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Preorganized, Macromolecular, Gene-Delivery Systems

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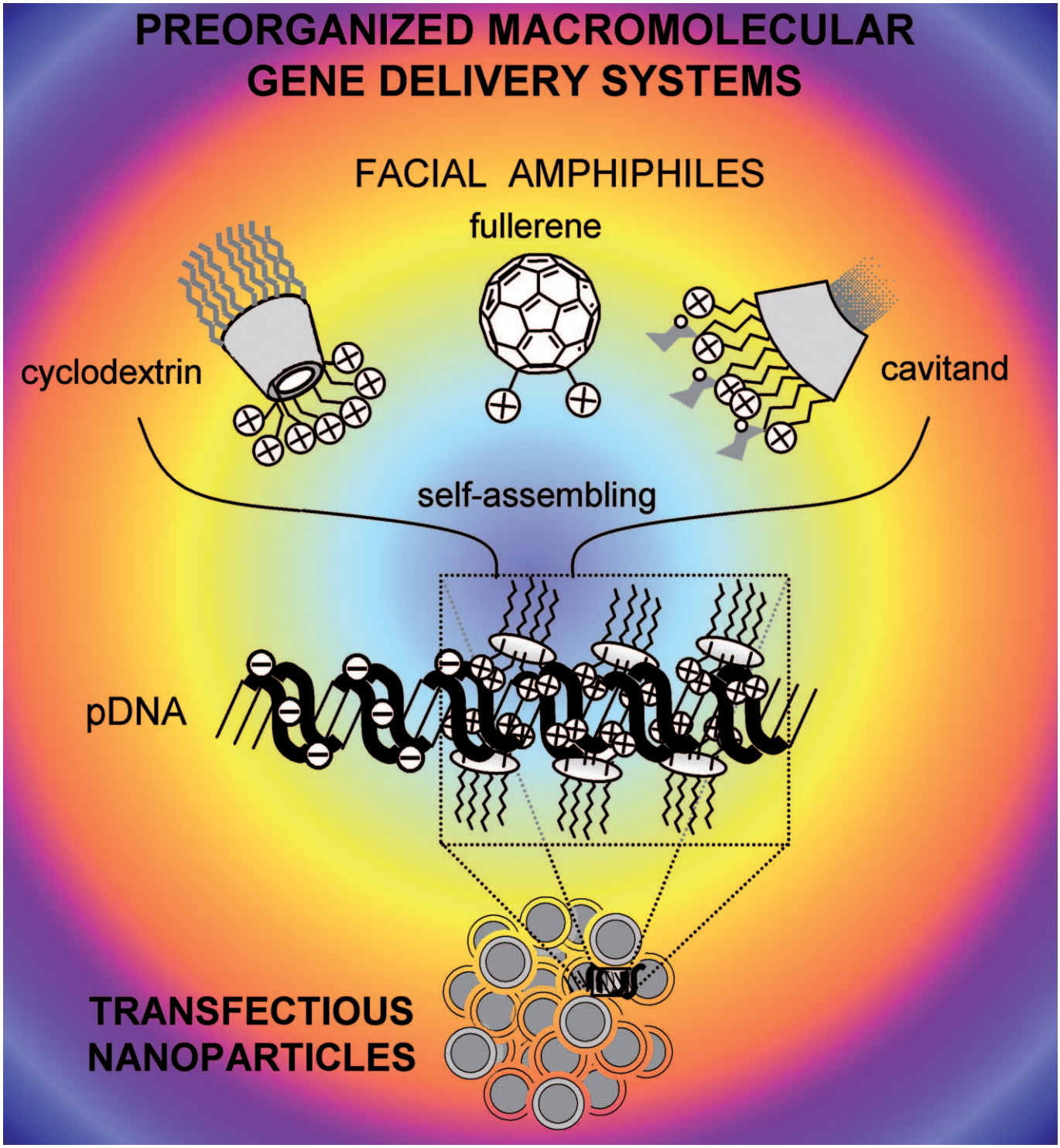

Abstract: Viruses represent a paradigmatic example of multicomponent, self-organized supramolecular systems specialized in the delivery and replication of their genetic material. Mimicking their functioning by artificial synthetic molecules represents a fantastic challenge that will lead to the future development of gene therapy. This is only possible if general approaches towards the construction of nanoscale vehicles for DNA are developed and the key rules governing their capacity to compact genetic material and its active transport/delivery through cell membranes are understood. In this area of research, synthetic organic chemistry plays an important role by providing tools to create tailor-made molecules of increasing complexity. Preorganization of functional elements onto macromolecular platforms has the potential to allow control of the self-assembling behavior of discrete architectures to produce nanometric objects that can be programmed to complex, compact, deliver, and release plasmid DNA in a target cell.

Keywords: calixarenes · cyclodextrins · dendrimers · drug delivery · fullerenes · gene delivery · resorcarenes

Introduction

Controlled and efficient gene delivery has implications in many fields ranging from basic science to clinical medicine. Genes embedded in plasmid DNA (pDNA) provide a stable source for therapeutic proteins and RNA provided they can enter the cell and reach the nucleus. Although naked pDNA has been shown to transfect cells both in vitro and in vivo, it is easily degraded by nucleases in biological fluids and the transfection efficiency is generally low, pointing to the need of specialized vehicles. For the introduction of a foreign DNA into a target cell, two different types of delivery systems can be distinguished: those that are synthetic and those derived from viruses. The latter can be considered as self-assembled biological machines adept at entering host cells and exploiting the cellular machinery to facilitate their replication. In spite of this very efficient mechanism, the FDA has not approved any viral-vector-based therapeutics up to date due to immunogenicity, oncogenicity, and potential virus recombination concerns.[1] During the last decade, under the advent of nanotechnology, a broad diversity of creative materials featuring promising properties for nonviral gene-delivery applications has emerged, including dendrimers,[2] gold nanoparticles,[3] silica nanoparticles,[4] nanogels,[5] carbon nanotubes,[6] carbon nanofibers,[7] and organic nanoparticles.[8]

Efficient complexation of DNA is only the first step of the whole process leading to expression of an exogenous gene in a cell (Figure 1). Internalization, escape of the intracellular endosomal compartments, dissociation of plasmid and carrier, nuclear translocation of the nucleic acids and, finally, transcription of the transfected pDNA and protein expression must be overcome to obtain a successful transfection. Internalization, which generally proceeds through endocytosis, and endosome escape are often the most critical steps. Because there is no clear understanding of what happens to functionally active transfecting particles at the membrane surface, it is difficult to use the information obtained on the transfection efficiency from different systems for the design of better chemical delivery agents.

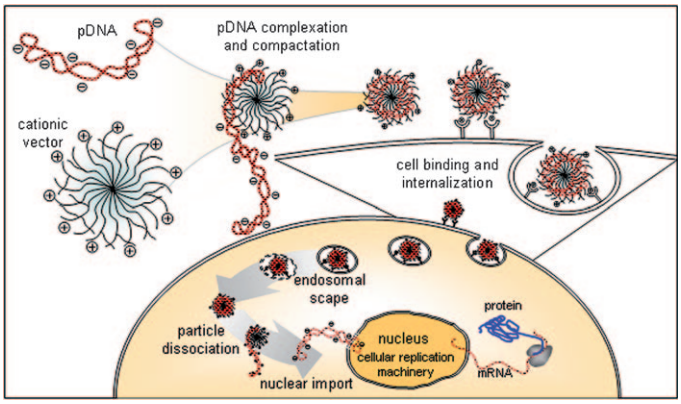

Figure 1. Schematic representation of the different steps comprising gene delivery using nonviral vectors.

Most of the currently available nonviral gene vectors belong to two main groups: cationic lipids (Figure 2A) and cationic polymers (Figure 2B).[9] Both types of compounds can condense pDNA into multimolecular complexes named lipoplexes or polyplexes, respectively, which show a range of sizes and physicochemical properties in solution.

Lipoplexes and polyplexes usually are positively charged particles that efficiently enter the cell after binding to negatively charged proteoglycans on the outer face of the membrane, resulting in improved pharmacokinetics and pharmacodynamics and, eventually, active intracellular delivery.[10] Further functional elements (e.g., targeting ligands, biocompatibility enhancing oligomers, such as polyethylene glycol,

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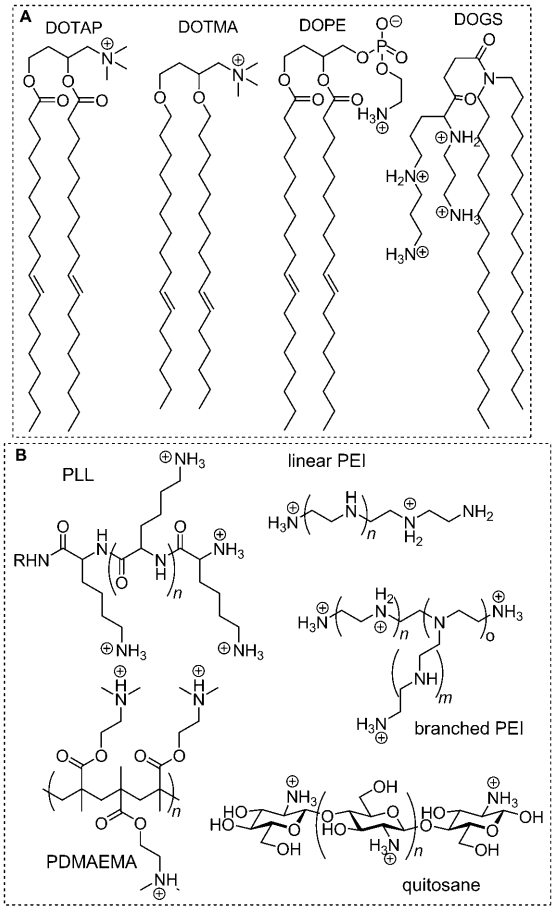


Figure 2. Structure of some representative cationic lipids (A) and cationic polymers (B) used for gene delivery (DOTAP: 2,3-dioleoyloxy trimethylammonium propane; DOTMA: 2,3-di-(oleyloxy)propyl trimethyl ammonium; DOPE: 2,3-di-(oleolyloxy)propyl phosphatidyl ethanolamine; DOGS: dioctadecylamidoglycyl spermine; PLL: poly-L-lysine; PEI: polyethyleneimine; PDMAEMA: poly[2-(dimethylamino)ethyl methacrylate].

fusiogenic peptides, or nuclear localization signals) can be incorporated onto the vector or the preformed pDNA–vector complex by means of covalent or supramolecular interactions to help the system to overcome the cellular barriers and the immune defense mechanisms, prevent undesired side effects, or targeting specific tissues. As an alternative, discrete macromolecules based on rigid frameworks, allowing the installation of spatially separated functional elements have emerged as an appealing alternative. Actually, the control of the architecture of multifunctional macromolecules is a major determinant in the rational design of successful nonviral gene delivery systems.

This paper will display the most significant examples of homogeneous architectures conceived for the delivery of genetic material into cells, but it is not an exhaustive review (more than 3500 papers were published with "gene delivery" as a keyword entry in 2009; about 500 with this term in the title!). The present article shall focus on the potential of polyfunctional macrocyclic platforms with respect to the design of artificial viruses for gene therapy. It starts by the introduction of pioneer contributions that illustrates the importance of macromolecular topology control to optimize gene vector design, and follows by the highlighting of recent work on original macromolecular gene-delivery systems that incorporate a variety of functional elements in a well-defined geometry. Most of the work relevant to this topic concerns four main frameworks, namely fullerenes, resorcarenes, calixarenes, and cyclodextrins.

The Facial Amphiphilicity Concept

Although a cationic component is necessary in most effective gene delivery agents, there is no prescription for what other structural features should be included in such a molecule. In a seminal work, Kahne and co-workers[11] designed a new family of compounds based on the idea that facially amphiphilic components known to destabilize membranes might increase the fusiogenic potential of the transfecting particles and thereby enhance DNA uptake. After observing some abstract similarities between certain amphiphilic peptides that promote DNA uptake[12] and polyhydroxylated steroids, they designed several amphiphilic bile acid derivatives (e.g. **1**, Figure 3) that worked significantly better than standard cationic lipid-based formulations.

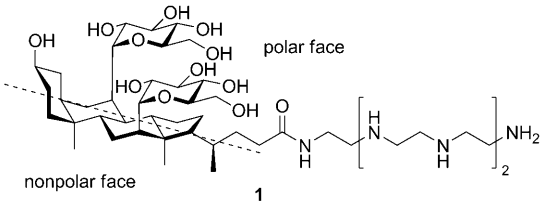


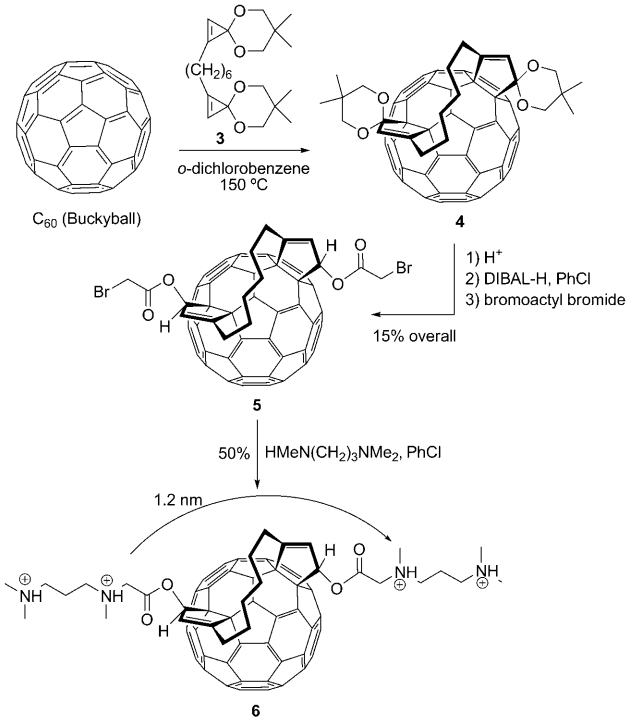
Figure 3. Representative example of a bile acid-based facial amphiphile (**1**) highlighting the segregation of polar and non-polar domains.

Kahne's approach using bile acids has been further developed by other groups.[13] Most importantly, this work suggests that the use of anisotropic preorganized scaffolds to achieve a precise alignment of functional elements represents a promising biomimetic alternative for gene vector design. The need for strict control of the conformational properties and chemical reactivity, while preserving molecular homogeneity, represents a considerable challenge that grows exponentially with molecular size and complexity. Nevertheless, the last years have witnessed a blossom of original multifunctional monodisperse gene delivery systems based in this concept. This approach is part of the general trend of organic chemistry taking control of total macromolecular synthesis to produce well-defined constructs, which could become more and more important in future drug applications.

Buckyballs Meet DNA

The C_{60} fullerene (Buckminsterfullerene or Buckyball, **2**), the most representative among fullerene carbon allotropes, is a remarkable stable compound consisting of a polygon with 60 vertices and 32 faces, 12 of which are pentagonal and 20 hexagonal. Due to the electron-withdrawing effect, fullerenes can undergo nucleophilic addition to the C=C double bonds by a variety of chemical groups that can donate a pair of electrons and, therefore, numerous reagents can be added to the fullerene cages.[14] The unique chemical and physical features of C_{60} have aroused the hope of a successful use in many fields both in biological and material chemistry.[15] For instance, the lipophilicity of the sphere can make Buckyballs able to intercalate into biological membranes, destabilizing them. Chemical modification breaks the spherical symmetry, creating two well-differentiated poles and thereby offering the opportunity to generate facial amphiphilicity. By connecting functional groups that are charged in water, such as carboxylate or amine groups, the extremely hydrophobic C_{60} can be endowed with surfactant functionality and made water soluble, leading to derivatives that can interact with biomolecules, including DNA, and form aggregate structures.[16]

Interactions of organofunctionalized fullerenes (organofullerenes) and DNA have attracted much interest since the discovery in 1993 that a water soluble fullerene cleaves DNA upon irradiation with visible light.[17] Further research led to the observation that organofullerene derivatives tightly binding to DNA cause formation of aggregates that resist electrophoresis.[18] Based on these results, Nakamura and coworkers consider the possibility that a cationic fullerene might bind to duplex DNA and deliver it to the cell nucleus. The molecular prototype **6**, which possesses two diamine side chains separated by 1.2 nm from each other, was designed as a complementary element of the two phosphate backbones flanking the DNA major groove.[19] To obtain the target molecule **6**, the doubly functional cyclopropene annulating agent **3**, incorporating a hexamethylene tether, was reacted with C_{60} to give the double [3+2] cycloadduct **4** with complete regiocontrol. The acetal groups in **4** were deprotected, reduced and converted to bis-bromoacetate **5**. Nucleophilic displacement of the bromo groups by *N,N,N′*-trimethyl-1,3-diaminopropane afforded the two-handed Buckyball **6** in 8% overall yield (Scheme 1).



Scheme 1. Synthesis of the two-handed tetracationic fullerene-based gene vector **6**.

The tetracationic compound **6** was found to condense plasmid DNA into micrometer-size fullerene–DNA particles through a process that implies synergistic effects of the large hydrophobic core and the two positively charged side chains. After incubation with African green monkey COS-1 cells, the complexes were internalized by endocytosis inside the cytoplasm in the form of endosomes, as supported by microscopic observation. Expression of the encoded gene took place over several days, indicating that the plasmid DNA was released without being damaged by the fullerene complexation. It was suggested that the cation-bearing ester linkage in **6** might be slowly cleaved either chemically or enzymatically to release the DNA from the fullerene core, though this idea remains to be proved. The efficiency of gene transfer of **6** was comparable to that of commercial cationic lipid formulations such as Lipofectin®. Interestingly, the transfection efficiency increased in the presence of 10% serum, which is opposite to what happens with Lipofectin® and most cationic lipids. Moreover, the cytotoxicity of the fullerene derivative **6** is negligible both in the presence or in the absence of ambient light, in spite of the photoreactivity of (weakly DNA binding) fullerenes.[20]

Atomic force microscopy (AFM),[21] static and dynamic light scattering (SLS and DLS), and scanning electron microscopy (SEM) studies[22] of both the homogeneous aggregates obtained upon dissolution of **6** in water and the heteroaggregates formed in the presence of DNA led to the advancement of a hypothesis for fullerene–DNA complex formation. In view of the high tendency of water-soluble fullerenes to form robust vesicles,[23] the process of fullerene–DNA condensation probably involves collisions between a fullerene vesicle and a DNA molecule, rather than a molecule–molecule interaction. Molecules of **6** could then bind DNA in the major groove of the DNA duplex to maintain the structural integrity of the helix. At low concentrations, fullerene **6**, condenses DNA into compactly folded single-DNA disks, and then to multi-DNA objects as the amount of the fullerene in solution increases (Figure 4).

Structure–activity relationship (SAR) studies regarding the ability of aminofullerenes for transient transfection, performed on a collection of 22 compounds sharing a common

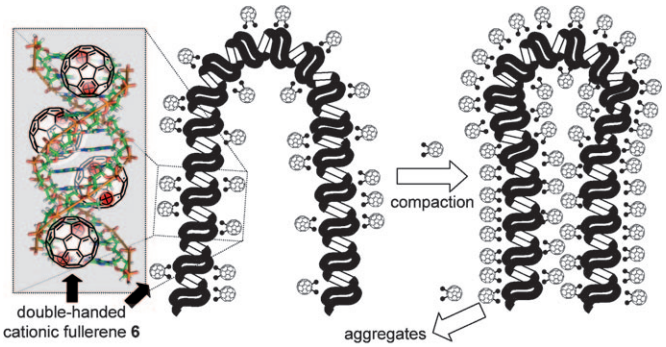


Figure 4. Schematic representation of the proposed DNA complexation, compaction and aggregation process promoted by the polycationic fullerene derivative **6**.

structural motif, indicated that any water-soluble fullerene-bearing amino group would bind to double-stranded DNA.[24] For these molecules to be effective transfection mediators, however, they require additional structural features. Thus, an appropriate hydrophobic–hydrophilic balance is essential to cross the cell membrane. Once the fullerene–DNA complex enters the cytoplasm, DNA must be released before it can enter the nucleus for production of the protein. The SAR analysis suggested that the insertion of cleavable segments (e.g., ester functionalities) connecting the protonated amine groups and the fullerene or, alternatively, the presence of amine groups that can undergo reactions that cause them to become neutral (e.g., acylation) are necessary to lose the DNA-binding ability. Accordingly, compounds **6** (Scheme 1), **7** and **8** (Figure 5) were identified as the most

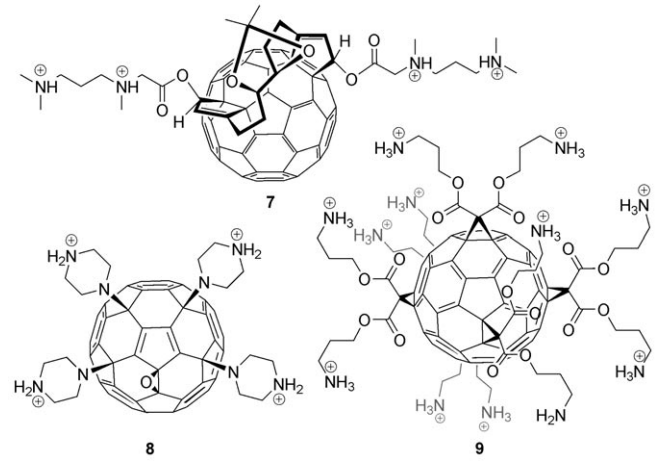


Figure 5. Representative examples of polycationic fullerene derivatives.

efficient representatives. It is worth noting that compound **8**, the best transfection agent among them, could be synthesized in just two steps with an overall yield of 80% and was more efficient and much less toxic to COS-1 cells than Lipofectin®. It caused no morphological changes in the cells either.

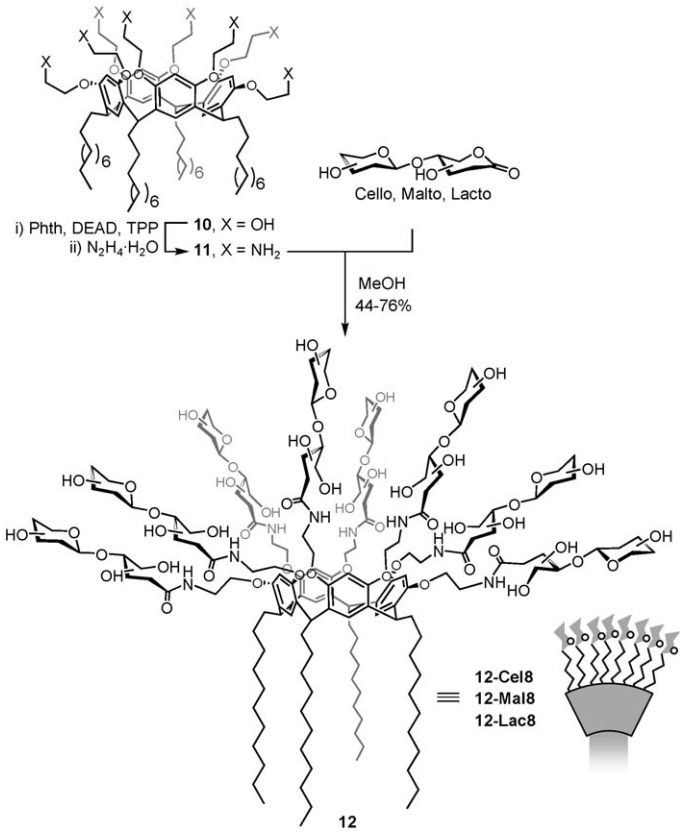
The fact that the isotropically functionalized polyaminofullerene **9** (Figure 5) was fully inactive as gene delivery agent and that no or poor transfection capabilities have been demonstrated for randomly multifunctionalized cationic fullerene adducts[25] underlines the importance of an appropriate preorganization with well-differentiated poles. Moreover, cell viability was problematic when mixtures of regioisomers were present, with toxicity increasing in a dose-dependent manner.[26] As a matter of fact, the in vivo biology and toxicology of fullerenes and their derivatives is still a matter under investigation. Few toxicological studies have addressed repeated dose exposures, reproductive toxicity, and carcinogenic effect that are mandatory information for adequate risk assessment. The current information suggests that fullerenes may be a "double-edged sword", which may have beneficial effects at low concentrations, but at high concentrations they may be able to induce inflammation and, if chronic, may promote development of cancer.[27]

Resorcarene-Centered Transfectious Glycoviruses

Unlike spherical fullerenes, cavitands are synthetic host molecules with open-ended enforced cavities. Among them, polyaromatic containers of the calixarene type and their relative resorcinarenes (resorcarenes) have been largely used for the construction and application of novel architectures, because of their nanometric three-dimensional rigid structure, concave and potentially extendable polyaromatic surfaces, commercial availability, and extremely rich preparative chemistry.[28] The existence of a lipophilic face opposite to a functionalized rim in an axial–symmetric arrangement makes them convenient platforms for directional synthetic elaboration. They have been used, for instance, as scaffolds for the construction of glycocluster motifs with a precise spatial orientation to explore multivalent interactions between carbohydrates and proteins.[29]

The potential of cavitands in the construction of biomimetic facial amphiphiles was pioneered by Aoyama in a work that aimed at developing characterizable unimolecular mimics of the cell-membrane sphingoglycolipid clusters.[30] For such purpose, the bowl-shaped calix[4]resorcarene framework, readily available by the condensation of resorcinol and dodecanal, was employed.[31] Transformation of octaol **10** into octaamine **11** and subsequent coupling with sugar lactone derivatives afforded glycolipid bundle compounds **12** composed of a definite number of saccharide moieties and four undecyl tails located in the opposite side of the macrocycle in a well-defined geometry (Scheme 2).

Despite the presence of the long alkyl chains in addition to the benzene rings, compounds **12** exhibit very high water solubility. Dynamic light scattering (DLS) as well as transmission electron microscopy (TEM) revealed that they form small spherical nanoparticles, termed glycocluster nanoparticles (GNPs) by the authors, in aqueous media over a wide concentration range. The particles are of micellar size, with a diameter of 4–5 nm and an aggregation number of ≈ 6.



Scheme 2. Synthesis of amphiphilic glycoresorcarenes (**12**).

Remarkably, the micellar nanoparticles manifest themselves in gel permeation chromatography (GPC). Actually, surface activity measurements revealed that the micelles do not dissociate into monomers; that is, micellization of these systems in water is irreversible. Strong intermolecular hydrophobic entangling of the alkyl groups, possibly coupled with lateral or intercluster hydrogen bonding in the glycocluster domain, may be at the origin of the unusual stability.[32] In the GNPs, the hydrophobic alkyl chains are effectively masked, exposing the sugar epitopes at the outer surface, readily available for interactions with solvent, surfaces, proteins, ions, and DNA. In fact, the marriage of GNPs and DNA is a notable case of synergistic effect of serendipitous observation and rational design (not so unusual in supramolecular chemistry!).[33] When a solution of GNPs in aqueous phosphate buffer was left for a long time, agglutination occurred, with the phosphate ions acting as a glue as seen from microscopic evidence.[34] NMR supported the existence of sugar-to-phosphate O–H···$^{-}$O–P hydrogen bonds in the aggregates (50–100 nm), which may further induce inter-GNP sugar–sugar hydrogen bonding and ultimately results in desolvation and entropy driven complexation. This unexpected phenomenon led Aoyama's group to take up DNA, a natural phosphate polymer, as an interaction partner of GNPs.

GNPs derived from the cellobiose-calix[4]resorcarene octaconjugate (**12**-Cel8) derivative were found to bind to a 7040 bp plasmid pCMVluc, which has a reporter gene for firefly protein luciferase and a cytomegalovirus promoter, to form what the author's called a "glycovirus".[35] Formation of the glycovirus was monitored by agarose gel electrophoresis, DLS, TEM, and surface plasmon resonance (SPR). The results evidenced that at a host/phosphate (or base) ratio of $\approx$0.6 saturation is reached and the size of the resulting DNA complex remains nearly constant thereof ($\approx$54 nm). The surface potential (ζ) of the complex, which is negative in the presaturation region, then becomes neutral. The 0.6:1 (i.e., 12:20) **12**-Cel8/nucleobase ratio indicates that two particles of GNP, composed of six molecules of **12**-Cel8, accommodate in every helical pitch of the DNA, composed of 10 bp (Figure 6). The strong complexation is proba-

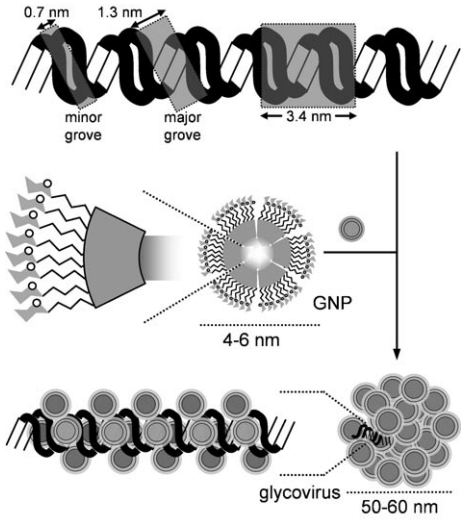


Figure 6. Hierarchical formation of glycoviruses from glyconanoparticles (GNPs) derived from glycoresorcarenes.

bly driven by multiple hydrogen bonding between OH groups of Cel8 and phosphate groups in the plasmid, which would be maximized when GNPs ($d \approx 4$ nm) are aligned along the major groove of DNA (1.3 nm with and 3.4 nm pitch length). Molecular models built assuming this scenario showed that four GNPs could be bound in two pitches without steric interferences, which is consistent with the observed stoichiometry. The resulting glycoviruses contain a single DNA molecule as a template and are highly efficiently packed, in agreement with the observed effective charge shielding.

Further studies showed that the monomolecularity of glycoresorcarene-based glycoviruses for a wide range of GNP/pDNA ratios is met only when cellobiose is the coating sugar. Maltose (Mal)- and lactose (Lac)-derived GNP equally complexed pDNA, but in that case the resulting glycoviruses experienced self-aggregation, which was more pronounced for maltose than for lactose.[36] The differing aggregation (rather adhesion, as seen from TEM images) tendencies in the order Mal > Lac $\gg$ Cel probably involve interviral

saccharide–saccharide interactions. This sugar-dependent process has important consequences in the size of the resulting particles which, in turn, drastically influence their cellular uptake and trasfection capabilities. Thus, transfection data indicated that, whatever the mechanistic details may be, monomeric viruses are by far more active than their aggregates. For instance, in HeLa cells only Cel viruses are practically active.

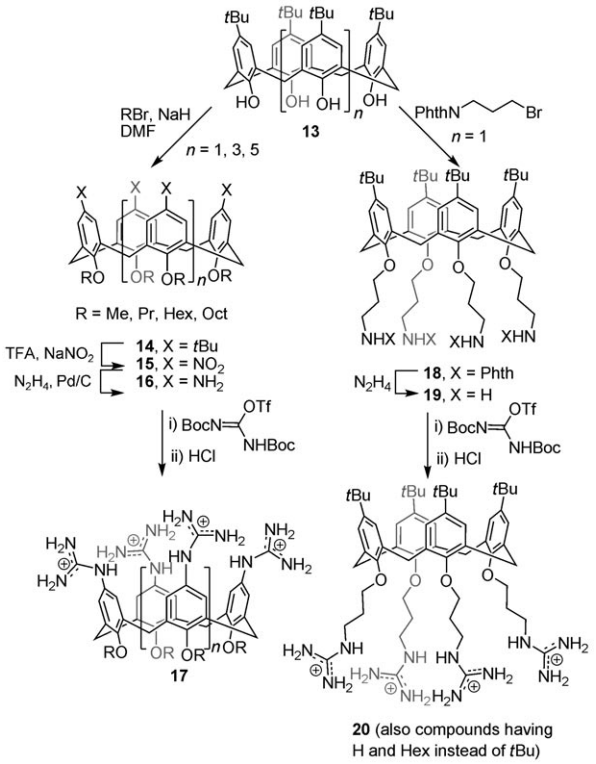
In the case of the hepatic cell line HepG2, Lac viruses exhibited a much higher activity than expected from the size correlation. This extra activity is due to specific interactions between the galactose residues and the asialoprotein receptors present at the cell surface, which could be exploited for targeted gene delivery. In neutral **12**-Lac8-derived glycoviruses this effect is compensated by the above-commented detrimental impact of self-aggregation in transfection. This effect can be minimized by decreasing the number of lactose residues onto the octaamine scaffold. A comparative study for $(Lac)_n(NH_3^+)_{8-n}$ resorcarene derivatives showed that the lactose moieties introduced mask charge, promote aggregation, and lower toxicity. The overall gene delivery to hepatocytes (HepG2 cells), while keeping high cell viability, is optimize when using a partially glycosylated derivative with $n=3$.[37]

The remarkable gene-delivery capacity of glycoviruses obtained from neutral resorcarene-scaffolded glycoclusters is the result of a hierarchical self-assembly process that involves irreversible micellization to form glyconanoparticles in the first step. Nevertheless, the transfection efficiency of the parent, unsubstituted octaamine amphiphile **11**, which does not form stable micelles, was found to be higher. Compound **11** additionally behaved as a very efficient small interfering RNA (siRNA) carrier.[38] However, cell viability was much lower for **11** as compared with the corresponding glycoclusters **12**. Modulating the molecular topology and the finite macromolecular association by acting not only on the nature and density of the head and tail groups, but also on the size and shape of the macrocyclic nucleus, offers further opportunities to optimize both transfection efficiency and cell viability parameters. Significant contributions in that direction are discussed in the next paragraphs.

Cationic Calixarene-Based DNA Cargos

Calixarene macrocyclic scaffolds have proven very useful to construct preorganized multitopic ligands for a variety of purposes.[39] They are readily obtained by cyclooligomerization of phenol and formaldehyde. Depending on reaction conditions, calixarene ring size (up to eight repeating units) and functional group spatial orientation can be finely tuned, which additionally allows controlling their conformational behavior.[40] For instance, calix[4]arenes, featuring four repeating phenol-formaldehyde units, may exist in four different conformations that, depending on the functional decoration pattern, may not be interconvertible.

Chemical derivatization of calixarenes has developed to a point that a broad variety of functional epitopes can be installed onto either the lower (bearing the phenolic oxygen atoms) or the upper rim on the macrocycle in the cone conformation. Not surprisingly, installation of polar saccharides onto calixarene cores imparts significant amphiphilicity and self-aggregation properties, which lies on the basis of the abnormal lectin recognition behavior of certain multivalent glycocalixarenes.[41] Following this work, Ungaro and Sansone envisioned that calixarene derivatives bearing anion-binding moieties with high phosphate avidity, such as guanidinium functionalities, might find application as nonviral gene vectors.[42] Based on this concept, polycationic calixarene-based facial amphiphiles with different shape, valency, and conformational constrains were developed (Scheme 3).



Scheme 3. Synthesis of amphiphilic guanidinocalixarenes.

For instance, alkylation of the phenolic hydroxyls in the tetra-*t*Bu derivative **13** (→**14**) followed by nitration (→**15**), reduction (→**16**) and guanidinylation afforded upper-rim guanidinocalixarenes **17**. By employing terminal phthalimidoalkyl bromides as alkylating agents (→**18**), the distribution of polar and lipophilic groups can be reversed. Deprotection (→**19**) and guanidinylation provided the corresponding lower-rim guanidinocalixarenes **20**.

Transmission electron microscopy revealed that upper-rim guanidinocalixarenes **17** are able to complex pDNA even at very low concentration in water. However, pDNA compaction exclusively takes place in the presence of the more

rigid calix[4]arene representatives ($n=1$ in Scheme 3), for which considerable reinforcement of the electrostatic interaction is expected due to hydrophobic contributions. Accordingly, transfection experiments on rhabdomyosarcoma cell line RD-4, using the gene encoding for the green fluorescent protein (GFP), was efficient only for aggregates obtained from the macrocyclic tetramers, underlining the importance of an efficient preorganization of polar and nonpolar domains.[43] Addition of the lipid helper DOPE to pDNA–calixarene formulations contributed to enhance transfection efficiency, in particular for calixarene derivatives bearing guanidinium groups on the lower rim (**20**).[44]

Although coformulation with DOPE was favorable, guanidinium–calixarene vectors suffered from low gene-delivery efficiency and high toxicity at the concentrations needed to observe cell transfection. Amino-substituted amphiphilic calixarenes represents an attractive alternative (Figure 7). Thus, compound **21** has been shown to self-assemble, with

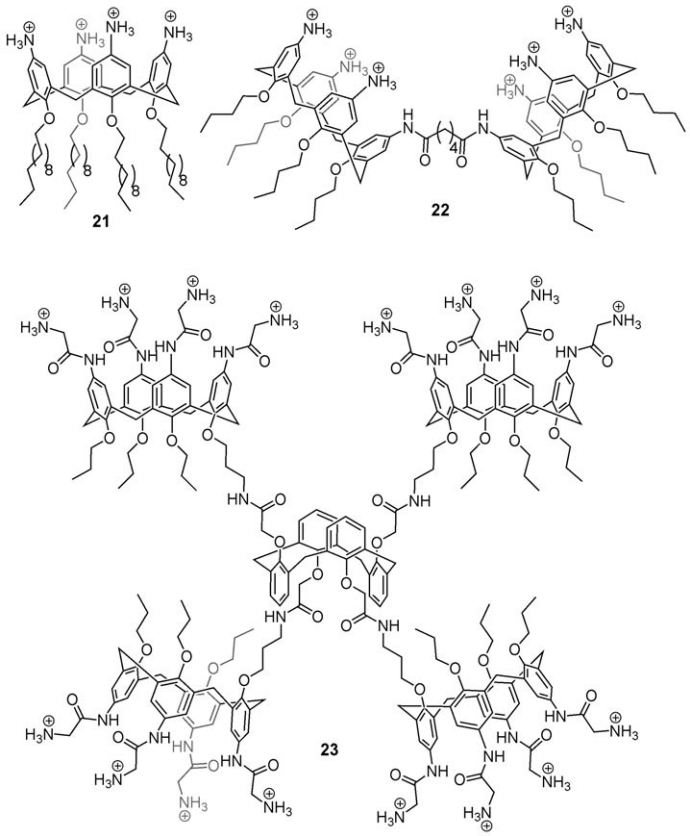


Figure 7. Structure of amphiphilic aminocalixarenes with different valencies. At physiological pH, only a fraction of the amino groups will be protonated.

the absence of a co-surfactant, to form positively charged solid lipid nanoparticles (SLNs) that interact with double stranded DNA.[45] The dimeric derivative **22**, with a higher degree of preorganization, had been previously shown to interact with DNA, probably by targeting the major groove, though transfection abilities were not demonstrated.[46] Matthiews and co-workers proposed an alternative macromolecular design based on the installation of four amino-functionalized amphiphilic calixarene motifs onto rigid calix[4]arene scaffolds.[47] Multicalixarene–pDNA binding turned out to be much stronger than that of their monomeric analogs, indicating the existence of cooperativity effects. Multicalixarenes blocked pDNA electrophoretic mobility, therefore efficiently condensing them, and are virtually nontoxic up to mM concentrations. Transfection experiments carried on Chinese hamster ovary (CHO) cells revealed that the presence of aliphatic amino groups, as in **23**, was necessary to efficiently promote GFP-encoding gene expression. pDNA complexes from multicalixarenes with arylamino groups were ineffective irrespective of the alternating or cone conformation of the central scaffold.

Polycationic Cyclodextrins–pDNA Complexes (CDplexes)

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 toroidal-cone structure features a relatively hydrophobic cavity well-fitted to harbor organic molecules of appropriate size.[48] 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.[49]

Most of the research in the cyclodextrin field has focused on their molecular container character, which is intrinsically limited by the internal volume defined by the macrocyclic ring. 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 (Figure 8). By using either specific-position or facial-selective functionalization methodologies, it is possible to incorporate cyclodextrin moieties in macromolecular constructs, for example, polymers[50] and dendrimers,[51] to install a variety of functional elements with a precise spatial orientation and density onto a CD scaffold,[52] or

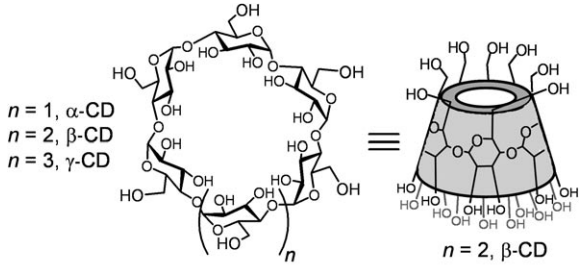


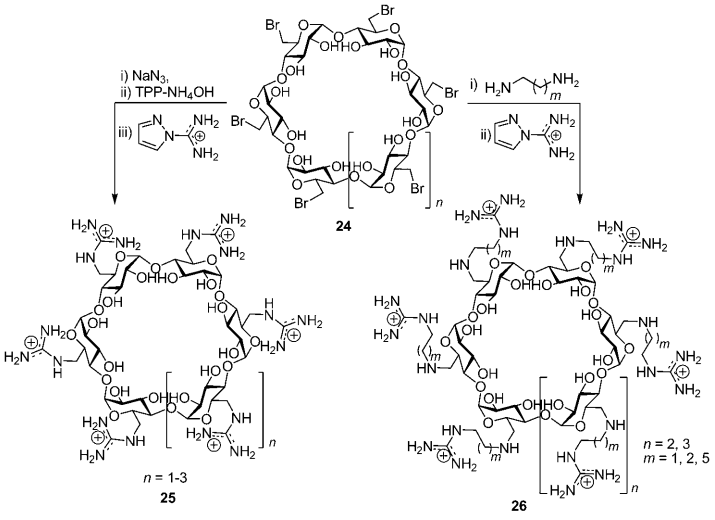
Figure 8. General structure of cyclodextrins.

even to cover linear chains by forming rotaxane complexes.[53] The resulting CD-based superstructures can be tailored to interact with biomacromolecules, including oligonucleotides and genes, and programmed to act as molecular shuttles for their delivery to target cells.[54]

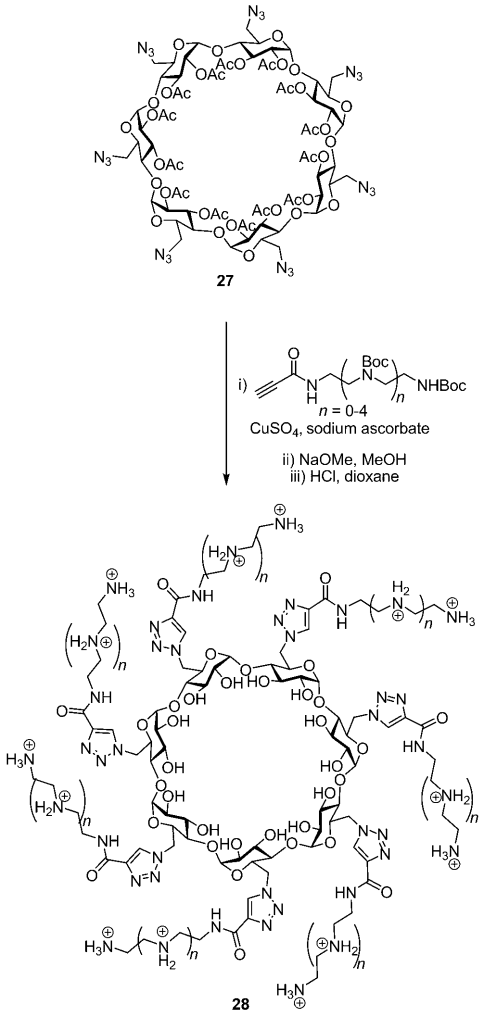
CD moieties have been shown to impart biocompatibility and to behave as transfection enhancers when incorporated to polycationic vectors; for example, grafting CDs onto PAMAM dendrimers,[55] threading CDs around polyethyleneimine (PEI) chains[56] or inserting CD motifs in cationic copolymers.[57,58] Very recently, several groups have turned their attention to the development of discrete, monodisperse CD derivatives that could self-organize in the presence of DNA to promote its compaction and safe delivery to cells.

Engineering homogeneous molecular polyfunctional ligands based on cyclodextrins critically depends on the development of efficient methods to manipulate their topology and recognition features with the environment. The higher accessibility of the primary hydroxyl groups facilitates homogeneous functionalization at the narrower rim, which has been used to create different types of polycationic bundles. Thus, O'Driscoll and Darcy reported that CD derivatives bearing alkyl and arylamine antennae on their primary rim can complex genes and moderately mediate transfection in COS-7 cells.[59] Yannakopoulou and co-workers have demonstrated that per-(C-6)-guanidino-CDs (**25**), prepared after regioselective bromination of the commercial CDs at the primary positions (→**24**) followed by nucleophilic addition of azide anion, reduction, and guanidinylation of the resulting amino groups (Scheme 4), tightly bind phosphorylated substrates with a much greater efficiency than per-(C-6)-amino-CDs. The guanidinocyclodextrins induced condensation of calf thymus DNA into nanoparticles in which the double helix was inaccessible to the intercalating agent ethidium bromide.[60] The same authors have elaborated a set of guanidinioalkylamino-CDs (**26**, Scheme 4) that, in addition to improved DNA binding avidity, exhibited cell-penetrating capabilities, which was ascribed to their resemblance to membrane-permeable polyarginine-type peptides. Interestingly, the transfection efficiency of these vectors against human embryonic kidney HEK 293T cells favorably compared with that of the commercial cationic lipid formulation Lipofectamine 2000®.[61]

Reineke and co-workers took advantage of the copper(I)-catalyzed Huisgen 1,3-dipolar azide–alkyne cycloaddition reaction[62] between the per-(*O*-2,*O*-3)-acetylated heptaazide **27** and acriloyl amide derivatives to synthesize a family of polycationic β-CD "click clusters" (**28**) bearing seven linear oligoethyleneimine branches with variable, but controlled, number of protonable amino groups (Scheme 5).[63] Agarose gel electrophoresis, DLS, and TEM experiments revealed that the click polycations complexed pDNA and protected it from nuclease degradation by forming nanoparticles with an average diameter of 80–130 nm. Notably, transfection experiments towards immortal human cervical cancer HeLa cells and rat heart H9c2 cells led authors to correlate the increase in gene expression efficiency with the length of the



Scheme 4. Synthesis of guanidinocyclodextrins.



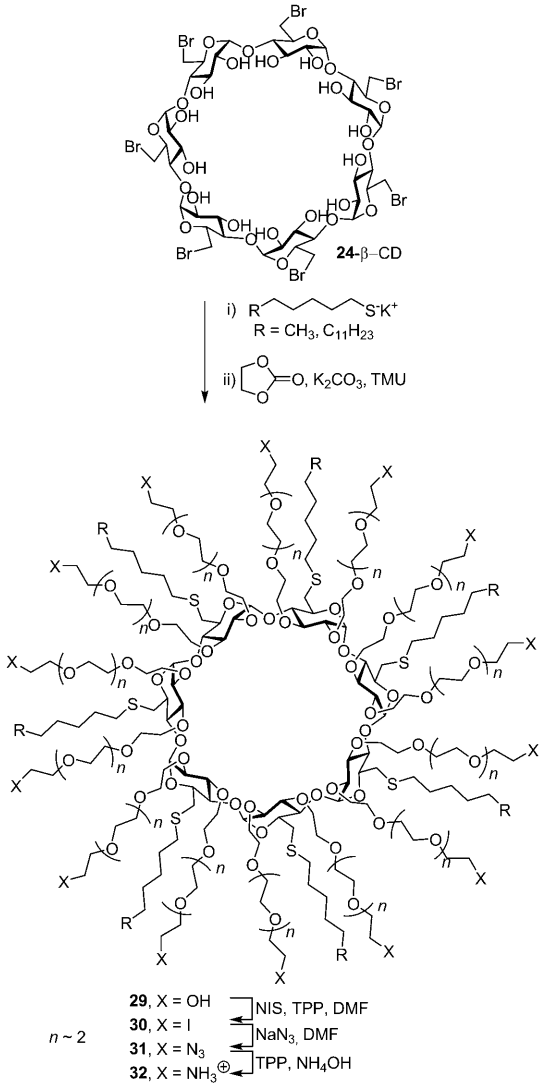
Scheme 5. Synthesis of polycationic β-CD click clusters.

oligoethyleneimine chains. Optimal transfection efficiency was reached for derivatives incorporating four or five protonable amino groups per chain ($n=3$ or 4, respectively, in Scheme 5), with expression levels that paralleled that of the commercial cationic polymer Jet-PEI® or the cationic dendrimer Superfect®, but featuring a far less toxic profile in both cell lines.

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 that, however, remain largely unexplored. In a pioneering work, Darcy and Ravoo took advantage of the differential chemical reactivity between the primary and secondary hydroxyls to install alkyl chains and polar groups at the primary and secondary positions, respectively.[64] Thioalkylation of per-6-brominated β-CD (**24**-β-CD) and subsequent hydroxyethylation with ethylenecarbonate furnished amphiphilic derivatives (**29**) that self-organized in water solution to form bilayer vesicles (Scheme 6). The same authors reported the first examples of polycationic amphiphilic CDs by converting the terminal hydroxyls to amino groups through iodination (→**30**), azide substitution (→**31**), and reduction (→**32**).[65] These compounds were shown to entrap pDNA by forming nanoparticles that behaved similarly to Lipofectamine®-derived lipoplexes in terms of transfection efficiency and toxicity against COS-7 and Hep G2 cells.[66]

The authors correlated the gene-delivery capability of the vectors with the length of the hydrocarbon chain. However, structure/transfection-efficiency relationship studies in these systems are seriously handicapped by the difficulty to obtain monodisperse samples. In fact, homogeneous functionalization at the secondary rim has consistently been a sticking point in CD chemistry. Even, a priori, simple derivatization reactions of all OH-2 and OH-3 hydroxyl groups (fourteen in total in the case of β-CD), such as acylation or etherification, have been reported to yield inseparable mixtures of under or overreacted products under standard conditions when sterically demanding reagents were involved.[67] Recently, we encountered that esterification of CD hydroxyls with long-chain acyl anhydrides in the presence of DMAP ensures homogeneous products independently of their location at the primary or secondary rim, opening a very convenient route to monodisperse multihead, multitail, facial amphiphiles through bidirectional manipulation strategies. Both possible orientations of the putative cationic and lipophilic groups onto the CD macrocycle are conceivable, namely the skirt-type (Figure 9A) or the medusa-type arrangement (Figure 9B).[68]

The polyamino amphiphilic CD (*paCD*) derivative **33**, accessible in only three steps from **24**-β-CD (i.e., four steps from commercial β-CD), as depicted in Scheme 7, already



Scheme 6. Synthesis of thioalkylated polycationic amphiphilic CDs.

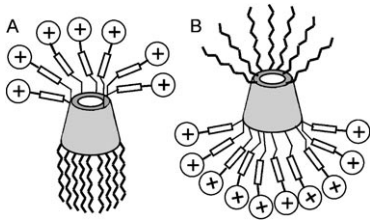
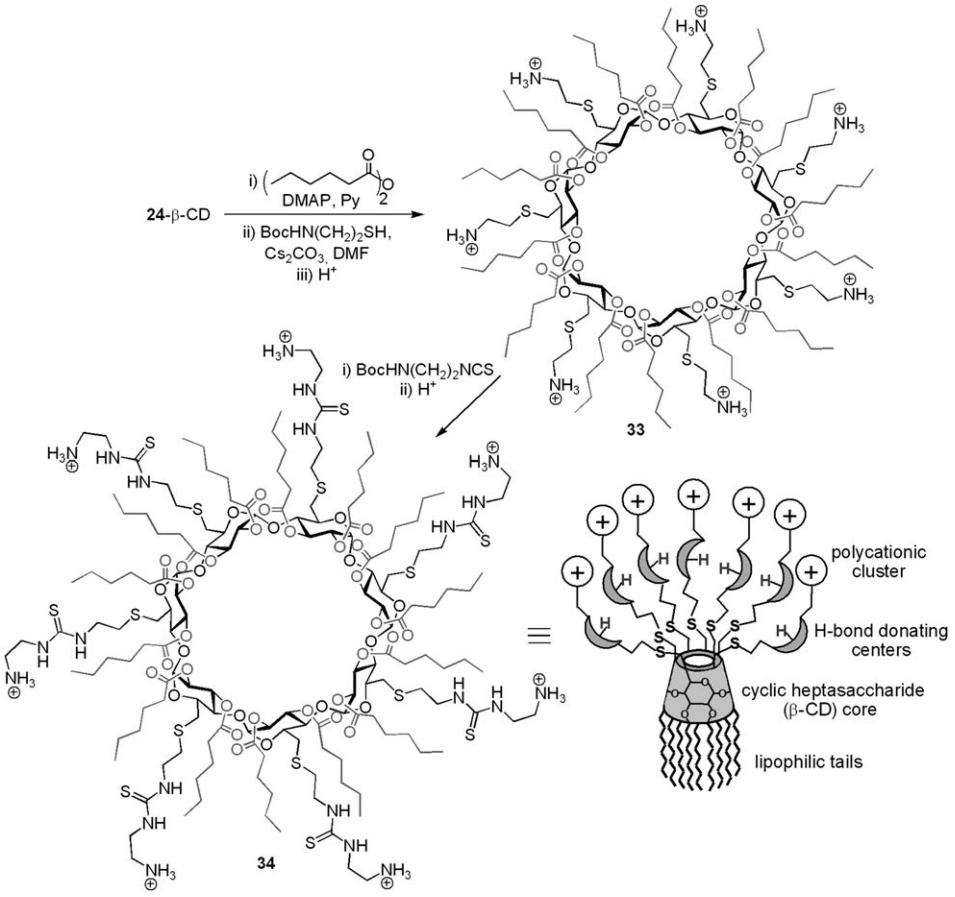


Figure 9. Relative orientation of the polycationic and hydrophobic domains in skirt-shaped (A) and medusa-shaped (B) polycationic amphiphilic CDs (paCDs).

formed stable complexes with pDNA (*CDplexes*) that fairly promoted gene expression in the murine hepatocyte BNL-CL2 cell line and human nasopharynx carcinoma KB cells, but at much lower rate than polyplexes prepared from commercial 25 kD branched PEI.[69] Most importantly, com-



Scheme 7. Synthesis of skirt-shaped cysteaminyl and thioureidocysteaminyl paCDs.

pound **33** can serve as a pivotal intermediate for further optimization through chemical elaboration. The amino groups in cysteaminyl CD derivatives have been previously shown to be particularly apt to participate in nucleophilic addition reactions, even in hyperbranched environments.[70] Inspired in the mechanisms of phosphate anion reversible recognition in nature, which implies cooperative electrostatic and hydrogen bonding interactions,[71] and keeping in mind the proven hydrogen-bond-donating capabilities of pseudoamide groups,[72] a belt of thiourea segments was inserted in the structure by multiple amine–isothiocyanate coupling reaction (e.g., →**34**). This structural modification boosted gene delivery efficiency by two orders of magnitude, paralleling that of bPEI.

The above example illustrates the utmost importance of developing modular, diversity-oriented, synthetic strategies suitable for structure–activity relationship studies to correlate modifications at the atomic level in discrete vectors and gene-delivery efficiencies for the corresponding supramolecular CDplexes. Factors such as the density and arrangement of the cationic groups and the thiourea NH hydrogen bond donor centers, the flexibility of the linkers, or the length of the lipophilic chains were evaluated.[73] Transfection efficiencies that surpass by tenfold those of bPEI and JetPEI have been achieved for BNL-CL2 and COS-7 cell lines by using paCDs possessing a dendritic arrangement of cationic elements, for example, **35**, while preserving much lower cytotoxic profiles (Figure 10).

Transmission electron microscopy evidenced the small size (≈40 nm) and homogeneous distribution of CDplex formulations from **35** (Figure 11). At high magnification, a snail-like ultrastructure, probably made of alternating lamellar arrangements of paCDs and electron-dense regions corresponding to the pDNA molecule, was observed. The rather small size and homogeneity of the CDplexes and the fact that they maintained high transfection levels in the presence of 10% serum makes them promising candidates for the development of systemic applications in vivo.

The superiority of amphiphilic tetradecacationic paCD vectors over heptacationic derivatives led to consider the possibility of "inverting" the relative orientation of the multicharged and multitail domains on the β-CD macrocycle, so as to take advantage of the full set of secondary hydroxyls, which already matches the optimal 14-valency. Transient regioselective protection of the primary hydroxyls as the corresponding *tert*-butyldimethylsilyl (TBDMS) ethers was then necessary. Sequential exhaustive 2,3-*O*-allylation (→**36**), hydroboration (→**37**), mesylation of the generated primary OH groups (→**38**), and substitution by cysteamine (→**39** to **41**) furnished a fully symmetric medusa-shaped multifunctional platform (Scheme 8).[74] By appropriately choosing the length of the acyl chains installed at the primary rim and finely adjusting the phosphate binding avidity of the functional elements at the secondary rim, a gene vector that behaved as efficiently as the skirt-type counterparts in transfection experiments using BNL-CL2 cells were obtained. A complementary strategy, that exploits photochemical addition of mercaptopropionic esters to per-2,3-*O*-allylated β-CD derivatives, has been reported by Darcy and co-workers.[75]

The awareness that the future development of molecular vectors depend to a great extent on our capacity to disclose simple and efficient synthetic routes to achieve the necessary preorganization of functional elements, led us to further explore the copper(I)-catalyzed azide–alkyne coupling reaction in the generation of CD-based facial amphiphiles. Thus,

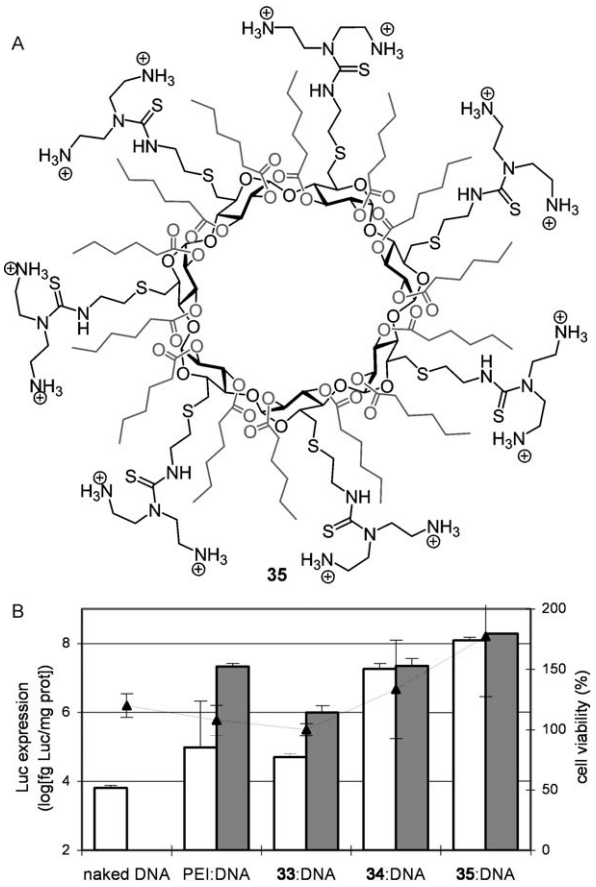


Figure 10. Structure of the dendritic paCD **35** (A) and in vitro gene expression efficiency (B, bars) and cell viability (B, line) in BNL-CL2 cells of CDplexes obtained from paCDs **33**, **34** (Scheme 7) and **35** versus naked DNA and PEI-based polyplexes at N/P 5 (unfilled bars) and 10 (filled bars).

a series of polycationic amphiphilic click clusters were prepared in which the triazole segments were either directly linked to the primary C-6 carbon of the β-CD core (rigid clusters) or separated by an acetamidocysteaminyl spacer (flexible clusters; Figure 12). In both cases, dendritic tetradecaamino derivatives were much more efficient at compacting pDNA and protecting it from degradation by nucleases than heptavalent analogues. Unexpectedly, the rigid cluster arrangement proved to be the most efficient at promoting gene transfection in CHO-k1 cells, offering 100% protection of DNA and sustained transfection levels, comparable to Lipofectamine® 2000, in the whole range of N/P values from 5 to 50. Most probably, the transfection efficiency is related to the ability of the individual molecules to self-organize in the corresponding CDplexes and the capability of the later to cross biological membranes, processes that are both intimately related to the stability of the interactions between the clusters and the pDNA chain.[76]

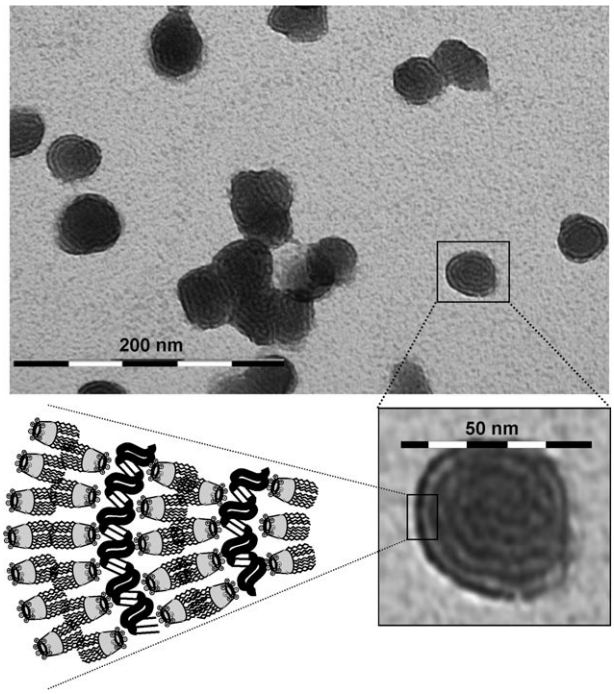


Figure 11. TEM micrograph of paCD **35**:pDNA CDplexes with amplification of the ultrafine structure of the particles and a schematic representation of the proposed arrangement of paCDs and the DNA double helix.

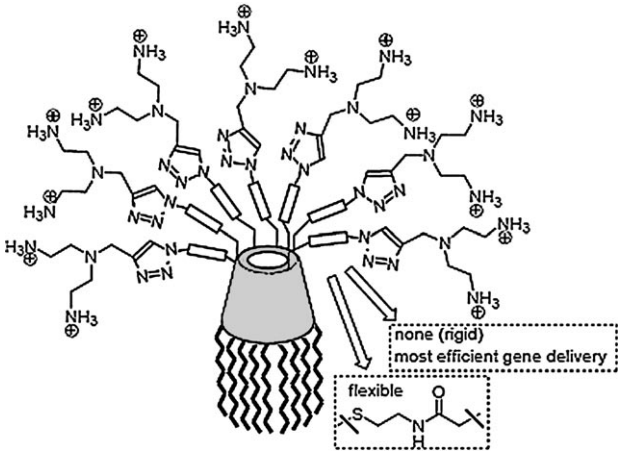
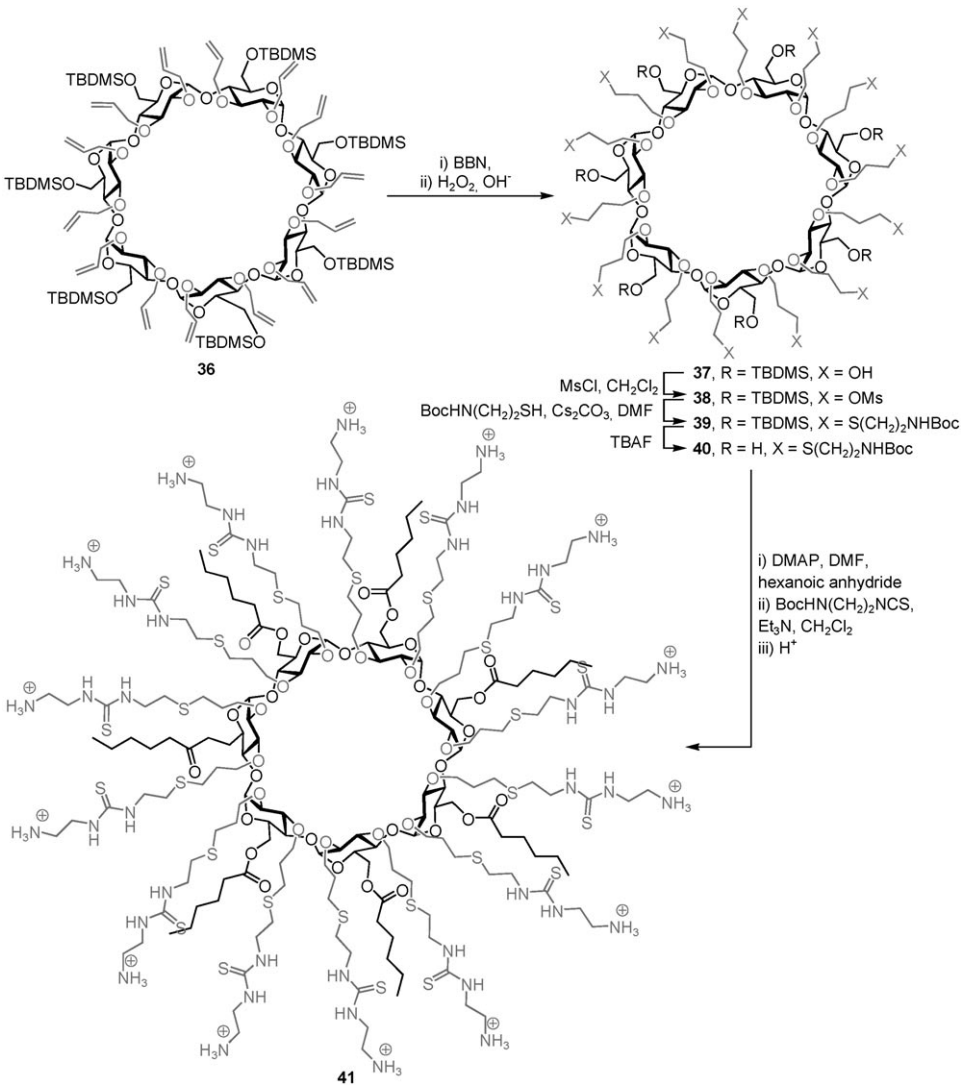


Figure 12. Schematic representation of the structure of polycationic amphiphilic β-CD click clusters.

Conclusions and Perspectives

Gene therapy is perceived as a revolutionary technology with the promise to cure almost any disease. The major limiting factor for those channels is the lack of efficient, nontoxic, and nonimmunogenic gene-delivery systems.[77] Cationic lipid formulations and cationic polymer-based nonviral vectors stand for safer and less costly alternatives to viral vectors. However, the frequent need for additional co-lipids



Scheme 8. Synthesis of the medusa-shaped thioureidocysteaminyl paCD **41**.

and the polydispersity of polymeric compounds complicates evaluation of dose scheduling and treatment thresholds and may represent a serious problem to get approval from national health authorities. Genetically engineered polymers have been proposed as a way to impart homogeneity and control over stereotacticity and full architecture.[78] Preorganized macromolecular gene vectors represent a promising alternative that, at the least, should be better suited for purity control and rational optimization.

Facial amphiphiles obtained by space-oriented functionalization of suitable platforms have already proven to be a viable approach for gene delivery. Axial–symmetric cage compounds appear particularly versatile scaffolds, since they allow organizing the space at the nanometric scale at two levels, namely the north and south faces and the inside and outside regions, thereby habilitating covalent as well as supramolecular routes for the installation of functional elements. In addition to aromatic cavitands and cyclodextrins, other macrocyclic compounds such as cyclic peptides[79] or non-natural cyclooligosaccharides[80] could be used as core structures. It is also conceivable that different platforms could be assembled by the current technologies to allow a precise control of composition, size and multifunctionality of the delivery system. Multiplatform architectures have the potential to hybridize the strengths of polymeric and modisperse vector in order to overcome the extra- and intracellular barriers to efficient, safe and cost-effective gene delivery.[81] The incorporation of targeting ligands or/and fluorescent probes is also an attractive option for site-specific gene delivery and mechanistic studies.[82] While statistical functionalization approaches are relatively immediate, keeping full structural control in the construction of artificial viruses will require the implementation of imaginative synthetic strategies. In addition to pDNA delivery, chemical control of vector architecture could be exploited for the development of nanovehicles for therapeutic siRNA, thus enlarging the potential for medical applications by inhibiting the translation pathway of a specific gene.[83] The following years will certainly witness remarkable progress in that direction.

Acknowledgements

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