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Cyclodextrin-scaffolded glycotransporters for gene delivery*

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Abstract: Conventional drugs consist of a formulation of a bioactive species and a carrier, the former accounting for most of the sophistication of the design. In the case of biomolecular drugs, however, the role of the carrier becomes decisive in enabling the load to reach its target to carry out its designed therapeutic function. Thus, the clinical success of gene therapy, where the active principles are nucleic acids, critically depends on the use of efficient and safe delivery systems. Carbohydrates have proven particularly useful in this regard. Glycocoating, similarly to poly(ethylene)glycol (PEG)-coating (*pegylation*), can stabilize colloidal aggregates by improving solvation and preventing nonspecific interactions, for example, with serum proteins. Moreover, glycoconjugates can drive specific recognition and receptor-mediated internalization in target cells. Actually, the inherent flexibility of carbohydrate and glycoconjugate chemistry has greatly contributed to enlarging the range of functional materials that can be rationally conceived for gene delivery. Herein, this is illustrated with selected examples that focus on controlling the architectural parameters of the vectors to make them suitable for structure–activity relationship (SAR) and optimization studies. The members of the cyclomaltooligosaccharide (cyclodextrin, CD) family will be the central actors of the story.

Keywords: carbohydrates; carbohydrate–protein interactions; cyclodextrins; DNA; drug delivery; gene delivery; lectins; nanodevices; nanoparticles.

INTRODUCTION

In 2011 the scientific community celebrated the 10th anniversary of the publication of the sequence of the human genome in the journals *Nature* [1] and *Science* [2]. This has been probably the most influencing achievement in the biomedical research area after the elucidation of the DNA double-helix structure, meeting an unprecedented echo in the mass media. The systematic analysis of such a huge amount of information opened new horizons in the understanding of the human machinery, with the promise, among others, of the development of personalized solutions for genetic as well as acquired diseases, including those for which no current satisfactory treatments are available. Most pathologies relate to the malfunctioning of one or more proteins. Establishing the connection between a given illness, the

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involved protein(s), and the encoding gene(s) represents an invaluable tool to understand the molecular basis of disease and develop appropriate drugs to attack not just the symptoms, but the origin of the problem. One of the more exciting approaches in this context would consist in providing a nucleic acid drug to supplement or alter genes within the patient's cells in order to produce the functional protein endogenously; this is the ultimate goal of gene therapy.

Gene therapy was first conceptualized in 1972 [3], well before the sequence of the human genome was accomplished, by proposing that "good" genetic material could be introduced into disabled cells of those who suffer from genetic defects to attenuate or correct expression of a disease. But cells are programmed to preserve their genetic information, even if it is wrong, and avoid invasion by exogenous nucleic acid. The need for a suitable vehicle, a vector, that will protect the genetic material from the environment and help it to overcome the different obstacles in its way to the final destination, either in the cytoplasm (for RNA) or in the cell nucleus (for DNA), is imperative. The clinical success of gene therapy critically depends on the use of efficient and safe delivery systems capable of reversibly complexing the relevant nucleic acid and facilitate cell membrane crossing, endosome escaping, cytoplasm trafficking, and nuclear membrane pore passing.

Because of their natural ability to infect cells, viruses were the first logical choice to deliver genes to the right spot. Recombinant viruses have been constructed by replacing the genes essential for the replication phase of their life cycles with the therapeutic genes of interest. In 1990, the first clinical trial based on this concept was performed in a four-year-old girl suffering from adenosine deaminase deficiency-severe combined immunodeficiency (ADA-SCID) [4]. Although the beneficial effects were only temporary, the results were successful. However, in further multicenter trials some of the children developed a leukemia-like condition, leading to suspension and revision of the regulatory protocols. These cases featured uncontrolled proliferation of T cells caused by deregulated expression of oncogenes, as a result of integration of the vector provirus in the gene region [5]. A thorough investigation to assess and then minimize risks, made possible by knowledge of the human genome sequence and the availability of adequate ligation-mediated polymerase chain reaction (PCR) technologies, was conducted. Substantial advances towards safe viral vector-mediated gene therapy have been made, but for the most part they remain to be validated in vivo. Meanwhile, genotoxicity remains a serious concern and is the main reason why the U.S. Food and Drug Administration (FDA) has not yet approved any viral gene vector and only very recently the European Medicines Agency (EMA) gave the green light to the first viral-based therapy (uniQure will be allowed to market Glybera to prevent pancreas inflammation in individuals affected by lipoprotein lipase deficiency [6]).

The design of artificial (nonviral) carriers for gene delivery has emerged as a safer and promising alternative to viral vectors [7]. The advent of nanotechnology has boosted the field, and nowadays literally hundreds of nonviral gene vectors have been proposed [8]. Most of them can be classified into either the cationic lipid [9,10] or the cationic polymer category (Fig. 1) [11,12]. Both types of compounds feature functional groups that electrostatically neutralize nucleic acids and cooperatively promote compaction into colloidal nanoparticles termed *lipoplexes* and *polyplexes*, respectively, with increased metabolic stability and membrane permeability [13]. In contrast to their viral counterparts, nonviral vectors are, in principle, invisible to the immune system and, since they can be tailored for a particular purpose following a "bottom-up" design, there are no restrictions on the size and amount of the cargo to be delivered [14]. Most interestingly, those systems can be endowed with biorecognition properties through covalent or supramolecular chemical manipulation. The potential of this approach for site-specific gene delivery to the brain was demonstrated in 2003 by using lipoplexes labelled with a human insulin receptor monoclonal antibody (HIRMAb), which represented a hallmark in the area [15]. However, the delivery efficiency and selectivity of lipoplexes and polyplexes, despite few exceptions [16], are far from that of their viral counterparts, and they are not fully devoid of cytotoxicity.

Implementing better-performing gene vector designs requires improving our understanding of the mechanisms involved in cell and systemic traffic of vector:pDNA complexes. Regulating nucleic acid condensation, enhancing bioavailability and biocompatibility of the DNA-vector assemblies, shielding

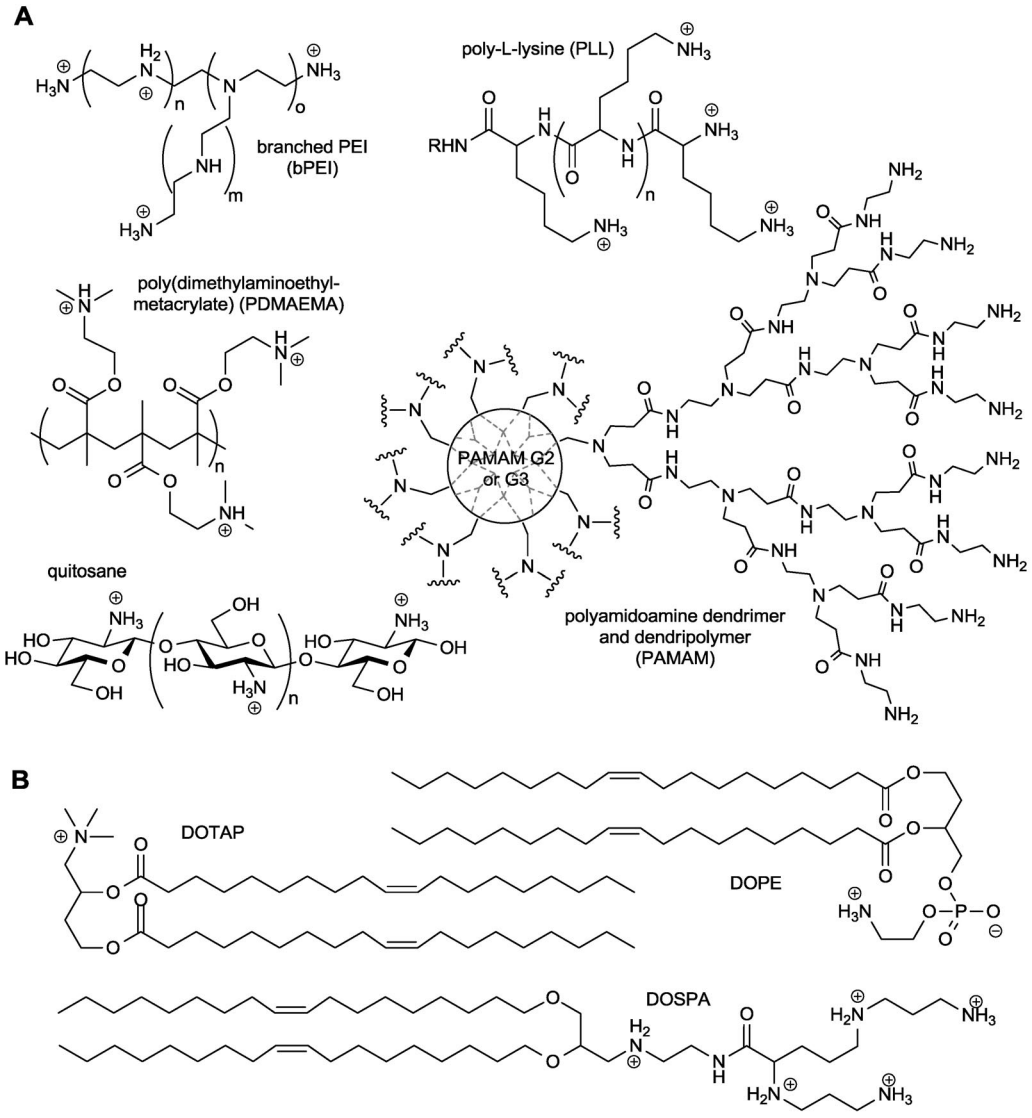


Fig. 1 Representative examples of cationic polymers (A) and cationic lipids (B) used for nonviral gene delivery.

the aggregates from nonspecific interactions, and avoiding off-target delivery or promoting selective cellular uptake are some of the aspects that must be taking into consideration for those channels. Carbohydrates have proven particularly useful in this regard. Glycocoating, similarly to poly(ethylene)glycol (PEG)-coating (*pegylation*) [17], can stabilize colloidal aggregates by improving solvation and preventing nonspecific interactions (e.g., with serum proteins). Moreover, glycoconjugates can drive specific recognition and receptor-mediated internalization in target cells [18,19]. Actually, nonviral gene delivery has benefited from the large structural and functional diversity of carbohydrates, offering broad opportunities to interfere and manipulate gene transfer capabilities through glycodecoration of first-generation lipidic or polymeric nonviral vectors [20]. The intrinsic polydispersity of most of these formulations and their random conformational properties represent important limitations that make it difficult to undertake a systematic investigation of the influence of structural modifications on the transfecting properties, however. In addition, their generally flexible character may give rise to self-

folding, which decreases the binding ability towards the gene material and forces the use of higher vector:nucleic acid ratios to achieve full complexation and protection [21]. In any case, the inherent flexibility of carbohydrate and glycoconjugate chemistry has greatly contributed to enlarge the range of functional materials that can be rationally conceived for gene delivery. In the next sections, selected examples that focus at controlling the architectural parameters of the vectors in order to make them suitable for structure–activity relationship (SAR) and optimization studies will be discussed. The members of the cyclomaltooligosaccharide (cyclodextrin, CD) family will be the central actors of this story.

WHY CYCLODEXTRINS?

CDs represent a paradigmatic example of carbohydrate derivatives exhibiting a close relationship between molecular status and supramolecular properties. Their macrocyclic skeleton, composed of α-(1 → 6)-linked D-glucopyranoside units, features a hydrophobic cavity that endows these compounds with guest inclusion capabilities [22]. This characteristic has been largely exploited in the solubilization and stabilization of organic molecules in aqueous media for applications in areas such as pharmaceutical technology [23], cosmetics [24], foods [25], or materials [26]. But CDs can also be perceived as nanometric platforms with two well-differentiated faces, the primary and secondary hydroxyls rims, susceptible of selective chemical manipulation. A battery of methods for regioselective monofunctionalization, face-selective functionalization, or even differentiation of several precise positions is currently at hand [27]. Their chemical versatility, in addition to the commercial availability of the native compounds in three different sizes, namely, the hexa- (αCD), hepta- (βCD), and octameric (γCD) homologues, provides a unique toolbox for the purpose of engineering molecular devices capable of performing sophisticated tasks (Fig. 2). Particularly interesting is the possibility to tailor their recognition properties towards biomolecules by the incorporation of functional elements in a precise spatial arrangement. The following two examples, selected from our own laboratories, illustrate this approach.

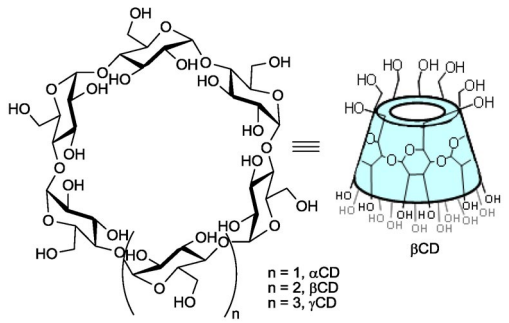


Fig. 2 Chemical structure and schematic representation of CDs.

In the frame of a project aimed at providing a molecular shuttle for the solubilization of the taxane antimitotic drug docetaxel (Taxotere®) and its specific delivery to macrophages, a βCD dimer was constructed from the corresponding 6^I-amino-6^I-deoxy derivative (Fig. 3) [28]. The spacer length was adapted to span the distance between the two aromatic rings in the docetaxel molecule, which fit fairly well in the βCD cavity, thereby promoting the formation of a chelate-type complex. The linker was further armed with a reactive group allowing the incorporation of a hexavalent α-D-mannopyranosyl (αMan) ligand exhibiting high affinity towards the specific mannose receptor at the surface of macrophages (macrophage mannose receptor, MMR). Cell adhesion experiments confirmed that the CD dimer:drug complex was able to bind to the MMR to form a ternary complex that elicited internalization, supporting the validity of the concept.

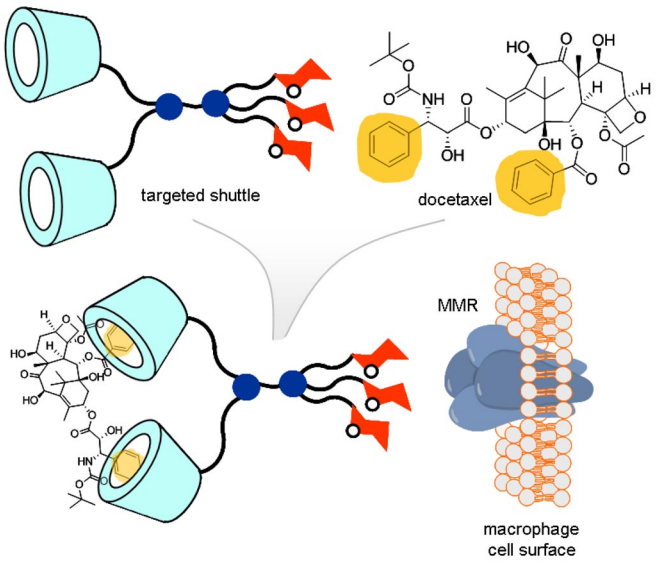


Fig. 3 Schematic representation of an MMR-targeted taxane drug carrier based on a glycodendritic dimeric CD derivative [28].

In the above example, association of the inclusion complex to the biomacromolecular partner is driven by a multivalent glycoligand appended to the CD core. It is also conceivable that the CD molecule itself could be shaped to make it complementary of a given biological receptor. Thus, the installation of a positively charged cluster at the primary or secondary rim of βCD, having a C_7-symmetry and an external diameter complementary of the heptameric pore of anthrax toxin, has been put forward to design pore binders that inhibit the translocation of the toxin lethal factor to the cytosol in cell assays, preventing cell death at μM concentrations (Fig. 4) [29]. In this case, the binding process relies on electrostatic interactions between the negatively charged amino acids at the interior of the pore and the cationic centers on the CD platform.

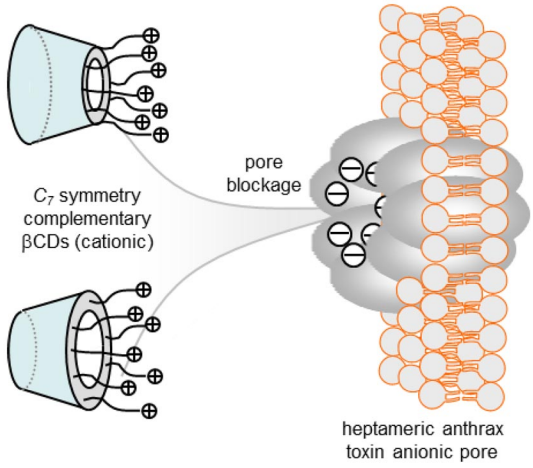


Fig. 4 Schematic representation of βCD-based anthrax toxin inhibitors with symmetry complementarity with the toxin heptameric pore.

CATIONIC CYCLODEXTRINS AND CYCLODEXTRIN:DNA COMPLEXES (CDplexes)

From the above-commented results, and considering that the most relevant examples of polyanionic biomolecules are nucleic acids, it can be intuited that a combination of cationic CD shaping and ligand appending could be implemented in the rational design of site-specific gene delivery systems. It is fair to start by saying that we have been neither the first nor the only group to realize and exploit the potential of CDs in gene delivery. βCD has been long known to behave as a transfection enhancer when present in vector formulations [30], which is ascribed to its ability to complex cholesterol and increase cell membrane permeability [31]. Selectively functionalized CDs have also been integrated in cationic polymers, dendrimers, and pseudorotaxanes, among others, to modulated nucleic acid complexation and delivery [32]. The work by M. E. Davis on a cationic polymer with βCD blocks inserted in the polymer backbone deserves special mention [16,33]. This construct efficiently complexed siRNA, and the resulting nanoparticles could be further decorated with elements imparting bioavailability (PEG chains) and targeting properties (transferrin) through supramolecular ligation, taking advantage of the strong affinity of the βCD cavity towards adamantane moieties.

Although very successful [34], the above system still suffers from the inconveniences associated with polydispersity in view of advanced SAR studies. Several groups have tried to overcome this limitation by developing monodisperse CD-scaffolded polycationic clusters [35]. In all cases, the cationic centers (amine, guanidine, amino acids) were installed at the primary positions after face-selective functionalization, keeping unmodified OH-2 and OH-3 hydroxyls, taking advantage of the direct accessibility of the corresponding per-(C-6)-halogenated precursors (Fig. 5). The resulting polycationic CDs (pCDs) formed nanocomplexes (CDplexes) with plasmid DNA (pDNA) that promoted transfection in vitro to some extent. The highly hydrophilic character of these materials somewhat hampers self-organization of the monomers at the surface of the oligonucleotide chain, however. Consequently, relatively big excesses are necessary for most representatives to achieve full protection from the environment. Nevertheless, in some cases the resulting CDplexes exhibited remarkable transfection capabilities while featuring much less toxic profiles than commercial cationic polymers.

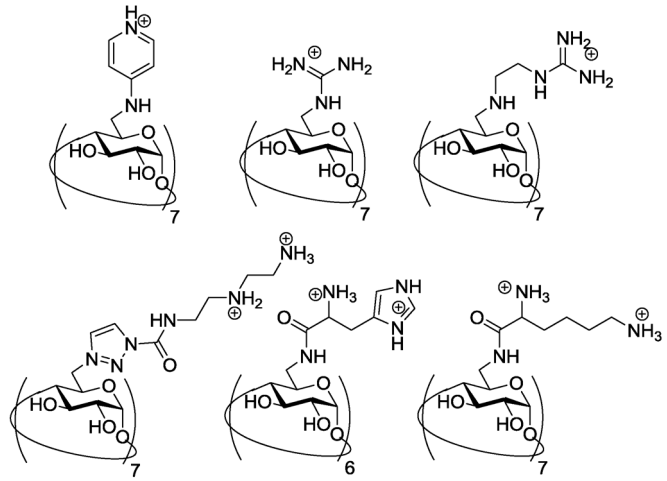


Fig. 5 Representative examples of homogeneous primary rim-functionalized pCDs with gene vector capabilities [35].

INTRODUCING FACIAL AMPHIPHILICITY: POLYCATIONIC AMPHIPHILIC CYCLODEXTRINS (paCDs)

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 vector design. A particularly promising clan of monodisperse systems has been inspired by the facial amphiphilicity concept [36]. The basic hypothesis is that compounds presenting segregated cationic and hydrophobic domains might increase the fusogenic potential of the transfecting particles, analogously to some natural components known to destabilize membranes, thereby enhancing DNA uptake. Several biomimetic prototypes have been thus proposed based on rigid platforms susceptible of selective chemical functionalization. Examples on record include fullerene [37], resorcarene [38], and calixarene derivatives [39]. Our laboratories and others [40] have focused on the implementation of this notion in the CD field for gene delivery purposes, with the permanent challenge of developing molecular diversity-oriented approaches that warrant sample homogeneity.

The facial anisotropy of the truncated-cone CD torus is amenable to the installation of cationic and hydrophobic elements in two distinct relative orientations, namely, the "skirt" and the "jellyfish" architectures (Figs. 6 A and B, respectively), with the cationic and lipophilic domains at the primary and secondary rims, respectively, or vice versa [40]. Indeed, we have prepared both types of paCD architectures [42,43] and shown that in either case the molecular parameters (charge density, spacer length, functional groups nature, hydrophilic–hydrophobic balance) can be finely adjust to achieve efficient nucleic acid complexation and protection as well as productive transfection in several cell lines after CDplex formation [44]. Aliphatic chains were attached to the CD core through the secondary or primary hydroxyls by ester or ether linkages, whilst nucleophilic addition of amines to isothiocyanates to afford thioureas, copper(I)-catalyzed azide-alkyne coupling (CuAAC) to give 1,4-substituted triazoles or the combination of both "click"-type reactions have been privileged for the attachment of the cationic elements [45]. Overall, the "skirt"-type arrangement, in combination with multiple thiourea-forming coupling approaches, presented advantages in terms of synthetic straightforwardness and optimization of self-assembling properties of the resulting paCDs in the presence of pDNA. To avoid an unduly long discussion, we will concentrate on this family of paCD derivatives.

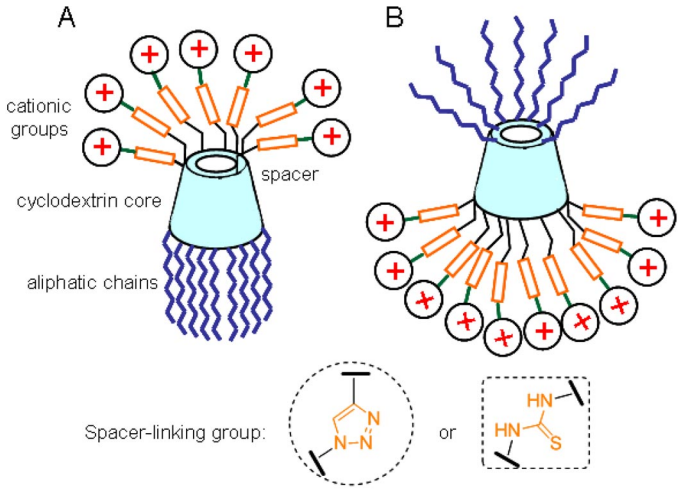
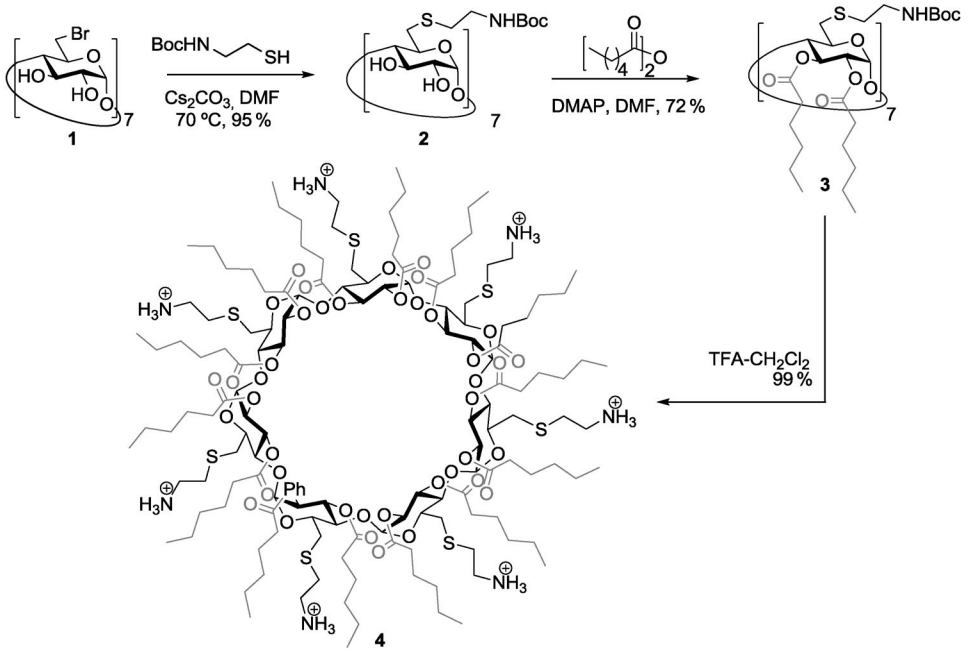


Fig. 6 Relative orientation of the polycationic and hydrophobic domains in skirt-shaped (A) and jellyfish-shaped (B) paCDs, with indication of the preferred linking groups to connect the functional elements to the CD core.

Cysteamine-based paCDs

Our first paCD vector candidate was a βCD-centered heptacationic cluster having cysteaminyl groups at the primary C-6 positions and hexanoyl chains at the secondary hydroxyls **4** [43]. It was obtained in only three steps from the known per-(C-6)-bromo functionalized βCD precursor **1** [46] by nucleophilic reaction with Boc-protected cysteamine (→ **2**), followed by acylation with hexanoic anhydride in *N*,*N*-dimethylformamide (DMF) in the presence of dimethylaminopyridine (DMAP; → **3**) and final acid-catalyzed carbamate hydrolysis (Scheme 1). The acylation conditions are critical: other reagent/solvent combinations (e.g., the corresponding acyl chloride in pyridine) led to over- or under-acylation products that hampered purification. Sample homogeneity was extensively checked by NMR, MS, and microanalytical techniques, fully confirming preservation of the C_7-symmetry of the final adduct. This multitechnique purity control protocol has been and continues to be rigorously applied to all paCDs prepared within this project to ensure molecular monodispersity.

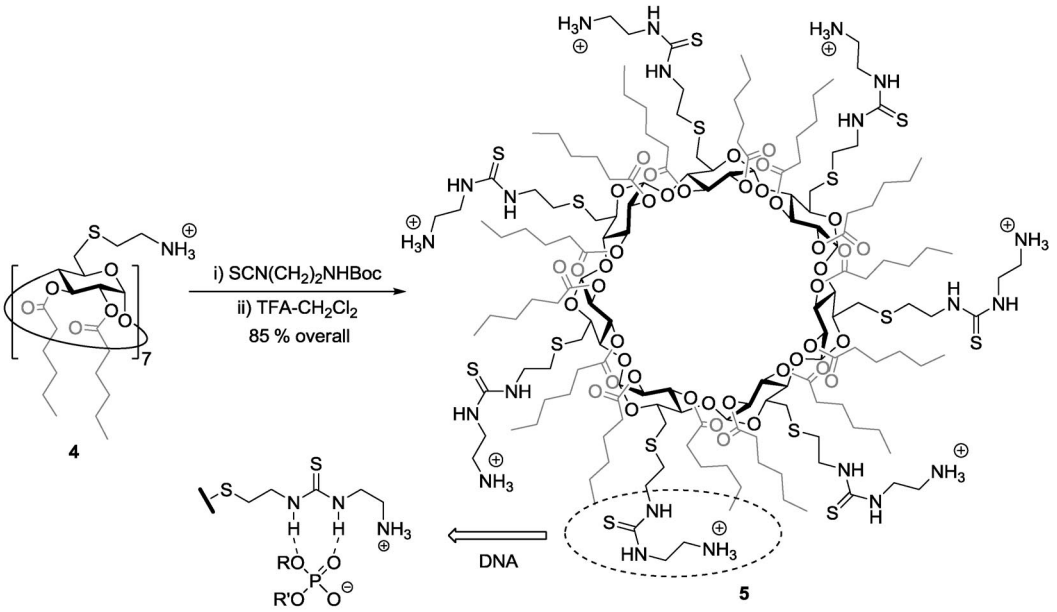


Scheme 1 Synthesis of the skirt-type cysteaminyl paCD **4** [43].

Compound **4** was found to form stable nanocomplexes (hydrodynamic diameter 50–80 nm) with positive surface potential (ζ-potential +24 to +28 mV) when formulated with luciferase-encoding pDNA (pTG11236, 5739 base pairs) at protonable nitrogen/phosphorous (N/P) ratios ≥5. The transfection efficiency in COS-7 (green monkey epithelial kidney cells) and BNL-CL2 (murine hepatocytes) cell lines was found to be 100-fold less efficient as compared with polyethyleneimine (PEI; the gold standard in nonviral gene delivery)-based polyplexes at N/P 10, the optimal for PEI. Although cell viability was much favourable for the CDplexes (100 vs. 40 %), it became evident that further optimization was required before real applications in gene therapy could be envisaged.

Thioureido-functionalized paCDs

We conceived that the insertion of a belt of thiourea groups between the CD core and the cationic cluster in the "skirt"-type archetype will help to improve reversible complexation of DNA through the interplay of hydrogen bonds and electrostatic interactions, a mechanism that mimics phosphate complexation by biological receptors. Actually, thiourea-based lipoplexes (lipopolythioureas) have also been developed for nonviral gene delivery [47]. Accessing this new family of paCDs was immediate from the cysteaminyl derivative **4** by multiple amine-isothiocyanate coupling [48]. Thus, reaction with 2-(*N*-Boc)aminoethyl isothiocyanate and subsequent carbamate removal afforded the corresponding hepta(aminoethylthioureido) adduct **5** (Scheme 2). CDplexes formulated with this compound at N/P 10 displayed a 100-fold increase in transfection efficiency as compared with the previous cysteaminyl paCD-based nanoparticles, equaling the performance of PEI-based polyplexes and validating the hypothesis. Most importantly, the synthetic protocol was purposely conceived to be molecular diversity-oriented, with a relatively low synthetic cost. It was, thus, very well-suited for vector library generation and studies on the relationships between vector structure and transfection efficiency, which is quite exceptional in the field of gene delivery.



Scheme 2 Synthesis of the thioureido-functionalized paCD **5** with indication of the proposed cooperative complexation of phosphate groups by aminothiourea segments [48].

In a rather comprehensive work, conducted in collaboration with Drs. P. Vierling, C. Di Giorgio (CNRS and University of Nice, France), and J. Defaye (CNRS and University of Grenoble, France), the different functional elements in the vector (aliphatic chain length, spacer between thiourea and amino groups, disposition and number of hydrogen bonding and cationic centers) were systematically modified and the capacity to transfect COS-7 and BNL-CL2 cell lines was evaluated [44a]. It was concluded that aminoethylthioureido motifs combined with hexanoyl tails led to optimal pDNA complexing and transfection properties and that dendritic presentations of the cationic heads, as in compound **6**, further increased transfection levels, overpassing PEI performance by ten-fold, even at lower N/P ratios (5 vs. 10) with no toxicity implications. The resulting nanocomplexes were very homogeneous, with a

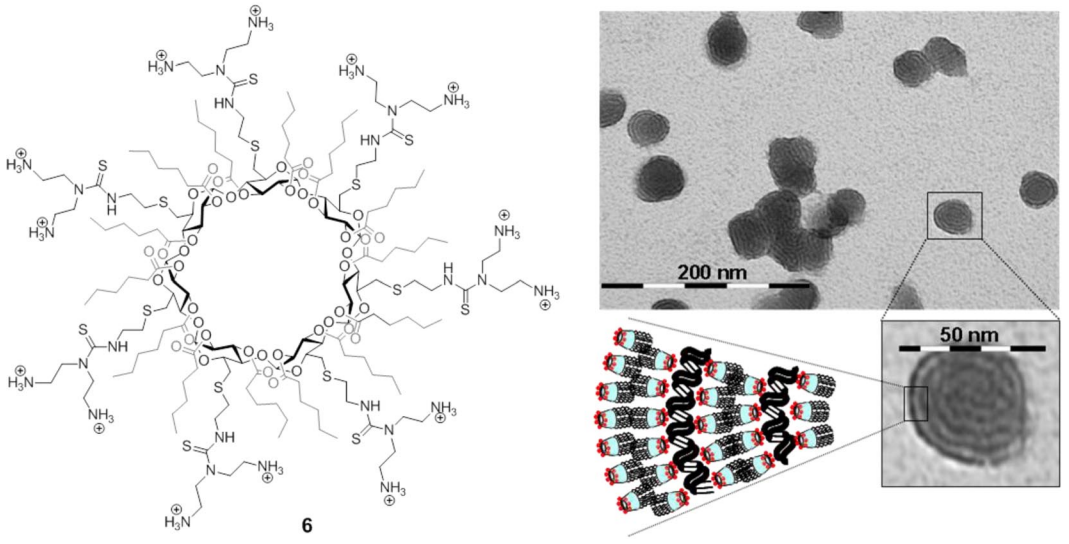


Fig. 7 Structure of the dendritic thioureido-functionalized paCD **6** (left) and TEM micrograph of the CDplexes obtained by mixing **6** with pDNA, with an schematic illustration of the probable arrangement of paCD and pDNA layers (right).

diameter of 40–50 nm as measured by transmission electron microscopy (TEM, Fig. 7). Considering that the plasmid has a size of about 1 μm, this implies a compaction ratio of about 20 times, meaning that the particles are probably monomolecular in DNA and well suited for systemic application [49]. An ultra-thin structure could be observed evidencing a snail-like arrangement with dark regions of high electronic density that correspond to the DNA chain and lighter bands that probably arise from bilayers of the paCDs.

In collaboration with Prof. Tros de Ilarduya, at the University of Navarra, Spain, the potential of CDplexes prepared from the dendritic paCD vector **6** and a gene encoding for interleukine-12 (IL-12) in gene therapy against cancer was explored [51]. Initial in vitro experiments were carried out in the presence of 60 % serum using human cervix cancer HeLa and cellular hepatocarcinoma Hep-G2 cells. The data indicated the superiority of the CDplexes formulated with **6** as compared with PEI-formulated polyplexes in terms of both transfection efficiency and toxicity. Results in hepatocarcinome cells were particularly interesting because this cancer has a high rate of mortality. Moreover, the CDplexes exhibited a certain tropism to the liver in a mouse model, with the same efficacy as hepatocyte-targeted liposomes, further supporting the promise for cellular hepatocarcinoma gene therapy strategies.

To investigate the possible differential uptake of CDplexes by specific cell types, the mechanisms of internalization were studied in collaboration with Prof. De Smedt, at Ghent University, Belgium. We prepared a fluorescently labelled derivative of a dendritic vector (**7**; Fig. 8) by conjugation with rhodamine-lissamine and monitored the trafficking of the resulting CDplexes in African green monkey epithelial VERO cells by confocal microscopy. Internalization was found to occur very fast: after 40 min almost 100 % of the cells contained CDplexes, which then proceed through the cytoplasm to accumulate at the vicinity of the nucleus in a short time (2 h). This observation discarded passive diffusion and was instead compatible with active-mediated endocytosis and transport. By using specific inhibitors of the different endocytic routes, it was concluded that caveolae-mediated endocytosis was the more productive one [51]. Since caveolae are present in most cell lines, this means that CDplexes must be considered as broad-range transfection systems. Their use in site specific gene delivery strategies will require the incorporation of biorecognition elements complementary of receptor partners at the target cells.

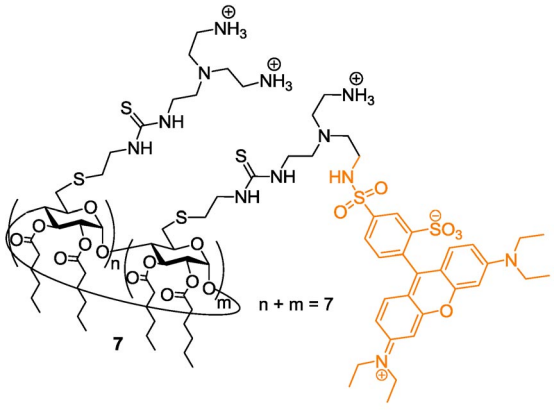


Fig. 8 Structure of the fluorescently labelled paCD vector **7** used to assess CDplex trafficking by confocal microscopy [51].

GLYCOTARGETED GENE DELIVERY: POLYCATIONIC GLYCOAMPHIPHILIC CYCLODEXTRINS (pGaCDS)

Having already developed a sugar carrier for the DNA drug, and considering our carbohydrate chemistry background, exploring glycotargeting for site-specific gene delivery appeared quite logical. Preliminary attempts to access glycocoated CDplexes by co-formulation of pDNA with a mixture of a paCD and a neutral GaCD conjugate failed. Proportions of the neutral glycoamphiphiles as low as 2 % completely disrupted the paCD:pDNA nanoparticles, probably by creating neutral microdomains that weakened the association and prevented self-association of the CD units onto the nucleotide chain. To avoid this unwanted effect, the preparation of conjugates having regular arrangements of cationic and sugar displays, namely, pGaCDs was undertaken. Two different prototypes (Fig. 9), one in which the cationic centers and the sugar ligands are located in separate branches at the primary rim of βCD (**8**)

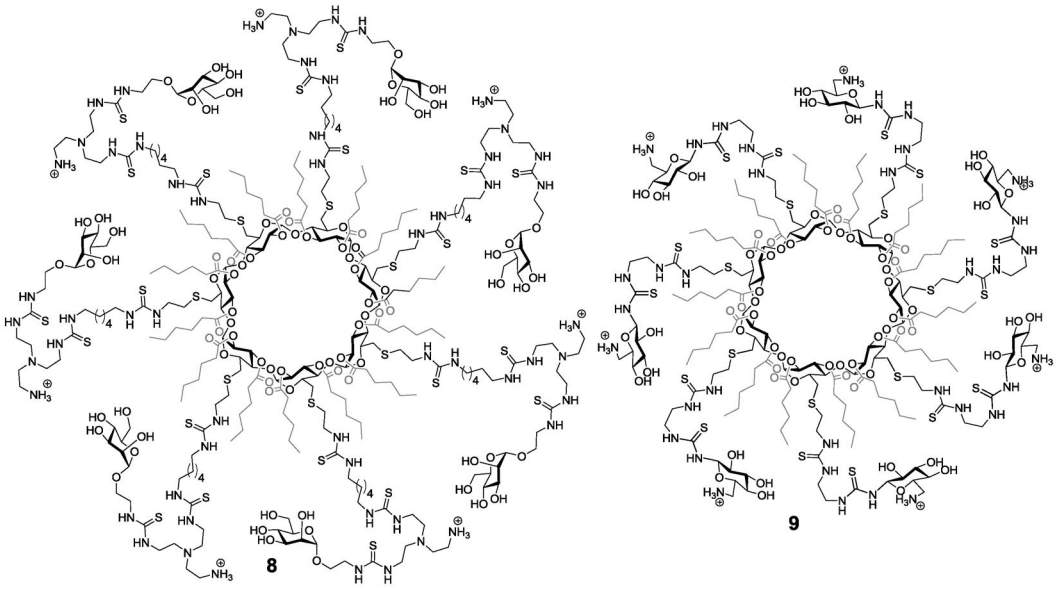
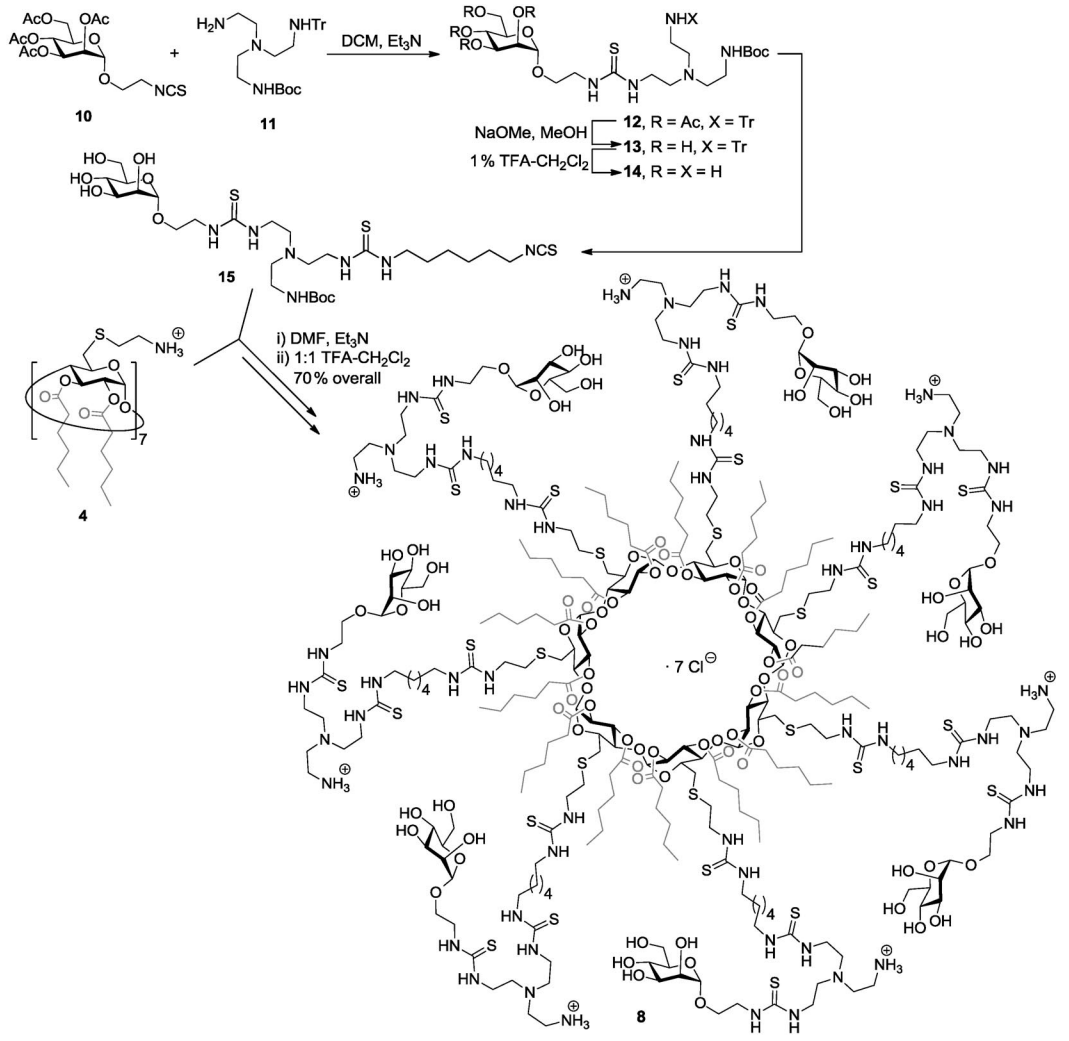


Fig. 9 pGaCDs **8** and **9**.

and another one bearing aminosugar substituents (**9**), were considered. In the first case, αMan glycotopes were considered, whereas the 6-amino-6-deoxy-β-D-glucopyranose motif was chosen for the last molecular design since this aminosugar is encountered in aminoglycoside antibiotics known to interact very efficiently with DNA [52].

The higher structural complexity of pGaCDs as compared with paCDs implied a higher chemical effort. As an example, the synthetic route developed for the preparation of the cationic mannosyl conjugate **8** is illustrated in Scheme 3. A convergent approach, based in the use of an orthogonally protected tris(2-aminoethyl)amine (TREN) precursor (**11**), was conceived in which the αMan glycotope was first installed from isthiocyanate **10** by using thiourea ligation chemistry (→ **12** to **14**). After selective detritylation, an isothiocyanate-armed spacer was incorporated (→ **15**). Multiple amine-isothiocyanate coupling with the cysteaminyl paCD **4** and final deprotection afforded the requested monodisperse heptamannosyl-heptacationic amphiphilic βCD derivative **8** [53].



Scheme 3 Synthesis of the heptamannosylated pGaCD **8**.

We were delighted to confirm that both pGaCDs **8** and **9** recovered the self-assembling properties in the presence of DNA and were able to form stable glycoCDplexes with an ordered ultrastructure analogous to that previously observed for paCD-based CDplexes [44a]. These nanoparticles must expose the glycoligans at their surface in a multivalent manner, which should result in high affinity towards specific lectins by virtue of the so-called multivalent or cluster effect [54]. This was first confirmed by enzyme-linked lectin assay (ELLA) [55] using concanavalin A (Con A) as a model mannose-specific lectin. Thus, the mannosylated CDplexes obtained from **8** were found to be extremely efficient at inhibiting the binding of the lectin to immobilized yeast mannan, while CDplexes formulated with the non-mannosylated paCD **5** or the aminoglucosylated pGaCD **9** did not (Fig. 10).

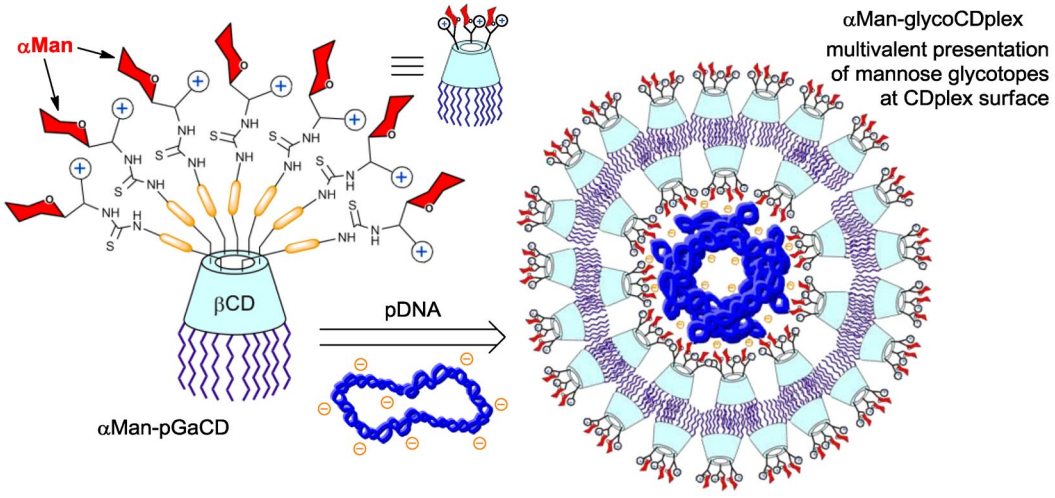


Fig. 10 Schematic representation of CDplexes obtained from pGaCD **8** exposing a multivalent display of αMan glycotopes at their surface available for specific lectin recognition [53].

A similar selectivity pattern was expected towards the mannose/fucose-specific MMR present at the cell membrane of macrophages, which could be exploited for site-specific gene delivery [56]. However, evaluation of the transfection capabilities of the paCD **5** and the two pGaCDs **8** and **9** in macrophages (RAW264.7 cells), in comparison with hepatocytes (BNL-CL2) and endothelial cells (COS-7), not expressing the MMR, showed that at the optimal N/P value of 10 the three vectors were equally efficient at promoting transfection in all three cell lines. Moreover, addition of D-mannose to the medium did not affect transfection of macrophages by the αMan-decorated CDplexes, which seemed to discard MMR-mediated internalization. Notwithstanding, at N/P 5 the mannosylated CDplexes selectively transfected the macrophages and transfection was inhibited to a large extent in the presence of yeast mannan, as confirmed by fluorescent absorption cell sorting (FACS) experiments using fluorescently labelled pDNA. These someway contradictory results casted some doubts about the glycotargeting hypothesis and the mechanism at work that required additional experimental confirmation [53].

A main concern when using Con A as a model lectin to evaluate the binding efficiency of multivalent αMan displays, in the context of selecting potential candidates for macrophage targeting, is that the human MMR (hMMR) has a completely different structure. Even though we have been unable to find a single example in the literature where a multivalent mannosyl ligand was efficiently recognized by Con A and was inefficient against the hMMR, direct correlations must be taking with care. To ascertain whether or not the mannosylated CDplexes were recognized by the hMMR, an enzyme-linked immunoabsorbent assay (ELISA)-type assay using recombinant soluble hMMR was developed The

data unequivocally confirmed that the mannosylated CDplexes strongly bound to hMMR, whereas only a background response was observed for non-mannosylated CDplexes. Actually, the data fully paralleled those obtained for the much less-costly Con A lectin under ELLA conditions [52]. Recent results on the relative binding affinities of a series of high-mannose oligosaccharides, known to be putative ligands of the hMMR, to Con A impinged on this parallelism [57].

Binding of a sugar ligand to a soluble lectin might significantly differ from binding to the same lectin anchored in the cell membrane. By using fluorescently labelled DNA, we further confirmed that the manosylated CDplexes exhibited a high avidity towards the surface of macrophages. Yet, control experiments showed that non-mannosylated CDplexes also did attach to the macrophage membrane to a significant degree (Fig. 11). This nonspecific binding background was considerably more pronounced at N/P 10 that at N/P 5 and probably arises from electrostatic interactions with negatively charged proteoglycans at the cell surface. At N/P 5 the binding of αMan glycoCDplexes to macrophages is almost fully inhibited by mannosylated-bovine serum albumin (Man-BSA). In combination with the results obtained by FACS, these data strongly supports that using this charge ratio internalization of the αMan-nanocomplexes exclusively occurs through the MMR-mediated route [53].

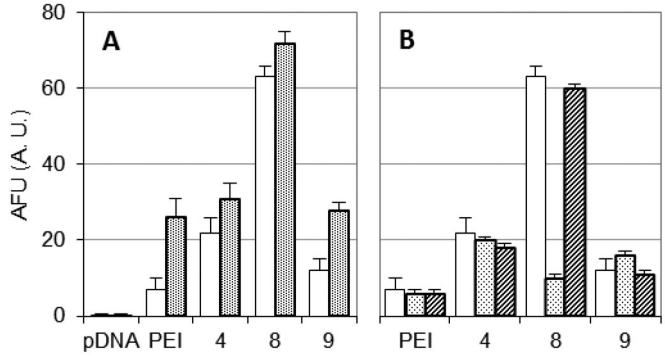
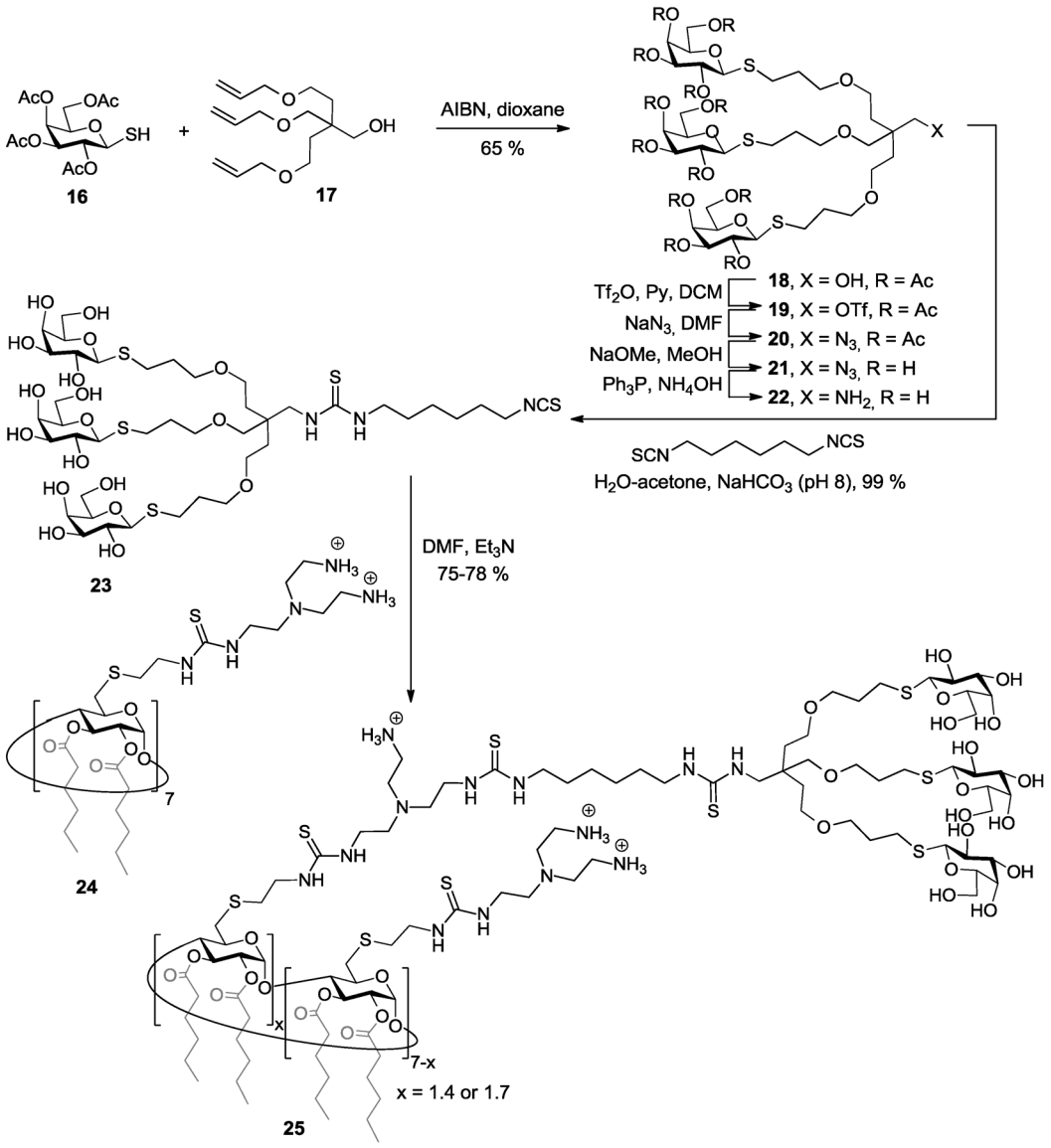


Fig. 11 (A) Adhesion to mice peritoneal macrophages of nanocomplexes obtained from **4**, **8**, and **9** vs. naked pDNA and PEI (25 kDa) at N/P 5 (blank bars) and 10 (solid bars). (B) Adhesion data at N/P 5 in the absence (blank bars) and in the presence of mannosylated-BSA (1 mg·mL^{-1}, dotted bars) or native BSA (1 mg·mL^{-1}, stripped bars).

The above results illustrate the promise of pGaCDs as vectors for site-specific gene delivery, provided the formulation is optimized to minimize electrostatic-driven nonspecific internalization. In the optics of addressing human hepatocarcinoma through gene therapy strategies, the logical approach would be targeting the asialoglycoprotein receptor (ASPR) at the surface of hepatocytes, a lectin that recognizes β-D-galactopyranosyl (βGal) motifs. According to our proposed molecular design, a CD derivative exposing βGal ligands and cationic centers at the same space region, in a facial amphiphile global architecture, would be requested. In order to lower the synthetic cost, statistic incorporation of preformed trivalent βGal dendrons onto a dendritic paCD derivative was explored. Thiol-ene "click" reaction [58] involving per-*O*-acetylated 1-thio-β-D-galactopyranoside (**16**) and tri-*O*-allylated pentaerythritol (**17**) was implemented for the preparation of the βGal-coated dendron **22** (via **18** to **21**; Scheme 4). After functional group manipulation, the glycodendron was armed with a spacer bearing an isothiocyanate group (**23**) and engaged in multiple thiourea-forming reactions with the dendritic βCD derivative **24**. The proportion of βGal motifs in the final product **25** could be modulated by acting in the relative proportion of reagents. The galactosylated pGaCD adducts self-assembled in the presence of pDNA to afford the corresponding nanocomplexes. Disappointingly, these βGal-coated CDplexes displayed very low transfection levels in hepatoblastome Hep G2 cells [59].



Scheme 4 Synthesis of statistically galactosylated pGaCDs **25** [59].

By using fluorescently labelled DNA and confocal microscopy, it was confirmed that the βGal-CDplexes were efficiently internalized in the Hep G2 cell line, being clearly observable in the cytoplasm. A comparative experiment in the absence and in the presence of a photosensitizer known to disrupt endosomes (photochemical internalization, PCI) did not evidence any increase in protein expression, discarding endosome escaping as the main obstacle for transfection. Moreover, the release of pDNA from the nanocomplexes was found to reach more than 60 % after only 3 min, confirming reversibility of pDNA complexation. We then hypothesized that low nuclear internalization rates might be at the origin of the deceivingly low transfection levels. To overcome this limitation, replacement of pDNA into mRNA, which does not need to enter the nucleus to produce the encoded protein, was next considered.

To explore the potential of the new glycovectors for mRNA-base gene therapy, the PEG-coated (pegylated) and galactopegylated adducts **26** and **27**, respectively, were prepared (Fig. 12), in addition to the galactodendron-coated pGaCD **25**. A similar convergent statistic strategy using the tetradecaamine βCD derivative **6** as a polyfunctional scaffold and isothiocyanate-armed building blocks was followed. Next, the mRNA complexing abilities and the delivery capabilities of the resulting CDplexes were evaluated using Hep G2 cells. We were happy to see that very high transfection efficiencies, much higher than those obtained with the reference compound JetPEI-hepatocytes (supposed to be specifically designed for hepatocyte transfection), were achieved for the βGal-coated glycoCDplexes. Up to 25 % of cells were transfected when using formulations containing 12 % of βGal ligand at N/P > 20 compared to 2.5 % for the reference polyplexes formulated with the non-glyosylated paCD **6**. Pegylation (as in **27**), known to increase bioavailability, almost totally abolished the transfection capacity. However, this was fully recovered when the galactosylated dendrons were installed at the end of the PEG chains (as in **26**). Finally, we confirmed that mRNA transfection using the βGal- and βGal-PEG-CDplexes obtained from **26** and **27**, respectively, was specifically mediated by the ASPR by performing the transfection assay in the absence and presence of a polyclonal antibody that blocks this lectin receptor. In both cases, the transfection levels decreased dramatically, strongly supporting that specific recognition of the βGal motifs by the ASPR is required for hepatocyte internalization of the nanocomplexes [59].

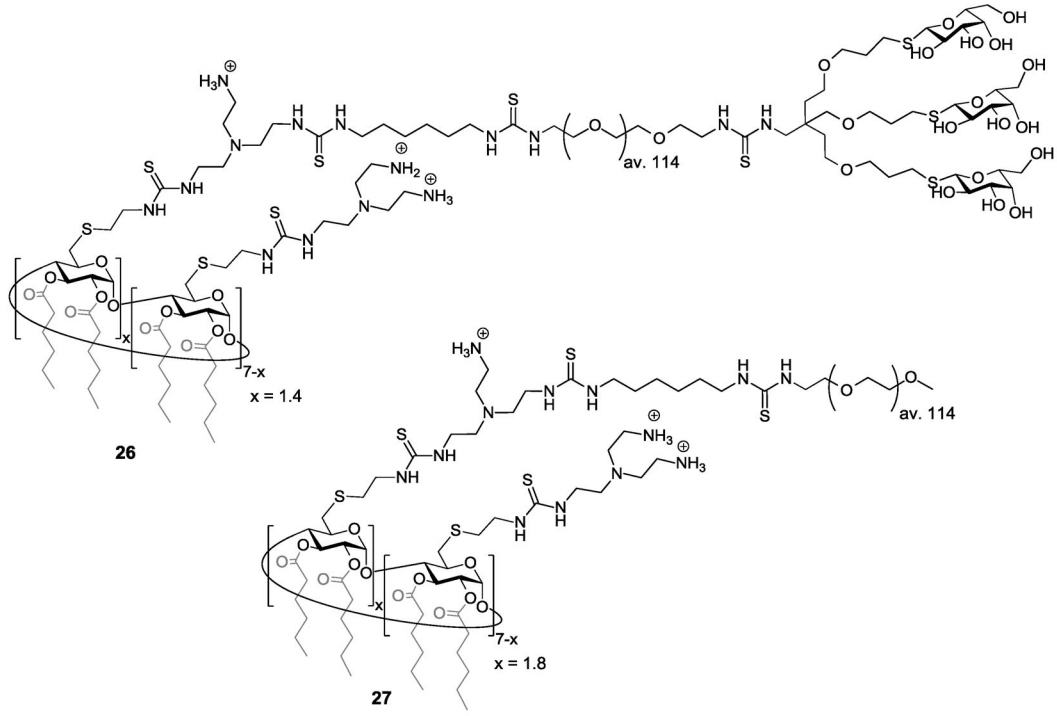


Fig. 12 Structure of the (galacto)pegylated paCDs **26** and **27** [59].

CONCLUSION

The body of results accumulated on the use of paCDs as gene vectors, in a relatively short period of time, has already provided candidates that rival the most popular commercial systems in terms of transfection efficiency, with much better toxicity profiles. A main advantage of the paCD family is the

monodisperse character of its members and their flexibility to undertake modifications, which is compatible with optimization strategies. Most interestingly, the incorporation of additional functional elements can be undertaken at the vector or at the CDplex level, after nucleic acid complexation. Covalent as well as supramolecular ligation chemistries can be conceived in order to tailor the properties of the transfectious nanoparticles for a particular application. Thus, sugar-based artificial viruses might be constructed by, first, creating a CD-based self-assembled enveloped around the nucleic acid drug and, second, installing glycotopes at the CDplex surface that can be recognized by pharmacologically relevant lectin partners. Although much research is still needed, the use of carbohydrates to establish a supramolecular bridge between nucleic acids and proteins may represent a sweet future for gene therapy.

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