Chapter 1

Gene therapy and non-viral vectors

1.1 The gene therapy

The recent collection of huge amount of data on human genoma offers to the medical research community an unparalleled opportunity to investigate the genetic basis of diseases and to operate on their activity to bring benefits on human health through the realization of the so called gene therapy..[1]

Gene therapy can be performed substituting (or integrating) an aberrant gene with a functional one, or inserting a suicide gene, or delivering gene for the synthesis of new therapeutic proteins, or introducing a nucleic acid able to block the transcription of an altered gene. It is also possible to insert a therapeutic gene to teach stem cells. The treatment of the cells can be done *in vivo* or *ex vivo*.

After the first enthusiasms that accompanied the gene therapy in its first developments and after a substantial slowdown of the research because of a series of failures and the death of a young patient in 1999 during a clinical trial, now it seems that, despite this is not yet a complete established clinical reality, first promising results could make it as integral part of the strategies to fight diseases in the next future. Some pathologies in fact have successfully been treated in the last years with this technique,[2] such as severe immunodeficiencies, the Wiskott-Aldrich syndrome, beta-thalassaemia, haemophilia, a congenital form of blindness, inherited skin adhesion disorders and adrenoleukodystrophy, a neurological disease affecting myelin. Moreover, gene therapy can in perspective find application also in the treatment of diseases with a wide social impact such as Hiv, leukemia and cardiovascular pathologies. Currently, the main problem to solve and at the same time the key for the success of gene therapy is to make available safe and efficient vectors for the delivery of the genetic material into the cell nucleus.

Several studies showed that free DNA (naked DNA) can be introduced into cells through electroporation,[3] by a “gene gun” (gold nanoparticles coated with DNA),[4] or direct injection into the target tissue,[5] but the clinical relevance of these methods is limited, due to inefficient uptake and poor biostability. In fact, free nucleic acids are rapidly degraded by serum nucleases [6] and their cellular uptake via plasma membrane permeation is hindered by the size and the negative charge of filaments. A vector is thus needed to carry them.[7] It must protect and compact oligonucleotides and satisfy certain requirements like specificity for the target cells, low immunogenicity and toxicity, and high transfection efficiency.

Research studies widely focused on viral carriers, including both retroviruses and adenoviruses, as these vectors exhibited high efficiency in delivering both DNA and RNA to numerous cell lines.[8] The applications mentioned above were performed using viruses as 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 severe limits and criticality among which the complex procedures for inactivation, the complicate and expensive preparation processes, the production in rather scarce quantities and, more importantly, the potential problems for the patient, since these vectors tend to induce a violent immune response and serious inflammatory processes. They are also potentially capable of generating replication competent viruses through various recombination events with the host genome and can produce insertional mutagenesis through random integration.[9]

These limitations stimulated the make synthetic vectors an attractive alternative to the viral ones. Advantages of non-viral vectors, in fact, include in general their non-immunogenicity, low acute toxicity, simplicity and feasibility to be produced on large scale. There are, however, some drawbacks, including their lower efficiency respect to viral vectors in gene transfer and the transient gene expression associated to their use, the substantial lack of cell selectivity and, in some cases, a not negligible toxicity that makes them not yet mature substitutes of viruses.

To develop winning strategies and obtain high level of transfection, there is not only a need for novel delivery systems; it is also needed to understand the mechanism of delivery how the vector can pass the cellular barriers to reach 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 right way of internalization and its transport into the nucleus to be expressed (**Fig. 1.1**). Essentially, the most effective systems are characterized by positively charged groups, able to interact electrostatically with the negative charges of the nucleic acid phosphates and of the cell membrane, and an hydrophobic part that determines the nucleic acid condensation and the interaction with lipophilic layers of the membrane. The morphology of vector-cargo ensembles is dictated by a combination of many

factors, primarily the type of vectors and its concentration, the DNA:vector ratio, defined also as N/P (N: generally ammonium ions, P: phosphates), the condensate diameter (smaller than 100 nm is of optimal size for transfection), the total charge of complexes (Z-potential), the pH, the ionic strength and the preparative details.[10] The resulting positively charged complex, where nucleic acid is masked and condensed, interacts with cytoplasmatic membranes and, generally, enters the cell via endocytosis in a non-specific way, if no specific ligands are present on the surface of the carrier. 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,[11] RGD (arginine-glycine-aspartate) motif[12] and saccharides.[13]

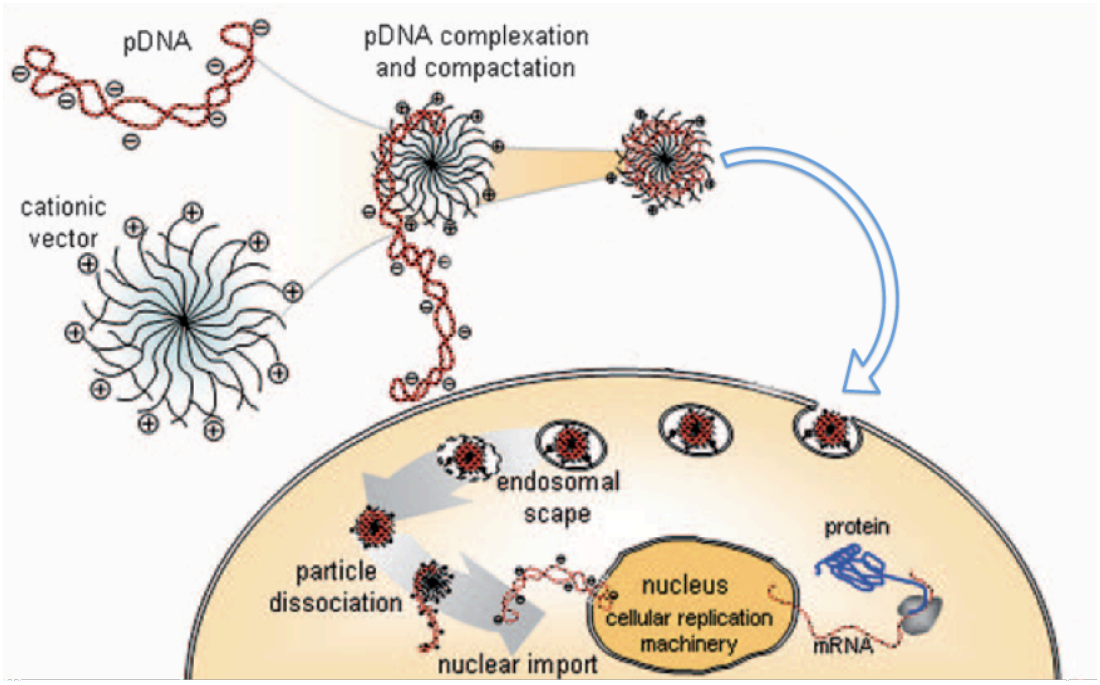

Fig. 1.1 Fundamental steps of the cell transfection process (adapted from ref. 14)

The vector-DNA complex, wrapped up into an endosomal compartment, must escape from it to avoid lysosomal degradation and proceed toward a successful transfection. The endosomal escape can occur by at least two mechanisms and it must be of course faster than degradation.[15] 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; on the other

hand, amphiphilic molecules may also interact directly with the endosomal membrane, destabilizing it, and releasing the genetic material into the cytosol.[16]

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[17] 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 complexes (NPCs). Macromolecules smaller than approximately 30 kDa and with appropriate size can passively diffuse into the nucleus through NPCs. On the other hand, larger macromolecules can be actively delivered into the nucleus by binding to nuclear transport proteins or during cell division accompanied by the disappearance of nuclear membrane.

Most recently, non-viral vectors have been also successfully used for RNA delivery,[18] in particular microRNA and small interfering RNA (siRNA), and are currently being evaluated in several clinical trials.[19] The possibility to complex and deliver RNA filaments has not, however, been investigated in this work.

1.2 Non-viral vectors

Usually the approach to the non-viral vectors starts from the synthesis of cationic lipids[20] and cationic polymers[21] that are able to complex DNA forming lipoplexes and polyplexes, respectively, or lipopolyplexes, when used in mixture containing molecules from both classes. Cationic polymers use multiple cationic sites to yield strong nucleic acid binding, whilst cationic lipids achieve this by assembling into highly charged aggregates.

However, more recently, under the advent of nanotechnology, a broad variety of materials with interesting biological properties, including for non-viral gene delivery applications, has emerged.

1.2.1 Cationic lipids

Cationic lipids are amphiphilic molecules composed of one or two acyl or alkyl lipophilic chains, a linker and a hydrophilic ammonium group (**Fig. 1.2**).

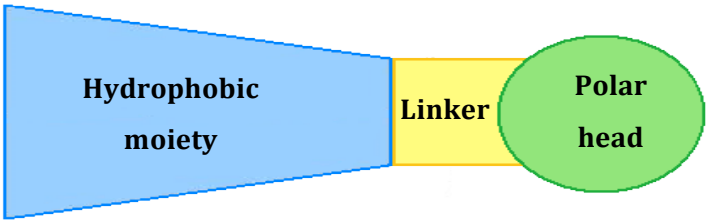

Fig. 1.2 Scheme of the three cationic lipid components.

They have received and are receiving a particular attention in researches about gene delivery. They present self-aggregation in aqueous media. It is possible to identify various supramolecular assemblies depending on their molecular structure and their critical packing parameter (C_{pp}), but, in general all of them offer positively charged interfaces for effective complexation with DNA (lipoplex formation) via electrostatic interactions (**Fig. 1.3**). Due to their bilayer membrane nature, the lipoplexes also favourably interact with negatively charged cell surfaces and assist delivery of DNA inside the cells, protecting it from enzymatic attack.

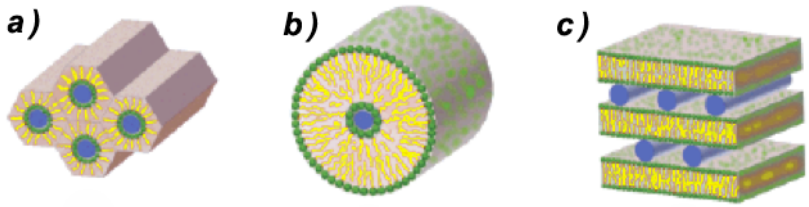

Fig. 1.3 Schematic representation of the three main cases of lipoplex geometry: a) hexagonal phase H_{II}^{C}, b) lipid bilayer-coated DNA strands, likely a metastable phase, and c) lamellar phase L_{a}^{C}. The blue cylinders are the DNA strands. [22]

Investigations focused on the variations of length of the carbon chains, the nature of the linker between head and tails, and the differences in the structure of the polar head and their effects on transfection efficiency. These structure-activity relationships elucidated two key trends: (1) the density and nature of the cationic headgroup affects the transfection properties of lipids, and (2) for a given headgroup, the

hydrocarbon moiety can be manipulated without predictably impacting gene transfer activity.[23]

Steric hindrance at nitrogen atom, electronic effects of substituents and the presence of hydrophilic groups often determine the transfection efficiency of lipids possessing quaternary ammonium head groups. Incorporation of a hydroxyalkyl chain onto the ammonium group (e.g. DORIE, **Fig 1.4**) decreases the hydration of the head group and promotes, instead, hydrogen bonding with neighbouring head groups, presumably optimizing the interaction of DNA with lipids. In addition, the ammonium group, generally present as polar head of cationic lipids, was replaced with phosphonium or arsenium groups[24,25], or more biocompatible amine derivatives (cationic nucleoside lipids)[26,27] to try to reduce cytotoxicity of some vectors, but without good results.

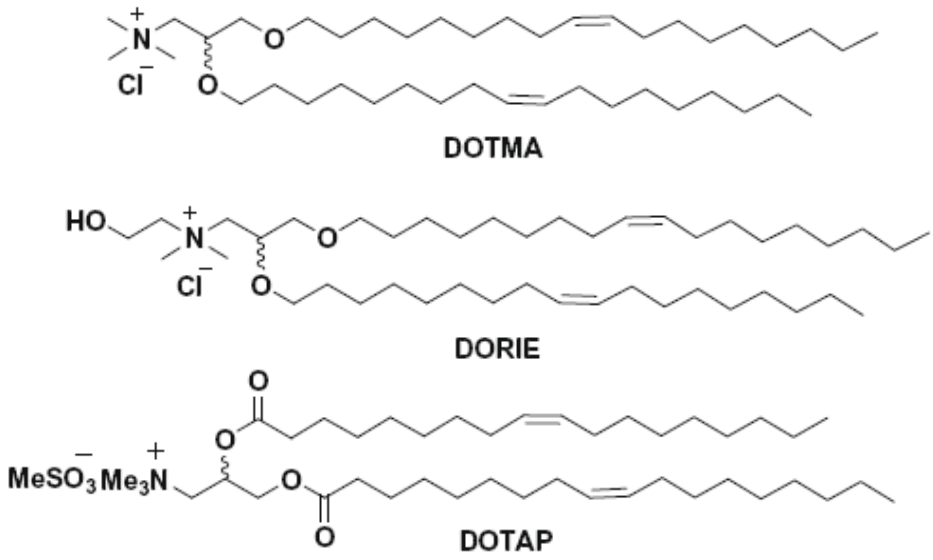

Fig. 1.4 Structural formulas of some cationic lipids.

As higher numbers of cationic groups increases DNA binding[28] a shift from single to multiple polar heads quickly became significant for lipid-mediated gene transfer. So lipids with linear or branched[29] or dendritic[30] multivalent head groups have been largely studied.

An interesting class of cationic lipids is constituted by dimeric surfactants,[31] 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.[32] Among these, the so called gemini surfactants, the most studied structures under the profile of biological activity and of physico-chemical properties, are bisquaternary ammonium salts (bisQUATS), and, among these, are the derivatives of N,N-bisdimethyl-1,2-ethanediamine.

The use of gemini surfactants (**Fig. 1.5**) as non-viral vectors in gene therapy has been proposed quite recently, on account of the possibility to take advantage of their cationic character necessary for binding and compacting DNA and of their superior surface activity.[33]

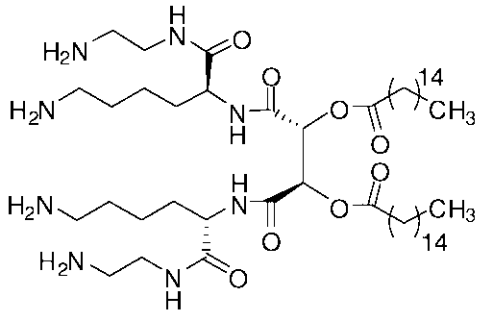

Fig. 1.5 Example of a gemini structural formula.

When at high concentration cationic lipids result cytotoxic, cholesterol and dioleoyl phosphatidylethanolamine (DOPE) (**Fig. 1.6**) are often co-formulated with lipoplexes as “helper lipids” that guarantee a good compromise between efficiency and toxicity.

The lipoplex packing was found to be a key parameter governing transfection properties. An improvement of efficiency for some cationic lipids in presence of DOPE was observed.[34] This behaviour is probably due to its capability to facilitate conversion of lipoplex lamellar organization to hexagonal phase, which would seem to maximize the endosomal escape[35] and, consequently, transfection.[36]

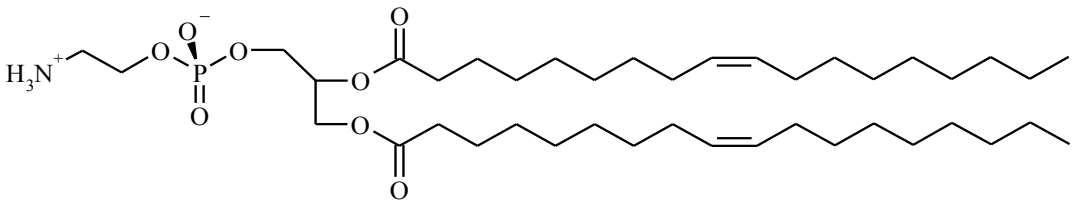

Fig. 1.6 Structural formula of DOPE.

1.2.2 Polimeric and Dendrimer-based vectors

As already mentioned, a critical requirement for a non-viral gene delivery vector is its ability to compact DNA and mask its negative charges. Many different polycationic molecules have been used for this purpose, including polymers, polysaccharides, polypeptides and dendrimers, which allow a high level of design flexibility.[2a] Polymer based non-viral gene delivery carriers, such as polyethyleneimine (PEI), chitosan (CS) and so on, have been widely used as vectors for gene delivery (**Fig 1.7**). Usually they interact with the DNA through electrostatic interactions by means of ammonium ions.

The transfection efficiency of PEI polyplexes increases with increased weight, ranging from 5 and 25 kDa,[37] and has been shown to be due, at least in part, to the "proton sponge" nature of the polymer. This buffering capacity allows PEI based polyplexes, in particular, when used in branched form, to avoid lysosomal trafficking and degradation once inside the cell, resulting in a very efficient transfection process.

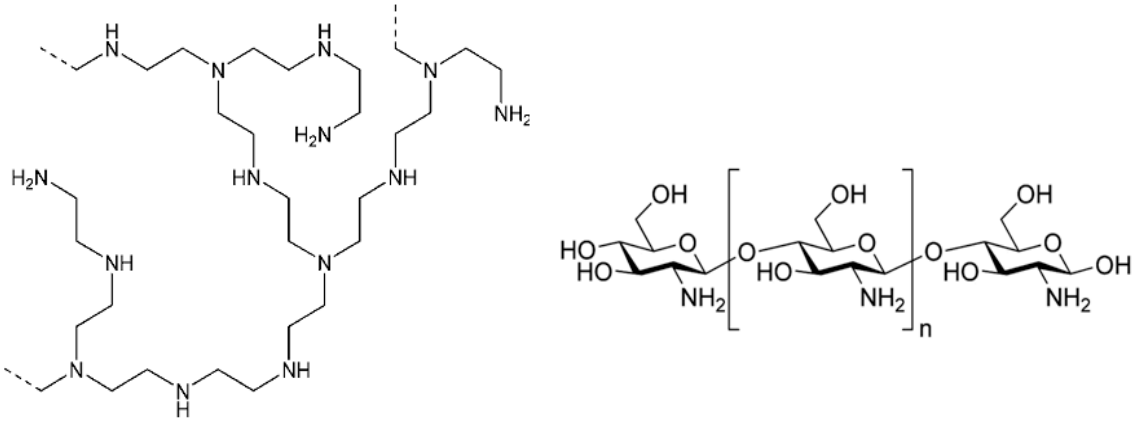

Fig. 1.7 Structural formula of branched polyethylenimine (left) and linear chitosan (right).

Especially high transfection levels in vitro can be achieved by using polylysine-based systems, with molecular weight ranging from 4000 to 30000.[38] For in vivo gene transfer, however, a number of parameters remain to be optimised; in particular, the formation of homogeneous, small, soluble and stable DNA–polylysine particles appears to be important.

Poly-L-lysines (PLLs) are biocompatible and can be easily degraded by cells.

In addition, the ε-amino group of lysine, positively charged at physiological pH, is a good target for many covalent chemical modification strategies. Various molecules

were grafted to PLL, such as poly(ethylene glycol) [(PEG)-grafted PLL] which can protect plasmid DNA (pDNA) from enzymatic degradation and reduce cytotoxicity, lipids or palmitic acid to facilitate membrane crossing of genetic cargo into cells. In addition, many target ligands, such as galactose or proteins, were also coupled to PLL to increase receptor-mediated endocytosis.[39]

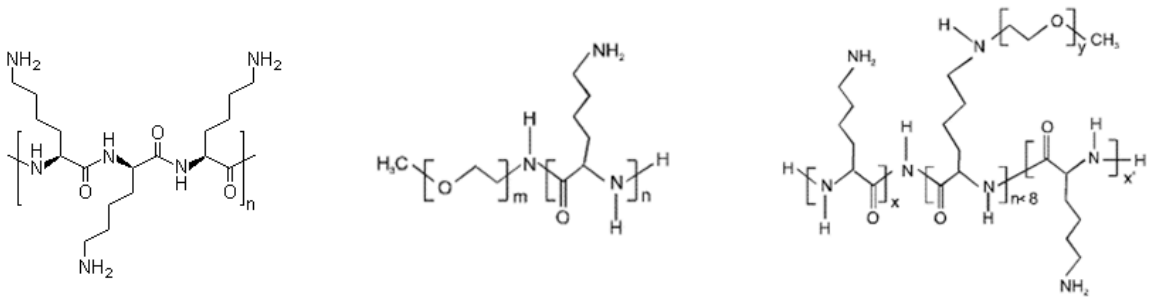

Fig. 1.8 Structural formulas of linear PLL and copolymers of PLL with poly(ethylene glycol) (PEG).

Other peptides, both natural and synthetic, and their analogues can result useful in this context because of their properties as molecular transporters and cell penetrating agents.[40] Covalently or noncovalently attached to an otherwise poorly bioavailable drug, drug candidate or probe, they enhance or enable its passage through biological barriers. These species and their application in gene delivery will be discussed in detail in the introduction of Chapter 2.

The poly(L-lysine), poly(ethylenimine), poly(propylenimine) (PPI) and poly(amidoamine) (PAMAM) motifs have also been widely exploited for the design of dendrimeric structures and investigated as a new class of vectors.

Dendrimers are versatile, derivatisable, well-defined and compartmentalised polymers and their molecular weight is stepwise increased via the repetition of a reaction sequence. So size and structure are highly controllable and molecular weight distribution is generally very narrow respect to the polymers featuring promising properties for non-viral gene delivery applications.[41]

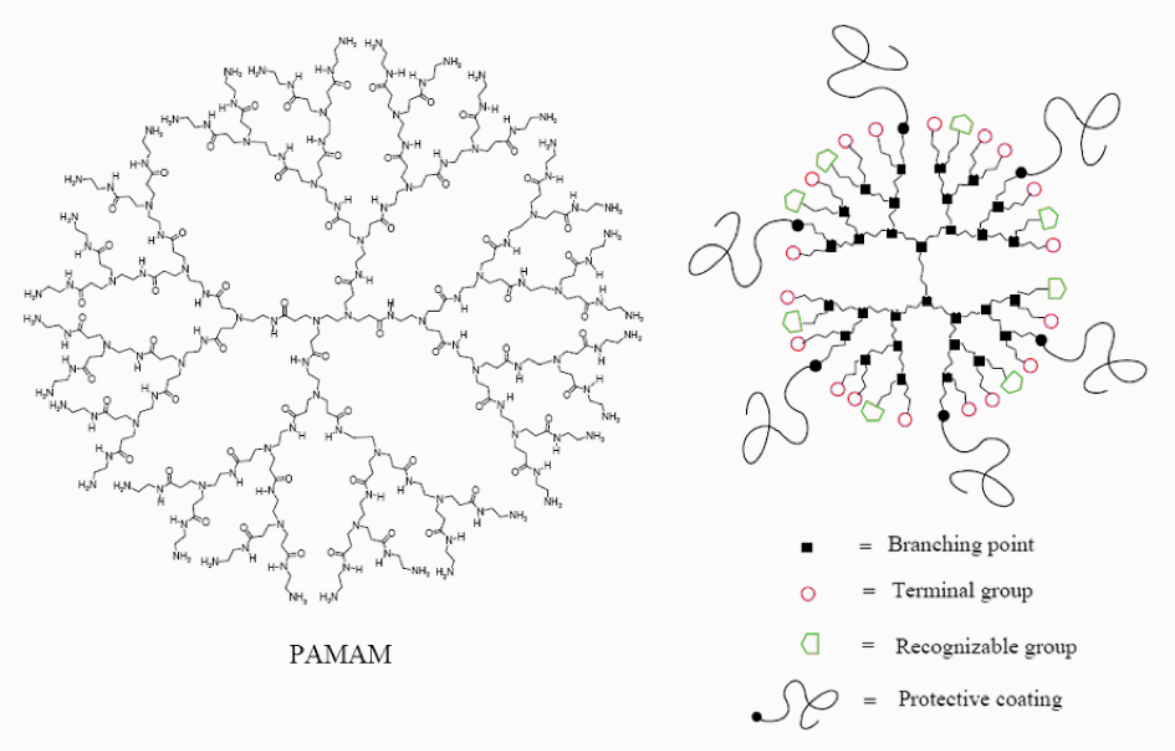

Fig 1.9 Third-generation PAMAM dendrimer and schematic representation of a multifunctional dendrimer.

Among dendrimer-based vectors, PAMAM and PAMAM derivatives have become the most utilized for transfection. Many modifications have been investigated on these structures to improve DNA complexation and transfection capabilities: conjugation with membrane-destabilizing peptides [42] and surface modifications with arginine units[43] or hydrophobic amino acids such as phenylalanine and leucine.[44]

1.2.3 Nanoparticles

Gold and Silica nanoparticles

Surface-modified gold and silica nanoparticles are attractive candidates for gene delivery. Their ability to interact with and enter cells has encouraged research to attach various compounds and biological macromolecules to these materials in effort to combine functionality and transport. In particular, in case of gold particles, research has been focused on modifying the surface to allow for cellular entry via endocytic pathway rather than particle bombardment, method applicable only to the treatment of skin deseases. Monolayer protected gold cluster obtained by reacting 2nm gold particles with PEI [45] or with various ratios of alkenethiols and

trimethylammonium thiols[46] were successfully tested in cellular trafficking studies. The most commonly used method to exploit silica for gene delivery is by functionalizing the surface of the nanoparticles with aminosilanes.[47]

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 longer fluorescence lifetime with numerous advantages over traditional organic dyes.[48] These characteristics, jointly with the possibility of bio-functionalising them, offer great potential for biological and medical applications, especially for imaging and sensoring. In some examples they have been also used as multifunctional delivery platform for gene therapy, especially by covalent conjugation of QD surface to plasmid DNA.[49]

1.2.4 Macrocycles

During the last years the control of the architecture of multifunctional structures became a major determinant in the rational design of successful non-viral gene delivery systems. In fact rigid frameworks that allow the installation of spatially separated functional elements have emerged as an appealing alternative.

Unlike the spherical nanostructure of fullerenes [50] or the cylindrical one of nanotubes,[51] which, however, have been employed as mulvalent platforms in DNA complexation, cavitands (calixarenes, resorcinarenes and cyclodextrins) have been largely used for the construction and application of novel architectures. Their subnanometric three-dimensional rigid structure shows a tunable preorganization with well-differentiated poles. The existence of a lipophilic face opposite to a functionalized rim in an axial–symmetric arrangement makes them convenient scaffolds for directional synthetic elaboration.

Cyclodextrins

Cyclodextrins (cyclomaltooligosaccharides, CDs) are C_n symmetric cyclic oligosaccharides composed by α(1→4)-linked glucopyranose units. The hexa-,

hepta- and octamer representatives (α-, β-, and γ-CD, respectively) are currently industrially produced by enzymatic degradation of starch.

Their truncated cone structure features a relatively hydrophobic cavity well-fitted to host organic molecules of appropriate size.[52] The ability of CDs to form inclusion complexes and their biocompatibility has led to a range of applications, including the protection of active principles in aqueous media and their controlled release.[53]

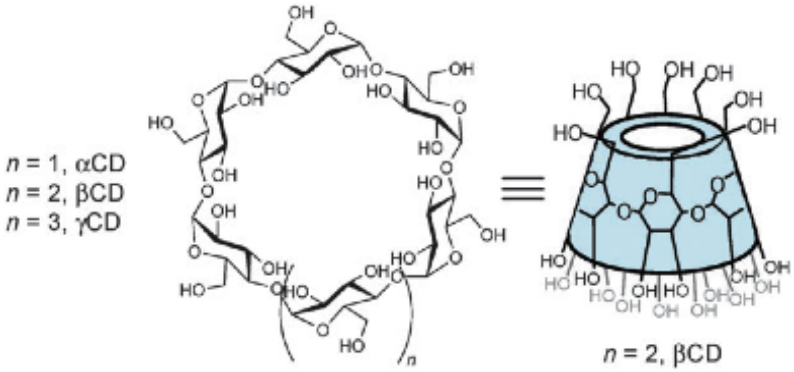

Fig. 1.11. General structure of cyclodextrins.

However, CDs can also be viewed as nanometric platforms with two well-differentiated faces: the narrower rim bearing the primary OH-6 hydroxyl groups and the wider rim, in which the secondary OH-2 and OH-3 hydroxyl groups are located (**Fig. 1.11**). By using facial-selective functionalization methodologies, it is possible to i) graft cyclodextrins to macromolecular constructs, for example, PAMAM dendrimers,[54] ii) thread CDs around PEI chains and iii) insert CD motifs in cationic copolymers,[55] to impart biocompatibility and to behave as transfection enhancers when incorporated to these polycationic vectors.

Very recently, several groups have turned their attention to the development of monodisperse CD derivatives that could self-organize in the presence of DNA to promote its compaction and safe delivery to cells.

The higher accessibility and reactivity of the primary hydroxyl groups facilitate homogeneous functionalization at the narrower rim, which has been used to create cationic clusters bearing guanidinium moieties [56] or linear oligoethyleneimine branches.[57]

Although monofacially functionalized polycationic CDs present an electrostatic and hydrophilicity gradient between the primary and secondary rims, hydrophobicity is

limited to the internal walls of the basket-shaped cavity, which is relevant for encapsulation of small guests, but, in principle, not to promote self-assembling and macromolecular interactions.

Elaboration of the secondary CD hydroxyls offers further opportunities for molecular tailoring and implementation of the facial amphiphilicity concept. So taking advantage of the differential chemical reactivity between the primary and secondary hydroxyls, alkyl chains and polar groups are installed at the primary and secondary positions, respectively, like in the case of polyaminothiourea CDs that form stable complexes with pDNA (*CDplexes*) and exhibit transfection efficiencies significantly higher than PEI-based polyplexes.[58]

Calix[n]arenes

Calix[n]arenes[59] are synthetic macrocycles obtained by condensation of phenols and formaldehyde. The shape, size and conformational properties of these molecules can be finely tuned by varying the reaction conditions and the length of functional groups linked to the phenolic hydroxyls.

Calixarene macrocyclic scaffolds have proven very useful to construct pre-organized multitopic ligands for a variety of purposes in bioorganic context. Some possible applications are like drug discovery,[60] as ion channel mimics,[61] as enzyme mimics,[62] as agents for the surface recognition of proteins[63]and inhibitors of medically relevant carbohydrate binding proteins (lectins), when functionalized with sugar units.[64] A calix[4]arene was also derivatized with a fluorophore and its cellular uptake was proved to be a non-specific process, not related to either of the main endocytic pathways, with accumulation of the molecule in the cell cytoplasm.[65] Some amino-substituted amphiphilic calixarenes are able to interact with double stranded DNA though transfection abilities were not investigated.[66]

Some years ago, our research group proposed the first examples of calixarene derivative for application in gene delivery. They bore anion-binding moieties with high phosphate avidity, such as guanidinium functionalities, at the upper rim and lipophilic tails at the lower rim (example in **Fig 1.10**, left). These guanidino-calix[n]arenes were able to condense plasmid and linear DNA, but they were also characterized by low transfection efficiency and high cytotoxicity, especially at the vector concentration required for observing cell transfection (10-20 μM). But these studies were significant

because evidenced that cell transfection, operated by these calixarene based vectors, is strongly dependent on the macrocycle size, lipophilicity, and conformation.[67]

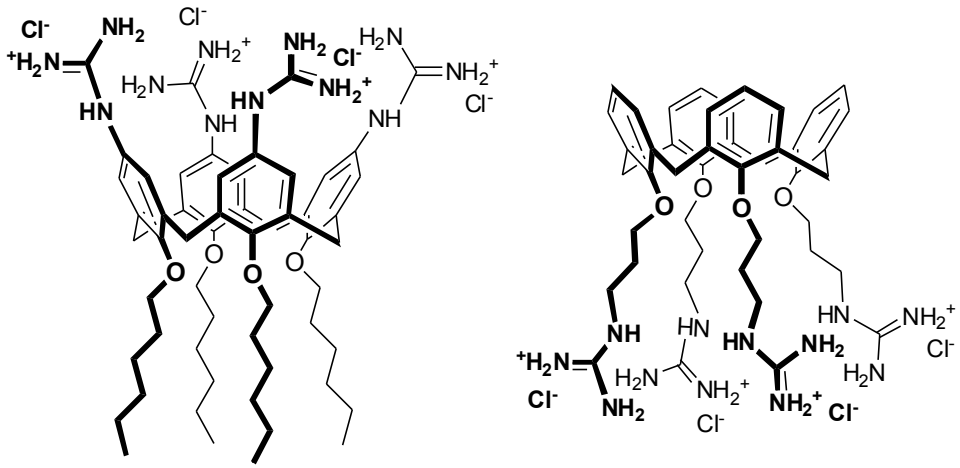

Fig. 1.10. Examples of upper and lower rim guanidino-calix[4]arenes synthesized in our group.

The transfection process resulted much more efficient using lower rim guanidino-calix[4]arenes (**Fig 1.10**, right), in particular when the helper lipid DOPE was added to pDNA-calixarene formulations.[68]

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