Design, Preparation and Characterisation of Enzyme-Triggerable Stealth Release of Targeted Nanoparticles for Cancer Genetic Therapy

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Declaration

I hereby declare that the work described here is entirely my own, except where specifically acknowledged in the text.

Peerada Yingyuad – Thesis submitted October 2010
Abstract

The use of cationic liposomes to deliver nucleic acids has shown great promise; however, their therapeutic potential is greatly limited by their in vivo instability. Upon systemic administration, cationic liposomes are prone to plasma protein adsorption, leading to RES recognition and rapid clearance from blood circulation. Surface modification using hydrophilic polymers, usually PEG, is known to prolong the circulation half-lives of liposomes in vivo. PEG is required for increased liposome stability, but it is undesirable once at a target tissue since its presence significantly inhibits the release of the encapsulated agent. The development of cleavable PEG that can be removed in response to a specific trigger once at the target site may be one strategy to overcome this problem.

Here, we report the development of PEGylated peptide-lipid conjugates sensitive to the proteolytic enzymes found in tumour cells, HLE and MMP-2. The synthesis was carried out by conjugating an enzyme substrate peptide between PEG and a lipid, enabling a series of enzyme-sensitive PEGylated peptide-lipid conjugates to be used for liposomal nucleic acid delivery. Although enzymatic degradation of the peptide linker resulting in the detachment of PEG was not observed with the analytical techniques used, the in vitro result showed enhanced pDNA transfection efficiency by the nanoparticles containing the PEGylated peptide-lipid conjugates in response to the enzymes, compared to the controls. Physicochemical characterisation showed the nanoparticles have small diameters and high nucleic acid encapsulation efficiency. The nanoparticles with lower zeta potential were found to exhibit enhanced stability in serum and minimum toxicity.

Further investigation in a siRNA system revealed the ability of the PEGylated peptide-lipid nanoparticles to significantly knockdown the target protein in response to enzymatic activation. Thus, the results from these studies demonstrated the possibility of using the enzyme-sensitive PEGylated peptide-lipid conjugates to improve nucleic acid delivery.
Acknowledgements

The work described in this thesis would not have been possible without the help and support of several persons.

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<thead>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>br</td>
<td>Broad</td>
</tr>
<tr>
<td>CDAN</td>
<td>$N'$-cholesteryloxy carbonyl-3,7-diazanonane-1,9-diamine</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
</tr>
<tr>
<td>DAPI</td>
<td>4-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC-Chol</td>
<td>3β-$(N'-(N',N'-Dimethylaminoethane)carbamoyl)cholesterol</td>
</tr>
<tr>
<td>DIPEA</td>
<td>$N,N'$-diisopropylethylamine</td>
</tr>
<tr>
<td>DODAG</td>
<td>$N',N'$-dioctadecyl-$N$-4,8-diaza-10-aminodecanoylglycine amide</td>
</tr>
<tr>
<td>DOGS</td>
<td>Dioctadecyglyclylspermine</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-Dioleoyl-$sn$-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-Dioleoyl-$sn$-glycerol-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-Dioleoyl-3-trimethylammonium propane</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMF</td>
<td>$N,N'$-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DU145</td>
<td>Human prostate carcinoma cell line</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELSD</td>
<td>Evaporative light scattering detector</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray mass spectrometry</td>
</tr>
<tr>
<td>Et2O</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
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<td>2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
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<td>HEK293T</td>
<td>Human embryonic kidney cell line</td>
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<td>Human ovarian carcinoma</td>
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<td>HEPES</td>
<td>Hydroxyethylpiperazine ethane sulfonic acid</td>
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<tr>
<td>HLE</td>
<td>Human leukocyte elastase</td>
</tr>
<tr>
<td>HOBT</td>
<td>1-Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>HT1080</td>
<td>Human fibrosarcoma cell line</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast adenocarcinoma cells</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
</tbody>
</table>

18
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>MLV</td>
<td>Multi lamellar vesicles</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>MTS</td>
<td>(3,(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>R_t</td>
<td>Retention time</td>
</tr>
<tr>
<td>s</td>
<td>Singlet</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small or short interfering ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
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1. Introduction
1.1 Gene therapy

Gene therapy is defined as the delivery of genetic material into cells for therapeutic purposes, either by replacing an existing defective gene with a therapeutic one, or by knockdown of disease-associated proteins. Gene therapy was initially aimed at treating inheritable diseases such as cystic fibrosis, adenosine deaminase deficiency, Gaucher’s disease, and Duchenne muscular dystrophy.\(^1\) However, as the field has grown, its potential for treating acquired diseases – in particular, cancer – has been realised and has gained increasing attention over the past two decades. Cancer is a term for diseases in which abnormal cells proliferate in an uncontrollable manner and have the ability to invade other tissues and spread to distant sites through the bloodstream (metastasis) ([http://www.cancerresearchuk.org/](http://www.cancerresearchuk.org/)). It is a leading cause of death worldwide. According to the World Health Organisation (WHO), 13% of all deaths in 2007 were caused by cancer; in that same year, the American Cancer Society reported total global deaths due to cancer of 7.6 million ([http://www.cancer.org/Research/CancerFactsFigures/index](http://www.cancer.org/Research/CancerFactsFigures/index)). For several decades, chemotherapy has been a predominant option for cancer treatment; however, it is associated with a number of drawbacks, including toxicity and poor bioavailability of anticancer agents. Non-specific distribution of chemotherapeutic drugs causes undesirable side effects on healthy tissues and also results in reduced quantities of drug accumulating at tumour sites, often to below therapeutic concentrations. Degradation of anticancer drugs before arriving at tumour sites, and drug resistance developed by the tumour cells, decreases their therapeutic efficiency. To increase bioavailability, administration of drugs in larger doses is required, which can further increase toxicity. In addition, the repeated use of chemotherapeutic agents is limited by toxicity. Doxorubicin is one of the most well-known anticancer drugs which has limited application due to its toxic side effects on the heart (cardiotoxicity) and on bone marrow activity (myelosuppression).\(^2\), \(^3\) To improve its safety profile, this has led to the development of PEGylated liposomal doxorubicin in which doxorubicin is contained in PEGylated liposomes (Doxil/Caelyx). Doxil has shown better accumulation in tumours than in normal cells and reduced toxicity compared to free doxorubicin.\(^4\), \(^5\) Abraxane®, albumin-bound nanoparticles of paclitaxel (a highly hydrophobic anticancer drug), was approved by the US Food and Drug Administration (FDA) in 2005 for treating metastatic breast cancer, showing higher efficiency in breast cancer treatment with
fewer toxic effects compared to paclitaxel on its own.\textsuperscript{6} These examples highlight the need to develop suitable delivery systems for efficient cancer treatments that enhance drug integrity, improve their bioavailability, and minimise their side effects and toxicity.

Cancer arises as a result of abnormalities or mutations occurred in the DNA sequence. The Cancer Genome Project’s success in identifying a large number of cancer genes (oncogenes) provides insight into the origins of cancer and suggests potential new avenues for treatment.\textsuperscript{7, 8} Cancer gene therapy, referring to the use of therapeutic nucleic acids to enhance the expression of tumour suppressors or to knockdown the expression of oncogenic proteins, may provide an effective and specific treatment, attacking the disease at its root cause. The use of RNA interference (RNAi) technology, including small interfering ribonucleic acid (siRNA) and microRNA (miRNA), to silence gene expression has recently gained the attention of many researchers. Its ability to render specific gene knockdown has led to the possibility of its use as a cancer therapy. siRNA-based formulations have been intensively studied and some are being investigated in clinical trials. ALN-VSP02, a stable nucleic acid lipid particle (SNALP) formulation containing two siRNAs directed against kinesin spindle protein (KSP) and vascular endothelial growth factor (VEGF), is currently being employed by Alnylam in a phase I clinical trial for the treatment of hepatocellular carcinoma (HCC) (http://clinicaltrials.gov). CALAA-01, cyclodextrin-based nanoparticles, has been developed by Calando to deliver the M2 subunit of the ribonucleotide reductase (RRM2) siRNA to solid tumours. RRM2 is involved in DNA replication process and inhibition of its activity has shown to reduce the growth of cancer cells.\textsuperscript{9} These nanoparticles are currently being studied in a phase I clinical trial.\textsuperscript{10} Finally, the restoration of metastasis suppressor miRNA-126 in human breast cancer \textit{in vivo} was shown to reduce tumour growth and proliferation.\textsuperscript{11}

Systemic administration of nucleic acids into blood circulation usually results in low efficiency of gene transfer due to the rapid degradation of nucleic acids by serum nucleases and their clearance by the mononuclear phagocyte system. The development of carriers that can selectively and efficiently deliver the nucleic acid therapeutics to target cells has been key to the success of genetic therapies. The types of carriers currently being studied fall into two categories: viral and nonviral. Viral
carriers have been the most effective delivery method for gene therapy due to their inherent ability to efficiently infect and transfer genetic material into host cells. To ensure safe gene transfer and avoid subsequent pathogenic effects, viral vectors derived from microorganisms (known as ‘wild type’) must have been modified by deleting their pathogenic genes, which are critical for viral replication, and replacing them with therapeutic foreign genes. Examples of viruses that have been developed for gene therapy include retroviruses\textsuperscript{12}, adenoviruses\textsuperscript{13}, adeno-associated viruses\textsuperscript{14} and herpes simplex viruses\textsuperscript{15}. These carriers are currently the most studied vectors in gene therapy clinical trials owing to their efficient gene transfer into a wide variety of cells (Figure 1-1).

\textbf{Figure 1-1:} Overview of vectors used in gene therapy clinical trials worldwide. Updated information available from The Journal of Gene Medicine Clinical Trial Database: http://www.wiley.com/legacy/wileychi/genmed/clinical/

Despite the success of viral vectors, many hurdles remain to be overcome before they can be extensively used in gene therapy applications. The major obstacles associated with viral carriers include toxicity and immunogenicity\textsuperscript{16}, random integration into host genomes and oncogenic effects\textsuperscript{17}, long-termed transgene expression\textsuperscript{16}, size limitations for transgene insertion\textsuperscript{18}, and ease of carrier preparation. These limitations, safety
concerns in particular, have led to the increased development of alternative carriers. Nonviral carriers, referring to the delivery of genes using either physical or chemical approaches, have advantages over viral vectors: low toxicity, ease of large-scale production, and simplicity of use. In the gene delivery history, nonviral vectors can be divided into two broad categories: delivery of nucleic acids by physical methods, such as gene guns and electroporation; and delivery of nucleic acids by chemical methods, such as protein-based, polymer-based, and liposome-based carriers.

1.2 Nonviral carriers

1.2.1 Delivery of nucleic acids by physical methods

1.2.1.1 Gene guns

Particle-mediated gene delivery, called “gene guns”, involves the bombardment of tissues or cells by DNA-coated gold particles which are accelerated either by a high voltage electric discharge or pressurised helium pulse. These gold particles are typically 0.5-2.0 μm in diameter. Owing to their small sizes, this approach allows the direct penetration of the microcarriers into cytoplasm, bypassing the endosomal/lysosomal membrane, thus diminishing the degradation of DNA by nuclease enzymes. Successful transfection in subcutaneous tumours and muscles has been demonstrated using this delivery method, without apparent toxicity. Although the technique has demonstrated advantages in gene therapy applications – such as ease of DNA microparticle production, controllable dosing of delivered DNA, and no observable toxicity – it is limited by short-term gene expression, low efficiency of transfection, and ability to deliver DNA to only small areas.

1.2.1.2 Electroporation

Electroporation, also known as electropermeabilisation, is the combination of naked DNA injection along with a pulsed, controlled electric field that facilitates nucleic acid
uptake into cells.\textsuperscript{20} Applied electric pulses open pores in the cell membrane, permitting macromolecules to pass through the membrane into the cell interior. The pores close after initial permeabilisation, and DNA is trapped inside the cells. Compared to injection of naked DNA without electric pulses, this technique increases gene expression in melanoma cells up to 21- to 42-fold.\textsuperscript{21} Transfection of the p53 gene by electroporation was able to suppress tumour growth.\textsuperscript{22} The gene expression, however, was limited to only the central part of the xenografts and multiple electroporations were therefore required in order to obtain apparent retardation of the tumour growth. Although the technique has shown great promise in nucleic acid delivery, the conditions of delivery, such as DNA dose, physical arrangement of electrodes, electrical field strength, and duration of electrical pulses, need to be well-optimised in order to achieve high transfection efficiency and long-term gene expression with minimum damage to target tissues and low toxicity.

Other techniques, such as hydrodynamic injection\textsuperscript{23} and ultrasound\textsuperscript{24} has also shown an improvement in gene expression compared to the delivery of naked DNA. The physical methods may be efficient in local delivery of nucleic acids but they are limited to target cells that are at remote sites, such as metastasised tumours. The use of synthetic carriers may provide a more viable approach toward effective cancer treatment.

1.2.2 Delivery of nucleic acids by chemical methods

1.2.2.1 Protein-based carriers

One example of a protein-based carrier is atelocollagen, a pepsin-digested product of type I collagen from which a telopeptide, an amino acid sequence attributed to collagen's antigenicity, is removed (Scheme 1-1).\textsuperscript{25} Atelocollagen therefore possesses low immunogenicity. Generally, atelocollagen is obtained from pepsin digestion of bovine demis. Due to its isoelectric point (pl) above 8, atelocollagen possesses positive charge at physiological pH, and can electrostatically interact with nucleic acids, such as plasmid DNA, antisense oligonucleotides (antisense ODNs), and short
interfering RNA (siRNA). At low temperature, atellocollagen is in liquid state and can easily mix with nucleic acids; however, at physiological temperature, it becomes fibrous and solid, protecting nucleic acids from nuclease digestion and thereby enhancing circulation times \textit{in vivo}. Delivery of anti-VEGF siRNA by atellocollagen showed suppression of both tumour angiogenesis and tumour growth.\textsuperscript{26} In addition, effective gene silencing in bone-metastatic tumours using atellocollagen-mediated siRNA delivery has been demonstrated with no observable side effects.\textsuperscript{27}

\begin{center}
\begin{tikzpicture}
  \node at (0,0) {\includegraphics[scale=0.5]{atelocollagen.png}};
  \node at (-2,2) {Telopeptides};
  \node at (2,2) {Telopeptides};
  \node at (0,-2) {Pepsin digestion};
  \node at (0,-1) {Atellocollagen};
\end{tikzpicture}
\end{center}


Protein-based carriers have been a preferred option for gene delivery due to their low immunogenicity, biocompatibility, and biodegradability. However, chemical manipulations of the proteins are not easy, limiting their potential for functionalisation, such as the incorporation of targeting ligands, which could improve the efficacy of this delivery system. The vectors capable of chemical modifications – for example, polymers and liposomes – are more widely used for gene therapy applications.
Polymers exhibit good potential as gene delivery vectors owing to their ease of preparation and chemical modification, providing flexibility for development according to their application. Polymer-based vectors are made to carry positive charge, which allows them to interact electrostatically with negatively charged nucleic acids and form stable nanoparticles known as polyplexes. One of the first polymers employed for gene therapy was poly (L-lysine) (PLL) (Figure 1-2). PLL is a linear polypeptide with a repeating unit of amino acid lysine, thus possessing biodegradable properties, which is an advantage for in vivo applications. However, PLL vectors have limited use as transfection agents due to their relatively poor level of gene expression and cytotoxic side effects. The inclusion of additional agents, or further synthetic modification, is usually required to improve transfection efficiency and reduce cytotoxicity.\textsuperscript{28,29}

![Figure 1-2: Structure of poly (L-lysine) (PLL)](image)

A more versatile polymeric vector is poly (ethylene imine) (PEI) (Figure 1-3), which is a synthetic linear or branched polymer with varying molecular weight.\textsuperscript{30} It bears a high cationic charge density, which allows for strong binding to nucleic acids. Low molecular weight PEI (5-25 kDa) is considered the most suitable for gene transfer as it has been shown to be less toxic compared to higher molecular weight PEI, and is yet still capable of transfection.\textsuperscript{31} The increased cytotoxicity associated with high molecular weight is probably due to the precipitation of cationic polymers in huge clusters that adhere to the outer cell membrane, which leads to necrosis.\textsuperscript{32,33} PEI (22 kDa) coupled to targeting-ligand transferin (Tf) showed enhanced gene expression in tumours and lowered toxicity compared to 800 kDa PEI.\textsuperscript{34} Linear PEI of low molecular weight is already
commercially available as transfection agents, including ExGen500® (Fermentas) and jetPEI® (Polyplus Transfection).

![Figure 1-3: Structure of linear poly (ethylene imine) (PEI)](image)

Although the toxicity of PEI appears to decrease with decreasing polymer size, total lack of toxicity would be an ideal characteristic for gene delivery vectors. The recent development of analogous biodegradable polymers has gained much attention. Unlike non-biodegradable PEI, degradable polymers decompose to low molecular weight constituents in cytosol and hence facilitate the release of nucleic acids, resulting in lower toxicity and higher transfection efficiency. Forrest et al. reported that biodegradable PEI derivatives, synthesised by coupling 800 Da PEIs via short diacrylate linkages, of which ester bonds are susceptible to hydrolysis at physiological condition and in acidic environment of endosomes, to form conjugates of 14-30 kDa. The degradable PEI enhanced gene expression 2- to 16-fold (depending on cell lines) compared to 25 kDa, nondegradable PEI, and showed minimal toxicity.\textsuperscript{35} siRNA delivery using a biodegradable poly (amido ethyleneimine), containing multiple disulfide bonds which can be cleaved by cytosolic glutathione, demonstrated higher gene suppression than that of 25-kDa PEI, with a more favourable toxicity profile.\textsuperscript{36}

Another well-characterised polymer is cyclodextrin-containing polymer. Cyclodextrins are cup-shaped, cyclic oligosaccharides comprising 6, 7 or 8 glucose units (Figure 1-4), obtained by degradation of starch by mean of enzymatic conversion.\textsuperscript{37} They have several characteristics that are beneficial for gene delivery including low toxicity, lack of immune stimulation, and biocompatibility. The cyclodextrin-based polymer is a short polycation that can assemble with nucleic acids via electrostatic interactions, resulting in the encapsulation of nucleic acids within their hydrophilic cores. In addition, via van
de Waal's interactions, the cavities within cyclodextrins can form inclusion complexes with small hydrophobic molecules, such as adamantane (AD). Davis et al. utilized this feature to develop a new class of cyclodextrin-based polymer in that the cyclodextrin was part of the linear polymer backbone and the cores within cyclodextrins that reside on the nanoparticle surface were used for inclusion of AD-PEG and AD-PEG-Tf conjugate for steric stabilisation and tumour-targeting effect (Figure 1-5). The surface modified cyclodextrin-containing polymer was shown to be an effective and versatile method of both DNA and siRNA delivery in vitro. The efficacy of the cyclodextrin-containing polycations for siRNA delivery was also demonstrated in vivo, showing efficient silencing of oncogenes, inhibition of tumour growth, and the possibility of multiple dose administration. In addition, the particles were well-tolerated in non-human primates and no sign of immune response was observed. A targeted, cyclodextrin polymer-based nanoparticle formulation of siRNA, known as CALAA-01, developed by Calando Pharmaceuticals has recently reached phase I clinical trial. The interim results of this trial demonstrate that systemic administration of anti-RRM2 siRNA in patients with melanomas can produce specific gene silencing, shown by a reduction in both RRM2 (the M2 subunit of ribonucleotide reductase) mRNA and the translated protein. This finding is the first to provide mechanistic evidence of RNAi in humans by the detection of mRNA fragments as a result of siRNA-mediated specific mRNA cleavage.

Figure 1-4: Representation of cyclodextrin and its cup-shaped structure
a) chemical structure of cyclodextrin-containing polymer (CDP) in which cyclodextrin is conjugated as part of the linear polymer backbone \((n = 5-6)\). b) (left) delivery components including CDP, siRNA, Adamantane (AD)-PEG conjugate (AD-PEG), and AD-PEG conjugated to targeting-ligand transferin (Tf) (AD-PEG-Tf), (middle) self-assembled nanoparticles containing siRNA and (right) nanoparticles entering the cell via receptor-mediated endocytosis.

The natural origin polymer chitosan is gaining increasing interest as a gene delivery vector due to its biodegradability, biocompatibility, and non-toxicity. It is a natural, linear polysaccharide produced by the deacetylation of chitin, a polysaccharide found in the exoskeletons of crustaceans and insects (Figure 1-6). It possesses a high cationic
charge, providing a strong electrostatic interaction with nucleic acids. Numerous reports have demonstrated efficient gene delivery using chitosan/DNA nanoparticles.\textsuperscript{43-45} The potential of chitosan-mediated siRNA delivery highlighted by the work of Howard \textit{et al.} showed effective gene silencing both \textit{in vitro} and \textit{in vivo}.\textsuperscript{46} However, transfection efficiency seems to depend on several factors: for example, the molecular weight of chitosan, the charge ratio of chitosan to nucleic acids, and transfection conditions.\textsuperscript{44, 47} Optimisation of these factors is different for different types of cells.

\begin{figure}[h]
\centering
\includegraphics[width=0.2\textwidth]{chitosan_structure.png}
\caption{Structure of chitosan}
\end{figure}

Other polymers, such as block copolymers and dendrimers, are also being investigated as efficient vehicles for gene delivery. One example of a block copolymer is poly (lactic-co-glycolic acid) (PLGA), which has been approved by the FDA as safe for human use\textsuperscript{48} (Figure 1-7). PLGA is a biodegradable copolymer obtained by the copolymerisation of two different monomers, lactic acid and glycolic acid, which are linked together by ester bonds. PLGA-mediated delivery has shown increased \textit{in vitro} transfection efficiency as well as sustained release of DNA.\textsuperscript{49} Murata \textit{et al.} recently demonstrated suppression of tumour growth and sustained suppressive effect after intratumoural injection by PLGA microspheres encapsulating anti-VEGF siRNA.\textsuperscript{50} Administration of PLGA nanoparticles loaded with siRNA to the reproductive tracts of female mice has also shown a significant level of gene knockdown and sustained gene silencing for at least 14 days.\textsuperscript{51}
Dendrimers are spherical, highly branched polymers. One of the most commonly used dendrimers for nucleic acid delivery is polyamidoamine (PAMAM). PAMAM contains a high density of amine, enabling electrostatic condensation of nucleic acids. Transfection efficiency appears to depend on the size and shape of the dendrimers. The modified dendrimers have been shown to enhance transfection efficiency and gene silencing in mammalian cell lines.

1.2.2.3 Liposome-based carriers

Liposomes are composed of amphiphilic lipids that, upon hydration, self-assemble into spherical bilayers enclosing an aqueous compartment. Amphiphilic lipids have both hydrophilic and hydrophobic regions in the same molecules. In an aqueous environment, lipids arrange themselves in order to minimise hydrophobic interaction and maximise hydrophilic interaction with aqueous phase, resulting in a variety of self-assemblies depending on geometrical structures and the concentration of the lipids in solution. Lipids involved in liposome preparation usually have a cationic head group on the hydrophilic region, allowing for nucleic acid encapsulation within the inner core of the liposome via electrostatic interaction (Figure 1-8). Liposomes, like polymer-based vectors, are capable of condensing nucleic acids; however, they have several advantages over cationic polymers as gene delivery carriers. The physical properties of liposomes, such as their size, surface charge, and the rigidity of bilayers, can be simply manipulated by adding and tuning lipid compositions according to their application. In addition, functionalisation of liposomes – including incorporation of stealth layers,
targeting ligands, fluorescent probes, and metallic-lipid amphiphiles, for stabilisation, targeting, tracking, and imaging purposes – can be easily performed. The organisation of all these functional molecules is feasible on a liposome and the resulting assembly is usually a nanoparticle. These features allow greater flexibility of nanoparticle design compared to polymers. Another attractive advantage of liposomes is their low toxicity and immunogenicity. Since liposomes are composed of lipid or amphiphile constituents which resemble cell membranes, they are biocompatible and easily cleared from cells after cellular uptake. Liposomes have been shown to be more versatile as carriers for nucleic acid delivery compared to the other delivery systems described above.

Figure 1-8: Representation of liposome formation and nucleic acid encapsulation. Amphiphilic lipids self-assemble into liposomes and nucleic acids can then be electrostatically entrapped within the aqueous compartment of liposomes.

Liposomes are typically categorised by their size and the number of lamellars or bilayers. Liposomes composed of multiple bilayers are termed multilamellar vesicles (MLV) and are typically larger than 500 nm in diameter. Liposomes that consist of a single bilayer are termed unilamellar vesicles (ULV). Their size ranges between 50-500 nm for large unilamellar vesicles (LUV) and 20-50 nm for small unilamellar vesicles (SUV). Diameters of liposomes are typically determined using dynamic light scattering.
Traditionally, liposomes have been used as drug carriers by encapsulating hydrophilic drugs in their core in order to improve drug pharmacokinetics. A number of liposomal drugs have been approved and some are in clinical trials. The potential of liposomes as efficient carriers for nucleic acid delivery was first recognised when Felgner et al. published their study in 1987. The authors demonstrated successful in vitro DNA transfection with the use of cationic lipid 1,2-di-O-octadecenyl-3-trimethylammonium propane hydrochloride (DOTMA) and neutral phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in a 1:1 molar ratio. Following its success in DNA delivery, this lipid formulation became commercially available under the trade name Lipofectin®. A more recently developed liposome-based transfection agent is Lipofectamine®, which has been widely used in several biological protocols. Cationic liposomes composed of cationic lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and neutral lipid cholesterol has also shown efficient transgene expression in tumour cells both in vitro and in vivo. Liposome-based delivery systems have been extensively studied and some formulations of cationic liposome/DNA nanoparticles – for example, Allovecin-7 – are being investigated in phase II clinical trials in patients with metastatic melanomas. The use of liposomes to mediate siRNA delivery has shown great promise for cancer gene therapy in recent years. Many publications have reported successful inhibition of tumour growth by siRNA-containing nanoparticles. Currently being investigated in phase I clinical trial is Atu-027, a liposomal formulated 2'-O-methyl modified siRNA directed against protein kinase N3 (PKN3), which has been identified as a downstream effector of the phosphoinositide-3-kinase (PI3K) signalling pathway, and is involved in tumour angiogenesis and development. Preclinical data regarding the use of this formulation in mouse models of prostate and pancreatic tumours showed significant inhibition of tumour growth and reduction of lymph node metastasis.
1.3 Barriers to liposome delivery to tumours

One major problem associated with the delivery of nanoparticles is that they are treated as foreign molecules by the body and subsequently cleared out of circulation before they can complete their therapeutic function. Understanding biological barriers encountered in the delivery process is essential for the design of viable nanocarriers. The hurdles to successful delivery of therapeutic nucleic acids \textit{in vivo} can be divided into two categories: extracellular barriers and intracellular barriers. Extracellular barriers describe the obstacles that nanoparticles meet after administration into blood circulation, up to their arrival at the tumour site. Intracellular barriers refer to obstacles faced after cellular internalisation, up to the release of the encapsulated agent inside the cell. The details of these biological barriers are described below.

1.3.1 Extracellular barriers

One of the unique anatomical and pathophysiological characteristics of tumours is their abnormal vasculature. Unlike normal blood vessels, tumour vasculatures are not uniform: they are leaky and their endothelial cells are disorganised with large fenestrations (Figure 1-9).\textsuperscript{72, 73} These defective architectures are a result of angiogenesis, the formation of new blood vessels from the existing microvascular beds due to elevated levels of vascular mediators such as vascular endothelial growth factor (VEGF). Angiogenesis occurs when the tumour reaches a size of 2-3 mm, supplying nutrients and oxygen to the growing tumour.\textsuperscript{74} The leaky vasculatures allow passive transport of circulating nanoparticles, a process termed extravasation, into tumour interstitium. This, combined with impaired lymphatic drainage in tumours, allows enhanced accumulation and retention of nanoparticles in solid tumours, and is known as the enhanced permeability and retention (EPR) effect,\textsuperscript{73, 75} which was initially described by Maeda \textit{et al.} in 1986.\textsuperscript{76} It was found that the maximum nanoparticle size that can cross vasculature fenestrations in tumours is around 400 nm,\textsuperscript{77} so only nanoparticles with diameters up to 400 nm can extravasate into tumour tissues. Nanoparticles less than 200 nm in diameter were shown to effectively accumulate in tumours.\textsuperscript{78-81} Nanoparticles less than 100 nm in diameter were susceptible to uptake by
hepatocytes due to their ability to penetrate through hepatic fenestrations and interact with hepatocytes. This established the significance of nanoparticle diameter in tumour accumulation: nanoparticle size is a key consideration of optimal design.

**Figure 1-9:** A schematic representation of nanoparticles’ localisation to tumour tissues by the enhanced permeability and retention (EPR) effect. Nanoparticles are unable to diffuse through normal vasculature due to the tight junction of endothelial cells, while leaky vasculatures in tumours enhance extravasation and increase accumulation of nanoparticles, contributing to the EPR effect. Modified from Lyer et al., *Drug Discov. Today*, 2006, 11 (17-18), 812-818.

In biological fluids, nanoparticles are exposed to high concentrations of salt and various plasma proteins in the bloodstream. Such conditions can dramatically alter the stability of the nanoparticles. Adsorption of plasma protein on the surface of the nanoparticles, specifically high-charge particles, leads to charge neutralisation, which induces nanoparticle aggregation. The increase in nanoparticle diameter as a result of nanoparticle aggregation reduces the opportunity for nanoparticles to diffuse into tumour sites, as extravasation of large macromolecules is limited to the size of tumour-vasculature fenestrations. In addition, the deposition of opsonins, plasma proteins involved in immune response of the reticuloendothelial system (RES), marks the positively charged nanoparticles for recognition by the cells of the mononuclear phagocyte system, which is mainly localised in the liver (specifically the Kupffer cells) and spleen. Identification by mononuclear phagocytes results in rapid removal of
the nanoparticles from circulation, preventing them from localising in tumour cells. Furthermore, this opsonisation can activate the complement system which in turn initiates an inflammatory response against the charged nanoparticles. To reduce their clearance rate, the surface characteristics of the nanoparticles are some of the most crucial factors to be considered in the nanoparticle design. According to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, the stability of colloidal particles in solution is explained by two types of interparticle interaction forces: repulsive forces generated by electrostatic and/or steric repulsion, and van der Waals attractive forces. To maintain dispersion of particles, the repulsive forces must be greater than the attractive forces in order to prevent nanoparticles from coming into contact and aggregating. In biological system, steric stabilisation is more crucial than electrostatic stabilisation since highly charged nanoparticles are prone to the RES recognition and rapidly cleared from circulation. Steric stabilisation is generally achieved by the introduction of a stealth layer (Figure 1-10), which is a layer of hydrophilic polymers such as polyethylene glycol (PEG). A PEG layer provides stability to nanoparticles by increasing repulsive forces between nanoparticles, preventing them from coming into close contact with one another. In addition, PEG creates a hydrophilic layer on the nanoparticle surface, which repels plasma proteins and slows down RES uptake.

To overcome extracellular barriers posed by systemic delivery and be able to accumulate in tumours, nanoparticles must resist aggregation in blood in order to maintain a small diameter and also resist plasma protein adsorption in order to avoid recognition by RES and clearance from circulation. After arriving at tumour cells via the circulation, nanoparticles encounter further challenges in the form of intracellular barriers.
Figure 1-10: According to DLVO theory, colloidal stability of nanoparticles is maintained when repulsive force is greater than attractive force. Stabilisation of nanoparticles in solution can be achieved by electrostatic repulsion, the nanoparticles are surrounded by a highly charged double layer, (left) or by steric repulsion, the surface of the nanoparticles is functionalised with polymer, (right). Adapted from http://www.malvern.com/LabEng/industry/colloids/dlvo_theory.htm

1.3.2 Intracellular barriers

Once at the tumour site, cellular entry of the nanoparticles is generally achieved by endocytosis. Endocytosis is the cellular uptake mechanism by which a cell membrane invaginates and pinches off to engulf extracellular substances into the cell. Cellular internalisation of non-targeted nanoparticles mainly relies on electrostatic interactions between cationic nanoparticles and anionic glycoproteins, such as proteoglycans, present on the cell surface, followed by the clathrin-coated pit mechanism (Figure 1-11).89, 90 For receptor-targeted nanoparticles, their internalisation occurs by receptor-mediated endocytosis.91 Clathrin is a cytosolic coat protein that plays a critical role in the formation of membrane-bounded vesicles for endocytosis. After being internalised, clathrin-coated vesicles become uncoated (depolymerisation), resulting in early endosomes. These endosomes can recycle their contents, such as plasma proteins or nanoparticles, back to the cell surface, or they can progress to late endosomes and
eventually fuse with lysosomes for degradation of endocytosed materials. In late endosomes, the acidity of the compartment increases from neutral to pH 5-6 due to ATPase proton-pump enzyme on the membrane transporting protons into the vesicles. The internalised macromolecules that are destined for the lysosome – a membrane-bounded vesicle, pH 5-5.5, containing various hydrolytic enzymes – are digested and unable to produce a therapeutic effect. Therefore, endosomal disruption of the endocytosed nanoparticles is essential to avoid lysosomal degradation and also transportation back to the cell surface.
Figure 1-11: Schematic representation of intracellular hurdles encountered by nanoparticles. Cationic nanoparticles first interact with anionic glycoproteins, such as proteoglycans, found on the cell surface. This leads to accumulation of clathrin proteins at the binding site for endocytosis. After internalisation, the clathrin coat depolymerises, resulting in early endosomes. The plasma proteins then recycle back to the cell surface. Endocytosed nanoparticles must escape from endosomes to be able to release their contents into the cytoplasm. The released DNA must then traffic through the cytoplasm and cross the nuclear membrane to perform their therapeutic effects in the nucleus, whereas siRNA functions in the cytoplasm. Internalised macromolecules that fail to escape from endosomes will degrade in lysosomes.

Endosomal disruption of liposomal nanoparticles was proposed to occur as a result of lipid mixing, the so-called flip-flop mechanism (Figure 1-12a). According to this mechanism, internalised cationic nanoparticles electrostatically induce lateral diffusion of anionic lipids, predominantly located on the cytoplasmic side, to form charge-neutralised ion pairs with cationic lipids in the liposomes. The ion pairing leads to
destabilisation of the endosomal membrane, allowing for the penetration of DNA into the cytoplasm. Unlike liposomal nanoparticles, polyplexes such as PEI are believed to escape from the endosomes through a different mechanism, termed the proton-sponge mechanism (Figure 1-12b). PEI possesses a high buffering capacity as it contains a large number of amine groups. This hypothesis suggests that at the low pH in endosomes, PEI becomes more protonated, causing ATPase to transport more protons to maintain the pH in the vesicles. The excessive accumulation of protons leads to an influx of chloride counter ions, causing osmotic swelling and eventually rupturing of the endosomal membrane, releasing the polyplexes into the cytoplasm.

Figure 1-12: Representation of hypothesis of endosomal escape of a) lipoplexes and b) polyplexes.
After endosomal escape, DNA must traffic to the nucleus through the cytoplasm. Several proteins and organelles in cytoplasm restrict the mobility of DNA, constituting another significant barrier for efficient DNA delivery. In addition, due to their small pore sizes (~ 55 Å), nuclear pores in the nuclear envelope permit the passive transport of molecules up to a molecular weight of 70 kDa or a diameter of ~ 10 nm. This size limit is much smaller than the size of DNA (e.g. a 5 kb DNA has a molecular weight of approximately 3.3 million Da), providing it little chance of entering the nucleus. It is widely accepted that the nuclear translocation can occur either via cell division during mitosis, when the nuclear membrane loses its integrity, allowing the entry of DNA, or via an active transport mechanism, requiring the use of nuclear localisation sequences (NLS) to facilitate DNA transportation by binding to cytosolic receptors – importins. Unlike pDNA, the interference mechanism of siRNA molecules occurs in cytoplasm, eliminating the obstacle associated with nuclear entry. However, siRNA still needs to escape the endosomal barrier.

1.4 Components of liposomal nanoparticles

In order to overcome the delivery barriers previously described, liposomal nanoparticles are generally composed of three main layers: the nucleic acid core (A), lipid envelope layer (B), and stealth layer (C) (Figure 1-13). Nucleic acids (A) are condensed within a lipid envelope (B) in order to protect them from the undesirable effects of external conditions. AB nanoparticles are themselves capable of nucleic acid delivery; however, their instability under physiological conditions usually results in aggregation and subsequent loss of their therapeutic function. A stealth layer (C), commonly made of a hydrophilic polymer such as PEG, is therefore required for ultimate protection of the nanoparticles. To enhance specificity of the delivery system, targeting ligands (D) having high affinity to receptors expressed on tumour cells can be incorporated on the external layer of the ABC nanoparticles.
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Figure 1-13: Schematic representation of ABCD nanoparticle paradigm. Nucleic acids (A) are condensed and protected within lipid bilayers (B). A stealth layer (C) is necessary for nanoparticles’ stability and long circulation in vivo. Targeting ligands (D) can be included on the exterior layer of the nanoparticles for selective delivery and enhanced cell internalisation.

1.4.1 Nucleic acids (A)

Nucleic acids employed in cancer gene therapy can be either DNA or RNA, with the aim of altering the production of specific proteins for therapeutic benefits, either by augmentation of tumour suppressor genes or inhibition of dominant oncogenes. Delivery of DNA is intended to enhance the expression of proteins involved in tumour growth suppression – the so-called tumour suppressors. One example of a tumour suppressor gene is p53, which encodes for a transcription factor that regulates the cell cycle and induces programmed cell death (apoptosis). In patients with cancer, p53 genes have been found to be mutated, leading to uncontrollable cell growth and tumourigenesis. Restoration of the gene function might re-establish the balance between cell cycle and apoptosis. The p53 gene has therefore gained much interest from many researchers as an attractive target for cancer therapy. The use of a p53-based adenoviral vector (Gendicine) has been approved for the treatment of head and neck cancer in combination with radiotherapy.
Emerging antisense technology has led to increasing focus on the use of antisense oligodeoxynucleotides (AS-ODNs) and RNA molecules for therapeutic purposes. Unlike DNA, antisense molecules are designed to inhibit the translation and expression of proteins that are involved in cancer growth – the so-called oncogenic proteins. Antisense oligodeoxynucleotides (AS-ODNs) are short, synthetic, single-stranded deoxynucleotide (DNA) molecules, usually composed of 12-25 nucleotides. They can stably complex to complementary sequences of messenger ribonucleic acids (mRNA), preventing mRNA from being translated to specific proteins. Fomivirsen (Vitravene) was the first antisense drug to be approved by the FDA for the treatment of viral infections of the eye caused by cytomegalovirus (CMV). Oblimersen is one of the antisense drugs that are being investigated in clinical trials. It is designed to target B-cell lymphoma protein 2 (Bcl2), which inhibits apoptosis. Overexpression of the BCL2 gene, found in many kinds of tumours, and the chemotherapy resistance of tumours is thought to be correlated. Despite significant progress in clinical trials, the use of ODNs has been reported to be associated with non-antisense effects, such as binding to non-targeted mRNA, receptors, or other proteins, which impairs their therapeutic effect and may cause un-intended consequences.

The use of short interference ribonucleic acid (siRNA) as a powerful tool in silencing specific gene expression has held great promise in cancer research since the discovery of the RNA interference (RNAi) pathway in 1998. siRNA is a short sequence of double stranded RNA, usually 21-23 nucleotides long. Similar to AS-ODNs, siRNA is able to bind to complementary mRNA sequences and inhibit the expression of coded proteins. However, siRNA has shown to be more stable in physiological fluids and more efficient in gene silencing than AS-ODNs. Once inside the cells, siRNA is incorporated within the RNA-induced silencing complex (RISC). The activated RISC then binds to and cleaves perfectly matched mRNA sequences, preventing protein from being translated. Like siRNA, micro RNA (miRNA) is capable of inducing RNAi-mediated gene suppression. When incorporated into RISC, the activated complex can bind to corresponding mRNA sequences. mRNA is cleaved and subsequently degraded if the complex is fully complementary to the mRNA target, whereas incomplete base pairing leads to inhibiting mRNA translation. Due to their ability to prevent specific proteins from being translated, the technology has been extensively
used for silencing of the oncogenic proteins being expressed. Vascular endothelial growth factor (VEGF) is an example of a widely studied oncogenic protein for RNAi-induced therapeutics. It is a tumour-secreted growth factor protein that plays a key role in angiogenesis, the process necessary for tumour growth. Blockade of the protein expression mediated by VEGF siRNA has been shown to result in inhibition of tumour growth.\textsuperscript{26, 111, 112} In one recent study, delivery of siRNA targeting the E6/7 oncogenes, which is found to bind and degrade p53 tumour suppressors, resulted in inhibition of tumour growth with a 50% reduction in tumour size.\textsuperscript{66}

All nucleic acids contain anionic phosphodiester bonds, which are highly sensitive to nucleases. Systemic administration of nucleic acids on their own (so-called naked nucleic acids) is therefore vulnerable to enzyme degradation, resulting in inefficient gene expression at target cells. In addition, phosphate backbones in their structure give nucleic acids negative charge, causing poor interaction with the anionic cell membrane. Nucleic acids therefore need to be packaged in a suitable carrier in order to increase their nuclease resistance and ultimately improve their interaction with the cell.

1.4.2 Lipid envelope layer (B)

In order to enhance cellular interaction with the negatively charged cell membrane, liposomes bearing positive charge are typically employed. Cationic liposomes generally consist of cationic lipids and neutral helper lipids. Cationic lipids are made of a cationic polar head group (usually polyamines) linked to a hydrophobic moiety (typically hydrocarbon chains or cholesterol), and are therefore positively charged amphiphiles. The cationic head groups are necessary for interaction with the negatively charged phosphate backbone of nucleic acids. This characteristic also provides electrostatic interaction with the anionic cell surface, resulting in enhanced cellular uptake of nanoparticles. Cationic lipids may be either monovalent lipids, containing a single amine functional group, or multivalent lipids, containing several amine functions. Examples of cationic lipids commonly employed for liposomal formulation are presented in Table 1-1.
<table>
<thead>
<tr>
<th>Cationic lipid</th>
<th>Structure</th>
<th>Maximum charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTAP</td>
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<td>+1</td>
</tr>
<tr>
<td>DOGS</td>
<td><img src="image" alt="DOGS structure" /></td>
<td>+4</td>
</tr>
<tr>
<td>DC-Chol</td>
<td><img src="image" alt="DC-Chol structure" /></td>
<td>+1</td>
</tr>
<tr>
<td>CDAN</td>
<td><img src="image" alt="CDAN structure" /></td>
<td>+3</td>
</tr>
</tbody>
</table>

**Table 1-1:** Examples of most commonly used cationic lipids$^{113}$

In addition to the commonly used cationic lipids shown in Table 1-1, design and synthesis of cationic lipids has been substantially conducted in order to enhance cellular binding and internalisation of cationic nanoparticles. Semple et al. recently
described the preparation of a new series of cationic lipid, DLin-K-DMA. The lipids was optimised from the prior DLinDMA cationic lipid, which was used as a key lipid in stable nucleic acid particle (SNALP) and was shown to be effective for siRNA delivery. The DLin-K-DMA lipids contained the same linoleyl hydrocarbon chain as found in DLinDMA, dioxolane linker and dimethylamino head group (Figure 1-14). The pKa of the head group was optimised to range between 6-7 in order to have minimal charge density (or be unprotonated) in blood circulation but become protonated in the acidic environment of the endosome (pH ~5.5), and this should promote fusion with the endosomal membranes. Incorporation of a new cationic lipid, DLin-KC2-DMA (pKa ~ 6.7), within SNALP formulation resulted in efficient hepatic gene silencing in vivo at low siRNA doses.

\[ \text{DLin-KC2-DMA} \]

**Figure 1-14: Structure of DLin-KC2-DMA**

A recent report by Mevel et al. demonstrated the synthesis of the novel cationic lipid \( N',N' \)-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide (DODAG), by conjugating a polyamine head group to a long chain hydrocarbon dialkylglycylamide moiety (Figure 1-15). The lipid structure was designed based on the hydrophilic head group of the previously developed cationic lipid CDAN and the hydrophobic tail of cationic lipid DOGS. The head group of CDAN contains three amine groups, with a distribution of pKa value, rendering it high capacity for nucleic acid encapsulation and aiding in cellular binding. DODAG-containing nanoparticles showed efficient pDNA delivery to various cancer cell lines and reasonable in vivo activity of anti-hepatitis B virus (HBV) siRNA in murine livers.
Apart from cationic lipids, which are a main component of liposome formulation, inclusion of neutral helper lipids is believed to aid in phase transition of lipid bilayers, promoting endosomal escape of encapsulated nucleic acids, which in turn enhances transfection efficiency. Neutral helper lipids generally employed for liposome preparation are shown in (Figure 1-16). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) is the most common helper lipid used in liposome formulation. When used with cationic lipids, it has been shown to enhance gene expression. The increased transfection efficiency may contribute to the fusogenic property of the lipid, which is related to its geometry. DOPE has a small polar head group and larger hydrophobic diacyl chains. Such a structure tends to adopt an inverted hexagonal (H$_{II}$) phase, which is believed to aid in endosomal destabilisation and facilitate the release of nucleic acids into cytosol. Other common phase structures of lipids include the lamellar (bilayers) and micellar phases. A lamellar phase is formed if the lipid possesses similarly sized head group and lipidic tails (i.e. it is cylindrical in shape), such as (DOPC). If lipid has a head group larger than its hydrophobic tail (i.e. it is conical in shape), it will assemble into a micellar phase. The different phase structures of lipids are presented in Figure 1-17.
Figure 1-16: Structures of common neutral lipids employed in liposome formulation\textsuperscript{113}
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Figure 1-17: Schematic representation of lipid phase structures a) a cone-shaped lipid assembles into a micellar (H\textsubscript{i}) phase, a cylindrical-shaped lipid forms lamellar (L\textsubscript{\alpha}) phase, and a lipid with inverse conical shape self-assembles into an inverted hexagonal (H\textsubscript{II}) phase; b) 2D structure of L\textsubscript{\alpha} and H\textsubscript{II} phases.\textsuperscript{123} Lipids such as DOPE can adopt the H\textsubscript{II} phase, which is thought to aid in endosomal disruption and facilitate the escape of nucleic acids into the cytoplasm.
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Cholesterol is another neutral co-lipids commonly employed in liposome formulation. Inclusion of cholesterol is known to increase the packing densities of phospholipids and reduce membrane permeability, resulting in bilayers with greater resistance to plasma protein binding. Cholesterol-containing liposomes have been shown to exhibit increased stability and prolonged circulation half-lives.124, 125

Preparation of cationic liposomes can be achieved by combining cationic lipids and helper lipids in an appropriate ratio.126 The vesicles are typically formulated by the dehydration-rehydration (DRV) method and then mixed with nucleic acids to acquire the desired nanoparticles. The DNA encapsulation model was proposed by Templeton et al.127 The condensation of DNA in liposomes results in entrapment of DNA within the aqueous layer of multilamellar vesicles by electrostatic interaction, forming a lipid-DNA sandwich (Figure 1-18). The encapsulation process is driven entropically by the release of counter ions associated with lipids and nucleic acids as one approaches the other, inducing the formation of a lipid-DNA complex.128 Other nucleic acids including ODNs and siRNA can be entrapped within liposomal membrane through a similar mechanism.129 The condensation efficiency can decrease when a PEG layer is introduced. The efficiency of siRNA entrapment can vary according to the preparation method. Hydrating a lipid film with a solution of siRNA has been shown to increase encapsulation of siRNA in the liposome core, whereas the classic method of adding siRNA to the preformed liposome usually results in most of the siRNA becoming bound to the outer layer of the PEGylated liposomes.130
Figure 1-18: Proposed model showing DNA encapsulation within cationic liposomes. Lipid bilayers electrostatically interact with negatively charged DNA and wrap around to form vesicles in which DNA is completely entrapped. Modified from Templeton et al. Nat. Biotech. 1997, 15, 647-652.

Although cationic nanoparticles are necessary in order to increase cell uptake by electrostatic interaction with plasma membranes, their positive charge also leads to detrimental effects for systemic delivery. Positively charged nanoparticles induce opsonisation and enhance uptake by the mononuclear phagocyte system, resulting in their rapid clearance from blood circulation. In addition, interaction with serum proteins leads to aggregation of the nanoparticles. These effects prevent the accumulation of nanoparticles in tumour tissue. To overcome these difficulties, nanoparticles should have low positive charge while still being capable of condensing nucleic acids. In addition to this, a stealth layer is an important component that confers stability to liposomes. A PEG layer renders steric stabilisation to nanoparticles and hinders their surface charges, resulting in decreased RES recognition and hence prolonging their half-lives in blood circulation.
1.4.3 **Stealth layer (C)**

Covering the liposomal surface with hydrophilic polymers has been shown to extend the blood circulation lifetime of nanoparticles by sterically hindering the binding of plasma protein onto the nanoparticle surface and thereby slowing down its recognition by RES.\textsuperscript{55, 131-134} Polymers that have been used as stealth layers are poly(vinyl pyrrolidone) (PVP), poly(acrylamide) (PAA), poly(vinyl alcohol) (PVA) and, in particular, poly(ethylene glycol) (PEG). PEG is the most commonly used protective polymer owing to its high water solubility and the high flexibility of its polymer chains.\textsuperscript{135-137} These characteristics provide a protective cloud over the surface of the liposome, repulsing plasma components out of the PEG layer and making it impermeable to serum opsonin adsorption. In addition, PEG exhibits low toxicity and does not form toxic metabolites.\textsuperscript{136} It was found that PEG with molecular weight less than 40 kDa can be rapidly removed from the body through the kidneys. PEG used for liposomal preparations is usually conjugated to lipids to allow facile incorporation within lipid bilayers. Currently, three main strategies have been described for the inclusion of stealth layers, known as PEGylation, into liposomes: pre-modification, post-insertion, and post-conjugation. Firstly, pre-modification refers to the incorporation of PEG-lipid conjugates during liposome formation prior to the addition of nucleic acids.\textsuperscript{138, 139} Although this technique is considered the simplest method for the preparation of stealth liposomes, it has some disadvantages. Introduction of the PEG layer prior to liposomal formulation renders an equal possibility that the polymer chains will not only be present on the liposome surface but also trapped inside the lipid bilayers. This might result in insufficient protection of the nanoparticles from opsonisation. In particular, using this technique to incorporate targeting moieties reduces the number of exposed ligands on the outer surface of the liposomes, significantly impairing their targeting function. Secondly, post-insertion implies the incorporation of PEG-lipids in preformed nanoparticles. The nanoparticles are prepared by condensation of nucleic acids into cationic liposomes, and then incubation with PEG-lipid micelles. The spontaneous insertion of PEG-lipid micelles onto the external surface of liposomes occurs as a result of relieving micellar strain.\textsuperscript{140, 141} Examples of commercially available PEG-lipid conjugates commonly employed in this method are 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (MeO-PEG\textsubscript{2000}-DSPE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (MeO-PEG\textsubscript{2000}-}
DPPE). The post-insertion technique allows for the presence of the polymer on the exterior surface of liposomes. However, further purification is required, especially when modifying with targeting ligands. Non-incorporated ligands can block the receptors, preventing binding of the ligand-bound nanoparticles to the receptors, and therefore need to be removed.\textsuperscript{142} Lastly, post-conjugation involves the chemical conjugation of PEG molecules to preformed nanoparticles via corresponding functional groups. Similar to post-insertion, this method allows PEG polymers to be located on the outer layer of the nanoparticles. The coupling efficiency relies on the selectivity and reactivity of the coupling reaction. Examples of conjugating methodologies are amide coupling, aminooxy coupling, and maleimide coupling.\textsuperscript{143} Similar to post-insertion, this technique requires further purification.

To maximise the stealth effect, the molecular weight and grafting density of PEG are important criteria. Incorporation of a PEG layer should provide sufficient coverage for the nanoparticles to resist aggregation, reduce opsonisation, and prevent consequent uptake by RES. The PEG layer is known to exhibit two different conformations on the liposome surface, which are called “mushroom” and “brush” (Figure 1-19).\textsuperscript{144-146} These regimes are governed by the Flory dimension ($R_f$), which refers to the volume occupied by each PEG cloud and is dependent on distance (D) between the adjacent chains. When $D > R_f$, the PEG grafting density is low, the polymer chains are far from each other and able to fold in on themselves to adopt a random coil-like configuration, called the mushroom regime. With an increased PEG grafting density, when $D < R_f$, each polymer chain encounters a packing pressure and is forced to adopt a more extended conformation, referred to as the brush regime. In the case of PEG\textsubscript{2000}, at lower than 4 mol\% grafting density, PEG exhibits the mushroom conformation, whereas the brush configuration is formed when the grafting density is higher than 4 mol\% of total lipid content.\textsuperscript{147}
Of the two regimes, the brush conformation is believed to provide the most resistance to plasma protein adsorption owing to the extension of the polymer chain away from the grafting surface, generating greater protein repulsion out of the PEG layer.\textsuperscript{134, 148, 149} Inclusion of PEG beyond its maximum loading is known to cause disruption of PEG-lipids from the lipid bilayers due to increased repulsion forces between each PEG chain.\textsuperscript{150-152} This dissociation usually results in the formation of micellar aggregates, either as small disks or spherical micelles. For PEG\textsubscript{2000}-lipid, 5-7 mol\% of total lipid concentration is the saturated density at which PEG can be incorporated into bilayers before being transformed into a micellar-like structure. The dissociation results in a liposome surface partially covered by a lesser PEG density, which markedly diminishes the delivery potential of the nanoparticles.
1.4.4 Targeting layer (D)

Apart from the three main layers of liposomal constitutions – the nucleic acid core, lipid envelope layer, and stealth layer – the targeting layer (D) also offers a potential advantage for site-specific binding to target cells to selectively improve cellular internalisation and reduce possible toxic side effects to non-target cells. Targeting ligands should be designed to exhibit high specificity to the receptors that are uniquely expressed or overexpressed on the cell of interest, but minimally expressed on normal cells. In addition, ligands should also have high affinity for the corresponding receptors and be able to induce receptor-mediated endocytosis. Ligands/receptors employed for nucleic acid delivery can be antibodies, growth factors, cytokines, proteins, and peptides. For cancer targeting, examples of receptor targets that have been extensively studied include the transferrin receptor (TfR), the human epidermal growth factor receptor 2 (HER-2), the folate receptor (FR) and the prostate-specific membrane antigen (PSMA) receptor. The targeting moiety should be stable while circulating in vivo so that it is able to interact with the receptor and accumulate in target tissues.

The modification of liposomes with the layer of protective polymer and targeting ligand demonstrates several advantages including increased circulation lifetime, decreased macrophage uptake, specific targeting to certain pathological sites, and enhanced cellular uptake. Long-circulating nanoparticles may be preferable for efficient accumulation at the target tissue (tumour); however, the presence of a stealth layer on liposomes inhibits cell binding and subsequent release of the internalised nanoparticles from the endosome into the cytoplasm. This observation suggests that the stealth layer may not always be favourable, especially for cellular internalisation of the nanoparticles. A strategy that enables the triggerable detachment of PEG is necessary to overcome steric hindrance in cell membrane binding and endosomal membrane fusion which may lead to improved therapeutic efficacy.
1.5 Triggerable modification of stealth liposomes via stimuli sensitivity

Surface modification with a PEG layer has been shown to be essential to prolong the circulation half-lives of nanoparticles. This characteristic, in conjunction with limited nanoparticle sizes (between 100-200 nm), enables them to preferentially accumulate in tumour cells. However, PEG has been found to inhibit the internalisation and endosomal escape of the nanoparticles, resulting in significant loss of transfection efficiency.\textsuperscript{157, 158} The use of PEG therefore creates a dilemma: it is required for altering the pharmacokinetics of the nanoparticles, but it poses a problem for cellular interaction with tumours. A potential triggerable mechanism capable of detaching PEG once deposited at the target tissues (pre-internalisation) or after internalisation (post-internalisation) would restore to it the therapeutic effect of nucleic acid delivery.

Recently, work has been published concerning the development of long-circulating liposomes by attaching PEG in a detachable fashion in order to improve endocytosis and endosomolysis of the nanoparticles. One approach is the use of diffusible PEG-lipid conjugates\textsuperscript{159, 160}, which can be dissociate from liposomes over time once presence in blood circulation, revealing their surface positive charges that allow for interaction with anionic cell membranes. The rate of diffusion varies according to half-life of lipid anchor. Shorter lipid anchors are found to dissociate more quickly from the bilayer. Optimisation of the length of lipid anchor, conjugated to PEG, is required in order to achieve a controllable rate of dissociation that benefits both stability and transfection efficiency of the nanoparticles. Other approaches for triggered release of the PEG coat that are more extensively studied include the use of extrinsic physical stimuli, such as heat, light, and ultrasound\textsuperscript{161, 162} and intrinsic stimuli associated with pathological sites, such as pH, redox conditions, and specific enzymes. Physical stimuli or triggers, usually combined with an imaging technique, are useful in the design of treatments for primary tumours, but less for metastases. This leaves the intrinsic stimuli as the preferable choice for triggerable release of the stealth layer at tumour tissues. Triggerable liposomes are usually achieved by conjugation of a suitable sensitive linker between the PEG polymer and lipid anchor within liposomes. This modification results in a multifunctional nanoparticle that is able to exhibit long circulation \textit{in vivo} and a stealth layer that is removable when exposed to appropriate stimuli.
1.5.1 pH triggered release

An acidic environment is found in tumours and endosomal compartments. The pH in tumours may drop to 6.5, further decreasing inside the endosomes to around 5.\textsuperscript{161} This difference in pH from the physiological value (pH 7.4) is sufficient to be utilised as a triggerable mechanism for PEG detachment through a labile linker capable of degradation in acidic conditions. A number of pH-sensitive liposomes have been developed. Examples of acid labile linkers that have been reported include hydrazones, diorthoesters, vinyl ethers, and cis-aconitic and oxime moieties.

A series of diorthoester-linked PEGylated lipid conjugates (POD) (Figure 1-20) with three different PEG chain lengths (750, 2000, and 5000 Da) was reported by Szoka et al.\textsuperscript{163} The POD-stabilised nanoparticles were shown to be relatively stable at pH 8.5, but rapidly destabilised at pH 5 in approximately 10 min. Improved \textit{in vitro} transfection efficiency was mediated by POD-contained nanoparticles. Among the diorthoester derivatives being tested, PEG\textsubscript{2000} nanoparticles were shown to be the most effective system with respect to particle size, stability, transfection efficiency, and cytotoxicity.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{diorthoester.png}
\caption{General structure of the diorthoester described by Szoka et al.}
\end{figure}

Boomer and co-workers reported the successful detachment of the PEG coat from liposomes by acid catalysed hydrolysis of vinyl ether linker (Figure 1-21) at pH 4, while minimal degradation of the linker was observed at pH 7.4.\textsuperscript{164} The degradation half-life of the sterically stabilised liposomes containing vinyl ether linker was reported to be 25
min. The nanoparticles showed improved release of the encapsulated content compared to non-hydrolysable PEGylated conjugates.

\[
\begin{align*}
\text{Figure 1-21: Structure of the vinyl ether reported by Boomer et al.}
\end{align*}
\]

A recent work by Carmona et al. demonstrated the preparation of pH-triggerable PEGylated-siRNA nanoparticles utilising chemoselective reactions for the post-coupling of a PEG polymer, PEG_{2000}(CHO)_2, to an aminoxo functional group of lipid-anchoring nanoparticles (Figure 1-22).\textsuperscript{143} The conjugation resulted in the formation of oxime bonds which are relatively stable at pH 7 but labile at pH 5.5 and below. The pH-sensitive PEGylated nanoparticles containing anti-HBV siRNA were able to mediate significant gene silencing \textit{in vivo}.
Figure 1-22: a) Structure of CPA lipid containing aminoxy functional group b) Formation of pH-sensitive oxime bond via conjugation of PEG$_{2000}$(CHO)$_2$ and aminox group of CPA.

A pH-triggerable release mechanism can be utilised for the preparation of multifunctional nanoparticles with temporarily “hidden” functions, such as cell-penetrating peptides (CPPs). CPPs, such as TAT and polyArg, have been demonstrated to facilitate intracellular internalisation.$^{165, 166}$ Temporary shielding by PEG shells of the cell penetrating function should confer long circulation times, in order to accumulate nanoparticles in target tissues as well as reduce nonspecific uptake in non-target cells. However, the protective PEG should be removed once it experiences an acidic environment at the target cell, to allow for effective internalisation. A study by Sawant et al. demonstrated that incorporation of hydrazone-linked PEGylated lipid conjugates within antibody-targeted nanoparticles containing TAT peptides resulted in relative stability at physiological pH with limited internalisation in cells. In acidic conditions (pH 5-6), loss of the PEG layer was observed due to hydrolysis of hydrazone linkers, exposing the hidden TAT peptides and thereby resulting in effective internalisation.$^{167}$
1.5.2 Redox trigger

The redox potential difference between reducing intracellular space and oxidising extracellular space can be utilised as a potentially useful stimulus for stealth layer detachment. The most well-established redox-triggered linker is that of the disulfide bond (S-S). The disulfide bond is relatively stable in the mildly oxidising environment of the bloodstream but is easily reduced to two thiols in the presence of high glutathione (GSH) concentrations inside cells. Glutathione is a cellular reducing agent, the function of which is to prevent cellular components from oxidative damage. The intracellular concentration of glutathione is approximately 100-fold higher than its normal extracellular level.\(^\text{168}\)

Kirpotin and colleagues reported the synthesis of disulfide-linked PEGylated lipid conjugate (Figure 1-23).\(^\text{169}\) The sterically stabilised DOPE liposomes containing disulfide linker were found to be stable in a serum environment for 36 h at 37 °C. However, upon incubation with a thiolytic reducing agent, dithiothreitol (DTT), the liposomes were destabilised and the entrapped dye was released. The loss of bilayer stability was presumably due to the thiolytic cleavage of disulfide bonds, leading to the detachment of PEG moiety.

![Figure 1-23](image)

Figure 1-23: The thiolytically cleavable PEGylated lipid conjugate described by Kirpotin et al.

Further *in vivo* study was carried out by Ishida *et al*. The sterically stabilised liposomes containing disulfide linker, targeting moiety, and loaded doxorubicin showed improved
therapeutic efficacy compared to the control PEGylated nanoparticles in mice. The nanoparticles, however, exhibited relatively short half-lives. It was suggested that this was due to rapid cleavage of disulfide bonds by blood components, such as cysteine, and consequent PEG removal. The loss of nanoparticle stability led to enhanced uptake by the mononuclear phagocyte system.\textsuperscript{170}

Like pH stimuli, the redox strategy can be employed to temporarily mask the ligand function. Under normal conditions, the ligands are shielded by a stealth layer but become unmasked after the protective PEG is removed by certain stimuli (Scheme 1-2). This allows for the protection of targeted nanoparticles from RES clearance and thus enhanced circulation times. McNeeley and co-workers reported that the insertion of cysteine-cleavable PEG-lipid conjugates, containing disulfide bonds, into folate-targeted nanoparticles showed prolonged circulation times of the nanoparticles \textit{in vivo}, compared to targeted nanoparticles without PEGylated disulfide lipid conjugates. The PEG coat was successfully cleaved by cysteine, and the hidden ligands were exposed to promote binding, resulting in improved cellular uptake of the nanoparticles.\textsuperscript{171}

\textbf{Scheme 1-2:} Schematic representation of multifunctional nanoparticles described by McNeeley \textit{et al.} A cleavable PEG mask targets ligands while in circulation, but is able to be detached once arriving at the target site through cleavage at a disulfide linker.\textsuperscript{171}
Chapter One: Introduction

1.5.3 Enzyme trigger system in cancer tissues

Elevated enzyme levels, of proteases in particular, are one of the unique characteristics found in tumour cells. High concentration of the enzymes plays a crucial role in tumour metastasis by degrading the basement membrane, leading to extravasation of the tumour cells to distant sites. The increased enzyme levels are also involved in the process of angiogenesis. The enzymes are capable of recognising and cleaving selectively at particular bonds or domains. This site-specific feature is considered a potentially attractive candidate for the removal of a stealth layer via a linker sensitive to enzymatic cleavage in target tissues. Some enzymes have been extensively studied to serve this purpose, such as phospholipase $A_2$, elastase, and matrix metalloproteinases.

1.5.3.1 Phospholipase $A_2$

Phospholipase $A_2$ belongs to a group of membrane-active enzymes that hydrolyse the ester linkage of the $sn$-2 acyl chain of phospholipids, liberating free fatty acids and 1-acyl-lysophospholipids (Figure 1-24). This enzyme has been reported to be upregulated in inflammatory sites and many tumours, such as breast, pancreatic, prostate, and lung cancer\textsuperscript{172}, making it an interesting site-specific trigger for PEG removal from sterically stabilised liposomes at cancer tissues. Reported by Jorgensen and co-workers, phospholipase $A_2$-mediated cleavage of various PEG-PE conjugates within liposomes has been shown to be feasible.\textsuperscript{173, 174} Their recent work has demonstrated the efficient release of encapsulated doxorubicin from liposomes containing PEG-DSPE when incubated with culture media of colon cancer cell lines containing secreted phospholipase $A_2$, whereas the doxorubicin released from Doxil® was minimal. The enzyme-degradable liposomes in the presence of the enzyme have shown higher cytotoxicity to the cancer cell lines compared to Doxil®, suggesting that degradation of the enzyme-sensitive liposomes has occurred, resulting in the release of the toxic drug.\textsuperscript{132} The use of phospholipase $A_2$ as a trigger may provide an efficient, site-specific activation leading to destabilisation of liposomal carriers and thus enhancing the delivery of therapeutic agents.
Chapter One: Introduction

The study in this thesis is concentrated on the following two enzymes: elastase and matrix metalloproteinase, which are the more well-established enzyme triggerable systems for PEG detachment at tumour sites. The following section will focus on the discussion of these enzymes with respect to their relations to cancer, their characteristics, and their application as site-specific triggers.

1.5.3.2 Elastase

Human neutrophil elastase (NE), also known as human leukocyte elastase (HLE), is one of the most widely studied enzymes for the triggered release strategy. Neutrophil elastase is a serine protease found in azurophil granules of neutrophils.\textsuperscript{175, 176} Its main function is to degrade foreign microorganisms which have invaded the body. It also has the ability to degrade a range of substrates, mainly elastin and other important connective tissue proteins such as collagen and fibronectin. While in blood circulation, the neutrophil does not cause any damage; however, on activation it rapidly secretes enzymes into the extracellular space to destroy the basement membrane in order to migrate and perform its function.\textsuperscript{177} Under normal conditions, its destructive feature is regulated by its natural protease inhibitor, $\alpha_1$-antitrypsin. The imbalance of these two counterparts, due to either a deficiency of $\alpha_1$-antitrypsin or elevated neutrophil elastase, leads to abnormally high levels of neutrophil elastase, increasing the possibility of tissue damage.\textsuperscript{175} Elevated levels of this enzyme have been detected in various types of cancers and are believed to promote tumour development and

\textbf{Figure 1-24:} Representation of the cleavage site by phospholipase A$_2$
progression by degrading the extracellular matrix around tumours and facilitating their invasion to sites of metastasis. The normal range of neutrophil elastase has been found to be 56-136 µg/L; however, its concentration varies under certain conditions, such as inflammation and some types of lung disease, and is dependent on the stage of cancer. Late stages of cancer (stage IIIIB and IV) have higher enzyme concentrations than earlier stages (stage I, II and IIIA). The enzyme level is therefore a significant prognostic factor for cancer. It has been reported that patients with breast cancer who have high levels of the enzyme have a lower chance of survival than those with low enzyme levels.\textsuperscript{175}

Neutrophil elastase is a glycoprotein composed of a single peptide chain of 218 amino acids and four disulfide bridges, with sugar chains linked to Asn-109 and Asn-159.\textsuperscript{178} It is a member of the chymotrypsin subfamily of serine proteases, which includes chymotrypsin, trypsin, and elastase. The structure of neutrophil elastase is shown in Figure 1-25.
Figure 1-25: The crystal structure of human neutrophil elastase (PDB ID 1EAS). The catalytic triad, His57, Asp102 and Ser195, is included.

The active site of the chymotrypsin subfamily typically contains three amino acid residues, termed a catalytic triad, including His57, Asp102, and Ser195, obtained from X-ray crystallography.\textsuperscript{178, 179} In some experimental studies, the catalytic triad of neutrophil elastase has been reported with different residue numbers (His41, Asp88, and Ser173).\textsuperscript{180} Each residue co-ordinately serves a catalytic role in peptide bond hydrolysis. The mechanism of the peptide hydrolysis reaction is composed of two major steps: acylation and deacylation.\textsuperscript{179} Acylation involves the cleavage of the amide bond of the peptide substrate and the formation of an ester bond between the carbonyl carbon of the peptide and the enzyme, forming an acyl-enzyme intermediate (Scheme 1-3). To enter and bind to the active site, the substrate is assisted by hydrogen bonds formed between the NH of Ser1195 and the carbonyl oxygen of the substrate, forming a non-covalent enzyme-substrate complex called the Michaelis complex. The OH group of Ser195 then performs a nucleophilic attack on the carbonyl carbon of the Michaelis complex to form a tetrahedral intermediate. The carboxylate group of Asp102 hydrogen
bonds to the imidazole ring of His57, making the N atom in the ring more nucleophilic. The electron pairs on the N atom of His57 that form hydrogen bonds to Ser195 accept protons (H⁺) from the OH group of Ser195. The intermediate reforms the carbonyl double bond and breaks the peptide bond, generating an acyl-enzyme intermediate and releasing an amine product. During the acylation step, the tetrahedral intermediate formed is stabilised by hydrogen bonding to the H atoms of the amide backbones of Gly193 and Ser195, known as the oxyanion hole. The oxyanion hole is believed to activate the cleavage of the peptide bond.

*Acylation step:*
Scheme 1-3: Reaction mechanism of the acylation step of peptide hydrolysis by proteases. This step involves the formation of an acyl-enzyme intermediate by the nucleophilic attack of the OH group of Ser195.

The second step, deacylation, involves the breakdown of the ester linkage of the acyl-enzyme intermediate and the regeneration of the enzyme active site (Scheme 1-4). The carbonyl carbon of the acyl-enzyme intermediate is attacked by a water molecule. The imidazole ring of His57 accepts a proton from the water molecule to produce another tetrahedral intermediate, which is stabilised by the oxyanion hole. The intermediate
recreates the carbonyl group, leading to the breakdown of the ester bond and the release of the carboxylic acid product. The oxygen anion of Ser195 withdraws a proton from the imidazole ring of the His57 and the hydrogen bond between His57 and Ser195 is reformed.

*Deacylation step:*
Scheme 1-4: Reaction mechanism of the deacylation step of peptide hydrolysis by proteases. In this step, an acyl-enzyme intermediate is attacked by water to form a tetrahedral intermediate and liberates carboxylic acid.

The nomenclature used for describing enzyme binding sites and substrate binding pockets was first introduced by Schechter and Berger in 1967. The individual amino acid residues of a peptide substrate are designated as Pn,...P2, P1, P1', P2',...,Pn' and the corresponding enzyme binding sites are designated as Sn,...S2, S1, S1', S2',...Sn'. The bond between P1 and P1' is called a scissile bond, where the enzyme cleavage occurs. The nomenclature demonstrated by Schechter and Berger is present in (Figure 1-26). The specificity of the enzyme generally relies on P1 and S1 interaction. S1 is a pocket located adjacent to Ser195, formed by amino acid residues. Individual chymotrypsin-like serine proteases exhibit varying specificity and this is mainly governed by the identity of the S1 pocket. Chymotrypsin shows a preference for Phe, Tyr, and Trp at the P1 position due to its hydrophobic S1 pocket formed by the combination of Ser189, Gly216, and Gly226. However, the negatively charged S1 site of trypsin created by Asp189, Gly216, and Gly 226 shows a strong favour for positively charged amino acids, such as Arg and Lys at the P1 pocket. Unlike chymotrypsin and trypsin, the presence of Val-190 and Val 216 near the top of the S1 pocket of elastase forms a relatively small hydrophobic hole, hence only small,
uncharged amino acids such as Ala and Val are preferable at the P1 position (Figure 1-27).  

![Diagram](image)

**Figure 1-26:** The Schechter and Berger Nomenclature for the description of substrate and protease interaction. The substrate subsites – each amino acid residue – are denoted as P, whereas enzyme binding sites are denoted as S. The peptide hydrolysis occurs at the scissile bond P1-P1'. The prime subsites of both substrates and enzymes are the residues of the carboxylic terminal sides.

![Representation of S1 site](image)

**Figure 1-27:** Representation of the S1 site of neutrophil elastase, showing Val190 and Val216 near the top of the pocket, allowing small, hydrophobic amino acids to fit in. Modified from Berg et al., *Biochemistry*, 2002.  

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Although the S2-Sn sites display less influence in enzyme specificity compared to the S1 site, their presence is important for the enzyme-substrate interaction. In particular, the hydrogen bonds formed between the carbonyl oxygen of Ser214 and the NH of the P1 residue, the NH of Phe215 and the carbonyl of P3, and the carbonyl of Val216 and the NH of P3, are vital for substrate hydrolysis efficiency, but to a lesser extent compared with the S1 pocket (Figure 1-28).\textsuperscript{178, 179} In the case of neutrophil elastase, the interaction between P3 and S3 was shown to improve the rate of substrate hydrolysis.\textsuperscript{187}

![Catalytic Triad Diagram](image)

**Figure 1-28:** Illustration of substrate and neutrophil elastase interaction, showing a catalytic triad (red), substrate recognition sites (blue and green), and interaction between amino acids at P1 subsite and S1 pocket.

Having similarity in structure to neutrophil elastase is its pancreatic counterpart. Pancreatic elastase is found in the pancreas and is secreted into the intestines upon activation by trypsin to participate in digestion. It has been shown to be mainly related to pancreatic diseases.\textsuperscript{188, 189} Unlike its counterpart, neutrophil elastase is widely known for its pathological role in various disease, such as cystic fibrosis, rheumatoid arthritis, inflammatory diseases, emphysema and, in particular, tumour invasion.\textsuperscript{175, 190, 191} Since
the main function of neutrophil is host defense, neutrophil elastase is essential for migration of neutrophil out of the vasculature and through the basement membrane for destruction of foreign invaders. To achieve this process, many connective tissue proteins are degraded – in particular, elastin, which is rich in small, hydrophobic amino acids, i.e. the preferable substrates for the enzyme. This destructive characteristic of the enzyme causes tissue damage which is associated with the diseases mentioned above.

In the case of cancer, degradation of the basement membrane and connective tissue by the enzyme facilitates tumour invasion and metastasis. The disease is usually observed alongside elevated levels of the enzyme, unlike in normal tissue. The high concentration of neutrophil elastase associated with tumour cells has been shown to be a suitable candidate for a site-specific trigger for the detachment of stealth layers at the tumour sites.\textsuperscript{192} The removal of PEG layers can be achieved through enzyme-mediated hydrolysis of a peptide linker, conjugated between PEG and lipid. So far, the use of neutrophil elastase for triggerable release of PEG coats to improve cell internalisation of nanoparticles has not yet been reported. However, the relevant studies that are worth mentioning are the works of Pak \textit{et al.}\textsuperscript{193} Known to enhance endosomal escape due to its ability to induce $\text{H}_\text{II}$ phase transition, DOPE does not form stable bilayers at physiological pH and is therefore too unstable for \textit{in vivo} application. In addition, DOPE-contained liposomes exhibit nonspecific fusion to cell membranes. Modification of the DOPE head group by the addition of a bulkier group renders the lipid to form a stable lamellar structure. DOPE was chemically conjugated to elastase substrate peptide, \textit{N}-acetyl-Ala-Ala (\textit{N}-Ac-AA), to generate a peptide-lipid, \textit{N}-Ac-AA-DOPE. The peptide linker not only altered the stability of DOPE but also temporarily inactivated its fusogenicity. While conjugated, the fusogenic property of the lipid was masked, and was unmasked upon activation by a specific stimulus, elastase, through the cleavage of the peptide linker. The enzyme degradation of the linker resulted in the conversion of \textit{N}-Ac-AA-DOPE to DOPE (Figure 1-29), creating a fusogenic property of liposomes on arrival at therapeutic sites.
Further study by Pak et al. demonstrated that a tetrapeptide linker, N-methoxy-succinyl-Ala-Ala-Pro-Val (MeO-suc-AAPV), exhibited greater sensitivity and selectivity for elastase cleavage compared to N-acetyl-AA. Liposomes containing Dioleoyl dimethylammonium propane (DODAP)/MeO-suc-AAPV-DOPE pretreated with HLE showed the successful delivery of an encapsulated fluorescence probe, calcein, into the cytoplasm of ECV304 endothelial cells.

Collectively, it can be postulated that conjugation of an enzyme substrate peptide between a PEG moiety and a lipid-anchoring liposome may render a strategy to detach PEG from the liposomal surface in response to specific enzyme at the site of interest, which could result in improved cell binding and promote endosomal escape of nucleic acids into cytosol.

1.5.3.3 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) have attracted much attention from researchers as a new target for cancer therapeutics owing to its direct association with tumours, which is
believed to play a major role in tumour invasion and metastasis. There has been a recent increase in the number of studies focusing on the use of the enzyme for activation of PEG detachment at tumour sites. To achieve metastasis, tumour cells have to overcome a series of sequential steps including detachment from the tumour mass, disruption of the basement membrane, entry of cells into the vascular or lymphatic system (intravasation), cell migration to distal sites and adhesion to endothelial cells, escape of cells from blood circulation (extravasation), cell proliferation, and induction of angiogenesis (Figure 1-30).\textsuperscript{195-197} The degradation of the basement membrane and extracellular matrix (ECM), consisting of connective tissues such as elastin, collagen, fibronectin, and other glycoproteins, is considered the key event in tumour metastasis. Since MMPs are capable of digesting various ECM components, the action of these enzymes is required for tumour invasion. In addition, MMPs play a crucial role in angiogenesis, which is an essential process for tumour growth. The basement membrane is degraded and the endothelial cells are induced to migrate through the digested membrane into tumour interstitium to create a lumen, which eventually turns into a blood capillary.\textsuperscript{198}

Figure 1-30: MMPs are involved in the process of tumour metastasis by degrading the basement membrane and facilitating intravasation and extravasation of tumour cells to proliferate at the distant site. MMPs are also thought to initiate angiogenesis.\textsuperscript{195}
Typically, MMPs are few or absent in normal cells; however, their elevated expression is associated with various tumours, such as breast, ovarian, and prostate tumours. An increased level of MMPs has been found to be related to the stage of tumour development. The enzyme concentration increases with increasing tumour progression.\textsuperscript{195-197} Regulation of MMP function can be achieved at multiple levels including transcription, which is in response to growth factors, cytokines, and hormones; activation of zymogen by a proteolytic process to release the active enzyme; and inhibition of the active enzyme by their natural inhibitors, which are the tissue-localised tissue inhibitors of metalloproteinases (TIMPs).\textsuperscript{195}

MMPs are a family of zinc-dependent endopeptidases that are synthesised as inactive proenzymes (zymogens) which require further proteolytic processing to become active enzymes. MMPs can be divided into subgroups on the basis of their structure and specificity including collagenases, stromelysins and stromelysin-like, matrilysins, gelatinases and membrane-type MMPs (MT-MMPs).\textsuperscript{195} Over 20 human MMPs and other homologues from other species have been reported to date (Figure 1-31).\textsuperscript{199, 200} Among the identified MMPs, MMP-2 (gelatinase A, 72 kD) and MMP-9 (gelatinase B, 92 kD) are thought to be key enzymes involved in tumour metastasis and angiogenesis. This is due to their ability to degrade type IV collagen, which is the major constituent of the basement membrane, and denatured collagen (gelatin).\textsuperscript{197} The expression of MMP-2 is more constitutive while the MMP-9 level is found to be restricted, but it can be in rapidly induced under certain conditions.\textsuperscript{196} The structure of MMP-2 is shown in Figure 1-32.
**Figure 1-31:** Schematic representation of the 24 human matrix metalloproteinases (MMPs).\(^{200}\) MMPs have similar domain structures with a signal peptide to target for secretion, a propeptide to maintain latency, a catalytic domain that contains a Zn(II) binding site, a hemopexin domain, and a fibronectin region for substrate recognition.
Figure 1-32: Representation of the pro-MMP-2/TIMP-2 complex. The domain structure of the enzyme is shown: propeptide, catalytic Zn(II) (coloured red), hemopexin, and fibronectin regions. The light blue circles indicate the interaction between the proenzyme and its inhibitor.²⁰¹

MMPs share similar structural domains with zinc-binding active sites. Three His residues at the active site are required for ligating with Zn(II) ions, which is essential for catalytic activity. Glu residue also plays a role in catalytic function. The mechanism of peptide hydrolysis starts with coordination of the scissile amide bond of the peptide substrate to the catalytic Zn(II) ligated to three residues of His (Scheme 1-5).²⁰²,²⁰³ The water molecule that is hydrogen bonded to the Glu residue (Glu404 in MMP-2) attacks the carbonyl carbon of the substrate and the carbonyl oxygen anion is stabilised by the Zn(II) ion. The water molecule donates one proton (H⁺) to the NH of the scissile amide bond. The carbonyl double bond is reformed and the scissile bond is disconnected,
releasing carboxylic and amine products. The conserved Ala residue (Ala192 in MMP-2) helps stabilise the positive charge at the nitrogen of the scissile amide bond.

**Scheme 1-5:** General reaction mechanism of peptide hydrolysis by MMPs.\(^{204}\)

Recently, MMP-2 has gained much interest in cancer research due to its critical role in tumour invasion, metastasis, and angiogenesis, and its constitutive expression in many cancers, such as fibrosarcoma, lung, bladder, and colon cancer.\(^{195}\) MMP-2 offers several attractive benefits for cancer therapeutics. Of particular interest is the exploitation of the enzyme as a potential site-specific trigger for PEG detachment at tumour sites. Unlike serine proteases, the selectivity of MMP-2 is dependent on the interaction between the S1' sites and the P1' residue, as the S1' pocket is relatively deep compared with the S3-S1 subsites that form the shallow region, thus having a greater possibility of recruiting amino acid substrates. This feature is also accounted for in other MMP enzymes in the family.\(^{202}\) MMP-2 has shown specificity toward collagen, the cleavage site of which has been found to be between Gly and Leu/Ile bonds.\(^{205, 206}\) The design of the MMP-2 substrate is usually based on modification around these amino acid residues.

Exploitation of MMP-2 in triggerable release strategies to detach PEG at tumour cells has been reported in some studies. Lee *et al.* has shown that conjugation of MMP-2 specific peptide substrates, Gly-Pro-Leu-Gly-Val (GPLGV) or Gly-Pro-Leu-Gly-Val-Arg-
Gly (GPLGVRG), to doxorubicin (DOX) and PEG\textsubscript{2000}, namely, P5D and P7D respectively (Figure 1-33), resulted in cleavable PEGylated peptide-DX conjugates.\textsuperscript{207} The micelles containing P5D, P7D, doxorubicin-loaded P5D, or doxorubicin-loaded P7D exhibited longer circulation in blood plasma compared to free doxorubicin, and efficiently inhibited mouse tumour growth by about 50-72%.

\textbf{Figure 1-33:} PEGylated peptide-DOX conjugates P5D and P7D.

A MMP-2 sensitive peptide sequence, Gly-Gly-Gly-Val-Pro-Leu-Ser-Leu-Tyr-Ser-Gly-Gly-Gly-Gly (GGGVPLSLYSGGGG), conjugated between DOPE and PEG\textsubscript{2000} has been reported on by Hatakeyama \textit{et al.}\textsuperscript{208} The PEGylated peptide-DOPE was proved to be sensitive to MMP-2 cleavage. Although liposomes consisting of PEGylated peptide-DOPE exhibited high transfection activity in a MMP-2 positive cell line, HT1080, the \textit{in vivo} study highlighted the need for improvements in order to increase nanoparticle stability and maximise their transfection efficiency.

A successful multifunctional delivery to hepatocellular carcinomas (HCC), exploiting a combined strategy of an MMP-2 triggerable release system and targeting moiety, has recently been reported by Terada and colleagues.\textsuperscript{209} HCC is known to highly express asialoglycoprotein receptors, which have high affinity for galactose (Gal) ligands. However, the problem of targeting delivery to HCC is that the galactosylated liposomes can also be taken up by healthy hepatocytes, which highlights the need for further
modification. MMP-2, found to be overexpressed in HCC tissues, was used to detach the PEG layer and elicit the ligand function at the target sites, improving the selectivity of the delivery system. A MMP-2 substrate peptide, Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln (GPLGIAQ), conjugated to DOPE and PEG, was hydrolysed by MMP-2. MMP-2 pretreated, Gal-targeted liposomes containing the cleavable PEG-peptide-lipid conjugate showed increased uptake in HepG2 cells compared with the non-cleavable counterpart and the targeted liposome, suggesting that the PEG layer was cleaved by MMP-2 and the liposomes were internalised via receptor-mediated endocytosis.

The exploitation of upregulated enzymes associated with tumours provides effective advantages not only for triggerable release of the stealth layer but also for targeting of tumour cells. The sterically stabilised nanoparticles bearing enzyme sensitive linker are able to retain their stability while in blood circulation, but are destabilised after arrival in tumour tissues as the PEG coat is removed as a result of peptide hydrolysis by the overexpressed enzymes, consequently improving cell uptake (endocytosis) and subsequent endosomal escape of the internalised nanoparticles. In addition, since the enzymes are expressed at high levels in tumour tissues, unlike in normal cells, this feature may be useful for site-specific delivery of the encapsulated agent, reducing non-specific interaction with non-target cells.
1.6 Thesis aim and objectives

The aim of this project is to design, synthesise, and characterise enzyme-sensitive PEGylated lipid conjugates that address the limitations of stealth liposomes: poor endocytosis and endosomolysis.

The use of a PEG layer presents a dilemma: it is necessary for prolonged nanoparticle stability, since it confers greater resistance to opsonisation and thus slows down RES recognition; however, its presence at the target site is unfavourable as it significantly inhibits cellular interaction and, as a result, diminishes transfection efficiency. To attain the beneficial stealth property while in the circulation, and maintain the efficacy of the therapeutic agent when it reaches the tumour tissues, a site-specific triggerable release mechanism for PEG detachment may improve cell trafficking of the nanoparticles. Of the several possible stimuli that could be employed for this purpose, an enzyme trigger is the preferred option in this study. The elevated enzyme levels associated with tumours, in particular leukocyte elastase (neutrophil elastase) and MMP-2, are found to facilitate tumour invasion and metastasis. This feature could be employed as a potential site-specific trigger for the removal of the stealth layer.

In this study, a series of enzyme-sensitive PEGylated peptide-lipid conjugates were synthesised by conjugation of PEG polymer to a lipid via a peptide linker, either AAPV or GPLGV, sensitive to either human leukocyte elastase (HLE) or MMP-2 respectively. The ability of the PEGylated compounds to detach the PEG layer through enzymatic cleavage of the peptide linker was investigated using analytical techniques. The physicochemical properties of the formulated nanoparticles containing the PEGylated peptide-lipid conjugates, optimised with respect to size, surface charge, and nucleic acid encapsulation efficiency, were examined in order to ensure desirable characteristics for nucleic acid delivery. The stability and cytotoxicity of the nanoparticles were also assessed, and their ability to improve in vitro nucleic acid delivery as a result of PEG detachment in response to the enzymes was then
investigated, with the hope of promoting cellular internalisation and endosomal escape of the liposomal content inside the cells.
2. Synthesis and degradation analysis of PEGylated peptide-lipid conjugates sensitive to HLE and MMP-2
Chapter Two: Synthesis and degradation analysis of PEGylated peptide-lipid conjugates

2.1 PEGylated peptide-lipid targets

PEGylated lipid conjugates bearing enzyme sensitive linkers have been incorporated within liposomes to enable both stability and, in particular, site-specific triggerable release of the stealth layer required for enhanced endocytosis. The design of the enzyme cleavable PEGylated lipid involves the coupling of the peptide linker between PEG and lipid. The peptide spacer should bear amino acid residues corresponding to the target sequence of the enzymes of interest, which are HLE and MMP-2. The peptide is conjugated to the head group of a fusogenic lipid for anchoring into lipid bilayers, and to a PEG chain for providing the stealth property when incorporated into a liposome.

According to the work reported by Pak et al. mentioned in the previous chapter, MeO-suc-AAPV-DOPE has shown great sensitivity to HLE cleavage; however, the enzyme-mediated cleavage of the PEGylated compound has not yet been demonstrated. Investigation of its enzyme cleavable ability may allow the possibility of utilising this system for enzyme triggerable release of the PEG layer at the target site. In this project, HLE sensitive PEG-peptide-lipid was synthesised by the conjugation of a short peptide sequence, AAPV, between a helper lipid and PEG. HLE shows preferential cleavage after V. Various peptide linkers have been shown to be sensitive to MMP-2 cleavage, as discussed previously. Of the peptide sequences that can be degraded by MMP-2, GPLGV is the preferred choice for the synthesis of a MMP-2 sensitive PEG-peptide-lipid target, due to the simplicity of its preparation and also due to it being the minimum sequence that matches the active site of MMP-2. MMP-2 shows specific cleavage between G and V. There are a variety of helper lipids that are suitable for incorporation within liposomes, including phospholipids and steroid-based lipids. Cholesterol, a steroid-based lipid, was recruited due to its ability to stabilise liposomal membranes by increasing packing density of phospholipid molecules, resulting in bilayers with greater resistance to destabilisation by plasma proteins. Cholesterol has minimal toxicity due to its endogenous nature. In addition, it has shown good stability in a wide range of chemical reactions. PEG polymers are commercially available in various sizes and
functionalities. The molecular weight of the polymer and its grafting density are proven to be crucial for maximising the stealth effect. Many studies have shown that longer PEG chains are more capable of preventing plasma protein adsorption and hence prolonging blood residence time of the nanoparticles\textsuperscript{213, 214}, but some studies have suggested that high grafting densities can be compensated for by low PEG molecular weight.\textsuperscript{146} The most commonly used PEG for liposome formulations is that with molecular weight of 2000, due to its ability to sufficiently prevent nanoparticles from aggregating and extend their circulation lifetimes, with varied grafting density to about 10 mol\% for \textit{in vivo} applications.\textsuperscript{84, 131, 215} PEG with molecular weight of 2000 Da is therefore a particularly good choice as a stealth layer in this study. These criteria led to the design of PEGylated peptide-cholesterol lipid targets sensitive to HLE and MMP-2, shown in Figure 2-1.

![Figure 2-1: PEGylated peptide-cholesterol lipid targets sensitive to HLE 1 and to MMP-2 2](image)

The retrosynthetic analysis of the target molecules 1 and 2 is presented in Scheme 2-1. Firstly, HLE and MMP-2 sensitive targets were obtained from the coupling of 6 to PEG acid 7. PEG acid is available in activated $N$-hydroxy succinimide (NHS) ester form, providing a facile approach when reacted with primary amines. Disconnection of the
amide bond on 6 results in the N-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5 and the peptide 3 or 4. The N-Fmoc-protected short peptides were prepared using solid phase peptide synthesis (SPPS). The lipid, N-cholesteryloxy-3-carbonyl-1,2-diaminoethane, was synthesised from commercial cholesteryl chloroformate and 1,2-ethylenediamine.

\[ \text{Scheme 2-1: The retrosynthetic analysis of target compounds 1 and 2} \]
2.1.1 Synthesis of N-Fmoc-protected peptide: substrates of HLE and MMP-2

The peptide substrates were synthesised on solid phase using H-Val-2-chlorotrityl resin 8 (Scheme 2-2). The coupling agents, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBT), and base, N,N-diisopropylethylamine (DIPEA), were used for each coupling step (Scheme 2-3). The Fmoc group was removed with 20% piperidine/DMF (Scheme 2-4) after each amino acid coupling, and deprotection was confirmed by a positive Ninhydrin test. The N-Fmoc-protected peptides were finally cleaved from the resin in mildly acidic conditions (Scheme 2-5), yielding 9 in good yield (82%) and 10 in reasonable yield (49%). The identities of the peptides were confirmed by electrospray ionisation (ESI) mass spectrometry and $^1$H and $^{13}$C NMR spectroscopy, and their purities (> 95%) were verified by reverse-phase HPLC.

Scheme 2-2: (i) Extension of peptide chain via standard Fmoc method$^{216}$ a) amino acid coupling using N-Fmoc-amino acid, HBTU, HOBT, DIPEA, DMF, rt, 45 min per coupling; b) Fmoc deprotection using 20% piperidine/DMF, rt, 10 min (twice); (ii) 0.5% TFA/DCM, rt, 4 h, 9 82%, 10 49%
Scheme 2-3: Mechanism of peptide bond formation using coupling agents
Chapter Two: Synthesis and degradation analysis of PEGylated peptide-lipid conjugates

Scheme 2-4: Mechanism of Fmoc deprotection using piperidine

Scheme 2-5: Mechanism of peptide cleavage from resin using TFA
2.1.2 Coupling of N-Fmoc protected peptide to cholesterol lipid

The protection of the amine functional group of the peptides is essential to prevent a side reaction (polymerisation of the peptide chains) that can occur otherwise, resulting in the loss of product yield. The Fmoc protection allows only a carboxylic acid terminus to react with the amine group of N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5 to yield peptide-cholesterol lipid conjugate. N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5 was prepared by a 1-step coupling reaction between commercial cholesteryl chloroformate 12 and excess 1,2-ethylenediamine 11 (Scheme 2-6).

Scheme 2-6: i) CHCl₃, rt, 18 h, 86%

The N-Fmoc-protected peptides 9 and 10 were then reacted with the free amine group of 5, using HBTU and 4-dimethylaminopyridine (DMAP) as carboxylic acid activators, to yield 13 and 14, respectively (Scheme 2-7). The reaction mixture was carried out with an acidic work-up using 4% citric acid solution, and afforded 13 and 14 in reasonable yield and purity. The presences of the conjugated products were confirmed by ESI mass spectrometry and ¹H and ¹³C NMR spectroscopy.
Chapter Two: Synthesis and degradation analysis of PEGylated peptide-lipid conjugates

Scheme 2-7: i) \( N \)-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5, HBTU, DMAP, dry \( CHCl_3 \), rt, 18 h, 13 68%, 14 74%

2.1.3 Synthesis of PEGylated peptide-cholesterol conjugates sensitive to HLE and MMP-2

To allow for PEGylation, the Fmoc-protected peptide-cholesterol conjugates 13 and 14 had to be first deprotected and then functionalised using the commercially available pre-activated PEG acid, \( \text{PEG}_{2000} \)-NHS ester (Scheme 2-8)
Fmoc deprotection of 13 and 14 were achieved using 20% piperidine/DCM solution, giving 15 and 16 in good yield. Thin layer chromatography (TLC), ESI mass spectrometry, and $^1$H NMR spectroscopy confirmed complete Fmoc removal and the formation of the deprotected products. The PEGylation was then carried out in basic conditions using DIPEA. The completion of the reaction was facilely monitored using HPLC due to the distinguishable retention time ($R_T$) of bound and unbound PEG moiety. Compounds 17 and 18 were obtained after purification in moderate yields and good purities. The reason for affording the products in moderate yield might be the
degradation of PEG-NHS ester during storage and handling, as PEG-NHS ester is sensitive to moisture and susceptible to hydrolysis. Hence, only active ester forms can react with the amine functionality of peptide-lipid conjugates, resulting in loss of product yield. The problem can be overcome by proper handling to minimise the compound’s exposure to moisture, or alternatively by use of PEG acid with activating agents such as HBTU and DMAP. The formation of 17 and 18 were confirmed by reverse-phase HPLC, matrix assisted laser desorption ionisation (MALDI) mass spectrometry, and $^1$H and $^{13}$C NMR spectroscopy. The PEGylated peptide-cholesterol (PPC) conjugates were named with respect to the specificity of the peptide attached, i.e. PP$_{HLE}$C and d PP$_{MMP-2}$C (Figure 2-2).

![Figure 2-2](image)

**Figure 2-2:** The synthesised PEGylated peptide-cholesterol conjugates: PP$_{HLE}$C 17 and PP$_{MMP-2}$C 18
Figure 2-3: a) MALDI mass spectrometry traces and b) HPLC traces of PP<sub>HLEC</sub> 17: column reverse phase C-4 protein, gradient mix A = H<sub>2</sub>O/ 0.1% TFA; B = MeCN/ 0.1% TFA; C = MeOH, 0.0 min [100% A], 15.0-25.0 min [100% B], 25.1-45.0 min [100% C], 45.1-55.0 min [100% A], flow: 1 mL min<sup>-1</sup>, ELSD detector (similar MALDI and HPLC patterns were observed for PP<sub>MMP2</sub>C 18).
2.1.4 Synthesis of control PEGylated cholesterol conjugate

In order to examine the hypothesis that incorporation of cleavable PEG within liposome should improve endocytosis of nanoparticles via enzymatic cleavage of the peptide linker, non-cleavable PEGylated cholesterol conjugate with the absence of the peptide linker was synthesised as a control for *in vitro* study. Similarly to 17 and 18, the PEGylation was carried out in DIPEA, using *N*-cholesteryl oxy-3-carbonyl-1,2-diaminoethane 5 and PEG<sub>2000</sub>-NHS ester, and afforded 19 in excellent yield (Scheme 2-9). The formation of 19 was confirmed by reverse-phase HPLC, MALDI mass spectrometry, and <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy. The resulting PEGylated cholesterol compound was named PC.

![Scheme 2-9](image_url)

**Scheme 2-9**: i) PEG<sub>2000</sub>-NHS, DIPEA, dry DCM, rt, 18 h, 98%

2.1.5 PEGylated peptide-dialkylglycylamide (C18) conjugates sensitive to HLE and MMP-2 and control PEGylated C18 conjugate

A new series of PEGylated peptide-lipid conjugates was synthesised using a saturated, long-chain hydrocarbon, dialkylglycylamide moiety (C18), in order to compare the effect of lipid anchor on enzyme-mediated cleavage, physicochemical properties, and cell uptake efficiency of nanoparticles. The PEGylated C18 conjugate series PEG<sub>2000</sub>-AAPV-C18, PEG<sub>2000</sub>-GPLGV-C18, and the control PEG<sub>2000</sub>-C18 were synthesised and supplied as a gift by Dr. Carla Prata in this project. The synthetic procedures of the PEGylated C18 conjugate series were quite similar to those of the PEGylated
cholesterol conjugate series described above; however, the synthesis of the peptides was performed by liquid phase peptide synthesis (LPPS) to scale up the synthesis, and the PEGylation was carried out using PEG acid with HBTU and DMAP, as carboxylic activators yielded in good yields and purities (Scheme 2-10).\textsuperscript{217} PEG\textsubscript{2000}-GPLGV-C18 21 and PEG\textsubscript{2000}-C18 22 were synthesised using similar synthetic procedures. The compounds 20, 21, and 22 were named as follows: PP\textsubscript{HLE}C18, PP\textsubscript{MMP-2}C18, and PC18 (Figure 2-4).

Scheme 2-10: Synthesis of PEG\textsubscript{2000}-AAPV-C18 20 i) HBTU, DMAP, dry DCM, rt, 90%, ii) 50% TFA/ dry DCM, quant., iii) HBTU, DMAP, dry DCM, iv) 20% piperidine/dry DCM, quant., v) NaHCO\textsubscript{3}, H\textsubscript{2}O/THF, 89%, vi) HBTU, DMAP, dry DCM vii) 50% TFA/ dry DCM, quant., viii) PEG\textsubscript{2000}-COOH, HBTU, DMAP, dry DCM, 20 80%
Chapter Two: Synthesis and degradation analysis of PEGylated peptide-lipid conjugates

Figure 2-4: The PEGylated peptide-C18 conjugates, PP$_{\text{HLE}C18}$ 20 and PP$_{\text{MMP-2}C18}$ 21 and the control PEGylated C18, PC18 22.

2.2 Determination of HLE and MMP-2 activities

Due to batch-to-batch variation of enzyme activity, a determination of enzyme activity is necessary to obtain reliable results in all experiments. The amount of enzyme required for cleavage studies can also be estimated from this activity.

Determination of HLE activity is based on a colorimetric assay using MeO-suc-AAPV-pNA 23 as an enzyme substrate$^{218}$ (Scheme 2-11). pNA 25 is released from the substrate as a result of enzymatic cleavage, which can be monitored at 410 nm. The HLE activity was calculated based on the calibration curve of pNA, previously established.
MMP-2 activity was determined using a fluorogenic substrate, Mca-PLGL-Dpa-AR-NH$_2$\textsuperscript{219} 26 (Scheme 2-12). The release of the product, Mca-PL-COOH 27, was monitored at $\lambda_{\text{ex}} = 328$ nm and $\lambda_{\text{em}} = 393$ nm. The MMP-2 activity was defined based on the calibration curve of 27, previously obtained.
2.3 Enzyme cleavage study of the synthesised PEGylated peptide-lipid conjugates

The PEGylated peptide-lipid conjugates, when incorporated within liposomes, should exhibit two features. Firstly, they should exhibit a stealth property, providing stability to liposomes while circulating in blood, a high salt/serum environment. Secondly, they should exhibit the ability to undergo PEG detachment by enzymes associated with tumours, promoting association/fusion with the cellular membrane and uptake, and hence enhancing the therapeutic efficacy of the encapsulated agent. To show their potential in improving endocytosis, the synthesised PEGylated peptide-lipid conjugates should undergo hydrolysis and subsequently detach the PEG layer in the presence of these specific enzymes. The ability of the compounds to be degraded by enzymes was therefore examined.

MeO-suc-AAPV-pNA 23 is a substrate generally employed for HLE activity assay, as the release of pNA after enzyme cleavage can be monitored by UV spectroscopy. To initially evaluate the effect of PEGylation on substrate selectivity, a new substrate, PEG$_{2000}$-AAPV-pNA 29, which is the PEGylated form of the original compound, was synthesised by Dr. Carla Prata (Figure 2-5) and the cleavage study was performed by Dr. Carla Prata using the original substrate 23 and the PEGylated substrate 29. The hydrolysis rates of these compounds were then compared.

![Figure 2-5: The synthesised PEG$_{2000}$-AAPV-pNA](image-url)
HLE was added to the substrates to a final concentration of 0.05 μM and the reaction was performed at 37 °C in 10 mM HEPES, 154 mM NaCl, 0.1 mM EDTA, pH 7.4. As expected, the hydrolysis rate of the PEGylated substrate 29 was significantly slower than that of the original substrate 23 (Figure 2-6). The result suggested that the presence of conjugated PEG probably affects the enzyme-substrate interaction by inhibiting the accessibility of the enzyme to the cleavage site, resulting in a decreased rate of substrate hydrolysis.

Figure 2-6: The kinetics of free pNA release from HLE catalysed hydrolysis reaction of MeO-suc-AAPV-pNA 23 and PEG2000-AAPV-pNA 29, monitored by UV spectroscopy. 0.12 mM of 23 or 0.20 mM of 29 was incubated with HLE (final concentration of 0.05 μM) at 37 °C and the reaction was carried out for 60 min.

The enzyme reactions of the synthesised PEGylated peptide-lipid conjugates were then examined. As the PEGylated compounds do not bear a UV-active group, the enzyme cleavage study was alternatively performed using reverse phase HPLC in conjunction with an evaporative light scattering detector (ELSD). ELSD is classified as a universal detector owing to its ability to detect most non-volatile compounds, including those that do not absorb UV light – for example, carbohydrates, lipids, amino acids, and polymers – like a refractive index detector (RID). However, ELSD offers advantages over RID:
Chapter Two: Synthesis and degradation analysis of PEGylated peptide-lipid conjugates

higher sensitivity, compatibility with gradient elution for faster separation and good resolution, and stable baselines that are not affected by changes in column temperature or solvent gradient. ELSD is therefore a more efficient method for analysis of multiple components.

HPLC allows the cleavage reaction to be facilely monitored due to distinguishable retention times of parent substrates and cleavage products. Since the PEGylated peptide-cholesterol conjugates, PP_{HLE}C 17 and PP_{MMP-2}C 18, were synthesised at the initial stage of the project, their enzyme hydrolysis results were discussed first. The enzyme cleavage reaction of 17 and 18 are shown below (Scheme 2-3 and Scheme 2-4).

Scheme 2-13: HLE cleavage reaction of PP_{HLE}C 17
The PEGylated compounds 17 and 18 were incubated with various concentrations of HLE and MMP-2, in appropriate buffers at 37 °C for various time intervals. After a certain incubation period, the reaction was stopped by adding TFA (to HLE reaction) or EDTA (to MMP-2 reaction) and aliquots were then subjected to HPLC for analysis. Only the peaks of the parent substrates, 17 and 18, were detected with no sign of the cleavage products. Several HPLC conditions were performed and the cleavage products, 30, 5, 31, and 32, were unable to be observed by ELSD. There are two possible explanations. First, the enzyme cleavage of the peptide linker might not happen due to the steric hindrance of the PEGylated substrates 17 and 18, which may prevent its interaction with the enzyme binding sites and thus inhibit peptide hydrolysis. Another possible explanation is that the structural arrangement of the PEGylated compounds in aqueous solution may limit the interaction with the enzymes. It is likely that the PEGylated peptide lipids form micelles, owing to the amphiphilic nature of the molecules, with cholesterol being hydrophobic and the PEG polymer being hydrophilic. Further evidence was demonstrated by the determination of the critical micelle concentration (CMC) of the compounds using a fluorescence probe, pyrene. The CMC of 17 and 18 were found to be 6 and 10 μM, respectively. The substrate concentrations employed in the enzyme reactions ranged between 0.36-0.37 mM (120-150 μL, 1
mg/mL in order to obtain good sensitivity and resolution from the technique. These concentrations are, however, above the CMC of the compounds, thus they tend to organise themselves in the micellar arrangement, where the PEG polymer forms a hydrated sphere around the cholesterol interior (Figure 1-27). In this arrangement, the cleavage site is greatly shielded by the PEG polymer due to the steric hindrance of the polymer and, consequently, prevents the enzyme from reaching to the cleavage site.

![Diagram of PEGylated peptide-lipid conjugate micelle](image)

**Figure 2-7:** An illustration of PEGylated peptide-lipid conjugate micelle

It was suggested that the introduction of the PEGylated substrates within a liposomal membrane would result in lower PEG density in a hydrated sphere compared to that of the micellar form, which may result in improved presentation of the substrate and better enzyme-substrate interaction. The compounds 17 and 18 were introduced in the lipid bilayers and their grafting densities were varied up to 10 mol% of total lipid concentration, which corresponded to the level of PEG typically employed for *in vivo* application. The stabilised liposomes were then incubated with HLE and MMP-2 respectively at different enzyme concentrations and to various time intervals. Displayed by HPLC analysis, the peaks of the parent substrates were very small compared to the peaks of other lipid compositions and buffer, owing to their relatively low percentages in
the liposome formulations. The tiny substrate peaks detected by the HPLC technique make the measurement of the degradation of the linkers difficult. The absence of hydrolysis products might be because the reaction did not occur in the first place, or because the HPLC and ELSD analysis lack the sensitivity to measure the degraded residues.

According to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory\textsuperscript{85}, for colloidal nanoparticles to maintain their stabilities and resist aggregation, the repulsive forces (electrostatic and/or steric repulsion) must be greater than the van der Waals attractive forces. Liposomes with large positive charge will repel each other and remain stable, while liposomes with low positive charge or neutral charge will tend to form aggregates. This aggregation results in increased nanoparticle sizes, which could be measured by dynamic light scattering. The theory therefore could be applied to the design of the enzyme cleavage study and the detection of the degraded products. In buffer solution, neutral liposomes containing the PEGylated substrates should retain their stability due to steric repulsion between PEG layers on the external surface of the liposomes. Conversely, in the presence of the enzyme, PEG detachment through the cleavage of the peptide linker exposes the neutral liposomes, which are susceptible to aggregation owing to the repulsive forces being less dominant than the van der Waals attractive forces\textsuperscript{87, 157}, resulting in an increase in size. The PEGylated substrates 17 and 18 incorporated into neutral liposomes at 1-5 mol% were incubated with various enzyme concentrations at 37 °C and their sizes were monitored at different time intervals. The diameters of the liposomes, however, were found to be stable while being incubated with the enzymes. This observation suggests that the enzyme-mediated cleavage of the peptide linker may not occur. According to Basanez and co-workers, the activity of phospholipase C was inhibited at PEG grafting densities above 1 mol%, which was probably because PEG hindered the enzyme from accessing its substrate.\textsuperscript{220} Terada \textit{et al.} showed that MMP-2-mediated cleavage of peptide linker was inhibited when PEGylated peptide lipid was incorporated within liposomes at 0.5-1 mol%.\textsuperscript{209} However, there was another possibility that the peptide hydrolysis might have occurred incompletely, and thus the remaining PEG may provide the nanoparticles with adequate resistance to aggregation. Torchillin \textit{et al.} demonstrated theoretically and experimentally that the presence of grafted PEG as low as 1 mol% could reduce protein
adsorption and hence confer stability to liposomes.\textsuperscript{137} In addition, Santos \textit{et al.} reported that circulation longevity of liposomes was enhanced with 0.5 mol\% of PEG grafting density compared with non-PEGylated liposomes.\textsuperscript{221}

ESI mass spectrometry was also employed, however the degradation of the PEGylated compound was not observed. Thereafter, another attempt to verify the enzymatic cleavage of the PEGylated substrates was carried out by Dr. Carla Prata using HPLC and ELSD analysis. PP\textsubscript{HLE}C\textsubscript{17} and PP\textsubscript{HLE}C\textsubscript{18}\textsubscript{20} were incubated with HLE for 2 h at 37°C. The denaturation of the enzyme was achieved by freezing the reaction mixture in N\textsubscript{2} (liq.) and aliquots were subjected to HPLC analysis. In the case of PP\textsubscript{HLE}C\textsubscript{18}\textsubscript{20}, a new tiny peak was detected around 25 and 30 min, similar to the retention time of free PEG acid (1 in Figure 2-8), suggesting that the compound might be degraded by the enzyme but to a very low extent. The retention time of the degraded residue observed was not exactly the same as that of free PEG acid, assuming that the degraded product might be corresponding to the peptide conjugated to PEG polymer. Although the peaks of both the degraded residue and the PEGylated compounds \textsubscript{20} were very tiny, this observation suggested the possibility of enzyme-mediated cleavage of the PEGylated compounds which may have occurred partially. The enzymatic degradation of the PEGylated compound \textsubscript{17}, however, was not observed.
Figure 2-8: The degradation of PP_{HLE}C18 20 after incubation with HLE (200 μL, 1.03 μM) for 2 h at 37 °C, monitored by HPLC. HPLC: column reverse phase C-4 protein; gradient mix A = H₂O/0.1% TFA; B = MeCN/0.1% TFA; C = MeOH, 0.0-10.0 min [100% A], 25.0 min [30% A and 70% B], 40.0-45.0 min [100% B], 46.0-55.0 min [100% C], 56.0-65.0 min [100% A]; flow rate 1.5 ml min⁻¹, ELSD detector.

Under the same HPLC condition, the MMP-2-mediated cleavage of PP_{MMP-2}C18 18 and PP_{MMP-2}C18 21 were not detected, suggesting that the peptide cleavage may not occur. This might be a consequence of poor substrate-enzyme interaction, which could result from the presence of PEG or unsuitable peptide linker. To examine if PEG inhibits the peptide hydrolysis, synthesis of the peptide substrate conjugated to chromophore, such as pNA, may provide a mean to facilely monitor the hydrolysis reaction. Since MMP-2 cleaves within amino-acid chain, GPLGV, resulting in di-peptide derivatives conjugated to pNA, which is unable to be detected by UV spectrometry. The synthesis of GPLGV-pNA or PEG₂₀₀₀-GPLGV-pNA might not be advantageous to the enzyme cleavage study.
In conclusion, the synthesis of the PEGylated peptide-lipid conjugates sensitive to HLE and MMP-2 was achieved and the enzyme cleavage study of the compounds was examined using analytical techniques. So far, only the enzyme cleavage of PEG$_{2000}$-AAPV-pNA 29 and PP$_{HLE}C18$ 20 were shown by UV and HPLC respectively, while the degradation of the rest of the PEGylated compounds was not observed. The presence of PEG in the peptide conjugate may inhibit the enzyme-substrate interaction, as seen in the case of MeO-AAPV-pNA 23 and PEG$_{2000}$-AAPV-pNA 29. The overall results obtained from the enzyme study may suggest two possibilities. First, the degradations of the PEGylated peptide-lipid conjugates were not feasible as PEG might prevent the accessibility of the enzyme to the substrate, resulting in inhibited peptide hydrolysis. In this case, modification of the peptide linker, either by coupling small amino acids that do not affect the catalytic activity of the enzymes to the original peptide linker or by conjugating repeated unit of the active peptide sequence between PEG and the lipid, may alter substrate availability and ultimately improve enzyme-substrate interaction. The micellar formation of the PEGylated peptide-lipid conjugates in aqueous solution could potentially block the accessibility of the enzyme to the cleavage site due to steric hindrance of the PEG layer. Another plausible explanation for no observation of the degraded linker was the detection limit of the techniques employed. According to the enzymatic cleavage of PP$_{HLE}C18$ 20, the degraded residues shown by HPLC were detected with very low intensities, suggesting that the degradation of the substrate may have occurred partially. It would be possible that the other PEGylated compounds might have been degraded by the enzyme but could not be detected. Future work on enzyme cleavage studies could involve the use of a colorimetric assay, such as TNBS (2,4,6-trinitrobenzenesulfonic acid) or fluorescamine to detect the cleavage of the PEGylated compounds. The assay is used for quantitating the amount of protein or peptides in solution by rapidly react with primary amine to form an adduct which can be detected by UV, or a fluorescent product and the assays could therefore be adopted for the detection of the degrade peptide linker. The technique may also allow for the use of the substrate concentration below its CMC in order to prevent micellar formation. The use of fluorescent PEG, such as Rhodamine-PEG, conjugated with peptide and lipid may provide an alternative means to detect the degradation of the PEGylated
compounds. The enzyme reaction of the fluorescently labelled PEGylated peptide-lipid may be carried out using a dialysis membrane with a certain molecular weight cut-off (MWCO), allowing only the degraded residue which is fluorescent PEG-peptide conjugate to pass through. This experiment will allow facile monitoring of the enzyme reaction by diffusion of coloured fluorescent residues out of the dialysis membrane.

**Figure 2-9:** Schematic illustration of enzyme-mediated cleavage of fluorescent PEGylated peptide-lipid in dialysis chamber that allows only for the passage of fluorescent PEG-peptide residue out of the dialysis membrane.
3. Biophysical and in vitro biological evaluation of PEGylated peptide-lipid conjugates: DNA system
3.1 Proof of concept study of enzyme-assisted pDNA delivery in vitro

Although the ability of the PEGylated peptide-lipid conjugates to undergo enzymatic degradation remained unclear using the analytical techniques, it was decided to further investigate their ability to promote pDNA delivery in vitro. PP\textsubscript{HLE}C was incorporated into DODAG/DOPE liposomes in order to obtain PEGylated liposomes bearing the short peptide linker sensitive to HLE cleavage. The choice of DODAG/DOPE formulation to test the cleavage hypothesis is based on the fact that this formulation has been shown to be optimum and generic for in vitro cell transfections in our lab. In these initial biological tests, 50 mol\% DODAG/DOPE lipoplexes containing either PP\textsubscript{HLE}C or control PC (1 and 5 mol\%) was examined for their pDNA transfection efficiency in the presence and absence of HLE in OVCAR-3 (human ovarian carcinoma cell lines). The pDNA used in this study was pEGFPLuc encoding for enhanced green fluorescence protein (EGFP) and luciferase protein. The detail of this pDNA will be discussed later in this chapter. Monocytic cell lines, such as U937 and THP-1, are found to secrete HLE upon PMA (phorbol 12-myristate 13-acetate) stimulation. However, these suspension cell lines are known to be difficult to transfect due to less contact of nanoparticles with cell membranes, leaving adherent cell lines, such as OVCAR-3 as an alternative choice for the in vitro experiment. The nanoparticles were divided into two groups: HLE-treatment and non-treatment, in order to verify the effect of the enzyme on the transfection efficiency of these nanoparticles. The result demonstrated that incorporation of PP\textsubscript{HLE}C into DODAG/DOPE nanoparticles resulted in increased gene expression in response to HLE compared to non-treated PP\textsubscript{HLE}C nanoparticles ($p < 0.05$), and this enhancement was more pronounced when compared to non-cleavable PC nanoparticles ($p < 0.05$) (Figure 3-1). The improved gene expression may be a consequence of PEG detachment via enzymatic cleavage of the peptide linker, thus promoting endocytosis. This encouraging result suggested that although the cleavage of the peptide linker was not observed with the techniques discussed in the previous chapter, the use of an enzyme triggerable system to mediate nucleic acid delivery may be possible in a biological system in vitro. We therefore decided to further investigate the possibility of using nanoparticles containing the PEG-peptide-lipid conjugates to improve pDNA delivery in response to specific enzymes.
Chapter Three: Biophysical and in vitro biological evaluation of PEGylated peptide-lipid conjugates: DNA system

Figure 3-1: Preliminary HLE transfection data of 50 mol% DODAG/DOPE nanoparticles, containing non-PEG, PP_{HLE}C (1 and 5 mol%), and PC (1 and 5 mol%), with HLE-treatment (black) and non-treatment (white), in OVCAR-3 cell lines. The transfection efficiency was expressed as % RLU/mg protein of non-PEGylated control with HLE-treatment. Each result represents the mean ± S.D. (n = 3), * indicates $p < 0.05$. (In collaboration with Dr. Mathieu Mevel)

3.2 Liposome formulation

For efficient nucleic acid delivery to tumours, nanoparticles should be formulated such that they have favourable properties including size, surface charge, nucleic acid encapsulation efficiency, and stability. However, these characteristics mainly allow for their long circulation and effective accumulation at the target sites. To maximise cell uptake, the stabilised nanoparticles may require a PEG–dissociation strategy to elicit the electrostatic ability required for endocytosis. Therefore, the PEGylated peptide-lipid conjugates sensitive to HLE and MMP-2 introduced into liposomes were assessed for their ability to improve endocytosis through an enzyme-cleavable linker. The immunogenicity and cytotoxicity of liposomal carriers in vitro might limit their viable...
application *in vivo*. Thus, the effect on cell viability of the added nanoparticles was also assessed in this study.

Cationic liposomes generally consist of a cationic lipid and a neutral co-lipid, each of which serves different functions essential for the delivery process. The cationic lipid incorporated into the formulation confers a positive charge to the liposomes, which is required to bind nucleic acids and promote cellular binding and internalisation via electrostatic interactions with anionic cell surfaces. The cationic lipid employed in this study was \( N',N'-\text{dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide} \), known as DODAG. It is a novel cationic lipid synthesised by our group, composed of a polyamine head group and dialkylglycylamide lipidic tails. It has demonstrated efficient *in vitro* pDNA delivery to many cell lines and also *in vivo* siRNA delivery to the liver.\(^{116}\) Unlike the conventional cationic lipid DOTAP, the polar head group of DODAG consists of three amine groups that can be protonated, allowing for more cationic charges available for condensing nucleic acids. Although a cationic lipid is critical for improved cellular entry, its high molar percentage has been associated with increased toxicity.\(^{222, 223}\) Another important lipid constituent is a neutral co-lipid. It is believed to facilitate cellular internalisation and the endosomal escape of nucleic acids into the cytoplasm. DOPE is the most commonly used neutral co-lipid, due to its fusogenicity which is associated with a tendency to adopt an inverted hexagonal (H\(_{\text{II}}\)) phase.\(^{121, 122}\) Despite being shown to enhance *in vitro* transfection efficiency, DOPE poses a problem for *in vivo* use as its fusogenic nature decreases nanoparticle stability as a result of increased aggregation in serum environments.\(^{224, 225}\) Other helper lipids, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol, are therefore more attractive candidates for the liposome formulation in this study. Owing to its cylindrical shape, DOPC is thought to allow the formation of the stable and small nanoparticles essential for long circulation.\(^{226}\) Cholesterol is known to increase liposome stability due to its ability to decrease membrane permeability and prevent plasma protein adsorption.\(^{124, 125}\) Replacement of DOPE with cholesterol has been shown to increase the level of gene expression *in vivo*.\(^{127, 224, 225}\) Because of these reasons, DOPC and cholesterol were chosen as co-lipids in this study. The structures of lipids employed in liposome formulation are presented in Figure 3-2.
Figure 3-2: The structures of lipids used in liposome formulation, a cationic lipid DODAG 33, and neutral co-lipids, DOPC 34 and cholesterol 35.

In this study, we compared the effect of a high concentration of the cationic lipid (50 mol% DODAG), termed a DODAG-high formulation, and a low concentration of the cationic lipid (20 mol% DODAG), termed a DODAG-low formulation, with respect to physicochemical characteristics, in vitro transfection efficiency, and stability. Since this study aims to improve endocytosis of the steric stabilised nanoparticles through enzyme-mediated cleavage of the peptide linker conjugated to PEG, the effect of PEG grafting densities of the PEGylated peptide-lipid conjugates (1 and 5 mol%) were also examined and compared to the PEGylated lipid control and non-PEGylated nanoparticles.
Figure 3-3: A schematic diagram illustrating detachment of the PEG layer in response to proteolytic enzymes overexpressed in tumour cells via the cleavage of the peptide linker (green diamond) leading to increased uptake (left and centre), whereas non-cleavable PEGylated nanoparticles, with no peptide linker, are unfavourably taken up by cells (right).

3.3 DODAG-high formulation with PEGylated peptide-cholesterol conjugates

In this formulation, the cationic lipid DODAG was introduced into lipid bilayers at 50 mol% of the total lipid concentration, as this concentration was shown to enhance gene expression \textit{in vitro} with minimal cytotoxicity.\textsuperscript{116} Inclusion of cholesterol at 30 mol% has found to increase liposome stability and extend their blood circulation half-lives.\textsuperscript{124} This amount of cholesterol is thus considered optimal for the formulation of long-circulating nanoparticles. Since the steric hindrance of PEG depends on its grafting density and could significantly affect both the enzyme-substrate interaction and the cellular uptake, different molar percentages of the PEGylated compounds (PP\textsubscript{HLE}C\textsubscript{17} and PP\textsubscript{MMP-2}C\textsubscript{18}, and the PEGylated control PC\textsubscript{19}), 1 and 5 mol%, were examined. These left the remaining molar percentage for the DOPC component. After detachment of the polymer through enzyme-mediated cleavage of the linker, the nanoparticles should show similar cell internalisation to their non-PEGylated counterparts. The non-PEGylated liposomes
were thus prepared as a control for in vitro study. The lipid compositions in a high-charge formulation are presented in Table 3-1.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Molar % of total lipid</th>
</tr>
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<tbody>
<tr>
<td>DODAG</td>
<td>50</td>
</tr>
<tr>
<td>DOPC</td>
<td>20 (or 19 or 15)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>30</td>
</tr>
<tr>
<td>PP&lt;sub&gt;HLE&lt;/sub&gt;C 17, PP&lt;sub&gt;MMP-2&lt;/sub&gt;C 18 or PC 19</td>
<td>0 (or 1 or 5)</td>
</tr>
</tbody>
</table>

**Table 3-1:** Lipid compositions in DODAG-high formulation

The PEGylated conjugates were introduced into liposomes using pre-modification technique. Although incorporation of PEG by this method might result in its partial entrapment in the liposomal interior, the technique is considered simple and adequate for a proof of concept study of enzyme triggerable release of the stealth layer to promote cell uptake in vitro. The liposomes were prepared using a standard technique of lipid film hydration. The combined lipid solutions were evaporated to obtain a thin lipid film, which was then hydrated in buffer and sonicated to yield unilamellar vesicles. The pDNA nanoparticles could typically be produced by mixing an appropriate volume of pDNA (pEGFPLuc, 6.4 kb) with the preformed liposomes under heavy vortex.
Figure 3-4: Illustration of the assembled PEGylated peptide-lipid nanoparticles.

3.3.1 Physicochemical characterisations of DODAG-high nanoparticles

Since the efficiency of nucleic acid delivery depends on the physicochemical properties of the delivery system, including nucleic acid encapsulation efficiency, size, and surface charge, optimisation of these properties is necessary to maximise the pharmacological activity of the nanoparticles. The physicochemical characteristics of the formulated nanoparticles were examined as a function of the lipid: pDNA weight ratio.

3.3.1.1 pDNA encapsulation efficiencies

To maximise the therapeutic effect of delivered pDNA, it is crucial to determine the optimal lipid to pDNA ratio required to fully protect pDNA from nuclease degradation and retain its integrity. Since pDNA condensation is facilitated by electrostatic interaction between positively charged liposomes and negatively charged nucleic acids, pDNA encapsulation is mainly dependent on the charge ratios of lipid: DNA, which corresponds to their weight ratios. The higher the amount of the lipid, the more the positive charges presence for interaction with pDNA. At optimal lipid: pDNA ratio, high
efficiency of pDNA entrapment will be achieved, indicating that most of pDNA is protected from external environment. The encapsulation of pDNA in liposomes was determined using propidium iodide (PI) assay. PI is a nucleic acid intercalating agent that exhibits enhanced fluorescence (20- to 30-fold) upon binding to DNA bases. Since the dye is membrane impermeant, the amount of non-entrapped pDNA, including surface-bound pDNA, can be quantified. This amount can then be converted to the amount of encapsulated pDNA, i.e. the encapsulation efficiency. pDNA nanoparticles containing PP_{HLE}C, PP_{MMP-2}C and PC, and non-PEGylated nanoparticles were prepared at various lipid: pDNA w/w. The encapsulation efficiency of pDNA is presented as a percentage of the amount of pDNA initially used in the preparation (Figure 3-5).

\[ \text{Figure 3-5: pDNA encapsulation efficiency of DODAG-high nanoparticles with non-PEG, 1 and 5 mol\% PP}_{HLE}C, 1 and 5 mol\% PP_{MMP-2}C, and 1 and 5 mol\% PC at various lipid: pDNA w/w (1 ug pDNA), using propidium iodide assay.} \]

The result demonstrated that the increased encapsulation efficiency was associated with increased lipid: pDNA w/w, owing to the higher cationic charge available for interaction with pDNA. 1 mol\% PP_{HLE}C, 1 mol\% PP_{MMP-2}C, and 1 mol\% PC nanoparticles showed almost complete pDNA entrapment at weight ratio of 4, showing that the inclusion of 1 mol\% PEG and the presence of the peptide linker did not affect
the nucleic acid encapsulation efficiencies. At 5 mol% PP\textsubscript{MMP-2}C and 5 mol% PC nanoparticles, the pDNA entrapment was nearly to completion at weight ratio of 12, suggesting that increased PEG density decreases surface charge presence to bind to pDNA. Thus, higher concentrations of cationic lipid are needed for complete pDNA encapsulation. Unlike their 5 mol%-PEGylated nanoparticle counterparts, 5 mol% PP\textsubscript{HLE-C} nanoparticles exhibited more than 80% encapsulation efficiency at weight ratio of 4. The only differences between these PEGylated liposomes are the types of peptide linkers (AAPV and GPLGV) and the presence and absence of the linkers. All amino acid residues have neutral hydrophobic side chains, suggesting that there is no contribution to the nanoparticle zeta potential from the presence of the peptide linker. However, the result suggested that there might be an interference of the peptide linker with the pDNA encapsulation that requires further investigation. For all formulations, pDNA encapsulation went to completion at weight ratio of 12, and no significant change in entrapment efficiency was observed at weight ratios higher than 12.

The pDNA encapsulation efficiency was further visualised and confirmed by agarose gel electrophoresis. During electrophoresis, non-encapsulated pDNA bearing negative charge due to their phosphate backbones migrate towards the positively charged electrode, which is shown by the migration band. A lack of migration band indicates complete encapsulation, which means that pDNA is retained in the liposomes (Figure 3-6).
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Figure 3-6: 0.8% agarose gel electrophoresis demonstrated pDNA encapsulation of DODAG-high nanoparticles with a) non-PEG, b) 1 mol% PP_{HLE}C, c) 1 mol% PP_{MMP-2}C, d) 1 mol% PC, e) 5 mol% PP_{HLE}C, f) 5 mol% PP_{MMP-2}C and g) 5 mol% PC at different lipid: pDNA w/w (gel a): lane 1 = DNA ladder, lane 2 = pDNA, lane 3 = empty liposomes, lane 4-7 = lipid: pDNA w/w of 1:1, 4:1, 8:1 and 12:1; gel b-g): lane 1 = empty liposomes, lane 2-5 = lipid: pDNA w/w of 1:1, 4:1, 8:1 and 12:1). The electrophoresis was performed at 65 mV for 180 min and the gels were visualized under UV light using Alliance 4.7 UVITEC Cambridge.
The result from gel electrophoresis was similar to the finding of the PI assay. In the case of non-PEGylated, 1 mol% $\text{PP}_{\text{HLE}}\text{C}$, 1 mol% $\text{PP}_{\text{MMP-2}}\text{C}$, and 1 mol% PC nanoparticles, encapsulation of pDNA was completed at a weight ratio of 4, whereas increased molar percentage of PEG required higher cationic lipid concentrations. 5 mol% $\text{PP}_{\text{HLE}}\text{C}$ showed almost complete encapsulation at weight ratio of 4, which was in line with the finding of the PI assay, while pDNA entrapment by 5 mol% $\text{PP}_{\text{MMP-2}}\text{C}$ and PC went to completion at weight ratio of 8, slightly lower than that obtained from the PI assay. Overall results demonstrated that pDNA was fully encapsulated in all liposome formulations at weight ratio of 12.

Preparation of pDNA nanoparticles by direct mixing of the preformed PEGylated liposomes and pDNA solution resulted in efficient entrapment of nucleic acids. According to the results obtained from the PI assay and agarose gel electrophoresis, 12:1 lipid: pDNA w/w was found to be optimal to achieve complete pDNA encapsulation. This ratio was therefore employed for all experiments involving DODAG-high formulations.

### 3.3.1.2 Size measurements

According to the EPR effect, only nanoparticles with small diameters, 100-200 nm, are capable of passage through the tumour-vasculature fenestrations, and are thus able to accumulate in tumour tissues. Nanoparticles with diameter above 300 nm (with smaller surface curvature) are susceptible to opsonisation and uptake by Kupffer cells and spleen macrophages. The size of the nanoparticles is therefore one of the most important characteristics required for effective delivery to tumours. pDNA nanoparticles were prepared as described at 12:1 lipid: pDNA w/w. Size measurements were then performed using a photon correlation spectrometer (PCS) by characterisation of scattered light as a result of the Brownian motion of spherical nanoparticles and size distribution obtained was intensity size distribution. The diameters of liposomes ranged from 90-130 nm (Figure 3-7). The sizes of 1 mol% $\text{PP}_{\text{HLE}}\text{C}$, 1 mol% $\text{PP}_{\text{MMP-2}}\text{C}$, and 1 mol% PC nanoparticles appeared similar to that of their non-PEGylated counterparts. 5 mol% $\text{PP}_{\text{HLE}}\text{C}$, 5 mol% $\text{PP}_{\text{MMP-2}}\text{C}$ and 5 mol% PC exhibited similar size ranges and
were approximately 20-25 nm larger than their non-PEGylated counterparts. These observations might be related to conformation of PEG on nanoparticle surfaces, which is related to its grafting density. PEG is said to adopt a mushroom conformation at low surface PEG densities (< 4 mol%), while at higher PEG densities (> 4 mol%), the polymer arranges in a brush configuration.\textsuperscript{147, 152} For PEG\textsubscript{2000}, the polymer length, defined by the Flory dimension (R\textsubscript{F}), is reported to be approximately 5.6 nm.\textsuperscript{228} The extension of PEG, incorporated at 5 mol%, into a brush regime thus increased the overall diameter compared with 1 mol% PEG, which collapses into a mushroom regime. After pDNA encapsulation, the nanoparticle diameters were determined to be within 120-180 nm and, in all cases, were larger than those of empty liposomes. This observation was supported by the work of Wasan \textit{et al.}, which found that pDNA-liposome complexes had greater diameters, ranging from 200-2000 nm, than that of their initial liposomes (\textasciitilde100 nm).\textsuperscript{229} This was probably due to the sandwiching of pDNA between lipid bilayers (predicted thickness of 10.5 nm), resulting in the formation of multilamellar vesicles, as proposed by Templeton \textit{et al.}\textsuperscript{127} The mechanism has been known for the formation of non-PEGylated cationic liposome-pDNA complexes, called lipoplexes. The formation of multilamellar vesicles was also shown to be possible in the case of PEGylated pDNA nanoparticles, demonstrated by the study of Martin-Herranz \textit{et al.} using x-ray diffraction technique.\textsuperscript{230} Variation in diameter of both liposomes and pDNA nanoparticles was observed from preparation to preparation; however, the size of liposomes and pDNA nanoparticles was found to fall in the same range as indicated above, which are 90-120 nm in the case of liposomes and 120-180 nm in the case of pDNA nanoparticles with polydispersity index of 0.2-0.7 for liposomes and 0.5-1.2 for pDNA nanoparticles.
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Figure 3-7: Average diameters of DODAG-high liposomes containing non-PEG, 1 mol% PP_HLE C, 1 mol% PP_MMP-2C, 1 mol% PC, 5 mol% PP_HLE C, 5 mol% PP_MMP-2C and 5 mol% PC, and their pDNA nanoparticles (12:1 lipid: pDNA w/w), determined by PCS. Each result represents the means ± S.D. of three measurements.

3.3.1.3 Zeta potential measurements

The surface charge exhibited by nanoparticles can affect both their stability and longevity in blood circulation. The determination of the surface charge is therefore crucial to establish a stable delivery system and is usually referred to as a measurement of zeta potential. When dispersed in solution, nanoparticles generally acquire surface charges that influence the distribution of the surrounding ions. This results in an electrical double layer surrounding the nanoparticles, termed the Stern layer and diffuse layer. Stern layer refers to an inner layer where counter ions are closely and tightly bound to the nanoparticle surface, while diffuse layer is an outer region where the ions of the opposite charges are associated with the Stern layer (Figure 3-8). Within the diffuse layer, there is a notational boundary where the nanoparticles and the ions form a stable entity, called slipping plane. When the nanoparticles diffuse under Brownian motion, the ions within this layer move as part of
the nanoparticles with the same velocity. The potential at this boundary is the zeta potential. The charged nanoparticles travel toward the electrode in an applied electric field with velocity corresponding to the magnitude of their apparent surface charges. When the nanoparticles are illuminated with laser beam, the moving nanoparticles cause the fluctuation of light intensity detected, which is proportional to the velocity of the nanoparticles. The electrophoretic mobility of the nanoparticles is then converted to the zeta potential. Based on the DLVO theory that the stability of colloidal nanoparticles is dependent on repulsive forces and van der Waals attractive forces, the magnitude of zeta potential could be an indicator of the stability of the colloidal system. The nanoparticles with large zeta potential, either positive or negative, will repel each other and resist aggregation, whereas the nanoparticles with low zeta potential are prone to aggregation since their repulsive forces are less dominant than their van der Waals attractive forces. Although high zeta potential is assumed to be advantageous for in vitro gene transfer, as it enhances interaction between nanoparticles and the cell surface, nanoparticles with low zeta potential are more preferable for in vivo applications since they are less susceptible to opsonisation, which can result in rapid uptake by RES, and the nanoparticles therefore exhibit increased blood circulation lifetimes.
Figure 3-8: Schematic representation of zeta potential of colloidal nanoparticles. In suspension, nanoparticles are associated with electrical double layers. The outer layer has a notational boundary where particles and ions form a stable entity and the zeta potential is defined as the potential at this boundary. Adapted from http://www.malvern.com.

A PEG layer enhances liposome stability by providing steric repulsion and minimises its surface charge by forming a protective shell, resulting in nanoparticles with greater resistance to aggregation and reduced recognition by macrophages. Here, the effect of PEG and its density on apparent surface charges, compared to non-PEGylated nanoparticles, was examined. pDNA nanoparticles were prepared at 12:1 lipid: pDNA w/w. The pH of the solution was adjusted to 7 and the zeta potential measurement was then performed using a zetasizer.
Figure 3-9: Zeta potentials of DODAG-high liposomes containing non-PEG, 1 mol% \( \text{PP}_{\text{HLE C}} \), 1 mol% \( \text{PP}_{\text{MMP-2C}} \), 1 mol% PC, 5 mol% \( \text{PP}_{\text{HLE C}} \), 5 mol% \( \text{PP}_{\text{MMP-2C}} \) and 5 mol% PC, and their pDNA nanoparticles (12:1 lipid: pDNA w/w), determined by zetasizer. Each result represents the means ± S.D. of three measurements.

The result in Figure 3-9 showed that the zeta potential decreased as the degree of PEGylation increased, and non-PEGylated liposomes exhibited the highest zeta potential. The reduction in zeta potential is presumably because PEG shifts the slipping plane to a greater distance from the liposome surface, resulting in a reduction in zeta potential. Further decrease in the zeta potential of liposomes was observed for pDNA nanoparticles. This reduction in surface charge after pDNA encapsulation was presumably a result of charge neutralisation between nucleic acids and cationic liposomes, indicating the formation of DNA-liposome complexes.

### 3.3.2 In vitro pDNA delivery of DODAG-high nanoparticles

The DODAG-high nanoparticles were optimised to achieve desirable physiochemical properties required for efficient nucleic acid delivery, as demonstrated previously. The
next step would be to assess the potential of PP_\text{HLE}C and PP_\text{MMP-2}C nanoparticles in promoting cellular uptake (endocytosis) and in improving the endosomal release of liposomal contents in the presence of specific enzymes, compared to non-cleavable PC nanoparticles. The ability of the nanoparticles to mediate pDNA delivery can be examined by means of transfection using a reporter pDNA encoding for a protein. The level of the protein expressed in the cells post-transfection represents the transfection efficiency of the nanoparticles. Here, the pDNA used was pEGFPLuc (Figure 3-10), which encodes for enhanced green fluorescence protein (EGFP) and luciferase protein (Luc), isolated from the firefly \textit{Photinus pyralis}. The plasmid contains human cytomegalovirus (CMV) as a promoter sequence, which is necessary for the expression of the encoded genes, and this promoter is commonly used for the expression of genes in many cell lines. The pDNA also carries gene encoding for kanamycin/neomycin antibiotic resistance.

![Figure 3-10: The pEGFPLuc plasmid](http://www.clontech.com/images/pt/dis_vectors/PT3347-5.pdf)

The efficiency in transfection of the nanoparticles was determined by measuring the level of luciferase protein expressed, using the luciferase assay system (Promega). The luciferase protein produced complexes with the added luciferin substrate, in the
presence of cosubstrates Mg\(^{2+}\) and ATP, to form luciferase-luciferyl-AMP complex. This complex is subsequently oxidised by molecular O\(_2\), resulting in the production of oxyluciferin, CO\(_2\), AMP, and light (detectable at 560 nm) (Scheme 3-1). The amount of light emitted is proportional to the amount of luciferase protein expressed in cells, i.e. the transfection efficiency, which is reported in terms of relative light units (RLU). Since the number of cells can vary in each experiment, the comparable transfection efficiency can be achieved by normalising the total protein content per experiment using a bicinchoninic acid (BCA) protein assay\(^{235}\) (Thermo Scientific). The result is expressed as RLU/mg protein.

Scheme 3-1: The reaction catalysed by the firefly luciferase.\(^{233}\)

To assess the transfection activity of the nanoparticles containing PEGylated peptide-lipid conjugates in response to the specific enzymes, the cell lines used should be able to secret the enzymes of interest. HLE is found to be secreted from monocytic cell lines, such as U937 and THP-1 upon PMA stimulation. However, transfection of these suspension cell lines is known to be difficult due to less contact of nanoparticles with cell membranes, and therefore adherent cell lines were used in this study. Elevated levels of HLE are found to be associated with tumour progression and development in many solid cancers, including breast cancer. MCF-7 (a human breast adenocarcinoma cell line), which is an adherent cell line, was chosen here for this reason and also because the cells are robust and require minimum handling, making it adequate for a proof of concept study of enzyme-assisted gene delivery. Since HLE is mainly secreted from neutrophils associated with cancer cells, the nanoparticles were pretreated with HLE before being used for transfection. For the MMP-2 experiment, HT1080 (a human fibrosarcoma cell line) was chosen because MMP-2 is highly expressed from this cell line.\(^{208}\)
In the HLE experiment, $\text{PP}_{\text{HLE}}\text{C}$ nanoparticles were assessed for their ability to mediate gene transfer in response to the enzyme, and their transfection efficiency was compared to that of non-PEGylated counterpart, non-cleavable PC control and naked pDNA. The effect of PEG density on transfection activity was also examined. To ensure that the gene expression was a result of enzyme-assisted delivery, the nanoparticles were divided into two groups: HLE-treatment and non-treatment. The nanoparticles were incubated with MCF-7 for 6 h. After this incubation period, the cells were washed and incubated with fresh complete medium for a further 24 h. The cell lysates were then analysed for luciferase expression.

![Figure 3-11](image_url)

**Figure 3-11**: The HLE transfection data of DODAG-high nanoparticles containing non-PEG, $\text{PP}_{\text{HLE}}\text{C}$ (1 and 5 mol%) and PC (1 and 5 mol%), with HLE-treatment (black) and non-treatment (white), in MCF-7 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control with HLE-treatment. Each result represents the mean ± S.D. (n = 3), * indicates $p < 0.05$.

The result of the transfection study showed that $\text{PP}_{\text{HLE}}\text{C}$ nanoparticles after pretreatment with HLE (both 1 and 5 mol%) exhibited higher transfection efficiency compared with non-treated $\text{PP}_{\text{HLE}}\text{C}$ nanoparticles and non-cleavable PEGylated...
cholesterol (PC) nanoparticles with HLE treatment and non-treatment ($p < 0.05$) (Figure 3-11). The finding suggested that $\text{PP}_\text{HLEC}$ nanoparticles might be cleaved by HLE and the resulting nanoparticles, with PEG removal, were favourably taken up by MCF-7 cells. However, there might be other biophysical changes to the nanoparticles that lead to this observation. The transfection efficiency of the nanoparticles after PEG detachment should be restored to the same level as that of their non-PEGylated counterpart. However, it was found that $\text{PP}_\text{HLEC}$ nanoparticles exhibited a significantly increased level of gene expression compared to that of non-PEGylated nanoparticles. This is possibly due to the instability of non-PEGylated nanoparticles in the transfection medium (normal cell growth medium containing 10% FBS). It was demonstrated by Zelphati et al. that binding of negatively charged FBS components, such as bovine serum albumin (BSA), lipoproteins, fibrinogen, and heparin, to cationic nanoparticles dramatically decreased the level of gene transfer. Two possible mechanisms were suggested: (1) destabilisation of nanoparticle structures upon interaction with serum components, thus inducing dissociation of pDNA from the complexes; (2) charge neutralisation leading to colloidal destabilisation and aggregation. The reduction in surface charge, as a result of adsorption of serum proteins, weakened the electrostatic interaction required for cell association and pDNA delivery was thereby reduced. However, nanoparticles with negative charge were shown to be capable of binding to cell membranes with a lesser degree of transfection efficiency, suggesting the interference of the nanoparticle components with the endosomal release of pDNA, since both the endosomal membrane and the complexes bear negative charge. Inclusion of PEG, as in the case of $\text{PP}_\text{HLEC}$ nanoparticles, should delay the adsorption of serum components and therefore reduce aggregation. The enhanced gene expression by $\text{PP}_\text{HLEC}$ nanoparticles, compared to that by non-PEGylated nanoparticles, also suggests that the nanoparticles may undergo partial peptide hydrolysis, leading to partial PEG detachment from the surface of the nanoparticles. The remaining PEG layer may allow the nanoparticles to maintain their stability in the transfection medium, and the partial surface exposure may render them to re-obtain their positive charge (or part of it) sufficient to facilitate interaction with negatively charged cell membranes that leads to cell uptake.
Transfection with naked pDNA did not show improvement in gene expression, possibly due to its degradation by nucleases in FBS. This highlighted the importance of carriers to retain nucleic acid integrity as well as their therapeutic function. The transfection efficiency of the HLE-pretreated PP_{HLE:C} nanoparticles decreased with increasing molar percentage of PEG. This observation was in line with several other studies using PEGylation systems, which is believed to result from reduced cell internalisation and also decreased level of endosomal escape.\textsuperscript{238} This result also suggests the lesser extent of PEG detachment in 5 mol\% PP_{HLE:C} compared with that of 1 mol\%, shown by the decrease in gene expression level. The PEG effect was also seen for the other PEGylated nanoparticles. Non-treated PP_{HLE:C} nanoparticles and non-cleavable PC nanoparticles, with HLE treatment and non-treatment, showed increased level of gene expression but to a lesser extent than HLE-treated PP_{HLE:C} nanoparticles, suggesting that PEGylated nanoparticles were capable of internalisation into the cell but may inhibit the endosomal escape of nucleic acids. The study of Song et al. showed that PEG-lipid had minimal effect on cell binding and subsequent endocytosis of cationic nanoparticles, but instead substantially inhibited the endosomal disruption of the lipid complexes, possibly by preventing fusion with endosomal membranes.\textsuperscript{158} Keller et al. also demonstrated that although PEG-lipid could be taken up by cells, its presence severely inhibited transgene expression.\textsuperscript{239} In the non-treatment group, 5 mol\% PP_{HLE:C} nanoparticles showed a similar level of gene expression to that of their 5 mol\% PC counterparts, presumably because of the steric hindrance effect. However, non-treated 1 mol\% PP_{HLE:C} exhibited increased transfection efficiency compared to that of 1 mol\% PC ($p < 0.05$) which might be due to nonspecific hydrolysis of the peptide linker. Although the serum does not affect the cleavage of the peptide linker as seen by experiment using HLE chromogenic substrate, MeO-Suc-AAPV-pNA in cell culture medium, it is possible that the peptide linker may be cleaved by other nonspecific peptidases at different positions in the peptide chain. The effect of non-specific hydrolysis, however, was minimal when 5 mol\% PEG was incorporated, possibly due to steric hindrance of PEG. Non-cleavable PC nanoparticles with HLE treatment and non-treatment showed comparable transfection efficiencies, and a similar finding was observed for non-PEGylated nanoparticles, indicating that the enzyme had minimal effect on cellular internalisation of the control PEGylated nanoparticles. The transfection efficiency of HLE-treated 1 mol\%-PP_{HLE:C} nanoparticles was found to be as efficient as that of the commercial transfection agent Lipofectamine™ 2000 (data not
shown), demonstrating the efficiency of the developed nanoparticle system and its possibility for \textit{in vivo} use.

In the MMP-2 experiment, the ability of PP_{MMP-2}C nanoparticles to mediate pDNA delivery was assessed on the MMP-2 positive cell line, HT1080, and their transfection efficiency was compared to that of naked pDNA, non-PEGylated counterpart, and PEGylated lipid control (PC). Transfection study of PP_{MMP-2}C and the control nanoparticles was carried out on HT1080 for 24 h. Then, the cells were washed and incubated with fresh complete medium for a further 24 h before cell lysates were analysed for luciferase expression.

![Graph](image.png)

**Figure 3-12**: The MMP-2 transfection data of DODAG-high nanoparticles containing non-PEG, PP_{MMP-2}C (1 and 5 mol\%) and PC (1 and 5 mol\%), in HT1080 cell lines. The transfection efficiency is expressed as \% RLU/mg protein of non-PEGylated control. Each result represents the mean \(\pm\) S.D. \((n=3)\), \(*\) indicates \(p < 0.05\).

The transfection data obtained showed that transfection efficiency of PP_{MMP-2}C nanoparticles (1 and 5 mol\%) was significantly higher than that of non-cleavable PC
nanoparticles (Figure 3-12). This result suggested that enzyme-mediated cleavage of the peptide linker on the surface of the nanoparticles might occur, leading to the detachment of the PEG layer and the subsequent restoration (i.e. increase) of positive charge required for enhanced cellular internalisation. The resulting nanoparticles, after PEG removal, should exhibit comparable levels of gene expression to that of their non-PEGylated counterpart. However, as with the HLE transfection study, non-PEGylated nanoparticles exhibited relatively lower transfection efficiency, which was presumably due to their instability (aggregation and destabilisation) in the transfection medium (normal cell growth medium). No improvement in gene expression was observed when using naked pDNA, suggesting its inability to enter the cells and its degradation by nucleases in the cell culture medium and thereby loss of gene expression function. The transfection efficiency of PP\textsubscript{MMP-2}C nanoparticles was shown to decrease as molar percentage of PEG increased. This result indicated that PEG might be cleaved to a lesser extent from 5 mol% PP\textsubscript{MMP-2}C compared with that of 1 mol%, and the remaining stealth layer might inhibit the cellular uptake and/or the release of nucleic acids from endosomes. In contrast to PP\textsubscript{MMP-2}C nanoparticles, non-cleavable PC nanoparticles exhibited similar gene expression levels irrespective of PEG concentrations, indicating that in this condition (normal cell growth medium) this 1% level of stealth might be sufficient to prevent transfection. PC nanoparticles also showed decreased levels of transfection compared with their non-PEGylated counterpart, presumably due to the effect of steric hindrance. Although the transfection efficiency of PP\textsubscript{MMP-2}C nanoparticles was found to be considerably lower than that of Lipofectamine\textsuperscript{TM} 2000 (data not shown), they exhibited a significant increase in gene expression levels compared with their non-cleavable controls, indicating the feasible use of enzyme-assisted delivery in improving cellular uptake and subsequent pDNA escape from endosomes.

According to the transfection data obtained from the HLE and MMP-2 experiments, the transfection efficiency was markedly enhanced when PP\textsubscript{HLE}C and PP\textsubscript{MMP-2}C nanoparticles were used in the presence of the enzymes, compared to that of the control PC nanoparticles with no peptide linkers. Overall data suggested that partial peptide hydrolysis by the specific enzymes may have occurred, leading to partial detachment of the PEG layer. The resulting nanoparticles then reveal more of their positive charges, facilitating interaction with anionic cell membranes and leading to
more favourable uptake by cells, whilst the remaining PEG helps to maintain the nanoparticle stability in the transfection medium. However, it is possible that other biophysical changes to the nanoparticles may have occurred, leading to an increase in transfection efficiency, for example the enzymes may require a certain PEG conformation found only on the surface of these nanoparticles in order to interact with the substrate or the association of the enzyme at the cleavage site may increase sedimentation of the complexes onto the cell surface and thereby enhance cell association. In the future, more information on the mechanism of enzyme-assisted delivery and cellular uptake of these nanoparticles should be accumulated.

For \textit{in vivo} applications, the overall therapeutic efficiency of nanoparticles could be hampered by their instability in blood circulation. Interaction of charged nanoparticles with plasma proteins may cause aggregation, dissociation, and increased recognition by the circulating macrophages, consequently diminishing the therapeutic efficacy of the nanoparticles. In addition, aggregation could activate the complement system, leading to the rapid clearance from blood circulation and increased toxicity of the administered nanoparticles. It is therefore crucial that nanoparticles should exhibit good colloidal stability, a characteristic required for extended circulation lifetimes and enhanced accumulation at target tissues. Inclusion of a stealth layer has been shown to increase the colloidal stability of nanoparticles. To investigate the effectiveness of PEG in preventing aggregation, 1 and 5 mol\% of \textit{PP}_{HLE}C and \textit{PP}_{MMP-2}C nanoparticles, which were previously used in transfection experiments, were examined for their stability in transfection medium (10\% FBS) compared to that of the non-PEGylated system. The nanoparticles were incubated in transfection medium at 37 °C and their increase in size (aggregation) was monitored as a function of time over a period of 4 hours.
The non-PEGylated nanoparticles were found to be unstable, and rapidly aggregated with diameter increasing to almost 300 nm compared with their original size (Figure 3-13). Increasing stability was observed when PEG was incorporated, shown by a smaller increase in diameter. 1 mol%-PP\textsubscript{HLE}C nanoparticles showed a level of aggregation comparable to that of the non-PEGylated counterpart after 120 min of incubation, and the aggregation decreased when the molar percentage of PEG was increased to 5 mol%. Similar to the PP\textsubscript{HLE}C counterpart, PP\textsubscript{MMP-2}C nanoparticles exhibited greater resistance to aggregation with increasing PEG concentration. From this result, it can be postulated that the inclusion of PEG provided steric repulsive barriers between two approaching nanoparticles and thus reduced interparticle van der Waals attraction. The polydispersity index of the nanoparticles was found to be 1.4-2.0 over the incubation period.

Although the PEGylated nanoparticles showed increased stability compared with the non-PEGylated system, a significant change in size over the course of the experiment,
indicating aggregation, was observed. In higher protein concentrations, such as in blood, high-charged nanoparticles would be rapidly aggregated leading to increased RES recognition and accumulation in the liver and lungs.\textsuperscript{240, 241} Although the PEGylated nanoparticles enhanced transfection efficiency in the presence of the enzymes, their instability in the transfection medium (containing 10\% FBS) limited their use \textit{in vivo}. Thus, the nanoparticles with lower surface charge (zeta potential) are more favourable due to their lower susceptibility to opsonisation and uptake by macrophages and thereby increased blood residence time.\textsuperscript{242, 243} The effects of DODAG-low nanoparticles on transfection activity and stability are discussed in the following section.

### 3.4 DODAG-low formulation with PEGylated peptide-cholesterol conjugates

Nanoparticles with high zeta potential are associated with efficient gene expression due to increased cell membrane association. Although a decrease in effective charge would significantly diminish their transfection activity, low-charge nanoparticles exhibit extended circulation half-lives.\textsuperscript{222, 244} From a therapeutic point of view, an efficient nucleic delivery system should be a compromise between good stability in blood circulation and high transfection ability. Here, the effects of charge on the transfection efficiency of \( \text{PP}_{\text{HLE}} \) and \( \text{PP}_{\text{MMP-2C}} \) nanoparticles as well as on their stability were investigated. The minimisation of the surface charge could be generally achieved by decreasing the amount of the cationic lipid incorporated in the liposome formulation, as long as there is good association with nucleic acids. This also reduces the cytotoxicity associated with using high concentrations of the cationic lipids. In the DODAG-low formulation, the cationic lipid, DODAG, was reduced to 20 mol\% since this percentage was previously found by our group to be a favourable minimum to obtain pDNA nanoparticles on the order of 100 nm in diameter with good encapsulation efficiency. The molar percentage of cholesterol was 30 mol\%, the same as that of the DODAG-high formulation since this amount was shown to be optimal for extended half-lives of nanoparticles. The PEGylated compounds \( \text{PP}_{\text{HLE-C}}, \text{PP}_{\text{MMP-2C}}, \) and PC were incorporated at 1 and 5 mol\%, which left the remaining percentage to DOPC lipid. The lipid compositions in the DODAG-low formulation are shown in Table 3-2.
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### Lipid compositions in DODAG-low formulation

<table>
<thead>
<tr>
<th>Lipid</th>
<th>mol % of total lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>DODAG</td>
<td>20</td>
</tr>
<tr>
<td>DOPC</td>
<td>50 (or 49 or 45)</td>
</tr>
<tr>
<td>Chol</td>
<td>30</td>
</tr>
<tr>
<td>PP$<em>{HLEC}$ 17, PP$</em>{MMP-2C}$ 18 or PC 19</td>
<td>0 (or 1 or 5)</td>
</tr>
</tbody>
</table>

**Table 3-2**: Lipid compositions in DODAG-low formulation

#### 3.4.1 Physicochemical characterisations of DODAG-low nanoparticles

The cationic lipid in the DODAG-low formulation was decreased by 2.5 times compared to that in the DODAG-high formulation. This should have led to a reduction in the overall surface charge, which may affect the ability of the nanoparticles in nucleic acid association. Similar physiochemical characterisations, including pDNA encapsulation efficiency, size, and surface charge, were examined for the DODAG-low nanoparticles and compared with the DODAG-high system.

#### 3.4.1.1 pDNA encapsulation efficiencies

Similar to the DODAG-high formulation, pDNA nanoparticles containing PP$_{HLEC}$, PP$_{MMP-2C}$, and PC, and non-PEGylated nanoparticles were prepared at various lipid: pDNA w/w. The encapsulation efficiency was determined by PI assay and the result was expressed as a percentage of the amount of pDNA initially used in the preparation (Figure 3-14). Like the DODAG-high formulation, encapsulation efficiency increased as lipid: pDNA w/w increased, presumably due to the greater amounts of cationic lipid available for binding to pDNA. Most of the DODAG-low nanoparticles showed almost complete pDNA entrapment at weight ratio of 12 and no significant change in encapsulation efficiency was observed at higher weight ratios. However, as previously demonstrated, pDNA entrapment was almost to completion at weight ratio of 4 for most DODAG-high nanoparticles. In addition, the DODAG-high non-PEGylated counterpart
showed more than 80% encapsulation efficiency at weight ratio of 2 compared with weight ratio of 8 for the DODAG-low system. These results suggest that the ability of the nanoparticles to condense nucleic acids is dependent on the amount of the cationic lipid, since the DODAG-low formulation consisted of 2.5 times less cationic lipid than the DODAG-high formulation. 1 mol% PP\textsubscript{HLE}C and 1 mol% PP\textsubscript{MMP-2}C nanoparticles exhibited higher encapsulation efficiencies compared to that of their 5 mol% PEGylated counterparts, suggesting less cationic charge presence for interaction with pDNA due to higher PEG concentrations. Compared to their 1 mol% PP\textsubscript{HLE}C and 1 mol% PP\textsubscript{MMP-2}C counterparts, 1 mol% PC nanoparticles required higher weight ratio (weight ratio of 12) for almost complete pDNA entrapment. This result suggested that the presence of the peptide linkers may facilitate the entrance of pDNA into liposomes or prevent it from leaving the core of the liposomes.

![Graph showing pDNA encapsulation efficiency](image)

**Figure 3-14:** pDNA encapsulation efficiency of DODAG-low nanoparticles with non-PEG, 1 and 5 mol% PP\textsubscript{HLE}C, 1 and 5 mol% PP\textsubscript{MMP-2}C, and 1 and 5 mol% PC at various lipid: pDNA w/w (1 ug pDNA), using propidium iodide assay.

Agarose gel electrophoresis was then performed to visualise and confirm the data obtained from PI assay. The result from gel electrophoresis was similar to the finding of
the PI assay (Figure 3-15). pDNA encapsulation by 1 mol% PP<sub>HLE</sub>C went to completion at slightly lower weight ratio compared to that from PI assay. 1 mol% PP<sub>MMP</sub>-2C and 1 mol% PC nanoparticles exhibited complete pDNA encapsulation at weight ratios of 8 and 12 respectively, while pDNA entrapment by their 5 mol% PEGylated counterparts, including PP<sub>HLE</sub>C, went to completion at weight ratio of 12. This result was in agreement with the finding of PI assay. For all low-charge nanoparticles, pDNA was fully encapsulated at weight ratio of 12.
Figure 3-15: 0.8% agarose gel electrophoresis demonstrated pDNA encapsulation of DODAG-low nanoparticles with a) non-PEG, b) 1 mol% PP_{HLE}C, c) 1 mol% PP_{MMP-2}C, d) 1 mol% PC, e) 5 mol% PP_{HLE}C, f) 5 mol% PP_{MMP-2}C and g) 5 mol% PC at different lipid: pDNA w/w. (gel a) lane 1 = DNA ladder, lane 2 = pDNA, lane 3 = empty liposomes, lane 4-7 = lipid: pDNA weight ratio of 1:1, 4:1, 8:1 and 12:1; gel b-g) lane 1 = empty liposomes, lane 2-5 = lipid: pDNA weight ratio of 1:1, 4:1, 8:1 and 12:1). The electrophoresis was performed at 65 mV for 180 min and the gels were visualized under UV light using Alliance 4.7 UVITEC Cambridge.
Overall pDNA encapsulation data obtained from both PI assay and agarose gel electrophoresis showed that complete encapsulation of pDNA was achieved at 12:1 lipid: pDNA w/w. The ratio was the same as the finding from the DODAG-high system, although slight differences in entrapment efficiency were observed in each formulation. The weight ratio of 12 was thus used for all experiments related to the DODAG-low formulation.

### 3.4.1.2 Size measurements

The diameter of the liposomes and pDNA nanoparticles (12:1 lipid: pDNA w/w) were measured using PCS. The diameters of the DODAG-low liposomes were found to be between 80-100 nm (Figure 3-16), slightly smaller than that of the DODAG-high liposomes (90-130 nm). At higher molar percentages of PEG, the PEGylated nanoparticles exhibited smaller sizes compared with their non-PEGylated counterparts. This was presumably due to the lateral steric repulsion between PEG chains, causing nanoparticles to increase their surface curvatures in order to relieve the strain, thus reducing their sizes. After complexing with pDNA, the diameters of the nanoparticles were greater than that of the initial liposomes, indicating nucleic acid encapsulation. The polydispersity index of both liposomes and pDNA nanoparticles in each preparation was found between 0.2-0.8.
Figure 3-16: Average diameters of DODAG-low liposomes containing non-PEG, 1 mol% PP_{HLE}C, 1 mol% PP_{MMP-2}C, 1 mol% PC, 5 mol% PP_{HLE}C, 5 mol% PP_{MMP-2}C and 5 mol% PC, and their pDNA nanoparticles (12:1 lipid: pDNA w/w), determined by PCS. Each result represents means ± S.D. of three measurements.

3.4.1.3 Zeta potential measurements

The zeta potential measurement of the DODAG-low liposomes showed a decrease in zeta potential as PEG concentration increased, with the effect being more pronounced at 5 mol% PEG (Figure 3-17), indicating greater shielding of the surface charge. All DODAG-low liposomes exhibited smaller zeta potentials compared to the DODAG-high formulation due to the reduced amount of the cationic lipid in the formulation. After nucleic acid encapsulation (12:1 lipid: pDNA w/w), the nanoparticles showed a further decrease in zeta potential owing to charge neutralisation, indicating the complexation between liposomes and pDNA. The zeta potential was found to be less than 10 mV for 1 mol%-PEGylated and non-PEGylated nanoparticles, and almost neutral for 5 mol%-PEGylated systems.
Figure 3-17: Zeta potentials of DODAG-low liposomes containing non-PEG, 1 mol% PP<sub>HLE</sub>C, 1 mol% PP<sub>MMP-2</sub>C, 1 mol% PC, 5 mol% PP<sub>HLE</sub>C, 5 mol% PP<sub>MMP-2</sub>C and 5 mol% PC, and their pDNA nanoparticles (12:1 lipid:pDNA w/w), determined by zetasizer. Each result represents means ± S.D. of three measurements.

3.4.2 MTS cell proliferation assay with DODAG-low nanoparticles containing PEGylated peptide-cholesterol conjugates

The effect of the nanoparticles on in vitro cell viability can be assessed by the determination of the cell population’s response to the nanoparticles using MTS assay. MTS is a colorimetric method for assessing the number of viable cells (balance between cell proliferation and cell death), which decreases if apoptosis or necrosis occurs. Similar to MTT, the assay involves the reduction of a tetrazolium compound by the dehydrogenase enzyme found in metabolically active cells (Figure 3-18). However, MTS requires fewer steps than MTT. The formazan product from MTT reduction is a crystalline precipitate that requires solubilisation before recording the absorbance, while the formazan product from MTS assay is soluble in tissue culture medium and its absorbance can be read immediately. The conversion of the yellow tetrazolium MTS (3,(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
tetrazolium, inner salt) to the soluble purple product, formazan, by dehydrogenase enzyme can be measured spectrophotometrically at 490 nm. The absorbance of formazan is proportional to the number of living cells, allowing for measuring and quantifying the viability of cells in the presence of the nanoparticles.

**Figure 3-18:** The reduction of tetrazolium compounds a) MTT and b) MTS to formazan product as a result of the metabolic activity of dehydrogenase enzyme.
The nanoparticles containing PP_{HLEC} and PP_{MMP-2C} (1 and 5 mol%) at three lipid: pDNA w/w (8:1, 12:1, and 16:1; 1 ug pDNA/well) were assessed for their effects on cell viability. The nanoparticles were incubated with MCF-7 and HT1080, respectively, for three incubation periods (4, 8, and 24h). Cells with no nanoparticles added were employed as positive controls and the measured cell viability is expressed as a percentage.
Figure 3-19: MTS cell viability results of DODAG-low nanoparticles containing 1 and 5 mol% PP_{HLE} nanoparticles incubated with MCF-7 for 4, 8 and 24 h (a-c) and 1 and 5 mol% PP_{MMP-2} nanoparticles incubated with HT1080 for 4, 8, and 24 h (d-f). The cell viability data are expressed as a percentage of the positive control cells without nanoparticles added to them.

The result of the MTS assay (Figure 3-19 a-c) showed that the cell viability was maintained above 80% for PP_{HLE} nanoparticles regardless the molar percentages of PEG, weight ratios, and incubation times. A similar finding was observed for PP_{MMP-2} nanoparticles, of which the cell viability was found to be greater than 70% (Figure 3-19 d-f). The results suggested that the added nanoparticles had a minimal effect on cell viability and that the cells continued normal growth despite the presence of the nanoparticles. A second assay that was used to confirm the results of the MTS assay was the lactate dehydrogenase (LDH) cytotoxicity assay, which was further carried out to quantify the level of cell death due to the PEGylated nanoparticles.

3.4.3 Lactate dehydrogenase (LDH) cytotoxicity assay with DODAG-low nanoparticles containing PEGylated peptide-cholesterol conjugates

To assess the toxic effect of the nanoparticles, LDH cytotoxicity assay was carried out using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). LDH assay is a
colorimetric assay that quantitatively measures a stable cytosolic enzyme, lactate dehydrogenase (LDH), released upon cell lysis during cell death. The LDH enzyme assay results in the conversion of a tetrazolium salt, 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), with the aid of diaphorase (an enzyme catalysed dehydrogenation of NADH to NAD\(^+\)), to a red formazan product, the absorbance of which can be measured at 490 nm (Figure 3-20). The amount of colour formed (formazan) is proportional to the number of lysed cells (dead cells). This allows for measuring the level of cell death due to the cytotoxicity of the added nanoparticles.

![General chemical reactions of the LDH cytotoxicity assay.](image)

**Figure 3-20:** General chemical reactions of the LDH cytotoxicity assay.

Similar to the MTS assay, PP\(_{HLEC}\) and PP\(_{MMP-2C}\) nanoparticles (1 and 5 mol%) were assessed for their cytotoxicity at three lipid: pDNA w/w (8:1, 12:1, and 16:1; 1 μg pDNA/well). The nanoparticles were incubated with MCF-7 and HT1080, respectively, for 4, 8, and 24 h. The cells with no nanoparticles added were used as a positive control and the cell death from cytotoxicity was measured and expressed as a percentage (Figure 3-21).
Figure 3-21: LDH assay results showing % cytotoxicity as a result of the added DODAG-low nanoparticles containing: a-c) 1 and 5 mol% PP_{HLEC} nanoparticles incubated with MCF-7 for 4, 8, and 24 h and d-f) 1 and 5 mol% PP_{MMP-2} nanoparticles incubated with MCF-7 for 4, 8, and 24 h.
incubated with HT1080 for 4, 8, and 24 h. The result is expressed as a percentage normalised against control cells without added nanoparticles.

PP_{HLEC} nanoparticles showed no significant differences in toxicity irrespective of PEG concentrations, lipid: pDNA weight ratios, and incubation times over the incubation period of 4 and 8 hours. However, LDH release from the cells increased to 10-12% after 24 h incubation for all formulations and weight ratios. For 1 mol% PP_{MMP-2C} nanoparticles, LDH release was less than 8% during all three periods of incubation. When PEG concentration was raised to 5 mol%, enhanced LDH release with increased lipid: pDNA weight ratio and incubation times was observed. At the conclusion of 24 h, the weight ratio of 16 showed approximately 20% cell death whereas around 15% and 10% were observed at weight ratios of 12 and 8, respectively. This result suggests that enhanced toxicity was associated with the concentration of PP_{MMP-2C} incorporated into the liposome formulation, the weight ratio of lipid: pDNA, and also the duration of time over which the nanoparticles were exposed to the cells. This observed effect on cell viability may indicate an impurity from the synthesis, or that the amphiphile has an effect on cell viability. Although increased LDH release was observed in some formulations, the release levels were minimal, <15% in most cases, suggesting that the added nanoparticles do not greatly affect cell proliferation.

From MTS cell viability and LDH toxicity data, it was concluded that PP_{HLEC} and PP_{MMP-2C} nanoparticles (1 and 5 mol%) for all weight ratios are not associated with significant toxicity. Along with high pDNA encapsulation efficiency, the weight ratio of 12 exhibited an acceptably low level of toxicity and is therefore optimal for cell uptake study.
3.4.4 In vitro pDNA transfection of DODAG-low nanoparticles containing PEGylated peptide-cholesterol conjugates

The DODAG-low nanoparticles containing PP_{HLE}C and PP_{MMP-2}C were then investigated for their ability to mediate pDNA delivery, and the effect of charge on transfection efficiency was also examined and compared with that of the DODAG-high formulation.

3.4.4.1 Transfection study of PP_{HLE}C nanoparticles

Transfection study of PP_{HLE}C nanoparticles was carried out under the same conditions and in the same cell line as that of the high-charge formulation. The transfection data showed enhanced transfection efficiency when using 1 and 5 mol% of PP_{HLE}C nanoparticles after pretreatment with HLE, compared with non-treated PP_{HLE}C nanoparticles and non-cleavable PC nanoparticles with HLE treatment and non-treatment (\(p < 0.05\)) (Figure 3-22a). These findings are similar to those of the DODAG-high nanoparticles. Once more, the transfection efficiency of non-PEGylated nanoparticles was markedly lower than that of the PEGylated nanoparticles, indicating that non-PEGylated nanoparticles with lower zeta potential were still susceptible to aggregation, leading to less electrostatic interaction with cell membranes. The inhibiting effect of PEG on endosomal disruption as the molar percentage of PEG increased was also observed for both PP_{HLE}C and PC nanoparticles with both HLE-treatment and non-treatment. Similar transfection efficiency was observed for non-cleavable PC nanoparticles with HLE-treatment and non-treatment as well as in the case of the non-PEGylated system, suggesting minimum effect of the enzyme on cellular uptake of the nanoparticles. In the non-treatment group, similar levels of transfection was observed for 5 mol% PP_{HLE}C and PC nanoparticles irrespective of the presence or absence of the peptide linker, presumably due to steric effects. The effect of the peptide linker, however, was more pronounced when using 1 mol% PEG, showing increased transfection efficiency by PP_{HLE}C nanoparticles compared with PC nanoparticles (\(p < 0.05\)). This observation was similar to the finding of the DODAG-high, non-treated 1 mol%-PP_{HLE}C nanoparticles, which might be due to non-specific peptide hydrolysis by non-specific peptidases in serum.
In comparison with the DODAG-high formulation, what was firstly observed was a considerably lower level of transfection by the DODAG-low nanoparticles, demonstrating a decrease in cell membrane association and uptake due to a lower amount of cationic lipid in the liposome formulation.\(^{246}\) Considering the HLE treatment group, 1 mol% PP\(_{\text{HLE}}\)C high-charge nanoparticles exhibited increased transfection efficiency of 2.6-fold, similar to that of the low-charge nanoparticles (3.7-fold) (Figure 3-22b). This result indicated that PP\(_{\text{HLE}}\)C nanoparticles may undergo partial peptide hydrolysis, leading to partial exposure of cationic surface charges that facilitate cellular binding. Similar findings were observed for 5 mol% PP\(_{\text{HLE}}\)C high-charge and low-charge nanoparticles (Figure 3-22c).

a)
a) The HLE transfection data of DODAG-low nanoparticles containing non-PEG, PP$_{HLE}$(1 and 5 mol%) and PC (1 and 5 mol%), with HLE-treatment (black) and non-treatment (white). The transfection was performed in MCF-7 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control with HLE-treatment. Each result represents the mean ± S.D. (n = 3), * indicates $p < 0.05$. Comparison of transfection efficiencies of DODAG-high and DODAG-low nanoparticles after pretreatment with HLE: b) 1 mol% PP$_{HLE}$ and PC, and c) 5 mol% PP$_{HLE}$ and PC.

Figure 3-22: a) The HLE transfection data of DODAG-low nanoparticles containing non-PEG, PP$_{HLE}$(1 and 5 mol%) and PC (1 and 5 mol%), with HLE-treatment (black) and non-treatment (white). The transfection was performed in MCF-7 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control with HLE-treatment. Each result represents the mean ± S.D. (n = 3), * indicates $p < 0.05$. Comparison of transfection efficiencies of DODAG-high and DODAG-low nanoparticles after pretreatment with HLE: b) 1 mol% PP$_{HLE}$ and PC, and c) 5 mol% PP$_{HLE}$ and PC.
3.4.4.2 Transfection study of $PP_{\text{MMP-2}C}$ nanoparticles

Similar to the DODAG-high formulation, the transfection efficiency of low-charge $PP_{\text{MMP-2}C}$ nanoparticles was assessed on the MMP-2 positive cell line, HT1080. Moreover, the effect of $PP_{\text{MMP-2}C}$ nanoparticles on MMP-2 negative cell lines was also examined to compare the specificity of the nanoparticles. Cell lines including HT1080, MCF-7, HEK293T, DU145, and HeLa were characterised for their levels of MMP-2 expression in culture medium using MMP-2 human ELISA assay kit (Invitrogen). HT1080 was shown to secrete high levels of MMP-2 (Figure 3-23), in line with other reported studies involving MMP-2 study. The other cell lines showed relatively poor expression of MMP-2. Of these cell lines, MCF-7 was chosen as the MMP-2 negative cell line for use in this study since it was already being used in the HLE experiment.

![Figure 3-23](image)

**Figure 3-23:** The expression of MMP-2 levels in cell culture medium, evaluated by ELISA MMP-2 assay. HT1080 was shown to have higher levels of MMP-2 expression compared with other tested cell lines.

The transfection of $PP_{\text{MMP-2}C}$ nanoparticles was first carried out on HT1080 and the level of gene expression was compared to that of the DODAG-high nanoparticles. The transfection level was significantly enhanced by $PP_{\text{MMP-2}C}$ nanoparticles (1 and 5 mol%)
compared to non-cleavable PC \( (p < 0.05) \) (Figure 3-24a), suggesting that \( \text{PP}_{\text{MMP}-2}\text{C} \) nanoparticles were activated in response to MMP-2 expression. The \textit{in vitro} MMP-2 level was less than the amount of the enzyme used in the enzyme cleavage study (chapter 2); however, the molar ratio of the substrate to the enzyme in both experiments was found to be similar. The effect of PEG on preventing the endosomal escape of pDNA was seen for PEGylated nanoparticles and the effect increased as the degree of PEGylation increased.

As expected, considerably lower transfection efficiency by DODAG-low nanoparticles was observed compared to that of the DODAG-high formulation (Figure 3-24b and c). Since binding of cationic nanoparticles to the cell membrane was purely via electrostatic interaction, the decrease in gene expression demonstrates the reduction in cellular binding and internalisation as a result of lowering the amount of the cationic lipid. The level of gene expression was 9.0-fold enhanced by 1 mol\% \( \text{PP}_{\text{MMP}-2}\text{C} \) DODAG-high nanoparticles; however, a dramatic increase in cell transfection was observed when using 1 mol\% \( \text{PP}_{\text{MMP}-2}\text{C} \) DODAG-low nanoparticles. Further enhancement in the gene expression level was observed for 5 mol\% \( \text{PP}_{\text{MMP}-2}\text{C} \) low-charge nanoparticles. This observation clearly demonstrates that the decrease in cationic charge limits the non-specific uptake of the nanoparticles to the cell membrane and thereby dominates the effect of enzyme-mediated gene transfer.
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a)

![Bar graph showing % RLU/mg protein of non-PEGylated control for different formulations: non-PEG, naked DNA, 1% PP<sub>MM</sub>, 1% PC, 5% PP<sub>MM</sub>, and 5% PC. The graph indicates significant differences between formulations, marked with asterisks.]

b)

![Bar graph comparing RLU/mg protein x 1000 for DODAG-high nanoparticles (50% DODAG) and DODAG-low nanoparticles (20% DODAG) with 1% PP<sub>MM</sub>C and 1% PC. The graph shows a 9.0-fold increase for 1% PP<sub>MM</sub>C and a 15.4-fold increase for 1% PC.]

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c)

**Figure 3-24:** a) The MMP-2 transfection data of DODAG-low nanoparticles containing non-PEG, PP<sub>MMP-2</sub>C (1 and 5 mol%), and PC (1 and 5 mol%) in HT1080 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control. Each result represents the mean ± S.D. (n = 3), * indicates p < 0.05. Comparison of transfection efficiencies of DODAG-high and DODAG-low nanoparticles: b) 1 mol% PP<sub>MMP-2</sub>C and PC and c) 5 mol% PP<sub>MMP-2</sub>C and PC.

The ability of PP<sub>MMP-2</sub>C nanoparticles to mediate transfection in MCF-7, a cell line that poorly expresses MMP-2, was then examined. The transfection was performed under the same conditions as those of HT1080. In the MMP-2 negative cell line, enhanced efficiency in transfection by PP<sub>MMP-2</sub>C nanoparticles was also observed (p < 0.05) (Figure 3-25), but to a lesser extent than that obtained from HT1080, the MMP-2 positive cells. In HT1080 cells, the transfection activity of 1 mol% PP<sub>MMP-2</sub>C nanoparticles was 15-fold higher than that of 1 mol% PC (Figure 3-24b), whereas the enhancement was less prominent, 1.8-fold, in MCF-7 cells. Similar to their 1 mol% counterparts, a substantial increase in gene expression, 92.5-fold, by 5 mol% PP<sub>MMP-2</sub>C nanoparticles was observed in HT1080 cells (Figure 3-24c), while the efficiency was enhanced 2.5-fold in MCF-7 cells. This result suggests that PP<sub>MMP-2</sub>C nanoparticles were activated by the secreted MMP-2, and this activation was more pronounced in...
MMP-2 positive cell lines, demonstrating that the transfection efficiency was related to the cellular expression level of MMP-2. The data were in agreement with the study of Hatakeyama et al. which showed a remarkably higher transfection activity by nanoparticles containing MMP-2 substrate peptide in the cell lines that highly express MMP-2, with a significantly lower enhancement observed in MMP-2 negative cells.\textsuperscript{208}

\textbf{Figure 3-25:} The MMP-2 transfection data of DODAG-low nanoparticles containing non-PEG, \(\text{PP}_{\text{MMP-2}}\) (1 and 5 mol\%) and PC (1 and 5 mol\%) in MCF-7 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control. Each result represents the mean ± S.D. (\(n = 3\)), * indicates \(p < 0.05\).

The stability of the DODAG-low nanoparticles containing 1 and 5 mol\% \(\text{PP}_{\text{HLE}}\) and \(\text{PP}_{\text{MMP-2}}\) in the transfection media (10% FBS) was then assessed by measuring the particle sizes over time. Non-PEGylated nanoparticles showed a marked increase in size during the course of the experiment, whereas no significant increase in diameter due to aggregation was observed for all PEGylated nanoparticles (Figure 3-26). This result suggests that PEG is crucial for conferring stability to the nanoparticles and preventing their aggregation. 1 mol\% PEG was shown to be sufficient to enhance nanoparticle stability in 10% serum, shown by a small increase in size. The aggregation

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was more pronounced in the case of non-PEGylated nanoparticles. This may contribute to their decreased transfection activity in MCF-7 and HT1080 cells compared to the other PEGylated nanoparticles. Unlike the DODAG-high formulation, the DODAG-low nanoparticles exhibited greater resistance to aggregation, presumably due to a decrease in the effective surface charge of the nanoparticles as a result of reduced molar percentage of the cationic lipid in the formulation, confirmed by the reduction in zeta potential compared to the DODAG-high system. The lowered surface charge resulted in less interaction with counter ions and serum components, thus reducing aggregation. The polydispersity index of the nanoparticles was found between 1.5-1.9 for non-PEGylated nanoparticles and 0.9-1.4 for the PEGylated nanoparticles.

**Figure 3-26**: The stability of DODAG-low nanoparticles containing non-PEG, PP_{HLEC} (1 and 5 mol%), and PP_{MMP-2C} (1 and 5 mol%) in transfection media (10% FBS), assessed by monitoring the change in nanoparticle diameter as a function of time (every 30 min over 4 h).
3.4.5 **Fluorescence microscopy of rhodamine labelled DODAG-low liposomes containing PEGylated peptide-cholesterol conjugates**

Fluorescence microscopy was employed to visualise the internalisation of the PEGylated nanoparticles inside the cells. To analyse the degree of intracellular uptake, the PEGylated liposomes were labelled with 1 mol% of rhodamine-DOPE. The incorporation of a small amount of the fluorescently labelled lipid should prevent it causing significant changes to the overall properties of the liposomes. The labelled PP<sub>HLE</sub>C and PC liposomes were incubated with MCF-7 for 6 h (the same uptake period as in the transfection study) and non-internalised liposomes were removed at the end of the incubation period. After the cells were fixed, their nuclei were further stained using 4-6-diamidino-2-phenylindole (DAPI), resulting in blue fluorescence upon binding to nuclear double stranded DNA.\(^{249}\) The fluorescence images of 1 mol% PP<sub>HLE</sub>C and 1 mol% PC liposomes with HLE treatment and non-treatment are shown below (Figure 3-27). The fluorescence microscopy revealed that the PEGylated liposomes were successfully uptaken by cells and dispersed throughout the cytoplasm. However, the degree of internalisation varied significantly. 1 mol% PP<sub>HLE</sub>C liposomes pretreated with HLE showed higher rhodamine intensities inside the cells, compared to that of non-treated 1 mol% PP<sub>HLE</sub>C and the PEGylated control PC liposomes with HLE treatment and non-treatment. This result was in agreement with the transfection data that HLE-pretreated PP<sub>HLE</sub>C nanoparticles were preferably taken up by cells, resulting in enhanced gene expression. This could be attributed to enzyme-mediated detachment of the stealth layer, leading to increased cell association. Cellular internalisation was observed for the other PEGylated liposomes, but to lesser extent.

![Fluorescence images](image)

**a)** 1% PP<sub>HLE</sub>C with HLE treatment

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Figure 3-27: Fluorescence microscopy images (40x magnifications) of MCF-7 cells after incubation with rhodamine labelled DODAG-low liposomes: a) 1% PP_{HLE}C with HLE treatment, b) 1% PP_{HLE}C with non-treatment, c) 1% PC with HLE treatment, and d) 1% PC with non-treatment, showing i) bright field ii) DAPI stained nuclei and iii) rhodamine labelled liposomes images.

Fluorescence microscopy of PP_{MMP-2}C liposomes was also carried out by incubating the rhodamine labelled liposomes with HT1080 for 24 h, similar to the uptake period in the transfection experiment. The fluorescence images of 1 and 5 mol% of PP_{MMP-2}C and the
PEGylated control PC liposomes are presented in Figure 3-28. Similar to PP_{HLE}C, PP_{MMP-2}C and PC liposomes were successfully delivered into the cytoplasm of the cells. Both 1 and 5 mol% PP_{MMP-2}C liposomes showed significantly higher fluorescence intensities than that of their PEGylated controls, and 1 mol% PP_{MMP-2}C liposomes exhibited increased rhodamine intensity compared to their 5 mol% counterparts. This result suggests that the presence of the peptide linker may contribute to enhanced cell uptake through PEG removal by the enzyme action, hence facilitating cellular internalisation. This finding was in agreement with the transfection data of the PP_{MMP-2}C system.

i) Bright field  ii) DAPI  iii) Rhodamine

a) 1 mol% PP_{MMP-2}C

b) 1 mol% PC

c) 5 mol% PP_{MMP-2}C
To further examine the localisation of the fluorescently labelled liposomes inside the cells, confocal microscopy was employed to provide serial imaging throughout the cells, producing ‘z’ stack images that can be used to confirm the internalisation of the labelled liposomes. The confocal image revealed that the liposomes were internalised and located around the nucleus as well as throughout the cytoplasm (Figure 3-29). The appearance of localised hyperintense fluorescence dots may indicate the enclosure of the rhodamine-DOPE probe by endosomes.
3.4.6 Stability of DODAG-low nanoparticles containing PEGylated peptide-cholesterol conjugates in 80% serum

Although the previous stability data in the transfection medium showed that 1 mol% PEG was adequate to obtain good nanoparticle stability in 10% serum, this concentration may not be adequate at higher concentrations of serum, such as in blood circulation. The effect of high serum concentration (80% FBS) on the stability of the nanoparticles (1-5 mol%) was therefore examined. FBS contains small protein aggregates, which makes size measurement using PCS unsuitable for monitoring aggregation at high serum contents, so the turbidity assay was employed instead. The change in turbidity of the nanoparticles in solution can be observed by measuring their absorbance at $\lambda = 600$ nm. 1 and 2 mol% PP_{HLE}C nanoparticles showed rapid increases in absorbance over the experiment period, indicating the formation of large aggregates which increased the turbidity of the solution (Figure 3-30a). Small changes
in absorbance were observed as PEG concentration was increased to 3 mol%, and similar levels of absorbance were shown by 4 and 5 mol% PEG, indicating that the nanoparticles were capable of maintaining their initial size during the course of the experiment. 3-5 mol% PEG exhibited comparable levels of protection against aggregation. Similar findings were observed in the case of PP\textsubscript{MMP-2C} nanoparticles. It was found that 1 and 2 mol% PP\textsubscript{MMP-2C} showed immediate increases in absorbance, in contrast to 3-5 mol% PEG (Figure 3-30b). The smallest change in absorbance was demonstrated by 5 mol% PEG, suggesting the most resistance to aggregation. This stability study highlighted the importance of the PEG layer in reducing inter-particle attraction by providing a repulsive barrier between nanoparticles.

\begin{center}
\includegraphics[width=\textwidth]{graph.png}
\end{center}

\textbf{a)}
Figure 3-30: The stability of DODAG-low nanoparticles in 80% FBS assessed by measuring the absorbance at 600 nm, a) 1-5 mol% PP_{HLE}C and b) 1-5 mol% PP_{MMP-2}C. The UV absorbance was monitored every 30 min over 4 h using a UV spectrometer.

pDNA delivery by PP_{HLE}C and PP_{MMP-2}C nanoparticles was shown to be activated by the enzymes, shown by a considerable increase in transfection efficiency compared with non-cleavable PC nanoparticles. It was reported that PEG-cholesterol in serum environments can be transferred between lipid bilayers and biological lipids, presumably due to ineffective anchoring associations with the liposomal phospholipids provided by the hydrophobic moiety of cholesterol.\textsuperscript{250, 251} The transfer of PEG-cholesterol from liposome bilayers before enzymatic activation may interfere with cell transfection by increasing the transfection efficiency of the PEGylated nanoparticles. To investigate this effect, a new series of PEGylated lipid conjugates was synthesised by conjugating a saturated long-chain hydrocarbon, a dialkylglycylamide moiety, to PEG, since a saturated long-chain lipid exhibits stronger anchoring due to its ability to increase the rigidity of the liposomal membrane, and it was shown to be associated with liposomes for an extended period after \textit{in vivo} injection.\textsuperscript{252} Incorporation of these PEGylated lipid conjugates should prevent lipid transfer and may result in a decrease in transfection efficiency.
3.5 **DODAG-low formulation with PEGylated peptide-C18 conjugates**

The synthesised PEGylated peptide-C18 conjugates, PP<sub>HLE</sub>C18 20 and PP<sub>MMP</sub>-2C18 21, and the PEGylated lipid control PC18 22 were incorporated into the same DODAG-low formulation as that of the PP<sub>HLE</sub>C and PP<sub>MMP</sub>-2C nanoparticles, and at the same PEG densities, 1 and 5 mol%. The physiochemical properties of PP<sub>HLE</sub>C18 and PP<sub>MMP</sub>-2C18 nanoparticles with respect to nucleic acid encapsulation efficiency, size, and surface charge were examined before their transfection abilities were assessed.

3.5.1 **Physicochemical characterisation of DODAG-low nanoparticles containing PEGylated peptide-C18 conjugates**

3.5.1.1 **pDNA encapsulation efficiencies**

PP<sub>HLE</sub>C18, PP<sub>MMP</sub>-2C18, and PC18 nanoparticles were prepared at different lipid: pDNA w/w and their encapsulation efficiency were examined using PI assay. Similar to their cholesterol counterparts, encapsulation efficiency increased as lipid: pDNA w/w increased (Figure 3-31). A marked increase in pDNA encapsulation efficiency was shown at weight ratio of 8 for 1 and 5 mol% PEGylated nanoparticles, and no significant change was observed at weight ratios higher than 12.
Figure 3-31: pDNA encapsulation efficiency of DODAG-low nanoparticles with 1 mol% PP<sub>HLE</sub>C18, 1 mol% PP<sub>MMP-2</sub>C18, 1 mol% PC18, 5 mol% PP<sub>HLE</sub>C18, 5 mol% PP<sub>MMP-2</sub>C18, and 5 mol% PC18 at various lipid: pDNA w/w (1 ug pDNA), using propidium iodide assay.

The pDNA entrapment was further visualised and confirmed by agarose gel electrophoresis. The gel electrophoresis result was in agreement with the finding of the PI assay. pDNA encapsulation of most PEGylated nanoparticles was to completion at weight ratio of 8. At weight ratio of 12, all nanoparticles exhibited complete pDNA encapsulation (Figure 3-32).
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Figure 3-32: 0.8% agarose gel electrophoresis demonstrated pDNA encapsulation of DODAG-low nanoparticles with a) 1 mol% PP_{HLE}C18, b) 1 mol% PP_{MMP-2}C18, c) 1 mol% PC18, d) 5 mol% PP_{HLE}C18, e) 5 mol% PP_{MMP-2}C18, and f) 5 mol% PC18 at different lipid: pDNA w/w (gel a) lane 1 = DNA ladder, lane 2 = pDNA, lane 3 = empty liposomes, lane 4-7 = lipid: pDNA w/w of 1:1, 4:1, 8:1, and 12:1; gel b-g) lane 1 = empty liposomes, lane 2-5 = lipid: pDNA w/w of 1:1, 4:1, 8:1, and 12:1. The electrophoresis was performed at 65 mV for 180 min and the gels were visualized under UV light using Alliance 4.7 UVITEC Cambridge.
The findings of both PI assay and gel electrophoresis were in agreement with the data obtained from the cholesterol-anchored PEGylated nanoparticles that the optimal pDNA encapsulation efficiency was found at a weight ratio of 12. This result suggests that different types of lipid anchor – cholesterol-based and long-chain lipids – have minimal affect on the pDNA entrapment ability of DODAG-low liposomes, indicating that the cationic lipid is the main component that controls the pDNA association.

3.5.1.2 Size and zeta potential measurements

The sizes of the liposomes and pDNA nanoparticles (12:1 lipid: pDNA w/w) were examined using PCS. The diameters of the liposomes were found to be approximately 80-100 nm (Figure 3-33), similar to that of their cholesterol-anchored PEGylated counterparts. An increase in size was observed after pDNA encapsulation, with the diameters ranging from 160-210 nm, indicating the complexation between liposomes and pDNA. The polydispersity index of both liposomes and pDNA nanoparticles in each preparation was found to be 0.2-0.8.
Figure 3-33: Average diameters of DODAG-low liposomes containing 1 mol% PP\textsubscript{HLE}C18, 1 mol% PP\textsubscript{MMP-2}C18, 1 mol% PC18, 5 mol% PP\textsubscript{HLE}C18, 5 mol% PP\textsubscript{MMP-2}C18, and 5 mol% PC18, and their pDNA nanoparticles (12:1 lipid: pDNA w/w), determined by PCS. Each result represents the means ± S.D. of three measurements.

The zeta potential data showed decreased zeta potential as PEG was increased to 5 mol% (Figure 3-34), similar to the finding of the cholesterol-anchored PEGylated nanoparticles. Further reduction in the surface charge was observed for pDNA nanoparticles (12:1 lipid: pDNA w/w) due to charge neutralisation, suggesting the formation of liposome: pDNA complexes. The zeta potential of 5 mol% PEGylated nanoparticles was found to be lower than that of their 1 mol% PEGylated counterparts, presumably due to greater shielding of the surface charge associated with higher PEG concentration.
Figure 3-34: Zeta potentials of DODAG-low liposomes containing 1 mol% PP_{HLE}C18, 1 mol% PP_{MMP-2}C18, 1 mol% PC18, 5 mol% PP_{HLE}C18, 5 mol% PP_{MMP-2}C18 and 5 mol% PC18, and their pDNA nanoparticles (12:1 lipid: pDNA w/w), determined by zetasizer. Each result represents the means ± S.D. of three measurements.

3.5.2 MTS cell proliferation assay and LDH cytotoxicity assay with DODAG-low nanoparticles containing PEGylated peptide-C18 conjugates

Like their PEGylated peptide-cholesterol counterparts,PP_{HLE}C18 and PP_{MMP-2}C18 (1 and 5 mol%) were assessed for their effect on cell viability (MTS assay) and toxicity (LDH assay). The nanoparticles were prepared at three lipid: pDNA w/w (8:1, 12:1, and 16:1; 1 μg pDNA/well) and incubated with MCF-7 and HT1080, respectively, for the periods of 4, 8 and 24 h.
Figure 3-35: MTS cell viability result of DODAG-low nanoparticles containing 1 and 5 mol% PP_{HLE}C18 nanoparticles incubated with MCF-7 for 4, 8 and 24 h (a-c) and 1 and 5 mol% PP_{MMP-2}C18 nanoparticles incubated with HT1080 for 4, 8, and 24 h (d-f). The
cell viability data is expressed as a percentage of the positive control cells without the nanoparticles added to them.

Like PP_{HLE}C nanoparticles, PP_{HLE}C18 nanoparticles showed little difference in cell viability irrespective of the molar percentages of PEG, weight ratios, and incubation times (Figure 3-35 a-c). Similar to PP_{MMP-2}C18 nanoparticles, greater than 70% of cell viability was observed for PP_{MMP-2}C18 nanoparticles (Figure 3-35 d-f). These results indicated that with similar liposomal lipid compositions, both C18 and cholesterol-based lipids showed minimal effect on the viability of the cells.

The results obtained from LDH assay showed no significant difference in toxicity for PP_{HLE}C18 nanoparticles regardless of PEG concentrations, weight ratios, and incubation times after 4 and 8 h of incubation; however, an increase in the amount of LDH released of approximately 15% was observed after 24 h (Figure 3-36: a-c). The LDH released due to 1 mol% PP_{MMP-2}C18 was found to be less than 10% for all incubation periods (Figure 3-36: Figure 3-36 d-f). By contrast, for 5 mol% PP_{MMP-2}C18, the level of LDH released increased as the weight ratio and incubation period increased. After 24 h of incubation, over 20% cell death was observed at the weight ratio of 16 while less than 15% was observed for the weight ratio of 12. This cytotoxicity profile is similar to that obtained from 5 mol% PP_{MMP-2}C. The observed effect on cell viability may be due to an impurity from the synthesis and nanoparticle preparation, or the potential effect of the amphiphile itself. The overall result obtained from LDH assay showed that PP_{HLE}C18 and PP_{MMP-2}C18 nanoparticles exhibited similar toxicity profiles to those of PP_{HLE}C and PP_{MMP-2}C nanoparticles, indicating that the type of anchoring lipid did not affect cell proliferation.
Figure 3-36: LDH assay results showing % cytotoxicity as a result of the added DODAG-low nanoparticles containing: a-c) 1 and 5 mol% PP_{HLE}C_{18} nanoparticles incubated with MCF-7 for 4, 8, and 24 h, d-f) 1 and 5 mol% PP_{MMP}C_{18} nanoparticles
incubated with HT1080 for 4, 8, and 24 h. The result is expressed as a percentage normalised against control cells without added nanoparticles.

From the findings of MTS and LDH assay, the lipid: pDNA weight ratio of 12 was found to be optimal for transfection study due to its high pDNA entrapment efficiency and minimal toxicity. This result corresponds to that of their cholesterol-anchored counterparts.

The physiochemical characteristics and toxicity profiles of the PEGylated peptide-C18 system were found to be similar to those of the cholesterol-anchored PEGylated nanoparticles. The result suggested that the incorporation of cholesterol or C18 anchoring lipids, conjugated to PEG up to 5 mol% in the liposome formulation, did not significantly change the overall liposomal properties.

3.5.3 In vitro pDNA transfection of DODAG-low nanoparticles containing PEGylated peptide-C18 conjugates

3.5.3.1 Transfection study of \( \text{PP}_{\text{HLE}} \text{C18} \) nanoparticles

Transfection study of \( \text{PP}_{\text{HLE}} \text{C18} \) nanoparticles was carried out using the same procedure as that of their \( \text{PP}_{\text{HLE}} \text{C} \) counterparts. A marked increase in the transfection efficiency of MCF-7 cells was observed for HLE-treated \( \text{PP}_{\text{HLE}} \text{C18} \) nanoparticles compared with that of non-treated \( \text{PP}_{\text{HLE}} \text{C18} \) nanoparticles and non-cleavable PC18 nanoparticles with HLE treatment and non-treatment \((p < 0.05)\) (Figure 3-37). The gene expression level decreased as PEG concentration increased, presumably due to the effect of steric hindrance. Non-cleavable PC nanoparticles with HLE treatment and non-treatment exhibited comparable efficiencies in transfection and a similar result was also observed for non-PEGylated nanoparticles, suggesting that HLE had a minimal effect on cellular uptake of the nanoparticles. Compared with transfection data obtained from their cholesterol counterparts (Figure 3-22a), C18-anchored PEGylated nanoparticles showed a similar trend of transfection but with a significantly lower
transfection efficiency. This result suggests that PP_{HLE}C18 nanoparticles may undergo partial peptide hydrolysis as in the case of cholesterol-anchored nanoparticles. Incorporation of C18 lipid anchors may well reduce the potential effect of PEG-lipid transfer that might occur in the case of PEG-cholesterol, resulting in more PEG remaining attached to the nanoparticle surface with less cationic surface exposure, and thereby a reduction in transfection efficiency.

Figure 3-37: The HLE transfection data of DODAG-low nanoparticles containing non-PEG, PP_{HLE}C18 (1 and 5 mol%) and PC18 (1 and 5 mol%), with HLE-treatment (black) and non-treatment (white). The transfection was performed in MCF-7 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control with HLE-treatment. Each result represents the mean ± S.D. (n = 3), * indicates p < 0.05.

3.5.3.2 Transfection study of PP_{MMP-2}C18 nanoparticles

In the MMP-2 positive cell line, HT1080, transfection efficiency was markedly increased by PP_{MMP-2}C18 nanoparticles compared to their non-cleavable PC18 counterparts and
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the non-PEGylated system ($p < 0.05$) (Figure 3-38). The steric effect of PEG on cellular uptake and endosomal destabilisation was observed when the molar percentage of PEG was increased. Compared with the transfection results obtained from their cholesterol counterparts (Figure 3-22a), a similar trend of transfection was observed for C18-anchored nanoparticles but with a significant increase in transfection level. This result was opposite to that of the HLE system, in which the level of gene expression was significantly lower when using C18-anchored nanoparticles. The results from both the HLE and MMP-2 systems suggested that the transfer of PEG-cholesterol out of the liposomal membrane may not occur, otherwise a lower level of transfection by C18-anchored nanoparticles should have been observed in both systems. The difference in transfection efficiency observed is likely to be due to different cell types.

Figure 3-38: The MMP-2 transfection data of DODAG0low nanoparticles containing non-PEG, PP$_{MMP-2}$C18 (1 and 5 mol%), and PC18 (1 and 5 mol%) in HT1080 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control. Each result represents the mean ± S.D. (n = 3), * indicates $p < 0.05$. 

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Transfection in the poorly MMP-2 expressing cell line, MCF-7, showed an enhanced level of gene expression by PP\textsubscript{MMP-2}C18 nanoparticles ($p < 0.05$) (Figure 3-39), but to a lesser extent than that obtained in HT1080, highly MMP-2 expressing cells. This result suggests a correlation between transfection efficiency and the cellular expression level of MMP-2. A similar finding was also observed for cholesterol-anchored nanoparticles.

Figure 3-39: The MMP-2 transfection data of DODAG-low nanoparticles containing non-PEG, PP\textsubscript{MMP-2}C18 (1 and 5 mol%), and PC18 (1 and 5 mol%) in MCF-7 cell lines. The transfection efficiency is expressed as % RLU/mg protein of non-PEGylated control. Each result represents the mean ± S.D. ($n = 3$), * indicates $p < 0.05$.

1 and 5 mol% PP\textsubscript{HLE}C18 and PP\textsubscript{MMP-2}C18 were assessed for their stability in the transfection media (10 % FBS). The size measurement showed that all PEGylated nanoparticles exhibited good stability over the incubation period, with very little change in diameter (Figure 3-40). Similar to their cholesterol counterparts, 1 mol% PP\textsubscript{HLE}C18 and PP\textsubscript{MMP-2}C18 were sufficient to prevent aggregation of the nanoparticles. The polydispersity index of the nanoparticles was found between 0.9-1.3 over the course of the experiments.
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Figure 3-40: The stability of DODAG-low nanoparticles containing non-PEG, PP_{HLE}C18 (1 and 5 mol%), and PP_{MMP-2}C18 (1 and 5 mol%) in transfection media (10% FBS), assessed by monitoring the change in nanoparticle diameter as a function of time (every 30 min over 4 h).

3.5.4 Fluorescence Microscopy of rhodamine labelled DODAG-low nanoparticles containing PEGylated peptide-C18 conjugates

Similar to their cholesterol counterparts, cellular uptake studies of PP_{HLE}C18, PP_{MMP-2}C18, and PC18 liposomes were carried out by incorporating 1 mol% rhodamine-DOPE into the liposome formulation. The labelled PP_{HLE}C18 and PC18 with HLE treatment and non-treatment were incubated with MCF-7 cells for a period of 6 h. Figure 3-41 showed that the PEGylated liposomes were successfully taken up by the cells, and HLE-pretreated 1 mol% PP_{HLE}C18 liposomes exhibited a significantly higher level of internalisation compared to non-treated 1 mol% PP_{HLE}C18 and the PEGylated control PC18 liposomes with HLE treatment and non-treatment. This observation was similar to that of the PP_{HLE}C system, presumably due to the dissociation of PEG from the liposome surface, leading to enhanced electrostatic interaction with the cell membrane.
The cell uptake study correlated well with the transfection data of $\text{PP}_{\text{HLE C18}}$ nanoparticles.

<table>
<thead>
<tr>
<th>i) Bright field</th>
<th>ii) DAPI</th>
<th>iii) Rhodamine</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Bright field" /></td>
<td><img src="image2" alt="DAPI" /></td>
<td><img src="image3" alt="Rhodamine" /></td>
</tr>
<tr>
<td>a) 1% $\text{PP}_{\text{HLE C18}}$ with HLE treatment</td>
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<tr>
<td><img src="image4" alt="Bright field" /></td>
<td><img src="image5" alt="DAPI" /></td>
<td><img src="image6" alt="Rhodamine" /></td>
</tr>
<tr>
<td>b) 1% $\text{PP}_{\text{HLE C18}}$ with non-treatment</td>
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</tr>
<tr>
<td><img src="image7" alt="Bright field" /></td>
<td><img src="image8" alt="DAPI" /></td>
<td><img src="image9" alt="Rhodamine" /></td>
</tr>
<tr>
<td>c) 1% PC18 with HLE treatment</td>
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Figure 3-41: Fluorescence microscopy images (40x magnifications) of MCF-7 cells after incubation with rhodamine labelled DODAG-low liposomes a) 1% PP\textsubscript{HLE}C18 with HLE treatment, b) 1% PP\textsubscript{HLE}C18 with non-treatment, c) 1% PC18 with HLE treatment, and d) 1% PC18 with non-treatment, showing i) bright field ii) DAPI stained nuclei and iii) rhodamine labelled liposome images.

The cellular internalisation of rhodamine labelled PP\textsubscript{MMP-2}C18 and PC18 liposomes is presented in Figure 3-42. Similar to the cellular uptake of PP\textsubscript{MMP-2}C liposomes, 1 and 5 mol% of PP\textsubscript{MMP-2}C18 showed a marked increase in fluorescence intensities compared to that of their PEGylated controls. 1 mol% PP\textsubscript{MMP-2}C18 nanoparticles showed greater rhodamine intensity compared with their 5 mol% counterparts. This result is in agreement with the transfection efficiency of PP\textsubscript{MMP-2}C18 nanoparticles.
Figure 3-42: Fluorescence microscopy images (40x magnifications) of HT1080 cells after incubation with rhodamine labelled DODAG-low liposomes: a) 1 mol% PP_{MMP-2}C, b) 1 mol% PC, c) 5 mol% PP_{MMP-2}C, and d) 5 mol% PC, showing i) bright field ii) DAPI stained nuclei and iii) rhodamine labelled liposome images.
3.5.5 Stability of DODAG-low nanoparticles containing PEGylated peptide-C18 conjugates in 80% serum

The stability of PP_{HLE}C18 and PP_{MMP-2}C18 nanoparticles (1-5 mol%) in high serum content (80%) were further assessed using a turbidity assay by monitoring the change in the turbidity of the solution containing the nanoparticles at $\lambda = 600$ nm. A rapid increase in the absorbance of 1 mol% PP_{HLE}C18 was observed after the first 30 min of the experiment (Figure 3-43), indicating an increase in the turbidity of the solution due to nanoparticle aggregation. At 2 and 3 mol% PEG, the nanoparticles showed slower rates of aggregation as seen by gradual increases in absorbance. 4 and 5 mol% PEG exhibited constant levels of absorbance over the course of the experiment, indicating no aggregation. In the case of PP_{MMP-2}C18 nanoparticles, 1 and 2 mol% PEG exhibited significant increases in absorbance. The aggregation was significantly reduced as PEG concentration increased, in particular at 4 and 5 mol% PEG. These observations were in agreement with the findings of PP_{HLE}C and PP_{MMP-2}C nanoparticles: that 4-5 mol% PEG appeared to be optimal to ensure sufficient nanoparticle stability in high serum concentration.
Chapter Three: Biophysical and in vitro biological evaluation of PEGylated peptide-lipid conjugates: DNA system

In this chapter, the effect of the PEGylated peptide-lipid conjugates in liposome formulations was assessed with respect to physicochemical properties, toxicity, and stability. In addition, their ability to mediate pDNA delivery in the presence and absence of the specific enzyme was discussed.

The effect of cationic charge on cell association was investigated using two types of formulations, DODAG-high (50% DODAG) and DODAG-low (20% DODAG). Both DODAG-high and DODAG-low formulations exhibited high pDNA encapsulation efficiency at 12:1 lipid: pDNA w/w and the pDNA nanoparticles formed at this ratio were found to be approximately 130-200 nm in diameter. The zeta potential measurement revealed that the surface charge of the liposomes could be altered by manipulating the

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**Figure 3-43:** The stability of DODAG-low nanoparticles in 80 % FBS assessed by measuring the absorbance at 600 nm, a) 1-5 mol% PP_HLE_C18 and b) 1-5 mol% PP_MMP-2_C18. The UV absorbance was monitored every 30 min over 4 h using UV spectrometer.

### 3.6 Chapter summary

In this chapter, the effect of the PEGylated peptide-lipid conjugates in liposome formulations was assessed with respect to physicochemical properties, toxicity, and stability. In addition, their ability to mediate pDNA delivery in the presence and absence of the specific enzyme was discussed.
amount of the cationic lipid in the formulation as well as the degree of PEGylation. Incorporation of the same molar ratio of PP_{HLE}C, PP_{MMP-2}C, PC, PP_{HLE}C18, PP_{MMP-2}C18, and PC18 within liposomes did not result in any significant change in the physicochemical characteristics of the nanoparticles, suggesting that the peptide linkers and type of lipid conjugate had minimal effects on nanoparticle properties.

PP_{HLE}C and PP_{MMP-2}C DODAG-high nanoparticles were shown to enhance transfection efficiency in the presence of HLE and MMP-2, respectively, compared with non-cleavable PC nanoparticles, demonstrating that the nanoparticles may be activated in response to the presence of the enzymes. The level of gene expression decreased when transfecting with the DODAG-low nanoparticles due to the reduction in electrostatic interaction with cell membranes. Despite having lower transfection efficiency, the DODAG-low nanoparticles exhibited greater resistance to aggregation in the serum-contained medium compared with the DODAG-high system. The increased stability was also observed when PEG was incorporated. This indicated that the surface charge of the nanoparticles played a crucial role in both the transfection ability and the stability of the nanoparticles. Aggregation of cationic nanoparticles caused by adsorption of negatively charged serum proteins, leading to increased size and subsequent removal by RES, is an unfavourable event since this can limit the therapeutic efficacy of nanoparticles. To prolong blood circulation half-lives and enable maximum accumulation at tumour tissues via the EPR effect, nanoparticles with lower zeta potential are more preferable, although such a characteristic is often associated with decreased cellular binding. The introduction of PEG that can be detached by tumour-associated enzymes could be a strategy to improve nanoparticle stability while in circulation by masking their surface charge and restore electrostatic interaction with the cell membrane once PEG is detached by the enzymes, facilitating cellular uptake as well as endosomal escape.

HLE-treated PP_{HLE}C and PP_{HLE}C18 nanoparticles showed remarkably enhanced gene expression levels on the tested cell lines compared with non-treated PP_{HLE}C and PP_{HLE}C18 nanoparticles and non-cleavable PC and PC18 nanoparticles with HLE treatment and non-treatment. A significant increase in transfection efficiency in MMP-2 positive cell lines was observed for PP_{MMP-2}C and PP_{MMP-2}C18, and the enhancement in
gene expression was lower in MMP-2 negative cells. Non-cleavable PC and PC18 nanoparticles showed increased levels of gene expression but to a lesser extent than the nanoparticles bearing peptide linkers, demonstrating the ability of PEGylated nanoparticles to be internalised into the cells but with reduced levels of endosomal escape. A similar trend of transfection was observed in both the HLE and MMP-2 systems; however, the transfection level was found to vary for each cell line and was not correlated with the types of lipid conjugates. Collectively, the transfection data suggested the possibility of partial peptide hydrolysis by specific enzymes leading to partial PEG detachment. The resulting nanoparticles may then have re-obtained part of their positive charges, facilitating interaction with anionic cell membranes and leading to enhanced cellular uptake and endosomal escape. Although the mechanism of the enzyme-mediated pDNA delivery is not yet understood and requires further investigation, the enhancement of gene expression by the nanoparticles containing PEGylated peptide-lipid in the presence of enzymes highlighted the possibility of the use of enzyme-mediated nucleic acid delivery to promote endocytosis and subsequent release of pDNA from endosomes into cytosol. These nanoparticles were found to be non-toxic to the tested cell lines as assessed by MTS cell viability and LDH toxicity assays. The nanoparticles also exhibited good stability in higher serum concentrations (80%), in particular when 4-5 mol% PEG was incorporated.
4. Biophysical and in vitro biological evaluation of PEGylated peptide-lipid conjugates: siRNA system
It was shown in the previous chapter that the transfection efficiency of the nanoparticles being studied could be assessed using pDNA encoding for luciferase as a reporter gene. However, for medical applications, the use of therapeutic nucleic acids encapsulated in nanoparticles would be required. siRNA has been increasingly recognised over the last ten years as a potential therapeutic tool for many diseases including cancer due to its ability to induce specific gene silencing. siRNA is a short sequence of double stranded RNA, usually 21-23 nucleotides long, of which 19-21 nucleotides form base pair duplexes and 2 nucleotides on each of the 3' ends overhang (Figure 4-1a). siRNA mediates gene silencing through sequence-specific cleavage of the corresponding mRNA (Figure 4-1b). After being taken up by cells, siRNA is incorporated into a multi-enzyme complex, consisting of Argonaute 2 (AGO2) and the RNAi-induced silencing complex (RISC). AGO2 unwinds the siRNA and cleaves the sense strand (or passenger strand). The activated AGO2-RISC complex containing only the antisense strand (or guide strand) then binds to the complementary mRNA. The target mRNA is cleaved at a single site in the centre of the duplexes, 10 nucleotides from the 5' end of the siRNA, resulting in rapid degradation and thereby inhibiting the expression of the corresponding protein.
Figure 4-1: a) Structure of siRNA. Two strands of the siRNA, around 21 nucleotides, form approximately 19 nucleotide duplexes with 2 nucleotides overhanging on each 3’ end. The cleavage position is indicated by the black arrow and the seed region indicates the position where interaction with the target mRNA begins.256 b) Representation of the siRNA mechanism. On cellular internalisation, siRNA is loaded onto RISC and its sense strand is cleaved by AGO2. The activated RISC-antisense strand complex binds to and degrades the complementary target mRNA, leading to target gene silencing.257
As with DNA, siRNA is prone to nuclease digestion in blood circulation, leading to the loss of its therapeutic function. In addition, its negative charge due to its anionic phosphate backbone prevents efficient association with cell membranes. siRNA therefore needs to be encapsulated in a carrier in order to protect it from enzymatic degradation and also facilitate cellular internalisation.\textsuperscript{257, 258} For specific delivery to occur, the carriers should have properties that aid their long circulation and accumulation in tumour tissues. After administration, the delivery of siRNA faces similar hurdles to those of DNA delivery, except for the nuclear barrier. The mechanism of siRNA-mediated gene silencing occurs primarily in the cytoplasm, unlike DNA which requires further transportation across the nuclear membrane for transcription. The use of siRNA therefore avoids one of the major obstacles encountered by DNA delivery. Since the final destination of siRNA and DNA delivery are the cytoplasm and nucleus, respectively, of the target cells, being endocytosed is not sufficient. The most challenging part of effective gene silencing or gene expression is endosomal escape, which usually requires the use of helper lipids which can induce phase transition of lipid bilayers in order to promote the release of encapsulated nucleic acids from endosomes. PEGylation of nanoparticles is usually found to inhibit the interaction between cationic nanoparticles and anionic endosomal membranes due to the steric hindrance of PEG, which prevents liposomal content from being released into cytosol. The presence of a PEG layer appears to be problematic at the intracellular level in both DNA and siRNA systems. A PEG dissociation strategy, such as the enzyme-triggerable detachment of the PEG layer, which can be employed to promote the cellular uptake and endosomal escape of DNA nanoparticles may also be applied to siRNA delivery. Following the investigation of the low-charge PEGylated peptide-lipid nanoparticles for mediating pDNA delivery in response to specific enzymes, we continue to evaluate the use of this triggerable system for siRNA-mediated knockdown of specific proteins.

### 4.1 DODAG-low siRNA nanoparticles

Here, the same DODAG-low formulation (20% DODAG) as for pDNA delivery was employed for siRNA (Table 4-1). The encapsulation of siRNA was carried out using the same procedure as for pDNA encapsulation.
Table 4.1: Lipid components in low-charge formulation for siRNA delivery

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Molar % of total lipid</th>
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<tbody>
<tr>
<td>DODAG</td>
<td>20</td>
</tr>
<tr>
<td>DOPC</td>
<td>50 (or 49 or 45)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>30</td>
</tr>
<tr>
<td>PP_{HLE}C, PP_{MMP-2}C, PC,</td>
<td>0 (or 1 or 5)</td>
</tr>
<tr>
<td>PP_{HLE}C18, PP_{MMP-2}C18, or PC18</td>
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</table>

**4.1.1 Physicochemical characterisation of DODAG-low siRNA nanoparticles**

**4.1.1.1 siRNA encapsulation efficiencies**

As with pDNA, siRNA has anionic phosphate backbones and can therefore interact electrostatically with positively charged liposomes to form liposome-siRNA complexes. However, siRNA is much smaller than pDNA with 19-21 base pair (bp) duplexes, whereas the minimal size of pDNA for condensation is approximately 4000 bp. siRNA therefore has far fewer negative charges – a maximum of 42 negative charges per molecule – while pDNA has approximately 8000 negative charges. The smaller number of siRNA charges allows the charge ratio of nanoparticles and siRNA to be easily manipulated. The charge ratios or N/P ratios refer to the relative numbers of cationic charges from the lipids in the formulation (N) (1.7 per molecule of DODAG) and anionic charges from phosphate groups of siRNA molecules (2 per bp). siRNA nanoparticles were prepared at different charge ratios by fixing the amount of siRNA and increasing the amount of the preformed empty liposomes. Their encapsulation efficiency was then assessed using PI assay.
**Figure 4-2:** siRNA encapsulation efficiency of DODAG-low nanoparticles with a) non-PEG, 1 and 5 mol% PP_{HLE}C, 1 and 5 mol% PP_{MMP-2}C, and 1 and 5 mol% PC b) with 1 and 5 mol% PP_{HLE}C_{18}, 1 and 5 mol% PP_{MMP-2}C_{18}, and 1 and 5 mol% PC_{18} at different lipid: pDNA charge ratios, using propidium iodide assay. The encapsulation efficiency
of siRNA is presented as a percentage of the amount of siRNA initially used in the preparation.

The data from the PI assay (Figure 4-2a and b) demonstrated an increase in entrapment efficiency as the charge ratio increased, due to a greater amount of cationic lipid being present for interaction with the siRNA. For all formulations, a lower degree of siRNA encapsulation was found at higher PEG concentrations, suggesting that PEG may shield the surface charge available for binding to siRNA. A significant increase in entrapment efficiency was observed at charge ratios of 2 and 4. At charge ratio of 4, the nanoparticles exhibited more than 80% siRNA encapsulation, and no significant change in entrapment efficiency was found at the charge ratio higher than 4.

Agarose gel electrophoresis revealed similar findings to those of the PI assay, showing almost complete siRNA encapsulation at a charge ratio of 4 for most of the formulations (Figure 4-3). However, 1 and 5 mol% PP_HLE C nanoparticles showed some migration of siRNA at the charge ratio of 4, compared to the 99% siRNA encapsulation observed in the PI assay, suggesting that some siRNA may transverse at the external layer of liposomes, and upon the application of an electric field these surface-bound siRNAs may disassociate from the liposomes. This observation was supported by the study of Buyens et al., which demonstrated that classical mixing of siRNA with preformed PEGylated liposomes could result in the binding of some siRNA to the outer surface of the liposomes. It is interesting that this occurs with PP_HLE C siRNA nanoparticles, indicating that the siRNA translocation to the surface might be affected by the peptide sequence.
Chapter Four: Biophysical and in vitro biological evaluation of PEGylated peptide-lipid conjugates: siRNA system

a) Lipid: siRNA charge ratio

Non PEG

1% PP_{HLE}\ C

1% PP_{MMP2}\ C

d) Liposomes

0.5:1  2:1  4:1  8:1

1% PC

5% PP_{HLE}\ C

5% PP_{MMP2}\ C

g) Liposomes

0.5:1  2:1  4:1  8:1

5% PC

1% PP_{HLE}\ C18

1% PP_{MMP2}\ C18
Chapter Four: Biophysical and in vitro biological evaluation of PEGylated peptide-lipid conjugates: siRNA system

Figure 4-3: 1% agarose gel electrophoresis demonstrated siRNA encapsulation of DODAG-low nanoparticles with a) non-PEG, b) 1 mol% PP_{HLE}C, c) 1 mol% PP_{MMP-2}C, d) 1 mol% PC, e) 5 mol% PP_{HLE}C, f) 5 mol% PP_{MMP-2}C, g) 5 mol% PC, h) 1 mol% PP_{HLE}C18, i) 1 mol% PP_{MMP-2}C18, j) 1 mol% PC18, k) 5 mol% PP_{HLE}C18, l) 5 mol% PP_{MMP-2}C18, m) 5 mol% PC18 at different lipid: siRNA charge ratios (gel a): lane 1 = siRNA, lane 2 = empty liposomes, lipid: siRNA charge ratios: lane 3 = 0.5:1, lane 4 = 2:1, lane 5 = 4:1 and lane 6 = 8:1; gel b-m): lane 1 = empty liposomes, lipid: siRNA charge ratios: lane 2-5 = same as lane 3-6 of gel a)). The electrophoresis was performed at 65 mV for 30 min and the gels were visualized under UV light using Alliance 4.7 UVITEC Cambridge.
According to the result obtained from the PI assay and agarose gel electrophoresis, all liposome formulations showed almost complete siRNA encapsulation at a charge ratio of 4. As a result, lipid: siRNA was kept at this ratio for all experiments related to DODAG-low siRNA nanoparticles.

### 4.1.1.2 Size and zeta potential measurements

PCS measurement (Figure 4-4a) showed increases in nanoparticle sizes after siRNA loading, indicating the encapsulation of siRNA. The diameters of siRNA nanoparticles were found to be within 93-120 nm, which is advantageous for in vivo systemic delivery since nanoparticles with diameter less than 200 nm are capable of bypassing the fenestrations in tumour vasculature and thereby accumulating in tumour tissue. The polydispersity index of siRNA nanoparticles was found to be 0.2-0.7.

The zeta potential of siRNA nanoparticles was found to decrease after siRNA entrapment (Figure 4-4b), suggesting charge neutralisation as a result of the complexation between liposomes and siRNA. At higher PEG concentrations, the nanoparticles exhibited lower surface charges as a result of the shielding effect of PEG. The zeta potential was almost neutral when 5 mol% PEG was incorporated. This low zeta potential is a favourable characteristic for a nucleic acid delivery system for in vivo application owing to the ability of nanoparticles to avoid RES uptake, therefore increasing their blood circulation half-lives.
Figure 4-4: a) Average diameters and b) zeta potentials of DODAG-low siRNA nanoparticles (4:1 lipid: siRNA charge ratio) containing non-PEG, 1 mol% PP<sub>HLEC</sub>, 1 mol% PP<sub>MMP-2C</sub>, 1 mol% PC, 5 mol% PP<sub>HLEC</sub>, 5 mol% PP<sub>MMP-2C</sub> and 5 mol% PC, 1 mol% PP<sub>HLEC18</sub>, 1 mol% PP<sub>MMP-2C18</sub>, 1 mol% PC18, 5 mol% PP<sub>HLEC18</sub>, 5 mol%
PP_{MMP-2}C18 and 5 mol% PC18, compared to their empty liposomes. Each result represents means ± S.D. of three measurements.

### 4.1.2 Exogenous luciferase gene knockdown using PEGylated peptide-lipid conjugates

Although siRNA nanoparticles were shown to have the optimal physiochemical properties required for efficient nucleic acid delivery in vivo, the most important feature is their ability to deliver the siRNA and render specific gene knockdown. The biological activity of the siRNA nanoparticles was evaluated in vitro using a luciferase reporter gene. MCF-7 and HT1080 cells were pretransfected with pDNA encoding for luciferase (pEGFPLuc) using a commercially available transfection agent jetPEI™ to induce transient luciferase expression. Here, Lipofectamine™ 2000 was not used since it was found to be potentially toxic to HT1080, causing a high level of cell death during a period of incubation. The nanoparticles were prepared to encapsulate either a specific anti-luciferase or a control nonspecific siRNA at the charge ratio of 4. Once the cells were expressing the luciferase gene, the siRNA nanoparticles were administered. Prior to using PEGylated peptide-lipid nanoparticles for functional siRNA delivery, the optimal siRNA concentrations required for maximum gene knockdown were examined using non-PEGylated nanoparticles. The non-PEGylated nanoparticles encapsulating siRNA were incubated with luciferase-pretransfected MCF-7 and HT1080 cells for 6 and 24 h, respectively. The cells were incubated for further 36 h before analysis for luciferase protein content.
Figure 4-5: Knockdown of transient luciferase expression using anti-luciferase siRNA encapsulated in non-PEGylated nanoparticles (at charge ratio of 4) in a) MCF-7 and b) HT1080 cells with different siRNA concentrations. The result is presented as % differential knockdown between anti-luciferase siRNA and nonspecific siRNA delivery calculated using the following formula: \([1 - (\text{luciferase activity}_{\text{anti-luciferase siRNA}}/ \text{luciferase activity}_{\text{nonspecific siRNA}})] \times 100.\)
Figure 4-5 shows a concentration dependent pattern of gene knockdown efficiency. The optimal dose of siRNA for maximising luciferase knockdown was found to be 15 nmol/well (30 μM) in both MCF-7 and HT1080 cells. Increasing siRNA concentrations above this level showed no significant enhancement in gene knockdown efficiency. This amount of siRNA, 30 μM, was considered a high dose since siRNA concentrations typically employed to achieve maximum gene silencing in vitro have been found to be within a range of nM, depending on formulations, target genes, cell types, and transfection conditions.\textsuperscript{120, 212, 260, 261} The requirement for an increased quantity of siRNA may be due to two possibilities: the efficiency of the nanoparticles to mediate siRNA delivery; and a high level of transient luciferase expression (\textasciitilde 10^7-10^8 RLU/mg protein) so that low siRNA doses (nM) were not capable of rendering significant gene knockdown. To investigate these possibilities, a commercially available siRNA transfection agent, Dharmafect\textsuperscript{®}, was used for siRNA delivery and its knockdown efficiency was compared to that of the non-PEGylated nanoparticles. Two siRNA doses, 15 pmol (30 nM) and 15 nmol (30 μM), were chosen as low and high siRNA concentrations. Interestingly, both Dharmafect\textsuperscript{®} and the non-PEGylated nanoparticles showed comparable levels of luciferase knockdown at the same siRNA doses in both MCF-7 and HT1080 cells (Figure 4-6). This result suggests that the ability of non-PEGylated nanoparticles to mediate siRNA delivery were as efficient as Dharmafect\textsuperscript{®}, and therefore we assumed that it was likely that a high level of transient luciferase expression required high siRNA doses to effectively induce gene silencing. Increasing the siRNA concentration is often observed to have undesirable effects including nonspecific gene knockdown and toxic side effects.\textsuperscript{262-264} These effects are dose-dependent and can be avoided by using lower siRNA concentrations. However, other studies demonstrated that nonspecific effects had a similar dose response to that required for the silencing of the target gene.\textsuperscript{265}
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Figure 4-6: Comparison of siRNA-mediated luciferase knockdown using a commercially available siRNA transfection reagent, DharmaFect®, and DODAG-low non-PEGylated nanoparticles in a) MCF-7 and b) HT1080 cells using two siRNA concentrations: 15 pmol/well and 15 nmol/well, 48-well plate. The result is presented as % knockdown of luciferase protein by anti-luciferase siRNA normalised by luciferase activity obtained from control nonspecific siRNA.
As siRNA concentrations increased, nonspecific siRNA was found to mediate luciferase gene knockdown to some extent, leading to downregulation of the luciferase protein (data not shown). The data further suggested that the expression of other cellular proteins might be inhibited by the nonspecific effect, and therefore high siRNA concentrations should be avoided. The siRNA concentration in nM when delivered using non-PEGylated nanoparticles, however, resulted in low knockdown efficiency (<15 %). We postulated that when using PEGylated peptide-lipid nanoparticles and their PEGylated controls for gene knockdown studies, it would be more difficult to clearly see the effect of enzyme-mediated delivery of siRNA. No significant detachment of cells from the transfection wells due to toxicity was observed at a siRNA concentration of 15 nmol/well and therefore the toxic effect induced by increased siRNA doses was not investigated. In the future, it would be useful to further assess the effect of siRNA quantity on cellular viability and toxicity using MTS, LDH, or apoptosis assay to ensure the suitability of the siRNA dose for mediating gene knockdown with minimal toxicity. Because of the reasons mentioned above, we decided to use 15 nmol of siRNA/well to examine the effect of enzyme-assisted PEG detachment on promoting endocytosis of the nanoparticles and ultimately enhancing the level of luciferase knockdown as a result of improved endosomal escape of encapsulated siRNA.

4.1.2.1 Transient luciferase gene knockdown using \( PP_{HLEC} \) and \( PP_{HLEC18} \) nanoparticles

In the HLE experiment, nanoparticles encapsulating either anti-luciferase or nonspecific siRNA were divided into two groups, HLE treatment and non-treatment, as were those prepared for pDNA delivery. MCF-7 cells were pretransfected with pDNA encoding for luciferase prior to the administration of siRNA nanoparticles. After 6 h of transfection and a further 36 h of incubation, it was found that \( PP_{HLEC} \) nanoparticles after HLE treatment were able to silence the expression of luciferase protein, although with relatively modest efficiency, compared to that of non-treated \( PP_{HLEC} \) nanoparticles and PC nanoparticles with HLE treatment and non-treatment (\( p < 0.05 \)) (Figure 4-7a). This result suggests that \( PP_{HLEC} \) nanoparticles may be activated by the enzyme, ultimately promoting the endosomal escape of siRNA into the cytosol where it functions. The silencing effect of the \( PP_{HLEC} \) nanoparticles was found to be similar to that of the non-
PEGylated nanoparticles ($p > 0.05$). Both 1 and 5 mol\% PC nanoparticles were shown to inhibit protein expression at lower levels compared to PP\textsubscript{HLE}C nanoparticles, demonstrating that PEGylated nanoparticles did not inhibit cellular uptake but greatly interfered with the release of nucleic acids from endosomal compartments. This observation was in agreement with the findings of the pDNA system. The level of protein knockdown in the case of non-PEGylated nanoparticles and 1 and 5 mol\% PC nanoparticles were found to be similar in both HLE treatment and non-treatment groups, suggesting that the cellular uptake of these nanoparticles was not affected by the presence of the enzyme.

An enhancement in knockdown efficiency by HLE-pretreated PP\textsubscript{HLE}C18 nanoparticles was also observed, compared to that of PP\textsubscript{HLE}C18 with non-treatment and HLE-treated and non-treated PC18 nanoparticles (Figure 4-7b). The trend of luciferase silencing mediated by C18-anchored PEGylated nanoparticles in response to enzyme treatment and non-treatment was found to be similar to that of the cholesterol-anchored system with slight differences in knockdown efficiency.
Figure 4-7: Knockdown of transient luciferase expression by anti-luciferase siRNA encapsulated in DODAG-low nanoparticles containing a) non-PEG, PP$_{HLEC}$ (1 and 5 mol%) and PC (1 and 5 mol%) b) non-PEG, PP$_{HLEC18}$ (1 and 5 mol%) and PC18 (1 and 5 mol%), with HLE-treatment (black) and non-treatment (white). The transfection was performed on MCF-7 cell lines and the data are presented as % differential.
knockdown between anti-luciferase siRNA and nonspecific siRNA delivery. Each result represents the mean ± S.D. (n = 3), * indicates p < 0.05.

4.1.2.2 Transient luciferase gene knockdown using $PP_{\text{MMP-2C}}$ and $PP_{\text{MMP-2C18}}$ nanoparticles

In the MMP-2 experiment, HT1080 cells were pretransfected with pDNA encoding for luciferase prior to siRNA delivery. After 24 h of transfection and a further 36 h of incubation, it was shown that $PP_{\text{MMP-2C}}$ nanoparticles were able to inhibit protein expression at significantly higher levels compared to that of non-cleavable PC nanoparticles ($p < 0.05$) (Figure 4-8a), suggesting the activation of $PP_{\text{MMP-2C}}$ nanoparticles in response to the secreted MMP-2 in the culture medium. The knockdown efficiency of both 1 and 5 mol% $PP_{\text{MMP-2C}}$ nanoparticles was found to be higher than 50%. Non-cleavable PC nanoparticles were able to mediate siRNA delivery, as shown by increases in knockdown efficiency, indicating their capability of cellular uptake.

An enhancement in the level of luciferase knockdown was also observed when using $PP_{\text{MMP-2C18}}$ nanoparticles, compared with non-cleavable PC18 nanoparticles ($p < 0.05$) (Figure 4-8b). The inhibiting effect of PEG was clearly seen with increasing degrees of PEGylation. C18-anchored PEGylated nanoparticles showed a similar trend of protein knockdown to that of the cholesterol-anchored system, but to a lesser extent of efficiency.
Figure 4-8: Knockdown of transient luciferase expression by anti-luciferase siRNA encapsulated in DODAG-low nanoparticles containing a) non-PEG, PP$_{\text{MMP-2C}}$ (1 and 5 mol%) and PC (1 and 5 mol%) b) non-PEG, PP$_{\text{MMP-2C18}}$ (1 and 5 mol%) and PC18 (1 and 5 mol%). The transfection was performed on MMP-2 positive HT1080 cell lines and the data was presented as % differential knockdown between anti-luciferase siRNA
and nonspecific siRNA delivery. Each result represents the mean ± S.D. (n = 3), * indicates p < 0.05.

According to transfection data obtained, the PEGylated peptide lipid nanoparticles were shown to mediate significant luciferase knockdown in response to the specific enzymes, compared with their control PEGylated counterparts. Although this system was somewhat remote from the knockdown of an endogenous gene since pDNA transfection is required prior to the delivery of siRNA, this fact should not interfere with the formulation parameters that were examined.

4.1.3 Knockdown of luciferase gene in stably transfected cells using PEGylated peptide-lipid conjugates

Previously, it was demonstrated that the PEGylated peptide-lipid nanoparticles were capable of silencing transient expression of luciferase gene in the presence of the enzymes. To confirm the significant of this data in a system that is more physiologically relevant, we continued our investigation with stably transfected luciferase cell lines, seeking to the knockdown of luciferase gene. Cell lines stably-expressing luciferase were obtained by mean of transfection using pDNA containing luciferase reporter gene, G418 antibiotic resistant gene and S/MAR sequence, which was kindly provided by Dr. Orestis Agyros and Dr. Richard Harbottle. Inclusion of S/MAR sequence into plasmid construct was shown to confer higher and more sustained transgene expression in both in vitro and in vivo. The transfected cells were kept under antibiotic selection to obtain stably luciferase expressed cell lines, which were then used for siRNA knockdown experiment. The level of luciferase expression was found to be ≈10⁸ RLU/mg protein.
4.1.3.1 Knockdown of luciferase gene in stably transfected luciferase cell lines using PP\textsubscript{HLE}C and PP\textsubscript{HLE}C18 nanoparticles

In HLE experiment, the nanoparticles encapsulating either anti-luciferase or nonspecific siRNA were prepared at charge ratio of 4 and were administered to MCF-7 cell lines stably-expressing luciferase (MCF-7-luc). The transfection data showed that HLE-treated PP\textsubscript{HLE}C nanoparticles were able to significantly inhibit luciferase expression although with modest efficiency, compared with that of non-treated PP\textsubscript{HLE}C nanoparticles and non-cleavable PC nanoparticles with HLE treatment and non-treatment ($p < 0.05$) (Figure 4-9a). Similar finding was also observed when using C18-anchored PEGylated nanoparticles, showing increased luciferase knockdown by HLE-treated PP\textsubscript{HLE}C18 nanoparticles in comparison with their control PC nanoparticles ($p < 0.05$) (Figure 4-9b). These observations were in line with the data obtained from transient luciferase expression experiment although with some variations, confirming the ability of the PEGylated peptide-lipid nanoparticles to promote siRNA delivery in response to the specific enzyme.
Figure 4-9: Knockdown of luciferase expression in stably transfected luciferase cell lines by anti-luciferase siRNA encapsulated in DODAG-low nanoparticles containing a) non-PEG, PP<sub>HLE</sub>C (1 and 5 mol%) and PC (1 and 5 mol%) b) non-PEG, PP<sub>HLEC18</sub> (1 and 5 mol%) and PC18 (1 and 5 mol%), with HLE-treatment (black) and non-treatment (white). The transfection was performed in MCF-7-luc cell lines and the data are
presented as % differential knockdown between anti-luciferase siRNA and nonspecific siRNA delivery. Each result represents the mean ± S.D. (n = 3), * indicates p < 0.05.

4.1.3.2 Knockdown of luciferase gene in stably transfected luciferase cell lines using PP\textsubscript{MMP-2}C and PP\textsubscript{MMP-2}C18 nanoparticles

To ensure that HT1080 cell lines stably expressing luciferase (HT1080-luc) were able to secrete the enzyme, the transfected cells were characterised for their levels of MMP-2 expression in cell culture medium using MMP-2 human ELISA assay. The cells were found to secrete high levels of MMP-2 (approximately 9 ng/mL), similar to that of normal HT1080 cells as shown in the previous chapter, and therefore were suitable to assess the ability of PP\textsubscript{MMP-2}C and PP\textsubscript{MMP-2}C18 nanoparticles to mediate siRNA delivery in response to the enzyme.

\textit{In vitro} transfection data showed that PP\textsubscript{MMP-2}C nanoparticles were able to silence the expression of luciferase protein in response to MMP-2 compared to non-cleavable PC nanoparticles (p < 0.05), although the knockdown efficiency was found to be modest (Figure 4-10a). The inhibition of luciferase expression was significantly enhanced by PP\textsubscript{MMP-2}C18 nanoparticles in comparison with their control PC counterparts (p < 0.05) (Figure 4-10b). The result was in agreement with that of the transient luciferase expression experiment, with some variation in knockdown efficiency. The data obtained confirmed that PP\textsubscript{MMP-2}C and PP\textsubscript{MMP-2}C18 nanoparticles were capable of improving siRNA delivery in response to the MMP-2 enzyme.
Figure 4-10: Knockdown of luciferase expression in stably transfected luciferase cell lines by anti-luciferase siRNA encapsulated in DODAG-low nanoparticles containing a) non-PEG, PP$_{MMP-2}$C (1 and 5 mol%), and PC (1 and 5 mol%), b) non-PEG, PP$_{MMP-2}$C18 (1 and 5 mol%), and PC18 (1 and 5 mol%). The transfection was performed in HT1080-luc cell lines and the data are presented as % differential knockdown between anti-luciferase siRNA and nonspecific siRNA delivery. Each result represents the mean ±
S.D. of three different experiments. Each result represents the mean ± S.D. (n = 3), * indicates p < 0.05.

The use of anti-luciferase siRNA served as a proof of concept to assess the ability of nanoparticles for functional siRNA delivery in vitro. The transfection data of all PEGylated peptide-lipid nanoparticles emphasises the importance of incorporating cleavable PEGylated lipid conjugates within nanoparticles to improve siRNA delivery. The luciferase knockdown efficiency was found to be variable depending on cell types and lipid anchoring system, but no direct relation among these parameters could be made. This variation may be partially attributed to the high levels of luciferase expression in both transient and stably transfected cell lines, making accurate analysis difficult in cases of low gene knockdown efficiency. In the case of transient gene transfection, lower gene expression may be achieved by optimising the amount of pDNA used for transfection and also the duration that the transfection reagent is exposed to cells. For stably transfected cell lines, using a cell batch with lower luciferase expression or seed cells at a lower density may decrease the level of gene expression. The reduced gene expression level may enable the use of lower siRNA concentrations and thus the nonspecific effects and toxicity associated with higher siRNA doses may be reduced.

4.2 Chapter summary

In this chapter, the viability of PEGylated peptide-lipid nanoparticles to enhance nucleic acid delivery was further investigated in a siRNA system. The nanoparticles showed a high siRNA encapsulation efficiency at a charge ratio of 4, and siRNA nanoparticles prepared at this charge ratio were small with diameters approximately less than 120 nm. The zeta potential of the nanoparticles decreased as the degree of PEGylation increased, and the surface charge was found to be almost neutral when 5 mol% PEG was incorporated within the nanoparticles. These features of the siRNA nanoparticles were advantageous for in vivo applications with respect to small size, low charge, and the ability to efficiently encapsulate nucleic acids. The optimisation of siRNA doses was investigated by performing protein knockdown experiments: non-PEGylated
nanoparticles produced efficient knockdown of the target gene and 15 nmol of siRNA/well (48 well-plate) was found to be optimal to maximise gene silencing. Although increasing siRNA concentrations was observed to result in some extent of nonspecific effects, this amount of siRNA was used since concentrations in the pmol range resulted in low gene knockdown efficiency, which may hinder the investigation of the effect of enzyme-mediated siRNA delivery. The low level of gene knockdown observed at low siRNA concentrations may be due to high levels of transient gene expression ($\sim 10^7$-$10^8$ RLU/mg protein), making efficient gene silencing difficult when using low amounts of siRNA. This problem may be solved by optimising the amount of pDNA used for transfection as well as the incubation time. Toxic effects (off-target effects) associated with the use of high siRNA concentrations should be further assessed in order to obtain a siRNA concentration that confers both high knockdown efficiency and minimum toxicity. In vitro transfection demonstrated that PEGylated peptide-lipid nanoparticles were able to significantly inhibit transient luciferase expression in response to the enzymes when compared to their PEGylated lipid controls. A similar finding was observed when further investigating their knockdown ability in stably transfected luciferase cell lines. Although the knockdown efficiency was modest and some variation was observed depending on cell lines and lipid anchors, no relationship was observed between these groups, and the incorporation of the PEGylated peptide-lipid conjugates sensitive to the specific enzymes was shown to promote siRNA delivery compared to non-cleavable PEGylated systems.
5. Conclusion and future directions
Nucleic acid therapeutics are administered mainly intravenously and preferably in the form of nanoparticles. When in systemic circulation, unmodified cationic liposomes are prone to plasma protein adsorption leading to the aggregation and rapid clearance by the mononuclear phagocyte system. PEGylation of the liposome surface by the incorporation of a synthetic PEG functionalised lipid is known to confer liposomal stability by providing steric repulsion, resulting in reduced interaction with plasma components. The use of PEG may be essential for the colloidal stability of all types of nanoparticles while in circulation; however, its presence is undesirable once at a target site (i.e. tumour cells) as it significantly inhibits the cellular uptake of nanoparticles as well as the release of the encapsulated agent. The development of stealth systems that could be removed in response to specific stimuli at target cells may be one strategy to overcome this problem.

PEGylated lipid conjugates sensitive to the enzymes secreted in solid tumours were developed. The synthesis was achieved by the conjugation of a PEG polymer to a lipid, either a cholesterol-based or C18 lipid, via an enzyme substrate peptide, resulting in a series of cholesterol- and C18-anchored PEGylated peptide-lipid conjugates bearing an HLE or MMP-2 substrate sequence: PP_{HLE}C_{17}, PP_{MMP-2}C_{18}, PP_{HLE}C_{18}^{20}, and PP_{MMP-2}C_{18}^{21}. The ability of these compounds to be degraded by the enzymes was not observed using a series of analytical methods except in the case of PP_{HLE}C_{18}^{20}, the degradation of which was detected by HPLC, but to a very low extent, as shown in chapter 2. There are two possible explanations for this. First, the substrates may not have been degraded by the enzymes at all, presumably due to micelle formation by the conjugates, in which the PEG polymer forms a hydrated sphere around the cholesterol or lipid interior. In this arrangement, PEG greatly hinders cleavage due to its steric effect, thereby preventing the access of the enzymes to the peptide substrates. Second, it might be that the techniques used lack the sensitivity to measure small amounts of degraded products.

Given preliminary *in vitro* data showing that the incorporation of PP_{HLE}C within liposomes was able to enhance gene expression in the presence of the specific enzyme compared to non-cleavable PC nanoparticles, it was decided to further investigate the ability of nanoparticles containing PEGylated peptide-lipid conjugates to
improve nucleic acid delivery in response to enzyme activation. First, the effect of nanoparticle surface charge was examined by introducing different molar percentages of the cationic lipid in the liposome formulation. The ability of the nanoparticles to condense pDNA was examined to ensure high pDNA encapsulation efficiency, and their sizes and surface charges were also measured. DODAG-high (50% DODAG) nanoparticles containing either PP\textsubscript{HLE}C or PP\textsubscript{MMP-2}C were able to increase transfection efficiency in response to the enzymes compared with non-cleavable PC nanoparticles and non-PEGylated control. Although the mechanism of enzyme-assisted pDNA delivery is not yet understood, the result suggested the possibility of partial peptide hydrolysis leading to partial PEG detachment. This enabled the nanoparticles to reveal more of their positive charges, facilitating interaction with negatively charged cell membranes and thus leading to cell uptake. The DODAG-high system, however, was found to rapidly aggregate in 10% serum and was thus unsuitable for \textit{in vivo} use. Formulation of nanoparticles with lower charges was achieved by reducing the amount of the cationic lipid in the formulation. Improved gene expression by PP\textsubscript{HLE}C and PP\textsubscript{MMP-2}C nanoparticles was also observed in the DODAG-low (20% DODAG) system, although with lower transfection efficiency. However, their stability in serum was found to increase, in particular when 4-5 mol% PEG was incorporated. Fluorescence and confocal microscopy revealed that the nanoparticles were internalised and dispersed throughout the cytoplasm. Furthermore, the toxicity of these low-charge nanoparticles was found to be minimal. The effect of a lipid-anchoring system on transfection activity was then investigated using C18-lipid anchored nanoparticles. Both PP\textsubscript{HLE}C\textsubscript{18} and PP\textsubscript{MMP-2}C\textsubscript{18} nanoparticles were shown to mediate pDNA delivery at significant levels in the presence of the enzymes, compared with their control nanoparticles. The correlation between types of lipid anchors and the mechanism of enzyme-assisted pDNA delivery was not observed in either the HLE or MMP-2 system.

The PEGylated nanoparticles were further investigated for their ability to deliver siRNA. The physicochemical characterisation of siRNA nanoparticles was assessed. The PEGylated nanoparticles containing PP\textsubscript{HLE}C, PP\textsubscript{MMP-2}C, PP\textsubscript{HLE}C\textsubscript{18}, or PP\textsubscript{MMP-2}C\textsubscript{18} were able to render protein knockdown in both transient luciferase expressing and stably transfected luciferase cells in response to the enzymes in comparison with their control PC nanoparticles. Although gene knockdown was observed, the dose of siRNA used
was high and this could be associated with nonspecific effects. The experiment therefore requires further optimisation as discussed in chapter 4. However, assuming that nonspecific effects are similar when using the same siRNA dose, the transfection data obtained showed a proof of concept for the study of enzyme-assisted siRNA delivery.

Future work on PEGylated peptide-lipid conjugates should involve enzyme cleavage studies utilising fluorescent PEG conjugated to peptide and lipid as discussed in chapter 2 or colorimetric assay such as TNBS and fluorescamine, which allows for the detection of primary amine functional groups of the product as a result of enzymatic degradation. These methods may allow for the use of substrate concentrations below CMC to prevent micellar formation. For TNBS and fluorescamine assay, to remove the enzyme and other buffer component, the sample mixture could be centrifuged at 50000 xg for 10 min, washed with saline, and then the lipid pellet resuspended in chloroform before the assay analysis. The liposome formulation being tested here was limited to one formulation using DODAG, DOPC, and cholesterol. Future work might include the further optimisation of liposome formulations capable of more efficient cellular uptake in the presence of serum. In the case of MMP-2 \textit{in vitro} experiments, to ensure that the preferential uptake of \textit{PP}_{\text{MMP-2}C} and \textit{PP}_{\text{MMP-2}C18} nanoparticles are due to the enzyme action, MMP-2 inhibitors such as Galardin (GM6001) may be preincubated with the cell culture before the addition of the nanoparticles and the transfection efficiency could be compared with that of the cells with no added inhibitor. In addition, to verify that the enzyme-assisted delivery is due to the specific sequence of the peptide linker, nonspecific peptide sequences could be conjugated to PEG and the lipid and their enzymatic cleavage as well as transfection efficiency could be compared to that of specific peptide sequences.

Further development of enzyme-assisted delivery systems could involve increasing the sensitivity of the peptide linker required for efficient PEG detachment which would ultimately increase transfection efficiency. The length of the linker may be extended to improve the interaction with enzymes, probably by introducing small amino acids that do not affect the catalytic activity of enzymes, such as glycine, to the original peptide linker, or by conjugating repeated sequences of the active unit between PEG and the
lipid. Furthermore, peptide sequences including GPLGIAGQ\textsuperscript{209} and GPLGVRGC\textsuperscript{267} have been recently reported to be effectively degraded by MMP-2. The use of these octapeptides may allow for more efficient removal of PEG via enzymatic cleavage of the peptide linker. Since cellular internalisation of this delivery system relies purely on electrostatic interactions between nanoparticles and cell membranes which can lead to nonspecific uptake, further development could involve the incorporation of a targeting moiety. Exposure of a targeting ligand for receptor binding once the PEG layer is removed could result in reduced nonspecific uptake and more efficient cell association and internalisation of the nanoparticles.
6. Experimental
6.1 General information

6.1.1 Materials

**Chemical reagents:** H-Val-2-chlorotrityl resin, 9-Fluorenylmethoxycarbonyl (Fmoc) protected amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-Hydroxybenzotriazole (HOBr) were purchased from Novabiochem (UK). Methoxypolyethylene glycol succinamate N-hydroxysuccinimide (PEG-NHS ester) (MW 2000) was obtained from Rapp Polymere (Germany). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol lipid, elastase from human leukocytes (HLE), N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (a chromogenic substrate for HLE), agarose gel, LB broth, kanamycin, N,N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), all buffer reagents, and all other chemicals and solvents were purchased from Sigma-Aldrich (UK). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-Rho) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). MMP-2, active, human, recombinant, CHO cells, MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (a fluorogenic substrate for MMP-2), and MCA-Pro-Leu-OH (a fluorogenic substrate control for MMP-2) were purchased from Calbiochem (UK). Propidium iodide and SYBR® green II RNA gel stain were obtained from Molecular Probes (UK). SYBR® safe DNA gel stain and 10X Tris-borate-EDTA (TBE) electrophoresis buffer were purchased from Invitrogen. DNA ladder, 6X orange DNA loading dye, 50X Tris-acetate-EDTA (TAE) electrophoresis buffer were obtained from Fermentas (UK). E.Z.N.A. ® endo-free plasmid kit was purchased from Omega Bio-Tek (VWR international LTD, UK).

**Biological reagents:** Human fibrosarcoma cell line (HT1080) and Human breast adenocarcinoma cell line (MCF-7) were obtained from the European Collection of the Cell Cultures (ECACC) (Salisbury, UK). Dulbecco’s Modified Eagle Medium (DMEM), Opti-MEM® I reduced serum media, fetal bovine serum (FBS), penicillin and streptomycin, phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from Gibco (Invitrogen, UK). Lipofectamine™ 2000 reagent and MMP-2 human ELISA
kit were obtained from Invitrogen (Paisley, UK). Transformed *E.Coli* containing pEGFPLuc, pUbC-Luc-S/MAR DNA, antibiotic G418 and jetPEI were kindly supplied by Dr Richard Harbottle and Dr Orestis Agyros. CytoTox 96® Non-Radioactive Cytotoxicity Assay, CellTiter 96® AQeuous One Solution Cell Proliferation Assay, Luciferase assay system and cell culture lysis buffer 5X reagent were purchased from Promega (Southampton, UK). BCA protein assay kit was obtained from Pierce (Thermo Scientific). DharmaFECT was purchased from Dharmacon (Thermo Scientific). Anti-luciferase siRNA (GL3) (target sequence 5′-AACTTACGCTGAGTACTTCG-3′) was obtained from Qiagen. Silencer® negative control siRNA was purchased from Applied Biosystems/ Ambion.

### 6.1.2 General Procedures

Solid phase peptide synthesis was performed on a manual peptide synthesis shaker. All reactions were carried out under a nitrogen atmosphere. CH$_2$Cl$_2$ was distilled over phosphorus pentoxide (P$_2$O$_5$). Flash column chromatography was performed using Merck silica gel 60 (230-240 mesh). Thin layer chromatography (TLC) was performed on Merck silica gel 60 F$_{254}$ aluminum backed plates and visualized with UV light (254 and 366 nm) and/or stained with acidic ammonium molybdate (IV) or potassium permanganate (IIV). $^1$H and $^{13}$C NMR were recorded on a 400 MHz Bruker Avance 400, using residual chloroform (CDCl$_3$, $\delta$H = 7.27 ppm, $\delta$c = 77.0 ppm) as integral standard. Data is reported as follows: chemical shift, (integration; br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; coupling constants (J) in hertz (Hz); assignment).
The numbering system for the assignment of cholesterol is shown below.

Mass spectra were performed using Bruker Esquire 3000 ESI or Micromass MALDI micro MX instrument. Analytical HPLC was conducted on Hitachi-LaChrom L-7150 pump system equipped with a Polymer Laboratories PL-ELS 1000 evaporative light scattering detector. The HPLC gradient assigned as follow: A = H2O/ 0.1% TFA; B = MeCN/ 0.1% TFA; C = MeOH. UV spectroscopy was conducted on a Pharmacia Biotech Ultrospec 4000 spectrometer. Fluorescence measurements were performed on a Varioskan flash microplate reader (Thermo Scientifc). Concentration of plasmid DNA was measured on Nanodrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific). Particle sizing were performed using a Coulter Delta N4 plus photon correlation spectrophotometer (PCS). Zeta potentials were recorded on a Nanoseries Nano-ZS zetasizer (Malvern instruments). Microscopy experiments were conducted on a Nickon Eclipse E600 microscope and confocal microscopy was performed with an upright Leica instrument.

6.2 Chemical Synthesis

**General Procedure for the synthesis of the N-Fmoc protected-peptide:** H-Val-2-Chlorotrityl resin (500 mg, 0.35 mmol) was added to a solid phase vessel and swelled in DMF (10 mL) and the vessel was agitated on a peptide synthesis shaker for 2h. DMF was removed and replaced with a solution of N-protected Fmoc-amino acid (1.05 mmol), HBTU (386.89 mg, 1.02 mmol), HOBt (141.88 mg, 1.05 mmol), and DIPEA (180 μl, 1.05 mmol) in 10 ml DMF. The mixture was agitated for 45 min. The resin was
washed twice with DMF and Fmoc group was deprotected with 10 mL of 20% (v/v) piperidine in DMF for 10 min. Cycles of amino acid coupling and Fmoc deprotection were carried out under the same condition. When last coupling was achieved, the resin was washed successively with DMF, DCM, MeOH, and Et$_2$O and allowed to dry under vacuum for 2 h. The peptide was then cleaved off the resin using 10 mL of 0.5% TFA in DCM for 4 h. The solution was filtered and the solvent was removed in vacuo. The residue was recrystallised in cold Et$_2$O and the precipitate was freeze-dried overnight to afford the N-Fmoc protected-peptide as a white solid.

**Fmoc-AAPV-OH 9**

Synthesised as described in the general procedure for the synthesis of the N-Fmoc-protected peptide, using Fmoc-Pro-OH (354.27 mg, 1.05 mmol) and Fmoc-Ala-OH (326.86 mg, 1.05 mmol), respectively, to afford 9 as a white solid (166.4 mg, 82%); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$H 0.87, 0.90 (6H, d, $J=6.8$ Hz, 2 x CH$_3$ Val), 1.33, 1.34 (6H, d, $J=6.8$ Hz, 2 x CH$_3$ Ala), 1.93-2.27 (5H, m, CH($\beta$) Val, CH$_2$($\beta$) Pro, CH$_2$($\gamma$) Pro), 3.60-3.80 (2H, m, CH$_2$(=) Pro), 4.18 (1H, t, $J=7.0$ Hz, CH (α) Val), 4.34-4.37 (2H, m, CH (α) Ala), 4.40-4.51 (2H, m, CH (α) Pro, Fmoc CH), 4.60-4.85 (2H, m, Fmoc CH$_2$), 5.9 (1H, d, $J=7.6$ Hz, NH), 7.25-7.77 (8H, Fmoc aromatic CH), 7.93 (1H, NH), 10.61 (1H, COOH); $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$C 17.3 (2C, 2 x CH$_3$ Val), 18.5 (2C, 2 x CH$_3$ Ala), 25.0 (CH$_2$(=) Pro), 28.0 (CH$_2$(=) Pro), 31.2 (CH (β) Val), 37.5 (Fmoc CH), 47.0 (CH (α) Ala), 47.7 (CH$_2$(=) Pro), 50.3 (CH (α) Ala), 57.5 (CH(α) Val), 60.4 (CH (α) Pro), 67.5 (Fmoc CH$_2$), 120.0, 125.1, 127.1, 127.8 (8C, Fmoc aromatic CH), 141.3, 143.7 (4C, Fmoc aromatic C), 156.3 (CO carbamate), 164.2, 171.7, 172.9 (3C, CO), 174.2 (COOH); HPLC: $R_t = 17.8$ min, column reverse phase C-4 protein, gradient mix: 0.0 min
Chapter Six: Experimental

Fmoc-GPLGV-OH 10

Synthesised as described in the general procedure for the synthesis of the N-Fmoc-protected peptide, using Fmoc-Gly-OH (312.16 mg, 1.05 mmol), Fmoc-Leu-OH (371.07 mg, 1.05 mmol), Fmoc-Pro-OH (354.27 mg, 1.05 mmol), and Fmoc-Gly-OH(312.16 mg, 1.05 mmol), respectively, to afford 10 as a white solid (114 mg, 49%); 1H NMR (400 MHz, CDCl₃) δH 0.86-0.97 (12H, m, 2 x CH₃ Leu, 2 x CH₃ Val), 1.53-1.85 (3H, m, CH₂ (β) Leu, CH (γ) Leu), 1.98-2.22 (5H, m, CH (β) Val, CH₂ (β) Pro, CH₂ (γ) Pro), 3.5 (2H, m, CH₂ (δ) Pro), 3.61-3.86 (2H, m, CH₂ (α) Gly), 4.0 (2H, s, CH₂ (α) Gly), 4.12-4.27 (4H, m, CH (α) Val, CH (α) Leu, CH (α) Pro, Fmoc CH), 4.30-4.51 (2H, m, Fmoc CH₂), 7.30-7.78 (8H, m, Fmoc aromatic CH); 13C NMR (400 MHz, CDCl₃) δC 17.8 (2C, 2 x CH₃ Leu), 19.0 (2 x CH₃ Val), 21.6 (CH₂ (β) Leu), 23.0 (CH₂ (β) Pro), 25.0 (CH₂ (β) Pro), 29.0 (CH (γ) Leu), 30.2 (CH (β) Val), 39.5 (Fmoc CH), 43.0 (CH₂ (α) Gly), 43.6 (CH₂ (α) Gly), 46.9 (CH₂ (δ) Pro), 52.2 (CH (α) Leu), 58.1 (CH (α) Val), 61.2 (CH (α) Pro), 67.3 (Fmoc CH₂), 119.9, 125.1, 127.0, 127.7 (8C, Fmoc aromatic CH), 141.2, 143.7 (4C, Fmoc aromatic C), 157.2 (CO carbamate), 169.9, 170.5, 172.4, 173.4 (4C, 5 x CO), 173.8 (COOH); HPLC: Rᵣ = 18.7 min, column reverse phase C-4 protein, gradient mix: 0.0 min [100% A], 15.0-25.0 min [100% B], 25.1-45.0 min [100% C], 45.1-55.0 min [100% A], flow: 1 mL min⁻¹; m/z (ESI +ve) 665 (M+H)⁺.
To a stirred solution of ethylene-1,2-diamine (150 mL) at room temperature, was added cholesteryl chloroformate (2 g, 4.45 mmol) in CHCl₃ dropwise. After 18 h, the reaction was quenched with H₂O (300 mL) and extracted with CH₂Cl₂ (3 x 150 mL). The organic extract was dried over MgSO₄ and the solvent was evaporated in vacuo. The compound was purified by flash column chromatography on silica gel (CH₂Cl₂: MeOH: H₂O: 92: 7: 1 v/v) to yield 5 as a white solid (1.71 g, 86%); ¹H NMR (400 MHz, CDCl₃) δH 0.66 (3H, s, 18-CH₃), 0.84 (3H, d, J = 6.4 Hz, 27-CH₃), 0.85 (3H, d, J = 6.8 Hz, 26-CH₃), 0.90 (3H, d, J = 6.4 Hz, 21-CH₃), 0.92 (3H, s, 19-CH₃), 1.02-1.63 (21H, m, 1-CH₂), 1.76-2.04 (5H, m, 2-CH₂, 7-CH₂, 8-CH), 2.22-2.36 (2H, m, 4-CH₂), 2.79-2.81 (2H, m, 3'-CH₂), 3.19-3.21 (2H, m, 4'-CH₂), 4.52 (1H, m, 3'-CH₂), 5.31 (1H, s, 6-CH); ¹³C NMR (400 MHz, CDCl₃) δC 11.8 (18-C), 18.6 (21-C), 19.3 (19-C), 21.0 (11-C), 22.5 (26-C), 22.8 (27-C), 23.7 (23-C), 24.2 (15-C), 27.9 (25-C), 28.2 (2C, 2-C, 16-C), 31.8 (2C, 8-C, 7-C), 35.7 (20-C), 36.1 (22-C), 36.5 (10-C), 36.9 (1-C), 38.5 (24-C), 39.4 (4-C), 39.6 (12-C), 39.8 (4'-CH₂), 40.6 (3'-CH₂), 42.2 (13-C), 49.9 (9-C), 56.0 (17-C), 56.6 (14-C), 74.2 (3-C), 122.4 (6-C), 139.8 (5-C), 156.4 (CO carbamate); MS (ESI +ve) 473 (M + H)+.

**General Procedure for the synthesis of the N-Fmoc-protected peptide-cholesterol conjugate:** To a solution of N-Fmoc-protected peptide, DMAP and HBTU in dry CHCl₃ (5 mL) was added N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5 in dry CHCl₃ (3 mL). The mixture was stirred at room temperature under a nitrogen atmosphere for 18 h. The reaction was quenched with 4% citric acid (4 mL) and washed with CHCl₃ (3 x
20 mL). The organic extract was dried over MgSO₄, filtered, and concentrated in vacuo to afford the desired N-Fmoc-protected peptide-cholesterol conjugate.

\[
Fmoc-AAPV-(N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane) 13
\]

Synthesised as described in the general procedure for the N-Fmoc-protected peptide-cholesterol conjugate, using N-Fmoc-protected peptide 9 (108.6 mg, 0.19 mmol), N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5 (88.2 mg, 0.19 mmol), DMAP (68.6 mg, 0.56 mmol), and HBTU (72.6 mg, 0.19 mmol) to afford 13 as a pale yellow solid (131.4 mg, 68%); \(^1\)H NMR (400 MHz, CDCl₃) \(\delta\) 

<table>
<thead>
<tr>
<th>(\delta)</th>
<th>0.66 (3H, s, 18-CH₃)</th>
<th>0.86 (3H, d, (J = 6.8) Hz, 27-CH₃)</th>
<th>0.87 (3H, d, (J = 6.8) Hz, 26-CH₃)</th>
<th>0.89-1.02 (12H, m, 2 x CH₃ Val, 19-CH₃, 21-CH₃)</th>
<th>1.04-2.38 (41H, m, 2 x CH₃ Ala, CH (β) Val, CH₂ (β) Pro, CH₂ (γ) Pro, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH, 3'-CH₃)</th>
<th>3.20-3.90 (2H, m, 4’-CH₂)</th>
<th>4.19-4.85 (7H, m, 1H, s, 6-CH)</th>
<th>7.25-7.76 (8H, Fmoc aromatic CH)</th>
</tr>
</thead>
</table>
| 13C NMR (400 MHz, CDCl₃) \(\delta\) | 11.8 (18-C), 17.7 (2C, 2 x CH₃ Val), 18.5 (2C, 2 x CH₃ Ala), 19.4 (21-C), 21.0 (19-C), 21.3 (11-C), 22.5 (26-C), 22.8 (27-C), 23.0 (CH₂ (γ) Pro), 23.8 (23-C), 24.2 (15-C), 25.1 (CH₂ (β) Pro), 28.0 (25-C), 28.2 (16-C), 28.4 (2-C), 30.1 (CH (β) Val), 31.8 (8-C), 33.6 (7-C), 35.7 (20-C), 36.1 (22-C), 36.5 (10-C), 36.9 (1-C), 38.9 (24-C), 39.5 (2C, 4-C, Fmoc CH), 39.6 (12-C), 39.9 (4’-CH₂), 40.4 (3’-CH₂), 42.2 (13-C), 47.1 (CH (α) Ala), 47.5 (CH₂ (δ) Pro), 47.8 (9-C), 50.2 (CH (α) Ala), 56.1 (17-C), 56.6 (14-C), 57.5 (CH (α) Val), 60.4 (CH (α) Pro), 67.0 (Fmoc CH₂), 74.5 (3-C), 122.5 (6-C), 119.9, 125.1, 127.1, 127.8 (8C, Fmoc aromatic CH), 139.7 (5-C), 142.1, 143.8 (4C, Fmoc aromatic C), 155.8,
156.7 (2C, 2x CO carbamate), 171.3, 171.5, 172.1, 172.5, (4C, 4x CO); m/z (ESI +ve) 1033 (M+H)^+.

**Fmoc-GPLGV-(N’-cholesteryloxy-3-carbonyl-1,2-diaminoethane) 14**

Synthesised as described in the general procedure for the N-Fmoc-protected peptide-cholesterol conjugate, using N-Fmoc-protected peptide 10 (105.0 mg, 0.16 mmol), N’-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5 (75.6 mg, 0.16 mmol), DMAP (58.6 mg, 0.48 mmol), and HBTU (60.7 mg, 0.16 mmol) to yield 14 as a white solid (132.8 mg, 74%).

**1H NMR (400 MHz, CDCl₃)** δ 0.69 (3H, s, 18-CH₃), 0.88 (3H, d, J = 6.8 Hz, 27-CH₃), 0.89 (3H, d, J = 6.8 Hz, 26-CH₃), 0.91-1.01 (18H, CH₃), 1.05-2.38 (38H, CH₂), 3.25-3.93 (6H, CH₂), 4.05-4.48 (9H, CH₂), 5.34 (1H, CH), 7.31-7.78 (8H, Fmoc aromatic CH);

**13C NMR (400 MHz, CDCl₃)** δ 11.8 (18-CH), 17.7 (2x CH₃ Leu), 18.7 (2x CH₃ Val), 19.3 (21-C), 21.0 (19-C), 21.3 (11-C), 21.5 (CH₂ (β) Leu), 22.5 (26-C), 22.8 (27-C), 23.0 (CH₂ (γ) Pro), 23.8 (23-C), 24.2 (15-C), 25.1 (CH₂ (β) Pro), 28.0 (25-C), 28.2 (16-C), 29.0 (2-C), 29.4 (CH (γ) Leu), 29.7 (CH (β) Val), 31.8 (2C, 8-C, 7-C), 35.8 (20-C), 36.1 (22-C), 36.5 (10-C), 36.9 (1-C), 38.6 (24-C), 39.5 (2C, 4-C, Fmoc CH), 39.7 (12-C), 39.8 (4'-CH₂), 40.6 (3'-CH₂), 42.3 (13-C), 43.7 (CH₂ (α) Gly), 44.3 (CH₂ (α) Gly), 47.0 (CH₂ (δ) Pro), 49.9 (9-C), 50.0 (CH (α) Leu), 56.1 (17-C), 56.6 (14-C), 59.1 (CH (α) Val), 61.5 (CH (α) Pro), 67.4 (Fmoc CH₂), 74.3 (3-C), 122.5 (6-C), 119.9, 125.0, 127.1, 127.8 (8C, Fmoc aromatic CH), 139.8 (5-C), 141.2, 143.6 (4C, 15-CH, 16-CH, 17-CH, 20-CH, 22-CH, 23-CH, 24-CH, 25-CH, 3'-CH₂), 3.25-3.93 (6H, CH₂), 4.05-4.48 (9H, CH₂), 5.34 (1H, CH), 7.31-7.78 (8H, Fmoc aromatic CH);
Fmoc aromatic C), 156.7, 157.4 (2C, 2 x CO carbamate), 169.8, 170.5, 172.2, 172.6, 174.7 (5C, 5 x CO); m/z (ESI +ve) 1119 (M+H)⁺.

**General Procedure for the formation of the amine deprotection of N-Fmoc-protected peptide-cholesterol conjugate:** To a solution of N-Fmoc-protected peptide-cholesterol conjugate in dry CH₂Cl₂ (4 mL) was added piperidine (1 mL). The reaction was stirred at room temperature under a nitrogen atmosphere for 4 h. The crude mixture was concentrated *in vacuo* and purified using flash column chromatography ((CH₂Cl₂: MeOH: NH₃: 25: 7.3: 1): CH₂Cl₂ 1:2, v/v) to afford the peptide-cholesterol conjugate as a white solid.

**H₂N-AAPV-(N’-cholesteryloxy-3-carbonyl-1,2-diaminoethane)** 15

Synthesised as described in the general procedure for the peptide-cholesterol conjugate, using N-Fmoc-protected peptide-cholesterol conjugate 13 (97.7 mg, 0.095 mmol) to afford 15 as a white solid (63.4 mg, 83%); **¹H NMR** (400 MHz, CDCl₃) δH 0.66 (3H, s, 18-CH₃), 0.86 (3H, d, J = 6.8 Hz, 27-CH₃), 0.87 (3H, d, J = 6.8 Hz, 26-CH₃), 0.88-1.03 (12H, m, 2 x CH₃ Val, 19-CH₃, 21-CH₃), 1.07-2.37 (41H, m, 2 x CH₃ Ala, CH (β) Val, CH₂ (β) Pro, CH₂ (γ) Pro, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH, 3'-CH₂), 3.20-3.85 (2H, m, 4’-CH₂), 4.21-4.80 (4H, m, CH (α) Ala, CH (α) Val, CH (α) Pro, 3-CH), 5.36 (1H, s, 6-CH); **¹³C NMR** (400 MHz, CDCl₃) δC 11.8 (18-C), 17.4 (2C, 2 x CH₃ Val), 18.6 (2C, 2 x CH₃ Ala), 19.4 (21-C), 21.0 (19-C), 21.5 (11-C), 22.6 (26-C), 22.7 (27-C), 23.0 (CH₂ (γ) Pro), 23.7 (23-C), 24.2 (15-C), 25.2 (CH₂ (β) Pro), 27.9 (25-C), 28.1 (16-C), 28.2 (2-C), 29.8 (CH (β) Val), 31.8 (8-C), 33.6 (7-C), 35.7 (20-C), 36.1 (22-
C), 36.5 (10-C), 36.9 (1-C), 38.5 (24-C), 39.4 (4-C), 39.7 (12-C), 40.1 (4'-CH₂), 40.4 (3'-CH₂), 42.2 (13-C), 46.2 (CH (α) Ala), 47.5 (CH₂ (β) Pro), 47.7 (9-C), 50.2 (CH (α) Ala), 56.1 (17-C), 56.6 (14-C), 58.5 (CH (α) Val), 60.9 (CH (α) Pro), 74.4 (3-C), 122.5 (6-C), 139.7 (5-C), 156.7 (CO carbamate), 171.1, 172.0, 173.1,175.5, (4C, 4 x CO); m/z (ESI +ve) 811 (M+H)+.

H₂N-GPLGV-(N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane) 16

Synthesised as described in the general procedure for the peptide-cholesterol conjugate, using N-Fmoc-protected peptide-cholesterol conjugate 14 (102.9 mg, 0.092 mmol) to afford 16 as a white solid (68.3 mg, 83%); ¹H NMR (400 MHz, CDCl₃) δ H 0.74 (3H, s, 18-CH₃), 0.93 (3H, d, J = 6.8 Hz, 27-CH₃), 0.94 (3H, d, J = 6.8 Hz, 26-CH₃), 0.96-1.09 (18H, m, CH₃ (β) Leu, CH (γ) Val, CH (β) Pro, CH₂ (γ) Pro, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH₂, 9-CH₂, 11-CH₂, 12-CH₂, 14-CH₂, 15-CH₂, 16-CH₂, 17-CH₂, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH, 3'-CH₂), 3.21-3.95 (6H, m, CH₂ (γ) Pro, CH₂ (α) Gly, 4'-CH₂), 4.10-4.50 (6H, m, CH₂ (α) Gly, CH (α) Val, CH (α) Leu, CH (α) Pro, 3-CH₂), 5.38 (1H, s, 6-CH₂); ¹³C NMR (400 MHz, CDCl₃) δ C 11.8 (18-C), 17.8 (2 x CH₃ Leu), 18.7 (2 x CH₃ Val), 19.3 (21-C), 21.0 (19-C), 21.4 (11-C), 22.6 (CH₂ (β) Leu), 22.7 (26-C), 22.8 (27-C), 23.0 (CH₂ (γ) Pro), 23.8 (23-C), 24.3 (15-C), 25.0 (CH₂ (β) Pro), 28.0 (25-C), 28.2 (16-C), 29.3 (2-C), 29.5 (CH (γ) Leu), 29.7 (CH (β) Val), 31.8 (2C, 8-C, 7-C, 35.8 (20-C), 36.2 (22-C), 36.6 (10-C), 37.0 (1-C), 38.6 (24-C), 39.5 (4-C), 39.7 (12-C), 40.6 (2C, 3'-CH₂, 4'-CH₂), 42.3 (3C, 13C, 2 x CH₂ (α) Gly), 46.6 (CH₂ (β) Pro), 50.0 (2C, 9-C, CH (α)
Leu), 56.1 (17-C), 56.7 (14-C), 58.3 (2C, CH (α) Val, CH (α) Pro), 75.0 (3-C), 122.5 (6-C), 139.8 (5-C), 156.8 (CO carbamate), 169.8, 171.0, 172.4, 172.6, 173.0 (5C, 5 x CO); m/z (ESI +ve) 897 (M+H)+.

**General Procedure for the synthesis of the PEGylated peptide-cholesterol conjugate:** To a solution of peptide-cholesterol conjugate and DIPEA in dry CH₂Cl₂ (5 mL) and under a nitrogen atmosphere, was added PEG₂₀₀₀-NHS ester in dry CH₂Cl₂ (3 mL). The reaction was stirred at room temperature for 18 h. The solvent was removed *in vacuo* and the crude mixture was purified using flash column chromatography ((CH₂Cl₂: MeOH: H₂O: 34.5: 9: 1): CH₂Cl₂ 1:1, v/v) to yield the desired PEGylated peptide-cholesterol conjugate as a white solid.

**PEG₂₀₀₀-AAPV-(N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane) 17**

Synthesised as described in the general procedure for the PEGylated peptide-cholesterol conjugate, using peptide-cholesterol conjugate 15 (59.2 mg, 0.073 mmol), DIPEA (12.5 µL, 0.073 mmol), and PEG₂₀₀₀-NHS ester (148.6 mg, 0.073 mmol) to obtain 17 as a white solid (109.6 mg, 55%); ¹H NMR (400 MHz, CDCl₃) δH 0.68 (3H, s, 18-CH₃), 0.86 (3H, d, J = 6.8 Hz, 27-CH₃), 0.87 (3H, d, J = 6.8 Hz, 26-CH₃), 0.88-1.03 (12H, m, 2 x CH₃ Val, 19-CH₃, 21-CH₃), 1.06-2.37 (41H, m, 2 x CH₃ Ala, CH (β) Val, CH₂ (β) Pro, CH₂ (γ) Pro, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₃, 24-CH₂, 25-CH, 3'-CH₂), 3.38 (3H, s, CH₂O), 3.63 (173H, s, PEG CH₂), 3.24-3.85 (2H, m, 4'-CH₂), 4.29-4.74 (4H, m, CH (α) Ala, CH (α) Val, CH (α) Pro, 3-CH), 5.36 (1H, s, 6-CH); ¹³C NMR (400 MHz, CDCl₃).
CDCl$_3$ $\delta$ C 11.8 (18-C), 17.3 (2C, 2 x CH$_3$ Val), 18.6 (2C, 2 x CH$_3$ Ala), 19.2 (21-C), 19.5 (19-C), 21.0 (11-C), 22.5 (26-C), 22.7 (27-C), 23.0 (CH$_2$ ($\gamma$) Pro), 23.7 (23-C), 24.2 (15-C), 25.2 (CH$_2$ ($\beta$) Pro), 27.9 (25-C), 28.1 (16-C), 28.2 (2-C), 29.8 (CH ($\beta$) Val), 31.5 (8-C), 31.7 (7-C), 35.7 (20-C), 36.1 (22-C), 36.5 (10-C), 36.8 (1-C), 38.5 (24-C), 39.4 (4-C), 39.7 (12-C), 40.0 (4'-CH$_2$), 40.4 (3'-CH$_2$), 42.2 (13-C), 47.4 (CH ($\alpha$) Ala), 48.1 (CH$_2$ ($\delta$) Pro), 48.8 (9-C), 49.9 (CH ($\alpha$) Ala), 56.1 (17-C), 56.6 (14-C), 58.4 (CH ($\alpha$) Val), 60.9 (CH ($\alpha$) Pro), 70.5 (PEG CH$_2$), 74.3 (3-C), 122.5 (6-C), 139.7 (5-C), 156.6 (CO carbamate), 171.1, 171.5, 172.0, 172.4, 172.6, 173.0 (6C, 6 x CO); HPLC: $R_t = 25.6$ min, column reverse phase C-4 protein, gradient mix: 0.0 min [100% A], 15.0-25.0 min [100% B], 25.1-45.0 min [100% C], 45.1-55.0 min [100% A], flow: 1 mL min$^{-1}$; m/z (MALDI +ve): 2753 (M+Na)$^+$. 

PEG$_{2000}$--GPLGV-(N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane) 18

Synthesised as described in the general procedure for the PEGylated peptide-cholesterol conjugate, using peptide-cholesterol conjugate 16 (68.3 mg, 0.076 mmol), DIPEA (13.1 $\mu$L, 0.076 mmol), and PEG$_{2000}$-NHS ester (155.1 mg, 0.076 mmol) to obtain 18 as a white solid (100 mg, 47%); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ H 0.66 (3H, s, 18-CH$_3$), 0.84 (3H, d, J = 6.8 Hz, 27-CH$_3$), 0.86 (3H, d, J = 6.8 Hz, 26-CH$_3$), 0.89-1.02 (18H, m, 2 x CH$_3$ Leu, 2 x CH$_3$ Val, 19-CH$_3$, 21-CH$_3$), 1.0-2.33 (38H, m, CH$_2$ ($\beta$) Leu, CH ($\gamma$) Leu, CH ($\beta$) Val, CH$_2$ ($\beta$) Pro, CH$_2$ ($\gamma$) Pro, 1-CH$_2$, 2-CH$_2$, 4-CH$_2$, 7-CH$_2$, 8-CH, 9-CH, 11-CH$_2$, 12-CH$_2$, 14-CH, 15-CH$_2$, 16-CH$_2$, 17-CH, 20-CH, 22-CH$_2$, 23-CH$_2$, 24-CH$_2$, 25-CH, 3'-CH$_2$), 3.38 (3H, s, CH$_3$O), 3.64 (173H, s, PEG CH$_2$), 3.20-3.83 (6H, m, CH$_2$ ($\gamma$) Pro, CH$_2$ ($\alpha$) Gly, 4'-CH$_2$), 4.10-4.50 (6H, m, CH$_2$ ($\alpha$) Gly, CH ($\alpha$) Val, CH ($\alpha$) Leu, CH ($\alpha$) Pro, 3-CH), 5.34 (1H, s, 6-CH); $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ C 11.8 (18-C), 17.8
(2C, 2 x CH₃ Leu), 18.7 (2C, 2 x CH₃ Val), 19.3 (21-C), 21.0 (19-C), 21.3 (11-C), 22.5 (CH₂ (β) Leu), 22.8 (26-C), 23.0 (27-C), 23.2 (CH₂ (γ) Pro), 23.8 (23-C), 24.3 (15-C), 25.0 (CH₂ (β) Pro), 28.0 (25-C), 28.2 (16-C), 28.9 (2-C), 29.6 (CH (γ) Leu), 30.3 (CH (β) Val), 31.8 (2C, 8-C, 7-C), 35.8 (20-C), 36.1 (22-C), 36.5 (10-C), 37.0 (1-C), 38.6 (24-C), 39.5 (4-C), 39.7 (12-C), 40.0 (2C, 3'-CH₂, 4'-CH₂), 42.3 (3C, 13C, 2 x CH₂ (α) Gly), 47.5 (CH₂ (δ) Pro), 50.0 (2C, 9-C, CH (α) Leu), 56.1 (17-C), 56.6 (14-C), 59.0 (3C, CH (α) Val, CH (α) Pro, CH₃O), 70.5 (PEG CH₂), 74.2 (3-C), 122.4 (6-C), 139.8 (5-C), 156.6 (CO carbamate), 169.4, 170.5, 172.0, 172.5, 173.0, 173.3, 174.2 (7C, 7 x CO); HPLC: $R_t = 25.5$ min, column reverse phase C-4 protein, gradient mix: 0.0 min [100% A], 15.0-25.0 min [100% B], 25.1-45.0 min [100% C], 45.1-55.0 min [100% A], flow: 1 mL min⁻¹; m/z (MALDI +ve): 2838 (M+Na)⁺.

**PEG₂₀₀₀-(N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane) 19**

![Chemical structure](image)

To a stirred solution of N'-cholesteryloxy-3-carbonyl-1,2-diaminoethane 5 (60 mg, 0.127 mmol) and DIPEA (21.8 μL, 0.127 mmol) in dry CH₂Cl₂ (5 mL) at room temperature and under a nitrogen atmosphere, was added PEG₂₀₀₀-NHS ester (258.5 mg, 0.127 mmol) in dry CH₂Cl₂ (3 mL). After 18 h, the crude mixture was concentrated in vacuo and recrystallized in cold Et₂O to afford 19 as a white solid (298 mg, 98%); ¹H NMR (400 MHz, CDCl₃) δₙ 0.68 (3H, s, 18-CH₃), 0.86 (3H, d, J = 6.4 Hz, 27-CH₃), 0.87 (3H, d, J = 6.4 Hz, 26-CH₃), 0.91 (3H, d, J = 6.4 Hz, 21-CH₃), 1.00 (3H, s, 19- CH₃), 1.05-1.62 (21H, m, 1-CH₂, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH), 1.78-2.04 (5H, m, 2-CH₂, 7-CH₂, 8-CH), 2.06-2.15 (2H, m, 4-CH₂), 2.25-2.38 (2H, m, 3'-CH₂), 3.25-3.35 (2H, m, 4'-CH₂), 3.38 (3H, s, CH₃O), 233
3.64 (1H, s, PEG CH₂), 4.48 (1H, m, 3-CH), 5.36 (1H, s, 6-CH); ¹³C NMR (400 MHz, CDCl₃) δ 11.8 (18-C), 18.7 (21-C), 19.3 (19-C), 21.1 (11-C), 22.5 (26-C), 22.8 (27-C), 23.8 (23-C), 24.2 (15-C), 28.0 (25-C), 28.2 (2C, 2-C, 16-C), 31.8 (2C, 8-C, 7-C), 35.7 (20-C), 36.1 (22-C), 36.5 (10-C), 37.0 (1-C), 38.6 (24-C), 39.5 (4-C), 39.7 (12-C), 41.0 (2C, 3'-CH₂, 4'-CH₂), 42.3 (13-C), 50.0 (9-C), 56.1 (17-C), 56.6 (14-C), 59.0 (CH₃O), 70.5 (PEG CH₂), 74.2 (3-C), 122.4 (6-C), 139.8 (5-C), 170.8, 172.4 (2C, 2 x CO); HPLC: Rᵳ = 25.5 min, column reverse phase C-4 protein, gradient mix: 0.0 min [100% A], 15.0-25.0 min [100% B], 25.1-45.0 min [100% C], 45.1-55.0 min [100% A], flow: 1 mL min⁻¹; m/z (MALDI +ve): 2415 (M+Na)+.

### 6.3 Enzyme Activity Assay and degradation Study

#### 6.3.1 Preparation of enzyme stock solutions

Lyophilized powder of HLE was resuspended in 10 mM HEPES, 154 mM NaCl, 0.1 mM EDTA, pH 7.4 to a final concentration of 1.03 μM. Aliquots of the stock solution were stored at -20 °C.

MMP-2 in the original vial (1.52 μM) were diluted in 25 mM HEPES, 10 mM CaCl₂, 10 μM ZnCl₂, pH 8.0 to prepare a stock solution of 15.2 nM. Aliquots of the stock solution were stored at -20 °C and the remaining original MMP-2 was stored at -80 °C.

#### 6.3.2 Determination of enzyme activity

MeO-suc-AAPV-pNA was employed as a substrate for the determination of HLE activity. MeO-Suc-AAPV-pNA solution (500 μL, 0.51 mM) dissolved in 10 mM HEPES, 154 mM NaCl, 0.1 mM EDTA, pH 7.4, were mixed with HLE (100 μL, 1.03 μM). The release of the pNA, as a result of enzyme reaction, was monitored at 410 nm for 2 min. The unit of HLE was defined based on pNA calibration curved, previously obtained, e.g. 1 unit releases 48 nmol of pNA per min at room temperature. The enzyme activity unit varies from batch-to-batch.
Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ was employed as a substrate for the determination of MMP-2 activity. The stock solution of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ (2.7 µL, 0.92 mM) in DMSO was diluted in 25 mM HEPES, 10 mM CaCl$_2$, 10 µM ZnCl$_2$, pH 8.0 to a final concentration of 0.05 mM in a 96-well microtiter plate. MMP-2 (50 µL, 15.2 nM) was added to the solution and the fluorescence intensity of Mca-Pro-Leu-OH, a cleavage product from the enzyme cleavage, was monitored at $\lambda_{ex} = 328$ nm and $\lambda_{em} = 393$ nm for 15 min. The unit of MMP-2 was defined based on Mca-Pro-Leu-OH calibration curve, previously obtained.

6.3.3 Degradation of MeO-suc-AAPV-pNA and PEG$_{2000}$-AAPV-pNA by HLE monitored by UV spectroscopy

MeO-suc-AAPV-pNA (0.12 mM) and PEG$_{2000}$-AAPV-pNA (0.20 mM) prepared in 10 mM HEPES, 154 mM NaCl, 0.1 mM EDTA, pH 7.4, was added HLE (10 µL, 1.03 µM). The absorbance of the released pNA was then monitored at 410 nM for 60 min.

6.3.4 Cleavage of PEG$_{2000}$-AAPV-pNA and PEG$_{2000}$-AAPV-C18 conjugates by HLE monitored by HPLC

PEG$_{2000}$-AAPV-pNA (0.20 mM) and PEG$_{2000}$-AAPV-C18 (0.18 mM) prepared in 10 mM HEPES, 154 mM NaCl, 0.1 mM EDTA, pH 7.4, were mixed with HLE (200 µL, 1.03 µM) and the mixtures were incubated at 37 °C for 1 to 2 h. The enzyme was denatured by freezing the mixtures in nitrogen (liq). Analytical HPLC was performed using reverse phase protein C4 column, ELSD detector and the following gradient mix: 0.0-10.0 min [100% A], 25.0 min [30% A and 70% B], 40.0-45.0 min [100% B], 46.0-55.0 min [100% C], 56.0-65.0 min [100% A]; flow rate 1.5 ml min$^{-1}$
6.4 Preparation and characterization of pDNA nanoparticles

6.4.1 Preparation of pEGFPLuc DNA

Transformed E. coli containing pEGFPLuc (pDNA, 6.4 kb) was grown in sterilised LB medium (5 mL) with kanamycin (5 µL) overnight at 37 °C with shaking. Next day, the culture was transferred to sterilised LB medium (500 mL) with kanamycin (500 µL) and was grown at 37 °C with shaking overnight. Isolation of pDNA was carried out using E.Z.N.A.® Endo-Free Plasmid Kit (Omega Bio-Tek) for preparation of plasmid with low endotoxin levels. The contamination of endotoxin, lipopolysaccharides (LPS) released from E. coli cell membrane during lysis step of plasmid purification, leads to low transfection efficiency in vitro and inducing inflammatory reaction in vivo. With the use of this kit, endotoxins are effectively removed from lysate with endo-toxin removal (ETR) buffer. Briefly, the culture was transferred to 4x 250 mL GSA centrifuge tubes. The cell pellets were harvested by centrifuging at 5,000 rpm for 10 min and the medium was removed. Resuspension buffer with RNase A (2.5 mL) was added to bacterial pellet and vortexed until the resuspension was complete. Lysis buffer (2.5 mL) was added, mixed gently by inverting and rotating the tube 10 times and incubated at room temperature for 2 min. Ice-cold precipitation buffer (1.25 mL) was added, mixed gently and thoroughly by inverting the tube 7 times and incubated at room temperature for 2 min. The lysate was immediately poured into the barrel of the Lysate Clearance Filter Syringe and left for 2 min. The end cap of the syringe was removed and the plunger was gently inserted into the barrel to expel the clear lysate into a 15-mL tube. The equal volume of ETR binding buffer was added to the lysate and mixed gently by inverting the tube 10 times. pDNA was then purified with HiBind DNA midi column. Equilibration buffer (1 mL) was added to HiBind DNA midi column and centrifuged at 3,800 rpm for 3 min. The flow-through liquid was discarded. The lysate (3.5 mL) was added to the column and centrifuged at 3,800 rpm for 3 min. The process was repeated until all the lysate was passed through the column. The flow-through liquid was discarded. ETR wash buffer (2 mL) was added to the column and centrifuged at 3,800 rpm for 3 min. Buffer HB (3 mL) was added to the column and centrifuged at 3,800 rpm for 3 min. The flow-through liquid was discarded. 2x DNA wash buffer (3.5 mL) were added to the column and centrifuged at 3,800 rpm for 3 min. The flow-
through liquid was discarded. The empty column was centrifuged at 3,800 rpm for 10 min to dry the column. The HiBind DNA midi column was placed over a new 15-mL tube and endo-free elution buffer (10 mM Tris, pH 8.5) (0.5 mL) was added and the column was left for 2 min. pDNA was eluted by centrifuging at 3,800 rpm for 5 min. pDNA concentration was determined using Nanodrop ND-1000, which was found to be 0.5-0.6 μg/μL. The pDNA quality was also determined by agarose gel electrophoresis.

6.4.2 Preparation of pDNA Nanoparticles

DODAG, DOPC, Chol and the synthesised PEGylated peptide-lipid conjugates were prepared as stock solutions in CHCl₃ and stored at -20 °C. Appropriate volumes of each lipid stock were combined in a round bottom flask (5 mL) containing CHCl₃ (500 μL). The solvent was slowly removed in vacuo to form an even lipid film which was then purged with nitrogen (g) to remove residual traces of CHCl₃. The film was re-hydrated with 4mM HEPES (pH 7) at a defined volume to obtain total lipid concentration of 1 mg mL⁻¹. The solution was subsequently subjected to sonication at 40 °C for 40 min. pDNA nanoparticles were prepared by mixing the appropriate volume of pDNA with the resulting liposome solutions under heavy vortex to obtain the desired lipid: pDNA w/w.

6.4.3 Determination of pDNA encapsulation efficiency

Propidium Iodide (PI) assay: The pDNA nanoparticles were prepared, as described previously, at 1:1, 2:1, 4:1, 8:1, 12:1 and 16:1 lipid: pDNA w/w. After 10 min incubation at room temperature, the nanoparticles were diluted in 4mM HEPES buffer to a total volume of 100 μL. PI solution (100 μL, 2.5 μM) was added and the mixtures were incubated at 37 °C for 5 min. The fluorescence intensity of each mixture was measured at λₑₓ = 535 nm, λₑₘ = 617 nm using a Varioskan flash microplate reader. The fluorescence intensities of empty liposome (with no pDNA) incubated with PI solution and PI solution in buffer were employed as background measurements.
% pDNA encapsulation = \[1-\left( \frac{F_{\text{nanoparticles}}}{F_{\text{control free pDNA}}} \right) \] \times 100

Where \( F_{\text{nanoparticles}} \) and \( F_{\text{control free pDNA}} \) represent measured fluorescence intensities of the nanoparticles and free pDNA incubated with PI solution, respectively.

**Agarose gel electrophoresis:** The nanoparticles were prepared, as described previously, at 1:1, 2:1, 4:1, 8:1, and 12:1 lipid: pDNA w/w. After 10 min incubation at room temperature, the nanoparticles were diluted in 4mM HEPES buffer to a total volume of 50 \( \mu \)L. 0.8% Agarose gel was prepared in 1X Tris-acetate-EDTA (TAE) buffer and heated in a microwave on high power (800W) for 2 min until completely dissolved. The gel solution was cooled down in the water bath at 50°C for 20 min. SYBR® safe DNA gel stain (10 \( \mu \)L of 10000X) was added and the gel solution was swirled to mix. The solution was then poured slowly into the tray (137 x 120 mm, gel thickness 1 mm) and the bubbles were removed using disposable pipette before the comb (2mm x 24 wells) was inserted. After left to solidify for 35 min, the gel was placed into the buffer tank containing 1X TAE buffer and SYBR® safe DNA gel stain (5 \( \mu \)L of 10000X). The nanoparticles solutions (20 \( \mu \)L, 0.4 \( \mu \)g pDNA) mixed with 6X orange DNA loading dye (1 \( \mu \)L) were loaded into each well. O’GeneRuler™ Ladder mix was used as the DNA marker. The electrophoresis was performed at 65 mV for 180 min and the gel was visualized under UV light using Alliance 4.7 UVITEC Cambridge.

### 6.4.4 Size and zeta potential measurement

Liposomes and nanoparticles sizes were measured by dynamic light scattering using Coulter Delta N4 plus 440SX particle analyser. All measurements were performed at 90 degree and 25 °C. The mean diameter was determined from the calculated unimodal size distribution of the particles. The zeta potential measurements were performed on a NanoZerises Nano-ZS zetasizer equipped with a 4 mW He–Ne laser (633 nm) and avalanche photodiode detector. The samples (total lipid concentration of 0.5 mg mL\(^{-1}\)) were prepared in 4 mM HEPES buffer, pH 7.
6.5 *In vitro study of pDNA nanoparticles*

6.5.1 **Cell culture and maintenance**

MCF-7 and HT-1080 cell lines were grown in a 75 mL tissue culture flask containing DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (complete medium), at 37 °C in 5% CO₂ atmosphere. The cells were grown until 80% confluence. The media was removed by aspiration and washed with PBS (2 x 5 mL) before trypsin-EDTA (2 mL) was added. The cells were then incubated for 10 min at 37 °C with 5% CO₂. The detached cells were diluted with complete media (8 mL) and the cell suspension (2 mL) was transferred to a new 75 mL tissue culture flask containing fresh complete media (8 mL). Both cell lines were used up to the passage numbers of 30. After that the cells were discarded and new cell batches were defrosted.

6.5.2 **Determination of MMP-2 level in cell culture media using MMP-2 human ELISA assay kit**

HT1080, MCF-7, HEK293T, DU145, and Hela cells were seeded at 4 x 10⁴, 6 x 10⁴, 4 x 10⁴, 4 x 10⁴, and 6 x 10⁴ cells per well, respectively, in 24-well plates (500 μL of complete medium). The cells were grown until 60% confluence for HT1080 and 80% confluence for MCF-7, HEK293T, DU145, and Hela at 37°C in 5% CO₂ atmosphere. The media was removed and replaced with fresh complete media (300 μL) and the cells were incubated at 37°C with 5% CO₂ for further 24 h. The cell supernatants were collected and centrifuged (5000 rpm for 2 min) to separate cellular debris. The determination of MMP-2 level in the cell culture media was carried out using MMP-2 human Elisa assay kit (Invitrogen). In brief, incubation buffer (50 μL) was added to microtiter wells precoated with MMP-2 antibody. MMP-2 human standard was reconstituted in standard diluents buffer and serial dilutions were made according to the manufacturer's protocol. The collected cell supernatants were diluted 5-fold in the
standard diluent buffer. After that the diluted MMP-2 standards and the diluted cell supernatants (50 μL) were added to each microtiter well, and the plate was incubated at the room temperature for 2 h. The mixture in each well was then removed by aspiration and the wells were washed with wash buffer (4 x 400 μL). A biotinylated secondary detection antibody (100 μL) was added to each well and the plate was incubated at room temperature for 1 h. The solution was removed by aspiration and the wells were washed with wash buffer (4 x 400 μL). Streptavidin-peroxydase solution (100 μL) was added to each well to bind to the biotinylated antibody. The plate was incubated at the room temperature for 30 min, and the solution was removed by aspiration, and the wells were washed with wash buffer (4 x 400 μL). Substrate solution (100 μL) was added to the wells and the plate was incubated in the dark at room temperature for further 30 min. Stop solution (100 μL) was then added to the wells and the absorbance of each well was recorded at 450 nm. The concentrations of MMP-2 in the supernatants were calculated from the calibration curve constructed with the MMP-2 human standard.

### 6.5.3 MTS cell proliferation assay

MCF-7 cells were seeded in 48-well plates (3.5 x 10⁴ cells per well, 250 μL of complete medium) for 72 hours prior to the assay. The cells were incubated at 37 °C in a humidified, 5% CO₂ incubator until 80% confluency. The media was then removed and replaced with fresh media. The nanoparticles with PEG₂₀₀₀-AAPV-lipid conjugates were added to each well at three lipid doses (8, 12 and 16 μg/well; 1 ug pDNA) and the cells were incubated at 37°C in 5% CO₂ atmosphere for 4, 8 and 24 h. The cell proliferation assay was performed using the CellTiter 96® AQ אילוסתורי One Solution Cell Proliferation Assay (Promega, USA). In brief, the media was removed, after each uptake period, and the cells were washed with PBS (2 x 250 μL). Fresh media (100 μL) was added to each well, followed by the addition of CellTiter 96® AQ אילוסתורי One Solution Reagent (20 μL). After 2 h incubation, the lysates were transferred to 96-well plates and the absorbance of each well was recorded at 490 nm using a microplate reader.
HT1080 cells were seeded in 48-well plates (2.5 x 10^4 cells per well, 250 μL of complete medium) for 24 hours prior to the assay. The cells were grown until 60% confluency at 37°C in a humidified, 5% CO₂ incubator. The media was then removed and replaced with fresh media. The nanoparticles with PEG_2000-GPLGV-lipid conjugates were added to each well at three lipid doses (8, 12 and 16 μg/well; 1 μg pDNA) and the cells were incubated at 37°C in 5% CO₂ atmosphere for 4, 8 and 24 h. The cell proliferation assay was carried out as described above.

### 6.5.4 Lactate dehydrogenase (LDH) cytotoxicity assay

MCF-7 cells were seeded as described in the MTS cell proliferation assay. The cells were grown until 80% confluence, and the media was then removed and replaced with fresh media. The nanoparticles with PEG_2000-AAPV-lipid conjugates were added to each well at three lipid doses (8, 12 and 16 μg/well; 1 μg pDNA) and the cells were incubated at 37°C in 5% CO₂ atmosphere for 4, 8 and 24 h. The LDH assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA). Briefly, the cell supernatants (150 μL) were transferred to microcentrifuge tubes and the tubes were spun down in a centrifuge (2000 rpm, 10 min) to separate cellular debris. The remaining supernatants were removed by aspiration and the cells were washed with PBS (2 x 250 μL), and the lysis buffer (100 μL) was added to each well. The plate was incubated at 37°C for 30 min. The cell supernatants, after centrifuged, were transferred to a new 96-well plate and the cell lysates (50 μL) were transferred to the other half of the plate. The LDH substrate mix was added to the wells and the plate was incubated in the dark at room temperature. After 30 min incubation, the stop solution (50 μL) was added and the absorbance of each well was measured at 490 nm using a microplate reader. % cell death from cytotoxicity was calculated as followed: % cell death = % LDH release from the cells incubated with the nanoparticles - % LDH release from control cells

HT1080 cells were seeded as described in the MTS cell proliferation assay. The cells were grown until 60% confluency at 37°C in a humidified, 5% CO₂ incubator. The media
was then removed and replaced with fresh media. The nanoparticles with PEG\textsubscript{2000}-GPLGV-lipid conjugates were added to each well at three lipid doses (8, 12 and 16 \( \mu \)g/well; 1 ug pDNA) and the cells were incubated at 37\(^\circ\)C in 5\% CO\textsubscript{2} atmosphere for 4, 8 and 24 h. The LDH cytotoxicity assay was carried out as described above.

6.5.5 pDNA transfection

**HLE experiment:** MCF-7 cells were seeded at 6 x 10\(^4\) per well in 24-well plates (500 \( \mu \)L of complete medium) for 72 hours prior to transfection, respectively. The cells were grown until 80\% confluency at 37\(^\circ\)C in a humidified, 5\% CO\textsubscript{2} incubator. The media was then removed and replaced with fresh media. Two sets of pDNA nanoparticles containing PEG\textsubscript{2000}-AAPV-lipid conjugate and control PEG\textsubscript{2000}-lipid conjugate were prepared at 12:1 lipid: pDNA w/w, as described previously. HLE (10 \( \mu \)L, 1.03 \( \mu \)M) was added to the first set and incubated at room temperature for 10 min, while the other set contained no HLE. The mixtures were added to each well (1 \( \mu \)g pDNA per well) and the plates were then incubated at 37\(^\circ\)C in 5\% CO\textsubscript{2} atmosphere for 6 h. The media was removed, the cells were washed with PBS (2 x 500 \( \mu \)L) and then fresh media was added (500 \( \mu \)L). After further incubation at 37\(^\circ\)C for 24 h, the cells were washed with PBS (2 x 500 \( \mu \)L) and 1 x cell culture lysis reagent (80 \( \mu \)L) was added to each well. One freeze-thaw cycle was carried out to ensure the complete lysis. The cells were scraped from the wells and the lysates were centrifuged (5000 rpm, 2 min) to separate cellular debris. The supernatants were then analysed for luciferase gene expression.

In initial biological test, in collaboration with Dr. Mathieu Mevel, 50 mol\% DODAG/DOPE lipoplexes containing PEG\textsubscript{2000}-AAPV-cholesterol and control PEG\textsubscript{2000}-lipid were divided into two sets: HLE-treatment and non-treatment, as described above. The mixture was incubated with OVCAR-3 cells in complete growth media at 37\(^\circ\)C in 5\% CO\textsubscript{2} atmosphere for 24 h. The media was removed, the cells were washed with PBS (2 x 500 \( \mu \)L) and then fresh media was added (500 \( \mu \)L). The cells were then incubated for a further 24 h before luciferase assay analysis.
**MMP-2 experiment:** MCF-7 and HT1080 cells were seeded as described above. The cells were grown until 80% confluency for MCF-7 and 60% confluency for HT1080, at 37°C in a humidified, 5% CO₂ incubator. The media was then removed and replaced with fresh media. pDNA nanoparticles containing PEG\textsubscript{2000}-GPLGV-lipid conjugate and control PEG\textsubscript{2000}-lipid conjugate were prepared at 12:1 lipid: pDNA w/w, as described previously, and added to each well (1 μg pDNA per well). The plates were incubated at 37°C in 5% CO₂ atmosphere for 24h. The media was removed, the cells were washed with PBS (2 x 500 μL) and the fresh media was added (500 μL). The cells were incubated for a further 24 h before luciferase assay was carried out.

**6.5.6 Luciferase reporter assay**

The luciferase assay system (Promega, USA) was used to measure luciferase gene expression. The assay was performed on a Berthold Lumat LB 9507 luminometer. The transfection efficiency was measured in relative light units (RLU) and normalised to total protein content determined with bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific). The data was reported as RLU/mg protein.

**6.5.7 Total protein assay**

Total protein assay was carried out using bicinchoninic acid (BCA) protein assay. BCA assay is a highly sensitive and selective assay for the detection and quantitation of total protein. The assay is based on the reduction reaction of Cu\textsuperscript{2+} to Cu\textsuperscript{1+} by protein in the alkaline medium. In the first step, Cu\textsuperscript{2+} chelates to the protein in the alkaline environment to form a blue-coloured complex. Two molecules of BCA then chelates with Cu\textsuperscript{1+} to form a purple-coloured complex, giving a strong absorbance at 562 nm. To carry out this assay, briefly, BCA assay reagent (200 μL) was added to the cell lysate supernatant (20 μL) in a 96-well plate. The mixtures were incubated at 37°C for 30 min and the absorbance of each well was recorded at 570 nm using a microplate reader. The protein content was calculated based on the calibration curve of known concentration of Bovine serum albumin standard, previously obtained.
6.5.8 Fluorescence Microscopy

**HLE experiment:** MCF-7 cells were seeded in a 6-well plate (1.2 x 10^5 cells per well, 2 mL of complete medium) containing microscope coverslips (1 mm, thickness) for 72 hours prior to the fluorescent labelling. The cells were grown until 80% confluency at 37 °C in a humidified, 5% CO_2 incubator. The media was then removed and replaced with fresh media. Preparation of fluorescent liposomes was achieved by incorporation of 1 mol% DOPE-Rhodamine into liposome formulation. Two sets of fluorescently labelled liposomes with PEG2000-AAPV-lipid conjugate and PEG2000-lipid conjugate (48 µg of total lipid) were prepared. HLE (10 µL, 1.03 µM) was added to the first set and incubated at room temperature for 10 min, while the other set contained no HLE. The mixtures were added to each well and the plates were incubated at 37°C in 5% CO_2 atmosphere for 6h. The media was then removed and the cells were washed with PBS (2 x 2 mL). The cells were treated with paraformaldehyde (1 mL, 4% v/v) and incubated at 37 °C for 20 min. The cells were then washed with PBS (2 x 2 mL) and incubated with glycine solution (1 mL, 20 mg mL⁻¹) at 37 °C for 20 min. The cells were washed with PBS (2 x 2 mL) and stained with DAPI (1 mL, 100 nM) at 37 °C for 5 min. The cells were washed with PBS (2 x 2 mL) one final time, then the coverslips were removed from the wells, and mounted face-down on microscope slides using PBS: glycerol (10 µL, 1: 1 v/v). Microscopy images were obtained on a Nikon Eclipse E600 microscope.

**MMP-2 experiment:** HT1080 cells were seeded in 6-well plates (8 x 10^4 cells per well, 2 mL of complete medium) containing microscope coverslips (1 mm, thickness) for 24 hours prior to the fluorescent labelling. The cells were grown until 60% confluency at 37 °C in a humidified, 5% CO_2 incubator. The media was then removed and replaced with fresh media. Fluorescently labelled liposomes with PEG2000-GPLGV-lipid conjugate and PEG2000-lipid conjugate (48 µg of total lipid) were prepared and added to the wells. The plates were incubated at 37 °C in 5% CO_2 atmosphere for 24 h. The coverslips were fixed and mounted as described above. Microscopy images were taken on an Olympus 251 scope and confocal images were obtained using an upright Leica instrument.
6.6 Serum aggregation analysis of pDNA nanoparticles

6.6.1 Stability in transfection medium (10% serum)

pDNA nanoparticles were prepared at 12:1 lipid: pDNA w/w, as described previously. The same volumes of the nanoparticles as employed in transfection were diluted with complete media (500 µL). The particles sizes were recorded immediately using dynamic light scattering. The mixtures were incubated at 37 °C and their sizes were measured at 30 min intervals over 4 h.

6.6.2 Stability in 80% serum: Turbidity assay

pDNA nanoparticles were prepared at 12:1 lipid: pDNA w/w, as described previously. FBS (400 µL) were added to the nanoparticle solutions (100 µL) and the absorbance of the mixtures was recorded immediately at 600 nm using UV spectrometer. The mixtures were incubated at 37 °C and their absorbance was measured at 30 min intervals over 4 h.

6.7 Preparation and characterization of siRNA nanoparticles

6.7.1 Preparation of siRNA stock solutions

Upon receipt, the siRNA tube was briefly centrifuged to ensure that the dried siRNA was at the bottom of the tube. The siRNA was resuspended in the Nuclease-free water to a final concentration of 50 µM. When handling, gloves should be worn and RNase-free reagents, tubes, and barrier pipette tips should be used to minimize the degradation of the siRNA by exogenous ribonucleases. Once reconstituted, the siRNA was ready to use and the resuspended siRNA was stored at -20 °C.
6.7.2 Preparation of siRNA nanoparticles

siRNA nanoparticles were prepared by mixing an appropriate volume of siRNA with the liposome solutions under heavy vortex to obtain the desired lipid: siRNA charge ratios.

6.7.3 Determination of siRNA encapsulation efficiency

Propidium Iodide (PI) assay: The nanoparticles were prepared, as described previously, at 0.5:1, 2:1, 4:1, and 8:1 lipid: siRNA charge ratios. The propidium iodide assay was carried out as described in 6.4.3, propidium iodide (PI) assay section. The fluorescence intensities of empty liposome (without siRNA) incubated with PI solution and PI solution in buffer were employed as background measurements.

\[
\text{% siRNA encapsulation} = \left[1 - \frac{F_{\text{nanoparticles}}}{F_{\text{control free siRNA}}} \right] \times 100
\]

Where \(F_{\text{nanoparticles}}\) and \(F_{\text{control free siRNA}}\) represent measured fluorescence intensity of nanoparticles and free siRNA incubated with PI solution.

Agarose gel electrophoresis: The nanoparticles were prepared, as described previously, at 0.5:1, 2:1, 4:1, and 8:1 lipid: siRNA charge ratios. After 10 min incubation at room temperature, the nanoparticles were diluted in 4mM HEPES buffer to a total volume of 100 µL. 1% Agarose gel was prepared in 1X Tris-borate-EDTA (TBE) buffer and heated in a microwave on high power (800W) for 2 min until completely dissolved. The gel solution was cooled down in the water bath at 50°C for 20 min. SYBR® green II RNA gel stain (5 µL of 10000X) was added and the gel solution was swirled to mix. The solution was then poured slowly into the tray before the comb (2mm x 24 wells) was inserted. After left to solidify for 35 min, the gel was placed into the buffer tank containing 1X TBE buffer and SYBR® green II RNA gel stain (5 µL of 10000X). The nanoparticles solutions (10 µL, 0.06 µg siRNA) mixed with 6X orange DNA loading dye (1 µL) were loaded into each well. Free siRNA was used as siRNA marker. The
electrophoresis was performed at 65 mV for 30 min and the gel was visualized under UV light using Alliance 4.7 UVITEC Cambridge.

6.7.4 Size and zeta potential measurements

siRNA nanoparticles were prepared, as described previously, at 4:1 lipid: siRNA charged ratio. Size and zeta potential measurements were performed as described in 6.4.4.

6.8 In vitro siRNA knockdown of transient luciferase expression

6.8.1 pDNA Transfection

MCF-7 and HT1080 cells were seeded at 3.5 x 10^4 and 2.5 x 10^4 cells per well in 48-well plates (250 μL of complete media) for 72 and 24 hours prior to transfection, respectively. The cells were grown until 80% for MCF-7 and 60% confluency for HT1080, at 37°C in a humidified, 5% CO₂ incubator. The media was then removed and replaced with fresh media. The transfection of pEGFPLuc DNA was carried out using jetPEI™. According to the manufacturer’s protocol, briefly, pDNA (0.5 μg) and jetPEI™ (1 μL) were diluted separately in NaCl buffer (150 mM, 25 μL). jetPEI™ solution was added to pDNA solution, the mixture was vortexed, and incubated at room temperature for 30 min. The mixture (50 μL) was then added to the wells and the cells were incubated at 37°C in 5% CO₂ atmosphere for 2 h. The media was removed and the cells were washed with PBS (2 x 250 μL) before being used for siRNA knockdown experiment.
6.8.2 Optimisation of anti-luciferase siRNA concentrations for luciferase knockdown

Non-PEGylated liposomes were prepared to encapsulate siRNA, anti-luciferase and negative siRNA, at 10, 20, 30, and 50 nM and 10, 20, 30, and 50 μM (4:1 lipid: siRNA charge ratios). The mixtures were added to each well of the transfected cell lines, MCF-7 and HT1080 in 6.8.1, (250 μL of OptiMEM). MCF-7 cells were incubated for 6 h and HT1080 cells were incubated for 24 h at 37°C in 5% CO₂ atmosphere. The media was removed, the cells were washed with PBS (2 x 250 μL) and fresh media was added (250 μL). The cells were incubated for a further 36 h before analysis for luciferase activity.

\[
\% \text{ luciferase knockdown} = \left[1 - \frac{\text{luciferase activity}_{\text{anti-luciferase siRNA}}}{\text{luciferase activity}_{\text{nonspecific siRNA}}} \right] \times 100
\]

6.8.3 Transient luciferase knockdown using anti-luciferase siRNA

**HLE experiment:** Two sets of liposomes containing PEG₂₀₀₀-AAPV-lipid conjugate and control PEG₂₀₀₀-lipid conjugate were prepared to encapsulate siRNA, anti-luciferase siRNA and negative siRNA, at 4:1 lipid: siRNA charge ratios. HLE (10 μL, 1.03 μM) was added to the first set and incubated at room temperature for 10 min, while the other set contained no HLE. The mixtures were added to the wells of the transfected MCF-7 in 6.8.1 (30 μM siRNA per well, 250 μL of complete media) and the plates were then incubated at 37°C in 5% CO₂ atmosphere for 6h. The media was then removed, the cells were washed with PBS (2 x 250 μL) and the fresh media was added (250 μL). The cells were incubated for a further 36 h before analysis for luciferase activity.

**MMP-2 experiment:** The liposomes containing PEG₂₀₀₀-GPLGV-lipid conjugate and control PEG₂₀₀₀-lipid conjugate were prepared to encapsulate siRNA, anti-luciferase siRNA and negative siRNA, at 4:1 lipid: siRNA charge ratios. The mixtures were added to the wells of the transfected HT1080 in 6.8.1 (30 μM siRNA per well, 250 μL of
complete media) and incubated at 37°C in 5% CO₂ atmosphere for 24 h. The media was removed, the cells were washed with PBS (2 x 250 μL) and the fresh media was added (250 μL). The cells were incubated for a further 36 h before analysis for luciferase activity.

6.9  **In vitro siRNA knockdown of luciferase expression in stably transfected cell lines**

6.9.1  **Preparation of stably transfected luciferase cell lines**

MCF-7 and HT1080 cells were seeded at 1.2 x 10⁵ and 8 x 10⁴ in 6-well plates (2 mL of complete growth media) for 72 and 24 hours prior to transfection, respectively. The cells were grown until 80% confluency for MCF-7 and 60% confluency for HT1080, at 37°C in 5% CO₂ atmosphere. The media was removed and replaced with fresh media. The transfection of pUbC-Luc-S/MAR DNA was carried out using jetPEI™. Briefly, pDNA (3 μg) and jetPEI™ (6 μL) were diluted separately in NaCl buffer (150 mM, 100 μL). jetPEI™ solution was added to pDNA solution, vortexed, and incubated at room temperature for 30 min. The mixture (200 μL) was added to the wells and incubated at 37°C in 5% CO₂ atmosphere for 2 h. The media was removed, washed with PBS (2 x 2 mL) and the fresh media was added (2 mL). The cells were incubated for a further 48 h. The media was removed and washed with with PBS (2 x 2 mL). The fresh media containing G418, a selection drug, (1 mg/mL) was added (2 mL) to each well and the plate was maintained at 37°C in 5% CO₂ atmosphere. The antibiotic-containing media should be replaced every 3-4 days. The drug-resistant colonies of MCF-7 appeared in 4 weeks and for HT1080 the drug-resistant colonies appeared in 2 weeks, after transfection. The media was removed and washed with PBS (2 x 2 mL). The large end of pipette tip (1000 μL) was snipped off, dipped into Vaseline and stuck around the colonies. Trypsin-EDTA (300 μL) was added to detach the cells and the individual colonies were transferred into a new 6-well plate containing complete media. The cells were incubated at 37°C in 5% CO₂ atmosphere and the media were replaced every 3-4 days. When confluency, the cells were trypsinized and transferred to a 75 mL tissue
culture flask containing complete media. The cell lines were analysed for luciferase expression before they were used for siRNA knockdown experiment.

6.9.2. **Luciferase knockdown in stably transfected cell line using anti-luciferase siRNA**

**HLE experiment**: MCF-7-luc was seeded in 48-well plates (3.5 x 10^4 cells per well, 250 µL of complete growth media) for 72 hours prior to transfection. The cells were grown until 80% confluency at 37°C in 5% CO₂ atmosphere. The media was then removed and replaced with fresh media. The luciferase knockdown using anti-luciferase siRNA was carried out as described in 6.8.3, **HLE experiment section**.

**MMP-2 experiment**: HT1080-luc was seeded in 48-well plates (2.5 x 10^4 cells per well, 250 µL of complete media) for 24 h prior to transfection. The cells were grown until 60% confluency at 37°C in 5% CO₂ atmosphere. The media was then removed and replaced with fresh media. The luciferase knockdown using anti-luciferase siRNA was carried out as described in 6.8.3, **MMP-2 experiment section**.
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