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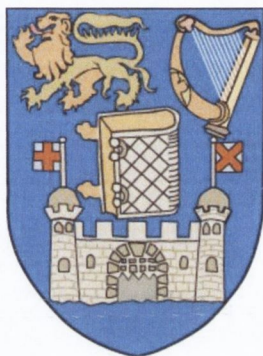
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***Investigation of novel photosensitisers for
photodynamic cancer therapy***



Submitted by

Edyta Paszko

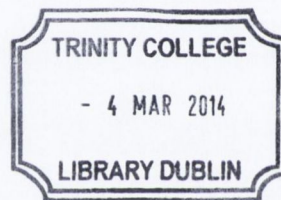
M.Sc. (Laboratory Medicine), Medical University of Bialystok, Poland

A thesis submitted to the University of Dublin, Trinity College for degree of

Doctor of Philosophy

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2013



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Summary

Photodynamic therapy (PDT) has developed over last century and is now becoming a more widely used medical tool having gained regulatory approval for the treatment of various diseases such as cancer and macular degeneration. It is based on photochemical reactions between light and tumour tissue with exogenous photosensitising agents (PS), most often porphyrins. Photochemical reactions lead to the conversion of molecular oxygen ($^3\text{O}_2$) into singlet oxygen ($^1\text{O}_2$) which is a key cytotoxic agent that damages cells.

The first photosensitiser officially approved was Photofrin® (Porfimer Sodium), but unfortunately has limitations for use in PDT due to prolonged photosensitivity (poor clearance) and lack of long wavelength absorption. As a result, a number of new second-generation photosensitisers have been developed. One of the most promising ones is 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*-THPC, Temoporfin, Foscan®), which is activated at a wavelength of 652 nm. Foscan appears to be among the most effective photosensitisers in PDT but still has some disadvantages such as photosensitivity and hydrophobicity.

Development of safe and highly selective photosensitising agents led to the investigation of newer PS, called third generation photosensitisers. These new drugs are characterized by conjugation with ligand which specifically targets the PS to the cancer cells, resulting in minimized accumulation in healthy tissues.

Recently, research is mainly focused on improving drug delivery with introducing of new delivery methods, formulations and enhanced targeting approaches, especially using of existing photosensitisers in conjunction with nanoparticles and a move to “nanomedicine”.

Several types of delivery vehicles have been applied to carry the photosensitiser to malignant cells, where lipid based delivery system such as liposomes offer many advantages. One of the most important is their ability to encapsulate both hydrophilic and hydrophobic drugs due to phospholipids typically used for preparing liposomes which are comprised of a hydrophilic head group and two hydrophobic chains. Liposomes are widely used in many areas in medicine. They have been also applied in photodynamic therapy achieving promising results. To improve the effect of Temoporfin, delivery to the target tissue, water solubility and also to prolong sensitiser circulation in the body we use liposomal formulations of Temoporfin, which is a more recently developed third generation photosensitiser based on a DPPC/DPPG lipids.

First, different formulations on liposomal Temoporfin were prepared according the thin lipid film method and extrudation in order to obtain a proper size of our vehicles. In order to further enhance activity of the liposomal formulation of Foscan and obtain specifically targeted vehicles, polyethylene glycol (PEG)-grafted, transferrin (Tf)-conjugated liposome formulations of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin, were prepared. Physicochemical properties of liposomal formulations, phospholipid quantification, encapsulation efficiency and transferrin binding efficiency in case of targeted liposomes were performed.

Expression of transferrin receptors (CD71) in the oesophageal cancer cell line, OE21, was confirmed by immunoblot and confocal laser scanning microscopy. The anti-proliferative effect of Foscan liposomes was evaluated using MTT reduction assay and compared with plain formulations (*i.e.*, without Tf) as well as with free drug. In addition, the intracellular accumulation was studied using high content analysis. Tf-targeted drugs and drug delivery systems have shown improved the therapy of many cancers; however, our study did not corroborate these findings.

We also investigate a class of newly synthesized porphyrin derivatives designed for photodynamic therapy against Human Caucasian esophageal carcinoma cell lines (OE33) and human esophageal adenocarcinoma (SKGT-) cell lines. Light and dark toxicity of porphyrin derivatives was evaluated using MTS assay and the intracellular accumulation was studied using high content analysis as previously. Novel, promising, water soluble photosensitiser were found.

Publications

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Paszko E, Vaz GMF, Davies A, Ehrhardt C, Senge MO. (2011): Liposomal formulations of photosensitisers and novel high content screening approach for the advancement of photodynamic therapy (PDT). The CSCB Annual Symposium 2011. Recent Advances in Synthesis and Chemical Biology X. 9/12, Health Sciences Centre, University College Dublin, Dublin, Ireland.

Paszko E, Vaz GMF, Ehrhardt C, Senge MO. (2011): Liposomal formulations of photosensitisers for photodynamic cancer therapy. FEBS Advanced Lecture Course on Translational Cancer Research. 27/09-4/10, Algarve, Portugal.

Paszko E, Vaz GMF, Ehrhardt C, Senge MO. (2011): Liposomal formulations of photosensitisers for photodynamic cancer therapy. 14th Congress European Society for Photobiology. 01/09-6/09, Geneva, Switzerland.

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Abbreviations

Ab – antibody;

ALA – aminolevulinic acid;

AIPcS₄ – aluminium phthalocyanine tetrasulfonate;

AMD – age-related macular degeneration;

APC – argon plasma coagulation;

BBB – blood brain barrier;

BCA – bicinchoninic acid;

BSA – bovine serum albumin;

Chol – cholesterol;

DPPC – 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine;

DPPG – 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt);

DSPC – 1,2-distearoyl-*sn*-glycero-3-phosphocholine;

DSPG – 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)];

DSPE-mPEG – distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt);

DSPE-PEG₂₀₀₀-COOH – 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (ammonium salt);

DSPE-PEG₅₀₀₀-COOH – 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-5000] (ammonium salt);

EDC – *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride;

EGF – epidermal growth factor;

EMR – endoscopic mucosal resection;

EPR – enhanced permeability and retention effect;

ER – estrogen receptor;

FRET – fluorescence resonance energy transfer;

GORD – gastroesophageal reflux disease;

HCS – high content screening;

HDL – high density lipoprotein;

HER2 – human epidermal growth factor receptor 2;

Hp – Hematoporphyrin;

HPMA – N-(2-hydroxypropyl)methacrylamide;

KTP – potassium-titanyl-phosphate laser;

LDL – low density lipoproteins;

LUV – large unilamellar vehicles;

mAb – monoclonal antibody;

M-ALA – methyl aminolevulinic acid;

MB – methylene blue;

MC40 – merocyanine 540;

MLV – multilamellar vesicle;

MV – multivesicular vehicle;

m-THPC – 5,10,15,20-tetra-(*m*-hydroxyphenyl)chlorin;

N₂O₃ – dinitrogen trioxide;

NC – naphthalocyanines;

Nd-YAG – neodymium-doped yttrium aluminium garnet laser;

NLS – nuclear localization sequence;

NO₂ – nitrogen dioxide;

NO – nitric oxide;

NOS – nitric oxide synthase;

NPC – nuclear pore complex;

NPe6 - Talaporfin sodium, mono-*L*-aspartyl chlorin *e*6;

OLV – oligolamellar vehicle;

ONOO⁻ – peroxyxynitrite;

PC – phosphatidylcholine;

PdI – polydispersity index;

PDT – photodynamic therapy;

PEG – polyethylene glycol;

PGA – poly(glycolic acid);

PLA – poly(lactic acid);

PLGA – poly(lactic-coglycolic acid);

PS – photosensitiser;

PVAL – poly(vinyl alcohol);

RA – rheumatoid arthritis;

RES – reticuloendothelial system;

RME – receptor mediated endocytosis;

RNS – reactive nitrogen species;

SA – sterylamine;

S-NHS – *N*-hydroxysulfosuccinimide;

SUV – small unilamellar vesicle;

TAM – tamoxifen;

Tc – transition temperature;

Tf – transferrin;

TfR – transferrin receptor (CD71);

TPC – 5-[4-carboxyphenyl]-10,15,20-triphenyl-2,3-dihydroxychlorin;

VEGF – vascular endothelial growth factor;

VLDL – very low density lipoprotein;

CHAPTER 1 Introduction

1.1 Oesophageal cancer

Cancer is abnormal and uncontrolled growth of the cells. The normal cells transform to the abnormal, cancer cells due to changes in their DNA which accumulate over the time. Alteration in the genetic information cause abnormal function of the cells. Cancer cells divide rapidly and uncontrollably forming accumulation of the abnormal cells called tumor [1].

The main difference between cancer and normal cells is loss of growth control by cancer cells which do not response to signals that their normal counterparts to cease growth and division. Transformed cells, compared to the normal cells, lack intracellular adhesion and their cell surface becomes convulted, the nucleus is enlarged and irregular and presents increase in nucleo-cytoplasmic ratio, chromosomes swell up and the number of chromosome sets increase owing to the growth of cancer cells which is known as aneuploidia. Besides, cytoplasm alterations, such as reduced Golgi apparatus, cytoskeletal elements such as microtubules and microfilaments tend to be disorganised and degree of this disorganisation depends on the degree of differentiation of cancer cells. [2,3].

There are four major types of cancer: carcinomas, which originate from skin or epithelium of the internal organs, glands and body cavities; sarcomas which originate from transformed cells in connective tissue such as cartilage, bone, muscle or fat; lymphomas which originate from cells which are the part of immune system and leukemias which is the cancer of the blood cells [1].

Oesophageal adenocarcinoma is the fast increasing cancer and has really poor prognosis [4]. Oesophageal adenocarcinoma develops as a consequence of chronic gastroesophageal

reflux disease (GORD) and Barrett's oesophagus is a precursor lesion of this cancer. In Barrett's oesophagus stratified squamous type mucosa of the normal oesophagus is replaced by specialised intestinal type columnar mucosa [5,6,7]. The incidence of Barrett's metaplasia has also an increasing incidence, and since many patients with Barrett's are in surveillance program, there are more patients with high-grade dysplasia and superficial adenocarcinoma presenting for therapy [5,7,8].

The only potentially curative option still remains surgery, but is it strictly associated with significant morbidity and mortality. During the past years, it has been demonstrated new technologies which allow endoscopic therapy with oesophageal preservation, minimize the morbidity and mortality of traditional forms of oesophagectomy. Individualization of therapy would lead to increasing successful outcome and quality of life with minimization of complications [9].

Ablation of the abnormal mucosa is associated with the regrowth of squamous epithelium, which can reduce the risk of progression to malignancy and has potential advantage to avoiding surgery. Due to poor prognosis with oesophageal carcinoma, the successful ablation of Barrett's oesophagus would be an attractive concept. There are several different techniques which lead to produce successful ablation of Barrett's oesophagus [9,10,11].

One of these techniques is thermal therapy, which apply the heat with one of the following specialized devices: electrocoagulation, argon plasma coagulation (APC), heat probe or neodymium-doped yttrium aluminium garnet (Nd-YAG) or potassium-titanyl-phosphate (KTP) laser [11].

Electrocoagulation was one of the earliest techniques used in treatment of Barrett's oesophageal [12,13]. It is safe, inexpensive, and easy to perform ablation method with positive results [14,15]. However, one of the disadvantages is relatively small surface area that can be treated with current probes and multiple procedures are necessary to achieve ablation [12,15].

Argon plasma coagulation (APC) is a convenient method for coagulating tissue which employs a high frequency electric current and ionised argon gas. The main advantage is safety; it is simple to perform, inexpensive and has minimal risk of perforation and bleeding. This treatment is characterised by non-contact coagulation, making it possible to provide tangential irradiation to coagulate a target site [9,16]. However, thermal coagulation of a larger segment of Barrett's is usually time consuming and repeated treatment sessions are necessary [9]. The significant variation in the regression of intestinal metaplasia and the formation of new squamous epithelium has been also noticed [11,17].

Another technique is **neodymium-yttrium-aluminium-garnet (Nd-YAG) laser** treatment which is considered a "coagulating laser". It emits at a wavelength of 1064 nm (invisible) and requires an aiming beam of use which is widely used also in treatment patients with gastric cancer as a curative or palliative therapy. **Potassium-titanyl-phosphate (KTP)** laser emits at a wavelength in the visible light range – 532 nm. This treatment does not require an aiming beam, is minimally invasive which limit the risk of bleeding [11,18].

From nonthermal methods we can mention endoscopic mucosal resection (EMR), photodynamic therapy, radiofrequency energy ablation or cryotherapy.

Endoscopic mucosal resection (EMR) has recently become the most investigated method than any other endoscopic technique and showed promising results [9,11]. EMR is the only endoscopic treatment in Barrett's that provides a specimen for histologic investigation. However, EMR is associated with persistent high grade dysplasia which is the critical step before invasive oesophageal carcinoma, persistent Barrett's epithelium and serious recurrence rates of dysplasia or neoplasia in the residual Barrett's epithelium. Complete removal of intestinal metaplasia is obligatory, thus combination of EMR with other therapeutic modalities can be applied, for example combinations of EMR and

photodynamic therapy with porfimer sodium, 5-ALA, or 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin [11,19,20,21] which showed successful results.

Radiofrequency energy ablation is a novel therapeutic technique using energy emitted from a controlled radiofrequency (RF) source, a sizer balloon catheter, which is introduced into the oesophagus and measures oesophageal width and an RFA balloon catheter (where A stands for ablation) [11,22]. After the oesophagus is measured with the sizer ballon, RFA balloon which consists of 60 electrode rings spaced narrowly in a bipolar fashion is placed in the oesophagus and inflated and RF source delivers energy on the oesophageal surface in a 360-degree [22]. This method allows for control of depth ablation and minimizes the risk of damaged beyond the desired limits [11,22].

Cryotherapy is a noncontact treatment method which uses endoscopically guided application of low pressure liquid nitrogen or liquid carbon dioxide in spray form [23,24] and shows promising early data [23,25,26,27]. However, because it is a new method and studies are still limited, long term outcome studies are necessary [12].

Photodynamic therapy (PDT) is also one of the method which leads to produce successful ablation of Barrett's oesophagus. PDT is a minimally invasive, nonthermal treatment modality which activates a previously administered photosensitising agent with light. It is one of the most promising techniques but still not optimized. There are still significant problems associated with photodynamic treatment [9,11]. This method will be covered in more details in the next chapter.

1.2 Photodynamic therapy

Photodynamic therapy (PDT) has been developed in past decades as an alternative method to the traditional treatment of cancer and non-cancer diseases. This method is based on the photochemical reactions between light and tumor tissue with exogenous photosensitising

agents (PS), most often porphyrins. Subsequent irradiation with light at the proper dose and wavelength leads to the photochemical conversion of molecular oxygen ($^3\text{O}_2$) into singlet oxygen ($^1\text{O}_2$). The latter is a key cytotoxic agent that damages cells *via* apoptosis or necrosis. During apoptosis, often called “programmed cell death”, cells undergo shrinkage, cells surface blebbing, chromatin condensation and chromosomal DNA fragmentation, whereas during necrosis, cells swell and lyse without previous compartmentalization. Necrosis usually occurs only in response to a pathologic form of cellular injury. Oxidative stress causes damage to cellular (macro)molecules such as nucleic acids, proteins and lipids [28,29,30].

The photochemical mechanism in PDT is represented by the Jablonski diagram [**Figure 1**]. The photochemical reaction is initiated by the absorption of a photon of light by a photosensitiser molecule which have ground state (P^0), promoting it to the first excited single state ($^1\text{P}^*$). The singlet excited porphyrin can decay back to the ground state with release of energy in the form of fluorescence – enabling identification of tumor tissue. If the singlet state lifetime is suitable, the ($^1\text{P}^*$) photosensitisers can be converted into the triplet excited state ($^3\text{P}^*$) via intersystem crossing (ISC). In the ($^3\text{P}^*$) photosensitiser can transfer energy to molecular oxygen ($^3\text{O}_2$), exciting it to its highly reactive singlet state ($^1\text{O}_2$). Most photosensitisers have high quantum efficiency for this transition and this is a key characteristic of a good PS. The ($^3\text{P}^*$) photosensitisers can also return to the ground state by emitting phosphorescence [28,31,32].

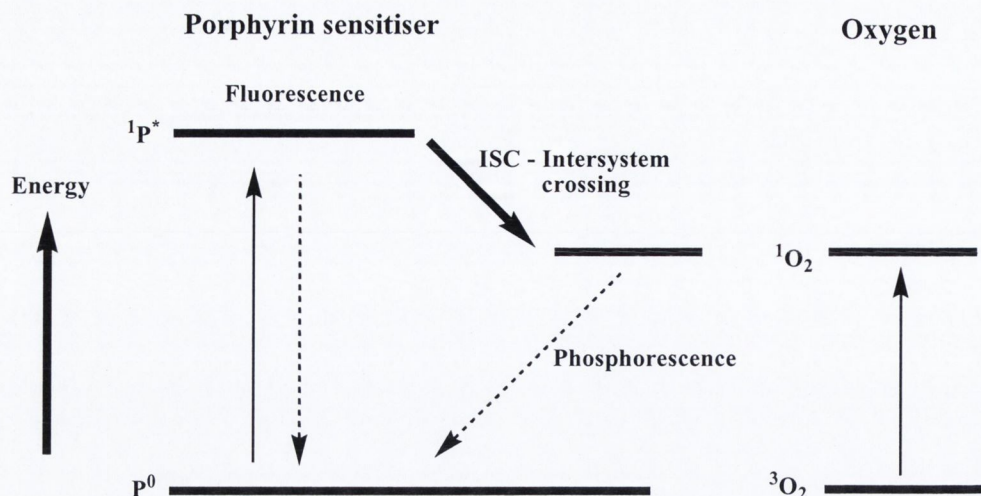


Figure 1. Jablonski diagram. Photophysics of PDT.

There are three types of photodynamic process that promote chemical reactions, they are called type I, II and type III photochemical reactions. Type I photochemical reaction involves electron (or hydrogen) transfer reaction between ($^3P^*$) photosensitizer and oxygen or other molecules producing a radical anion or (cation) respectively. These radicals react with molecular oxygen and produce reactive oxygen species (ROS) which are harmful to the cells. One example is superoxide anions (O_2^-) forming by transfer electron from the photosensitizer to molecular oxygen. Superoxide anions are not very reactive but can react and produce hydrogen peroxide (H_2O_2), which is able to easily penetrate biological membranes. Hydrogen peroxide can react with superoxide anions and produce highly reactive hydroxyl radicals ($\cdot OH$) [28,31,33].

It has been also demonstrated that PDT influences the NO (nitric oxide) level, as the activated photosensitizer can cause production of NO. For the first time, Gupta et.al [34] reported an increase level of NO after PDT treatment of hman epidermoid carcinoma using Pc4 in vitro. The increase of NO correlated with an enhanced constitutive expression of nitric oxide synthase (NOS). No is a free radical, can react with other highly reactive

radicals, after reaction with O_2 or O_2^- , to give reactive nitrogen species (RNS) such as N_2O_3 , NO_2 and $ONOO^-$ (RNS), which can impair the functions of biomacromolecules [35].

The type II of photochemical reaction involves transfer energy from ($^3P^*$) to molecular oxygen (3O_2) which represent the ground state of the molecule and form singlet oxygen (1O_2), a very reactive molecule which is responsible for damage of cells membrane including plasma, mitochondria, lysosomal and nuclear membranes as well as protein modification [28,31,33]. Type III is a less common pathway, where the triplet state PS directly reacts with biomolecule which lead to destroy of photosensitiser and damage of biomolecule [36]. Most known photosensitisers used in PDT represent type II photochemical reaction and quantum yield of singlet oxygen production is one of the most important determinants for photosensitiser activity. Efficiency of PDT depends on the type of photosensitiser, the pharmaceutical formulation, the amount of PS delivered to target cells, the physical localization, time of light exposure and light doses, time between administration of the drug and irradiation with light and amount of oxygen [28,31,33].

The classic PDT treatment of cancer utilizes the administration of a photosensitiser drug to the patient. The drug then accumulates in the tumor tissue and tumor area and next is illuminated with light which activates the photosensitiser inducing cell death.

1.3 Photosensitisers

Photosensitisers are generally classified as porphyrins and non-porphyrin PS. The compounds under investigation have changed over the years in order to find an ideal photosensitising agent. There are many properties which a photosensitiser should posses. One of them is high absorption of the PS between 650 nm and 850 nm where the tissue

penetration is quite high [31]. It should have high singlet oxygen quantum yield for the photochemical reaction, be a single and pure compound with a stable composition, be soluble in water and should not form aggregates. An ideal photosensitisers should be selective to the target tissue, be safe, *i.e.*, should not result in side effects such as mutagenic, carcinogenic or allergic effects, should not be toxic in therapeutic doses and not damage normal healthy cells. Moreover, metabolism and excretion after administration should be as rapid as possible to minimize photosensitivity. In addition, it should be inexpensive, easy to synthesize and commercially available [37,38].

1.3.1 Porphyrin photosensitisers

1.3.1.1 Porphyrins

The term porphyrin originates from the Greek word *porphura* which means “purple”. Porphyrins belong to the tetrapyrroles class which contains four pyrrole-subunits connected by four methine bridges [**Figure 2**] and these are naturally occurring compounds, present in all living cells and playing an important role in biological processes. Metal complexes of porphyrins are involved in essential biological processes such as transport oxygen around the body and storage in muscle tissue, catalysis of biochemical reactions (haemoglobin, myoglobin and cytochrome P450 respectively, which contain iron), photosynthetic process in plants and bacteria (chlorophyll which contain a central magnesium iron) [39].

Porphyrin **1** can undergo reduction of double bond and form hydroporphyrins such as chlorin **2**, bacteriochlorin **3** or isobacteriochlorin **4** without substantial loss of the macrocyclic aromaticity. These compounds absorb further into the red which make them advantageous in biological uses. Bacteriochlorins are even stronger absorbers than

porphyrins and chlorins, but are generally less stable in solution [40]. *In vivo* studies indicated that activity increases in the sequence porphyrin, chlorin, bacterochlorin [40,41,42].

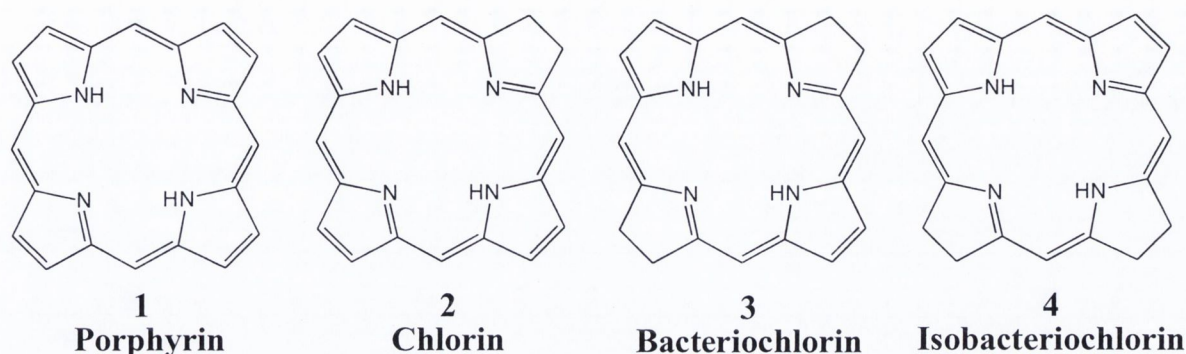


Figure 2. Different classes of tetrapyrroles.

Porphyrin-based photosensitisers are also often classified as a first, second, and third generation PS.

1.3.1.2 First generation photosensitisers

The first generation photosensitisers include hematoporphyrin derivative (HpD) and Photofrin®. Hematoporphyrin (Hp) [**Figure 3a**] was isolated from haemoglobin during research on the nature of blood in 1841 [28,37]. Schwarz *et al.* showed that Hp consists of several porphyrins with different properties [43]. Purification and solubilization of Hp led to preparation of HpD, a complex mixture of several components where about 50% are identified as oligomeric haematoporphyrins and protoporphyrins which had low photosensitising activity *in vivo*. Further purification led to preparation of porfimer sodium (Photofrin®) [**Figure 3b**], a lyophilized and concentrated form of monomeric and oligomeric porphyrins and a reduced fraction of not photoactive components [28,37].

Photofrin® was the first photosensitiser approved for use in the clinic (in 1993 in Canada) with long wavelength absorption maxima between 625 and 630 nm, which allows for a penetration of about 5—10 mm in therapeutic PDT [28,44]. To date, Photofrin® has been approved in most countries for the treatment of various cancer indications, for example, oesophageal cancer, superficial bladder cancer, early and late stage lung cancers as well as malignant and non-malignant skin disease [37,38,45]. Porphimer sodium unfortunately has limitations for use in PDT because of its poor water solubility, prolonged photosensitivity (low clearance rate) and it also lacks long wavelength absorption [38,45,46]. Due to the drawbacks of first generation photosensitisers, a number of the ‘second-generation’ photosensitisers have been developed.

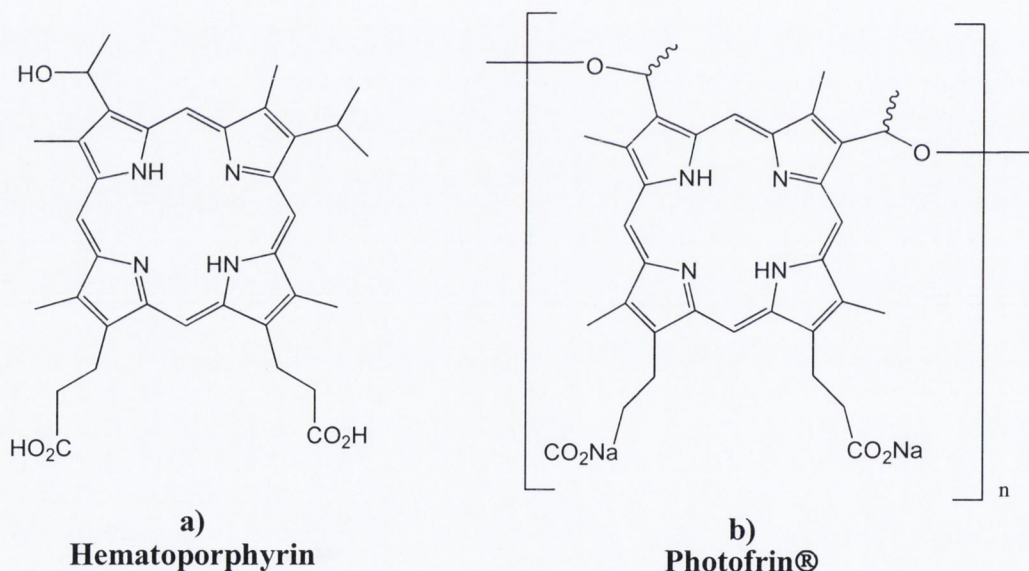


Figure 3. Chemical structures of first generation photosensitisers.

1.3.1.3 Second generation photosensitisers

Unlike first generation PS, these are chemically synthesised as pure compounds with a constant composition, have strong long wavelength absorption in the range of 650—800 nm where tissue penetration is optimal and have higher extinction coefficients of absorption in the red. Their tissue accumulation is much shorter and therefore, the treatment can be carried out on the same day as the administration of the drug. Moreover, second generation photosensitisers show lower toxicity, but most of these agents are still very hydrophobic and show poor tumor selectivity [37,45].

A typical example of a second-generation photosensitisers is **5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin** (Foscan®, Temoporfin) [**Figure 4a**] (approved in 2002 as an agent for the palliative treatment of head and neck cancer), a dark purple, non-hydroscopic, single pure chlorin with a high quantum yield of singlet oxygen generation which is activated at a wavelength of 652 nm. This results in a depth of light penetration of at least 1 cm. Light with a wavelength longer than 850 nm is not used due its weak energy which is not enough to initiate a photochemical reaction. It is practically insoluble in all aqueous media and only soluble in alcohol, acetone and ethyl acetate which present a highly hydrophobic drug [46,47,48]. Foscan® is a member of the chlorin family and one of the promising reduced porphyrins which has been investigated for many years in various research groups [46,48,49,50]. However, in spite of many benefits, it still presents serious drawbacks. One of the most important effects that limitates using Temoporfin is skin photosensitivity. Use of Foscan in PDT requires a long term interval between drug administration and light illumination. This interval is several days, and the patients are restricted to limited light exposures. Due to of the hydrophobic character of the photosensitiser, aggregates are formed which is another drawback of this molecule.

Aggregation results in the localization of only low levels of the substance in the target tissue in comparison to the injected dose. This leads in poor selectivity and limited treatment outcome [37,38,48].

Verteporfin [Figure 4b] is a benzoporphyrin derivative monoacid ring A BPD-MA, which is clinically active when formulated with liposomes under the trade name Visudyne® [51]. It is showing maximal absorption at 690 nm allowing for deep penetration and activation. Verteporfin exhibit also rapid accumulation in the desired tissue and fast clearance from circulation which allow for minimal skin photosensitisation. Treatment can occur 15-30 min after administration [38]. Verteporfin is an approved drug for treatment of age-related macular degeneration (AMD) [52,53,54].

Aminolevulinic acid [Figure 4c] (ALA, Levulan®) is another PS which belongs to second generation photosensitisers and was approved for the treatment of basal cell carcinomas, Paget's disease, squamous cell carcinomas, T-cell lymphomas and other cancer types such as lung, bladder, oral cavity, oesophagus and brain tumors. ALA is particularly effective in dermatology for treating neoplastic cutaneous tissues [30,55]. Aminolevulinic acid is the first committed intermediate in the biosynthesis of tetrapyrroles. This naturally occurring amino acid is enzymatically converted to photoactive protoporphyrin IX (PpIX) in mitochondria of tumor cells leading to their damage after light exposure. The activation of the drug occurs at 630 nm [38,55]. ALA can be administrated intravenously (*i.v.*) or topically. Moreover, oral ALA administration is also acceptable and even though this route is preferred by patients, the bioavailability after oral administration of ALA is generally lower due to presystemic drug elimination [55,56]. ALA demonstrates quick metabolism and high selectivity for malignant lesions. Rapid systemic elimination of ALA-induced PpIX within 24 h reduces prolonged photosensitivity and allows treatment to be repeated at regular intervals without cumulative effects and risk of damage to normal tissue. However, ALA shows many limitations in clinical PDT. It is a hydrophilic compound and does not

penetrate easily through intact skin, cell membranes or biological barriers such as stomach or intestinal walls which causes production of PpIX after topical administration only to 2-3 mm of skin surface, which can be insufficient to receive satisfactory photosensitisation. Moreover, limited depth of tumor penetration, individual variations between the patients which affect ALA absorption and pharmacokinetics parameters as well as pain during the treatment are other disadvantages which can limit the clinical application [37,38,55,56,57,58].

Methyl ester of ALA, **methyl aminolevulinic acid** [Figure 4d] (M-ALA, Metvix®), shows greater lipophilicity than ALA which allows for better penetration through the skin. Methyl aminolevulinic acid is demethylated to ALA through the action of intracellular esterases once inside the target cells and then the standard process of biochemical reactions associated with ALA appeared [37]. After application the drug, red light illumination can take place about 3 h later. Methyl aminolevulinic acid has been approved in many European countries for treatment of basal cell lesions and actinic keratosis, but pain during treatment is still a common side effects [37,38,59,60].

NPe6 [Figure 4e] (Talaporfin sodium, mono-*L*-aspartyl chlorin *e*6, LS11, Laserphyrin®) is another second generation photosensitiser. It is a hydrophilic chlorin, a chemically pure compound clinically approved in Japan for treatment of early-stage lung cancer. It shows significant absorption at 664 nm, high singlet oxygen yield production and tendency to accumulate in lysosomes [37,61,62,63]. Optimal illumination time for tumor areas is about 2–4 h post administration. Recent data suggest that NPe6 is a promising, effective photosensitiser with low skin photosensitivity due to rapid elimination from circulation after administration of the drug [62,64,65,66].

Tookad [Figure 4f] (palladium bacteriopheophorbide, WST09) which is currently under clinical investigation is a palladium-metalated bacteriopheophorbide derived from bacteriochlorophyll (BChL) [37,67]. Maximum absorption wavelength occurs near infrared

at a wavelength of 762 nm which allows treatment with a longer wavelength source and a deeper penetration of the irradiation light. Tookad appears to be promising second generation photosensitisers which shows fast clearance from the circulation after administration [68], short time between administration of drug and light exposure, minimal skin photosensitivity, target the vasculature of the tumor initiating inflammation, hypoxia, necrosis and tumor regression [67,69,70,71].

Purlytin [Figure 4g] (tin ethyl etiopurpurin, rostoporfin, SnET2), is a synthetic purpurin structurally analogous to chlorophyll with maximum absorption at 664 nm [37,38]. Purlytin shows promising results in clinical trials in the treatment of basal cell, squamous cell, chest wall metastasis, and Kaposi's sarcoma in terms of responding tumor to a single dose of a drug [72,73] but still is not devoid of side effect, which one of the most important is photosensitivity caused by accumulation photosensitiser in skin [37,72,73].

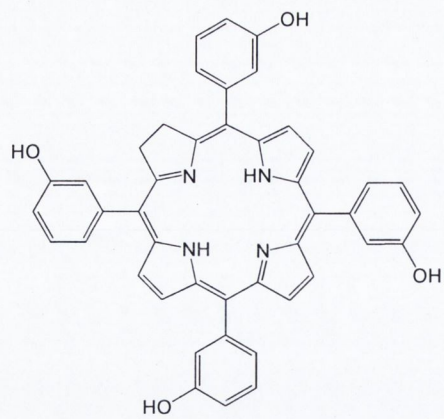
Lutrin [Figure 4h] (motexafin lutetium, lutetium texaphyrin) is a member of texaphyrin molecules family working through tumor and vascular targeting mechanisms. It is a pure, water-soluble photodynamic therapy agent showing a strong absorption at 732 nm, good singlet oxygen production upon irradiation, reduced photosensitivity [37,74,75] and intracellular localization in the lysosomes causing lysosomal degradation, cytoplasmic blebbing and cell death [74]. Clinical trials demonstrate promising results of Lutrin as photosensitising agent for treatment of prostate cancer [76,77] or coronary atherosclerosis [78] with minimal photosensitivity.

Other examples are **porphycenes [Figure 4i]**, a group of second generation photosensitisers which are modifiable isomers of porphyrins [37,79]. Porphycene derivatives show high absorption in the red spectral region (> 600 nm) and efficient production of reactive oxygen species after light irradiation. Photophysical and photobiological properties of porphycenes make them good candidates for photosensitiser agents demonstrating fast uptake and various subcellular localizations mainly in

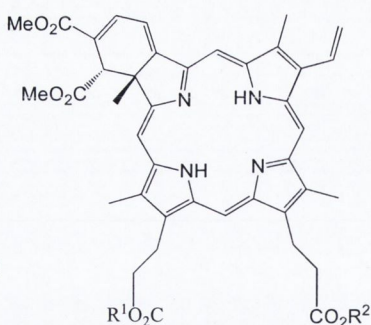
membranous organelles [37, 80]. One of the examples of wide range of porphycenes is hydrophobic, tetra-*n*-propyl-porpycene (TPP) which exhibit efficient and selective transport to malignant tissue [81,82]. Another example, 9-acetoxy-2,7,12,17-tetrakis-(β -methoxyethyl)-porphycene (ATMPn) shows good uptake and lysosomal localization in the cancer cells, minimal dark toxicity and high photodynamic effect [83].

Phthalocyanines (PC) [Figure 4j] are group of second generation photosensitisers which differ from porphyrin by having nitrogen atoms link the individual pyrrole units. They are suitable agents for photodynamic therapy due to their intense absorption in the red (650-680 nm) where appreciable light penetration occur, high triplet state quantum yields and long triplet lifetimes, especially when a suitable central atom such as zinc, aluminium or silicon is included [37,40,84,85,86,87]. For example, aluminum phthalocyanine tetrasulfate demonstrated promising clinical response in nasal and aural squamous cell carcinoma in cats [88], zinc phthalocyanine tetrasulfonate showed high photodynamic effect on rat brain mitochondria [89], whilst recent studies with silicon phthalocyanine photosensitisers show that this agent can be effectively delivered into human skin via topical application [90] and is a safe, tolerable PS with no significant local toxicity or increased photosensitivity, which effectively triggers apoptosis in cutaneous neoplasms such as mycosis fungoides in phase 1 clinical trial [91].

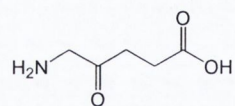
Naphthalocyanines (NC) [Figure 4k] are extended phthalocyanine derivatives which have an additional benzene ring attached on the periphery of the phthalocyanine structure. They have stronger absorption than phthalocyanines (approximately 740–780 nm) which further increase the depth NC photosensitisers. However, a NC photosensitisers present number of problems, such as instability and tendency to form aggregates which are photoinactive [40,92]



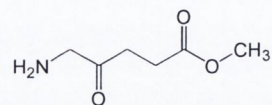
a)
5,10,15,20-Tetrakis(*m*-hydroxyphenyl)chlorin, Foscan



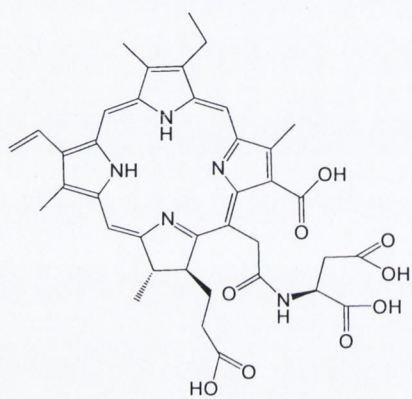
b)
benzoporphyrin derivative
Verteporfin
regioisomeric mix of
 $R^1 = H \quad R^2 = Me$
 $R^1 = Me \quad R^2 = H$



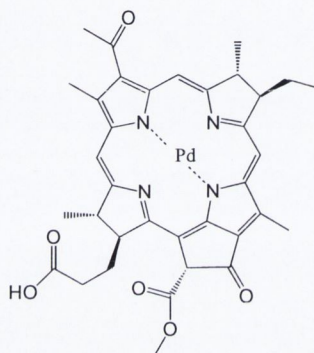
c)
ALA - δ -aminolevulinic acid



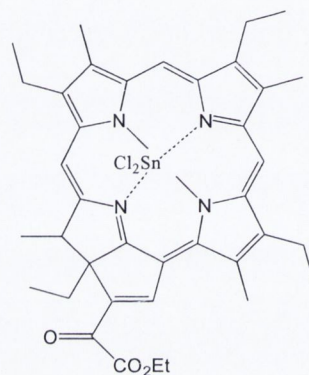
d)
M-ALA -methyl aminolevulinic acid



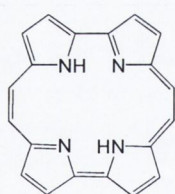
e)
NPe6, Talaporfin sodium



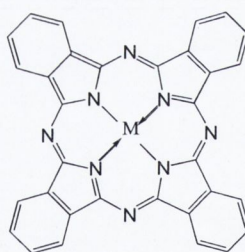
f)
Tookad



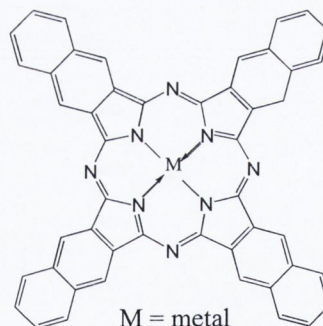
g)
Purlytin



i)
Porphycene



M = metal
j)
Phthalocyanine



M = metal
k)
Naphthalocyanine

Figure 4. Chemical structures of some second generation photosensitisers.

1.3.1.4 Third generation of photosensitisers

PDT with various photosensitisers has been clinically approved for the treatment of several malignant and non-malignant diseases. Each of the currently commercially available photosensitisers has specific characteristics, but none of them is an ideal agent. Most of the PS are hydrophobic and can aggregate very easily in aqueous media which can affect their photophysical, chemical and biological properties and show poor tumor selectivity [93]. Thus, development of safe and highly selective photosensitising agents led to the investigation of newer PS, called third generation photosensitisers. These new drugs are characterized by conjugation with ligand which specifically targets the PS to the cancer cells, resulting in minimized accumulation in healthy tissues [37,38,87,94].

There are several strategies to enhance PDT efficacy and selectivity by specific delivery of photosensitiser to the target cells. One of the methods is coupling the drug with specific proteins which are expressed in or on the cancer cells in comparison to normal cells due to their transformed nature [95]. Several tumor cells overexpress epidermal growth factor (EGF) receptors, thus it is an attractive molecule for specific targeting which is internalized into the cancer cells *via* receptor-mediated endocytosis (RME). One of the example is EGF conjugated with disulfochloride aluminum phthalocyanine [Pc(Al)] and disulfochloride cobalt phthalocyanine [Pc(Co)] which showed higher cytotoxic activity than free phthalocyanines *in vitro*, against the human breast carcinoma cell line MCF-7 and demonstrated inhibition of tumor development, increased mean life spans and mean survival time *in vivo* in murine melanoma B16 unlike free phthalocyanines which had no effect [96].

Photosensitisers coupled with transferrin (Tf) specific to transferrin receptor (TfR) also target cancer cells through receptor mediated endocytosis, for example uptake of hematoporphyrin (Hp) conjugated with Tf by murine NIH-3T3 fibroblast and human HT29

colon cancer cells [97]. Hematoporphyrin coupled with Tf also significantly improved the specificity and efficiency of PDT for erythroleukemic cells by almost 7-fold than free photosensitiser. Moreover, including the compound luminol together with PS-Tf initiated intracellular chemiluminescence which activated Hp and showed cytotoxicity in 95% of cells. These combined approaches which were used to obviate the use of external radiation for PS provide an opportunity to develop new, more effective applications of PDT [98].

Another strategy to improve specificity as well as solubility of PS is to conjugate the drug with amino acid and one of the example is second generation photosensitiser NPe6 (mono-*L*-aspartyl chlorin *e*6) which is a chlorin *e*6 functionalized with aspartic acid [95]. Recent clinical data suggest that NPe6 is promising, effective photosensitiser with low skin photosensitivity and rapid elimination from circulation after administration of the drug [66,99].

Polymer-bound photosensitisers such as polyethylene glycol (PEG), *N*-(2-hydroxypropyl)methacrylamide (HPMA) or poly(vinyl alcohol) (PVAL) also facilitate delivery to target tissue [33]. The improved tumor localization was observed for meta-tetra(hydroxyphenyl)chlorin (*m*-THPC) conjugated with polyethylene glycol in nude mice grafted with a human colon carcinoma [100], whilst meso chlorin *e*6 (Mce6) conjugated with *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer showed enhanced photocytotoxicity in SKOV-3 human ovarian carcinoma cells [101].

Photosensitisers coupled with cationic peptides are another promising method for improving transport which is related to their interaction with the negatively charged surface of most cells [95]. Membrane-penetrating arginine oligopeptide was conjugated to 5-[4-carboxyphenyl]-10,15,20-triphenyl-2,3-dihydroxychlorin (TPC). Such complex enhanced intracellular uptake of the photosensitiser, improved water solubility of TPC and increased phototoxicity in human MDA-MB-468 breast cancer cells [102].

Non-proteic ligands such as folic acid (also called vitamin B9) have also been evaluated as the targeting ligands for selective binding the folate receptor [95]. The folate receptor is a membrane protein highly overexpressed of many cancer cells with no expression in normal healthy tissue [95, 103]. Temoporfin conjugated to folic acid enhanced accumulation of the PS *in vivo* in mice xenografted with FR- α -positive KB human nasopharyngeal cells but not in mice xenografted with HT-29 human colon adenocarcinoma cells lacking FR- α suggesting a good selectivity of the PS-folic acid conjugates [104].

Photosensitisers such as pyropheophorbide coupled with tamoxifen (TAM) [105], chlorin *e6*-dimethyl ester conjugated with C17- α -alkynylestradiol [106] or tetraphenylporphyrin coupled with C(11)- β -derivative of estradiol [107] show specific binding to estrogen receptor (ER) and cause selective cell-killing in MCF-7 breast cancer cells upon light exposure making them potential candidates for ER-targeted photodynamic therapy of hormone sensitive cancers.

Another approach is conjugations PS with saccharides. These complexes demonstrate increased uptake, accumulation and phototoxicity of photosensitiser agents in tumor tissue [95] since rapidly proliferating cancer cells overexpress monosaccharide transporters at the surface [108]. An example is a fluorinated tetraphenylporphyrin coupled with glucose which showed increased binding and phototoxicity against breast cancer cells [109] or glycoconjugated tri and tetra(*m*-hydroxyphenyl)chlorins which demonstrated promising results in human adenocarcinoma cells [110].

A different strategy employed monoclonal antibodies (mAbs) or their fragments as targeting ligands which can specifically bind to epitopes on cancer cells, called photoimmunotherapy [95]. First study with a photosensitiser conjugated with monoclonal antibody applied hematoporphyrin chemically coupled to monoclonal antibodies directed to the DBA/2J myosarcoma and showed effective drug delivery and suppression of tumor growth in experimental animals compared with the free photosensitiser [111]. A chlorin

e6 conjugated to mAb OC125, which recognize the antigen CA 125, showed selectivity accumulation in murine ovarian cancer *in vivo* [112] whilst coupled with murine monoclonal antibody 17.1A showed efficient and selective treatment of hepatic metastases of colorectal cancer in an orthotopic murine xenograft [113]. Other examples of photosensitisers coupled with antibodies or antibody fragments showing great potential in selective delivery to cancer cells *in vitro* are for example pyropheophorbide-a coupled with two different mAb (HER50 and HER66) targeted human epidermal growth factor receptor 2 (HER2) in SK-BR-3 breast and SK-OV-3 ovarian cancer cells expressing HER2 [114], and *in vivo*, *m*-THPC conjugated to murine mAb 425, specific for head and neck squamous cell carcinoma [115]; hematoporphyrin, conjugated with antibodies to vascular endothelial growth factor (VEGF) in Lewis lung carcinoma murine models [116] or bis(triethanolamine)Sn(IV) chlorin *e*(6) attached to L19 human antibody in mice with subcutaneous tumors [117];

Target specific organelles in the cells also show promising results [95]. One of the examples is conjugation of photosensitisers with NLS, a short peptide, nuclear localization sequence specifically recognizing nuclear pore complex (NPC), one of the organelle in the cell [95]. A novel zinc(II) phthalocyanine conjugated with a short peptide with a nuclear localization sequence, Gly-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val, showed enhanced the cellular uptake, increased efficiency in generating intracellular reactive oxygen species, and higher photocytotoxicity against HT29 human colorectal carcinoma cells *in vitro* and enhanced tumor-retention in tumor-bearing nude mice [118].

Photosensitisers coupled with peptides, which are specific for enzymes overexpressed in cancer cell, use photosensitising agent which is quenched by a quencher placed near the photosensitiser or by self-quenching when two PS are close to each other. Hydrolysis of the peptide leads to release PS from quenched state [95]. Multiple units of photosensitiser pheophorbide a, were tethered to a polymeric backbone via short thrombin-cleavable

peptide linkers to target thrombin, a protease upregulated in tissues of rheumatoid arthritis (RA) patients. Interaction between neighbouring PS units efficiently quenched photoactivity, which was restored by exogenous and endogenous thrombin both *in vitro* and *in vivo* in a collagen-induced arthritis model showing selective accumulation in diseased joints [119].

1.3.2 Non porphyrin photosensitisers

The development of non-porphyrin photosensitisers lacks considerably behind that of the respective porphyrin PS. A typical example is hypericin [**Figure 5a**], a naturally occurring compound from *Hypericum perforatum* (commonly known as St.John's Wort) plants with an absorbance around 590 nm [37]. It shows high singlet oxygen production, minimal dark toxicity, and high clearance from the body, after administration [120]. Its mechanisms of action strongly depend on oxygen, with no photocytotoxicity occurring in hypoxic environments [37,121,122] whilst efficient singlet oxygen production under normal oxygen conditions [37,123,124]. Hypericin exhibits lipophilic nature and localizes in cell's membrane [37]. *In vitro* and *in vivo* studies demonstrate their antidepressant [125], antiviral [126], antibacterial [127] and promising antitumor effects [128,129]. Although hypericin shows good tumor localization and minimal dark toxicity, pain associated with treatment has been reported [37,130].

Methylene blue (MB) [**Figure 5b**], a tricyclic phenothiazine drug is another example of non-porphyrin photosensitisers widely used in medicine, showing maximum absorption at 666 nm, high singlet oxygen production and destroying nucleic acids in a nuclease-like manner [37]. Initially, it was applied as a drug for treatment of malaria and Guttman and Ehrlich were the first who report the antimalarial properties of methylene blue in 1891 [131,132]. As a photosensitizer, methylene blue was effectively used for example in

patients with oesophageal cancer [133], in *in vivo* studies with experimental colonic tumours [134], in *in vitro* studies with melanoma cells [135], to inactivate *Candida albicans* [136] or *Helicobacter pylori* using endoscopic white light and MB [137]. However, there are still many drawbacks associated with MB as a PS, such as lack of activity associated with intravenous administration due to poor tumor localization of highly hydrophobic agent [37].

Other examples are cationic photosensitisers such as cyanine dyes which demonstrate maximum absorption at wavelengths longer than 600 nm [138]. Cyanine dyes consist of two nitrogen atoms linked by a conjugated polymethine chain containing an odd number of carbon atoms [139]. They have been applied to dyeing textiles, in silver halide photography, as mode-locking compounds in laser technology, organelle stains, labels for neuron pathway tracing and as probes for membrane structure and dynamics and photodynamic therapy [139]. Most of these compounds are cationic and accumulate in mitochondria of neoplastic cells with greater selectivity in comparison with normal cells [37,140]. One of the example of cyanine dyes used in PDT is merocyanine 540 (MC40) [Figure 5c], a negatively charged oxacyanine derivative, used for example for selective purging of ocular leukemia, lymphoma and neuroblastoma cells in autologous bone marrow grafts [140].

Xanthene dyes such as Rose Bengal and eosin show intense absorption in the green area of the visible spectrum (480-550 nm) and high yield of singlet oxygen production [40]. An anionic water-soluble Rose Bengal has been considered a promising sensitizer in photodynamic therapy of tumors, with minimal side effects [141]. It has been applied as a photosensitising agent also for destroying *Candida albicans* [142], Enterobacteriaceae [143] or for topical delivery to the skin [144,145].

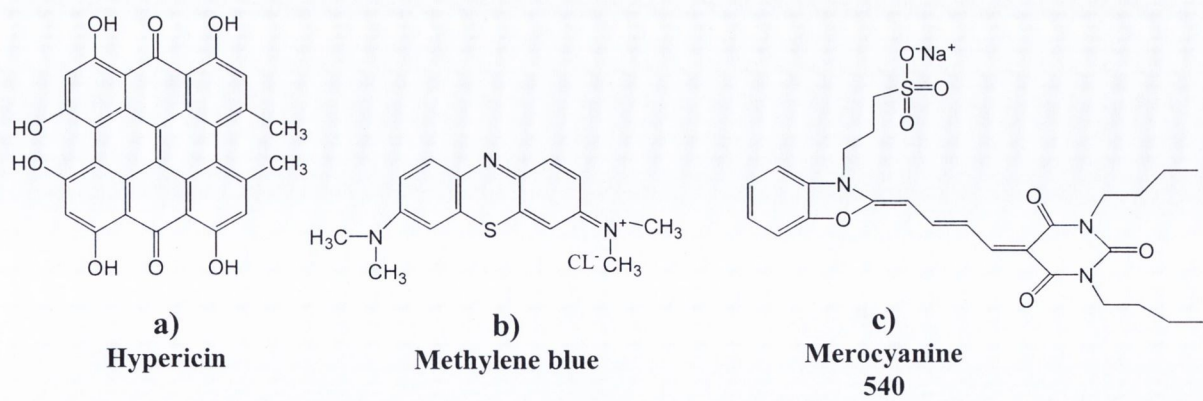


Figure 5. Chemical structures of some non-porphyrin photosensitisers.

CHAPTER 2 Nanomedicine

2.1 Nanomedicine and nanoformulations

Nanomedicine is the medical application of nanotechnology for the diagnosis and treatment of human diseases. It uses precisely engineered materials, known as nanoparticles, generally in the 1—100 nm dimension range which have unique physicochemical properties, such as small size, large surface area to mass ratio and high reactivity which are used to overcome some limitations found in traditional therapeutic agents [146].

Among the most important opportunities of nanomaterials we can mention the potential for improvement of drug delivery to the target area to provide the maximum therapeutic efficacy, improve the solubility of poorly water soluble drugs, prolong the circulation half-life in the blood, minimize degradation of the drug after administration, decrease side effects and increase bioavailability. Other putative benefits are lower toxicity, better biocompatibility and safety. They show a high ability to specifically recognize and bind to target areas *via* surface attached specific ligand, for example monoclonal antibodies, folate, transferrin (Tf) or antibodies against the transferrin receptor (TfR) [146, 147]. In the last years, many varieties of nanoparticle therapeutic and diagnostic agents have been developed for medical applications, for example, cancer treatment, infections, pain, allergy and almost every branch of medicine. Another significant application of nanomedicine is the delivery of drugs across the blood—brain barrier and transport into specific cellular compartments including the nucleus [148,149]. While many advances have been made in nanomedicine in general, ‘nano’ applications in PDT have only slowly emerged in the last two decades and are not always superior to traditional methods.

PDT is limited due to several issues. The effectiveness of this method is determined by many properties such as singlet oxygen production, the degree of selectivity to the target tissue with therapeutic concentrations without damage healthy cells, the pharmaceutical formulation, the amount of PS delivered to target cells, the physical localization. There are many factors which can have influence how efficiently a photosensitiser works. The synthesis of more direct and specifically localized photosensitising agents may be executed by active targeting, which is under investigation at the moment [150]. An ongoing problem is skin photosensitivity and associated pain [151]. Reducing photosensitivity would improve the comfort of life for all patients and improve the outpatient protocols. Moreover, another important problems to overcome in photodynamic therapy is water solubility of many photosensitisers and their tendency to aggregate under physiological conditions. Accumulation and selective recognition of target tissue is still not high enough for many clinical applications. Focusing on the use of new delivery methods, formulations and enhanced targeting approaches offers significant potential and presents an intriguing possibility [152].

The ideally delivery system should be biodegradable, has small size and high loading capacity, minimum immunogenicity and be non-toxic, should not cause side effects, and demonstrates prolonged circulation in the body after administration, minimal self-aggregation tendency and should selectively accumulate in required area in therapeutic concentration with little or even no uptake by non-target cells [147,150].

Several types of delivery vehicles can be applied to carry the photosensitiser to malignant cells. Great potential has lipid based delivery system such as liposomes or oil emulsions [33,153,154]. Liposomes with various lipid compositions encapsulated different types of photosensitisers will be covered in more details in next chapter. Emulsions prepared with the polyethoxylated castor oil, Cremophor-EL (CR) is other alternative carrier [33,154]. Tin etiopurpurin (SnET_2) prepared as a CR emulsion induced 100 % of tumor regression in

rats with an implanted urothelial tumor [155], whilst bis(methyloxyethyleneoxy)silicon-phthalocyanine (SiPc) in a Cremophor showed promising results in mice bearing transplanted Lewis lung carcinoma [156]. Low density lipoproteins (LDL) have been suggested as another naturally occurring carrier system for photosensitisers since many tumors overexpress LDL receptor on their surface because of high demand for cholesterol for synthesis of cell membrane [33, 154]. As a drug delivery system they have many advantages, such as natural components of lipoproteins keep them away from macrophages of reticuloendothelial system which results a long circulation life time in the bloodstream, they are non immunogenic, due to their small size can diffuse through the vascular membrane, they can encapsulate hydrophobic drug due to their lipophilic core, which also do not alter their recognition by the cell receptors [33]. For example hematoporphyrin associated with LDL appear to allow a more specific delivery of the complexed Hp to the tumor tissue as compared with HDL, VLDL or free Hp in mice affected by MS-2 fibrosarcoma [157]. Biodegradable and non-biodegradable nanoparticles encapsulated photosensitive drugs have a variety of advantages. They are not removed from the body by the RES system due to their small size, have a strong ability to protect encapsulated agents, are compatible with biological systems, and their surface can easily be modified with functional groups such as antibodies or other ligands to improve selectivity [148]. One example of PS encapsulated in nanoparticles is for example water insoluble 2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide entrapped in spherical, monodispersed and stable in aqueous system silica based nanoparticles which showed efficient generation of singlet oxygen, the active uptake of PS into the cytosol of tumor cells and significant damage tumor cells [158]. 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*-THPC) and 5,10,15,20-tetra(*m*-hydroxyphenyl)porphyrin (*m*-THPP) loaded into human serum albumin nanoparticles exhibited more efficient singlet oxygen generation as well as the photocytotoxicity in Jurkat cells compared with free photosensitisers [159]. Poly(lactic

acid) (PLA), poly(glycolic acid) (PGA), and their copolymer (PLGA) based nanoparticles encapsulated photosensitiser were also used. Zinc(II) phthalocyanine (ZnPc) encapsulated in nanoparticles formulated from poly(lactic-co-glycolic acid) (PLGA) demonstrated significantly smaller mean tumor volume, increased tumor growth delay and longer survival in tumor-bearing mice compared with free ZnPc [160]. Studies with photosensitiser, meso-tetra(hydroxyphenyl)porphyrin (p-THPP) incorporated into nanoparticles of poly(*D,L*-lactide-co-glycolide) (PLGA) and poly(*D,L*-lactide) (PLA) showed that lower incubation times and lower drug dosages were required to achieve the same photo damage in EMT-6 mammary tumor cells as the free drug [161]. Alternatively, polymeric micelles are used as nanoparticles which resist elimination by the RES and thus increase their circulation in the body and ability to deliver drug to the target cells and encapsulate pharmaceuticals poorly soluble in water [148]. One example, protoporphyrin IX encapsulated in methoxy poly(ethylene glycol)-*b*-poly(caprolactone) micelles, has potential as a drug delivery system for photosensitisers and leads to enhance photodynamic therapy efficiency, show higher intracellular accumulation of the drug and higher photocytotoxicity in comparison to free PpIX [162].

Quantum dots are semiconductor nanoparticles, which have several characteristics that make them a potentially new class of photosensitisers, such as small size range of 1—6 nm, high photoluminescence quantum yield, high photostability, high molar extinction coefficient and constant composition, lack of cytotoxicity in the absence of light, but the potential to induce cytotoxicity under UV irradiation [163, 164, 165]. Quantum dots have also been used as energy donors and acceptors in various biological studies based on FRET (fluorescence resonance energy transfer) which is a non-radiative energy transfer from photoexcited donor to a nearby acceptor molecule after absorption of a higher energy photon [166], in the combination with photosensitising agents for photodynamic therapy

[167]. For example, quantum dots conjugated to (phthalocyaninato)silicon can generate singlet oxygen via FRET from nanocrystals to photosensitiser [168].

Photosensitisers can also be modified using dendrimers, highly complex molecules with a core, branches and end groups which can be conjugated or loaded with drug molecules [148]. They have the ability to control and modify the size and lipophilicity of the dendrimer-conjugate for optimizing uptake by cells and possess a high drug payload like for example ALA dendrimers which was more efficient than ALA for porphyrin synthesis at low concentrations in good correlation with higher cellular ALA dendrimer accumulation. *In vivo*, dendrimer induced sustained porphyrin production for over 24 h whilst ALA exhibited an early peak between 3 and 4 h in most tissues [169]. The dendrimer phthalocyanine (DPc)-encapsulated polymeric micelle (DPc/m) induced efficient and rapid cell death, significantly higher *in vivo* PDT efficacy than clinically used Photofrin® and no skin phototoxicity in mice bearing human lung adenocarcinoma A549 cells [170]. Another possibility to deliver photosensitiser to required tissues is carbon nanotubes which are synthesized by rolling sheets of carbon into hollow tubes that are single-walled, double-walled or multi-walled. They are removed rapidly from the body, and solubilised nanotubes have no significant cytotoxicity [171]. Carbon nanotubes absorb light in the near-infrared region and can cause cell death inside living cells due to of excessive local heating [172].

Fullerenes have been also suggested as potential agents in PDT due to their high degree of photostability and lower photobleaching, producing more reactive oxygen species type I, such as superoxide or hydroxyl radicals or increased selectivity for microbial cells over than mammalian cells [173, 174]. Currently they are used in anti-microbial applications [173, 175].

To further increase the selectivity and specific localization of the photosensitiser in the tumor, targeting ligands were also attached to the surface of vehicles. As a examples we

can mention anti-EGFR antibody coupled with verteporfin nanoparticles [176], zinc-phthalocyanine-polyethylene glycol-gold nanoparticle conjugate with anti-HER mAb [177], pyropheophorbide-a-fullerene with mAb rituximab [178], indocyanine-green-loaded calcium phosphosilicate nanoparticles coupled with anti-CD117 antibody [179], quantum dot-folic acid conjugates [180], protoporphyrin IX entrapped in folic acid-conjugated chitosan nanoparticles [181], methylene blue conjugated hydrogel nanoparticles coated with F3 peptides [182] or 5,10,15,20-tetraphenylporphyrin loaded into polymeric micelles modified with tumor specific monoclonal antibody 2C5 (mAb 2C5) against murine Lewis lung carcinoma [183], all showing potential in improving effective of PDT.

2.2 Liposomes

Liposomes are lipid vehicles of one or more concentric phospholipid bilayers, containing an aqueous phase inside and between bilayers. The phospholipids typically used for preparing liposomes are comprised of a hydrophilic head group and two hydrophobic chains which assure that liposomes are able to encapsulate both hydrophilic and hydrophobic drugs [**Figure 6**] [184,185]. There are several means by which liposomes act within and outside the body: they can attach to cell membranes and fuse with them and then release their content into the cells. In the case of phagocytotic cells liposomes are taken up, phospholipids walls are acted upon by lysosomes (which are used for the digestion of macromolecules via their enzymes) and the active drug is released. This mechanism is known as phagocytosis [185]. Liposomes are often distinguished according to their size.

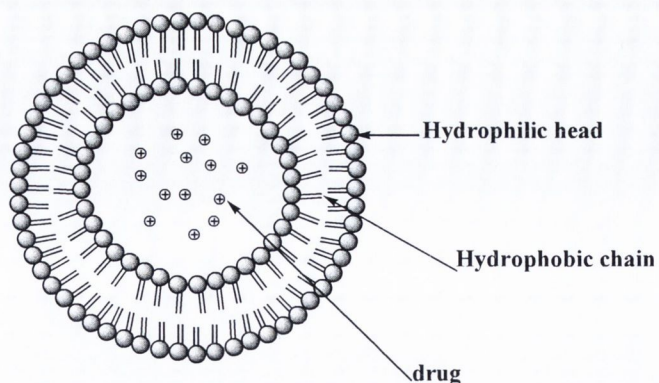
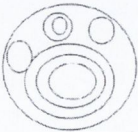


Figure 6. Structure of liposome.

Table 1. A classification of liposomes based on structure [185].

Type of vesicle	Abbreviation	Size	Number of lipid bilayers
Small unilamellar vesicles	SUV	< 100 nm	1 lipid bilayer 
Large unilamellar vesicles	LUV	100 > 1000 nm	1 lipid bilayer 
Multilamellar vesicles	MLV	> 0.5 μ m	5 – 25 lipid bilayers 
Oligolamellar vesicles	OLV	> 1 μ m	Aprox. 5 
Multivesicular vesicles	MV	> 1 μ m	Multicompartment structure 

The pharmacokinetics of liposomes depends on their physicochemical characteristics such as size, surface charge, steric stabilization, membrane lipid packing or route of administration [184].

Liposomes are good carriers of chemotherapeutics since they are able to overcome the intrinsic problems such as hydrophobicity or low solubility through a simple formulation strategy. In contrast, many of the other nano approaches require dedicated chemical steps that increase the developmental costs. Conventional liposomes consist of naturally occurring phospholipids and cholesterol which is essential for controlling the membrane fluidity and imparting better stability. Cholesterol also modulates membrane-protein interactions [186]. These plain liposomes are rapidly removed from the circulation after systemic administration by macrophages of the reticulo-endothelial system (RES). The circulatory half-life of conventional liposomes can be significantly augmented using functionalized lipids. The resulting long circulation liposomes, sterically stabilized liposomes or STEALTH® liposomes demonstrate less reactivity with serum proteins and hence evade opsonization and RES uptake. The most widely used polymeric steric stabilizer is polyethylene glycol (PEG), a water-soluble polymer that exhibits protein resistance, low toxicity, non-immunogenicity and antigenicity and can be prepared synthetically with high purity and in large quantities which has led to their acceptance for clinical applications [184,187].

2.2.1 Passive liposomes

The major feature of the liposomes is the size. The small dimension of vehicles make them capable to accumulate in pathological areas, such as many solid tumors or infarcted sites *via* enhanced permeability and retention effect (EPR), which stems from the fact that the vasculature in pathological areas is “leaky”, unlike in normal healthy tissue. The pore size

in tumors varies from 100 to 780 nm and allows the spontaneous accumulation of nanomaterials in an interstitial tumor tissue [147,184].

Liposomes are widely used in many areas in medicine. They have been applied as carriers for delivery the drugs for tumor therapy such as breast cancer [188], colon cancer [189], lung cancer [190] and others [191,192,193], drugs for respiratory disorders like for example asthma [194], delivery system of anti-infectious agents [195,196,197], gene therapy [198,199] or delivery the drug through blood brain barrier [200]. They also have been found to be useful as vaccines adjuvant [33] or contrast agents for diagnostic images [201].

In photodynamic therapy they have been applied achieving promising results. Visudyne®, a semi synthetic porphyrin derived from protoporphyrin, is the first liposomal photosensitiser approved by FDA in 2000 for the treatment of AMD [52] and in 2001, for the treatment of pathological myopia [53,54].

Foslip is a more recently developed third generation photosensitiser based on a DPPC/DPPG liposomal formulation of 5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin [202]. Studies on Foslip include studies on the putative absence of side effects [203], efficacy and reduced damage of healthy tissue compared to the non-liposomal formulation Foscan® [204, 205], the potential use of an intratumoral injection of a liposomal formulation of Foslip in a mouse model of local recurrence of breast cancer [206], its photothrombic activity [207] or delivery to the skin [208].

For ALA entrapped in liposomes with phosphatidylethanoamine (PE)/cholesterol/sodium stearate composition improved skin penetration was reported [209]. Topical delivery ALA to skin is even higher when liposomes contain ethanol (ethosomes) [210]. Likewise, inclusion of ALA esters, especially, of ALA hexyl esters, seemed to result in higher stability upon dilution with cell culture medium [211].

Many other studies using liposomes were published, for example on coproporphyrin I and (coproporphyrinato I)zinc(II) [212,213], Photofrin® [214], phthalocyanines [215], naphthalocyanines [216], porphycenes [217], hematoporphyrin [218] or non-porphyrin photosensitisers [219] showing great potential in delivery PS and improving efficiency of PDT.

Fullerenes have also been used for incorporation into liposomes, as a means to increase their water solubility. For example, lipid membrane incorporated fullerenes were prepared *via* the fullerene exchange method from γ -cyclodextrin and showed low toxicity in the dark and good activity against HeLa cells after irradiation. Notably, the C70 derivatives showed a higher photodynamic activity than the C60 derivatives [220].

2.2.2 Actively targeted liposomes

A promising strategy to enhance the therapeutic effect of pharmaceutical agents to tumor tissue and the same time keep them away from normal, healthy cells is specifically deliver liposomes to the target area. Thus, targeted liposomes with ligands attached to their surface, which recognize cell surface antigens or receptors in the target tissue, have been engineered. Several types of properties have been used such as antibodies or antibody fragments, vitamins, glycoproteins, peptides, oligonucleotides, polysaccharides [147,184,186,221]. The biological properties of the ligands such as target recognition, binding efficiency, drug loading efficiency should not be substantially altered. Moreover, the integrity of liposomes should not be changed by surface modification, targeted liposomes should be stable and have circulation half-lives long enough to allow them to reach and interact with the target cells [222,223].

Antibody (Ab) based drug delivery system is promising approach to improve delivery photosensitisers to target tissue. This strategy employs immunoglobulins (Ig), especially, of the IgG class and their fragments used as a targeting moieties for drug delivery system. Monoclonal antibodies possess a high degree of specificity which makes them advantageous over other ligands. However, they are expensive, production is time consuming and most important, they might cause immune reaction [33, 186]. Liposomes conjugated with antibodies are called immunoliposomes (IL) and their action of delivery to cancer cells contain two phases: the transport phase, when immunoliposomes travel from the administration site to the desired tissue, and the effector phase, where the specific binding of immunoliposomes to tumor cells and subsequent release of the entrapped drugs occur. In effector phase we can distinguish two different means. First is the fusion of immunoliposomes with the plasma membrane of the tumor cells, followed by immunoliposome binding to the target cells where the encapsulated drug is released directly into the cytosol. This method does not add a restriction to the size of immunoliposomes. The second mean is a pH-dependent fusion with the endosomal or lysosomal membrane after internalization by the target cell via receptor-mediated endocytosis which requires a suitably size of immunoliposomes ($\varnothing < 100$ nm) [221]. Antibody can be combined with liposomes via direct conjugation with surface of plain liposomes [**Figure 7a**] which demonstrated rapid elimination from the circulation because of lack of functionalized lipids. Binding ligand *via* direct insertion into the lipid bilayer parallel with PEG chain [**Figure 7b**] showed longer circulation time but decreased coupling efficiency of the ligand and target recognition due to steric hindrance of PEG. To overcome this problem, targeting ligands can be attached to the terminal ends of PEG chains [**Figure 7c**], enhancing antibody accessibility. Such compounds show much better binding to specific target cells [184,221,224]. Ligands can be attached to the membrane lipids either before or after formation of liposomes and both covalent and non-covalent

conjugation is possible. For non-covalent conjugation biotin-modified lipids are used to attach a variety of avidin/streptavidin-linked targeting proteins. For covalent bonding attachment of proteins or peptides to liposomes via *N,N*-dicyclohexylcarbodiimide mediated amide bonding of amino groups with free carboxylate groups on the terminal end of a PEG chain have been used [222].

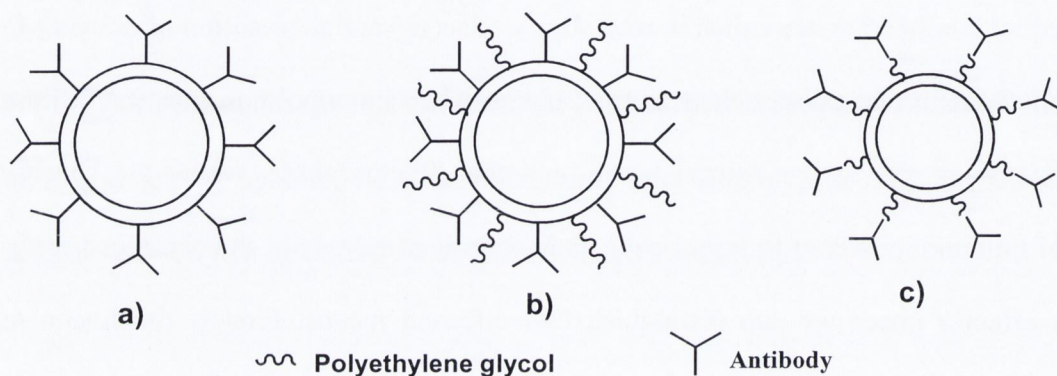


Figure 7. Three types of immunoliposomes.

Studies using antibody targeted liposomes for selective delivery photosensitisers to tumor tissue present for example pheophorbide encapsulated into liposomes conjugated with a monoclonal antibody against the bladder tumor cell line T-24, showing antibody dependent uptake of immunoliposomes and subsequent photo-activated killing of the tumor cells [225]. Monoclonal antibody against an antigen on the surface of T lymphocytes, BE3, were covalently attached to liposomes containing pyrene and demonstrated specific binding and cytotoxicity toward T cells in human peripheral blood lymphocytes [226]. Liposomes containing the photosensitive dye sulphonated aluminium phthalocyanine (AlSPc) coupled to anti-B-cell antibody (8A) showed selective targeting and significant phototoxicity in DW-BCL cells (an Epstein Barr virus immortalised B-cell line) [227]. In another study, AlSPc was encapsulated in liposomes and linked with monoclonal antibody 791T/36. The

photocytotoxic effects of the liposomes determined on two cell lines, 791T, an osteosarcoma and C170, a colorectal carcinoma, was proportional to the number of antigens on the cells, concentration of photosensitiser and the time of exposure to light. No significant toxicity was observed in case of untargeted liposomes or free AlSPc [228]. AlSPc encapsulated in liposomes bearing antibody E7 (IgM subclass), which recognize an antigenic determinant expressed on human bladder carcinoma cell line 647V were compared with aluminum tetra-3 sulfonyl chloride phthalocyanine (PC) directly coupled to antibody by a sulfonamide linkage. Results demonstrated that at equimolar AlSPc doses absolute toxicity was similar for the two conjugate types, but at equimolar antibody doses, the liposomal conjugate was more effective by up to 13-fold [229].

In addition to antibodies, various other ligands are used to target liposomes to specific tissues in treatment many cancers [224, 230], but very few of them were used to selectively delivery of photosensitisers. Examples include folate [231,232], peptides [233,234,235], growth factors [236], or transferrin (Tf) [223,237,238]. However, these ligands are cheaper than antibodies and present a minimal risk of immune reaction, their specificity for cancer cells are weaker [186].

Another example of specific liposomes can be vehicles with a triggered release mechanism [239]. For example, thermosensitive liposomes which release their content as a result of increase in the membrane lipid disorder and consequently in the permeability of the liposomal bilayer which is possible through heating of the target tissue above the phase transition temperature of the liposome membrane [186]. There are two types of thermosensitive liposomes, one contain temperature-sensitive lipids such as dipalmitoylphosphatidylcholine and the other one which may or may not contain thermosensitive lipids and have a surface coating with thermo-sensitive polymers. Thermosensitive liposomes that consist of thermosensitive lipids have a gel to liquid crystalline phase transition temperature of about 42 °C, thus, the permeability of

phospholipid bilayer of liposomes increases at this temperature which is also the upper temperature limit for hyperthermia. The liposomes are stable after administration into the body and release their content at a specific site when the temperature is raised above 42 °C [240].

The other type is liposomes coupled with pH-sensitive moieties. In order to obtain pH-induced interliposomal destabilization or fusion of the liposomes with biomembranes, acid-sensitive molecules, for example, phosphatidylethanolamine (PE) are added to the liposomal membrane, which act as a membrane stabilizer at neutral pH [186,240]. In general, the bilayer of pH-sensitive liposomes is designed to destabilize between pH 5 and 6.3 and these liposomes can be used to release their cargo into the cytoplasm or into the environment of tumor cells [186]. An example of photosensitizer used in pH-sensitive liposomes composed of phosphatidylethanolamine and cholesteryl hemisuccinate is 9-acetoxy-tetra-*n*-propylporphycene which demonstrated significantly more photokilling effect than the non-pH-sensitive liposomes [241].

CHAPTER 3 Materials and Methods

3.1 Materials

Egg phosphatidylcholine (PC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-[Phospho-rac-(1-glycerol)] (DSPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoglycerol (DPPG), distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-mPEG), 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG₂₀₀₀-COOH) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-5000] (ammonium salt) (DSPE-PEG₅₀₀₀-COOH) was purchased from Nanocs (Boston, MA, USA). All lipids had a purities $\geq 99\%$ and were stored at $-20\text{ }^{\circ}\text{C}$.

Cholesterol (Chol), sterylamine (SA), Human holo-transferrin (Tf) and FITC-labelled phalloidin were purchased from Sigma-Aldrich (Dublin, Ireland).

5,10,15,20-Tetra-(*m*-hydroxyphenyl)chlorin (*m*-THPC, Temoporfin, Foscan) was provided by Professor Senge.

N-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) was purchased from Fluka (Dublin, Ireland).

N-Hydroxysulfosuccinimide (S-NHS) and the BCA microprotein assay kit were obtained from Pierce (Rockford, IL, USA).

The OE21, OE33 and SKGT-4 cell lines was obtained from the Health Protection Agency (Salisbury, UK).

RPMI 1640 medium, foetal bovine serum, penicillin/streptomycin mixture and trypsin/EDTA solution were purchased from Hyclone (Dublin, Ireland).

The anti-CD71 primary antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and the secondary anti-mouse IgG antibody and MTT and MTS assay were obtained from Promega (Dublin, Ireland).

Alexa Fluor 647 labelled secondary antibody and Hoechst 33342 were purchased from Invitrogen (Dun Laoghaire, Ireland).

Paraformaldehyde was obtained from Acros Organic (Dublin, Ireland).

3.2 Methods

3.2.1 Liposome preparation

Liposomes based PC lipids

Liposomes encapsulating Foscan were prepared according the Bangham method [242]. Temoporfin was encapsulated by adding the proper volume of Temoporfin stock solution (50 μ M) in ethanol to the chloroform solution of the lipids prior to evaporation. The organic solvents were removed by rotary evaporation to prepare a dry thin lipid film. The homogeneous lipid films were then hydrated with HEPES buffer (pH 7.4) under shaking above the phase transition temperature of the lipids. Here, the following compositions of liposomes were used: DSPC: Chol (neutrally charge vesicles), DSPC: Chol: DSPG (negatively charged) and DSPC: Chol: SA (positively charged) in molecular ratios of 2:1, 7:2:1 and 7:2:1, respectively.

Then, the influence of surface charge of the lipids and the influence of the buffer composition on the liposomal characteristics was investigated. Compositions of liposomes PC: Chol (neutrally charge vehicles), PC: Chol: DPPG (negatively charged vehicles) and

PC: Chol: SA (positively charged vehicles) was prepared. The lipid films were hydrated using either Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) (containing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl, KCl, NaH_2PO_4 , NaHCO_3 , glucose and HEPES) or 10 mM HEPES buffer containing 140 mM NaCl (pH 7.4) under shaking above the phase transition temperature of the lipids ($\geq 50^\circ\text{C}$). The final lipid concentration was $10\ \mu\text{mol mL}^{-1}$. The liposomal suspensions were then subjected to size reduction at $\geq 50^\circ\text{C}$ using a Lipex thermobarrel extruder (Northern Lipids, Burnaby, BC) fitted with polycarbonate filters (Whatman, Batavia, IL, USA) of pore sizes ranging from 100 nm to 800 nm.

Gel filtration was carried out to remove non-encapsulated components using desalting columns (Econo-Pac 10DG Desalting Columns, BioRad, CA, USA) or polyacrylamide desalting column (D-SaltTM Polyacrylamide Desalting Columns, BioRad, CA, USA).

Liposomes based DPPC DPPG lipids

Conventional (*i.e.*, non-PEGylated) liposomes were composed of $18\ \text{mg mL}^{-1}$ of dipalmitoylphosphatidylcholine (DPPC) and $2\ \text{mg mL}^{-1}$ of dipalmitoylphosphatidylglycerol (DPPG). For preparation of PEGylated liposomes, 6 mol% of distearoylphosphatidylethanolamine-methoxy-(polyethylene glycol)-2000 (DSPE-mPEG) were added to the aforementioned lipids. The photosensitiser 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (Foscan) was used at a concentration of 0.5, 1, 1.5 and $2.5\ \text{mg mL}^{-1}$. The respective lipids were mixed and dissolved in chloroform. Foscan was encapsulated by adding the relevant volume of stock solution in ethanol to the lipid solution prior to evaporation. The organic solvents were removed by rotary evaporation to prepare a dry thin lipid film. The homogeneous lipid films were then hydrated with glucose solution 5% (w/w) or PBS buffer (pH 7.4) under shaking above the phase transition temperature of the lipids ($\geq 55^\circ\text{C}$). The extrusion was carried out at $\geq 55^\circ\text{C}$. Non-encapsulated drug was

separated by gel filtration on desalting columns (Econo-Pac 10DG Desalting Columns, BioRad, CA, USA) using glucose solution 5% or PBS (pH 7.4) as elution buffer.

Empty liposomes were prepared using the same method with corresponding concentrations of lipids.

3.2.2 Physicochemical characterisation of liposomes

Physicochemical properties of liposomes *i.e.*, particle size and ζ -potential were characterised before and after gel filtration. The particle size was determined by dynamic light scattering with a Zetasizer Nano Series (Malvern Instruments, Malvern, UK). The results give information of the mean diameter of the liposomes population (z-average) and the width of distribution *via* the polydispersity index (Pdl). Pdl ranges from 0-1 with the higher value representing the highest degree of heterogeneity. The ζ -potential was determined also with a Zetasizer Nano Series (Malvern Instrument, Malvern, UK). For measuring the size and zeta potential the liposomal suspension was mixed with respect buffer (pH 7.4). All measurements were carried out at a temperature 25 °C with automatic duration.

3.2.3 Phospholipid quantification

After obtaining a liposomal suspension with the desired particle mean size, the quantification of phospholipids was performed using the colorimetric Stewart assay [243]. The method is based on the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution. A solution of proper lipids (PC or DPPC) in chloroform at a concentration of $0.125 \mu\text{mol mL}^{-1}$ was prepared. Increasing volumes ranging from 0 (for blank) to 1.0 mL of the standard solution were added into 15 mL conic glass tubes and chloroform was added to reach a final volume of 2 mL in all tubes. Then, 2

mL of ferrothiocyanate solution was added to all tubes and the mixture was vortexed for 15 s following centrifugation for 5 min at 1500 rpm. After centrifugation, the lower layer (chloroform) was separated using a Pasteur pipette and the optical density of all samples was read at 485 nm using an UV spectrophotometer (UV1700 Pharma Spec UV Visible Spectrophotometer SHIMADZU). The concentration of the test sample was determined by comparison with the standard curve.

3.2.4 Encapsulation efficiency

To investigate the encapsulation of Foscan inside liposomes, the fluorescence intensity of Foscan was measured using a microplate reader FluorOptima, (BMG Labtech, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 405 and 650 nm, respectively. Non-ionic surfactant Triton X-100 was added to the liposomes to a final concentration 1% in order to solubilise membrane proteins and pure ethanol. The concentration of the test sample was determined by comparison with the standard curve.

3.2.5 Conjugation of transferrin to liposomes

The conjugation of Tf to liposomes was achieved using a previously published method of EDC and S-NHS-mediated amide bond formation between the carboxyl groups of DSPE-PEG-COOH and free amine groups of the transferrin molecule [244]. Briefly, to 1 mL of liposomes (lipid concentration: 10 $\mu\text{mol mL}^{-1}$), 1 mL of PBS, 180 μL of S-NHS and 180 μL of EDC, both freshly prepared 0.25 M in PBS, were added. This mixture was allowed to incubate for 10 min at room temperature. Then, 125 μg of Tf per μmol of lipid was added and gently agitated overnight at 4 °C. The unbound protein was separated from the liposomes by ultracentrifugation at 250,000 g for 3 h (15 °C) in a Beckman LM-80 ultracentrifuge. After ultracentrifugation, the pelleted liposomes were re-suspended with

PBS buffer and Tf binding efficiency was determined using a micro BCA protein assay according to the manufacturer's instructions.

3.2.6 Protein assay and Tf binding efficiency

Liposomes were also analyzed in terms of Tf conjugation efficiency according to procedure applied in previous studies [237,245]. Briefly, 400 μL of methanol was added to the liposome suspension (100 μL) and the mixture was centrifuged for 10 s at 9000 g . Then, 200 μL of chloroform was added and the sample was centrifuged again for 10 s at 9000 g . For phase separation, 300 μL of water was added to the mixture, followed by one min centrifugation at 9000 g . The upper phase was removed and a further 300 μL of methanol was added to the sample and centrifuged for 10 s at 9000 g in order to obtain a protein pellet. The supernatant was removed and the protein pellet was dried and resuspended in PBS buffer. The concentration of Tf was determined using a bicinchoninic acid protein assay kit [246] and BSA as a standard. The absorbance was measured at 595 nm in a plate reader (FLUOstar OPTIMA). The coupling efficiency was calculated as $\mu\text{g Tf } \mu\text{mol PL}^{-1}$.

3.2.7 Cell culture

Human oesophageal squamous cell carcinoma (OE21), Human Caucasian esophageal carcinoma cell lines (OE33) and human esophageal adenocarcinoma (SKGT-) cell lines were cultured in RPMI 1640 medium supplemented with 10% inactivated foetal bovine serum and 1% penicillin/streptomycin. Cells of passage numbers 7-35 were grown in culture flasks (Nunc, Denmark) and subcultured using a 0.25% trypsin/EDTA solution once confluence of 80-90% was reached. The culture medium was changed every other day.

3.2.8 Western blot

Cell lysate was collected from OE21 cells grown in 6-well plates until 80-90% of confluence. Cells from three different passages were used in this study. Cells were washed three times with ice-cold PBS followed by cell homogenisation in freshly prepared lysis buffer containing 6% aprotinin and 1% leupeptin. Cell lysates were then sonicated twice for 10 s and centrifuged for 20 min at 10,000 rpm (4 °C). The total amount of protein was determined by micro BCA protein assay according to the manufacturer's instructions. Samples were then diluted to equal concentration of protein. Six microlitres of loading buffer were added to each sample and the mixture was heated at to 95 °C for 5 min, followed by loading samples (18 µL) onto a 10% SDS gel. Electrophoresis was carried out at 110 V. The protein bands were then transferred onto Immuno-Blot PVDF membranes (Bio-Rad) at 25 V for 30 min. Blots were blocked in PBS containing 5% (w/v) BSA for at least 1 h at room temperature. After blocking, blots were incubated overnight with the anti-CD71 primary antibody (1:200) at 4 °C. Membranes were then washed with PBS and incubated with the secondary anti-mouse IgG antibody (1:12,500) for 1 h at room temperature. Peroxidase activity was detected using the Immobilon Western Chemiluminescent HRP substrate and blots were captured using a ChemiDoc system (Bio-Rad) [247].

3.2.9 Confocal laser scanning microscopy

OE21 cells were seeded in 96-well glass bottom plates and left overnight to provide sufficient time for the cells to attach. Transferrin-modified liposomal formulations (at a concentration of 1.5 µM of Foscan) were added to the cells for 24 h. Untreated cells were used as control. Cells were fixed with 4% paraformaldehyde solution washed twice with PBS, and then 0.1% of Triton X-100 was added in order to permeabilise cell membranes.

Cells were washed again twice with PBS and blocked in PBS-BSA (1%) solution for 15 min, followed by washing with PBS. Next, cells were incubated with anti-CD71 primary antibody (1:50) for 1.5 h at room temperature. Cells were then blocked in PBS-BSA (1%) solution for 15 min and washed with PBS. Secondary anti-mouse antibody labelled with Alexa Fluor 647 was added (1:300) and incubated for 1 h at room temperature, followed by washing with PBS. Subsequently, cells were stained with FITC-labelled phalloidin to visualise F-actin and Hoechst 33342 to counterstain the nuclei, respectively. Images were obtained using 63× oil immersion objectives on a Zeiss LSM Meta 510 confocal laser scanning microscope with the instrument settings adjusted so that no positive signal was observed in the channel corresponding to fluorescence for the isotypic controls.

3.2.10 Phototoxicity assay

The cytotoxicity of Foscan-loaded liposomal formulations was determined in OE21 cell lines using an MTT assay [248]. The cytotoxicity of tested porphyrin derivatives was determined in OE33 and SKGT-4 cell lines using an MTS assay. Cells were seeded at 3000 cells/well into 96-well plates and incubated at 37 °C in a 5% CO₂ incubator for 24 h respectively, in order to provide sufficient time for the cells to attach and resume growth. Trypan blue was used when cells were counted using a haematocytometer. Foscan® and tested porphyrin derivatives was prepared by dissolving the photosensitiser in ethanol:propylene glycol (60:40) (v/v) [249]. Free Foscan/free tested porphyrin derivatives and liposomal formulations of the Foscan were diluted with medium to obtain proper final concentrations and were administered to the cells. Control wells were treated with equivalent volumes of liposome-free medium or tested porphyrin derivatives-free medium respectively, as well as with empty liposomes. Cells were incubated with non-encapsulated Foscan and its liposomal formulation of for 2, 4, 8, 12 and 24 h at 37 °C in a 5% CO₂ incubator in the dark, whilst with tested porphyrin derivatives for 24 h in the same

conditions. Following, the incubation solution was exchanged for freshly prepared, pre-warmed medium and one (of two equal) plate(s) was illuminated with light (2 min) of a fluence rate of 1.7 mW cm^{-2} [250], the second plate was kept in the dark. Twenty-four hours post irradiation, cell survival was assessed by MTT/MTS assay according to manufacturer's instruction. The resulting absorbance, indicative of remaining cellular activity, was read at 570 nm and 490 nm respectively, using a Wallac Victor2 plate reader (Perking Elmer, Singapore).

3.2.11 Intracellular uptake analysis by high content analysis

OE21, OE33 or SKGT-4 cells were grown and treated exactly as described above minus the MTT/MTS assay. Instead, cells were washed with PBS and fixed with 4% paraformaldehyde solution and then stained with FITC-labelled phalloidin to visualise F-actin and Hoechst 33342 to stain the nuclei for 1 h. After that time, cells were washed three times with PBS and then plates were resuspended with 100 μl of PBS and stored at 4 °C in the dark until analysis. Images were obtained using an InCell 1000 high content system (GE Healthcare, Piscataway, NJ) using a total of 10 fields per well at 10 $\times$ magnification using three filters: blue (excitation 345 nm and emission 435 nm), green (excitation 475 nm and emission 535 nm) and red (excitation 560 nm and emission 680 nm) in case of liposomes encapsulating Foscan and blue (excitation 345 nm and emission 435 nm), green (excitation 475 nm and emission 535 nm) and red (excitation 620 nm and emission 700 nm) in case of tested porphyrin derivatives. Image analysis was performed using the InCell 1000 image analyser (GE Healthcare, Piscataway, NJ). At least 3000 individual cells were imaged and analysed using InCell Foscan parameters such as Foscan intensity determined by F-actin stain and Foscan. Stain parameters were recorded numerically for individual cells in the field as well as average values per field and per well.

3.2.12 Statistical analysis

Each experiment was repeated a minimum of three times. Data are presented as mean $\pm$ SEM (n), where n is the number of observations. Difference between two groups means were determined by two-tailed Student's t -tests. Differences among more than two group means were determined by one-way analyses of variance (ANOVA) followed by Tukey multiple comparison tests. P value < 0.05 was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, USA). Averages and SEM were plotted using Graphpad Prism.

CHAPTER 4 Characterization of liposomes based on phosphatidylcholine lipids (PC)

4.1 Introduction

Phospholipids which are the major component of the liposomes, when placed in the water, spontaneously form a spherical structure with an aqueous phase enclosed by a phospholipid bilayer [185,242]. Due to the features of the phospholipids typically used for preparing liposomes, which are composed of a hydrophilic head group and two hydrophobic chains, liposomes are able to encapsulate both hydrophilic and hydrophobic drugs and thus, they are often used to solubilise the hydrophobic drugs [184,185,186]. Liposomal vehicles can be prepared also from cholesterol, non-toxic surfactants, glycolipids, sphingolipids or long chain fatty acids [185,186].

The pharmacokinetics of liposomes depends on their physicochemical characteristics such as size, surface charge, membrane lipid packing but also can depend on techniques of preparation [184].

Different methods are used for preparing liposomes. The simplest procedure is **lipid hydration method**, originally developed by Bangham *et al.* [242], where liposomes are prepared by hydrating the thin lipid film in an organic solvent which then is removed by film deposition *via* rotary evaporation. All residual solvent is removed and the solid lipid mixture is hydrated later with an aqueous buffer at a temperature above the gel-liquid crystalline transition temperature (T_c) of the lipid. Lipids spontaneously swell and hydrate which leads to the formation of liposomes. This method is used to produce MLVs (multilamellar vesicle) [185,251,252].

The ultrasound method is used for the preparation of SUVs (small unilamellar vesicle). The two types of sonicators for ultrasonication of an aqueous dispersion of phospholipids can be used: probe sonicator for small volumes which requires high energy or bath sonicator for larger volumes. Possibly degradation of phospholipids and compounds which is encapsulated is one of the drawback [185,251,252].

LUVs (large unilamellar vesicle) and OLV (oligolamellar vesicle) can be prepared by **reverse phase evaporation**, where lipids are added to a round bottom flask and the solvent is removed using distillation. Then, lipids are re-dissolved in the organic phase. In this phase the reverse phase vesicle will form. After the lipids are redissolved the emulsion is obtained and the solvent is removed by evaporation to a semisolid gel. Non-encapsulated material is then removed. Liposomes prepared via this method are called reverse phase evaporation vesicles (REV). This method has the ability to encapsulate large macromolecules with high efficiency [185,251,252]. Different method to prepare LUV's liposomes uses detergents at their critical micelles concentrations to solubilise lipids. Detergent is removed by dialysis and then micelles become richer in phospholipids and form large unilamellar vesicles [251,252]. Adding calcium to small unilamellar vesicles induces fusion and cause the formation of multilamellar structures in spiral configuration. LUV's are formed by adding EDTA to these preparations. This method can be use to receive LUV's only from acidic phospholipids [251].

Another widely used method for encapsulation of proteins is called **freeze thaw extrusion**, where liposomes are formed by the thin lipid film with the solute to be entrapped until the film is suspended. The resulted MLVs (multilamellar vesicle) are frozen in water and vortexed. After two cycles, the sample is extruded three times, followed by six freeze thaw cycles and eight extrusions. This process ruptures and defuses SUVs, liposomes fuse themselves and increase in size to form LUVs by the extrusion technique [185,251,252]. In Dehydration-Rehydration method empty buffer containing SUVs is mixed with a marker

or with drug which needs to be entrapped. This mixture is dried by vacuum or freeze-dried. This process leads to form large aggregates of small vesicles with the marker or drug enclosed. This method is used to obtain mainly oligo lamellar vesicles (OLV) [185,252]. The aim of this study was preparing different formulations of Foscan encapsulated liposomes and investigates the influence of surface charge of the lipids and buffer composition on their physicochemical characteristics

4.2 Results and discussion

Initially we used liposomes encapsulating calcein to learn and practice preparation of liposome vesicles. We carried out this experiment according to Bangham method [242] and we used three different compositions of liposomes: DSPC: Chol (neutrally charged vehicles), DSPC: Chol: DSPG (negatively charged vehicles) and DSPC: Chol: SA (positively charged vehicles).

Table 2 and 3, as well as Figure 8 and 9 show physical properties of liposomes encapsulated calcein and Foscan which were carried out for practice.

Table 2. Measurements of ζ -potential and polydispersity index (PdI) of liposomes encapsulated calcein. Data are presented as mean of three measurements of sample (only one experiment).

	Z-potential (mV)		PdI	
	Before	After	Before	After
	gel filtration	gel filtration	gel filtration	gel filtration
DSPC:Chol	-2.41	-2.92	0.18	0.21
DSPC:Chol:DSPG	-15.70	-17.03	0.26	0.23
DSPC:Chol:SA	3.09	0.51	0.80	0.10

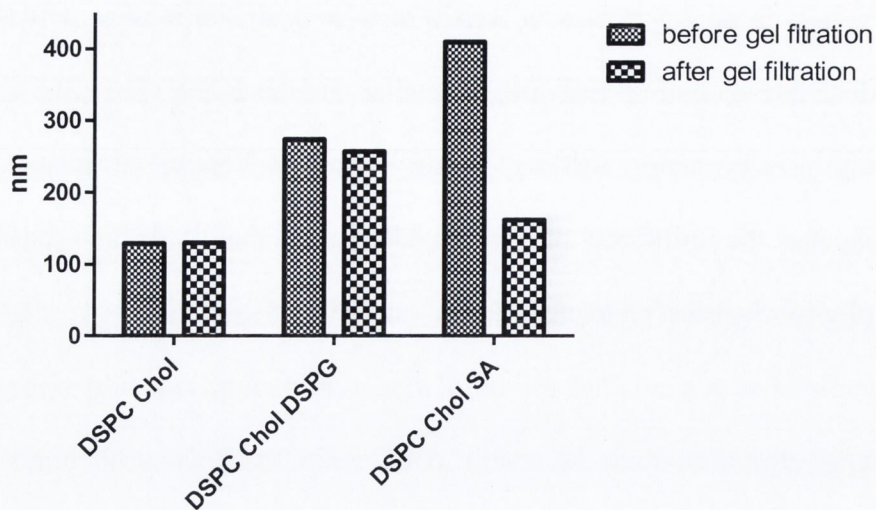


Figure 8. Particle size of liposomal formulation encapsulated calcein before and after gel filtration.

Table 3. Measurements of ζ -potential and polydispersity index (PdI) of liposomes encapsulated Foscan. Data are presented as mean $\pm$ S.E.M. ($n = 3$), where n is the number of observations.

	Z-potential (mV)		PdI	
	Before gel filtration	After gel filtration	Before gel filtration	After gel filtration
DSPC:Chol	-1.66 $\pm$ 0.04	-1.64 $\pm$ 0.35	0.18 $\pm$ 0.09	0.12 $\pm$ 0.06
DSPC:Chol:DSPG	-16.23 $\pm$ 2.06	-16.36 $\pm$ 0.13	0.12 $\pm$ 0.01	0.10 $\pm$ 0.02
DSPC:Chol:SA	18.73 $\pm$ 2.03	18.76 $\pm$ 1.76	0.18 $\pm$ 0.03	0.23 $\pm$ 0.08

Student's t-tests were used to compare ζ -potential before and after gel filtration for each individual formulation ($p > 0.05$). One way analysis of variance was used to compare PdI values of three different formulations before and after filtration ($p > 0.05$).

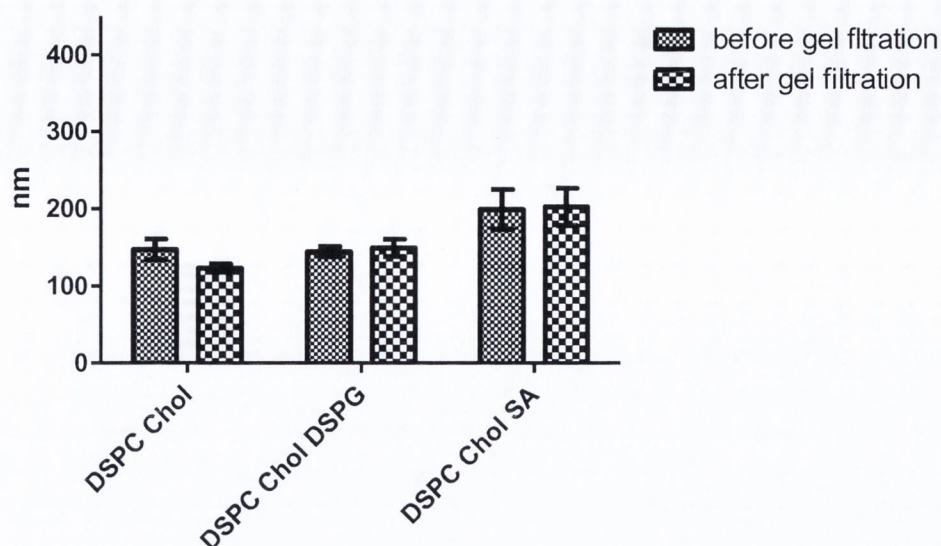


Figure 9. Particle size of liposomal formulation encapsulated Foscan before and after gel filtration. All values are presented as the mean $\pm$ S.E.M of $n=3$. One way analysis of variance was used to compare particle size between three different formulations before and after filtration ($p>0.05$).

There was no significant difference in particles size among three different liposomal vehicles encapsulating Foscan, before as well as after filtration process ($p>0.05$). The average size of liposomes was 147.20 ± 13.40 nm and 123.20 ± 5.30 nm for neutrally charge vehicles before and after gel filtration, respectively; 144.30 ± 6.60 nm and 149.40 ± 11.10 nm for negatively charged vehicles before and after gel filtration, respectively; whilst for positively charged formulations it was 199.13 ± 26.05 nm and 202.50 ± 24.10 nm before and after gel filtration, respectively. No significant difference in PDI results before and after gel filtration were also observed ($p>0.05$) which was always below 0.2 for neutral and negatively charged liposomes and slightly higher for DSPC: Chol: SA compositions. There was no significant change in ζ -potential after filtration process in all formulations ($p>0.05$). The relevant data are in -1.66 ± 0.04 and -1.64 ± 0.35 mV for DSPC: Chol composition before and after gel filtration, respectively; -16.23 ± 2.06 and -16.36 ± 0.13 mV for DSPC: Chol: DSPG composition and 18.73 ± 2.03 and 18.76 ± 1.76 mV for DSPC: Chol: SA composition before and after gel filtration, respectively. The average size of liposomes

encapsulated calcein was higher for positively (409.80±18.60 and 161.66±0.67 nm) and negatively (274.0±0.70 nm and 257.03±0.71 nm before and after gel filtration respectively) charged vehicles than liposomes encapsulated Foscan, whilst in case of DSPC: CHOL no difference was noticed. The same observation was made for PdI value which was higher in DSPC: Chol: SA and DSPC: Chol: DSPG compositions, whilst DSPC: Chol formulation had similar value as vehicles with Foscan. Differences in ζ -potential were observed only in positively charged vehicles, which was higher in Foscan liposomes than vehicles entrapped calcein. However, only one experiment with Calcein was performed.

Next, liposomal formulations were prepared using different lipids and the influence of surface charge was studied as well as the influence of buffer compositions on liposomal characteristics. Two different buffers, KRB and HEPES, both of pH 7.4, were used. The charge of neutral lipid mixtures composed of PC and cholesterol, the negatively charged vesicles were prepared using PC, cholesterol and DPPG, and positively charged formulations comprised PC, cholesterol and SA. From all mixtures we prepared liposomal formulations by extrudation.

The results of the physicochemical analysis are summarised in Table 4, as well as in Figure 10.

Table 4. Measurements of ζ -potential and polydispersity index (PdI) of liposomes. Data are presented as mean $\pm$ S.E.M. (n = 4), where n is the number of observations.

		Z-potential (mV)		PdI	
		Before	After	Before	After
		gel filtration	gel filtration	gel filtration	gel filtration
KRB	PC:Chol	-1.37 $\pm$ 0.62	-1.47 $\pm$ 0.37	0.30 $\pm$ 0.01**	0.27 $\pm$ 0.03**
	PC:Chol:DPPG	-5.66 $\pm$ 3.25	-5.69 $\pm$ 3.47	0.41 $\pm$ 0.25***	0.41 $\pm$ 0.31***
	PC:Chol:SA*	12.50	14.30	0.36	0.42
HEPES	PC:Chol	-1.69 $\pm$ 0.44	-1.52 $\pm$ 0.64	0.34 $\pm$ 0.05**	0.23 $\pm$ 0.05**
	PC:Chol:DPPG	-15.84 $\pm$ 0.66	-6.21 $\pm$ 0.46	0.12 $\pm$ 0.03***	0.08 $\pm$ 0.01***
	PC:Chol:SA*	13.30	14.20	0.37	0.45

*only one experiment was carried out. Student’s t-tests were used to compare ζ -potential before and after gel filtration for each individual formulation (p>0.05) and one way analysis of variance to compare PdI of vehicles prepared with two different buffers before and after filtration (**p>0.05; ***p<0.0001).

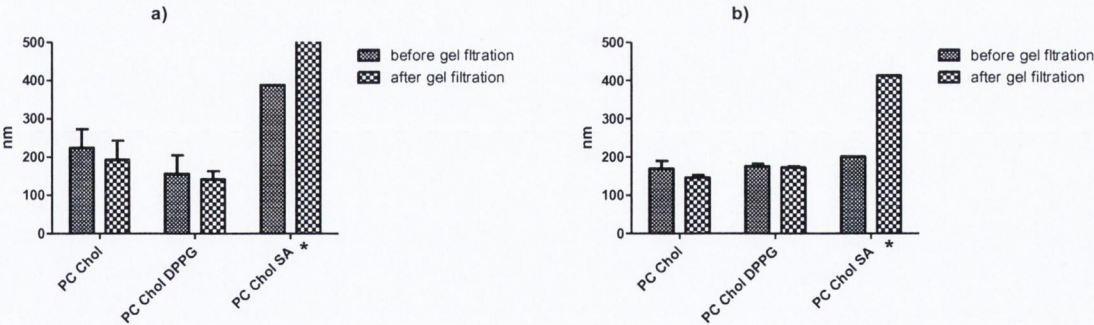


Figure 10. Particle size of liposomal formulation encapsulated Foscan before and after gel filtration using HEPES (a) and KRB (b) buffer.

All values are presented as the mean $\pm$ S.E.M of n=4 (*only one experiment were performed). Student’s t-tests were used to compare particle size between PC Chol and PC Chol DPPG composition prepared with one type of buffer (p>0.05) and one way analysis of variance between two different buffers (p>0.05).

No significant difference in average size of neutrally and negatively charged liposomes prepared with KRB buffer were noticed ($p>0.05$) [Figure 10]. Particle size of PC Chol composition before and after gel filtration was 169.80 ± 14.20 nm and 146.3 ± 4.75 nm, respectively. For PC Chol DPPG compositions values of 175.60 ± 5.0 nm and 172.50 ± 2.50 nm were obtained, and 202.60 ± 1.50 nm and 417.10 ± 3.50 nm, respectively, for PC Chol SA lipid composition (only one experiment were performed). There was also no significant difference in size between neutrally and negatively charged vehicles prepared with HEPES buffer ($p>0.05$) [Figure 10]. The size of liposomes was 224.24 ± 16.16 nm and 192.51 ± 16.69 nm for PC: Chol liposomes, before and after gel filtration, respectively; 156.17 ± 16.03 nm and 141.58 ± 7.34 nm for PC:Chol:DPPG and > 400 nm for PC: Chol: SA formulations before as well as after gel filtration (only one experiment were performed). Liposomal vehicles showed similar results in case of both buffers ($p>0.05$).

Table 4 summarises ζ -potential and PDI values for the systems under investigation before and after gel filtration and for the two different buffers. It can be seen that values of ζ -potential did not change significantly during the filtration process in all formulations ($p>0.05$).

PDI values for neutrally charged vesicles were 0.30 ± 0.01 and 0.27 ± 0.03 before and after gel filtration, respectively, 0.36 and 0.42 for positively charged formulations, before and after gel filtration, respectively and 0.41 for negatively charged ones in both cases. PDI values for negatively charged liposomes were significantly lower when prepared with HEPES buffer than in vehicles prepared with KRB ($p<0.0001$) and values were always below 0.2 which indicates a narrow size distribution, whilst PDI results for neutral and positively charged vehicles were similar to data received with KRB buffer ($p>0.05$).

PDI values were higher in the case of neutrally and positively charged formulations in both buffers, whilst significantly lower for negatively charged vehicles when prepared with

HEPES buffer. Figure 10 also shows that the particle size of positively charged vehicles was higher than neutral and negatives ones in case of both buffers, before and after gel filtration.

As HEPES buffer showed better results, PC: Chol and PC: Chol: DPPG formulations were prepared and compared according to phospholipids and Foscan content after gel filtration using two different columns: desalting columns with cut off 6000 Da or polyacrylamide desalting column with a cut off 1800 Da. The physical properties of liposomes encapsulated Foscan after filtration through two different columns are summarized in Table 5 as well as in Figure 11.

Table 5. Measurements of ζ -potential and polydispersity index (PdI) of liposomes. Data are presented as mean $\pm$ S.E.M. (n = 4), where n is the number of observations.

	Z-potential (mV)		PdI	
	Before gel filtration	After gel filtration (1800 cut off column)	Before gel filtration	After gel filtration (1800 cut off column)
PC:Chol	-1.69 $\pm$ 0.44	-2.16 $\pm$ 0.21	0.55 $\pm$ 0.07	0.12 $\pm$ 0.02*
PC:Chol:DPPG	-15.84 $\pm$ 0.66	-15.35 $\pm$ 0.98	0.12 $\pm$ 0.03	0.05 $\pm$ 0.008
		After gel filtration (6000 cut off column)		After gel filtration (6000 cut off column)
PC:Chol	-1.69 $\pm$ 0.44	-1.52 $\pm$ 0.64	0.55 $\pm$ 0.07	0.42 $\pm$ 0.08*
PC:Chol:DPPG	-15.84 $\pm$ 0.66	-16.21 $\pm$ 0.46	0.12 $\pm$ 0.03	0.08 $\pm$ 0.15

Student's t-tests were used to compare ζ -potential before and after gel filtration for each individual formulation ($p>0.05$) and one way analysis of variance to compare PdI value between vehicles after filtration using 2 different columns ($p>0.05$, * $p<0.05$)

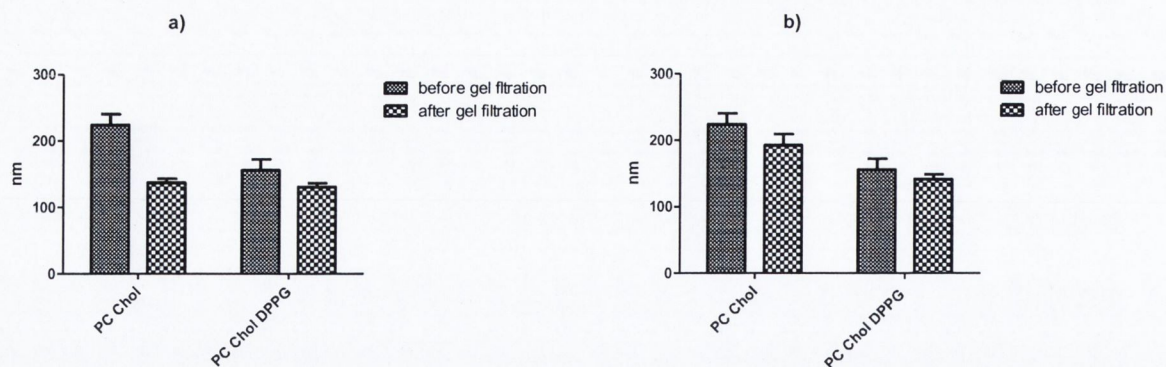


Figure 11. Particle size of liposomal formulation encapsulated Foscan before and after gel filtration using 1800 (a) and 6000 (b) cut off column.

All values are presented as the mean $\pm$ S.E.M of $n=4$. One way analysis of variance was used to compare particle size between PC Chol and PC Chol DPPG composition after filtration through 2 different columns ($p>0.05$).

The amount of phospholipids and Foscan was determined before and after the filtration process. Figures 12 and 13 demonstrate percentage content of both components after filtration through two different columns. Initial concentration of phospholipids was $10 \mu\text{mol mL}^{-1}$ whilst the initial amount of Foscan was $0.05 \mu\text{mol mL}^{-1}$.

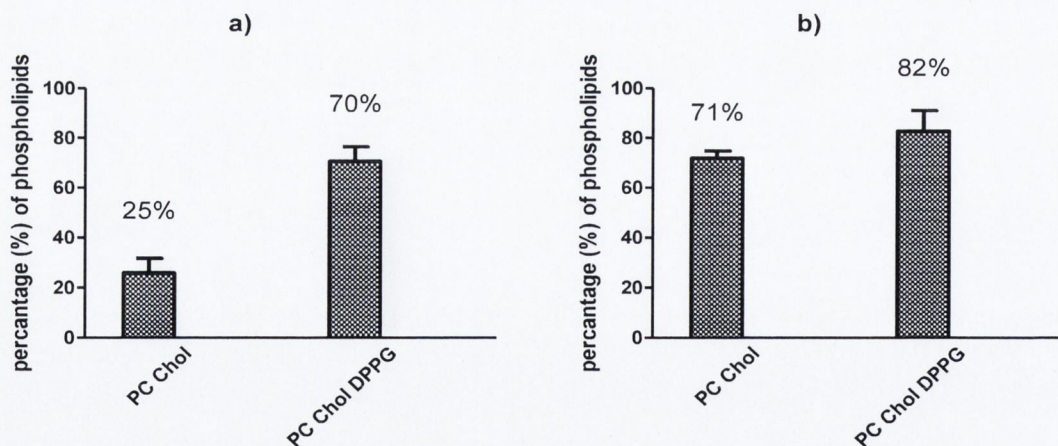


Figure 12. Content of phospholipids in PC: Chol and PC: Chol: DPPG compositions of liposomes encapsulated Foscan after gel filtration using 1800 cut off (a) and 6000 cut off (b) columns.

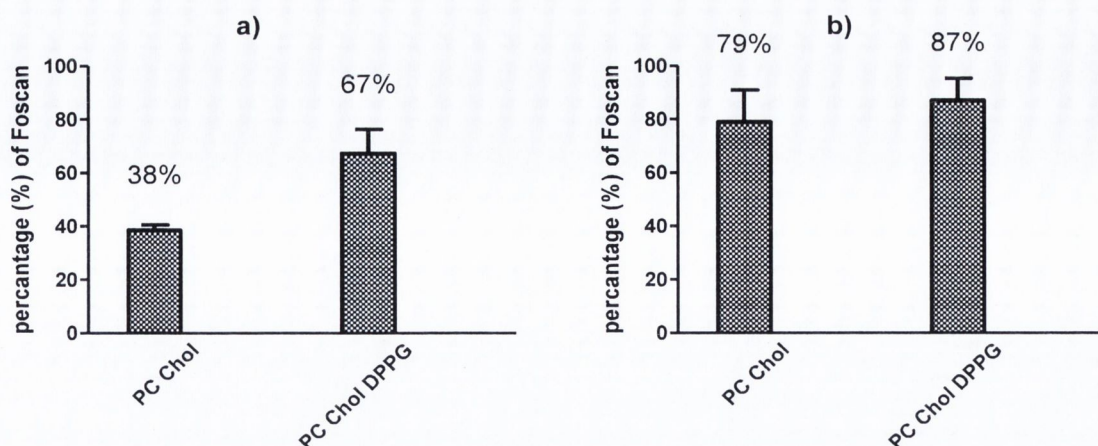


Figure 13. Content of Foscan in PC: Chol and PC: Chol: DPPG compositions of liposomes after gel filtration using 1800 cut off (a) and 6000 cut off (b) columns.

There was no significant difference in particle size after the filtration process through two different columns ($p > 0.05$). No significant changes in ζ -potential after gel filtration was observed in both cases ($p > 0.05$). PdI values were significantly smaller when vehicles were filtered through 1800 cut off column ($p < 0.05$) in case of neutral formulations and no significant difference was observed in negatively charged vehicles ($p > 0.05$). However, the quantification of phospholipids as well as photosensitiser after passing through the two columns clearly indicated that the 1800 cut off column caused more phospholipid and Foscan loss. The differences are more pronounced in PC: Chol compositions. Filtration through a 6000 cut off column resulted in a loss of 29% and 18% of phospholipids in PC: Chol and PC: Chol: DPPG composition, respectively, whilst filtration through a 1800 cut off column showed 75% and 30% loss in PC: Chol and PC: Chol: DPPG formulation, respectively. Loss of drug was 21% in neutrally charged vehicles and only 13% in negatively charged liposomes when a 6000 cut off column was used, while 62% and 33% in neutrally and negatively charged vehicles, respectively, with an 1800 cut off column.

Liposome encapsulated Foscan has also been described by others. Bombelli *et al.* reported that cationic liposomes of Foscan, which had a large positive ζ -potential, increased biological activity and demonstrated high drug encapsulation [253,254,255,256,257]. However, this group studied liposomes formulated with different percentages of 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and any of the cationic surfactant (1S,2S)-N-hexadecyl-N-methylprolinolinium bromide, (1R,2S)-N-hexadecyl-N-methylprolinolinium bromide, N-hexadecyl-1-prolinolinium hydrobromide and N,N-dihexadecyl-1-prolinolinium bromide. Moreover, the liposomes were prepared with different buffer solutions and a combination of two methods. In their experiment they used PBS buffer at pH 7.4 and freeze-thawed method followed by extrusion for preparing liposome vehicles. Their data suggest that high entrapment efficiency also depends on the value of surface charge. Lower (positive) values corresponded to lower activity whereas higher values correspond to toxicity.

On the other hand, Dragicevic-Curic *et al.* demonstrated promising results of negatively charged vehicles loaded Foscan with soybean phosphatidylcholine as a main component in skin penetration studies [258,259,260]. They found that invasomes (a liposomes containing ethanol) containing 3.3% of ethanol and 1% of terpene mixture with particle size < 130 nm and PdI 0.094 present an effective drug carrier system for delivering Temoporfin into the deep layers of skin [258]. In further studies, they noticed that negatively charged liposomes entrapped *m*-THPC containing ethanol led to a higher overall skin deposition compared to *m*-THPC-liposomes with no ethanol and the highest amount of photosensitiser was delivered to the skin by liposomes containing 20% ethanol [259]. The invasomes had sufficiently small particle size (64–128 nm), which was necessary for the penetration of vesicles into the skin and PdI values < 0.3. They utilized PBS buffer at 7.4 pH and the extrusion method for preparing vehicles.

In recent studies, they investigated *m*-THPC-loaded liposomes in order to enhance the skin delivery of Foscan [261]. They compared neutrally, positively and negatively charged Foscan loaded vehicles with flexible membranes (*i.e.* flexosomes) composed of PC as the main component and edge activator polysorbate 20 which provided the deformability of these vesicles. Neutral flexible liposomes contained phosphatidylcholine (PC) and polysorbate 20; cationic flexosomes were composed of PC, stearylamine (SA) and polysorbate 20 whilst anionic flexosomes contained PC, dicetyl phosphate (DCP) and contained polysorbate 20. Liposomes were prepared with the same method as in previous studies. There was no significant difference in particle size between all formulations and PDI ranged from 0.062 for neutral flexosomes to 0.264 for negatively charged vehicles. Their data indicated that cationic flexosomes delivered the highest amount of Foscan into the skin.

However, a recently developed liposomal formulation of Foscan based on DPPC/DPPG lipids shows promising results [203,204,205,206,207]. Kuntsche *at al.* reported a broad study on the physicochemical characterization of Temoporfin liposomes using conventional vehicles composed of DPPC/DPPG and long circulation liposomes containing additional DSPE-mPEG lipids [262]. All vehicles had a particle size between 100-130 nm and PDI < 0.2, indicating a narrow size distribution. Liposomes were prepared with conventional lipid film method followed by extrusion and demonstrated good stability over 8 months with respect to size and macroscopic appearance.

While other liposomal formulations of Foscan have been reported before, this was the first use of phosphatidylcholine in combination with DPPG and HEPES buffer. Our data indicate that positively charged systems tends to become unstable and lack a sufficient encapsulation efficiency of the drug. Hence, we suggest that the ζ -potential has a strong influence on physicochemical parameters of Foscan-loaded liposomes. We found that

encapsulation was higher and PdI values were lower when liposomes were negatively charged.

4.3 Conclusion

Liposomes have been extensively studied since their discovery by Bangham in 1965 [242] and have been applied in many areas of medicine [185,187]. Because of their ability to encapsulate hydrophilic as well as hydrophobic drugs with good efficiency, the interest in lipid vehicles is high and many drugs have been entrapped in liposomal formulations [185,187] including Foscan® [203-207,253-261], with clinical approval several of liposomal drugs such as: verteporfin (Visudyne®), photodynamic drug approved for treatment of age-related macular degeneration (AMD) [52,53,54], Daunorubicin (Daunoxome®) for treatment of Kaposi's sarcoma [263], Doxorubicin (Mycet®) for combination therapy of recurrent breast cancer [264] or Amphotericin B (AmBisome®) for fungal infections [265]. Various different methods of preparation, physicochemical properties and applications have been proposed in several studies. The size of liposomes for example depends on their composition and method of preparation whilst the encapsulation efficacy can differ in various liposomes depending on their composition, size, charge and preparation method [266]. Our data indicate that also buffer composition can have significant influence on physicochemical characteristics of liposomes and drug encapsulation.

CHAPTER 5 Liposomes based on DPPC DPPG lipids

5.1 Introduction

Liposomes have the ability to accumulate in tumour tissues *via* the enhanced permeability and retention (EPR) effect as a result of their size [267]. However, also as a result of their size, conventional liposomes are rapidly removed from the circulation by macrophages of the reticulo-endothelial system (RES). As a countermeasure, sterically stabilised systems have been developed called long-circulation or STEALTH® liposomes [187]. Such vehicles show longer circulation in the blood and demonstrate less reactivity towards serum proteins and are susceptible to RES uptake. The most widely used polymeric stabiliser is polyethylene glycol (PEG), a water-soluble polymer that exhibits low toxicity, is non-immunogenic and can be prepared synthetically with high purity and in large quantities [184,187].

Foslip, is a recently developed liposomal formulation of Foscan based on DPPC/DPPG lipids [203,204,205,206,207]. A study provided by Kuntsche *at al.* presents a detailed physicochemical characterization of Temoporfin liposomes using conventional vehicles composed of DPPC/DPPG and long circulation liposomes containing additional DSPE-mPEG lipids [262]. Liposomes prepared with conventional lipid film method followed by extrusion demonstrated good stability over 8 months with respect to size and macroscopic appearance.

All vehicles had a particle size between 100-130 nm and PDI < 0.2, indicating a narrow size distribution.

The aim of our study was preparing a conventional and long circulating liposomes encapsulated Foscan based on DPPC/DPPG lipids followed by investigation of their

physicochemical characteristics and estimate encapsulation efficiency of the photosensitiser.

5.2 Results and discussion

Plain and PEGylated liposomal formulations of Foscan were successfully prepared by conventional lipid film method using dipalmitylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) lipids according to a method adapted from Kuntsche *et al.* [262]. Different concentrations of Foscan (in a range between 0.5 – 2.5 mg mL⁻¹) were used for the preparation of conventional liposomes. Physicochemical properties of the obtained vehicles are shown in Table 6 and Figure 14. Liposomes were always negatively charged with values for the ζ -potential of approximately -60 mV and did not significantly change after the filtration process ($p>0.05$). PDI values did not differ significantly ($p>0.05$) and were always below 0.2, which indicated a narrow size distribution. The size of plain liposomes consistently ranged from 100 nm to 120 nm, both before and after gel filtration process. However, when 2.5 mg mL⁻¹ of Foscan was used, the particle size increased to 150.57 $\pm$ 12.54 nm and 130.97 $\pm$ 6.81 nm, before and after gel filtration respectively. The highest concentration of photosensitiser resulted in aggregation during preparation of the vehicles and led to blocking of the extruder; thus only two experiments were carried out. Loss of phospholipids and Foscan after gel filtration was similar in all formulations [Figure 15 and 16]. Liposomes encapsulating 0.5, 1 and 1.5 mg mL⁻¹ resulted in a loss of < 30% of phospholipids and approximately 20% of Foscan. The encapsulation efficiency was $\geq 90\%$ in all cases and was defined as the ratio of $[(\text{Fos/Lip})_f/(\text{Fos/Lip})_i]$ in percent, where $(\text{Fos/Lip})_f$ was the final Foscan to lipid ratio and $(\text{Fos/Lip})_i$ was the initial Foscan to lipid ratio. Liposomes with 2.5 mg mL⁻¹ of

photosensitiser showed 34% loss of phospholipids and about 50% of Foscan after filtration and lacked a sufficient encapsulation efficiency of the drug. Incorporation of PEGylated lipids did not significantly change the physicochemical properties of liposomes ($p>0.05$). The PdI values as well as ζ -potential were comparable with conventional vehicles. Foscan was used at a concentration of 1.5 mg mL^{-1} since it was the highest amount of drug encapsulated with an encapsulation efficiency of $\geq 90\%$.

Table 6. Measurements of ζ -potential and polydispersity index (PdI) of liposomes prepared with different concentrations of Foscan. Data are presented as mean $\pm$ S.E.M. ($n = 4$), where n is the number of observations.

		PdI		Zeta Potential (mV)	
		Before gel filtration	After gel filtration	Before gel filtration	After gel filtration
DPPC: DPPG	0.5 mg/ml	0.16 $\pm$ 0.02	0.14 $\pm$ 0.007	-59.72 $\pm$ 7.60	-55.22 $\pm$ 9.33
	1 mg/ml	0.15 $\pm$ 0.01	0.13 $\pm$ 0.01	-56.78 $\pm$ 4.78	-65.80 $\pm$ 3.75
	1.5 mg/ml	0.08 $\pm$ 0.004	0.08 $\pm$ 0.01	-63.54 $\pm$ 2.92	-62.36 $\pm$ 4.02
	*2.5 mg/ml	0.21 $\pm$ 0.06	0.10 $\pm$ 0.03	-67.16 $\pm$ 3.75	72.62 $\pm$ 5.25
DPPC: DPPG: mPEG	1.5 mg/ml	0.14 $\pm$ 0.01	0.16 $\pm$ 0.02	-62.50 $\pm$ 6.46	-57.32 $\pm$ 7.88

*Only 2 experiments were carried out. One way analysis of variance was used to compare ζ -potential after gel filtration ($p>0.05$) and PdI values of all formulations before and after filtration ($p>0.05$).

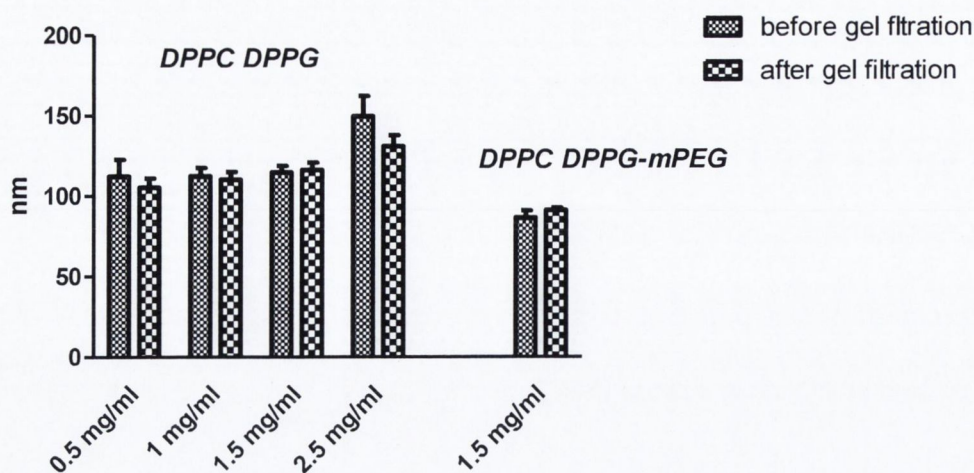


Figure 14. Particle size of liposomal formulations prepared with different concentration of Foscan before and after gel filtration.

All values are presented as the mean $\pm$ S.E.M of $n=4$. One way analysis of variance was used to compare all formulation ($p>0.05$).

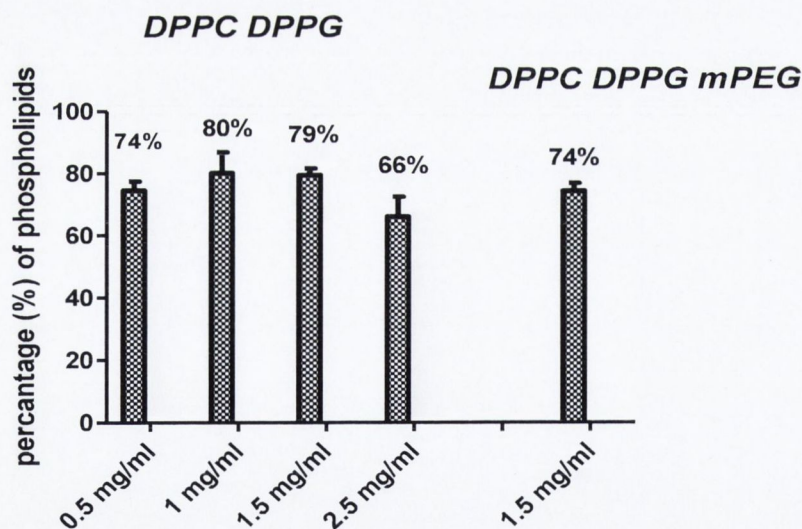


Figure 15. Content of phospholipids in liposomes prepared with different concentrations of Foscan after gel filtration.

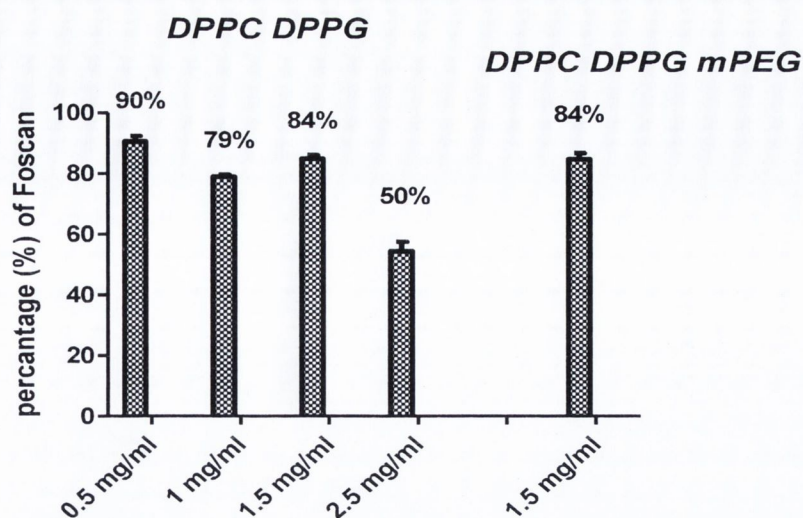


Figure 16. Content of Foscan in liposomes prepared with different concentration of Foscan after gel filtration.

Liposomal formulations of Temoporfin based on DPPC DPPG lipids have been extensively studied recently. Buchholz *et al.* studied liposomal *m*-THPC in cats with spontaneous squamous cell carcinoma [203]. Their data showed that the liposomes were well tolerated and did not exhibit side effects during and after drug injection, whilst the bioavailability in the tumor was 2-4 times higher compared to free Foscan [203]. Similar results were observed in cats with cutaneous squamous cell carcinomas [208]. No systemic adverse effects and a complete response of all cats to the therapy were observed. Kiesslich *et al.* [205] demonstrated evidence that Foscan encapsulated in liposomal vehicles is an equivalent water-soluble formulation of Temoporfin and represent an efficient photosensitizer for the treatment of human biliary tract cancer cells. Moreover, no difference in intracellular localisation between free and liposomal formulation of Foscan was observed. A valuable alternative of DPPC DPPG based liposomes encapsulated Foscan were carried on EMT 6 xenograft nude mice which exhibited faster tumor accumulation of photosensitizer compared to the free drug [204]. In a different study,

Bovis *et al.* showed improved selectivity of PEGylated liposomes encapsulated Foscan in comparison to free photosensitiser to the target tissue *in vivo* in fibrosarcoma tumor bearing rats [268]. Sterically stabilised *m*-THPC loaded liposomes also demonstrated less toxicity in absence of light than free drug in human lung carcinoma (A549) [269]. The data also suggest that encapsulated Foscan inside PEGylated liposomes did not change the intracellular localization of photosensitiser. The results were consistent with the free drug and accumulated mainly in the Golgi apparatus and endoplasmic reticulum as reported for other type of cell lines [205,270]. Pegaz *et al.* evaluated the photothrombic efficiency of Temporfin in plain and PEGylated liposomes based on DPPC DPPG lipids using the chick chorioallantoic membrane as *in vivo* model and demonstrated that, in spite of a similar pharmacokinetic profile for both liposomes, the PEG modified formulation showed higher vascular damage and was more potent delivery system for the treatment of choroidal neovascularization associated with age-related macular degeneration than conventional vehicles [207].

5.3 Conclusions

A various different studies reported promising results on a liposomal formulation of Foscan based on DPPC/DPPG [203-207]. These include studies on the putative absence of side effects [203], efficacy and reduced damage of healthy tissue compared to the non-liposomal formulation Foscan® [204,205], the potential use of an intratumoral injection of a liposomal formulation of Foslip in a mouse model of local recurrence of breast cancer [206] and its photothrombic activity [207].

We were able to successfully prepared the liposomal vehicles encapsulated Temporfin and our data were consistent with results obtained by Kuntsche in terms of particles size,

polydispersity index or encapsulation efficiency. However, the authors did not mention ζ -potential of their vehicles. All our liposomal formulations were negatively charged, demonstrated small size (between 100 – 150 nm) which is efficient to accumulate in pathological areas *via* enhanced permeability and retention effect (EPR) [147, 184], narrow size distribution, and high encapsulation efficiency resulted in $\geq 90\%$.

CHAPTER 6 Tf-conjugated liposomes

6.1 Introduction

Liposomes have been used as vehicles for anti-cancer drug delivery for many years, mainly due to their biocompatibility and the opportunity to incorporate both hydrophilic and hydrophobic compounds [187]. The addition of moieties such as vitamins, glycoproteins, peptides, oligonucleotide aptamers and antibodies enhances tumour delivery and reduces toxicity to healthy tissues [147]. When adding such groups, it is pivotal that biological properties of the ligands (*e.g.*, target recognition and binding efficiency) and drug loading efficiency of the carries remain unaffected [222]. In this context, the covalent modification using polyethylene glycol (PEG)-linked lipid residues has become a promising strategy [222,223,237,238,271).

One of the most widely used ligands for anticancer delivery is transferrin [272,273], which is exploiting receptor mediated endocytosis followed by release of the drug into the cell [274]. The transferrin receptor (TfR) is ubiquitously expressed on healthy cells; however, many cancers exhibit vastly increased quantities of TfR [275]. Moreover, the transferrin receptor shows a high turnover on tumour cells due to their increased iron consumption [276].

The goal of our successive studies was to obtain specifically targeted vehicles by attaching ligand to the liposomal surface in order to improve delivery of Foscan to oesophageal cancer cell via receptor mediated endocytosis [Figure 17].

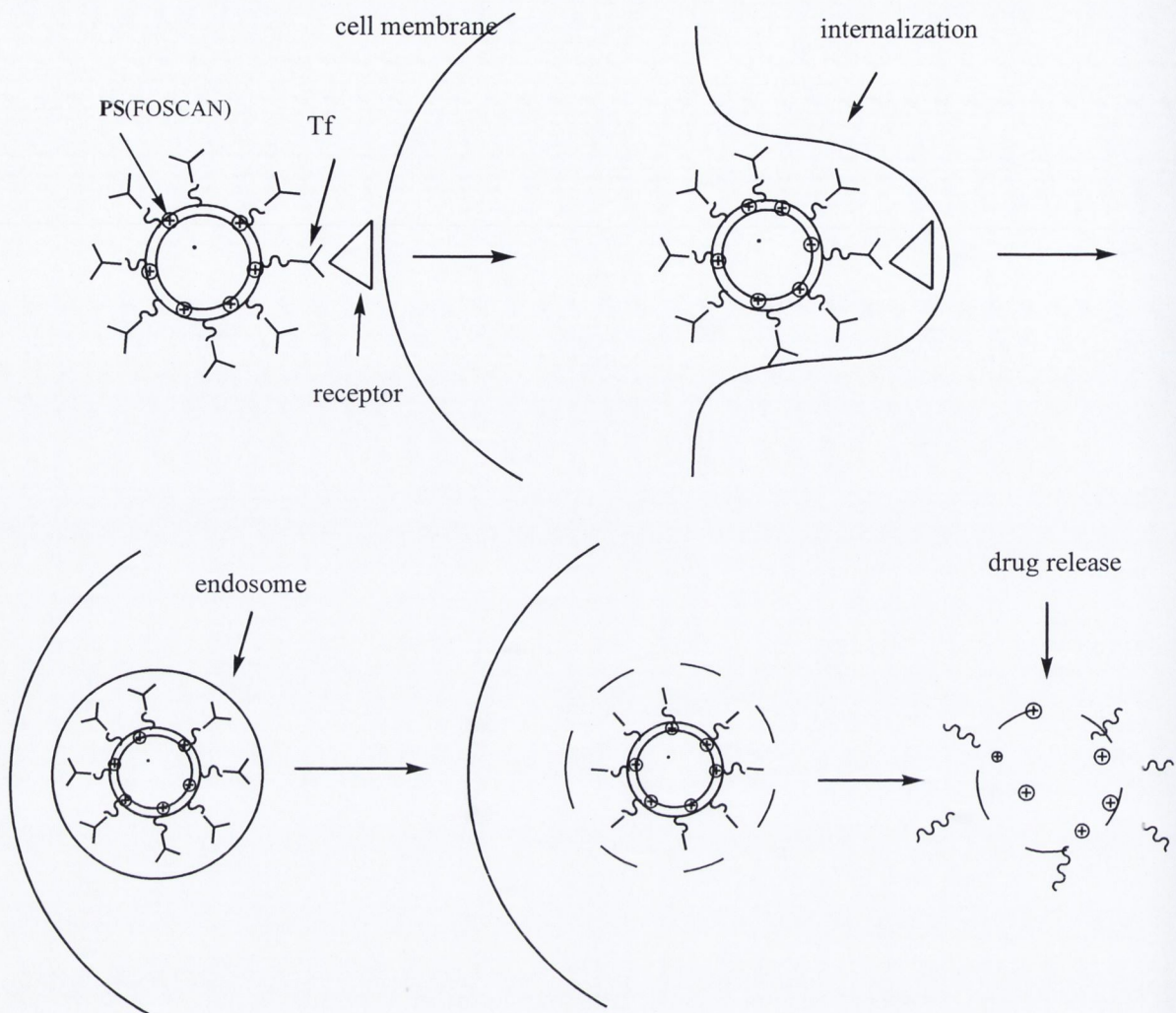


Figure 17. Receptor mediated endocytosis.

6.2 Results and Discussion

Plain and PEGylated liposomal formulations of Foscan were prepared by the conventional lipid film method using DPPC and DPPG lipids according to the method described in a previous chapter. Foscan was encapsulated at a concentration of 1.5 mg mL⁻¹. The encapsulation efficiency was ≥ 90 % in both cases. Physicochemical properties of liposomes are listed in Table 7 as well as in Figure 18. Liposomes were always negatively charged with values for the ζ-potential ranging from -15 mV to -3 mV for the conventional and PEGylated liposomes, respectively. Polydispersity index (PdI) were generally ≤ 0.22. The size of liposomes consistently was in 142.41±8.28 nm and 122.26±5.36 nm for conventional liposomes before and after gel filtration, respectively, whilst for the PEGylated formulation it was 131.18±4.16 nm before gel filtration and 129.91±1.80 nm after gel filtration, respectively.

Table 7. Physicochemical parameters of conventional and PEGylated liposomes. Data represent means ± S.E.M (*n* = 3).

	Z-potential (mV)		Polydispersity index (PdI)	
	Before	After	Before	After
	gel filtration	gel filtration	gel filtration	gel filtration
DPPC:DPPG	-14.18±0.80	-15.25±1.50	0.22±0.02	0.17±0.08
DPPC:DPPG:mPEG	-2.70±0.50	-2.80±0.50	0.17±0.05	0.15±0.07

Student’s t-tests were used to compare ζ-potential after gel filtration for each individual formulation (*p*>0.05) and PdI values for DPPC: DPPG and DPPC: DPPG: mPEG (*p*>0.05).

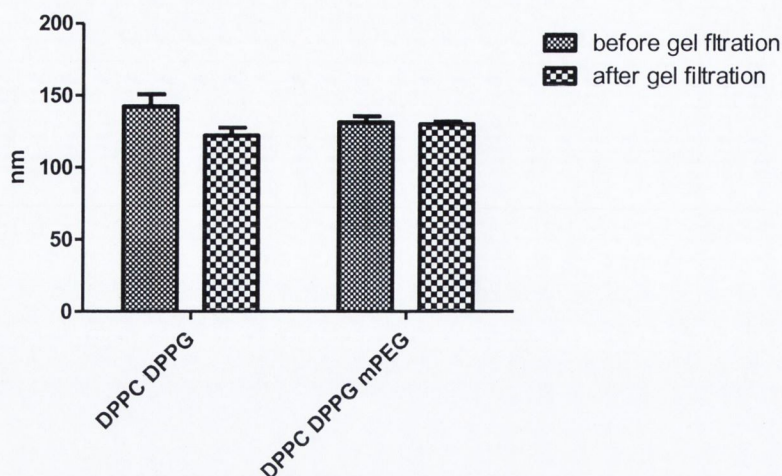


Figure 18. Particle size of conventional and PEGylated liposomes.

All values are presented as the mean $\pm$ S.E.M of $n=3$. Student's t -tests were used to compare DPPC: DPPG and DPPC: DPPG: mPEG ($p>0.05$).

The conjugation of Tf to liposomes was achieved using of EDC and S-NHS-mediated amide bond formation between the carboxyl groups of DSPE-PEG-COOH and free amine groups of the transferrin molecule. The reaction of carboxyl groups in the presence of EDC forms an amine-intermediate which then reacts with S-NHS to yield a reactive NHS ester capable of reacting with amino groups of Tf to yield a stable amide bond [Figure 19] [245,277].

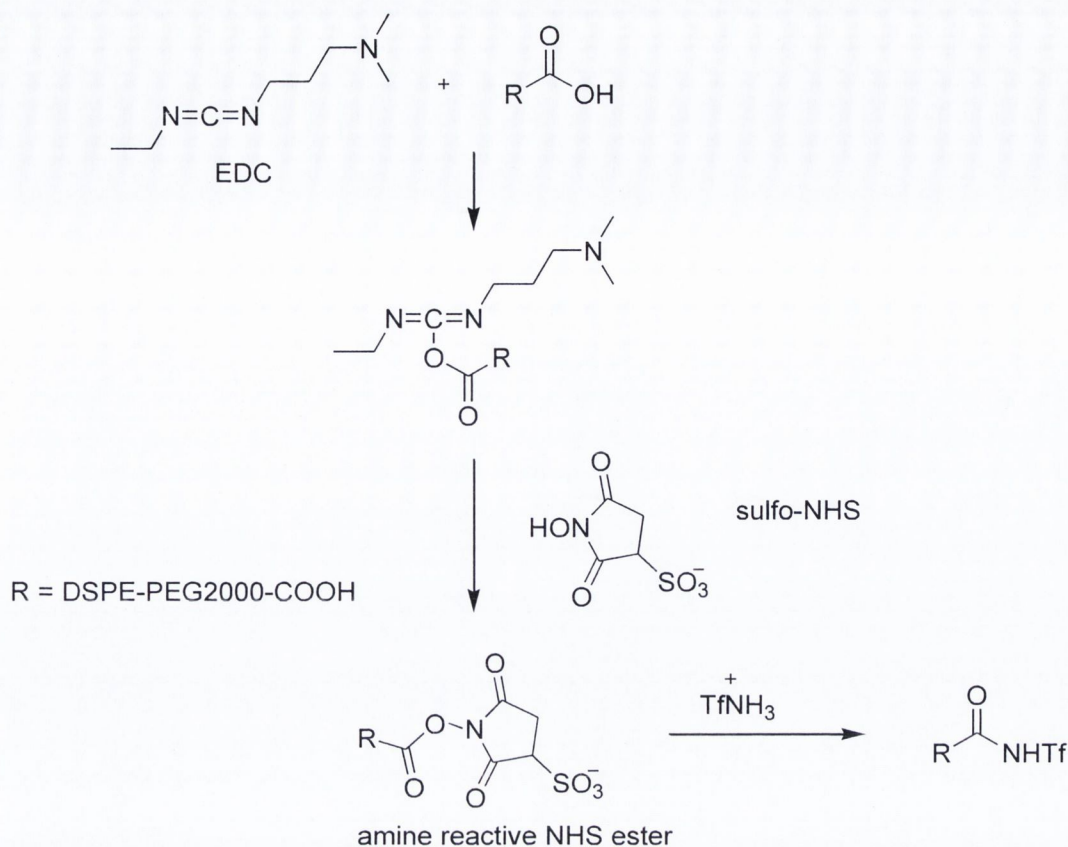


Figure 19. Reaction of coupling Tf with carboxylic group of the linker lipid DSPE-PEG-COOH.

The physicochemical properties of these new liposomes, *i.e.*, particle size, PdI and ζ -potential, before and after coupling of Tf, are shown in Table 8. No significant changes in any of the measured parameters were observed after conjugation of Tf ($p > 0.05$). Approximately 40 μg Tf were conjugated per 1 μmol of phospholipids, which corresponded to a coupling efficiency of 31%. No differences in physicochemical properties of liposomes were observed, when two linkers with different molecular weight DSPE-PEG₂₀₀₀-COOH and DSPE-PEG₅₀₀₀-COOH were used.

Table 8. Physicochemical parameters of transferrin-modified liposomes. Data represent means $\pm$ SEM ($n = 3$).

DPPC:DPPG:cPEG	Before Tf conjugation	After Tf conjugation
Size (nm)	137.00 $\pm$ 4.82	126.01 $\pm$ 3.02
Polydispersity index (Pdl)	0.155 $\pm$ 0.10	0.150 $\pm$ 0.09
Zeta Potential (mV)	-10.52 $\pm$ 1.10	-11.53 $\pm$ 1.20
Coupling efficiency (%)	125 μ g Tf/1 μ mol PL (n/a)*	31.01 $\pm$ 4.20

*125 μ g Tf/1 μ mol PL – the initial amount of transferrin added to the liposomal formulation; Student’s t-tests were used to compare_ physicochemical parameters before and after filtration process ($p>0.05$).

Functional expression of TfR has previously been demonstrated in the oesophageal adenocarcinoma cell lines, OE33 and SEG-1 [4]. To assess if the OE21 cells used in our study also express TfR, Western blot analysis and immunocytochemical staining were performed. Figure 20A shows TfR expression (*red*) in OE21 cells as obtained by confocal laser scanning microscopy. In Figure 20B OE21 cells treated with Tf-modified liposomes are depicted. High abundance of TfR is visible, and since TfR has a high turnover rate in tumour cells, incubation with Tf-modified liposomes resulted in increased TfR association to the membrane. The immunoblot detected a single band at 80 kDa [Figure 20C] and confirmed the presence of the transferrin receptor in OE21 cells. Three different passage numbers were used to ensure that TfR is expressed on protein level independent of time in culture.

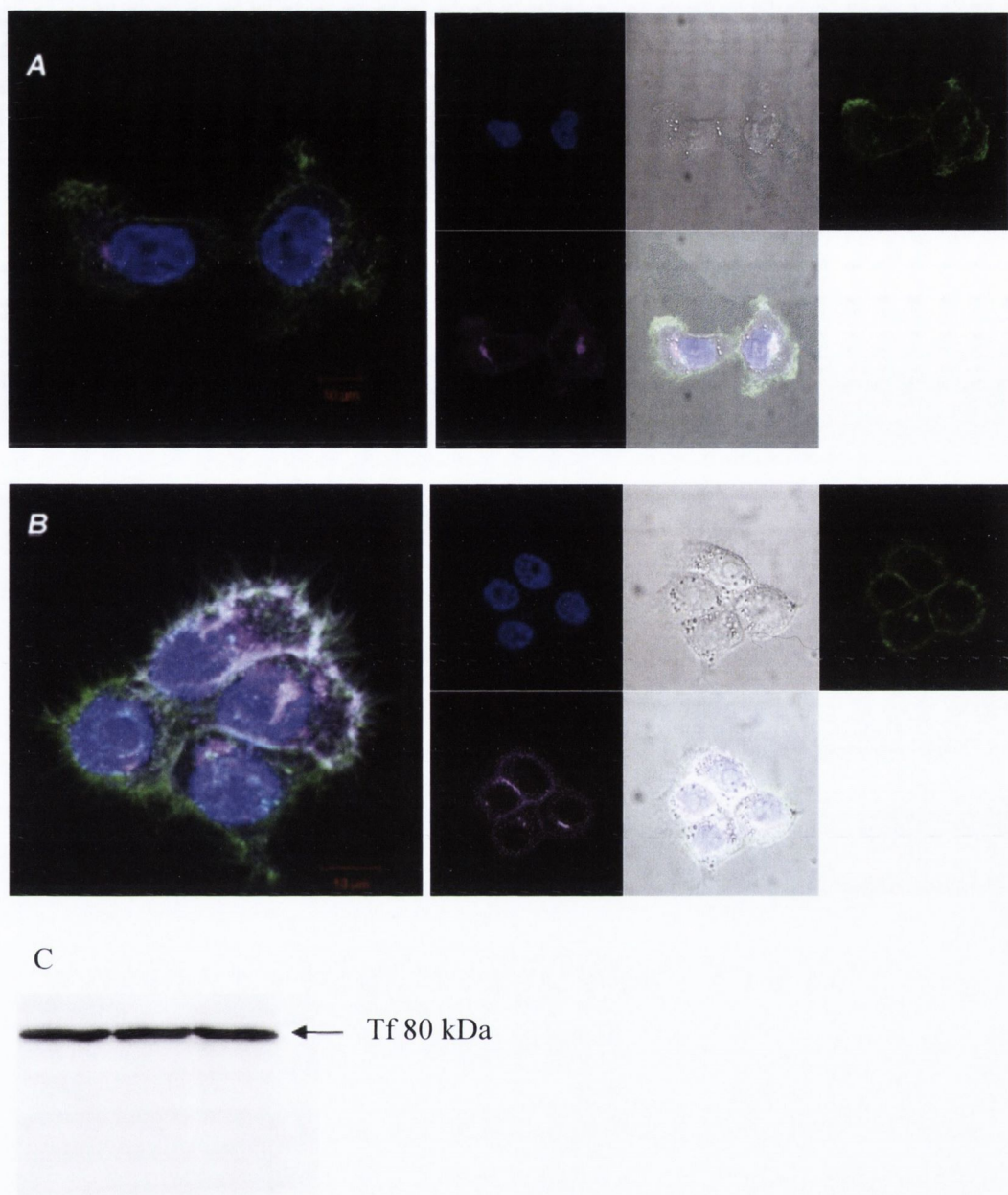


Figure 20. Expression of transferrin receptor (CD71) in OE21 human oesophageal tumour cells. (A&B) Immunocytochemistry for transferrin receptor (red). F-actin was stained with FITC-labelled phalloidin (green) and cell nuclei were counterstained with Hoechst 33342 (blue). Shown are untreated cells (A) as well as cells incubated with Foscan containing transferrin-modified liposomes for 24 h (B). (C) Representative immunoblot of transferrin receptor in OE21 oesophageal cancer cells from three different passages (lanes 1 – 3).

Next, dark and photocytotoxic effects of various Foscan liposomes were measured in OE21 cells in dependence of time, and compared with Foscan solution. Empty control liposomes did not have an influence on cells survival, indicating that the observed cytotoxic effects were only caused by the photosensitiser. Liposomal formulations of Foscan showed a concentration-dependent anti-proliferating effect on OE21 cells after irradiation [Figure 21]. Photocytotoxicity exhibited by non-encapsulated Foscan did not differ significantly than corresponding concentrations of plain and PEGylated liposomes ($p > 0.05$). Phototoxic effect was generally more pronounced at the highest concentration (*i.e.*, 2 μM). Surprisingly, transferrin-modified liposomes, did not show an improved cytotoxic effect upon irradiation, even after 24 h of incubation. No dark toxicity was observed in any of the formulations used [Figure 22].

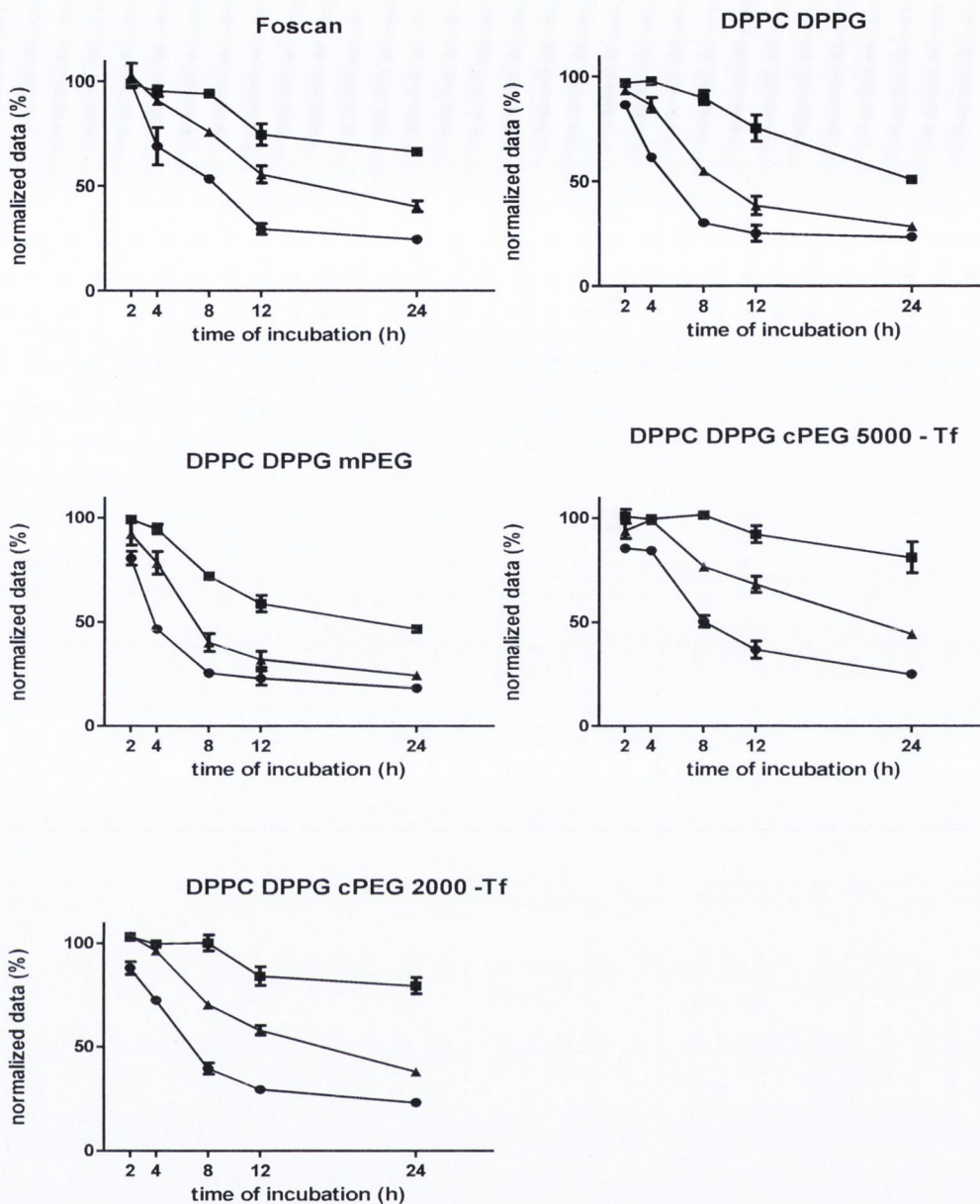


Figure 21. Cytotoxicity of free and liposomally encapsulated Foscan after illumination investigated by MTT assay 24 h post illumination in OE21 oesophageal cancer cells. The liposomal formulations studied were plain liposomes (DPPC DPPG), PEGylated liposomes (DPPC DPPG mPEG) and PEGylated liposomes modified with transferrin using either PEG linkers of 2000 Da or 5000 Da molecular weight (DPPC DPPG cPEG₂₀₀₀ Tf and DPPC DPPG cPEG₅₀₀₀ Tf, respectively). ■ – 0.5 μM; ▲ – 1 μM; ● – 2 μM; Results are expressed as means ± SEM ($n = 3$). One way analysis of variance to compare liposomal formulations with non-encapsulated Foscan ($p > 0.05$).

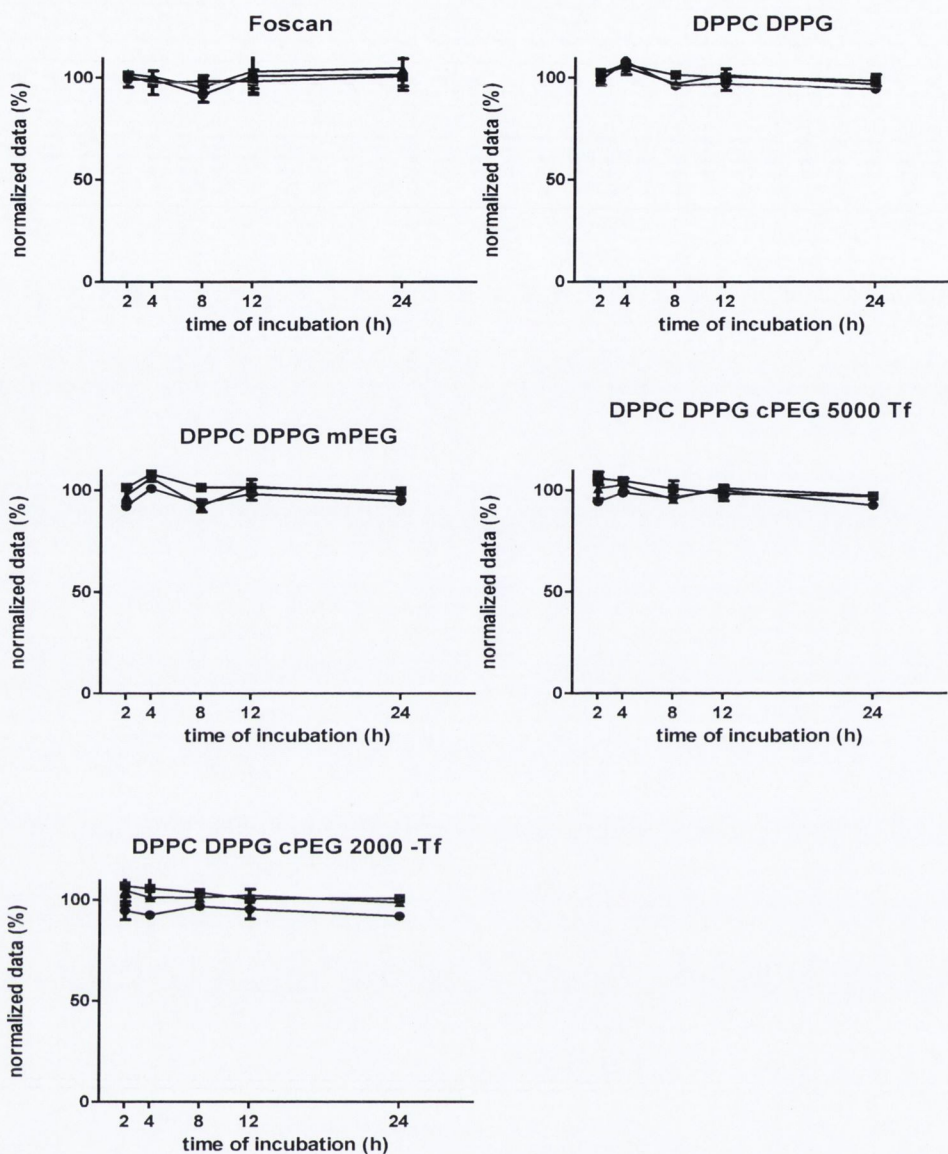


Figure 22. Cytotoxicity of free and liposomally encapsulated Foscan in dark conditions measured by MTT assay in OE21 oesophageal cancer cells. The liposomal formulations studied were plain liposomes (DPPC DPPG), PEGylated liposomes (DPPC DPPG mPEG) and PEGylated liposomes modified with transferrin using either PEG linkers of 2000 Da or 5000 Da molecular weight (DPPC DPPG cPEG₂₀₀₀ Tf and DPPC DPPG cPEG₅₀₀₀ Tf, respectively). ■ – 0.5 μM; ▲ – 1 μM; ● – 2 μM; Results are expressed as means ± SEM (*n* = 3).

One way analysis of variance to compare liposomal formulations with non-encapsulated Foscan (*p*>0.05).

It was previously shown that Tf modification of drugs or drug delivery systems can improve their efficiency in cancer therapy [223,244]. Several different cancer types were used in these studies, and it was demonstrated that Tf-targeted liposomes were indeed promising vehicles for delivering chemotherapeutic agents that can be encapsulated at high levels over extended periods of time such as doxorubicin to, *e.g.*, non-small cell lung cancer [244,272], hepatocellular carcinoma [273], leukaemia [278] and metastatic mammary carcinoma [279]. Derycke *et al.* previously studied the influence of transferrin-targeted, sterically stabilised liposomes as vehicles for the selective delivery of photosensitisers such as AlPcS4 and hypericin in the treatment of bladder and cervix carcinoma [223,237,238]. Whilst data obtained with AlPcS4 were quite promising [223,238], the liposomally encapsulated hypericin was much less effective [237]. It was suggested that significant amounts of hypericin were leaking from the PEGylated liposomes, thus, reducing their efficiency [237].

Our results also indicated that Tf-modified liposomes did not improve the efficiency of Foscan in oesophageal cancer. Consequently, we attempted to determine whether the anti-proliferative effects could be correlated to the intracellular accumulation of Foscan. The signal intensity of Foscan within OE21 cells, when measured by high content analysis, showed that in the case of Tf-liposomes, the length of incubation indeed did not significantly increase the intracellular accumulation. To the contrary, Tf modified vehicles at 2 μ M concentration of photosensitiser after 24 h led to lower intracellular levels ($p < 0.05$), when compared to free photosensitiser as well as plain and PEGylated liposomes [Figure 23]. The images of OE21 cells treated with different liposomal formulations of Foscan and free photosensitiser obtained by InCell analyzer are presented in Table 9.

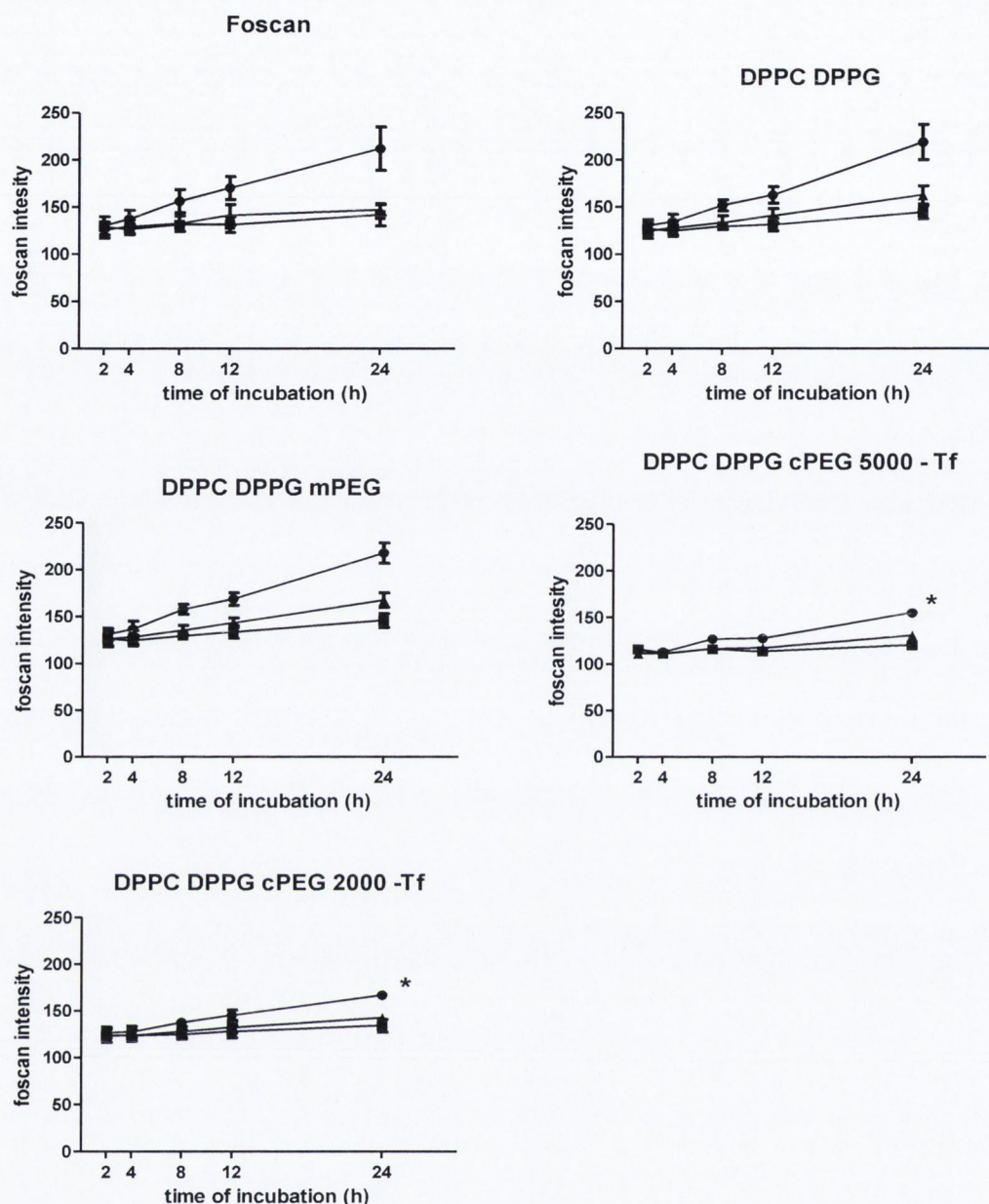


Figure 23. High content analysis of Foscan intensity of various liposomal formulations and as free drug in dependence of time of incubation in OE21 oesophageal cancer cells. The liposomal formulations studied were plain liposomes (DPPC DPPG), PEGylated liposomes (DPPC DPPG mPEG) and PEGylated liposomes modified with transferrin using either PEG linkers of 2000 Da or 5000 Da molecular weight (DPPC DPPG cPEG₂₀₀₀ Tf and DPPC DPPG cPEG₅₀₀₀ Tf, respectively). Concentrations used were: ■ – 0.5 μM; ▲ – 1 μM; ● – 2 μM. Results are expressed as means ± SEM ($n = 3$). One way analysis of variance to compare liposomal formulations with non-encapsulated Foscan ($p > 0.05$, $*p < 0.05$).

Results obtained with free Foscan were consistent with data measured for non-conjugated liposomes. The differences were less pronounced at lower concentrations, *i.e.*, 0.5 and 1 μM , but well defined at the highest (2 μM) concentration of Foscan. No differences in Foscan intensity were observed when comparing differently sized PEG-linkers, *i.e.*, DSPE-PEG₂₀₀₀-COOH and DSPE-PEG₅₀₀₀-COOH, suggesting that the chain length of PEG did not have an influence on the targeting ability of transferrin modified liposomes.

On the other hand, the Foscan area within OE21 cells measured by high content analysis [Figure 24], demonstrated similar uptake rates and no significant differences after 24 h in all formulations. Taken together, this may suggest that photosensitiser was released from the Tf-modified liposomes and taken up by the cells in form of aggregates where fluorescence is quenched.

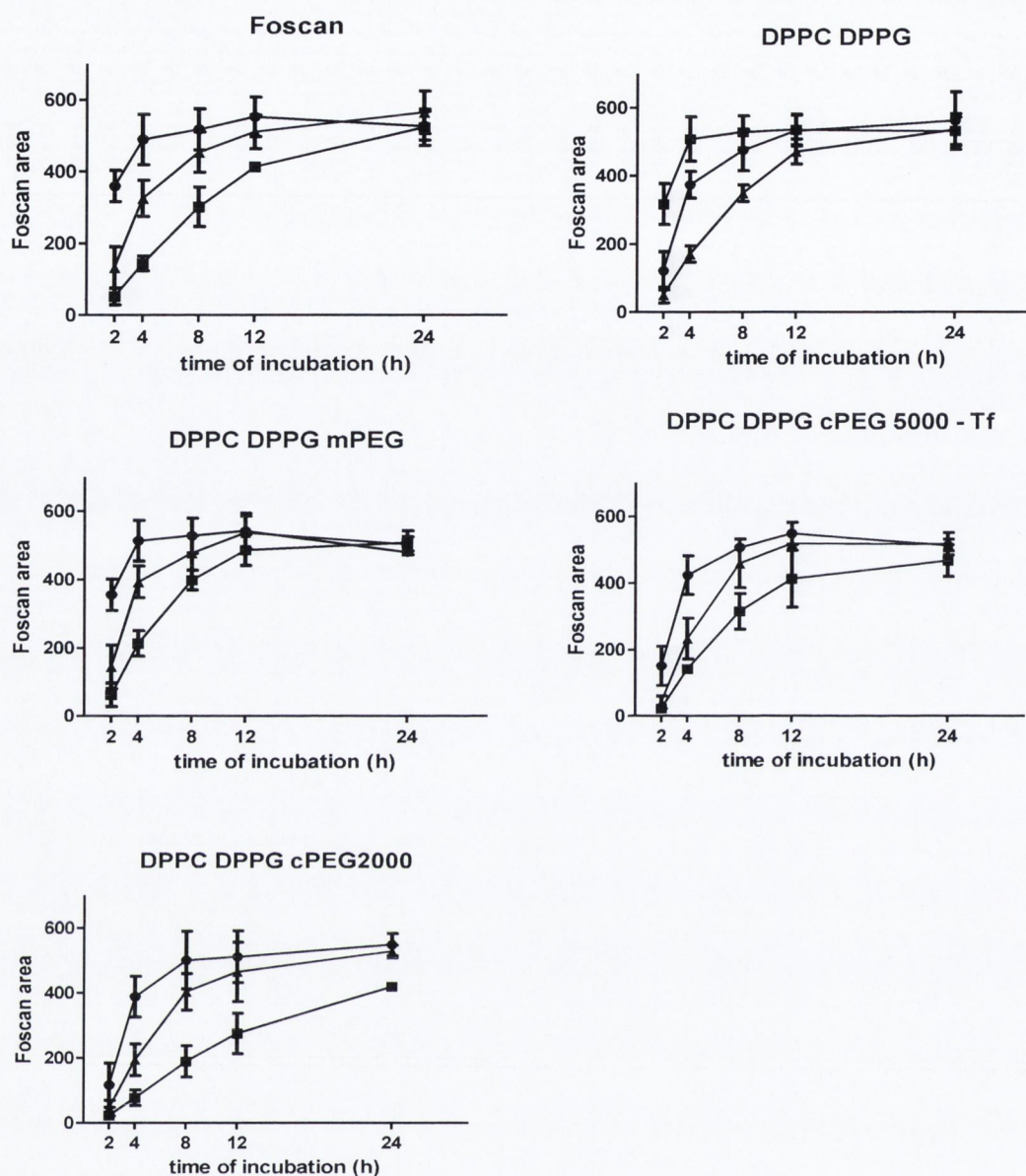


Figure 24. High content analysis of Foscan area in various liposomal formulations and as free drug in dependence of time of incubation in OE21 oesophageal cancer cells. The liposomal formulations studied were plain liposomes (DPPC DPPG), PEGylated liposomes (DPPC DPPG mPEG) and PEGylated liposomes modified with transferrin using either PEG linkers of 2000 Da or 5000 Da molecular weight (DPPC DPPG cPEG₂₀₀₀ Tf and DPPC DPPG cPEG₅₀₀₀ Tf, respectively). Concentrations used were: ■ – 0.5 μM; ▲ – 1 μM; ● – 2 μM. Results are expressed as means ± SEM ($n = 3$). One way analysis of variance to compare liposomal formulations with non-encapsulated Foscan ($p > 0.05$).

Our data demonstrated that Tf-modified liposomal formulations of Foscan did not improve the photocytotoxicity or intracellular accumulation of the photosensitiser in oesophageal cancer cells. There are a number of possible explanations for this observation. Foscan might have leaked from the Tf-vehicles as mentioned above as a result of a membrane destabilising effect of Tf. Foscan as a highly lipophilic drug may form aggregates in an aqueous environment, but may still be taken up by the cells, which would explain the observed lower Foscan intensity.

Hefesha *et al* [280] in their studies investigated the influence of total lipid content, temperature, charge of donor liposomes, acyl chain length and saturation of the lipids on the transfer mechanism of Foscan. Their data showed that transfer rates depended strongly on the temperature and charge of the lipids. At 37 °C donor liposomes exhibited an increase in the transfer rate compared with 15 and 22 °C, but after 24 h this effect had leveled off. The authors suggested that molecular motion and acyl chain mobility increased with the temperature, resulting in a more fluid bilayer, thus at 37 °C Foscan was rapidly released. However, all our experiments were performed at 37 °C only. As shown in Figure 24 the amount of photosensitiser within the cells was similar in all cases and after 24 h, no differences were visible in the maxima. Even if Foscan was released from liposomes at 37 °C, Figure 24 indicated that the photosensitiser was still taken up by the cells at the same rate, thus destabilisation of Tf-liposomes is the only explanation. Hefesha *et al.* also noticed that positively charged liposomes had a faster transfer rate than negative ones, but the maximum amount transferred was almost identical. In our experiments all formulations were negatively charged and the differences between them were not significant. Moreover, the saturation degree or acyl chain length may have had an influence. Saturated lipids showed faster transfer rate than unsaturated in experiment performed at 37 °C (all our

lipids were saturated), and the transfer rate was also faster for phospholipids with longer chains. Here, however, only DPPC was used.

It has been reported that the size of Tf-modified liposomes can regulate the selectivity of these vehicles to different tissues [281]. Small size, *i.e.*, less than 80 nm, was an important factor for the tissue targeting of Tf-targeted liposomes to the liver and the brain, whilst the heart was able to take up both small and large (> 140nm) liposomes. In a different study, Sakaguchi and colleagues described that the internalisation of Tf-modified, pH-sensitive, fusogenic liposome-lipoplex hybrid complexes was inversely correlated to expression levels of TfR in a variety of cell lines [282]. Hence, it might be possible that our vesicles were either too large to be internalised or that TfRs expressed in the OE21 cell line do not recycle very fast and hence, Tf modification does not offer an advantage. Finally, it cannot be ruled out that under the chosen experimental conditions, iron ions dissociated from the holo-transferrin molecules, resulting in inactive apo-transferrin conjugated to the liposomes. Similar observations were reported by van Rooy *et al.*, when they observed that only a transferrin receptor antibody was able to significantly increase brain uptake *in vitro* and *in vivo*, but not Tf-modification [283].

Figure 25. Images collected from InCell analyzer. Images present OE21 cells treated with different liposomal formulations of Foscan and free photosensitizer with different times of incubation. Blue color present nuclei stained by Hoechst; green color present F-actin stained by phalloidin; red color present Foscan.

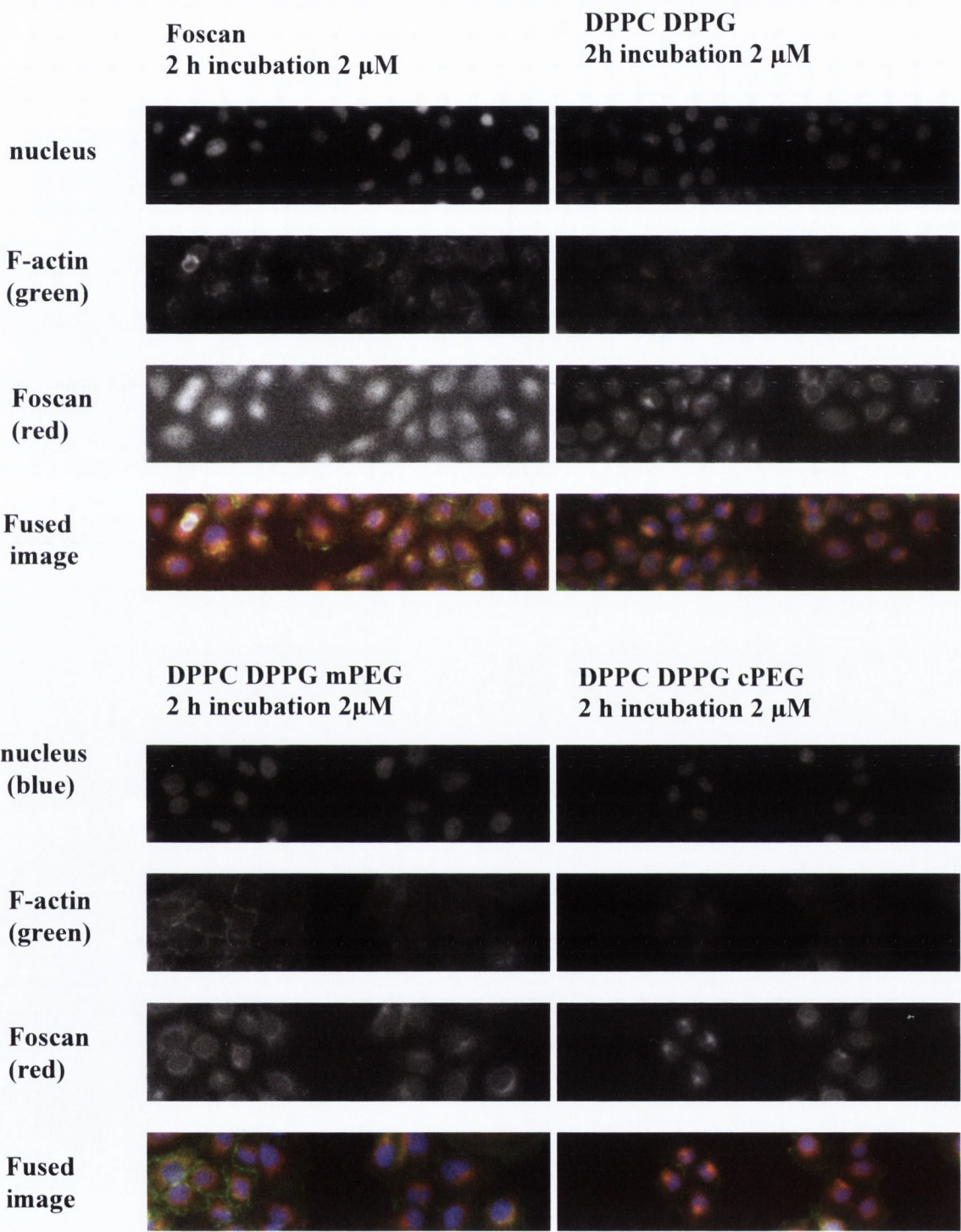
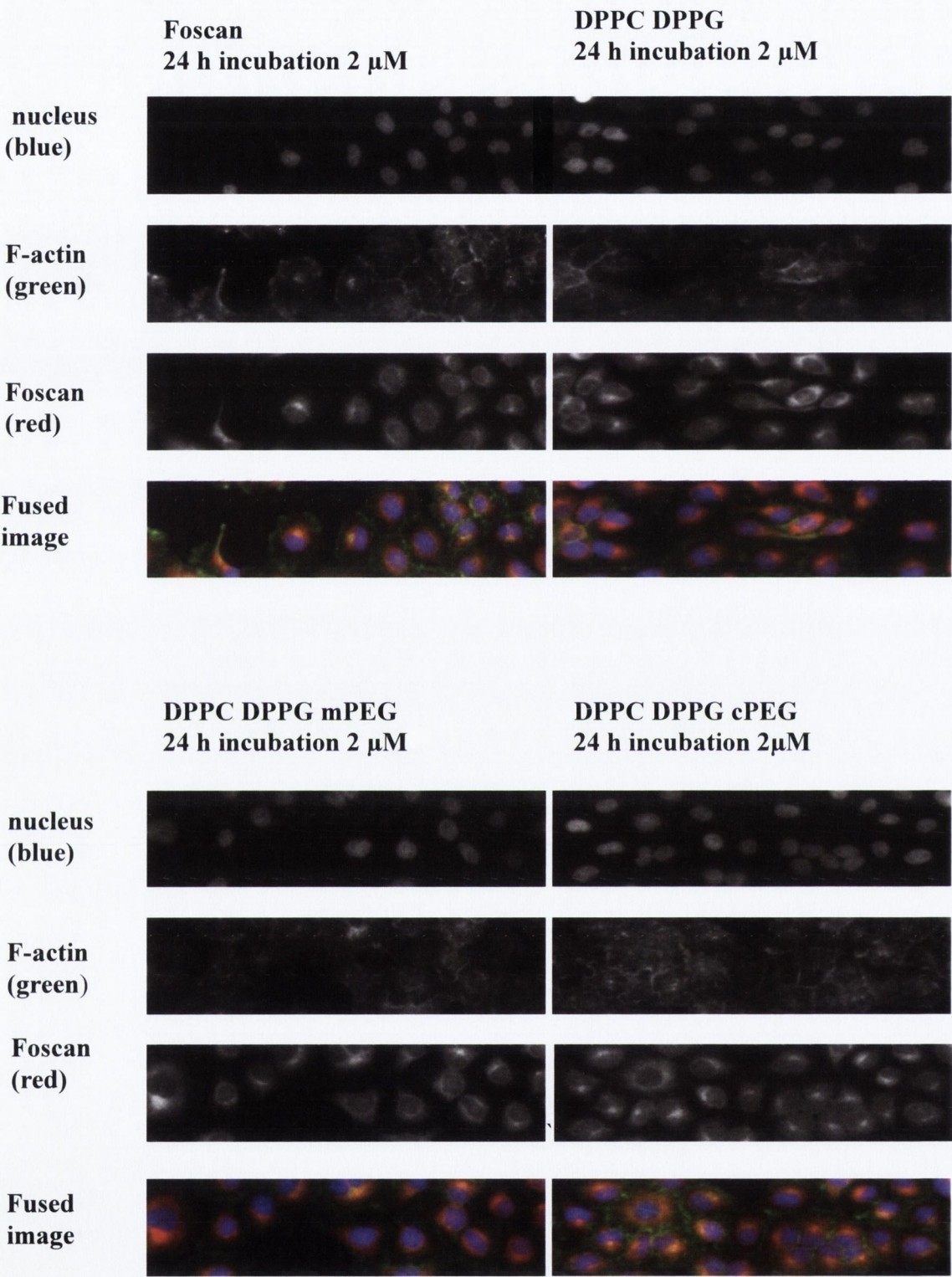


Figure 25. continued



6.3 Conclusions

Improved efficiency of cancer therapy with transferrin modified liposomes have been demonstrated in several different studies including examples as delivery doxorubicin to lung cancer cells [244,272], hepatocellular carcinoma [273], leukemia cells [278] or metastatic mammary carcinoma cells [279]. In addition, they exhibited potential in gene therapy in leukemia cells [284], osteoblastic cell lines [285], osteosarcoma cells [286], cervical cancer cells [287] or prostate cancer cells [288]. They have also been used for combination therapy to deliver imatinib and anti-BCR-ABL siRNA to leukaemia cells [289] or Bcl-2 antisense and cisplatin to breast cancer cells [290].

Studies provided by Derycke *et al.* are the only publications on photosensitisers encapsulated in Tf conjugate liposomes as vehicles for the selectively delivery to the target tissue [186,237,238]. Unlike AlPcS4 [223,238], hypericin entrapped in Tf-liposomes demonstrated much less effective results than non modified vehicles [237].

This is the first study reporting the effect of Tf-targeted liposomes on an oesophageal cancer cell line. Our data clearly indicate that PEGylated liposomes based on DPPC/DPPG lipids coupled with Tf did not significantly change any physicochemical parameters of the formulation, however, intracellular intensity of PS 8 in case of targeted vehicles is significantly lower than other formulations. A possible explanation may be instability of Foscan incorporated in Tf targeted liposomal formulation or that our vehicles were too large to be internalised into the cells. It might be also possible, that TfRs expressed in the OE21 cell line do not recycle very fast and hence, Tf modification is not an advantage.

CHAPTER 7 Porphyrin derivatives

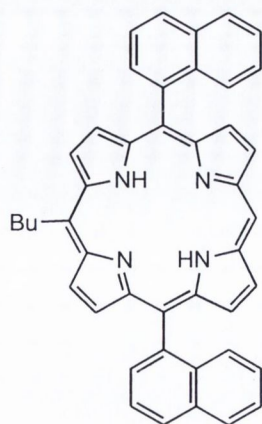
7.1 Introduction

The search for the ideal photosensitiser has been ongoing and many new compounds have been synthesised in order to find perfect compound with properties such as high absorption between 650 nm and 850 nm, minimal dark toxicity, high singlet oxygen production, stable composition, safety, solubility in water or selectivity to the target tissue [31,37,38]. Several new potential photosensitisers have been reported and various new strategies have been developed in order to enhance their photodynamic effect and overcome drawbacks associated with the drugs. The main disadvantage to overcome is water solubility, selectivity of the photosensitisers to target tissue and thus minimal side effects such as photosensitivity. The main strategy is enhance they selectivity and minimize side effects by chemical modification of existing photosensitisers or target with ligands which specifically targets the cancer cells and decrease accumulation in healthy tissues [94, 95] such as the conjugation of the PS with transferrin (Tf) specific to transferrin receptor (TfR) [97], EGF specific to epidermal growth factor (EGF) receptors [96], specific antibodies or their fragments [111], polymer-bound photosensitisers [100, 101], photosensitisers with carbohydrates moieties [291], amino acid residues [292] or steroids [106, 107]. The aim of our next study was investigate a class of compounds designed for photodynamic therapy.

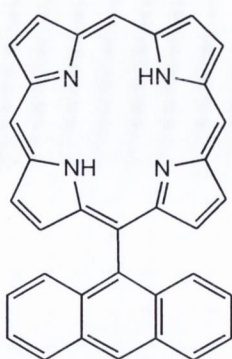
7.2 Results and discussion

7.2.1 Porphyrin analogues

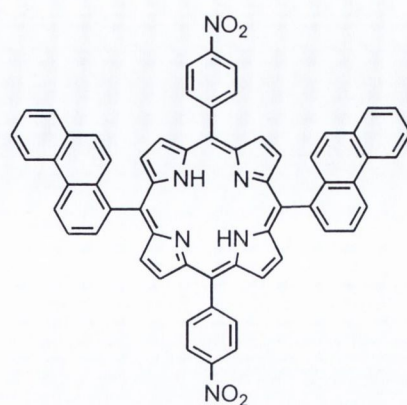
Compounds provided by our chemistry laboratory were analysed in terms of their cytotoxicity. Photosensitiser solutions were prepared by dissolving the compounds in ethanol:propylene glycol (60:40) (v/v). Solutions were then diluted with medium to obtain final concentrations in a range 1-50 μM . However, often due to formation of aggregates in solutions, the concentrations were decreased to the range 0.25-5 μM which showed clearer solution. Among 42 compounds, which are listed in Figure 26 below, only compounds **24**, **30**, **32** and **38** showed photodynamic activity [Table 9], similar in both cell lines, OE33 and SKGT-4.



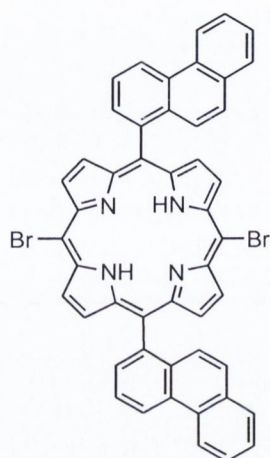
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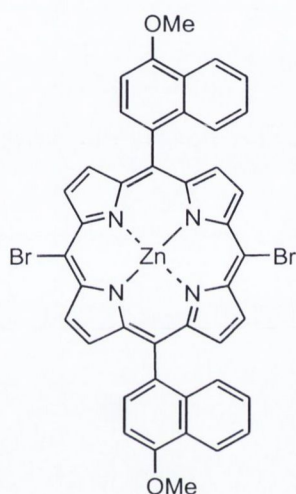
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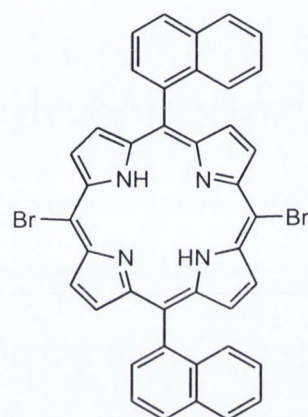
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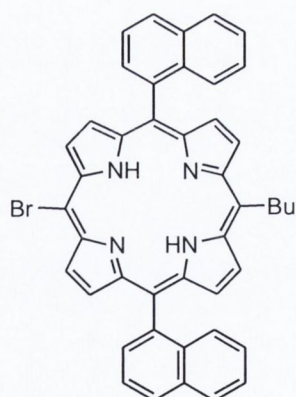
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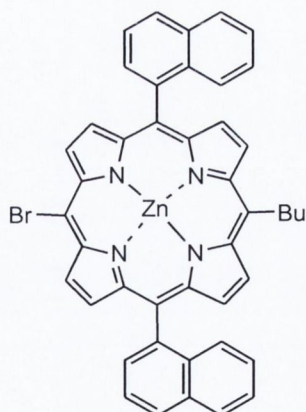
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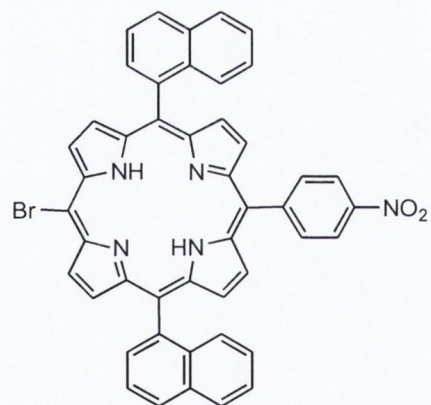
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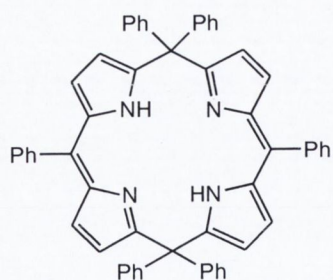
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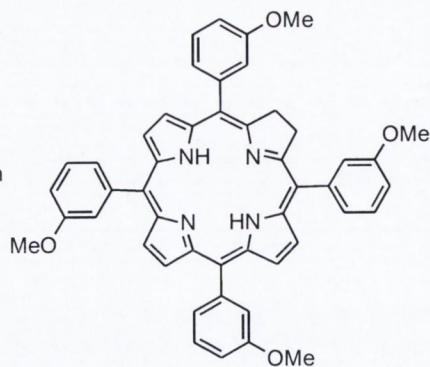
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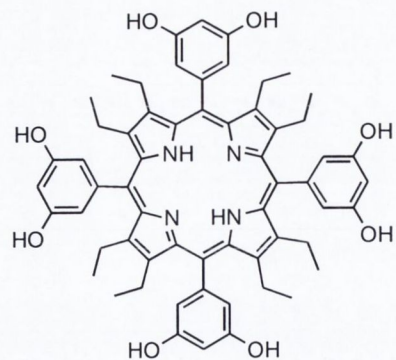
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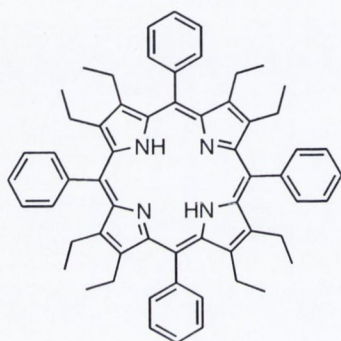
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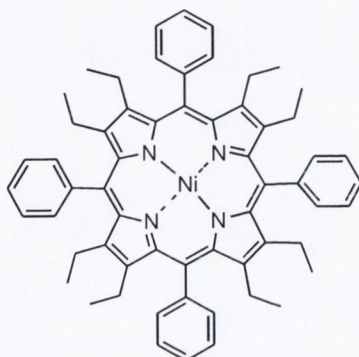
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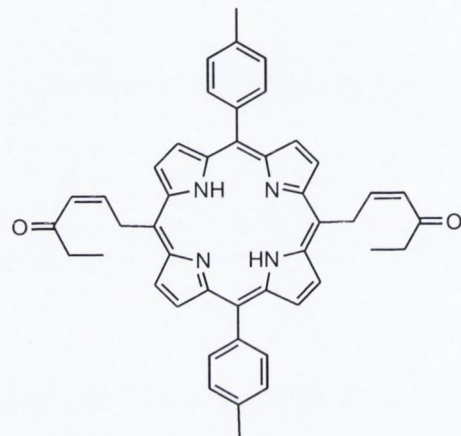
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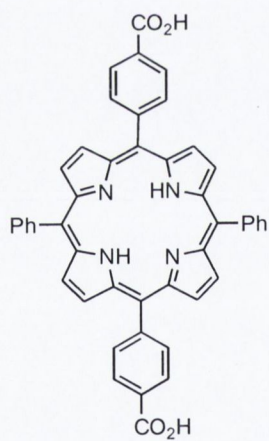
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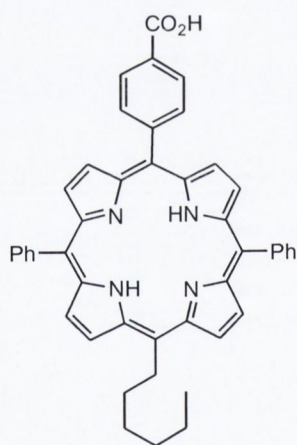
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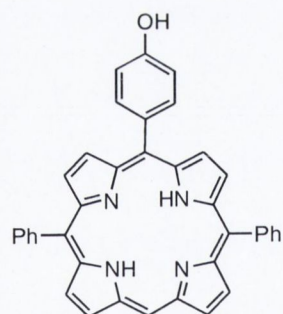
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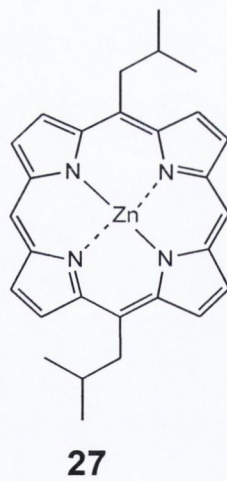
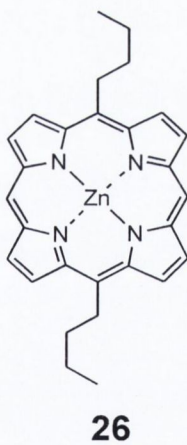
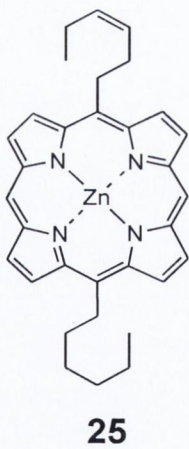
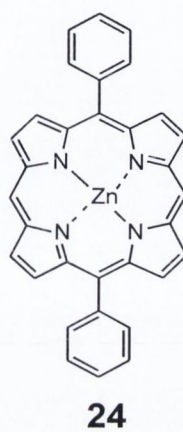
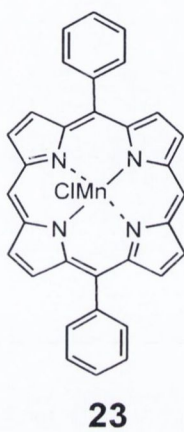
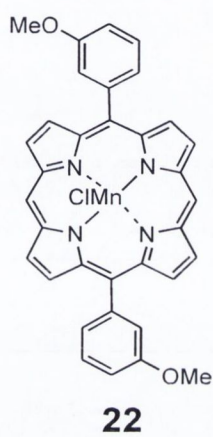
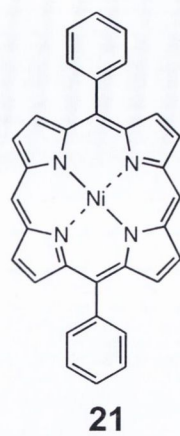
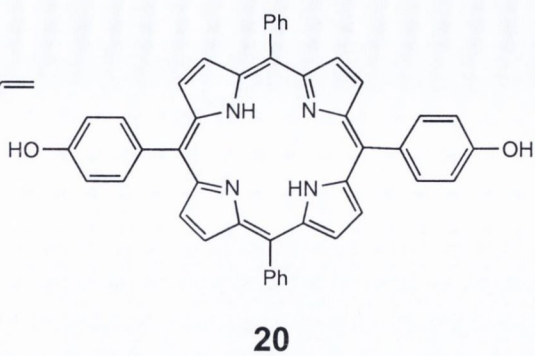
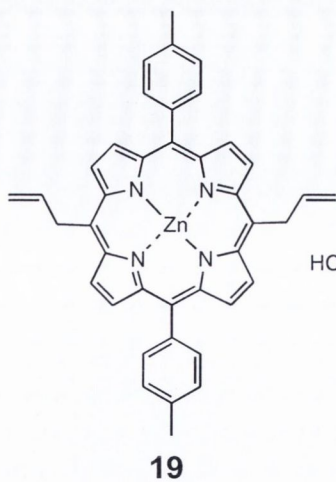
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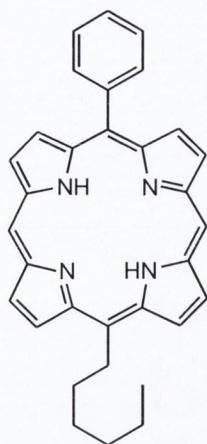


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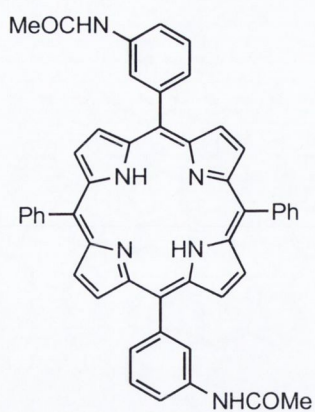


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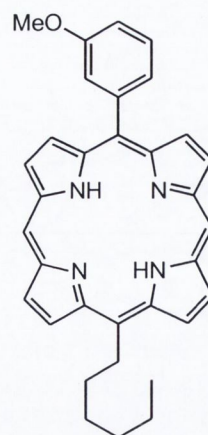




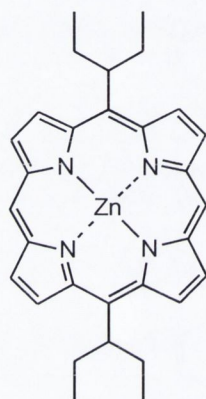
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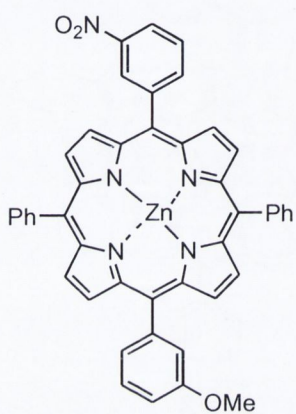
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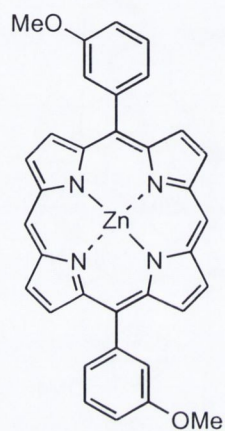
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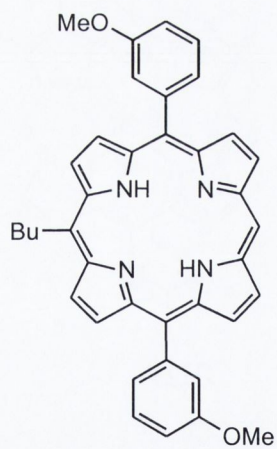
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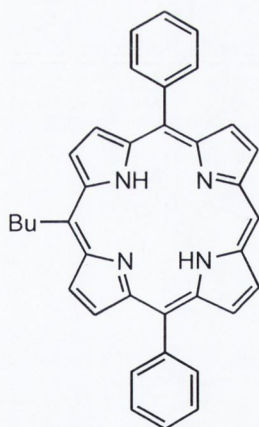
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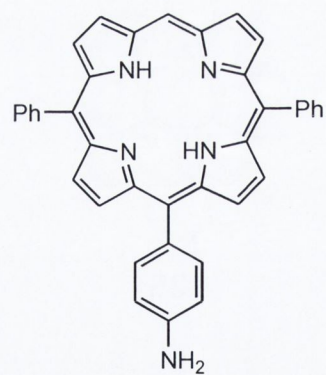
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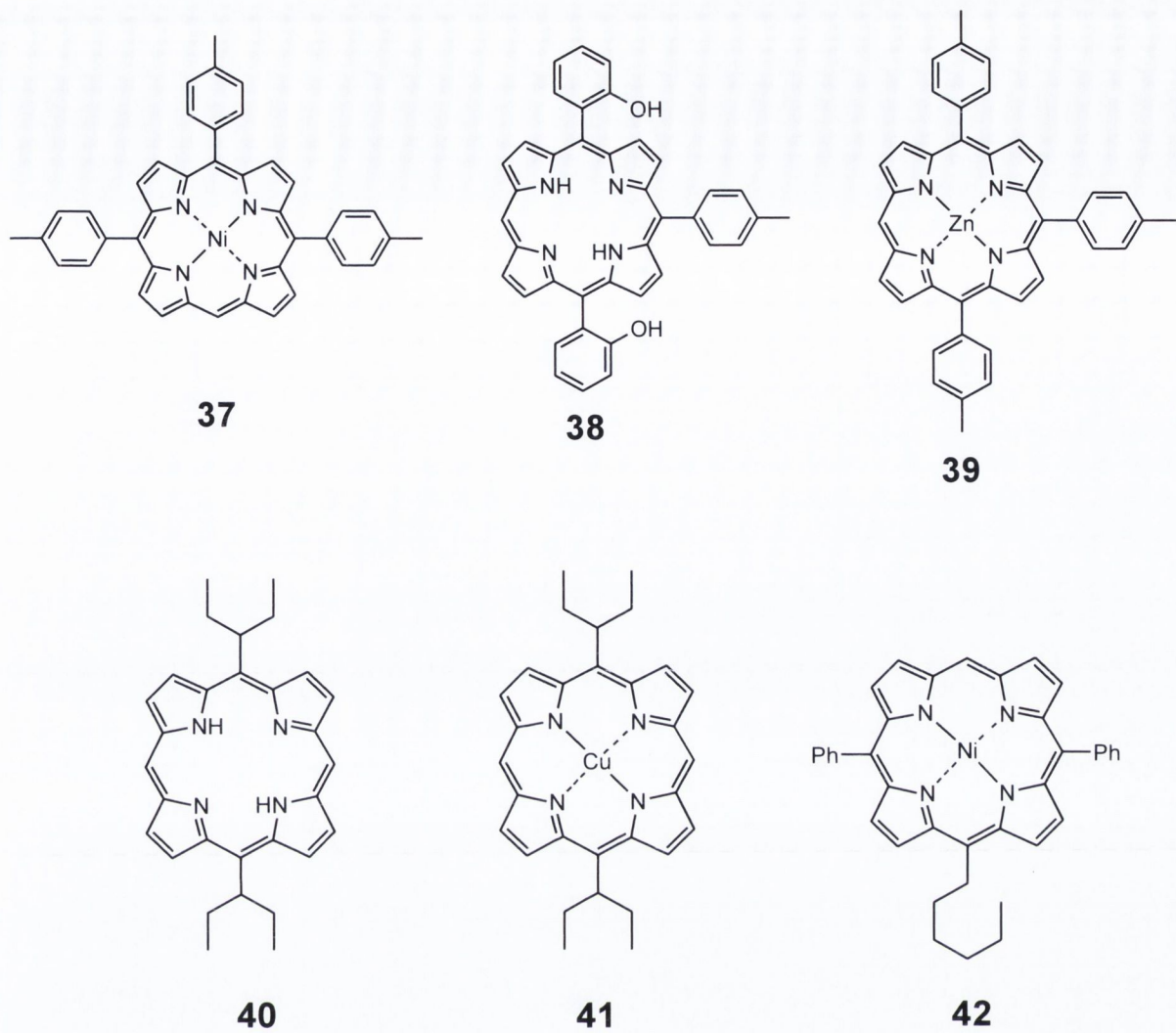


Figure 26. Chemical structures of tested porphyrin derivatives.

Table 9 . PDT effect of the tested compounds in dark and after illumination with 1.75 mW cm⁻² light using MTS assay.

Compound number	PDT effect	Light, cell survival (%) at 5 µM PS	Dark, cell survival (%) at 5 µM PS	Good solubility in ethanol:polypropylene glycol
1	no	88	93	+
2	no	83	87	+
3	no	-	-	no
4	no	79	89	+
5	no	80	84	+
6	no	84	79	+
7	no	-	-	no
8	no	97	100	+
9	no	93	100	+
10	no	-	-	no
11	no	62	67	+
12	no	83	86	+
13	no	90	94	+
14	no	100	100	+
15	no	97	100	+
16	no	-	-	no
17	no	94	87	+
18	no	100	100	+
19	no	100	100	+
20	no	-	-	no
21	no	-	-	no
22	no	74	66	+
23	no	89	80	+
24	yes	52	92	+
25	no	50	52	+
26	no	68	66	+
27	no	72	66	+
28	no	-	-	no
29	no	100	100	+
30	yes	57	97	+
31	no	71	54	+
32	yes	49	97	+
33	no	92	92	+
34	no	84	97	+
35	no	-	-	no
36	no	-	-	no
37	no	-	-	no
38	yes	44	100	+
39	no	93	97	+
40	no	88	89	+
41	no	98	93	+
42	no	-	-	no

Only compound number **24**, **30**, **32** and **38** among 42 porphyrin derivatives showed photodynamic activity. All 4 compounds demonstrated approximately 50% of cellular survival after 24 h incubation and illumination with 1.75 mW cm^{-2} light and no significant dark cytotoxicity at $5 \text{ }\mu\text{M}$ concentration measured with MTS assay. None of the compounds show good uptake by OE33 and SKGT-4 cell lines in experiment with High Content analysis.

7.2.2 Bile acid porphyrin conjugates

Bile acid porphyrin conjugates [Figure 27] were successfully synthesised in department of Organic Chemistry. Certain bile acids have shown to be selectively taken up by tumors and have also been proven to induce apoptosis [293,294]. By linking these compounds to the porphyrins, it was hoped to impart these desired attributes of bile acids and therefore create a photosensitiser for PDT with minimum side effects and maximum efficacy.

m-THPP and *m*-THPC were chosen as they are highly conjugated systems with an extensive delocalized π -electron system absorbing strongly in the UV-visible region. Test solutions were prepared by the same method as previously, by dissolving in ethanol: propylene glycol (60:40) (v/v). Solutions were then diluted with medium to obtain final concentrations in a range $1\text{-}50 \text{ }\mu\text{M}$. Photodynamic activity of the compounds were measured with MTS assay 24 h post irradiation with 1.7 mW cm^{-2} of light. However, none of the four compounds show dark or light cytotoxicity. Figures 28 and 29 demonstrate toxicity test in OE33 cell lines. The same results were obtained in SKGT-4 cells.

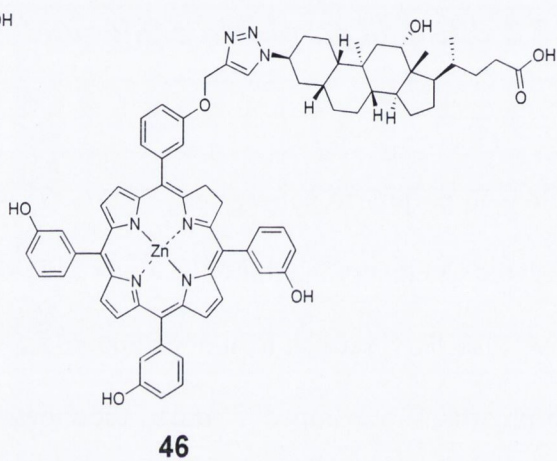
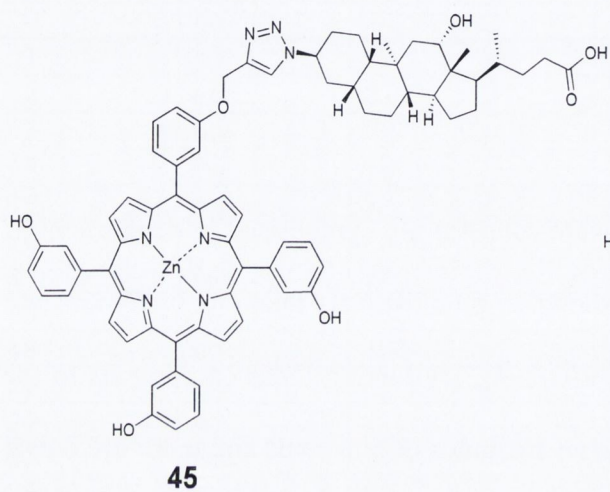
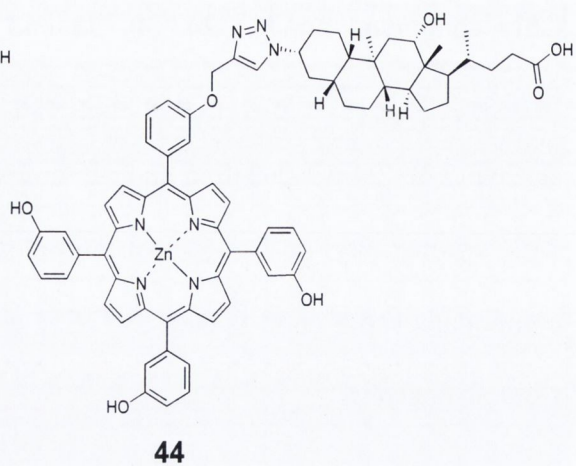
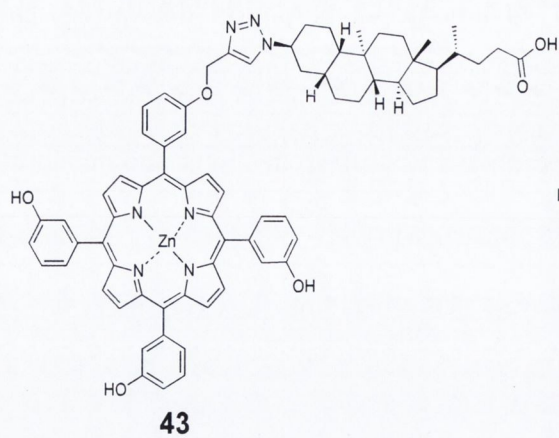


Figure 27. Structures of bile acid porphyrin conjugates.

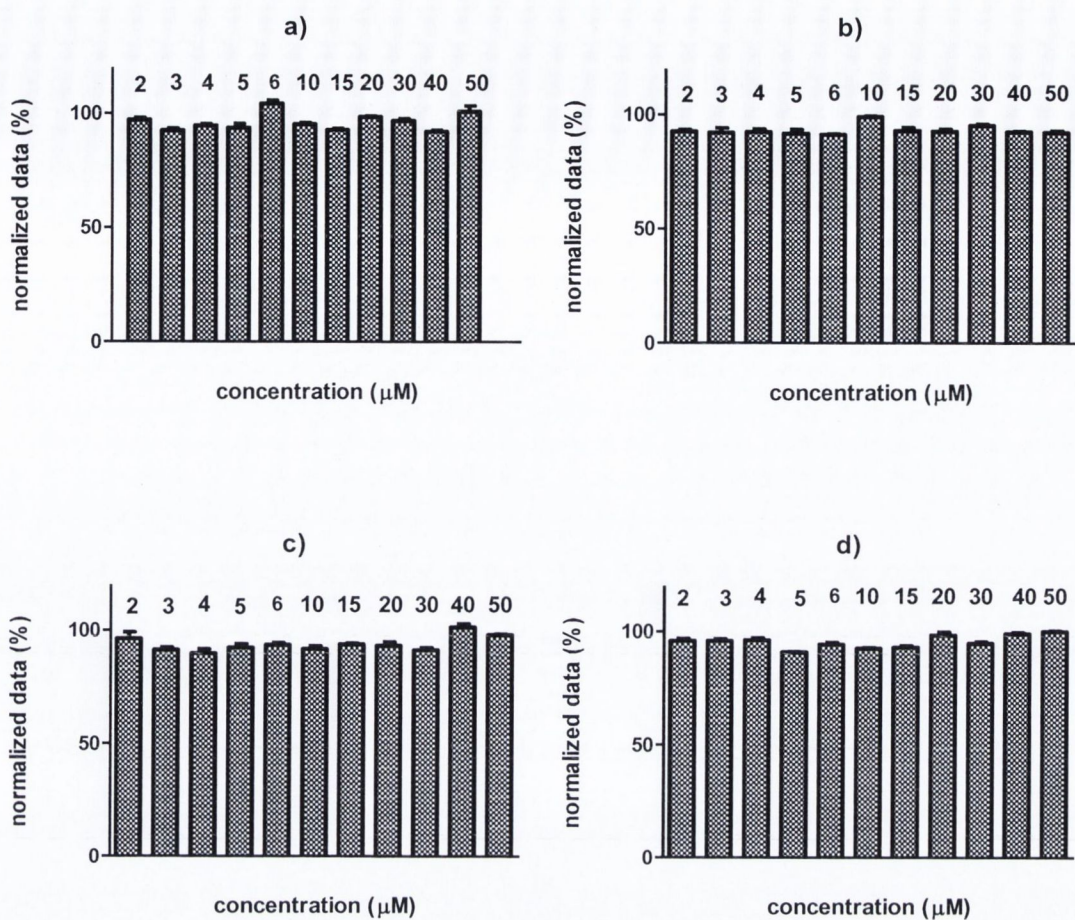


Figure 28. Cytotoxicity of porphyrin derivatives investigated by MTS assay in OE33 oesophageal cancer cells. The phototoxic effect was assessed in dependence of concentration of the photosensitiser and measured 24 h after illumination; a) compound 43; b) compound 44; c) compound 45; d) compound 46.

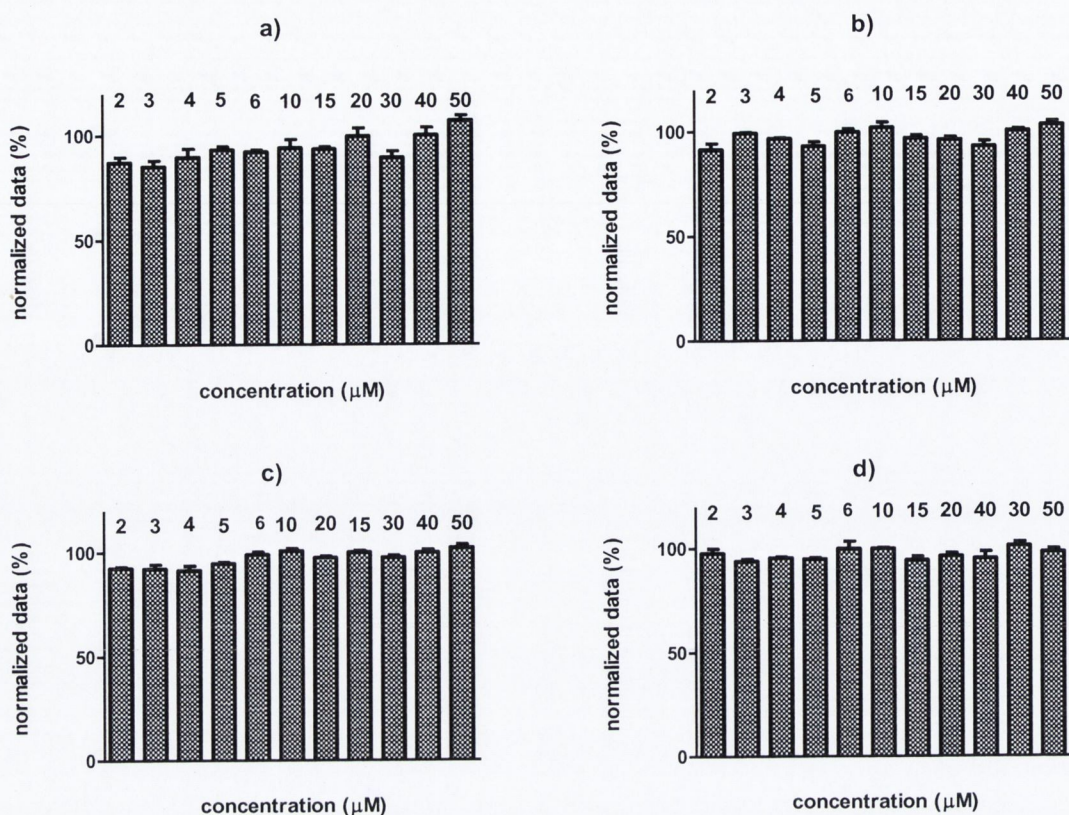


Figure 29. Cytotoxicity of porphyrin derivatives measured by MTS assay in OE33 oesophageal cancer cells. The cytotoxic effect was measured in dependence of concentration without illumination; a) compound **43**; b) compound **44**; c) compound **45**; d) compound **46**.

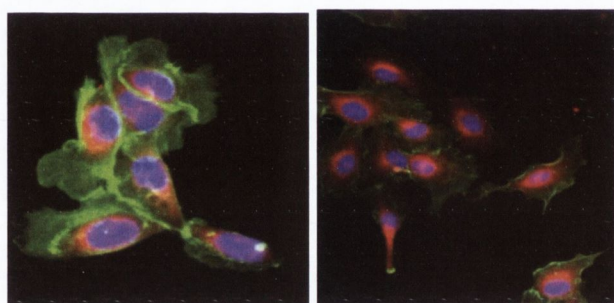
All bile acid conjugates demonstrated clear uptake by OE33 and SKGT-4 cell lines after 24 h incubation. Images obtained by InCell analyzer are present below in Figure 30.

Figure 30. Images collected from InCell analyzer. Images present OE33 and SKGT-4 cells treated with compounds number 43, 44, 45 and 46 after 24 h of incubation in the dark. Blue color presents nuclei stained by Hoechst; green color presents F-actin stained by phalloidin; red color presents photosensitiser.

OE33

SKGT-4

Compound 43:



Compound 44:

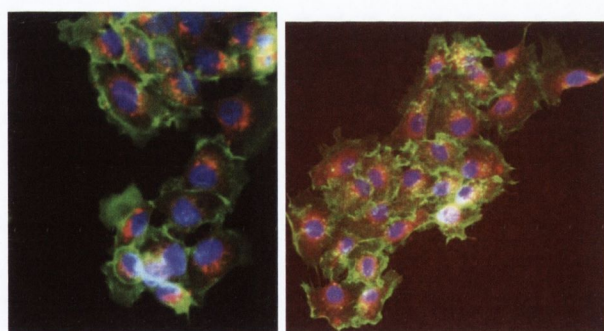
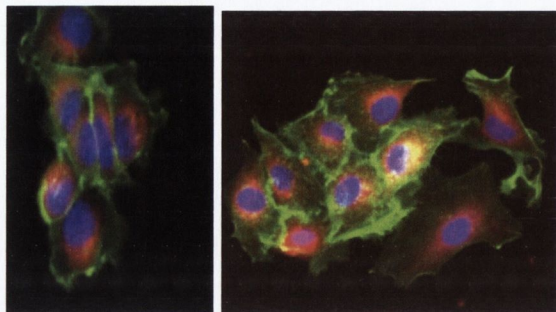
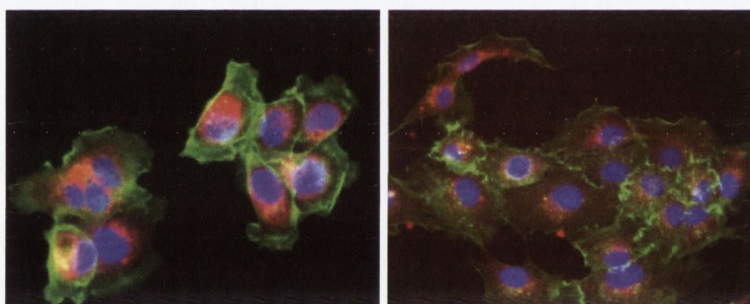


Figure 30. continued.

Compound 45:



Compound 46:



7.2.3 New drug candidates

New potential water soluble porphyrin photosensitisers were successfully synthesised in our chemistry lab and show promising results against OE33 and SKGT-4 oesophageal cancer cells. PDT effect and cellular uptake of the 3 novel photosensitisers were tested in concentrations range between 0.5 – 50 μM . Compounds were readily taken up by living cells and no visible toxicity in the dark were observed. Table 10 shows PDT effect of the compounds against OE33 and SKGT-4 cancer cell line.

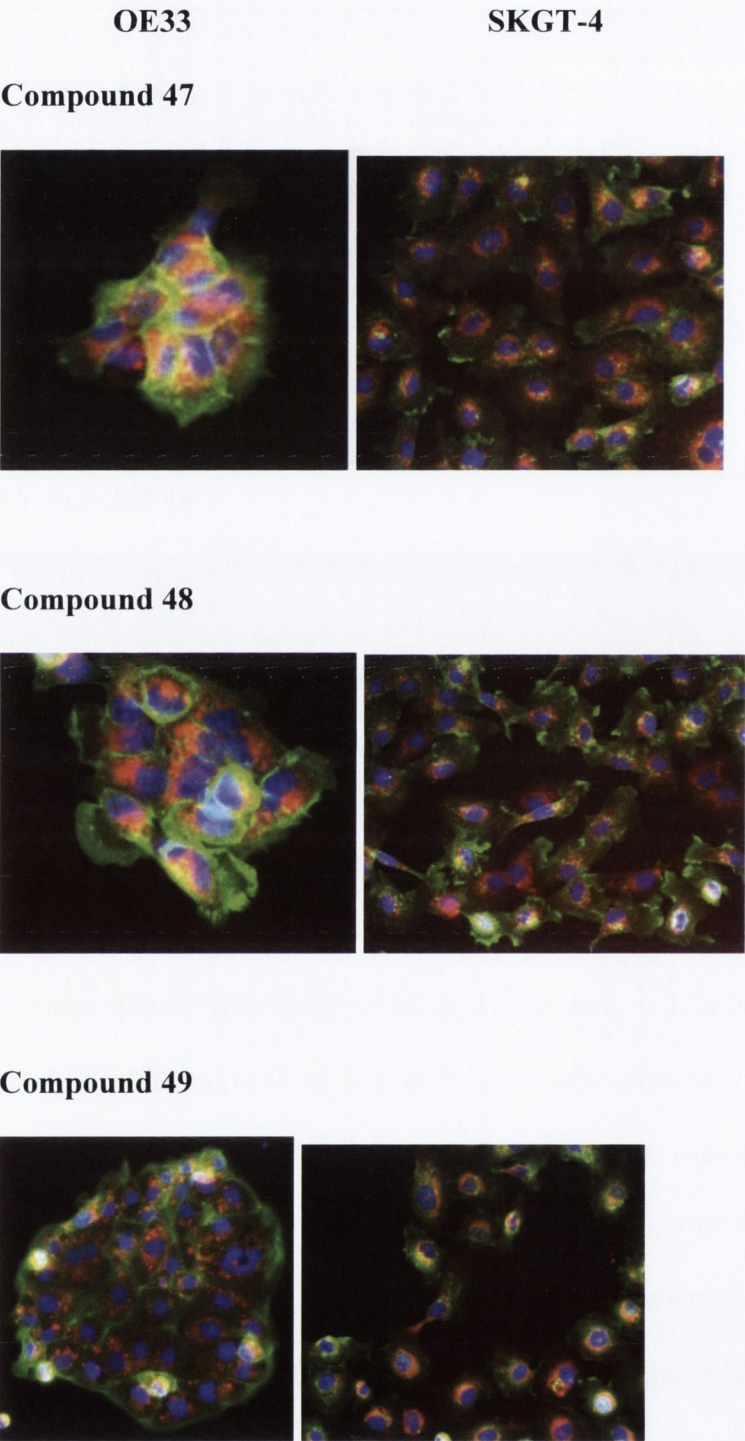
Table 10. PDT effect of new drug candidates using MTS assay.

	SKGT-4		OE33	
	IC ₅₀ light, μM ± SD	IC ₅₀ dark, μM ± SD	IC ₅₀ light μM ± SD	IC ₅₀ dark, μM ± SD
Compound 47	6.5 μM (±0.011)	42.5 μM (±0.022)	7 μM (±0.01)	43.5 μM (±0.023)
Compound 48	45 μM (±0.022)	> 50 μM	48 μM (±0.021)	> 50 μM
Compound 49	5.5 μM (±0.01)	28 μM (±0.02)	6 μM (±0.014)	24.5 μM (±0.016)

Novel photosensitisers demonstrated promising photodynamic effect against OE33 and SKGT-4 oesophageal cancer cells. IC₅₀ of compound **47**, after illumination with 1.7 mW cm⁻² light was 6.5 μM and 7 μM in SKGT-4 and OE33 cell lines, respectively, whilst IC₅₀ in dark was approximately 42 μM in SKGT-4 and 43 μM in OE33 cells. Compound **49** showed similar IC₅₀ after illumination which was approximately 5.5 μM in SKGT-4 cells and 6 μM in OE33 cells, whilst IC₅₀ in the absence of light was significantly lower resulted in 28 μM and 24.5 μM in SKGT-4 and OE33 cells, respectively. Compound **48** demonstrated meaningful lower cytotoxic, with IC₅₀ of 45 μM in SKGT-4 and 48 μM in OE33 cell lines after illumination and no significant dark toxicity up to 50 μM concentration.

All three photosensitisers demonstrated good uptake by OE33 and SKGT-4 cell lines after 24 h incubation. Images obtained by In Cell analyzer are present below in Figure 31.

Figure 31. Images collected from InCell analyzer. Images present OE33 and SKGT-4 cells treated with 3 posphorous porphyrin after 24 h of incubation. Blue color presents nuclei stained by Hoechst; green color presents F-actin stained by phalloidin; red color presents photosensitiser.



Numerous studies on *in vitro* cytotoxicity have been determined using different potential new photosensitisers in a various different experiments. However, Foscan still remain one of the most powerful, clinically approved photosensitiser [48]. Many studies on Temoporfin and related compounds, such as its polymer-conjugate *m*-THPC-PEG (also known as Fospeg®) and liposomal formulation Foslip®, have been reported demonstrating promising results.

Teiten *et al.* examined toxicity of *m*-THPC in MCF-7/DXR human breast cancer cells (doxorubicin resistant subline), expressing P-glycoprotein (P-gp) and the wild-type parental cell line, MCF-7. They did not observe any significant difference in intracellular accumulation of Foscan in two cell lines up to 3h incubation, whilst the photodynamic activity measured with MTT assay and using red laser light 650 nm was higher in MCF-7/DXR cell lines than in MCF-7. Cell survival was about 38.3 and 5 % for MCF-7 and MCF-7/DXR, respectively with energy rate 4.7 J cm^{-2} and $1.5 \text{ }\mu\text{M}$ concentration of PS [295]. In another study on MCF-7 human breast carcinoma cell line incubated 6 days with Foscan, PS demonstrated IC_{50} of $4.55 \text{ }\mu\text{g mL}^{-1}$ after illumination with 2.5 J cm^{-2} light using ATP cell viability assay and [296].

In human colon adenocarcinoma cell line (Colo 201 cells), *m*-THPC showed light toxicity of $0.45 \pm 0.15 \text{ }\mu\text{g mL}^{-1}$ after 24 h incubation and illumination with 3 J cm^{-2} light dose and no significant dark toxicity in concentrations ranged between 0.0625 and $4 \text{ }\mu\text{g mL}^{-1}$ measured with MTT assay [49] whilst in HT29 human colon adenocarcinoma cells, the IC_{50} of *m*-THPC resulted in $0.2 \text{ }\mu\text{g mL}^{-1}$ after 24 h incubation and irradiation with 10 J cm^{-2} of light dose [297].

Studies on ovarian cancer cell lines SK-OV3 incubated with *m*-THPC for 24 h, demonstrated the light toxicity of $0.9 \text{ }\mu\text{M}$, and $1.25 \text{ }\mu\text{M}$ after 2 h incubation and illumination with approximately 15 J cm^{-2} of light [298]. In murine leukemia cells L1210, *m*-THPC reached the IC_{50} of $0.88 \text{ }\mu\text{g mL}^{-1}$ after 2 h incubation and irradiation with light

514 nm and 25 J cm^{-2} energy, whilst $6 \mu\text{g mL}^{-1}$ in darkness performed with MTT reduction assay [299]. *m*-THPC incubated 18 h with Lewis lung carcinoma (LLC1) cells, demonstrated IC_{50} of 400 ng mL^{-1} at energy 60 mJ cm^{-2} or approximately 600 ng mL^{-1} at 30 mJ cm^{-2} assessed by the MTT assay [300].

In the cell line KYSE 510, derived from human oesophageal squamous carcinoma, standard solution of *m*-THPC showed a significant reduction of cellular viability at the $0.2 \mu\text{M}$ concentration after 24 h incubation and irradiation with 0.12 J cm^{-2} of light dose, and no cell survival at the concentration $1.25 \mu\text{M}$ assessed by MTS assay and the trypan blue exclusion test. In the absence of light, *m*-THPC was toxic to oesophageal carcinoma cells at a concentration above $1.75 \mu\text{M}$ [301].

m-THPC and *m*-THPC methoxyPEG₂₀₀₀ derivative (*m*-THPC MD) were tested for their dark and light toxicity in MCF-7 (human breast carcinoma) and V-79 (Chinese hamster lung fibroblast) cell lines. No dark toxicity up to $1 \mu\text{g mL}^{-1}$ was observed for both PS. Above that, the cells survival incubated with *m*-THPC decreased rapidly, whilst *m*-THPC-MD₂₀₀₀ demonstrated no dark toxicity up to $100 \mu\text{g mL}^{-1}$. Light toxicity for *m*-THPC methoxyPEG₂₀₀₀ derivative was 10 times lower than *m*-THPC using different energy doses [302].

The studies performed on human epidermoid carcinoma cell line A431 showed, that Foscan did not demonstrated dark toxicity up to $3.13 \mu\text{M}$ and decreases cell survival to $< 6 \%$ at concentration higher than $25 \mu\text{M}$. Fospeg on the other hand, did not show dark toxicity up to $6.25 \mu\text{M}$, followed by a decrease at higher concentration and reaching approximately 40% of cell survival at a concentration of $400 \mu\text{M}$ measured with MTT assay. When cells were incubated 20 h with $0.4 \mu\text{M}$ Foscan followed by illumination with 0.07 J cm^{-2} energy dose, exhibited a decrease in cellular survival to about 80% compared to untreated control. Increasing light dose resulted in decreasing cell survival to $< 5\%$. Cells incubated with Fospeg at the same concentration and illuminated with 0.07 J cm^{-2}

light resulted in a decrease of cellular survival to about 90 % with decreasing up to 10% with increasing light dose [303].

Compagnin *et al.* studied dark toxicity of *m*-THPC loaded in DPPC DPPG liposomes stabilized with poly(ethylene glycol) of different length and density and compared with *m*-THPC delivered as a standard solution in CCD-34Lu human normal fibroblasts and A549 lung cancer cells. Cells were incubated 24 h with the PS and cellular availability was measured with MTS assay. All Fospeg formulations showed lower phototoxicity than the standard solution and exhibited decreasing phototoxicity with increasing PEGylation. No dark toxicity was observed with 8% of PEG up to the 5 μ M concentration of *m*-THPC in both cell lines [269]. Foscan® and Foslip® investigated in two biliary cancer cell lines, GBC, gall bladder cancer and BDC, bile duct cancer cells showed, that Foscan above 2 μ g mL⁻¹ concentration, was toxic to the both cell lines after 20 h of incubation in the absence of light. Foslip on the other hand, showed dark toxicity at the 100 μ g mL⁻¹ concentration. IC₅₀ for standard solution after irradiation with 1.5 J cm⁻² of light was approximately 45 and 44 ng mL⁻¹ for GBC and BDC cell lines, respectively, whilst for liposomal formulation showed 61 and 56 ng mL⁻¹ for GBC and BDC cell lines, respectively [205]. Liposomal formulation of Foscan, investigated in the metastatic 143B osteosarcoma cell lines showed similar cytotoxicity, after illumination with 2.5 J cm⁻² of light dose and 5 h incubation was 047 μ g mL⁻¹. Dark toxicity, however, was observed at a concentration of 2.5 μ g mL⁻¹ [304].

Vrouenraets *et al.* developed a new generation photosensitisers by adding the 3 different monoclonal antibodies (a humanized mAb BIWA 4, which recognizes the v6 domain of CD44 splice variants; IgG1 mAb E48, recognizing a 16-22 kDa glycosylphosphatidylinositol (GPI)-anchored surface antigen; and IgG2a mAb 425 recognizing an epitope localized on the external domain of the epidermal growth factor receptor (EGFR)) to the hydrophobic *m*-THPC and to the hydrophilic aluminium(III)

phthalocyanine tetrasulfonate (AlPcS₄). They studied photodynamic activity *in vitro* with 5 different SCC cell lines, UM-SCC-22A, UM-SCC-22B, OE, A431 and SCV-7 using 25 J cm⁻² as a illumination light dose and the sulforhodamine B (SRB) assay for toxicity determination.

Unlike free AlPcS₄ with the IC₅₀>700 nM, mAb conjugations demonstrated a high phototoxicity in all 5 cell lines with lower IC₅₀, especially the mAb BIWA 4 conjugate with IC₅₀ in range between 0.006-5.4 nM for different cell lines. However, Temoporfin with antibody modification was hardly effective and free photosensitiser was more potent for each cell line [305].

Very recently Aicher *et al.* synthesised novel $\hat{\alpha}$ -functionalized derivatives of the Temoporfin by nucleophilic addition reaction to corresponding deketo chlorine. The phototoxicity of the new photosensitisers evaluated in human colon adenocarcinoma cells HT-29 showed similar level of activity as compared to *m*-THPC after 24 h incubation and irradiation with 50 J cm⁻² of light dose. None of the new PS exhibited dark toxicity. They possessed comparatively strong absorption in the red spectrum and singlet oxygen quantum yield as Temoporfin, however, novel $\hat{\alpha}$ -functionalized derivatives, demonstrated increased chemical stability [306].

Four new phthalocyanine peptide conjugates photosensitisers were developed recently by Ongarora *et al.* They were designed to target the epidermal growth factor (EGFR) and conjugated with two peptide ligands, EGFR-L1 and EGFR-L2 bearing 6 and 13 amino acids residues, respectively and evaluated in human squamous cell carcinoma HEp2, human epidermoid carcinoma A431, human colorectal adenocarcinoma HT-29, and kidney Vero (negative control) cells. All formulations were non toxic up to concentration 100 μ M in all cell lines, both in the dark and upon light activation 1 J cm⁻². Only one compound, conjugated with EGFR-L1 peptide, showed toxicity to A431, HEp2 and Vero cell at 15.8

μM , $17.0\ \mu\text{M}$ and $47.0\ \mu\text{M}$ respectively. None of the PCs were toxic to the human HT-29 cell [307].

The library of glycosylated porphyrins (glycoporphyrins) was evaluated for their photodynamic therapy (PDT) activity against the oesophageal squamous-cell carcinoma cell line OE21. The number and relative orientation of the carbohydrates was varied and a bis-modified glycoporphyrin with two different types of carbohydrate were newly synthesised. However, no significant decrease in cells survival treated with any of the tested glycoporphyrins was observed and no difference between illuminated and dark cultures was noticed when MTT assay were used [308].

Three new amphiphilic porphyrin derivatives, containing a guanidine, a biguanidine or an MLS peptide, were tested toward human carcinoma HEP2 cells. All tested compounds showed low dark toxicity, $84\ \mu\text{M}$, $86\ \mu$ and $>100\ \mu\text{M}$ for guanidinium-porphyrin, MLS peptide porphyrin and biguanidinium-porphyrin respectively, whilst the toxicity after irradiation with $1\ \text{J cm}^{-2}$ of light dose and 24 h incubation with photosensitisers were $4.8\ \mu\text{M}$, $8.2\ \mu\text{M}$ and $9.8\ \mu\text{M}$ for guanidine-, biguanidine- and MLS peptide porpyrin respectively [309].

However, there is no single standard method for measuring phototoxicity of a photosensitiser. There are a large number of factors which can determine the efficiency of PDT such as concentration of the photosensitiser, time of incubation, time and intensity of irradiation, the cell type used and other conditions [310].

7.3 Conclusions

Numerous novel compounds have been developed to find ideal photosensitiser and overcome drawbacks associated with currently existing drugs. The main research for improving the activity and selectivity of the photosensitisers focus on conjugation with ligands which specifically targets the PS to the cancer cells and minimize accumulation in healthy tissues. Another challenge to overcome is water solubility of many photosensitising drugs and their tendency to aggregate under physiological conditions. Thus, new delivery methods and formulations have been adapted. Many offers significant potential and possibilities, however, they not always are benefit and not always demonstrate more potent results than non modified compounds and most of the bioconjugates, like for example antibodies are expensive, require a long time production and most important, they might cause immune reaction. Our study show that bile acid porphyrin conjugates which were synthesised specifically for PDT, due to proven ability of certain bile acids to be selectively taken up by tumors and induce apoptosis [293, 294], after all show no phototoxicity against oesophageal cancer cell lines. However, cytotoxicity investigation of series newly synthesised porphyrin derivatives as a potential novel photosensitisers for photodynamic therapy led to found that 3 of them show promising results against OE33 and SKGT-4 oesophageal cancer cells. Novel photosensitising agents which are not ligand conjugated demonstrate water solubility, good cell uptake, significant toxicity after irradiation with light and minimal dark cytotoxicity which make them a potential, inexpensive candidates for photodynamic therapy competitive to other 3rd generation photosensitisers.

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