

Chapter 1

Non-Viral Gene Therapy

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This chapter is meant to serve as an introduction to non-viral gene transfer by highlighting therapeutic applications that have transitioned from preclinical research into the clinic. Non-viral gene therapy is the administration of plasmid DNA encoding a transgene gene locally or systemically yielding expression of a therapeutic protein, thereby correcting a disease state. Local administration of plasmid DNA results in gene transfer to cells at the site of injection. Gene transfer efficiency can be increased by applying electric current (electroporation) or sound waves (sonoporation). Alternatively, the plasmid DNA can be formulated with cationic lipids or polymers to increase gene transfer. All of these methods result in increased uptake by cells and therefore in increased gene expression. Clinical applications of this technology include: treatment of peripheral vascular disease following local administration at the sites of muscle ischemia; development of genetic vaccines resulting in immune activation against the specific expressed antigen; development of therapeutic cancer vaccines that induce surveillance and killing of tumor cells by the immune system; correction of genetic disease by expressing a functional wild type protein in cells that lack a functional protein.

1. Introduction

It is difficult to pinpoint a specific discovery that initiated the field of plasmid DNA based gene therapy. There have been several milestones that led to its development. Table 1 lists a series of events that have impacted the development of the field.

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Table 1.1. Scientific milestones that impacted the field of non-viral gene therapy.

Scientific Milestone	Year	Refs.
First Liposome Based DNA Delivery Patent filed	1983	1
First publications describing the use of cationic lipids to transfect cells	1987–89	2–4
Demonstration that “Naked DNA” can Transfect muscle cells <i>in vivo</i>	1990	5
First human clinical trial conducted for development of melanoma cancer vaccine using cationic lipid formulated plasmid DNA	1996	6, 7
First indications of clinical efficacy demonstrated for treatment of Chronic Limb Ischemia following IM administration of VEGF Naked pDNA	1996	8, 9
Electroporation yields order of magnitude increase in gene expression following local administration	1998	10
Aqua Health (Novartis) anti-viral vaccine for salmon receives approval in Canada.	2005	
Successful demonstration of efficacy for treatment of chronic limb ischemia following IM administration of pDNA expressing hepatocyte growth factor.	2007	
Merial receives conditional USDA approval of canine melanoma therapeutic genetic vaccine.	2008	

The discovery of cationic lipids was the segue into therapeutic applications of plasmid based gene delivery. It provided a methodology for getting DNA into cells resulting in expression. This was not a new gene transfer concept in that calcium phosphate and poly cationic polypeptides, such as poly-lysine and poly-L-ornithine had been used to introduce plasmid DNA and RNA into cells.¹¹ However, this methodology had many uncontrolled variables resulting in a high degree of variability and not being applicable for clinical development.

The transition of cationic liposomes from an *in vitro* transfection reagent to a clinical application was first realized with the testing of the first cancer vaccine where a non-self major histocompatibility antigen, HLA-B7 was encoded along with β -2 microglobulin in an expression plasmid, complexed

with cationic liposomes composed of DC-Chol/DOPE and injected into tumors. Gene transfer of the foreign major histocompatibility antigen complex triggers a T cell mediated immune response that not only results in the killing of antigen expressing tumor cells but also results in the killing of non-antigen expressing tumor cells. This local priming of the immune system against tumor cells activates immune surveillance of the body to seek and destroy neoplasms distal to the initial tumor immunization site.

As in any new therapy, the initial clinical trials provided lessons that would impact the design of future clinical trials and focus improvements in the technology that increased performance and safety. Two major technology improvement categories included increased expression of the therapeutic protein and increased duration of expression. The subsequent sections will describe the basic features of the plasmid DNA, the formulations and the gene transfer techniques that have been employed to overcome technology deficiencies. Though these deficiencies have not completely been overcome, the lessons learned have been applied to yield commercialization of animal health products and produce successful late stage human clinical trials.

2. Plasmid DNA

Plasmid DNA is a closed circular double stranded helix DNA molecule. When isolated from bacteria, pDNA is in a supercoiled, dimer or concatamer form. The isolation conditions can cause single strand or double strand nicks producing relaxed or linear forms. Isolation conditions are optimized to yield the highest percentage of supercoiled pDNA and minimize the production of the other forms because there are studies that show increased supercoil content yields higher levels of transgene expression.¹² Furthermore, the FDA has deemed the percent supercoil content a product shelf life determinant. The fundamental features of pDNA are shown in Fig. 1.1. These are: the expression cassette, the origin of replication and the drug resistance gene. The origin of replication (ORI) is a DNA sequence of 13 mer and 9 mer repeats that initiates plasmid DNA replication in bacteria. The drug resistant gene, Kanamycin resistance gene or Ampicillin resistance gene, allows for the selection of plasmid transformed bacteria.

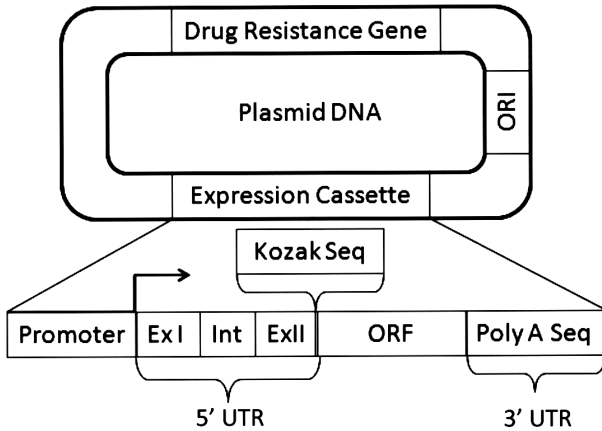


Fig. 1.1 Diagram of Plasmid DNA. Diagram shows feature of plasmid DNA. Abbreviations: ORI-Origin of Replication; ExI and ExII-Exon I and Exon II; Int-Intron; Poly A Seq-Poly Adenylation Sequence; Arrow-Transcription Initiation Sequence.

The expression cassette can be divided into the following components: the promoter; the 5'untranslated coding sequence (5'UTR) containing at least 1 intron and the kozak sequence; the open reading frame (ORF) encoding the gene to be expressed; the 3' untranslated coding sequence (3'UTR) containing the poly-adenylation sequence (PolyA). The promoter contains a DNA sequence that recruits RNA polymerase II for initiation of transcription. The promoter can contain additional sequences, known as enhancer sequences that bind proteins termed transcription factors that further facilitate the recruitment of RNA polymerase II.

The inclusion of at least one intron in the 5'UTR ensures the entry of the transcript into the pre-mRNA/mature mRNA processing pathway and export of the mRNA into the cytoplasm.¹³ Located in the 5'UTR is the Kozak sequence which is a signal for the ribosome to start translation. The Kozak consensus sequence is (gcc)gccRccAUGG, where R is an adenine three bases upstream of the AUG start codon which is followed by another 'G'.¹⁴ The AUG codon encodes methionine which is the first amino acid of the transgene. The open reading frame can be codon optimized to increase gene expression. Due to the redundancy of the genetic code, rarely used codons can be replaced with more commonly used codons, especially those codons more commonly used by the target cell; the purpose being to increase protein synthesis. This strategy can also be used to

reduce the amount of CpG sequences that can activate the immune system. Activation of the immune system has been shown to reduce the amount and duration of gene expression.¹⁵ However, in developing genetic vaccines, codon optimization can be used to increase CpG content thereby increasing immune activation.¹⁶ The 3'UTR contains the polyadenylation sequence that is a binding site for a multi-protein complex that cleaves the end of the mRNA transcript and polyadenylate polymerase adds approximately 250 adenine nucleotide monophosphates. The poly adenylation takes place in the nucleus and promotes nuclear export of the mRNA and translation, and inhibits degradation.

Endogenous microRNA cleavage sequences approximately 20 base-pairs in length are located in the 3'UTR of endogenous mRNAs. Transfection of cells with endogenous microRNA activity against a latent target in the 3'UTR of a therapeutic gene could inhibit protein synthesis by removing the poly A tail resulting in immediate degradation of the mRNA. Databases, such as the Wellcome Trust Sanger Institute siRNA database (<http://microrna.sanger.ac.uk/>), are continually being updated for microRNA target sequences as they are identified. Scanning the sequence of the 3'UTR using these microRNA target sequence databases will avoid inactivation of the transcript by endogenously expressed microRNAs.

2.1 Plasmid DNA Manufacture

The therapeutic gene is ligated into the plasmid backbone and standard microbiological protocols are used to identify a bacterial clone that contains the plasmid DNA. The bacterial clones are also selected for the highest specific activity with regard to plasmid DNA/bacterium. Master cell banks are created using this clone. The bacteria are fermented at lab scale in a shaker flask or can be fermented using a fermenter. The bacteria are pelleted by centrifugation and resuspended in resuspension buffer. The bacteria are lysed opened by alkaline lysis, neutralized and then centrifuged. The supernatant is extracted with phenol/chloroform followed by ethanol precipitation of the pDNA. The precipitant is resuspended in buffer and double banded using CsCl equilibrium centrifugation with a vertical rotor. The ethidium bromide is extracted with buffer saturated butanol followed by dialysis of the DNA against buffer. There are commercially available kits

to purify pDNA from bacteria. However, the quality of the pDNA can vary. The method outlined above yields highly purified plasmid DNA with regard to elimination of bacterial protein, RNA and endotoxin. This is especially important when formulating the pDNA with polymers and cationic lipids. Low-speed and high-speed centrifugation are not suitable for gram scale pharmaceutical manufacture. Substitution of filters for low speed centrifugation, and replacement of high speed CsCl density gradient centrifugation with anion exchange chromatography combined with hydrophobic interaction chromatography (HIC) makes this process amenable to pharmaceutical scale pDNA manufacture.^{17,18} Depending upon the bacterial strain and the plasmid DNA backbone, a single 500 L fermentation run can yield 10 to 20 grams of plasmid DNA.

3. Plasmid DNA Gene Transfer Methods

3.1 *Plasmid DNA or “Naked DNA” as a Gene Delivery System*

As stated in the introduction, plasmid DNA can be injected by itself and yield gene expression. This was first discovered by intramuscular injection⁵ expressing a reporter gene resulting in the marking of muscle cells at the site of injection. The expression levels are low compared to other forms of non-viral gene therapy. However, the DNA is not toxic and low levels of expression can be compensated for by increasing the dose and dosing frequency.

Two late stage clinical applications take advantage of this form of plasmid based gene delivery. Both treat peripheral vascular disease by expressing a therapeutic gene encoding for angiogenic growth factors, basic fibroblast growth factor (FGF-1)¹⁹ or hepatocyte growth factor (HGF),^{20,21} to induce new blood vessel growth in ischemic limbs. Both non-viral gene therapies have similar pDNA doses but different dosing schedules and different endpoints. For the FGF Phase 2 clinical trial, 125 patients, where revascularization surgery was not an option and had non-healing ulcers, were randomized and double blind placebo controlled for 2.5 ml injection of FGF-1 pDNA, [pDNA] = 0.2 mg/ml, on days 1, 15, 30 and 45. The primary end point was healing of at least one ulcer and secondary end

points were ankle brachial index, amputation and death. The gene therapy was well tolerated. There was no significant difference in ulcer healing between treatment and placebo group. However, there was a significant reduction in risk of amputations and there was a trend in the reduced risk of death.

For expression of HGF, multiple clinical trials were conducted showing that the therapy was well tolerated and no severe complications or adverse events were observed for any of the patients.^{20,21} A multicenter Phase 3 trial was conducted in Japan comprised of patients with arteriosclerosis obliterans with critical limb ischemia that could not undergo revascularization and did not respond to conventional drug therapies. Patients were randomized 2:1 therapy (55 patients) to placebo (26 patients) groups and received two intramuscular injections at the site of ischemia at 4 week intervals. Patients were followed for 8 weeks after the last administration. The primary endpoints were decreased rest pain or improvement in ischemic ulcer. The treatment group showed a 70% response in rest pain reduction and ulcer improvement whereas the placebo group showed a 30% response rate. At this time, these two therapeutic applications are in the latest stages of clinical development for plasmid DNA delivery.

Another active area of plasmid DNA therapy is the development of genetic vaccines. Purification of protein antigen can be problematic with regard to the yield and purity, often giving rise to antibody responses to the impurity rather than the intended protein antigen. This can be especially challenging for water insoluble proteins, such as integral membrane proteins. Also, combinations of different protein antigens can be prohibitive due to formulation incompatibility. Expression of the protein antigen following intramuscular, intradermal or subcutaneous administration of pDNA dramatically simplifies the immunization process and insures immune response to the native protein antigen. Genetic vaccines using pDNA alone are being developed for pandemic flu, HIV and Hepatitis C. In some cases, more than one gene is being expressed, such as HIV where six to 7 different genes are being expressed at the same time.²²⁻²⁴

One technique that has been used as a research tool is to systemically administer plasmid DNA in a large volume of vehicle, termed “hydrodynamic” gene delivery. This was initially discovered using a mouse animal model in which the pDNA is administered via the tail vein in a 1 ml injection

volume rapidly (10 seconds). The result is a high gene transfer efficiency to the liver. Hydrodynamic gene delivery while not practical for clinical development of systemic non-viral gene therapies has been used to screen for biological activity of secreted proteins into the blood, such as blood clotting factors. Therapeutic applications for Naked DNA are limited by the low level of gene expression. The level of gene expression can be improved by increasing the amount of DNA that gets into the cells, enters the nucleus and is expressed. The following section will describe technologies designed to increase gene transfer efficiencies.

3.1.1 *Electroporation of Naked DNA*

Applying energy following local administration of pDNA results in large increases in gene expression. The forms of energy can be electrical — used in “electroporation” or ultrasound — used in “sonoporation”. Electroporation consists of applying voltage to the site of administration in a series of electrical pulses lasting microseconds for each pulse. The hypothesis is that the electrical pulses induce a transient depolarization of the smooth muscle plasma membrane allowing the pDNA to enter the cell. The number of pulses, duration of pulses and the electrical strength of the pulse are of a particular magnitude to minimize permanent damage to the cell membrane and maximize gene transfer. Voltage was initially applied through calipers and the muscle was sandwiched between the plates. However the administration technology has been modified to use needles arranged in a hexagonal array with the electrical field alternating between opposing needles creating an electrical field around the injection site. Electroporation can increase gene expression of pDNA from one to several orders of magnitude compared to pDNA alone.²⁵ The fold increase is dependent on the transgene to be expressed, the administration route and the optimization of the electrical field pulse. A phase 1 clinical trial has been conducted involving the electroporation of an IL-12 expression plasmid into surface accessible tumors of melanoma patients.²⁶ There were 24 patients in the study. This was a dose ranging study with the most serious adverse event being pain at the site of injection. IL-12 and interferon gamma were observed at the tumor injection site. Also induction of infiltrating lymphocytes into the tumor was observed.

3.1.2 Sonoporation of Naked DNA

Substitution of high frequency sound waves in sonoporation, for electricity can achieve similar effects as electroporation. The hypothetical mechanism for facilitating gene transfer is similar to electroporation, in that the sound waves produce a very short lived transient breach in the integrity of the plasma membrane facilitating entry of pDNA into the cell. The key components are development of a probe that can effectively deliver the sound waves to the site of administration and application of pDNA effectively to maximize transfection efficiency.^{27–29} Gene transfer efficiency can be increased by applying ultrasound contrast reagent along with the ultrasound.^{30,31}

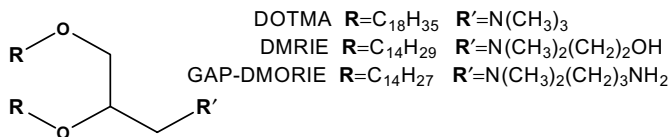
3.2 Plasmid DNA Formulations

3.2.1 Cationic Lipids

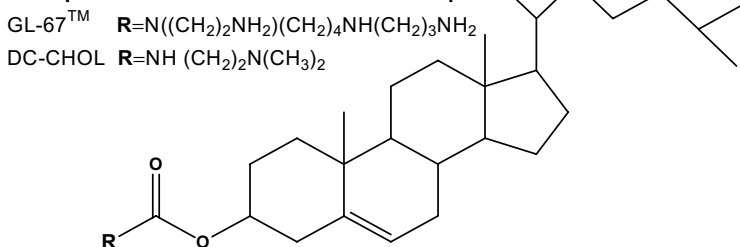
Cationic lipids are synthetic amphiphiles comprised of a hydrophobic domain (R), a linker and a hydrophilic domain (R'). For the cholesterol based cationic lipids, the hydrophobic domain is cholesterol and the cationic head group is denoted by R. Chemical structures of cationic lipids used in non-viral gene therapy clinical trials used are shown in Fig. 1.2. The lipid anchor is linked to the head group by either an ether linkage or a carbamate linkage, as shown for DC-CHOL and GL-67.

Cationic lipids bind to pDNA by electrostatic interactions between the cationic lipid moiety and the phosphate pDNA backbone or through hydrogen bonding between the amines and hydroxyl groups of the cationic lipids and the pDNA. The cationic lipids shown in Fig. 1.2 require a helper lipid, DOPE or cholesterol for the diacyl cationic lipids, and DOPE for the cholesterol based cationic lipids, to transfect cells. Cationic liposomes are prepared by first mixing the lipids in an organic solvent; the solvent is removed by evaporation producing a lipid film and the lipid film is hydrated with a buffer to form liposomes. The liposome size can be reduced by extrusion through a filter with fixed pore size, or energy can be applied to the suspension by strong bath or probe sonication to reduce the diameter of the liposomes.

Examples of Acyl Chain Based Cationic Lipids



Examples of Cholesterol Based Cationic Lipid



Helper Lipids-Phosphatidylethanolamine (DOPE) and Cholesterol

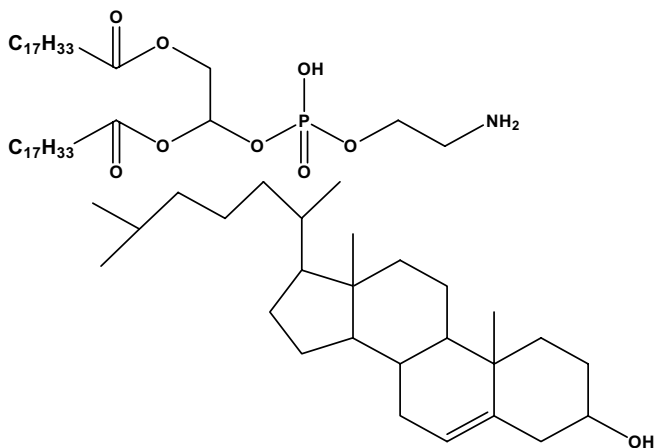


Fig. 1.2 Chemical structures of cationic lipids and helper lipids used in non-viral gene therapy clinical trials.

3.2.1.1 *In vitro* transfection

For *in vitro* transfections, the pDNA is usually in 10 mM Tris, 1 mM EDTA and the commercially available liposomes are in water. Complexes are formed at 10–50 ug pDNA per ml and diluted to 1 to 5 ug/ml with serum

free media and added to cells also in serum free media. After 4 hrs, the transfection complexes are aspirated off and replaced with serum containing media. The amount of cationic lipid to pDNA phosphate is titrated over a range of 2/1 to 8/1. The cationic lipids can be toxic to certain cells so there a balanced optimal ratio is required to achieve maximal gene expression with minimal toxicity. The biological activity of the transfection complexes has a short half life (hours) and maximal gene expression is achieved within 0.5 hr to 1 hr after formation.

3.2.1.2 Systemic *in vivo* gene transfer

Formulation of cationic lipids with pDNA for *in vivo* gene transfer is more complicated. First, there is very little correlation between optimal parameters, such as cationic lipid to helper lipid, ratio of cationic lipid to pDNA, liposome size or vehicle composition, developed for *in vitro* gene expression and *in vivo* gene expression. Secondly, the resulting transfection complexes are dependent on the type of cationic lipid used and how the complexes are formed. Binding of endogenous polyamines, such as spermine and spermidine, to plasmid DNA transforms supercoiled pDNA from a random coil into a toroid. Several approaches have been taken in developing cationic lipids for systemic gene transfer.

The first is to use cationic moieties known to bind to pDNA as the cationic head group. The resulting lipids are formulated with a helper lipid, such as DOPE or cholesterol, to form liposomes. The mole ratio of cationic lipid to helper lipid can vary from 100/0 to 10/90; most commonly it is set at 50:50. The mole ratio of cationic lipid to DNA phosphate can vary from 2/1 to 6/1 depending on the affinity of the cationic lipid in the context of a liposome or micelle to pDNA yielding a positively charged transfection complex. Non-reducing carbohydrates, such as sucrose, can be substituted for salt, such as NaCl, to maintain isogenicity.

An alternative approach is the synthesis of cationic lipid libraries, complex the cationic amphiphiles to the plasmid DNA and then test for gene expression.³² Assays can use expression of reporter genes such as green fluorescent protein (GFP) or β -galactosidase (Lac-Z), or a secreted protein, such as human placental secreted alkaline phosphatase. It is impractical to screen the library other than by *in vitro* tissue culture due to the number of animals needed and the sample processing. Formulation

of active cationic lipid candidates can then be optimized for *in vivo* gene transfer using the same reporter genes or a more popular *in vivo* screen is to detect expression by expression of a bioluminescent active reporter gene, such as firefly luciferase. This technique is excellent for liver, lung, spleen and heart imaging but poor for brain and muscle due to the limited biodistribution of the luciferase substrate following systemic administration.

The ideal particle size should range between 50 nm to 200 nm. Particles with diameters greater than 200 nm result in the reduction of the endocytotic index, except for monocyte derived cells such as macrophage, Kupffer cells or dendritic cells, which are specialized phagocytic cells. Once inside the endocytotic vesicle, the transfection complexes are disassembled and the pDNA must escape the internal vesicle and migrate to the nucleus for transcription of the transgene.

Modifications to the lipid composition have been made to increase systemic gene transfer efficiency. The circulation half life of the transfection complexes can be increased by covering the surface of the transfection complex with high molecular weight polyethylene glycol (2kdalt–10kdalt) inhibiting opsonization of the transfection complexes, thereby reducing non-specific uptake by the reticuloendothelium system (RES). Internalization of the transfection complex can be restricted to a specific cell type by derivatizing ligands to the surface of the transfection complex that bind to a receptor expressed on a specific cell type.^{33–35} Intracellular release of the pDNA from either the endosome or lysosome can be achieved by changing the cationic lipid composition to one that is susceptible to fusion at acidic pH.³⁶ Lastly, nuclear uptake of the pDNA can be increased by adding nuclear localization sequences in the form of DNA sequences that bind proteins manufactured in the cytoplasm that contain nuclear localization sequences allowing the pDNA to “hitch a ride” into the nucleus.³⁷ There are currently no clinical trials using systemically administered cationic lipid/plasmid DNA transfection complexes.

3.2.1.3 Local administration of cationic lipid/pDNA transfection complexes

Local administration of cationic lipid/pDNA transfection complexes refers to intratumoral administration and intramuscular administration. From a

safety perspective, the bulk of gene delivery and gene expression is limited to the site of administration. Therapeutic applications of intratumoral administration have focused on expression of molecules, such as cytokines³⁸ and self antigens³⁹ for the purpose of priming the immune system to kill not only the transfected cells but in so doing, prime the immune system to kill non-transfected tumors, thus creating a systemic therapy from a local administration.

With regard to the expression of self antigens, the first non-viral clinical trial was conducted by Dr. G. Nabel in collaboration with Dr. L. Huang. Intratumoral administration of cationic lipid formulated HLA B-7 pDNA was shown to be safe and tumor regression was observed. Subsequent clinical trials modified the cationic lipid formulation from the use of DC-Chol/DOPE to DMRIE/DOPE, increased the pDNA dose and tested different dosing schedules. A phase 2 dose ranging study identified a 2 mg per intratumoral injection as an effective antitumor dose and administration once a week for 6 weeks in a single tumor to be the dosing cycle. The clinical results from the phase 2 dose escalation study were used to define the patient entry criteria for a Phase 3 clinical study. The Phase 3 clinical study compares the cationic lipid/pDNA non-viral gene therapy (Allovectin-7) to Dacarbazine or Temozolomide. This therapy is currently in Phase 3 clinical trial.⁴⁰ Results from this trial should be available in 2012.

Expression of foreign antigens by administration of pDNA results in an immune response to the expressed protein creating the potential for development of genetic vaccines. However, mg quantities of pDNA injected multiple times have been required to sustain an immune response. Certain cationic lipids when formulated with pDNA not only increased transfection efficiency but were also immunostimulatory. A cationic liposome formulation composed of GAP-DMROIE and diphytanolylphosphatidylethanolamine (DPyPE) was shown to be immunostimulatory for several expressed antigens in rodent and non-human primates.^{41,42} Multiple demonstrations of proof of concept advanced this formulation into a phase 1 human clinical trial for a pandemic influenza vaccine. The phase 1 trial evaluated tolerability and immunogenicity. These parameters were tested for this clinical trial included a comparison of a needle free device, Biojector 2000, vs. needle; a single plasmid expressing the H5 hemmagglutinin viral envelope

protein vs. the H5 plasmid plus two additional plasmids expressing viral proteins whose amino acid sequences are highly conserved amongst multiple pandemic influenza virus clades. One hundred and three patients were enrolled and 86 were evaluable for immunogenicity. A 65% response rate was observed for neutralizing antibody equivalent to or exceeding protecting titers of 1/40. This was a durable response being observed out to 182 days (unpublished results).

3.3 Polymer

Polymer based plasmid DNA gene therapy can be divided into two categories: cationic polymer and neutral polymers. Examples of cationic polymers are polylysine, polyethyleneimine, or panamdendrimers. Examples of neutral polymers are Polylactide glycolic anhydride (PLGA), poloxamer and polyvinylpyrrolidone (PVP).

3.3.1 Cationic Polymers

Cationic polymers behave similarly to cationic lipids in that they contain a cationic moiety which either electrostatically bonds with the phosphate backbone of the pDNA or hydrogen bonds to the pDNA. The first cationic polymers were naturally derived from poly amino acids such as poly-L-ornithine and poly-L-lysine. Utility was derived from the virology field that first used poly-L-ornithine to facilitate viral infection of cells.¹¹ It was later discovered that transfection of cells with viral RNA complexed to poly-L-ornithine produced infectious virus. Lessons learned from the virology field were applied to pDNA delivery with the substitution of poly-L-ornithine for poly-L-lysine. The polymer provided a versatile backbone for chemical modification of ligands to target the complexes to cells by additional peptide sequences or chemical modification of small molecular weight ligands, such as folate or carbohydrates.⁴³ One application that has evolved from the bench to the clinic is the use of poly-L-lysine/pDNA complexes for the treatment of cystic fibrosis.⁴⁴ Several key developments in transitioning from the research lab to the clinic were the development of an aerosolized transfection complex, reduction in the CpG content of the plasmid to avoid secondary cytokine activation,¹⁵ use of a polyethylene glycol-substituted 30-mer lysine peptides.⁴⁵

Improvements in polycationic amino acids polymers have included other positively charged amino acids such as arginines and histidines.⁴⁶ The latter not only serves as an alternate DNA binding moiety but also has a pK that can impact the transfection complex when the pH environment acidifies, as is found in the lysosome and endosome, facilitating decomplexation and potentially lysing open the endocytic vacuole.⁴⁷

Polyethyleneimine (PEI) is a completely synthetic polymer that has similar intracellular release properties as the poly-L-histidine, first introduced into the field of nonviral gene therapy by JP Behr.⁴⁸ The imine moiety of the polymer provides electrostatic bonding and hydrogen bonding to pDNA and also serves as a protonatable buffer that prevents acidification of the endosome/lysosome resulting in intracellular plasmid DNA release. This polymer has been modified with polyethylene glycol and targeting ligands. The polymer/pDNA complex is currently in preclinical development.

3.3.2 Neutral Polymer

Neutral polymers are defined as polymers carrying no net charge. Examples are poloxamers, polyvinylpyrrolidone (PVP), polyanhydride (PLGA) polymers. The first two form simple mixtures of polymer and pDNA whereas the PLGA polymer forms nanoparticles in which the pDNA is packaged. Both PVP^{49,50} and poloxamer have been used in clinical trials for local administration of pDNA. The applications have been in cancer, cardiovascular disease and vaccine development against infectious disease. For cancer applications, plasmid DNA encoding cytokines, such as IL-2, IL-12, or interferon- α in combination with IL-12,^{49,50,51} is formulated with PVP and injected intratumorally resulting in expression and secretion of the cytokines from the transfected cells. The chemoattracting cytokine gradient causes immune cells to migrate to the tumor and kill the tumor cells. The therapeutic hypothesis is similar to that of the cationic lipid based immunotherapy, in that T cell mediates tumor cell killing programs and activates tumor surveillance of the immune system, thus preventing new tumor formation.

Poloxamers are block copolymers of polyethylene oxide (a) and polypropylene oxide (b) that are linked by ether linkages shown in Fig. 1.3 with molecular weights ranging from 1,000 daltons to 12,000 daltons. In

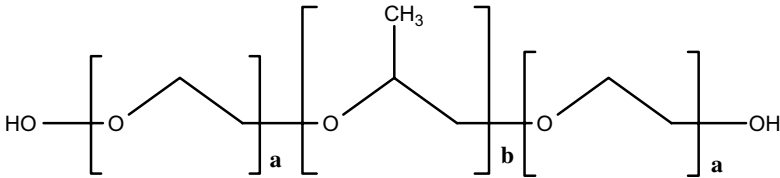


Fig. 1.3 Poloxamer chemical structure. α -Hydro- ω -hydroxypoly (oxyethylene)_a poly(oxypropylene)_b poly(oxyethylene)_a block copolymer in which a values can range from 12 to 101 and b values from 20 to 56.

aqueous media, the polymers assemble into micelles. These micelles can be induced to aggregate to form hydrogels by increasing the temperature. The ratio of ethyleneoxide to propyleneoxide determines the gelling temperature range (cloud point). The poloxamer is formulated with plasmid DNA in saline and injected into muscle resulting in gene expression from muscle cells. Clinical applications take advantage of the local expression of therapeutic genes, specifically cardiovascular and genetic vaccines.

The human developmentally regulated endothelium locus (Del-1) gene was identified by T. Quertermous to be a potential angiogenic factor.⁵² In pursuit of restoring blood flow to claudified muscle, formulation of a Del-1 expressing pDNA formulated with poloxamer 188 was shown to yield muscular gene expression that was sufficient to induce angiogenesis and be well tolerated in mice and rabbits. A Phase II multicenter clinical trial conducted with the Del-1 pDNA/poloxamer 188 formulation in 105 patients with peripheral arterial disease was shown to be safe and well tolerated.⁵³ The execution of the trial showed that the formulation could be scaled up to cGMP pharmaceutical manufacture, have an acceptable shelf life and meet FDA approval. Although there was no statistical significance in outcome compared to poloxamer 188 alone, these results showed that this formulation was suitable for clinical applications and identified areas for improvement to achieve therapeutic efficacy.

A modified poloxamer formulation was used for the development of a therapeutic vaccine for prevention of cytomegalovirus induced pneumonia in hematopoietic stem cell (HSC) transplant patients.⁵⁴ Poloxamer was combined with benzylalkonium chloride (BAK) to create mixed micelles that were positively charged. A series of freeze and thaw cycles were used to reduce particle size with 500 nm diameters. A mixture of 2 pDNAs encoding

the surface glycoprotein B (gB) and the internal matrix protein (pp65) was added to the poloxamer/BAK and stored frozen.^{55,56} A phase I clinical trial was conducted involving 22 CMV seronegative and 22 seropositive healthy subjects. The vaccine was well tolerated with no serious adverse events. Immunogenicity determined by *ex vivo* interferon (IFN)-gamma enzyme-linked immunospot assay yielded 45.5% of CMV seronegative subjects and 25% of CMV seropositive subjects in subjects receiving the full vaccine series. The safety and immunogenicity results supported further evaluation in a phase 2 clinical trial.

Conclusions

Local administration of plasmid DNA based gene therapies seem to be the most promising and have the least potential for any side effects. pDNA alone has advanced into phase 3 clinical trials for cardiovascular applications. Success of this clinical application is based upon the potent angiogenic stimulation produced by a very low level of expressed protein that remains localized at the site of administration. Improvements in gene transfer efficiency from the result of pDNA formulation with cationic liposomes and polymers are also combined with selection of expressed genes that produce a potent secondary effect, such as the case of immunostimulation against tumor antigens and pathogenic antigens. Research focused on increasing the transfection activity of plasmid DNA based gene delivery systems is very active. The hope is to combine the development of cell specific targeted gene delivery along with tissue specific promoters that further restrict gene expression to a specific cell type with the application for systemic administration.

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