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Supporting Information

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Supporting Information

for

The Influence of Ligand Valency on Aggregation Mechanisms for Inhibiting Bacterial Toxins

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1. Isothermal Titration Calorimetry – curve fitting at high c-values

Thermodynamic parameters are usually extracted from ITC data by non-linear least squares curve fitting using the so-called Wiseman Isotherm.[1, 2] The shape of the titration curve is dependent on the "c-parameter" which is defined as:

$$c = nM_tK_a = \frac{nM_t}{K_d}$$

where n is the binding stoichiometry, M_t is the initial receptor concentration, K_a is the association constant and K_d is the dissociation constant. When c > 10 the curve is sigmoidal.[2] As the value of c increases, the curve becomes steeper until it becomes a vertical step. Under such conditions it is still possible to determine the enthalpy and binding stoichiometry with high accuracy, but the affinity can no longer be determined. Therefore, when working with high affinity systems, it is preferable to use lower concentrations of the receptor. However, lower concentrations of receptor usually mean poorer ratios of signal to noise, and thus more uncertainty in the data.

In the current study the ligands all bind with high affinity (low nM K_d) and so the titration curves have steep transitions (Fig. S1-S3). However, the CTB-GM1 interactions have high enthalpy values (ca. -20 kcal/mol) and the level of signal-to-noise is thus still very good, even at 4-10 μM binding site concentration. In all cases the titration curves were fitted using the standard "one-site" model supplied with the MicroCal system. The Origin software returned errors < 10% for each of the variable parameters used in the curve fitting (ΔH, K_a, n), which indicated that the curve fitting was converging at a unique solution.[3]

If values of parameters are mutually dependent on one another the "parameter dependency" values will be close to 1 (the parameter dependency for the i^{th} parameter is defined as: $1-\frac{1}{C_{ii}C_{ii}^{-1}}$ where C_{ii} is the diagonal element of the variance-covariance matrix).[4] In all cases, the parameter dependency values were between 0.15 and 0.25; i.e., there was little mutual dependency of individual parameters, which also suggests that the curve fitting was reaching a unique solution.

Data simulations were conducted to determine how well our experimental data could be used to distinguish between similar values of K_d. In each case, the data were simulated in Origin using the values of n and ΔH that were returned by Origin curve-fitting tool when all three fitting parameters were allowed to float freely.

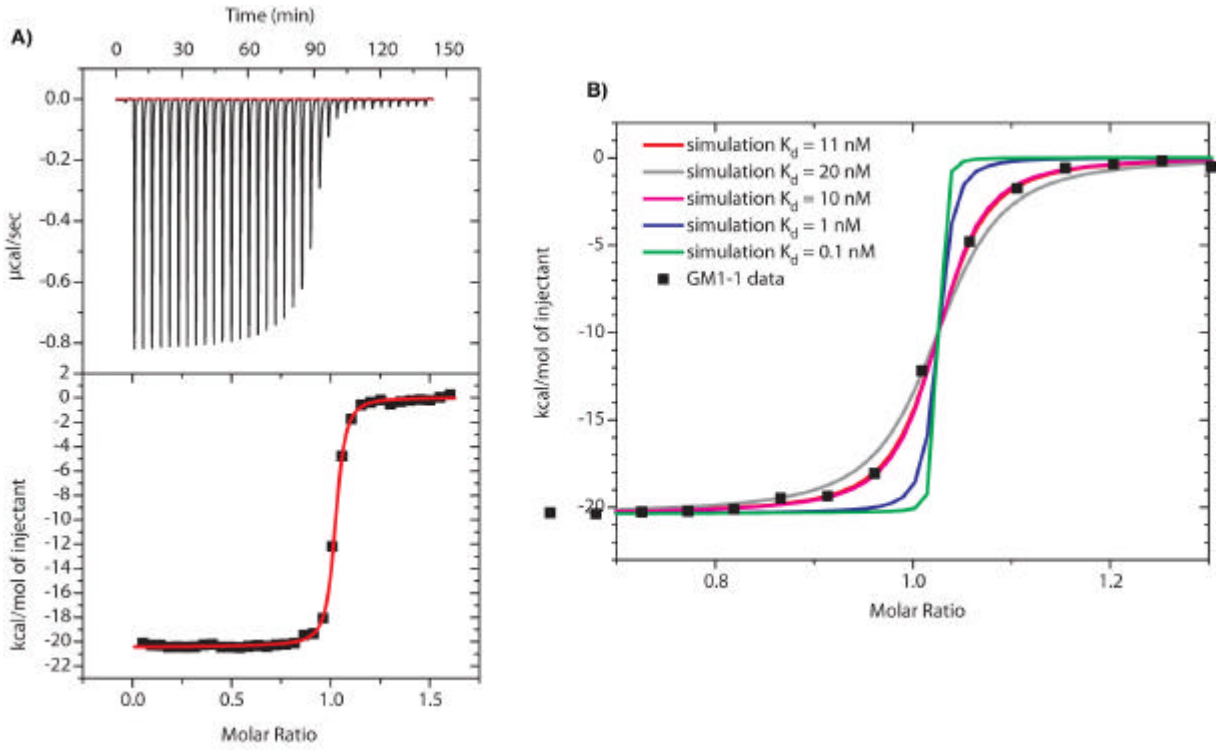

Figure S1. Typical **GM1-1** / LTBh titration. A) raw and processed data; B) simulated curves.

Figure S1b shows the transition region of a typical titration curve for the monovalent **GM1-1** ligand (c = 1800). The line of best fit is shown in red and simulated lines for K_d = 20, 10, 1 and 0.1 nM are shown for comparison. From a visual inspection it is clear that the data fit the red line (11 nM) very well, and that it is easy to distinguish an 11 nM K_d from 20 nM or 1 nM, even though the c-value lies outside the range usually recommended for accurate curve fitting.[1, 2]

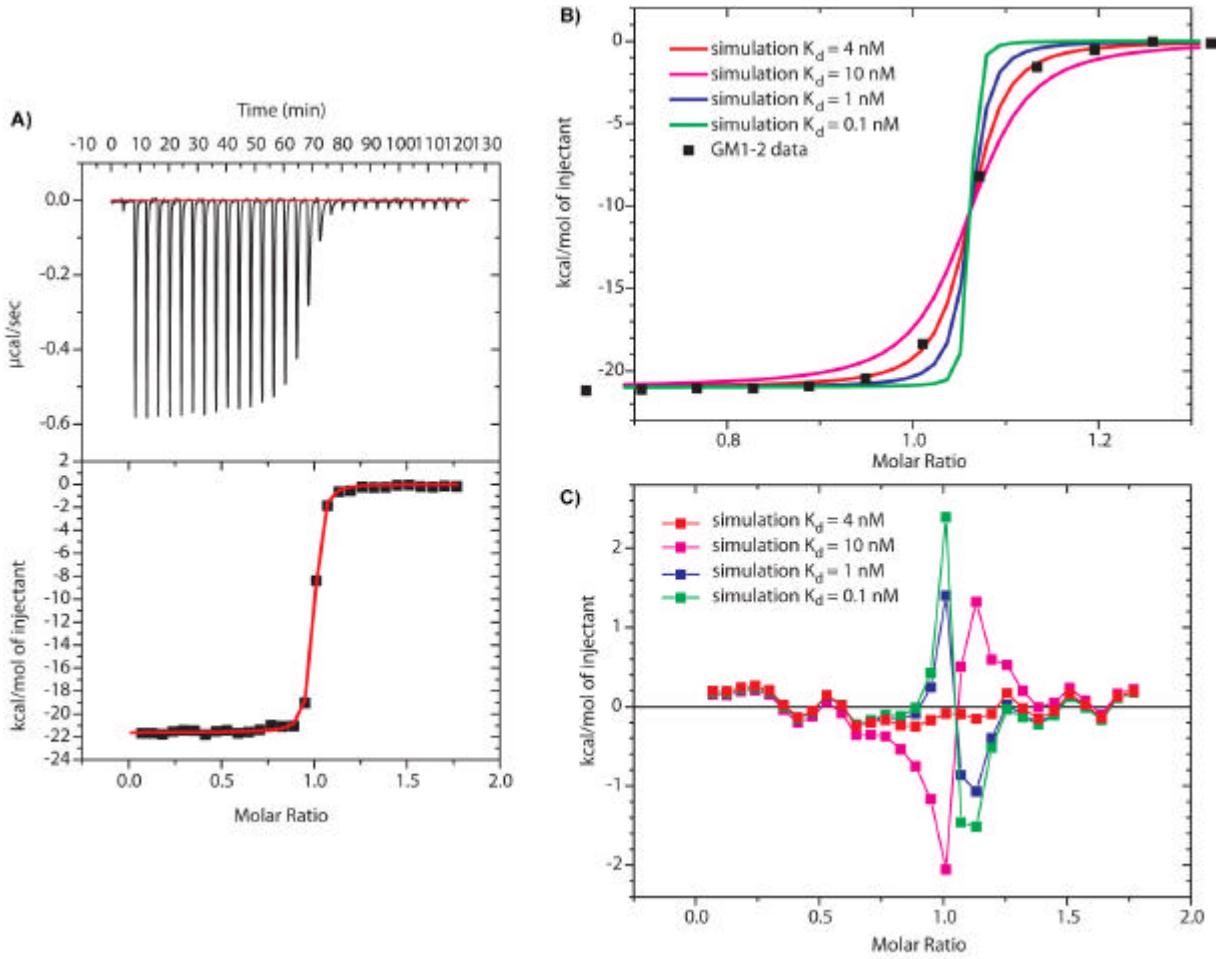

Figure S2. Typical **GM1-2** / LTBh titration. A) raw and processed data; B) simulated curves; C) residuals from curve fitting (red) and data simulations (pink, green, blue)

In Figure S2 the **GM1-2** ligand gives a steeper transition corresponding to its higher affinity (K_d = 4 nM; c = 2500). This c-value lies outside the range usually recommended for accurate curve fitting, yet the data in the transition region allow us to distinguish clearly between K_d = 4 nM and simulated curves for K_d = 10, 1 and 0.1 nM (Figure S2b). A plot of the residuals (data minus simulated curve values; Figure S2c) highlights how the simulated curves deviate significantly from the data around the equivalence point, while the line of best fit (in red) gives small residuals across the full titration. Addi-tional titrations at lower LTBh concentration returned the same value of K_d.

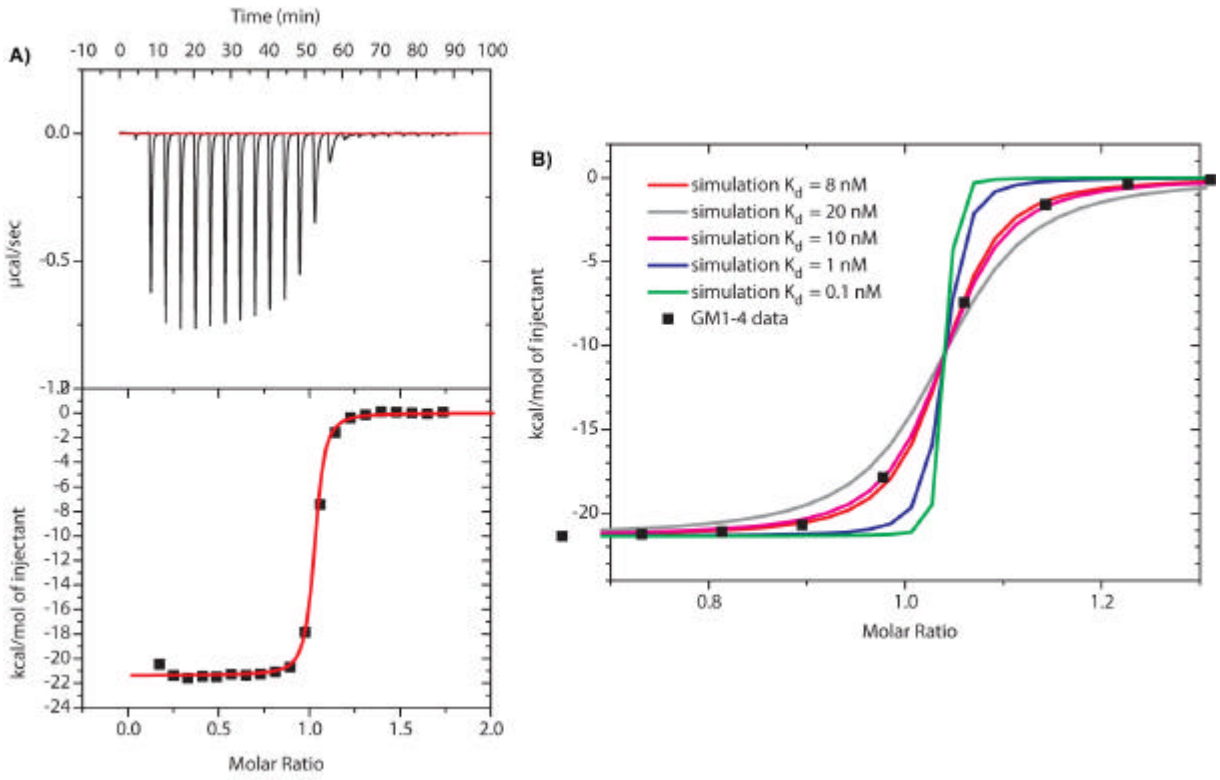

Figure S3. Typical **GM1-4** / LTBh titration.

Figure S3 shows a typical dataset for the tetravalent ligand **GM1-4** (K_d = 11 nM; c = 1100). In this case, it was found that the first two injections of ligand always gave less heat than the subsequent injections. This data could be consistent with a co-operative mechanism in which either the enthalpy or affinity of the interaction change during the course of the titration. However, it can not be completely excluded that there was some instrumental error that led to a reduced delivery of ligand during these titrations. Nevertheless, the curve fitting assuming a simple 1:1 binding model (Figure S3b), once again shows that the data can distinguish between K_d = 8 nM, and simulated curves with K_d = 20, 1 and 0.1 nM.

Therefore, we can conclude that the large enthalpy for the GM1-LTBh interaction allows us to work accurately at high c-values and the multivalent ligands do not bind substantially more tightly than the monovalent ligand **GM1-1**.

2. Specific vs non-specific protein aggregation

Control experiments were undertaken to determine if the aggregation processes resulted from specific interactions of the multivalent GM1-ligands, or through nonspecific aggregation induced by protein-dendrimer interactions. Divalent and tetravalent galactosyl ligands based on the same dendritic scaffolds as **GM1-2** and **GM1-4** have been reported previously (**Gal-2** and **Gal-4**; Fig S4).[5] Although these multivalent compounds are also effective inhibitors of LTB/CTB adhesion to GM1-coated plates, they can be competitively displaced from the LTBh binding sites with **GM1-1** (vide infra).

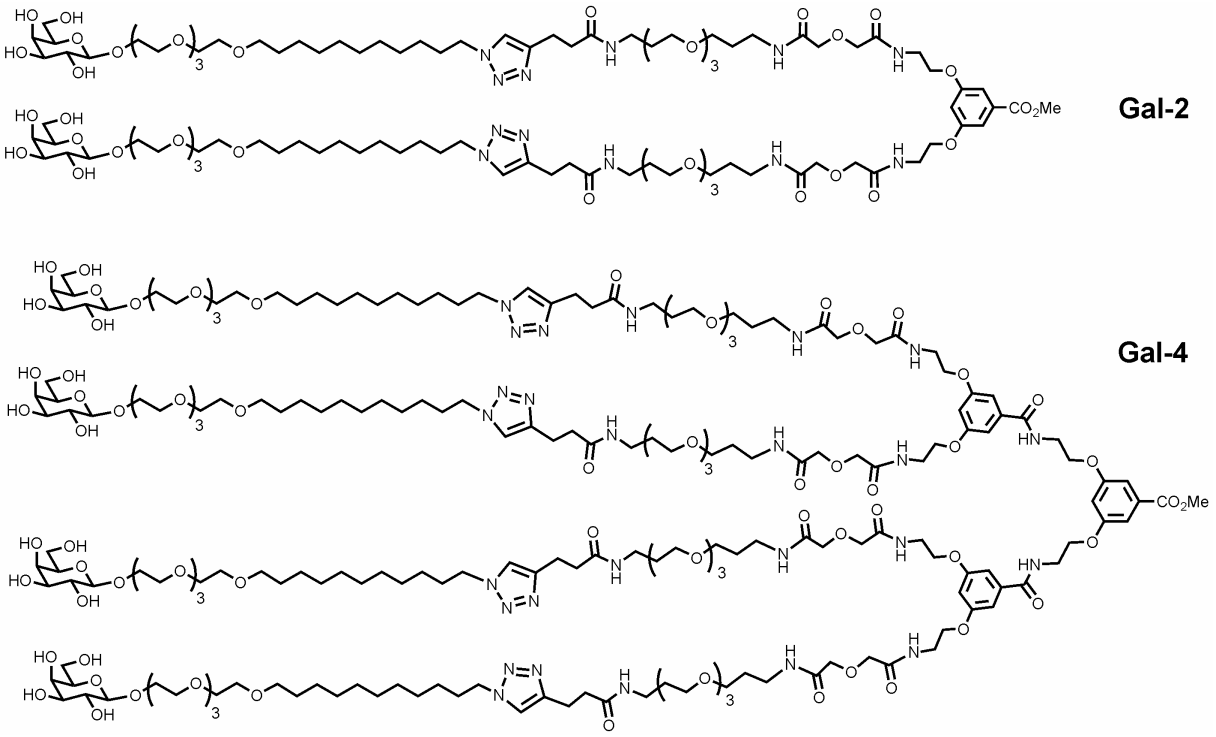

Figure S4. Multivalent galactosyl ligands **Gal-2** and **Gal-4**.

Solution preparation. Solutions of LTBh (2 μM pentamer concentration) and either **Gal-2** (6 mM) or **Gal-4** (24 mM) were titrated with **GM1-1** to give a final **GM1-1**:LTBh subunit ratio ca. 2:1 (Fig S5). In each case the ITC curves displayed a reduced apparent enthalpy of interaction relative to a titration in the absence of the galactosyl ligands. These results indicated that the galactosyl groups binding to the LTBh protein were displaced by **GM1-1**.[6, 7] Analysis using Sigkurskjold's displacement model[6] suggested K_d values of approximately 1.6 mM for both the divalent and tetra-

valent ligands, which is again in contrast with their greatly enhanced inhibitory potencies relative to the monovalent compound as determined by ELISA.[5] The resulting solutions thus constitute the LTBh/**GM1-1** complex mixed with either free **Gal-2** or **Gal-4**. We would attributed the small decrease in the stiochiometry of the competition titrations to differences in the LTBh concentration between the direct titration and the competition experiments. However, even if a small fraction of the **Gal-2** or **Gal-4** ligands remain bound to the LTBh protein, there is no impact on the conclusions of the following aggregation experiments.

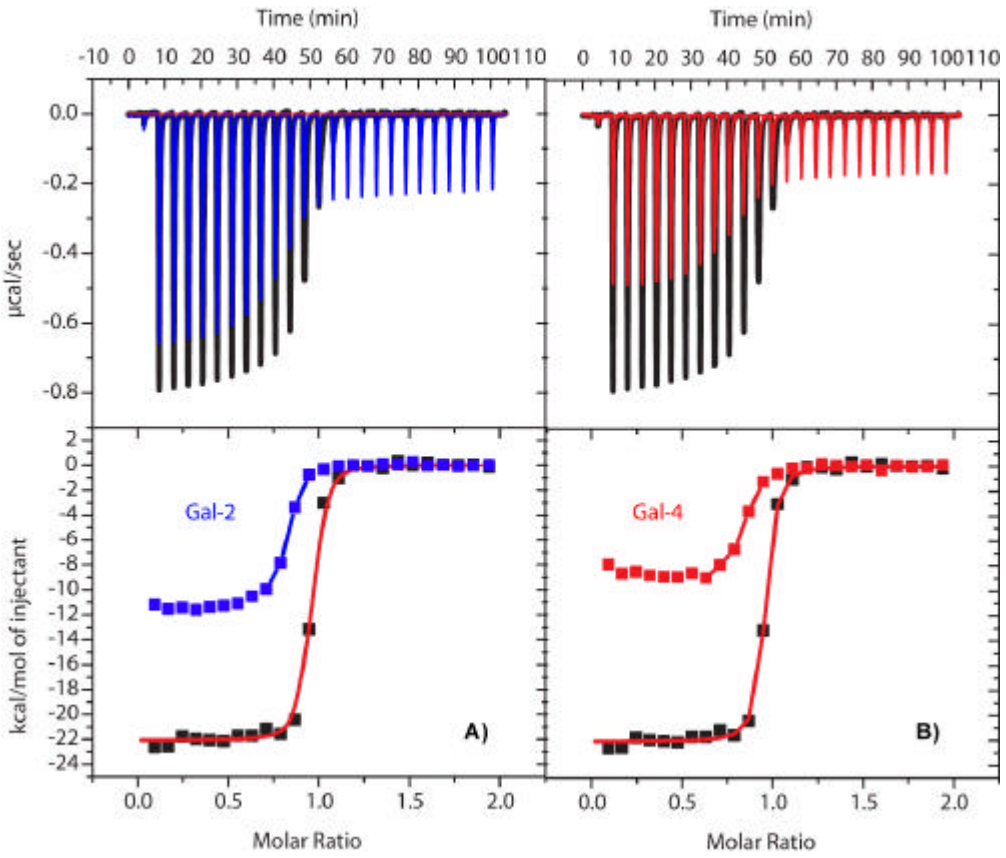

Figure S5. ITC titrations of GM1-1 into LTBh (2 µM pentamer concentration) in the presence of A) 6 mM **Gal-2** (blue data) and B) 24 mM **Gal-4** (red data). In each case the black titration data is for a titration in the absence of any competing multivalent ligand.

Aggregation experiments. Mixtures of LTBh (2 µM pentamer concentration) and **GM1-1** (20 µM) showed no aggregation after incubation at 4 ºC for several weeks in the absence (Figure S6, well A1) or presence of the divalent **Gal-2** ligand (6 mM; well

A3) or tetravalent **Gal-4** ligand (24 mM; well A5). In contrast, solutions of LTBh mixed with **GM1-2** or **GM1-4** to give the same GM1-oligosaccharide concentration displayed visible aggregation (Figure S6, wells A2 and A4, respectively). The lack of aggregation in the presence of high concentrations of non-binding ligands demonstrates that aggregation arises from specific interactions with the **GM1-2** and **GM1-4** ligands, and does not result through non-specific interactions with the dendritic scaffolds or "salting-out" mechanisms associated with the phosphate buffer.

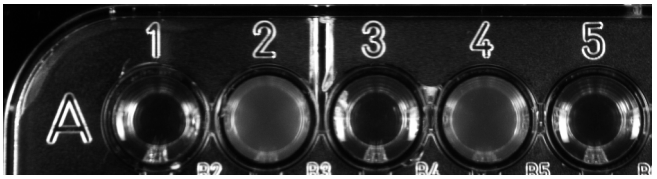

Figure S6. Comparison of the aggregative properties of different mixtures monovalent and multivalent ligands. In all cases the total concentration of GM1 oligosaccharide groups is 20 μM and the LTBh pentamer concentration is 2 μM. Ligands added to each well: A1 – **GM1-1**; A2 – **GM1-2**; A3 – **GM1-1** + 6 mM **Gal-2**; A4 – **GM1-4**; A5 – **GM1-1** + 24 mM **Gal-4**.

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