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Synthetic *In Vitro* Delivery Systems for Plasmid DNA in Eukaryotes

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Success for gene therapy clinical protocols depends on the design of safe and efficient gene carriers. Nature had already designed efficient DNA or RNA delivery devices, namely virus particles. However, they have a propensity to trigger neutralizing and other immune responses and insertional mutagenesis have limited their clinical use. Alternatively, safer approaches involving non-viral carriers have been and continue to be developed although they have not reached the transfection levels achieved by viruses. Those methods can be broadly classified into two categories: chemical and physical methods. In this review we present the most common and recent chemical non-viral methods to introduce, *in vitro*, pDNA into eukaryotic cells.

KEYWORDS: *Non-Viral Gene Delivery, Cationic Lipids, Liposomes, Polymers, Gold, Nanotubes, Peptides.*

CONTENTS

INTRODUCTION

Not until the early 1960's was foreign DNA introduced as-permanent, stable, functional, and hereditable elements into mammalian cells.[1] With this achievement, new approaches for treating human diseases became imaginable. Theoretically, the insertion of DNA to correct genes that are either absent or miscoded could ameliorate many genetic disorders. Since then, an increasing number of methods have been and are currently being developed for delivering DNA into cells.

Besides plasmid DNA (pDNA), various types of RNA molecules have been introduced into cells, such as oligodeoxyribonucleotides (ODNs) and small interference RNA (siRNA).[2] In contrast to pDNA, which is used to introduce a new or corrected copy of the defective gene, RNA is used to inhibit translation of mRNAs,[3] in a transient fashion.[4] Plasmid DNA can also be used as genetic vaccines leading to activation of specific immune responses either preventing or treating infectious or degenerative diseases.[5–7]

The delivery of foreign genetic material involves overcoming several extracellular and cellular barriers. For mammalian cells *in vitro*, nucleic acids must cross the cell membrane, then avoid lysosomal degradation and then traverse the nuclear membrane, and achieve therapeutic levels of expression without disrupting other genes.[8] For *in vivo* delivery addition challenges must be overcome. The added genetic material has to be protected from circulating nucleases present in the organism, phagocyte degradation, aggregation with serum proteins and finally target specific tissues.[4] Furthermore, when used to target CNS

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nervous tissues, the genetic material must efficiently cross the blood-brain barrier to reach the targeted cells.[9]

In vivo, cells can take up naked DNA however the mechanism is not fully understood. Injections of naked DNA into skeletal muscle may result in the expression of the proteins coded by the added DNA however at low and extremely variable levels.[10] For this reason the development and use of molecular carriers that both coat and shield the nucleic acids from hydrolysis by nucleases and target the negatively charged DNA to specific tissues is vital. Nature had already designed efficient DNA or RNA delivery devices, namely virus particles. Viruses effectively package, protect and deliver genetic material. Researchers seized on this approach and began working with certain viruses for use as human gene delivery systems. Adenovirus and retrovirus were the first carriers used to delivery therapeutic genes. At present, they remain the vectors of choice, showing the highest efficiency.[11] One of the advantages of viral gene delivery system is that a gene can be integrated into the host's genome. However, the death of a patient receiving an adenoviral vector triggered safety objections and questioned the further use of viral vectors.[12] Also, other issues including the potential risk of insertional mutagenesis, previously established immunity to the virus vector, and the size limitation on the DNA that can be packaged have limited clinical applications.[13] Despite these risks, researchers continue working to improve the efficacy and safety of these vectors. In recent years, research has focused on the use of lentivectors, which, like their retrovirus counterparts, are devoid of viral proteins, not replication competent, and able to transduce non-dividing cells.[14] Currently, these lentivectors are being used in about 3.3% ($n = 67$) of clinical trials (http://www.wiley.co.uk/genmed/clinical/ [July 2013]).[15]

Alternatively, non-viral biological methods are being investigated for DNA delivery as safer systems.[16] While somewhat less efficient than their viral counterparts, significant progress has led to improved transfection efficiencies and made them feasible alternatives. With the newly developed genome editing, such as CRISPR/Cas9 and

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zinc-finger nuclease (ZFN) systems, integration of the corrected gene into the genome can be site specific.[17,18] When incorporating genome editing elements to pDNA, non-viral gene delivery can provide a significant refinement for gene therapy.

Non-viral synthetic systems employ biocompatible molecules that facilitate the uptake of nucleic acids into cells. Synthetic carrier methods first require the complexation of cationic amphiphilic molecules with anionic nucleic acids followed by cellular uptake through a facilitated membrane translocation. The application of these newer methods ranges from preclinical studies to human trials. Lipid base vectors now account for the 5.5% ($n = 112$) of gene therapy clinical trials (http://www.wiley.co.uk/genmed/clinical/ [July 2013]). The biggest challenge for non-viral gene therapy will be translation from bench to clinical applications.[19] This review focuses on the most recent (2010-present) non-viral pDNA delivery systems *in vitro* and compares their transfection efficiencies and potential.

SYNTHETIC NONVIRAL DNA CARRIERS

Synthetic non-viral DNA carriers are cationic molecules that bind to DNA, predominantly through electrostatics. Cationic lipids, polysaccharides, peptides, synthetic polymers, and metals are examples of molecules that are currently used as chemical DNA carriers (Fig. 1). The electrostatic interactions between the chemical vectors and pDNA generate complexes with different topologies; either spherical vesicles, rod-like or ordered multi-laminar structures.[20] These complexes even with incorporated DNA retain their net positive charges, facilitating interactions with negatively cell membrane surfaces.[21,22] Natural Glycosaminoglycan chains such the sulfated forms of heparin, dermatan and chondroitin, are responsible for the eukaryotic cell membrane's negative potential.[23] Once bound, endocytosis serves as the principle route of uptake of thepolycation/DNA complexes.[24–26] Endocytosis in itself is a broad term that encompasses specific pathways. They can be broadly classified in four types: phagocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) and macropinocytosis.[27] According to several reports there is no evidence linking the mode of entry to transfection efficiency.[24,28] It is generally assumed that low to moderate gene expression is due mostly to the trapping and degradation of the pDNA within intracellular vesicles and limited translocation to the nucleus.[16,29]

Due to their cationic nature, non-viral nanocarriers can be toxic.[30] The integrity of organelle membranes can be compromised and undesirable interactions with other negatively charge compounds such nucleic acids and proteins could affect the protein expression and other natural cellular process.[31] Factors that have a direct impact in cytotoxicity are: charge density, molecular weight, presence of free carrier and degree of biodegradability.[32] Currently, research on how to improve the transfection efficiency without compromising cell viability remains the largest obstacle for this type of non-viral DNA/RNA delivery *in vitro*.

CATIONIC LIPIDS

Cationic lipids are the most commonly used synthetic gene delivery molecules due to their high transfection efficiency. The most common systems for gene delivery using lipids are: liposomes, solid lipid nano particles and micelles.[33] Lipids are a broad class of molecules that number in the thousands depending on hydrocarbon chain length, degree of unsaturation and nature of any adducted molecules. Cationic lipids used for DNA delivery generally contain four functional domains: a hydrophilic head-group, a linker, a backbone domain, and a hydrophobic domain[34] (Fig. 2). The most commonly hydrophilic head groups are primary-, secondary-, tertiary-amines, or quaternary ammonium salts. However, guanidino, imidazole, pyridinium, can be present.[35,36] The hydrophobic tails are usually made of two types of hydrophobic moieties, aliphatic chains or steroids. The most common linkages between the hydrophilic head and hydrophobic moieties are ethers, esters, carbamates, or amides.[37] The structure of cationic lipids is a major factor for their transfection activity and toxicity.[38] Masotti et al.[39] have compared different parameters of some commercially available cationic lipids influencing toxicity and transfection efficiency on Rat Glioma Cell Line (C6) (Table IA).

A pioneering design by Felgner et al.[40] was the glycerol backbone-based cationic transfection lipid-DOTMA

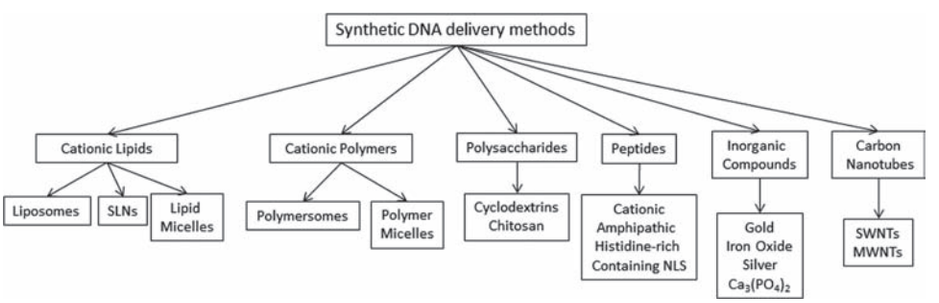

Figure 1. **Current synthetic DNA delivery vectors.**

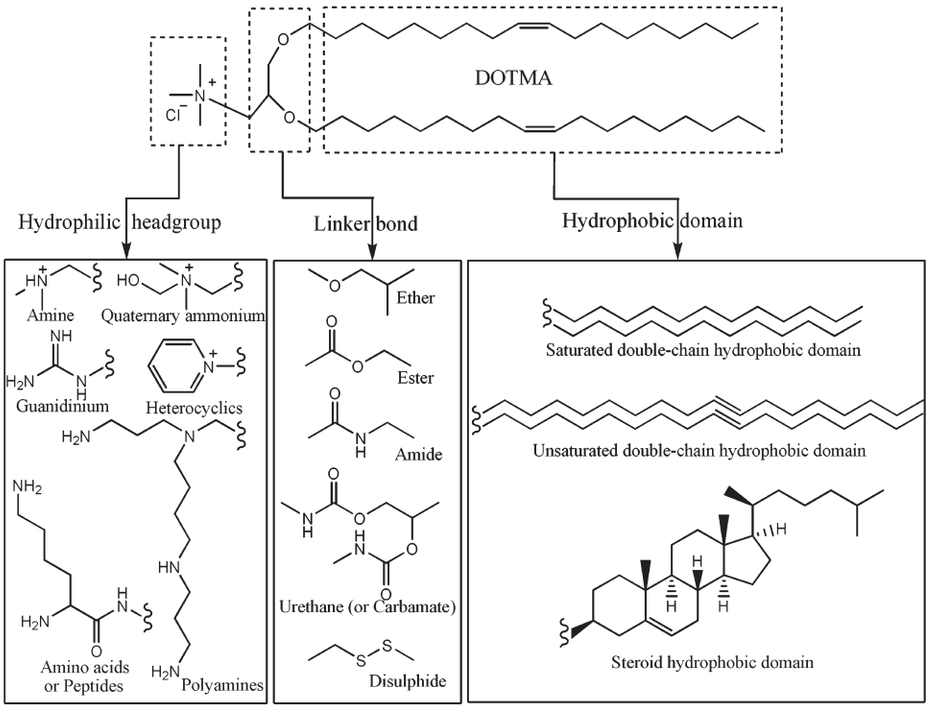

Figure 2. Different structural components of cationic lipids: Hydrophilic head group, linker bond, and hydrophobic domain. Reprinted with permission from [38], D. Zhi, et al., The head group evolution of cationic lipids for gene delivery, *Bioconjugate-Chem.* 24, 487 (2013). © 2013, American Chemical Society.

(*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride). Since then a number of compounds have been developed and display considerable diversity in structure, number of aliphatic chains, asymmetry, chain length and degrees of unsaturation. Often they are combined with neutral helper lipids such dioleoylphosphatidylethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) to yield higher transfection efficiencies in many cell types.

Mevel et al.[41] reported on the synthesis of a novel cationic lipid: *N*′,*N*′-dioctadecyl-*N*-4,8-diaza-10-aminodecanoylglycine amide (DODAG). This cationic

Table I(A). Luciferase expression in various mammalian cell lines transfected with cationic lipids commonly used for gene transfer. Transfection efficiency is expressed in Relative Light Units (RLU). The comparison was done at the optimal charge/ratio (that one that shows be more efficient) of each liposome formulation after 24 h of transfection.

	Transfection efficiency (RLU/per 96-well)	Lipid/DNA charge ratios	Incubation time (h)	Serum	Ref.
Rat glioma cell line (C6)					
DOTAP/DOPE	5×10^5	1:1	4	+	[39]
DC-Chol/DOPE	4×10^5	5:1	4	–	[39]
DDAB/DOPE	1×10^5	2.5:1	4	+	[39]
FUGENE^{++}	4×10^6	–	4	+	[39]
DMRIE/Chol	8×10^4	2.5:1	4	–	[39]
LIPOFECTIN	4×10^5	5:1	4	–	[39]
LIPOFECTAMIN 2000	1.1×10^5	4:1	4	–	[39]
CELLEFECTIN	6×10^4	2.5 :1	4	–	[39]
CHO					
DOTAP/DOPE	1.1×10^5	5:1	4	–	[45]
OLON/DOPE	2×10^6	5:1	4	–	[45]
LHON/DOPE	2.5×10^6	3:1*	4	–	[45]
CTAB/DOPE	5×10^4	2:7*	4	–	[45]
DOGSH/DOPE	4×10^5	3:1	4	–	[45]

Notes: *Represents the *w*/*w* ratio; $^{++}$Fugene has a protected formulation; Abbreviations: DC-Chol:cholesteryl-3*b*-*N*-(dimethyl-aminoethyl)carbamatehydrochlorideDDAB: dimethyldioctadecylammonium bromide; DMRIE: 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; CELLEFECTIN: 1:1.5 molar mixture of the cationic lipid *N*,*N*′,*N*″,*N*‴-tetra-methyltetrapalmityl-spermine (TM-TPS) and DOPE; DOGSH: 19,29-dioleyl-*sn*-glycero-39-succinyl-1, 6-hexanediol; CHO: Chinese Hamster Ovary.

Table I(B). Luciferase expression in various mammalian cell lines transfected with cationic lipids commonly used for gene transfer. Transfection efficiency is expressed in Relative Light Units (RLU). The comparison was done at the optimal charge/ratio (that one that shows be more efficient) of each liposome formulation after 24 h of transfection.

	Transfection efficiency (RLU/per mg/protein)	Lipid/DNA charge ratios	Incubation time (h)	Serum	Ref.
HeLa					
LIPOFECTIN	3.3×10^6	5:1	4	–	[41]
LIPOFECTAMINE	4.8×10^7	2.5:1*	4	+	[41]
DODAG/DOPE	1.3×10^8	4:1*	4	+	[41]
CDAN/DOPE	5×10^7	8:1*	4	+	[41]
CAPG/DOPE	2×10^7	2:1*	4	+	[41]
KLN47	1×10^7	2:1	4	–	[46]
BSV10	1×10^6	2:1	4	–	[46]
BSV36	1×10^7	2:1	4	–	[46]
DDCTMA	3.2×10^5	2:1			[37]
SWB-95					
DOTAP/Chol	8×106	1:1	1	+	[36]
2Oc	1×10^7	1:1	1	+	[36]
HEK-293					
Lipofectamine	10×10^{10}	N/A	4	+	[47]
5c	10×10^8	8:1	4	+	[47]

Notes: *Represents the *w*/*w* ratio; ++Fugene has a protected formulation; Abbreviations: CDAN: *N*^{1}-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; CAPG: *N*1-cholesteryloxycarbonyl-3-aza-pent-1-amino-5-guanidiniumchloride; DDCTMA: *N*-[1-(2,3-didodecylcarbamoyloxy)propyl]-*N*,*N*,*N*-trimethylammoniumiodide; 2Oc: 1-(2,3-dioleoyloxy-propyl)-2,4,6-trimethylpyridinium; KLN47: trimethylarsoniumLipophosphoramidate polar head; BSV36: trimethylammoniumpolar head; BSV10: guanidinumpolar head; DDCTMA: *N*-[1-(2,3-didodecyl carbamoyloxy)propyl]-*N*-ethyl-*N*,*N*-dimethylammonium iodide; SWB-95: Brain Glioma; HEK-293: Human Embryonic Kidney 293.

lipid contains two chains in the hydrophobic domain and a guanidinium functional group. Using DODAG in combination with the helper lipid DOPE three cell lines were transfected obtaining high levels of luciferase expression compared to Lipofectamine 2000 (Table IB). They reported as well moderate cytotoxicity; only around 5% of LDH release was found using immortalized adherent cell lines. For the same type of cell line, around 20% lactate dehydrogenase (LDH) release (equivalent to cell death) has been reported for Lipofectamine 2000. Safinyaand co-workers[42] have design a cationic lipid (CMVL5) that contains a disulfide bond spacer between the head group and the hydrophobic tails. The objective of this modification was to facilitate the degradation in the reductive environment of the cytoplasm and with this decrease the cytotoxicity. Although, the transfection efficiency was similar than Lipofectamine 2000, cell viability was 10% more than what was reported for the commercial reagent. Zhi et al.[43] have reported similar results in terms of transfection and cytotoxicity but using carbamate linkages between the ammonium heads and the hydrocarbon chains. Medvedeva et al.,[44] synthesized a biodegradable cholesterol-based (CcHPB), obtaining 22% GFP expression on HEK 293 (Human Embryonic Kidney 293) cells. While significant transfection efficiencies have also been reported for single-tailed cationic lipids, for to most part single-tailed and three-tailed cationic lipids appear to be more toxic and less efficient than their double-tailed counterparts.[38] Examples of single-tailed cationic lipids include: cetyltrimethylammonium bromide (CTAB), oleoylornithinate (OLON), and 6-lauroxyhexyl ornithinate (LHON)[45] (Table IA).

Comparison of transfection efficiencies of lipophophoramidates[46] and Biotinylated[47] cationic lipids formulations are show in Table IB.

Liposomes

Liposomes are lipid vesicles that are usually formed through the self-assembly of cationic di-acyl phospholipids which are amphiphilic in nature. The first lipid vesicle reported was formed by the dispersion of phospholipids in water by Bangham and coworkers.[48] Since then, a number of structurally diverse lipid vesicles have been developed. Unlike polypeptides, polysaccharides and polynucleotides, lipids are not polymers, by definition, however they are rarely found as monomers.[49] They prefer to assemble into macromolecular assemblies to escape expose of the hydrophobic segments to water. Lipid vesicles will have different sizes depending on the method of preparation. Vesicle sizes fall into the nanometer to micrometer range.[50] Liposomes have found uses in many biological and pharmaceutical applications including diagnostic agents and drug delivery vehicles.

A major limitation for liposomes, *in vivo,* is the short circulating half-life.[51] Systemic elimination of cationic lipids takes place upon formation of larger aggregates via their interactions with the negatively charged serum molecules or cellular components (primarily erythrocytes and platelets). Surface shielding through the use of hydrophilic and charge neutral polymers such as

polyethylene glycol (PEG) to reduce excessive charge–charge interaction appears very effective in prolonging the circulation half-life of lipoplexes.[49] However, some reduction in the overall transfection efficient has been reported.[52]

DNA Complexation, Cellular Uptake and DNA Release

Cationic liposomes when mixed with pDNA, in aqueous solutions, are capable of self-assembly into different liquid crystalline structures. These structures are called "lipoplexes" and the morphology of the phase depends on the packing parameter of the lipid molecules. Two phases appear to be more efficient for mammalian cell transfection: lamellar (L_α) phase with alternating lipid bilayer and DNA monolayers and inverted hexagonal (H_{II}) phase consisting of DNA coated with a lipid monolayer in a hexagonal lattice. It has been argued that the H_{II} phase complexes have a much higher transfection rates than do the L_α phase ones.[53] One explanation could be that this phase promotes the lipoplex fusion with cellular membranes facilitating the cellular uptake and the endosomal release.[54]

A number of recent publications indicate that the usual endocytic routes of uptake for lipoplexes are: clathrin- and caveolae-mediated endocytosis and/or macropinocytosis.[55–57] It appears that lipoplexes are not limited to a single uptake pathway; rather they can utilize several of these pathways. It was observed that the inhibition of one pathway produced an increase in uptake though the other one (compensatory effect).[59] Also, modifications in particle size, shape of and exposed ligands on the lipoplexes can alter the route of uptake.[56] Nevertheless, the method of entry of these structures does not appear to contribute to transfection efficiency, no direct relationship has been observed.[58, 59] For instance, for two lipoplex formulations, i.e., DOTAP/DOPC and DCChol/DOPE the same endocytic route (macropinocytosis) was observed in CHO cells, however the sub-cellular co-localization and the transfection efficiency was remarkable different. One explanation is that DCChol/DOPE lipoplexes or their cargos were capable of escaping the endosomes while the DOTAP formulation appeared to accumulate within these sub-cellular compartments.[60] These results suggest that transfection efficiency correlation more directly with the ability of a lipoplex to escape the lysosome than its method of cellular uptake.[61]

It has been proposed that for lipid-base systems escape from the endosomal/phagosomal vesicles is facilitated by membrane fusion and formation of transient pores in the organelles with the concomitant release of nucleic acids into the cytosol.[62] The presence of the helper lipid DOPE in some formulations promotes the transition from lamellar (L_α) phase to hexagonal (H_{II}) phase thus helping the insertion of lipoplexes into endosomal membranes.[48] This mechanism has been explained by the phenomenon called "ion paring." In this scenario the cationic and anionic phospholipid head groups neutralized each other thereby favoring the transition to the hexagonal (H_{II}) phase. Upon neutralization, dissociation of the DNA from the complex occurs.[63] The results suggest that strong lipid-DNA binding will interfere with the dissociation process and decrease transfection efficiencies. The incorporation of some modifications to the cationic lipids such pH sensitive linkers between the head group and the acyl portion of the molecules can promote enhanced DNA release.[64] Others have proposed that shorter hydrocarbon chains in the lipoplexes will increase the fluidity of the bilayer and favor higher rates of inter-membrane delivery and lipid membrane mixing, resulting in the increased disruption of the endosomal membrane and DNA escape.[48]

Cytotoxicity of Lipoplexes

An important issue to address when considering *in vivo* studies is the toxicity of a specific nano carrier. Various cellular and tissue responses could lead to altered signaling or physiology, cell death and induced immune responses.[65] One cause of lipid cytotoxicity is the interaction of the tertiary or quaternary nitrogen functional groups that bind to and inhibit enzymes such as PKC.[66] In addition, cationic liposomes are known to affect the function of membrane proteins involved in signal cascades implicated in immune responses.[67] It was shown that some certain liposomes will induce immune response in the absence of antigens such as DOTAP liposomes which can induce expression of monocyte chemo-attractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1a) and macrophage inflammatory protein-1 beta (MIP-1b) together with transcription of a dendritic cell maturation marker, CD11c.[65, 68] Nevertheless, a novel strategy has been proposed to take advantage of this specific immune-stimulating(adjuvant) feature to enhance the development of an anticancer vaccine using the peptide antigen derived from E7 on coprotein of human papilloma virus(HPV) type 16 mixed with the cationic lipid DOTAP.[69] While several reports have described this phenomenon these effects have not been studied in great detail.

Also cationic lipids have been reported to activate several kinases implicated in immune responses and regular cell signaling.[70,71] In addition, cationic lipids fusing with cell membranes could lead to the insertion of foreign lipids into the membrane.[72] Having foreign lipids in a membrane could alter the physio-chemical properties of the bilayer thereby affecting membrane protein structure, cell signaling and interactions between lipids and protein membranes. It has been reported that this process can cause non specific inflammatory responses.[73]

POLYMERIC DNA CARRIERS

Polymers used in gene delivery, like their lipid counterparts, are generally cationic in character. They are

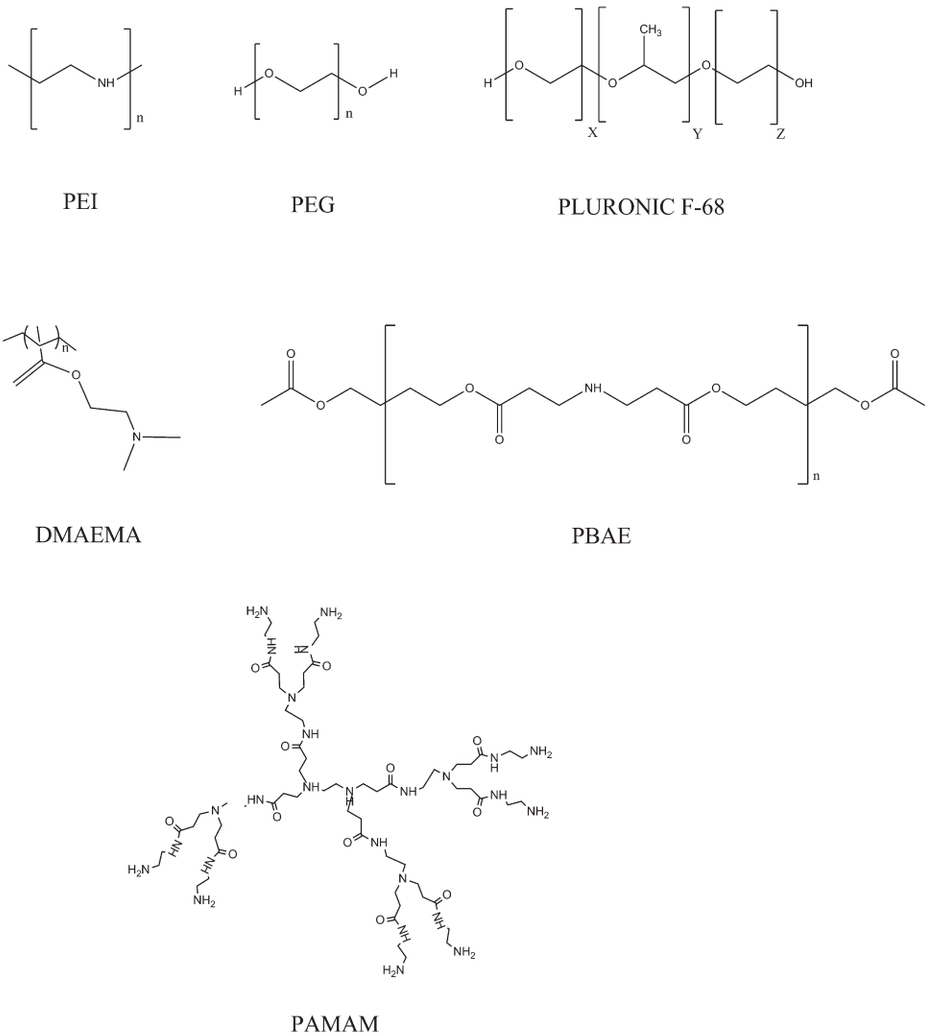

Figure 3. **Structures of the most common synthetic polymers used for gene delivery.**

made up of a wide variety of polymerized monomers.[74] The most frequently used pDNA carriers include: Polyethylenimine (PEI),[75] poly(a-[4-aminobutyl]-l-glycolic acid (PAGA),[76] Poly(β-Amino Ester) (PBAE),[77] poly(amidoamine) (PAMAM), poly-propylenimine (PPI), Poly(2-(dimethylamino)ethyl methacrylate (DMAEMA),[78] Pluronic and polyethylene glycol (PEG) (Fig. 3). They are easy to synthesized, and some of them including PBAE and PAGA, contain biodegradable linkages designed to reduce cell toxicity.[76, 77] PEI, is one of the most widely studied non-viral vectors due to its high transfection efficiency. Its highly cationic surface strongly associates with and helps condense DNA efficiently, however it is one of the most toxic agents being investigated.[79] Dendrimers, such PAMAM and PPI are synthetic, hyper-branched spherical molecules that are capable of entrapping small molecules within their structures.[80] Their large size and multivalent surfaces provide an excellent platform for the attachment/associate of different drugs and therapeutic genes. DMAEMA is a cationic polymer offering the advantage of being a water-soluble cationic polymer.[81] PEG it is commonly conjugated to some of the polymers mentioned above to prevent aggregated complexes and to increase stability and circulation half-life, *in vivo*.[82, 84] Natural occurring polymers in complexation with other molecules, such as collagen with DMPC[83] and gelatin with PEI[84] have been tested also as pDNA delivery vehicles where they have shown promising results and having the advantage of good cyto-compatibility. These properties have made these molecules them popular choices in areas outside of gene delivery namely, tissue engineering scaffolding applications.[85]

Recently, Newland et al.[86] reported on a new polymeric gene vector formed predominantly through internal cyclization reactions within the polymer instead the usual hyper-branching between polymer chains. Ethylene glycol dimethacrylate (EGDMA) was the cyclizing unit while

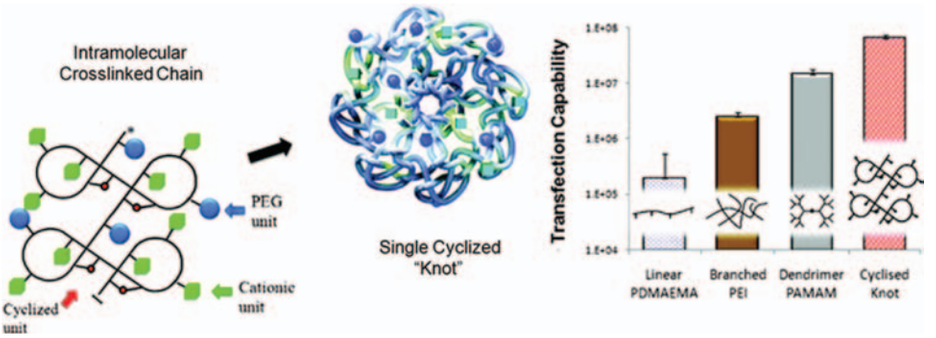

Figure 4. **The structure of the single cyclized polymer knot. This knotted polymer shows better transfection capability compared to linear, branched or dendritic polymers. Reprinted with permission from [86], B. Newland, et al., Single cyclized molecule versus single branched molecule: A simple and efficient 3D "knot" polymer structure for nonviral gene delivery. *J. Am. ChemSoc. USA* 134, 4782 (2012). © 2012, American Chemical Society.**

2-(Dimethylaminoethyl) methacrylate (DMAEMA) was the cationic unit. Despite the multi-step and synthesis of the polymer involving a dialysis process for several days, significant transfection efficiencies and moderate toxicity was observed when compared to commercial agents such as SuperFect®(partially degraded PAMAM) and PEI (Fig. 4, Table III). Zhou et al.[84] synthesized a family of biodegradable poly(amine-co-esters) formed through enzymatic copolymerization of di-esters with amino-substituted diols. They reported very high transfection efficiencies and low toxicity in comparison to polyethylenimine and Lipofectamine 2000. However, the formation of the poliplexes has to be done under slightly acidic conditions and in the presence of DMSO. These conditions could be a drawback for some sensitive cell lines. Keenet et al.[87] reported on a poly(β-amino ester) biodegradable system for the efficient delivery of Mini Circle MC-DNA that gave significant GFP expression (Table III). MC-DNA it is a small supercoiled DNA molecule where the bacteria cassette commonly used in plasmids has been removed. They created a small library of 18 poly(β-amino ester) polymers differing in the backbone structure and the end-group chemistry. Subsequently, they were tested in embryonic kidney 293 cells and mouse embryonic fibroblasts obtaining excellent transfection rates in some of them. In this study they demonstrated that higher transfection efficiency can be achieved for HEK cells using the MC-DNA instead the conventional plasmid.

Different materials have been combined with polymers to form hybrid gene delivery nanoparticles in order to improve the stability, targeting and the circulating half-life in blood.[88–91] Majoros et al. reported folate-mediate targeting[92] where the poly(amidoamine) (PAMAM) dendrimer was conjugated to folic acid. These folate residues bind to folate receptors that mediate receptor medicated endocytosis and are over expressed in cancer cells.[93] Comparisons of some commercial available polymeric-base transfection reagents[94] are shown in Table II.

Anderson and co-workers[95] reported a high-throughput method for assessing polymer-mediated transfection. Hundreds of polymers can be tested for gene delivery using 96-well plates in one day. One limitation of this

Table II. **Luciferase expression in various mammalian cell lines transfected with the indicated cationic polymers commonly used for gene transfer. Transfection efficiency is expressed in Relative Light Units (RLU). The comparison was done at the optimal charge/ratio (that one that shows be more efficient) of each polyplex formulation after 24 h of transfection.**

	Transfection efficiency (RLU/per 96-well)	Polymer/DNA charge ratios	Incubation time (h)	Serum	Ref.
HeLa					
Arrest In	5.4×10^5	5:1	4	+	[94]
Express Fect	2.9×10^5	1:1	4	+	[94]
JetPEI	6.5×10^5	2:1	4	+	[94]
SuperFect	2.2×10^6	5:1	4	+	[94]
Dextran-Spermine	4×10^5	5:1	4	–	[94]
Collagen	$1. \times 10^5$	4:1	4	–	[94]
CHO					
PEI 800 Da	4×10^6	18	4	–	[116]
PEI 25 kDa	3×10^{10}	9	4	–	[116]
PEI-DSP	8×10^9	9	4	–	[116]
PEI-DTBP	3×10^8	9	4	–	[116]

Notes: Abbreviations: DSP: cross-linking reagent, di-thio-bis(succinimidylpropionate); DTBP: cross-linking reagent dimethyl 3,3′-dithiobispropionimidate 2HCl.

Table III. Transfection efficiency using the reporter plasmid pEGFP (Enhanced Green Fluorescence Protein) in various mammalian cell lines transfected with cationic polymers commonly used for gene transfer. The comparison was done at the optimal charge/ratio (that one that shows be more efficient) of each lipoplex formulation.

	% Cells transfected (%)	Polymer/DNA charge ratios	Incubation time (h)	Serum	Ref.
HT 1080					
Chitosan/y-PGA	54	10:1:14*	2	–	[130]
Lipofectamin 2000	33	N/A	2	–	[130]
PMSC's					
Fugene	15	N/A	4	–	[124]
PEI-β-CD	12	N/A	4	–	[124]
TAT-PEI-β-CD	16		4	–	[124]
HEK-293					
PEI 25 KDa	10	15	4	+	[122]
PEI 1.8 KDa	4	90	4	+	[122]
PAMAM G5	22	90	4	+	[122]
PAMAM G1	18	10	4	+	[122]
PAMAM G2	8	50	4	+	[122]
EA-G1	18	50	4	+	[122]
EA-G2	43	50	4	+	[122]
PEI-25KDa	12	15	4	+	[122]
Lipo-2k	23	6	4	+	[122]
HeLa					
PEI	30	5:1	2	–	[121]
CTS	18	7	2	–	[121]
NMCTS-graft-PAMAM	36	5:1	2	–	[121]
MWCNT-CS-FA-NPs	4.1	5:1	6	–	[178]
MC/PBAE-1445	82	10:1	4	+	[87]
3T3 fibroblasts†					
PEI	3	N/A	4	–	[86]
dPAMAM	10	N/A	4	–	[86]
PD-E 8 PEG	13	3:1*	4	–	[86]

Notes: *This represent *w*/*w* ratio; ++Fugene has a protected formulation; †Normalized to total parent events; Abbreviations: NMCTS-graft-PAMAM: *N*-maleyl chitosan-graft-polyamidoamine; CTS: Chitosan; MWCNT-CS-FA-NPs: Multi-Walled Carbon Nanotubes (MWCNTs) of different functionalized with chitosan–folic acid nanoparticles; MC: Minicircle DNA; G: Generation of dendrimer; EA-G2 (or EA-G1) was prepared by aminolysis of poly(ethylene glycol)-poly(L-benzyl glutamate) (PEG-PBLG) using PAMAM G2 (or G1); HT-1080: Fibrosarcoma. PMSC's: Parthenogenetic Mesenchymal Stem Cell.

high-throughput method is the small volumes that were used for the transfections where polymer-DNA complexation could not be guarantee. Nevertheless, this method has been already tested with some polymeric based transfection reagents. From these experiments there appear to be several parameters involved in the successful design and effectiveness of polymeric transfection reagents: cell type, carrier/DNA ratio, particle size, toxicity, solubility and stability in serum.

DNA Complexation, Cellular Uptake and DNA Release

Polymers due to their cationic nature are capable of interacting and condensing pDNA to generate nano-sized complexes called poliplexes.[96] Polymers bind DNA through electrostatic interactions between the phosphate groups present in the DNA and the cationic groups present in the polymer reagent,[97] This is an entropic process, with counter ions being displaced from both from the DNA and the polymer Surface charges and the "nitrogen to phosphate" (N:P) ratios are the primary factors controlling the size and morphology of the poliplexes.[97,98] They adopt structures that are typically blends of toroids and rods, with diameters ranging from 50–100 nm.[97]

Regardless of the complex's topology, it's the net positive charge that facilitates the binding interactions with negatively charged cell surface. Subsequently, they are internalized, in most cases, through endocytosis.[99] It has been reported most internalization of poliplexes (<500 nm in diameter) occurs through receptor-mediated endocytic routes such as clathrin-mediated endocytosis (CME) and caveolae-mediated endocytosis (CvME). Particles sizes >500 nm are taken up by other internalization pathways.[100] Polymers can be internalized as well by non-endocytic pathways. Studies carried out by Hong et al.[101,102] demonstrated that PAMAM forms small holes in the cell membrane with its subsequently diffusion into the cell. However, modifications to this polymer such the conjugation to folic acid switches its enter route to receptor-mediated endocytosis. This indicates that the attachment of certain ligands can be used for targeting purposes to trigger a specific endocytic route and manipulate the intra-cellular localization of the poliplexes.[100]

It is generally assumed that low to moderate gene expression is due to the trapping and degradation of the pDNA within intracellular vesicles and limited translocation to the nucleus.[16, 103] For cationic polymers it is hypothesizes that tertiary amine groups become protonated inside the endosome or (phagolysome) due to normal acidification processes leading to an influx of Cl^- counter ions to restore charge neutrality. Subsequently, through osmosis excess water enters the endolysosomes causing membrane rupture.[104] This controversial process is referred to as "proton sponge escape.[105,106] In order for this process to occur the pKa(s) of the *tert*-amines must be low enough such that they are unprotonated at physiological pH and only upon acidification acquire the quaternary form. Some reports using simulations or direct measurements suggest that more than 50% are already protonated under physiological conditions.[107] In the original paper describing this phenomenon, all of the structures that displayed this property were true imines containing a double bond at the bridging nitrogens.[21] Nearly all of the plasmid delivery structures produced through the polymerization of imines have been reduced to *tert*-amines at the bridging nitrogens, hence no double bonds. The most recent studies suggest that proton sponge escape is probably not the driving force for the rupture of the endosomes.[108] The exact mechanism of endosomal escape is still open to debate.

Some studies suggest that poliplexes must escape the endosomes and release the DNA into the cytosol. The released DNA then makes its way to the nucleus for transcription.[19] However, the translocation of the DNA molecules through the nuclear pores it reported to be a relatively inefficient process with only 0.1% translocating into the nuclei. It has been proposed that the DNA is degraded in the cytoplasm and has difficulty traversing the small diameter of the nuclear pores.[109] One study found pDNA in the nucleus still complexed with its carrier. Breuzard et al.[110] found using FRET and FRAP analyses that LPEI-DNA complexes are present in the nucleus. This has raised the question of how the poliplexes cross the nuclear membrane. The authors suggested that the poliplexes can undergo modifications in order to pass and fit through the nuclear pores. Also, they proposed that the plasmid is unpacked in the cytosol and repacked in the nuclei. In contrast to some polymers, lipids are never found in the nuclei suggesting that only the pDNA is translocate across nuclear pores.[111]

While the mechanism of nuclear import of pDNA in complexes remains controversial, clearly the rate-limiting step, which determines transfection efficiency, lies between endosomal escape and translocation across the nuclear pore. Incorporation of nuclear localization signals and bimetallic nanorods made of Ni/Cu[112] that can associate with compacted DNA and target ligands simultaneously to the nucleus could be potential tools to enhance the DNA translocation step.

Cytotoxicity of Polyplexes

Among all of the features of polymers, molecular weight and chain length have the most impact on transfection efficiency and toxicity. Generally, high-molecular weight polymers display better transfection rates yet are found to be more toxic for most of cell lines.[53] The inherent toxicity of some polymers is an issue that still needs to be addressed. Researchers are constantly developing novel cationic structures or optimizing existing ones with a variety of motifs hoping to reduce toxicity.[113]

PEI, alone, can cause cell necrosis and apoptosis[114] and in some cases long-term expression is not achieved.[65] Hunter et al.[65] presented two phases of PEI cytotoxicity: Phase 1, cell membrane destabilization and trigger of apoptotic pathways and Phase 2, apoptosis and loss of Mitochondrial Membrane Potential (MMP), leading to drop the ATP production and loss of mitochondrial membrane integrity. It was found that *N*-acylation of a branched 25 kDa PEI can increase its gene delivery efficiency and at the same time reduce its cytotoxicity.[115] Conjugation of PEI with homo-bifunctional amine reactive reducible cross-linking reagents has improved the *in vitro* gene delivery efficiency in Chinesehamster ovary (CHO) cells, with a reduction in cytotoxicity[116] (Table II). The incorporation of cyclodextrins and targeting peptides has also raised their transfection efficiency and decreased cytotoxicity.[117]

Poly-amidoamine (PAMAM) and poly-propylenimine (PPI) are dendrimers that show high transfection efficiency.[118] However, the toxicity of dendrimers is the major concern limiting their medical use. Dendrimers interact nonspecifically with negatively charged biological membrane forming transient nano-holes leading to leakage of cytoplasmic elements and cell death.[119] Several approaches have been tried to reduce their toxicity including the design of more biodegradable and/or biocompatible dendrimers comprised of peptide and sugars. Surface modifications such the addition of PEG or Chitosan significantly reduces toxicity of these complexes.[120] Recently, Sarkar et al.,[121] conjugated *N*-maleyl chitosan (NMCTS) by Michael type addition reaction to improve solubility, transfection efficiency and low the toxicity (Table III). Pan et al.[122] reported as well high transfection activity with moderate toxicity of a copolymer made of poly(ethylene glycol)-poly-(L-glutamine) mixed with high molecular weight PAMAMs (Table III).

Outstanding issues regarding the bioactivity, metabolism and cytotoxicity of free polymers in the cytoplasmor in sub-cellular components needs to be fully characterized in order to design a less toxic poly-cations.[65] There are indications that these molecules could be used in medicine where cytotoxicity is desired such as cancer chemotherapy. Such cationic polymer organelle interactions may afford new routes to mitochondrial-mediated apoptosis.

POLYSACCHARIDE-BASED DNA CARRIERS

Cyclodextrins

Cyclodextrins (CDs) have been extensively studied as non-viral gene delivery vehicles due to their exceptional biocompatibility and high cation density.[123] Cyclodextrins by itself does not form stable complexes with pDNA and this has limited their application as a transfection system. For this reason they are being use mostly as enhancers with other cationic polymers or dendrimers dueto their ability to make the cell membrane more permeable by depleting or affecting membrane cholesterol levels[124,125] (Table III). However, CDs have shown potential for gene delivery due to their amphiphilic character, low immunogenicity and multiple reactive sites available for the attachment of target groups and cations. Cryan et al.,[126] modified CDs with pyridylamino, alkylimidazole, methoxyethylamino or primary amine groups at the 6-position of the glucose and reported significant luciferase expression in COS-7 cells. Darcy[127] and coworkers converted the terminal hydroxyls groups of CDs into amino groups and obtained transfection efficient on COS-7 cells comparable to Lipofectamine 2000®.

Chitosan

Chitosan is a polysaccharide obtained through the chemical treatment of the naturally occurring cross-linked polymer- chitin, the primary constituent of crustacean exoskeletons.[66] Chitosan has been extensively studied for non-viral gene therapy due to its exceptional biocompatibility and high cation density.[128] Gene delivery efficiency of chitosan is affected by a number of factors, including molecular weight, counter ions, degree of deacetylation, and the pH of the culture.[12] Even with optimized formulations, the application of chitosan-based gene delivery system is still limited by reduced water solubility, inefficient gene unpacking and low gene transfection efficiency. In recent years, various chitosan derivatives have been generated aiming to resolve these problems. The structural modification of these newer chitosan derivatives can be divided into hydrophilic and hydrophobic modifications.[129] Incorporation of negatively charged agents such as hyaluronic acid (HA) or poly(L-glutamic acid) (-PGA) with chitosan has increased its transfection efficiency significantly[130,131] (Table III). Such improvements in the transfection ability of chitosan-based carriers has be attributed to the reduced charge density upon the addition of HA chains or the formation of -PGA/chitosan/DNA complexes that can dissociate into smaller sub-particles after cellular internalization, both of which could improve the release of bound DNA. It should be noted that the choice of the type and the amount of anionic polymer incorporated to chitosan/DNA complexes greatly influences the transfection ability through changes in cellular uptake, stability, the size of the nanoparticles, and condensing and dissociating ability of the DNA. Chitosan has also been conjugated to diverse molecules and polymers such: poly-L-lysine (PLL), arginine, guanido groups, PEG (Polyethylene glycol), histidine, cysteine, glutathione, glutamic acid, galactose, targeting peptides and proteins, biotinylated, chondroitin sulfate, chitosan nanobubbles, PEI (poly(ethylene-imine), lipid shells and spermine, with the purpose of lowering the cytotoxicity and increasing DNA delivery efficiency.[75,132]

PEPTIDE DNA CARRIERS

The ability of some peptide sequences to translocate across cell membranes was discovered serendipitously through the observations of virologist working on the HIV-1 Tat trans-activating factor and by neurobiologists working on the Drosophila Antennapedia transcription factor.[133] Short basic amino acid sequence segments, rich in arginine residues, were identified as the critical component responsible for membrane translocation. This region has been given the term "peptide transduction domain" (PTD). After this discovery several variations of cell penetrating peptides (CPPs) have been reported in literature.[134] They vary significantly in their sequence, hydrophobicity, and polarity. However, common features are their amphipathic character and net positive charge. CPPs can be broadly classified into different structural categories: cationic peptides, amphipathic peptides, histidine-rich peptides and peptides containing nuclear localization signal (NLS).[135] Older examples of cationic peptides (or peptides with low amphiphilic character) are poly-L-lysine (PLL),[136] polyarginines,[137] penetrain[138] and TATp.[139] Examples of amphiphilic CPPs include MAP,[140] transportan[141] and KALA.[142] Pep-1[143] it is a well know amphiphilic peptide but it is has been used mostly as a protein carrier rather than DNA carrier. Histidine containing peptides, due to the presence of the imidazole group, can produce membrane destabilization and promote endosomalrelease.[144] A nuclear localization signal sequence has been incorporated to some peptides (NLS)[145] in order to promote the nuclear localization of CCPs upon escaping the endosome. NLSs are characterized by short clusters of basic amino acids that are recognized and bind to cytoplasmic receptors knows as imporntins. The most well know NLS comes from the large tumor antigen of the simian virus 40 (S40) made of only 7 amino acids PKKKRKV.

The conjugation of proteins or peptides to cationic liposomes appears to be a promising method for improving pDNA delivery *in vitro*.[146] Several publications have reported significant advances in transfection efficiency using theses combined complexes.[147–149] While the liposomes can efficiently interact with pDNA and promote endosomal escape, peptides can aid in targeting the complex to a specific type of cell and even the nuclei. *In vivo* delivery still has drawbacks due to inactivation by serum components, renal excretion and rapid clearance by immune phagocytic cells if the size of the complexes is

below or above 100 nm. Peptide adducts can affect the size and overall surface of the complexes making them more suitable for *in vivo* deliver. Satya et al.[150] synthesized 100 nm arginine-based cationic lipid nanoparticles. They reported that these nanoparticles, when mixed with DNA, formed large aggregates in the absence of serum but in the presence of serum small vesicles were observed. Transfection of HeLa cells with these complexes showed higher efficiency and less toxicity when compare to Lipofectamine 2000.

To further enhance gene delivery peptides have been conjugated to several synthetic polymers as well. The, pTAT sequence has been couple to PAMAM, PEG and PEI.[151] Kwot et al.[152] explored peptide dendrimers as a new type of transfection reagent. They synthesized a collection of dendrimers conjugated to different cationic and hydrophobic peptides motifs in conjunction with the lipid DOTMA/DOPE (Table III). The best results were observed for G1,2,3-KL ($(LysLeu)_8(LysLysLeu)_4(LysLysLeu)_2LysGlySerCys-NH_2$) with reported transfection efficiency up to 10-fold higher than commercial reagents.

Recently we developed a new type of cationic peptide particle that is comprised of two self-assembling branched amphiphilic peptides bis-(FLIVIGSII)-K-KKKK (h_9) and bis-(FLIVI)-K-KKKK (h_5).[153–155] In water, a 1:1 mixture of these two peptidesform water-filled bilayer delimited capsules. Solutes are encapsulated during the assembly process. When prepared at 25 °C and then cooled to 4 °C, extremely stable 20–30 nm capsules are formed that are resistant to temperature, chaotropes, proteases, detergents and the cell's degradative machinery. Initial investigations involving the entrapment and delivery of pDNA failed as the peptides appear to coat the surface of the DNA and cause its linearization. Under these conditions the N:P ratio was 131. Very low transfection rates were observed using this method. Subsequent lowering the N:P ratio to near unity results in compaction of the DNA with the peptides acting like histones. This lower ratio gives much higher transfection rates (~20% GFP). Recent refinements are pushing the transfection rates even higher and with no measurable toxicity.

Peptide-based gene delivery systems have some advantages over other gene therapy strategies.[156] For instance, peptides are more stable, easier to synthesize on a large scale than lipids, less prone to oxidation, less toxic and are easy to covalently modify with cell specific recognition ligands.[157] These ligands include vitamins, cholesterol, metals and antibodies. These adducts can be added through the formation of amines, esters and disulfide linkages.[158] Nevertheless, the transfection efficiencies are low compared with the liposomes and some polymers. Endosomal entrapment and poor nuclear import has been suggested as the main causes for reduced transfection efficiencies for pure peptide systems.[159]

DNA-Complexation, Cellular Uptake and DNA Release

Cationic peptides can efficient packed DNA into nanoparticles and prevent their enzymatic degradation. Peptides containing lysine and arginine have been extensively used for gene delivery, however the mechanism of how they bind and condense DNA is poorly documented in comparison to lipoplexes and polyplexes. Mann et al.[160] in a recent publication studied the different patterns of DNA condensation between lysine and arginine based homopeptides and correlated these structures with gene delivery. By varying the charge ratio and the length of the homopeptides they characterized six different types of DNA-peptide structures ranging in a size between 30–350 nm using atomic force microscopy. Results showed that a lower peptide to DNA charge ratios the tendency is to form road-like structures. Whereas, at higher peptide to DNA charge ratios compact spheres were observed. The authors proposed two structures for the arg- and lys-containing complexes, with a multi-molecular condensation pattern proposed for arginine containing peptides, with multiple pDNA molecules associated with the peptide and a monomolecular structure proposed with the incorporation of a single pDNA molecule with the lysine containing peptides.

There are many factors that are involved in determining the peptide cellular uptake including: exposed ligands, particle size, particle shape, cell type, presence of cargo and even culture conditions.[104] Currently, it is accepted that peptides can be internalized by two pathways: non-endocytic and endocytic.[161] The non-endocytic pathway has three prominent modalities to explain peptide internalization: fusion, inverted micelle and formation of transient pores in the cell membrane. Some references propose that a tight ionic interaction between the basic groups of the peptide side chains and the negative charges of the phospholipid heads induce a local invagination of the plasma membrane.[162] The local reorganization of the phospholipid bilayer would then lead to the formation of inverted micelles with the peptide enclosed in the hydrophilic cavity and ultimately resulting in cytoplasmic release. There are few reliable methods available to definitively identify non-endocytic pathways. For the energy depend pathways, macropinocytosis seems to be the primary route responsible for CPP-mediated intracellular delivery of DNA.[28, 163] However, as previously discussed nanoparticles can simultaneously enter cells through more than one pathway. Antp, nona-arginine, and the TAT peptide simultaneously used three endocytic pathways: macropinocytosis, clathrin-mediated endocytosis, and caveolae/lipid raft-mediated endocytosis.[164]

For cationic peptides, the mode of escape from the lysosome is thought to occur through anendosomallysis mechanism similar to that seen for lipoplexes.[28, 165] Histidine-rich peptides contain ionizable imidazole groups

that are partially unprotonated at physiological pH. Protonation through endosomal acidification could induce rupture of the endosomes as previously described. It has been suggested that TAT fusion proteins can enter the cell through endocytosis but escape the endosomal rout similarly thus evading the lysosomal degradation. Subsequently, they became localize near the perinuclear area.[166] The relatively low transfection level of PLL-DNA complexes is due possibly to the poor release of the complexes from endosomal compartments. Incorporation of histidine residues and lipids such a myristic, palmitic and stearic acids have shown improved release into the cytoplasm with a concomitant increase in transfection efficiency.

Peptide Cytotoxicity

Peptides are made of natural occurring aminoacids and display low toxicity at the concentrations typically employed. Very little has been reported in the literature associated peptide carriers with cell necrosis or apoptosis. This fact and the ability to easily penetrate cell membranes, could make them potentially the preferred carriers for gene delivery *in vitro* and *in vivo*. As far as we know, alterations in the cell physiology or immune responses have not been reported for pure peptide gene delivery systems.One report indicated that poly-lysines can induce mitochondrial-mediated apoptosis.[65] Cardoso et al.[167] have reported that some amphiphilic peptides can produce membrane perturbations and induce transient influxes of calcium ions however the report indicates that cell membranes rapidly recovered and no permanent cell damage was observed.

INORGANIC NANOPARTICLES

Inorganic nanoparticles are usually prepared from metals (e.g., iron, gold, silver), inorganic salts, or ceramics (e.g., phosphate or carbonate salts of calcium, magnesium, or silicon).[168] The metal ion salts produce complexes with a typical size range of 10–100 nm in diameter. The surfaces of these nanoparticles can be coated to facilitate DNA binding or target gene delivery. The small particle size offers several advantages including that they usually bypass most of the physiological and cellular barriers and produce higher gene expression. They can also be transported through the cellular membranes via specific membrane receptor or nucleolin, which delivers nanoparticles directly to the nucleus skipping the endosomal–lysosomal degradation. Nanoparticles have the ability to efficiently transfect post-mitotic cells *in vivo* and *in vitro*.[169] Additionally, they tend to show no to low toxicity and are immune silent. Supra-paramagnetic iron oxide-based nanoparticles display magnetic properties when placed in magnetic fields thereby allowing magnetic field guided delivery.[170] Progress in (*in vivo*) applications of inorganic nanoparticles has accelerated recently. However, extensive studies are still required to assess the effect of their types, sizes, and shapes on transfection efficiency. It is certain that further studies focusing on long-term safety and surface functionalization will foster future clinical applications.

Gold Nanoparticles

The ability of gold nanoparticles (GNPs) to interact with and enter cells has prompted researchers to attach various compounds and biological macromolecules to gold in an effort to combine functionality with cellular uptake. The loading of gold nanoparticles with drugs or genes offers the prospect of greater control and increased therapeutic efficacy. In particular, the combination of gold nanoparticles and laser irradiation to control the release of drugs and genes has the potential to provide useful therapeutic benefits.[171] The attractive features of gold nanoparticles include their monitoring by surface plasmon resonance, the controlled manner in which they interact with thiol groups, and their low-toxicity. Gold nanoparticles were functionalized with cationic quaternary ammonium groups and then

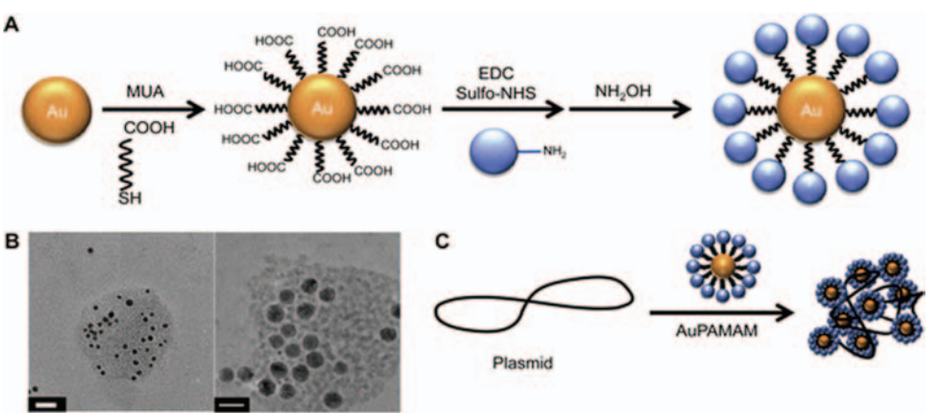

Figure 5. Biocompatible multi-walled carbon nanotube-chitosan–folic acid nanoparticle hybrids as GFP gene delivery materials. The scheme shows the synthesis of MWCNT-CS–FA NP hybrids. Reprinted with permission from [174], E. R. Figueroa, et al., Optimization of PAMAM-gold nanoparticle conjugation for gene therapy. *Biomaterials* 35, 1725 (2014). © 2014, Elsevier.

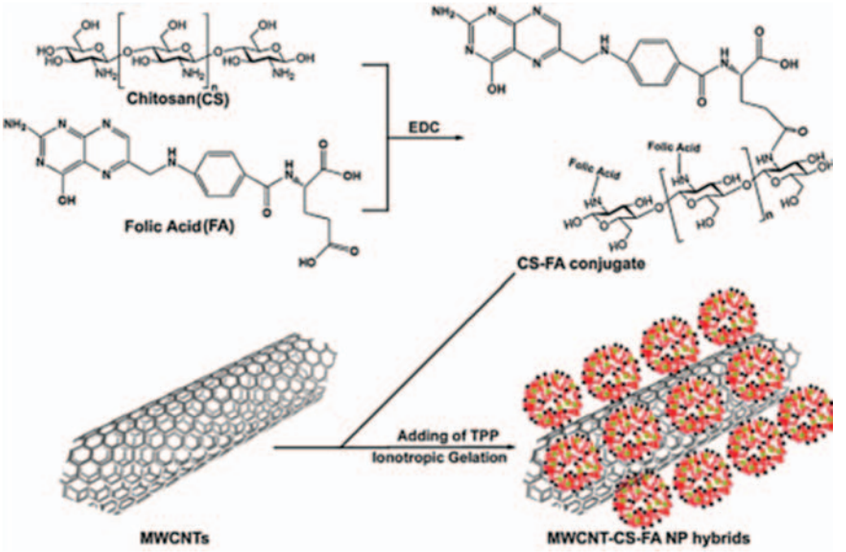

Figure 6. Biocompatible multi-walled carbon nanotube-chitosan–folic acid nanoparticle hybrids as GFP gene delivery materials. The scheme demonstrating the synthesis of MWCNT-CS–FA NP hybrids. Reprinted with permission from [178], X. Liu, et al., Biocompatible multi-walled carbon nanotube-chitosan–folic acid nanoparticle hybrids as GFP gene delivery materials. *Colloids Surf. B Biointerfaces* 111, 224 (2013). © 2013, Elsevier.

electrostatically bound to plasmid DNA. This composite particle could protect the DNA from enzymatic degradation and could regulate DNA transcription of T7 RNA polymerase.[172] In another report, cationic gold nanoparticles prepared by $NaBH_4$ reduction in the presence of 2-aminoethanethiol formed a complex structure with plasmid DNA expressing a luciferase gene.[171,173] Figueroa et al.[174] conjugated gold nanoparticles (AuNP) to polyamidoamine (Fig. 5). AuPAMAM conjugates have been synthesized by crosslinking PAMAM dendrimers to carboxylic-terminated AuNPs. This new hybrid system was capable of condensing and delivering pDNA in an efficient manner with low cytotoxicity.

CARBON NANOTUBES

Carbon nanotubes (CNTs) consist of carbon atoms symmetrically arranged in sheets of graphene. Bianco et al.[175] were one of the earliest practitioners in the use of carbon nanotubes for gene delivery. They synthesized a modified carbon nanotube using the 1,3-dipolar cyclo-addition of azomethineylides. Both single walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) were functionalized with a pyrrolidine ring bearing a free amine-terminal oligo-ethylene glycol moiety attached to the nitrogen atom. This functional group increased the solubility of carbon nanotubes and enhanced their ability to bind and condense DNA. CHO cells were transfected using these functionalized carbon nanotubes, delivering pDNA containing β-galactosidase as a marker gene.[176] Nunes et al.[177] have transfected lung epithelial (A549) using carboxylated MWNTs conjugated to the cationic polymers polyethylenimine (PEI), polyallylamine (PAA), or a mixture of the two polymers. Liu et al.[178] have reported the conjugation of MWNT with chitosan-folic acid nanoparticles and transfected cells using the gene reporter GFP (Fig. 6). They observe an increase on transfection efficiency and reduction of cytotoxicity with this functionalization. Compared to the traditional delivery vehicles, the major advantages provided by carbon nanotubes are the following:

(1) they are easily translocated across cell membrane
(2) are capable of achieving spatially- and temporally-controlled release for targeted gene silencing due to their strong adsorption in (near infra-red) NIR range,
(3) their influence on conformation and conformational transitions of DNA/siRNA due to their unique shape, modifiable surface chemistry, and their remarkable flexibility, and
(4) their ability to be monitored for therapeutic effects of DNA/siRNA due to their extremely stable and strong Raman signal and NIR fluorescence emission.[179]

SUMMARY

A significant number of diverse methods are currently available for pDNA delivery *in vitro*. Reports are emerging with great frequency describing modified or completely new transfection materials and protocols that do not require virus particles. For chemical carriers, there are several factors that can affect the transfection efficiency: cell type, carrier/DNA ratio, particle size, toxicity, solubility and stability in serum. Lipid and polymer base systems conjugated to peptides or inorganic nanoparticles seem to be the some of the most promising non-viral vectors for

pDNA gene delivery *in vitro*. It is important to critically analyze combination of these elements in order obtain high delivery efficiencies using the current generation of biomolecules. Researchers are undoubtedly trying to generate the next generation of amphiphilic cationic molecule capable of overcoming the extracellular and intracellular obstacles for *in vitro* and *in vivo* gene delivery taking into account the advantages and disadvantages of the current systems. Using current screening technologies this will be a time- and energy-intensive endeavor. The development of high-throughput methods for assessing transfection systems is needed and will be vital for optimizing the numerous parameters involve in this complex process.

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