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FULL PAPER

Multivalent thiosialosides and their synergistic interaction with pathogenic sialidases.

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Abstract: Sialidases (SA) hydrolyze sialyl residues from glycoconjugates of the eukaryotic cell surface and are virulence factors expressed by pathogenic bacteria, viruses and parasites. The catalytic domain of SA are often flanked with carbohydrate-binding module(s) previously shown to bind sialosides and to enhance enzymatic catalytic efficiency. Here we designed non-hydrolyzable multivalent thiosialosides as probes and inhibitors of *V. cholerae*, *T. cruzi* and *S. pneumoniae* (*NanA*) sialidases. *NanA* was truncated from the catalytic and lectinic domains (*NanA-L* and *NanA-C*) to probe their respective roles when interacting with sialylated surfaces and the synthetically designed di-, and polymeric thiosialosides. *NanA*-L domain was shown to fully drive *NanA* binding, improving affinity for the thiosialylated surface and compounds by more than two orders of magnitude. Importantly, each thiosialoside grafted onto the polymer was also shown to reduce *NanA* and *NanA-C* catalytic activity with a 3000-fold higher efficiency compared to the monovalent thiosialoside reference. These results extend the concept of multivalency for designing potent bacterial and parasitic sialidase inhibitors.

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Introduction

In the exoglycosidase classes, the sialidases (SA), or neuraminidases, that occur in animals, virus, pro- and eukaryotic microorganisms, are of particular interest for developing new anti-infective therapies. In a wide range of virus and pathogenic bacteria, SA have been reported as virulence factors removing the sialic acid from sialoglycan molecules of host cells, in the primary step of infection. SA activity unmasks underlaying ligands in which the bacteria and their toxins can adhere and/or be internalized.[1] Bacterial SA have also been shown to promote the formation of biofilms, although the exact mechanism has not been elucidated.[2] Parasites such are Trypanosomes are unable to synthesize sialic acids but express *trans*-sialidases that are used to transfer sialic acids from the host cells to coat glycans on the pathogen surface. This dampens the host immune system and helps parasite survival.

Although all *exo*-sialidases from glycoside hydrolase 33 family have a common catalytic domain (CAT) with a six-bladed β-propeller fold, most of them also contain various additional domains including membrane-binding domains, carbohydrate-binding modules (CBM), or lectin-like domains (LD). This structural diversity has been poorly studied and has not yet been exploited to design efficient and specific SA inhibitors. In a pionneering study, Boons and co-workers showed that large SA from *C. perfringens* or *V. cholerae* flanked with a lectin domain, were more efficient in hydrolyzing sialic acid residue from a polyvalent 3'-sialyl *N*-acetyllactosamine substrate, than to a monovalent sialyl analogue.[3] This effect was not observed with the small SA from *S. typhimurium* that lack a CBM, highlighting the crucial role of the lectin domain in SA catalytic efficiency. On the other hand, previous reports described the efficiency of glycoclusters[4] bearing multiple sialic acids or transition state sialosyl cations analogues for the synergistic detection[5,6] and inhibition of sialic acid binding proteins[7,8] or viral neuraminidase,[9–12] respectively.

Inspired by these results, we reasoned that multivalent thiosialosides could act as potent inhibitors of pathogenic SA by binding the CAT and/or CBM domains. Thiosialosides should be stable to enzymatic hydrolysis thanks to the anomeric sulfur atom, and able to interact in the CAT and CBM when multivalently displayed on an appropriate scaffold and linker. Furthermore these glycoclusters are interesting probes to study the contribution of the CAT and CBM domains in the SA catalytic efficiency in more depth.

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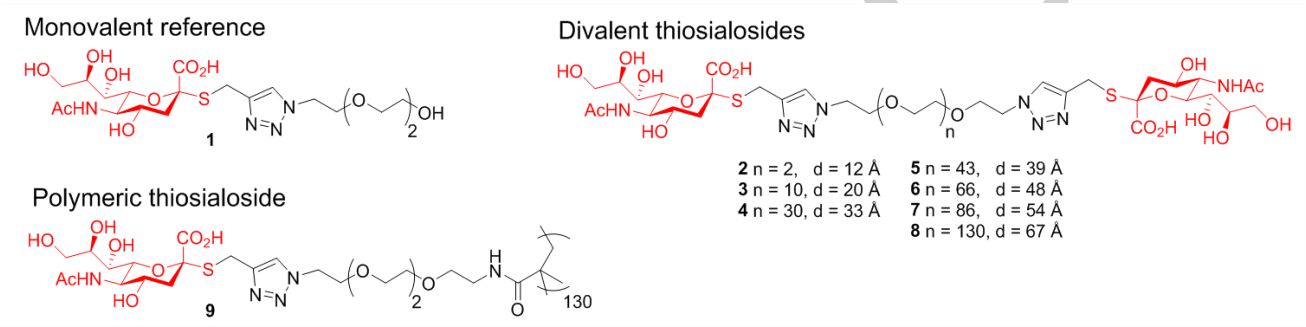
Divalent sugars with a fine-tuned spacer arm length have proved efficient binders for the multimeric cyclic nucleotide-gated channels[13] and lectin such as wheat germ agglutinin (WGA),[14] dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN),[15] and LecA from the bacteria *Pseudomonas aeruginosa*.[16,17] The synergistic effects observed were ascribed to a highly favorable protein chelation by the second sugar ligand due to the dramatically increased sugar concentration in the second binding site proximity. To evaluate the possibility of reaching such a chelation on the CAT+CBM, we designed divalent sialosides **2**-**8** using different sized PEG linkers (from n=2 to n=130, Figure 1A). The largest length, n=130, is within 5% of the theoretically maximal multivalent effect according to the statistical physics model which assumes a Gaussian chain for polymer (Figure S1).[18] The effective length of the PEG spacers in solution is much shorter than the extended conformation and proportional to the root mean square of the PEG molecular weight.[13] Using this polymer theory we empirically estimated the average sugar distance for **2**-**7** which varies from 12 to 67Å.

A)



B)

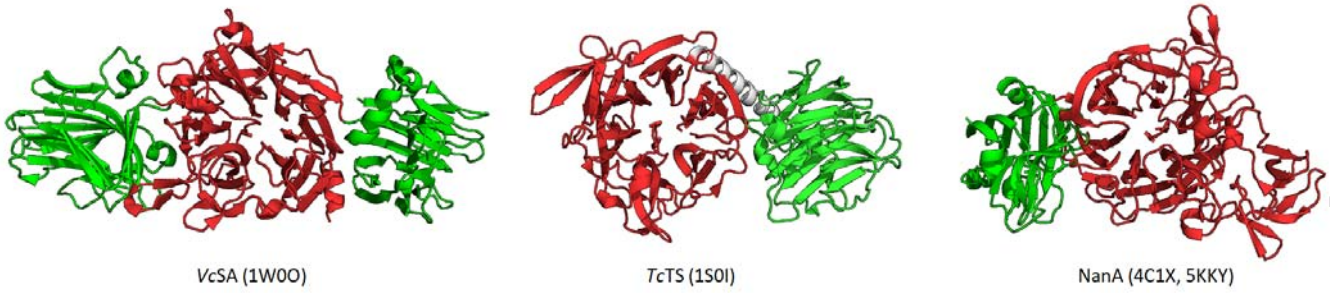


Figure 1. A) Structure of the synthetic thiosialosides **1**-**9**. B) Structure of the SA targets where the catalytic domain (in red) is flanked with a lectinic domain (in green).

Polymeric sugars can surpass the inhibitory potency of their monovalent references by several orders of magnitudes.[19–22] This was explained by a model where the multimeric lectin jump from one epitope to another along the polymer chain, reducing the kinetic off-rate and thus enhancing the affinity.[23] To estimate if such "bind and jump" mechanism can occur with the targeted SA, we designed the glycopolymer **9** with an average of 130 thiosialoside ligands per chain. To compare the potential improvement in affinity or inhibition due to multivalency, a monovalent reference is required with chemical groups in the binding site environment structurally as close as for the multivalent **2**-**9**. Consequently, we designed thiosialoside **1** bearing a triazol group and a short PEG spacer. The enzyme targets selected were three different *exo*-sialidases (*Vibrio cholerae* sialidase- *VcSA*, *Trypanosoma cruzi* trans-sialidase – *TcTS*, *Streptococcus pneumoniae* Neuraminidase A - *NanA*) which are representative of the structural variety found in the GH33 family in terms of an additional carbohydrate binding module flanked to the catalytic site (Figure 1B). The structural domains of these SA have been solved and are available from the Protein Data Bank (PDB),[24–26] which will be used for interpretation of the inhibition and binding data.

VcSA removes sialic acid from higher order ganglioside to reveal GM1 ganglioside, a ligand for the cholera toxin, which causes acute infection of the intestine. *VcSA* contains a catalytic domain flanked by two lectin-like domains (Figure 1B). For the LD at the C-terminal end of the catalytic domain, the ligand is unknown, but the other LD at the terminal end binds to the sialic acid with a K_d of 30 µM, which is one of the highest

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affinity reported for the recognition of a monosaccharide by a CBM.[24]

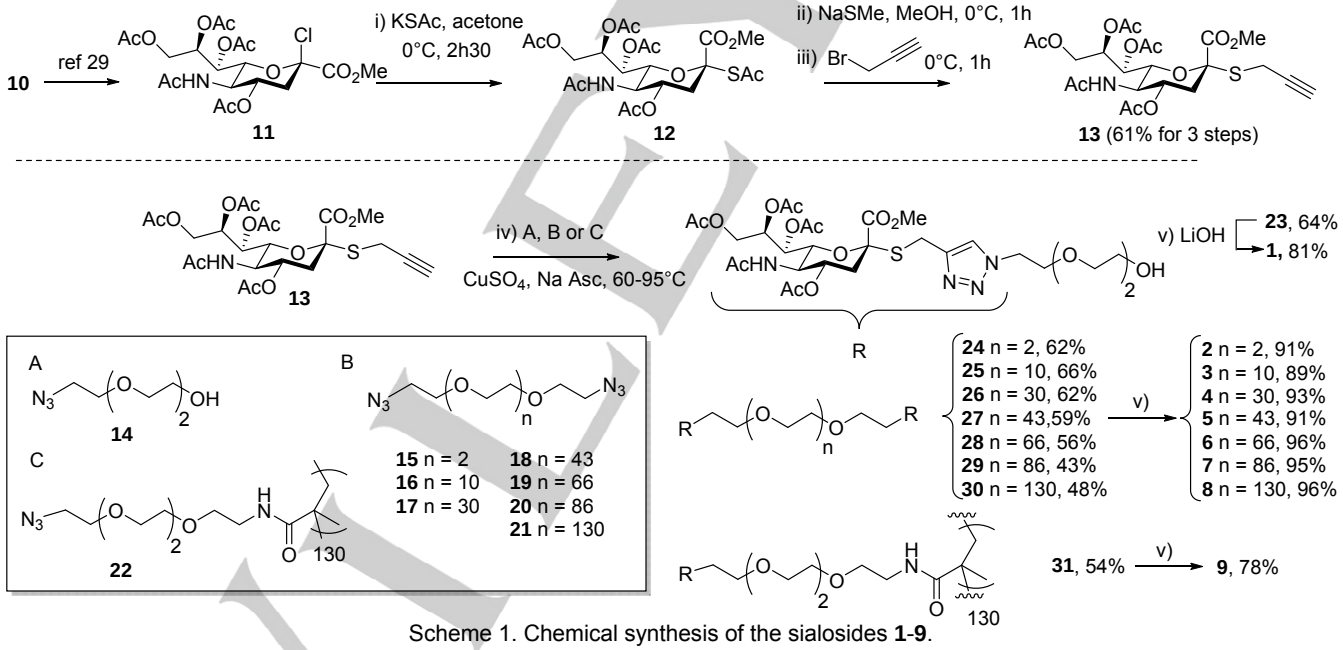
TcTS from the protozoan catalyze the transfer of sialic acid residue from host to parasite glycoconjugates, which is critical for *T. cruzi* survival and cell invasion capability. *T. cruzi* is the causative agent of Chagas'disease, which has affected over 20 million people in South America. *TcTS* displays a lectin-like domain (LD) fused to the C-terminal end of the catalytic domain. Little is known about the function and ligand specificity of these LDs but glycan array studies indicate that mannose-containing oligosaccharides and Gal, GalNAc and LacNAc containing oligosaccharides are binding partners of these LDs.[25]

This work mainly focuses on *NanA*, a virulence factor present in 100% of strains from *S. pneumoniae*,[27,28] causing meningitis, septicemia and respiratory tract infections. The catalytic domain of *NanA* is flanked by a *N*-terminal CBM40 domain previously shown to be critical in *S. pneumoniae* adherence and invasion of the blood-brain-barrier.[29]

To date, inhibitors of *NanA* were only directed towards the catalytic domain,[30] but in recent cloning, ligand analysis and crystallization of the CBM domain revealed a specificity for sialosides which may open new perspectives in terms of drugs design.[26,31] Here, the affinity and inhibitory activity of the synthetic multivalent thiosialosides were assessed against full length *NanA*, the catalytic domain (*NanA-C*) and lectin domain (*NanA-L*). The production of the truncated domains offers a unique opportunity to decipher their relative role in sugar binding and catalytic efficiency.

Results and Discussion

Chemical synthesis of the thiosialosides. The chemical synthesis (Scheme 1) starts with the hydroxyl protection and anomeric activation of *N*-acetylneuraminic acid **10** in two steps to form **11**.[32] The anomeric sulfur atom of **12** can be easily introduced by a nucleophilic substitution of the chlorine atom with potassium thioacetate in acetone. We failed to obtain **13** by chemoselective thioacetate deprotection protocol with sodium methanolate at low temperature, followed by propargylation of the thiol.[33] **13** was obtained from crude **12** after acetate deprotection and thioalkylation with propargyl bromide. The ligand could be efficiently grafted to scaffold azides **14**-**22** (synthesis in SI) by copper-catalyzed azide-alkyne cyclization (CuAAc) with yields ranging from (43 to 66%) for compounds **23**-**31**. Residual copper was removed after washing with EDTA or using a Chelex resin. After acetate deprotection under Zemplèn conditions, the high molecular weight compounds **3**-**9** were purified by size-exclusion chromatography.



Scheme 1. Chemical synthesis of the sialosides **1**-**9**.

Inhibitory activity of the thiosialosides. *VcSA* is commercially available, the full procedure to produce *TcTS* and the *NanA*, *NanA-C* and *NanA-L* constructs is described in the supplementary material.

The inhibitory activity of **1**-**9** against *VcSA*, *TcTS* and the *NanA* constructs was studied with 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU-NANA) as the enzymatic substrate releasing sialic acid and fluorescent 4-methylumbelliferone (λex = 360 nm and λem = 455 nm) after enzymatic hydrolysis (Figure 2A). The IC_{50}^{mol} of the compound is classically reported and defined as the molecule concentration required to inhibit the initial hydrolysis velocity of the enzyme by 50%. However, for multivalent ligands this led to inflated inhibitory potencies and a more objective comparison of the inhibition requires to correct this value by

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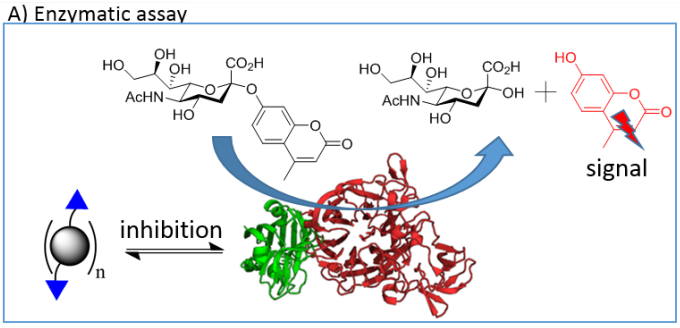
the ligand valency. All the IC_{50} values are reported herein in mole of sialosides (IC_{50}^{si}) instead of molecules (IC_{50}^{mol}), the former being corrected by the valency V (IC_{50}^{si} = V x IC_{50}^{mol}). The relative inhibitory potency (RIP) compared to our monovalent reference **1** (RIP = IC_{50}^{1} / IC_{50}^{si}) will also be provided to directly assess the level of a potential synergistic (or multivalent) effect (if RIP>1).

IC_{50}^{si} values obtained for *VcSA*, *TcTS*, *NanA* and *NanA-C* after Dixon plots analysis (Figures S3) are presented in Figure 2B. The monovalent ligand **1** did not show any inhibition (n.i.) for *VcSA* at high concentration (4 mM) and only a moderate inhibition of *TcTS* (IC_{50}^{si} = 666 µM). These results confirmed that a monovalent interaction of simple sialosides with *VcSA* or *TcTS*, lacking the lactose unit of the natural substrates α-2,3-sialyllactose and α-2,6-sialyllactose is weak. Sialyllactose was previously crystallized in the catalytic domain of *VcSA* and a non-hydrolysable analog was shown to interact strongly (K_d of 30 µM) with the CBM.[24] For *TcTS*, lactose displayed a much greater affinity (K_m = 10 µM) compared to sialic acid (K_m = 6 mM).[25] The low inhibition observed here was not improved with divalent **2**-**8**.

A) Enzymatic assay



B) Inhibitory activity

		IC_{50}^{si} (µM, valency-corrected)			
Cpds	$V_{(n)}$	VcSA	TcTS	NanA	NanA-C
1	$1_{(2)}$	n.i.	666 ± 22	1042 ± 20	426 ± 53
2	$2_{(2)}$	n.i.	1194 ± 208	1005 ± 29	1485 ± 58
3	$2_{(10)}$	n.i.	1879 ± 226	413 ± 46	517 ± 5
4	$2_{(30)}$	n.i.	2542 ± 133	125 ± 7	58 ± 4
5	$2_{(43)}$	n.i.	1189 ± 97	48 ± 2	20 ± 1
6	$2_{(66)}$	n.i.	1065 ± 112	113 ± 7	41 ± 3
7	$2_{(86)}$	n.i.	1766 ± 66	16 ± 2	15 ± 1
8	$2_{(130)}$	n.i.	269 ± 39	14 ± 1	4 ± 1
9	$130_{(2)}$	103 ± 7	3.2 ± 1.4	0.343 ± 0.048	0.143 ± 0.044

IC_{50}^{si} expressed in µM of sialosides (valency-corrected). n.i. no inhibition at 4 mM. Results from triplicates.

C) Relative inhibitory potency (RIP) per thiosialoside

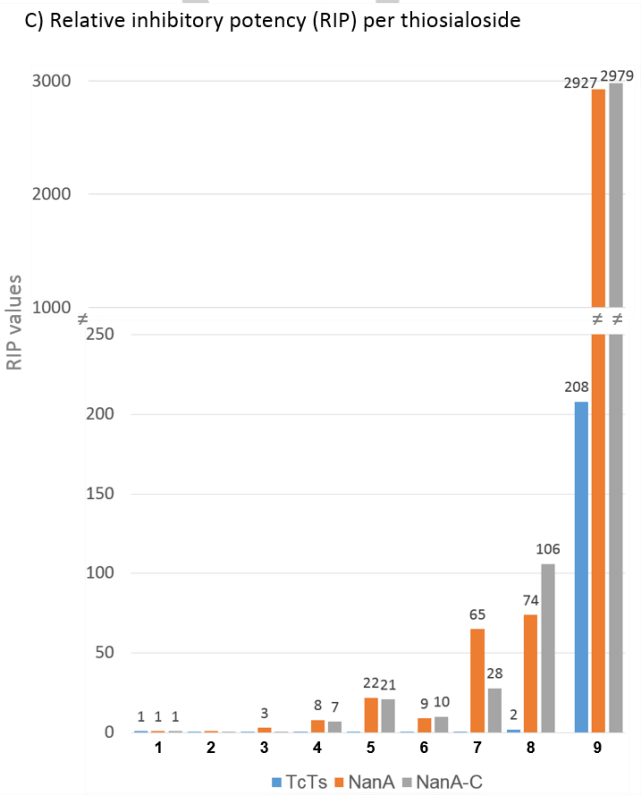


Figure 2. A) Illustration of the enzymatic assay. B) Inhibitory activity of thiosialosides **1**-**9** against *VcSA*, *TcTS*, *NanA* and *NanA-C*. IC_{50} values are expressed in mol of thiosialosides (valency-corrected). Experiments were performed in triplicate. C) Valency-corrected inhibitory potency of multivalent thiosialosides **2**-**9** compared to **1** (RIP values).

If rebinding effects and chelate binding modes are potentially operating, they do not seem to significantly impact catalytic activity. In stark contrast, sialosides attached to the polymer **9** proved to be highly potent inhibitors of both SA. Their inhibitory activity against *VcSA* or *TcTS* was improved by at least 39-fold, and by 208-fold (Figure 2C), respectively. The low IC_{50}^{si} of 3.2 µM with *TcTS* surpasses previously reported IC_{50} value for the sialic acid transition state analogue DANA,[34] and are the best *TcTS* inhibitors described so far.[35]

When assessed against *NanA* and *NanA-C*, the compounds showed a highly similar IC_{50} variation on the full and truncated domains. RIP of divalent inhibitors **2**-**8** consistently improved as the spacer arm length is elongated from n = 2 to n = 130 (Figure 2C). This effect was fully ascribed to the sialoside ligands as PEG spacers of similar size did not inhibit the enzymes (results not shown). The maximal RIP and minimal IC_{50}^{si} achieved within the series was 106 and 4 µM when compound **8** was tested against *NanA-C*. As previously observed with *VcSA* and *TcTS*, the polymer **9** was by far the most potent of the series, showing outstanding IC_{50}^{si} values of 343 nM and 143 nM for *NanA* and *NanA-C*, respectively. We subsequently determined the corresponding inhibitory constants (Ki^{Si}) by Dixon and Lineweaver-Burk plots analysis and found 409 ± 65 nM and 335 ± 40 nM for *NanA* and *NanA-C* with an inhibition profile in accordance with a competitive binding mode in both cases (Figures S4 and S5). Thus far, this

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represents the best inhibitor for this enzyme and an affinity improvement per sialosides of around 3000-fold. Surprisingly, the high affinity improvement observed is therefore not related to the presence of the lectin domain and **9** showed very similar RIP value whether tested with *NanA-C* or *NanA* (2979 vs 2927).

Aggregative behavior of NanA-C. We first hypothesized that a possible multimerization of the *NanA-C* domains in solution may compensate the absence of the CBM and dramatically improve polymer affinity through chelate effects. Engineered *V. cholerae* CBM40 into tetramers was previously shown to improve avidity for a sialic-acid rich surface compared to the single CBM40 by around three orders of magnitude.[36] A multimeric *NanA-C* domain may potentially provide such synergistic effect and we carefully studied the oligomerization state of the full and truncated *NanA* forms by dynamic light scattering (DLS), small-angle X-ray solution scattering (SAXS), Multi Angle Light Scattering (MALS), and size-exclusion chromatography (SEC).

DLS data indicated that solutions of NanA, NanA-C (with initial concentrations respectively at 19µM and 43µM) have very similar profiles and contain a major species for NanA and NanA-C with respectively 83% and 74% of intensity scattered by the smaller molecular weight (Figures S6 and S7). The three protein samples were further analyzed by SAXS and MALS and the experimental molecular weight values found for the molecular envelopes closely matched the calculated values of the monomeric constructs (Figures S8 and S9, Table S1).To estimate the concentration limit at which NanA-C started to form higher molecular weight structures, different sample concentrations were analyzed by SEC (Superdex 200pg 16/600, Thermo Scientific®). The unique peak attributed to NanA (Vr = 84 mL, C=13 µM) was shown to decrease when the protein concentration was increased to 64 µM (5 mg/ml), and higher molecular weight structures appeared at Vr = 37.49 mL and Vr = 47 mL (Figure 3).

Thus a significant quantity of *NanA-C* aggregates is only partially formed at the high protein concentration of 125 µM (7 mg/ml). Consequently, self-associative processes are unlikely during the measurement of the inhibitory activity due to the very low protein concentration used (~ 1-5 nM).

Binding affinity by Surface plasmon resonance (SPR). We studied the affinity of *NanA*, *NanA-C* and *NanA-L* for a surface coated with thiosialosides mimicking cell-surface sialoglycans, using surface plasmon resonance (SPR). Thiosialoside **34** (structure and synthesis in SI) appended with a terminal amino-group instead of a primary alcohol was coated through the formation of an amide bond. Dissociation constants from the three constructs highlighted the benefit of the lectin domain to improve *NanA* affinity for a sialylated surface (Figure 4A). Indeed, *NanA* showed a 283-fold lower dissociation constant (K_d = 0.755 µM) compared to *NanA-C* (K_d = 214 µM). This affinity improvement is mainly due to the higher intrinsic affinity of *NanA-L* (K_d = 10.3 µM) for the sialylated surface. Thus, *NanA* binding to a sialylated surface is mainly promoted by the lectin domain, illustrating its crucial role in anchoring the sialidases to a sialylated membrane.

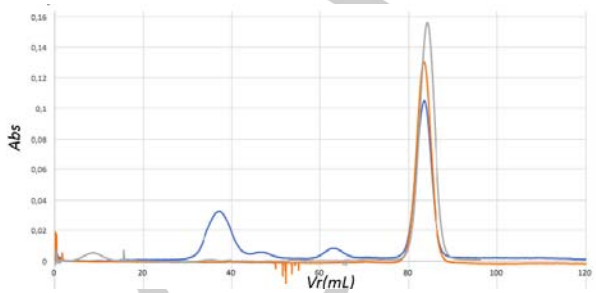

Figure 3. Analysis of the aggregation behavior of *NanA* and *NanA-C*. SEC analysis showed the formation of protein aggregates only at high concentrations of 125 µM (blue curve) and a unique peack (Vr = 84 mL) at 13µM (grey curve) and 64 µM (orange curve).

Next, we used SPR to study the faculty of the compounds **1**-**9** to disrupt *NanA* adherence in a competition assay. Inhibition constants expressed per mol of sialosides (K_I^{si}) are reported in Figure 4B. This SPR assay reflects the relative affinity of the compounds for the *NanA* constructs which differs from and complements the measurement of the inhibition of the catalytic enzyme activity (Table 1). Divalent compounds **2**-**8** showed a moderate affinity improvement with a maximal RIP value of around 5 (compound **4** K_I^{si} = 9.4 µM for *NanA*). Again, the most potent compound was polymer **9** showing K_I^{si} values of 3.1, 61 and 2.3 µM for *NanA*, *NanA-C* and *NanA-L*, respectively. Although significant, this improvement in terms of affinity remains moderate compared to the very high improvement in inhibiting the catalytic activity of *NanA* (RIP = 2927) and *NanA-C* (RIP = 2979). The two assays measure very different characteristics of the enzymes, which are the inhibition of its hydrolyzing activity and its binding potency. In the SPR assay, the compounds should prevent and disrupt *NanA* attachment from a heavily functionalized thiosialoside surface.

All the compounds, including **9** showed much weaker affinity for *NanA-C* compared to *NanA-L* or *NanA*. Coherently to the K_d values obtained with the sialylated SPR surface alone, the lectin domain *NanA-L* was shown to improve the polymer binding for *Nan-A* significantly. The highly similar K_I^{si} recorded for the compounds with *NanA* and *NanA-L* but not *NanA-C* (Figure 4B) suggests that they predominantly bind to the lectin domain.

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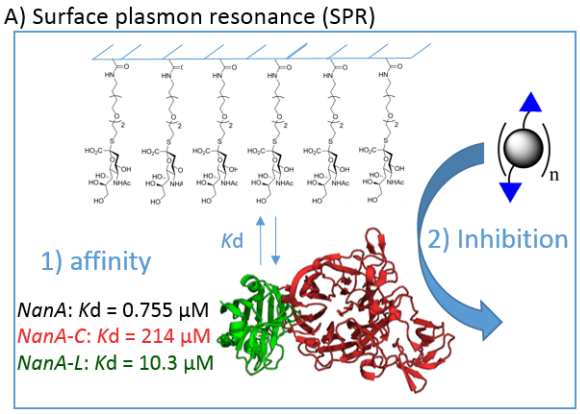

B) Binding inhibition against NanA, NanA-C, NanA-L

		Binding inhibition K_i^{si} (µM)		
Cpds	$V_{(n)}$	*NanA*	*NanA-C*	*NanA-L*
1	$1_{(2)}$	44	601	65
2	$2_{(2)}$	28	209	17
3	$2_{(10)}$	36	1170	26
4	$2_{(30)}$	9.4	377	14
5	$2_{(43)}$	30	1170	20
6	$2_{(66)}$	55	>2000	46
7	$2_{(86)}$	31	562	17
8	$2_{(130)}$	133	306	56
9	$130_{(2)}$	3.1	61	2.3

Figure 4. A) Illustration of the SPR assay. 1) The dissociation constants of the *NanA* constructs for the thiosialylated surface were first measured without compounds 2) Competitive experiments were subsequently performed to determine K_i^{si} values for compounds **1**-**9**. B) Inhibition values (K_i^{si}) expressed in µM of thiosialoside residues.

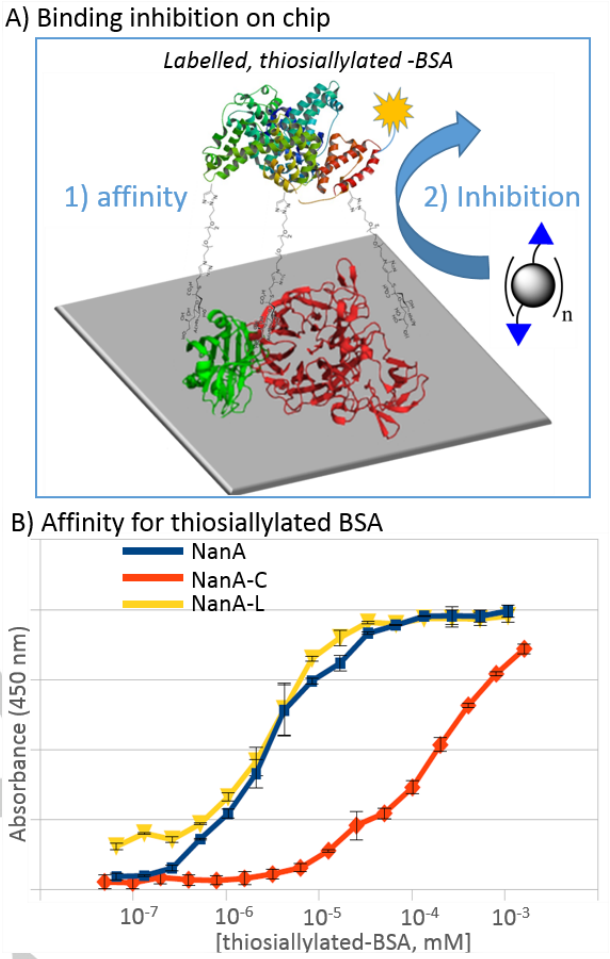

Figure 5. A) Illustration of the chip assay. *NanA* constructs were grafted on the plate and the interaction with thiosialylated BSA was measured. Competitive experiments were subsequently performed to determine IC_{50}^{si} values for compounds **1**-**9** (reported in Table 1). B) Binding inhibition of *NanA*, *NanA-C* and *NanA-L* for the thiosialylated BSA.

Binding affinity on chip. The binding inhibition of the compounds **1**-**9** was subsequently evaluated using a very different system adapted from "lectin glycoprofiling" technologies.[37] First, *NanA* constructs were coated on a microplate surface. Labelled-BSA coated with azido-functionalized thiosialosides by click-chemistry (see supporting information for detailed procedure) was then added to the plate (Figure 5A). In the first set of experiments serial dilutions of thiosialylated BSA were applied and the recorded absorbance determined the interaction profiles with *NanA*, *NanA-C* and *NanA-L*. As seen in Figure 5B *NanA* and *NanA-L* have very similar inhibition profiles and exceed by around two orders of magnitude *NanA-C* affinity for the thiosialylated-BSA. This is fully coherent with the SPR results and further highlights the key role of lectin domain in driving the sialoside binding.

Competitive experiments with serial dilutions of compounds **1**-**9** were performed to obtain IC_{50} values (Table 1). The binding assay could not be performed with *NanA-C* due to its much lower affinity for the thiosialylated-BSA probe. Monovalent reference **1** was shown to be a significantly more potent inhibitor for *NanA* and *NanA-L* compared to **Neu5Ac**, highlighting the positive impact of the anomeric residues of **1**. Significant synergistic effects were not observed with divalent **2**-**8**. All compounds showed poor IC_{50}^{Si} values ranging from 440 to 2160 µM. In contrast polymer **9** showed low IC_{50}^{si} values of 3.5 and 2.3 µM for *NanA* and *NanA-L*, which represents high RIP values of 254 and 939-fold, respectively. Again, the polymer binding affinity for the sialidase is largely promoted by the lectin domain in view of the nearly identical IC_{50}^{si} values observed for *NanA* and *NanA-L*.

High levels of affinity improvements have been previously reported with glycopolymers and lectins.[20] We anticipated that

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polymer **9** could also act as a potent inhibitor of sialoside-binding lectins. We selected Wheat Germ Agglutinin (WGA) as a model lectin, which was previously shown to be successfully inhibited by multivalent *N*-acetylglucosamine derivatives.[14,38] As seen in Table 1, WGA can also accommodate sialosides such as **Neu5Ac** and **1**, and with a similar range of affinity (mM) compared to *NanA* and *NanA-L*. In the divalent series **2-8**, the IC_{50}^{si} values were shown to gradually decrease from 4300 μM to 130 μM when the spacer arm length was increased from 2 to 130 EG units. This significant affinity improvement was, however exceeded by polymer **9** showing IC_{50}^{si} of 1.2 μM and calculated RIP values 917, respectively. Thus, polymer **9** showed similar and high levels of affinity improvement on WGA, *NanA* and *NanA-L*, blurring the line between the lectins and sialidases.

Table 1. Binding inhibition against *NanA* and *NanA-C* determined on a chip.

Cpds	$V_{(n)}$	WGA	NanA	NanA-L
Neu5Ac	1	9500 ± 500	8420 ± 100	6840 ± 300
1	$1_{(2)}$	1100 ± 100	890 ± 7	2160 ± 200
2	$2_{(2)}$	4300 ± 200	800 ± 200	1560 ± 80
3	$2_{(10)}$	1500 ± 200	880 ± 200	1200 ± 140
4	$2_{(30)}$	540 ± 40	440 ± 160	540 ± 200
5	$2_{(43)}$	330 ± 40	760 ± 40	480 ± 180
6	$2_{(66)}$	320 ± 20	720 ± 100	520 ± 40
7	$2_{(86)}$	182 ± 10	580 ± 80	560 ± 200
8	$2_{(130)}$	130 ± 10	1040 ±140	780 ± 200
9	$130_{(2)}$	1.2 ± 0.3	3.5 ± 0.3	2.3 ± 0.2

Conclusions

We have designed non-hydrolysable multivalent thio-sialosides as probes and inhibitors of biologically relevant sialidases. The polymeric compound **9** was by far the most active showing unprecedented levels of inhibitory capacity and binding affinity against the biologically relevant *TcTS* and *NanA* sialidases. Each thiosialoside ligand attached to the polymer backbone surpassed the inhibitory capacity of the corresponding monovalent reference by more than three orders of magnitude. Thus, polymeric thiosialosides and sialidases can reach the high level of inhibition previously reported for glycopolymers and lectins. This is relevant considering the difficulty in designing potent sialidases and trans-sialidases inhibitors. Importantly, the expression of the *NanA* truncated domains *NanA-C* and *NanA-L* allowed a better understanding of the respective functions of the catalytic and lectinic domains of the pathogenic sialidases. The lectinic domain improves *NanA* binding to a sialylated SPR surface by more than two orders of magnitude (Figure 4A, NanA k_d = 0.755 μM, NanA-C k_d = 214 μM). A similar scenario was observed when the *NanA* constructs were immobilized on a chip. The affinity profile for the thiosialylated BSA was nearly identical for *NanA* and *NanA-L*, and two-orders of magnitudes lower for *NanA-C* (Figure 5B). Thus, the binding to a sialylated surface is almost exclusively promoted by the lectin domain. The blend of assays also shed light on the binding interaction with the polymer **9**. Results obtained from the binding inhibition experiments with **9** showed high and very similar binding potency against *NanA* and *NanA-L* with K_i^{si} and IC_{50}^{si} values of 3.1 vs 2.3 and 3.5 vs 2.3 μM, respectively. The binding of the polymer to *NanA* is also fully promoted by the lectin domain and a chelate binding mode with thiosialosides ligands embracing both the catalytic and lectinic domains, as initially hypothesized, seems irrelevant. Unexpectedly, **9** exerted very high inhibition of the enzymatic activity (Figure 2B, 2C) on both *NanA* and *NanA-C* (K_i^{si} = 409 ± 65 nM and 335 ± 40 nM). This suggests that the activity of sialidases lacking a lectin domain can be efficiently inhibited by polymeric thiosialosides such as **9**. The strong inhibition observed did not result from *NanA-C* aggregation as evidenced by DLS, SEC and MALS. We propose that the enhanced affinity and inhibitory activity observed with **9** results from a "bind and jump" process were the *NanA* constructs diffuse from one thiosialoside ligand to another along the polymeric chain, as previously described by Brewer on GalNAc binding lectins.[23] This "zipping" mechanism is not accessible to divalent compounds **2-8** that are indeed poor sialidase inhibitors.

To summarize, the design of polymeric thiosialosides led to highly potent sialidase inhibitors with high sygnergistic multivalent effects. Binding affinities were significantly increased with sialidase lectin domains, but strong inhibition of the enzymatic activity was observed on the catalytic domain. The design of multivalent glycosidase modulators is a promising emerging field[39–43] as illustrated by the continuous reports of potent glycosidase activators[44,45] or inhibitors based on clusterized iminosugars [46,46–49] or sugar substrates.[50] This concept is now extended to the inhibition of bacterial and pathogenic sialidases for which transition-state inhibitors fail to reach the submicromolar level.

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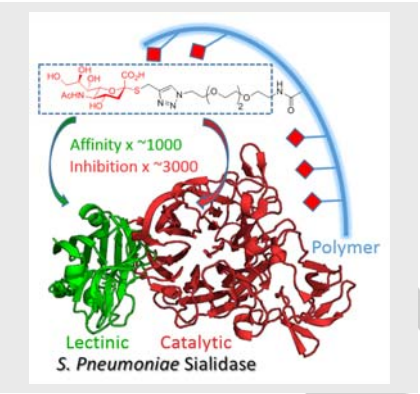
FULL PAPER

High synergy: Sialidases (SA) are virulence factors expressed by pathogenic bacteria, viruses and parasites. Di- and polymeric thiosialosides were designed to inhibit *V. cholerae*, *T. cruzi* and *S. pneumoniae* sialidases. Each thiosialoside grafted on the polymer was shown to reduce SA catalytic activity with much higher efficiency compared to their monovalent analogues. This extend the multivalent concept to this class of enzymes.



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