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Cyclodextrin-based multivalent glycodisplays: covalent and supramolecular conjugates to assess carbohydrate–protein interactions†

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Covalent attachment of biorecognizable sugar ligands in several copies at precise positions of cyclomaltooligosaccharide (cyclodextrin, CD) macrocycles has proven to be an extremely flexible strategy to build multivalent conjugates. The commercial availability of the native CDs in three different sizes, their axial symmetry and the possibility of position- and face-selective functionalization allow a strict control of the valency and spatial orientation of the recognition motifs (glycotopes) in low, medium, high and hyperbranched glycoclusters, including glycodendrimer–CD hybrids. ''Click-type'' ligation chemistries, including copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC), thiol–ene coupling or thiourea-forming reactions, have been implemented to warrant full homogeneity of the adducts. The incorporation of different glycotopes to investigate multivalent interactions in heterogeneous environments has also been accomplished. Not surprisingly, multivalent CD conjugates have been, and continue to be, major actors in studies directed at deciphering the structural features ruling carbohydrate recognition events. Nanometric glycoassemblies endowed with the capability of adapting the inter-saccharide distances and orientations in the presence of a receptor partner or capable of mimicking the fluidity of biological membranes have been conceived by multitopic inclusion complex formation, rotaxanation or self-assembling. Applications in the fields of sensors, site-specific drug and gene delivery or protein stabilization attest for the maturity of the field.

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1. Introduction

The ability of carbohydrate ligands to participate in biological recognition processes is strongly related to their density and presentation mode. Many synthetic polyconjugates with various copies of the individual recognition motif onto molecular, dendritic, polymeric, self-assembled or nanometric scaffolds have been developed aiming at mimicking and matching the arrangement of their complementary protein receptors (lectins) in the natural mode of affinity enhancement.[1–6] A consistent body of results has been accumulated over the last twenty years, amply demonstrating that ligand multivalency increases protein-binding avidities dramatically. The geometrical characteristics of the multivalent assembly also exert an important influence that is dependent on the glycotope, the lectin partner and the respective densities.[7] Adjusting the architectural parameters, including ligand placing, orientation and active positioning is critical to maximize the activities of multivalent glycoconstructs. Well-defined molecular platforms allowing a precise control of these features have contributed decisively to unravel the mechanisms at work, leading eventually to biotechnological[8,9] or therapeutic applications.[10–12] Among them, the multivalent cyclooligosaccharides of the cyclodextrin family (CDs) occupy a prominent position, their development being historically related to progress and maturation of the multivalency concept.[13–16] The close relationship between molecular features and supramolecular status of these compounds makes them particularly well-suited not only to optimize carbohydrate–protein interactions, but also to further programming the system to perform recognition-dependent specific tasks.

CDs are naturally occurring cyclic oligosaccharides derived from starch composed of six, seven or eight α(1–4)-linked

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† Part of the multivalent scaffolds in glycosciences themed issue.

glucopyranose units (α, β and γCD, respectively). They feature a toroidal truncated-cone topology in which the glucose elements, in the $^4C_1$ chair conformation, orient the hydroxyls to the outer space flanking the upper (OH-6) and lower rims (OH-2 and OH-3), keeping an axial symmetric arrangement. Differences in the steric accessibility and acidity of the three types of hydroxyls in the molecule have been taken into account to conceive efficient position-selective and face-selective chemical functionalization methodologies.[17–27] A variety of multivalent conjugates with diverse architectures becomes then accessible through appropriate ligation chemistries (Fig. 1).

Álvaro Martínez

Alvaro Martinez received his BSc (First class honours) degree in Chemistry from the University of Seville (Spain) in 2011. He was granted an international scholarship by Caja Madrid Foundation to pursue an MSc by research at the University of York in 2012 and is currently enrolled in the Master of Crystallography and Crystallization imparted by the International University Menéndez Pelayo (UIMP) and the Spanish National Research Council (CSIC), granted with a national scholarship from La Caixa Foundation. His doctoral project focuses on the synthesis and evaluation of cyclodextrin-based platforms for glycotargeted gene delivery.

The inner nanometric cavity of the cyclooligosaccharide core, comparatively hydrophobic, remains essentially unaltered after conjugation, preserving the distinct inclusion capabilities of CDs already profusely exploited by the pharmaceutical industry for drug encapsulation purposes.[28–33] Multivalent CDs can be thus considered as hybrid ligand–host molecules bearing two orthogonal recognition domains, namely the glycoligand display and the hydrophobic cavity. The potential of such unique arrangement for receptor-mediated site-specific delivery of active guests or probes was soon recognized and has been a permanent motivation for the design and optimization of multivalent CD conjugates.[34] Interestingly, this ligand–host duality can also be exploited to build guest-mediated supramolecular assemblies, *e.g.* through inclusion complex formation or rotaxanation (Fig. 2A and B), thereby altering the formal valency and the dynamic rearrangement of the biological association. The spontaneous organization of CD conjugates into well-defined structures held together by non-covalent interactions can also be promoted by judicious installation of additional functional elements in the structure *via* covalent or supramolecular approaches, which typically seek at imparting amphiphilicity, thereby broadening the range of available CD-based multivalent systems and the potential for more sophisticated applications (Fig. 2C).[35,36]

This review outlines the potential of CDs to build multivalent systems through molecular diversity- and purpose-oriented strategies. The contribution of CD conjugates to improve our knowledge on the mechanisms involved in multivalent interactions and the way this information can be optimized and directly applied in the design of molecular and

Carmen Ortiz Mellet

Carmen Ortiz Mellet received her PhD degree in Chemistry from the University of Seville (Spain) in 1984, where she was appointed as Tenure Professor of Organic Chemistry in 1987. In 1990 and 1995 she joined the group of Professor Jacques Defaye (Centre d'Etudes de Grenoble, France) to work on the synthesis of complex thiooligosaccharides and pursue synthetic and supramolecular studies on cyclodextrins. Since 1998 she has been responsible for the Carbohydrate Bioorganic Chemistry Group at the University of Seville, being promoted to Full Professor in 2008. Her ongoing projects include the implementation of the concept of multivalency to glycosidase inhibition, the development of pharmacological chaperones for the treatment of lysosomal storage disorders and the design of self-assembled glycomaterials with dual nucleic acid and lectin recognition abilities for site-specific gene delivery. The laboratory also develops a research line on prebiotic oligosaccharides.

José M. García Fernández

Jose Manuel García Fernández received his Doctor of Chemistry degree from the university of Seville (Spain) in 1988. Between 1990 and 1995 he pursued postdoctoral research at the Centre d'Etudes de Grenoble, entering the field of cyclodextrins under the guidance of Dr Jacques Defaye. In 1996 he joined the Spanish National Research Council (CSIC) at the Institute for Chemical Research (CSIC – University of Seville), where he currently serves as a Research Professor and the Director. He authored above 150 scientific articles in peer-reviewed journals, review articles and book chapters and is a co-inventor of 16 patents. Current targets of the laboratory include the implementation of carbohydrate–protein and carbohydrate–nucleic acid interactions in the design of glycocarriers and glycodrugs for applications in nanomedicine, gene delivery, anticancer therapy, the treatment of lysosomal storage disorders and the management of inflammatory bowel diseases.

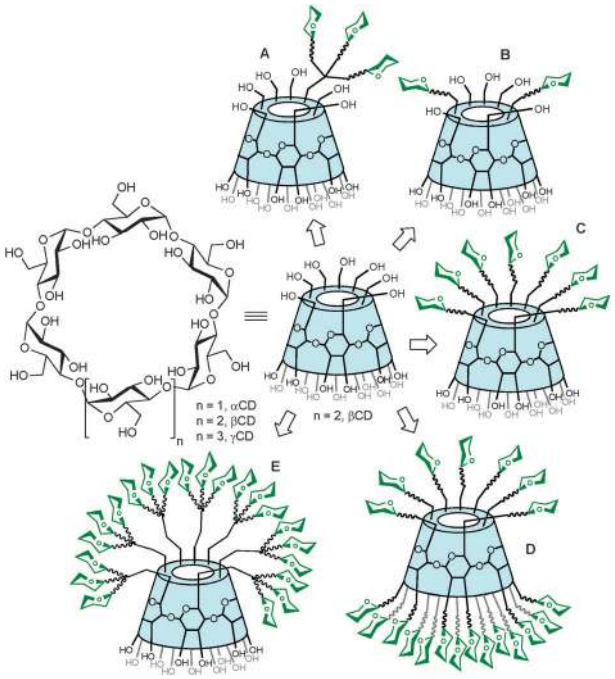


Fig. 1 Structure of the CDs and examples of multivalent conjugate architectures (the βCD platform is represented): (A) CD–glycodendrimer conjugate; (B) position-selective CD-bis conjugate; (C) face-selective glycocluster; (D) dual-face glycocluster; (E) hyperbranched glycocluster.

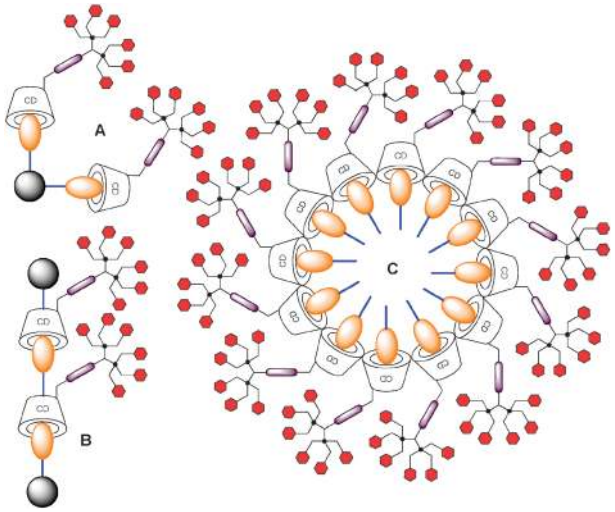


Fig. 2 Examples of self-assembled multivalent architectures from CD–glycoligand (in red) conjugates promoted by either a guest partner or a functional moiety (in orange): (A) multitopic inclusion complex; (B) polyrotaxane; (C) micelle, vesicle or nanocomplex.

macromolecular devices will be highlighted. For the sake of congruity, the classical "static" glycocluster-type systems are revisited and updated, but the accent will be placed in the opportunity of combining multivalency, host–guest and self-organization properties to conceive multifunctional "dynamic" CD nanometric glycoassemblies.

2. Position-selective multivalent cyclodextrin conjugates

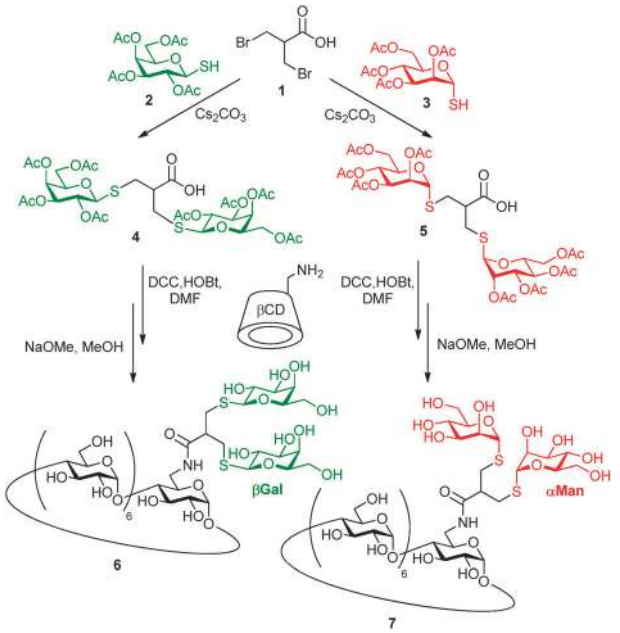
Selective mono-(*O*-6)-*p*-toluenesulfonylation (tosylation) of commercial CDs by reaction with *p*-toluenesulfonyl chloride or other *p*-toluenesulfonyl-transferring reagents under basic catalysis is a classical methodology to access single-position functionalized derivatives.[37–42] Tosylation at O-6^{I} is favored on steric grounds and, in the particular case of βCD when using aqueous media, by inclusion of the sulfonylating reagent in the cavity. This pathway competes, however, with tosylation at the more acidic O-2 position, which strongly complicates the purification step and represents a serious handicap for the final yield. This drawback has been largely mitigated by pre-formation of a sandwich-type complex with Cu^{2+} under strict pH control.[25] The 6^{I}-*O*-tosyl functionality can be replaced by other functional groups through classical nucleophilic displacement reactions, allowing the implementation of different strategies for the construction of CD–glycodendron or –glycodendrimer conjugates (see Fig. 1A for a schematic representation). Simultaneous regioselective differentiation of O-6^{I} and O-6^{IV} positions in βCD has also become accessible after di-(isobutyl)-aluminium (DIBAL)-promoted di-debenzylation of the fully benzylated derivative,[20] which provides an excellent scaffold for the construction of di-branched prototypes with a controlled separation between the coating sugar ligands (se Fig. 1B for a schematic representation).

2.1. CD-glycodendrimer hybrids

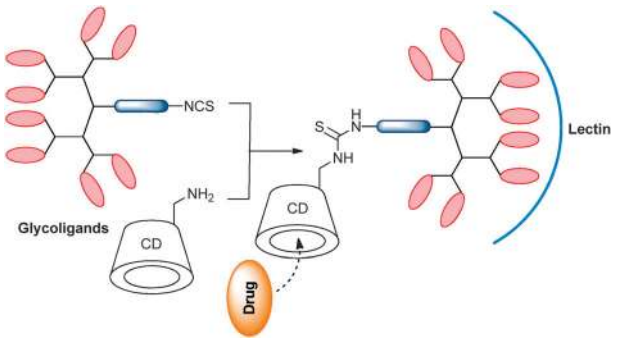
In a seminal work published in 1992, Lancelon-Pin and Driguez reported the synthesis of monosubstituted divalent derivatives by conjugation of carboxylic acid-armed β-D-galactopyranosyl (βGal) and α-D-mannopyranosyl (αMan) dendrons (**4** and **5**) with mono-(C-6)-amino βCD.[43] The branching unit was prepared from dibromide precursor **1** by nucleophilic displacement with the per-*O*-acetylated thiosugars **2** and **3**, after *in situ* generation of the corresponding thiolates with cesium carbonate (Scheme 1). Preliminary studies showed specific recognition of the *galacto*- and *manno*-conjugates (**6** and **7**) by *Ricinus communis* and concanavalin A (Con A) lectin, respectively.

The prototype consisting of a CD moiety connected through a single primary position to the focal point of a glycodendritic structure, *e.g.* through a thiourea bridge, seemed to be particularly well adapted for the design of molecular shuttles for site specific drug delivery. First, the access of the drug guest to the CD cavity should not be much altered after monosubstitution, whereas conjugation is expected to increase water solubility. Second, the peripheral multivalent sugar display will remain fully accessible to participate in recognition phenomena by specific receptors at the cell surface, imparting targeting capabilities through the formation of drug:CD:lectin ternary complexes (Scheme 2).

In a collaborative work, the groups of Defaye, García Fernández and Ortiz Mellet provided a proof of concept for the above hypothesis. By exploiting the thiourea-forming coupling reaction[44,45] between isothiocyanate-armed glycodendrons and



Scheme 1 Synthesis of monosubstituted divalent βCD conjugates according to Lancelon-Pin and Driguez.[43]



Scheme 2 Schematic representation of CD–glycodendrimer conjugates (their construction by using the thiourea-forming reaction is shown) and their involvement in the formation of ternary complexes with drug and lectin partners.

mono-(C-6)-amino βCD, they prepared a series of αMan-coated conjugates comprising valencies between 2 and 6 (Fig. 3).[46,47] Enzyme-linked lectin assay (ELLA) comparative binding studies against the mannose-specific lectin Con A,[48–50] using monovalent conjugates and βCD-devoid glycodendrons as control, let confirm that (i) increasing the αMan valency significantly enhanced Con A binding avidity, (ii) the presence of the CD moiety did not interfere with lectin recognition and (iii) conjugation was not detrimental regarding βCD inclusion capabilities. Interestingly, they additionally found that the internal structure of the glycodendrimer had a strong impact on the magnitude of the multivalent effect. Thus, grouping the ligand units in triads was particularly favourable.

Based on the above commented results, the authors designed a molecular device purposely conceived to complex the antimitotic drug docetaxel (Taxotère®),[51] consisting of a ditopic βCD host expanding the distance between the two

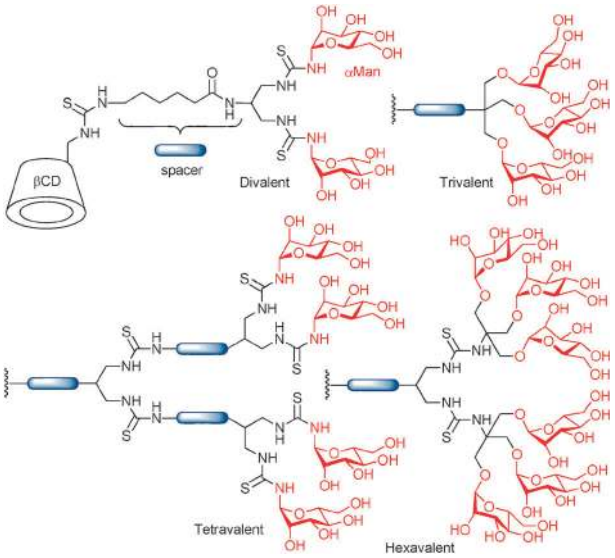


Fig. 3 Set of αMan-coated βCD–glycodendrimer conjugates prepared by Defaye, García Fernández and Ortiz Mellet for comparative binding to Con A lectin.[46,47]

phenyl rings in the drug and an appended hexavalent glycodendrimer incorporating two trimeric αMan subunits (Fig. 4). The system was shown to specifically bind at the membrane of mouse alveolar macrophages *via* the macrophage mannose receptor (MMR).[47]

Hattori and co-workers developed a chemoenzymatic approach for the preparation of monosubstituted CD derivatives that incorporated the sialoglycopeptide (SGP) branched oligosaccharide as a multivalent biorecognizable ligand (Scheme 3).[52] They first attached a unit of 4-hydroxyphenyl β-D-glucopyranoside (arbutin) at a single primary position onto βCD and then employed the endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (Endo-M) and SGP from hen egg yolk to obtain the transglycosylation product. The presence of the arbutin moiety in the βCD acceptor warranted a high yield (65–67%) in the last step and,

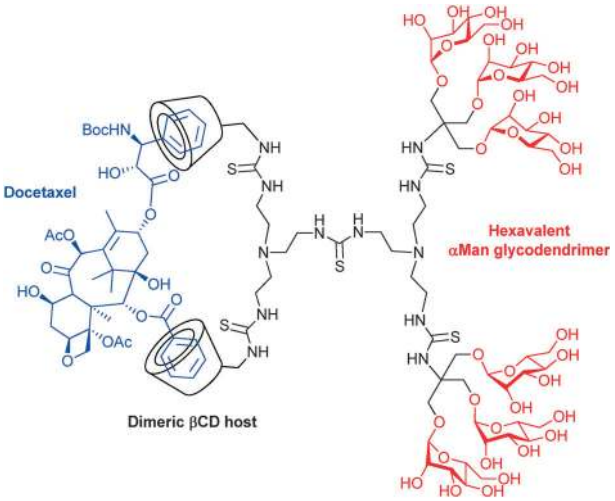
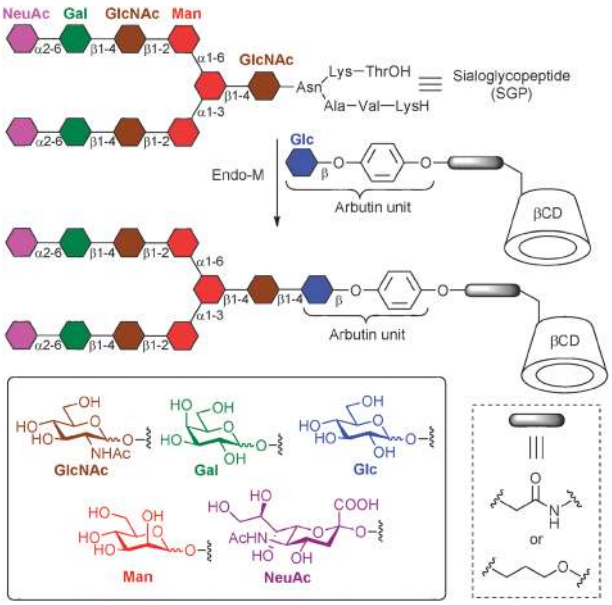


Fig. 4 Dimeric hexavalent CD–glycodendrimer conjugate design for MMR-mediated delivery of docetaxel (Taxotère®) to macrophages.[47]



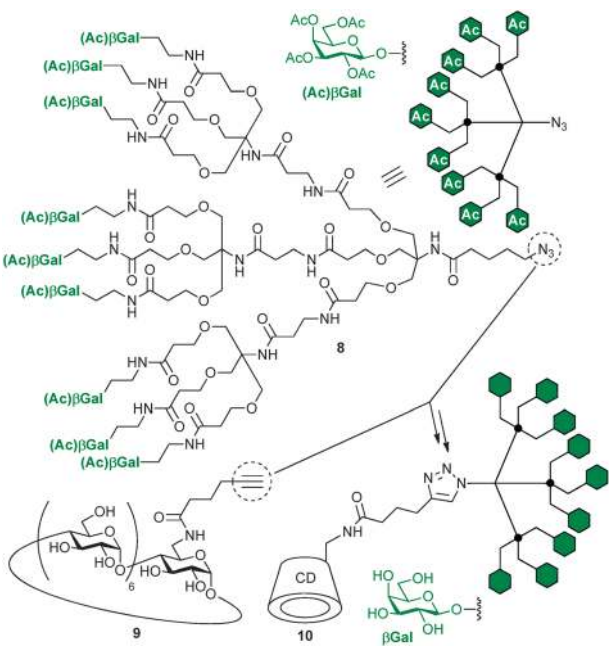
Scheme 3 Synthesis of monosubstituted arbutin-functionalized βCD conjugates bearing the natural SGP branched oligosaccharide.[52]

moreover, enhanced the stability of the corresponding inclusion complexes with the anticancer drug doxorubicin (DXR).

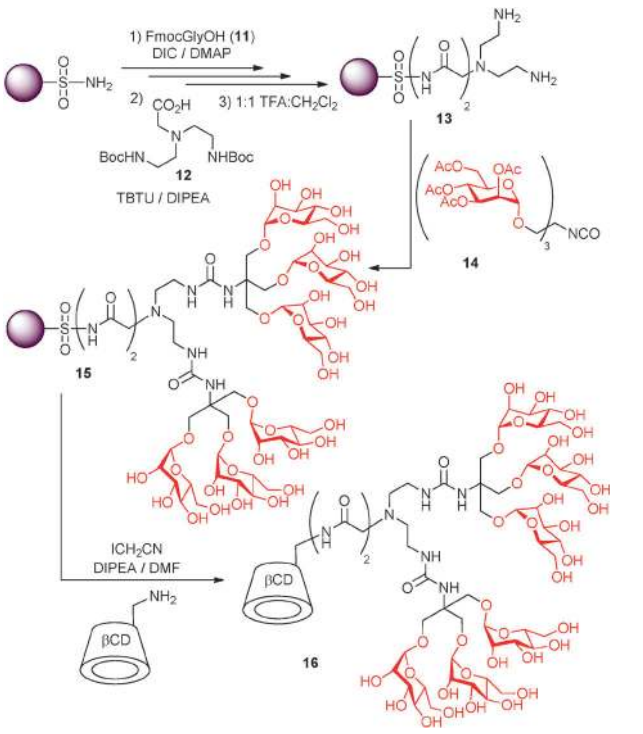
More recently, Seeberger[53] and co-workers implemented the copper(I)-catalyzed alkyne–azide 1,3-dipolar cycloaddition (CuAAC), the paradigmatic "click"-type reaction,[54–57] for the conjugation of an azide-armed glycodendrimer incorporating nine copies of per-*O*-acetylated βGal (**8**) and a monosubstituted βCD derivative equipped with a terminal alkyne group (**9**) (Scheme 4). The fully unprotected adduct **10** acted as a target-driven delivery platform to hepatocytes (HepG2 cell line) expressing the asialoglycoprotein receptor (ASGPr). In agreement, the corresponding complex with DXR produced chemically induced apoptosis in this cell line. The βGal-mediated character of the delivery process was confirmed by using an analogous nonavalent αMan conjugate as negative control.

The promise of CD–glycodendrimer conjugates for drug glycotargeting led Benito and co-workers to explore alternative synthetic routes based on solid-phase strategies. A successful resin-assisted procedure, employing Ellman safety-catch sulfonamide linker, was developed.[58] The methodology is exemplified in Scheme 4 for the preparation of a hexa-αMan derivative. Fmoc-protected glycine (FmocGlyOH) **11** was first anchored to the solid support, followed by peptide coupling with diBoc-protected *N*-bis(2-aminoethyl)glycine **12** and TFA-catalyzed hydrolysis of the carbamate groups (→**13**). Thiourea-coupling of the resulting diamine with the trivalent isocyanate-armed dendron **14** provided a hexavalent glycodendrimer attached to the resin (→**15**). Linker activation by reaction with iodoacetonitrile and diisopropyl ethyl amine (DIPEA) followed by addition of mono-(C-6)-amino-βCD transferred the multivalent branch from the resin to the cyclooligosaccharide (→**16**; Scheme 5). The approach minimizes purification steps and the final product could be isolated in an excellent 68% yield on the basis of the initial resin loading.

Many of the reported examples of multivalent sugar constructs succeed in emulating the increase in binding affinity towards specific protein receptors encountered in natural systems. Yet, only a few of them can fully mimic the switching between the "on" and "off" states and the regulation of the



Scheme 4 Nonavalent βGal-coated βCD–glycodendrimer conjugate prepared by Seeberger and co-workers for hepatocyte-targeted drug delivery.[53]

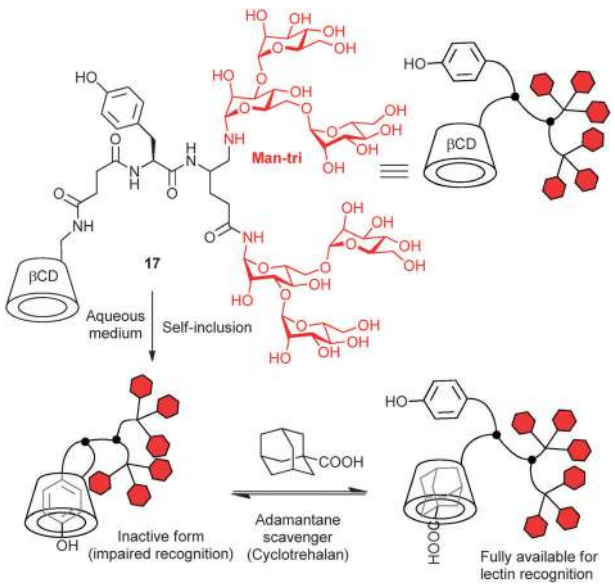


Scheme 5 Solid-phase supported synthesis of CD–glycodendrimer conjugates.[58]

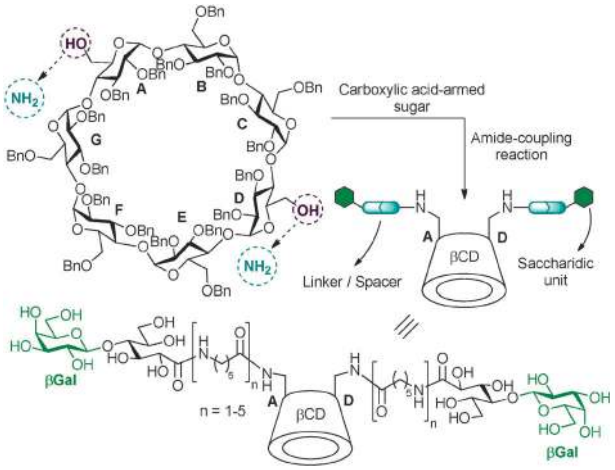
binding intensity after an external stimulus characteristic of carbohydrate–protein binding-mediated processes. Djedaïni-Pilard and García Fernández *et al*. conceived a system based on the "reversible structure-dependent binding" concept by designing conformationally switchable glycoligands that are sensitive to chemical inputs. They exploited the ability of L-tyrosine moieties anchored at a primary position of βCD to undergo self-inclusion of the aromatic ring into the CD cavity. Installing this segment in the bridge connecting the βCD core to a glycodendritic branch, containing either one or two copies (**17**) of the branched trisaccharide 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranose (Man-tri), led to formation of the corresponding intramolecular complex, in which the external sugars were not accessible to Con A lectin recognition processes. The addition of a guest molecule having strong affinity towards the βCD cavity, *e.g.* an adamantane derivative, disrupted self-inclusion and activated lectin binding capabilities.[59,60] Full reversibility of the process was proven by addition of an α,α′-trehalose-based host (cyclotrehalan)[61–66] that behaved as an adamantane scavenger, restoring the initial state (Scheme 6). The involvement of self-inclusion as the key step was supported by spectroscopic evidence as well as by molecular dynamics simulations.[67]

2.2. Regioselectively substituted CD bis-glycoconjugates

Hattori, Yamanoi and co-workers reported the use of βCD platforms selectively differentiated at two primary positions in the cycloheptasaccharide to prepare a series of divalent conjugates in which the branches are located at the α-D-glucopyranosyl subunits in A and D relative disposition. In these compounds, the distance between the two anchored glycotopes can be modulated by varying the spacer link connecting the external sugars and the CD core (Scheme 7).[68–71] This feature was exploited by the authors to estimate the separation between two βGal binding sites in peanut agglutinin (PNA) lectin by employing surface plasmon resonance (SPR). They also confirmed that the inclusion properties of βCD remained operative in the bis-conjugates, using doxorubicin as a model guest. The possibility of optimizing lectin binding and inclusion capabilities illustrates the potential of the system for site-specific drug delivery.



Scheme 6 CD–glycodendrimer conjugate with supramolecularly switchable Con A binding abilities prepared by Djedaïni-Pilard and co-workers.[60]



Scheme 7 General strategy to access βCD bis-glycoconjugates developed by Hattori, Yamamoi and coworkers. Ether-linked and αGlc-coated divalent derivatives were also prepared.[68–71]

3. Face-selective functionalized CD-scaffolded glycoclusters

The facial anisotropy of the CD platform warrants that glycotope motifs in multivalent conjugates built from face-selective functionalized precursors share an equivalent orientation and the same space region, being ideal models to test the influence of microclusterization in carbohydrate–protein interactions. The use of high-yielding ligation chemistries is mandatory for those channels in order to keep the C_n symmetry of the starting CD. Although only a few approaches have proved compatible with the preparation of monodisperse multiconjugates, their high versatility provides opportunity for virtually any molecular design that might be conceived.

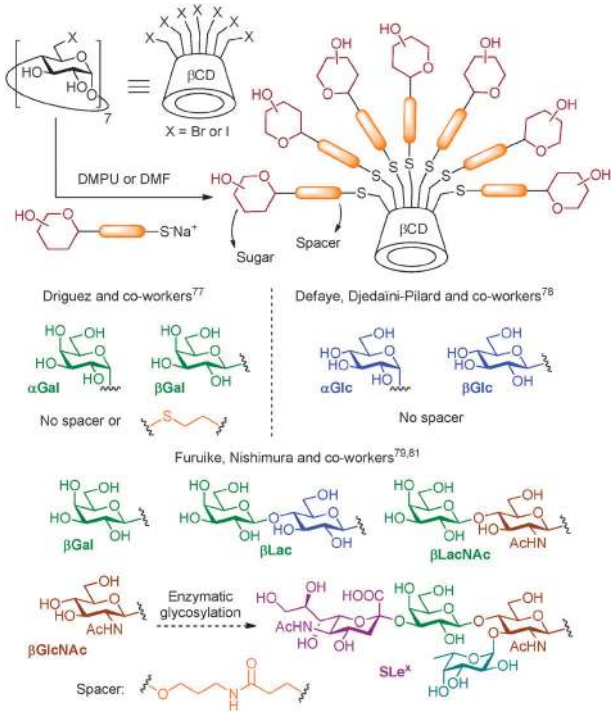
3.1. Primary face-anchored "jellyfish-type" glycoclusters

Selective replacement of the primary hydroxyl groups in cyclodextrins by halogen (I, Br) represents the most efficient strategy to access homogeneously per-(C-6)-functionalized CD derivatives.[72–76] The reproducibility and high yield of these transformations have provided an excellent model system to test the suitability of different methodologies towards glycocluster synthesis, which are itemized hereinafter. Moreover, in the resulting "jellyfish-type" arrangement the wider secondary rim of the CD platform remains open for the entrance of suitable guests to the internal cavity, potentially retaining the capacity for drug encapsulation and delivery.

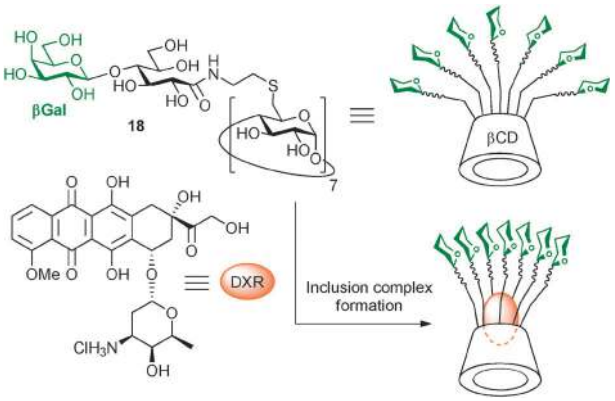
Coupling reactions involving sulfur nucleophiles: S_N2 displacements and thiol–ene photoaddition reactions. In 1994

Driguez and co-workers communicated the preparation of heptavalent CD-centred glycoclusters by S_N2 displacement in per-(C-6)-Br βCD by thiolate-functionalized fully-unprotected galactose derivatives of all bromo groups.[77] The reaction proceeded in dimethylpropyleneurea (DMPU) at 70 °C with moderate to good yields (50–80%) using either glycosyl thiolates (α and βGal) or thiol-terminated spacer-armed derivatives (βGal) as nucleophiles (Fig. 8). Essentially the same synthetic strategy was further implemented by the groups of Defaye and Djedaïni-Pilard[78] (α and βGlc), Furuike[79] and Nishimura[80] (βGal, βLac, βGlcNAc, βLacNAc) to broaden the range of homogeneously glycocoated heptabranched conjugates. Per-(C-6)-I βCD was alternatively used as the substrate in either DMPU or DMF as the solvent. Interestingly, the peripheral carbohydrates are susceptible for further enzymatic glycosylation using the appropriate glycosyltransferase-sugar nucleotide-donor pair. This chemoenzymatic approach was implemented by Nishimura and co-workers for the synthesis of a heptavalent array of Sialyl-Lewis^X (SLe^x) tetrasaccharide (Scheme 8).[81]

The above pioneering work already evidenced three important general features of primary face-anchored multivalent CD–glycoclusters that make them attractive candidates for site-specific drug delivery, namely high water solubility, an improved binding affinity towards complementary lectins (*e.g.* mammalian galectins for βGal derivatives),[82] and encapsulation capabilities that are retained for guests entering the CD cavity through the secondary rim (*e.g.* prednisolone).[78] Eventually, the incorporation of functionalized spacers between the CD platform and the coating sugars may result in additional



Scheme 8 Synthesis of heptavalent βCD-centred glycoclusters from per-(C-6)-halo derivatives by S_N2 reaction with fully unprotected sugar thiolates.



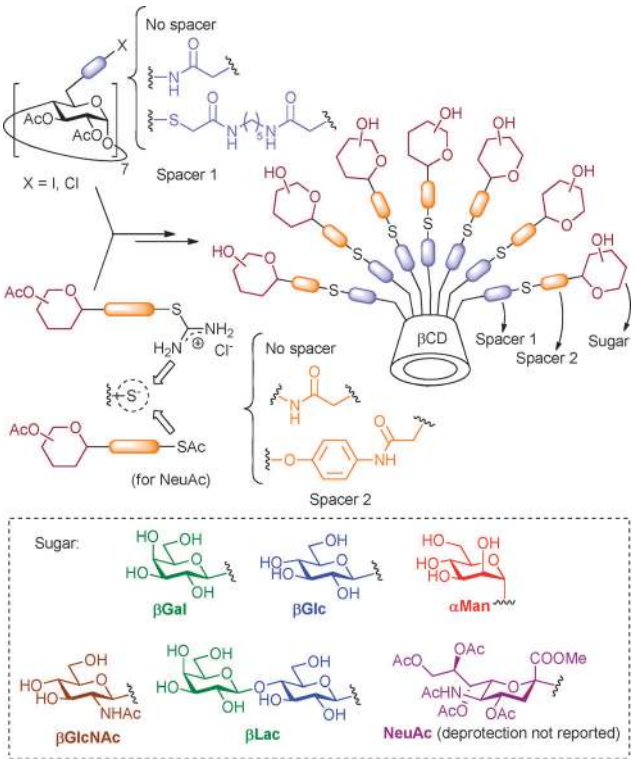
Scheme 9 Guest-induced mechanism for preorganization of βGal ligands in jellyfish-like heptavalent βCD conjugates proposed by Hattori and co-workers.[83]

interactions with an included guest following an induced-fit process that, at its turn, may preorganize the glycotopes for lectin recognition. Such a mechanism has been proposed by Hattori and co-workers to account for the unexpected high association constants of the amidolactitol-derived βGal conjugate **18** towards both the anticancer drugs doxorubicin (DXR) and PNA lectin (Scheme 9).[83]

Purification of fully unprotected macromolecular CD conjugates bears considerable difficulty. To overcome this problem, the groups of Santoyo-González, Roy and Vargas-Berenguel favoured the use of acetylated per-(C-6)-I βCD as the substrate and sugar thiouronium salts (βGal, βGlc, αMan, βGlcNAc, βLac) or S-acetyl derivatives (neuraminic acid, NeuAc) as S-nucleophile precursors. They also explored the incorporation of halogen-armed spacers at the primary positions of the CD platform to generate molecular diversity at the bridging segments (Scheme 10).[84–87]

Extensive specific lectin recognition studies were performed by different techniques, including classical ELLA, two-site ELLA (sandwich assay), turbidimetry and isothermal titration calorimetry (ITC).[86,88] It was demonstrated that the spacer arm was not a passive element regarding lectin binding or guest inclusion. Longer bridges often led to more efficient lectin recognition, whereas the effect of persubstitution at the primary face could be positive or detrimental for inclusion complex formation depending on the guest partner. Thermodynamic evidence for the formation of a ternary complex involving a heptavalent βLac conjugate with thioacetamido linkers, PNA lectin and 2-naphthalenesulfonate was obtained, supporting an induced-fit mechanism analogous to that depicted in Scheme 9.[86]

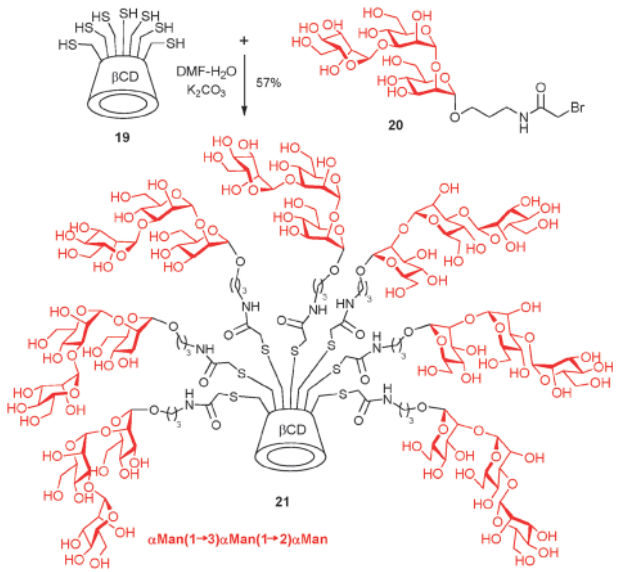
Carpenter and Nepogodiev employed per-6-thio cyclodextrins as sulfur multinucleophiles and halogen-armed carbohydrates as substrates for the preparation of primary face-anchored glycoclusters, thereby reversing the location of the functional groups involved in the key multiple S_N2 reaction.[89] Per-6-thio-αCD and -βCD (**19**) were obtained from the corresponding per-(C-6)-Br and per-(C-6)-I derivatives, respectively, by reaction with thiourea in the absence of oxygen followed by decomposition of the resulting thiouronium salts with aqueous NaOH.[90]



Scheme 10 Synthesis of heptavalent βCD-centred glycoclusters by nucleophilic substitution from protected precursors.[84–87]

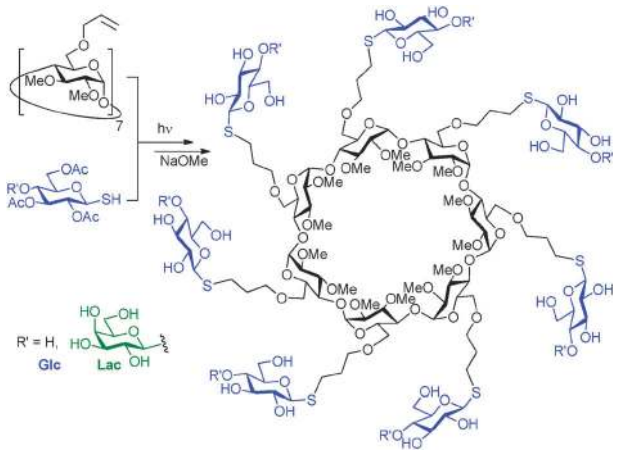
The fact that they are solid compounds insoluble in water facilitated their isolation, preventing air oxidation. The corresponding hexa (αCD) and heptathiolate (βCD) were subsequently generated in DMF–water mixtures by treatment with potassium carbonate. To test the suitability of the synthetic strategy, 4-bromoacetamidobutyl glycosides of αMan and βGlc were first selected as reaction partners, affording the corresponding multivalent conjugates in 57–75% yield after purification by gel permeation chromatography. The procedure was next extended to the preparation of an heptavalent conjugate **21** incorporating the αMan-(1–3)αMan-(1–2)αMan mannotrioside (**20**) as a mimic of the outer chains of the mannoproteins of yeast (*Saccharomyces cerevisiae*), responsible for their allergenicity and antigenicity (Scheme 11).[89]

In the search of a high yielding and flexible synthetic strategy to access multivalent CD-based glycoclusters, Fulton and Stoddart investigated the photochemical addition of thiols to allylated-βCD derivatives. Starting from heptakis(6-*O*-allyl-2,3-di-*O*-methyl)βCD and per-*O*-acetylated 1-thiosugars (from βGlc and βLac) the corresponding heptavalent conjugates were obtained in 67 and 69% yield, respectively (Scheme 12).[91,92] Homogeneity was fully established by NMR and MALDI-TOF mass spectrometry. Molecular modeling supported "time averaged" conformations with no significant deviations from the C_7 symmetry. Although each carbohydrate appendage exhibited a significant degree of freedom, they remained situated close enough to each other to expose a highly dense patch of the external glycotopes.[91,92]

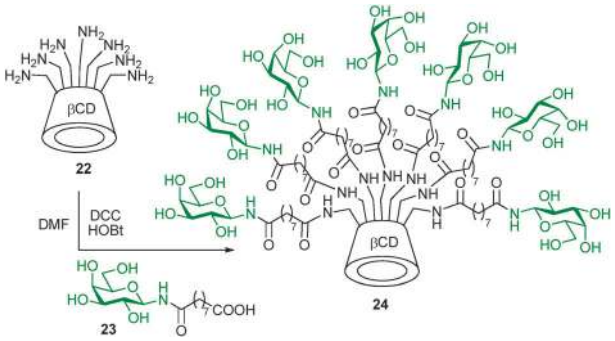


Scheme 11 Synthesis of thioether-linked hexa and heptavalent glycoclusters from per-6-thio α- and βCD.[89]

Coupling reactions involving amine nucleophiles: amide, urea, thiourea and reductive amination ligation chemistries. Given the relevance of peptide bonds in biomolecules and the broad availability of methods for their elaboration, attempting the construction of CD–glycoclusters through amide-linking strategies seemed to be a logical development. Parrot-Lopez and Bonaly *et al.* reported the use of per-(C-6)-amino βCD **22**, available in two steps from per-(C-6)-halogeno precursors *via* the corresponding heptaazide, for that purpose. Condensation with the carboxylic acid-armed βGal derivative **23** was carried out in DMF using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazol (HOBt) as coupling reagents. The target hepta-βGal βCD derivative **24** was thus obtained, but in a modest 35% yield (Scheme 13).[93,94] Recognition by the galactose specific lectin from *Kluyveromyces bulgaricus* KbCWL was observed, but a real cluster effect could not be evidenced.



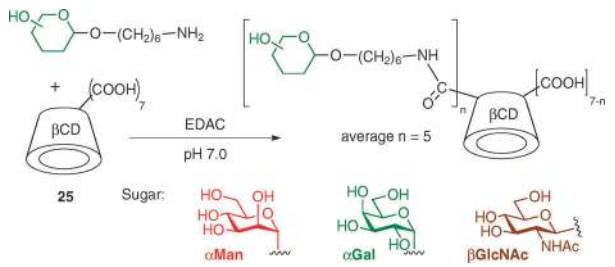
Scheme 12 Synthesis of heptavalent glycoclusters by photochemical addition of thiosugars to the per-(*O*-6)-allyl βCD derivative.[91,92]



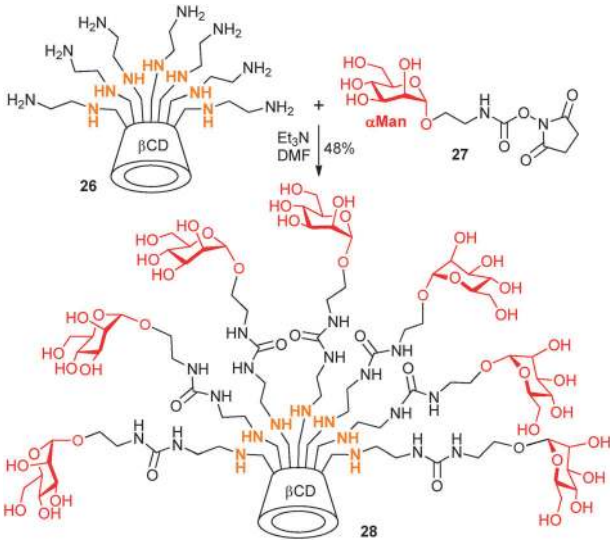
Scheme 13 Amide-coupling synthesis of hepta-βGal glycocluster **24**.[93,94]

Heptaamine **22** and its per-(*O*-2,*O*-3)-methyl derivative have been further employed by the groups of Hattori *et al.*[95] and Stoddart *et al.*,[96] respectively, as multifunctional scaffolds in the preparation of primary-face glycoclusters following essentially the same approach. In all cases, the yield of the target homogeneous heptaconjugates was handicapped by the yield of each individual peptide coupling reaction. Inserting an spacer between the CD core and the polyamine rim, thereby releasing the steric strain, did not improve the efficiency of the multi-*N*-acylation reaction.[95,97] Ichikawa an co-workers[98] explored the possibility of reversing the location of the reacting functionalities, taking advantage of the direct oxidation of the primary hydroxyls in native βCD with the system 2,2,6,6-tetramethylpyridine 1-oxyl (TEMPO) radical-sodium perchlorate to access the corresponding heptacarboxylic acid precursor **25**. However, further multiacylic nucleophilic reaction with amine-armed αGal, αMan and βGlcNAc derivatives afforded a distribution of adducts with an average valency of five from which no pure compounds were isolated (Scheme 14).

The steric constrain at the primary CD rim might be partially responsible for the low efficiency of coupling reactions involving amine or carboxylic acid. Yannakopoulou's group proposed the use of heptakis[6-(2-aminoethyl)amino-6-deoxy]-βCD (**26**) as a multifunctional polyamine platform for the construction of glycoclusters.[97] In principle, the more accessible primary amino groups should engage preferentially in nucleophilic addition reactions with suitable electrophiles. The corresponding hepta-adducts would keep seven protonable amino groups, being potentially capable of interacting with negatively charged proteoglycans at the cell surface and impart



Scheme 14 Statistical amide bond conjugation of heptacarboxylic acid–βCD **25** with amine-armed glycosides.[98]

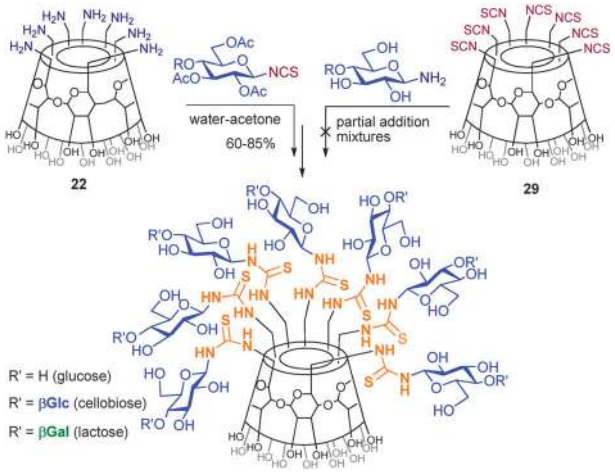


Scheme 15 Urea-forming reaction in the synthesis of heptaamino-heptamannosyl cluster **28**. An analogous αGlcNAc-coated heptaconjugate was also prepared following the same route.[97]

cell penetrating properties to the system, as already observed for the non-glycosylated βCD precursor.[99] As an alternative to the amide-bond forming strategy, they examined the reaction of **26** with *O*-succinimidylcarbamate-armed glycosides (*e.g.* the αMan derivative **27**) to give the corresponding heptaureas (*e.g.* **28**; Scheme 15). Nevertheless, the coupling yields remained low (48% for **28**; 19% for an analogous αGlcNAc cluster) and the homogeneity of the conjugates could not be fully warranted.

In 1998 García Fernández, Defaye and co-workers anticipated the potential of the thiourea-forming reaction for the synthesis of multivalent CD-scaffolded glycoclusters.[100] They examined the reactivity of both the βCD heptaamine **22** and the corresponding heptaisothiocyanate **29** towards per-*O*-acetylated glycosyl isothiocyanates or glycosylamines, respectively. Whereas the later protocol failed at providing C-6 heptaantennated glycoclusters, high yields (60–80%) of the fully homogeneous conjugates were obtained in the first case when the coupling reactions with βGlc, βCel and βLac glycosyl isothiocyanates were conducted in water–acetone mixtures at pH 8. Notably, purification of the hemiacetylated adducts could be carried out by simple column chromatography, affording the pure glycoclusters after conventional Zemplén deacetylation (Scheme 16). The heptathioureas exhibited enhanced water solubility as compared with native βCD and retained inclusion capabilities. For instance, the water solubility of Taxotère® (0.004 g L^{-1}) was raised up to 1.5 g L^{-1} in a 50 mM solution of the βGlc cluster, meaning a 350-fold increase.

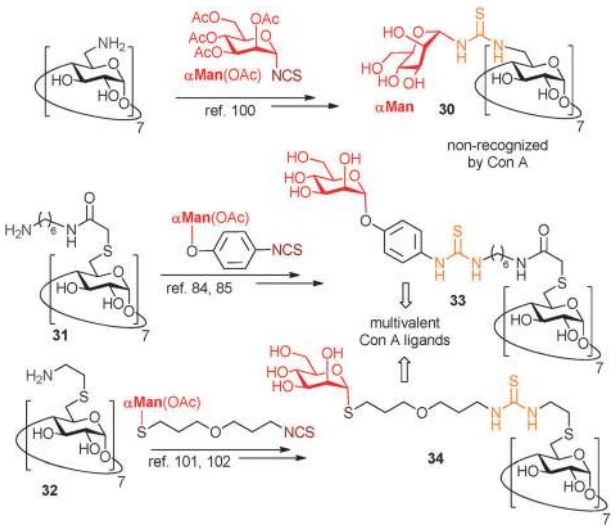
The combination of molecular inclusion and multivalency was expected to find application in site-specific drug delivery through the formation of ternary drug:glycocluster:lectin complexes. Notwithstanding, the βCD-centred heptakis(α-D-mannopyranosylthioureido) cluster **30** (Scheme 17) proved to be unable to bind Con A under the ELLA test conditions, which was ascribed to the impaired access of the convergent



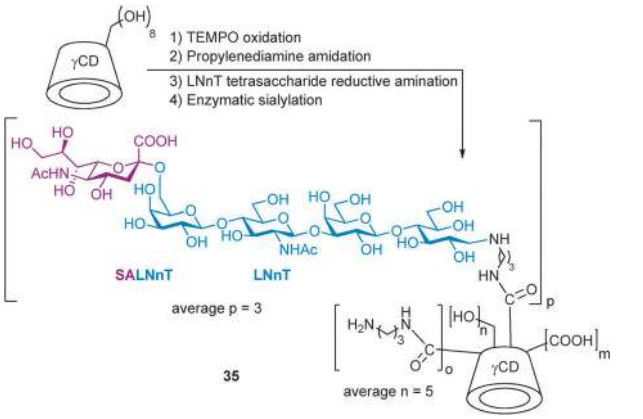
Scheme 16 Heptaglycosylthioureido-CD clusters prepared by García Fernández, Defaye and coworkers.[100]

mannosyl display to the mannose-binding sites in the lectin.[101] To overcome this limitation, the incorporation of different spacer segments between the CD core and the polyamino groups as well as between the glycoligands and the isothiocyanate functionality has been proposed. Santoyo-González, Roy and co-workers implemented the use of *p*-isothiocyanatophenyl glycosides in reaction with the amino-terminated per-(C-6)-thioalkyl derivative **31**,[85,86] whereas Ortiz Mellet, Defaye, García Fernández and coworkers coupled aliphatic isothiocyanate-armed thioglycosides and the per-(C-6)-cysteaminyl βCD derivative **32** as a heptanucleophile.[102,103] In both cases multiconjugation reaction proceeded in high yield. The corresponding hepta-αMan glycoclusters **34** and **35** exhibited Con A binding affinities according to an operative multivalent effect (Scheme 17).

Glycocluster homogeneity is essential for fundamental studies regarding multivalent carbohydrate–protein interactions. Yet, for some practical applications statistic classical bioconjugation



Scheme 17 Synthesis of heptamannosylated βCD glycoclusters with different spacer arms and comparative Con A binding abilities.



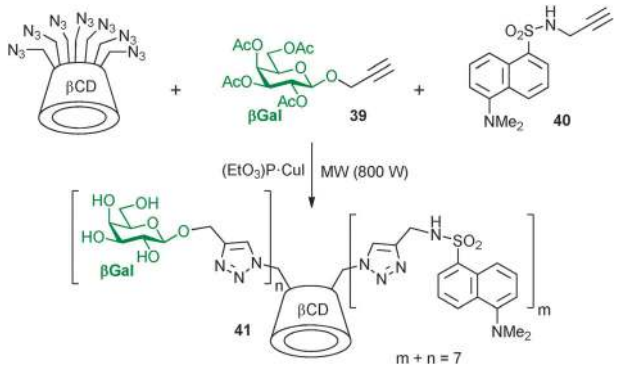
Scheme 18 Synthesis of randomly substituted multivalent sialyl TNnT–γCD conjugates through reductive amination.[104]

methods might be acceptable. Thus, Helin and co-workers used reductive amination to anchor the human milk tetrasaccharide LNnT to a γCD platform partially oxidized at the primary positions and modified by amidation with 1,3-diaminopropane. The mixture of LNnT conjugates was enzymatically sialylated using an α2,6-sialyltransferase to obtain the corresponding sialyl-TNnT (SALNnT)-appended γCD glycoclusters (**35**; Scheme 18), intended for antiadhesive therapies against *Helicobacter pylori* infection.[104]

Coupling reactions involving alkyne derivatives: 1,3-dipolar cycloadditions. Santoyo-González and co-workers pioneered the application of 1,3-dipolar cycloadditions between different 1,3-dipolar functions and propargyl derivatives as dipolarophiles for the preparation of multivalent glycoconstructs onto CD platforms. Already in 2000 they reported a first example consisting in the reaction of heptakis(2,3-di-*O*-methyl-6-*O*-propargyl)βCD **36** and per-*O*-acetylated galactopyranosyl nitrile oxide **37** to give the corresponding isoxazole hepta-adduct **38** in 78% yield (Scheme 19).[105]

In a later work, the same authors employed per-(C-6)-azido βCD as the dipolar partner for CuAAC click multiconjugation. The reactions with propargyl glycosides were conducted in organic solvents with either $(Ph_3P)_3$·CuBr or $(Et_3O)_3P$·CuI as Cu(I) catalyst. The simultaneous use of catalytic amounts of CuI (10% mol) and microwave irradiation notably shortened the reaction times. Under these conditions, the heptasubstituted derivatives were obtained in 73–94% yield.[106] The approach is compatible with co-clicking strategies to access multifunctional conjugates. Thus, by combining peracetylated propargyl β-D-galactopyranoside **39** and the *N*-propargylated dansyl derivative **40**, the corresponding fluorescently labelled multivalent

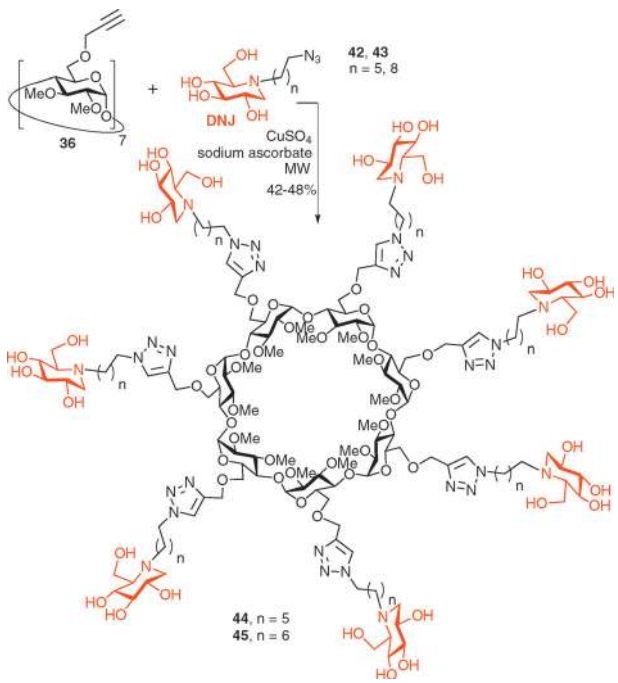
Scheme 19 Synthesis of an isoxazol-bridged βCD galactosyl glycocluster.[105]



Scheme 20 Synthesis of fluorescently labelled multivalent βGal conjugates by co-clicking using CuAAC. The method was also applied to the preparation of homogeneous heptavalent glycoclusters.[107]

cyclodextrin **41** was obtained (Scheme 20). This compound was found to act as a synthetic activator of the monocyte/macrophage RAW264.7 cell line by promoting cell adhesion and production of the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α). However, comparison with monovalent analogous showed that this response was valency-independent.[107]

More recently, Compain and Ortiz Mellet *et al.* extended the CuAAC methodology for the preparation of jellyfish-like βCD-centred clusters (**44**, **45**) in which the coating unit was the iminosugar 1-deoxynojirimycin (DNJ), a nitrogen-in-the-ring glycomimetic with a hydroxylation profile of structural complementarity with D-glucose. The coupling reaction involved the heptapropargylated CD derivative **36** and the *N*-(ω-azidoalkyl)-DNJ derivatives **42** and **43**, differing in the length of the spacer, as the alkyne and azide partners, respectively (Scheme 21).[108]



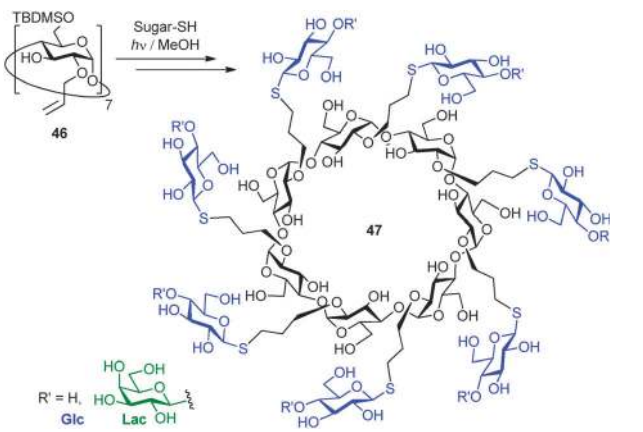
Scheme 21 Click-coupling synthetic step to obtain triazolalkyl-spaced heptavalent glycosidase inhibitors.[108,110]

Evaluation of the inhibitory activity towards a panel of glycosidases revealed striking multivalent effects that were dependent on both the enzyme and the glycomimetic cluster architecture. Thus, up to 200-fold enhancements in the inhibition potency, compared with monovalent analogues, were observed for human β-glucocerebrosidase (GCase). The nonamethylene-spaced compound was further tested for its chemical chaperone activity against GCase mutants associated with the lysosomal storage disorder known as Gaucher disease.[109] The results indicated a GCase activity increase of 1.6-fold in homozygous N370S fibroblasts at 10 μM concentration of **45**, which however did not represent a significant difference with respect to a monovalent control.[110]

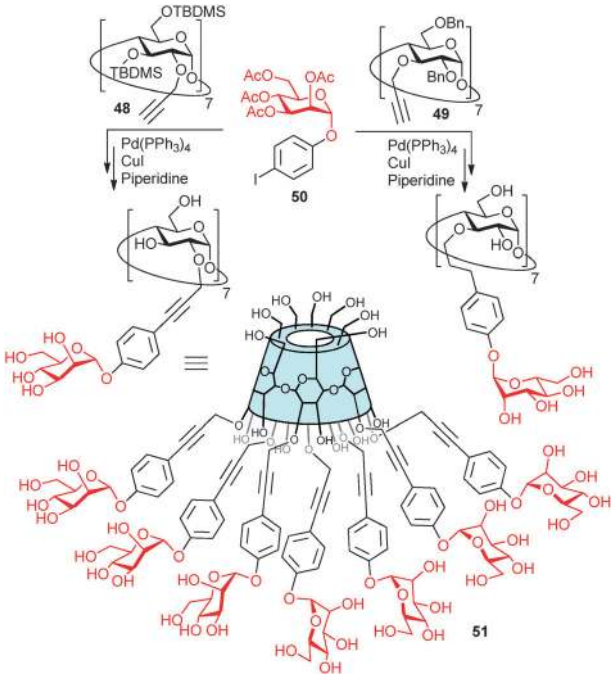
3.2. Secondary face-anchored "skirt-type" glycoclusters

No examples of CD-scaffolded glycoclusters fully decorated at the secondary rim seem to be on record, even though efficient syntheses of suitable precursors for click-type multiconjugation involving simultaneously the O-2 and O-3 positions have been reported.[111] Selective homogeneous functionalization of the OH-2 or OH-3 hydroxyl groups on the secondary face of the CDs requires prior protection of the primary hydroxyls, which is generally accomplished by *tert*-butyldimethylsilyl (TBDMS) etherification. Regioselective alkylation of the seven OH-2 groups in βCD can then be effected by taking advantage of their higher acidity as compared with the OH-3 groups, allowing their differentiation. This reaction scheme was implemented by Fulton and Stoddart to access the selectively per-(*O*-2)-allylated βCD derivative **46**, which was subsequently engaged in thiol–ene coupling with per-*O*-acetylated 1-thio-β-D-glucopyranose and 1-thio-β-lactose to afford the first examples of skirt-type CD-scaffolded glycoclusters **47** (Scheme 22).[91,92]

Vargas-Berenguel and co-workers implemented the use of the Sonogashira cross-coupling reaction between terminal alkynes and iodophenyl derivatives, with formation of a C–C bond per reacting pair, to access heptavalent secondary face αMan–βCD conjugates **51**. Derivatives in which the branching units are appended either at the O-2 or O-3 positions were prepared starting from heptakis(3,6-di-*O*-*tert*-butyldimethylsilyl-2-*O*-propargyl)βCD **48** or heptakis(2,6-di-*O*-benzyl-3-*O*-propargyl)βCD



Scheme 22 Thiol–ene synthesis of per-(*O*-2)-anchored skirt-type glycoclusters.[91,92]

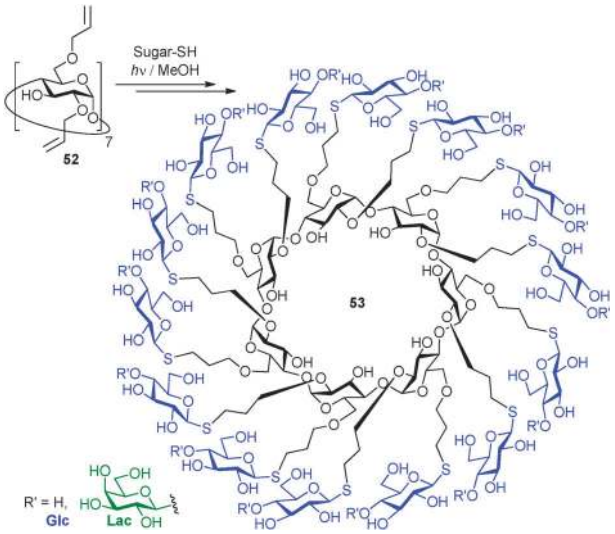


Scheme 23 Synthesis of secondary-face substituted heptamannosylated βCD derivatives *via* Sonogashira cross-coupling.[112]

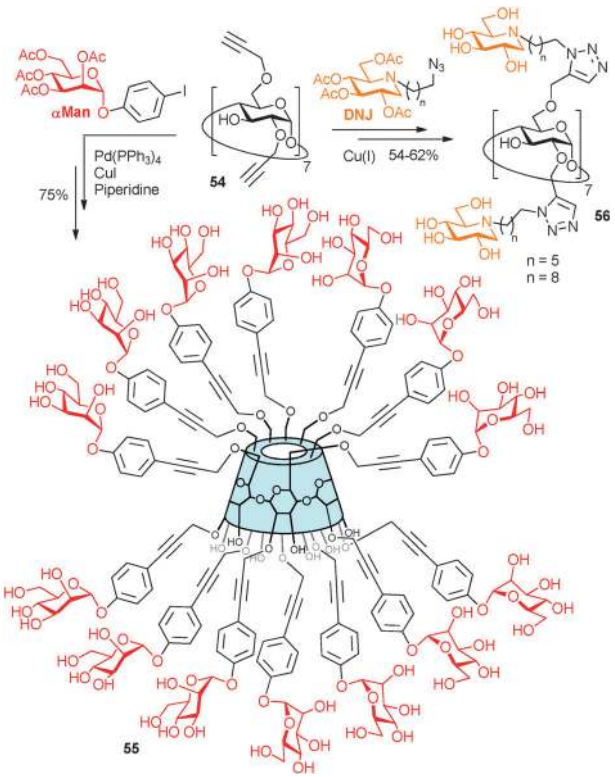
49, respectively, upon reaction with peracetylated *p*-iodophenyl-α-D-mannopyranoside **50** in the presence of catalytic amounts of $Pd(PPh_3)_4$ and CuI in piperidine (60–66% yield). In the first case, deprotection of the adduct involved fluorolysis of the silyl ether groups. In the second case, hydrogenolysis removed the benzyl protecting groups with simultaneous reduction of the triple bonds (Scheme 23).[112] The availability of per-(*O*-2)-propargyl-βCD was also exploited by Santoyo-Gonzalez and co-workers to prepare skirt-type triazol-linked βGal and βLac heptavalent conjugates through CuAAC using the azide-armed glycosides.[107]

3.3. Dual-face substituted "bouquet-type" glycoclusters

The much lower reactivity of the OH-3 hydroxyl groups in the CD platform allows simultaneous regioselective alkylation at O-6 and O-2, opening the door to the synthesis of glycoclusters in which the appendages are symmetrically distributed in two opposite domains, separated by the cyclooligosaccharide skeleton. Starting from βCD, the corresponding tetradeca-*O*-allyl and -*O*-propargyl derivatives (**52** and **54**, respectively) can be easily accessed in this manner. The first one was employed as a precursor for the preparation of tetradecavalent βGlc and βLac conjugates **53** *via* thiol–ene ligation chemistry (Scheme 24).[91,92] The second one was engaged in Sonogashira cross-coupling[112] or in CuAAC[108] to afford the corresponding bouquet-type clusters incorporating αMan glycotopes **55** (Scheme 24) or DNJ motifs **56**, respectively (Scheme 25). The iminosugar clicked-cluster with the C_9 spacer (**56**, $n = 8$) exhibited the highest multivalent effect reported up to date against a glycosyl hydrolase: an inhibition constant (K_i) against Jack bean α-mannosidase of 0.022 μM was determined, as compared with 188–204 μM for monovalent DNJ controls, meaning a four orders of magnitude affinity enhancement.[108]

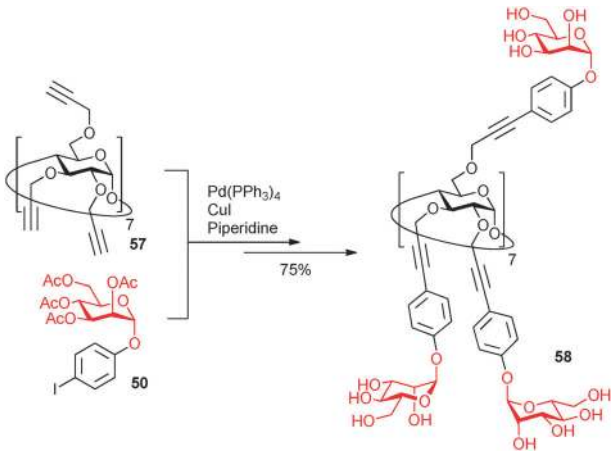


Scheme 24 Synthesis of dual-face substituted βCD-scaffolded glycoclusters by thiol–ene coupling.[91,92]



Scheme 25 Synthesis of "bouquet-type" tetradecavalent glycoclusters by Sonogashira[112] and CuAAC[108] ligation chemistries.

The efficiency of the Sonogashira cross-coupling reaction in multiconjugation schemes was further illustrated by the synthesis of the 21-valent αMan conjugate **58** from heptakis(2,3,6-tri-*O*-propargyl)βCD **57** (Scheme 26).[112] Unfortunately, no systematic evaluation of lectin binding properties has been reported up to date for glycoclusters obtained using this strategy.



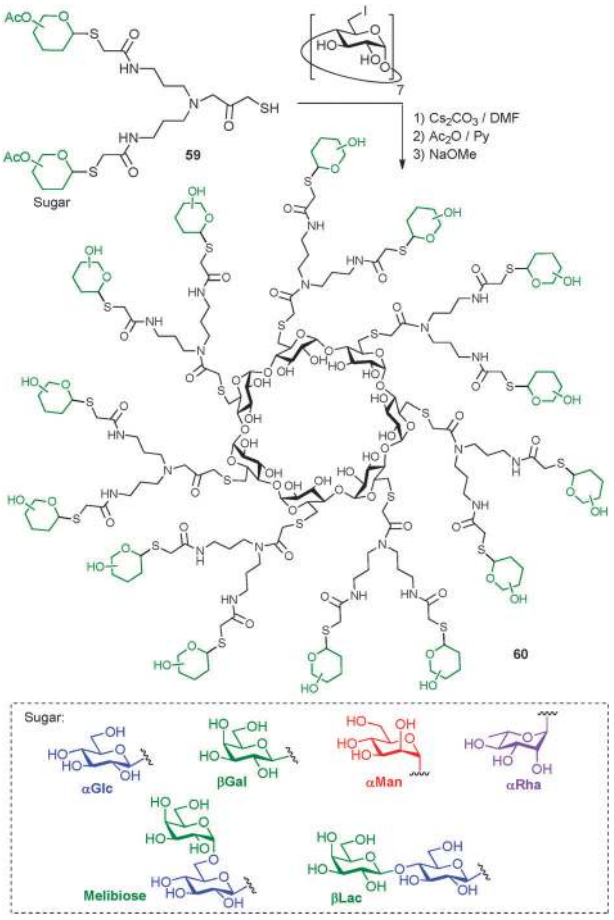
Scheme 26 Synthesis of the 21-valent ''bouquet-type'' glycocluster **58**.[112]

3.4. Hyperbranched "star-type" CD glycoconjugates

Multiconjugation of face-selective modified CDs with dendronized branches allows increasing the total valency of the glycoclusters keeping a symmetrical star-like arrangement around the cyclooligosaccharide core. Not surprisingly, all reported syntheses of hyperbranched CD glycoconjugates involve the coupling of primary position-functionalized precursors, readily accessible from the commercial CDs, with preformed glycodendrons bearing a complementary functional group at the focal point. This convergent approach warrants significant molecular mass differences between the target fully substituted cluster and any secondary product arising from undersubstitution, facilitating the purification step.

Santoyo-González, Vargas-Berenguel and co-workers prepared an extensive series of hyperbranched tetradecavalent glycoconjugates **60** bearing different saccharidic antennae by S_N2 reaction between heptakis(6-deoxy-6-iodo)βCD and thiol-armed divalent glycodendrons **59** (Scheme 27).[86,87] Hyperbranching almost prevented the access of guest molecules to the internal CD cavity, which was not the case for the primary face-anchored heptavalent analogues. The higher steric hindrance imposed by the multisaccharidic ligands, which fully block access to the cavity through the primary rim and can partially fold down toward the opposite secondary face, was probably at the origin of this observation, although specific blocking of inclusion mechanism pathways can also operate. Concerning specific lectin recognition, a valency increase from 7 to 14 did not implied enhanced binding affinity. Surprisingly, in the case of the tetradecavalent αMan conjugate binding to the αMan-specific lectin Con A was fully cancelled, stressing the tremendous impact that architectural parameters may have on carbohydrate–lectin recognition phenomena.[87]

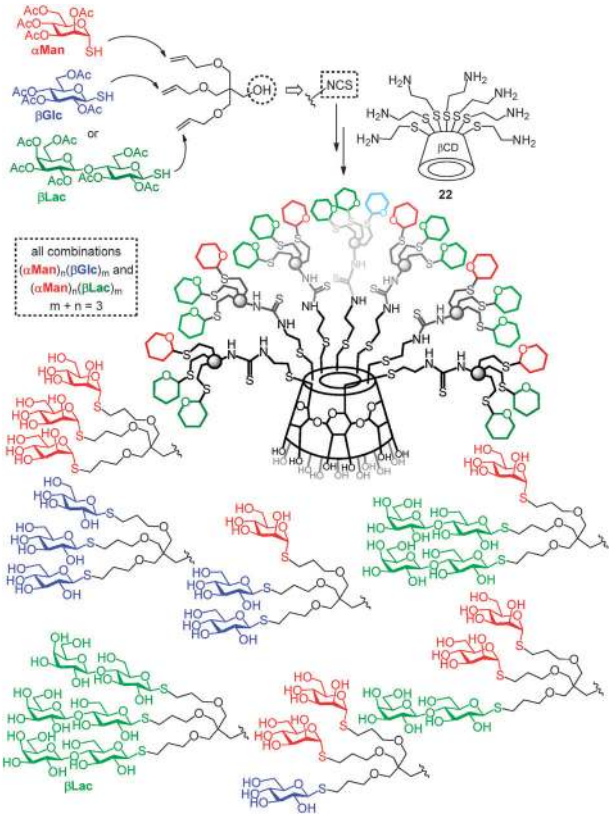
García Fernández, Defaye, Ortiz Mellet and coworkers exploited the thiourea-forming reaction as the key step in the preparation of a series of 21-valent hyperbranched homo and heteroglycoclusters with αMan, βGlc and βLac glycotopes intended to assess the effect of highly dense heterogeneous glycoenvironments in the recognition of sugar ligands by specific lectins.[102,103,113] The isothiocyanate-armed trivalent



Scheme 27 Synthesis of hyperbranched tetradecavalent glycoconjugates by S_N2 displacement involving sulphur nucleophiles.[86,87]

heteroglycodendron building blocks were synthesized by sequential thiol–ene coupling onto triallylated pentaerythritol followed by functional group manipulation. After conjugation with per-(C-6)-amino βCD **22** and deacetylation of the adducts, all combinations of $(\alpha Man)_{7n}(\beta Glc \text{ or } \beta Lac)_{7m}$ $(m + n = 3)$ were obtained (Scheme 28).

A comprehensive investigation of the binding properties towards Con A and PNA lectins by means of different techniques, including ELLA, ITC, SPR, two-site sandwich-type ELLA and turbidimetric assays, in comparison with low density glycoclusters, revealed striking synergistic effects. Thus, αMan was significantly more efficiently recognized by Con A in the presence of βGlc or βLac even though these sugars are not recognized by the lectin either in low or high valency homodisplays. Comparison of the binding avidities of tetradecavalent αMan–βCD conjugates having either no other sugar or seven additional βLac units in the structure towards Con A illustrates this fact: the $Man_{14}Lac_7$ heteroglycocluster exhibited a 5-fold higher affinity in a mannose molar basis. A similar scenario was observed for βLac recognition by PNA in the presence of αMan. This phenomenon was termed by the authors the *heterocluster effect*,[103] its biological significance remaining still uncertain.[114]



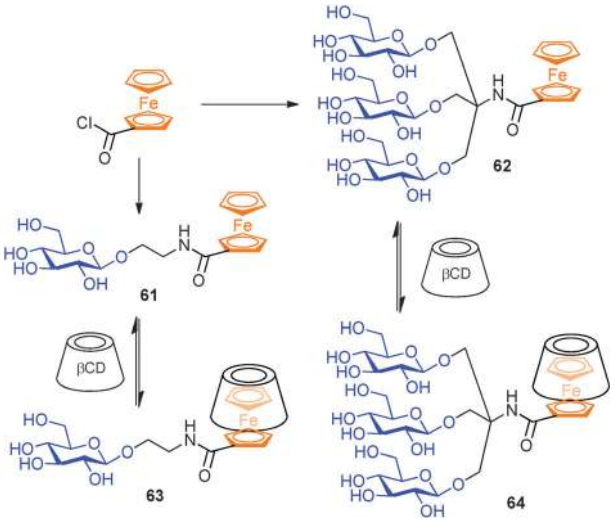
Scheme 28 Synthesis and general structure of βCD-scaffolded hyperbranched homo and heteroglycoclusters by thiourea-forming reactions.[102,103,113]

4. Multivalent CD-based supramolecular architectures

The versatility of the CD scaffold allows conceiving a range of non-covalent or mixed covalent—non-covalent multivalent architectures that broaden the potential of CD conjugates in fundamental as well as applied fields. The diverse examples of multivalent CD-based supramolecular architectures, going from bimolecular glycocomplexes to hybrid CD-biomacromolecule glycoassemblies, are presented in the following subsections.

4.1. Supramolecular glycoclusters

Conjugation of a multivalent glycoligand and a functional element having strong affinity towards the CD cavity affords multivalent guests that can be exploited to assemble supramolecular glycoclusters after inclusion complex formation. In an early example, Credi, Raymo and coworkers reported the synthesis of amide-linked glycoferrocene conjugates **61** and **62** as redox-active ligands for βCD.[115] Monosubstituted conjugates with either a β-D-glucopyranosyl unit or a trivalent β-D-glucopyranosyl dendron formed 1 : 1 complexes with βCD (**63** and **64**) in D_2O at room temperature, with stability constants (K) of 2000 and 1300 M^{-1}, respectively (Scheme 29). The interaction with the cyclodextrin cavity induced changes in the electrochemical properties of the ferrocene moiety that were used to sense the inclusion process. The authors focused their work on the possibility of using



Scheme 29 Supramolecular glycoferrocene–CD conjugates.[115]

carbohydrate substitution and inclusion complex formation to protect the ferrocene core from solvent interactions or from interactions with an electrode or a partner in an encountered complex. Although the results illustrate the potential of the CD inclusion capabilities for designing multivalent supramolecular architectures, this aspect was someway disregarded at that time.

Further work by the group of Vargas-Berenguel deepened on the characterization of glycoferrocene–βCD complexes.[116] Divalent 1,1′-bis(glycosyl)ferrocenes bearing β-D-glucopyranosyl (βGlc), α-D-mannopyranosyl (αMan) and β-lactosyl (βLac) residues (**65** and **66**) were synthesized by either thioglycosylation or copper(I)-catalysed alkyne–azide cycloaddition (CuAAC) ligation chemistries. The association constants with βCD were determined from calorimetric and voltammetric titration experiments both in their neutral and oxidised forms. A main conclusion of this work was that the disubstituted ferrocene derivatives formed stable complexes with βCD through inclusion of the ferrocene core into the cyclodextrin cavity. Whereas for monovalent conjugates the ferrocene moiety adopts an axial orientation, as shown in Scheme 29, in the case of the 1,1′-disubstituted derivatives it adopts an equatorial disposition in the supramolecular complex (Fig. 5). In no case rotaxane-type complexes, *i.e.*, complexes having the ferrocene moiety axially oriented inside the cavity with the sugar units protruding from opposite faces, were formed. The potential of these systems to probe multivalent carbohydrate–protein interactions by electrochemical techniques was mentioned by the authors, but no data seem to be available up to date.

In the above commented cases, the valency of the 1 : 1 inclusion complex system is already defined by the valency of the guest glycoconjugate. If the CD host already bears a sugar ligand, inclusion complex formation will produce a supramolecular homoglycocluster with increased formal valency if both guest and host are substituted by the same saccharide motif and a heteroglycocluster if they are different. An example of this approach has been reported by Hayashida and Hamachi using a cyclophane platform modified with three β-D-galactopyranosyl (βGal) substituents and a dansyl group (**67**), responsible

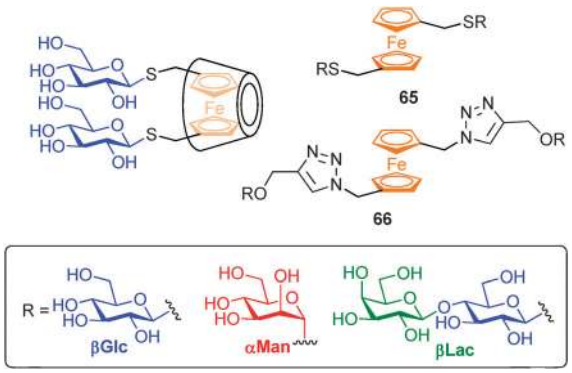
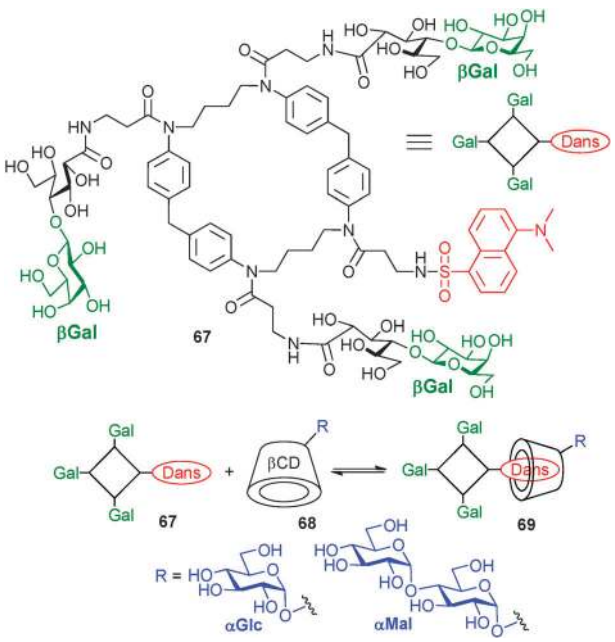


Fig. 5 Example of a divalent supramolecular conjugate based on a glycoferrocene–βCD inclusion complex (left) and structure of the diglycosylated ferrocene derivatives prepared by Vargas-Berenguel and co-workers.[116]

for the fluorescence of the molecule and well-adapted to inclusion in the βCD cavity.[117] This conjugate formed heterodimers (**69**) with 6-*O*-β-D-glucopyranosyl- and 6-*O*-maltosyl-βCD (**68**) with *K* values of 770 and 600 M^{-1}, respectively (Scheme 30). The ability of the dansyl-labelled trivalent galactocyclophane guest to bind specifically to peanut agglutinin (PNA) was confirmed by turbidimetry and fluorescence techniques. The high versatility of the preparation method, that involves just mixing the complementary partners, makes it suitable for assembling supramolecular clusters having various kinds of sugar residues with a well-defined chemical structure and stoichiometry.

The combination of CD conjugates and complementary multitopic guests represents an alternative strategy to assemble supramolecular complexes with increased formal valency. The potential of this approach to improve the affinity of a CD-centered glycocluster towards specific lectins was first pointed



Scheme 30 Glycocyclophane–glycoβCD supramolecular multivalent heterodimers.[117]

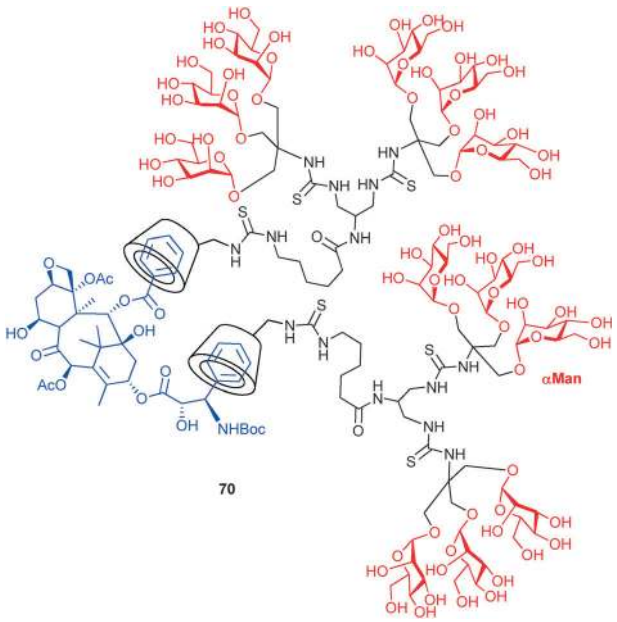


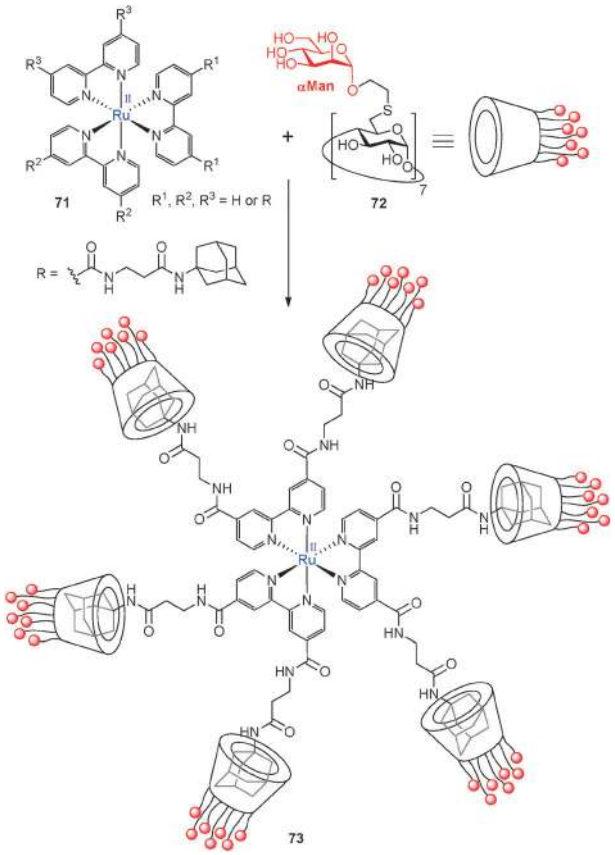
Fig. 6 Docetaxel (in blue)-promoted clusterization of hexamannosylated glycodendritic βCD conjugates.[47]

out by Defaye, García Fernández, Ortiz Mellet and co-workers in the frame of a project aiming at developing site-specific delivery systems for the taxane anticancer drug docetaxel (Taxotère®).[47] They observed clusterization of tri- and hexa-mannosyl dendron–βCD conjugates in the presence of the drug through formation of 1 : 2 inclusion complexes implying the aromatic rings in the docetaxel molecule (*e.g.* **70**), which translated into enhanced binding affinity towards Con A (Fig. 6).

More recently, Seeberger and coworkers have extended the concept of guest-promoted clusterization of multivalent CD conjugates to assemble fluorescent sensors with varied architectures.[118] The multivalent supramolecules are comprised of a fluorescent ruthenium(II) core functionalized with 2, 4 or 6 adamantyl units (**71**) that act as anchor elements for βCD conjugates. Heptavalent glycoclusters in which the βCD platform was homogeneously substituted at the primary C-6 positions with 2-(α-D-mannopyranosyloxy)ethylthio branches (**72**) were self-assembled onto this core to form complexes exposing 14, 28 or 42 mannopyranosyl units (**73**) with a unique spatial orientation (Scheme 31). These systems proved to be very well-suited to probe the structural requirements for efficient binding to immobilized Con A as a function of lectin density by surface plasmon resonance (SPR). Additionally, the inherent red fluorescence of the Ru(II)-containing supramolecular clusters allowed direct visualization of their association with *E. coli* (strain ORN178), expressing wild-type mannose-binding FimH lectin in the pili, by confocal microscopy. The possibility of tuning the saccharide ligands, the cluster valency and the supramolecular spatial arrangement in a very flexible system provides a streamline method to generate collections of multivalent probes.

4.2. Multivalent polyrotaxane glycodiplays

Polyrotaxanes are supramolecular assemblies consisting of several cyclic compounds threaded onto linear polymeric



Scheme 31 Ru(II)-centred supramolecular multivalent mannosides prepared by Seeberger and co-workers; the 42-valent construct is shown.[118]

chains capped with bulky end-groups. If the capping groups are missing, therefore allowing dethreading processes to take place, the term pseudopolyrotaxane is used. The most characteristic feature of these "beads-on-a-string" dynamic systems is that the cyclic components can spin around the axes of the polymer as well as move back and forth along the polymer chain. The cavity of cyclodextrins, particularly of αCD and βCD, has been shown to be well adapted to pseudopolyrotaxane formation with linear polymers[119–121] such as poly(ethylene glycol) (PEG), poly(propylene glycol) (PPG) or poly(tetrahydrofuran) (PTHF).[122] If the CD beads are part of a glycoconjugate, then a "mobile" display of the coating sugar ligand is obtained, with the potential to self-fit the intersaccharide distance and orientation in the presence of a given receptor partner for an optimal interaction (Fig. 7A). Alternatively, threading CD "beads" onto a glycopolymer "string" can be exploited to modulate the dynamic properties of the polymer backbone and, likewise, influence the molecular recognition behaviour of the supramolecular system (Fig. 7B).

With the aim to investigate how ligand mobility affects multivalent interactions, Yui and co-workers prepared a series of maltose–αCD–polyrotaxane (Mal–αCD–PRXs) conjugates by sequential (i) rotaxanation of αCD onto α,ω-diamino-PEG (M_n = 4000) (**74**), (ii) condensation with *N*-benzyloxycarbonyl (Z)-protected L-tyrosine (Z-TyrOH), thereby capping polyrotaxane

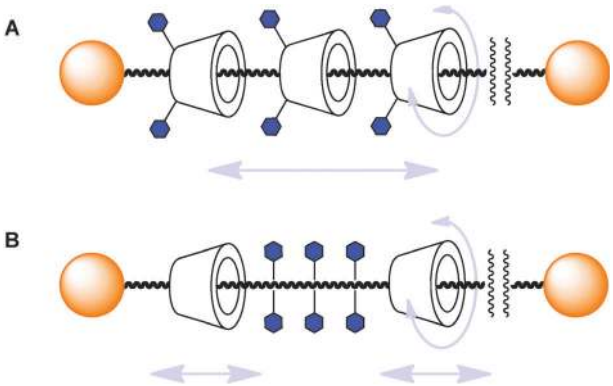
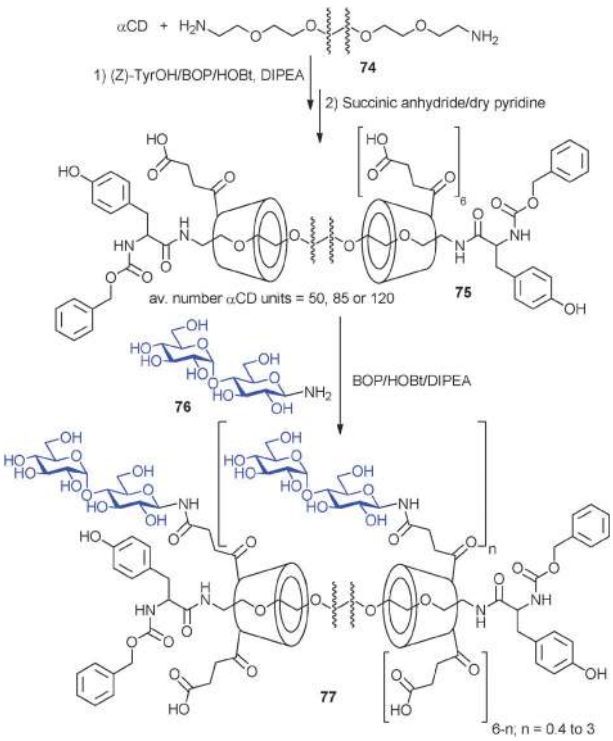
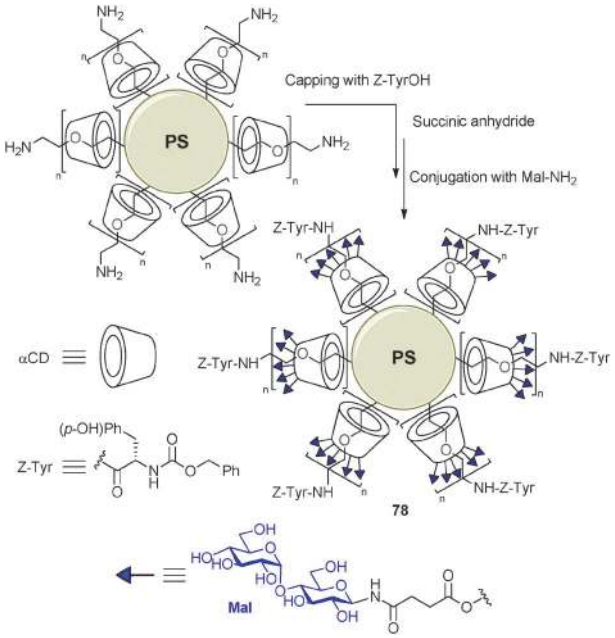


Fig. 7 Schematic representation of multivalent polyrotaxane glycodisplays based on CD-conjugates (A) and on glycopolymer–CD polyrotaxane hybrids (B). The mobile character of both types of supramolecular constructs is indicated.

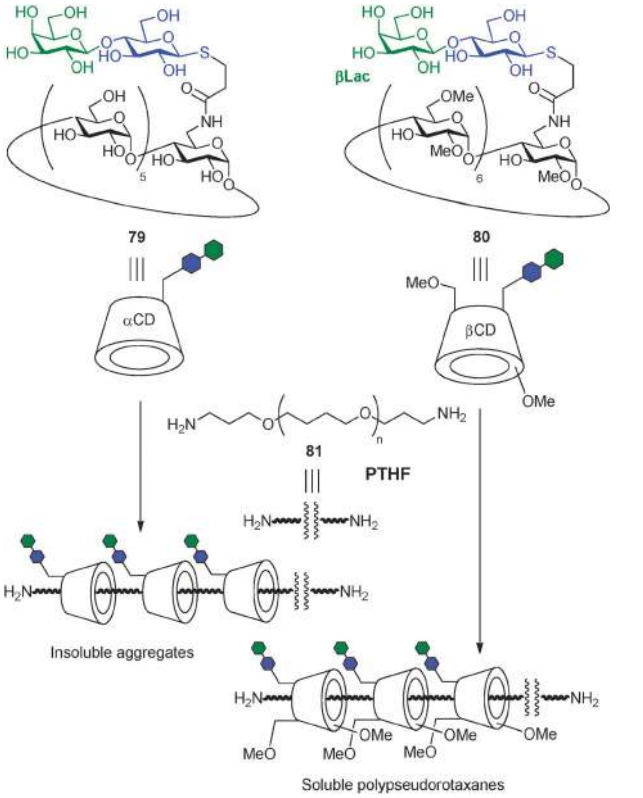
ends, (iii) reaction of the resulting polyrotaxane with succinic anhydride to convert all primary OH groups in the αCD units into the corresponding 2-carboxyethyl esters (→**75**) and (iv) statistic incorporation of β-maltosylamine (**76**) through amide bond-forming reaction (→**77**; Scheme 32).[123–125] Structural variability was introduced based on the CD threading percentage (22, 38 or 53%; corresponding to 50, 85 or 120 αCD beads per polymer string) and the maltose conjugation degree (from 0.4 to 3 Mal units per αCD bead, meaning from 42 to 244 Mal ligands per polyrotaxane supramolecule). Glyco-PRXs with different CD loads and glycosyl units per CD but similar amounts of total maltose were thus obtained.



Scheme 32 Synthesis of maltose–αCD–polyrotaxanes according to Yui and co-workers.[123–125]



Scheme 33 Mal–αCD–PRX-coated polystyrene microspheres.[127]



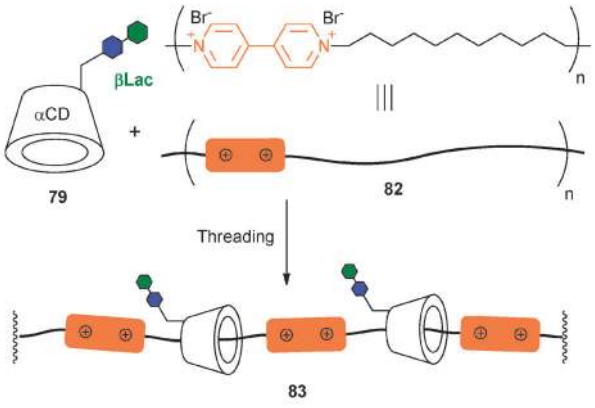
Scheme 34 Schematic representation of multilactosylated pseudorotaxane assembling from monolactosylated CD conjugates.[128]

A systematic evaluation of the binding affinity of the Mal–αCD–PRXs against Con A was carried out by hemagglutination inhibition assay. Maltose, an unthreaded αCD derivative bearing three maltosyl substituents and maltose-poly(acrylic acid) glycopolymers bearing 42, 55, 117 or 240 βMal units per polymer chain were used as standards for comparative purposes. The results revealed that in the case of polyrotaxanes bearing 50 or 85 αCD beads per PEG chain, the lectin binding affinity increased with the number of Mal units, whereas for the most heavily charged Mal–αCD–PRX, with 120 αCD beads per chain, as well as for the glycopolymers, a maximum in affinity was reached when 117 Mal units were exposed, then decreasing dramatically for higher Mal densities. The authors interpreted these results in terms of optimal mobility for the first constructs that allows maximizing binding while minimizing steric mismatching upon lectin aggregation. Experimental evidence to support this hypothesis was obtained initially by Raman spectroscopy[126] and further confirmed by advanced NMR studies.[125] The relatively high mobility of the Mal ligand in the rotaxane architecture additionally prevents the formation of ordered clusters of water molecules, avoiding the unfavourable energy term derived from breaking those clusters upon lectin binding.

In 2010, Sasabe and coworkers extended Yui's approach for the synthesis of multivalent Z-protected tyrosine-capped glycopolyrotaxanes for the preparation of Mal–αCD–PRXs immobilized onto polystyrene (PS) microspheres (1–50 μm), with an average load of five maltosyl groups per αCD threaded unit (**78**, Scheme 33).[127] The interaction between the glycoparticles and biotinylated Con A was studied by fluorescence microscopy employing a quantum dot–streptavidin (QD–SV) conjugate. The red fluorescence observed at 655 nm suggested specific recognition of the maltosyl moieties by the lectin. It should be noted, however, that the αCD polyrotaxane itself, prior to Mal conjugation, also elicited a Con A-dependent weak response in this assay.

Nelson and Stoddart implemented an alternative strategy to multivalent pseudopolyrotaxane preparation that employed monovalent, therefore monodisperse, amide-linked βLac–CD conjugates as the initial threading components.[128] Rotaxanation of the αCD derivative **79** onto bis-3-aminopropyl-terminated PTHF (M_n = 1100) was first considered, but the system was found to be unstable in water solution, precipitating on standing. This problem could be avoided using instead the per-(*O*-2,*O*-6)-methylated βCD conjugate **80**, which threaded onto bis-3-aminopropyl-terminated PTHF (**81**) or bis-2-aminopropyl-terminated PPG (M_n = 2000) to afford dynamic water-soluble pseudopolyrotaxanes (Scheme 34). Unfortunately, the threading/dethreading of the CDs was fast on the ^{1}H NMR timescale to the extent that isolating the pseudopolyrotaxanes was not possible, preventing lectin binding evaluation studies.

In order to lower the rate of translational motion of the CD beads along the polymer chain of pseudopolyrotaxanes, the same laboratory investigated the use of polycationic polyviologen AB-copolymers (**82**), comprising alternating decamethylene segments and positively charged bipyridinium moieties as strings. The authors hypothesized that the mono-Lac–αCD conjugate **79** should thread onto the polymer chain in aqueous solution and rest preferentially on the hydrophobic decamethylene segments (**83**), which was confirmed by ^{1}H NMR



Scheme 35 Schematic representation of polyviologen (n = 8, 17 or 21)-βLac–αCD pseudorotaxane neoglycoconjugates **83** with translational restricted mobility.[129]

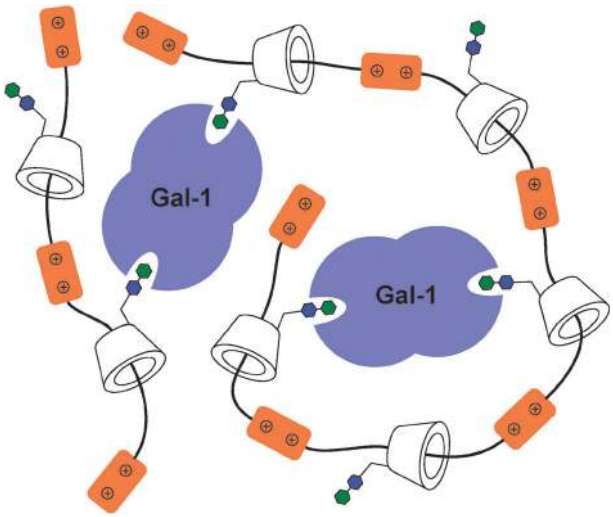


Fig. 8 Possible binding mechanism for two Gal-1 lectin molecules and two multilactosylated polypseudorotaxane chains.[129]

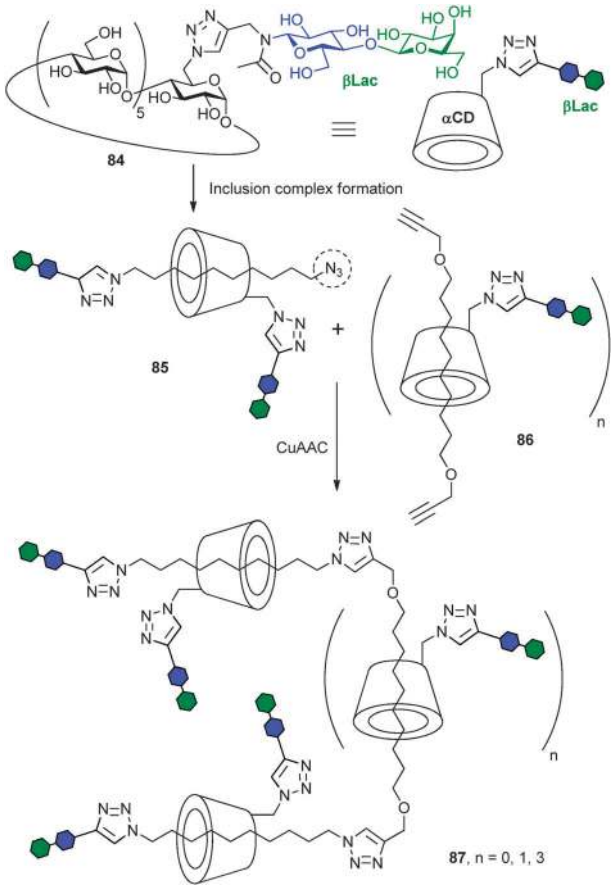
spectroscopy, whereas the cationic aromatic moieties should act as electronic "speed bumps" (Scheme 35).[129]

Interestingly, the threading percentage could be determined for different pseudo-PRX constructs, varying in the polyviologen chain length (average number of repeating units 8, 17 or 21) and in the initial Lac–βCD conjugate : polyviologen ratio by using the Bradford assay, towards which the "uncovered" polymer was sensitive.[130] The resulting supramolecular species were challenged for their ability to inhibit agglutination of T-cells induced by recombinant human galectin-1 (Gal-1), a ditopic lactose-recognizing lectin involved in tumor development, as well as in quantitative Gal-1 precipitation assays.[129,131] Valency corrected binding enhancements up to 30-fold over native lactose in the agglutination assay were observed, with pseudo-PRX : Gal-1 ratios close to 1 : 1 in the quantitative precipitation assay and binding affinity enhancements per rotaxanated polymer that tend to increase in a statistic manner with the number of Lac recognition motifs. The results were compatible with a one-to-one binding mechanism in which only one of the lactoside-binding sites is occupied at a time. Nevertheless, deviations from the statistic trend for PRX prepared from the longer polyviologen chains (21 repeating units) with the lower Lac–CD load (one forth of the theoretical full coverage), leading to the highest per-lactose binding enhancements, suggested that chelation also operates. Most probably, the dynamic character of multivalent PRXs allows a variety of binding modes with the lectin whose distribution changes with the degree of threading (Fig. 8).

In 2009, Fort and co-workers re-investigated the potential of using a monoLac–αCD conjugate **84** to afford multiLac neoglycoconjugates **87** through rotaxanation, taking advantage of the affinity of the αCD cavity towards linear decamethylene segments.[132] The authors implemented CuAAC "click" chemistry to incorporate the lactosyl glycotope onto the αCD beads as well as at the end of the terminal repeating unit of the oligomer string. The same ligation chemistry was further used to connect the clickable oligorotaxane portions (**85** and **86**), after inclusion complex formation, to ensemble the target mechanically interlocked multivalent system. A main advantage of the approach is that the density of lactosyl moieties can be efficiently controlled by adjusting the excess of the monoLac–αCD host before clicking (Scheme 36).

The resulting oligorotaxanes were evaluated as inhibitors of asialofetuin–PNA binding by ELLA. The lectin binding affinity clearly improved with valency. However, if the interaction is analyzed on a lactosyl unit basis, only a 2 to 4-fold improvement is achieved, meaning a rather modest multivalent effect. Insufficient mobility of the threaded cyclodextrin conjugates to allow an optimal complementarity between the displayed Lac units and the lectin binding sites may be at the origin of this result. Nevertheless, it must be noted that, in the absence of the chelate effect, ELLA is considered to provide information about multivalent ligands and a single binding site of the lectin. In such a scenario, sliding of recognition motifs over the binding site and rebinding effects are usually the predominant mechanisms at work, which might not be particularly favoured in the polyrotaxane architecture.

Dong and co-workers conceived a completely different type of multivalent CD-based pseudopolyrotaxane architecture consisting of a glycosylated triblock copolymer, with two edge blocks made of poly(D-gluconamidoethylmethacrylate) (PGAMA) separated by a poly(ε-caprolactone) pseudopolyrotaxane (PPR) threaded with native αCD.[133] (Fig. 9). In this particular construct, the CD motif acts by masking the crystallinity of the biodegradable poly(ε-caprolactone) block, thereby preventing precipitation of the glycopolymer. The compounds were found to self-assemble spontaneously in water, with critical aggregation concentrations between 46.1 and 51.2 μg mL^{-1}. DLS and TEM studies showed spherical micellar aggregates with 81–121 nm radius, where the central PPR segments adopted a channel type structure. Specific interactions with Con A were evidenced by the formation of bigger aggregates, in the μm range, in the presence of the lectin, leading the authors to conclude that these new biohybrids might found broad application in targeted drug delivery.



Scheme 36 Synthesis of multilactosylated polyrotaxanes by CuAAC.[132]

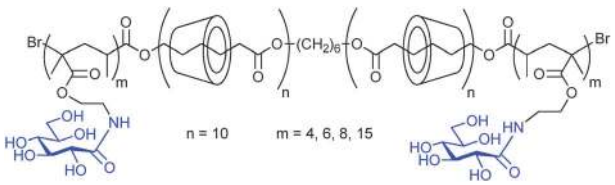
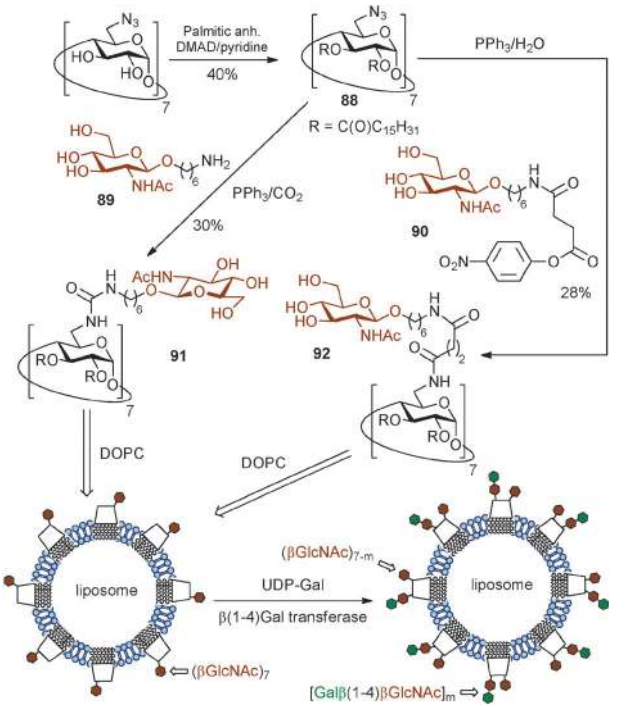


Fig. 9 Glycopolymer–pseudopolyrotaxane biohybrids.[133]

4.3. Self-assembled glycocoated liposomes, micelles and vesicles based on amphiphilic CDs

Chemical modification at one or both rims of CD platforms has been largely exploited to achieve amphiphilic derivatives with the capability of self-assembling into nanoparticles or liposomes that can be loaded with drugs.[134] Endowing those systems with targeting properties has been a main motivation for the development of glycoamphiphilic CD (GaCD) constructs. In 2004 Sallas, Niikura and Nishimura reported the first representatives of this new family of compounds.[135] Starting from per-(C-6)-azido-βCD, the corresponding tetradeca-(*O*-2,*O*-3)-palmitoyl derivative **88** was first prepared by reaction with palmitoyl anhydride and 4-(*N*,*N*-dimethylamino)pyridine in anhydrous pyridine, though in modest (40%) yield. Transformation of the azido groups into isocyanate or amine functionalities allowed subsequent installation of

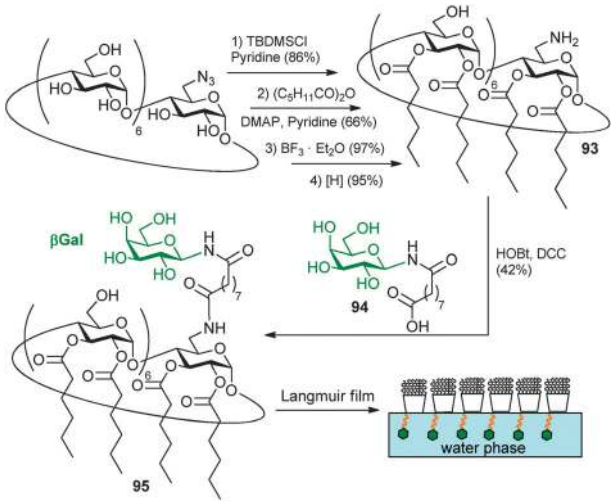


Scheme 37 Synthesis of multivalent liposomes from glycoamphiphilic cyclodextrins.[135]

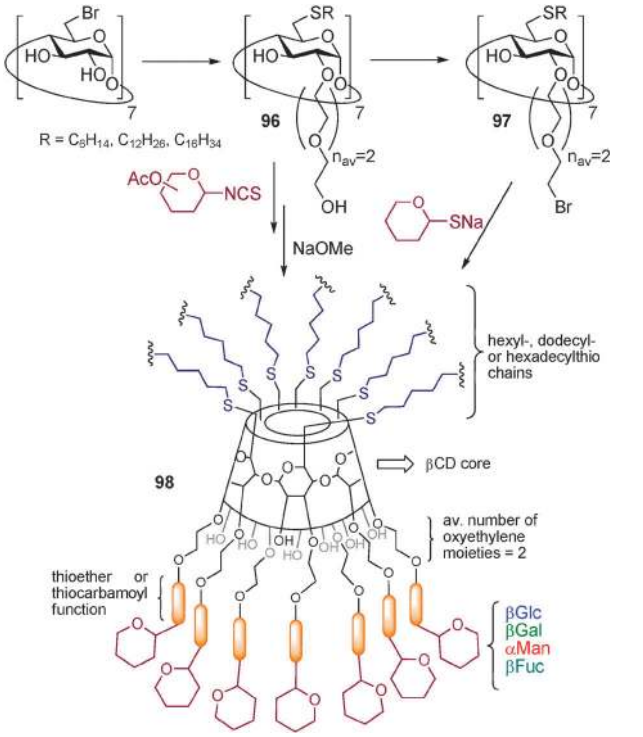
2-acetamido-2-deoxy-β-D-glucopyranosyl (βGlcNAc) units **89** and **90** through urea- (short arm) or amide-forming reactions (long arm) (Scheme 37). None of the amphiphilic glycoclusters **91** and **92** thus prepared was water-soluble, but they could be incorporated into liposomes by using 1 : 9 mixtures with dioleyl phosphatidylcholine (DOPC). The accessibility of the coating monosaccharide residues to protein partners was assessed by monitoring the reaction with β(1–4)galactosyltransferase and UDP-Gal. In the case of the liposomes prepared from the shorter-arm GaCD derivative **91**, a single galactopyranosyl residue per GaCD cluster was incorporated, whereas for the longer-arm counterpart **92** a mixture of differently substituted compounds was observed by MALDI-TOF mass spectrometry

In principle, self-assembling of GaCDs should lead to a multivalent presentation of the glycotopes attached to the CD core even in the case of monosubstituted derivatives. With this idea in mind, Parrot-Lopez and coworkers conceived the synthesis of a GaCD derivative containing a single βGal antenna at the C-6 position and lipophilic hexanoyl groups at the O-2 and O-3 positions.[136] The synthetic route implied protection of the primary hydroxyl groups in (C-6)-monoazido-βCD as the corresponding *tert*-butyldimethylsilyl (TBDMS) ethers, followed by acylation of the secondary hydroxyls with hexanoic anhydride and DMAP in pyridine (66% yield). Catalytic hydrogenation of the azido group and amide condensation of the resulting monoamine **93** with a carboxylic acid-armed βGal derivative **94** afforded the target conjugate **95**, for which its ability to form stable Langmuir films was demonstrated (Scheme 38).

The groups of Darcy and Mazzaglia developed a novel prototype of GaCDs in which the hydrophobic tails are installed



Scheme 38 Synthesis of a mono-βGal amphiphilic βCD **95** and schematic representation of its self-association into multivalent Langmuir films.[136]

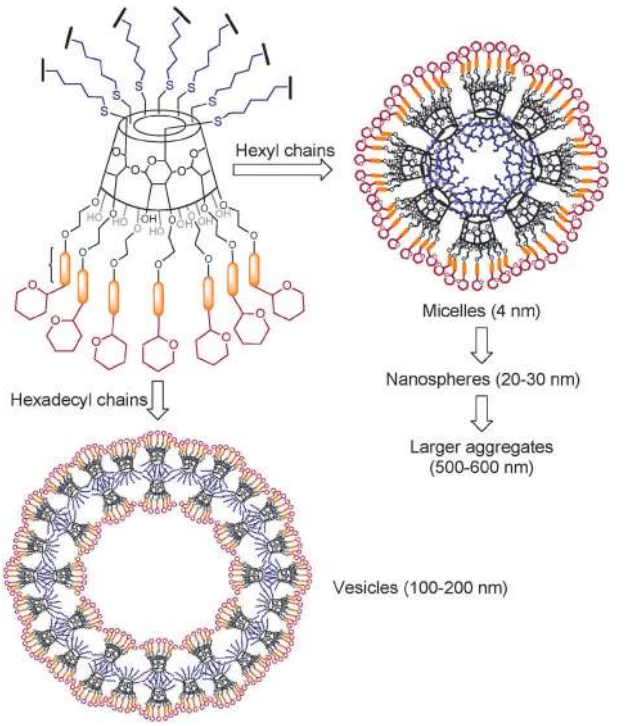


Scheme 39 Synthesis and schematic representations of "jellyfish-type" GaCDs **98**.[140]

at the primary face of the βCD platform and the glycosyl residues at the secondary rim, a "jellyfish-type" arrangement.[137] The synthetic approach benefits from the well-established efficiency of the nucleophilic displacement of (C-6)-bromo groups by thiolate to introduce alkylthio chains of different length, followed by etherification of the more acidic OH-2 groups with ethylene carbonate. This latter reaction creates a new cluster of seven primary hydroxyls (**96**) that were selectively brominated (**97**) by reaction with triphenylphosphine/*N*-bromosuccinimide in DMF. A second multiple nucleophilic displacement round, using now sugar thiolates as nucleophiles, afforded a set of thioether-linked GaCDs **98**.[138,139] Alternatively, the primary hydroxyls in the key precursor **96** can be reacted with glycosyl isothiocyanates to give heptaconjugates in which the glycotopes are linked to the CD core through thiocarbamoyl bridges (Scheme 39).[140]

While the synthetic approach is very flexible, it sacrifices homogeneity and symmetry by affording a polydisperse mixture of heptavalent compounds in which the spacer between the coating sugar and the βCD core can contain a variable number of oxyethylene fragments (average two). Nevertheless, the mixtures were shown to self-organize in water solution after sonication to provide either small micelles (~4 nm) or nanospheres (20–30 nm), that eventually aggregated into larger objects (500–600 nm), or vesicles (100–200 nm). The first trend was preferentially observed for GaCDs bearing hexylthio chains at the hydrophobic domain, whereas hexadecylthio tails favoured the formation of vesicular structures (Scheme 40).[137–140]

The presence of an aqueous compartment both in micellar aggregates and vesicles was evidenced by encapsulation of the fluorescent dye Rhodamine 6G. In any case, a multivalent display of the sugar epitope at the surface of the nanoobject is expected. Binding to specific lectins was investigated by fluorescence techniques, taking advantage of the modification of the triptophane emission after complex formation. In the case of hexylthio conjugates, specific recognition of βGal-coated micellar aggregates by the lectin PA-I from *Pseudomonas aeruginosa*[136–139] and of αMan- and βFuc-modified analogues by *Lens culinaris* LcH lectin[140] was demonstrated. Unexpectedly, selective interactions of vesicles formulated from hexadecyl



Scheme 40 Schematic representation of the self-assembling processes of "jellyfish-like" GaCDs.[137–140]

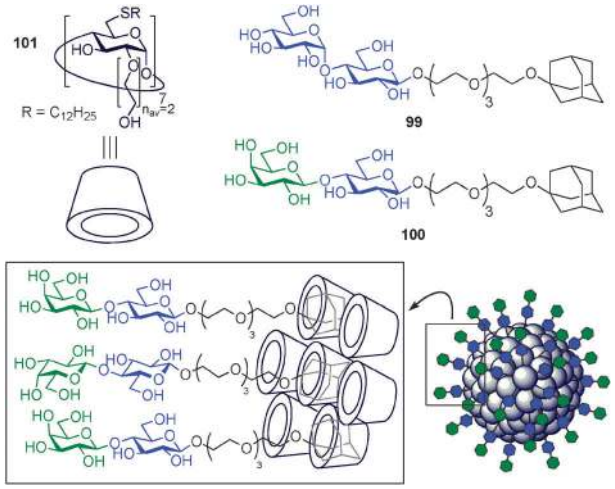


Fig. 10 Amphiphilic CD **101** and glycoadamantyl conjugates **99** and **100** prepared by Ravoo and coworkers (top) and schematic illustration of the multivalent glycovesicles formed by supramolecular ligation.[141]

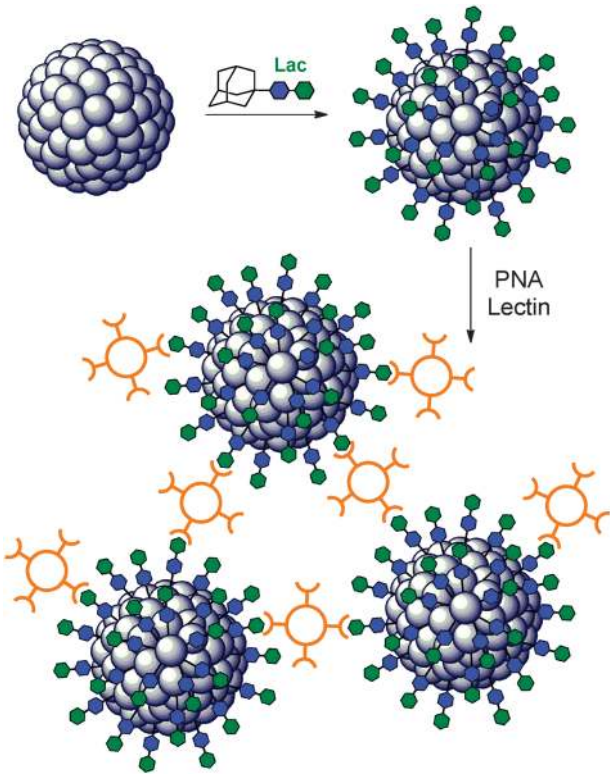
GaCDs exposing the same glycotopes could not be ascertained. The authors argued that binding can be hindered in this case by the vesicular architecture, but a more rigorous analysis would require the implementation of alternative evaluation techniques.

The approach based on (C-6)-thioalkylation followed by (*O*-2)-etherification with ethylene carbonate, prior to glycoconjugation, gives rise to amphiphilic βCD derivatives with a high tendency to self-organize into unilamellar vesicles in water. These CD vesicles exposed the hydrophilic secondary face, the wider entry to the internal cavity, to the external environment. This feature was exploited by Ravoo and coworkers to install glycoligands at the vesicle surface through supramolecular ligation, taking advantage of the high affinity of the βCD cavity towards adamantane moieties.[141] Thus, they prepared β-maltoside (Mal) and β-lactoside (Lac) tetraethyleneglycol–adamantane conjugates (**99**, **100**) and studied their association with CD vesicles obtained from the dodecylthio amphiphilic βCD derivative **101** by ITC (Fig. 10). Association constants of 4.4×10^4 M^{-1} and 4.0×10^4 M^{-1} for **99** and **100**, respectively, were determined, with a very favourable entropic term.

Optical density (400 nm) and DLS studies of the sugar-decorated nanovesicles in the presence of Con A and PNA lectins evidenced the presence of aggregates for those ternary systems where the CD vesicles were present in conjunction with matching pairs of sugar ligand/lectin, *i.e.* Mal/Con A or Lac/PNA (Scheme 41). No aggregation occurred in any other case, demonstrating the specificity of the interaction and the need for a multivalent presentation.[141,142]

4.4. Biomacromolecule-cyclodextrin multivalent assemblies

Multivalent cyclodextrin conjugates are susceptible to interact with biomacromolecules (proteins, nucleic acids) through, *e.g.*, encapsulation of hydrophobic moieties into the CD cavity or electrostatic interactions, to form supramolecular assemblies. Eventually, poly(CD-conjugate)–biomacromolecule complex



Scheme 41 Schematic representation of the aggregation of Lac–CD vesicles with PNA lectin. An analogous process was observed for Mal–CD vesicles with Con A.[141,142]

formation may lead to profound changes in the structure and physicochemical properties of the biomacromolecular partner, often resulting in increased stability in biological media. The glycosyl residues in the CD conjugate exert a notable influence in this process. Moreover, upon self-assembling their presentation mode is also drastically affected, generally leading to increased glycotope densities and, consequently, to enhanced affinities towards lectin receptor partners. The potential of this concept for site-specific delivery of biomacromolecular drugs has spurred intense research in the field in the last few years.[143–146]

In 2006, Uekama and coworkers reported the stabilization of lysozyme and basic fibroblast growth factor by a series of CD derivatives including (*O*-6)-maltosyl α, β and γCD (Mal–CD), being the βCD derivative specially efficient (Fig. 11).[147] The authors rationalized the result in terms of the better fitting of aromatic amino acid residues inside the βCD cavity, favoring the formation of poly(Mal–βCD):protein inclusion complexes. Complexation was actually found to destabilize the native conformation of the protein, favouring a partially unfolded state. The maltosyl substituents exerted a decisive role in preventing interactions with other protein molecules and avoiding the formation of aggregates. This behaviour is consistent with their exposure to the environment, which should make them accessible for lectin recognition events. In spite of the appealingness of the strategy for glycotargeted protein delivery, no research in that direction seems to have been communicated so far.

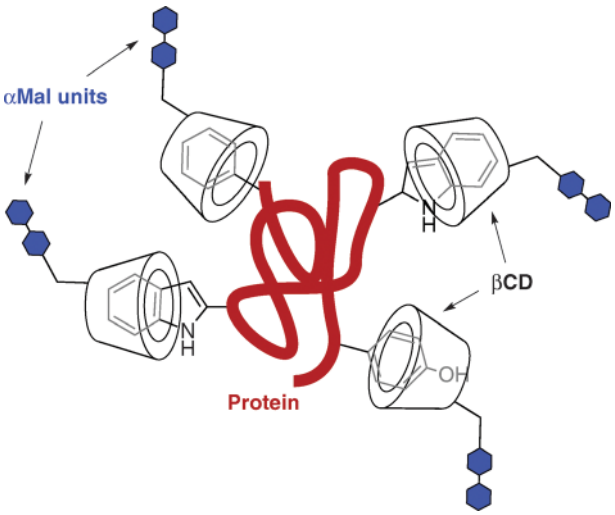
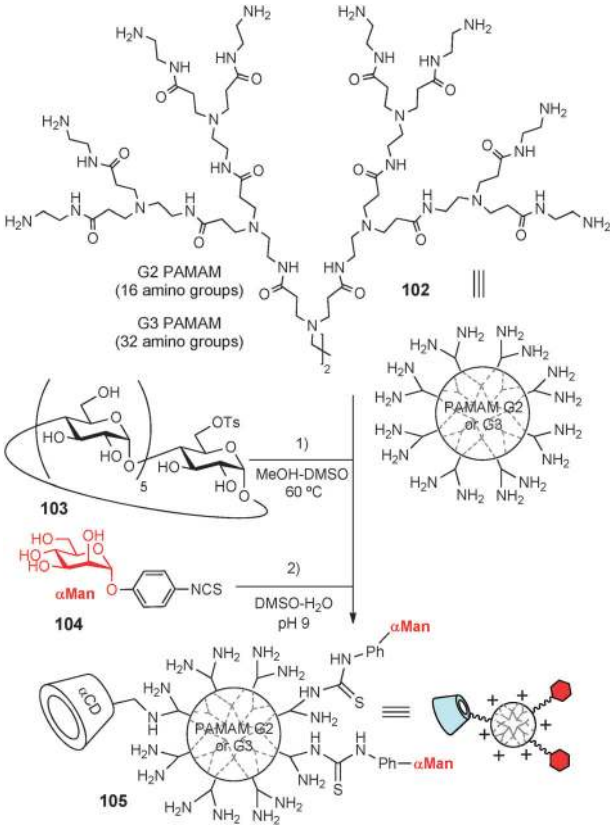


Fig. 11 Schematic representation of the poly(maltosyl-βCD):protein complexes reported by Uekama and co-workers.[147]

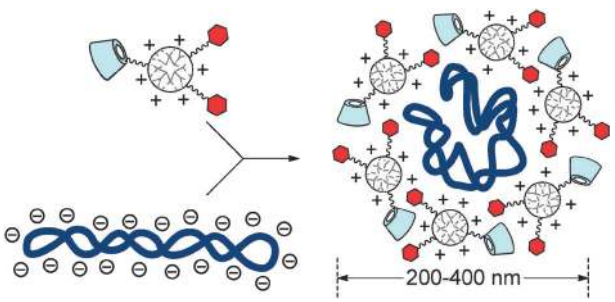
The increasing awareness of the potential of polycationic CD derivatives in the design of nonviral gene delivery systems has led several groups to explore the suitability of polycationic CD conjugates to impart, simultaneously, stability and targeting capabilities of the corresponding complexes with plasmid DNA (pDNA), for which the term CDplexes has been coined.[148–157]

Uekama, Arima and co-workers conceived a series of multifunctional CD conjugates consisting of a poly(amidoamine) (PAMAM) starburst dendrimer **102** onto which one-to-two αCD units (**103**) were randomly attached through a single primary (C-6) position by reaction with the corresponding 6^I-tosyl derivative **103**. Some of the remaining primary amino groups of the dendrimer were subsequently engaged in thiourea forming reactions with isothiocyanate-armed αMan (**104**) and αGal derivatives to afford the corresponding polycationic αCD–PAMAM glycoconjugates. Second (G2) and third (G3) generation PAMAM derivatives (**105**) with different substitution degrees of either αMan or βGal motifs were thus prepared (Scheme 42).[158–166]

In the presence of pDNA, the G2 and G3 αCD–PAMAM conjugates formed nanocomplexes (glycoCDplexes; 200–400 nm) whose stability was dependent on the coating sugar substitution degree (Scheme 43). In general, replacement of PAMAM amino groups by glyco-thiourea moieties was detrimental regarding DNA compaction and protection abilities. However, enhanced serum-resistant transfection capabilities were encountered in several cell lines after optimization of the αMan or αGal loading. Thus, the G3 αMan–αCD–PAMAM system with a substitution degree of 10 sugar units per dendrimer was as efficient as the αCD–PAMAM unglycosylated vector, but with a much more favorable toxicity profile. Unexpectedly, the transfection properties were independent of the expression or not of specific αMan receptors at the cell surface. Competition experiments revealed that, even though receptor-mediated internalization did not operate, the multivalent αMan display at the surface of the glycoCDplexes imparted endosome escaping,



Scheme 42 Synthesis of αMan–αCD–PAMAM (G2 and G3) conjugates prepared by Uekama and coworkers. Conjugates incorporating αGal instead of αMan units were analogously prepared.[158–166]



Scheme 43 Schematic representation of glycoCDplex formation from αMan–αCD–PAMAM conjugates and plasmid DNA.[165,166]

trafficking and nuclear localization abilities, which might arise from interactions with intracellular lectins.[165,166]

The inability of the αMan-coated CDplexes formulated with αMan–αCD–PAMAM conjugates to elicit specific internalization in macrophages, expressing the macrophage mannose receptor (MMR) at their surface, might arise from impaired accessibility of the αMan residues to participate in molecular recognition events. Actually, SPR experiments indicated a rather low affinity of these constructs towards Con A. The corresponding αGal-coated CDplexes similarly behaved as broad range transfection systems, with no selectivity for cell lines expressing Gal-specific lectins such as the asialoglycoprotein receptor (ASGPr) at the

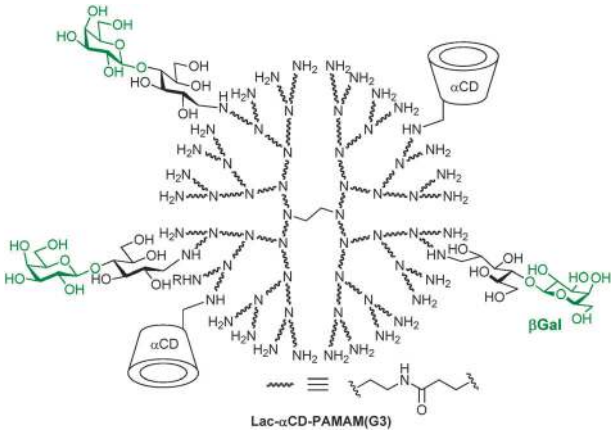
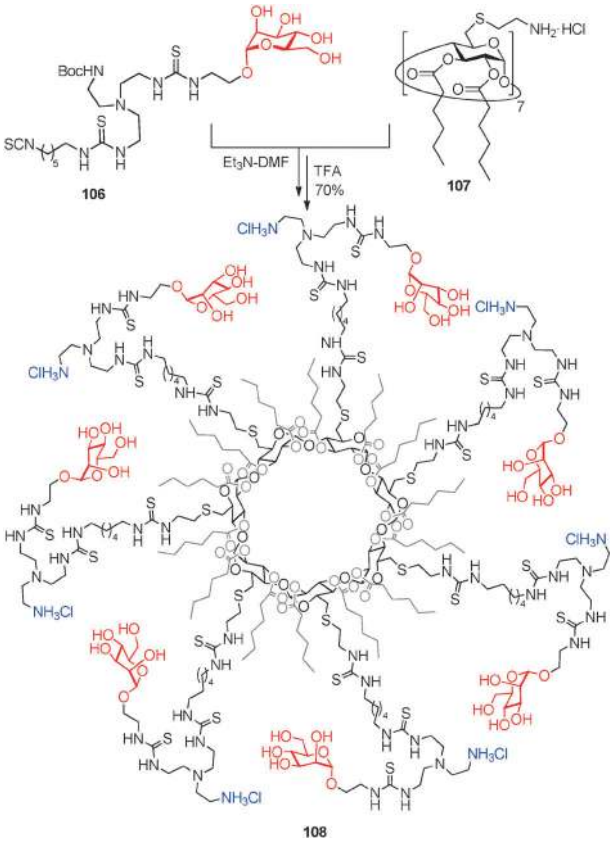


Fig. 12 Lactosylated αCD–PAMAM conjugates (the G3 generation with an αCD loading of 2 and a Lac substitution degree of 3 is shown), obtained by reductive amination, for hepatocyte-specific gene delivery.[163,164]

cell surface of hepatocytes. Looking for constructs well-suited for site-specific gene delivery, direct conjugation of αCD–PAMAM with lactose through reductive amination, to give Lac–αCD–PAMAM conjugates, was next considered (Fig. 12). In this case, the βGal-coated nanoparticles obtained after glycoCDplex formulation exhibited high affinity towards PNA lectin and enhanced hepatocyte (HepG2 cell line) binding ability as compared with non-galactosylated vectors, as evidenced by SPR and flow cytometry, respectively. Both Lac–αCD–PMAM (G2) and (G3) reported high and selective hepatocyte transfection levels, with no appreciable cytotoxicity.[163,164] *In vivo* studies with the G2 glycoconjugate revealed enhanced transfection in the liver and higher biocompatibility than the commercial reference compound JetPEI™-Hepatocyte.[163]

In the frame of a collaborative project aiming at developing CD-based molecular systems for site-specific gene delivery, García Fernández, Ortiz Mellet, Vierling and Defaye *et al.* proposed a new multivalent vector prototype (**108**), characterized by incorporating (i) a cluster of alternated amino groups and glycosyl units at the primary rim of the βCD platform and (ii) a multitail hydrophobic domain at the secondary face (Scheme 44).[167] The synthesis followed a convergent scheme involving, as the key step, the multi-thiourea coupling reaction of an isothiocyanate-armed bifunctional dendritic element bearing an αMan residue and a Boc-protected amino group (**106**) with the per-(C-6)-cysteaminyl-per-(*O*-2,*O*-3)-hexanoyl βCD derivative **107**. Final carbamate hydrolysis afforded the target C_7-symmetric αMan-coated polycationic glycoamphiphilic CD (pGaCD) **108** in 70% yield.

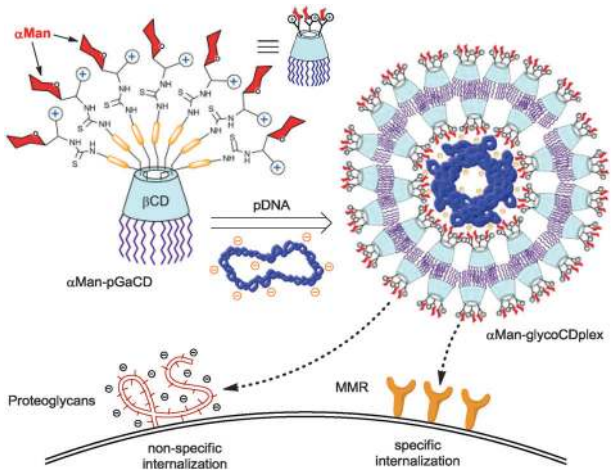
The αMan–pGaCD derivative was found to self-assemble in the presence of pDNA to give nanometric glycoCDplexes (hydrodynamic radius 80 nm) with positive ζ-potential (40 mV). The accessibility of the αMan glycotopes at the surface of the nanoparticles to lectin binding events was confirmed by ELLA using Con A as well as recombinant human MMR (rhMMR) lectins. Adhesion to macrophages was also evaluated *in vitro* using a fluorescently labelled plasmid. The data indicated that binding specificity was dependent on the vector : pDNA ratio



Scheme 44 Synthesis of the αMan–pGaCD gene vector prototype proposed by García Fernández and co-workers.[167]

used for glycoCDplex formulation. At a protonable nitrogen/phosphorous (N/P) ratio of 10, nonspecific binding, probably due to electrostatic interactions with negatively charged proteoglycans at the cell surface, competed with MMR-mediated binding, but the latter was largely predominant at N/P 5 (Scheme 45). Using this formulation, selective macrophage internalization was achieved as evidenced by fluorescent activated cell sorting (FACS), leading to macrophage-specific transfection.[167]

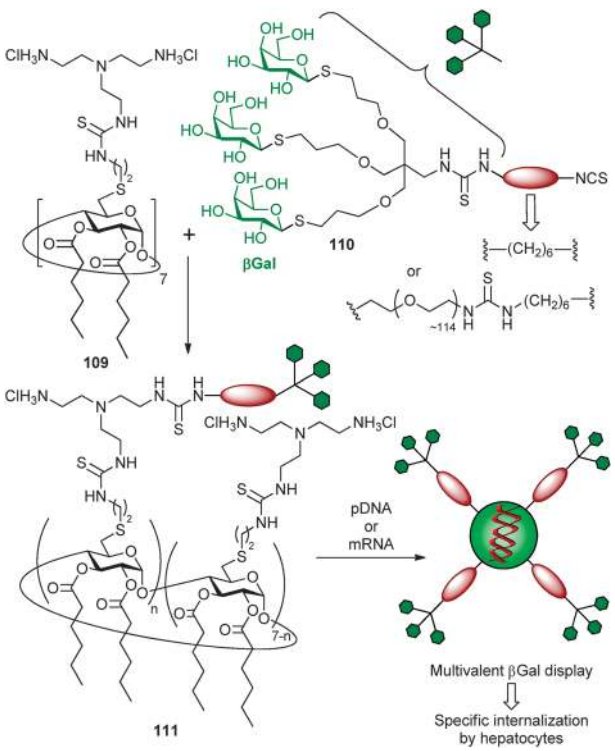
Statistic incorporation of biorecognizable glycotopes onto a polycationic amphiphilic cyclodextrin (paCD) platform represents an alternative strategy to impart targeting abilities to molecular CD-based gene vectors. In principle, the pDNA complexing and lectin binding abilities could be optimized by varying the proportion of the coating sugar. Rejman, García Fernández and co-workers explored this approach for the preparation of βGal-coated pGaCDs (**111**) intended for hepatocyte-specific nucleic acid delivery.[168] Since replacement of cationic centres by sugar motifs in paCDs was detrimental for CDplex stability, grouping the recognition elements into multivalent glycodendrons prior to paCD conjugation was judged convenient. The coating moiety consisted of a trivalent β-D-galactopyranosyl antenna, built on a pentaerythritol scaffold, armed with an isothiocyanate group (**110**) for thiourea-forming conjugation. A tetradecacationic dendritic paCD (**109**) that already had proven to be highly efficient as gene vector was



Scheme 45 Schematic representation of αMan-glycoCDplex formation by self-assembling of αMan–pGaCD in the presence of pDNA, with indication of the two competing internalization mechanisms in macrophages.[167]

chosen as the polyamine partner. Insertion of poly(ethyleneglycol) chains between the glycodendrons and the CD core was additionally considered to improve the bioavailability of the system (Scheme 46).

The βGal-coated polycationic glycoamphiphilic CDs formed stable glycoCDplexes with pDNA and mRNA that were internalized by HepG2 cells through specific interactions with the asialoglycoprotein receptor. Competition experiments demonstrated that the complexes escaped from the endosomal compartments



Scheme 46 Convergent synthesis of βGal–pGaCD conjugates **111** from trivalent βGal dendrons and schematic representation of the corresponding glycoCDplexes after self-association in the presence of pDNA or mRNA.[168]

and released the nucleic acid material into the cytosol. Deceivingly, very low transfection levels were achieved in the case of pDNA, which is consistent with recent results of O'Driscoll and co-workers in a related family of galactosylated CD vectors.[169] The experimental evidence suggested that the nuclear envelop represented the major barrier for efficient pDNA transfection with pGaCDs **111**. The fact that high transfection levels were achieved with mRNA, which is translated in the cytoplasm, much higher as compared with mRNA complexes formulated with the reference compound JetPEI™-Hepatocyte, supported this assumption.[168]

5. Conclusions and perspectives

The increasing awareness of the importance of multivalency in carbohydrate recognition and the number of unanswered questions regarding the mechanisms at work warrants sustained fundamental research in this topic in the following years. In any case, it is now well-established that multivalency amplifies and modulates the biological information encoded by carbohydrates.[170,171] Taking full advantage of this notion requires suitable supports capable of presenting the code signs to the reader partner in a proper manner. Cyclodextrins have largely demonstrated their suitability for those channels. The functional character of the CD scaffold further allows exploiting this knowledge to interfere, control or program carbohydrate binding to biological receptors. By taking advantage of the inclusion capabilities of CDs, receptor-targeted molecular delivery can be achieved through the guest:multivalent CD:lectin ternary complex formation provided that the corresponding supramolecular processes exhibit appropriate equilibrium constants. The magnitude of apparent stability constants for several drug:CD complexes, K in M^{-1}, ranges from 0 to 10^5, the value of $K_{1:1}$ being most often between 50 and 2000 M^{-1}.[172–174] Higher stability constants may lead to drug sequestering.[175] Regarding affinity towards therapeutically useful lectins, association constants in the μM to nM range, which can generally be achieved by exploiting multivalency, may warrant efficient glycotargeting by eliciting receptor-mediated internalization mechanisms.[176] Although hydrophilic cyclodextrins are considered nontoxic at low to moderate oral dosages, parenteral administration may involve toxicity issues that need to be investigated in depth.[174] Chemical tailoring can be further put forward to promote the formation of glycomicelles, films, vesicles or nanoparticles for sensing, diagnosing or biomacromolecule transport. New developments will certainly arise in the near future from the intimate merging of multivalency, supramolecular chemistry and nanotechnology through the cyclodextrin connection.

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