Chapter 1

Non-viral vectors for gene therapy

1.1 The gene therapy

"There are only three problems in gene therapy - delivery, delivery, and delivery"

M. Verma in Jaroff, L. TIME, 1999; Jan 11

With the prompt conclusion of the project related to the definition of the human genome sequence the principle to treat genetic disorders delivering the correct genes to the target cells emerged as an alternative to the use of synthetic drugs.

This strategy is known as gene therapy, an experimental treatment that can be used to correct the sequences of altered genes responsible for the development of many human deseases, because almost all have a genetic component.[1] Among these, some have a very high social impact, such as tumours, cystic fibrosis, AIDS, and cardiovascular and neurological ailments.[2] These premises constitute an important reason to contribute to research in this sector, which could have big effects in the future therapeutic field.

A key element at the basis of the gene therapy development concerns the transport of the genetic material, which is defined cell transfection and allows to express specific proteins which the sick cell necessitates and it is not able to synthesize, or causes the death of the cell itself, if it is degenerated, as for example for tumour cells or again, can silence a defected gene. Therefore, the gene therapy can be performed[3] substituting or integrating the aberrant gene with a functional one, or by the delivery of a suicide gene,[4] or via transfer of genes for the synthesis of new therapeutic proteins, or by introducing a nucleic acid able to block the transcription of an altered gene. Although the efforts of many international research groups, the complete knowledge on the molecular mechanisms of an efficient and safe cell transfection is still missing.

An easy cellular uptake of free DNA via plasma membrane permeation is hindered by the size and negative charge of the genetic material, so a vector is needed to carry it.[5] The ideal vector should satisfy certain requirements, like specificity for the target, low immunogenicity and toxicity, and high transfection efficiency. Systems which function as vectors for genetic material *in vivo* in a way efficient and selective enough are not common and at the moment very few examples of effective application of this therapy exist.[6]

The vectors used up to now can be divided in two fundamental classes: the viral and non-viral vectors. The viral vectors, in which the own genome is manipulated in order to eliminate the pathogenic component (inactivation) and substituted with the therapeutic gene, are characterized by a great transfection capacity in terms of efficiency and selectivity, which are

inherent to the nature of virus, that commonly attacks the cells and transfers inside its genetic material. Nevertheless, the use of viral vectors presents some limits among which the complex procedures of inactivation, the complicate and expensive preparation process, the production in rather scarce quantities and the potential problems for the patient, since these vectors tend to induce a not negligible immune response and inflammatory processes. They are also potentially capable of generating replication competent virus through various recombination events with the host genome and can produce insertional mutagenesis through random integration.[7]

To avoid these issues, as an alternative, non-viral gene delivery protocols make use of physical methods or synthetic chemical vectors or both to deliver the gene of interest. While several studies have shown that free DNA can be introduced into cells through electroporation,[8] by a "gene gun",[9] or direct injection into the target tissue,[10] the clinical relevance of these methods is limited, due to inefficient uptake and poor biostability.

Synthetic chemical and biotechnological methods are more extensively used, based on non-viral vectors. While being at the moment less efficient and selective than viral ones, non-viral vectors result less immunogenic, can be obtained in big quantities and are synthetically versatile. Given these extremely valuable advantages offered by non-viral compared to viral delivery systems, the realization of new non-viral vectors able to overcome, or at least minimize, the drawbacks of those currently known is considered a fundamental result for the possible success of the gene therapy.[11]

1.2 The cellular barriers

To design an ideal non-viral system for gene delivery, it is necessary to identify and understand the cellular barriers and obstacles the vector has to pass to arrive to the cell nucleus with its precious cargo. Several steps must be successfully completed to achieve efficient transfection and they include the binding to the cell surface, the crossing of the plasma membrane, the escape from the lysosomal degradation, and the overtaking of the nuclear envelope (**Fig. 1.1**).[12] Essentially, most working systems are characterized by positively charged groups, able to interact electrostatically with the negative charge of the phosphates of the nucleic acids and of the cell membrane, and a lipophilic part, which determines the nucleic acid condensation. Also high concentrations of neutral polymers such polyethylene glycol and polyvinylpyrrolidone can compact DNA through nonspecific interactions by excluding solvent volume.[13] The morphology of vector-cargo ensembles is

dictated by a combination of many factors,[14] primarily the type of vectors, but even concentration, DNA:vector ratio defined also as N/P (N: positive charges, P: phosphates), pH, ionic strength and preparative details.[15] With a diameter of 10-100 nm, the resulting complex where nucleic acid is masked and condensed generally enters the cell via endocytosis in a non-specific way, if no specific ligands are present on the surface of the carrier. Condensates with diameters smaller than 100 nm are of optimal size for transfection as this value corresponds to the diameter of the coated pit in the receptor mediated endocytosis. A receptor-mediated pathway, more useful for *in vivo* delivery, can be promoted by functionalizing the vector-DNA surface with specific ligands, such as folic acid, transferrin, epidermal growth factor,[16] and RGD motif.[17]

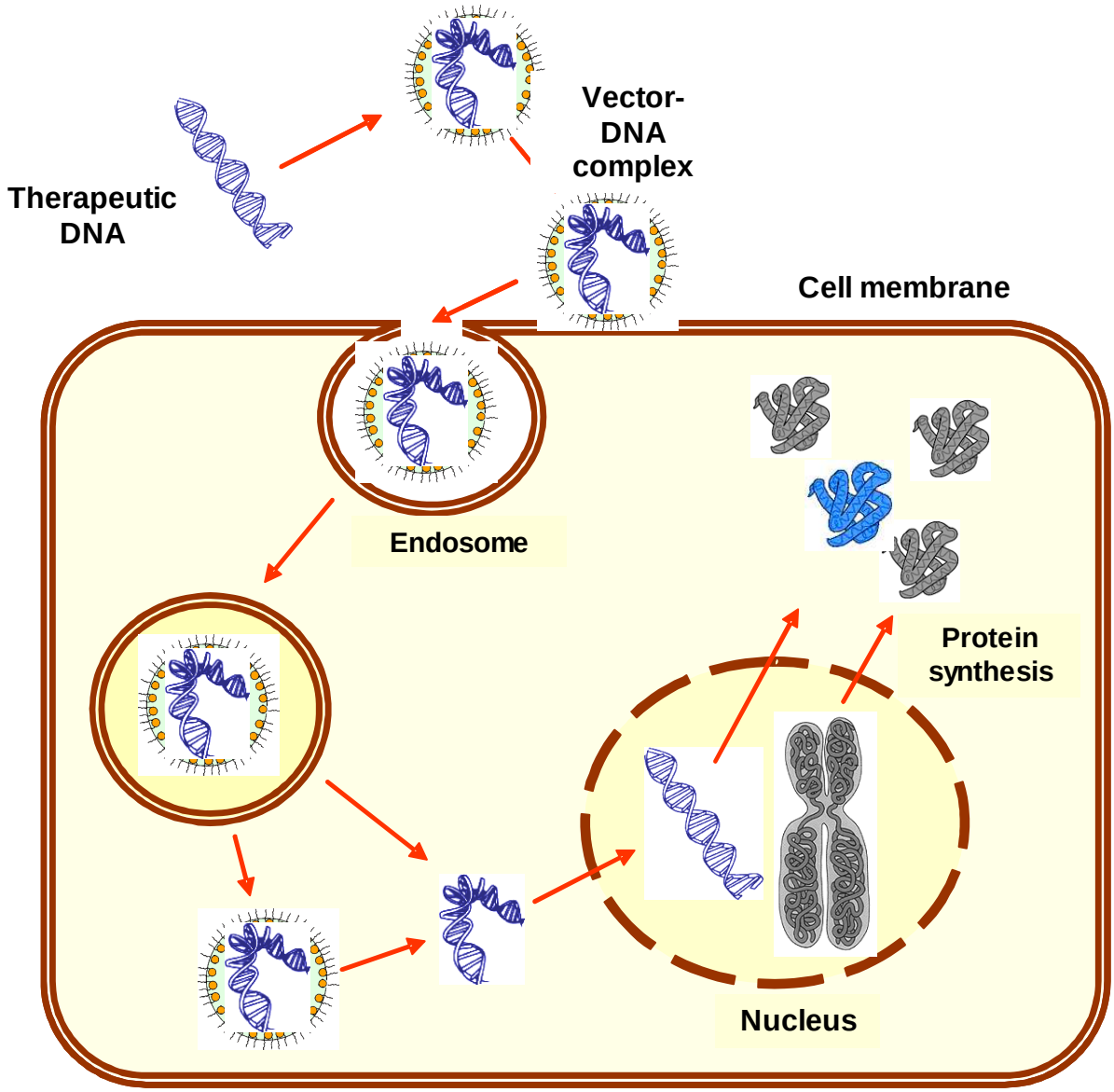


Fig. 1.1 Fundamental steps of the cell transfection process.

In the whole process, DNA has also to be protected from nucleases. Viruses achieve this by packaging their genetic material within a viral capsid, capable of preserve it from damages by many degrading agents. The condensation into a compact form by the non viral vectors is an approach to simulate this phenomenon and overcome degradative enzymes protecting the sites vulnerable to cleavage.[18]

Once internalized, the vector-DNA complex is wrapped up into an endosomal compartment, from which it must escape not to be degraded for a successful transfection. The endosomal escape can occur by at least two mechanisms and it must naturally be faster than degradation.[19] In the case of polyamine vectors, the buffer capacity of the vector-DNA complex that counteracts endosomal acidification (pH 5) may lead to high osmotic pressure, swelling, and rupture of the vesicle;[21] on the other hand, amphiphilic molecules may also interact directly with the endosomal membrane, destabilizing it, and releasing the genetic material into the cytosol.[21]

Regardless of the route of entry, complexes would first be brought into the early endosomes, which would fuse with other endocytic vesicles, more commonly late endosomes that exocytose internalized products. The process of fusion between early and late endosomes is brought about by lowering pH to 5 within the micro environment of the former.[22] 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, where complexes would be immersed in an environment of acidity and degrading enzymes that would destroy the DNA. Hence, for a successful gene transfer to take place, complexes have to escape from the endosome before they fuse with lysosomes. A strategy to facilitate endosomal escape is to use amines with different pKa. (See **Introduction** of **Chapter 4**).

For the delivered DNA to be functional, it has to be transported into the nucleus where it can be transcribed into mRNA and ultimately translated into protein. The movement through the cytosol and into the perinuclear region is very difficult by active transport. It has been popularly believed that DNA complexes arrive at the perinuclear region by slow diffusion, but some molecules are helped by microtubule motor functions[23] or using nuclear localization sequences. The entry from the perinuclear region into the nucleus is governed by the nuclear membrane, one of the most challenging barriers to cross. 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 limits the traffic of larger

molecules of more than 50 kD. The nuclear entry is thought to occur mainly during cell division, when nuclear membrane is broken for mitosis.[24]

1.3 Non-viral vectors

Usually the approach to the non-viral vectors starts from the synthesis of cationic polymers[25] and cationic lipids,[26] which are able to complex DNA forming polyplexes and lipoplexes, respectively, or lipopolyplexes, when used in mixture containing molecules from both classes. However, more recently, new series of non-viral carriers have been developed, taking advantage from new techniques and/or technologies, such as metallic nanoparticles and carbon nanotubes (see below).

Cationic polymers use multiple cationic sites to yield strong nucleic acid binding, whilst cationic lipids achieve this by assembling into highly charged aggregates.

1.3.1 Cationic polymers and dendrimers

In this class of vectors we can include polymers, polysaccharides, polypeptides and dendrimers, which allow a high level of design flexibility.[5] Usually they interact with the DNA through electrostatic interactions by means of ammonium ions. Poly(L-lysine), polymethacrilates and polyethylenimine (PEI) (**Fig. 1.2**), also variably modified and derivatized, are widely used as vectors. PEI, in particular, when used in branched forms results very efficient in transfection.

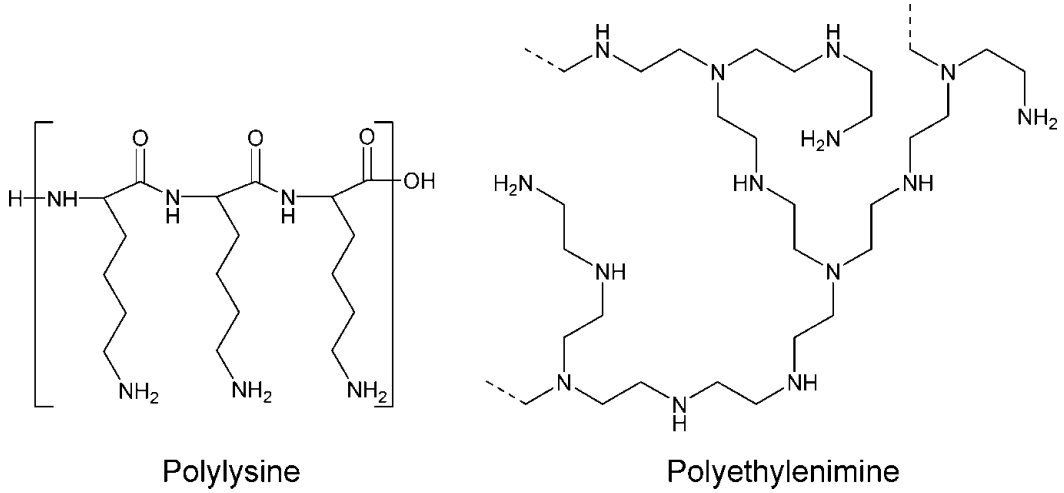


Fig. 1.2. Structural formula of "off the shelf" gene delivery polymers.[1b]

Chitosan, dextrans and carbohydrates containing polymers are also used for these purposes. Natural polypeptides and analogues can result useful in this context because of their

properties as molecular transporters and cell penetrating agents.[27] Covalently or non covalently attached to an otherwise poorly bioavailable drug, drug candidate or probe, enhance or enable its passage through biological barriers. Among these, very interesting are the guanidinium rich transporters,[28] the polyarginine[29] and the amino acid sequences specific for the central nervous system.[30]

Dendrimers are versatile, derivatisable, well-defined, compartmentalised chemical polymers with sizes and physicochemical properties resembling those of biomolecules.[31] In the field of gene delivery, cationic dendrimers are an important class of vectors.[32] Those based on poly(L-lysine),[33] PEI and poly(amidoamine) PAMAM have been widely investigated and have shown a relatively good efficiency in transfection of various animal cells.[34]

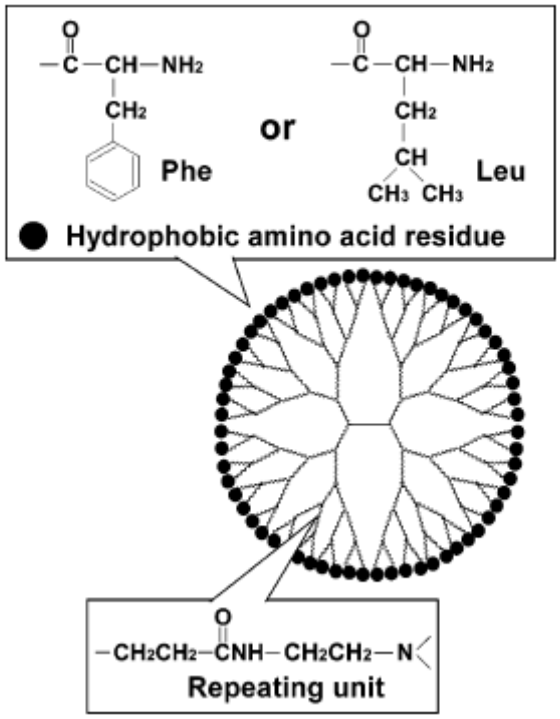


Fig. 1.3. Structure of phenylalanine- or leucine-modified PAMAM G4 dendrimer.[39]

Molecular weight of the dendrimers is stepwise increased via the repetition of a reaction sequence, so their size and structure are highly controllable and their molecular weight distribution is generally very narrow.[35] Dendrons attached via the focal point to hydrophobic units, such cationic lipid-like systems, exhibit good transfection activities.[31c,36] Many modifications have been performed on these structures: Haensler and Szoka attempted to improve their transfection activity by conjugation of a pH-sensitive membrane active peptide that destabilizes the endosomal membrane and promotes the escape of DNA into the cytosol.[34a] Arima and colleagues prepared conjugates of PAMAM with cyclodextrins, and this conjugation greatly enhanced the transfection activity of this type of dendrimers.[37] In

addition, modification of PAMAM dendrimers with various molecules, such as poly(ethylene glycol),[38] demonstrably improves their efficiency for gene and oligonucleotide delivery. Poly(amidoamine) dendrimers with phenylalanine or leucine residues at their chain ends were also designed (**Fig. 1.3**)[39] and efficient gene transfection of cells through synergy of the proton sponge effect was achieved, which is induced by the internal tertiary amines of the dendrimer, and hydrophobic interactions by the hydrophobic amino acid residues in its periphery.

1.3.2 Cationic lipids

Cationic lipids present three main parts: a polar head group, which allows the binding of DNA, a lipidic chain, which helps self-association through hydrophobic interactions forming micelles or liposomes, depending on its packaging (**Fig. 1.4**) and a linker to interconnect the two functional

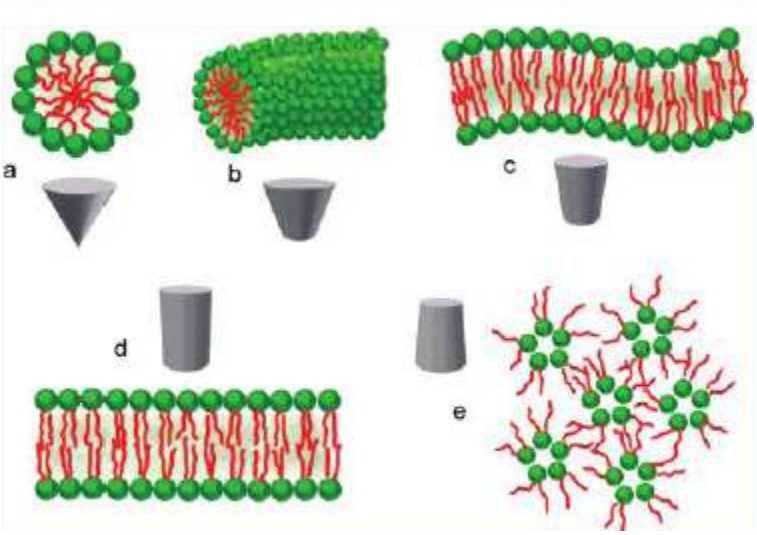


Fig. 1.4. Schematic description of the supramolecular assemblies formed by amphiphiles as a function of their critical packing parameter C_{pp}. a) $C_{pp} < 0.33$, formation of micelles; b) $0.33 < C_{pp} < 0.5$, formation of cylindrical micelles; c) $0.5 < C_{pp} < 1$, formation of vesicles and flexible bilayers; d) $C_{pp} \sim 1$, formation of planar bilayers; e) $C_{pp} > 1$, formation of inverted micelles.[31e]

components (**Fig. 1.5**). It is important to recognize that the transfection efficiency is not determined solely by one part of the cationic lipid but by a combination of all of them.[3a]

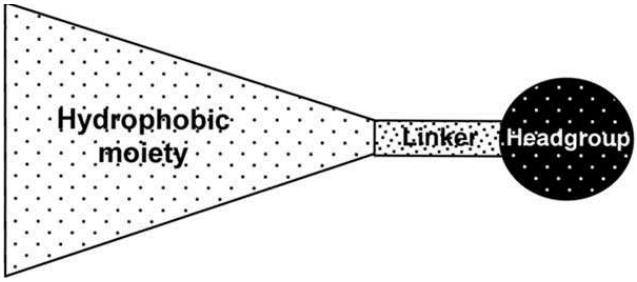
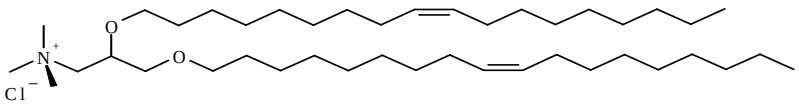
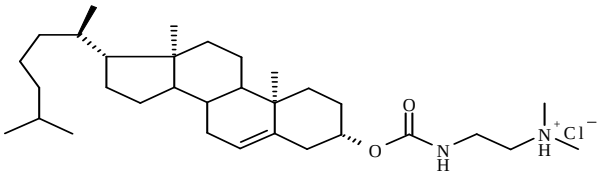


Fig. 1.5. Scheme of the three cationic lipid components.

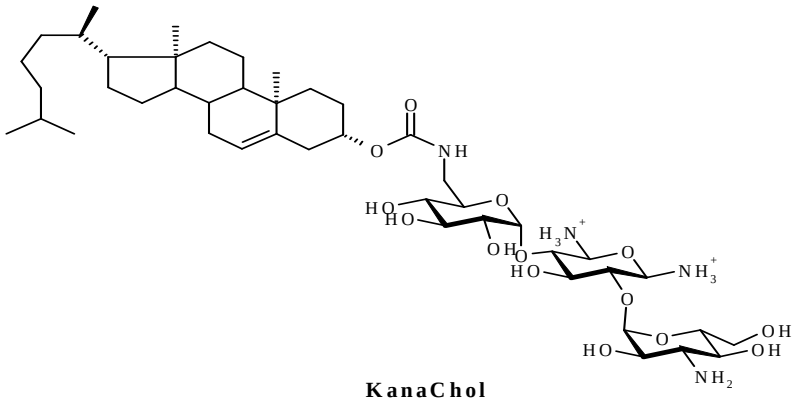
Cationic lipids and DNA interact forming the so called lipoplexes. In general, these delivery systems present high transfection efficiency *in vitro* although it lowers when used *in vivo*, but the relatively ease of preparation and the availability of a variety of cationic heads and lipophilic tails gives the advantage to allow the generation of a wide variety of molecules (**Fig. 1.6**).



DOTMA



DC-Chol



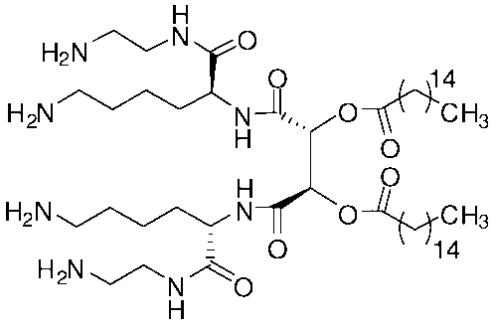
KanaChol

Fig. 1.6. Structural formula of some cationic lipids.

An interesting class of cationic lipids is constituted by dimeric surfactants,[40] where two identical moieties are covalently joined together at the polar head level, and have been shown to possess unusual aggregation and biological properties. Their use as surfactants has developed very quickly for their advantages over the monomeric ones owing to their increased surface activity, lower critical micelle concentration (CMC), and useful viscoelastic properties.[40a,41] Among these so called gemini surfactants, the most studied structures under the profile of biological activity and of chemico-physical properties are the bisquaternary

ammonium salts (bisQUATS), and, among these, derivatives of N,N-bisdimethyl-1,2-ethanediamine.

Quite recently, the use of gemini surfactants as non-viral vectors in gene therapy has been proposed, on account of the possibility to take advantage of their cationic character necessary for binding and compacting DNA and of their superior surface activity (**Fig. 1.7**).[42]

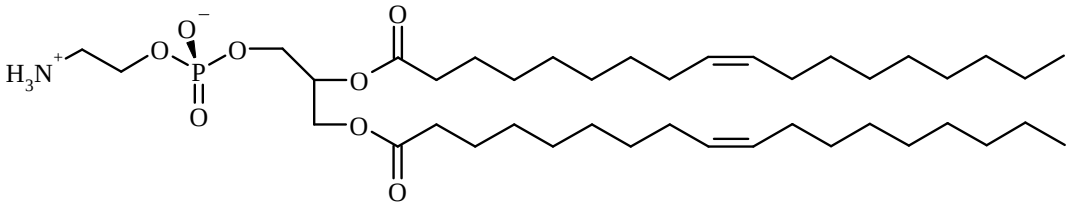


I

Fig. 1.7. Structural formula of Gemini **I**.[43]

At high concentration sometimes the cationic lipids result quite cytotoxic, so another approach is their use in presence of a neutral lipid.[44]

Cholesterol and DOPE (**Fig. 1.8**) are some of the most used neutral co-lipids, also called helper lipids, which guarantee a good compromise between efficiency and toxicity.



DOPE

Fig. 1.8. Structural formula of DOPE.

To clarify the mechanistic role of cationic lipids in gene delivery, apart being an efficient template for lipoplex assembly, an understanding of their properties in terms of the packing parameter *P* has proven highly useful.[45] *P* is defined as the ratio of the cross-sectional areas of the hydrophobic hydrocarbon chains and the polar head group.[46] Cationic lipids with a relatively small head group and unsaturated alkyl- or acyl hydrocarbon chains, occupying an extended area in a wedgelike manner, have a tendency to adopt the inverted hexagonal H_{II} phase (**Fig. 1.9**). A similar cone-shaped structure is preferred by the DOPE ($P > 1$), as

opposed to the lamellar L_α structure, adopted by most other phospholipid species, which show a cylindrical shape (P ~1).

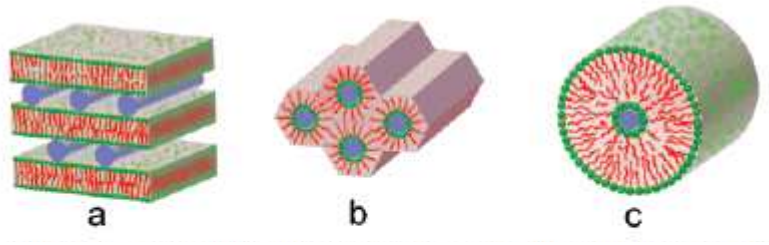


Fig. 1.9. Schematic representation of the three main cases of lipoplex geometry: a) Lamellar phase L_α^C; b) hexagonal phase H_{II}^C, and c) lipid bilayer-coated DNA strands, likely a metastable phase. The blue cylinders are the DNA strands. The names of the first two phases come from the lyotropic phases of pure amphiphiles, the superscript C standing here for "complex".[31e]

In contrast, micellar (non-inverted) structures are preferred by lipids with a head group that occupies a relative large surface area as compared to the cross-sectional area taken by the hydrophobic chains (P < 1), such as in the case of lysophospholipids. Near neutral or acidic pH.[47] DOPE becomes zwitterionic. Thus, around neutral pH, when P > 1, DOPE alone undergoes a lamellar to H_{II} phase transition, which occurs at temperatures above 10-15 °C. Cationic lipids like DOTAP and SAINT-2 (**Fig. 1.10**) prefer a bilayer organization. However, mixing the cationic lipid with DOPE will affect the packing parameter and when this additive average packing parameter becomes sufficiently large (>1), the system will revert to an hexagonal phase as observed for DOTMA,[48] DOTAP,[49] and SAINT-2[50] when mixed with an equimolar amount of DOPE.

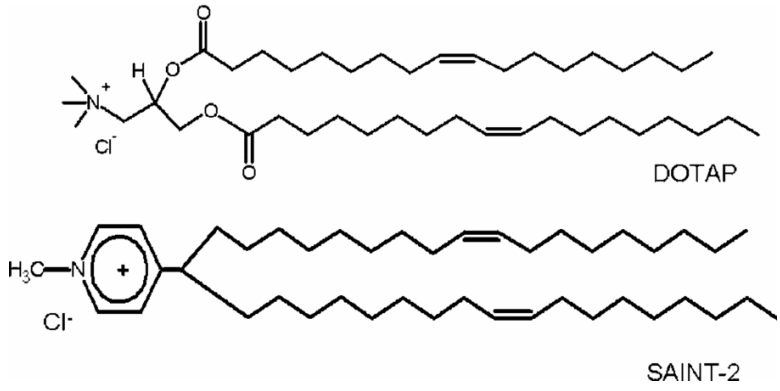


Fig. 1.10. Structural formula of DOTAP and SAINT-2. From www.biochemsoctrans.org

Within the hexagonal phase adopting lipoplexes, lipid-coated DNA strands are arranged on a hexagonal lattice, contrasting the sandwich organization of DNA between lipid bilayers for

lipoplexes in a lamellar organization.[49a] The efficiency of DOPE to promote the hexagonal phase in cationic lipid containing systems may depend on conditions. This approach is often taken to "stabilize" lipoplexes, providing stealth properties that preclude (extensive) clustering of the complexes, which is obviously undesirable for in vivo application of these devices

Nanoparticles

Quantum Dots

Quantum dots (QDs) are semiconducting nanomaterials that, due to their physical size and composition, present bright fluorescence, narrow emission, broad UV excitation, and high photostability with numerous advantages over traditional organic dyes.[51] These characteristics, jointly with the possibility of bio-functionalising them, offer great potential for biological and medical applications, especially for imaging and sensoring.[14] Some examples have been also used in gene therapy, like functionalized silanised CdSe/ZnS QDs with a nuclear localisation signal peptide[52] and fluorescent silica nanotubes, incorporating CdSe/ZnS QDs, functionalized with 3-(aminopropyl)-trimethoxysylane, to generate a polycationic surface.[53]

Silica nanoparticles

Surface-modified silica nanoparticles are attractive candidates for gene delivery for several reasons. Compared to cationic carriers, colloidal silica nanoparticles are inert and exhibit less cytotoxicity, and compared to lyposomes, silica particles are more stable with respect to physical stresses such as aerosolisation.[54] Finally, they are not affected by freeze-drying so they can be stored conveniently.[55] The most commonly used method to exploit silica for gene delivery is by functionalizing the surface of the nanoparticles with aminosilanes.[56]

Nanotubes

Carbon nanotubes are allotropes of carbon with a cylindrical nanostructure. For their intrinsic nature it is important to functionalise their surface to get them water-soluble and biocompatible. Carbon nanotubes were exploited *in vitro* and *in vivo* for their high surface area, their easy functionalisation, and their ability to cross the cell membranes, and recently have been applied to drug and gene delivery.[57] They were also shown to transport RNA into mammalian cells[58] and to perform gene silencing.[59]

Fullerenes

A fullerene is a pure carbon molecule composed of at least 60 atoms of carbon, with the shape of a hollow sphere. Biological applications of fullerenes continue to attract increasing attention,[60] and in drug delivery they resulted useful systems because can be multifunctionalized, act as drug absorbents and form particles in the nanoscale range.

There are some examples in the field of gene delivery of fullerenes derivatized with ammonium and alkyl ammonium groups.[61]

Macrocycles

There are very few examples of non-viral vectors based on macrocyclic scaffolds, some are of natural origin, like cyclodextrins, other unnatural, like calixarenes and porphyrins.[62] The first two scaffolds are going to be discussed in more details.

Cyclodextrins

To overcome the toxicity of many non-viral gene delivery agents, β-cyclodextrin intrinsic availability, biocompatibility, tubular symmetric framework with well differentiated faces, and various functionalization patterns that can be modified in a tunable manner, were exploited together with their non-immunogenicity, and biodegradability.[63]

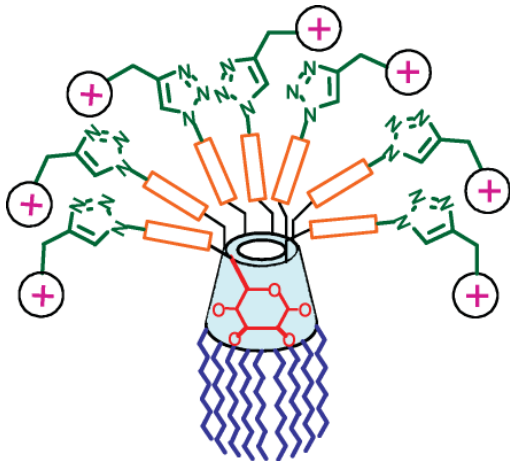


Fig. 1.11 Schematic representation of βCD-scaffolded amphiphilic polycationic "click clusters". The rectangular boxes account for additional spacer elements.[67b]

CD-DNA nanoparticles (*CDplexes*) have been prepared with polyaminothiourea and lipophilic tails, and exhibit transfection efficiencies significantly higher than PEI-based polyplexes.[64] Some cyclodextrins have been decorated with the guanidinium group,[65] obtaining a vector with a transfection capacity comparable to Lipofectamine 2000.[66] It was shown that β-cyclodextrin polymers created with alternating saccharide and

oligoethyleneamine monomers, obtained via click chemistry, (**Fig. 1.11**) can effectively complex, condense, and deliver DNA with exceptional biocompatibility and efficacy.[67]

These molecules were also incorporated into cationic polymers, derivatized by ammonium and amidine cations[68] and have been exploited as rings blocked in a polymer chain, building a cationic polyrotaxane which could condense DNA in small nanoparticles showing low cytotoxicity and good gene-transfection efficiency.[69] Besides, they have been used as drug-delivery agents for their ability to protect drugs from physical, chemical and enzymatic degradation, and even to solubilize hydrophobic drugs,[70] and to synthesize gold nanoparticles covered with a targeting antibody for non-covalent incapsulation of an anti-cancer drug.[71]

Calix[n]arenes

Calix[n]arenes[72] are synthetic macrocycles derived from the condensation of phenols and formaldehyde.

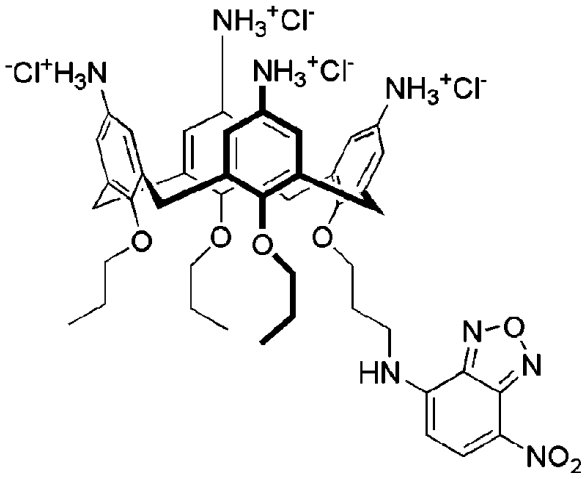


Fig. 1.12. Structural formula of the fluorescent calixarene **II**.[85]

The shape, size and conformational properties of these molecules can be fine-tuned by varying the procedures adopted for their synthesis. Calixarenes have recently been shown to have interesting potential in a wide range of biological process and systems. They have been derivatized with hydrophilic molecules to make them soluble in water and suitable for applications, like drug discovery,[73] as ion channel mimics,[74] as enzyme mimics,[75] as agents for the surface recognition of proteins[76] as platforms for magnetic resonance imaging agents,[77] as antitubercolosis agents,[78] as drug delivery systems, including solid lipid nanoparticles,[73c,79] antimicrobials,[78a,78c,80] antibacterial,[81] antifungal.[82] Some calixarene derivatives are able to interact and recognize the DNA,[83] and also to perform gene transfection.[84] With this aim, multicalixarenes bearing glycine units were obtained, but they

showed a low transfection efficiency.[84] A calix[4]arene was derivatized with a fluorophore (**Fig. 1.12**), and its cellular uptake was proved to be a non-specific process, not linked to either of the main endocytic pathways, with an accumulation of the molecule in the cell cytoplasm.[85]

Actually the first examples of calixarene-based gene vectors were prepared in our research group. Upper rim guanidinium calix[n]arenes were prepared (**Fig. 1.13**), able to condense plasmid and linear DNA, and to perform cell transfection in a way which is strongly dependent on the macrocycle size, lipoph **II** , and conformation.[86]

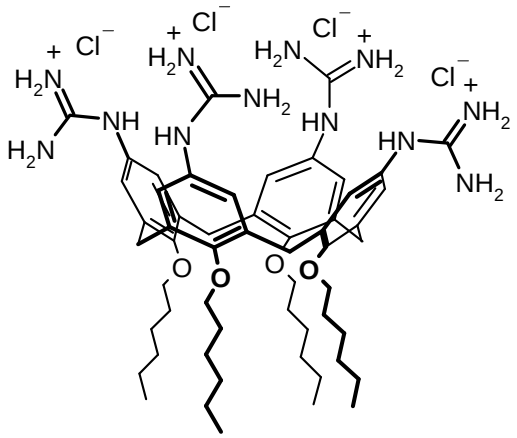


Fig. 1.13. Example of a structural formula of one of the guanidinium upper rim Calix[n]arenes synthesized in our group.

However these compounds resulted characterized by low transfection efficiency and high cytotoxicity, especially at the vector concentration required for observing cell transfection (10-20 μM), even in the presence of the helper lipid DOPE.

1.4 Conclusions

As outlined at the beginning of this chapter over the last few years the development of several methods for delivering genes into cells has stimulated great interest in the possibility of treating human disease by gene-based therapies. However, despite substantial progress, a number of key technical issues remain to be solved that gene therapy can be safely and effectively applied clinically.

Cationic lipid-mediated DNA delivery is one of the most promising approaches for gene delivery and much progress has been made in their development for *in vitro* and *in vivo* applications. Various parameters affecting the aggregation properties, the complexation with nucleic acids and their further application toward gene delivery have been optimized by different research groups. The factors such as lipid architecture composition, lipid/DNA

charge ratios, different cell types, ionic strength, and lipoplex structures need to be optimized for the successful application of gene delivery. The future of synthetic non-viral vectors is also the creation of systems that have been self-assembled in a modular and sequential fashion from tool-kits of well-defined chemical components and they should be formulated with nucleic acids in a reproducible and scalable manner giving discrete and well-defined narrow particles.[87]

Because diverse kinds of cationic lipids have been designed, which still have a low transfection efficiency compared to their viral counterparts, there remains a need for a continous improvement in the design and synthesis of more efficient gene delivery systems to different kinds of cells *in vivo* and their ultimate use in gene therapy,[3a] together with a deeper understanding of the barriers to efficient transfection.

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