CHAPTER 16

SPERMINE AND LIPOPOLYAMINES AS GENE DELIVERY AGENTS

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1. INTRODUCTION TO SPERMINE AND LIPOPOLYAMINES

Polyamines are polycationic at physiological pH and play a variety of important biological roles. Many mammalian cells also possess an active polyamine uptake system, although little is known about its function. Spermine (Fig. 1i) (3.4.3 methylene count between the amine groups) and spermidine (Fig. 1ii) (4.3 methylene spacings) were first discovered in the nuclei of sperm, where these polycations help histone proteins to package DNA by charge neutralization of the phosphate anions along the DNA backbone. Most cells use histone proteins and polyamines to condense DNA in the nucleus. The histones found in chromatin are categorized as core histone (subunit H2A, H2B, H3, H4) (Fig. 1) and linker histone (H1), forming an octomer (108kDa) that binds to DNA. Lysine (Lys, K) and arginine (Arg, R), the most important of the positively charged amino acids, are found in significant sequences along most histone proteins. Lys has its (CH₂)₄NH₃⁺ (basic) side-chain, and Arg is the naturally occurring (mammalian) amino acid containing a guanidine functional group, the most basic functional group in biological chemistry. Thus, the side-chains of these amino acids (together with ornithine and histidine) can be positively charged in the cell. The pKₐs of Lys and Arg (side-chains) are 10.5 and 12.5 respectively. DNA-histone complexes are dissociated on treatment with acids or dilute salt solutions. This is evidence of ionic and non-covalent interactions; the phosphate anions are titrated by the ammonium cations. Of course, there also has to be some consideration of spermine (i) and spermidine (ii) and their conjugates as neurotoxins; such neurotoxication can be due to high levels of free polyamines, or similar effects achieved by polyamine amides in the mammalian brain by mechanisms involving glutamate receptors. This latter process parallels the modes of action of the spider and wasp polyamine amide (invertebrate) neurotoxins.

2. OVERVIEW OF NON-VIRAL GENE THERAPY (NVGT)

Gene therapy is a new treatment strategy for some difficult-to-cure diseases, introducing a therapeutic gene into body cells and aiming for desired gene expression. The therapeutic outcome is not only limited to missing proteins in patients (such as a chloride ion channel in...
the lungs of patients with cystic fibrosis), but also an inhibitory protein signal to stop the progression of disease, especially as a potential treatment for certain cancers.\(^4\) The design and synthesis of efficient DNA delivery vectors are major research areas in non-viral gene therapy (NVGT). Lentiviral and other viral vectors show efficient transfection, however, there is the possibility of severe toxicity from the viral genome, as well as immunogenicity, and there is the drawback of a limited DNA payload. Naked DNA is used even in current clinical trials. NVGT vectors have been produced to retain or to improve upon the performance of viral vectors and to provide a safe alternative for clinical gene therapy.\(^4\) Recently, Felgner et al. reported a system of NVGT nomenclature guidelines to aid in NVGT research, dividing the vectors into the two major synthetic gene delivery systems: lipoplex (cationic lipid-nucleic acid complex) e.g. lipopolyamines, cationic liposomes, and polyplex (cationic polymer-nucleic acid complex) e.g. polyethylenimine (PEI), poly-L-lysine (PLL) etc.\(^5\) Gene therapy is a complex drug (DNA) delivery strategy which must overcome intracellular barriers. The contributions from these barriers in blocking efficient NVGT are not well understood. We are aiming to understand (Fig. 2) and, thereby, to design a significant improvement over current transfection yields.

![Figure 1](image1.png)

**Figure 1** Spermine (i), spermidine (ii) and histone 2A (iii) K(13)AKTRSSR(20) (underlined amino acids representing some DNA contact sites of histone 2A are shown).\(^3\)

![Figure 2](image2.png)

**Figure 2** Barriers to NVGT (I – DNA condensation and particle formation, II – endocytosis, III – endosome escape, IV - nuclear localization, V - nuclear entry and VI - gene expression).
Negatively charged DNA (due to the phosphate anions), when neutralized by salt formation with positively charged lipopolyamine vectors, results in condensed nanometer-sized particles typically 50-150nm in outer diameter (Fig. 4). Nanoparticles, toroidal in shape, were observed in polyamine-induced DNA condensation. This DNA compaction facilitates its stability in extracellular compartments, cellular uptake, and other intracellular process such as nuclear entry. The electrostatic interactions between the surface of DNA particles and cell membranes is expected to be an initial process leading to internalization by endocytosis of DNA nano-complexes, with the involvement of clathrin-coated pits on the cell membrane. It has been shown that endosome escape of the DNA is one major critical barrier to efficient transfection. Early endosomes are formed by the inverted cell membrane upon DNA particles endocytosis, which are eventually degraded into late endosomes by internalization into lysosome vesicles. The “proton sponge” hypothesis has been proposed for endosome escape of NVGT cationic polymer. The protons are pumped into endosomes (at pH 7.4) by V-ATPase proton pump at their membrane, while polycations (the pK_a of a primary amine is around 10.5) work as a pH buffer material. This results in an increased proton/water flux and lower pH at 5.5. Membrane disruption by swelling and finally osmotic lysis allows DNA complexes to escape from these vesicles prior to enzymatic degradation. Alternatively, the mechanism of lipid membrane mixing between endosomal membrane and lipidic gene carrier has also been hypothesized as an alternative means by which cationic lipids/liposomes might facilitate the endosome escape process, although this process is not well understood.

Also, the mechanism of DNA nuclear translocation is not well understood. However, the transport through nuclear pores has been considered as the most likely possible route in conjunction with help from nuclear localization signals (NLS). NLS are peptides that help DNA complexes (which are typically bigger than 50kDa and cannot pass through the nuclear pore complex) to reach the nucleus. NLS binds with the α-subunit of the cytosolic importin receptor, then the β-subunit of the importin binds to the nuclear pore complex with the help of GTP binding protein (called Ran). The β-subunit will be retained at the inner face of pore complex. Based on amino acid sequence analysis of NLS such as SV40 T-antigen, HIV-tat protein etc., positively charged amino acids are also found as their unique characteristic. The nuclear envelope contains double phospholipid layers, with the membrane space directly connecting to the endoplasmic reticulum lumen. The inner and outer membranes join together at the nuclear pore complex (NPC). The NPC is a large structure with an assembly of eight spokes attached to rings on the cytoplasmic and nuclear sides of the nuclear envelope, accordingly named “the cytoplasmic ring” and “the nuclear ring”, with a formed central channel approximately 40nm in diameter for the transportation of large particles across the nuclear membrane. The pore diameter is 120nm and the molecular mass is about 125 million Da. The small molecules (size of 9nm diameter, size less than 50kDa), for example, ions, metabolites and small proteins, use passive diffusion to get in and out of the nucleus freely. Bigger molecules, including DNA, are transported by an energy-dependent mechanism through this NPC. The selective transport of these macromolecules to and from the nucleus requires a NLS to direct their traffic through the NPC.

3. SPERMINE MEDIATED DNA CONDENSATION AND PARTICLE FORMATION

Spermine (i) is a natural biomolecule involved in cell growth and differentiation control. In vitro studies have shown that the binding of spermine to DNA induces structural changes in DNA. The spermine cations neutralize the negatively charged phosphodiester groups of DNA, initiating the DNA helix’s axis bending resulting in DNA condensation. This gene
packing enables efficient gene therapy by non-viral vectors. The results show that the nanoparticle (50-150nm) is able to go across the cell membrane and lead to the gene expression of delivered DNA.\textsuperscript{7,15} The ethidium bromide (EthBr) assay was used to measure DNA condensation. EthBr is a cationic dye (Fig. 3) that intercalates between DNA base pairs. This intercalation increases the fluorescence yield of EthBr (excitation 546nm, emission 595nm). Many compounds that bind to DNA, including spermine, can displace EthBr from the EthBr-DNA complex. The EthBr displacement by these chemicals is related to DNA bases as well as environmental conditions (e.g. buffer composition, ionic strength). So, EthBr fluorescence quenching can be used to measure the DNA-polyamine binding efficiency.\textsuperscript{15,16}

![Figure 3 Ethidium bromide.](image)

The EthBr assay method is a modified literature protocol of Cain and co-workers\textsuperscript{17}, regarded as an “exclusion assay” requiring the pre-forming of DNA and DNA binding drugs, before EthBr is added. The excitation wavelength used is 546nm, which is based on the excitation of EthBr. In 2000, the modified reproducible EthBr “displacement assay” was reported by Geall and Blagbrough.\textsuperscript{16} The excitation wavelength was optimized at 260nm, so (only) intercalated EthBr is indirectly excited by energy transfer from the DNA. The proposed protocol also allows the experiment to be run without pre-complexing DNA and its binding molecule. This method is named as “displacement assay”.\textsuperscript{16} Additionally, the EthBr-DNA complex formation restricts the internal rotational freedom of EthBr. The fluorescence polarization is calculated from the emission intensity from polarizer and analyzer in a spectrofluorimeter. The polarization increase is observed when DNA is condensed due to the limited movement of EthBr. The measurement of steady-state fluorescence polarization can be used to indicate the EthBr-DNA complex conformation upon binding of spermine.\textsuperscript{15}

In addition to the intercalating assay using EthBr, the UV absorbance at over 300nm (e.g. 320nm) was useful in confirming the formation of nanoparticles. The double helix DNA was bound by polyamines and formed the nanoparticles which scatter the light resulting in the UV absorbance increase above 300nm.\textsuperscript{18} So, light scattering (LS) is being measured rather than (apparent) UV absorption. Some precipitation of the DNA may be visible, but it does not increase the absorption above 300nm. However, the DNA concentration used in this assay was in a 10-fold excess compared to the EthBr assay given the low sensitivity of this experiment and the lack of fluorescent indicator.\textsuperscript{11}

DNA used in gene therapy research is normally prepared as plasmids, usually with a protein marker that can be followed in the gene expression quantification. pEGFP\textsuperscript{19} (4.7 kilobasepairs) is the plasmid encoding for enhanced green fluorescent protein, under CMV (cytomegalovirus) promoter, obtained from Clontech. Successfully transfected cells with pEGFP shows the distinct fluorescence detectable by a Fluorescent Activated Cell Sorter (FACS). Modified firefly (Photinus pyralis) luciferase pGL3 control vector\textsuperscript{20} (5.2 kilobasepairs), with SV-40 promoter, was from Promega. In this luciferase assay, luciferin was added to transfected cells that underwent oxidation and generated the yellow light, measured by a luminometer.
Figure 4 Detailed NVGT mechanism. A: endocytosis process. B: nuclear entry and gene expression process.
pEGFP and pGL3 were condensed by spermine to about 50%, measured using the EthBr assay (Fig. 5). Particle formation was also confirmed by the LS assay, with significantly increased apparent absorbance at an N/P ratio > 3.00. We conclude that (simple) spermine-mediated condensation may provide less compacted particles; also these are only really significant at high N/P ratios. Additionally, the condensation profiles of both plasmids were found to be similar, possibly relative to their similar plasmid sizes. We and others have shown that lipopolyamines are more efficient DNA condensers (though not necessarily NVGT vectors) than these simple polyamines.21

![Figure 5](image)

**Figure 5** DNA condensation and light scattering of nanoparticles (above – pEGFP plasmid vs spermine, below – pGL3 vs spermine).

4. DEVELOPMENT OF POLYCATION VECTORS IN NVGT

A variety of polycations, but nevertheless small molecules compared to histones or DNA, have now been reported for studies in NVGT (Fig. 6). The examples chosen serve to illustrate the current range of molecular diversity within lipopolyamines. From one of the earliest steroid conjugates, DC-Chol containing only one positively charged N-atom, through
Genzyme’s GL#67, Behr’s Transfectam (DOGS), Scherman and co-workers’ RPR-120535 and DOSPA (all containing spermine in different ways), through to vitamin D and modified buckyball (C60) conjugates. The most recent lipopolyamine is KanaChol from Lehn and co-workers.\textsuperscript{23}

**Figure 6** A selection of lipopolyamine-based NVGT vectors (lipoplex).\textsuperscript{21}
Polymer polycationic NVGT vectors (Fig. 7) for the formation of polyplexes are based upon linear and branched polyethylenimines, PLL, poly-L-arginine and poly-L-histidine. These representative examples demonstrate the distribution of positive charge in the presence of a lipid region, cationic lipids.

**Linear and branched polyethylenimine (PEI)**

![Linear and branched polyethylenimine (PEI)](image)

**Poly-L-lysine (PLL)**

![Poly-L-lysine (PLL)](image)

**Poly-L-arginine**

![Poly-L-arginine](image)

**Poly-L-histidine**

![Poly-L-histidine](image)

*Figure 7* Polymer polycationic NVGT vectors (polyplex).

5. PHYSICOCHEMICAL PROPERTIES OF POLYAMINES-BASED DNA CONDENSING AGENTS

The effects of the regiochemical distribution of positive charges along the polyamine moiety in DNA condensing agents were studied by Geall *et al.*[^11][^27] DNA condensation is dependent upon
three characteristic properties of polyamines: the number of positive charges, the regiochemical distribution of charges (determined by the $pK_a$ of each amino group), and the local salt concentration. From the $pK_a$ investigation and calculation, spermine (i) carries 3.8 charges and its cholesterol conjugate has a positive charge distribution similar to that found in spermidine (ii) (4.3) (Fig. 8). Such spermidine mimics (lipospermidines) are still effective as DNA condensing agents, significantly more so than spermidine itself, which is but a weak DNA condensing agent.

![Figure 8](image)

**Figure 8** $pK_a$ of spermine and a cholesteryl lipopolyamine.\textsuperscript{11}

We have designed and prepared lipopolyamines (Fig 9) and monitored their efficiency in DNA condensation into particles by LS and their salt-dependent binding affinity for DNA by fluorescence quenching.\textsuperscript{11,27} Lipopolyamines were prepared from cholesterol, lithocholic and cholic acids (5β-cholanes) by acylation of tri-Boc-protected tetra-amines spermine and thermine. These ligands are polyammonium ions at physiological pH.\textsuperscript{28}

![Figure 9](image)

**Figure 9** Lipopolyamines based on cholesterol\textsuperscript{11}, bile acids\textsuperscript{28} and fatty acids.
LipoGen (from InvivoGen) is an example of a lipopolyamine with two oleoyl groups at $N^2$ and $N^3$ of spermine, therefore with only two positively charged nitrogens. Modifying the lipophilicity of spermine (3.4.3) led to efficient DNA condensation (15% residual fluorescence at N/P charge ratio 2.5) comparing to the tetracationic spermine (50% at charge ratio more than 3.50) (Fig 10). According to Vijayanathan et al.\textsuperscript{29} the $\lambda$-phage DNA condensed particles (by spermine) are in the size range 50-100 nm. Lipospermine was shown to condense pSfiSVneo, pSfiSV19 and pCISfi-$\gamma$IFN DNA to smaller particle sizes at 50-70nm.\textsuperscript{30} The efficient condensation of DNA by lipospermine over spermine, resulting in smaller sized particles, may promote more effective transfection.

Figure 10 pEGFP condensation by LipoGen vs spermine.

6. TRANSFECTION IN HUMAN SKIN FIBROBLAST CELLS (FEK4)

LipoGen (Invivogen) is a lipospermine with two oleoyl groups at $N^2$ and $N^3$ of spermine (i) and, therefore, only two positively charged primary amines. It was prepared as a non-liposomal formulation. The lipophilic modification aims to facilitate the transfection process (e.g. potentially through enhanced DNA condensation, cell entry, endosomal escape). This lipophilic modification of the spermine structure resulted in a more efficient pEGFP DNA condensation (15% residual fluorescence, in the EthBr assay, at N/P charge ratio 2.5) compared to tetracationic spermine (50% at N/P charge ratio 3.0). The \textit{in vivo} transfection of pEGFP (2$\mu$g/well) with LipoGen was carried out using FEK4\textsuperscript{31} (2.5 x 10$^4$ cells/well at 50% confluence), incubated for 4h, then the transfection was stopped by removal of the DNA complex and replacement with foetal-calf serum containing media; FACS analysis was performed 48h post-transfection. By using pEGFP as delivered DNA, the EGFP chromophore (a substituted 4-hydroxybenzylidene imidazolidinone) was detected in successfully transfected cells by FACS (fluorescent-activated cell sorting). The EGFP amino acid sequence L(64)TYGV(68) transcribed from pEGFP was cyclised in the post-translational
steps (at amino acids 65-67) forming an EGFP fluorophore with $\lambda_{\text{ex}} = 488\text{nm}$ (red-shifted from wild GFP protein) and $\lambda_{\text{em}} = 507\text{nm}$. Lipofectin (DOTMA/DOPE = 1:1 w/w, Invitrogen), the commonly used transfection liposomal reagent containing cationic lipid (with one positive charge) and helper lipid (DOPE), was also used in this experiment for a comparison. The fluorescent cell counts observed in all N/P ratios were higher in LipoGen-mediated transfection. Thus, the lipid moiety in lipospermine is playing an important role in in vitro transfection. The optimal charge ratio for FEK4 transfection with pEGFP-LipoGen complex is around 2.5, which corresponds to the optimal DNA condensation N/P ratio (from the EthBr assay). However, from Fig. 11 it appears that increased condensation leads to higher transfection efficacy. The high N/P ratio (more than 5.0) results in less efficient gene delivery overall. A similar relationship between N/P charge ratio and transfection efficiency was also observed in Lipofectin-mediated transfection.

![Graph](image1.png)

**Figure 11** FEK4 Transfection of pEGFP complexed by LipoGen (above) and Lipofectin (below).
7. CONCLUSIONS

Our research has advanced neurotoxic polyamine amides from spider and wasp natural products, with their exquisite sensitivity in modulating glutamate receptors, from pharmacological tools towards therapeutic neurochemistry, thereby highlighting their potential as therapeutic agents. So, whilst polyamines and polyamine amides are potent and selective receptor probes for a variety of voltage- and especially for ligand-gated cation channels, they also are now seen to have potential as synthetic vectors and can be exploited in NVGT. Using our designed steroidal lipopolyamine probes (Fig. 9), we are studying DNA-lipopolyamine complexes with respect to their formation by DNA condensation. Comparable DNA-binding efficiency to that of the unlabelled lipopolyamines and robust fluorescent spectral properties across the varying cellular pH range are desirable properties in fluorescent ligands, but this requires experimental verification. Have we designed and prepared fluorophore-substituted lipopolyamines or lipopolyamine-substituted fluorophores? Only time and experimental results will tell. However, we have achieved a controlled chain extension of suitably protected polyamines using reductive alkylation to mimic spermine (i) rather than spermidine (ii) in the target molecules. A practical method for the efficient hydroboration of cholesteryl carbamates has allowed us to prepare our designed trans-AB steroidal lipopolyamines, together with the corresponding cis-AB ring junction as the minor product of hydroboration or from naturally occurring bile acids. We are able to introduce fluorophores of choice by our Fmoc-chemistry route. The design in our versatile synthetic route to Fmoc protected aminoesters of cholesteryl carbamate and lithocholic acid polyamine amides allows a range of selected fluorophores to be readily incorporated. The design and synthesis of these novel fluorescent lipopolyamines allows us to study the intracellular events during transfection. As well as our studies on lipopolyamine-mediated DNA condensation, we are optimising these probes to employ them monitoring the intracellular hurdles to NVGT. At present, a poor understanding of the molecular mechanisms of action of non-viral vectors remains an important, but unresolved issue. With a greater knowledge of these mechanisms, new (possibly spermine based) lipopolyamine vectors with improved transfection efficiency can be rationally designed and used as gene delivery agents in vivo.

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References