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Synthesis, Characterization, and Remarkable Biological Properties of Cyclodextrins Bearing Guanidinoalkylamino and Aminoalkylamino Groups on Their Primary Side

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Abstract: The introduction of aminoalkylamino and guanidinoalkylamino substituents on the primary side of β- and γ-cyclodextrin (CDs) resulted in a series of novel compounds that were extensively characterized by NMR spectroscopy and mass spectrometry. Bromination of the primary side of β- and γ-CD, and reaction with neat alkylene diamines at a pressure of 7 atm afforded aminoalkylamino derivatives that were then guanylated at the primary amino group to give the corresponding guanidinoalkylamino-CDs. These compounds are water soluble and display pK_a values that allow them to be mostly protonated at neutral pH; for example, pK_{a_1} ~6.4 and pK_{a_2} ~9.5 for the aminoethylamino-β-CD and pK_{a_1} ~7.8 and pK_{a_2} ~11.0 for the guanidinoethylamino-β-CD. The title CDs are rigid, cyclic α-D-glucopyranose oligomers (heptamers or octamers) with branches that resemble lysine and arginine side chains that enable multiple interactions with suitable substrates. Thus, they bear similarities to known cell-penetrating peptides. Indeed, the compounds were found to cross the membranes of HeLa cells and penetrate inside the cytoplasm quickly, the guadinylated ones within 15 min, as shown by fluorescence microscopy using fluorescein-labeled derivatives. The toxicity of the compounds, measured by performing MTT tests, ranged from 50 to 300 μM. Furthermore, some of the aminated CDs could facilitate the transfection of DNA expressing the green fluorescent protein (GFP) in HEK 293T cells, with effectiveness comparable to the commercial agent Lipofectamine 2000. Circular dichroism, atomic force microscopy and electrophoresis experiments confirmed the strong interaction of the compounds with DNA. Because of their carbohydrate, non-peptide nature the title compounds are not anticipated to be enzymatically labile or immunogenic, and thus they fulfill many of the criteria for non-hazardous transport vectors in biological and pharmaceutical applications.

Keywords: cyclodextrins · DNA · membranes · supramolecular chemistry · transfection

Introduction

Cyclodextrins (CDs) are cyclic oligomers of α-D-glucose that possess a set of primary hydroxyl groups on the narrow side and a set of secondary hydroxyl groups on the wider side (Scheme 1a). The uniform and position-selective introduction of substituents onto the macrocycles has been pursued for many years[1] because the derivatives obtained can act as functional host molecules. Reliable procedures affording per-substituted cyclodextrins bearing charged groups have received particular attention,[1c–f] given that a geometrically defined charged region on one side of the macrocycle may present, in addition to the cavity, another significant receptor able to bind suitable substrates through multiple electrostatic interactions. Per(6-amino-6-deoxy)-cyclodextrins, known for some time, are compounds that provide both free

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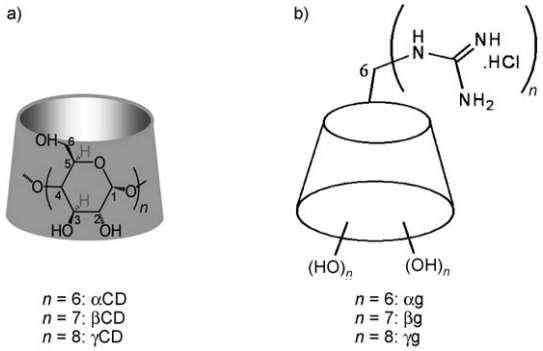
amine and ammonium groups at neutral pH ($pK_a \sim 8$) and behave as biomimetic catalysts.[2] Recently, the efficient synthesis, characterization and properties of per(6-guanidino-6-deoxy)CDs, **αg**, **βg**, or **γg** (Scheme 1b), was published by us.[3] It was shown that these products bound preferentially onto phosphorylated substrates. DNA was such a substrate, which upon incubation with either **αg**, **βg**, or **γg** underwent structural change that eventually led to its packing into nanoparticles, a very desirable feature for direct delivery of DNA through cell membranes.[4]

There is an exceedingly large amount of recent research directed toward the interactions of positively charged macromolecules, mainly polymers, but also liposomes and dendrimers with DNA, that result in DNA transfection.[5] Some



Scheme 1. a) Representation of the α-, β-, γ-cyclodextrin macrocycles corresponding to $n = 6$, 7, 8, respectively, and b) per(6-guanidino-6-deoxy)-cyclodextrins.

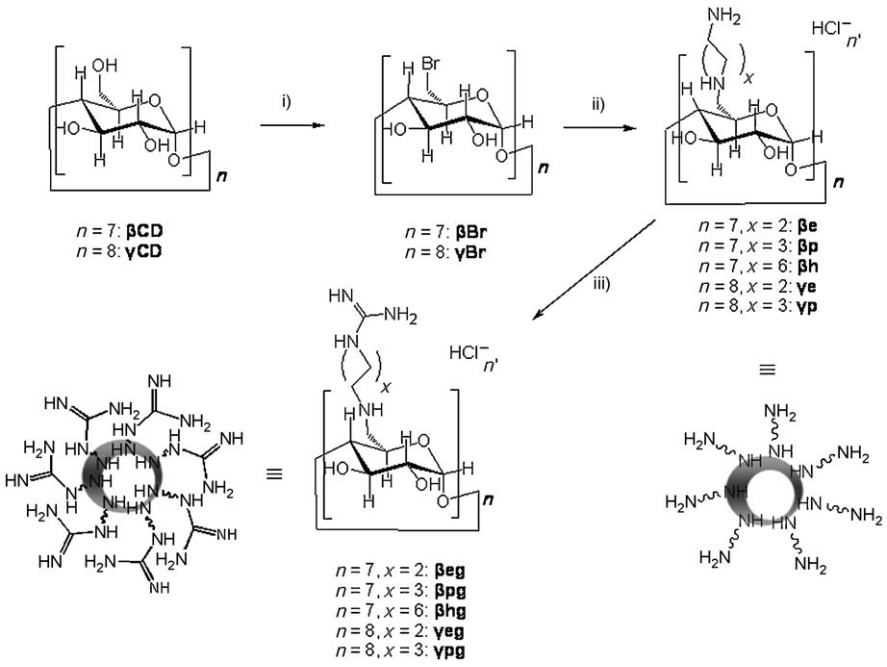
Abstract in Greek:

Η σύνθεση νέων, πλήρως υποκατεστημένων κυκλοδεξτρινών (CDs) στην πρωτοταγή τους πλευρά επετεύχθη σε συνθήκες υψηλής πίεσης με χρήση αλειφατικών διαμινών ως διαλύτες οπότε παρήχθησαν οι περι(6-αμινοαλκυλαμινο-6-δεοξυ)CDs. Επακόλουθη γουανυλίωση των πρωτοταγών αμινομάδων έδωσε τις αντίστοιχες περι(6-γουανιδινοαλκυλαμινο-6-δεοξυ)CDs. Οι νέες ενώσεις χαρακτηρίσθηκαν πλήρως με φασματοσκοπία NMR και φασματομετρία μάζας. Μετρήθηκαν τα $pKa_1 \sim 6.4$ και $pKa_2 \sim 9.5$ για μία αμινο-β-CD και $pKa_1 \sim 7.8$ και $pKa_2 \sim 11.0$ για μία γουανιδινο-β-CD, συνεπώς σε συνθήκες ουδέτερου pH οι λειτουργικές ομάδες τους εμφανίζονται πολλαπλώς φορτισμένες, ομοιάζουν δε με εκείνες των πλευρικών αλυσίδων των αμινοξέων λυσίνη και αργινίνη ενώ επισης παρουσιάζουν υψηλή χωρική συγκέντρωση θετικών φορτίων. Αυτό το δομικό χαρακτηριστικό τους προσδίδει την ικανότητα να αλληλεπιδρούν με αρνητικώς φορτισμένα συστήματα, όπως κυτταρικές μεμβράνες και DNA. Στην πρώτη περίπτωση εντάσσεται η ταχεία εισαγωγή των παραγώγων επισημασμένων με ισοθειοκυανική φλουορεσκεΐνη στο κυτταρόπλασμα καρκινικών κυττάρων HeLa όπως πιστοποιήθηκε με μικροσκοπία φθορισμού. Η αλληλεπίδραση με το DNA είναι ισχυρή και επιβεβαιώθηκε με ηλεκτροφόρηση, φασματοσκοπία κυκλικού διχρωϊσμού και μικροσκοπία ατομικών δυνάμεων. Η αλληλεπίδραση αυτή αποτελεί βασική προϋπόθεση για τη μεταφορά γενετικού υλικού εντός του κυττάρου και την έκφραση της κωδικοποιούμενης πρωτεΐνης. Όντως, όταν κύτταρα HEK 293T επωάσθηκαν παρουσία του πλασμιδίου της πρωτεΐνης GFP και ορισμένων αμίνο CDs, παρατηρήθηκε έκφραση της GFP σε επίπεδα συγκρίσιμα με εκείνα που επιτυγχάνει η εμπορικώς διαθέσιμη Lipofectamine 2000. Η κυτταροτοξικότητα των νέων ενώσεων προσδιορίσθηκε με τη μέθοδο MTT (50 έως 300 μM). Λόγω της υδατανθρακικής φύσης τους, οι νέες κυκλοδεξτρίνες αναμένεται να παρουσιάζουν χαμηλή ανοσογονικότητα και να είναι στα-θερές έναντι πρωτεολυτικών ενζύμων.

of the most successful polymeric agents[5c–e] bear cyclodextrin rings linked together by cationic spacer chains. In contrast to polymers, cationic oligomers have been shown to act as molecular transporters, namely to cross readily cell membranes carrying a covalently linked molecular cargo, such as a drug, that itself is unable to cross the cellular barrier. A prominent place among such oligomers is occupied by peptides comprising entirely or partially arginine or lysine residues.[6] These amino acids are known to be the primary constituents of peptide sequences found in proteins that are characterized by cell-membrane permeation properties (cell-penetrating peptides, CPPs).[7] Some literature systems bearing guanidino groups on linear backbones indicated that structural rigidity and spatial organization of the positive charges to achieve stable helical arrangement were important for cell entry.[6e] Therefore, the introduction of seven or eight lysine- and arginine-like side groups on one side of a CD, combined with its rigid cyclic structure and its suitable size (diameter ~1 nm), was envisaged as a promising way to construct cell-penetrating CDs (CPCDs). The present work describes the successful preparation of such derivatives, that is, novel per(6-aminoalkylamino-6-deoxy)- and per(6-guanidinoalkylamino-6-deoxy)-CDs (Scheme 2) that were spectroscopically characterized in detail, including pK_a measurements. Interestingly, the title products displayed the dual properties of cell-penetrating and DNA-transfection agents.

Results and Discussion

Synthesis and characterization: The synthesis of heptakis- and octakis(6-aminoalkylamino-6-deoxy-)-β- and -γ-cyclodextrins, and of heptakis- and octakis(6-guanidinoalkylamino-6-deoxy)-β- and -γ-cyclodextrins, is shown in Scheme 2. The natural β- and γ-cyclodextrins were converted to the corresponding per(6-bromo-6-deoxy) derivatives (**βBr** and **γBr**),[1f,8] which reacted with the appropriate alkylene diamine as the solvent in an autoclave under a 7-atm nitrogen atmosphere at 75 to 80°C to afford within 3–4 d the per-(6-aminoalkylamino-6-deoxy)-cyclodextrins **βe**, **βp**, **βh**, **γe**, and **γp**.



Scheme 2. i) Ph_3P/DMF/Br_2, 75–80°C, 18 h; ii) $H_2N(CH_2)_xNH_2$, $x=2$, 3, 6, 80°C, 7 atm N_2, 3–4 d; iii) 1*H*-pyrazole-1-carboxamidine·HCl, DMF, DIPEA (*N*,*N*-diisopropylethylamine).

The ^{1}H NMR and especially the ^{13}C NMR spectra of the products in D_2O corresponded to per-substituted cyclodextrins having C7 and C8 symmetry, respectively, as they displayed one peak for each type of proton (or carbon) in the repeating glucopyranose unit. The change in chemical shifts of carbon atoms C6 of the primary side from ~62 ppm in the natural CDs to ~34 ppm in **βBr** and **γBr**, and to ~47–51 ppm in the aminoalkylamino products **βe**, **βp**, **βh**, **γe**, **γp** was proof that all products were aminated fully, as no residual peaks of the brominated precursors were detected. Complete NMR assignment was carried out by recording 2D COSY and HSQC spectra. In addition, the attachment of only one nitrogen of each $H_2N(CH_2)_xNH_2$ to the C6 of the cyclodextrin (Scheme 2) was confirmed by 2D HMBC (Supporting Information Figure S1). The MALDI-TOF mass spectra displayed strong molecular ions, $[M+H]^+$ (~100%) (Supporting Information Figures S2, S3, S4), as well as two or three fragments of appreciable intensity corresponding to sequential loss of $H_2N(CH_2)_xNH_2$ groups. This kind of fragmentation seems to take place in the spectrometer rather than arise from the presence of incompletely substituted oligomers, contrary to what was reported for the MALDI-TOF spectra of imperfect carbosilane dendrimers:[9] there are no mass peaks corresponding to brominated- or hydroxylated-oligoaminoalkylated CDs (a sign of incomplete substitution), confirming the previously described ^{13}C NMR data for the C6 peaks (absence of C6–Br at ~34 ppm and C6–OH at ~62 ppm). Further, in the MALDI-TOF spectra of the corresponding guanidines (Supporting Information) there are no low-molecular-weight peaks that would arise from partially guadinylated CDs that could arise from incompletely aminoalkylated precursors. No peaks corresponding to dimers (i.e., bridging of two CD macrocycles) or higher oligomers were detected either, only very small peaks (<5% intensity) of mass higher than M^+, attributable to the adduct $[M+H_2N(CH_2)_xNH_2]^+$, were visible. On the other hand, the ESI mass spectra showed multicharged fragments, $[M+7H^+]$ or $[M+8H^+]$, for β-or γ-CD derivatives, respectively, corresponding to the expected molecular ions. The derivative **βh** was obtained as a 30–40% mixture with the starting hexylene diamine, probably held strongly in the cavity or among the aminohexylamino sustituents that branch out. The latter supramolecular entity was not found in the MALDI-TOF spectrum, maybe due to the acidic matrix used. Dialysis or extensive chloroform washing, or even reverse-phase column chromatography, were unsuccessful in removing the free diamine, therefore, **βh** was not used for the biological tests that follow.

Attempts to react **βBr** or **γBr** or the per-iodinated analogue, **βI**, with the alkylene diamines (10–40 equivalents) in DMF at 80°C for four days following a previously reported[1f] procedure afforded partially substituted products displaying multiple or broad ^{1}H and ^{13}C peaks in the NMR spectra. An alternative approach using either mono-protected *N*-Ac- or *N*-Boc-ethylene (Boc = *tert*-butoxycarbonyl) diamine in reaction with **βBr** in DMF, as described above, did not result in uniform substitution. Further, attempts to obtain the desired products by preparation of the known[1f] per[6-(2-hydroxyethylamino)-6-deoxy]-β-CD, followed by conversion of the terminal hydroxy to amino groups using Gabriel amination conditions[10] also gave poor results. Therefore, the best methodology was to use the reacting amine as the solvent at 80°C under elevated pressure. The reverse approach, involving reaction of per(6-amino-6-deoxy)-β-cyclodextrin with 2-bromoethylamine hydrobromide, was also ineffective.

The per(6-guanidinoalkylamino-6-deoxy)-cyclodextrins **βeg**, **βpg**, **βhg**, **γeg**, **γpg** were obtained after treatment of the corresponding amines with 1*H*-pyrazole-1-carboxamidine hydrochloride as the guanylating reagent and were characterized spectroscopically. The ^{1}H and ^{13}C NMR spectra showed single peaks although broadened, not an unusual observation for per-substituted cyclodextrins.[11] The assignment was performed by obtaining 2D NMR spectra, and the connectivity of the tertiary guanidino group carbon with the protons of the ω-carbon atom of the alkylene chain was established (Supporting Information Figure S5). The MALDI-

MS spectra showed the molecular ions, but also many additional peaks due to loss and even gain of a guanidino group, its protonated ion, or its radical (*m*/*e* 59, 60, 58), and of $NH_2^{\bullet}$ (*m*/*e* 16), typically observed in the EI-MS of guanidines.[12]

The present methodology provides the title amino derivatives in two steps from the parent CDs. Compound **βe**$_x$ was obtained previously after heating **βBr** with ethylene diamine in DMF solution, but the degree of substitution, *x*, calculated from the NMR spectra was 5.[13] Analogous aminoalkyl (and consequently guanidinoalkyl) derivatives connected to the CD macrocycle through a sulfide moiety have been reported, obtained in four steps.[14] Finally treatment of the compounds with a chloride-anion exchange resin ensured the presence of a constant number of anions. In fact, the guanidinylated derivatives showed that they can hold up to two chloride ions per guanidino group.

Both ^{1}H and ^{13}C NMR signals in all new compounds were very much pH dependent, as reported previously for the per(6-amino-6-deoxy)cyclodextrins.[3] The pH did not only affect the chemical shifts, as expected,[15] but also the number of peaks as well as their line width. In the ^{13}C NMR spectra of a pure compound, for example, **βp**, the sharp signals of C1 and C4 at pH 1.2 (Supporting Information Figure S7) changed to broad and shifted signals at pH 11.5, and new peaks appeared, evidently due to varying degrees of protonation, possibly associated also with macrocyclic deformation. Titration of a solution of **γe** with dilute DCl and recording of the ^{13}C chemical shifts of the carbon atoms Cα and Cω neighboring -NH- and -NH$_2$ groups, in the presence of benzene as the external reference, yielded a plot of δ_{obs} as a function of pH (Figure 1a) with two plateaus. This was then plotted according to the Henderson–Hasselbach Equation [Eq. (1)] (Figure 1b),

$$pH = pK_a + \left\{\log[(\delta_{acidic}-\delta_{obs})/(\delta_{obs}-\delta_{basic})]\right\} \quad (1)$$

The monitored atom Cα is at position β to the primary amino group, whereas Cω is β to the secondary amino group in **γe**. It is well known that protonation of amines causes considerable shielding of neighboring carbon atoms, predominantly those at the β position (up to ~5 ppm), an effect attributed to the electric field *E* of the charged group and its gradient $\partial E/\partial r$ on the observed atom.[15]

The titration plot of Cα (Figure 1a) shows a small increase of shielding around pH 6 and a large increase of shielding around pH 9, whereas Cω displays the reverse behavior. Linear fit of the points *at* the pH change yields the pK_a values 6.4 and 9.5, corresponding to the secondary and primary amino groups, respectively. In fact, these values parallel the ones of ethylene diamine, that is, 7.6 and 10.7,[16] but are smaller by one pK_a unit.

These apparent pK_a values show that the amino groups at neutral pH are partly protonated, contrary to normal amines that are fully protonated at neutral pH. As a consequence, **γe** is less basic than the lysine side chain (pK_a ~ 10.5). Low pK_a values have been observed for lysine active sites of natural enzymes, a fact that allows their partial protonation at pH 7 and thus gives them the ability to operate as nucleophiles or general base catalysts.[17] In the present CDs the grouping of the eight aminoalkylamino groups

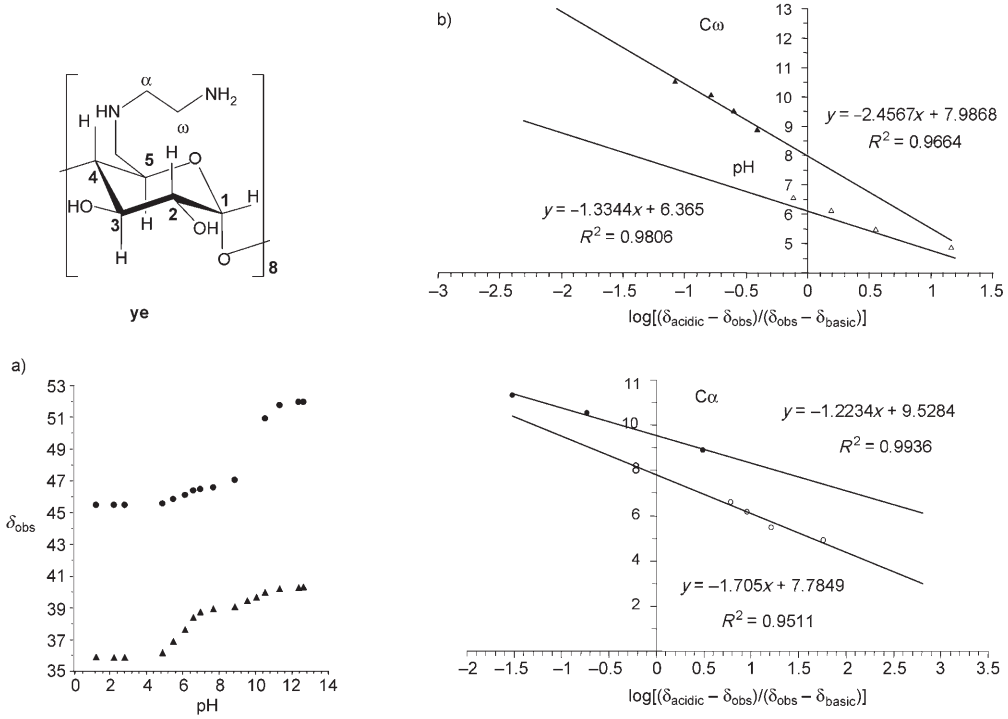
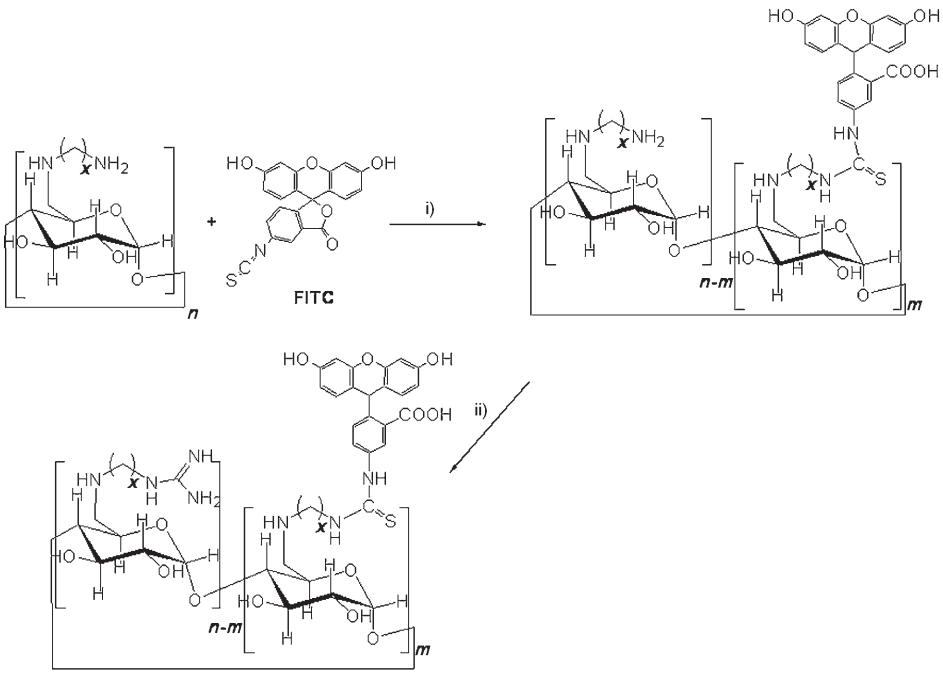


Figure 1. a) δ_{obs} = f(pH) and b) pH = f{log[$(\delta_{acidic}-\delta_{obs})/(\delta_{obs}-\delta_{basic})$]} for carbons Cα (●/○), Cω (▲/△) of **γe**.

within 1 nm^2 on the primary CD side sets up a favorable environment for the persistent protonation of the amino groups. Analogously, pH titration of the corresponding guanylated compound **γeg** afforded pK_a values of 7.8 and 11.0, corresponding to the secondary amino and guanidino groups, respectively. Again, the basicity of the present guanidino group is lower than that of the arginine side chain (pK_a = 12.5). These values are in accord with the reported pK_a values of per(6-amino-2,3-di-*O*-methyl)-α-, -β-, and -γ-CDs, which range from 7.9 to 8.2.[2d]

Synthesis and characterization of fluorescein-labeled aminoalkylamino and guanidinoalkylamino cyclodextrins: The new aminoalkylamino derivatives were labeled with fluorescein isothiocyanate (FITC) to give the corresponding derivatives (Scheme 3), whose fluorescein content ranged from 2 to 10%, as seen from the ^{1}H NMR spectra. The labeled derivatives, even with this low percentage of fluorescein, appeared typical of mono-substituted cyclodextrins. They showed several signals arising from diverse anomeric protons H1, evidently due to the breaking of molecular symmetry by attachment of fluorescein, in addition to the signals arising from unlabeled molecules. Mono(6-amino-6-deoxy)-β-CD[18] was also labeled (fluorescein content ~20%). The labeled compounds were further guanylated to obtain the corresponding guanidinoalkylamino derivatives (Supporting Information Figures S8–S10). The final products showed, in addition to the fluorescein-NMR peaks, the characteristic signal of guanidine at ~160 ppm in their ^{13}C NMR spectra.



Scheme 3. Fluorescein labeling of the title compounds (x = 2, 3, n = 7, 8, m < 0.1): i) DMF, DIPEA, FITC; ii) 1*H*-pyrazole-1-carboxamidine/DIPEA.

Cell-penetration experiments: HeLa cells were incubated with the fluorescein-labeled title compounds and the results were examined under a fluorescent and a confocal microscope. All compounds were found to enter the cytoplasm, as revealed by intense fluorescent spots inside the cells, forming possibly aggregates at high concentrations. The intensity of the fluorescence increased with time (15 min, 1 h, and 24 h) and concentration (50—100 μM). In addition, the guanidino derivatives entered cells faster than the amino derivatives (Figure 2). Control experiments, carried out with either fluorescein alone or fluorescein/βCD complex or even with fluorescein-labeled mono(6-amino-6-deoxy)-β-cyclodextrin, showed absolutely no fluorescence under identical concentrations and incubation periods (Supporting Information Figure S11).

Cells were also examined under a confocal microscope (Figure 3). Images of different slices from the surface showed strong cytoplasmic fluorescence. The observed nucleolus staining could be due to the fixative procedure, as reported previously.[7b]

The above results clearly demonstrate the ability of the prepared compounds to cross the cell membranes of HeLa cells. The CDs form clusters inside the cells (Figure 2), especially at high concentrations, giving rise to strong, spotted autofluorescence. A careful inspection of the images of the HEK293 cells in the transfection experiments below also revealed autofluorescence, indicating that the compounds entered those cells, too. This latter behavior was observed during the transfection experiments in live (unfixed) cells and thus, combined with results of the cell-penetration experiments visualized after cell fixation (Figures 2 and 3), shows the true penetration effect.[19]

The new compounds share characteristic structural features: i) they bear substituents on the primary cyclodextrin side that are similar to the side chains of lysine and arginine amino acids; ii) they are heptamers and octamers, considered ideal for carrying covalently bound molecules into cells;[6] iii) they have structural rigidity and spatial organization of the positive charges to achieve a stable arrangement, considered important for cell entry.[6e,7] Therefore, the title compounds can act as cell-penetrating CDs (CPCDs). One of the most efficient cell-penetrating peptides (CPPs) is the well-known TAT peptide and related hepta-arginine oligopeptides, which enter cells by a passive transfer mechanism through the lipid bi-

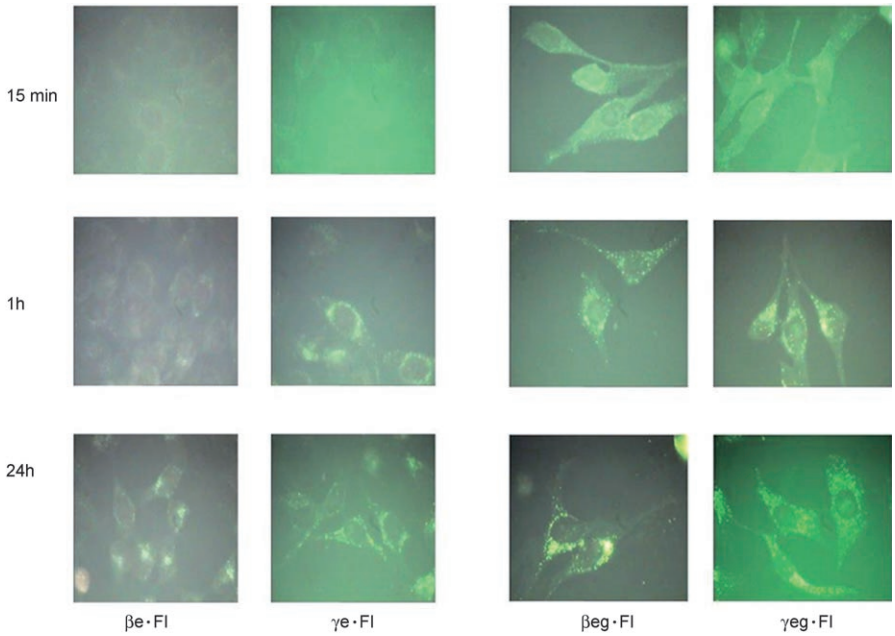


Figure 2. Fluorescent microscopy images of HeLa cells incubated in the presence of the indicated compounds (100 μM) for different incubation times.

layer.[7b] It is possible that the present compounds use the same penetration pathway, as their functional elements are similar to those of TAT. However, because it is well known that CDs are able to relax membranes due to encapsulation of the lipophilic components inside their cavity,[20] a related additional mechanism cannot be excluded in the present process.

MTT assays: The toxicity of the compounds was evaluated by performing MTT assays on HeLa cells. The experimental IC_{50} values vary between 50 and 300 μM. There was no apparent relation between the IC_{50} values and structures of the compounds. These results are not directly comparable with those of previously reported MTT tests[21] in which pyridyl- and imidazolyl-substituted CDs were used, because the latter tests were conducted using the CDs as complexes with DNA. On the other hand, other toxicity tests carried out on natural CDs and pharmacologically approved derivatives (hydroxypropyl-, sulfobutyl-CDs) employed methods quite different from the present one.[22]

DNA-binding experiments

Circular dichroism: The CD spectra of circular plasmid DNA (Figure 4, curve a) in the presence of an increasing concentration of **βe** (curves b, c, d) showed that up to a ratio **βe**/DNA ~1:5 (curve b) there is no change in the spectrum of DNA, that is, in the positive band around 270 nm and the negative band around 240 nm, arising from the stacking interactions of the bases in the helix. A sudden change, seen as shift and reduction of the positive band at 260–280 nm, is observed at a ratio 1:3 (curve c) and remains the same thereafter, up to the ratio **βe**/DNA 1:1 (curve d), showing a permanent restructuring of the macromolecule. The structural change is visible in the presence of a less-than-equimolar quantity of **βe**, and this is also shown in the description of the electrophoresis experiments below.

Electrophoresis: A decrease of DNA migration up to complete inhibition was observed during agarose gel electrophoresis in the presence of various quantities of the new aminoalkylamino and guanidinoalkylamino cyclodextrins. Previous experiments[3] with the per(guanidino)CDs showed that the compounds inhibited migration of DNA in the agarose gel at a mass/charge ratio DNA/CD of 1:1 or better, whereas the parent CDs, as well as guanidine hydrochloride, had no effect. Inhibition of DNA was also observed with the present compounds. Figure 5 displays the migration of calf

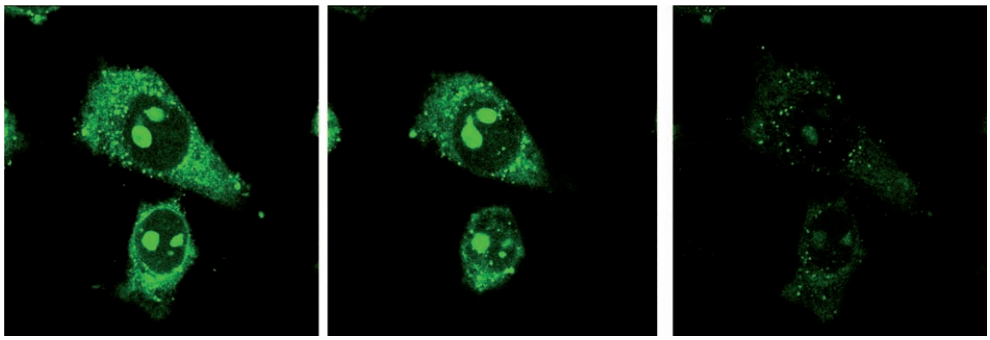


Figure 3. Confocal microscopy slices at three different levels from the surface (from left to right: deeper to superficial) of immobilized HeLa cells incubated for 1 h with **βeg·Fl** (50 μM).

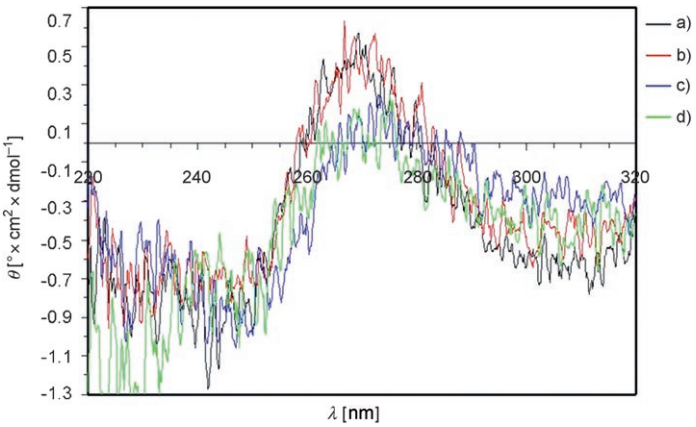


Figure 4. CD spectra of pcDNA (5.3 kbp) 100 μM in the presence of **βe**: a) 0 μM, b) 19.6 μM, c) 38.5 μM and d) 107 μM.

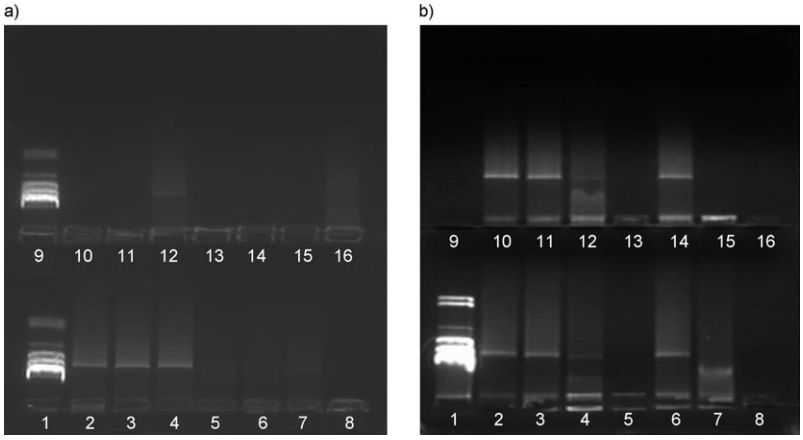


Figure 5. Agarose gel electrophoresis of the mixtures listed in Table 1 (gel a) and Table 2 (gel b).

thymus DNA in the various lanes, visualized by the fluorescence of the intercalated ethidium bromide, using solutions of compounds as indicated in Tables 1 and 2 were incubated with DNA solution in order to achieve the DNA/compound mass/charge ratio[23] indicated. In gel a it is shown that βCD has no effect on DNA migration, whereas per(6-amino-6-deoxy)-β-CD (**βpNH$_2$**) results in partial reduction of the observed fluorescence. Compounds **βe** and **βp** completely inhibit migration of DNA, and only at DNA/**βe** or **βp** mass/charge = 15 is DNA migrating.

These results show that at physiological pH the newly prepared **βe** and **γe** bear more positive charges than the amino CDs (pK_a ~ 8).[2d] Similar results were obtained upon electrophoresis of DNA in the presence of the guanidino derivatives **βeg**, **γeg**, **βpg**, **γpg** (Table 2, gel b). Qualitatively, comparing the same mass/charge ratios among the compounds in several gels, the DNA inhibition effect increases in the order **βNH$_2$** < **βe** < **βp**, **γNH$_2$** < **γe** < **γp**. Of all the prepared compounds tested, those with more functional groups were the most active, that is, γCD derivatives more than βCD derivatives and guanidino more than amino derivatives. This behavior is expected because the guanidine–phosphate interaction is widely acknowledged to be a specific and strong interaction. The high local concentration of positively charged groups on the CDs defines an area for strong, cooperative, multivalent interactions,[24] presumably with the phosphate groups of DNA, a feature frequently observed in biological systems. As mentioned previously,[3] an example of biological molecules that bear some similarity to the present compounds are protamines. These small proteins found in sperm heads have sequences of five, six or seven consecutive arginine residues[25] and form core α-helices[25d,e] that interact strongly with the major groove of DNA, thus resulting in its very efficient packing. The present compounds feature 14–16 amino/guanidino groups clustered in a fixed area of ~1 nm diameter, therefore, it is reasonable to propose a mode of interaction with DNA similar to that of protamines.

Table 1. Compounds and the corresponding ratios of DNA/compound used for electrophoresis.[a]

Lane	Compd	V [μL]	Mass/Charge	Lane	Compd	V [μL]	Mass/Charge
1	λHindIII	–	–	9	λHindIII	–	–
2	**βCD**	15	–	10	**βe**	10	2
3	**βCD**	10	–	11	**βe**	5	3
4	**βCD**	5	–	12	**βe**	1	15
5	**βpNH$_2$**	15	1	13	**βp**	15	1
6	**βpNH$_2$**	10	2	14	**βp**	10	2
7	**βpNH$_2$**	5	3	15	**βp**	5	3
8	**βe**	15	1	16	**βp**	1	15

[a] Concentrations: [**βCD**] = [**βpNH$_2$**] = 0.0844 mM, [**βe**] = 0.0778 mM, [**βp**] = 0.0955 mM. Mass/Charge ratio refers to DNA/compound.

Table 2. Compounds and the corresponding ratios of DNA/compound used for electrophoresis.[a]

Lane	Compd	V [μL]	Mass/Charge	Lane	Compd	V [μL]	Mass/Charge
1	λHindIII	–	–	9	–	–	–
2	DNA	–	–	10	DNA	–	–
3	**βeg**	0.1	100	11	**βpg**	0.1	100
4	**βeg**	1	10	12	**βpg**	1	10
5	**βeg**	10	1	13	**βpg**	10	1
6	**γeg**	0.1	100	14	**γpg**	0.1	100
7	**γeg**	1	10	15	**γpg**	1	10
8	**γeg**	10	1	16	**γpg**	10	1

[a] Concentrations: [**βeg**] = 0.0886 mM, [**βpg**] = 0.0844 mM, [**γeg**] = 0.0886 mM, [**γpg**] = 0.0844 mM. Mass/Charge ratio refers to DNA/compound.

Atomic force microscopy (AFM): Double-stranded linear DNA (5 kbp) buffered with HEPES/NaOH at pH 7.4 was immobilized on freshly cleaved mica substrates. The images obtained (Figure 6a) for DNA alone showed fibre-like formations producing a "network" on the surface. Incubation of the same DNA in the presence of **βe** for 1 h followed by AFM imaging revealed the formation of globular nanoparticles (Figure 6b) of sizes ranging from ~40–140 nm, in complete contrast to the previous image. Scanning in several different areas of the surface did not show presence of the previously observed fibres. Clearly, the presence of the title compounds results in packing of the DNA in particles of size suitable for cell entry.[4]

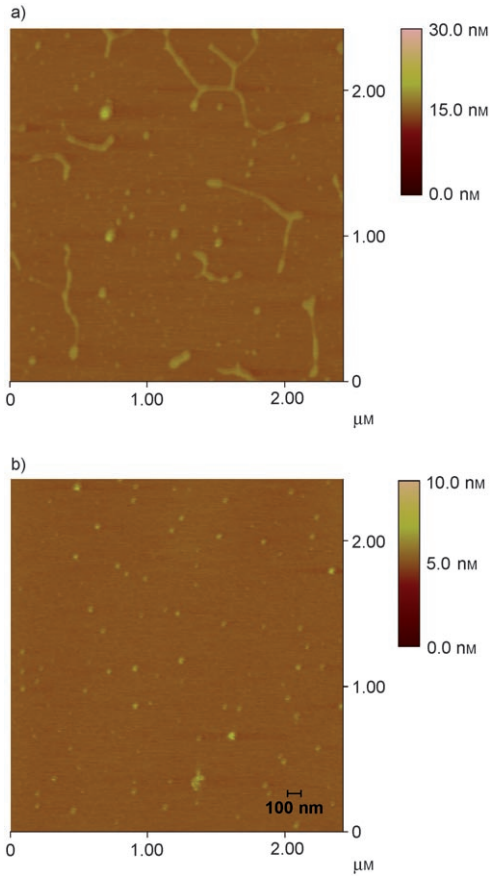


Figure 6. AFM images of a) double-stranded linear DNA 5 kbp and b) the DNA/**βe** complex at 1:1000 ratio.

Transfection experiments: The compounds prepared were tested as potential DNA-transfection agents on human embryonic kidney (HEK) 293 and 293T cells, by using pAdTrack (adenoviral plasmid expressing green fluorescent protein, eGFP). In an initial screen on HEK 293 cells, no transfection was observed at several low concentrations of CD derivatives (1 nM to 10 μM), in the presence or absence of serum. However, in all concentrations tested, varying degrees of spotted autofluorescence was observed, which was particularly evident upon using the guanidino compounds. The use of higher concentrations of CD reagents (0.4 to 2.2 mM) resulted in cell transfection with compounds **βe**, **γe**, and **βp** only, in which one or two cells per field were observed. Given that **βe**, **γe**, and **βp** were evidently non-toxic to recipient cells at concentrations up to 2.2 mM, the concentration of these compounds in the CD/DNA mixture was increased. This led to an increase in the transfection efficiency, although to only a small extent (images not shown). Consequently, HEK 293T cells were chosen as the target cell line, given that their efficiency of transfection, achievable using standard commercial reagents, is known to be higher than that of parental HEK 293 cells. The optimal concentration of each CD reagent required to mediate transfection was first determined by dose response experiments. Concentrations ranging from 4 to 40 mg mL^{-1} of the CD derivative were complexed to pAdTrack and the mixture then added to HEK 293T cells. Expression of GFP was determined by fluorescence microscopy 48 h later (Figure 7a).

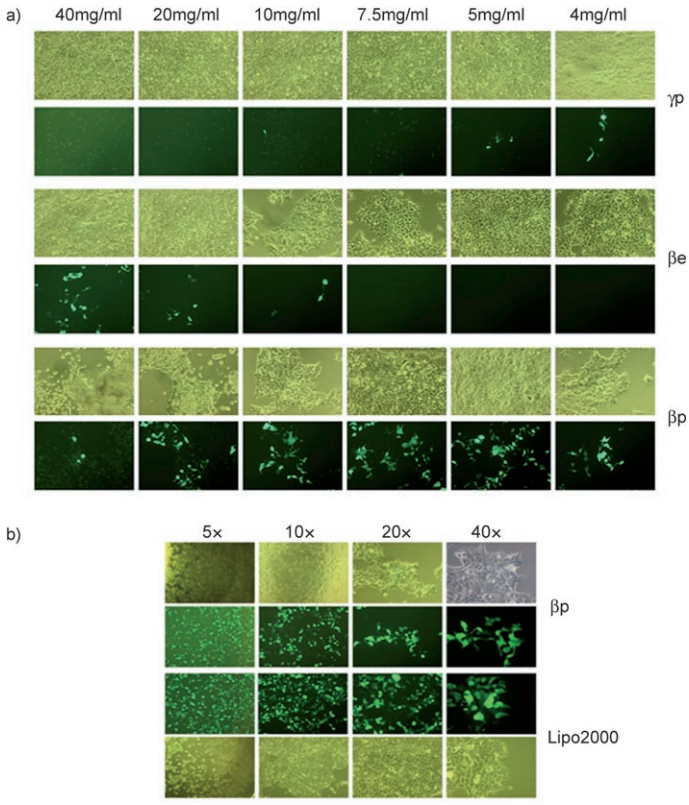


Figure 7. a) Optical and fluorescence microscopy images showing expression of eGFP protein in HEK 293T cells in the presence of selected CD derivatives and b) magnification and comparison with commercially available Lipofectamine 2000 as control.

The three reagents, **γp**, **βp**, and **βe**, mediated much higher transfection under these conditions, giving optimal expression of GFP at concentrations of 4 mg mL^{-1} (2.4 mM), 7.5 mg mL^{-1} (4.2 mM) and 40 mg mL^{-1} (20.8 mM), respectively. Compound **βp** mediated the most efficient transfection in

HEK 293T cells, as well as in the HEK 293 cell line. Indeed, evaluation of transfection efficiency by fluorescence microscopy at a range of magnifications revealed that **βp** mediated the transfection of HEK 293T cells to an extent similar to that obtained by using Lipofectamine 2000 (Figure 7b). In a previous report, heptakis(6-amino-6-deoxy)-β-cyclodextrin and several pyridylamino- and imidazoyl-β-CDs were shown[21] to complex with plasmid DNA and be effective in DNA transfection, however, direct comparison of the present compounds with these literature compounds cannot be made as the latter were tested on different cells.

Conclusion

The present novel compounds, which were shown to penetrate cell membranes and mediate satisfactory DNA transfection, combine several desirable features:

1) structurally, they possess invariant spatial arrangement of positively charged groups and a cross-section of ~1 nm^2;
2) they are oligomers prepared in two and three steps from inexpensive parent CDs in moderate to good yields, using processes that could be easily scaled up;
3) they are not peptidic in nature, therefore, they are not expected to be immunogenic and simultaneously unlikely to be enzymatically cleaved by, for example, trypsin, as opposed to CPPs that are enzymatically labile under physiological conditions;[7d]
4) they possess aqueous solubility within the mM range;
5) they allow cell survival and viability combined with fast cell entry;
6) they possess amino groups that can be readily attached to a cargo molecule (e.g., a suitable drug), or even decorated with a targeting functionality, providing many possibilities for future applications.

Moderate efficiency of DNA transfection was reported previously for otherwise excellent heptarginine molecular transporters,[26] therefore, observation of appreciable transfection levels for some of the present compounds is an interesting result. Cell-penetrating CDs fulfill most criteria for efficient, non-hazardous transport vectors, and may provide a significant tool in pharmaceutical research.

Experimental Section

CDs were products of Cyclolab or Sigma–Aldrich and were used as received. DMF was dried and distilled over molecular sieves. Benzoylated cellulose dialysis tubing, 32 mm, cut-off MW 1100, was obtained from Aldrich. Reactions requiring pressure were carried out in a stainless steel Parr pressure reactor equipped with mechanical stirring and temperature controller. The starting materials, per(6-bromo-6-deoxy)-derivatives **βBr** and **γBr**, were prepared as described previously.[1f,3] All moisture-sensitive reactions were carried out under a nitrogen atmosphere. 1D and 2D NMR spectra were acquired either at 250 or at 500 MHz with Bruker spectrometers. ^{1}H NMR spectra recorded in D_2O were referenced to HOD at $\delta = 4.79$, unless otherwise stated. MALDI-TOF spectra were obtained with an Autoflex (Bruker Daltonics) instrument using 2,5-dihydroxybenzoic acid in ethanol (80%), at the Istituto di Ricerche Chimiche e Biochimiche "G. Ronzoni", Milano, and ESI-MS spectra with an AQA Navigator, Finnigan, at "Demokritos".

Syntheses:

*Heptakis[6-(2-aminoethylamino)-6-deoxy]-βCD (**βe**)*: Heptakis(6-bromo-6-deoxy)-βCD (**βBr**) (0.316 g, 0.2 mmol) was added to 1,2-diaminoethane (3.7 mL, 56 mmol) and the mixture was placed inside an autoclave and stirred under N_2 atmosphere (7 atm) at 80 °C for 72 h. The excess 1,2-diaminoethane was removed by evaporation under reduced pressure and the oily residue was dissolved in doubly distilled water (5 mL). The resulting solution was dialysed using cellulose tubing for 72 h to remove the low-molecular-weight, water-soluble impurities. Subsequent removal of the solvent by lyophilization afforded a pale-yellow solid (0.267 g, 93%). ^{1}H NMR (500 MHz, D_2O, 25 °C): $\delta = 5.06$ (brs, 7H; H1), 3.90 (brm, 14H; H3, H5), 3.58 (brm, 14H; H2, H4), 3.04 (brs, 14H; -CH_2NH$_2$), 2.90–2.84 ppm (br, 28H; H6, -NHCH_2-); ^{13}C NMR (63 MHz, D_2O, 25 °C): $\delta =$ 100.6 (C1), 81.2 (C4), 72.0 (C3), 71.1 (C2), 69.6 (C5), 47.7 (C6), 46.7 (-NHCH_2-), 38.0 ppm (-CH_2NH$_2$); ESI-MS: *m/z* (%): 205.3 (100) $[M+7H]^{7+}$, 716.0 (20) $[M+2H]^{2+}$; MALDI-TOF MS: *m/z* (%): 1429.9 (100) $[M+H]^+$, 1451.9 (88) $[M+Na]^+$, 1391.8 (42) ($[M+Na]^+ - H_2NCH_2CH_2NH_2 = 60$), 1369.8 (73) $[M^+ - 60]$, 1309.7 ($[M+H]^+ - 2\times60$).

*Heptakis[6-(3-aminopropylamino)-6-deoxy]-βCD (**βp**)*: Heptakis(6-bromo-6-deoxy)-βCD (**βBr**) (0.316 g, 0.2 mmol) was added to 1,3-diaminopropane (4.7 mL, 56 mmol) and the mixture was reacted and treated as above. Lyophilization afforded a pale-yellow solid (0.123 g, 40%). ^{1}H NMR (500 MHz, D_2O, 25 °C): $\delta = 5.08$ (br, 7H; H1), 3.89 (br, 14H; H3, H5), 3.58 (br, 7H; H2), 3.51 (br, 7H; H4), 3.00 (br, 14H; -NHCH_2-), 2.92 (br, 14H; H6), 2.72 (br, 14H; -CH_2NH$_2$), 1.84 ppm (br, 14H; -NHCH$_2$CH_2-); ^{13}C NMR (63 MHz, D_2O, 25 °C): $\delta = 104.1$ (C1), 84.4 (C4), 75.4 (C3), 74.7 (C2), 72.8 (C5), 51.7 (-NHCH$_2$-), 49.5 (C6), 40.7 (-CH$_2$NH$_2$), 29.2 ppm (-NHCH$_2$CH$_2$-); ESI-MS: *m/z* (%): 219.3 (28) $[M+7H]^{7+}$, 510.4 (12) $[M+3H]^{3+}$, 765.0 (11) $[M+2H]^{2+}$; MALDI-TOF MS: *m/z* (%): 1549.9 (15) $[M+Na]^+$, 1528.0 (100) $[M+H]^+$, 1453.9 (95) ($[M+H]^+ - H_2NCH_2CH_2CH_2NH_2 = 74$), 1379.8 (19) ($[M+H]^+ - 2\times74$).

*Heptakis[6-(6-aminohexylamino)-6-deoxy]-βCD (**βh**)*: Heptakis(6-bromo-6-deoxy)-βCD (**βBr**) (0.316 g, 0.2 mmol) was added to 1,6-diaminohexane (6.51 g, 56 mmol) and the mixture was treated as above. Lyophilization afforded a white solid containing approximately 35% free diamine that persisted despite extensive washings with chloroform and dialysis (0.250 g, 35%). ^{1}H NMR (500 MHz, D_2O, 25 °C): $\delta = 5.03$ (brs, 7H; H1), 3.82 (brs, 14H; H3, H5), 3.57 (br, 7H; H2), 3.45–3.31 (brm, 7H; H4), 2.83 (br, 28H; H6, -CH_2NH$_2$), 2.54 (br, 14H; -NHCH_2-), 1.55 (br, 14H; -NHCH$_2$CH_2-), 1.46 (br, 14H; -CH_2CH$_2$NH$_2$), 1.30 ppm (br, 28H; -CH_2CH_2CH$_2$CH$_2$NH$_2$); ^{13}C NMR (126 MHz, D_2O, 25 °C): $\delta =$ 102.8–101.0 (C1), 83.7–82.4 (C4), 73.3 (C2), 72.2 (C3), 70.7–70.2 (C5), 49.8–49.6 (C6, -NHCH$_2$-), 40.0 (-CH$_2$NH$_2$), 30.2 (-NHCH$_2$CH$_2$-), 29.0 (-CH$_2$CH$_2$NH$_2$), 28.6–27.7 (-NHCH$_2$CH$_2$CH$_2$-), 26.9–26.4 ppm (-CH$_2$CH$_2$CH$_2$NH$_2$); ESI-MS: *m/z* (%): 261.4 (35) $[M+7H]^{7+}$, 912.3 (14) $[M+2H]^{2+}$; MALDI-TOF MS: *m/z* (%): 1860.0 (7) $[M+K]^+$, 1844.2 (60) $[M+Na]^+$, 1822.3 (58) $[M+H]^+$, 1706.2 (100) $[([M+H]^+ - H_2N(CH_2)_6NH_2 = 116) \equiv M1]$, 1590.3 (21) $[M1-116]$, 1612 (2) $[M1-116+Na]^+$.

*Octakis[6-(2-aminoethylamino)-6-deoxy]-γCD (**γe**)*: Octakis(6-bromo-6-deoxy)-γCD (**γBr**) (0.360 g, 0.2 mmol) was added to 1,2-diaminoethane (3.85 mL, 64 mmol) and the mixture was reacted and treated as above. Lyophilization afforded a pale-yellow solid (0.320 g, 98%). ^{1}H NMR (500 MHz, D_2O, 25 °C): $\delta = 5.12$ (brm, 8H; H1), 3.88 (br, 16H; H3, H5), 3.58 (br, 16H; H2, H4), 3.02 (br, 16H; -CH_2NH$_2$), 2.88 ppm (br, 32H; H6, -NHCH_2-); ^{13}C NMR (63 MHz, D_2O, 25 °C): $\delta = 103.7$–103.1 (C1), 83.6–83.0 (C4), 75.1 (C3), 74.7 (C2), 73.0 (C5), 51.1 (C6), 49.0 (-NHCH$_2$-), 41.2 ppm (-CH$_2$NH$_2$); ESI-MS: *m/z* (%): 205.3 (100) $[M+8H]^{8+}$, 818.0 (12) $[M+2H]^{2+}$; MALDI-TOF MS: *m/z* (%): 1633.8 (100) $[M+H]^+$, 1655.8 (92) $[M+Na]^+$, 1573.8 (58) $[([M+H]^+ - H_2NCH_2CH_2NH_2 = 60) \equiv M1]$, 1513.7 (18) $[M1-60]^+$, 1453.6 (3) $[M1-2\times60]$.

*Octakis[6-(3-aminopropylamino)-6-deoxy]-γCD (**γp**)*: Octakis(6-bromo-6-deoxy)-γCD (**γBr**) (0.450 g, 0.25 mmol) was added to 1,3-diaminopropane (6.6 mL, 80 mmol) and the mixture was reacted and treated as above.

Lyophilization afforded a pale-yellow solid (0.421 g, 79%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 5.21 –5.11 (brs, 8H; H1), 3.90 (brt, 16H; H3, H5), 3.58 (brd, 8H; H2), 3.52 (brt, 8H; H4), 3.01 (brt, 16H; -CH_2NH$_2$), 2.95 (br, 16H; H6), 2.76 (br, 16H; -NHCH_2-), 1.87 ppm (brt, 16H; -NHCH_2CH_2-); ^{13}C NMR (63 MHz, D_2O, 25°C): δ = 101.0–99.6 (C1), 81.4–80.1 (C4), 72.5 (C3), 71.9 (C2), 69.8 (C5), 49.1 (C6), 46.6 (-NHCH_2), 37.9 (-CH_2NH$_2$), 26.2 ppm (-NHCH$_2$CH_2-); ESI-MS: *m*/*z* (%): 219.3 (28) [*M*+8H]$^{8+}$; MALDI-TOF MS: *m*/*z* (%): 1768.0 (50) [*M*+Na]$^+$, 1746.0 (100) [*M*+H]$^+$, 1671.9 (77) [([*M*+H]$^+$–H$_2$NCH$_2$CH$_2$CH$_2$NH$_2$ = 74) ≡ *M*1], 1597.9 (24) [(*M*1+H$^+$–74) ≡ *M*2], 1523.8 (5) [*M*2+H$^+$–74].

Heptakis[6-(2-guanidinoethylamino)-6-deoxy]-βCD (***βeg***): Heptakis-[6-(2-aminoethylamino)-6-deoxy]-βCD (**βe**) (0.208 g, 0.12 mmol) was dispersed in dry dimethylformamide (10 mL) and to the mixture 1*H*-pyrazolecarboxamidine hydrochloride (1.23 g, 8.4 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (1.65 mL, 9.7 mmol) were added in three equal portions over a period of 72 h. During this period the mixture was stirred continuously at 70°C under a N_2 atmosphere. After cooling, diethyl ether (150 mL) was added drop-wise and the suspension formed was stirred for 2 h at RT. The solvent was decanted and the collected sticky solid was dissolved in water (2 mL). Addition of ethanol (200 mL) resulted in the precipitation of a white substance that was filtered off, washed with ethanol (50 mL) and dried under vacuum. This precipitate was dissolved in doubly distilled water (3 mL), the pH was adjusted to 7 with hydrochloric acid (1 N) and the solution was treated with Dowex Type I resin (Cl^- exchanger) for 1 h. The resin was removed by filtration and the solution was kept inside dialysis tubing for 72 h to remove the low-molecular-weight impurities. Subsequent lyophilization afforded a pale-yellow solid (0.070 g, 27%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 5.04 (brs, 7H; H1), 3.89 (brs, 14H; H3, H5), 3.58–3.41 (brm, 21H; H2, H4, H6), 3.27 (brs, 14H; -CH_2NH-C=NH), 2.81 ppm (brt, 21H; H6′, -NHCH_2-); ^{13}C NMR (126 MHz, D_2O, 25°C): δ = 159.9 (NH=*C*-NH$_2$), 104.3 (C1), 85.0 (C4), 75.5 (C3), 74.7 (C2), 73.3 (C5), 51.5 (C6), 50.7 (-NH*C*H$_2$-), 43.6 ppm (-*C*H$_2$NH-C=NH); ESI-MS: *m*/*z* (%): 247.3 (37) [*M*+7H]$^{7+}$; MALDI-TOF MS *m*/*z* (%): 1781 (78) ([*M*+H]$^+$ + 2 × [CNH$_2$$^{\bullet}$ = 28])$^+$, 1752.1 (100) [([*M*+H]$^+$ + CNH$_2$$^{\bullet}$ = 28) ≡ *M*1], 1736.0 (52) (*M*1–NH$_2$$^{\bullet}$)$^+$, 1724.1 (34) [*M*+H]$^+$, 1665 (33) ([*M*–H$_2$NCH(NH)NH)$^{\bullet}$]$^+$ = 58).

Heptakis[6-(3-guanidinopropylamino)-6-deoxy]-βCD (***βpg***): Heptakis[6-(3-aminopropylamino)-6-deoxy]-βCD (**βp**) (0.200 g, 0.11 mmol) was dispersed in dry dimethylformamide (5 mL) and to the mixture 1*H*-pyrazolecarboxamidine hydrochloride (0.678 g, 4.6 mmol) and DIPEA (0.93 mL, 5.5 mmol) were added in three equal portions over a period of 72 h. The reaction mixture was then reacted and treated as above. Lyophilization afforded a pale-yellow solid (0.027 g, 13%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 5.34–4.90 (br, 7H; H1), 3.84 (br, 14H; H3, H5), 3.50 (br, 7H; H2), 3.36 (br, 7H; H4), 3.16 (br, 14H; -CH_2NH-C=NH), 2.94–2.73 (br, 14H; H6), 2.61 (br, 14H; -NHCH_2-), 1.72 ppm (br, 14H; -NHCH$_2$CH_2); ^{13}C NMR (126 MHz, D_2O, 25°C): δ = 157.9 (NH=*C*-NH$_2$), 101.6 (C1), 83.6 (C4), 73.1 (C3), 72.7 (C5), 72.3 (C2), 49.2 (C6), 45.7 (-NHCH_2-), 38.7 (-*C*H$_2$NH-C=NH), 27.8 ppm (-NHCH$_2$CH_2-); ESI-MS: *m*/*z* (%): 261.3 (97) [*M*+7H]$^{7+}$, 304.3 (22) [*M*+6H]$^{6+}$; MALDI-TOF MS *m*/*z* (%): 1851.0 (70) [*M*+CNH$_2$ = 28]$^+$, 1823.1 (100) [*M*+H]$^+$, 1806.1 (46) [*M*–NH$_2$$^{\bullet}$]$^+$, 1790 (35) [*M*–2 × NH$_2$$^{\bullet}$)$^+$, 1764 (23) [*M*+H$^+$–guanidine = 59], 1734, 1692.

Heptakis[6-(6-guanidinohexylamino)-6-deoxy]-βCD (***βhg***): Heptakis[6-(6-aminohexylamino)-6-deoxy]-βCD (**βh**) (0.091 g, 0.05 mmol) was dispersed in dry dimethylformamide (3 mL) and to the mixture 1*H*-pyrazolecarboxamidine hydrochloride (0.205 g, 1.4 mmol) and DIPEA (300 μL, 1.75 mmol) were added in four equal portions over a period of 96 h. The reaction mixture was then reacted and treated as above. Lyophilization afforded a white solid that includes approximately 50% of free diamine (0.075 g, 30%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 5.05 (br, 7H; H1), 4.00–3.76 (br, 14H; H3, H5), 3.61 (br, 7H; H2), 3.46 (br, 7H; H4), 3.10 (br, 14H; -CH_2NH-C=NH), 2.95 (br, 14H; H6), 2.71 (br, 14H; -NHCH_2-), 1.52 (br, 28H; -NHCH$_2$CH_2CH$_2$CH$_2$CH_2CH$_2$NH-C=NH), 1.30 ppm (br, 28H; -NHCH$_2$CH$_2$CH_2CH_2CH$_2$CH$_2$NH-C=NH); ^{13}C NMR (126 MHz, D_2O, 25°C): δ = 156.6 (NH=*C*-NH$_2$), 100.8 (C1), 83.6–82.4 (C4), 71.9 (C3), 70.7 (C2), 68.4 (C5), 48.5 (-NHCH_2-), 48.1 (C6), 40.3 (-*C*H$_2$NH-C=NH), 27.0 (-NHCH$_2$*C*H$_2$CH$_2$CH$_2$*C*H$_2$CH$_2$NH-C=NH), 25.0 ppm (-NHCH$_2$CH$_2$*C*H$_2$CH$_2$CH$_2$CH$_2$NH-C=NH); ESI-MS: *m*/*z* (%): 303.5 (50) [*M*+7H]$^{7+}$; MALDI-TOF MS: *m*/*z* (%): 2159.5 (43) ([*M*+H]$^+$ + C(NH)NH$_2$ = 43)$^+$, 2117.5 (29) [*M*+H]$^+$, 1959.4 (61) ([*M*+H]$^+$–H$_2$N-C(NH)HN(CH$_2$)$_6$NH$_2$ = 158 ≡ *M*1), 1917.3 (100) [*M*1+H$^+$–C(NH)NH$_2$ = 43].

Octakis[6-(2-guanidinoethylamino)-6-deoxy]-γCD (***γeg***): Octakis[6-(2-aminoethylamino)-6-deoxy]-γCD (**γe**) (0.163 g, 0.1 mmol) was dispersed in dry dimethylformamide (5 mL) and to the mixture 1*H*-pyrazolecarboxamidine hydrochloride (0.352 g, 2.4 mmol) and DIPEA (547 μL, 3.2 mmol) were added in three equal portions over a period of 72 h. The reaction mixture was then reacted and treated as above. Lyophilization afforded a pale-yellow solid (0.120 g, 61%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 5.15 (br, 8H; H1), 3.88 (br, 16H; H3, H5), 3.58 (br, 16H; H2, H4), 3.29 (br, 16H; -CH_2NH-C=NH), 3.06 (br, 8H; H6), 2.88 ppm (br, 24H; -NHCH_2-, H6′); ^{13}C NMR (126 MHz, D_2O, 25°C): δ = 157.1 (NH=*C*-NH$_2$), 99.6 (C1), 79.9 (C4), 71.7 (C3), 71.3 (C2), 69.6 (C5), 47.7 (C6), 46.9 (-NHCH_2-), 39.9 ppm (-*C*H$_2$NH-C=NH); ESI-MS: *m*/*z* (%): 247.2 (44) [*M*+8H]$^{8+}$; MALDI-TOF MS: *m*/*z* (%): 1992.2 (42) [*M*+Na]$^+$, 1970.2 (100) [*M*+H]$^+$, 1954.2 (79) ([*M*+H$^+$–NH$_2$$^{\bullet}$), 1869.1 (73) [*M*+H$^+$ –[H$_2$N-C(NH)HN(CH$_2$)$_2$NH$_2$$^{\bullet}$] = 102 ≡ *M*1], 1852 (44) [*M*1–16].

Octakis[6-(3-guanidinopropylamino)-6-deoxy]-γCD (***γpg***): Octakis[6-(3-aminopropylamino)-6-deoxy]-βCD (**γp**) (0.106 g, 0.05 mmol) was dispersed in dry dimethylformamide (5 mL) and to the mixture 1*H*-pyrazolecarboxamidine hydrochloride (0.236 g, 1.6 mmol) and DIPEA (370 μL, 2.1 mmol) were added in three equal portions over a period of 72 h. The reaction mixture was then reacted and treated as above. Lyophilization afforded a pale-yellow solid (0.0125 g, 13%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 5.24–5.14 (br, 8H; H1), 3.99 (br, 8H; H5), 3.90 (br, 8H; H3), 3.59 (br, 8H; H2), 3.55 (br, 8H; H4), 3.23 (br, 16H; -CH_2NH-C=NH), 3.03 (br, 16H; H6), 2.87 (br, 16H; -NHCH_2-), 1.93–1.88 ppm (br, 16H; -NHCH$_2$CH_2-); ^{13}C NMR (126 MHz, D_2O, 25°C): δ = 156.8 (NH=*C*-NH$_2$), 98.5 (C1), 79.3 (C4), 71.1 (C3), 70.7 (C2), 68.0 (C5), 48.1 (C6), 45.3 (-NHCH$_2$-), 37.9 (-*C*H$_2$NH-C=NH), 25.0 ppm (-NHCH$_2$CH_2-); ESI-MS: *m*/*z* (%): 261.3 (5) [*M*+8H]$^{8+}$; MALDI-TOF MS: *m*/*z* (%): 2082.4 (23) [*M*+H]$^+$, 1966.0 (36) [*M*+H$^+$–H$_2$N-C(NH)HN(CH$_2$)$_3$NH$_2$ = 116].

Fluorescein labeling

Mono(6-amino-6-deoxy)-βCD·Fl (***βNH$_2$·Fl***): Mono(6-amino-6-deoxy)-βCD[18] (0.057 g, 50 μmol) was dispersed in dry DMF (5 mL) mixed with DIPEA (9 μL, 50 μmol), to which a solution of fluorescein isothiocyanate (isomer I) (0.001 g, 2.5 μmol) in DMF (5 mL) was added drop-wise over 1 h. The mixture was stirred and heated at 70°C under N_2 for 3 h. Cooling to RT and solvent removal in vacuo gave a solid that was dissolved in doubly distilled water (5 mL) and the pH was adjusted to 11 using 1 N ammonium hydroxide. The solution was extracted with chloroform (3 × 50 mL) to remove DIPEA and the pH was adjusted to 7 with 1 N HCl, so that unreacted fluorescein precipitated and could be removed carefully after centrifugation. The final solution was concentrated to dryness, dissolved in doubly distilled water (5 mL) and dialysed. After water removal a yellow solid was obtained (0.055 g) (fluorescein content ~20%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 7.85 (brs, H$_{fl}$), 7.75 (brm, H$_{fl}$), 7.20 (brs, H$_{fl}$), 6.83 (brd, H$_{fl}$), 6.72 (brd, H$_{fl}$), 4.99 (d, *J* = 3.4 Hz, 7H; H1), 3.84 (t, *J* = 9.5 Hz, 7H; H3), 3.80–3.75 (brm, 21H; H5, H6), 3.57 (dd, *J* = 3.4, 9.5 Hz, 7H; H2), 3.50 (t, *J* = 9.5 Hz, 7H; H4), 3.12 (brs, 14H; H8), 3.04–2.93 ppm (br, 28H; H6, H7).

Heptakis[6-(2-aminoethylamino)-6-deoxy]-βCD·Fl (***βe·Fl***): The amine **βe** (0.084 g, 50 μmol) was dispersed in dry DMF (5 mL) containing DIPEA (60 μL, 0.35 mmol), to which a solution of fluorescein isothiocyanate (0.001 g, 2.5 μmol) in DMF (10 mL) was added drop-wise over a period of 1 h. After identical treatment to that described above an orange-red product was obtained (0.080 g) (fluorescein content ~5%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 8.28–8.22 (m, H$_{fl}$), 8.14 (brs, H$_{fl}$), 7.93 (d, *J* = 7 Hz, H$_{fl}$), 7.60 (brt, H$_{fl}$), 7.51 (brt, H$_{fl}$), 5.15 (brs, 7H; H1), 3.97 (br, 14H; H3, H5), 3.68 (brd, 14H; H2, H4), 3.12 (brs, 14H; H8), 3.04–2.93 ppm (br, 28H; H6, H7).

Heptakis[6-(3-aminopropylamino)-6-deoxy]-βCD·Fl (***βp·Fl***): The amine **βp** (0.036 g, 20 μmol) was dispersed in dry DMF (6 mL) containing DIPEA (24 μL, 0.14 mmol) and to it, fluorescein isothiocyanate was added (0.0004 g, 1 μmol) in DMF (2 mL). After identical treatment to

that described above, an orange-red solid was obtained (0.030 g) (fluorescein content ~5%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 8.00 (brm, H_{fl}), 5.03 (brs, 7H; H1), 3.89 (br, 14H; H3, H5), 3.56–3.32 (brm, 14H; H2, H4), 2.92 (brm, 28H; H6, H7), 2.64 (brs, 14H; H9), 1.78 ppm (brs, 14H; H8).

*Oktakis[6-(2-aminoethylamino)-6-deoxy]-γCD·Fl (**γp·Fl**)*: The amine **γp** (0.065 g, 40 μmol) DMF (5 mL), DIPEA (55 μL, 0.32 mmol) and fluorescein isothiocyanate (0.0008 g, 2 μmol) in DMF (10 mL) were treated as described above. Finally, 0.058 g of an orange-red solid was obtained (fluorescein content ~2%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 8.16–8.12 (brm, H_{fl}), 8.04 (brs, H_{fl}), 7.84 (brd, H_{fl}), 5.13 (brs, 8H; H1), 3.87 (br, 16H; H3, H5), 3.56 (brm, 16H; H2, H4), 3.02 (brm, 16H; H8), 2.87 ppm (brs, 32H; H6, H7).

*Heptakis[6-(2-guanidinoethylamino)-6-deoxy]-βCD·Fl (**βeg·Fl**)*: The amine **βe** (0.084 g, 50 μmol) was dispersed in dry DMF (3 mL) containing DIPEA (120 μL, 0,7 mmol) and to this a solution of fluorescein isothiocyanate (0.001 g, 2.5 μmol) in dry DMF (2 mL) was added drop-wise over a period of 1 h. The mixture was stirred and heated at 70°C under N_2 for 3 h. Then, 1*H*-pyrazole-1-carboxamidine hydrochloride (0.153 g, 1.05 mmol) and DIPEA (180 μL, 1.05 mmol) were added in three equal portions over a period of 3 d at 70°C, under N_2. The mixture was then cooled and concentrated to dryness, the solid obtained was dissolved in doubly distilled water (5 mL) and the pH adjusted to 12 using 1 N ammonium hydroxide. Excess DIPEA was removed by extraction with $CHCl_3$ (3×50 mL), the pH was readjusted to 7 using 1 N HCl and centrifugation removed unreacted fluorescein. The final solution was concentrated under vacuum, dissolved in doubly distilled H_2O (5 mL) and dialysed. Evaporation of water in vacuo afforded an orange-red solid (0.078 g) (fluorescein content ~5%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 8.13–8.02 (brm, H_{fl}), 5.03 (brs, 7H; H1), 3.88 (br, 14H; H3, H5), 3.57–3.47 (brm, 14H; H2, H4), 3.25 (brs, 14H; H8), 2.87–2.74 ppm (brm, 28H; H6, H7); ^{13}C NMR (63 MHz, D_2O, 25°C): δ = 159.6 (H_2N-*C*=NH), 104.1 (C1), 84.9 (C4), 75.2 (C3), 74.5 (C2), 73.1 (C5), 51.3 (C6), 50.4 (C7), 43.3 ppm (C8).

*Heptakis[6-(3-guanidinopropylamino)-6-deoxy]-βCD·Fl (**βpg·Fl**)*: The amine (0.036 g, 20 μmol) in DMF (2 mL) and DIPEA (24 μL, 0.14 mmol) was labeled using fluorescein isothiocyanate (0.0004 g, 1 μmol) in dry DMF (4 mL) as described above. The product was then immediately guanylated without isolation by adding 1*H*-pyrazole-1-carboxamidine hydrochloride (0.062 g, 0.42 mmol) and DIPEA (72 μL, 0.42 mmol) in three equal portions as above. Identical treatment as described previously afforded an orange-red solid (0.031 g) (fluorescein content ~5%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 8.20–7.80 (brm, H_{fl}), 5.20–5.11 (brs, 7H; H1), 4.11 (br, 7H; H5), 3.91 (br, 7H; H3), 3.60 (brs, 14H; H2, H4), 3.25 (brs, 14H; H9), 3.05 (brs, 28H; H6, H7), 2.04 ppm (brs, 14H; H8); ^{13}C NMR (63 MHz, D_2O, 25°C): δ = 160.5 (H_2N-*C*=NH), 103.2 (C1), 83.5 (C4), 74.4 (C3), 74.0 (C2), 70. 5 (C5), 51.0 (C6), 48.4 (C7), 39.4 (C9), 26.8 ppm (C8).

*Octakis[6-(2-guanidinoethylamino)-6-deoxy]-γCD·Fl (**γeg·Fl**)*: The amine **γe** (0.095 g, 58 μmol) in DMF (10 mL) with DIPEA (78 μL, 0.46 mmol) was labeled using fluorescein isothiocyanate (0.0012 g, 3 μmol) in dry DMF (3 mL) as described above. The product was then immediately guanylated without isolation by adding 1*H*-pyrazole-1-carboxamidine hydrochloride (0.204 g, 1.4 mmol) and DIPEA (234 μL, 1.4 mmol) in three equal portions as described above. Identical treatment as described afforded an orange-red solid (0.086 g) (fluorescein content ~5%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 8.01 (brm, H_{fl}), 5.14 (brs, 8H; H1), 3.88 (br, 16H; H3, H5), 3.58, (brm, 16H; H2, H4), 3.31 (brm, 16H; H8), 3.06–2.90 ppm (brs, 32H; H6, H7); ^{13}C NMR (63 MHz, D_2O, 25°C): δ = 159.5 (H_2N-*C*=NH), 103.4 (C1), 83.3 (C4), 74.6 (br, C3, C2), 72.5 (C5), 51.2 (C6), 50.1 (C7), 42.8 ppm (C8).

*Octakis[6-(3-guanidinopropylamino)-6-deoxy]-γCD·Fl (**γpg·Fl**)*: The amine **γp** (0.051 g, 25 μmol) in dry DMF (2 mL) with DIPEA (34 μL, 0.2 mmol) was labeled using fluorescein isothiocyanate (0.0005 g, 1.25 μmol) in dry DMF (4 mL) as described above. The product was then immediately guanylated without isolation by adding 1*H*-pyrazole-1-carboxamidine hydrochloride (0.088 g, 0.6 mmol) and DIPEA (137 μL, 0.8 mmol) in three equal portions as described above. An orange-red solid was finally obtained (0.086 g) (fluorescein content ~5%). ^{1}H NMR (500 MHz, D_2O, 25°C): δ = 8.30–7.80 (brm, H_{fl}), 5.31 (brs, 8H; H1), 4.12 (br, 7H; H5), 3.94 (br, 7H; H3), 3.61 (brs, 14H; H2, H4), 3.26 (brs, 14H; H9), 3.06 (brs, 28H; H6, H7), 2.04–1.98 ppm (brm, 14H; H8); ^{13}C NMR (63 MHz, D_2O, 25°C): δ = 159.2 (H_2N-*C*=NH), 101.2 (C1), 81.9 (C4), 74.3 (C3), 73.8 (C2), 70.2 (C5), 51.1 (C6), 48.2 (C7), 39.4 (C9), 26.8 ppm (C8).

pK_a determination: Determination of the pK_a values of compounds **γe** and **γeg** was performed by titrating their basic D_2O solutions (0.05 mM in D_2O, pD > 12 by NaOD addition) with 1 N or 5 N DCl and monitoring the induced chemical shifts of certain carbon nuclei ($\Delta\delta_{obs}$) by ^{13}C NMR spectroscopy, using benzene in a sealed tube as internal reference. The pD of the solution was measured after every DCl addition and just before the spectrum acquisition. If the peak of interest overlapped with another peak, a 2D HSQC spectrum was acquired in order to determine its exact chemical shift. For the ionization equilibrium of a protonated amine [Eq. (2)],

$$AH^+ \overset{K_a}{\rightleftharpoons} A + H^+ \tag{2}$$

with

$$K_a = [H^+][A]/[AH^+] \tag{3}$$

$$-\log K_a = -\log[H^+] - \log[A] + \log[AH^+] \tag{4}$$

$$pH = pK_a + \log([A]/[AH^+]) \tag{5}$$

The plots δ_{obs} = f(pD) have the shape of a double sigmoidal curve, thus showing that each of these compounds has two discrete K_a constants. The pK_a values were obtained by plotting according to Equation (6),

$$pD = f\{\log[(\delta_{acidic} - \delta_{obs})/(\delta_{obs} - \delta_{basic})]\} \tag{6}$$

in which δ_{acidic} and δ_{basic} are the chemical shifts of the observed signal of the fully protonated and deprotonated compound, respectively, and linear fitting in the two rising portions of the curve.

Cell experiments

Cell culture: The human adenocarcinoma HeLa cell line (ATCC) was maintained in high-glucose DMEM medium with L-glutamine (PAA Laboratories GmbH), supplemented with 10% foetal calf serum (PAA Laboratories GmbH) and penicillin/streptomycin (PAA Laboratories GmbH) at 100 UI per 100 μg per mL, in a 5% CO_2 incubator at 37°C. Cells were subcultured every two or three days.

For cell-penetration experiments, cells were seeded on coverslips. The day after seeding, the compounds were added at various concentrations (50–100 μM) and incubated for various time periods (15 min, 1 h and 24 h). After incubation and intensive washing with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 1 L distilled water, subsequent pH adjustment (7.4) with dilute HCl and sterilization) cells were fixed in cold methanol and mounted with *p*-phenylenediamine (PPD) anti-fade solution (Aldrich). For microscopic observation, a Nikon Eclipse E400 fluorescence microscope with a triple filter (DAPI-FITC-Texas Red) was used.

MTT assay: HeLa cells were seeded in 96-well plates at a density of 3000 cells per 100 μL per well and incubated for 24 h for attachment. The day after seeding, exponentially growing cells were incubated with the prepared compounds in nine different concentrations in quadruplicate over a period of 24 h. Controls consisted of wells without the compounds. The medium was removed and the cells were incubated for 4 h in the presence of 1 mg mL^{-1} MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Sigma) in RPMI (PAA Laboratories GmbH) without phenol red at 37°C (100 μL per well). The MTT solution was removed and 100 mL per well of isopropanol was added. After thorough mixing, absorbance of the wells was read in an ELISA reader at test and reference wavelengths of 540 and 620 nm, respectively. The mean of the optical density of different replicates of the same sample and the percentage of each value was calculated (mean of the OD of various replicates/OD

of the control). The percentage of the optical density against drug concentration was plotted in a semi-log chart and the IC_{50} from the dose response curve was determined.

DNA experiments

Agarose gel electrophoresis: For the electrophoresis experiments, ultrapure calf thymus DNA (~8.6 MDa, >13 kb, Sigma) was used. The working solution was prepared by dissolving 12.5 μg of DNA in 50 μL of doubly distilled, autoclaved water. For the preparation of each sample, 1 μL of the working solution was mixed with increasing volumes (2, 5 or 10 μL) of a solution of the CD derivative in order to achieve a DNA/derivative mass/charge ratio $[(M/Q)_{DNA}/(M/Q)_N]$ of ≫1:1 to 1:1. The concentration C_N of the CD-derivative solution was calculated by using the equation: $m_N/Q_N = m_{DNA}/Q_{DNA}$ for a 1:1 ratio, in which m is the mass and Q is the absolute total charge of the molecule. C_{DNA} is 7.6×10^{-4} M, V_{DNA} is 1 μL and $(M/Q)_{DNA}$ is 330 (every base pair has a molecular weight of ~330 g mol^{-1} and charge of −1). Each mixture was diluted with doubly distilled, autoclaved water to a total volume of 20 μL and incubated for approximately 45 min at RT. Control samples with guanidine hydrochloride [10 μL of 0.1706 mM solution, $(M/Q)_{DNA}/(M/Q)_N = 1$] and βCD and γCD (10 μL of 0.1045 mM solutions) were prepared in the same way. After incubation, 2 μL of a dye (0.4% bromophenol blue, 67% sucrose) were added to each sample, which was then loaded onto a 1% agarose gel containing 10 μg ethidium bromide [10 mg mL^{-1} per 100 mL TAE buffer (40 mM Tris-acetate, 1 mM EDTA)] and electrophoresed, using λ*Hin*dIII as the molecular-weight marker.

Atomic force microscopy: Samples were examined by using a Digital Instruments Multimide AFM with Nanoscope III controller, operating in Tapping mode. The AFM tips, having a typical radius of curvature of 7 nm, and mounted on cantilevers with a resonant frequency of approximately 300 kHz, were supplied by NANOSENSORS. The images had a 512×512 pixel resolution with the scan range varying between 1 μm× 1 μm and 6 μm×6 μm. The tip was operated at a scan rate of 1–3 Hz (lines/sec). AFM images of the linear DNA (6 kbp) alone and in the presence of **βe** were collected. The 5 μL of DNA solution was diluted to 100 μL with HEPES/NaOH buffer (pH 7.4), resulting in a ~3×10^{-11} μM solution. The buffer solution was prepared with ultrapure water (18.2 MΩ) and filtered through a 0.22 μm Millipore filter prior to use. For the DNA condensation studies, 10 μL of DNA solution were mixed with 10 μL solution (~10^{-8} M) of **βe** and left for incubation for 1 h at RT. Then 5 μL of the above mixture were placed on a mica surface and left to dry under a stream of air for 30 min. For the control experiment, 5 μL of linear DNA solution were used in the same way.

Circular dichroism: Spectra were obtained with a single-beam JASCO spectrophotometer at 320–200 nm. Cells of 1 mm were used and the plasmid DNA (5.3 kb) solutions were prepared in buffered sodium chloride/sodium citrate (pH 7.0). The concentration of DNA was determined to be 10^{-4} M by measuring the UV absorption (maximum at 258 nm). Initially, the spectra of buffer and DNA solution (150 μL) were obtained and then the spectra of DNA/**βe** at different ratios ($[\mathbf{\beta e}] = 10^{-3}$ M). For each spectrum eight scans were acquired at 50 nm min^{-1}.

Transfection experiments: The title compounds were complexed with pAdTrack (adenoviral plasmid expressing eGFP, available from Stratagene). Target cells were human embryonic kidney (HEK) 293T cells (modified to express the SV40 large T antigen). To establish the most efficient transfection, the CD derivatives were used at a range of concentrations. Typically, 25 μL (for 24-well plates) or 100 μL (for 12-well plates) of each CD solution at concentrations ranging from 4 to 40 mg mL^{-1} in sterile water were aliquoted into separate eppendorfs and 0.8 μg of reporter plasmid was added to each CD and incubated for 20 min at RT. The CD–DNA mix was then added drop-wise to 293 cells in 12- (or 24-) well plates that had been plated at a density of 5×10^4 (or 1×10^5) cells per well one day prior to transfection in a volume of 0.5 mL (or 1.0 mL) growth medium per well. Control transfection experiments were also conducted by using Lipofectamine–DNA complexes (Invitrogen) according to the manufacturer's instructions. Expression of eGFP was then monitored after 48 h by fluorescence microscopy.

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