Maximising multivalency effects in protein–carbohydrate interactions

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Multivalent carbohydrates are currently produced in many forms ranging from dendrimers, polymers, micelles, vesicles, nanoparticles to functionalized nanotubes, in order to enhance the potency of the carbohydrates as ligands or inhibitors. Variations in valency range from systems containing two carbohydrate units to those containing more than 2000. In this perspective a number of popular target proteins for multivalent binding/inhibition have been selected. The optimal systems displaying the largest multivalency effects are discussed with respect to their mechanism of multivalent binding.

1 Introduction

Carbohydrates tend to bind only weakly to their complementary proteins. Stronger binding or enhanced inhibition is often achieved by the use of multiple interactions by multivalent carbohydrates. This so-called cluster effect[1] is highly prevalent in nature and has inspired the design of multivalent inhibitors to block protein–carbohydrate interactions. There are many possible designs for multivalent inhibitors. First of all, the valency can be varied from two to very high numbers. Another factor is the relative orientation of the ligands, which might be linear, circular or in a multibranched system. With the recent advent of various types of nanoparticles, new platforms are available with distinct and attractive features. One of the goals for the use of multivalent carbohydrates is to create systems with an enhanced potency relative to a monovalent carbohydrate. True enhancement only occurs if the enhancement holds when it is expressed as a relative potency per sugar ligand. This perspective describes recent studies in multivalency on several popular targets that have been approached *via* different types of multivalent carbohydrates. The focus is on studies that clearly quantify the potency enhancements due to multivalency. Several popular targets have been selected. The potency enhancements of a number of conceptually different multivalent carbohydrates are compared for each of the targets. The outcomes are discussed in relation to the likely mechanism of the potency enhancement for each particular target. Attempting to rationalise data *e.g.* on polymers, dendrimers or nanoparticles in the binding to various protein targets, will lead to useful guidelines for the design of inhibitors with maximised multivalency effects for each particular type of multivalent protein or cell surface.

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2 Mechanisms of potency enhancement

Clearly, one way that can lead to enhanced binding or inhibition is *chelation.* If the protein (aggregate) allows simultaneous binding of more than one (sub)ligand of a multivalent system to more than one binding site of the protein target, the binding of the second (sub)ligand should be enhanced, since translational and rotational entropic penalties were already paid by the first binding event and need to only be paid once (Fig. 1a). The chelation can also occur by two non-identical ligands to two non-identical binding sites. The nature of the spacer separating the (sub)ligands is of great importance. Some of the largest multivalency effects observed are attributed to chelation, especially for the inhibition of AB_5 toxins (*vide infra*) and other multi-site lectins such as the asialoglycoprotein receptor from human liver pioneered by Y. C. Lee[2] or the wheat germ agglutinin (WGA).[3] Enhancements can be as high as 10^3–10^6, for both carbohydrate-based systems and non-carbohydrate systems.[4] Another type of mechanism must be operative in cases where the tether between the carbohydrate (sub)ligands is too short to allow chelation or where the protein contains only a single binding site, but where multivalency enhancements are observed nonetheless. One such mechanism has been named *statistical rebinding*[5] and the effects have been called *proximity/statistical* effects. The effect is caused by the slower off-rate of the multivalent carbohydrate in comparison with a monovalent ligand, due to the close proximity of additional (sub)ligands that can take the place of the first after it releases, resulting in a net increased affinity (Fig 1b). While it is also clear that aggregation occurs in many cases involving multivalent carbohydrates, it is less clear to what extent this enhances the affinity or inhibitory potency and whether

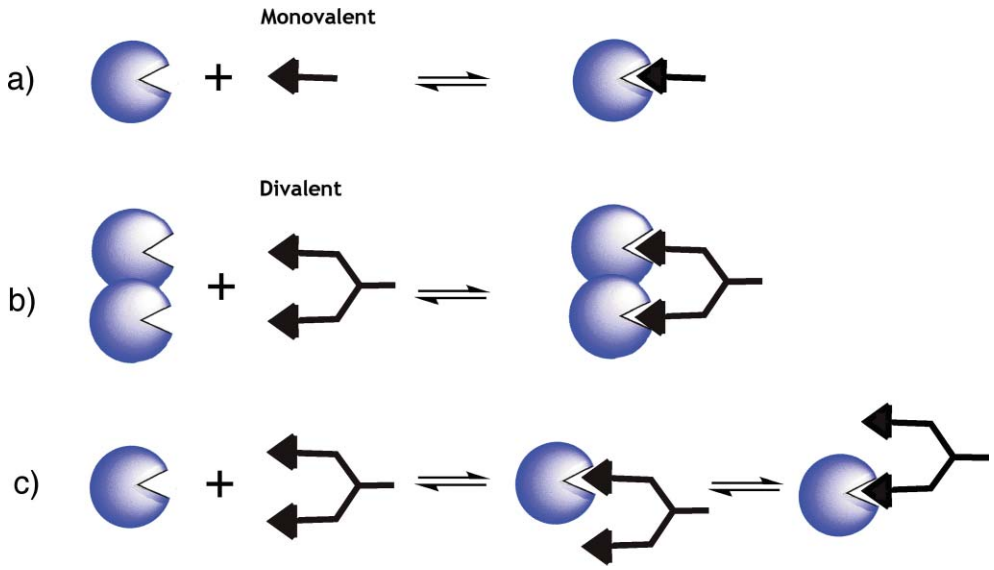

Fig. 1 a) Monovalent binding b) a divalent ligand binding *via* a chelation mechanism, c) a divalent ligand binding *via* a statistical rebinding mechanism.

aggregation phenomena can be separated from the statistical rebinding effects. These non-chelation effects are typically smaller than the effects cases where a chelation mechanism is operative. Nevertheless, when the multivalent ligands are large and contain many (sub)ligands, the effects can be large as well. This was shown *e.g.* for large polyamidoamine (PAMAM) dendrimers and also for large linear mucin derived systems in the binding of lectins. The mechanism in the latter cases has been named the internal diffusion mechanism and also the *bind and slide* mechanism, which is also operative in the binding of protein ligands to DNA.[6]

3 Multivalent carbohydrate systems

The most familiar multivalent systems are the small organic compounds of low valency, based either on a particular organic scaffold, such as a cyclodextrin, a cyclotriveratrylene (CTV), a calixarene, or dendritic systems based *e.g.* on polylysines.[2,7] Dendritic systems can also be significantly larger, such as the PAMAM framework that is available with 128 and even more end groups. Linear polymeric systems also exist and have been made by numerous polymerisation methods. Stoddart *et al.* introduced a variation by threading cyclodextrin units onto a polymer. The cyclodextrin contained the carbohydrate ligands which have increased mobility in this system.[8] Inorganic frameworks have appeared in the literature in the form of nanoparticles. These include gold glyconanoparticles with attached thio-linked carbohydrates. Additional particles include the so-called quantum dots.

Gold glyconanoparticles were first described by Penades *et al.* in 2001 as an alternative multivalent presentation form of carbohydrates.[9] The carbohydrate-containing appendages are attached to the gold surface *via* Au-S covalent bonds. They are typically prepared in a reduction reaction, in which glyco-linked thiols are present along with tetrachloroauric acid. The resulting highly water-soluble particles can be characterized by several conventional techniques such as ^{1}H NMR but also by transmission electron microscopy (TEM) to reveal the particle size. Carbohydrate structures that have been attached to gold nanoparticles include glucose, lactose (see **1**, Fig. 2), maltose, the Lewis X trisaccharide and the Lewis Y tetrasaccharide.[10] A clearly positive point of the gold glyconanoparticles is the ease with which several different components can be attached. Reports have appeared with application of the particles as a cancer vaccine,[11] as a therapeutic to reduce an experimental metastasis,[12] and for the detection of carbohydrate binding proteins such as ricin[13] and cholera toxin.[14] Quantum dots are related particles to which carbohydrates can be appended. These are semiconductor nanoparticles, *e.g.* made up of CdS, which exhibit a strong fluorescence. The carbohydrates, linked to a thiol-terminating spacer, were used as their disulfides in a reaction with sodium sulfide and cadmium nitrate to yield particles with a diameter between 2 and 5 nm such as the maltose functionalized **2**.[15] Attachment of biotinylated glycopolymers to streptavidin-conjugated quantum dots, was demonstrated as an alternative.[16] CdSe-based quantum dots displaying *ca.* 210 GlcNAc moieties were also reported,[17] and also CdSe-ZnS quantum dots of *ca.* 4 nm that display between 2 and 35 sugar moeities.[18] These particles were used for the detection of lectins. The use of thiol-based carbohydrates for the synthesis of such particles also has some disadvantages, as they are incompatible with high temperatures and in some cases are less stable. Alternatively, silica coated quantum dots[19] and other nanoparticles have been developed that have a cross-linked shell surrounding the particle core. Outfitting the silica core with a layer of dextran yielded particles of 10–40 nm that were able to agglutinate concanavalin A (ConA).[19] Finally, another novel platform was described: single-walled carbon nanotubes have been prepared that were outfitted with mannose sugars as in **3**.[20]

4 Plant lectins

Most of the plant lectins are part of the class of simple lectins that are composed of a small number of subunits of moderate size.[21] Many of these have been well-studied[22] since they are

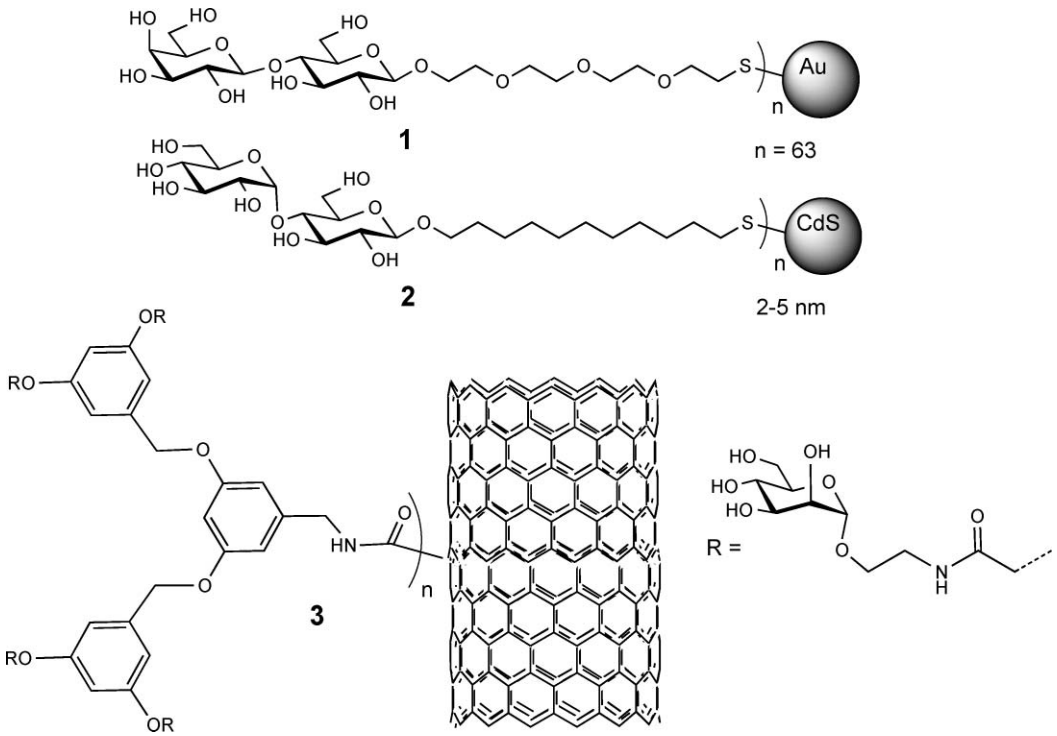

Fig. 2 Novel multivalent platforms displaying carbohydrates: gold glyconanoparticles (**1**), quantum dots (**2**) and single-walled nanotube (**3**).

readily available in large quantities. They show a wide variety of specificities, are useful tools in glycobiology and have functioned as model systems in the study of protein–carbohydrate interactions.

4.1 ConA

Concanavalin A is a popular target lectin from the Jack bean with a binding specificity for mannose but it also binds to glucose. The protein is a tetramer at neutral pH with four subunits in a tetrahedral orientation which leads to a separation between the binding sites of *ca.* 72 Å (Fig. 3).[23,24] Early on, Roy prepared divalent mannosides such as **4** that was 19-fold more potent per sugar in an enzyme-linked lectin assay (ELLA) than the most relevant monovalent reference compound studied: allyl α-D-mannopyranoside.[25] Similarly, relatively small multivalent mannosides were prepared based on metal coordination. A ruthenium complex linked to 6 mannose moieties **5**, showed a relative potency per sugar of 37 in a hemagglutination inhibition assay.[26] Kiessling and coworkers prepared larger systems, *i.e.* mannosylated polymers (**6**), obtained by ring opening metathesis polymerisation (ROMP) that varied in length.[27] The obtained polymers were evaluated as inhibitors of ConA-mediated hemagglutination. A gradual increase was seen in potency for the longer polymers, starting with a 200-fold enhancement for a decamer, with a maximum potency increase of 2000 for a 143-mer, relative to the monomer. This effect was attributed in part to chelation that is possible for the longer systems, along with statistical effects. With a current look at these striking results one can say that they are in line with Brewers observations with the long mucin polymers (*vide infra*)[6] and statistical rebinding mechanisms. Indeed chelation may contribute to some extent. Other polymers showed an enhancement of 500-fold.[28] The use of large dendrimers also yielded major potency enhancements. PAMAM dendrimers outfitted with mannose units (**7**) yielded enhancements of up to 600-fold per sugar in a hemagglutination inhibition assay.[29] Notably, the maximum effect was reached when only about half of the available linkage sites were functionalized with a mannose. Apparently, steric interactions make a denser functionalization less favorable. Since a 4th generation dendrimer seemed required for large multivalency effects, it was suggested that this size was needed to allow chelation, on top of the statistical effects. A study of Mangold and Cloninger[30] on monomeric and dimeric versions of ConA instead of the usual tetramer, helped to delineate the relative contributions of chelation and statistical rebinding effects. The studies involving the monomeric ConA clearly showed that the proximity/statistical effects can be very large. As determined by hemagglutination inhibition studies with monomeric ConA, enhancements of close to a factor of 200 were determined for a large dendrimer containing 172 endgroups. The effects drop off with the size of the dendrimer to only about a factor of three for the smallest dendrimer with 16 mannose ligands. With the dimeric ConA, chelation should be possible for the large dendrimers, but this only added an additional factor of *ca.* 3–4. Aggregation mechanisms can in principle also be the cause of potency enhancements or it can merely be a consequence of multivalent binding. Aggregation was ruled out by Mangold and Cloninger as no evidence was seen for it by dynamic light scattering studies (at 0.2 μM ConA). However, both Toone *et al.* and Brewer *et al.* showed that aggregation occurred with their dendrimers of low valency (up to hexavalent), although the ConA concentration they used was higher (0.09–0.2 mM) and they used tetrameric ConA.[31] Additional studies have emphasized the occurrence of aggregation, and also its importance in biological effects caused by multivalent protein–carbohydrate interactions.[32] Kiessling *et al.*

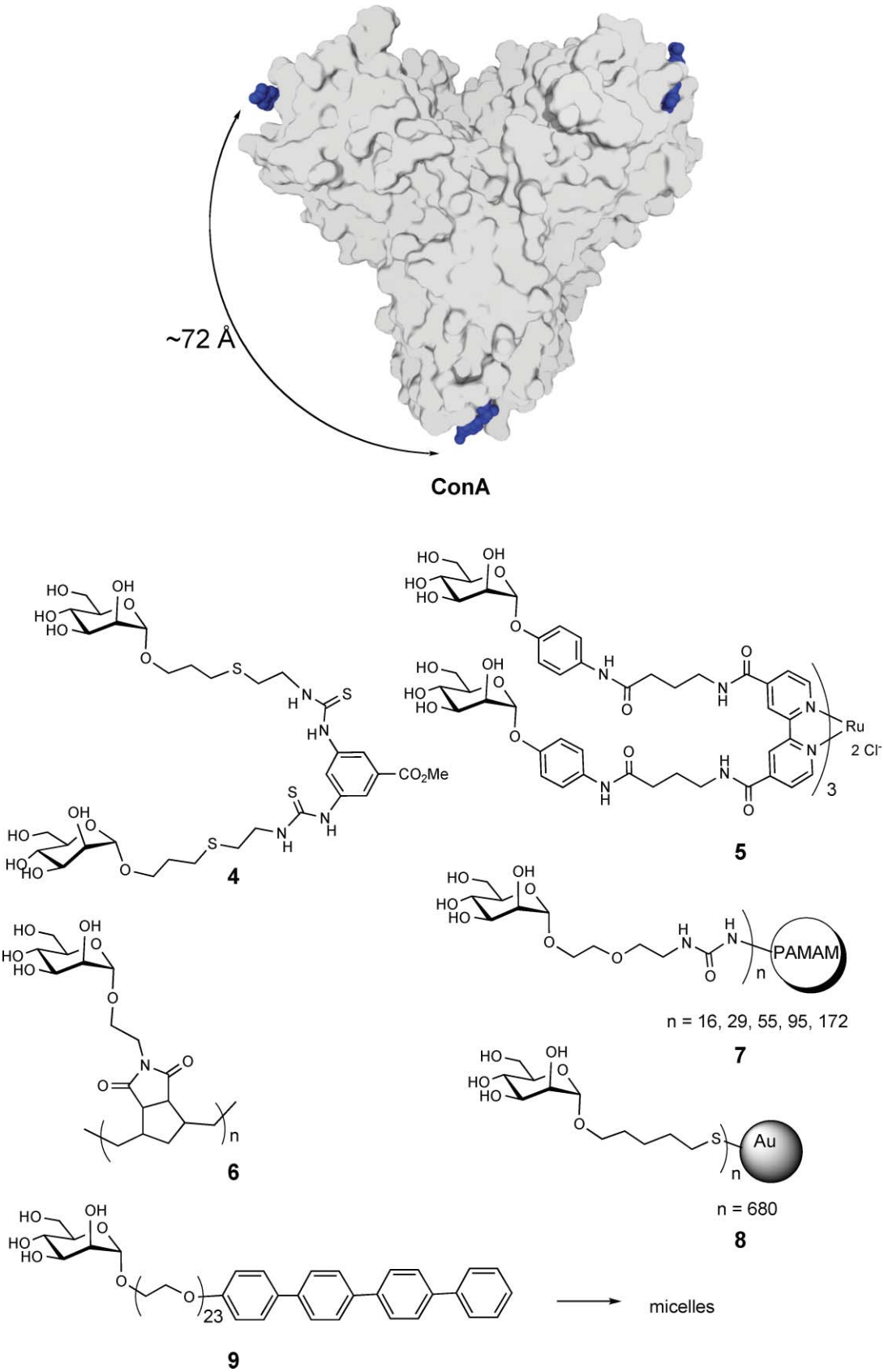

Fig. 3 Structure of ConA with bound mannose derivatives and the structures of selected ConA ligands/inhibitors.

actually detected aggregates of ConA with a trivalent mannoside, by surface plasmon resonance (SPR).[33]

Mannose- and glucose-linked gold nanoparticles were prepared and evaluated as multivalent ConA ligands. The most potent particle in this study was **8**, which was *ca.* 20 nm in diameter and contained *ca.* 680 mannose moieties on the surface.[34] Each sugar was more than a 100-fold stronger inhibitor than monovalent methyl α-D-mannopyranoside. The system is reminiscent of the large PAMAM dendrimers studied by Cloninger *et al.* although somewhat larger and a bit less effective, which could be caused by a more dense display of mannose moieties. More effective particles were prepared by Lee *et al.* and were based on the self-assembly of rod-shaped tetra(p-phenylene)-based vesicles and micelles. Micelles derived from **9**, with a diameter of 20 nm were found to be no less than 1800-fold more potent per sugar than α-D-mannopyranoside.[35] By TEM, sizeable (180 nm diameter) spherical aggregates of ConA molecules attached to the micelles were observed.

In summary, sizeable multivalency effects have been observed with ConA for multivalent ligands of high valency. These can be polymers, micelles, dendrimers or gold nanoparticles. The effects are mostly due to statistical rebinding with additional minor effects due to chelation. This is also consistent with reported experimental results in which ligands of various architectures and valencies were tested in an inhibition assay of ConA.[5b] Maximising the multivalency effects can be achieved by increasing the valency above *ca.* 100–150.

4.2 WGA

Wheat germ agglutinin (WGA) is a dimeric protein with eight binding sites for GlcNAc that are separated by distances of 14 Å and larger (Fig. 4).[23,36] For binding to WGA or its inhibition, several multivalent systems were developed. Two types of calixarenes were used: a tetravalent calix[4]arene fixed in the cone conformation and the more flexible octavalent calix[8]arene. The most potent compound was the tetravalent calix[4]arene compound **10**, which was 312-fold more potent on a per sugar basis than the monovalent GlcNAc. Notably, a compound with an additional branching in the arm linked to the calixarenes was found to be 15-fold less active, despite the doubling in valency, presumably due to steric interference. Wittmann *et al.* evaluated numerous multivalent systems for inhibition of WGA. They found the tetravalent **11** to be 360-fold more potent on a per sugar basis than monovalent GlcNAc.[37] This number was shown to be dependent on the assay, specifically the nature of the matrix on the ELLA plate, a phenomenon that was also observed by others.[38] Quantum dots with an 11 nm diameter displaying 210 GlcNAc units on the surface (**12**), showed strong interactions with the lectin. Addition of the lectin to the quantum dots led to a quenching of the fluorescence. This indicated a K_a of around 10^7, which was *ca.* 3–4 orders of magnitude higher than the K_a of the monomer.[17] For WGA the most potent inhibitors seem to be the quantum dots, but the small tetravalent systems are not far behind. Considering the many relatively closely spaced binding sites, chelation is likely playing a major role in the potency enhancement. The fact that relative low-valency systems already exhibited maximised multivalency effects supports this. The high-valent quantum dots may benefit from additional effects due to statistical rebinding as seen for ConA.

4.3 Other plant lectins

The mentioned work of Mangold and Cloninger[30] showed that non-chelation multivalency effects can be sizeable, but require large systems. Other examples of this were recently shown by Brewer who studied the inhibition of α-GalNAc-specific lectins by a very long linear mucin-derived inhibitor, containing *ca.* 2300 α-GalNAc residues.[39] The enhancement, which was determined by hemagglutination inhibition and isothermal titration calorimetry (ITC), was on average 10^6 for the soybean agglutinin (SBA) and the *Vatairea macrocarpa* lectin (VML) inhibition, or *ca.* 430 per sugar residue. For shorter systems of lower valency (39 sugars) the enhancements were much lower, *i.e.* 3- or 17-fold per sugar for the SBA and VML lectins respectively, clearly showing the need for high valencies to maximize the multivalency effects in non-chelation systems.

5 AB_5 toxins

The AB_5 toxins are a series of toxins that all contain a disease-causing A subunit that is surrounded by five carbohydrate binding B-subunits.[40] These B-subunits attach the toxins to cell surfaces as a first step towards disease. Inhibitors have been designed that block the attachment and can therefore have a therapeutic effect. Besides this, also sensitive detection systems can be developed based on this recognition.[14] A prominent member of the AB_5 toxins is the Cholera toxin (CT); see Fig. 5 for an X-ray structure of its B-subunits (CTB_5) bound to the GM1 oligosaccharide.[41] Another member is the heat-labile enterotoxin of *E.coli* (LT-I). They both bind to the ganglioside GM1. Distances between bound GM1 molecules in neighbouring binding sites are *ca.* 30 Å.[23,41] Furthermore, the Shiga toxins are also a subgroup of the AB_5 toxins and are produced by *Shigella dysenteriae* but also by *E. coli* that produces the Shiga like toxins (SLT-I and SLT-II). These toxins contain three binding sites of unequal affinity for its natural Gb3 ligand (Galα1,4Galβ1,4GlcβCer) per monomeric B-subunit. See Fig. 6 for an X-ray structure of the B-subunits of SLT-I bound to a derivative of the trisaccharide ligand.[42] Distances between binding sites are as short as 13 Å.[23] Since the AB_5 toxins can bind to multiple sugars simultaneously, they are ideal candidates for a multivalent ligand approach, where chelation, *i.e.* bridging adjacent binding sites should be the goal.

5.1 Cholera toxin and the heat labile enterotoxin of *E. coli*

Hol and Fan reported a series of galactose derivatives that were linked to a pentavalent core and found strong potency increases in the inhibition of the B-subunit of LT-I, especially when long spacer arms were used.[43] Their best inhibitor of this series **13** (Fig. 5), was *ca.* 1800-fold more potent than a relevant monovalent reference compound, however, it was still about a factor of 50 weaker than the natural ligand, the GM1 oligosaccharide (GM1os). Their work showed the importance of using a long spacer design, as previously explored by Kramer and Karpen.[44] The bottom line of their approach was that the average spacer length of flexible spacers (which can be calculated and is significantly shorter than the length of an extended chain) should match the distance

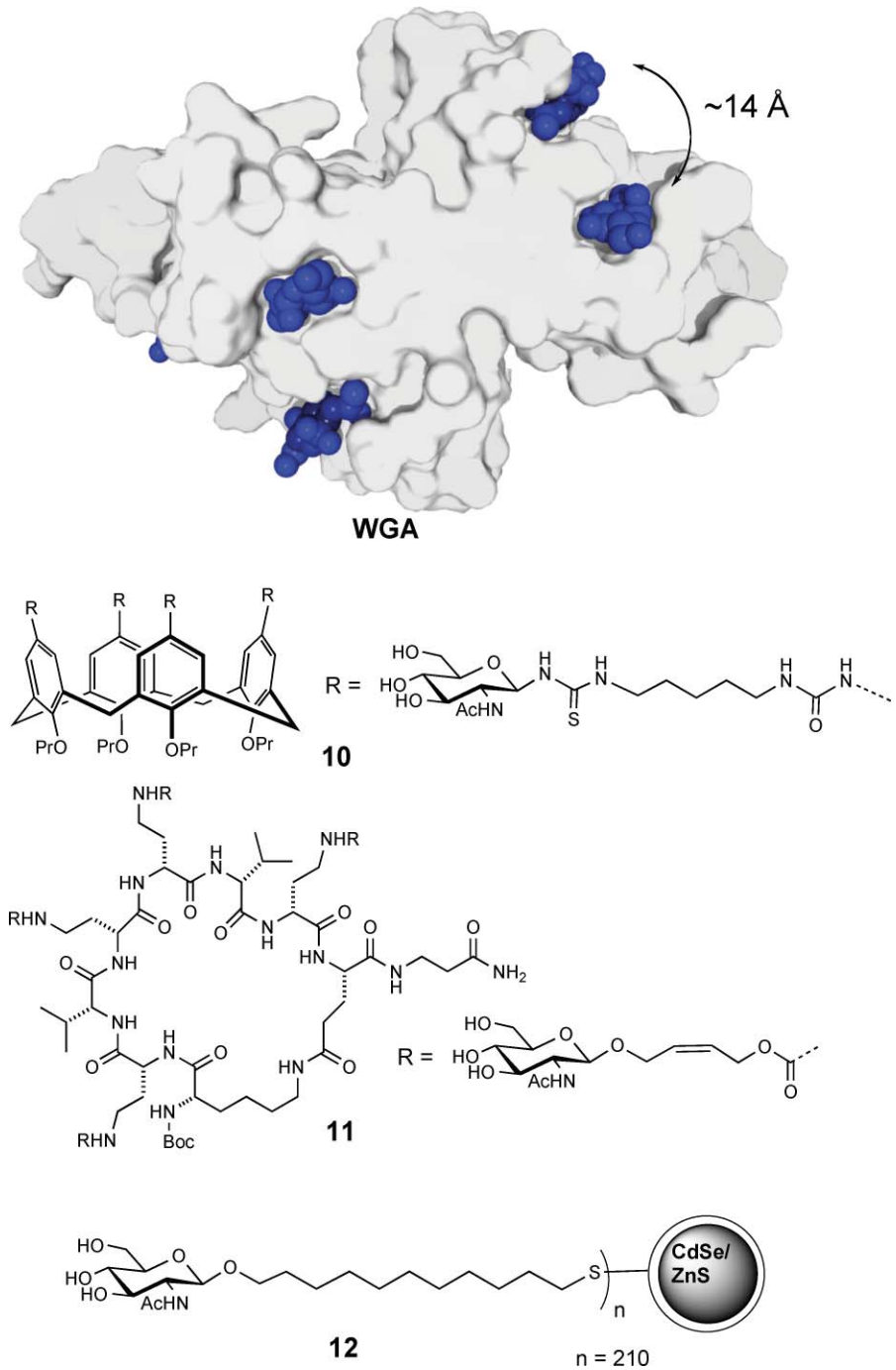

Fig. 4 Structure of WGA with bound GlcNAc ligands and selected WGA ligands/inhibitors.

between two ligand binding sites. Kitov *et al.* described a related method to predict optimal spacer length based on molecular dynamics.[45] In our group we prepared CT ligands based on a dendritic architecture while taking advantage of the long spacer arm design. The compounds were evaluated for inhibition of the toxin's B-subunit (CTB_5) in an ELISA type assay with immobilized GM1 on the plate. In the case where the GM1os was linked to the dendrimers, a multivalent potency enhancement of 47,500-fold per sugar was observed for an octavalent system over a monovalent GM1os derivative, while this number was 20,750 for the shown tetravalent derivative **14**.[46] In the case where galactose was the ligand, the tetravalent **15** was the most potent with a potency enhancement of 2500 per sugar,[47] which yielded compounds that were as potent as GM1os derivatives in this assay. Interestingly, while the first and subsequent systems of Hol and Fan did not show any detectable aggregation, our dendritic systems did strongly aggregate, as observed by multiple techniques,[48] although measured at higher concentration than used in the ELISA assay. Presumably the aggregation was due to a mismatch in the valency of the ligands (2, 4, 8) with that of the toxin (5). Nanoparticles

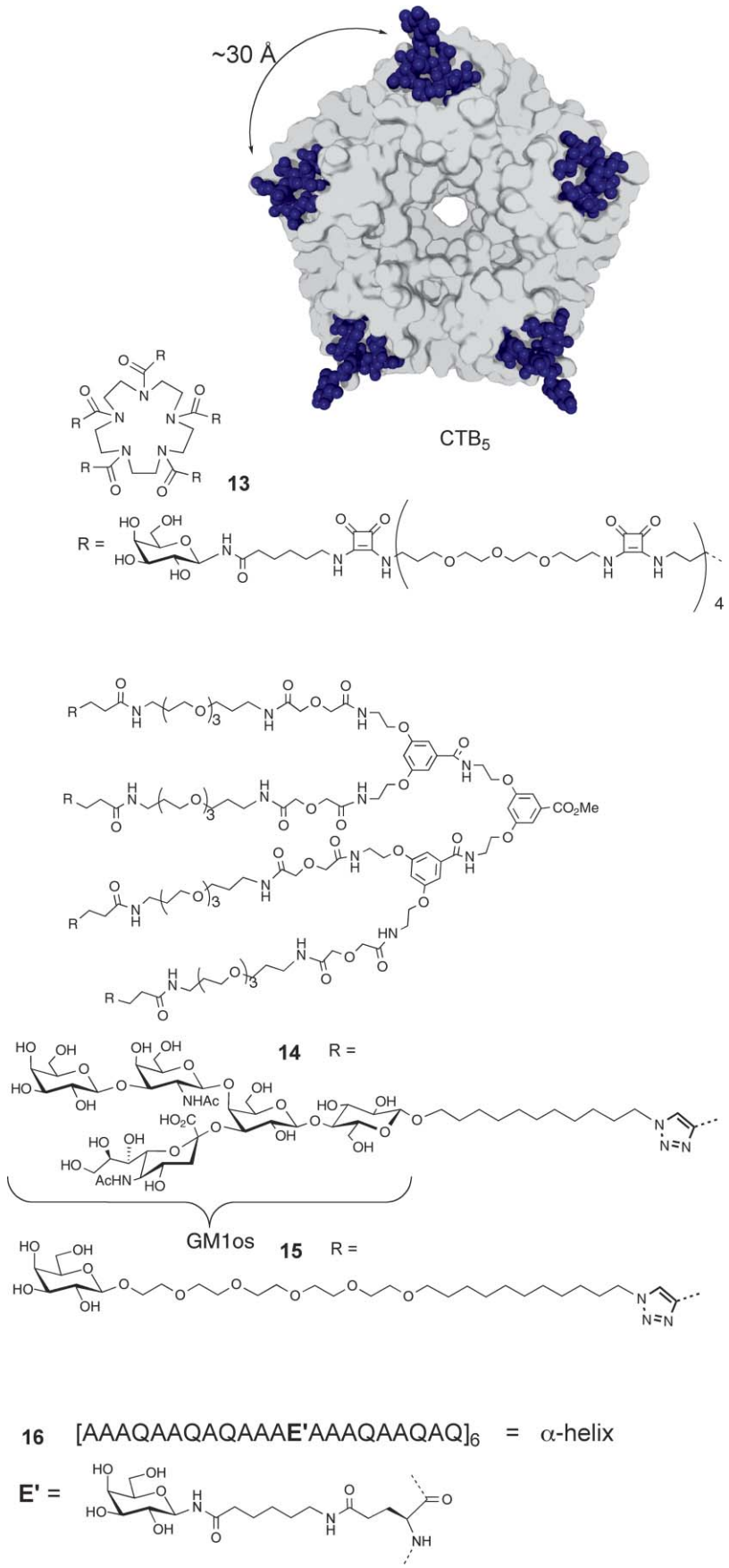

Fig. 5 Structure of cholera toxin B subunit (CTB_5) bound to the GM1-oligosaccharide and selected ligands/inhibitors of the toxin.

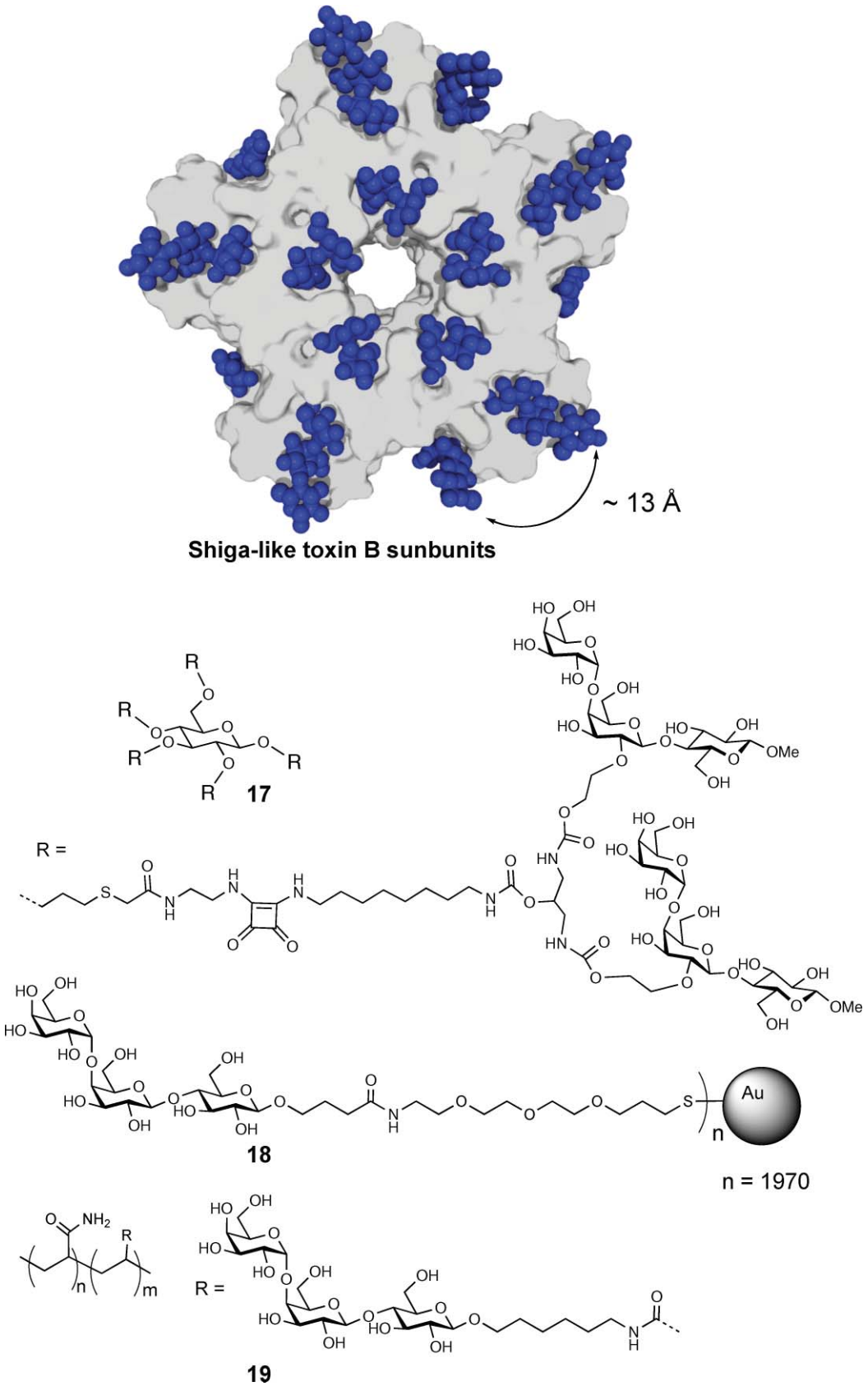

Fig. 6 Structure of the SLT-1 bound to its trisaccharide ligand and selected ligands/inhibitors of the shiga-like toxins.

were also used for the detection of CT but no indication on the multivalency effects was provided in that case.[14] Finally, a recent report appeared in which a well-defined peptide-based scaffold was described.[49] The peptide **16** was helical and contained attached galactose moieties at a defined distance of 35Å, which approximates the interbinding site distance in the toxin. The peptide showed a 340-fold enhancement per sugar in the inhibition of CTB_5 over free galactose. However, the system is not fully understood, since also a related random coil peptide was only a factor 2 less effective. For maximising multivalency effects for CT or LT a low valency is sufficient, but the spacer length is a crucial factor. Chelation seems the clear origin of the large multivalency effects, however additional aggregation phenomena observed for mismatched (with respect to valency) CT inhibitors were not observed for matched cases. It is not yet clear whether or not aggregation phenomena enhance the multivalency effects in CT inhibition.

5.2 Shiga-like toxins

For the Shiga-like toxins, Bundle *et al.* produced multivalent inhibitors using a pentavalent core structure.[50] In compound **17** (Fig. 6) ten globotriose moieties were attached to the five arms that were linked to the glucose-based core structure. Very high inhibitory potencies were observed for SLT-I, with a multivalency enhancement of 875,500, as determined in an ELISA-like assay with immobilized toxin. The design included two globotriose units per arm to bind to two of the binding sites per subunit, however in the crystal structure of the complex it was observed that a hamburger shaped 2:1 complex was formed in which two toxins bound to one inhibitor and the two globotriose units per arm each occupied a binding site in a different toxin. Bundle and coworkers also developed a theoretical model to explain the enhancements in the inhibition of AB_5 toxins. The model emphasized the importance of a statistical term, called the avidity entropy.[51] This term describes in how many ways a multivalent ligand can bind to multiple binding sites. For an octavalent dendrimer the term was very high in comparison to a pentavalent system that matches the valency of the toxin. Experimentally, the octavalent dendrimer indeed proved to be the best inhibitor of the SLT-1, while a pentavalent inhibitor was considerably less effective with relative potencies per sugar of *ca.* 3,000,000 and 8700 respectively, relative to a monovalent globotriose derivative. Gold glyconanoparticles have also been prepared that contain the globotriose sugar.[52] Particles of different sizes were prepared ranging from 4 to 20 nm and containing from 60 to 2000 sugars on the surface. These particles were tested as inhibitors of SLT-I B-subunit binding to an SPR chip surface. The particles proved to be very potent inhibitors with relative potencies per sugar ranging from 1300 for the smallest particles to 228,000 for the larger **18**. The enhanced potencies were also used for the purpose of a sensitive detection method. Polymers with attached globotriose units (**19**) were also prepared and tested as inhibitors of SLT-I.[53] Enhancements of 5000-fold per sugar were determined in this case. In a different study polymeric ligands were used on an SPR chip to detect the two toxins SLT-I and SLT-II, although no indication on the multivalency effects were provided.[54] The ligands contained galabiose/globobiose moieties (Gb2, Galα1,4Gal) and upon addition of a competitor ligand, differentiation between the toxins was possible.

In short, for the AB_5 toxins extremely effective inhibitors have been prepared of various types. Maximised multivalency effects were seen for glycodendrimers and related organic molecules of relatively low valency, although the nanoparticles are not far behind. Considering the topology of the toxins and the large potency enhancements by compounds of low valency, a chelation mechanism is likely.

6 Bacterial adhesion lectins

Bacterial attachment to tissue cell surfaces is often the first step in an infective process. The attachment is in many cases mediated by protein–carbohydrate interactions, where adhesion proteins are positioned on the bacteria. The adhesion determines the species specificity of bacterial pathogens and also the tissue tropism, the preference of bacterial pathogens for certain tissues, and is an attractive process for therapeutic intervention.[55]

6.1 FimH

The FimH protein is involved in the attachment of uropathogenic *E. coli* to the bladder cell surface. The protein is part of type 1 fimbriae, hair-like appendages on the bacterial cell surface. The adhesion protein that contains a single binding site for mannose derivatives, is present at the tip and along the fimbrial shaft at roughly 100–150 nm intervals (Fig. 7)[56,57] as was clearly visible by the attachment of the mannose-outfitted gold nanoparticles in a TEM image.[58] The *E. coli* bacteria as a whole bind in a multivalent manner to the bladder tissue surface. They attach themselves *via* the simultaneous binding of several fimbriae. This was demonstrated by Whitesides *et al.* with the use of force constant determinations using optical tweezers and an artificial mannose surface.[59] The bacterial detachment from a mannose-containing surface was shown to occur in a 'Velcro-like' manner. Monovalent inhibitors have been prepared that showed that a lipophilic aglycon part such as a heptyl group can strongly enhance the binding by a factor of 440-fold over methyl α-D-mannoside.[56] Numerous multivalent inhibitors based on dendrimers of relatively low valency have been prepared.[60] Major multivalency effects were not observed, but rather small enhancements in line with the modest statistical rebinding effects that can be expected for systems of this kind in the binding to monovalent binding sites, as recently seen for **20** (Fig. 7). The distances between the binding sites are too large to allow chelation. This is also true for the mentioned study of FimH-based adhesion by gold nanoparticles **21**.[58] The particles had a diameter of 6 nm and displayed *ca.* 200 mannose units. In a competition with free mannose the nanoparticles bound more strongly, in-line with a statistical rebinding effect, although it is not clear to what extent. Vesicles formed from the rod-shaped terphenyl compound linked to a mannose unit *via* a spacer (**22**) were also suitable binders of type 1 fimbriated *E. coli*.[61,35] The vesicles had a 36 nm diameter and were seen to attach to the fimbriae by TEM. Polymers with attached mannose moieties were also used to bind *E. coli*, with the pupose of detection.[28] To a poly(*p*-phenylene ethynylene) polymer with a number average molecular weight (Mn) of 128,000 and a polydispersity index (PDI) of 1.53, mannose units were coupled. The resulting glycopolymer **23** was able to aggregate bacteria. In a competition experiment with monomeric mannose it was

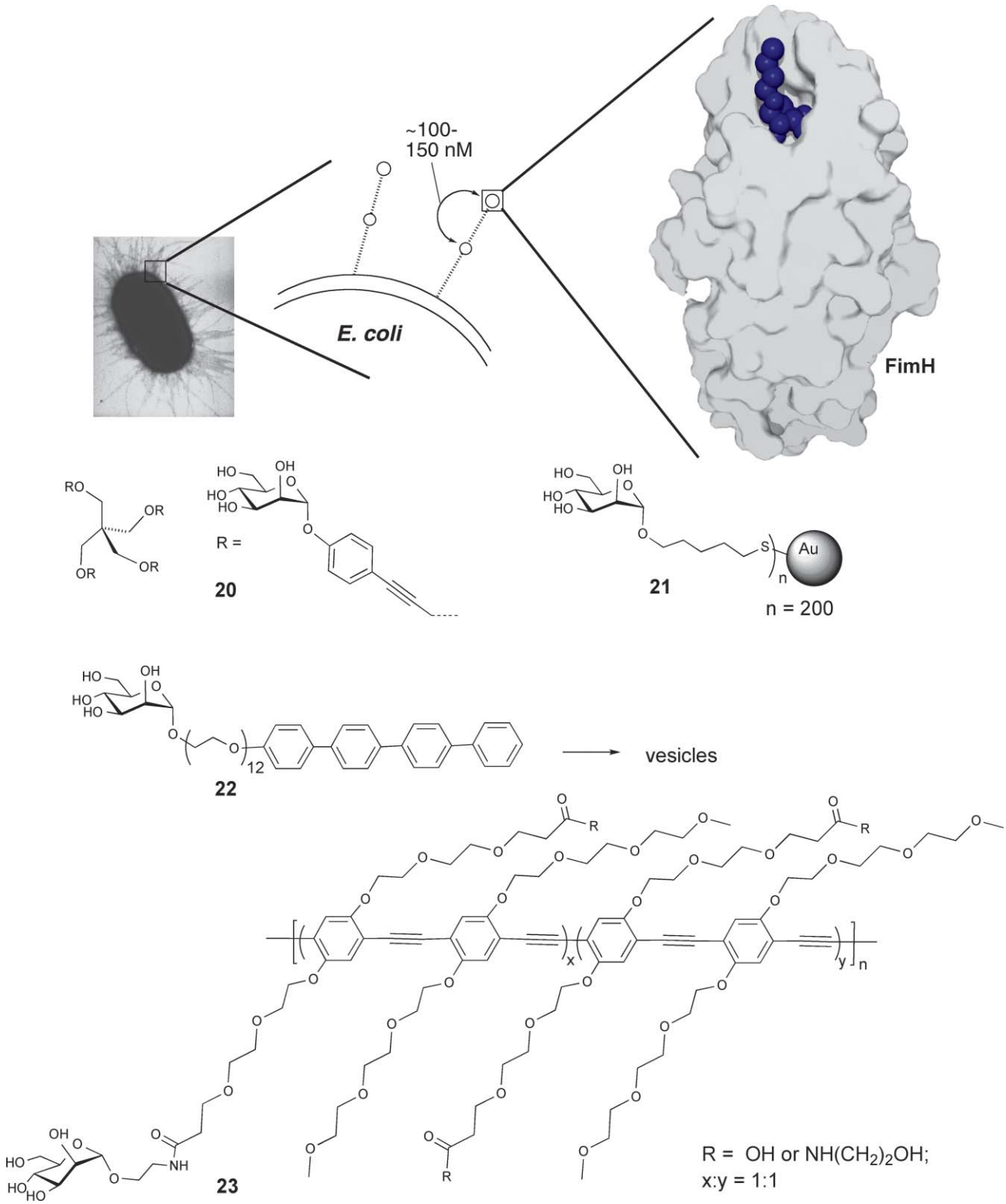

Fig. 7 The FimH adhesion protein as part of the fimbriae of uropathogenic *E. coli* and selected ligands/inhibitors thereof.

shown that a mannose concentration that was *ca.* 3500-fold higher than the polymer-linked mannose concentration was needed for noticeable competition. This is the largest multivalency effect for this target and may be due to bridging of binding sites by these lengthy polymers in addition to sizeable statistical rebinding effects. From the current data it seems that only long polymers are potent multivalent ligands for type 1 fimbriated *E. coli* where the FimH target proteins are far apart (100–150 nm). Maximising the multivalency effects should still be achievable by other high-valent systems such as large glycodendrimers or nanoparticles that can

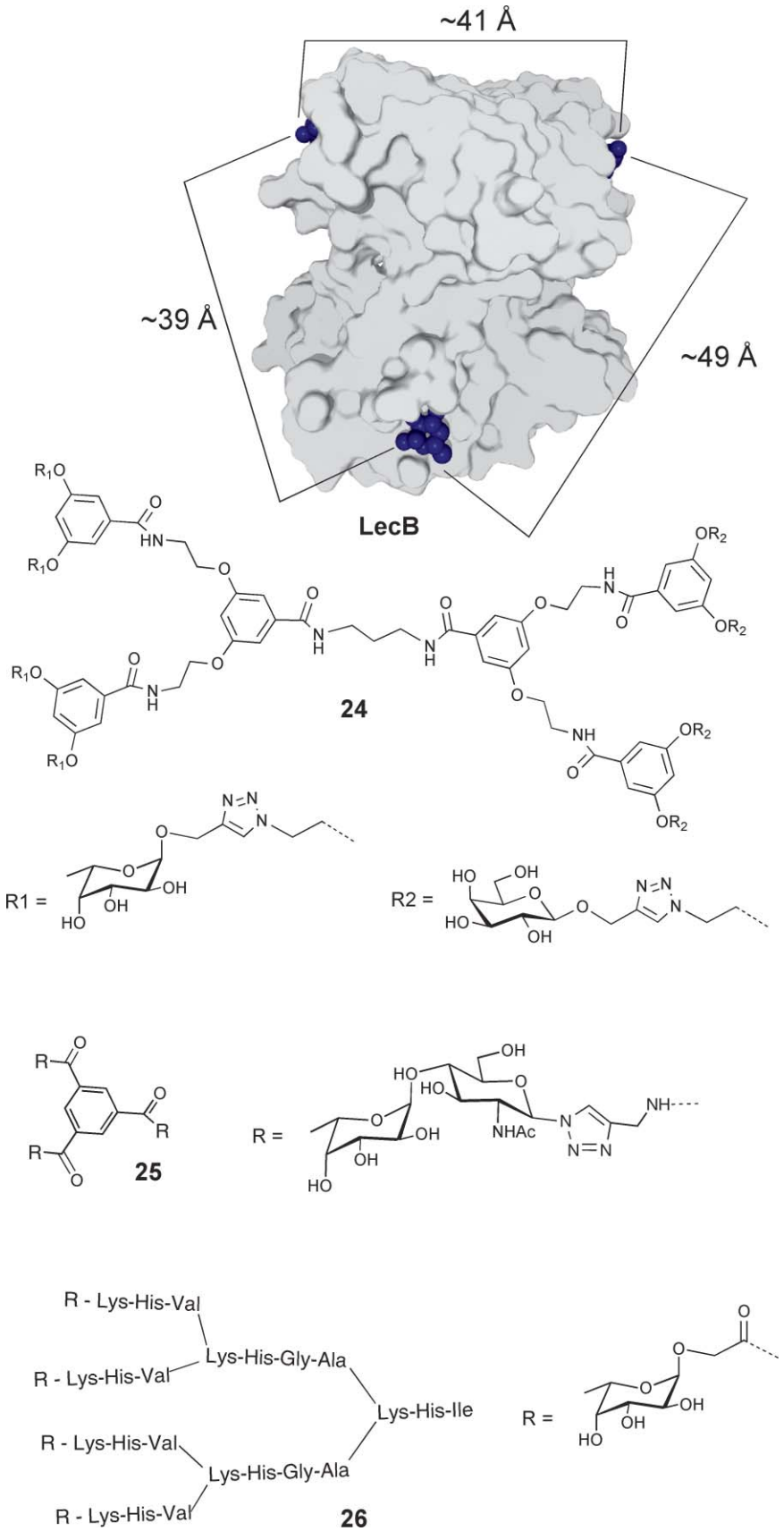

Fig. 8 Structure of LecB bound to L-fucose and selected ligands/inhibitors of lecA and/or LecB of *Pseudomonas aeruginosa.*

exhibit sizeable statistical rebinding effects as seen for conA, but these have not yet been reported.

6.2 LecA and LecB

The problematic pathogen *Pseudomonas aeruginosa* produces lectins that bind to galactose and fucose. These lectins are called lecA and lecB (also named PA-IL and PA-IIL). They are tetrameric lectins, and are positioned on the bacterial surface where they mediate interactions to surfaces of other bacteria, to components of the airway mucosa and to biofilm components. The orientation of the binding sites is such that no chelation is possible with relatively small dendrimers.[62] See Fig. 8 for the structure of LecB with bound L-fucose ligands, separated by 40–50 Å.[23,63] Multivalent ligands for both lectins have been reported. In one study a series of dendrimers was prepared that was functionalized with fucose and galactose residues and also hybrids such as **24** (Fig. 7) were prepared that contained both of them. These compounds were able to precipitate the two lectins, but no indication of multivalency effects was obtained.[64] In another study[62] small clusters of the LecB ligand α-L-Fuc*p*-(1→4)-β-D-GlcNAc such as **25** were studied with ELISA and ITC and no multivalency effect was observed. The compounds tend to aggregate the lectins that readily precipitate. Finally, larger fucosylated linear phosphodiester containing oligomers were prepared. These contained up to 10 fucose moieties. They exhibited only an affinity enhancement of a factor of 2 per sugar unit.[65] Reymond *et al.* prepared a 15,536 member library of fucosylated peptide-based dendrimers.[66] These were screened with LecB and one of their best ligands was **26**, a tetravalent system that was about 11-fold more potent per sugar in comparison to a single fucosylated dendrimer arm. According to the authors the binding enhancement for this compound, which was potent enough to interfere with biofim formation, was most likely caused by interactions between the dendrimer backbone and the lectin surface. As for FimH, maximising multivalency effects should still be achievable by high-valent systems to capitalise on statistical rebinding phenomena.

7 Discussion and conclusions

Many different types of multivalent carbohydrates have been prepared over the years. The objective was often to increase the affinity or the inhibitory potency of these systems. For a number of popular target proteins almost all of the conceivable multivalent platforms have been tested. Looking at these data it is possible to draw some conclusions about the efficacy of each of these platforms with respect to the multivalent nature of the target. Two main mechanisms of multivalent enhancements seem to be operative: *chelation*, in case binding sites can be bridged by a multivalent ligand, and *statistical rebinding*, in cases where this is not possible. Depending on the target protein and the multivalent nature of the ligand, combinations of the two are also possible. The largest multivalency effects have been reported for proteins that allow chelation (up to 3×10^6). In order to maximise the effects, very low valency (<10–12) is sufficient. However, the nature of the spacer is very important. The flexible spacers that are typically used need to be longer (in their extended theoretical conformation) than the distance they need to span. Several methods are available to estimate a suitable length.[43,44,45] Besides low valency systems, larger systems can also be effective for proteins that allow chelation, possibly with additional statistical rebinding effects. For multivalent carbohydrate binding proteins that do not allow chelation, multivalent ligands of low valency invariably show only moderate multivalency effects, typically below a factor of 20. Maximising the multivalency effects requires ligands of higher valency (>*ca.* 100–150), for which enhancements of up to 2000-fold per sugar have been obtained. The nature of these large multivalent systems seems to be less important as large effects have been observed for dendrimers, polymers, nanoparticles, micelles and vesicles. While for the two extreme mechanistic cases the situation seems clear, it should be kept in mind that systems often exhibit a combination of the mechanisms, and also aggregation phenomena that are frequently observed, may contribute to the overall picture, depending on the assay. Looking at the systems discussed here we can summarize the following: for ConA, the most studied target that only allows chelation for the larger systems, the effects are dominated by statistical rebinding effects for large dendrimers, polymers and nanoparticles. LecB of the bacterial pathogen *P. aeruginosa*, is a similar type of protein, but has not been studied as much. For the other bacterial protein, FimH of uropathogenic *E. coli*, the situation is similar, with low enhancements with small systems, and no large ones tested so far, except one polymeric system. The AB_5 toxins clearly allow chelation and small inhibitors have shown very large potency enhancements. For larger systems, such as polymers and nanoparticles very large potency enhancements were also seen. For WGA, a lectin that contains several binding sites that allow chelation, low valent systems already show sizeable potency enhancements, consistent with such a mechanism. For further development, multivalency effects should still be maximised for systems such as lecB and fimH and the many unexplored ones. Furthermore, to fully harness the potential of multivalency effects in ligand recognition, rapid screening methods will also increase the efficiency in the discovery process such as the use of glycodenrimers on microarays.[67]

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