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Transferrin conjugation does not increase the efficiency of liposomal Foscan during *in vitro* photodynamic therapy of oesophageal cancer

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Abstract

Photodynamic therapy (PDT) is based on the delivery of photocytotoxic agents to a target tissue, followed by irradiation. In order to increase the efficiency of PDT in oesophageal cancer therapy, polyethylene glycol (PEG)-grafted, transferrin (Tf)-conjugated liposome formulations of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (Foscan), a second-generation photosensitiser, were prepared. 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 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. Surprisingly, delivering Foscan by transferrin-conjugated PEG-liposomes to oesophageal cancer cells did not improve the photocytotoxicity or the intracellular accumulation of Foscan when compared to unmodified liposomes or indeed free photosensitiser. Tf-targeted drugs and drug delivery systems have shown improvedthe therapy of many cancers. Our study, however, did not corroborate these findings. If this is due to the tumour type, the choice of *in vitro* model or the delivery systems remains to be confirmed.

Key words: Drug targeting; *m*THPC; High content analysis; Encapsulation; Cytotoxicity.

Abbreviations: $AlPcS_4$ – aluminium phthalocyanine tetrasulfonate; DPPC – 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG – 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt); DSPE-mPEG – distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt); DSPE-PEG-COOH – 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene

glycol)-2000] (ammonium salt); EDC – *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; EPR – enhanced permeability and retention effect; *m*THPC – 5,10,15,20-tetra-(*m*-hydroxyphenyl)chlorin; PdI – polydispersity index; PDT – photodynamic therapy; PEG – polyethylene glycol; PS – photosensitiser; RES – reticuloendothelial system; S-NHS – *N*-hydroxysulfosuccinimide; Tf – transferrin; TfR – transferrin receptor (CD71).

Introduction

Photodynamic therapy (PDT) has been developed as a treatment for cancer and non-malignant diseases such as macular degeneration. It is based on the photochemical reaction between a photosensitising agent (PS), most often porphyrins (Kessel and Dougherty, 1999; Pandey and Zheng, 2000), and light of an appropriate dose and wavelength. The result is a photochemical conversion of molecular oxygen ($^3O_2$) into singlet oxygen ($^1O_2$), a key cytotoxic agent that damages cells via apoptosis or necrosis (MacDonald and Dougherty, 2001; Plaetzer et al., 2009).

One of the most promising second-generation photosensitisers is 5,10,15,20-tetra-(*m*-hydroxyphenyl)chlorin (*m*THPC, Foscan®), which was approved in 2002 for the palliative treatment of head and neck cancer. Foscan is activated at a wavelength of 652 nm which enables a light penetration of at least 1 cm and has a high quantum yield of singlet oxygen generation. (Bonnett et al., 1989; Senge and Brandt, 2011; Senge, 2012). Although Foscan is one of the most effective compounds for PDT of cancer lesions, including oesophageal cancer (Javaid et al., 2002; Lovat et al., 2005), it still has a number of disadvantages, *e.g.*, prolonged skin photosensitivity. The interval between injection and light administration is several days, and patients are restricted to limited light exposures during that time. Foscan is also prone to form aggregates, leading to low levels of the substance in the target tissue (Leung et al., 2002). On-going research is hence, focused on the development of new PS with optimised photophysicochemical and pharmacological properties (Castano et al., 2004; Nyman and Hynninen, 2004) and indeed the optimisation of formulation and delivery strategies for existing compounds (Paszko et al., 2011).

One means to improve drug delivery is the use of submicron liposomal formulations. These can accumulate in tumour tissues via the enhanced permeability and retention (EPR) effect as a result of their size (Maeda et al., 2000). 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 (Torchilin, 2005). 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 (Kepczynski et al., 2006).

To further enhance the therapeutic effect and to specifically deliver drugs to the tumour site, targeted liposomes can be prepared, using surface moieties recognising cell surface antigens or receptors primarily or over-expressed on cancer cells (Moghimi et al., 2001; Torchilin, 2006). One of the most widely used ligands for anticancer delivery is transferrin (Anabousi et al., 2006; Li et al., 2009), which is exploiting receptor mediated endocytosis followed by release of the drug into the cell (van Renswoude et al., 1982). The transferrin receptor (TfR) is ubiquitously expressed on healthy cells, however, many cancers exhibit vastly increased quantities of TfR (Daniels et al., 2006). Moreover, the transferrin receptor shows a high turnover on tumour cells due to their increased iron consumption (Singh, 1999). Whilst PDT can be used for many malignancies, we are mainly interested in oesophageal adenocarcinoma, one the fastest increasing cancer types with poor prognosis (Pohl and Welch, 2005). The major risk factor is Barrett's metaplasia which develops as a consequence of chronic gastro-oesophageal reflux disease (GORD) (Falk, 2001). Interestingly, the progression of Barrett's metaplasia to adenocarcinoma, correlates well with expression levels of iron import proteins including TfR (Boult et al., 2008).. New therapies have been developed including endoscopic resection with oesophageal preservation, which minimise the morbidity and mortality of traditional forms of oesophagectomy (Haringsma, 2002). PDT is also used in oesophageal cancer treatment, but irrespective of the photosensitiser used, still has drawbacks (Javaid et al., 2002; Kelty et al., 2002; Lovat et al., 2005).

In order to improve the targeting of established PS such as Foscan we aimed to develop plain and transferrin-modified liposomal formulations of the drug. Transferrin was conjugated to PEGylated liposomes and the light/dark cytotoxic effect of the systems was determined and compared with pure drug as well as plain and non-Tf-modified PEGylated liposomes.

Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (DPPG), distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-mPEG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}-COOH) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-5000] (ammonium salt) (DSPE-PEG_{5000}-COOH) was purchased from Nanocs (Boston, MA, USA). All lipids had a purities ≥99% and were stored at -20°C. Human holo-transferrin (Tf) and FITC-labelled phalloidin were obtained from Sigma-Aldrich (Dublin, Ireland). *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 cell line 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 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). Foscan was prepared as described previously (Berenbaum and Bonnett, 1982).

Experimental Methods

Liposome preparation

Liposomes were prepared using the lipid film method. Conventional (*i.e.*, non-PEGylated) liposomes were composed of 18 mg/ml of dipalmitoylphosphatidylcholine (DPPC) and 2 mg/ml of dipalmitoylphosphatidylglycerol (DPPG). For preparation of PEGylated liposomes, 6 mol% of distearoylphosphatidylethanolamine-metoxy-(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 1.5 mg/ml. The respective lipids were mixed and dissolved in chloroform. Foscan was encapsulated by adding the relevant volume of stock solution in methanol 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 PBS (pH 7.4) under shaking above the phase transition temperature of the lipids (Tc DPPC: 55ºC). The liposomal suspensions were then subjected to size reduction at ≥55ºC 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. Non-encapsulated drug was separated by gel filtration on desalting columns (Econo-Pac 10DG Desalting Columns, BioRad, CA, USA) using PBS of pH 7.4 as elution buffer. Empty liposomes were prepared using the same method with corresponding concentrations of lipids.

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 (Gaspar et al., 2012). Briefly, to 1 ml of liposomes (lipid concentration: 10 μmol/ml), 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 4ºC. The unbound protein was separated from the liposomes by ultracentrifugation at 250,000 *g* for 3 h (15ºC) in a Beckman LM-80 ultracentrifuge. After ultracentrifugation, the pelleted liposomes were re-suspended with buffer and Tf binding efficiency was determined using a micro BCA protein assay according to the manufacturer's instructions.

Physicochemical characterisation of liposomes

Liposomes were characterised in terms of mean size, ζ-potential, lipid concentration, Foscan content and Tf conjugation efficiency. Mean size and polydispersity index (PdI) were measured by laser light scattering and ζ-potential by laser Doppler electrophoresis using a ZetaSizer, Nano Series (Malvern Instruments, Malvern, UK), after appropriate dilution of the liposomal suspensions in buffer at 25ºC. The PdI ranges from 0-1 with the higher value representing the highest degree of heterogeneity. The phospholipid content of liposomal suspensions was determined using Stewart's assay (Stewart, 1980). The concentration of encapsulated Foscan was quantified via a fluorimetric assay. Triton X-100 was added to liposomal samples to a final concentration of 1% (v/v). Samples were measured in a plate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 405 and 650 nm, respectively. Samples were diluted with PBS when necessary. Tf conjugated to liposomes was quantified according to procedure applied in previous studies (Derycke and de Witte, 2002; Anabousi et al., 2005). 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 (Smith et al., 1985) and BSA as a standard. The absorbance was measured at 595 nm in a plate reader (FLUOstar OPTIMA). The coupling efficiency was calculated as µg Tf/µmol PL.

Cell culture

Human oesophageal squamous cell carcinoma OE21 cells were cultured in RPMI 1640 medium supplemented with 10% inactivated foetal bovine serum and 1% penicillin/streptomycin. Cells of passage numbers 10-35 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.

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. Cells 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 95ºC 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).

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.

Phototoxicity assay

The cytotoxicity of Foscan-loaded liposomal formulations was determined in OE21 cell lines using an MTT assay (Tim, 1983). OE21 cells were seeded at 3000 cells/well into 96-well plates and incubated at 37°C in a 5% CO_2 incubator for 48 h, in order to provide sufficient time for the cells to attach and resume growth. Tryptan blue was used when cells were counted using a haematocytometer. *m*THPC solution was prepared by dissolving the photosensitiser in ethanol:propylene glycol (60:40) (v/v). Free Foscan and liposomal formulations of the photosensitiser were diluted with medium to obtain final concentrations of 0.5, 1 and 2 μM, and were administered to the cells. Control wells were treated with equivalent volumes of liposome-free medium, as well as with empty liposomes. Cells were incubated with non-encapsulated PS or liposomes for 2, 4, 8, 12 and 24 h at 37°C in a 5% CO_2 incubator in the dark. Following this, 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 $mWcm^2$ (Feest, 2009), the second plate was kept in the dark. Twenty-four hours post irradiation, cell survival was assessed by MTT assay according to manufacturer's instruction. The resulting absorbance, indicative of remaining cellular activity, was read at 570 nm using a Wallac Victor2 plate reader (Perking Elmer, Singapore).

Intracellular uptake by high content analysis

OE21 cells were grown and treated exactly as described above minus the MTT 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× 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 700 nm). 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.

Statistical analysis

Each experiment was repeated a minimum of three times. Data are presented as mean±SEM (*n*), where *n* is the number of observations. Differences among more than two group means were determined by one-way analyses of variance (ANOVA). Averages and SEM were plotted using Graphpad Prism version 5.0 (GraphPad Software, USA).

Results and Discussion

Plain and PEGylated liposomal formulations of Foscan were prepared by conventional lipid film method using dipalmitylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) lipids according to a method adapted from Kuntsche *et al.* (Kuntsche et al., 2010). Foscan was encapsulated at a concentration of 1.5 mg/ml. The encapsulation efficiency, defined as the ratio of $[(Fos/Lip)_f/(Fos/Lip)_i]$ in percent, where $(Fos/Lip)_f$ was the final Foscan to lipid ratio and $(Fos/Lip)_i$ was the initial Foscan to lipid ratio, was ≥ 90% in all cases. Physicochemical properties of the thus obtained liposomes are listed in Table 1. 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. The size of liposomes consistently ranged from 100 nm to 150 nm, both before and after gel filtration. Polydispersity indices (PdI) were generally ≤ 0.22.

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 (Torchilin, 2005). The addition of moieties such as vitamins, glycoproteins, peptides, oligonucleotide aptamers and antibodies enhances tumour delivery and reduces toxicity to healthy tissues (Torchilin, 2007). 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 (Anabousi et al., 2005). In this context, the covalent modification using polyethylene glycol (PEG)-linked lipid residues has become a promising strategy (Derycke and de Witte, 2002; Gijsens et al., 2002; Derycke et al., 2004; Anabousi et al., 2005; Bakowsky et al., 2008). In order to couple transferrin to liposomes, we used the formation of an amide bound between the carboxylic groups of the linker lipid, DSPE-PEG-COOH, and free amino groups of the transferrin in the presence of a water-soluble carbodiimide (EDC). 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 (Ishida et al., 2001; Anabousi et al., 2005). 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 2. No significant changes in any of the measured parameters were observed after conjugation of Tf. Approximately 40 μg Tf were conjugated per 1 μmol of phospholipids, which corresponded to a coupling efficiency of 31%.

Functional expression of TfR has previously been demonstrated in the oesophageal adenocarcinoma cell lines, OE33 and SEG-1 (Boult et al., 2008). To assess if the OE21 cells used in our study also express TfR, Western blot analysis and immunocytochemical staining were performed.Figure 1A shows TfR expression (*red*) in OE21 cells as obtained by confocal laser scanning microscopy. In Figure 1B 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 1C) 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.

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 (data not shown) indicating that the observed cytotoxic effects were only caused by the photosensitiser. Liposomal formulations of Foscan showed a dose-dependent anti-proliferating effect on OE21 cells after irradiation (Figure 2). Non-encapsulated Foscan also exhibited a photocytotoxicity, although at slightly lower levels

than corresponding concentrations of plain and PEGylated liposomes. This effect was generally more pronounced at the highest concentration (*i.e.*, 2 μM). Surprisingly, transferrin-modified liposomes, showed a profoundly lower cytotoxic effect upon irradiation, even after 24 h of incubation. No dark toxicity was observed in any of the formulations used [data not shown]. We and others have previously shown that Tf modification of drugs or drug delivery systems can improve their efficiency in cancer therapy (Derycke et al., 2004; Gaspar et al., 2012). 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 (Anabousi et al., 2006; Gaspar et al., 2012), hepatocellular carcinoma (Li et al., 2009), leukaemia (Wu et al., 2007) and metastatic mammary carcinoma (Lopez-Barcons et al., 2005). 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 (Derycke and de Witte, 2002; Gijsens et al., 2002; Derycke et al., 2004). Whilst data obtained with AlPcS4 were quite promising (Gijsens et al., 2002; Derycke et al., 2004), the liposomally encapsulated hypericin was much less effective (Derycke and de Witte, 2002). It was suggested that significant amounts of hypericin were leaking from the PEGylated liposomes, thus, reducing their efficiency (Derycke and de Witte, 2002).

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 modification led to lower intracellular levels, when compared to plain and PEGylated liposomes (Figure3.

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-PEG_{2000}-COOH and DSPE-PEG_{5000}-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, Foscan area within OE21 cells measured by high content analysis (Figure , 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 which do not show a fluorescence signal.

Our data demonstrated that Tf-modified liposomal formulations of Foscan did not improve 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.* 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 levelled off. The

authors suggest 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 4 the amounts of photosensitiser within the cells were 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 4 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. In our studies, 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 (Hatakeyama et al., 2004). 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, Sakeguchi 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 (Sakaguchi et al., 2008). It might hence be possible that our vesicles were either too large to be internalised or that TfRs expressed in the OE21 cell line do no 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 (van Rooy et al., 2011).

Conclusions

This is the first study reporting the effect of Tf-targeted liposomes on an oesophageal cancer cell line. Whilst Tf-modification of drugs and drug delivery systems has in the past led to very promising results in other cancer types, our results indicated that in this specific case, targeting of the TfR did not improve the efficacy of the formulation. If the observation is due to the nature of the cell type (*i.e.*, TfR recycling) or indeed due to issues with the formulation (*i.e.*, size, leakage of the payload or transformation form holo- to apo-transferrin) needs to be determined.

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Table 1. Physicochemical parameters of conventional and PEGylated liposomes. Data represent means ± SEM (n = 3).

Lipid composition	**Size (nm)**		**Polydispersity index (PdI)**		**Zeta potential (mV)**	
	Before gel filtration	*After gel filtration*	*Before gel filtration*	*After gel filtration*	*Before gel filtration*	*After gel filtration*
DPPC:DPPG	142±25	122±16	0.222±0.020	0.170±0.080	-14.1±0.8	-15.2±1.5
DPPC:DPPG: mPEG	131±12	130±5	0.169±0.050	0.152±0.070	-2.7±0.5	-2.8±0.5

Table 2. Physicochemical parameters of transferrin-modified liposomes. Data represent means ± SEM (n = 3).

DPPC:DPPG:cPEG	**Before Tf conjugation**	**After Tf conjugation**
Size (nm)	137±18	126±10
Polydispersity index (PdI)	0.155±0.100	0.149±0.090
Zeta Potential (mV)	-10.5±1.1	-11.5±1.2
Coupling efficiency (%)	125 µg Tf/1 µmol PL (n/a)*	31.0±4.2

*125 µg Tf/1 µmol PL – the initial amount of transferrin added to the liposomal formulation

Figure Legends

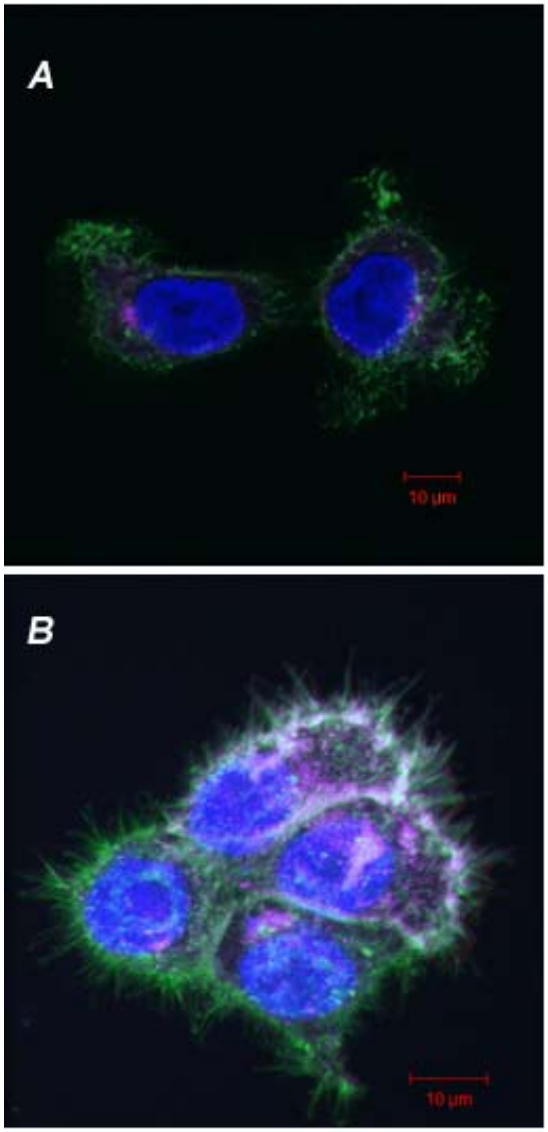
Figure 1. Expression of transferrin receptor (CD71) in OE21 human oesophageal tumour cells. A&B) Immunocytochemistry for transferrin receptor (red). F-actin was stained with FITC-labelled phalloidin (green) and cell nuclei were counterstained with Hoechst 33342 (blue). Shown are untreated cells (A) as 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).

Figure 2. Cytotoxicity of free and liposomally encapsulated Foscan investigated 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 cPEG 2000 Tf and DPPC DPPG cPEG 5000 Tf, respectively). The phototoxic effect was assessed in dependence of incubation time and measured 24 h after illumination; ■ – 0.5 μM; ▲ – 1 μM; ● – 2 μM; Results are expressed as means ± SEM (n = 3).

Figure 3. 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 cPEG 2000 Tf and DPPC DPPG cPEG 5000 Tf, respectively). Concentrations used were: ■ – 0.5 μM; ▲ – 1 μM; ● – 2 μM. Results are expressed as means ± SEM (n = 3).

Figure 4. 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 cPEG 2000 Tf and DPPC DPPG cPEG 5000 Tf, respectively). Concentrations used were: ■ – 0.5 μM; ▲ – 1 μM; ● – 2 μM. Results are expressed as means ± SEM (n = 3).

Figure 1.



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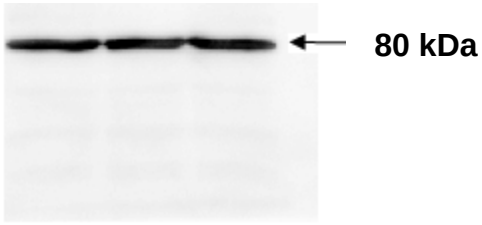


Figure 2.

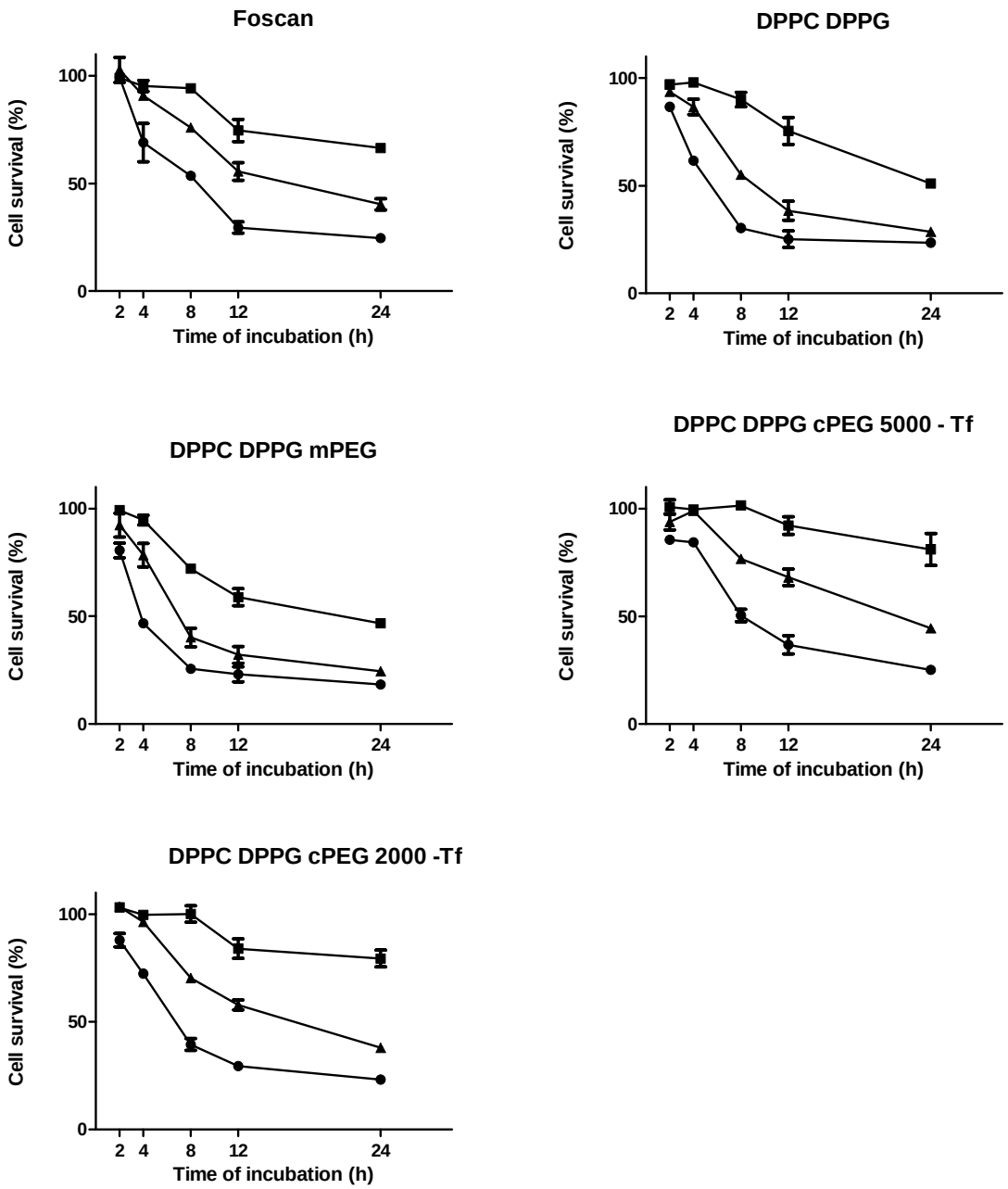

Foscan
DPPC DPPG
DPPC DPPG mPEG
DPPC DPPG cPEG 5000 - Tf
DPPC DPPG cPEG 2000 -Tf
Cell survival (%)
Time of incubation (h)
0
50
100
2
4
8
12
24

Figure3.

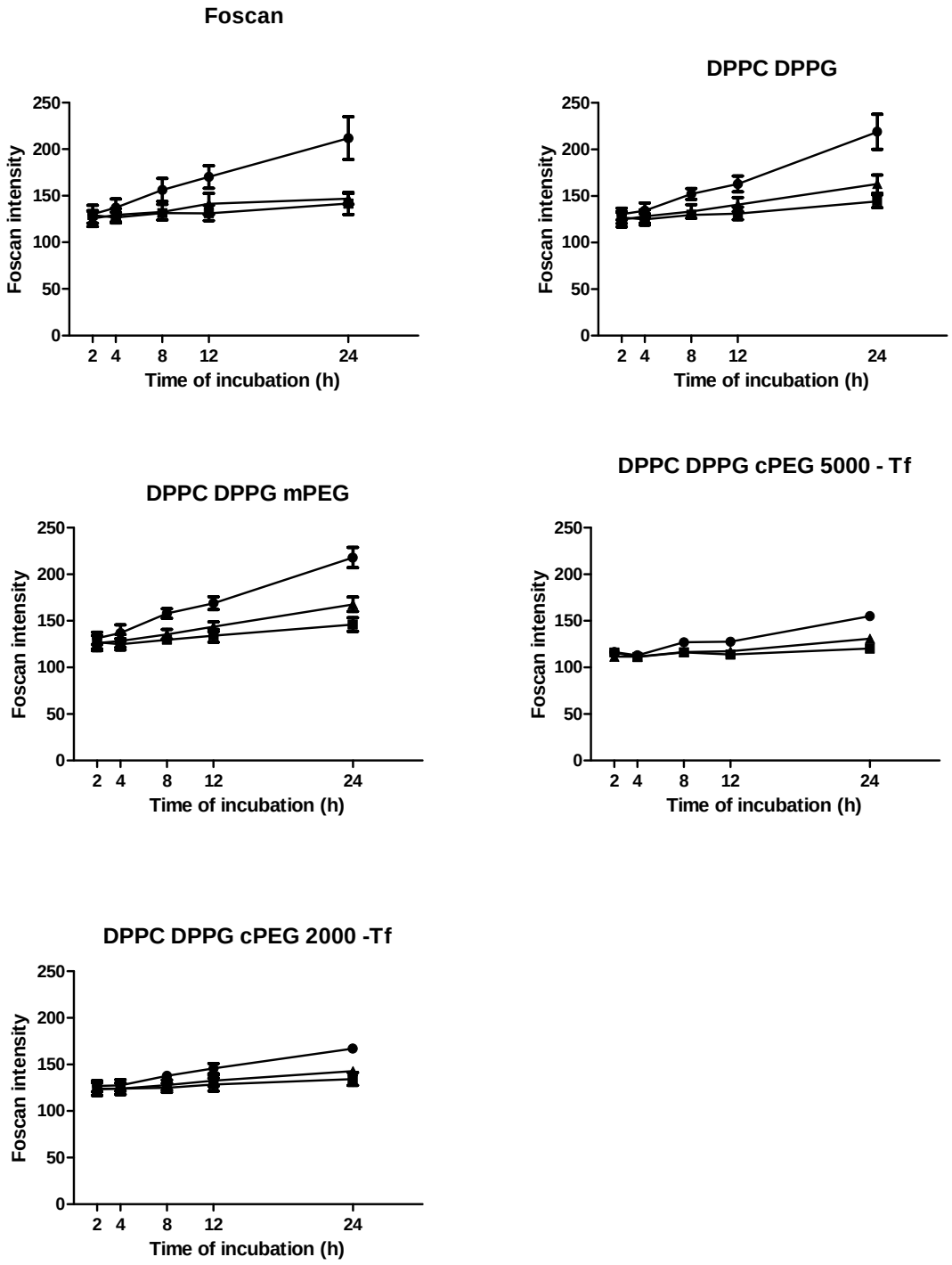
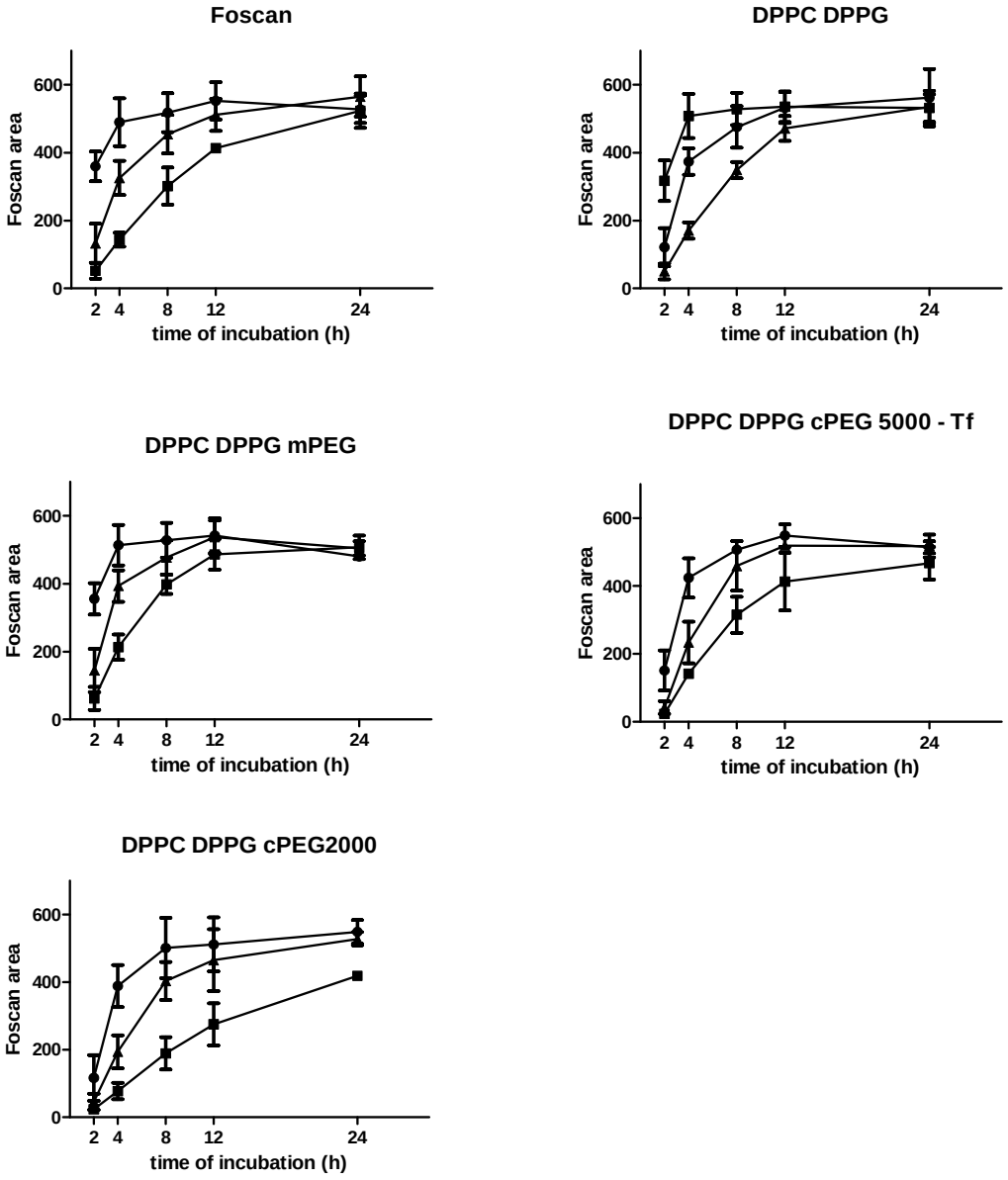

Foscan
Foscan intensity
Time of incubation (h)
DPPC DPPG
DPPC DPPG mPEG
DPPC DPPG cPEG 5000 - Tf
DPPC DPPG cPEG 2000 -Tf
0
50
100
150
200
250
2
4
8
12
24

Figure 4.


Foscan
DPPC DPPG
DPPC DPPG mPEG
DPPC DPPG cPEG 5000 - Tf
DPPC DPPG cPEG2000
Foscan area
0
200
400
600
2 4 8 12 24
time of incubation (h)