Rational design of cationic cyclooligosaccharides as efficient gene delivery systems†

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Self-assembled cyclodextrin (CD)–DNA nanoparticles (*CDplexes*) exhibiting transfection efficiencies significantly higher than PEI-based polyplexes have been prepared from homogeneous seven-fold symmetric polyaminothiourea amphiphiles constructed on a β-cyclodextrin scaffold.

The delivery of genetic materials into a target cell is a major challenge for gene therapy.[1] Viral vectors are far more effective when compared to non viral delivery systems, but there are safety concerns.[2] Synthetic vectors, which include polymers, lipids, nanomaterials and combinations thereof,[3] although highly effective *in vitro* have a low efficiency *in vivo*, exhibit cell toxicity and are still immunogenic.[4] Biocompatibility can be significantly enhanced by the incorporation of polycationic polysaccharides or conjugation with carbohydrate moieties,[5] and interestingly the corresponding DNA complexes—glycoplexes—often exhibit higher transfection efficiencies. Reproducibility is, however, problematic because of the high polydispersity of such systems. The design of artificial carriers that could prove as efficient as their viral counterparts but are safer to use, homogeneous, non-immunogenic and more readily adapted to tailor-made elaboration represents the ultimate challenge for the future development of gene therapy.

Cyclodextrins (cyclomaltooligosaccharides, CDs)[6] are a class of cyclic α-(1–4)-linked glucooligosaccharides that present specific features of interest as core molecules for the development of new gene vectors. They are non-immunogenic and biodegradable biomaterials and their inclusion capabilities have been widely exploited in therapeutics to protect drugs from physical, chemical and enzymatic degradation, to improve their bioavailability and also to enhance cell membrane permeability.[7] Notably, CDs have been incorporated into polycationic polymers, dendrimers and self-assembled systems both through covalent linkages[8] and non-covalent interactions,[9,10] playing the role of transfection enhancers. Herein we present an efficient and highly flexible synthesis of polycationic amphiphilic cyclomaltoheptaose (β-cyclodextrin, βCD) derivatives adapted to the complexation and delivery of DNA suitable for library generation. High transfection efficiencies with low toxicity profiles have been achieved by the incorporation of: (i) thiourea segments as hydrogen bond anchors; (ii) a dendritic display of amino groups and; (iii) a cluster of lipophilic chains in a defined spatial orientation with a seven-fold symmetry (Fig. 1).

Former results pointed at the superior DNA complexing and delivery capabilities of cationic lipids incorporating multiple positive charges in a compact arrangement as compared with spare dispositions.[11] Actually, compound **2**,[12] obtained in only two steps *via* the corresponding per-(C-6)-bromo derivative **1**,[13] was already able to tightly bind and condense DNA, although no transfection activity of the resulting complexes was observed. To endow the system with cell membrane fusogenic capabilities, the installation of fatty acyl chains at the fourteen secondary hydroxyl positions of the CD was undertaken. Clean and very fast conversion of **1** into the

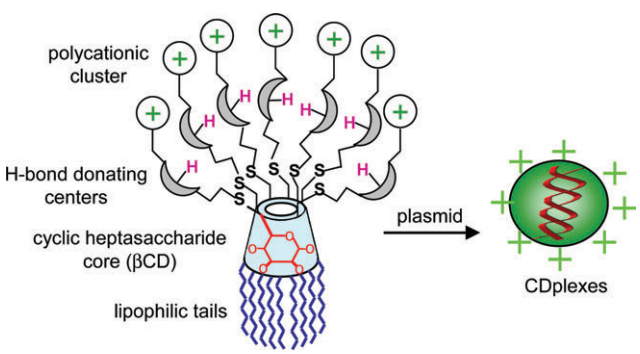

Fig. 1 Schematic representation of the CD-based gene vectors prepared in this work.

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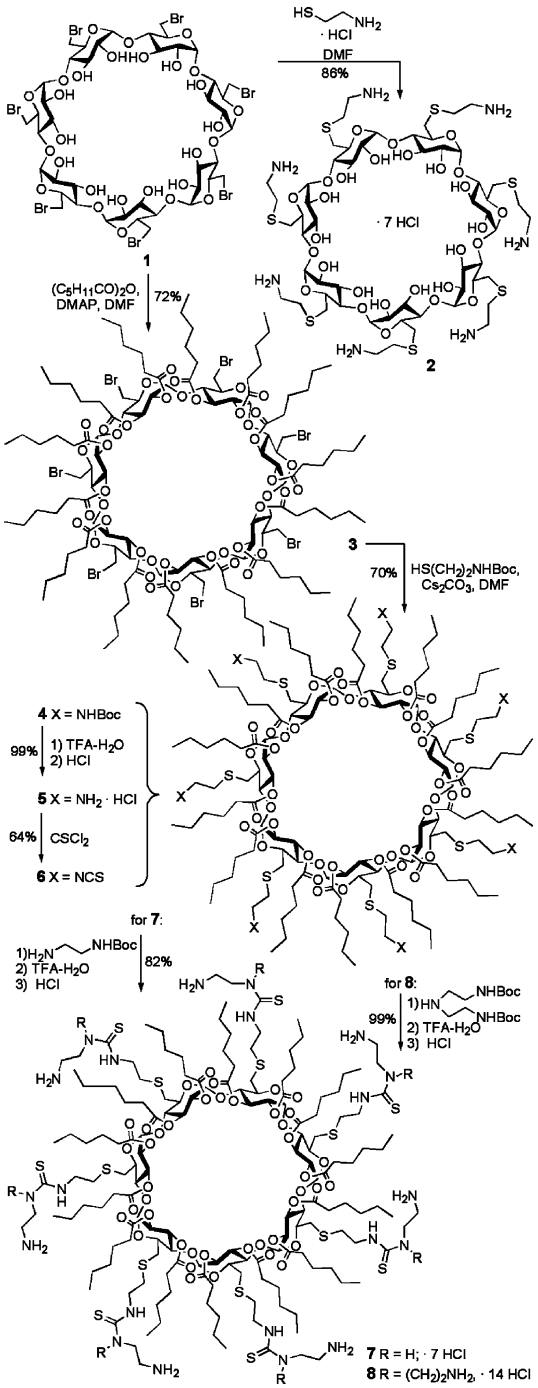
seven-fold symmetric tetradecahexanoate **3** was achieved by reaction with hexanoic anhydride in DMF. Further displacement of the bromo groups with Boc-protected cysteamine (→**4**) and final hydrolysis of the seven carbamate groups afforded the polycationic amphiphilic derivative **5** (Scheme 1).

We next considered the incorporation of thiourea segments as hydrogen bond anchoring points for biomimetic DNA complexation.[14] It was recently shown that carbohydrate derived thioureas can associate to phosphate even in water through cooperative hydrogen bonding.[15] As a matter of fact, DNA complexes made from neutral lipopolythioureas are able to transfect cells, though with moderate efficiency.[16] Cationic DNA complexes formulated from amphiphilic polyaminothioureas should interact more favorably with the negatively charged proteoglycans at the cell surface, facilitating internalization. Subsequent endosome escape is pH-controlled; densely packed polyamine clusters might act as proton sponges and promote this process.[17]



Scheme 1

To check the above working hypotheses, the preparation of the polyaminothiourea-βCDs **7** and **8**, bearing a cluster of seven and fourteen amino groups, respectively, *via* the corresponding hepta-isothiocyanate **6**, was accomplished (Scheme 1). Molecular integrity and homogeneity was checked for every intermediate, demonstrating the suitability of this convergent approach to generate molecular diversity in a sophisticated structural design with total control and through a limited number of synthetic steps.

Compounds **5**, **7** and **8** formed positively charged aggregates both in saline 1 mM solution (hydrodynamic radii 80–140 nm; ξ-potential of +42 to +63 mV) and in phosphate buffer (pH 7; 85–140 nm; ξ-potential of +19 to +29 mV), with critical aggregation concentrations <0.1 μM in all cases. Encapsulation experiments using copper(II) tetrasulfonated phthalocyanine as a chromophore (see ESI†) supported the reversible character of the self-association process, with relatively fast exchange rates.

The ability of the polycationic βCDs **5**, **7** and **8** to condense plasmid DNA (pDNA) into particulate structures (*CDplexes*) was next confirmed by agarose gel electrophoresis, particle size analysis and ξ-potential measurements. Polyethyleneimine (branched PEI, 25 kDa), one of the most efficient cationic polymer vectors,[17] was used for comparative purposes. All three derivatives could efficiently compact pDNA into stable nanoparticles of remarkably small dimensions and low polydispersity (50–75 nm) even at a N/P[18] ratio of 5 (to be compared with 100–150 nm in the case of PEI), making these vectors very promising for *in vivo* gene delivery. At N/P 5 and 10, all the complexes exhibited a net positive surface charge, indicating that they consist of cationic nanoparticles (ξ-potential of +18 to +46 mV). Moreover, pDNA in these nanoparticles was not accessible to ethidium bromide, confirming complete protection.

Direct interaction of the CD-aggregates, rather than the individual molecules, with pDNA cannot be discarded under the experimental conditions used in our study. In fact, the dynamic behaviour of the association events makes both phenomena indistinguishable. In the presence of pDNA, fast equilibration between CD aggregates and CD-DNA complexes probably occurs, to give the corresponding CDplexes. The fact that the CDplexes are smaller than the CD aggregates strongly support this mechanism, discarding an irreversible two-step aggregation process.[5e]

The transfection efficiency of the self-assembled polycationic CD–DNA complexes was assessed using the luciferase-encoding reporter gene as a marker in BNL-CL2 murine embryonic cells. Fig. 2a shows the luciferase expression mediated by **5**, **7** and **8** at N/P ratios 5 and 10 in comparison with data for branched PEI (25 kDa) and naked pDNA. Structure-activity relationship (SAR) analysis clearly evidences that inserting the thiourea segments in the cluster architecture results in a dramatic improvement in transfection efficiency. Thus, at N/P 5, the CDplexes made from

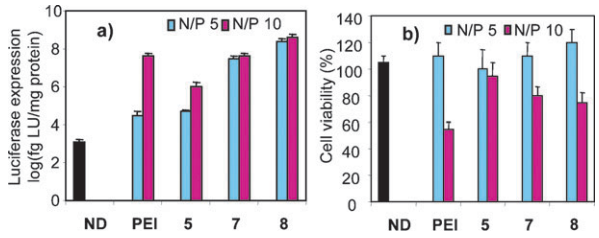


Fig. 2 *In vitro* gene transfection efficiency (a) and cell viability (b) of the CDplexes in BNL-CL2 cells in comparison with PEI (25 kDa)-based polyplexes and naked DNA (ND). Data represent mean standard deviation ($n = 3$).

aminothiourea **7** were over 100-fold more efficient than those obtained from heptamine **5**. Increasing the number of protonable amine groups in a dendritic arrangement represented a further significant improvement in vector design. Thus, the transfection efficiency for the tetradecaaminothiourea **8** CDplexes was up to 10-fold higher as compared with that for CDplexes from **7**. Noticeably, our studies showed further that the CDplexes were much less toxic than the PEI-based polyplexes in cell cultures (Fig. 2b).[19]

In conclusion, we have implemented a straightforward cyclodextrin-based design of homogeneous molecular systems for efficient gene delivery. A economic, diversity-oriented strategy to access face-differentiated polycationic amphiphilic CDs, suitable for SAR studies, has been developed. The overall architecture of the molecules can be controlled in terms of density of amino groups, flexibility and presence of additional hydrogen-bonding functionalities. High transfection efficiencies for homogeneous systems, that overpass that of the cationic polymer PEI (25 kDa), with very low toxicity profiles, have been achieved by combining amine and thiourea groups in a C_7-symmetric cluster arrangement. The presence of the CD cavity offers opportunities for the construction of "artificial viruses" by incorporation of further functional elements (*e.g.* for cell targeting, nuclear localization, visualization) to the DNA complexes as well as for the co-delivery of drugs and DNA by forming ternary complexes. Research in that direction is currently in progress in our laboratories.[20]

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18 N and P represent the number of amine and phosphate equivalents of the polycationic cyclodextrins and pDNA, respectively.

19 Similar transfection efficiency and cell viability trends were encountered in KB cells (human nasopharyngeal cancer cell line) for **8** as compared with PEI. See ESI†.

20 The extremelly low cmc values for **5**, **7** and **8** makes it difficult to assess their inclusion capabilities in pure water. Nevertheless, the formation of inclusion complexes with adamantane derivatives was evidenced in methanol or DMSO–water mixtures by NMR titration experiments, supporting the possibility of using adamantane-conjugated functional elements to further elaborate the corresponding CDplexes through supramolecular interactions.