

NJC

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.



www.rsc.org/njc

ARTICLE

Cyclodextrin-scaffolded amphiphilic aminoglycoside clusters: Self-assembling and gene delivery capabilities

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Eva M. Aguilar Moncayo,^a Nicolas Guilloteau,^b Celine Bienvenu,^b José L. Jiménez Blanco,^{*a} Christophe Di Giorgio,^{*b} Pierre Vierling,^b Juan M. Benito,^c Carmen Ortiz Mellet^a and José M. García Fernández^c

Precise control over the architecture of gene carriers is instrumental to manipulate gene delivery efficiency. Combining cationic centers and carbohydrates motifs onto monodisperse architectures has been proposed as a suitable strategy to impart nucleic acid condensation abilities while preserving biocompatibility. Herein, we have assessed the influence of the arrangement and orientation of cationic elements on the self-assembling and gene transfer capabilities of polycationic glycoamphiphilic cyclodextrins (pGaCDs). For such purpose, a series of cyclodextrin multiconjugates bearing aminoglycoside motifs at their primary rim and hexanoyl chains at the secondary positions were synthesized. In the presence of pDNA, pGaCDs self-assemble into nanoaggregates that promote cellular uptake and gene expression into COS-7 cells with efficiencies that are intimately associated to the arrangement of amino functionalities imposed by the aminoglycoside antennae onto the cyclodextrin-scaffolded cluster. Although transfection efficiencies were lower than those observed for polyethyleneimine (PEI)-based polyplexes and previously-reported polycationic amphiphilic cyclodextrins (paCDs), the results reported herein illustrate (i) the dramatic consequences that subtle architectural modifications exert on the supramolecular organization of pGaCDs and (ii) the virtues of monodisperse systems for tailoring gene transfer capabilities.

Introduction

Gene therapy, involving the introduction and expression of foreign gene material into cells, bears a high promise to cure a wide range of genetic as well as acquired diseases.^{1,2} Its effective development critically depends on the design of appropriate delivery systems to carry out compaction, protection, cell internalization and timely release of the gene payload. Viral vectors have proved to be highly efficient gene delivery agents. However, despite success,³ this approach is seriously limited due to immunogenicity and toxicity risks.⁴ Alternatively, research in synthetic gene delivery systems has gained momentum. Non-viral gene vectors, e.g. cationic polymers or lipids,⁵ bear important safety advantages over viral approaches,^{6,7} as well as lower cost and ease of production. They also offer alternative mechanisms for gene material delivery, eventually resulting in improved pharmacokinetics and pharmacodynamics.^{8,9} The overall positive surface electrostatic potential of the corresponding complexes with nucleic acids (polyplexes or lipoplexes) promotes adhesion to negatively charged proteoglycans on the outer face of the cell membrane, thus facilitating cellular uptake and transfection.^{10,11}

Yet, the application of non-viral vectors to humans has been, with remarkable exceptions,^{12,13} held back by their poorer delivery efficiency.

Lessons learned from the vast amount of research on non-viral gene carriers highlight the dramatic influence that minute architectural modifications exert on nucleic acid complex formation and, consequently, on nanoparticle trafficking, cellular uptake, payload release and gene expression.¹⁴⁻¹⁶ However, the inherent polydispersity and random conformations of many of these formulations turn into a hurdle to assess structure-activity relationships (SAR) and optimize carrier performance. Alternatively, discrete molecular frameworks, allowing the installation of spatially segregated functional elements, have emerged as an appealing option.¹⁷ Thus, multifunctional pre-organized platforms, such as calixerenes,¹⁸ fullerenes,¹⁹ pillar[5]arenes,²⁰ and cyclodextrins (CDs),²¹⁻²³ have recently been exploited as scaffolds to build up monodisperse architectures with the ability to condense DNA into transfectious nanoparticles. Among them, CD-based vectors have been so far the most profusely investigated due to

the inherent biocompatibility and ease of chemical tailoring of the cyclooligosaccharide core.^{24,25}

In the framework of a project aimed at engineering site-specific gene vectors,²⁶ we recently observed that installation of glycosyl antennae onto polycationic amphiphilic CDs (paCDs) did not only serve to mediate binding to specific receptors (lectins) on the target cells,^{27,28} but also dramatically influenced self-assembling capabilities in the presence of nucleic acids. It can be expected that DNA complexation by polycationic glycoamphiphilic CDs (pGaCDs) bearing aminoglycoside motifs will be particularly sensitive to structural modifications. Actually, the differential binding of aminoglycoside antibiotics to nucleic acids has already been exploited in the design of cationic lipid-type vectors.²⁹⁻³¹ Moreover, a number of glycosylated gene carriers have been shown to operate with transfection efficiencies that correlated with their glyco-dependent self-assembling capabilities in the presence of nucleic acids rather than with specific recognition events towards target protein receptors.³² To gain a deeper insight on the structural features governing gene delivery efficiency of pGaCDs, herein we report the synthesis of a series of representatives featuring different aminoglycoside motifs, and the assessment of (i) their self-assembling ability in the presence of pDNA and (ii) the transfection efficiency of the resulting pGaCD-pDNA nanoaggregates (CDplexes) towards COS-7 cells. The results indicate that subtle variations in the topology of the cationic elements significantly impact the stability and physicochemical properties of the corresponding glycoCDplexes and, consequently, cell transfection efficiency and cytotoxicity profiles.

Results and discussion

Design criteria and synthesis

It has been previously shown that the number and arrangement of the cationic elements in paCDs critically influence gene carrier capabilities.³³⁻³⁶ To pinpoint these effects in the pGaCD series, three aminoglycosylated β CD derivatives **2-4** (Fig. 1), featuring subtle differences in the disposition of the cationic elements but rather similar hydrophobic/hydrophilic balances have been now considered. pGaCDs **2-4** also share the same spacer arm linking the aminoglycoside motif to the CD core. The thiourea functionalities have been purposely included in the molecular design since their presence has been previously shown to be beneficial for promoting reversible nucleic acid complexation through hydrogen bonding.³⁷ Moreover, the thiourea-forming reaction has proven extremely useful in “click-type” multiconjugation schemes.^{38,39} The pDNA complexing capabilities and transfection efficiencies of **2-4** have been evaluated in comparison with paCD **1**, one of the most efficient cyclodextrins-based vector candidates reported to date.³³

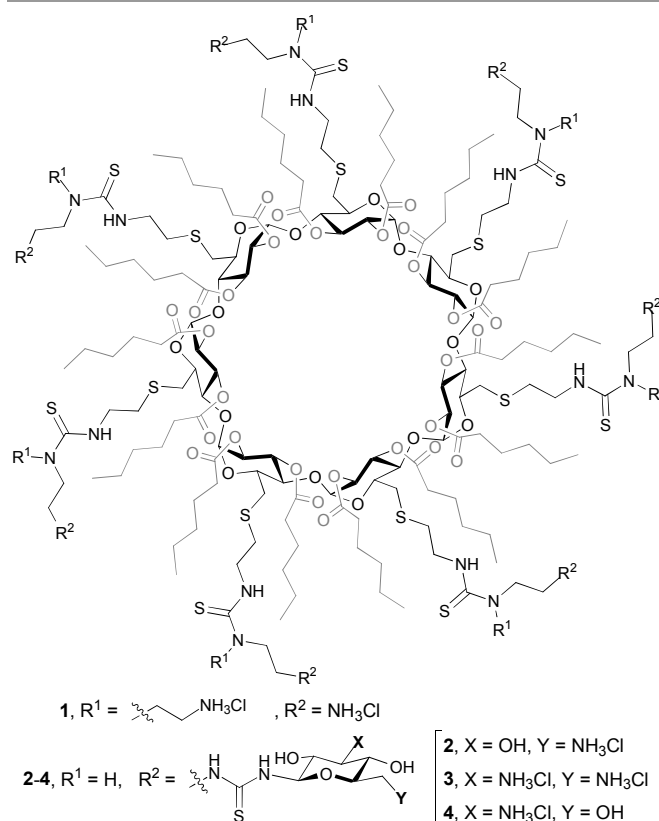


Fig. 1. Structure of paCD **1** and pGaCDs **2-4**.

For the preparation of the differently-substituted pGaCDs **2-4** a convergent synthetic scheme was designed in which the key step is the coupling reaction of the heptaisothiocyanate **17**³³ with a complementary amine-armed glycoconjugate (Scheme 1). For such purpose, the required isothiocyanates **5-7** were synthesized from the corresponding azidoglycosylenamine derivative following a previously described methodology.^{27,40} Isothiocyanate derivatives **5-7** were first coupled with *N*-tritylethylene-1,2-diamine⁴¹ (\rightarrow **8-10**), followed by sequential acetyl (\rightarrow **11-13**) and trityl cleavage to yield amines **29, 30** and **31** (71-40% over three steps, Scheme 1). Triethylamine-promoted nucleophilic addition of the resulting amine-armed aminoglycoside derivatives **14-16** to heptaisothiocyanate **17**³³ in DMF proceed slowly at room temperature to furnish the fully substituted β CD adducts **18-20**. Final acid-promoted carbamate hydrolysis yielded the target heptavalent aminoglycoclusters **2-4**, which were characterized as the corresponding perhydrochlorides (71-50% overall, Scheme 1). The structure and molecular homogeneity of the CD-centred glycoclusters **18-20** and **2-4** were confirmed by NMR, MS and combustion analysis, the ensemble of data being consistent with the expected C_7 -symmetry arrangement for homogeneously substituted β CD derivatives.

Table 1. Size (av. hydrodynamic diameter, nm), polydispersity index (P. I.) and ξ -potential (mV) of CDplexes of paCDs 1-4 and pDNA formulated at N/P 10 determined by DLS and M3-PALS analysis, respectively, in the absence and in the presence of serum.

Complex	av. size (nm)	P. I.	ξ -potential (mV)
1:pDNA	76 \pm 1	0.12	+46 \pm 1
1:pDNA ^a	160 \pm 15	0.13	n.d.
2:pDNA	100 \pm 20	0.16	+48 \pm 1
2:pDNA ^a	240 \pm 20	0.20	n.d.
3:pDNA	260 \pm 100	0.50	-12 \pm 1
4:pDNA	105 \pm 7	0.17	+54 \pm 2
4:pDNA ^a	270 \pm 25	0.22	n.d.

^aMeasurements in the presence of serum (10%).

The morphology of the glycoCDplexes was next evaluated by transmission electron microscopy (TEM). The corresponding micrographs confirmed the small size and homogeneous distribution of the nanoparticles obtained from pGaCDs 2 and 4 (Fig. 3). A snail-like ultra-thin structure alternating dark (high electron density) and light (low electron density) regions could be observed in some cases, suggesting the arrangement of the pGaCD molecules in bilayers in the confined space between pDNA segments. A similar topography has been previously observed for transfectious CDplexes.^{27,33} As expected, formulations with derivative 3 did not render well-defined particles, but rather polydisperse aggregates.

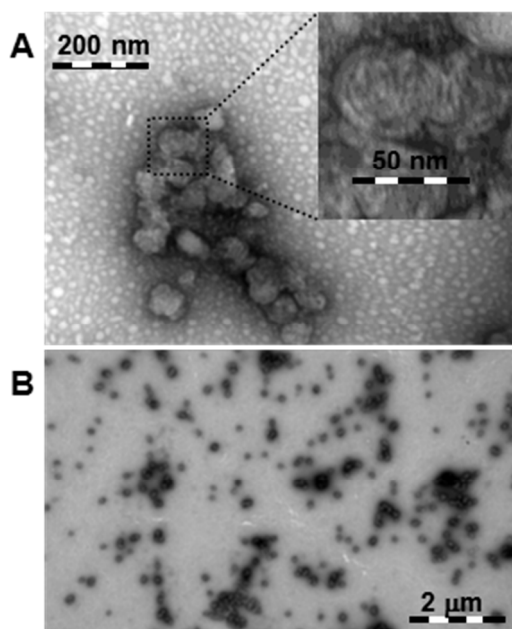


Fig. 3. TEM micrograph of (A) 2:pDNA and (B) 4:pDNA glycoCDplexes: The inset in panel A represents the amplification of the structure of the particles.

In vitro pDNA transfection into COS-7 cells

The transfection efficiency of the self-assembled pGaCD:pDNA nanocomplexes formulated with 2 and 4 at N/P 5 and 10 was evaluated using a luciferase-encoding reporter gene (pTG11236, pCMV-SV40-luciferase-SV40pA) in monkey fibroblast-like COS-7 cells in HEPES buffer (20 mM, pH 7.4, see experimental section for details) both in the absence and in

the presence of serum. Linear Jet-PEI (22 kDa, polyplexes formulated at N/P 10) as well as paCD 1 (CDplexes formulated at N/P 5 and 10) and naked pDNA were used as positive and negative controls, respectively.

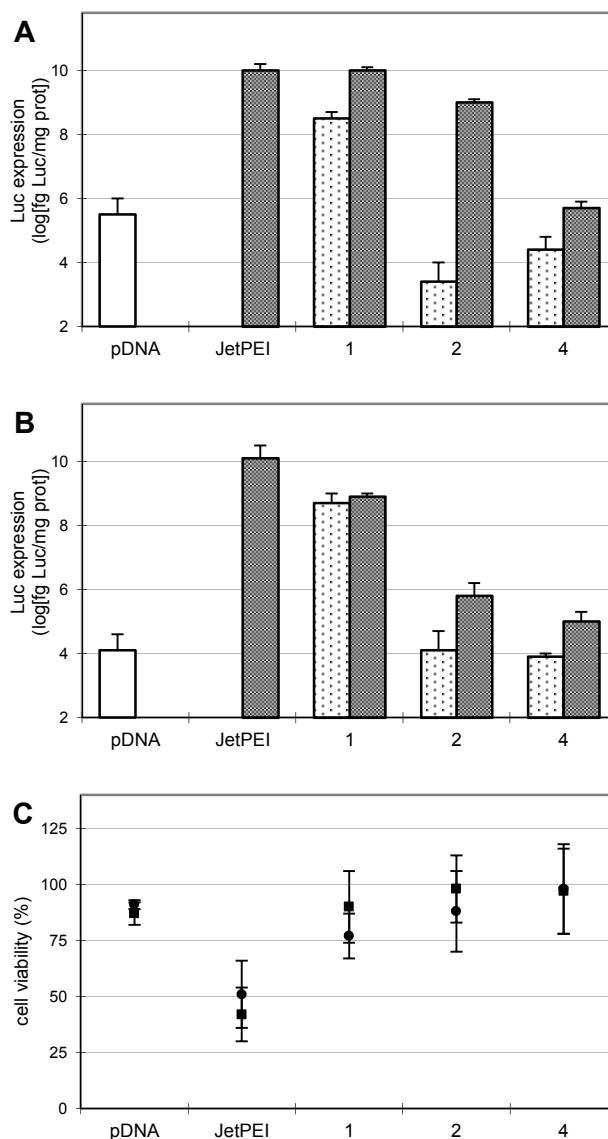


Fig. 4. In vitro transfection efficiency (bars) at N/P 5 (dotted bars) and 10 (filled bars) in COS-7 cells for CDplexes formulated with paCD 1 and pGaCDs 2 and 4 in the absence (panel A) and in the presence (panel B) of serum (10%). Naked pDNA and Jet-PEI-based polyplexes (formulated at N/P 10) were used as negative and positive controls, respectively. Cell viability in the absence (●) and in the presence (■) of serum (10%) is represented in panel C.

At N/P 5, pGaCDs 2-4 did not improve the expression of luciferase achieved with naked pDNA, neither in the absence nor in the presence of serum (Fig. 4). In the absence of serum, formulations at N/P 10 of the heptacationic pGaCDs 2 and 4 enhanced transfection efficiency by 4 and 2 orders of magnitude, respectively, implying that the corresponding nanoparticles are internalized to a significant extent through routes that allow endosome escape, DNA release and protein

expression. Indeed, previous studies on structurally-related paCDs have shown that CDplexes are rapidly internalised in cells by several endocytic routes⁴³ and the reversibility of the paCD-pDNA association.⁴⁴ Remarkably, the luciferase expression efficiency achieved with 2:pDNA glycoCDplexes is only one order of magnitude lower than that determined for paCD 1 and Jet-PEI, with a much more favourable toxicity profile than the later (Fig. 4, panel C). The large discrepancies between the performances at N/P 10 of pGaCDs 2 and 4, featuring similar hydrophilic/hydrophobic balances (7 protonable amino groups in each glucose antenna and 14 hexanoyl groups at secondary positions of β CD), reveal the dramatic influence exerted by the presentation mode of the protonable amine centers. The presence of the amino group at the primary position of the glucopyranose moiety probably favours the accessibility of the charged groups in the polycationic cluster to phosphate anions in the pDNA skeleton. A similar effect has been observed in cationic lipids furnished with aminoglycoside polar heads.²⁹

The presence of serum (10%) led to a generalized efficiency drop for both pGaCDs 2 and 4 when compare to paCD 1. Probably, the greater increase in size of the glycoCDplexes formed with 2 and 4 after interaction with serum proteins, is at the origin of this observation. Indeed, DLS measurements in serum-containing media (table 2) support this hypothesis. Such phenomena probably result into a weakening of the interaction with cell membrane components, limiting thus efficient internalization process of nanoparticles and/or cause less favourable cellular trafficking.

CDplexes formulated with pGaCD 3 were unable to promote pDNA expression under identical experimental conditions either in the absence or in the presence of serum. Although improved pDNA binding has been associated to higher cationic valencies for cationic CDs,⁴⁵ our results confirm that in the case of amphiphilic derivatives architectural features governing the self-assembling properties exert a much larger influence on the gene delivery capabilities.^{33,35,46}

Conclusions

In summary, the assessment of the self-assembling and gene transfer capabilities of this set of polycationic glycoamphiphilic β CD clusters illustrates the utmost relevance of structure-activity relationship analyses in the design of non-viral gene carriers. Small differences in the arrangement and display of the cationic elements responsible for the initial interaction of individual molecules with nucleic acid (pDNA) may have strong consequences in the transfection capabilities of the resulting nanocomplexes. All three pGaCDs clusters 2-4, similarly to the previously reported non-glycosylated paCD 1, are heavily cationic discrete species. Yet, while 2 and 4 render homogenous nanoparticles (glycoCDplexes) with cell transfection abilities, pGaCD 3 does not. Moreover, remarkable performance disparities are found for glycoCDplexes formulated with 2 and 4 in COS-7 cells, with 2:pDNA glycoCDplexes paralleling the golden standard Jet-PEI with

null cytotoxicity. Altogether the present results illustrate the usefulness of well-defined molecular vectors for mapping the structural requirements governing DNA complexation and delivery. The information thus obtained, in combination with robust synthetic methodologies, can be put forward in the optimization of vector architecture.

Experimental

General methods

All chemicals were obtained from Sigma-Aldrich (Germany) and Panreac (Spain) and were of analytical grade. No further purification steps were performed unless indicated. All solvents were used as obtained from the commercial sources. Optical rotations were measured at room temperature in 1-cm or 1-dm tubes on a Jasco P-2000 polarimeter. Ultraviolet-visible (UV) spectra were recorded in 1-cm tubes on a Beckman DU640 UV spectrophotometer. Infrared (IR) spectra were recorded on a Jasco FT/IR 6000-Series spectrophotometer and are reported in reciprocal centimetres (cm^{-1}). ^1H (and ^{13}C NMR) spectra were recorded at 500 (125.7) and 400 (100.6) MHz with a Bruker 500 and 400DRX instruments. Satisfactory resolutions were achieved after heating above 313 K. 1D TOCSY, 2D COSY, HMQC and HSQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with silica gel 60G F254 (E. Merck), with visualization by UV light and by charring with 10% H_2SO_4 . Column chromatography was carried out on Silica Gel 60 (E. Merck, 230- 400 mesh). FAB mass spectra were obtained with a Kratos MS-80 RFA instrument. The operating conditions were the following: the primary beam consisted of Xe atoms with a maximum energy of 8 keV; the samples were dissolved in thioglycerol, and the positive ions were separated and accelerated over a potential of 7 keV; NaI was added as cationizing agent. ESI mass spectra were recorded in the positive mode on a Bruker Esquire 6000 ion-trap mass spectrometer. Typically, samples were dissolved in appropriate solvent at low μM concentrations. Samples were introduced by direct infusion, using a Cole-Palmer syringe at a flow rate of 2 $\mu\text{L}\cdot\text{min}^{-1}$. Ions were scanned between 300 and 6000 Da with a scan speed of 13000 $\text{Da}\cdot\text{s}^{-1}$ at unit resolution using resonance ejection at the multipole resonance of one-third of the radio frequency ($\Omega = 781.25$ kHz). Elemental analyses were performed at the Instituto de Investigaciones Químicas (Sevilla, Spain). paCD 1 was prepared according to the reported procedure.³³ The starting materials 2,3,4-tri-*O*-acetyl-6-*N*-tert-butoxycarbonylamino-3-deoxy- β -D-glucopyranosyl isothiocyanate (5),²⁷ 2,4-di-*O*-acetyl-3,6-di-*N*-tert-butoxycarbonylamino-3,6-dideoxy- β -D-glucopyranosyl isothiocyanate (6),⁴⁰ 2,4,6-tri-*O*-acetyl-3-*N*-tert-butoxycarbonylamino-3-deoxy- β -D-glucopyranosyl isothiocyanate (7),⁴⁰ *N*-tritylethylene-1,2-diamine,⁴¹ and heptakis[6-deoxy-2,3-di-*O*-hexanoyl-6-(2-isothiocyanatoethylthio)]cyclomaltoheptaose (17)³³ were prepared as described previously.

Syntheses

N'-(*N*-Trityl-2-aminoethyl)-*N*-(2,3,4-tri-*O*-acetyl-6-*tert*-butoxycarbonylamino-6-deoxy- β -D-glucopyranosyl)thiourea (**8**). To a solution of *N*-tritylethylene-1,2-diamine⁴¹ (0.50 g, 1.65 mmol) in pyridine (20 mL) was added **5**²⁷ (0.49 g, 1.10 mmol) and the reaction mixture was stirred at rt for 15 min, then concentrated. The solvent was eliminated and pyridine traces were eliminated by co-evaporation with toluene. The residue was purified by column chromatography (1:1 EtOAc-petroleum ether). Yield: 0.79 g (96%); $R_f = 0.40$ (1:1 EtOAc-petroleum ether); $[\alpha]_D = -5.4$ (c 1.0, DCM); ¹H NMR (500 MHz, CDCl₃): $\delta = 9.09$ (bs, 2 H, NHCS), 7.41-7.17 (m, 15 H, Ph), 6.63 (bs, 1 H, NHTr), 5.62 (t, 1 H, $J_{1,NH} = J_{1,2} = 9.0$ Hz, H-1), 5.34 (t, 1 H, $J_{2,3} = J_{3,4} = 9.0$ Hz, H-3), 4.98 (t, 1 H, H-2), 4.80 (bs, 1 H, H-4), 4.57 (bs, 1 H, NH-6), 3.69 (bs, 1 H, H-5), 3.34 (bs, 1 H, H-6a), 3.14 (bs, 1 H, H-6b), 2.56 (m, 2 H, CH₂NHCS), 2.44 (m, 2 H, CH₂NHTr), 2.06, 2.05 (3 s, 9 H, MeCO), 1.41 (s, 9 H, CMe₃); ¹³C NMR (100.6 MHz, 313 K, CDCl₃): $\delta = 184.6$ (CS), 171.5, 171.0, 169.7 (3 CO ester), 155.7 (CO carbamate), 145.1-126.9 (Ph), 88.3 (C-1), 79.5 (CMe₃), 74.3 (C-3), 72.9 (C-5, CPh₃), 71.4 (C-2), 69.3 (C-4), 60.3 (C-6), 45.3 (CH₂NHCS), 40.8 (CH₂NHTr), 28.3 (CMe₃), 20.9, 20.6, 20.5 (3 MeCO); ESI-MS: m/z 771 [M + Na]⁺; Anal. Calcd for C₃₉H₄₈N₄O₉S: C, 62.55; H, 6.46; N, 7.48. Found: C, 62.71; H, 6.39; N, 7.33.

N'-(*N*-Trityl-2-aminoethyl)-*N*-(2,4-di-*O*-acetyl-3,6-di-*tert*-butoxycarbonylamino-3,6-dideoxy- β -D-glucopyranosyl)thiourea (**9**). To a solution of **6**⁴⁰ (0.12 g, 0.24 mmol) in dry pyridine (4 mL), *N*-trityl-1,2-ethylenediamine⁴¹ (0.11 g, 0.36 mmol, 1.5 eq) was added and the reaction mixture was stirred at rt for 1 h. The solvent was evaporated under reduced pressure and traces of pyridine were removed by co-evaporation with toluene. The residue was purified by flash column chromatography (2:3 \rightarrow 1:1 EtOAc-petroleum ether) to give **11**. Yield: 114 mg (58%); $R_f = 0.66$ (1:1 EtOAc-petroleum ether); $[\alpha]_D = -1.17$ (c 1.0, MeOH); UV (MeOH): $\lambda_{max} = 246$, 216 nm (ϵ_{mM} 10.1, 26.1); IR (NaCl): $\nu_{max} = 3350$, 2975, 1746, 1224, 1034, 706 cm⁻¹; ¹H NMR (400 MHz, CD₃OD, 323 K): $\delta = 7.44$ (m, 15 H, Ph), 5.72 (d, 1 H, $J_{1,2} = 9.3$ Hz, H-1), 4.99 (m, 1 H, H-2), 4.89 (m, 1 H, H-4), 3.90 (m, 1 H, H-3), 3.70 (m, 3 H, CH₂NHCS, H-5), 3.32 (dd, $J_{6a,6b} = 14.6$ Hz, $J_{5,6a} = 2.7$ Hz, H-6a), 3.15 (dd, $J_{5,6a} = 6.0$ Hz, H-6b), 2.43 (m, 2 H, CH₂NHTr), 2.09, 1.95 (2 s, 6 H, MeCO), 1.43 (s, 18 H, CMe₃); ¹³C NMR (100.6 MHz, CD₃OD, 313 K): $\delta = 186.0$ (CS), 172.0, 171.6 (CO ester), 158.1, 157.9 (CO carbamate), 147.2-127.4 (Ph), 84.3 (C-1), 80.5 (2 CMe₃), 80.4 (CPh₃), 76.9 (C-5), 72.3 (C-2), 71.0 (C-4), 57.1 (C-3), 45.9 (CH₂NHCS), 44.6 (CH₂NHTr), 42.5 (C-6), 28.8, 28.7 (2 CMe₃), 20.8, 20.7 (2 MeCO); FABMS: $m/z = 828$ ([M + Na]⁺); Anal. Calcd for C₄₂H₅₅N₅O₉S: C, 62.59; H, 6.88; N, 8.69. Found: C, 62.33; H, 6.61; N, 8.51.

N'-(*N*-Trityl-2-aminoethyl)-*N*-(2,4,6-tri-*O*-acetyl-3-*tert*-butoxycarbonylamino-3-deoxy- β -D-glucopyranosyl)thiourea (**10**). To a solution of **7**⁴⁰ (0.156 g, 0.35 mmol) in dry pyridine (6.4 mL), *N*-trityl-1,2-ethylenediamine⁴¹ (0.16 g, 0.53 mmol) was added and the reaction mixture was stirred at rt for 1 h. The

solvent was eliminated under reduced pressure and traces of pyridine were removed by co-evaporation with toluene. The residue was purified by column chromatography (2:1 EtOAc-petroleum ether) to give **10**. Yield: 0.27 g (quantitative); $R_f = 0.51$ (1:1 EtOAc-petroleum ether); $[\alpha]_D = -3.6$ (c 1.0, DCM); UV (DCM): $\lambda_{max} = 255$, 226 nm (ϵ_{mM} 16.0, 19.5); IR (NaCl): $\nu_{max} = 3351$, 3048, 2977, 1746, 1227, 1037, 741, 708 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 323 K): $\delta = 7.46$ (m, 15 H, Ph), 5.78 (d, 1 H, $J_{1,2} = 9.5$ Hz, H-1), 5.01 (bs, 2 H, H-2, H-4), 4.25 (dd, 1 H, $J_{6a,6b} = 12.5$ Hz, $J_{5,6a} = 5.0$ Hz, H-6a), 4.10 (dd, 1H, $J_{5,6b} = 6.0$ Hz, H-6b), 3.93 (m, 1 H, H-3), 3.86 (m, 1 H, H-5), 3.71 (m, 2 H, CH₂NHCS), 2.38 (m, 2 H, CH₂NHTr), 2.06, 2.05, 1.97 (3 s, 9 H, MeCO), 1.46 (s, 9 H, CMe₃); ¹³C NMR (125.7 MHz, CD₃OD, 323 K): $\delta = 186.0$ (CS), 172.4, 172.1, 171.5 (CO ester), 157.9 (CO carbamate), 147.3-127.3 (Ph), 84.6 (C-1), 80.6 (CMe₃), 79.5 (CPh₃), 75.8 (C-5), 72.3 (C-2), 70.2 (C-4), 63.6 (C-6), 57.1 (C-3), 46.0 (CH₂NHCS), 44.7 (CH₂NHTr), 28.7 (CMe₃), 20.7, 20.6 (3 MeCO); FABMS: $m/z = 771$ ([M + Na]⁺); Anal. Calcd for C₃₉H₄₈N₄O₉S: C, 48.97; H, 6.58; N, 8.57. Found: C, 62.41; H, 6.329; N, 7.37.

N'-(*N*-Trityl-2-aminoethyl)-*N*-(6-*tert*-butoxycarbonylamino-6-deoxy- β -D-glucopyranosyl)thiourea (**11**). Compound **11** was quantitatively obtained by treatment of **8** (0.76 g, 1.02 mmol) with methanolic MeONa (0.5 mol per mol of acetates) in MeOH (10 mL) at 0 °C followed by neutralization with Amberlite 120 (H⁺). Yield: 0.64 g; $R_f = 0.62$ (45:5:3 EtOAc-EtOH-H₂O); $[\alpha]_D = -11.2$ (c 1.0, MeOH); ¹H NMR (400 MHz, MeOD, 313 K): $\delta = 7.45$ -7.14 (m, 15 H, Ph), 5.22 (bs, 1 H, H-1), 3.66 (bs, 2 H, CH₂NHCS), 3.48 (bd, 1 H, $J_{6a,6b} = 13.6$ Hz, H-6a), 3.41 (t, 1H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H-3), 3.33 (ddd, 1 H, $J_{4,5} = 9.1$ Hz, $J_{5,6b} = 7.0$ Hz, $J_{5,6a} = 2.7$ Hz, H-5), 3.27 (bt, 1 H, H-2), 3.15 (m, 1 H, H-6b), 3.14 (dd, 1 H, H-4), 4.57 (bs, 1 H, NH-6), 2.37 (m, 2 H, CH₂NHTr), 1.40 (s, 9 H, CMe₃); ¹³C NMR (100.6 MHz, MeOD, 313 K): $\delta = 185.7$ (CS), 158.7 (CO carbamate), 147.3-127.4 (Ph), 85.2 (C-1), 80.4 (CMe₃), 78.7 (C-3), 77.7 (C-5), 74.4 (C-2), 72.9 (C-4), 72.2 (CPh₃), 46.1 (CH₂NHCS), 44.7 (CH₂NHTr), 42.9 (C-6), 28.8 (CMe₃); ESI-MS: m/z 645 [M + Na]⁺; Anal. Calcd for C₃₃H₄₂N₄O₆S: C, 63.64; H, 6.80; N, 9.00; Found: C, 63.62; H, 6.64; N, 8.78.

N'-(*N*-Trityl-2-aminoethyl)-*N*-(3,6-di-*tert*-butoxycarbonylamino-3,6-dideoxy- β -D-glucopyranosyl)thiourea (**12**). Compound **12** was obtained by treatment of **9** (89 mg, 0.11 mmol) with methanolic MeONa (0.5 mol per mol of acetates) in MeOH (10 mL) at 0 °C. The crude product was purified by column chromatography (22:1 DCM-MeOH). Yield: 71 mg (89%); $R_f = 0.67$ (22:1 DCM-MeOH); $[\alpha]_D = -5.2$ (c 1.0, MeOH); UV (MeOH): $\lambda_{max} = 243$, 214 nm (ϵ_{mM} 17.4, 42.2); IR (KBr): $\nu_{max} = 3412$, 3063, 2973, 1685, 1250, 1079, 706 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 313 K): $\delta = 7.36$ (m, 15 H, Ph), 5.21 (bs 1 H, H-1), 3.66 (m, 2 H, CH₂NHCS), 3.49 (dd, $J_{6a,6b} = 13.7$ Hz, $J_{5,6a} = 2.6$ Hz, H-6a), 3.41 (d, $J_{2,3} = J_{3,4} = 9.5$ Hz, 1 H, H-3), 3.38 (m, 3 H, $J_{4,5} = 9.5$ Hz, $J_{5,6b} = 6.5$ Hz, H-5), 3.34 (m, 1 H, $J_{1,2} = 9.5$ Hz, H-2), 3.14 (dd, 1H, H-6b), 2.43 (m, 2 H, CH₂NHTr) 1.43 (s, 18 H, CMe₃); ¹³C NMR (125.7 MHz, CD₃OD, 313 K): $\delta = 186.0$ (CS), 159.2, 158.6 (CO carbamate), 147.4-127.4 (Ph), 86.3 (C-1), 80.6 (2 CMe₃), 79.0 (C-5), 72.8

(C-2), 72.3 (CPh₃), 71.3 (C-4), 61.8 (C-3), 46.2 (CH₂NHCS), 44.8 (CH₂NHTr), 43.2 (C-6), 28.4 (2 CMe₃); FABMS: *m/z* = 745 ([M + Na]⁺); Anal. Calcd for C₃₈H₅₁N₅O₇S: C, 63.22; H, 7.12; N, 9.70. Found: C, 62.94; H, 7.00; N, 9.59.

N-(*N*-Trityl-2-aminoethyl)-*N*-(3-*tert*-butoxycarbonylamino-3-deoxy-β-D-glucopyranosyl)thiourea (**13**). Compound **13** was obtained by treatment of **10** (0.21 g, 0.28 mmol) with methanolic MeONa (0.5 mol per mol of acetates) in MeOH (3 mL) at 0 °C. The reaction mixture was stirred at rt for 35 min. The crude product was purified by column chromatography (6:1 EtOAc-petroleum ether → EtOAc). Yield: 127 mg (71%); *R*_f = 0.43 (EtOAc); [α]_D = -7.3 (*c* 1.0, DCM); UV (DCM): λ_{max} = 254, 228 nm (ε_{mM} 15.3, 18.6); IR (NaCl): ν_{max} = 3329, 3083, 2929, 1676, 1292, 1245, 1168, 1079, 1026, 748, 706 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 323 K): δ = 7.38 (m, 15 H, Ph), 5.23 (bs 1 H, , H-1), 3.85 (dd, 1 H, *J*_{6a,6b} = 12.0 Hz, *J*_{5,6a} = 2.5 Hz, H-6a), 3.67 (bs, 2 H, CH₂NHCS), 3.66 (dd, 1 H, *J*_{5,6b} = 5.0 Hz, H-6b), 3.48 (t, 1H, *J*_{2,3} = *J*_{3,4} = 10.0 Hz, H-3), 3.44 (ddd, 1H, *J*_{4,5} = 10.0 Hz, H-5), 3.38 (t, 1 H, H-4), 3.43 (m, 1 H, H-2), 2.41 (m, 2 H, CH₂NHTr), 1.48 (s, 9 H, CMe₃); ¹³C NMR (125.7 MHz, CD₃OD, 323 K): δ = 186 (CS), 159.2, (CO carbamate), 130.2-127.4 (Ph), 86.1 (C-1), 80.5 (CMe₃), 80.4 (C-5), 72.8 (C-2), 72.2 (CPh₃), 70.0 (C-4), 62.9 (C-6), 61.9 (C-3), 46.3 (CH₂NHCS), 44.6 (CH₂NHTr), 28.8 (CMe₃); FABMS: *m/z* = 646 ([M + Na]⁺); Anal. Calcd for C₃₃H₄₂N₄O₆S: C, 63.64; H, 6.80; N, 9.00. Found: C, 63.51; H, 6.67; N, 8.85.

N-(2-Aminoethyl)-*N*-(6-*tert*-butoxycarbonylamino-6-deoxy-β-D-glucopyranosyl)thiourea (**14**). Compound **12** (0.2 g, 0.32 mmol) was treated with 2% TFA in DCM (8 mL) at rt for 4 h. The organic phase was extracted with water (6 x 10 mL), and the aqueous solution was neutralized, freeze-dried and purification by column chromatography (10:1:1 MeCN-H₂O-NH₄OH). Yield: 88 mg (76%); *R*_f = 0.08 (10:1:1 MeCN-H₂O-NH₄OH); [α]_D = -19.2 (*c* 1.0 in H₂O); ¹H NMR (400 MHz, D₂O, 323 K): δ = 5.52 (bs, 1 H, H-1), 3.89 (bs, 2 H, CH₂NHCS), 3.78 (t, 1H, *J*_{2,3} = *J*_{3,4} = 9.0 Hz, H-3), 3.71 (m, 2 H, H-5, H-6a), 3.65 (t, 1 H, *J*_{1,2} = 9.0 Hz, H-2), 3.51 (dd, 1 H, *J*_{3,4} = 9.0 Hz, H-4), 3.43 (dd, 1 H, *J*_{5,6b} = 7.2 Hz, *J*_{6a,6b} = 13.6 Hz, H-6b), 3.17 (bt, 2 H, ³*J*_{H,H} = 6.0 Hz, CH₂NH₂), 1.64 (s, 9 H, CMe₃); ¹³C NMR (100.6 MHz, D₂O, 323 K): δ = 184.7 (CS), 159.4 (CO carbamate), 85.5 (C-1), 82.4 (CMe₃), 77.7 (C-3), 77.0 (C-5), 73.2 (C-2), 72.2 (C-4), 45.8 (CH₂NHCS), 42.5 (C-6), 40.6 (CH₂NH₂), 28.9 (CMe₃); ESI-MS: *m/z* 381 [M + H]⁺; Anal. Calcd for C₁₄H₂₈N₄O₆S·H₂O: C, 42.20; H, 7.59; N, 14.06; Found: C, 41.93; H, 7.24; N, 14.49.

N-(2-Aminoethyl)-*N*-(3,6-di-*tert*-butoxycarbonylamino-3,6-dideoxy-β-D-glucopyranosyl)thiourea (**15**). Compound **12** (69 mg, 95 μmol) was treated with 2% TFA in DCM (3 mL) at rt for 5 h. The organic phase was extracted with water (6 x 10 mL), and the aqueous solution was neutralized (diluted NH₄OH) and freeze-dried to give **15**. Yield: 35 mg (77%); *R*_f = 0.43 (10:1:1 MeCN-H₂O-NH₄OH); [α]_D = +1.3 (*c* 0.8, H₂O); UV (H₂O): 244 nm (ε_{mM} 3.6); IR (KBr): ν_{max} = 3117, 1668, 1202, 1137 cm⁻¹; ¹H NMR (500 MHz, 12:1 CD₃OD-D₂O, 323 K): δ = 5.34 (bs, 1 H, H-1), 3.96 (m, CH₂NHCS), 3.55 (dd, 2 H, *J*_{6a,6b} = 14.0 Hz, *J*_{5,6a} = 2.5 Hz, H-6a), 3.49 (m, 3 H, H-2, H-4,

H-5), 3.29 (t, 1 H, *J*_{2,3} = *J*_{3,4} = 9.3 Hz, H-3), 3.28 (m, 2 H, CH₂NH₂), 3.19 (dd, 1H, *J*_{5,6b} = 7.0 Hz, H-6b), 1.49 (s, 18 H, CMe₃); ¹³C NMR (125.7 MHz, 12:1 CD₃OD-D₂O, 323 K): δ = 186.5 (CS), 159.2, 158.7 (CO carbamate), 86.0 (C-1), 80.9 (2 CMe₃), 78.7 (C-5), 72.3 (C-2), 71.1 (C-4), 61.3 (C-3), 42.9 (C-6), 42.5 (CH₂NHCS), 40.6 (CH₂NH₂), 28.8 (2 CMe₃); ESIMS: *m/z* = 479.8 ([M + H]⁺); Anal. Calcd for C₁₉H₃₇N₅O₇S: C, 47.58; H, 7.78; N, 14.60. Found: C, 47.20; H, 7.45; N, 14.27.

N-(2-Aminoethyl)-*N*-(3-*tert*-butoxycarbonylamino-3-deoxy-β-D-glucopyranosyl)thiourea (**16**). Compound **13** (95 mg, 0.15 mmol) was treated with DCM-TFA (1%, 4 mL) at rt for 4 h. The organic phase was extracted with water (6 x 10 mL), and the aqueous solution was neutralized (NH₄OH aqueous) and freeze-dried to give **16**. Yield: 57 mg (quantitative); *R*_f = 0.88 (10:1:1 MeCN-H₂O-NH₄OH); [α]_D = -108.1 (*c* 1.0, MeOH); UV (MeOH): 248, 212 nm (ε_{mM} 10.8, 9.0); IR (KBr): ν_{max} = 3300, 3078, 2971, 1687, 1074, 1032 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 323 K): δ = 5.23 (bs, 1 H, H-1), 3.92 (m, 2 H, CH₂NHCS), 3.86 (dd, 1 H, *J*_{6a,6b} = 12.0 Hz, *J*_{5,6a} = 2.5 Hz, H-6a), 3.65 (dd, 1H, *J*_{5,6b} = 5.5 Hz, H-6b), 3.46 (t, 1 H, *J*_{2,3} = *J*_{3,4} = 9.5 Hz, H-3), 3.44 (m, 1 H, H-5), 3.38 (dd, 1 H, *J*_{1,2} = 9.5 Hz, H-2), 3.34 (dd, 1 H, *J*_{4,5} = 9.5 Hz, H-4), 3.20 (m, 2 H, CH₂NHTr), 1.45 (s, 9 H, CMe₃); ¹³C NMR (125.7 MHz, CD₃OD, 323 K): δ = 186.7 (CS), 159.3 (CO carbamate), 85.9 (C-1), 80.5 (CMe₃, C-5) 72.7 (C-2), 70.1 (C-4), 63.0 (C-6), 61.8 (C-3), 42.8 (CH₂NHCS), 40.9 (CH₂NH₂), 28.8 (CMe₃); ESIMS: *m/z* = 403 ([M + Na]⁺), 381 (63%, [M + H]⁺); Anal. Calcd for C₁₄H₂₈N₄O₆S: C, 44.20; H, 7.42; N, 14.73. Found: C, 43.88; H, 7.21; N, 14.52.

Heptakis[2,3-di-*O*-hexanoyl-6-[2-[*N*'-2-[*N*'-(6-deoxy-6-*tert*-butoxycarbonylamino-β-D-glucopyranosyl)thioureido]ethyl]-thioureido]ethylthio]cyclomaltoheptaose (**18**). To a solution of **17**³³ (44 mg, 13.6 μmol) in DCM (1 mL) Et₃N (16 μL, 0.11 mmol, 1.1 eq) and **14** (40 mg, 0.105 mmol, 1.1 eq) in DMF (2 mL) were added a solution of and the reaction mixture was stirred at rt for 7 days. The solvent was removed under vacuum and the residue purified by column chromatography (70:10:1 DCM-MeOH-H₂O). Yield: 61 mg (76%); *R*_f = 0.35 (70:10:1 DCM-MeOH-H₂O); [α]_D = +34.0 (*c* 1.0, MeOH); ¹H NMR (500 MHz, DMSO-*d*₆, 343 K): δ = 7.72 (bs, 7 H, NH-1_{Glc}), 7.64 (bs, 7 H, NH), 7.51 (bs, 7 H, NH), 7.39 (bs, 7 H, NH_{Cyst}), 5.27 (t, 7 H, *J*_{2,3} = *J*_{3,4} = 9.0 Hz, H-3), 5.09 (bs, 7 H, H-1), 5.04 (bs, 7 H, H-1_{Glc}), 4.76 (bs, 7 H, NH-6_{Glc}), 4.73 (dd, 7 H, *J*_{1,2} = 4.0 Hz, H-2), 4.15 (m, 7 H, H-5), 3.90 (t, 7 H, *J*_{4,5} = 9.0 Hz, H-4), 3.64 (bs, 42 H, 2 CH₂NHCS, CH₂N_{Cyst}), 3.38 (ddd, 7 H, *J*_{6a,6b} = 14 Hz, *J*_{NH,6a} = 6.0 Hz, *J*_{5,6a} = 3.5 Hz, H-6a_{Glc}), 3.25 (m, 7 H, H-5_{Glc}), 3.24 (t, 7 H, *J*_{2,3} = *J*_{3,4} = 9.0 Hz, H-3_{Glc}), 3.22 (m, 7 H, H-2_{Glc}), 3.09 (m, 14 H, H-6a, H6b), 3.01 (t, 7 H, *J*_{4,5} = 9.0 Hz, H-4_{Glc}), 3.00 (dd, 7 H, *J*_{5,6b} = 6.0 Hz, H-6b_{Glc}), 2.80 (bs, 14 H, CH₂S_{Cyst}), 2.36 (m, 14 H, H-2a_{Hex}), 2.21 (m, 14 H, H-2b_{Hex}), 1.54 (m, 28 H, H-3_{Hex}), 1.39 (s, 63 H, CMe₃), 1.29 (m, 56 H, H-4_{Hex}, H-5_{Hex}), 0.87 (m, 42 H, H-6_{Hex}); ¹³C NMR (125.7 MHz, DMSO-*d*₆, 313 K): δ = 184.2, 183.2 (CS), 172.9, 171.9 (CO ester), 156.3 (CO carbamate), 96.6 (C-1), 83.9 (C-1_{Glc}), 79.1 (CMe₃), 78.4 (C-4), 77.6 (C-3_{Glc}), 77.6 (C-5_{Glc}), 73.1 (C-2_{Glc}), 73.0 (C-6), 72.2 (C-4_{Glc}), 71.6 (C-5), 70.6 (C-2, C-3), 44.1,

43.8, 44.3 (CH₂NHCS), 42.4 (C-6_{Glc}), 33.9, 33.7 (C-2_{Hex}), 33.1 (CH₂N_{Cyst}), 31.3, 31.1 (C-4_{Hex}), 28.7 (CMe₃), 24.3, 24.2 (C-3_{Hex}), 22.2 (C-5_{Hex}), 14.0 (C-6_{Hex}); ESIMS: *m/z* 2979.7 [M + 2 K]²⁺; Anal. Calcd for C₂₄₅H₄₂₇N₃₅O₈₄S₂₁: C, 50.04; H, 7.32; N, 8.34. Found: C, 49.89; H, 7.22; N, 8.18.

Heptakis[2,3-di-*O*-hexanoyl-6-[2-[N⁷-[2-[N⁷-(6-amino-6-deoxy-β-D-glucopyranosyl)thioureido]ethyl]thioureido]ethylthio]cyclomaltoheptaose heptahydrochloride (**2**). Compound **18** (46 mg, 7.8 μmol) was treated with TFA-DCM (1:1, 2 mL) at rt for 2 h. Then solvent was evaporated and acid traces were removed by co-evaporation with water, and the residue was freeze-dried from diluted HCl. Yield: 37 mg (93%); [α]_D = +426.5 (*c* 0.75, MeOH); ¹H NMR (500 MHz, 5:1 CD₃OD-D₂O, 333 K): δ = 5.40 (m, 7 H, H-1_{Glc}), 5.33 (t, 7 H, *J*_{2,3} = *J*_{3,4} = 9.0 Hz, H-3), 5.19 (d, 7 H, *J*_{1,2} = 3.6 Hz, H-1), 4.86 (dd, 7 H, *J*_{1,2} = 4.0 Hz, H-2), 4.20 (m, 7 H, H-5), 3.94 (t, 7 H, *J*_{4,5} = 9.0 Hz, H-4), 3.70 (m, 7 H, H-5_{Glc}), 3.74 (bs, 42 H, 2 CH₂NHCS, CH₂N_{Cyst}), 3.56 (t, 7 H, *J*_{2,3} = *J*_{3,4} = 9.0 Hz, H-3_{Glc}), 3.51 (m, 7 H, H-2_{Glc}), 3.46 (ddd, 7 H, *J*_{6a,6b} = 14 Hz, *J*_{5,6a} = 2.8 Hz, H-6a_{Glc}), 3.31 (m, 7 H, H-4_{Glc}), 3.18 (m, 14 H, H-6a, H6b), 3.09 (dd, 7 H, *J*_{5,6b} = 8.8 Hz, H-6b_{Glc}), 2.97 (bs, 14 H, CH₂S_{Cys}), 2.45-2.28 (m, 28 H, CH₂CO), 1.66 (m, 28 H, CH₂CH₂CO), 1.37 (m, 56 H, CH₂CH₃, CH₂CH₂CH₃), 0.95 (m, 42 H, CH₃); ¹³C NMR (125.7 MHz, DMSO-*d*₆, 323 K): δ = 186.4, 185.7 (CS), 175.6, 174.5 (CO ester), 99.3 (C-1), 86.5 (C-1_{Glc}), 80.1 (C-4), 75.4 (C-3_{Glc}), 74.5 (C-5_{Glc}), 74.6 (C-6, C-2_{Glc}, C-4_{Glc}), 74.2 (C-5), 73.3 (C-2, C-3), 48.9 (C-6_{Glc}), 46.8, 46.5, 46.1 (CH₂NHCS, CH₂N_{Cyst}), 36.5, 36.4, 35.7 (C-2_{Hex}), 33.9, 33.8 (C-4_{Hex}), 32.0 (CH₂S_{Cyst}), 27.0, 26.9 (C-3_{Hex}), 24.9 (C-5_{Hex}), 16.7 (C-6_{Hex}); ESI-MS: *m/z* 1727.1 [M + 3 H]³⁺; Anal. Calcd for C₂₁₀H₃₇₈N₃₅O₇₀S₂₁: C, 46.41; H, 7.01; N, 9.02. Found: C, 46.29; H, 6.88; N, 8.87.

Heptakis[2,3-di-*O*-hexanoyl-6-[2-[N⁷-[2-[N⁷-(3,6-dideoxy-3,6-di-*tert*-butoxycarbonylamino-β-D-glucopyranosyl)thioureido]ethyl]thioureido]ethylthio]cyclomaltoheptaose (**19**). To a solution of **17**³³ (86 mg, 26.7 μmol) in DCM (1 mL) Et₃N (23 μL, 0.17 mmol, 1.1 eq) and **15** (78 mg, 0.206 mmol, 1.1 eq) in DMF (3 mL) were added a solution of and the reaction mixture was stirred at rt for 11 days, then concentrated. The solvent was removed under vacuum and the residue purified by column chromatography 70:10:1 → 70:20:1 DCM-MeOH-H₂O to give **19** as an amorphous solid. Yield: 91 mg (52%); *R*_f = 0.50 (70:10:1 DCM-MeOH-H₂O); [α]_D = +494 (*c* 1.0, MeOH); UV (MeOH): 285, 250 nm (ε_{mM} 50.5, 106.9); IR (KBr): ν_{max} = 3325, 2957, 2930, 2856, 1749, 1698, 150, 1167, 1040 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 333 K): δ = 5.46 (d, 7 H, *J*_{1,2} = 9.5 Hz, H-1_{Glc}), 5.36 (bs, 7 H, H-3), 5.19 (bs, 7 H, H-1), 4.86 (bs, 7 H, H-2), 4.57 (t, 14 H, ³*J*_{H,H} = 7.5 Hz, CH₂NHCS), 4.25 (m, 7 H, H-5), 3.96 (bs, 7 H, H-4), 3.96 (t, 14 H, ³*J*_{H,H} = 7.8 Hz, CH₂N_{Cyst}), 3.58 (dd, 7 H, *J*_{6a,6b} = 14.0 Hz, *J*_{5,6a} = 2.0 Hz, H-6a_{Glc}), 3.56 (m, 28 H, CH₂NHCS, CH₂S_{Cyst}), 3.52 (t, 7 H, *J*_{2,3} = 9.5 Hz, H-2_{Glc}), 3.46 (t, 7 H, *J*_{3,4} = 9.5 Hz, H-3_{Glc}), 3.41 (ddd, 7 H, *J*_{4,5} = 9.5 Hz, *J*_{5,6b} = 7.5 Hz, H-5_{Glc}), 3.29 (t, 7 H, H-4_{Glc}), 3.28 (m, 14 H, H-6a, H6b), 3.17 (dd, 7 H, H-6b_{Glc}), 2.52-2.24 (m, 28 H, H-2_{Hex}), 1.66 (m, 28 H, H-3_{Hex}), 1.47, 1.46 (2 s, 63 H

each, 2 CMe₃), 1.42-1.30 (m, 56 H, H-4_{Hex}, H-5_{Hex}), 0.96 (m, 42 H, H-6_{Hex}); ¹³C NMR (125.7 MHz, CD₃OD, 333 K): δ = 185.5, 181.5 (CS), 175.9, 174.7 (CO ester), 160.4, 159.8 (2 CO carbamate), 99.4 (C-1), 88.5 (C-1_{Glc}), 81.8 (CMe₃), 80.6 (C-4, C-5_{Glc}), 74.2 (C-5), 73.9 (C-2_{Glc}), 73.0 (C-6, C-2, C-3) 72.7 (C-4_{Glc}), 63.3 (C-3_{Glc}), 54.6 (CH₂NHCS), 53.9 (CH₂N_{Cyst}), 44.5 (C-6_{Glc}), 42.4 (CH₂NHCS), 36.4, 36.3 (C-2_{Hex}), 35.2 (CH₂S_{Cyst}), 33.8, 33.7 (C-4_{Hex}), 30.0 (CMe₃), 26.8 (C-3_{Hex}), 24.6 (C-5_{Hex}), 15.6, 15.5 (C-6_{Hex}); Anal. Calcd for C₂₈₀H₄₉₀N₄₂O₉₈S₂₁: C, 51.15; H, 7.51; N, 8.95. Found: C, 50.87; H, 7.29; N, 8.71.

Heptakis[2,3-di-*O*-hexanoyl-6-[2-[N⁷-[2-[N⁷-(3,6-diamino-3,6-dideoxy-β-D-glucopyranosyl)thioureido]ethyl]thioureido]ethylthio]cyclomaltoheptaose tetradecahydrochloride (**3**). Compound **19** (81 mg, 13.8 μmol) was treated with TFA-DCM (1:1, 2 mL) at rt for 4 h. Then solvent was evaporated and acid traces removed by co-evaporation with water, and the residue was solved in 10 mM HCl and freeze-dried to yield the unprotected compound **3**. Yield: 78 mg (quantitative); [α]_D = +470.6 (*c* 1.0, MeOH); UV (MeOH): 247, 270 nm (ε_{mM} 62.8, 35.5); IR (KBr): ν_{max} = 2959, 1789, 1747, 1676, 1286, 1039 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 333 K): δ = 5.46 (d, 7 H, *J*_{1,2} = 8.3 Hz H-1_{Glc}), 5.35 (bs, 7 H, H-3), 5.18 (bs, 7 H, H-1), 4.84 (bs, 7 H, H-2), 4.56 (m, 14 H, CH₂NHCS), 4.23 (bs, 7 H, H-5), 4.00 (t, 14 H, ³*J*_{H,H} = 7.3 Hz, CH₂N_{Cyst}), 3.77 (dd, 7 H, *J*_{2,3} = 9.8 Hz, H-2_{Glc}), 3.76 (m, 14 H, H-5_{Glc}), 3.61 (t, 7 H, *J*_{2,3} = *J*_{3,4} = 9.1 Hz, H-3_{Glc}), 3.58 (t, 28 H, ³*J*_{H,H} = 7.3 Hz, CH₂NHCS, CH₂S_{Cys}), 3.47 (dd, 7 H, *J*_{6a,6b} = 13.6 Hz, *J*_{5,6a} = 3.1 Hz, H-6a_{Glc}), 3.25 (m, 14 H, H-6a, H6b), 3.17 (t, 7 H, *J*_{4,5} = 9.1 Hz, H-4_{Glc}), 3.11 (dd, 7 H, *J*_{5,6b} = 8.8 Hz, H-6b_{Glc}), 2.43-2.29 (m, 28 H, H-2_{Hex}), 1.64 (m, 28 H, H-3_{Hex}), 1.36 (m, 56 H, H-5_{Hex}, H-4_{Hex}), 0.94 (m, 42 H, H-6_{Hex}); ¹³C NMR (125.7 MHz, CD₃OD, 323 K): δ = 185.9 (CS), 176.0, 174.8 (CO ester), 99.4 (C-1), 88.3 (C-1_{Glc}), 86.1 (C-4), 81.6 (C-5_{Glc}), 77.5 (C-5), 76.0-68.7 (C-6, C-4_{Glc}, C-2, C-3), 63.1 (C-2_{Glc}), 61.9 (C-3_{Glc}), 54.7 (CH₂N_{Cyst}), 49.4 (C-6_{Glc}), 45.4, 43.2, 42.5 (CH₂NHCS), 36.5, 36.4 (C-2_{Hex}), 33.7 (C-4_{Hex}), 31.9 (CH₂S_{Cyst}), 26.8 (C-3_{Hex}), 24.7 (C-5_{Hex}), 15.5 (C-6_{Hex}); Anal. Calcd for C₂₁₀H₃₉₂N₄₂O₇₀S₂₁: C, 44.38; H, 6.95; N, 10.35. Found: C, 44.01; H, 6.62; N, 9.97; S, 11.49.

Heptakis[2,3-di-*O*-hexanoyl-6-[2-[N⁷-[2-[N⁷-(3-deoxy-3-*tert*-butoxycarbonylamino-β-D-glucopyranosyl)thioureido]ethyl]thioureido]ethylthio]cyclomaltoheptaose (**20**). To a solution of **17**³³ (32 mg, 9.9 μmol) in DMF (1.3 mL) a solution of Et₃N (9.5 μL, 69 μmol, 1 eq) and **16** (29 mg, 76 μmol, 1.1 eq) in DMF (1.5 mL) were added and the reaction mixture was stirred at rt for 7 days, then concentrated. The solvent was removed under vacuum and the residue purified by column chromatography 70:20:1 → 70:10:1 DCM-MeOH-H₂O, to give **20**. Yield: 37 mg (64%); *R*_f = 0.53 (70:20:1 DCM-MeOH-H₂O); [α]_D = +59.0 (*c* 1.0, DCM); UV (DCM): 247 nm (ε_{mM} 206.1); IR (NaCl): ν_{max} = 3319, 2957, 1750, 1693, 1247, 1165, 1038 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 323 K): δ = 5.31 (bt, 7 H, H-3), 5.26 (bs, 7 H, H-1_{Glc}), 5.17 (d, 7 H, *J*_{1,2} = 3.0 Hz, H-1), 4.84 (m, 7 H, H-2), 4.18 (m, 7 H, H-5), 3.90 (t, 7 H, *J*_{4,5} = 8.5 Hz, H-4), 3.89 (bd, 7 H, *J*_{6a,6b} = 12.5 Hz, H-6a_{Glc}), 3.76 (bs, 42 H, 2 CH₂NHCS, CH₂N_{Cyst}), 3.73 (m, 7 H, H-6b_{Glc}), 3.53-3.40

(m, 28 H, H-5_{Glc}, H-3_{Glc}, H-2_{Glc}, H-4_{Glc}), 3.28 (m, 7H, H-6a), 3.17 (m, 7 H, H6b), 2.93 (bs, 14 H, CH₂S_{Cys}), 2.42 (m, 14 H, H-2_{Hex}), 2.33 (m, 7 H, H-2a_{Hex}), 2.25 (m, 7 H, H-2b_{Hex}), 1.63 (m, 28 H, H-3_{Hex}), 1.45 (s, 63 H, CMe₃), 1.32 (m, 56 H, H-5_{Hex}, H-3_{Hex}), 0.89 (m, 42 H, H-6_{Hex}); ¹³C NMR (125.7 MHz, CD₃OD, 323 K): δ = 183.9, 182.7 (CS), 173.4, 172.1 (CO ester), 157.8 (CO carbamate), 96.8 (C-1), 84.6 (C-1_{Glc}), 79.5 (CMe₃), 79.3 (C-4^{*}_{Glc}), 79.0 (C-4, C-5^{*}_{Glc}), 71.9 (C-5), 71.1 (C-3), 70.6 (C-2), 68.5 (C-2_{Glc}), 61.3 (C-6_{Glc}), 60.5 (C-3_{Glc}), 45.0, 44.0 (CH₂NHCS, CH₂N_{Cyst}), 33.8, 33.7 (C-6, C-2_{Hex}), 32.7 (CH₂S_{Cyst}), 31.2, 31.1 (C-4_{Hex}), 27.6 (C-3_{Hex}), 24.2 (CMe₃), 22.1 (C-5_{Hex}), 13.1 (C-6_{Hex}). Anal. Calcd for C₂₄₅H₄₂₇N₃₅O₈₄S₂₁: C, 50.04; H, 7.32; N, 8.34, S, 11.45. Found: C, 49.78; H, 6.98; N, 8.01; S, 11.03.

Heptakis[2,3-di-O-hexanoyl-6-[2-[N^o-[2-[N^o-(3-amino-3-deoxy-β-D-glucopyranosyl)thioureido]ethyl]thioureido]ethylthio]]cyclomaltoheptaose heptahydrochloride (**4**). Compound **20** (36 mg, 5.5 μmol) was treated with TFA-DCM (1:1, 2 mL) at rt for 4 h. Then solvent was evaporated and acid traces removed by co-evaporation with water, and the residue was solved in 10 mM HCl and freeze-dried to yield the unprotected compound **4**. Yield: 30 mg (quantitative); [α]_D = +27.8 (c 1.0, MeOH); UV (MeOH): 339 nm (ε_{mM} 1.1); IR (KBr): ν_{max} = 3288, 2959, 1760, 1667, 1035 cm⁻¹; ¹H NMR (500 MHz, CD₃OD, 333 K): δ = 5.44 (m, 7 H, H-1_{Glc}), 5.31 (bs, 7 H, H-3), 5.16 (bs, 7 H, H-1), 4.83 (bs, 7 H, H-2), 4.18 (m, 7 H, H-5), 3.90 (m, 7 H, H-4), 3.88 (bd, 7 H, J_{6a,6b} = 11.5 Hz, H-6a_{Glc}), 3.77 (bs, 42 H, 2 CH₂NHCS, CH₂N_{Cyst}), 3.72-3.59 (m, 21 H, H-2_{Glc}, H-4_{Glc}, H-6b_{Glc}), 3.53 (m, 7 H, H-5_{Glc}), 3.27 (m, 7 H, H-6a), 3.17 (m, 14 H, H-6b, H-3_{Glc}), 2.96 (bs, 14 H, CH₂S_{Cys}), 2.42-2.24 (m, 28 H, H-2_{Hex}), 1.63 (m, 28 H, H-3_{Hex}), 1.37-1.28 (m, 56 H, H-5_{Hex}, H-4_{Hex}), 0.91 (m, 42 H, H-6_{Hex}); ¹³C NMR (125.7 MHz, CD₃OD, 333 K): δ = 185.6, 183.4 (CS), 174.7, 173.4 (CO ester), 98.2 (C-1), 85.6 (C-1_{Glc}), 80.0 (C-5_{Glc}, C-4), 73.2 (C-5), 71.9 (C-3), 71.6 (C-2), 70.3 (C-4_{Glc}), 67.6 (C-2_{Glc}), 61.9 (C-6_{Glc}), 61.0 (C-3_{Glc}), 45.4, 45.0, 44.3 (CH₂NHCS, CH₂N_{Cyst}), 35.1, 35.0 (C-6, C-2_{Hex}), 34.0 (CH₂S_{Cyst}), 32.5, 32.4 (C-4_{Hex}), 25.5 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.4, 14.3 (C-6_{Hex}). Anal. Calcd for C₂₁₀H₃₇₈N₃₅O₇₀S₂₁: C, 46.41; H, 7.01; N, 9.02; S, 12.39. Found: C, 46.07; H, 6.73; N, 8.76; S, 12.10.

Preparation of nanocomplexes composed of pGaCD 2-4 and pDNA (pTG11236)

The plasmid pTG11236 (pCMV-SV40-luciferase-SV40pA), used for the preparation of the pDNA complexes and for transfection assay is a plasmid of 5739 bp (base pairs). The quantities of compound used were calculated according to the desired pDNA concentration of 0.1 mg·mL⁻¹ (303 μM phosphate), the N/P ratio, the molar weight and the number of protonable nitrogens in the selected CD derivative or Jet-PEI.^{47,48} Experiments were performed for N/P 5 and 10. Concerning the preparation of the DNA complexes from CD derivatives and Jet-PEI, pDNA was diluted in HEPES (20 mM, pH 7.4) to a final concentration of 303 μM, and then the desired amount of CD derivative was added from 10 or 20 mM stock solution (DMSO). For Jet-PEI polyplexes, pDNA was diluted in a 150 mM NaCl solution to a final phosphate concentration of 303 μM, and then the desired amount of Jet-PEI was added from a 7.5 mM water solution. The preparation was vortexed for 2 h and used for characterization or transfection experiments.

Agarose gel electrophoresis

Each CD derivative/pDNA formulation (20 μL, 0.4 μg of plasmid) was submitted to electrophoresis for about 30 min under 150 V through a 0.8% agarose gel in TAE 1X (Tris-acetate-EDTA) buffer and stained by spreading a solution of ethidium bromide (EtBr, Sigma) in TAE buffer (20 μL EtBr of a 10 mg·mL⁻¹ solution in 200 mL TAE). The DNA was then visualized after photographing on an UV transilluminator. The plasmid integrity in each sample was confirmed by electrophoresis after decomplexation with sodium dodecyl sulfate (SDS, 8%).

Measurement of CDplex size and of the ζ-potential by dynamic light scattering (DLS)

The average size of the glycoCDplexes were measured using a Zetasizer Nano (Malvern Instruments, Paris, France) with the following specification: sampling time, automatic; number of measurements, 3 per sample; medium viscosity, 1.054 cP; refractive index, 1.33; scattering angle, 173°; λ = 633 nm; temperature, 25 °C. Data were analyzed using the multimodal number distribution software included in the instrument. Results are given as volume distribution of the major population by the mean diameter with its standard deviation. Zeta potentials measurements on the glycoCDplexes were made with the same apparatus using "Mixed Mode Measurement" phase analysis light scattering (M3-PALS).

M3 consists of both slow field reversal and fast field reversal measurements, hence the name 'Mixed Mode Measurement' that improves accuracy and resolution. The following specifications were applied: sampling time, automatic; number of measurements, 3 per sample; medium viscosity, 1.054 cP; medium dielectric constant, 80; temperature, 25 °C.

Before each series of experiments, the performance of the instruments was checked with either a 90 nm monodisperse

* Assignment of signal can be exchanged.

latex beads (Coulter) for DLS or with DTS 50 standard solution (Malvern) for zeta potentials.

Transmission Electron Microscopy (TEM)

Formvar-carbon coated grids previously made hydrophilic by glow discharge were placed on top of small drops of the CDplex samples (HEPES 20 mM, pH 7.4, pDNA 303 μM phosphate) prepared as described above. After 1-3 min of contact, grids were negatively stained with a few drops of 1% aqueous solution of uranyl acetate. The grids were then dried and observed with a Philips CM12 electron microscope working under standard conditions. All these experiments were reproduced twice on each formulation.

Cell-based assays

Twenty-four hours before transfection, COS-7 cells were grown at a density of 2×10^4 cells/well in a 96-well plates in Dulbecco modified Eagle culture medium (DMEM; Gibco-BRL) containing 10% foetal calf serum (FCS; Sigma) and 100 units/mg penicillin and $100 \mu\text{g} \cdot \text{mL}^{-1}$ streptomycin in a wet (37 °C) and 5% $\text{CO}_2/95\%$ air atmosphere. The above described CDplexes and Jet-PEI:pDNA polyplexes were diluted to 100 μL in DMEM or in DMEM supplemented with 10% FCS in order to have 0.5 μg of pDNA in the well (15 μM phosphate). The culture medium was removed and replaced by these 100 μL of complexes. After 4 h and 24 h, 50 and 100 μL of DMEM supplemented with 30% and 10% FCS, respectively, were added. After 48 h, the transfection was stopped, the culture medium was discarded, and the cells washed twice with 100 μL of PBS and lysed with 50 μL of lysis buffer (Promega, Charbonnières, France). The lysates were frozen at -32 °C, before the analysis of luciferase activity. This measurement was performed in a luminometer (GENIOS PRO, Tecan France S.A) in dynamic mode, for 10 s on 20 μL on the lysis mixture and using the “luciferase” determination system (Promega) in 96-well plates. The total protein concentration per well was determined by the BCA test (Pierce, Montluçon, France). Luciferase activity was calculated as femtograms (fg) of luciferase per mg of protein. The percentage of cell viability was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells $\times 100$. The data were calculated from three or four repetitions in two fully independent experiments (formulation and transfection).

Statistical analysis

Statistical tests were performed with STATGRAPHICS Plus 5.0 software. Analysis of variance (Anova) was run on the logarithmic transformation of transfection levels (Log_{10} [fg luciferase/mg protein]) and on the cell viability to fit normal distributions of the data. Two factors, i.e. nature of the complexing agent (CD derivative and Jet-PEI) and N/P ratio, were analyzed as source of the variation of logarithmic transformation of the transfection levels and of cell variability percentages using a multiple comparison procedure. The Tukey's honestly significant difference (HSD) method was

used to discriminate among the means of cell viability percentages and the logarithmic transformation of luciferase expression levels.

Acknowledgements

This work was supported by the Spanish Ministerio de Economía y Competitividad (MinECO; contract numbers CTQ2010-15848 and SAF2010-15670), the Junta de Andalucía, the CSIC, the CNRS, and FUSINT (CNRS project).

Notes and references

^a Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, C/Prof. García González 1, E-41012 Sevilla, Spain.

^b LCMB UMR 6001, Université de Nice Sophia Antipolis – CNRS, 28, Avenue de Valrose, F-06100 Nice, France.

^c Instituto de Investigaciones Químicas, CSIC – Universidad de Sevilla, Américo Vespucio 49, E-41092 Sevilla, Spain.

† Electronic Supplementary Information (ESI) available: NMR spectra of compounds 8-20 and 2-4. See DOI: 10.1039/b000000x//

- 1 J. Grisham, *Nat. Biotechnol.*, 2000, **18**, 254.
- 2 S. Nayak and R. W. Herzog, *Gene Ther.*, 2010, **17**, 295.
- 3 S. Ylä-Herttuala, *Mol. Ther.*, 2012, **20**, 1831.
- 4 T. Wirth, N. Parker and S. Ylä-Herttuala, *Gene*, 2013, **525**, 162-169.
- 5 M. A. Mintzer and E. E. Simanek, *Chem. Rev.*, 2009, **109**, 259.
- 6 D. Glover, H. Lipps and D. Jans, *Nat. Rev. Genet.*, 2005, **6**, 299.
- 7 K. Miyata, N. Nishiyama and K. Kataoka, *Chem. Soc. Rev.*, 2012, **41**, 2562.
- 8 E. Wagner, *Pharm. Res.*, 2004, **21**, 8.
- 9 T. Niidome and L. Huang, *Gene Ther.*, 2002, **9**, 1647.
- 10 H. Akita and H. Harashima, *Expert Opin. Drug Deliv.*, 2008, **5**, 847.
- 11 B. Demeneix, Z. Hassani and J. Behr, *Curr. Gene Ther.*, 2004, **4**, 445.
- 12 M. E. Davis, *Mol. Pharm.*, 2009, **6**, 659.
- 13 M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. A. Alabi, Y. Yen, J. D. Heidel and A. Ribas, *Nature*, 2010, **464**, 1067.
- 14 D. Zhi, S. Zhang, S. Cui, Y. Zhao, Y. Wang, D. Zhao, *Bioconjugate Chem.*, 2013, **24**, 487.
- 15 J. Sunshine, J. J. Green, K. P. Mahon, F. Yang, A. A. Eltoukhy, D. N. Nguyen, R. Langer and D. G. Anderson, *Adv. Mater.*, 2009, **21**, 4947.
- 16 D. F. Zhi, S. B. Zhang, B. Wang, Y. N. Zhao, B. L. Yang and S. J. Yu, *Bioconjugate Chem.*, 2010, **21**, 563.
- 17 C. Ortiz Mellet, J. M. Benito and J. M. García Fernández, *Chem. Eur. J.*, 2010, **16**, 6728.
- 18 V. Bagnacani, V. Franceschi, M. Basi, M. Lomazzi, G. Donofrio, F. Sansone, A. Casnati and R. Ungaro, *Nat. Commun.*, 2013, **4**, 1721, and references therein.
- 19 D. Sigwalt, M. Holler, J. Iehl, J.-F. Nierengarten, M. Nothisen, E. Morin and J.-S. Remy, *Chem. Commun.*, 2011, **47**, 4640.
- 20 I. Nierengarten, M. Nothisen, D. Sigwalt, T. Biellmann, M. Holler, J.-S. Remy and J.-F. Nierengarten, *Chem. Eur. J.*, 2013, **19**, 17552.
- 21 L. Gallego-Yerga, M. J. González-Álvarez, N. Mayordomo, F. Santoyo-González, J. M. Benito, C. Ortiz Mellet, F. Mendicuti and J. M. García Fernández, *Chem. Eur. J.*, 2014, **20**, in press, DOI 10.1002/chem.201402026, and references therein.

- 24 S. Srinivasachari, K. M. Fichter and T. M. Reineke, *J. Am. Chem. Soc.*, 2008, **130**, 4618.
- 23 B. M. D. C. Godinho D. J. McCarthy, C. Torres-Fuentes, C. J. Beltrán, J. McCarthy, A. Quinlan, J. R. Ogier, R. Darcy, C. M. O'Driscoll and J. F. Cryan, *Biomaterials*, **35**, 489, and references therein.
- 24 C. Ortiz Mellet, J. M. García Fernández and J. M. Benito, *Chem. Soc. Rev.*, 2011, **40**, 1586.
- 25 J. M. García Fernández, J. M. Benito and C. Ortiz Mellet, *Pure Appl. Chem.*, 2013, **85**, 1825.
- 26 C. Aranda, K. Urbiola, A. Méndez Ardoy, J. M. García Fernández, C. Ortiz Mellet and C. Tros de Ilarduya, *Eur. J. Pharm.*, 2013, **85**, Pages 390.
- 27 A. Díaz-Moscoso, N. Guilloteau, C. Bienvenu, A. Méndez-Ardoy, J. L. Jiménez Blanco, J. M. Benito, L. Le Gourrierec, C. Di Giorgio, P. Vierling, J. Defaye, C. Ortiz Mellet and J. M. García Fernández, *Biomaterials*, 2011, **32**, 7263.
- 28 N. Symens, A. Méndez-Ardoy, A. Díaz-Moscoso, E. Sánchez-Fernández, K. Remaut, J. Demeester, J. M. García Fernández, S. C. De Smedt and J. Rejman, *Bioconjugate Chem.*, 2012, **23**, 1276.
- 29 L. Desigaux, M. Sainlos, O. Lambert, R. Chevre, E. Letrou-Bonneval, J. Vigneron, P. Lehn, J. Lehn and B. Pitard, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 16534.
- 30 A. Ghilardi, D. Pezzoli, M. C. Bellucci, C. Malloggi, A. Negri, A. Sganappa, G. Tedeschi, G. Candiani and A. Volonterio, *Bioconjugate Chem.*, 2013, **24**, 1928.
- 31 Y. Zhang, J. M. Pelet, D. A. Heller, Y. Dong, D. Chen, Z. Gu, B. J. Joseph, J. Wallas and D. G. Anderson, *Adv. Mater.*, 2013, **25**, 4641.
- 32 C. Ortiz Mellet, J. M. García Fernández and J. M. Benito, *Carbohydr. Chem.*, 2012, **38**, 338-375.
- 33 A. Díaz-Moscoso, L. Le Gourrierec, M. Gomez-García, J. M. Benito, P. Balbuena, F. Ortega-Caballero, N. Guilloteau, C. Di Giorgio, P. Vierling, J. Defaye, C. Ortiz Mellet and J. M. García Fernández, *Chem. Eur. J.*, 2009, **15**, 12871.
- 34 A. Méndez-Ardoy, N. Guilloteau, C. Di Giorgio, P. Vierling, F. Santoyo-González, C. Ortiz Mellet and J. M. García Fernández, *J. Org. Chem.*, 2011, **76**, 5882.
- 35 A. Méndez-Ardoy, M. Gómez-García, C. Ortiz-Mellet, N. Sevillano, M. D. Girón, R. Salto, F. Santoyo-González and J. M. García Fernández, *Org. Biomol. Chem.*, 2009, **7**, 2681.
- 36 Á. Martínez, C. Bienvenu, J. L. Jiménez Blanco, P. Vierling, C. Ortiz Mellet, J. M. García Fernández and C. Di Giorgio, *J. Org. Chem.*, 2013, **78**, 8143.
- 37 J. L. Jiménez Blanco, P. Bootello, J. M. Benito, C. Ortiz Mellet and J. M. García Fernández, *J. Org. Chem.*, 2006, **71**, 5136.
- 38 J. L. Jiménez Blanco, F. Ortega-Caballero, C. Ortiz Mellet and J. M. García Fernández, *Beilstein J. Org. Chem.*, 2010, **6**, 20.
- 39 Á. Martínez, C. Ortiz Mellet and J. M. García Fernández, *Chem. Soc. Rev.*, 2013, **42**, 4746.
- 40 J. L. Jiménez Blanco, P. Bootello, R. Gutiérrez Gallego, C. Ortiz Mellet and J. M. García Fernández, *Synthesis*, 2007, 2545.
- 41 J. Loccuffer, J. Crommen, J. Vandorpe and E. Schacht, *Makromol. Chem. Rapid Commun.*, 1991, **12**, 159.
- 42 N/P ratios were estimated considering all potentially ionizable groups on pGaCD and pDNA, respectively. For a revealing discussion of considering the effective charges when formulating transfectious NPs, see: M. Muñoz-Úbeda, S. K. Misra, A. L. Barrán-Berdón, C. Aicart-Ramos, M. B. Sierra, J. Biswas, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *J. Am. Chem. Soc.*, 2011, **133**, 18014.
- 43 A. Díaz-Moscoso, D. Vercauteren, J. Rejman, J. M. Benito, C. Ortiz Mellet, S. C. De Smedt and J. M. García Fernández, *J. Control. Release*, 2010, **143**, 318.
- 44 A. Díaz-Moscoso, P. Balbuena, M. Gomez-García, C. Ortiz Mellet, J. M. Benito, L. Gourrierec, C. Di Giorgio, P. Vierling, A. Mazzaglia, N. Micali, J. Defaye, and J. M. García Fernández, *Chem. Eur. J.*, 2008, 2001.
- 45 S. Srinivasachari, K. M. Fichter and T. M. Reineke, *J. Am. Chem. Soc.*, 2008, **130**, 4618.
- 46 A. Méndez-Ardoy, K. Urbiola, C. Aranda, C. Ortiz Mellet, J. M. García Fernández and C. Tros de Ilarduya, *Nanomedicine*, 2011, **6**, 1697.
- 47 M. Louis, S. Dutoit, Y. Denoux, P. Erbacher, E. Deslandes, J.-P. Behr, P. Gauduchon and L. Poulain, *Cancer Gene Ther.*, 2006, **13**, 367.
- 48 O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J.-P. Behr, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 7297.