS4 ($0.37 \pm 0.9$). The behaviour and interaction of these different macrocyclic compounds with cell membranes would likely be related to their hydrophilicity. Even though the obtained numbers describe PS1 as tenfold more hydrophobic than PS4 the localisation of PS4 in the lysosomes—a characteristic of cationic, hydrophilic compounds—only supports that these theoretical values are only an approximation to the environment of a cell membrane. Overall, PS1 localises in the ER, Golgi and mitochondria as well as the lysosomes. On the contrary, PS4 does not localise in the ER, and Golgi but localises somewhat in the mitochondria and extensively in the lysosomes. This raises the following questions: Are the ER and Golgi the key compartments to target for PDT? Are the lysosomes an unfavourable target for PDT? Does localisation not hold the answer but perhaps depends on how fast and efficiently the uptake occurs? Do these imaging studies explain the potency of the photosensitisers?

Overall, a compound must exhibit sufficient uptake by cells (which in turn depends on its lipophilicity and/or targeting species), have the ability to produce singlet oxygen and other ROS and must localise in suitable compartments/ organelles within the cells (which could vary from cell line to cell line) (Li et al., 2008, Zheng et al., 2009, Peng et al., 2010).

The subcellular localisation of a PS can change with increasing incubation period for a given compound. It has been shown that depending on the location of the drug at the time of irradiation apoptosis or necrosis can be triggered selectively (Oleinick et al., 2002). Furthermore, depending on the concentration of the drug the timelines of triggering apoptosis can also differ by one or two days (Oleinick et al., 2002). This can be important when looking at dose-response curves and that is why in this experimental setup the cells were allowed to grow for two days post treatment before measuring their viability. It is clear
that apoptosis can be triggered post PDT treatment however this is not true for all circumstances and is mainly dependent on the cell line, the PS used and its subcellular localisation (Oleinick et al., 2002). Producing free radicals or singlet oxygen within the same organelle can also change the way a cell dies (Oleinick et al., 2002).

Trying to establish a link between mode of cell death with subcellular localisation and PS structure is challenging. It appears that apoptosis can be triggered via both mitochondrial damage and lysosomal damage but with differing timelines (Noodt et al., 1999, Oleinick et al., 2002). PDT induced damage of the ER can also lead to triggering of apoptosis but this is less well studied (Rück et al., 2000, Oleinick et al., 2002). Should it ever be consistently shown that mitochondria are the optimal organelle to target, a photosensitiser that inherently localises to mitochondria can be conjugated to an antibody specific to the target of interest and using a suitable cleaving site released once within the cell.

Localisation of PS4 in the lysosomes could indicate a different mode of uptake by the cells such as phagocytosis/pinocytosis (Zheng et al., 2009). It is important to note that it is likely that different cell lines react differently to compounds targeting their mitochondria or lysosomes. Phthalocyanines described by Li et al. reached maximum internalization at 2 hrs with the most amphiphilic derivative showing the highest uptake (Li et al., 2008). However, the derivative with the highest singlet oxygen quantum yield showed lower potency than the rest. These cationic photosensitisers were found to localise primarily in the lysosomes similarly to other positively charged compounds (Li et al., 2008). These studies support our findings on PS4. Finally, it has been argued that a compound targeting the mitochondria can be potent in one cell line and not as potent in another. The same applies to the lysosomes and perhaps to all the organelles (Zheng et al., 2009).

It is important to note that the singlet oxygen quantum yields described in chapter 3 were measured in toluene, an organic solvent where the three photosensitisers are in monomeric form. When photosensitiser solutions are prepared for either cytotoxicity assays or microscopy they are diluted in aqueous based solutions. The PSs form oligomeric aggregates which could affect their singlet oxygen quantum yield disproportionally to the one they exhibited in toluene. In other words, PS4 has the highest singlet oxygen quantum yield in toluene but it doesn't mean it will have the highest value in physiological conditions.
5.3.9 Concluding remarks

Three structurally different photosensitisers were characterised *in vitro* and their subcellular localisation was determined. In this chapter the *in vitro* characterisation of the free photosensitisers using confocal microscopy and cytotoxicity assays was described following the photophysical characterisation given previously (chapter 3). Subsequently, the PICs were also characterised *in vitro*. The free PS characterisation was successfully completed (although some of the data require further validation). The PIC characterisation was incomplete due to the difficulties encumbered during imaging with the commercial anti-HER2 mAb and the tags of the scFv.

The uptake of our PSs may explain the unsuccessful attempts to replicate the microscopy protocol where 2 µM of PPa are incubated for 20 hrs leading to insufficient staining (Sun et al., 2002). Uptake could also explain the lower sensitivity of SKOV3 all PSs compared to KB (Savellano et al., 2005b, Bhatti et al., 2008). It has previously been shown that low uptake of chlorin HPPH derivatives with similar singlet oxygen yields by cells was associated with the low potency exhibited (Zheng et al., 2009). Overall, it is not surprising that a compound with a marked difference in lipophilicity will be uptaken less easily leading to lower potency irrespectively of its singlet oxygen quantum yields.

There is a discrepancy in our experimental setup which involves the two very different incubation periods that were used with the free photosensitisers. The confocal microscopy experiments were conducted following the *in vitro* cell kills and was thus difficult to return and re-evaluate the assay to conform with the 20 hrs incubation as for imaging. It is possible that the localisation changes following the initial 2 hrs and the patterns that we saw at 20 hrs did not represent the actual position and concentration of the PS at the time of irradiation.

The SkBr3 breast cancer cell line has been considered as a higher expresser of ErbB2/HER2 receptors (1.0 x 10⁶ per cell (Hynes et al., 1989, Kirpotin et al., 1997)) than SKOV3 although a recent publication indicated that the HER2 expression levels of the two cell lines are very similar (Lundberg et al., 2007). SKOV3 cells were chosen for this work as they are more suitable for setting up tumours *in vivo* than SkBr3. Nonetheless, a second positive control when testing targeted therapy and intracellular localisation would be useful.

In order to better characterise the PICs, the *in vitro* assays should be repeated using a
second positive cell line. In an even more ideal setup, the confocal microscopy experiments would be conducted before the cell kill assays to allow the determination of the time required for maximum internalisation of the standalone photosensitisers, the scFv and the PICs.

Photosensitisers can often redistribute within a cell following receptor mediated endocytosis and endosomal distribution. Theoretically, increased uptake of the conjugate should lead to increased potency. However, its localisation in the endosomes/lysosomes and possible redistribution to the cytoplasm might decrease the potency gap as the ER and mitochondria are likely to be more suitable targets than the cytoplasm. It is difficult to compare published studies since the methods of measuring cell death, the laser dose and the actual drugs differ throughout the literature.

5.3.10 Future Experiments

Important future experiments would include uptake characterisation (Mojzisova et al., 2007) of the 3 PSs to determine the uptake of PS4 and whether it is less than the other two PSs. A cell kill assay where the cells are incubated with the photosensitiser for 20 hrs as for the confocal in order to directly compare potency and localisation would also be carried out. It would be rational to determine whether, working at the same time frame as for the cell kill, the uptake of PS4 is lower and to what extent than the other two.

In order to complete this work cytotoxicity assays with the PS4 PIC and if possible with the pure C6.5(-k)-PPa and C6.5(-k)-PS1 PICs would be carried out. Correlating from the differences in potency between free and conjugated PSs as shown by Vrouenraets and discussed in section 5.3.5 it is difficult to predict whether the C6.5(-k)-PS4 PIC will exhibit higher or lower potency to the free photosensitiser. Equally important would be to visualise the internalisation of a pure PIC (where possible) and compare with one that contains significant amounts of non-covalently bound material in parallel with controls such as the commercial anti-HER2 antibody and the detection of the scFv. Further co-localisation studies with the PICs and the specific organelle stains might provide clear information regarding the nature of the fluorescent signal in the conjugate samples.
Finally, the pharmacokinetics and hence the clearance of both the free PSs and PIC on both antigen positive and antigen negative cell lines will be very different \textit{in vivo} to the \textit{in vitro} setting. \textit{In vivo} the working administered concentration of the PS as part of the conjugate is much lower than the corresponding concentrations that exhibited dark toxicity \textit{in vitro} and should not induce any side effects. Therefore, the effect of non-covalent binding \textit{in vivo} and the importance of purifying the PIC can only be determined following the \textit{in vivo} studies.
Chapter 6 *In vivo Characterisation*
6.1 Introduction

6.1.1 Previous work with C6.5(-k)-PPa PIC

In chapter 4, section 4.1, the synthesis and characterisation of an anti-HER2, C6.5(-k)-PPa PIC by Bhatti et al. (Bhatti et al., 2008) was described. This was followed by its in vivo biodistribution and therapy which was conducted in nude mice. In vivo studies are usually carried out in an effort to represent an approximation to a real tumour and fill in the gaps where the limitations of an in vitro study are too severe or the information obtained is of limited use or substance. In vivo studies are often conducted to primarily obtain a pharmacokinetic profile and biodistribution for a given drug followed by a therapy which is the nearest approximation to pre-clinical studies. In the case of PICs, the pharmacokinetics would be governed mostly by the antibody. This would lead to the PS clearing faster when conjugated to an antibody fragment than the free PS would. The biodistribution would also be governed by the antibody and exhibit significant differences in terms of specificity when compared to the free PS. The conjugated PS would be expected to show increased tumour targeting with minimal non-specific accumulation.

Bhatti et al. reported on the pharmacokinetics analysis for C6.5(-k) and MFE-23 (scFv) and their conjugates which showed a biphasic exponential clearance profile with an alpha and beta phase. The alpha phase corresponds to the distribution phase which is the rapid equilibration of the protein into all tissue compartments and the beta phase is the deposition phase which is the elimination via the kidneys and other organs from the blood (systemic) (Bhatti et al., 2008). The two scFvs had very similar PKs whereas an IgG used as a control had ten to twenty times slower clearance owing to its larger size. Free PPa exhibited slower clearance than both the scFvs due to its hydrophobicity and binding to LDL and lipoproteins (see chapter 1). The corresponding PICs had PKs in between those of the free PS and the scFv. The slower clearance rate of the PIC compared to the unconjugated scFv was attributed to the increased hydrophobicity of the PIC and not to the size increase due to bioconjugation (Bhatti et al., 2008). The specificity of the PIC was significantly higher than the free at 7:1 for the tumour than blood at 24 hrs which was less significant at 3:1 at 8 hrs. The spleen contained some PIC at 24 hrs but most other organs were clear by 24 hrs.

For the therapy experiment the control samples included PBS (saline) and free PS alongside single and two cycle therapies. Tumour specificity was at 3:1 at 8 hrs for the PIC and much less for the free PPa. Free PPas showed no tumour regression matching the saline control whereas the 3 dose therapy showed complete tumour eradication.
Bhatti et al. carried out 8-24 hr incubation periods for PIC therapies and 1 or 3 cycles of PDT (Bhatti et al., 2008). The best results were obtained with an 8 hr incubation with 100 µg of conjugate. Several cycles were necessary to observe complete tumour eradication (Bhatti et al., 2008). A 7 day interval between cycles was examined but no other dose regimens were investigated.

The initial work by Bhatti et al. nicely exhibited the benefits of targeting with free PPα and its PIC showing a significant difference in both pharmacokinetic and therapeutic outcomes. However, there is scope for improvement which could be addressed by improved PICs. These would require fewer therapy cycles to achieve complete tumour regression, they would be of higher concentration to allow higher PS dose per cycle which would be relevant in a clinical setting as well as higher specificity for the tumour.

In a study by Adams where C6.5(-k) was used in RIT, SKOV3 tumours were grown in nude mice. When tumour sizes reached 16 mm³, which is comparatively small, they treated them with a C6.5(-k)-²¹³Bi-CHX-A'' (CHX-A” is a chelating agent) RIC including control samples of a non specific scFv and unconjugated C6.5(-k). Their data showed severe toxicity of the RIC at higher doses (Adams et al., 2000). Lower doses that were tolerated by the animals showed comparable tumour growth as for the unlabeled scFv which showed no efficacy. Furthermore, the higher doses were shown to be non-tumour specific as the non-specific scFv showed similar efficacy (Adams et al., 2000). Adams et al. then proceeded to prepare a C6.5(-k) diabody-90Y-CHX-A” RIC, with yttrium-90 being a β-emitter, which they tested in two nude mice models of SKOV3 (ovarian cancer) and breast cancer xenografts. The mice bearing the SKOV3 tumours were given a higher dose to show minimal growth rate delay whereas the second cell line proved to be more sensitive showing more pronounced tumour growth delay. The diabody control was shown to have no efficacy (Adams et al., 2004). SKOV3 were verified (clonogenic assay) to be more resistant to radiotherapy treatment with this RIC than the second cell line which they attributed to the rate of HER2 internalisation in SKOV3, the susceptibility of SKOV3 to cytotoxic drugs including radiotherapy, tumour size and p53 status (Adams et al., 2004).

Perhaps repeated administrations of both RICs would have given improved responses including cures but the aggressive nature of radiotherapy does not allow for repeated doses to be given (Adams et al., 2004). This would not be an issue with PDT as its bimodality should allow several drug light cycles without affecting healthy tissue. Overall, C6.5(-k) coupled to a potent α-emitter showed limited specificity in SKOV3 tumours, high toxicities and limited efficacy. A higher affinity C6.5(-k) diabody coupled to a longer lived β-emitter
was also shown to delay tumour growth in the same cell line with slightly improved efficacy in a breast cancer cell line.

There are very few published targeted PDT in vivo therapy studies with these being mostly in mice. These are summarised in figure 6.1 showing active work up to 2008. To our knowledge other therapy studies have not been published since. The in vivo studies carried out by Mew et al. (Mew et al., 1983) described the use of the first PIC to treat tumours in mice. They obtained promising data with a haematoporphyrin-anti-M-1 mAb PIC in subcutaneous tumours showing high tumour specificity and tumour regression (Mew et al., 1983). Despite its limitations this work demonstrated the targeting benefits in efficacy and specificity potentially offered by the use of TPDT.

It took more than ten years for the second in vivo studies to be published by Goff et al. showing the further difficulties faced. Using a Ce6 derivative-mAb conjugate to treat mice, they observed therapy related toxicity leading to 50% of the animals dying. They observed tumour growth delay after four treatments (Goff et al., 1996).

An anionic PIC, Ce6 –murine mAb 17.1A was used to treat an orthotopic model for hepatic metastases of colorectal cancer. The PIC showed increased potency at reducing tumour size when compared to the free PS control (injecting equal amounts of free PS as that calculated for the PIC) with the mice dying at 102 and 62 days respectively (Del Governatore et al., 2000). Even though their anionic PIC was highly specific for the tumour when compared to controls and free PS, they observed unwelcomed liver toxicity. Finally, a linear correlation was made between laser dose and efficacy (Del Governatore et al., 2000).

The studies highlight the main issues faced by in vivo targeted PDT such as establishing the drug dose, the drug-to-light interval, and the laser dose. The work by Bhatti (Bhatti et al., 2008) showed that it is possible to observe tumour regression and cure, however this is not what is generally reported. Unwelcomed dark toxicity deviates from the “no healthy tissue damage” concept of PDT and particularly so for TPDT indicating that specificity needs to be further improved. Nonetheless, the aforementioned studies support the concept of TPDT with better selectivity for tumours compared to the free PSs being exhibited. Given the significant efficacy difference between the PICs and free PSs, it will also be possible to reduce the effective drug dose by using a PIC instead of a free PS.
Targeted immunotherapy

**Figure 6.1 Listing key PSs used as part of PICs in therapy studies.** These are the main in vivo therapy studies on TPDT published to date. Reproduced from (Kwitniewski et al., 2008).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Photosensitizer immunoconjugate</th>
<th>Cancera</th>
<th>Model</th>
<th>References</th>
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<tbody>
<tr>
<td>Anti-M-1 mAb</td>
<td>Hp</td>
<td>M-1</td>
<td>Mice</td>
<td>Mew et al., 1983</td>
</tr>
<tr>
<td>OC125 mAb (anti-CA125)</td>
<td>CMA</td>
<td>OVCAR-3</td>
<td>Mice</td>
<td>Goff et al., 1996</td>
</tr>
<tr>
<td>U36 mAb (anti-CD44v6), 425 mAb (anti-EGFR)</td>
<td>mTHPC</td>
<td>UM-SCC-11B, UM-SCC-22A, A431</td>
<td>Cells</td>
<td>Vrouenraets et al., 1999</td>
</tr>
<tr>
<td>17.1A mAb</td>
<td>Ce6</td>
<td>HT29</td>
<td>Mice</td>
<td>Governatore et al, 2000</td>
</tr>
<tr>
<td>U36 mAb, 425 mAb, E48 mAb</td>
<td>AIPcs4</td>
<td>UM-SCC-11B, UM-SCC-22A, A431</td>
<td>Cells</td>
<td>Vrouenraets et al., 2001</td>
</tr>
<tr>
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<td>Ce6</td>
<td>Chemically induced papillary tumours A431</td>
<td>Hamsters</td>
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<td>A431, OVCAR-5</td>
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<td></td>
<td>PPa</td>
<td>SKOV-3, SK-BR-3, MDA-MB-468</td>
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<td>C6.5 scFv (anti-HER2)</td>
<td>PPa</td>
<td>SKOV3</td>
<td>Mice</td>
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</table>


**Radiolabeling and biodistribution**

As there is not enough published studies to make a connection between pharmacokinetics, biodistribution and therapeutic efficacy, *in vivo* biodistribution will be briefly discussed here. Radiolabeling is generally used as the main method for tracing drugs in pre-clinical studies and not only to identify drug localisation, blood pharmacokinetics and so on. Iodine-125 is predominantly used when radiolabeling a sample for biodistribution studies. Theoretically, fluorescence imaging using the intrinsic fluorescence of the PS could be used instead of radiolabeling without the need to further modify the PS or PIC.

Conway et al. used unlabelled PS and conjugate to determine uptake and biodistribution of protoporphyrin IX and its conjugate. The PS blood content was determined using the fluorescence of protoporphyrin IX and a standard curve of fluorescence. However, this approach was not sensitive enough to obtain the required information (Conway et al., 2008). Although the hydrophobicity increase, due to the introduction of hydrophobic atoms such as iodine on the radiolabeled entity, could affect the pharmacokinetics, there is currently no reliable alternative to monitoring uptake and biodistribution of drugs.

**The importance of conjugate loading ratios**

Drawing a parallel from immunotherapy and ADCs where *in vivo* data are more readily available, an auristatin conjugate showed better overall response at lower loading ratios than
higher ones in murine xenografts which was the opposite of what was seen in vitro for the same conjugate (Hamblett et al., 2004). This is unlikely to occur in TPDT unless perhaps the photophysics of the PS or the specificity of the antibody are affected.

**Skin photosensitivity and side effects**

One of the major challenges faced by TPDT is to prove that it offers significant benefits compared to conventional PDT. Clinicians are calling for PSs with less dark toxicity to make PDT attractive to the patient. Therefore, systemic toxicity and skin photosensitivity should follow biodistribution and therapy studies.

To do so, various laser doses (intensity and time) would be administered to treat a specific area of skin followed by PS injection as shown with Cp6 by (Leach et al., 1992). Skin photosensitivity can be described on a scale of 0-6 and can include obvious effects such as blistering and skin necrosis (level 6) (Nelson et al., 1987).

### 6.1.2 Aims and objectives

Following the *in vitro* characterisation of the three main PSs and the two PICs, the aims of this part of the work were to characterise them *in vivo*. The biodistribution and blood pharmacokinetics would be determined for the PSs as well as for the scFv and the PICs. The C6.5(-k)-PPa PIC would be used for comparison. Lastly a therapy using the necessary controls would be run for the main PIC.

The *in vivo* studies were to be conducted in an ethical manner conducting only necessary – in our opinion- experiments.

The objectives were therefore to radiolabel and run biodistribution studies thus establishing a therapeutic window for the main C6.5(-k)-PS1 PIC. Subsequently therapies using the PIC in single and multiple cycles would be run and the animals observed for an extended amount of time monitoring for severe side effects and distress. A higher dose PIC would also be used to observe benefits of using such an alternative.
6.2 Results

Note: The synthesis of the PSs used throughout this chapter is described in chapter 3 and the synthesis of the PICs in chapter 4.

6.2.1 Radiolabeling and tumour implantation

Tumours were set up as described in section 2.3.10. The free PSs, PICs and C6.5(-k) were radiolabeled as described in the same section using Iodogen. Iodogen is 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril and acts as a mild oxidizing reagent (Pierce, 2009b). The protein’s tyrosines will be substituted with an iodine-125 at the ortho position. The radioactivity of the collected fractions following chromatographic purification was measured (gamma counting) to confirm labeling. The protein containing samples were labelled more efficiently than the PSs. PPα labelled better than PS1 and PS4.

SKOV3 cancer cells were implanted on the flanks of female BALB/c nude mice and when the growing tumours reached a diameter of 5-6 mm (~100 mm³) they were used for biodistribution or therapy studies (see chapter 2.2.9). This tumour size allowed sufficient penetration of the laser through the entire tumour even though generally tumours used for similar experiments were slightly bigger at 150-200 mm³ (Conway et al., 2008).

6.2.2 Pharmacokinetic and biodistribution studies of free PSs

The blood clearance of the three main PSs are shown in figure 6.2. The data fitted well to a biphasic exponential decay curve (see chapter 2 for equation) for PPα and PS1 and less so for PS4 exhibiting a fast α-phase and fast β-phase corresponding to the systemic elimination through the kidneys and other organs. The half lives for both phases are shown in table 6.1. The values for the α-phase illustrate that PS1 and PS4 distribute to the tissues equally faster (~3.5x) than PPα. The clearance from the blood (t₁/₂ β) appears fastest for PS4, the monocationic PS, as perhaps would be expected to be given its positive charge. However, looking at the data and not the fitted curve, PS4 does not appear to clear faster than PS1. So, PS1 and PS4 clear from the blood ~2.5 times faster than PPα which is attributed to their increased hydrophilicity. It is due to the fast β-phase that the PS4 curve does not fit well as the 24 hrs point had a very low hence inaccurate radioactivity count. Overall, the data indicate that the synthetic modifications to the PPα macrocycle leading to PS1 and PS4 have improved the pharmacokinetics as they distribute to the tissues faster and clear faster from the blood.
Figure 6.2 Blood pharmacokinetics of PPα, PS1 and PS4. The PSs were radiolabeled (\(^{125}\))I, injected (5 µg, 50 µg ml\(^{-1}\)) into the tail of SKOV3 tumour bearing nude mice over 24 hrs. The data (▲, △, ▲ symbols) were fitted to a double exponential decay four parameter equation. The data are an average of minimum 4 mice (n=2). Errors are standard deviation.

Table 6.1 Blood clearance half lives of the free PSs, PPα, PS1 and PS4. The values were obtained using the blood pharmacokinetic data (figure 6.2) which were fitted to a biphasic exponential decay curve (see chapter 2). Symbols (▲) refer to the data figure 6.2 SD is standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Half-life (t(_{1/2})) (hrs)</th>
<th>Half-life (t(_{1/2})) (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-phase ± SD</td>
<td>β-phase ± SD</td>
</tr>
<tr>
<td>PPα (▲)</td>
<td>1.51 ± 0.5</td>
<td>17.33 ± 8.6</td>
</tr>
<tr>
<td>PS1 (▲)</td>
<td>0.43 ± 0.06</td>
<td>8.72 ± 3.3</td>
</tr>
<tr>
<td>PS4 (▲)</td>
<td>0.41 ± 0.04</td>
<td>3.5 ± 1</td>
</tr>
</tbody>
</table>

The tumour uptake which describes how efficiently a drug is taken up by the tumour and tumour to blood ratio which is an indication of specificity and targeting, for the free PSs are shown in figures 6.3 and 6.4 and a summary of the data is shown in table 6.3. These data were important in defining a time window suitable for therapy where there is sufficient amount of drug in the tumour and little in other tissues and organs.

From the tumour uptake, blood clearance and tumour : blood ratio of the free PSs (figures 6.2, 6.3 and 6.4), the differences between the pharmacokinetic properties of the three PSs can be observed. For PS1, 2 hrs following injection tumour uptake is nearly identical to PS4
and 3-fold lower than PPa at where PPa reaches its peak uptake at 28 %. At the same time, the blood content for PPa at 2 hrs is at its second highest at 39 % which is 4-fold higher than PS4 and 2.6-fold higher than PS1. So whilst PPa highly accumulates in both tumour and blood at 2hrs, PS1 and PS4 also reach their peak accumulation. Overall, all three PSs reach their maximum tumour concentration at 2 hrs. Looking at the tumour to blood ratio, the selectivity of PS4 for the tumour is 2-fold higher than PPa and PS1. PPa and PS1 have very similar specificities. Theoretically, one would choose the time point 2 hrs for optimum potency of either of the 3 PSs and the time point at least 8 hrs for minimum side effects as the selectivity is also higher. The pharmacokinetics of PS4 as a standalone PS are better (if it was used as a standalone) than both PS1 and PPa as it is more specific, has a quick distribution to tissue ($t_{1/2 \alpha}$) of 41 minutes and the quickest of the three systemic elimination ($t_{1/2 \beta}$).

Figure 6.3 In vivo tumour uptake of $^{125}$I-labeled free PSs, PPa, PS1 and PS4 in SKOV3 tumour bearing nude mice over 24 hrs. The data (▲) were obtained as for figures 6.2 and are an average of minimum 4 mice (n=2). Errors are standard deviation.
Table 6.2 Summary of the tumour uptake and blood clearance. (% ID/g tissue) ± standard deviation for the free PSs as illustrated in figures 6.3.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PS</th>
<th>Time (hours- post injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tumour</td>
<td>PPa</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>PS1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>PS4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Blood</td>
<td>PPa</td>
<td>67.0 ± 0</td>
</tr>
<tr>
<td></td>
<td>PS1</td>
<td>67.0 ± 0</td>
</tr>
<tr>
<td></td>
<td>PS4</td>
<td>67.0 ± 0</td>
</tr>
</tbody>
</table>

Figure 6.4 In vivo biodistribution -tumour to blood ratio of free PSs. $^{125}$I-labeled free PSs, PPa, PS1 and PS4 in SKOV3 tumour bearing nude mice over 24 hrs. The points (▲, ▲, ▲ symbols) were obtained using the data in figure 6.2 and 6.3.

From the biodistribution data, figure 6.5 the two most important time points as discussed above, are 2 hrs and 8 hrs for uptake and specificity respectively. The tissue uptake basically follows the blood levels. As the blood levels decrease so do the normal tissue levels. The early high accumulation level in the kidneys is attributed to residual free Iodine-125 that clears through the kidneys.
Figure 6.5 In vivo biodistribution of free PSs. $^{125}\text{I}$-labeled free PSs, PPa (top), PS1 (middle) and PS1 (bottom) in SKOV3 tumour bearing nude mice over 24 hrs. The data are an average of 4 mice ($n=2$). Errors are standard deviation. Please note that the t=0 blood level = the level in the blood just after injection. This is very hard to determine and is an estimation.
6.2.3 Pharmacokinetic and biodistribution studies of PICs

Based on the overall data of the PSs and PICs so far, including their in vitro characterisation in chapter 5, C6.5(-k)-PS1 PIC was chosen for further studies as it appeared to be an improvement compared to PPa.

The blood clearance of the PICs and the corresponding control samples are shown in figure 6.6. The data fitted well to a biphasic exponential decay curve (see chapter 2 for equation) for PPa, PS1, and less so for C6.5(-k)-PPa PIC and unconjugated C6.5(-k), exhibiting a fast α-phase corresponding to the tissue distribution and a fast β-phase corresponding to the systemic elimination through the kidneys and other organs. The half lives for both phases are shown in table 6.3.

The data did not fit well for C6.5(-k) and C6.5(-k)-PS1 PIC due to their fast β-phase leading to less accurate low radioactivity counts. The $t_{1/2}$ is very similar for the three scFv containing samples as the actual data (and not the fitted curve) for C6.5(-k)-PS1 PIC and C6.5(-k) indicate. The small differences are probably due to the change in hydrophobicity of the scFv which could be governed by its loading ratio.

The free PSs have slower blood clearance ($t_{1/2}$) than the corresponding PICs and the scFv as expected due to their higher hydrophobicity. The clearance rate for the C6.5(-k)-PPa PIC is somewhere between that of the free PS and the scFv whereas for the C6.5(-k)-PS1 PIC it appears to be governed more by the scFv. Nonetheless, the C6.5(-k)-PS1 PIC clears faster from the tissue than the C6.5(-k)-PPa PIC supporting the evidence previously discussed for free PS1.

Table 6.3 Blood clearance half lives of the free PSs, and the corresponding PICs. The values were obtained using the blood pharmacokinetics data (figure 6.6) which were fitted to a biphasic exponential decay curve (see chapter 2). Symbols (●) refer to the data figure 6.6 SD is standard deviation. *not reliable as the fit to the curve was not as good.

<table>
<thead>
<tr>
<th></th>
<th>Half-life ($t_{1/2}$) (hrs) α-phase ± SD</th>
<th>Half-life ($t_{1/2}$) (hrs) β-phase ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPa (●)</td>
<td>1.2 ± 0.63</td>
<td>13.1 ± 6.5</td>
</tr>
<tr>
<td>PS1 (●)</td>
<td>0.39 ± 0.03</td>
<td>9.8 ± 1.65</td>
</tr>
<tr>
<td>C6.5(-k)-PPa PIC (●)</td>
<td>0.13 ± 0.26</td>
<td>4.5 ± 1.02</td>
</tr>
<tr>
<td>C6.5(-k)-PS1 PIC (●)</td>
<td>0.22 ± 0.09</td>
<td>1.38 ± 0.27</td>
</tr>
<tr>
<td>C6.5(-k) (●)</td>
<td>0.15*</td>
<td>1.54*</td>
</tr>
</tbody>
</table>
Figure 6.6 Blood pharmacokinetics of PICs. C6.5(-k), PS1, PPa and C6.5(-k)-PPa and C6.5(-k)-PS1 PICs. The samples were radiolabeled (\textsuperscript{125}I), injected (5 µg, 50 µg ml\textsuperscript{-1}) into the tail of SKOV3 tumour bearing nude mice over 24 hrs. The data (●, ●, ●, ● and ● symbols) were fitted to a double exponential decay four parameter equation. The data are an average of minimum 4 mice (n=2). Errors are standard deviation.

The tumour uptake of PPa remains the highest at 2 hrs which decreases between 24 and 48 hrs. The uptake of both PICs are lower than their corresponding free PSs due to faster blood clearance. However this is compensated by the specificity of the PICs which is 5-7: 1 for C6.5(-k)-PS1 PIC (6-24 hr) and 2-4: 1 for the C6.5(-k)-PPa PIC. Overall, the scFv exhibits the highest tumour specificity with uptake levels and clearance being faster than the C6.5(-k)-PS1 PIC (table 6.4).

From the rest of the tissue biodistribution for C6.5(-k)-PS1 PIC, in figure 6.9, it is clear that at 24 hrs there is distinct tumour specificity compared to other tissue (4 fold). At 1 hr, the PIC is mostly in the blood, lungs, tumour kidney, liver and spleen. It is also important to note the difference in the tumour uptake specificity for the C6.5(-k)-PS1 PIC (figure 6.7) at 24 hrs compared to PS1 at 24 hrs (figure 6.8). Detailed tissue biodistribution for the C6.5(-k)-PPa PIC was described by Bhatti et al. (Bhatti et al., 2008).

The reproducibility of the free PS data is also shown as the clearance and uptake are nearly identical to the free PSs experiments. Note that Adams reported the tumour uptake for C6.5 to be 1.47 %ID/g at 24 hrs following iodine-125 radiolabeling (Schier et al., 1995). We do not
have a direct comparison for C6.5(-k) as they did not show their biodistribution data (Adams et al., 2000).

Table 6.4 Summary of the tumour uptake and blood clearance of PICs. (% ID/g tissue) ± standard deviation for the PICs as illustrated in figures 6.7 and 6.8.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PS</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>6</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour</td>
<td>C6.5(-k)</td>
<td>0.0 ± 0</td>
<td>4.2 ± 0.08</td>
<td>6.7 ± 0.8</td>
<td>4.0 ± 0.4</td>
<td>2.7 ± 1.1</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>PPa</td>
<td>0.0 ± 0</td>
<td>21.0 ± 0.4</td>
<td>28.0 ± 1.8</td>
<td>25.0 ± 5.9</td>
<td>22.0 ± 0.7</td>
<td>7.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>C6.5(-k)-PPa PIC</td>
<td>0.0 ± 0</td>
<td>5.2 ± 0.4</td>
<td>9.2 ± 0.8</td>
<td>10.2 ± 0.3</td>
<td>6.1 ± 0.8</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>PS1</td>
<td>0.0 ± 0</td>
<td>11.0 ± 3.3</td>
<td>9.0 ± 0.5</td>
<td>7.0 ± 1.9</td>
<td>5.0 ± 0.4</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>C6.5(-k)-PS1 PIC</td>
<td>0.0 ± 0</td>
<td>4.8 ± 1.0</td>
<td>7.8 ± 0.7</td>
<td>6.7 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>3.1 ± 0.04</td>
</tr>
<tr>
<td>Blood</td>
<td>C6.5(-k)</td>
<td>67.0 ± 0</td>
<td>12.0 ± 3.9</td>
<td>8.0 ± 0.5</td>
<td>1.0 ± 0.1</td>
<td>0.2 ± 0.08</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PPa</td>
<td>67.0 ± 0</td>
<td>54.3 ± 4.2</td>
<td>39.4 ± 1.4</td>
<td>26.7 ± 0.5</td>
<td>9.4 ± 0.6</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>C6.5(-k)-PPa PIC</td>
<td>67.0 ± 0</td>
<td>21 ± 2.5</td>
<td>18 ± 1.6</td>
<td>19.2 ± 0.3</td>
<td>1.8 ± 1.1</td>
<td>0.87 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>PS1</td>
<td>67.0 ± 0</td>
<td>23.0 ± 1.9</td>
<td>15.3 ± 0.9</td>
<td>9.6 ± 2.3</td>
<td>3.1 ± 1.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C6.5(-k)-PS1 PIC</td>
<td>67.0 ± 0</td>
<td>19 ± 1.7</td>
<td>10.5 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>0.52 ± 0.2</td>
<td>0.32 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 6.7 In vivo tumour uptake of $^{125}$I-labeled free PSs and corresponding PICs in SKOV3 tumour bearing nude mice over 48 hrs. The data (●, ●, ●, ● and ⋄ symbols) were obtained as for figure 6.6 and are an average of minimum 4 mice (n=2). Errors are standard deviation.
Figure 6.8 *In vivo* biodistribution - tumour to blood ratio of PICs. $^{125}$I-labeled free PSs and corresponding PICs in SKOV3 tumour bearing nude mice over 48 hrs. The points (●, ●, ● and ● symbols) were obtained using the data in figure 6.6 and 6.7.

Figure 6.9 *In vivo* biodistribution of $^{125}$I-labeled C6.5(-k)-PS1 PIC in SKOV3 tumour bearing mice. The data are an average of 4 mice (n=2). Errors are standard deviation.
6.2.4 In vivo targeted photodynamic therapy with C6.5(-k)-PS1 PIC

Therapy experiments were set up for nude mice bearing SKOV3 subcutaneous tumours. The mice were injected with the C6.5(-k)-PS1 PIC in two sets per experiment and treated as described in chapter 2. One for single dose injection followed by one irradiation and a second set where two injections were followed by two irradiations. Tumour uptake was highest for C6.5(-k)-PS1 PIC at 2 hrs (table 6.4) and as specificity didn’t change significantly after 6 hrs (2 fold increase from 2 to 48 hrs) as the tumour to blood ratios indicate (figure 6.8) a 4 hours drug-to-light interval was chosen. Therapy experiments have been repeated thrice. It was important as with all PDT experiments to protect the mice from direct light exposure following injection with a photosensitising agent.

Therapy with 1 mg ml\(^{-1}\) C6.5(-k)-PS1 PIC

Using a 1 mg ml\(^{-1}\) C6.5(-k)-PS1 PIC solution in PBS loaded with an average of 6 PS1 molecules per scFv, the animals were injected (200 µg, 200 µl per mouse i.v (bolus)), treated and monitored for signs of toxicity, tumour growth (repeated measurements 2-3 times/week) (see chapter 2 for equation) and distress. No distress or side effects were seen during injection or irradiation.

The tumours response to the therapy are shown in figure 6.10 and the statistical analysis results are shown in table 6.5. Treatment with PBS (saline) and PS1 lead to the same response which is uninhibited tumour growth up to 33 and 38 days when the mice were culled due to the size of their tumours. A single round of treatment gave a partial response by delaying tumour growth which did not re-grow for 46 days. Two rounds of treatment three days apart gave complete tumour regression and cure was observed from day 31 to day 46 when the measurements stopped being taken.

The analysis of variance (ANOVA) shows that there is no significant difference between treatment with PBS and PS1 whereas both sets of PIC treatments were significant relative to both PBS and PS1. The significance between one and two rounds of treatment is also very important as it demonstrates the scope for improvement of the therapy upon optimisation of the different components.
Figure 6.10 *In vivo* PDT therapy using C6.5(-k)-PS1 PIC. Treatment of nude mice bearing SKOV3 tumours using 1 mg ml$^{-1}$ of C6.5(-k)-PS1 PIC. Arrows (—) indicate day of injection followed by illumination on the same day- second arrow does not apply for the single dose treatment (●). Samples were injected per animal as follows: 1 mg ml$^{-1}$ solution of PIC (● and ●) (200 µg of scFv, 50.6 µg, 4.13x10$^{-8}$ mol of PS1 assuming a 1:6 LR), control samples saline (●) and free PS1 (135 µg, 1.1x10$^{-7}$ mol) (●). All animals were illuminated 4 hrs later. Dotted line indicates 0 % tumour size. (n= 4 for each dose/sample) Error bars are standard deviation and shown in one direction for clarity. Individual mice responses in appendix 2.

Table 6.5 Analysis of variance values of the 1 mg ml$^{-1}$ therapy Therapy of SKOV3 tumour bearing nude mice with 1 mg ml$^{-1}$ of C6.5(-k)-PS1 PIC. P values are considered significant for P<0.05 (Del Governatore et al., 2000).

<table>
<thead>
<tr>
<th>Photosensitising agent</th>
<th>PS1 (double)</th>
<th>C6.5(-k)-PS1 PIC (single)</th>
<th>C6.5(-k)-PS1 PIC (double)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative to</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>P values</td>
<td>0.62</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PS1 (double)</td>
<td>C6.5(-k)-PS1 PIC (single)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
**Therapy with 4 mg ml\(^{-1}\) C6.5(-k)-PS1 PIC**

Using the 4 mg ml\(^{-1}\) C6.5(-k)-PS1 PIC solution in PBS loaded with an average of 1.4 PS1 molecules per scFv, the animals were treated and monitored after that for signs of toxicity, tumour growth and distress. Apart from the higher C6.5(-k)-PS1 PIC and PS1 concentration the rest of the therapy set up was maintained the same as for the 1 mg ml\(^{-1}\) rounds.

The tumour response to the therapy is shown in figure 6.11 and the statistical analysis results are shown in table 6.6. As previously shown PBS and PS1 do not exhibit any therapeutic response.

The two sets of PIC therapies showed tumour growth delay but tumour regression was not observed. The statistical analysis indicates that there is a significant therapeutic difference between the two injection/irradiation cycles compared to PBS, PS1 and the single round. The significance between the single round and the control samples is less notable.
**Figure 6.11** *In vivo* PDT treatment of nude mice bearing SKOV3 tumours in using 4 mg ml⁻¹ of C6.5(-k)-PS1 PIC. Arrows (—) indicate day of injection followed by illumination on the same day; second arrow does not apply for the single dose treatment (●). Samples were injected per animal as follows: 4 mg ml⁻¹ solution of PIC (+ and ●) (800 µg of scFv, 47.2 µg, 3.85x10⁻⁸ mol PS1 assuming a 1:1.4 LR), control samples saline (+) and free PS1 (135 µg, 1.1x10⁻⁷ mol) (●). All animals were illuminated 4 hrs later. Dotted line indicates 0 % tumour size increase. (n=4) Error bars are standard deviation and shown in one direction for clarity.

**Table 6.6** Analysis of variance values of the 4 mg ml⁻¹ therapy of SKOV3 tumour bearing nude mice with 4 mg ml⁻¹ of C6.5(-k)-PS1 PIC. P values are considered significant for P<0.05 (Del Governatore et al., 2000).

<table>
<thead>
<tr>
<th>Photosensitising agent</th>
<th>PS1 (double)</th>
<th>C6.5(-k)-PS1 PIC (single)</th>
<th>C6.5(-k)-PS1 PIC (double)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative to PBS</td>
<td>PBS</td>
<td>PS1 (double)</td>
<td>C6.5(-k)-PS1 PIC (double)</td>
</tr>
<tr>
<td>P values</td>
<td>0.12</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.036</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
6.3 Discussion

6.3.1 Radiolabeling and tumour implantation

The methodologies and experimental setup used in this part of the work closely followed the work of Bhatti et al. (Bhatti et al., 2008). As proposed by Bhatti, the iodide ($^{125}$I) acts as a nucleophile and inserts at either a meso position on the macrocycle or at the vinyl bond in PPa (Bhatti et al., 2008). The absence of the double bond (3a-3b) in both PS1 and PS4 due to its reduction to an alkane in the first synthetic steps (see chapter 3) makes use of that bond unlikely. The second proposed binding site would be the most reactive meso position which again is occupied by the hexynoic acid chain and pyridyl ring for PS1 and PS4 respectively. Therefore, it is possible that the iodide is introduced on the benzyl moiety of PS1 at one of the ortho positions or the triple bond whereas for PS4 that is likely to take place at one of the aromatic positions on the pyridyl ring (could it exchange with the iodide counter ion). Bhatti (Bhatti et al., 2008) reported that the protein labelled more efficiently than the free PS which was also observed here. The labeling for both the scFv and PICs was sufficient to allow the studies to be carried out.

Even though SKOV3 have generally been considered as moderate HER2 expressers when compared to SkBr3 breast cancer cell line, Lunberg showed by FACS and real time PCR that this is unlikely to be true as the HER2 receptor levels were found to be very similar and highest than several other HER2 expressing cell lines (Lundberg et al., 2007, Milenic et al., 2008). Therefore, considering that SKOV3 grow well and more efficiently than SkBr3 as tumour xenografts they are a suitable cell line for the initial set of studies.

Despite the lack of T cells which compromises their immune system, it is still hard to grow tumours in athymic/ nude mice. SCID mice are more severely immunocompromised as they lack both T and B cells, making it easier for tumours to grow (Robertson et al., 2009). However, nude mice were preferred for use in this project as they do not have hair which makes their use for PDT easier.

6.3.2 Pharmacokinetics and biodistribution of free PSs

It was important before evaluating the in vivo behaviour of the PICs to establish a comparison using the free PSs as similarly described in chapter 5.

As expected, free PSs have slow blood clearance especially when compared to the scFv. Photosensitisers being hydrophobic molecules clear more slowly from the blood as they
associate with cell membranes, LDL and albumin non-covalently during their circulation. PPα is more hydrophobic and less water soluble than both PS1 and PS4 as described in chapter 3. PS1 and PS4 are equally water soluble (visual observation) but the positive charge present on PS4 facilitates its faster clearance from the blood as these tend to go through the negatively charged glomerular membrane more efficiently (Deen et al., 2001, Tang et al., 2004).

From the observations regarding tissue localisation and tumour uptake, PPα exhibited the highest tumour uptake of the three which did not correlate with the highest tumour specificity. Even though neither the uptake nor specificity of PS1 have improved compared to PPα, it clears twice as fast as PPα (β-phase). Our PPα data are in agreement to that published previously (Bhatti et al., 2008). Producing PSs with higher tumour specificity in vivo for use as standalone PDT agents was never the scope nor aim of this work. Nonetheless, it is worth pointing out that PS4 exhibited marginally higher specificity than both PS1 and PPα and fastest blood clearance. Based on these observations, PS4 could be an ideal PS to use for non-targeted PDT as administration and illumination could be performed on the same day. Faster clearance should be associated with minimal skin photosensitivity as shown by the skin uptake in figure 6.5 (skin photosensitivity tests need to be conducted). Considering the in vitro data on PS4 (chapter 5), it would be interesting to see how it behaves when conjugated.

Summarising the behaviour of the free PSs

- Tumour uptake: PPα > PS1 = PS4
- Blood clearance: PS4 > PS1 > PPα
- Tumour specificity: PS4 ≥ PPα ≥ PS1

It is often reported that free PSs tend to accumulate in the liver which was observed here as well (Woodburn et al., 1992, Del Governatore et al., 2000). Lungs and kidneys were also amongst the highest accumulating tissues with the latter being attributed to the clearance of free iodine-125 as the levels decreased at later time points. Kessel reported that the distribution of PSs is related to the number of LDL receptors available on the tissues which is in the order of liver > kidney > lung > spleen (Kessel, 1986).

Finally, pointing out that the samples were prepared and injected in PBS without the need for formulation with the animals showing no signals of distress. This is important as the use of liposomes, PEGs and detergents to facilitate the solubility of free PSs for PDT is a downside of some of the currently studied PSs. As we have observed, Foscan® is highly insoluble in
water and required formulation using PEG to obtain a solution that could be used in vivo (data not shown nor discussed here).

6.3.3 Pharmacokinetics and biodistribution of PICs

Before proceeding to therapy experiments, it was crucial to understand the behaviour of the PIC. As expected, the scFv has a very fast blood clearance and therefore clears within 4-6 hrs of administration compared to a whole IgG that takes 3-5 days. This is mainly due to the scFv’s smaller size allowing it to go through the glomerular filtration and the lack of the neonatal Fc receptor. An accurate half life for the scFv or the C6.5(-k)-PS1 PIC could not be obtained as the data did not generate a good fit, but the data suggest that the clearance is a little faster than that of the C6.5(-k)-PPa (~4.5 hrs). This observation is consistent with that of Bhatti who showed that $t_{1/2}^\alpha= 0.14$ and $t_{1/2}^\beta= 4.1$ hrs (Bhatti et al., 2008).

Summarising the behaviour of these agents:

- Blood clearance: C6 ≥ C6.5(-K)-PS1 PIC > C6.5(-k)-PPa PIC > PS1 > PPa Tumour uptake: PPa > PS1 > C6.5(-k)-PPa PIC > C6 = C6.5(-K)-PS1 PIC
- Tumour specificity: C6 > C6.5(-K)-PS1 PIC > C6.5(-k)-PPa PIC > PPa = PS1

The scFv has the fastest blood clearance and that of the PS1 is faster than that of the C6.5(-k)-PPa PIC and in agreement with its increased hydrophilicity. Both PICs have faster clearance than their corresponding free PSs and exhibit the expected bi-phasic behaviour (Bhatti et al., 2008). The molecular size threshold for secretion through the kidneys is 60 kDa, well below any of our PICs (Beckman et al., 2007).

From the tumour uptake and tumour to blood ratios it is clear that the more hydrophobic species accumulate in increased amounts in the tumours (see chapter 1) than the more hydrophilic ones. C6.5(-k) maintains the highest tumour to blood ratio compared to the PICs. Overall, PPa has a more prominent effect on the in vivo behaviour of the scFv than PS1 as the PPa’s hydrophobic property overrides the hydrophilicity of the scFv-unlike PS1.

C6.5 was shown to have an uptake of ~1.4%ID/g tumour in SKOV3 tumours at 24 hrs (Schier et al., 1995, Adams et al., 1998) which is in good agreement with our data at 2.7% ID/g tumour. The biodistribution studies with C6.5 were one of the first such studies using fragments. Adams showed the scFv to accumulate in SKOV3 tumours at high tumour to organ ratios at 24hrs. C6.5 was shown to accumulate in the liver at 0.04 %ID/g and in the blood at 0.05 %ID/g at 24 hrs (Schier et al., 1995, Adams et al., 1998). They also showed
that the higher affinity C6.5 diabody was better retained in the tumour than the scFv (Adams et al., 1998) suggesting that our PIC could benefit from higher affinity.

One of the *in vivo* limitations and downside of PSs currently in the clinic or in trials is their high accumulation in the liver. From the biodistribution profiles of the free PSs and the PIC it can be observed that at 1 hr the liver accumulation of PPa is at ~40 %, for PS1 at ~30 %, for PS4 at ~25 % and for the C6.5(-k)-PS1 PIC at <5 %. An impressive 8-fold reduction in liver accumulation is observed going from PPa to the C6.5(-k)-PS1 PIC. Comparing the free PS (PS1) with its corresponding PIC, the reduction is 6-fold. Considering the tumour accumulation, the specificity of the PIC is governed by partially both components, for PS1 more so by the scFv which is very welcomed.

Hudson showed that cationic conjugates (especially ones with higher loading ratios) cleared much faster from the blood than a PIC with a neutral PS which compared to the unconjugated mAb (Hudson et al., 2005). The neutral PS PIC showed pharmacokinetics similar to the unconjugated antibody. However despite the faster clearance the PIC specificity was exceptionally high for the non-internalising antibody-cationic PS PIC (Hudson et al., 2005). This suggests that a PS4 conjugate could have improved specificity ratios than the C6.5(-k)-PS1 PIC.

Lastly, importantly, the C6.5(-k)-PS1 PIC showed minimal non-specific accumulation at 24 hrs in agreement with the data for C6.5 by Schier (Schier et al., 1995). One of the most significant parameters for PDT, is skin uptake and subsequent skin photosensitivity. From the biodistribution of the C6.5(-K)-PS1 PIC, the amount of radiolabeled material is half of that of the free PS1 at 24 hrs. Eliminating the uptake of PSs by healthy cells *in vivo* is on its own a great achievement.

### 6.3.4 Targeted PDT *in vivo*

Finally, two different therapy approaches were conducted using the C6.5(-k)-PS1 PIC at either 1 mg ml\(^{-1}\) or 4 mg ml\(^{-1}\) (scFv concentration) loaded with 6 and 1.4 molecules of PS1 per scFv respectively. The reproducible results from the first approach (1 mg ml\(^{-1}\)) are extremely encouraging and a verification of the concept and aims of TPDT. Both PIC therapies exhibited significant potency compared to free PS1. This conclusion partially disproves the need to have a pure PIC with no non-covalently bound PS in order to see a clear difference between targeted and non-targeted PDT *in vivo*. In chapters 4 and 5 the importance of removing the non-covalently bound PS was described and demonstrated with the cytotoxicity observed on the antigen negative cell line. We could assume that the NCB
material once \textit{in vivo} dissociates in the presence of albumin and LDL and behaves as a free PS thus having no therapeutic effect and clearing as described for the free PS. There are several reports in the literature to support the dissociation of non-covalently bound material from PICs in the presence of BSA (Sun et al., 2002, Sutton et al., 2002, Kuimova et al., 2007). Completion of the confocal microscopy studies to observe the co-localisation of the impure PIC with the unconjugated scFv might further support this as it could show the PIC to be more dispersed/distributed within the cell than the scFv. Another parallel that can be drawn from ADC is a study where a non-covalent mixture of auristatin with a mAb gave very slight tumour growth delay compared to the more efficacious conjugate (Hamblett et al., 2004) indicating that the non-covalently bound material had no significant effect and that covalent linkage to the mAb was crucial for its efficacy.

On the other hand, it is possible that the non-covalent binding remains non-covalently associated with the scFv thus increasing its loading ratio and contributing to the efficacy observed. To investigate, a study where conjugates with varying degrees of non-covalently bound material would be conducted to observe the effects, if any, of the NCB PS.

SKOV3 tumours were completely eradicated using two cycles of drug injection followed by illumination on the same day. The marked significant difference in efficacy between therapy using free PS1 and the PIC supports the fundamentals of TPDT. Uptake levels for PS1 were higher or similar to the PIC yet the \textit{in vivo} efficacy is completely different. The potency variation cannot be explained with respect to the tumour uptake levels. The C6.5(-k)-PS1 PIC exhibited increased specificity compared to the free PS1. However, this does not explain why PS1 did not show equal potency as the PIC \textit{in vivo} as the same amount of PS should be located in the tumour in both cases at the same time (figure 6.7). Intracellular tareting cannot be the reason as it would correlate with \textit{in vitro} efficacy. After all, comparing the two, \textit{in vitro} only showed a 3-fold potency increase for the PIC (see chapter 5). The discrepancy probably lies with the fact that the mouse is a multicellular living organism and has various types of cells able to communicate and affect the end result of the therapy in a way that is not possible in a plastic dish. It has been shown by others that increased and preferential uptake does not negate potency (Conway et al., 2008).

In order to reach tumours, systemically delivered drugs need to cross the vascular barrier through the interstitium by diffusion or convection (Jain, 2001). HER2 overexpressing tumours were imaged using Trastuzumab to observe that most of it remains in the vasculature and does not reach the tumour cells (Dennis et al., 2007). If this is an analogy that can be made with C6.5(-k) and it remains in the tumour vasculature it could explain the lack of potency for PS1 which would internalise through binding LDL and albumin. Targeting
the tumour vasculature and causing coagulation is a potent and efficient way to reduce blood supply to the tumour causing tumour infarction (Birchler et al., 1999).

Future experiments could include histology and immunohistochemistry to investigate tumour response mechanisms (Koudinova et al., 2003). Such a study was carried out with Tookad-PDT and identified tumour vasculature damage accompanied by coagulation and haemorrhage as the main cause that eventually lead to tumour cell death. Within 10 days an inflammatory response with inflammatory cells and necrotic tissue was observed. Tumour starvation by hypoxia is a likely mechanism of action of one of the most promising PSs. (Koudinova et al., 2003).

Overall, a $^{213}$Bi-C6.5(-k) RIC, the closest independent study similar to our PIC system showed no significant efficacy on SKOV3 tumours and high toxicities, albeit after one treatment cycle (Adams et al., 2000). Perhaps considering that an α-particle has a diffusion range of 70 µm (Adams et al., 2000) compared to singlet oxygen of <100 nm (Moan et al., 1991, Hatz et al., 2008) and a half life 47 mins (Mcdevitt et al., 1998) compared to 3.5 µs (Egorov et al., 1989), an alpha particle should be more potent overall. However, an alpha particle appears to be less cytotoxic than singlet oxygen or other ROS. Most importantly, there was no significant observed tumour response from mice treated once with unconjugated C6.5(-k) (Adams et al., 2000). The sensitivity of SKOV3 cells to radioactivity compared to ROS could partially explain the differences observed with our system. There are several reports of SKOV3 being resistant to chemotherapy drugs. We have observed the increased resistance of SKOV3 to cisplatin compared to other human cancer cell lines in vitro (data not shown). It is therefore possible that SKOV3 are more susceptible to ROS than radioactivity but at the same time it may be that our PIC is much more potent than Bi-213 radioactive RIC (Liu et al., 1998, Schmidt et al., 2001, Nowis et al., 2006, Milenic et al., 2008, Danhui et al., 2009).

For the C6.5(-k)-PPa PIC specificity increased 5-fold from 2 hrs to 48 hrs and uptake didn’t change much between 2 and 16 hrs (as clearance is slower for PPa) thus justifying the results by Bhatti showing lower potency at 24 hrs compared to 8 hrs which was found to be more appropriate (Bhatti et al., 2008). Using analogous reasoning, the 4 hour gap is probably ideal for the C6.5(-K)-PS1 PIC although it could be further investigated.

When treating the mice with the higher concentration C6.5(-k)-PS1 PIC, the observed response was worse than for the lower concentration PIC even though the antibody was four times higher in concentration. Response was more significant for the two round treatments.
indicating that some process occurs between first and second round consistent in both sets of experiments that enhances the efficacy, killing more resistant cells or cells that didn’t receive enough treatment during the first cycle. The higher concentration PIC has only 1.4 molecules of PS1 per scFv molecule making the injected dose of PS1 per mouse to be slightly less than the main therapy. It is also possible that as there is a larger amount of non-covalently bound material in the more concentrated sample, it is more heavily aggregated affecting its photophysical properties. Aggregation is known to decrease singlet oxygen generation. Overall, in order to achieve similar or increased efficacy with higher concentration PICs optimisation will be required including further studies and quantification of the photophysical properties of the PIC at the various concentrations.

Most importantly, the limitations described and supported by the data in chapter 5 regarding the non-specific cytotoxicity that was observed in vitro for the PICs does not apply in vivo. There is no significant correlation between the in vivo efficacy of the PIC compared to the free PS1. No adverse effects were observed. Inflammation, skin necrosis and animal distress were monitored.

This is a foremost achievement and verification of proof of concept. TPDT works well leading to complete tumour regression in a model where other therapies have failed to achieve similar results. The C6.5(-k)-PS1 PIC used in a double therapy showed improved efficacy compared to C6.5(-k)-PPa PIC when used with the same model and experimental set up. Tumour eradication was also observed by Bhatti with the C6.5(-k)-PPa PIC however that was achieved in 3 rounds of therapy (Bhatti et al., 2008). It also emphasises the need for further in vivo work to be carried out in order to understand the mechanisms tumour response.

6.3.5 Limitations of the work

Some of the limitations of this work include the inefficient way of quantifying the efficiency of radiolabeling of the PSs, the PICs and the scFv. However, it was not considered crucial as the data is presented in relation to the injected dose thus independent of other samples. As mentioned in section 6.1.1 it is possible that radiolabeling affects the hydrophobicity of the labelled molecule perhaps affecting the values of the PK and biodistribution.

The lack of good quality data for the blood clearance of some of the samples could be addressed by either increasing the number of mice used or increasing the initial radioactivity dose given to obtain higher gamma counts at the later time points. This should allow us to
generate better plots to obtain more accurate $\alpha$ and $\beta$ phases especially for the $\alpha$-phase which is more rapid.

C6.5(-k) was shown to be non-toxic and was therefore not considered as a control here (Adams et al., 2000, Bhatti et al., 2008). A non-specific antibody-PS1 PIC could be prepared to verify the significance of the results when comparing PS1 and C6.5(-K)-PS1 PIC.

The use of nude mice allows the use of a fully human tumour model to be tested in a living organism and is common in therapeutic cancer research. However, even though murine xenografts are widely used in preclinical testing, the fact that they are immunodeficient implies that there are limitations as to how reliable the response is. Many successful pre-clinical therapies have failed in humans. Could the nude mice fight the ROS as efficiently as non-immunodeficient normal mice? It would also not be possible to pick up the benefits of a PDT induced immune response that is believed to aid and improve the efficacy of PDT by eliciting an immune response. If PDT does indeed induce an inflammatory response, as was shown for example with Tookad (Koudinova et al., 2003), the nude mouse lacking T-cells (thus T cell helper cells) will not be able to demonstrate it. The use of a syngeneic animal model where a mouse tumour would be treated in a mouse would address this.

6.3.6 Concluding remarks

Overall, a significant advancement has been made with the development of a new PIC based on C6.5(-k) and PS1. The efficacy and clear discrimination between targeted and non-targeted PDT is evident. Importantly, the non-covalently bound impurity present in the PIC appears to have little significant effect in the therapeutic outcome especially as the free PS1 has no efficacy. Savellano states that “it is possible that thorough removal of non-covalently bound associated photosensitisers impurities may not be necessary to produce PIC preparations of clinical utility. Even the most widely used PS in clinical PDT Photofirin consists of a heterogeneous porphyrin mixture” (Savellano et al., 2003). Perhaps, this is not far from the truth. There are several issues that arise with antibody-drug conjugates and efficacy is not easy to achieve.

The PS1 based PIC is clearly more potent than the C6.5(-k)-PPa PIC requiring less cycles to achieve complete tumour eradication (Bhatti et al., 2008). It is also more potent than other published attempts to treat SKOV3 subcutaneous tumours (Adams et al., 2000). It was also shown that the loading ratio of a PIC is crucial for its efficacy for reasons that are somewhat unclear. Whilst injecting very similar effective doses for the free PS1 and the C6.5(-k)-PS1
PIC, very different responses were observed one leading to tumour growth suppression and the other to tumour annihilation.

Finally, the effective drug dose can be lowered as in order to get an analogous response with free PS1 as for the PIC it would require much higher doses of PS than that present in the PIC. There is a general lack of robustly designed clinical trials for PDT with research being restricted mostly to cells in vitro, animal tumours in vivo, with the latter being quite limited as indicated by the table entries in figure 6.1 (Kwitniewski et al., 2008). A major issue faced when carrying out in vivo studies with PICs is that there is no indication as to how potent these really are in humans making it even more difficult to explain and appreciate the newly emerging TPDT data!

6.3.7 Future work

Tumour volume was chosen such that it would allow sufficient laser penetration (Bhatti et al., 2008). Whether larger tumours can be efficiently treated it remains to be seen. Tumours grown orthotopically are difficult to successfully grow and even less so to treat but can also examine collateral damage. That could be a challenging next step.

Using a non-specific antibody as a control would be a further corroboration of the strength and validity of the therapy outcomes. The effect of the loading ratio in conjunction with high concentration PICs needs to be further explored by characterising the photophysical properties of the 4 mg ml$^{-1}$ lower ratio PIC.

Future work should then include skin photosensitivity studies (Pandey et al., 1991) as well as toxicology studies which are rarely undertaken for PDT (Del Governatore et al., 2000)
Chapter 7 Final Discussion
7.1 Overview

In summary, this thesis set the scene on conventional PDT. It is clear that PDT has advantages which have not yet been fully realised. However, limitations such as skin photosensitivity and poor potency of existing PSs need to be addressed before PDT can become a mainstream therapy. Targeted PDT promises to overcome these limitations and is now a developing and expanding area of research. While there are many ways to target PSs, antibodies are the main candidates as they are widely accepted and validated agents. Due to the relatively limited research of fully-characterised PICs, especially in vivo, we are only still learning about the important parameters. However, the more advanced area of ADCs has helped and could even facilitate the advance of TPDT even further.

The chemical synthesis of two new water-soluble PPα derivatives, PS1 and PS4, was described and the optimised routes to their synthesis were extensively discussed. Many other derivatives were examined but the proposed synthetic routes did not afford the desired compounds. Consequently, PS1, a neutral amphiphilic photosensitiser with an NHS ester for bioconjugation and PS4, a monocationic amphiphilic photosensitiser were obtained. The synthesis of PS1 and PS4 was followed by brief yet concise and informative photophysical measurements that confirmed that the two PSs possess equal or improved singlet oxygen quantum yields compared to the parent PPα. They are also characterised with enhanced water solubility. However, aggregation studies showed that they aggregate severely in aqueous solutions with PS1 being slightly improved compared to PPα.

Subsequently, the use of PS1 in the synthesis of C6.5(-k)-PS1 PIC was described highlighting the higher sample concentration (4-fold up to 16-fold) (compared to the C6.5(-k)-PPα PIC) which was loaded with an average of 6 PS1 molecules per scFv. The increased water solubility of PS1 led to increased non-covalent binding which was not resolved despite an extensive combination of purification efforts. When C6.5(-k)-PS1 PIC was examined in vitro, phototoxicity assays showed a 44-fold increase in potency on the antigen positive cell line compared to C6.5(-k)-PPα PIC. It was also shown that it was cytotoxic towards the antigen negative cell line which was attributed to the non-covalently bound PS. This was partially verified by confocal microscopy where both cell lines showed intense staining with the PS from the PIC. The free PS studies showed that PS4 was less potent than PPα on both cell lines which was further investigated by confocal microscopy. It was speculated that the ER co-localisation of PS1 and somewhat of PPα might explain the increased potency exhibited by PS1 and PPα compared to PS4.
Finally, PS1, PS4 and PPα were tested in vivo in SKOV3 tumour bearing nude mice, determining their biodistribution profile and pharmacokinetics. As expected, the cationic PS4 cleared slightly faster than PS1 which cleared faster than PPα. Slight increased tumour specificity was observed for PS4 compared to PPα. However, the true tumour specificity was observed when the C6.5(-k)-PS1 PIC was characterised showing distinct tumour localisation at 24 hrs. In the end, the therapy studies where C6.5(-k)-PS1 PIC was compared for efficacy against PBS and PS1 as controls, showed significant therapeutic effect when the PIC was compared against the free PS. The PIC caused complete tumour regression, further indicating that there is scope for improvement as a two cycle therapy was more significant than one. One of the more important findings was that loading ratio is more important than higher concentration PICs as shown by the in vivo therapy.

Meeting aims and objectives

Considering the objectives of this thesis outlined in chapter 1:

- Synthesis of water-soluble PPα derivatives by incorporating short polyethylene glycol chains and/or positive charges and their characterisation for aggregation and photophysical properties

PS1 and PS4 were made and characterised meeting the increased aqueous solubility criterion set out at the start of this project. It was also important that the absorption maximum $\lambda_{\text{max}}$ and photophysical characteristics of the compounds was at least the same as PPα but for both PS1 and PS4 they are slightly increased compared to PPα which is a welcomed outcome. PS1 was synthesised to contain a bioconjugation handle. This was not achieved in time for PS4 due to its long and cumbersome synthesis. Future PS design will contain a combination of the characteristics found on PS1 and PS4 trying to address the purification problems that arise with increased water solubility. The knowledge gained throughout this project regarding purification should help resolve any future issues more readily.

- Conjugate these to an anti-HER2 scFv to make high concentration conjugates and subsequently purify and characterise the loading ratio of the resulting PICs

The synthesis of C6.5(-k)-PS1 PIC at 1 and 4 mg ml$^{-1}$ met the aim of making higher concentration PICs. However these PICs contained significant amounts of non-covalently bound PS which could not be resolved. It is well accepted that the non-covalent forces that
occur between porphyrinic macrocycles and proteins are very strong interactions that are not easily disrupted. The options and combinations for further purification attempts are endless and can be further pursued. The characterisation of the PICs was achieved by spectroscopy and SDS-PAGE. Attempts to fully characterise the loading ratio by mass spectrometry failed. Mass spectrometry of a PIC has only been reported by one research group in the literature with the use of a pure PIC. It is therefore likely that the characterisation of our PIC by MS will be successful upon purification. The conjugation of PS4 to the scFv was not completed as the active ester was not made.

- Characterise both the PICs and free PSs in vitro using cell viability assays to estimate potency, confocal microscopy and FACS analysis

The in vitro characterisation of the free PSs using PPa as a control both by cytotoxicity assays and confocal microscopy were successfully completed, determining potencies and subcellular localisation respectively. The cytotoxicity assays for the C6.5(-k)-PS1 PIC were also completed showing some increased potency compared to the free PS. FACS analysis of the PICs was not addressed at all.

- Lastly, characterise both the free PSs and a chosen PIC in vivo using a HER2 expressing cancer tumour xenograft on nude mice. The potency of the PIC determined and either prove or disprove the benefits of TPDT.

The final objective set in chapter 1 was fully met, as it was shown that there is a significant potency difference between PDT (using free PS1) and TPDT using the C6.5(-k)-PS1 PIC. The use of the PIC showed that it is possible to lower the effective drug dose, minimising potential side effects. This result provides further proof of principle.

Synthetically, this thesis described the use of Sonogashira coupling to introduce a handle for bioconjugation and Suzuki coupling to introduce a positive charge on the 20-meso position.

By comparing PS4 and PS1 in vitro, we showed that their structural differences affect their cytotoxicity and subcellular localisation. It was also indicated that the ER may have an important role in PDT efficacy as it was the main point of variation in their subcellular localisation that could explain their marked potency differences. Photosensitiser structure
(charge, hydrophilicity etc) is also important when looking at free PS treatment because the structure can affect the “targeting” or preferential localisation site and efficiency of a free PS (Josefsen et al., 2008b). It may be interesting to examine the correlation between intracellular localisation, tumour localisation and efficacy in vivo using ex vivo tissue samples.

The loading ratio we achieved assuming there is a linear correlation between PICs and ADCs at 6 molecules PS1/scFv is in good accord with the results Senter discussed in a recent interview on their findings on auristatin conjugates (Hughes, 2010) where two-four drugs per mAb were found to be optimal (Hughes, 2010).

Finally, the complete tumour regression we observed with our model superseded previous studies by Adams where a RIC (using the same model and antibody) only exhibited tumour growth delay. Furthermore, we improved on the results previously reported by Bhatti for C6.5(-k)-PPa. RIT and ADCs both somewhat compare to TPDT but with the clear advantage of TPDT having (or potentially having) no severe side effects and no drug resistance as there is no known cellular defence mechanism for singlet oxygen, the main cytotoxic species in PDT.

**Advances and contribution**

The knowledge gained from the work we presented should facilitate the design of improved PPa derivatives both in terms of water solubility and aggregation. Both PS1 and PS4 have significant absorption maxima in the red making them more attractive as PDT agents than porphyrins which tend to have very low extinction coefficients in the red. Both PS1 and PS4 are amphiphilic (which has been shown to be advantageous for free PSs) and both contain or have the potential to contain a single carboxylic acid for bioconjugation. This makes them suitable for direct lysine coupling onto antibodies without the need to either introduce space linkers or modify the antibody’s amino acids as has been often reported in the literature.

Furthermore, the optimised conjugation conditions for PS1 to C6.5(-k) can form the starting point for synthesising new conjugates. We took our first steps to understand the requirements and limitations of a conjugatable photosensitiser and antibody system that can lead to PICs potentially suitable for treating tumours in humans. Our current PIC, at 1 mg ml$^{-1}$ was injected in mice at 8 mg/kg per animal which would correspond to 600 mg of antibody in a human. At 1 mg ml$^{-1}$ that would require a patient to be injected with 600 ml of solution.
which is not feasible. Instead, it could be infused, still successfully delivering the drug to the patient. The requirement for such high volumes would not be well received by clinicians and therefore the need for even higher concentration PICs remains. Increasing the concentration 6 fold would only require 100 ml infusion which would be more acceptable. With increased understanding on the effect of loading ratios, the effect and importance of non-covalent binding, spatial separation of the conjugated PS and the stability of the resulting PICs in vivo may allow for such higher concentration PICs to be ‘engineered’ as it would require both improved PS characteristics and an antibody stable at high concentrations.

The protocols developed for confocal microscopy imaging of these derivatives should be applicable to future PPa derivatives although they might require further optimisation depending on the solubility of the PS and the photophysical properties. Imaging the subcellular localisation of PSs whether free or as part of a PIC is key to understanding the mechanisms of PDT. A hydrophobic PS, PPa localises preferentially in the Golgi apparatus and the ER with PS1 localising mainly in the ER and mitochondria. However, PS4 was shown to localise in the lysosomes which is where a PIC is likely to deliver the PS to. Does PS1 as part of the PIC remain in the lysosomes when delivered by the scFv or does it detach and localise in the ER? If so, PS4 is likely to be a less potent PS regardless of being conjugated as its localisation would not be altered. If this is true then at least for our conditions, targeting the lysosomes is not effective and PS1 or ER targeting should be the preferred route. Understanding the mechanisms driving the localisation and potency in PDT is crucial in designing more potent PSs and PICs.

Lastly, the verification that TPDT has significant benefits to conventional PDT in vivo will provide the basis and reference to future experiments as other cancer models, and other PICs are tested.

Water-soluble PSs are constantly being developed for use in PDT such as porphyrins, chlorins and phthalocyanines. Water solubility is relatively easy to impart however as many have shown and we have discovered, minimising aggregation is not straight forward. There appears to be a systematic approach towards improving the absorption maximum of new PSs to make them more appealing for use in PDT. However, ‘engineering’ PSs to improve their characteristics for bioconjugation has not been widely addressed. Most research is focussed on improved PSs for use as free PSs or for conjugation with small molecules that are more stable to organic solvents reducing the need to significantly improve on the water solubility and aggregation of their PS. We consider the design and synthesis of a bioconjugatable PS that has water solubility such that it can undergo bioconjugation to form
PICs of therapeutic value in vivo, to significantly contribute to the field of TPDT. The PICs reported here were soluble and relatively stable in PBS without the need for PEG chains or high organic solvent formulations as reported by many. As far as we are aware, Alonso et al. led by Boyle and Neri, remain the only other group currently reporting on systematic synthesis of both PSs and PICs followed by their in vitro and in vivo characterisation. Their reports included well characterised PICs that are water-soluble and show efficacy in vitro. However the low loading ratio they report may prove to be insufficient for a corresponding efficacy in vivo as we have shown with our lower loaded PIC. Further to that, their porphyrins have poor absorption in the red making them less likely candidates for further pre-clinical studies for TPDT.

“Can you have your cake and eat it?” Is attempting to incorporate water solubility, absorption in the red, low non-covalent binding, potency and a bioconjugation handle into one single molecule an over-optimists idea?! It will be a while before this can be ascertained as more research is carried out but from these very early steps we can deduce that, it is unlikely that all the above components can be incorporated in one PS. We have shown that we can incorporate water solubility and potency while Boyle et al. have shown water solubility and no non-covalent binding. However, whereas non-covalent binding is likely to restrict the progress of our PSs, low absorption in the red is likely to restrict theirs. The synthesis of a PS4 PIC could clarify further requirements and criteria for the synthesis of an ideal bioconjugatable PS.

The TPDT area is still in its infancy with very few in vivo studies having been published. We have attempted to methodically characterise our PIC in vivo establishing a reference point for future studies. Other cancer models can be looked at such as prostate and breast, examples of HER2 positive tumours or by using a different scFv, target for example, tumour vasculature.

7.1.1 Future work

Immediate future work could include finishing the synthesis of PS4 and making conjugates with C6.5(-k). These could then be used in vitro to determine whether the potency of PS4 changes upon conjugation. Confocal microscopy can be used to further explore the internalisation of the PICs and subsequent fate of the PS as well as the effect of non-covalent binding on both antigen positive and antigen negative cell lines using C6.5(-k) and commercial anti-HER2 mAb as controls.
The next step in photophysical characterisation could be to obtain singlet oxygen quantum yields of the free PSs and PICs in aqueous/physiological buffers. An aqueous system would be a more biologically relevant system helping improve our understanding of the PDT process \textit{in vitro} and subsequently \textit{in vivo}.

More PPa derivatives, containing multiple charges based on short PEG chains could be further explored in an attempt to find more suitable PSs for synthesising a more homogeneous and fully characterised final PIC. The conjugation of PS1 to a different scFv to explore the non-covalent binding of PS1 in a different protein environment as well as exploring other tumour antigens to further realise the potential and limitations of our system could also be useful. Combining various PSs with various antibodies can give rise to endless studies.

Would it be unrealistic to imagine a scenario where a potent photosensitiser is conjugated to order to antigen specific antibodies to treat various cancers?

Finally, a longer term plan would involve establishing a mechanism of action of the C6.5(-k)-PS1 \textit{in vivo} using various drug doses or various loading ratios of PS/scFv. Non-covalent binding may be considered a problem when producing a pharmaceutical, but there are other heterogeneous mixtures in the clinic (Photofrin, Visudyne, ADCs) which show that a reproducible and well characterised drug is more important than purity. Scaling up the synthesis of the PIC, establishing its maximum tolerated dose in an animal model (MDT) would be required followed by identifying a process to make it on a clinical scale and of clinical purity under the good manufacturing practice (GMP).
Appendix

Appendix 1
Synthesis schemes for PS5 and PS6

Scheme 1 Proposed synthesis of PS5; a. addition of HBr on the vinyl bond followed by b. substitution using a large excess of the benzyl alcohol (34); c. would include acid/alcohol coupling as for PS1 to introduce the same benzyl alcohol (34) on the propionic acid chain followed by Sonogashira coupling to introduce a handle for bioconjugation and subsequently activation to obtain the NHS ester.
Scheme 2 Shows the complex attempts to make the key benzyl alcohol moiety (41) (in red) containing tertiary nitrogens (suitable for quaternisation) for PS6 (scheme 3, appendix 1). Looking at the alcohol (41) two routes appeared suitable. One was to produce as previously an activated tosylate (39) with which to alkylate (43) (which wasn’t successful) or the activation of the benzylic hydroxyl groups (43) (which was successful, (38)) and its subsequent use to alkylate (42) to obtain the benzyl ester (40) (which didn’t work). Use of (38) to obtain benzyl alcohol (34) was successful. Also successful was the synthesis of (45) from (42) and (32).
Scheme 3 Shows the proposed route to PS6 which could not be made. The proposed reactions followed the same path as for PS1. Obtaining the benzyl alcohol (41, appendix 1) building block was not possible in the given time. Theoretically, PS6 could have been obtained in 4 steps from the main derivative bromo-meso-PPa (10); a. alkylation, b. Sonogashira coupling, c. quaternisation and d. active ester.
Appendix 2

Chapter 6 *In vivo* PDT therapy- individual mice responses.

Individual Mice responses following C6.5(-k)-PS1 PIC therapy at 1 mg ml\(^{-1}\) -2 cycle treatment

![Graph showing individual mouse responses](image)
Publications and presentations associated with the work in this thesis

Talks and poster presentations

Division of Cell and Molecular Biology, Imperial College London, 20 minute final year talk. “Photoimmunoconjugates for use in Photodynamic Therapy (PDT) of cancer”, Ioanna Stamati.

Division of Cell and Molecular Biology, Imperial College London, poster presentation “Photoimmunoconjugates for Photodynamic therapy of cancer” Ioanna Stamati, Gokhan Yahioglu, David Phillips, Mahendra P. Deonarain.

Graduate Schools of engineering and physical sciences, Imperial College London, poster presentation “Photoimmunoconjugates for Photodynamic therapy of cancer” Ioanna Stamati, Gokhan Yahioglu, David Phillips, Mahendra P Deonarain.

237th American Chemical Society conference, Salt Lake City, Utah, March 2009, poster presentation, Targeted photodynamic therapy of cancer using novel photosensitizer derivatives based on pyropheophorbide-a (PPa). Stamati, Ioanna; Yahioglu, Gokhan; Phillips, David; Deonarain, Mahendra P.

Publications


Two further papers are currently in preparation based on the work described in chapters 4, 5 and 6.


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