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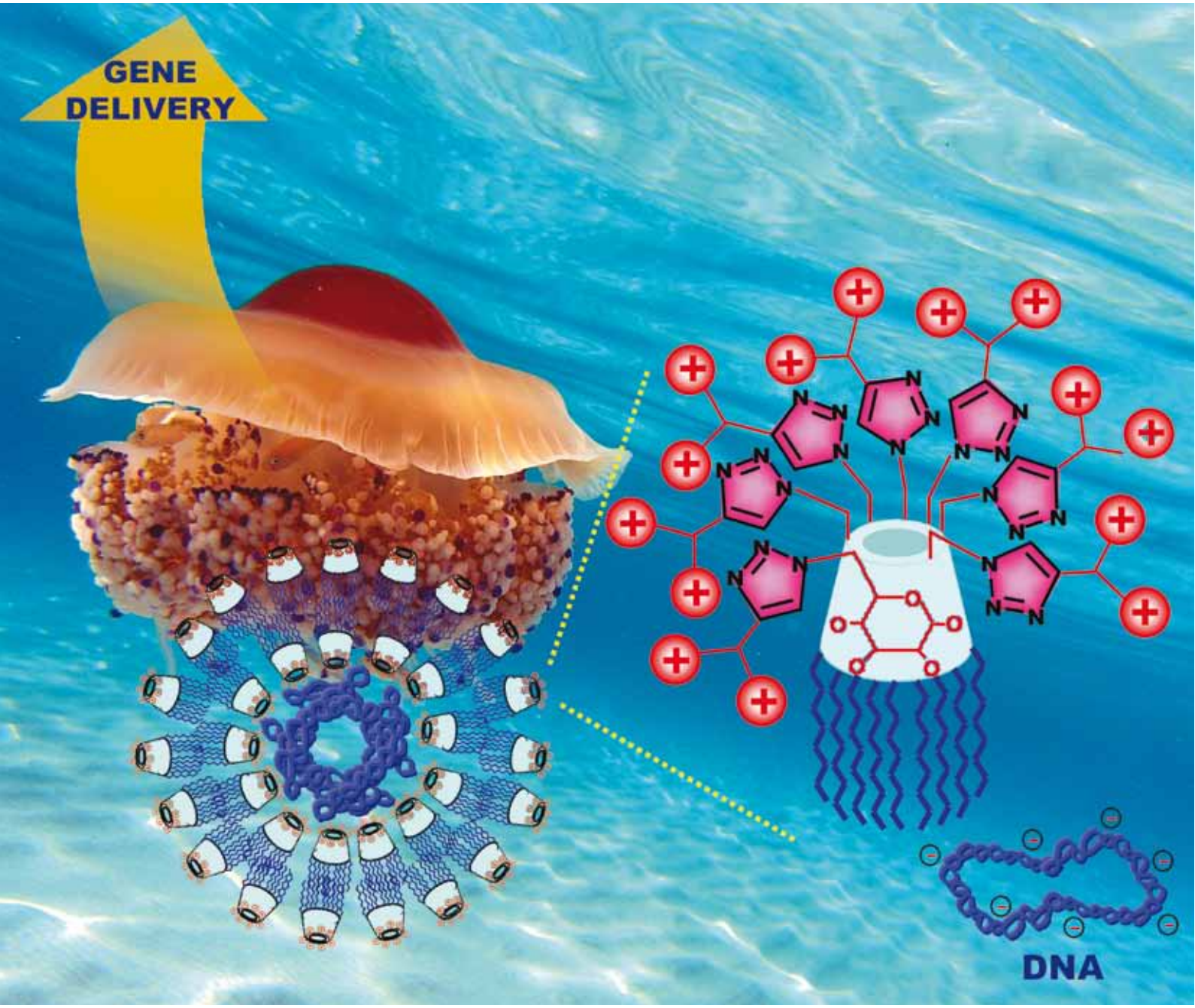

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Preorganized macromolecular gene delivery systems: amphiphilic β-cyclodextrin "click clusters"†

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Gene delivery systems based on the β-cyclodextrin scaffold have been synthesized by combining the copper(I)-catalyzed azide-alkyne coupling ("click chemistry") and an efficient acylation method of the secondary hydroxyls; molecular flexibility, charge density and hydrophobic-hydrophilic balance are critical parameters that can be fine-tuned by the click approach.

The use of preorganized macrocyclic scaffolds to achieve a precise alignment of functional elements has proven to be extremely useful over the years in the design of artificial receptors/ligands capable of emulating the supramolecular events occurring in living organisms.[1] The cyclic maltooligosaccharide (cyclodextrin, CD) nucleus is considered to be a privileged platform for these channels, as it combines biocompatibility, availability, a tubular symmetric framework with well differentiated faces, and various functionalization patterns that can be modified in a tunable manner.[2] CD-based architectures can additionally take advantage of their distinctive inclusion capabilities. Notably, chemically modified CDs have been incorporated into polycationic polymers that can effectively complex and deliver plasmid DNA (pDNA) with exceptional biocompatibility and efficacy.[3] The surface of the corresponding nucleic acid particles can be decorated with hydrophilic polymers for steric stabilization and/or targeting ligands for cell-specific delivery through non covalent attachment of chemical entities that can interact with the CD cavity.[4]

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. In this context, the copper(I)-catalyzed version of the Huisgen 1,3-dipolar cycloaddition ("click chemistry") between azide and alkyne precursors has been established as one of the most effective and versatile methodologies to introduce multiple functionalities onto a CD core.[5] Previous results showed, however, that the rigidity of the 1,2,3-triazolyl tether in click adducts can significantly alter the topical characteristics of the scaffold by limiting the conformational space available to the attached groups and, consequently, their ability to interact with complementary biomolecules.[6] Thus, the pDNA complexing and delivery abilities of primary-face ethyleneimine-type "click clusters" obtained from cyclomaltoheptaose (βCD) were shown to be satisfactory only for relatively long oligo(ethyleneimine) segments.[7]

Elaboration of the secondary CD hydroxyls offers further opportunities for molecular tailoring that, however, remain largely unexplored. We have recently reported a modular synthetic strategy for the preparation of polycationic amphiphilic CDs[8,9] that allows the installation of different building blocks onto either rim of the CD torus in a sequential and controlled manner, offering a unique opportunity for structure-activity relationship and optimization studies.[10] We have now implemented this bidirectional diversity-oriented concept for the construction of amphiphilic polycationic βCD "click clusters" (Fig. 1) as a new family of discrete and well-characterized macromolecular nucleic acid vehicles. The molecular construct benefits from the high yield of the "click" coupling, even in sterically hindered environments, and the possibility to control the self-assembling properties of the adducts, their ability to interact with pDNA, and the membrane-crossing and transfection capabilities of the corresponding CD-pDNA complexes (CDplexes).

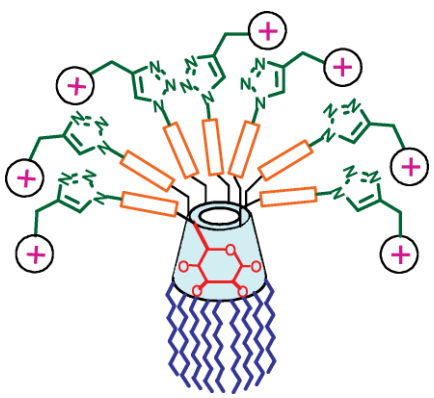

Fig. 1 Schematic representation of βCD-scaffolded amphiphilic polycationic "click clusters". The rectangular boxes account for additional spacer elements.

A main concern in the design of our prototype is the influence of conformational constraints in the ability of polycationic click clusters to form stable CDplexes. A second one is whether or not amphiphilicity in these homogeneous systems can be

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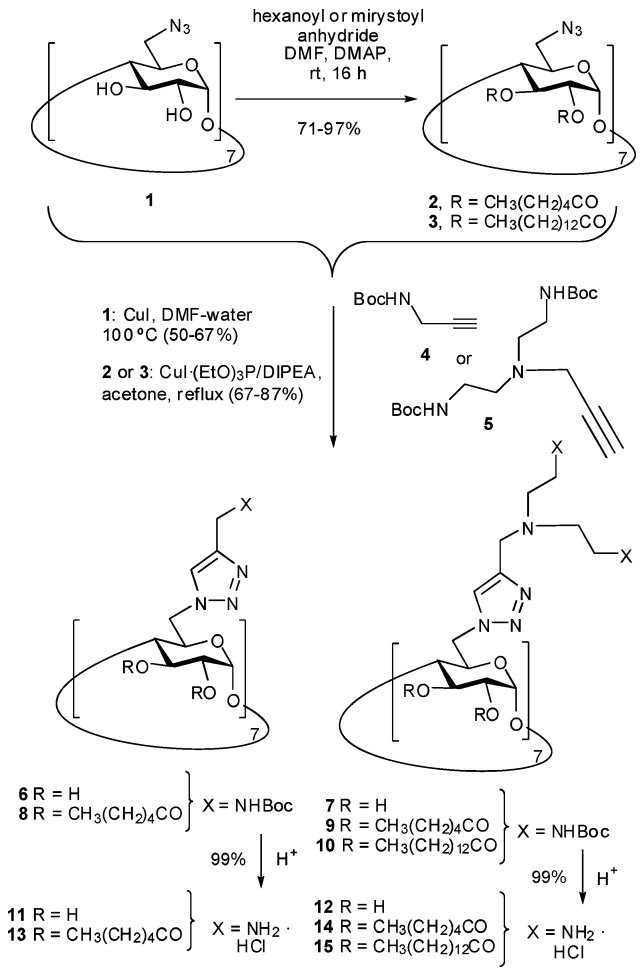
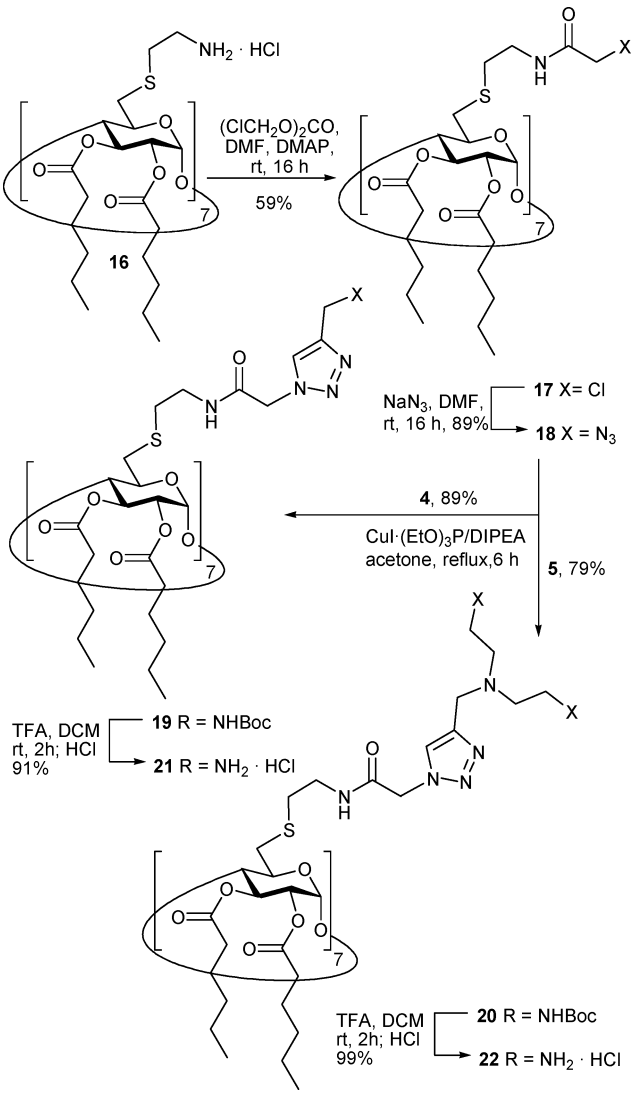
fine-tuned to control their self-assembling properties. The density of positive charges, their distance to the CD core and the length of the lipophilic tails are key parameters that can be systematically modified to address these questions. The synthesis of adducts devoid of any connector between the triazole rings and the CD nucleus started from the readily available heptakis(6-azido-6-deoxy) βCD **1**,[11] which was converted into the per(2,3-di-*O*-hexanoyl and per(2,3-di-*O*-mirystoyl) derivatives **2** and **3** by reaction with the corresponding fatty acid anhydride in *N,N*-dimethylformamide (DMF) in the presence of *N,N*-dimethylaminopyridine (DMAP). Boc-protected propargylamine **4** and the homologous second generation dendron **5** were considered as aminoalkyne counterparts. Click coupling proceeded cleanly in all cases to afford the 1,4-triazole regioisomers in high yield. Adducts **6–10** were prepared in this way and further transformed into the target polycationic derivatives **11–15** (Scheme 1).



Scheme 1

For the preparation of flexible spacer-armed azide precursors we took advantage of our previously reported methodology for the easy installation of the cysteaminyl segment at the primary rim of βCD.[12] *N*-Chloroacetylation of the seven amino groups in the tetradecahexanoate **16**[10a] (→**17**) and further nucleophilic displacement of the chloro groups by azide anion afforded the corresponding heptaazide **18**. Subsequent cycloaddition reaction with the amine-coated alkyne building blocks **4** and **5** (→**19** and **20**) and final acid-promoted carbamate hydrolysis afforded the polycationic amphiphilic βCDs **21** and **22** (Scheme 2).



Scheme 2

Data on the relative pDNA (GFP-encoding pEGFP-N3 plasmid) complexation capabilities of the new polycationic CDs at different N/P values (protonable nitrogens in the CD carrier/phosphate groups in the plasmid ratio), degree of protection of the plasmid in the corresponding CDplexes against the action of DNase, and efficiency to promote transfection (CHO-k1 cells) are presented in Figs. 2a–c, respectively. The gel retardation or gel electrophoresis shift assay (Fig. 2a) reflects the capability of the amphiphilic βCD click cluster to compact and protect pDNA. Formation of the corresponding CDplexes results in a significant decrease in the hydrodynamic size as compared with free pDNA and, therefore, in a lower mobility over the gel. Moreover, if pDNA is efficiently protected in the CDplexes, it becomes not accessible to the intercalating agent ethidium bromide used as staining reagent. The single-face modified βCD polycations **11** and **12** were used as control compounds to assess the influence of amphiphilicity in the interaction between rigid "click clusters" and pDNA. Although the dendritic architecture **12** was much

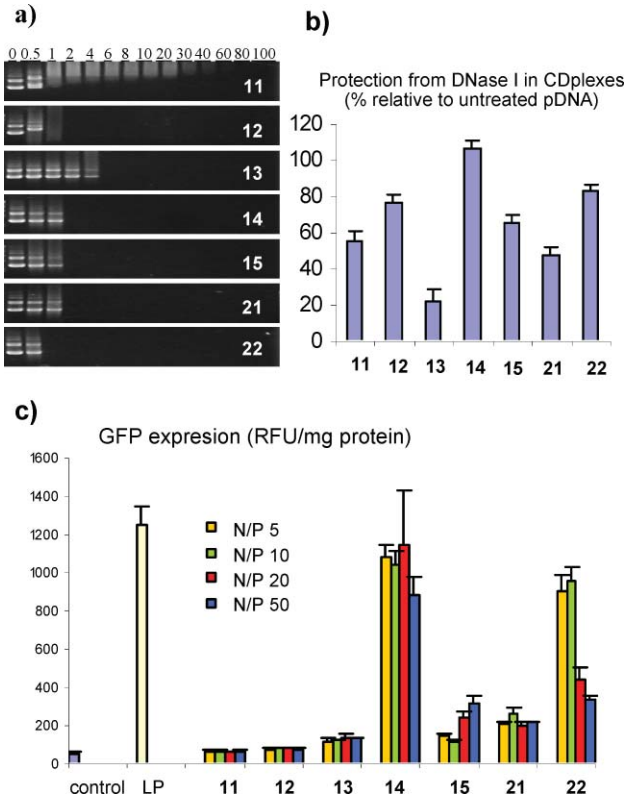


Fig. 2 (a) Gel electrophoresis shift assay showing amphiphilic βCD-pDNA binding at increasing N/P ratios. (b) Quantization of the relative intensity (untreated pDNA value equal to 100) of the sum of relaxed and supercoil electrophoretic pDNA bands corresponding to the pDNA samples complexed with amphiphilic βCD click clusters and treated with DNase I (data represent mean standard deviation; n = 4). (c) In vitro gene transfection efficiency of the CDplexes from **11–15**, **21** and **22** in CHO-k1 cells in comparison with Lipofectamine™ 2000-based polyplexes (LP) and naked DNA (control) (data represent mean standard deviation; n = 8).

more efficient than the heptamine derivative **11** in compacting pDNA (Fig. 2a) and protecting it against degradation by DNase (Fig. 2b), none of them was able to promote gene transfection to any significant extent (Fig. 2c).

The tetradecahexanoylated heptacationic cluster **13** exhibited enhanced pDNA complexing abilities as compared with the non-amphiphilic analog **11**. Yet, the plasmid was still accessible to DNase in the corresponding CDplexes and the transfection levels remained very poor. The benefits imparted by amphiphilicity appeared much more evident when comparing the behaviour within the branched series **12**, **14** and **15**. Compound **14**, bearing hexanoate tails, gave rise to stable CDplexes warranting full protection of pDNA from the environment for N/P values ≥5. Moreover, they showed remarkably high transfection efficiencies, analogous to that obtained using Lipofectamine™ 2000. Increasing the hydrophobicity of the molecular motif by replacing the hexanoate into tetradecanoate chains did not alter the complexing efficiency, as see from data for **15**, but drastically decreased the transfection capabilities, pointing out the utmost importance of a fine adjustment of the hydrophobic-hydrophilic balance not only to optimize the formation of CD-DNA nanoparticles, but also for the further processes leading to cell internalization and gene expression.

Compounds **21** and **22** reproduce the polyaminotriazole/tetradeca-*O*-hexanoyl decoration of **13** and **14** at the primary and secondary rim of βCD, respectively, but incorporating a flexible spacer arm between the triazole rings and the macrocyclic core. A comparative analysis of the corresponding data indicated moderate increases in CDplex stability, efficacy in protecting pDNA against the action of DNase, and efficiency of DNA transfection on moving from **13** to **21**. Unexpectedly, data for **14** and **22** did not show the same trend, the rigid motif being advantageous in this case as compared with the flexible architecture. Actually, the CDplexes obtained from **14** are the only ones in the whole set of "click clusters" synthesized to offer 100% protection of DNA and sustained transfection levels, comparable to Lipofectamine™ 2000, in the whole range of N/P values from 5 to 50.

The observed superiority of the rigid design in mediating DNA transfection does not seems to lay in the differences in the self-assembling properties of the amphiphilic polycationic CDs in the absence of the plasmid. Although they form relatively big positively charged aggregates (200–500 nm hydrodynamic diameter; see Supporting Information), a full reorganization process takes place in the presence of the plasmid leading to much smaller CDplexes (about 100 nm for N/P = 10) with positive ζ-potential (32–50 mV). Most probably, the transfection efficiency is related to the ability of the individual molecules to self-organize in the complex and the capability of the resulting CDplexes to cross membranes. Both processes are intimately related to the stability of the interactions between the clusters and the pDNA chain. Increasing charge density at every arm in the click cluster, thereby promoting cooperativity in their interaction with phosphate centers (*e.g.*, **14** and **22**), probably facilitates covering of the plasmid external surface. The lipophilic tails can then act as fusogenic elements, facilitating internalization and endosome escape as in classical lipoplexes.[13]

In summary, the current body of results illustrates the promise of amphiphilic CD-scaffolded "click clusters" as gene delivery systems. Most importantly, the combination of very efficient synthetic methods for accessing fully homogeneous macromolecules and a molecular diversity-oriented bidirectional strategy provides unprecedented opportunities for structure-activity relationship and optimization studies. Molecular flexibility, charge density and hydrophobic-hydrophilic balance have been shown to be critical parameters that can be fine-tuned by the click approach.

Acknowledgements

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