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INTRODUCTION

1.1 NOMENCLATURE AND DEFINITIONS

1.1.1 Valency

According to Mammen et al. [1], the *valency* of a molecule, or that of a biological entity such as a cell, virus, or bacterium, represents the number of separate structural units of the same or a similar type that are connected to the molecule or entity. Thus, if a molecule presents two tethered, identical copies of binding elements, such as a ligand, it is classified as a *divalent molecule (ligand)*. Schematic examples are provided in Figure 1.1 to describe the concept of valency in the context of receptor and ligand interaction. For example, divalent binding occurs when a divalent ligand associates with a divalent receptor through the simultaneous interaction established between two receptor–ligand pairs. Similarly, multivalent or polyvalent interactions are defined as specific simultaneous associations of multiple ligands present on a molecular construct or biological surface that bind to multiple receptors presented on a complementary entity.

The classification of multivalent molecules presented above is simply based on the structural aspects of molecules, such as the number of structurally identical ligands per multivalent construct; hence, the term *structural valency* is used to classify such molecules. However, not all ligands present on a multivalent molecule are involved in interactions with a multivalent receptor displaying

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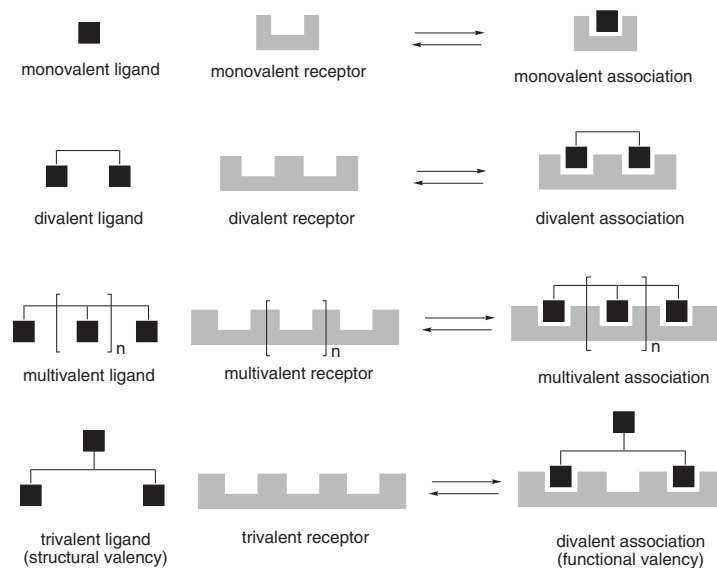


Figure 1.1 Definitions of structural and functional valency as illustrated in monovalent, divalent, and multivalent (polyvalent) interaction.

multiple sites, and a certain portion of the tethered ligands are functionally inactive (Figure 1.1). Thus, for a particular multivalent receptor–ligand association, the valency of a multivalent ligand is not necessarily equivalent to its structural valency. This property is described by Dam et al. [2] as *functional valency*. The concept of functional valency is of significant interest; however, it has been explored in only a limited number of studies. In this book the valency of a multivalent molecule normally refers to structural valency unless its functional valency is available otherwise.

Multivalent interactions are now understood to be a ubiquitous strategy that has evolved in nature for a wide range of functions, including selective recognition of multivalent antigens by antibodies [3] (e.g., bivalent anti-DNP IgG [4], decavalent IgM, bivalent anticardiolipin antibody binding to β_2 -glycoprotein I lipoprotein [5]), neutrophil adhesion and rolling on the surface of an activated endothelial cell [6], and the tight adsorption of a virus particle or bacterium to a host cell surface [7–9] (Figure 1.2). These multivalent interactions are more potent and selective over the analogous monovalent interaction and are therefore only weakly inhibited by most monovalent ligands, especially when the binding cleft for the monovalent ligand is shallow. When designing inhibitor molecules to interfere with multivalent interactions, the most effective strategy is to use multivalent molecules. Such multivalent molecules proved to be highly potent inhibitors: in particular, against surface–surface interaction as observed in virus–cell and cell–cell adhesion (Figure 1.2) [10,11].

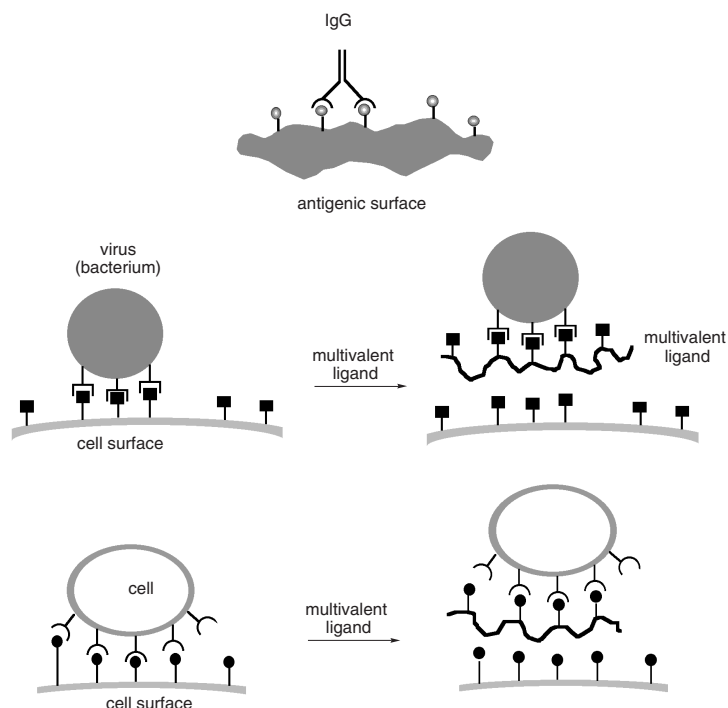


Figure 1.2 Representative examples of natural multivalent interactions and their synthetic multivalent inhibitors.

1.1.2 Linkers

The synthesis of multivalent molecules is performed by tethering multiple copies of a ligand or binding element with a *linker*. A linker provides not only a covalent connection but also appropriate spacing between tethered ligands such that multiple receptor–ligand pairs interact simultaneously without being forced to mismatch. A linker used for a multivalent molecule has to be stable chemically, biochemically, and enzymatically. In contrast, many linkers used in bioconjugate molecules, such as those designed into prodrugs or delivery tools [12], must be chemically or enzymatically labile in order to provide the release of monovalent drugs at a certain stage or site after delivery into a biological system. In addition, the use of linker in prodrugs is not intended for modulation of binding activity of prodrugs such that covalently attached ligands from prodrugs may not bind to its receptor in either monovalent or multivalent fashion until its ligands are released free. The linker must be at least a neutral contributor to biological activity, so as not to interfere with the intrinsic activity of the tethered ligand. In some cases, the linker may even contribute favorably provided that productive contacts are made between the linker and the target surface.

In this book, the terms *linkers* and *spacers* are used interchangeably. A linker needs to be designed taking a number of factors into account, such as linker length between connected ligands, conformational property (i.e., flexible, rigid), and the nature of linker functional groups (e.g., amide, ether, amine). As long as the linker is long enough to allow bivalent occupation of tethered ligands at receptor sites, a rigid linker contributes more favorably to tight association than does a flexible one, as predicted from considering the conformational entropy of linkers, examined by Mammen et al. [13]. This is because a large number of linker configurations existing in the unbound state are going to a single configuration in the bound state, a thermodynamic feature associated with an entropic cost. Therefore, the larger the initial number of configurations, the greater this cost. A very important assumption, however, is that the rigid linker orients the ligands such that multivalent binding is possible. With this assumption, an unsaturated or aryl-incorporated linker, for example, experiences a lower conformational entropic cost upon association than does a saturated or thioether-containing flexible linker. Amide- and ether-based linkers provide an intermediate flexibility. However, the entropic effect is often accompanied by an enthalpic contribution to the free energy of binding since the linker itself is able to participate in interaction with the receptor, or to contribute via a hydrophobic effect by favorable transfer from an aqueous medium to a hydrophobic receptor domain. The experimental verification of such linker variation is discussed in later chapters.

1.1.3 Scaffolds

Covalent linkage is a term generally associated with a scaffolding or framework that serves as a molecular anchoring system where multiple chemically reactive sites are expressed as handles for ligand attachment. The valency and shape of a scaffold exert a significant influence on the binding and functional ability of multivalent molecules that comprise such a scaffold. Numerous classes of scaffold are commonly used in the design of multivalent molecules, as shown in Figure 1.3. Molecules of low valency (e.g., di- and trivalent molecules) are designed using a one-dimensional linear or branched chain. Examples include oligo(glycine)-spaced divalent sialoside as an influenza inhibitor [14], alkane-spaced divalent sLe^x as an E-selectin inhibitor [15], and Tris-linked trivalent lactoside as a ligand to the asialoglycoprotein receptor [16].

In addition to linear scaffolding, multivalent molecules may also be built on a rigid and preorganized scaffold that is well defined in structure, orientation, and conformation. Such a rigid system presents a known number of pre-assembled attachment sites that enables one to achieve the proper positioning between attached ligands. Rigid scaffolds are found in diverse classes of molecular systems, ranging from small molecules [17] such as benzene and glucose, to macrocycles such as azacrown ether, to round molecules such as cyclodextrin and calix[*n*]arene. For example, azacrown ether proves to be an effec-

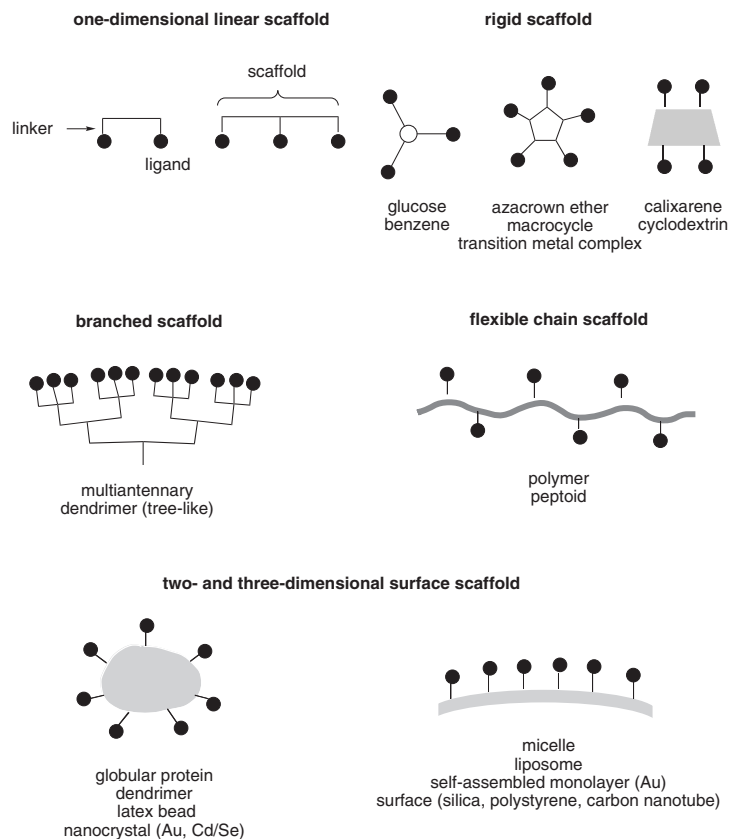


Figure 1.3 Shape and size of various scaffolds used in multivalent presentation.

tive framework for the activity of galactoside-presenting pentavalent ligands, which bind cholera toxin B pentamer very tightly [18]. Calix[n]arene consists of four ($n = 4$), six ($n = 6$), or eight ($n = 8$) phenol units joined to form a vase-like structure (*calix* = “vase” in Greek). Another well-known rigid system includes cyclodextrin, a cyclic oligosaccharide composed of six, seven, or eight α -D-glucose units, thus forming α -, β -, and γ -cyclodextrin, respectively [19]. Scaffolds such as cyclodextrin and calix[n]arene offer multiple repeats of a hydroxyl functional group located around the circumference of the narrow (primary) or the wide (secondary) face. These hydroxyl groups are readily derivatized for tethering ligands such as those based on carbohydrates [20–24]. The application of such rigid scaffolds is represented by a heptavalent galactoside anchored on the primary face of β -cyclodextrin [25] and a tetravalent sialoside displayed on calix[4]arene.

The surfaces of naturally occurring glycoproteins present multiple types of

complex carbohydrates clustered in di- and triantennary configurations [26]. Such branched scaffolds resemble a treelike structure that is classified as a dendrimer [27,28]. Compared to these natural glycodendrimerlike molecules, synthetic dendrimers offer templates that are more compact, with dense branching. Synthetic dendrimers allow the multivalent display of ligands in a medium range of valency (10- to <100-mer), thus filling the gap formed between relatively low valency (2- to 10-mer) and high valency (>100-mer in a polymer) [29–32]. Depending on branching pattern and core structure, some dendrimers adopt a large spherical or pseudospherical structure, whereas others take on the shape of a half sphere. For example, a hexadecavalent sialoside that strongly inhibits the adhesion of influenza virus to red blood cells is designed on the template of an oligo(lysine)-derived half-spherical dendrimer [33].

The synthesis of multivalent molecules designed on a polymer backbone is readily achieved by polymerizing a ligand monomer or by modifying a preformed polymer by conjugating it with the ligand [34]. A polymer scaffold provides a high-valency (>100-mer) system that is difficult to construct by using repeated connections of linear and branched scaffolding. Polymeric polyvalent molecules are highly effective in interfering with multivalent interactions, in particular those involving interactions of complementary micrometer-scale surfaces. This high level of interfering activity is attributed primarily to tight binding of one surface, but also partially to a steric effect, a second mode of action observed in certain multivalent systems [35]. This *steric occlusion* involves blocking the complementary surfaces from approaching one another due to the presence of the interfering ligand, even if that ligand is incompletely bound to one surface. Typical examples of polymeric polyvalent ligands are based on a flexible framework that includes poly(acrylamide). For instance, the sialic acid present in amide side chains of poly(acrylamide) acts as a potent inhibitor of virus–cell adhesion [36]. Other polyvalent examples are based on conformationally rigid scaffolds, such as polymers of unsaturated framework prepared by ring-opening metathesis polymerization. A specific example of the latter class includes polymeric mannoside, which provides multiple copies of mannoside as an inhibitor of lectin-mediated hemagglutination [37].

Unlike the natural peptide backbone, peptoid is made of synthetic α -amino peptide units in which the side chains are linked at the amide nitrogen rather than at the α -carbon. The resulting tertiary amide both removes a hydrogen bond critical to secondary structure and introduces a second stable rotamer of the amide bond. Both effects are probably responsible for the unique properties that distinguish peptoids from a peptide scaffold. An example peptoid-based multivalent molecule is illustrated by an N-substituted oligo(glycine) presenting multiple copies of lactoside or mannoside as side chains [38].

Several classes of scaffold mimic a two-dimensional plane or three-dimensional spherical surface, as shown in Figure 1.3. First, globular proteins such as human albumin constitute a type of adaptable scaffold because they contain reactive functional groups such as lysine, which are useful for amide coupling, and tyrosine, which is reactive to diazonium molecule for diazo coupling [39].

These proteins are soluble in water and lack intrinsic glycosylation, thus permitting neoglycoprotein preparation.

Self-assembled monolayers (SAMs) on gold comprise a two-dimensional planar surface that enables the presentation of multivalent molecules. This system mimics a cell surface in some aspects, but it lacks lateral mobility as observed in a cell surface. Sugar-displaying SAMs have been designed to study multivalent sugar–lectin interactions [40,41]. Similar systems may be used to model the surface for bacterium–mammalian cell adhesion [42,43].

Liposomes and vesicles allow multivalent display on a spherical surface and are commonly used to simulate cells. In such a system, lateral motion is permitted and allows a multivalent display to “adapt” to a complementary surface, much as may occur in cellular systems. The utility of liposomes and vesicles is demonstrated by several examples, including sialyl ganglioside displayed on a liposome as a potent inhibitor of influenza virus [44], and D-Ala-D-Ala peptide ligand presented on micelles to mimic bacterial cell surfaces [45]. Multivalent display on a larger round surface is possible by using nanometer-sized spheres made of latex, silica, or gold. For instance, polystyrene beads are available for derivatization with 6-sulfo sLe^x as a selectin ligand to produce multivalent nanoparticles that can effectively inhibit selectin-mediated attachment of a human embryo to the uterine wall [46].

1.1.4 Ligand Density

Two or more different types of molecules can be presented simultaneously, in multiple copies, on the surface of a liposome or on the side chains of a polymer. By varying, for instance, the proportions of bioactive ligand and inactive residues, it is possible to change the biological activity of such molecules. The properties of such molecules are described in terms of *surface density* or *ligand density* for liposomes and polymers, respectively. For polyvalent polymers, *ligand density* is defined as the number of attached ligands relative to the total number of side chains per polymer molecule. For liposomes bearing ligands, the *surface density* is defined as the average number of ligands relative to the total number of functional groups, including ligands, exposed on the surface of a liposome particle. Pertinent examples are illustrated using multivalent sialic acid, shown as either poly(acrylamide) [47] or liposome [48] in Figure 1.4. Ligand density constitutes a critical factor to be considered when designing multivalent molecules because it broadly affects ligand distribution, interligand distance, and the shape and conformation of the polymer. All of these properties help to modulate the biological activity of multivalent molecules.

1.1.5 Homo- and Heterovalent Molecules

Multivalent interactions can refer to the simultaneous association occurring between multiple, identical pairs of receptor and ligand; such interactions are *homovalent*. In contrast, multivalent interactions can occur between *more than*

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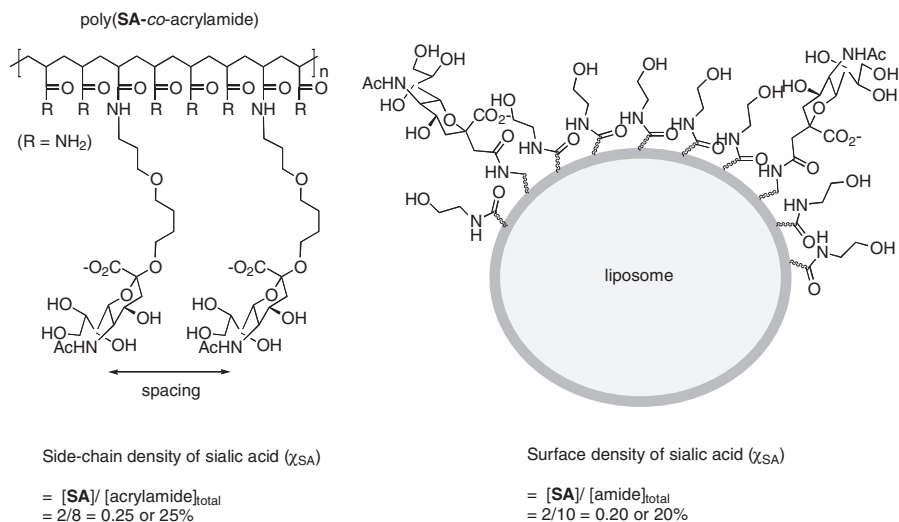


Figure 1.4 Definition of ligand density in polymer- and liposome-based multivalent molecules.

one kind of receptor and ligand; such interactions are *heterovalent*. As with homovalent interactions, formation of multiple ligand–receptor pairs should contribute to the increased association strength of the entire complex. Figure 1.5 illustrates the functionally bivalent association between a heterobivalent ligand and a heterobivalent receptor. Structurally, a heterodimeric molecule closely resembles a bifunctional molecule, as the latter is also composed of two different ligands tethered through a linker. However, a bifunctional molecule is designed to bind in a monovalent manner to two distinct target receptors widely separated or located in a different compartment of a cell.

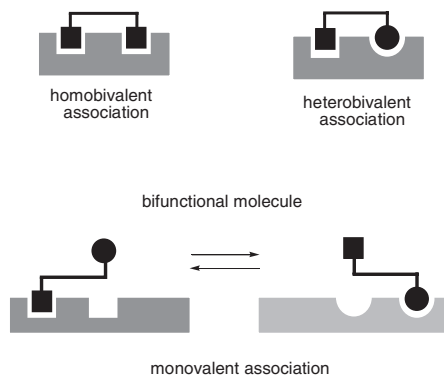


Figure 1.5 Representation of homovalent and heterovalent interaction.

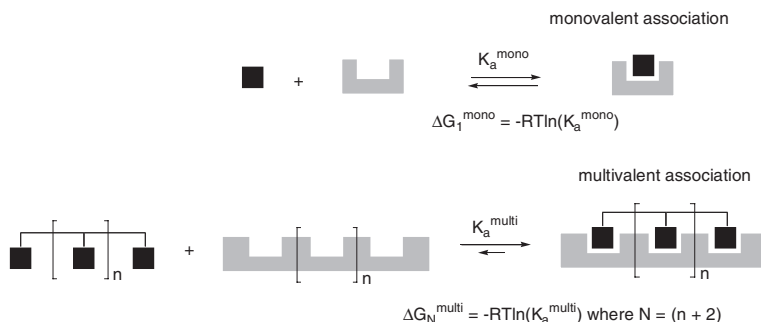


Figure 1.6 Comparison of thermodynamic parameters of association in monovalent and multivalent interaction.

1.2 MECHANISTIC ASPECTS OF MULTIVALENT INTERACTION

1.2.1 Affinity Constant and Avidity

Multivalent and monovalent interactions differ fundamentally in their respective definitions and calculations of association strength [1]. For a monovalent system composed of a receptor and a ligand, the *affinity constant* (K_a) is the binding strength for a monovalent complex (Figure 1.6). This term is related to the free energy of association (ΔG^{mono}) by the Gibbs equation below. In a multivalent interaction between two entities presenting N tethered ligands and N tethered receptors, the association constant is defined as *avidity* (K_a^{multi}). This constant is a collective association constant that takes into consideration multiple interactions between two multivalent entities, and it is related to the free energy of binding calculated as for monovalent binding:

$$\Delta G^{\text{mono}} = -RT \ln(K_a^{\text{mono}})$$

$$\Delta G_N^{\text{multi}} = -RT \ln(K_a^{\text{multi}})$$

In estimating *cooperativity* in multivalent association, the free energy of multivalent binding ($\Delta G_N^{\text{multi}}$) can be related to that of N monovalent associations: that is, $N\Delta G^{\text{mono}}$, given N independent receptor–ligand interactions. The ratio between the two indicates the degree of cooperativity, where the cooperativity coefficient (α) is defined as follows:

$$\alpha = \text{degree of cooperativity} = \frac{\Delta G_N^{\text{multi}}}{N \Delta G^{\text{mono}}}$$

Depending on the magnitude of α , multivalent interaction is positively cooperative or *synergistic* ($\alpha > 1$), noncooperative or *additive* ($\alpha = 1$), or negatively cooperative ($\alpha < 1$). The term *cooperativity* is often used in biological systems

as with the hemoglobin tetramer–oxygen interaction, which shows positive cooperativity [49]. However, the term is rarely used in multivalent systems, partly because few multivalent systems have been shown to demonstrate positive cooperativity. Moreover, multivalent interaction can be much tighter than monovalent binding, regardless of the size of the cooperativity constant. In practical terms, the contribution of a multivalent association is often expressed by the ratio (β) of multivalent avidity to monovalent affinity constant, introduced by Mammen et al. [1] (Figure 1.6):

$$\beta = \frac{K_a^{\text{multi}}}{K_a^{\text{mono}}}$$

Thus, β represents the enhancement factor, a term that reflects the strength of a multivalent association relative to the monovalent association.

The difference between α and β is illustrated by a trivalent system based on a vancomycin receptor and a D-Ala-D-Ala ligand (Figure 1.7) [50]. Vancomycin is an antibiotic belonging to the glycopeptide class of receptors. Antibiotics in

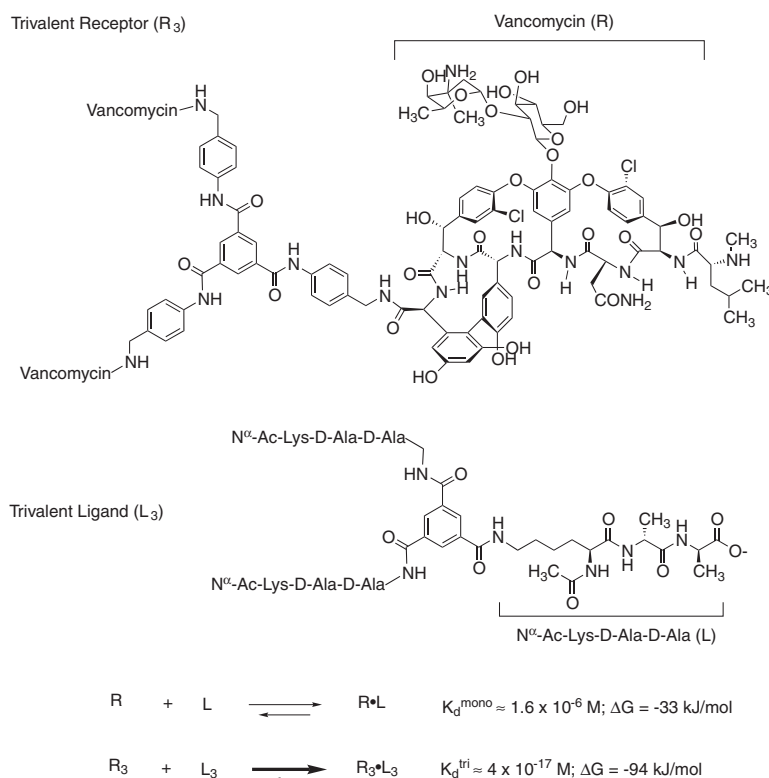


Figure 1.7 Interaction of a trivalent vancomycin with a trivalent D-Ala-D-Ala ligand.

this class target bacterial cell walls by binding to the D-Ala-Ala peptide precursor. In a monovalent system, vancomycin associates with D-Ala-D-Ala ligand with a dissociation constant (K_d) value of $1.6 \times 10^{-6} M$ ($\Delta G^{\text{mono}} = -33$ kJ/mol). In a trivalent system composed of trivalent vancomycin and trivalent D-Ala-D-Ala designed by Rao et al. at Harvard [50], the receptor–ligand association is extremely tight, with a K_d value of $4 \times 10^{-17} M$ ($\Delta G^{\text{tri}} = -94$ kJ/mol). The avidity of the trivalent complex is much higher than the affinity of the monovalent interaction. Based on the value of the cooperativity constant [$\alpha = 94 \div (3 \times 33) = 0.95$], this trivalent interaction is still negatively cooperative, although it is exceptionally tight. In terms of binding enhancement (β), the trivalent interaction leads to about a 4×10^{10} -fold increase in binding strength relative to that of the corresponding monovalent interaction. In fact, the binding strength is 25 times higher than that of the avidin–biotin system—one of the strongest monovalent interactions in biological systems—thus clearly demonstrating the practical value of multivalency for designing a high-affinity system.

Gargano et al. at Syracuse [51] proposed a simplified model that can be used in correlating the strength of multivalent association with that of monovalent association. This model makes it possible to estimate the multivalent enhancement factor (β) (Figure 1.8). The model is, however, designed under highly simplified conditions of multivalent systems that are not likely to fit real systems. The conditions include (1) equivalent binding sites on the multivalent receptor as a homobivalent system, (2) noncooperativity ($\alpha = 1$) such that first binding at one site is thermodynamically identical to second binding at neighboring site, (3) a flexible linker of optimal length to ensure bivalent association, and (4) no linker–receptor interaction, to avoid extra thermodynamic considerations. Because of such limiting conditions, this model should be used for

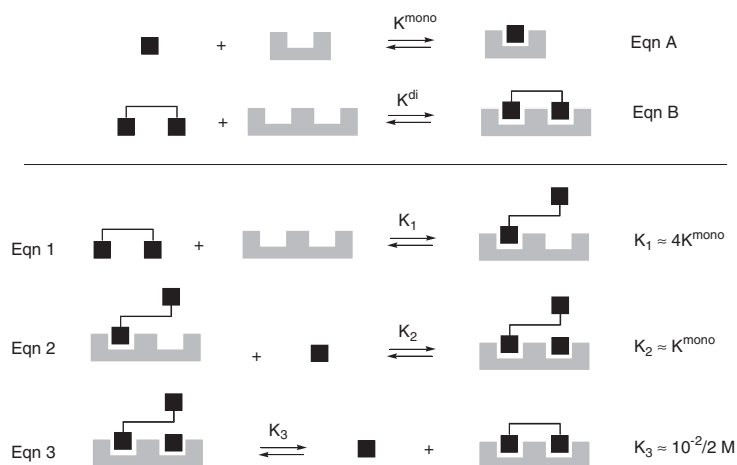


Figure 1.8 Theoretical model used for deriving an equation that would make it possible to estimate di(multi)valent association constant.

estimating the β term rather than predicting it accurately. It is illustrated by a simple divalent binding model composed of a dimeric receptor and a dimeric ligand, as described in Figure 1.8. This model suggests that the bivalent association constant (K^{di}) is expressed as three related association constants: K_1 , K_2 , and K_3 . Each equilibrium constant is derived based on certain approximations, such as the number of possible permutations assumed in receptor–ligand site association in Eqn 1 in the figure ($K_1 = 4K^{\text{mono}}$), monovalent binding in Eqn 2 ($K_2 = K^{\text{mono}}$), and effective local concentration (C_{eff}) of bound divalent ligand in Eqn 3 ($K_3 \approx C_{\text{eff}} = 10^{-2}/2 M$, where two tethered ligands are 30 Å apart [52]). An overall divalent association constant, which provides an estimate of the factor of divalent enhancement, is obtained as a product of the three equilibrium constants:

$$K^{\text{di}} = K_1 K_2 K_3 = 2 \times 10^{-2} (K^{\text{mono}})^2$$

$$\beta = \frac{K^{\text{di}}}{K^{\text{mono}}} = 2 \times 10^{-2} (K^{\text{mono}})$$

This type of prediction is generalized to the multivalent association constant in a higher-order system as follows:

$$K^{\text{multi}} = F(s \times 10^{-2})^{n-1} (K^{\text{mono}})^n$$

where n is the valency number, F is a statistical factor defined by the system, and $s = 30/[\text{interreceptor distance (Å)}]$. An application is illustrated by a monovalent P^{k} trisaccharide ligand, which binds to pentavalent Shiga toxin (AB_5) with a K_a value of $1 \times 10^3 M^{-1}$. If this monovalent ligand is converted to a polyvalent ligand that carries P^{k} trisaccharide as side chains of poly(acrylamide), a binding constant (K^{penta}) can be estimated for the multivalent interaction with the toxin pentamer as follows:

$$K^{\text{penta}} (M^{-1}) \approx 1(1 \times 10^{-2})^{5-1} (K^{\text{mono}})^5 = 10^{-8} (10^3)^5 = 10^7$$

where $F = 1$ and $s = 1$. The predicted value is close to the experimental value of the binding constant, $K^{\text{penta}} \approx 1.2 \times 10^7 M^{-1}$, obtained from a cell-based assay [51]. This theoretical equation for calculating K^{multi} indicates that the strength of the multivalent association increases exponentially as a function of valency. It also predicts that the intrinsic affinity of a monovalent system contributes significantly to the strength of multivalent association and that the intrinsic affinity is especially important in cases of low valency, such as for a bivalent or trivalent ligand.

1.2.2 Thermodynamics

The thermodynamic basis of multivalent interactions has been investigated by numerous groups of researchers [1,2]. Mammen et al. have provided a particu-

larly thorough description in their review article [1], and the following discussion borrows ideas from this review. To elucidate the thermodynamic basis of multivalent interactions, it is easiest to start by examining a simple, bivalent system. A bivalent association (Eqn B, Figure 1.8) can be compared with a monovalent association (Eqn A) under certain limiting conditions, as defined in Figure 1.8. The free energy of binding (ΔG) in a receptor–ligand association is made up of enthalpic (ΔH) and entropic (ΔS) components, in which the monovalent association is linked to the standard relationships between three thermodynamic parameters (ΔG^{mono} , ΔH^{mono} , and ΔS^{mono}), which are expressed as follows:

$$\begin{aligned}\Delta G^{\text{mono}} &= \Delta H^{\text{mono}} - T \Delta S^{\text{mono}} \\ \Delta S^{\text{mono}} &\approx \Delta S^{\text{mono}} (\text{translational}) + \Delta S^{\text{mono}} (\text{rotational})\end{aligned}$$

In the equations above, the total entropy change involved is derived from changes primarily in the translational and rotational entropies of the receptor and ligand upon association. The equation represents a qualitative summation. It assumes that the translational and rotational entropies make the greatest contribution and that other possible contributions, such as from the conformational, vibrational, and solvation entropies, are relatively insignificant (i.e., vibrational entropy of a water molecule is about 3000-fold smaller than its rotational or translational entropy) and too complicated to be treated here.

For a bivalent association, the free energy of binding (ΔG^{di}) is expressed similarly, using standard equations that relate thermodynamic components in qualitative terms:

$$\begin{aligned}\Delta G^{\text{di}} &= \Delta H^{\text{di}} - T \Delta S^{\text{di}} \\ \Delta H^{\text{di}} &\approx 2\Delta H^{\text{mono}} \\ \Delta S^{\text{di}} &\approx \Delta S^{\text{mono}} (\text{translational}) + \Delta S^{\text{mono}} (\text{rotational}) \\ &\quad + \Delta S^{\text{di}} (\text{conformational, linker})\end{aligned}$$

where the association of a discrete ligand of a bivalent molecule with each of the bivalent sites is assumed to occur in an unstrained and independent manner. Under such binding conditions, the bivalent enthalpy change is equal to twice the enthalpy of monovalent binding. The change of entropy in the bivalent association is considered to consist of three components. The first two terms are equal to translational and rotational components from monovalent association. Such an estimation is based on certain limited considerations: (1) translational and rotational entropies of divalent and monovalent particles are equal because these entropies are only weakly dependent on mass and size; and (2) in both bivalent and monovalent associations, two particles associate and become one particle, resulting in the net loss of the free translation and rotation

of a single particle. The third component results from a change in conformational entropy. This component takes into consideration the conformational entropy that is contributed by the change in entropy of the linker upon association.

For a simple estimation of the thermodynamic advantages of a multivalent system, the free energies of association can be compared between two ideal systems. One is a multivalent system in which one multivalent receptor, with n sites, binds to one multivalent ligand, also with n sites, to become one perfectly matched multivalent complex. The second system is represented by n independent pairs of monovalent complexes composed of n copies of the monovalent ligand and n copies of the monovalent receptor. Thus, the free energy of association for each system is given collectively as follows:

$$\begin{aligned} n\Delta G^{\text{mono}} &= n\Delta H^{\text{mono}} - nT\Delta S^{\text{mono}} \\ \Delta G^{\text{multi}(n)} &= \Delta H^{\text{multi}(n)} - T\Delta S^{\text{multi}(n)} \\ &\approx n\Delta H^{\text{mono}} - T\Delta S^{\text{mono}} - T\Delta S^{\text{multi}(n)} \text{ (conformational, linker)} \end{aligned}$$

Subtracting the two equations provides a free-energy difference between the two systems:

$$\Delta\Delta G = \Delta G^{\text{multi}(n)} - n\Delta G^{\text{mono}} = (n-1)T\Delta S^{\text{mono}} - T\Delta S^{\text{multi}(n)}$$

Therefore, if the multivalent association is tighter than the monovalent association, it is due largely to the entropic contribution of monovalent binding ($\Delta S^{\text{mono}} < 0$). This comparison suggests that multivalent binding is favored because it suffers a relatively smaller entropy loss.

In practical examples reported in the literature, the thermodynamic benefits of a multivalent system are estimated from a comparison with one pair of a monovalent system rather than with n pairs of a monovalent complex as a group. As an example, the free energy of bivalent association is compared with that of monovalent association, where the difference between ΔG^{di} and ΔG^{mono} can be approximated by two terms, the enthalpy of monovalent binding and the conformational entropy. Such a relationship is extended similarly to a multivalent system:

$$\begin{aligned} \Delta G^{\text{di}} &= 2\Delta H^{\text{mono}} - T\Delta S^{\text{mono}} - T\Delta S^{\text{di}} \text{ (conformational, linker)} \\ \Delta G^{\text{di}} - \Delta G^{\text{mono}} &= \Delta H^{\text{mono}} - T\Delta S^{\text{di}} \text{ (conformational, linker)} \\ \Delta G^{\text{multi}(n)} - \Delta G^{\text{mono}} &= (n-1)\Delta H^{\text{mono}} - T\Delta S^{\text{multi}} \text{ (conformational, linker)} \end{aligned}$$

Based on this estimation, a negative value of the free-energy difference ($\Delta\Delta G = \Delta G^{\text{multi}} - \Delta G^{\text{mono}}$) represents multivalently enhanced binding. There-

fore, the enthalpy term contributes significantly and positively to the free energy of the multivalent association, whereas the conformational entropy causes a negative effect because it is often associated with loss of conformational entropy of a linker upon receptor–ligand association. Such an enthalpy contribution has also been noted in ditopic binding of the cyclodextrin receptor dimer by Breslow et al. at Columbia [19]. In summarizing this section, thermodynamic aspect suggests that the enhancement factor ($\beta = e^{-\Delta\Delta G/RT}$) becomes greater in a system that involves a higher valency number (n), a tighter monovalent association (more negative ΔH^{mono}), and a rigid linker (a lower degree of conformational entropy loss), unless the linker interacts directly with the receptor.

The thermodynamic basis of a tight multivalent association is illustrated by an experimental system (Figure 1.7) that is based on the association of a trivalent vancomycin molecule to a trivalent D-Ala-D-Ala molecule [50]. The trivalent binding results in a favorable value of ΔG^{tri} (-94 kJ/mol), which is dissected to enthalpic and entropic contributions ($\Delta H^{\text{tri}} = -167$ kJ/mol; $T\Delta S^{\text{tri}} = -73$ kJ/mol). It is a highly favored process compared with the association of monovalent vancomycin and D-Ala-D-Ala, which leads to a ΔG^{mono} value of -33 kJ/mol, where ΔH^{mono} is composed of ΔH^{mono} (-50.2 kJ/mol) and $T\Delta S^{\text{mono}}$ (-17.2 kJ/mol). Thus, in this system, ΔH^{tri} has a value that is slightly more negative than three times the value of ΔH^{mono} and thus is a major, driving factor for tight association. On the other hand, the absolute value of $T\Delta S^{\text{tri}}$ is larger than that of $T\Delta S^{\text{mono}}$ by 56 kJ/mol, which might be related to the loss in conformational entropy of trivalent complex, including the entropic loss contributed by linker. Clearly, favorable enthalpy makes a strong contribution to the very tight association of the trivalent system ($K_d^{\text{tri}} = 4 \times 10^{-11}$ M).

The thermodynamic basis for benefits of multivalent ligand–receptor association can be influenced by the system itself. For example, a multivalent ligand can interact with either a multivalent receptor presenting multiple connected sites (such as trivalent vancomycin and trivalent D-Ala-D-Ala) or unconnected multiple receptor sites. A series of studies performed by Dam et al. at Albert Einstein School of Medicine [2,53,54] provide important insights underlying the thermodynamics of multivalent binding of the latter class. Their studies are based on the association of concanavalin A dimer (Con A at pH 5.2) and divalent trimannoside cluster (Figure 1.9). The trimannoside dimer can form a soluble, one-dimensional cross-linked complex with Con A, with a divalent association constant (K_a^{di}) of 2.5×10^6 M^{-1} , which is about sixfold tighter than that of trimannoside monomer to Con A ($K_a^{\text{mono}} = 3.9 \times 10^5$ M^{-1}). Such a slight enhancement in bivalent binding can be attributed to a combination of two separate, sequential association events where a trimannoside dimer cross-links to two lectins with a distinct binding affinity (first association: $K_{a1} = 1.6 \times 10^7$ M^{-1} ; second association: $K_{a2} = 8.8 \times 10^5$ M^{-1}). Therefore, the first association is 18-fold tighter than the second one, which translates into

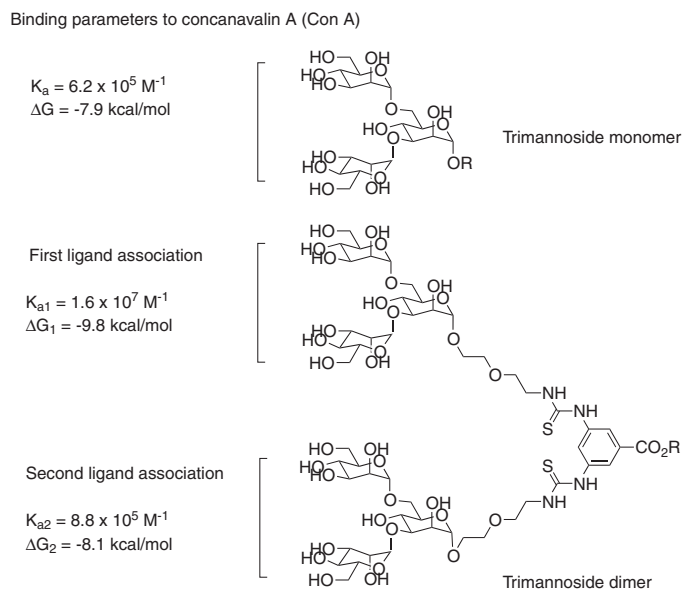


Figure 1.9 Thermodynamic binding parameters in divalent trimannoside–lectin interaction.

a more negative value for the free energy of binding ($\Delta G_1 = -9.8 \text{ kcal/mol}$; $\Delta G_2 = -8.1 \text{ kcal/mol}$), indicating negative cooperativity generated on bivalent association. The two separate association events display similar enthalpies of binding ($\Delta H_1 = -12.5 \text{ kcal/mol}$; $\Delta H_2 = -12.3 \text{ kcal/mol}$), whereas the first association shows a more favorable entropy of binding ($T \Delta S_1 = -2.7 \text{ kcal/mol}$) than the second ($T \Delta S_2 = -4.2 \text{ kcal/mol}$), or binding of a trimannoside monomer to a lectin molecule ($T \Delta S = -5.2 \text{ kcal/mol}$). This class of bivalent ligand–lectin association represents a multivalent system driven by entropy. Therefore, in the thermodynamic basis of binding, this system is clearly distinguished from a previous enthalpy-driven vancomycin/D-Ala-D-Ala system where a trivalent receptor presents three connected sites.

Drug–DNA interactions represent another well-studied multivalent ligand–receptor system [55]. This type of system is exemplified by the association of hydroxyrubicin and daunorubicin to double-stranded DNA (Figure 1.10). Hydroxyrubicin is an anthracycline antibiotic composed of two parts. The first part is an adriamycinone moiety that intercalates into nucleotide bases. The second part is an attached sugar moiety that is able to bind at a DNA minor groove. Chaires et al. [56] reported that the free energy of binding (ΔG) of hydroxyrubicin to DNA is -7.0 kcal/mol , while that of adriamycinone is -5.7 kcal/mol . The higher affinity of hydroxyrubicin is attributable to the bivalent mode of interaction (intercalation plus minor groove recognition) compared to the monovalent mode of interaction (intercalation only) by adriamycinone.

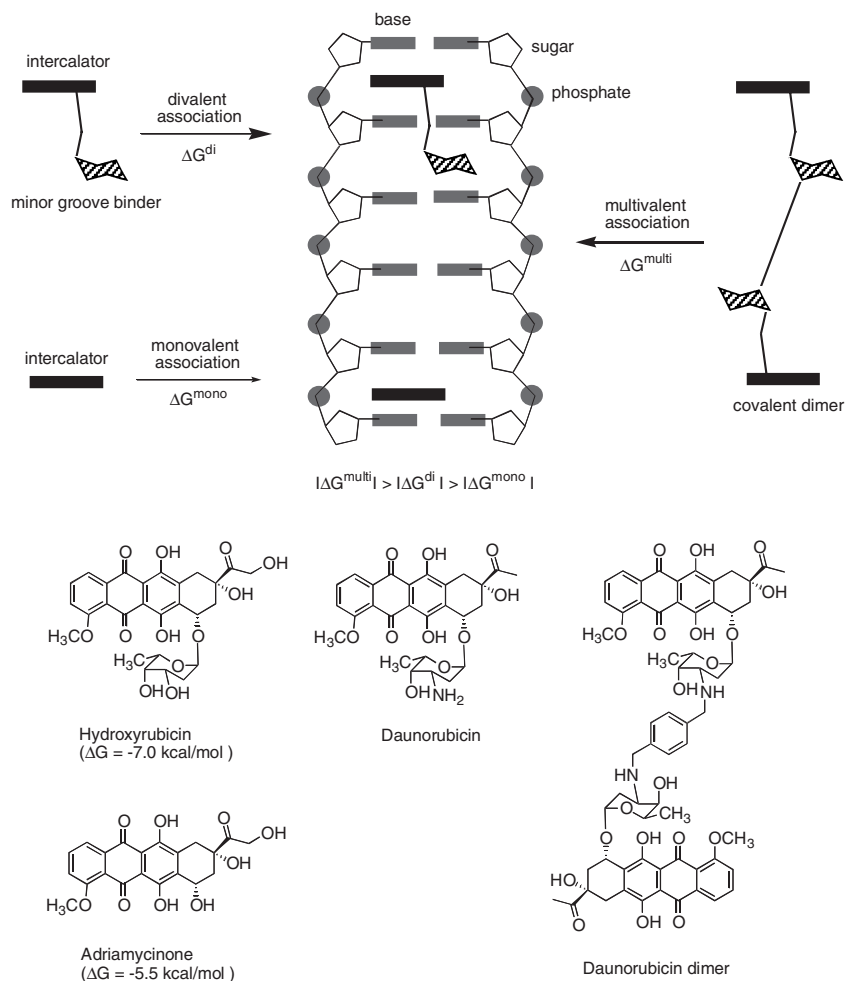


Figure 1.10 Mode of DNA binding displayed by daunorubicin and its dimer.

The involvement of multivalency in drug–DNA interactions is illustrated more dramatically by daunorubicin, a close analog of hydroxyrubicin. A daunorubicin dimer binds tightly to DNA with an association constant K_a of $2.7 \times 10^{11} M^{-1}$ ($\Delta G = -15$ kcal/mol), which is 16,000-fold greater than that of daunorubicin ($\Delta G = -10$ kcal/mol) [57]. This greatly enhanced affinity comes mostly from a large enthalpy contribution ($\Delta H = -30$ kcal/mol; $T \Delta S = -15$ kcal/mol) compared to daunorubicin ($\Delta H = -11$ kcal/mol; $T \Delta S = -1$ kcal/mol). The favorable enthalpy displayed by the daunorubicin dimer, which is close to three times that of the daunorubicin monomer, serves as a major driving force enabling the otherwise unfavorable entropic cost arising from multi-

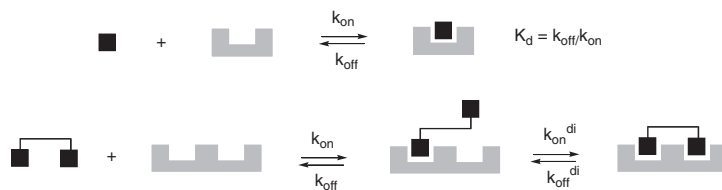


Figure 1.11 Comparison of association kinetics in monovalent and bivalent systems.

valent drug–DNA interaction to be overcome. This example illustrates the important role of enthalpy in the association of a multivalent ligand with a multivalent receptor. In summary the thermodynamic basis of multivalent association can be driven by enthalpy or entropy and depends on the nature of multivalent system as discussed with the three examples above, involving vancomycin, lectin, and DNA.

1.2.3 Kinetics

The thermodynamic aspects of multivalent receptor–ligand interactions are highlighted by enhanced binding affinity. These aspects are, however, inseparably related with the kinetic features of multivalent binding, which is characterized by a decrease in the rate of dissociation of the two interacting entities. The kinetic aspects of bivalent association are shown schematically in Figure 1.11, in which a bivalent ligand occupies two binding sites present on a divalent receptor. The dissociation of a bound dimer occurs in two stages, so that its dissociation rate ($k_{\text{off}}^{\text{di}}$) at the second site can be compared theoretically with the rate of dissociation (k_{off}) of a bound monovalent ligand, as suggested by Kramer and Karpen [52]:

$$k_{\text{off}}^{\text{di}} = \frac{2k_{\text{off}}K_d}{C_{\text{eff}} + K_d}$$

where C_{eff} refers to the effective local concentration of a ligand unit at the second site and K_d refers to the dissociation constant of the monovalent ligand. The effective concentration of a bound divalent ligand is calculated from the volume of the molecular hemisphere, with a radius of r equal to the length of the linker:

$$C_{\text{eff}} = \frac{1000}{N_A \left(\frac{2}{3}\right) \pi r^3}$$

where N_A is Avogadro's number. The effective concentration is closely associated with effective molarity as a kinetic parameter commonly used in enzymatic or intramolecular reactions where effective molarity contributes to rate acceleration relative to bimolecular reaction as investigated by Page and Jencks [58] and others. Thus, the rate of dissociation at the second site, $k_{\text{off}}^{\text{di}}$, is defined as a linear function of the monovalent dissociation rate k_{off} multiplied by both a statistical factor of 2 (because dissociation can occur at either of two sites) and the probability $K_d/(C_{\text{eff}} + K_d)$ that the second site is not occupied.

Approximating the divalent dissociation rate is illustrated by the interaction of a bivalent cyclic guanosinemonophosphate (cGMP) tethered with a variable poly(ethylene glycol) spacer of molecular weight 2000 kDa. The spacer includes a cyclic nucleotide-gated channel which presents four cGMP sites distributed equally on four subunits comprising the channel [52]. With a linker length of 39 Å, this bivalent ligand binds the tetrameric channel receptor with a K_d^{di} value of about 5 nM, which represents 600-fold tighter binding than that of the cGMP monomer ($K_d \approx 3 \mu\text{M}$). Assuming that the first site is occupied by a bivalent cGMP, the effective concentration of cGMP at the second site is calculated to be 13.4 mM, which is much higher than the monovalent dissociation constant ($K_d \approx 3 \mu\text{M}$). Under such circumstances, $k_{\text{off}}^{\text{di}}$ at the second site is predicted to be about 1700-fold slower than k_{off} by monovalent cGMP. Thus, the decrease in dissociation rate is linked to the enhanced binding affinity of bivalent cGMP.

Another good example of a slow dissociation rate by multivalent species involves a trivalent system composed of vancomycin and D-Ala-D-Ala (Figure 1.7). Compared to a monovalent system of vancomycin and D-Ala-D-Ala peptide ($K_d = 1.6 \times 10^6 \text{ M}$; $k_{\text{on}} = 9.3 \times 10^6 \text{ M}^{-1}/\text{s}$; $k_{\text{off}} = 14.88 \text{ s}^{-1}$), this trivalent system shows an exceptionally low dissociation constant K_d of $4 \times 10^{-17} \text{ M}$, and $k_{\text{off}}^{\text{tri}}$ is estimated to be $3 \times 10^{-9} \text{ s}^{-1}$ [50]. Thus, for any trivalent receptor-ligand with a very tight association, a slower rate of dissociation can be inferred.

1.2.4 Steric Effects

As discussed, to design any multivalent ligand intended to target a cell surface receptor, the linker is an important factor to consider. In addition to its normal role as a spacer for bridging, it is implicated in the occurrence of undesired steric effects caused by the size of receptor and the incompatible ligand spacing of multivalent ligands [59]. This unfavorable effect is illustrated in Figure 1.12, where the interligand distance (L_L) is smaller than the distance between adjacent receptor sites (L_R). Under these circumstances, a certain fraction of unbound ligand units of a multivalent ligand are no longer available for receptor binding because these units are either obstructed directly by a bound receptor or they are excluded sterically by adjacent bound receptors.

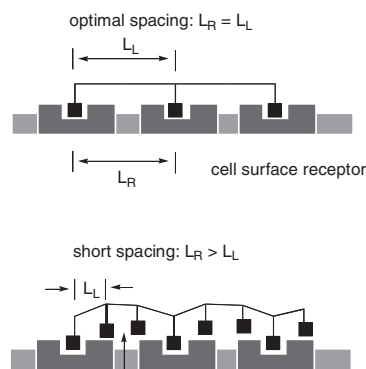


Figure 1.12 Definition of steric effect in multivalent receptor–ligand complexation.

sterically hindered, and unavailable for receptor binding

Hlavacek et al. [59] proposed a theoretical model defining the steric effect in a multivalent system that makes it possible to predict the relationship between the fraction of available ligand units and the fraction of bound receptor sites. It is illustrated using a linear multivalent ligand with a valency of 200 under arbitrary conditions where the number of ligand units covered by or in contact with bound receptor is 3 ($L_R > L_L$; Figure 1.12). This model predicts that if the fraction of bound receptor sites by such a multivalent ligand is 5% (i.e., there are 10 bound ligands), about 75% of ligand units are still available for receptor binding. If the fraction of bound receptors increases to 10% (20 bound ligands), the fraction of ligand units available for receptor binding decreases to 50%. Therefore, a moderate increase in the fraction of bound receptor sites leads to a much greater degree of steric hindrance.

In contrast, where linker spacing exceeds the optimal length between receptor sites, such that $L_L > L_R$, unbound ligand units are not covered or excluded sterically by bound receptors but instead are possibly available for receptor binding. However, the increase in linker length accounts for the decrease in effective local concentration of the ligand, and thus leads to a weaker contribution to multivalent binding.

1.3 BIOLOGICAL ROLES OF MULTIVALENT LIGANDS

In biological systems, multivalent binding provides a broad range of benefits and unique roles that are not achievable with monovalent interactions. Here, we introduce selected features of multivalency benefits; detailed coverage is left for upcoming chapters. First, multivalent interaction is collectively much tighter than monovalent interaction. There are numerous examples of multivalent ligands that bind more strongly to their respective multivalent receptors,

as demonstrated in a diverse array of targets, including molecules as diverse as cell surface receptors, enzymes, G-protein-coupled receptors, ion channels, lectins, toxins, and nucleic acids found in cells and pathogens (viruses, bacteria). Notable examples include multivalent sialic acid molecules that bind to the hemagglutinin receptor on the surface of the influenza virus, where the multivalent enhancement factor ranges from 10 to 10^6 , depending on the degree of valency and type of scaffold [60]. High-affinity multivalent ligands that target surface receptors show great potential as effective inhibitors of surface–surface interactions, including cell–cell and cell–pathogen interactions, that occur in biological systems. Second, multivalent interactions can enhance receptor selectivity, as demonstrated by studies using multivalent ligands that bind to lectins [61], acetylcholine esterase [62], monoamine transporters [63], and serotonin receptors [64]. Third, multivalent interactions induce receptor clustering on the cell surface, as illustrated by bacterial galactoside-sensing receptors [65] and galactosyl transferase receptors on the surface of sperm [66,67]. Fourth, multivalent interactions provide a strategy for controlling signal transduction pathways within cells, as represented by the FK506 dimer, a prototype member of chemical inducers of dimerization (CID) [68–70] and by the polyamide dimer as an artificial transcription factor [71].

Numerous excellent review articles deal with potential applications and characteristics of multivalent ligand–receptor interactions. The principles and biomedical applications of polyvalent interactions are discussed systematically by Mammen et al. [1]. The thermodynamic basis of multivalent binding is evaluated with respect to lectin–carbohydrate interaction by Brewer [54] and with respect to drug–DNA interaction by Chaires [55]. Classical examples of multivalent interactions, known as the *cluster effect*, are described by Lee and Lee [26] in the context of carbohydrate–protein (C-type lectin and animal lectin) interaction, and multivalent selectin inhibitors are reviewed by Simanek et al. [72]. The mechanism of action by dimers of the FK506 class and its biomedical uses are reviewed by Crabtree and Schreiber [68]. Accounts by Roy [29] and Kiessling and Pohl [60] deal with chemical and biological aspects of multivalent neoglycoconjugates, and those by Yarema and Bertozzi [73] and Bertozzi and Kiessling [74] describe the novel roles played by multivalent carbohydrates in a chemical approach to glycobiology. Polyamide dimers are treated as highly selective DNA binders and their use in transcriptional control is described by Dervan and Bürli [75] and Wemmer and Dervan [76]. The need for polyvalency when designing drugs to prevent pathogen–cell adhesion is described by Matrosovich [77]. Examples of multivalent drug candidates are documented in several therapeutic areas by Wright and Usher [11], while focused reviews include that of the use of multivalent glycan mimics as anti-infectives by Mulvey et al. [78], and rational design of a multivalent inhibitor of bacterial toxin by Fan and Merritt [79]. The role of noncovalent dimerization of vancomycin in its antibiotic action is reviewed by Williams [80]. Cyclodextrin is a molecule that plays a dual role: Its hydrophobic cavity serves as a receptor,

and multiple hydroxyl groups on its rims serve as handles for ligand attachment. Breslow et al. [19] reviewed the chelate effect observed in ditopic binding of covalent cyclodextrin dimer. Fulton and Stoddart [21] discuss neoglycoconjugates designed on a multivalent scaffold composed of cyclodextrin and calix[*n*]arene. Finally, the use of protein- and synthetic polymer-based polyvalent carbohydrates is reviewed by Bovin and Gabius [39].