Structure and Design of Polycationic Carriers For Gene Delivery

Geoffrey A. Pietersz[1], Choon-Kit Tang[2] and Vasso Apostolopoulos[2,*]

[1]*BioOrganic & Medicinal Chemistry Laboratory,* [2]*Immunology & Vaccine Laboratory, Burnet Institute at Austin, Studley Rd, Heidelberg, VIC 3084, Australia*

Abstract: The development of safe and effective gene delivery methods is a major challenge to enable gene therapy or DNA vaccines to become a reality. Currently there are two major approaches for delivery of genetic material, viral and non-viral. The majority of on-going clinical trials in gene therapy or DNA vaccines use retroviruses and adenoviruses for delivering genetic materials. Viral delivery systems are far more effective than non-viral delivery however there are concerns regarding toxicity, immunogenicity and possible integration of viral genetic material into the human genome. Given the negative charge of the phosphate backbone of DNA, polycationic molecules have been the major focus as carriers of DNA. There are several physiological barriers to overcome for effective systemic delivery of DNA. The ideal vector must be stable in the systemic circulation, escape the reticuloendothelial system, able to extravasate tissues, enter the target cell, escape lysosomal degradation and transport DNA to the nucleus to be transcribed. With increasing understanding of the physicochemical properties essential to overcome the various barriers, it is possible to apply rational design to the cationic carriers. A number of poly-amino acids, cationic block co-polymers, dendrimers and cyclodextrins have been rationally designed to optimize gene delivery. This review will discuss approaches that have been used to design various synthetic polycations with enhanced DNA condensing ability, serum stability and endosomolytic capability for efficient gene transfer *in vitro* and *in vivo*.

Key Words: Polyethyleneimine, poly-L-lysine, DNA, vaccine, gene delivery.

INTRODUCTION

The Human Genome Project, completed in 2003, revealed much into our genetic makeup. It yielded a blueprint that enables us to determine genes and types of mutations responsible for some human diseases. This new knowledge may allow us to formulate treatments for some diseases at the genetic level, by replacement of the dysfunctional genes in cells. However, the success of this form of treatment is impeded by lack of efficient and reliable methods of gene delivery that maintains stable and long-term expression of the foreign genetic material in host cells. Over the years, many have studied in depth the process of successful transfection and devised methods to overcome each limiting step. Viruses, which are naturally evolved to have innate transduction capabilities, were logically chosen to be tested for their potential use as gene delivery vehicles. Although effective, rising safety concerns of introducing viruses into humans have deviated many from electing viral mediated transfection as the gold standard in gene delivery. This has lead to the investigation of non-viral methods that could match transfection efficiency seen in viral delivery methods [1, 2]. A series of non-viral delivery approaches have arisen throughout the years, including the use of naked DNA, nanoparticles, liposomes, designer peptides and polymers [1, 2]. In this review, hurdles to successful non-viral gene delivery are first identified and chemical methods to overcome these obstacles are discussed.

*Address correspondence to this author at the Burnet Institute at Austin, Immunology and Vaccine Laboratory, Kronheimer Building, Studley Road, Heidelberg, VIC 3084 Australia; Tel./Fax: +613-92870666/00;
E-mail: vasso@burnet.edu.au

IDENTIFYING BARRIERS AND METHODS TO OVERCOME THESE, FOR SUCCESSFUL NON-VIRAL MEDIATED GENE TRANSFER

Barriers to successful non-viral gene transfer include a) surviving the extracellular environment and entering the target cell type, b) cytosolic delivery from the endosomes, c) traversing to the nucleus from the cytoplasm and finally d) dissociation of the DNA from carriers for transcription.

Extracellular Environment

The foreign DNA has to reach the targeted organ from the site of injection and be taken up by specific cells in order for the desired response to occur. The extracellular environment presents the first major hurdle the foreign DNA would have to overcome. The non-viral delivery vector will interact with body fluids and tissue matrix, where there are nucleases capable of digesting un-protected DNA into fragments, incapacitating its ability to express the encoded protein. In many non-viral methods of gene delivery, there is the need for the complex to be positively charged. These charges interact with the negatively charged red blood cells and aggregate which cause it to be accumulated in the first organ it encounters, which may not necessarily be the target organ [3]. There is also the possibility that the aggregates be trapped in capillaries where it would be cleared by passing phagocytes. The positively charged complex is also known to interact with albumin, fibrinogen and complement C3 in the bloodstream with negative effects on transfection efficiency [4]. These unwanted non-specific reactions and instability *in vivo* could be rectified by conjugating polyplexes to non ionic water soluble polymers, which act as brush like bristles extending from the particle. Polycation coupled DNA vaccine could be endocytozed by cells non-specifically. This is usually medi-

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ated by the interaction between negatively charged proteoglycans natively expressed on most cell surfaces and the net positive charged polycation/DNA complex [5]. Thus, the extracellular environment is an initial barrier to DNA immunization, which results in the therapeutic genetic material inefficiently delivered to the desired organ and cell type.

DNA has to be protected from nucleases present in the circulation or extracellular matrix between the site of injection and the target cell. Viruses achieve this by packaging its vital genetic information within a viral capsid, capable of resisting damage from many degrading agents. One approach of the DNA overcoming degradative enzymes is to condense it into a compact form such that sites vulnerable to cleavages could be protected from enzymes. This process, is triggered by the perturbation of electrostatic interactions within the α-helix structure of the DNA. This condensation process could be initiated by adding a polycation such as polyethylenimine (PEI) or poly-L-lysine (PLL), Fig. (**1A – C**), Fig. (**2A, B**), to the DNA. The level of protection would depend on how the molar charge ratio between polycation and DNA (N/P ratio). In poly-L-lysine, this protection was achieved at N/P ratio above 1 [6]. Cationic lipids also fuse with and condense DNA for gene delivery with good transfection efficiency [7]. DNA condensation in gene delivery serves two purposes, (i) to provide DNA protection, and, (ii) compact it into a nm size which would allow access through small openings [8]. It has been suggested that polyplexes with diameters smaller than 100 nm are of optimal size for transfection as it corresponds to the diameter of the coated pit in receptor mediated endocytosis. DNA complexes formed with PEI, was found to have a size of between 40 to 80 nm [4, 9], while polyplexes formed with PLL have a diameter of 25 to 50 nm [10], which is one of the characteristics that may explain their transfection capabilities. For PEI complexes, the size formed depends on many factors, including the form of PEI (branch or linear), Fig. (**1A, B**), Fig (**2A, B**), the amount of salt present, concentration of DNA and the rate and order of mixing the two components of the complex [11]. Interestingly, the size of complexes formed at lower concentrations of NaCl are similar regardless of the form of PEI used (branched or linear), however, it does matter when higher ionic solution is used [12].

Another commonly regarded pre-requisite of non-viral gene delivery is for the complex to have an overall positive charge. This was thought to improve transfection efficiency because it facilitates the interaction between the DNA complex and the overall negatively charged cell surface. This could be achieved by having complexes of a higher N/P ratio, which increases the amount of positively charged amine groups over the negatively charges of the phosphate groups of DNA [13].

Increasing the N/P ratio in polyplexes increases the level of DNA condensation, decreases the size and allows for a positively charged particle. Unfortunately, strongly positively charged polyplexes cause cell death and activate complement [14]. Furthermore, positively charged particles bind to and aggregate with proteins present in the circulation which would in turn be taken up by passing phagocytic cells [11]. PEI-DNA complexes could be seen aggregating in a matter of hours when in physiological saline conditions and transfection efficiency of the polyplex could be lost within a day if the sample is kept at room temperature [15].

Including amphiphilic molecules such as polyethylene glycol (PEG) into the polyplex decreases aggregate formation. Such molecules act as brush-like extensions from the polyplex core with hydrophilic heads containing oxyethylene groups exposed to the exterior, sterically prevent the com-

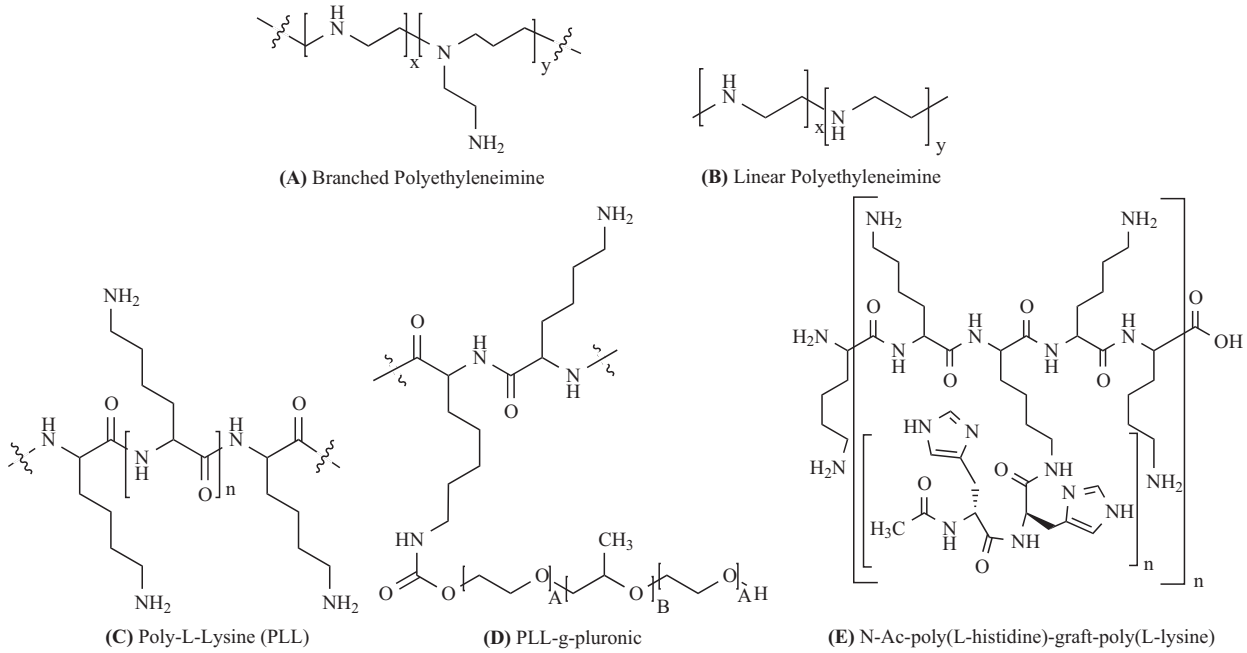


Fig. (1). Branched (**A**) and linear (**B**) polyethyleneimine and novel PLL-based polycations (**C-E**).

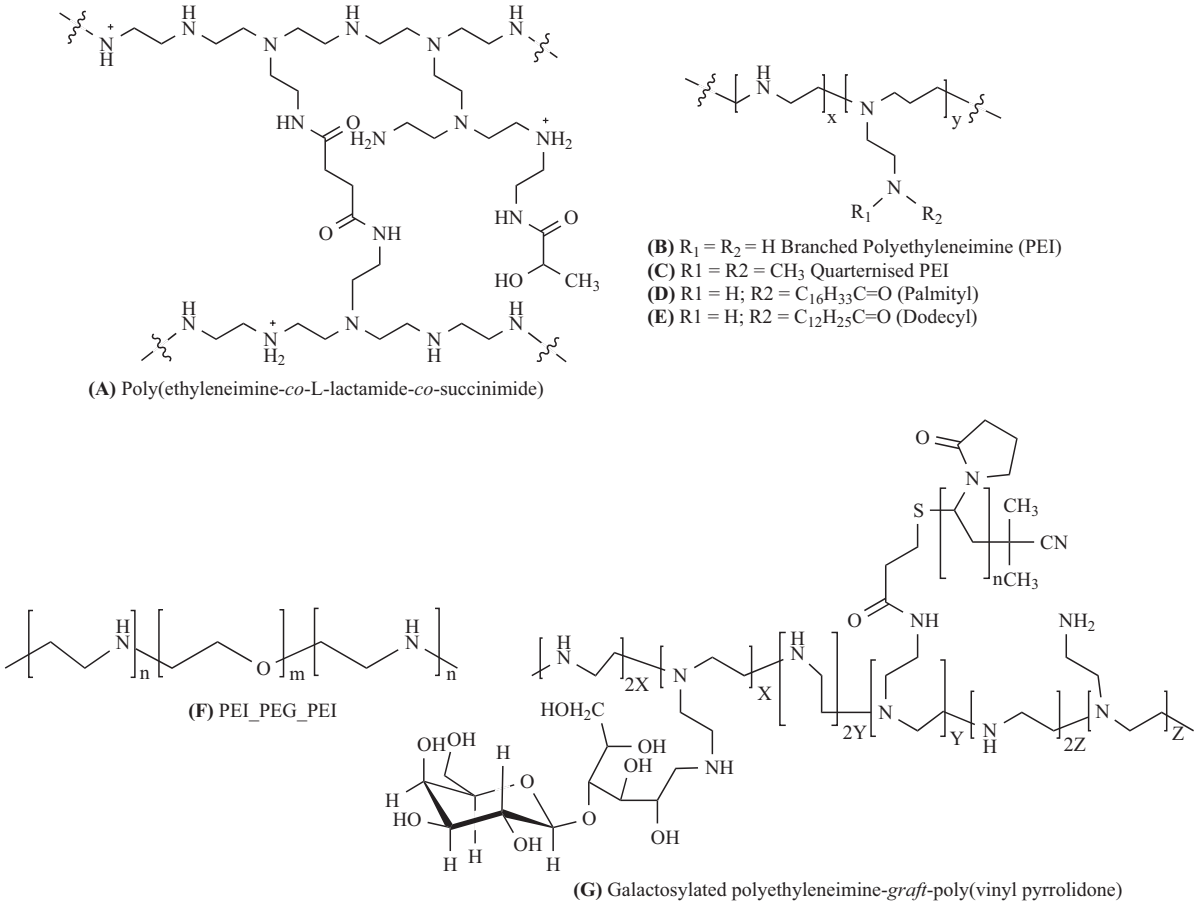


Fig. (2). Novel polyethyleneimine-based polycations (**A-G**).

plexes from coming together. These hydrophilic moieties also prevent non-specific uptake by cells in receptor mediated gene transfer. An emerging shielding reagent, Poly(vinyl pyrrolidone) (PVP), which is an amphipathic synthetic molecule with similar properties as PEG was investigated for its use in gene delivery [16]. PVP increased the specificity of galactose-PEI mediated gene transfer into hepatocytes. Unfortunately, in that study, it was not compared to Galactose-PEG-PEI transfections. PVP is better than PEG in several aspects such as longer circulation time in the bloodstream and was shown to provide better anti-tumor efficacy when conjugated to TNF-α than its PEG/TNF-α counterpart.

Endosomal Release

Polyplexes with cell targeting ligands enter cells *via* receptor mediated endocytosis while untargeted complexes enter *via* pinocytosis. Regardless of the route of entry, polyplexes would first be brought into the early endosomes, which would either fuse with other endocytic vesicles, more commonly late endosomes that exocytoze internalized products. The process of fusion between early and late endosomes is brought about by the sudden lowering pH to 5 within the micro environment of the former [17]. This pH drop is the result of active transport of protons from the cytosol by the ATPase proton pump present on the membrane of endosomes. Late endosomes would ultimately fuse with lysosomes. Once in the lysosome, polyplexes would be immersed in an environment of acidity and degrading enzymes that would degrade the DNA. Hence, for a successful gene transfer to take place, polyplexes have to escape from the endosomes before they fuse with lysosomes.

Several strategies have been used to facilitate endosomal escape such as properties of the polycation or by incorporation of other substances. Of the simple polycationic carriers PEI has an advantage over PLL in gene delivery because of its endosomolytic property. Unlike PLL which contains only primary amines in its structure, branched PEI has primary, secondary and tertiary amines, Fig. (**1A**). These secondary and tertiary amines present in PEI are protonated in the acidic vesicles and acts as a buffering system, hence the term "proton sponge" polymer. The endosomal pH has to be lowered by active transport of protons by ATPase to about pH 5 before fusing with vesicles from the Golgi to form lysosomes where its contents could be degraded by lysozymes. The presence of PEI in the endosome prevents such acidification which would result in the intake of even more protons to achieve the desired pH. Together with the influx of counterions at the same time, the increased ion concentration within the endosome would cause osmotic swelling and ultimately rupture of the organelle. PEI stripped of its "proton sponge"

property by N-quaternization of the amines reduced transfection efficiency by two orders of magnitudes [18].

To increase the endosomolytic property of PEI further, Melittin, a major component of bee venom known to lyse cell membranes was conjugated to branched 25kDa PEI, which showed augmented levels of endosomal release and nuclear transport [19]. Inactivated viruses (replication defective) have also been used in conjunction with non-viral gene delivery systems to mediate endosomal disruption. Adenovirus has been shown to be effective in such function. It could enhance gene transfer efficiencies by complexation to polyplexes [20] or be added directly to the transfection medium [21]. Although effective, the use of viruses still invokes fears of eliciting potent immune responses in humans and defeats the purpose of using non-viral methodologies. To overcome this problem, viral components with endosomal disrupting properties at the molecular level were identified and used. Fusogenic peptides is an active component in viruses that mediates endosomal release and have since been used as endosomolytic agents in gene delivery, usually used in conjunction with liposomes. Such peptides could be found naturally occurring in many organisms such as viruses [22, 23] and protozoans [24-26]. They are pH sensitive peptides that undergo conformational changes in the presence of low pH (~5) from random coils to amphipathic helices, which causes insertion into the lipid bilayer resulting in the release of its encapsulated contents [27-29]. Among the better characterized fusogenic peptides, is the NH_2-terminal domain of influenza virus hemagglutinin subunit HA-2 [30]. It has been shown that it is able to augment transferrin/poly-l-lysine mediated gene delivery 100 fold in HeLa cells using *Photinus pyralis* luciferase as a reporter gene [31].

Many synthetic forms of fusogenic peptides were also designed and tested, such as GALA, a 30 residue peptide was the first peptide designed synthetically [32] for liposomal delivery that mimics viral fusion with membranes [33]. Most of these compounds are synthesized to mimic peptides present on the viral coat such as the influenza virus which mediates endosomal release by becoming fusogenic with the endosomes resulting from protonation at acidic pH [34]. These compounds are usually hydrophilic at physiological pH and hydrophobic when in an acidic environment, which is membrane disruptive.

Traversing to the Nucleus

Despite its infinitesimal distance compared to migration of DNA from site of injection to the desired cell, traversing through the cytosol and into the peri-nuclear region is difficult without any active transport. It has been demonstrated that nucleotides larger than 2000 base pairs are too large to remain mobile in the cytosol filled with organelles, cellular products and a network of microtubules and thus, eliminates the possibility of DNA complexes making its way to the nucleus by random movements [35]. Furthermore, due to the action of intracellular nucleases, single and double stranded forms of DNA were shown to have a half-life of 50 to 90 minutes when microinjected into COS and HeLa cells [36].

It has been popularly regarded that DNA complexes arrive at the peri-nuclear region by slow diffusion. However, in a study using fluorescence recovery after photobleaching (FRAP) method, PEI-DNA complexes are shown to use the microtubule motor functions for fast intracellular trafficking to the peri-nuclear region [37]. Together with the established fact that PEI binds strongly to DNA even after entering the nucleus [38], this could be another reason for efficient gene delivery by PEI. Mixed reports were seen as to whether nuclear localization sequences (NLS) assist plasmid DNA to the perinuclear region. It was stated in one study which used NLS containing NFkappaB p50, that NLS was able to facilitate not only nuclear entry but also its transport towards the nucleus *via* the microtubules in a dynein-dependent manner [39]. At the same time however, it was demonstrated that adding NLS sequences to DNA had no effect in intra-cellular transport. The authors tried increasing the number of NLS insertions, time and spacer length between the DNA and NLS without further improvement [40]. Increasing spacer length could reduce charge interactions between the anionic DNA and cationic NLS, which could interfere with the docking of the latter with transport proteins. Another compound found to be effective in mediating nuclear delivery of foreign DNA is the adenovirus hexon protein [41]. When conjugated to PEI, hexon protein was found to have better nuclear transfer efficiency than NLS. Like NLS, the hexon protein delivers through the nuclear pore complex but using a different mechanism.

Entry into Nucleus and Transcription

For the delivered DNA to be functional, it has to be transported into the nucleus where it could be transcribed into mRNA and ultimately be translated into protein. This entry from the perinuclear region into the nucleus is governed by the nuclear membrane. Transport of molecules across this barrier is mediated through the nuclear pore complex. This nuclear envelope spanning complex allows passive passage of small molecules but severely limiting the traffic of larger molecules of more than 50 kD across the membrane [42, 43]. Small oligonucleotides of 18 to 28 base pairs are found to naturally accumulate in the nucleus post transfection [44] and this right of passage could be extended to nucleotides of up to 310 base pairs [45]. The transport of the larger molecules however requires a more complex active process which involves tagging of a NLS [46, 47]. Early studies demonstrating that NLS coupled with non-nuclear molecules or synthetic compounds could retain their normal function prompted many researchers to include NLS as a component in their gene delivery vector [48]. The process of nuclear localization of genetic material bound to polyplexes without NLS was commonly thought to happen only during cell division when the nuclear membrane is temporarily disintegrated and could include any free polyplex in the vicinity into the nucleus when a new membrane is formed.

Dissociation of Carrier/DNA Complex

To determine whether carriers need to be dissociated from the DNA before it can be transcribed and translated into protein gene expression levels from cells with DNA plasmids micro-injected into the nucleus and into the cytoplasm were compared [49, 50]. The former method was consistently found to give rise to higher expression levels and therefore regarded that dissociation of carriers from DNA

plasmid is an essential process for its efficient transcription. It was further demonstrated in another experiment where PLL of different sizes were complexed to plasmid DNA and expression noted [51]. Plasmid complexed to PLL of smaller molecular weights showed higher transfection efficiency but shorter term expression compared to DNA complexed to larger PLL. It was thought that this was due to the ability of smaller PLL being able to dissociate from the plasmid faster than its larger counterpart, hence, the faster transfer of plasmid into the nucleus to be transcribed. These findings are consistent with the fact that dividing cells have considerably higher transfection efficiency as during cell division between prophase and pro-metaphase there is a period where the nuclear envelope is temporarily disintegrated [52] and which allows envelopment of the foreign DNA molecule into the mitotic nuclei.

METHODS TO CHARACTERIZE THE DNA/CARRIER COMPLEX

Extensive studies have been performed to determine characteristics of polyplexes that are required for efficient gene delivery. Attributes of polyplexes that affect gene delivery efficiency include degree of DNA/carrier complexation, conformation, size and charge.

Light scattering (or laser diffraction) techniques have been a popular method of monitoring particle mass, size, and charge. Time-resolved multi-angle laser light scattering (TR-MALLS) is one such sophisticated method of monitoring polyplex formation [53]. This method allows real-time analysis of particle formation through monitoring of the time evolution in supramolecular complex mass and geometric size by determining the DNA-carrier mass concentration ratio. This is achieved without chemical or physical interference thus minimizing artifacts and allows measurement in its true form. Measurements of particle size could also be achieved using photon correlation spectroscopy [54] and quasi-elastic light scattering, which determines the size of sub-micron particles by measuring the Brownian motion as a function of time. This method sensitive enough to measure particle sizes of down to 3 nm. Surface charge of a particle could be estimated by using a zetasizer to measure its zeta-potential, which defined as the charge that develops at the interface between a solid surface and its liquid medium. Ethidium bromide fluorescence quenching is the most common method used to monitor DNA condensation [55]. It is a simple method of analysis based on the property of ethidium bromide producing a strong fluorescence signal upon intercalating with DNA. Therefore, the degree of DNA condensation could be determined from the level of fluorescence relative to DNA alone. The degree of complexation between positively charged particles and negatively charged DNA could be monitored using gel retardation assay, which analyses the overall charge of DNA complexes using agarose gel electrophoresis. Fully complexed DNA would have lost its negative charges and therefore loses its ability to migrate to the anode and would appear as a larger size band than DNA alone. Microscopy techniques at the atomic level are also popular methods enlisted to visualize the actual particle structure instead of inferring from results of data from sedimentation and light scattering techniques [56]. Images collected from electron microscopy are able to provide enough resolution that allows differentiation of the various DNA conformation the particle has adopted. Atomic force microscopy is also used to study particle structure formation [57]. Unlike electron microscopy, atomic force microscopy provides the advantage of not requiring the specimen to undergo any special treatments that may interfere with the carrier/DNA interaction, hence a better method of analysis.

With these methods of analysis available, we could now better characterize the various attributes of polyplexes, which is of utmost importance for devising reproducible and reliable method of forming specific DNA carrier complexes fit for use in clinical applications.

NON-VIRAL DELIVERY VECTORS

Non-viral delivery methods incorporate naked DNA, cationic lipids, cationic polymers, polymeric vesicles or nanoparticles. As described above in order to improve delivery of DNA with the use of non-viral vectors various strategies have to be utilized to modify the cationic carriers to optimize DNA complexation, nanoparticle formation, serum stability and endosomal escape. In this section various chemical strategies used to modify the polycations to achieve efficient delivery is discussed using specific examples from the recent literature.

Cationic Polymers

PLL and PEI

PLL and PEI are two of the first polycations to be used in gene delivery. The properties and their efficacy in *in vitro* and *in vivo* gene delivery has been extensively reviewed [5, 58]. Tables **1** and **2** outlines some of the recent studies with these two polymers. PEI is the most widely used polycation for gene delivery. However, there is significant cytotoxicity which limits its application. Since the toxicity of the PEI is related to the presence of primary amino groups, PEI has been further modified with chloroethylamine to alter the ratio of primary:secondary:tertiary amine groups. The reduction of linear-to-branched ratio from 1.17 to 0.70 yielded polymers which compacted DNA to particles of 70-100 nm at low N/P ratios [59]. Under optimal conditions these complexes transfected COS-1 cells 6 times more efficiently than a commercial dendrimer preparation. PLL based dendritic molecules with a hexamethylenediamine of generation 6 with 128 amine groups compacted DNA into particles with mean diameter of 200-250 nm at N/P ratios of 2.0 to 8.0 [60]. The G6 complex at N/P ratio of 4.0 showed significantly reduced toxicity to Superfect™ and simlar tranfection efficiency into CHO, HeLa, HuH-7 and COS-7 cells.

Degradable Cationic Polymers

Stabilization of polyplexes is important for the transport of DNA to the target cells. However, once polyplexes reach the cytoplasm or nucleus they need to release the DNA to be transcribed. Several polymers have been designed to efficiently compact DNA and retain it's integrity in the endosome/lysosomal pH of 4.5 - 5 but degrade at pH 7.4. Such polymers are based on having a stable backbone but cationic side chains are separated by a carbonate ester. One such polymer, based on the monomer carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacrylamino)-ethyl ester

Table 1. Modification of PLL to Augment Different Aspects of Gene Delivery

Modification	Mechanism	Response	Ref.
PLL-g-Pluronic Fig. (**1D**)	Composed of polyethylene oxide and polypropylene oxide, pluronic is a surfactant used to augment transport of biologically active compounds into cells	Two-fold increase of transfection efficiency over conventional PLL transfection	[90]
PLL modified iron oxide nanoparticles	Iron oxide nanoparticles have the potential to accumulate in tumor cells and tumor-associated macrophages. PLL is used in conjunction for its DNA binding, condensation and protection properties.	Intravenous injection resulted in distributed in organs such as lung (34.9%), brain (10.7%) and kidney (6.9%)	[91]
Poly(lactic-co-glycolic acid) (PLGA)-grafted-PLL	Formation of a self-assembled micelle containing a hydrophobic core composed of PLGA and cationic PLL chain as a hydrophilic surface corona	*In vitro* transfection efficiency of the PLL-g-PLGA was 10 times higher than that of PLL and was less toxic	[92]
DNA/PEG-g-PLL + KALA	KALA is a fusogenic peptide with membrane disruptive properties	Improved transfection efficiencies	[93]
PLL-DNA + Chitosan microspheres	Chitosan, a natural cationic polysaccharide, that has high positive charges and low cytotoxicity. It is used here to achieve long-term release of pDNA	Chitosan encapsulated PLL-DNA complexes achieved better protection from DNase I treatment and more sustained drug release compared to PLL. It has better transfection efficiency.	[94]
N-Ac-poly(L-histidine)-graft-PLL. Fig. (**1E**)	Poly-l-histidine is used to induce membrane fusion at endosomal pH values in combination DNA polyplex forming properties of PLL	Improved transfection efficiency over PLL but still not as good as chloroquine aided PLL transfection	[95]

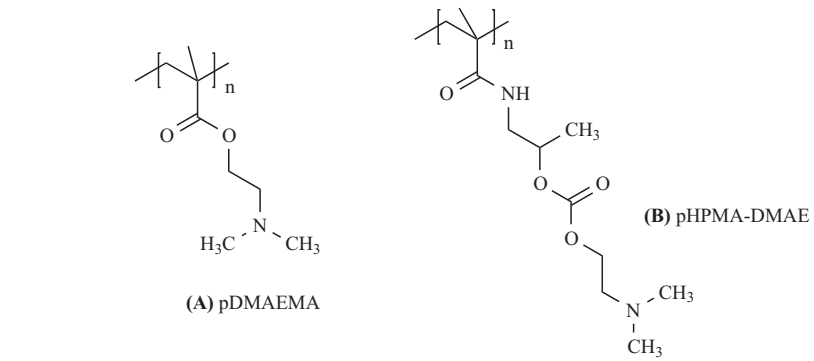
Table 2. Modification of PEI to Augment Different Aspects of Gene Delivery

Modification	Mechanism	Response	Ref.
Poly(ethylenimine-co-L-lactamide-co-succinamide). Fig. (**2A**)	Linkage of several low molecular weight PEI using an oligo(L-lactic acid-co-succinic acid) to form an soluble and easily degradable polymer	Have low toxicity as low MW PEI but with significant higher transfection efficiency	[96]
Covalent attachment of palmitic acid to bPEI 25kDa. Fig. (**2D**)	Creates an amphiphilic comb-polymer derivative of PEI	10 times less toxic compared to PEI but retain only 30% of the transfection efficiency. GFP expression in liver.	[97]
Dodecylation of primary amino groups of 2-kDa PEI. Fig. (**2E**)	Presence of long lipophilic substituents on PEI can increase the interaction of PEI/DNA complexes with cell membrane	Less toxic than parent PEI and transfects 400 times better	[98]
Linear Polyethylenimine-b-poly(ethylene glycol)-b-polyethylenimine Triblock Copolymers Fig. (**2F**)	Combination of low MW lPEI for high transfection efficiency, PEG for shielding and bPEI for high DNA condensation capability	3-fold higher luciferase reporter gene expression over linear PEI 25000/DNA complexes. Good colloidal stability. Serum does not inhibit transfection activity. Low cytotoxicity.	[99]
PEG-PEI-CHOL	Synergism of PEI/DNA condensation, PEG shielding and enhancing properties of cholesterol transfection	Injection of a murine IL-12 plasmid polyplexed with PPC into tumors in mice yield significant inhibition of tumor growth	[100]
Poly(L-lactic acid)-PEI and Poly(d,l-lactide-co-glycolide)-PEI	Combination of excellent drug delivery systems using nanoparticles and DNA condensation and endosomal releasing properties of PEI	Transfection efficiencies are 50% of PEI alone but showed reduced cytoxicty	[101]
Poly(lactide-co-glycolide)-PEI	A three-dimensional biodegradable scaffold, which encourages cell infiltration used to encapsulate PEI-DNA	Long-term (15 weeks) and high expression *in vivo* (55-60%)	[102]
Poly(vinyl pyrrolidone)-PEI. Fig. (**2G**)	A hydrophilic molecule much like PEG, dextran sulfate and serum albumin, which has been shown to reduce cytotoxicity and aggregation	Improved specificity to hepatocytes (HepG2) when coupled to galactose as a ligand and reduce cytotoxicity.	[16]

(Table 2. Contd....)

Modification	Mechanism	Response	Ref.
Full deacylation of polyethylenimine	N-acyl group present in bPEI and not lPEI as a result of different methods of synthesis, affects nucleic acid transfection. Removal of it should increase transfection efficiencies in bPEI	Deacylated bPEI 25kDa has 21 times and 10000 times enhanced gene delivery *in vitro* and *in vivo* respectively compared to native counterpart	[103]
Polyoxyethylene (100) stearate + PEI-DNA	A nonionic surfactant (hydrophobic and hydrophilic properties) used to prevent aggregation of polyplex	At 2.5% Polyoxyethylene stearate prevents polyplex aggregation completely and further enhances transfection efficiency even after prolong storage of polyplexes	[15]

(HPMA-DMAE), Fig. (**3B**). The half life of the polymer was 10 hr at 37 ^{o}C, at pH 7.4 whilst at pH 5.0 the half life was 380 hr [61]. These complexes had a low transfection efficiency on COS-7 cells when compared to the non-degradable poly(dimethylamin methacrylate) (pDMAEMA), Fig. (**3A**), however, at higher N/P ratios with the addition of a membrane disrupting peptide (INF) was similar. In another study, a series of polymers based on a degradable hyperbranched poly(ester amine)s containing primary, secondary and tertiary amino groups were synthesized for gene delivery, Fig. (**3C**) [62, 63]. These polymers incorporated secondary and tertiary amines for "proton sponge effect", primary amines in the periphery for complexeing to DNA, water solubility and a dendrimer like synthetic versatility. At 37 ^{o}C and pH 7.4, 10% of the ester bonds of the polymers were hydrolyzed in 4 hr. Furthermore, these polymers condensed DNA and were less toxic than PEI or pDMAEMA. The best complex had better or comparable transfection efficiency to PEI and pDMAEMA polyplexes.



(C) Polyester amines containing primary, secondary and tertiary amino groups.

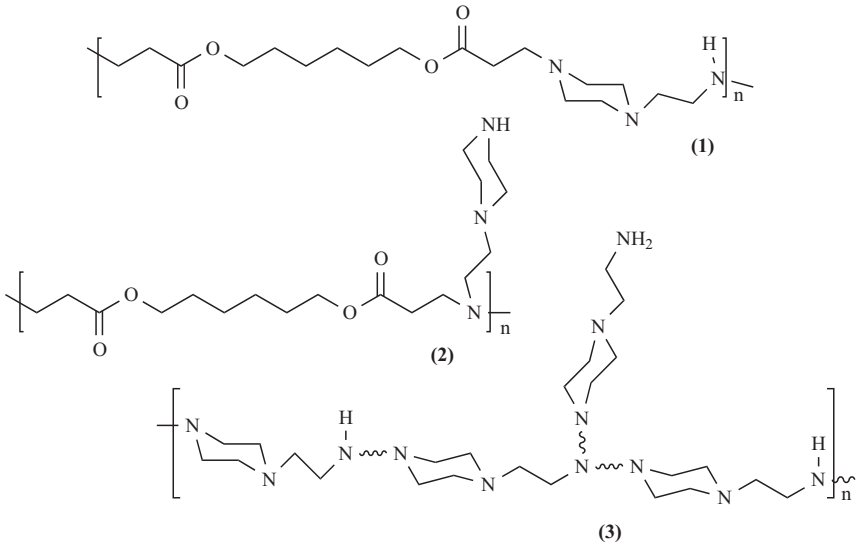


Fig. (3). Synthetic degradable polycationic polymers (**A-C**).

Peptide-Based Polymers

The successful use of PLL as a poly-cationic carrier for DNA has prompted the design of linear and branched synthetic cationic polymer based amino acids lysine and arginine. The disadvantage of PLL is the lack of endosomolytic properties and the need to use lysosomatropic agents for improved transfection efficiency. A series of linear and branched histidine and lysine co-polymers were made with DNA condensing and buffering properties for use with liposome mediated DNA delivery [64]. The branched histidine-lysine copolymers were more effective gene delivery agents than the linear analogs and the co-polymers with 4 branches were more efficient than the 2 co-polymers with 2 branches. The utility of polyplexes based on peptides enable the incorporation of NLS in the same polymer. A series of arginine-lysine containing peptides were made based on a DNA condensing peptide $YKAK_8WK$ [65, 66]. In contrast to PEI based polyplexes the Arginine-containing peptides (eg. $RA(KR)_4$-ARY formed smaller polyplexes (20 nm *vs* >100 nm). This polyplex was also stable in plasma and resistant to DNase I. The polyplexes also trasfected Hep2 cells and the transfection efficiency was further increased by incorporating a high affinity ligand for the asialoglycoprotein receptor [Tris$(GalNac)_3$]. The effect of side chain configuration and side chain spacing in a lysine based oligomer (18-mer) was studied utilizing L-Lys, D-Lys and β3-homolysine [67]. D-Lys and β3-homolysine oligomers were superior to L-Lys in transfection assays with hepa and COS-7 cells. However, it was interesting that when chloroquine was used, the transfection efficiency between the L-Lys and the D-Lys and β3-homolysine oligomers was significant and attributed to the protease inhibitor activity of chloroquine in addition to the buffering activity.

Dendrimers

Polyamidoamine (PAMAM) dendrimers, Fig. (**4A**), were first used for gene delivery in 1993 and were shown to be efficient gene transfer agents for a number of cell lines.

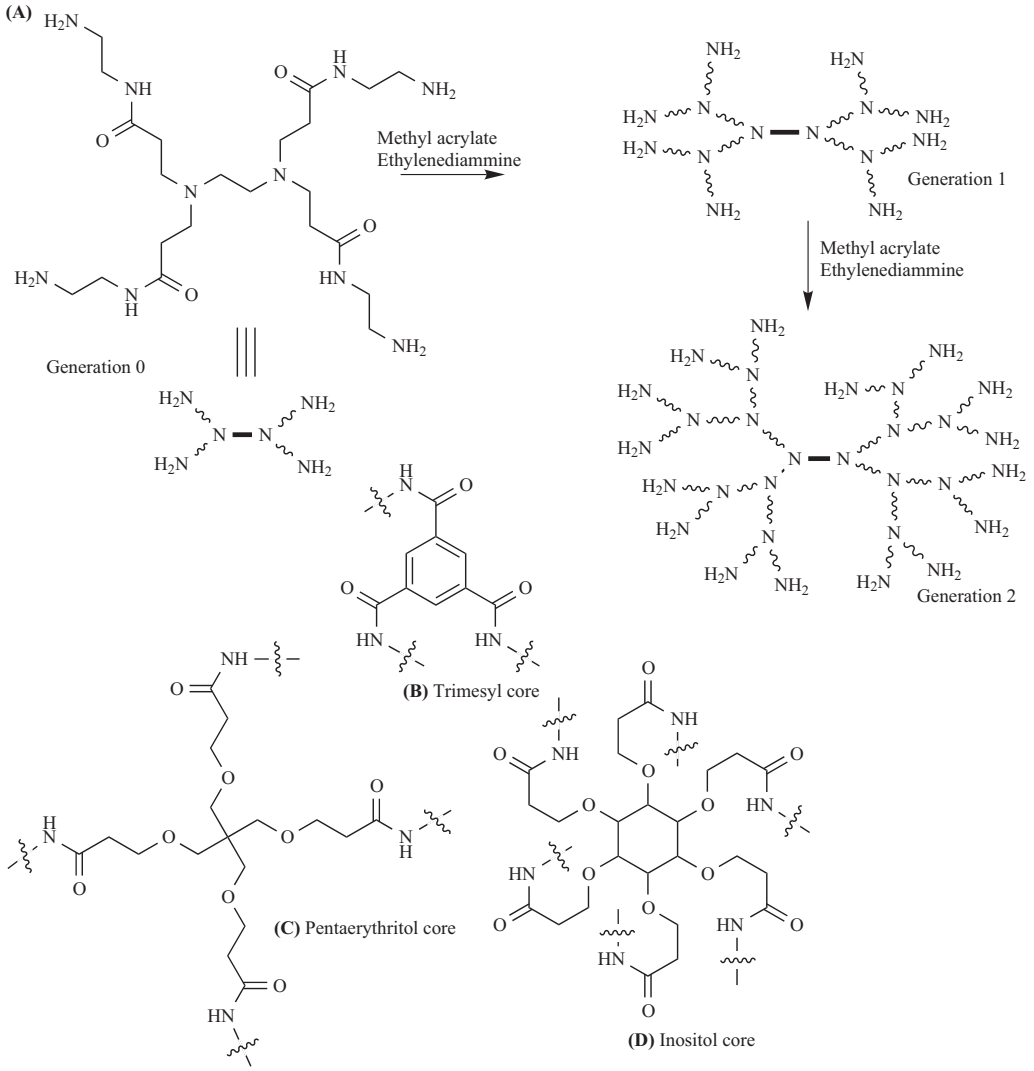


Fig. (4). (**A**) PAMAM dendrimer and (**B-D**) 3, 4 and 6 branched dendrimer cores.

Since then, various dendrimers have been used extensively and Superfect™ is a commercial transfection reagent based on a dendrimer preparation for transfection of cultured cells. PAMAM dendrimers are spherical, ordered, branched polymers with positively charged amino groups on their surface and also tertiary amines in the branches. The protonation of the amines (pKa 3.9 and 6.9) in weakly acidic conditions suppress the lowering of the pH in the endosomal/lysosomal compartment preventing degradation and rupturing of the endosome to release contents into the cytoplasm similar to PEI. Altering the core or surface of the dendrimer allows the manipulation of the charge density or hydrophobicity of the surface of the dendrimer and thus ideal for design of novel carriers with improved transfection efficiency.

Alteration of Core of Dendrimer

The number of amino groups on the dendrimer surface, size and generation of the dendrimer has an impact on the transfection efficiency and toxicity. PAMAM dendrimers derived from cores with 2, 3, 4, 5 and 6 reactive functional groups that can be utilized for chain elongation will yield 128, 192, 256, 320 and 384 amine groups [68]. The various properties of 4 to 8 generation PAMAM dendrimers were compared based on a trimesyl (3 branches), pentaerythritol (4 branches) and an inositol (6 branches) core [69], Fig. (**4B-D**). Only generation 6 of the trimesyl core dendrimer (DT6) condensed DNA to a complex of 100-300 nm in diameter whilst the generation 5 pentaerythritol (DP5) and inositol (DI5) core based dendrimers complexes were >600 nm. The highest transgene expression in COS-7 cells were with DP5 and DI5 but DT6 for the trimesyl core dendrimer. The cytotoxicity of the DT decreased with increasing generation [DT4 (628 μg/ml) to DT8 (77 μg/ml)] but still less toxic than PEI (18 μg/ml) or PLL (28 μg/ml). This study demonstrated the importance of flexibility of the dendrimers and how they influence the condensation with DNA and resulting transfection efficiency. A PAMAM G5 dendrimer with a poly(ethylene)glycol core did not precipitate and showed enhanced water solubility at all N/P ratios [70]. The copolymer showed little cytotoxicity with 94% 293 cell viability at 150 μg/ml. The transfection efficiency was greatly improved than PAMAM G4 and also more importantly did not alter in the presence of serum.

Surface Modification of Dendrimer - Amino Acid

Modification of the surface of PAMAM dendrimers with hydrophobic amino acids would change the stability of the interaction between DNA and carrier and also interaction with the cell surface. PAMAM G4 dendrimer (64 amino groups) modified with various numbers of phenylalanine residues (16.4-64.5) needed higher N/P ratios for complex formation than the unmodified dendrimer, Fig. (**5**) [71]. The phenylalanine modified dendrimers showed a reduced capacity to form complexes with DNA possibly due to the higher pKa of the phenylalanine α-amino group. PAMAM G4 containing 64 phenylalanine groups [(Phe)64-G4] hardly formed a complex at pH 7.4, however, the N/P ratio for complex formation decreased with decreasing pH. Five times more expression of luciferase gene expression in CV1 cells was noted with (Phe)64-G4 - DNA complexes with N/P ratio of 60 at pH 5 compared to (Phe)46-G4 - DNA complexes with N/P ratio of 250 at pH 7.4. The comparison of the transfec-

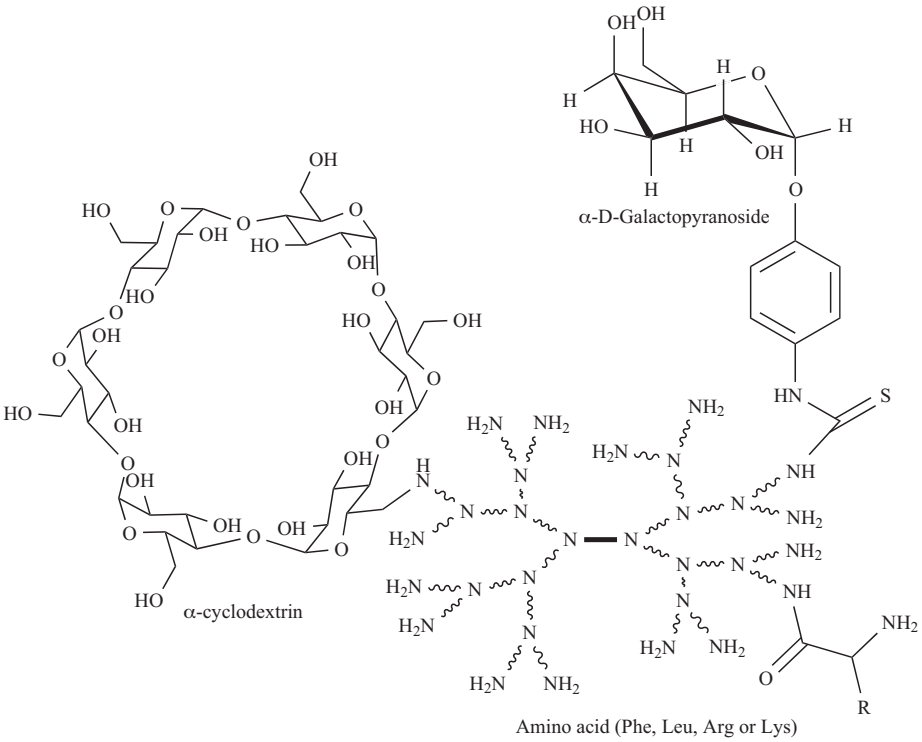


Fig. (5). Schematic representation of surface modification of PAMAM dendrimer with α-cyclodextrin, galactose and amino acids.

tion efficiency with commercially available reagents Lipofectamine and Superfect™ indicated 2.3 and 10-fold increase respectively in luciferase gene expression. Interestingly, modification of dendrimer with leucine did not increase the transfection efficiency at either pH. Multiple arginine and lysine amino acids are abundant in protein transduction domain peptides that display enhanced translocation into cells by endocytic or energy independent process. To enhance possible uptake into cells PAMAM G4 dendrimers were modified with arginine or lysine end groups [72]. These modified dendrimers showed complete complex formation at N/P ratios of 4.0 whilst unmodified dendrimers required an N/P ratio of 2.0. The mean particle size of the polyplexes incorporating PEI, PAMAM-Arg and PAMAM-Lys were slightly smaller (200 nm) than the particle size of PAMAM complexes (245 nm). The Luciferase gene transfection efficiency of the complexes were compared using 293, Hep2 and Neuro 2A cells. The transfection efficiency of all the complexes were similar when 293 cells were used, however, PAMAM-Arg was 10 fold higher than PAMAM and identical to PEI. When Neuro 2A cells were used, the PAMAM-Arg complexes yielded 100 fold higher gene expression than PAMAM or Lipofectamine and 10 fold higher than PEI [72].

Surface Modification of Dendrimer – Cyclodextrins/Sugars

Cyclodextrins (CyD) cyclic (α1,4)-linked oligosaccharides of α-D-glucopyranose containing a central hydrophobic cavity and external hydrophilic surface. Alpha (α), beta (β) and gamma(γ)-CyD contain 6, 7 or 8 α-D-glucopyranose units. CyD can form inclusion complexes with guest molecules and increase the permeability of drugs through biological membranes. α, β and γ-CyD, Fig. (**5**), have been linked to PAMAM dendrimers with the aim to modify it's DNA delivery properties. Functionalization of PAMAM G2 dendrimers with a single α, β or γ-CyD [73] resulted in only α-CyD demonstrating 100 times more transfection efficiency than the unfunctionalized PAMAM dendrimer. In another study, the physicochemical properties of α-CyD conjugates of PAMAM G2, G3 and G4 dendrimers were compared [74]. All the α-CyD conjugates (G2, G3 and G4) complexed DNA, particle size, had ζ-potentials similar the unmodified PAMAM dendrimers. In NIH3T3 cells the transfection efficiency was highest with the α-CyD G3 conjugates being 20 and 2 times more than α-CyD G2 and α-CyD G4 conjugate respectively and similar results were obtained in RAW264 cells. In attempts to further increase the transfection efficiency of α-CyD conjugated PAMAM dendrimer conjugates they were modified with various numbers of either galactose or mannose moieties [75, 76]. α-CyD PAMAM G2 incorporating 1, 4, 5, 8 and 15 galactose residues showed decreased DNA complexation and protection from DNase I digestion with increasing number of galactose residues [76]. Furthermore, there was no DNA compaction effect of Gal-α-CyD conjugates. Gal-α-CyD conjugates with 4 galactose residues had much higher transfection efficiency than native dendrimer or unmodified α-CyD conjugate on HepG2, NIH3T3 and A549 cells. This transfection efficiency was independent of the expression of the asialoprotein receptor. Similar results were obtained for the mannose conjugated α-CyD conjugate where the presence of 3.3 or 4.9 residues of mannose resulted in highest transfection efficiency and independent of the presence of mannose receptors [75]. The reasons for the increase transfection efficiency of the glycosylated α-CyD conjugate is not known.

POLYSACCHARIDES

Polysaccharides have also been used as carriers of DNA. These are attractive carriers in that they are from natural sources, non-toxic, biodegradable and biocompatible and chemically modified to introduce cationic pendant groups. Some polysaccarides such as chitosan have primary amino groups. Chitosan is a linear cationic copolymer of glucosamine and N-acetylglucosamine derived from natural polysaccharide chitin. To improve the DNA complexation ability chitosan was trimethylated to various degrees to form quaternary amine group [77]. The cytotoxicity of modified chitosan oligomers and polymers on MCF-7 and COS-7 cells increased with increasing degree of trimethylation but still lower than PEI. Trimethyl chitosan oligomers (44% modification) and polymers (57% and 93% modification) were more effective than PEI in transfecting MCF-7 cells. Polycationic pendant groups can be conveniently introduced into dextran by periodate oxidation of sugars. Dextran has been modified with PEI, spermine, spermidine or PEG for gene delivery [78-80].

CATIONIC LIPIDS

Cationic liposomes are an important class of compounds suitable for carrying negatively charged DNA. Several commercial transfection [DOTMA (Lipofectin), DOTAP, DOSPA, DOSPER, DDAB, DODAC, NeoPhectin (PCL-2), DMRIE, DC-Chol, DOGS(Transfectam)] reagents are based on cationic lipids, Fig. (**6, 7**). However, these have inherent toxicities. Cationic lipids consist of a positively charged head group, a hydrophobic tail and a linked connecting the head to the tail group. The charged head groups are quaternary amines, tails are saturated or unsaturated alkyl chains or cholesteryl groups. Structure activity studies of various cationic lipids indicated ideal hydrocarbon chain length, with a small cross-sectional area head group and larger hydrophobic chain cross-sectional area exhibiting a cone shape will have enhanced transfection efficiency [81, 82]. A series of cationic lipids (PCL-1, PCL-2, PCL-3, PCL-4 and PCL-5) were synthesized based on the natural phospholipid cardiolipin [83], Fig. (**7**). These cationic lipids were used to make liposomes incorporating the helper lipid DOPE. Of these PCL-4 showed low transfection efficiency but PCL-3 transfected CHO cells just as efficiently as the commercial reagent NeoPhectin (PCL-2). A group of cationic steroid antibiotics which were facial amphiphiles rather than having a conventional cationic head and hydrophobic tail transfected HEK293 and 911 cells, although not as efficient as DOTAP [84], Fig. (**7**). It had efficient endosomal release independent of the acidification process and indicates that the complex behaves like a cationic lipid than PEI. To study the effect of the head group structure of cationic lipid mediated DNA transfection a cationic lipid consisting of a tertiary amine (DADP), quaternary amine with a hydroxyl group (HE-DADP) or a quaternary amine with a mesylate group (EMS-DADP) with two C18 unsaturated fatty acid esters were made [85]), Fig. (**7**). The transfection efficiency of these cationic lipids depended on the ratio of cationic lipid to DNA, type of colipid and molar

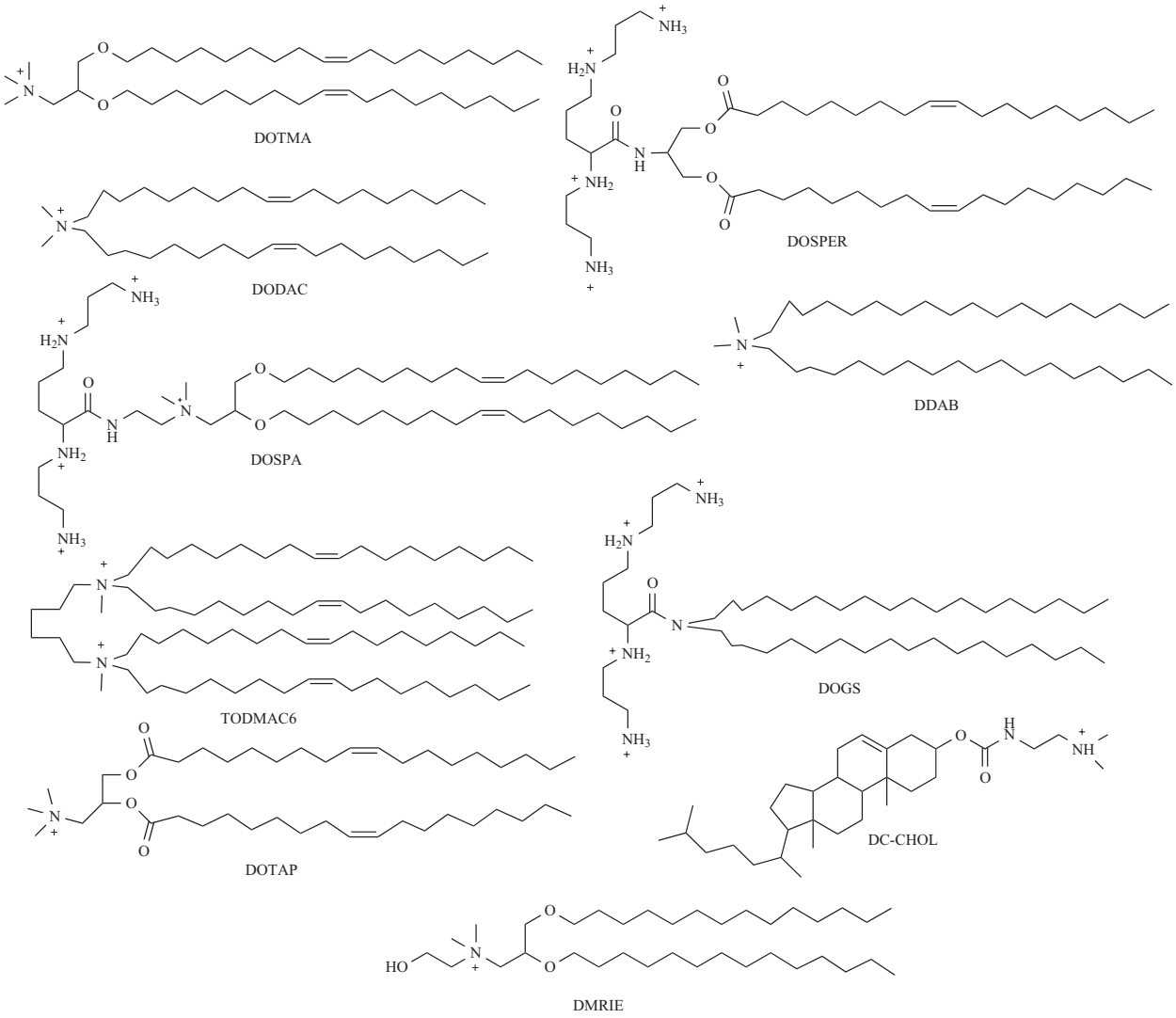


Fig. (6). Structures of commercial cationic lipid transfection reagents.

ratio of cationic lipid to colipid. HE-DADP and EMS-DADP both had higher transfection efficiencies than Lipofectamine and was presumed to be due to the additional hydrogen bond interaction of the hydroxyl group of HA-DADP with the DNA duplex and possible alkylation of DNA resulting in stabilization of the complex. Cationic lipids based on a PAMAM dendrimeric structure was synthesized incorporating two n-dodecyl groups by initially reacting n-didodecylamine with methyl acrylate followed by successive generations built with ethylenediamine and methyl acrylate [86]. The lipid containing PAMAM dendrimer (DL) formed more stable complexes with DNA than the PAMAM dendrimer. At the optimum N/P ratio the lipid containing dendrimers with the higher generation (DL-G2, DL-G3 and DL-G4) exhibited highest transfection efficiency in CV1 cells and were superior to the corresponding PAMAM dendrimer. Addition of helper lipid DOPE further increased the transfection efficiency, therefore, these cationic lipids have dual properties of endosome buffering due to dendrimer tertiary amine groups and membrane fusion with endosome due to the DOPE.

CONCLUSIONS / FUTURE DIRECTIONS

Clearly there is a need for safe and efficient gene delivery systems for gene therapy or for vaccination. Most of the limiting factors to efficient gene delivery have been identified and as described above chemical methods to overcome these are available. The requirements for DNA delivery for gene therapy and vaccines are different. In the latter case DNA will need to be delivered to APC for transcription and translation in these cells or antigen expressed by other cells taken up by APC and presented *via* cross presentation to T cells. The uptake of DNA by APC may be facilitated by targeting DNA to the mannose receptor on APC using mannose or mannan linked polycations [87-89]. Since immunizations are performed in skin there is ready availability of APC for

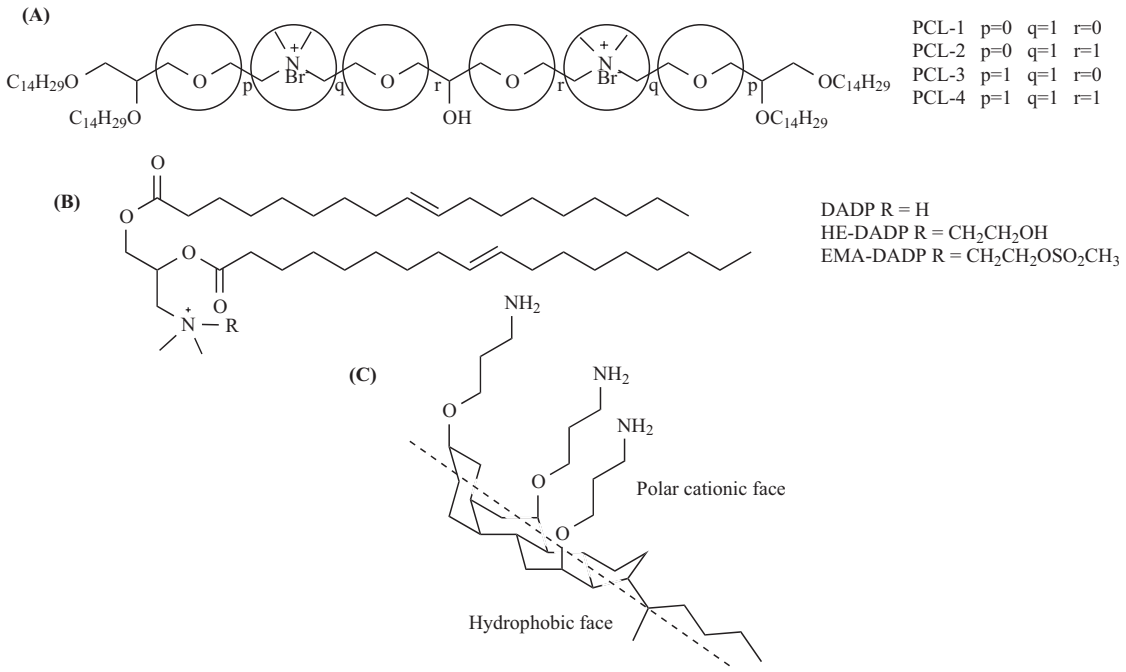


Fig. (7). Structures of cationic lipids based on (**A**) cardiolipin, (**B**) different head groups and (**C**) steroid antibiotic.

transfection. Furthermore, the requirement of sustained DNA expression is not so crucial for DNA vaccination *vs* gene therapy. Even in gene therapy if the non-viral vectors are non toxic and non immunogenic, repeated injections may be possible. Gene therapy to lung or liver is feasible with current technologies but more difficult if targeting to specific tissue by systemic delivery is required.

In vitro transfection data may not always mirror the responses *in vivo* and DNA vaccination although successful in small animal models has failed to yield therapeutic responses in larger animals and humans. Therefore, it is important to test the delivery of DNA in established animal models rather than *in vitro* transfection using reporter genes (luciferase, GFP or galactosidase). In addition, expression of gene of interest will depend on the promoter used and in most cases are from cytomegalovirus, Rous sarcoma virus or SV40. Since these are viral promoters transgene expression controlled by these promoters are inhibited by IFN and TNFα. It will be wise to use promoters that will induce enhanced gene expression when inflammatory cytokines are produced.

In the future new non-viral delivery vectors with optimum characteristics will be developed and combined with plasmids with optimized promoters and enhancers, which will slowly remove the negative aspects that set apart viral-based gene delivery from non-viral delivery.

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ABBREVIATIONS

CyD	=	Cyclodextrin D
DC-Chol	=	3ß-[N-(N',N'-Dimethylaminoethane) carbamoyl]Cholesterol Hydrochloride
DDAB	=	Dimethyl dioctadecylammonium bromide
DMRIE	=	1,2-dimyristoyloxypropyl-3-dimethyl-hydroxyethyl
DNA	=	Deoxyribonucleic acid
DODAC	=	Dioctadecyldimethylammonium chloride
DOGS	=	Dioctadecylamidoglycyl spermine
DOPE	=	1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine
DOSPA	=	2,3-dioleoyloxy-N-(2(sperminecarboxamide)ethyl)-N,N-dimethyl-1-propanammonium
DOSPER	=	1,3-Di-Oleoyloxy-2-(6-Carboxy-spermyl)-propylamid
DOTAP	=	1,2-dioleoyloxy-3-(trimethylammonio) propane
DOTMA	=	N-(2,3-(dioleoyloxy)propyl-N,N,N-trimethyl ammonium
HMPA-DMAE	=	Carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacrylamino)-ethyl ester
NLS	=	Nuclear localizing sequence
pDMAEMA	=	Poly(dimethylamin methacrylate)

PEG = Polyethylene glycol

PEI = Polyethyleneimine

PLL = Poly-L-lysine

PMAM = Polyamidoamine

PVP = Poly(vinyl pyrrolidone)

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