Modifying Polyethylene Glycol Effects Liposome Relaxivity and Enhances Tumour Cell Uptake for Drug Delivery

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Introduction: Liposomes are versatile tools that enable a host of applications ranging from drug and gene delivery to imaging agents. Bimodal liposomal agents for MR have utilized Gd(III) chelates conjugated to hydrophobic tail groups so that the chelate becomes part of the lipid bilayer as well as fluorescent probes[1]. These bimodal liposomes are of great interest in cancer research as they allow for the monitoring of drug delivery to tumour tissue. For *in vivo* applications liposomes are formulated with high concentrations of long polyethylene glycol (PEG) chains (7-10%) to minimize clearance by the reticuloendothelial system (RES)[2], thus increasing circulation time and accumulation within tumour tissue by the enhanced permeability and retention (EPR) effect[3]. However, highly PEGylated liposome formulations, especially with long PEG chains have limited uptake compared to targeted liposomes[4]. PEG may also hinder targeting moieties or effect relaxivity. Therefore, optimisation of passive bimodal liposomes for drug delivery requires modification of PEG within the lipid bilayer. In this study, we assess the MR functionality of bimodal liposome formulations containing novel lipid formulations of both Gd(III) chelated lipid, as well as modified short PEG chains to get a better understanding of how liposomes may be modified to enhance both MR relaxivity as well as cell uptake for drug delivery in cellular models of cancer.

Methods: As part of a collaboration, the UCL Department of Chemistry has designed a range of lipids that carry novel PEG chains, to A) enhance cell uptake but still provide protection from enzymatic degradation, and B) carry Gd(III) chelating and fluorescent probes, to allow imaging. Multimodal liposomes (Figure 1) can then be formulated using these lipids to allow either direct imaging of a liposomal contrast agent or as in this case, cellular labelling studies.

Liposome formulations: Appropriate amounts of each stock lipid (50% PEG lipid [either ME42[5] or CH300[6]], 30% Gd-lipid [NM65], 19% DOTMA, and 1% Fluorescein labelled Lipid [Invitrogen]) was added to a round-bottom flask and the solution evaporated to produce a thin film, which was then re-hydrated in sterile water and the solution sonicated to produce liposomes. For relaxivity values a range of concentrations (1-0.0625 mM) NM65 was compared to Gd.DOTA (Dotarem®) as a stock solution as well as within liposome formulation.

In vitro experiments: Hela, human epithelial cervical cancer cells, were plated at 5×10^5 in 6 well plates 24 hours prior to adding liposomes. The cells were washed with PBS and 2 ml of serum free media to which 100μl of liposome preparation (1-0.0625mM) or water was added. Cells were either incubated at 4°C (fridge) or 37°C (incubator) for 24 hours. After incubation, the cells were washed with PBS and fluorescence imaged using an inverted Zeiss Microscope. The cells were then harvested using trypsin-EDTA and either taken for FACS scanning using a FACS Calibre to assess the percentage uptake, or pelleted and scanned by MRI to assess T_1 changes. Cells were also re-plated in 6 well plates and the longevity of liposome uptake was assessed by fluorescence microscopy and FACS over a 7 day period (37°C incubation only).

Relaxivity and T_1 Measurements: Phantoms were placed into a quadrature ^1H volume coil and imaged using a 9.4T Varian VNMRS 20 cm horizontal-bore system (Varian Inc. Palo Alto, CA, USA). A fast spin-echo sequence with the following parameters was used to assess T_1 relaxation, TR = 200, 350, 500, 700, 1000, 3000, 5000, 7000, 10000, 15000 ms, TE = 17 ms, FOV = 40 x 40 cm, averages = 4: matrix size = 256 x 128: and a 1.0 mm thickness. The signal intensities were taken using a region of interest (ROI) on ImageJ and T_1 were estimated in each sample (cell pellets). The longitudinal relaxivity r_1 was determined from a linear fit of $1/T_1$ as a function of gadolinium (III) concentration.

Results: The r_1 of Gd-lipid NM65 ($2.67 mM^{-1}s^{-1}$) demonstrated that the conjugation of Gd.DOTA to the hydrophilic tail group had no effect on relaxivity compared to Dotarem® ($2.68 mM^{-1}s^{-1}$). Liposomes formulated CH300 PEG showed a slight reduction in r_1 ($2.23 mM^{-1}s^{-1}$), whereas liposomes formulated with ME42 PEG was greatly reduced ($1.75 mM^{-1}s^{-1}$), suggesting that PEG has some effect on relaxivity. Cells incubated with either CH300 or ME42 liposomes at 4°C showed halos of fluorescein fluorescence around the cell membrane (Fig 2a, b) indicating that the cationic nature of the liposome allows for static adherence to the cell membrane. Whereas cells incubated at 37°C showed clear internalization, with small pockets of fluorescence, rather than widespread distribution throughout the cyctoplasm consistent with incorporation into vesicles by pinocytosis (Fig 2c, d). FACs data indicated that although the same percentages of cells were labelled (~98%) there was less fluorescence at 4°C than at 37°C. Furthermore, there was less fluorescence by FACS and a reduced T_1 affect by MRI for the ME42 PEG liposome compared to CH300 PEG liposomes (Fig. 3), suggesting that PEG also affects the degree of cell uptake. Nonetheless, the T_1 results for cell pellets indicated that at 4°C there was a 40% reduction in T_1, whereas at 37°C there was only a 25% reduction in T_1 for cells incubated with CH300 PEG liposomes compared to controls. Therefore, although FACS data demonstrates a greater degree of cell uptake of CH300 PEG liposomes at 37°C compared to 4°C, MRI demonstrates a reduced effect on T_1. This is most likely due to limited water interaction within the vesicles of the cell at this time point, and should be considered when evaluating uptake *in vivo*.
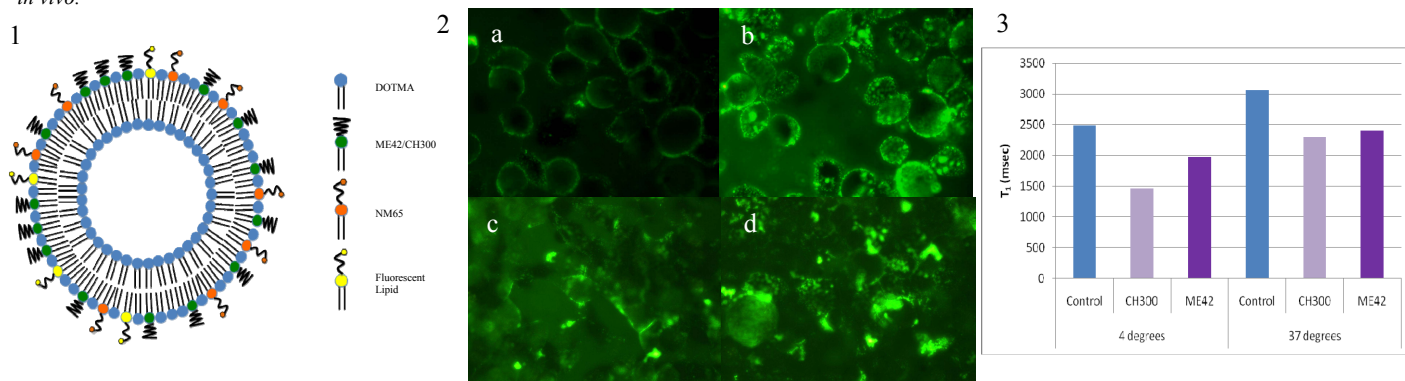
Figure 1: Illustration of a liposome composed of Gadolinium chelating, polyethylene glycol, cationic DOTMA and Fluoresein lipid. **Figure 2:** Fluorescent images of ME42 liposome incubated at 4°C (a), 37°C (c) and CH300 liposomes at 4°C (b), and 37°C (d). **Figure 3:** T_1 measurements of both CH300 and ME42 liposomes at both 4°C and 37°C with controls at each temperature.

Conclusions These results show that modification of PEG effects liposomal relaxivity and functionality. Optimization of PEG bimodal liposomes can be utilized to image and label cells, but most importantly they have an increased uptake in tumour cells for drug delivery without targeting. This will allow liposome nanoparticles to deliver drugs at higher concentrations to a wide range of tumours, bringing this technology one step closer to the clinic.

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