Multivalent Inhibition of AB_5 Toxins

Joseph M. Gargano, Thao Ngo,† Ji Young Kim,
David W. K. Acheson,† and Watson J. Lees*

Department of Chemistry, Syracuse University
Syracuse, New York 13244
Division of Infectious Diseases
New England Medical Center
750 Washington Street, Boston, Massachusetts 02111

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The recognition of specific carbohydrates by proteins is essential for the regulation of cellular activity, such as fertilization, homing of lymphocytes, and mediation of endocytosis. Unfortunately, the interaction between a single sugar unit and receptor protein is almost always weak ($K_a = 10^3-10^4$ M^{-1}).[1] In biological systems, this weak binding is often enhanced through multivalent interactions.[2] Multivalency occurs when one entity with multiple ligands binds to another entity with multiple receptors thus creating numerous ligand−receptor interactions. Multivalency is not limited to in vivo systems and has been applied with some success to drug development by the synthesis of hub-and-spoke systems (STARFISH/finger systems),[3,4] dendrimers,[5,6] and polymers,[2] all of which contain multiple copies of the ligand attached. Herein we describe a theoretical model for calculating binding enhancement due to multivalency and the strong multivalent binding of a synthetic glycopolymer to a soluble receptor, *Escherichia coli* Shiga toxin.

To illustrate the theoretical model, we selected the simplest multivalent system, which is two ligands connected by a linker binding to a dimeric receptor with two equivalent binding sites (Figure 1, eq D). To solve for the equilibrium constant of eq D, the association of the dimer with the receptor was viewed as the sum of three other equilibria, eqs A, B, and C. The choice of these three equilibria was based upon the fact that we could solve for the K_{eq} value of each of them in terms of the association constant of the monomeric ligand, K_a. The equilibrium constant for eq A, which represents the initial binding of a dimeric ligand to a dimeric receptor, contains K_a and a statistical factor of 4. The statistical factor represents the number of permutations that can convert the starting material to a product divided by the number of permutations that can convert the product back again. Equation A has four permutations of the forward reaction, two potential receptor sites, and two possible ligands, one at each end of the dimer ($2 \times 2 = 4$), but only one permutation of the backward reaction, loss of the one ligand bound to the receptor. The equilibrium constant for eq B, which represents the binding of a single monomer to the single open receptor site, has a statistical factor of 1. The equilibrium constant for eq C has a statistical factor of 0.5 because the forward reaction has one permutation but the backward reaction has two, replacement of either end of the dimer with the monomer. Equations B and C involve a monomeric ligand, but the overall equilibrium constant for eq D is independent of the presence of monomer. Equation C represents intra- versus intermolecular interactions, and its equilibrium constant is an effective concentration.[7] Effective concentrations are almost independent of the actual reaction involved.[7] In this case the effective concentration should be independent of the value of K_a. Equation B mathematically connects eqs A and C. Theoretical methods from the polymer literature can calculate the effective concentration, C_{eff}, of one polymer end at a given distance from the other end as a function of polymer length.[8,9] When the two polymer ends are 30 Å apart, the maximum C_{eff} is 10^{-2}/2 M.[10] Therefore, the K_{eq} for eq C is 10^{-2}/2 M, when the two receptor sites are 30 Å apart and the linker is of optimal length. Recent experimental papers have also reported a K_{eq} of approximately 10^{-2}/2 M for bivalent ligands with flexible linkers.[8,11] Multiplying the equilibrium constants for eqs A, B, and C together results in the overall equilibrium constant, K_a (dimer) $= 2(10^{-2})K_a^2$, for the binding of a dimeric receptor to a dimeric ligand, eq D. The binding enhancement (BE) of the dimeric ligand on a per ligand basis relative to the monomer is $(10^{-2})K_a$.

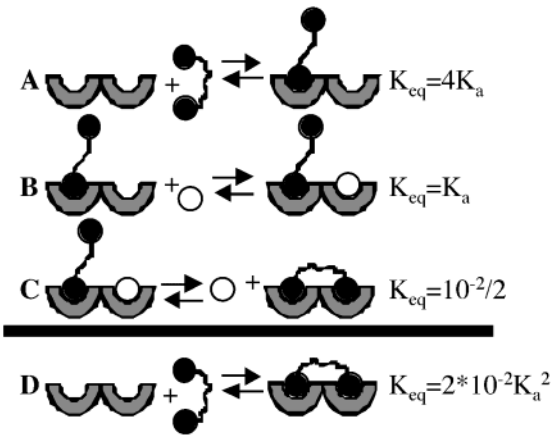

Figure 1. Polyvalent binding model. The simplest case involves a bivalent ligand (solid circles), its monomeric counterpart (open circles), and protein (double horseshoe). K_a is the association constant of the monomer with the receptor. The K_{eq} for reaction D is the product of K_{eq} values for the individual reactions A, B, and C.

The model can be extended to higher-order systems such as trimers binding to trimers and pentamers binding to pentamers using similar sets of eqs. For example, the general solution for binding enhancement is $BE = F[sK_a(10^{-2})]^{(n-1)}$ where n is the smaller of the number of sites on the receptor (trimeric receptor = 3 sites) or the number of sites on the ligand (pentameric ligand = 5 sites).[10] The term F is a system specific statistical factor, similar to those mentioned in the previous paragraph, which is ≥1.[10] The term s adjusts for distances between the receptors that are not 30 Å, $s = (30/(\text{distance in Å}))$.[3] The general solution is applicable under the following assumptions: (1) binding sites are equivalent, (2) no cooperative binding, (3) the linker is flexible and of optimal length, (4) no linker−receptor interaction, (5) binding enhancement is due to intramolecular binding. Due to the requirement of a linker of optimal length, the calculated value always overestimates the binding enhancement or is within a factor of 4.[2,10,11] The model makes suggestions about the design of multivalent ligands and provides rationale for the synthetic effort. If the monomer binds weakly ($K_a = 10^3$ M^{-1}) to a dimeric

† New England Medical Center.

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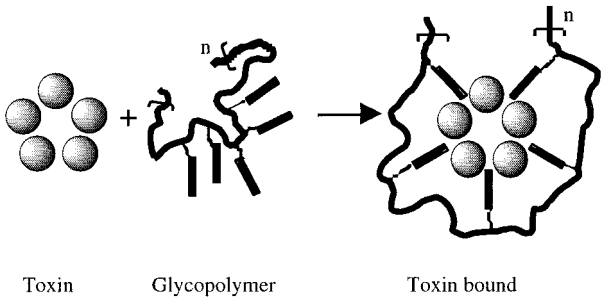
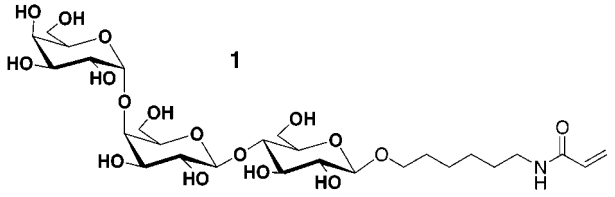
Scheme 1



receptor and a submicromolar inhibitor is desired, then synthesizing a dimer is not likely to succeed, BE = $(10^{-2})K_a = 10$ per ligand, K_a(dimer) = 2×10^4 M^{-1}.

According to our theoretical model, multivalent inhibitors should be ideal for inhibiting the binding of carbohydrates to multivalent receptor proteins such as AB_5 toxins, which have five identical B subunits each with at least one receptor site (Scheme 1). Recent studies on AB_5 systems, such as *E. coli.* enterotoxin and Shiga toxin with the STARFISH/finger[3,4] ligands show a large multivalent binding enhancement. On the basis of the model we expect polymers to produce similar results and have broader applicability. Polymers also are more facile to synthesize and minimize the requirement of a detailed knowledge of valency or structure for designing ligands.

Synthetic polymers have shown very large binding enhancement in a number of systems.[2] However, these systems have involved either a surface coated with receptors or proteins that naturally cross-link with multivalent ligands. Polymers of sialic acid inhibit the binding of influenza virus to cells but do not inhibit the binding of ligands to the isolated trimeric receptor protein, hemagglutinin.[12] Multimers and polymers of carbohydrates inhibit conconavalin A by cross-linking the protein intermolecularly not intramolecularly.[6] We sought to demonstrate that polymers, as calculated by the model, show large binding enhancements to soluble receptors that naturally bind multimers intramolecularly.[3,4] The AB_5 toxin, *E. coli* Shiga toxin, was chosen because of its medical relevance along with a reliable assay.[13] The theoretical model described above calculates a binding enhancement of $>10^4$ per ligand for the *E. coli* Shiga toxin binding to polymers of **1**. The P^k trisaccharide (monomer) has a K_a of 1×10^3 M^{-1},[14] and the binding sites on the receptor are approximately 30 Å apart, $s = 1$.[3] We conservatively assume a statistical factor of unity, $F = 1$, and one binding site per receptor, $n = 5$, BE = $F[sK_a(10^{-2})]^{(n-1)} = [s10]^4 = 10^4$ per ligand.



A polymerizable derivative, **1**, of a ligand for Shiga toxin, P^k trisaccharide,[14] was synthesized and copolymerized with acrylamide in varying ratios. Using a cell-based assay the IC_{50} for three different polymers (P^k monomer (**1**): acrylamide; 1:9, 1:4, 3:7) against Shiga toxin was determined to be from 800 to 900 nM in terms of trisaccharide concentration (Figure 2).[13] The assay involved incubating various concentrations of the polymers for 1 h at room temperature with 100 ng of Shiga toxin type 1 (Stx1). Residual Shiga toxin was determined using tritiated leucine incorporation into Vero cells.[13] The Shiga toxin alone and polymers alone were used as controls for these experiments. The inhibition constant for P^k trisaccharide itself, methyl P^k trisaccharide, or octyl P^k trisaccharide was determined to be greater than 5 mM. The enhancement due to multivalency, $>5 \times 10^3$, was determined by measuring the inhibition constants of polymer and monomer side by side, and compared well with the calculated value of greater than 10^4. Control experiments with polymers containing lactose showed no inhibition. The trisaccharide polymer exhibited no toxicity to mammalian cells in control experiments. The P^k trisaccharide was toxic to cells at 10 mM.

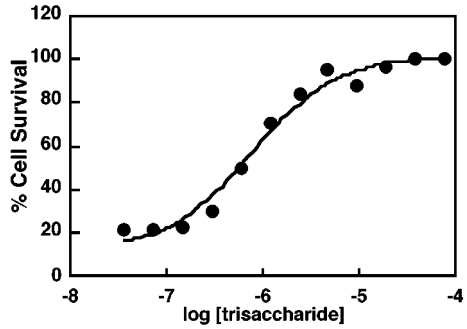


Figure 2. Inhibition of toxin (Shiga toxin 1) activity by a linear polyacrylamide polymer with P^k trisaccharide appendages (3:7, **1**:acrylamide). The curve is representative of all three trisaccharide polymers assayed.

In conclusion, we have provided a theory suitable for calculating binding enhancements (BE) due to multivalency; BE = $F[sK_a(10^{-2})]^{(n-1)}$. The theory suggested that an AB_5 toxin, which has at least one receptor per B subunit, would be an ideal target for multivalent inhibition. Recent work with hub-and-spoke (STARFISH/finger) systems has shown this to be the case.[3,4] We have demonstrated that synthetic polymers provide strong multivalent inhibition. Besides ease of synthesis, an additional benefit of polymers is that neither valency nor distance between binding sites needs to be known. With polymers an enhancement of $>5 \times 10^3$ was observed for the inhibition of *E. coli* Shiga toxin type 1 activity. Such a large enhancement has not been demonstrated previously for synthetic polymers inhibiting a soluble receptor that binds intramolecularly.

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Supporting Information Available: Mathematical details and application of the theoretical model, spectral data and synthetic procedures for **1**, assay procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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